UNIVERSITY OF CALIFORNIA SAN DIEGO

NLRP3 Inflammasome Products IL-1beta and IL-18 have Direct Effects on Cardiomyocytes

A Thesis submitted in partial satisfaction of the requirements for the degree Master of Science

in

Biology

by

Jacqueline Bella Anderson Enni

Committee in charge:

Professor Joan Heller Brown, Chair
Professor Nicholas Spitzer, Co-Chair
Professor Michael David

2020

The Thesis of Jacqueline Bella Anderson Enni is approved, and it is acceptable in quality and form for publication on microfilm and electronically:

Co-Chair

Chair

University of California San Diego

2020

TABLE OF CONTENTS

LIST OF ABBREVIATIONS

α-actinin	alpha-actinin
AMI	acute myocardial infarction
AMPK	AMP-activated protein kinase
BCA	bicinchoninic acid
CaMKII	calcium/calmodulin-dependent kinase II
CM	cardiomyocyte
CCL3	chemokine (C-C motif) ligand 3
cDNA	complementary DNA
COX IV	cytochrome c oxidase subunit 4
cT	cycle threshold
CXCL1	chemokine (C-X-C motif) ligand 1
CXCL2	chemokine (C-X-C motif) ligand 2
CXCL9	chemokine (C-X-C motif) ligand 9
CXCL10	chemokine (C-X-C motif) ligand 10
DAMP	damage-associated molecular pattern
DNA	deoxyribonucleic acid
EGTA	ethylene glycol tetraacetic acid
GAPDH	glyceraldehyde phosphate dehydrogenase
HCl	hydrochloride
IL-1	interleukin-1
IL-18	interleukin-18
IL-18R	interleukin-18 receptor

IL-1β	interleukin-1 beta
IL-1R	interleukin-1 receptor
IL-6	interleukin-6
JAK/STAT	janus kinase/signal transducer and activator of transcription
JNK	c-Jun N-terminal kinase
MAPK	mitogen-activated protein kinase
MAPKKK	mitogen-activated protein kinase kinase kinase
MCP-1/CCL2	macrophage chemoattractant protein-1/chemokine (C-C motif) ligand 2
mL	milliliter
mM	millimolar
mRNA	messenger ribonucleic acid
mTOR	mammalian target of rapamycin
NaCl	sodium chloride
Na_2EDTA	disodium salt dihydrate
Na_3VO_4	sodium vanadium oxide
NF-κB	nuclear factor kappa-light-chain-enhancer of activated B cells
ng	nanogram
nM	nanomolar
NP-40	nonyl phenoxypolyethoxylethanol-40
NRVM	neonatal rat ventricular myocyte
PLB	phospholamban
PVDF	polyvinylidene fluoride

qPCR	quantitative polymerase chain reaction
RIPA	radioimmunoprecipitation assay
ROS	reactive oxidant species
STAT3	signal transducer and activators of transcription 3
TAK1 Thr184/187	transforming growth factor-β-activated kinase 1 threonine 184/187
TGF-β	transforming growth factor beta
TNFα	tumor necrosis factor alpha
TLR	toll-like receptor
µg	microgram

LIST OF FIGURES

ABSTRACT OF THE THESIS

NLRP3 Inflammasome Products IL-1beta and IL-18 have Direct Effects on Cardiomyocytes

by

Jacqueline Bella Anderson Enni

Master of Science in Biology

University of California San Diego, 2020

Professor Joan Heller Brown, Chair
Professor Nicholas Spitzer, Co-Chair

We previously demonstrated a significant role for NLRP3 inflammasome activation in cardiomyocytes in cardiac inflammation, remodeling and contractile dysfunction in response to stressors such as Angiotensin II infusion and pressure overload. There is limited data regarding responses of cardiomyocytes to interleukin-1 β (IL-1β) and interleukin 18 (IL-18), which are generated as a result of NLRP3 activation during the cardiac inflammatory response. To determine the effects of IL-1β and IL-18 on cardiomyocytes, isolated neonatal rat ventricular myocytes (NRVMs) were treated for various times with 1ng/mL recombinant IL-1β or 10ng/mL recombinant IL-18. Western blotting was used to measure the activation state of protein kinases

and downstream signaling pathways. Quantitative Polymerase Chain Reaction (qPCR) was used to assess the expression of pro-inflammatory cytokines and chemokines in cardiomyocytes. Proteins and genes showed enhanced phosphorylation and expression when treated with either cytokine, but the majority of NRVMs responded with greater significance to IL-1β stimulation. After treatment with IL-1β, Western blotting showed a several-fold increase in nuclear translocation of NF-κB p65 subunit and phosphorylation of mitogen-activated protein kinases (MAPKs) and P-STAT3, which regulate inflammatory functions. qPCR data showed significant increases in gene expression of pro-inflammatory chemokines and cytokines CXCL1, TGF-β and IL-1β by exogenous IL-1β. We have previously shown that the cardiomyocyte is a source of proinflammatory cytokines and chemokines, including IL-1β and IL-18. Our results suggest the possibility that IL-1β and IL-18 activate a pro-inflammatory positive feedback loop in cardiomyocytes which enhances and sustains cardiac inflammation, contributing to cardiac remodeling under stress conditions.

Introduction

Heart disease became the leading cause of death in the United States in the mid-20th century and, as of 2017, has remained the number one cause of death worldwide [1, 2]. Heart disease includes adverse conditions of the blood vessels, muscles and valves of the heart, and therefore, is commonly interchanged with the term cardiovascular disease [3]. Cardiovascular disease can progress to heart failure, which occurs when the heart can no longer effectively pump enough oxygenated blood throughout the body [4]. Like its antecedent cardiovascular disease, heart failure has a high prevalence globally. The American Heart Association predicts that the number of heart failure diagnoses will continue to increase in the next 10 years to affect more than 8 million people [5].

Hearts that fail undergo physical changes and remodeling such as muscle enlargement, excess fibrosis, dilated ventricles, and apoptosis. Changes to heart muscle initially act as compensatory mechanisms to maintain continual function after stress or injury, such as a heart attack or excess arterial plaque [6]. To make up for heart cells lost during injury, cardiomyocytes, the muscle cells of the heart, undergo hypertrophy and enlarge. This process allows the heart to continue pumping the necessary volume of oxygenated blood throughout the body [4,7]. Likewise, another class of cells in the heart known as fibroblasts create connective tissue to repair damage and provide additional structural support to compensate for injured or dead cells [8]. However, this remodeling is ultimately adverse because accumulated fibrosis stiffens the heart, making it difficult for the enlarged, dilated organ to successfully pump and heart failure develops.

Hearts that undergo stress or injury also experience inflammation, which is a key initiator of physical remodeling [6]. Many studies have been conducted on inflammation and remodeling

in ischemic heart disease, in which the flow of oxygenated blood to the heart through vessels is obstructed, causing cardiomyocytes to die from lack of oxygen [9]. Cardiac inflammation is initiated when dead heart cells release damage-associated molecular patterns (DAMPs) which activate pro-inflammatory signaling cascades in living cardiomyocytes. DAMPs bind toll-like receptors (TLRs) on the surface of the healthy cardiomyocytes and activate nuclear factor NF-κB [10]. NF-κB is a complex found in the cytoplasm and is composed of two subunits which translocate to the nucleus upon activation. In the nucleus, NF-κB subunits bind to DNA and act as transcription factors for inflammatory mediator genes such as Macrophage Chemoattractant Protein-1 (MCP-1/CCL2), Interleukins, and Tumor Necrosis Factors [11, 12, 13]. DAMPs and stress signals released from dead cardiomyocytes also activate the NLRP3 inflammasome, a protein complex that activates and releases inflammatory cytokines Interleukin-1 β (IL-1β) and Interleukin 18 (IL-18) [11, 14, 15]. Increased transcription and production of these cytokines and chemokines attracts immune cells, such as macrophages and T-cells, to the heart. These recruited immune cells are sources of reactive oxidant species (ROS), enzymes, and metabolites that have been shown to damage endothelial cells and contribute to cardiac remodeling [16].

An association between inflammation, cardiac remodeling, and heart failure also occurs in non-ischemic cardiac injuries caused by increased pressure or mechanical stress on the heart [17]. Congenital heart disease, diabetes, and hypertension are forms of non-ischemic insult in which cell death does not occur because the flow of oxygenated blood is not impeded [18]. In the absence of cell death and release of DAMPs, work from our laboratory has shown that inflammation is initiated by non-ischemic injury such as pressure overload due to a calcium imbalance in cardiomyocytes, which activates Calcium/calmodulin-dependent Kinase II (CaMKII). CaMKII in turn activates NF-κB leading to its nuclear translocation and induces

subsequent transcription and release of chemokines such as MCP-1/CCL2, which recruit immune cells to the heart. CaMKII also primes and activates the NLRP3 inflammasome leading to generation and release of the pro-inflammatory cytokines IL-1β and IL-18 [19, 20]. Cardiomyocytes in CaMKII deficient mice experience significantly less fibrosis and contractile dysfunction after long-term surgical aortic constriction, a model of hypertension [19, 20, 21]. Likewise, downregulating CaMKII leads to a decrease in production of immune cell attracting chemokines, pro-fibrotic mediators, and pro-fibrotic genes [20, 21]. Thus, CaMKII and its downstream signaling pathways contribute to cardiac remodeling and heart failure.

The cardiomyocyte has been implicated as a source of inflammation after ischemic and non-ischemic injury, and inflammation is a key initiator of remodeling [21]. However, it is not known when and how initial inflammation after injury transitions to a chronic immune response that causes physical changes in the heart to accumulate and progress to heart failure. Specifically, it is not known if cytokines generated through the NLRP3 inflammasome act back upon the cardiomyocyte to induce changes that contribute to fibrosis, ventricular dysfunction, apoptosis, and adverse remodeling [20, 21].

Indeed, there is only limited data regarding the response of cardiomyocytes to IL-1β and IL-18, both of which are generated as a result of NLRP3 activation in cardiac injury. After initial stress-induced inflammation, these cytokines may not only act to recruit immune cells and alter fibroblast or endothelial cell function, but may also positively feedback on the heart cells from which they were released to further sustain cardiac remodeling and chronic inflammation, facilitating decompensation and the transition to heart failure [22]. Understanding whether IL-1β and IL-18 can re-activate pro-inflammatory cascades and gene expression in cardiomyocytes could explain what happens in the heart after injury and the initial generation and release of

inflammatory cytokines. Thus, the goal of this project was to investigate whether there are signaling pathways by which IL-1β and IL-18 act upon cardiac myocytes to potentiate expression of inflammatory genes and processes that contribute to cardiac remodeling and the progression to heart failure. Elucidating these mechanisms may provide targets for early intervention or disease prevention.

Methods

Ventricular myocytes were isolated from neonatal rats (1-2 days old) and cultured in serum-containing medium for 24 hours. Cells were washed three times and cultured for an additional 24 hours in the absence of serum prior to stimulation with recombinant IL-1β or IL-18. To determine the optimal agonist concentration, neonatal ventricular myocytes (NRVMs) were treated with 1ng/mL and 10ng/mL recombinant IL-1β or 1ng/mL, 10ng/mL, and 100ng/mL recombinant IL-18. Cells were cultured in the presence of each agonist concentration for 30 minutes or 24 hours. 2 cell cultures were left untreated to serve as controls. Whole cell lysates were obtained using 1X RIPA buffer (20nM Tris-HCL pH 7.4, 150mM NaCl, 1mM Na_2EDTA, 1mM EGTA, 1% NP-40, 1% sodium deoxycholate, 2.5 mM sodium pyrophosphate, 1mM β-glycerophosphate, 1mM Na_3VO_4, 1μg/mL leupeptin) and the proteins were run on a 4-12% BisTris gel with a molecular-weight size marker. Gels were transferred onto a PVDF membrane and exposed to primary antibodies of well-known pro-inflammatory molecules for overnight at 4 degrees Celsius. GAPDH, α-actinin, and COX IV antibodies were used as loading controls. Following washing of primary antibodies and exposure to secondary antibodies (for 1 hour at room temperature), blots were washed for 30 minutes (10-minute wash, three times) and the Western blots were developed using SuperSignal™ West Femto Maxium Sensitivity Substrate. Western blots showed that a concentration of 1ng/mL IL-1β and 10ng/mL IL-18

consistently produced bands of sufficient intensity compared to a variety of other cytokine concentrations. Likewise, signal strength was optimal at 30 minutes compared to 24 hours after cytokine stimulation (Fig. S1 and S2). Thus, concentrations of 1ng/mL IL-1β and 10ng/mL IL-18 applied to cells at early time points around 30 minutes were determined to result in ideal phosphorylation of protein kinases and were used to treat NRVMs in this study.

To investigate the downstream signaling pathways by which IL-1β and IL-18 act upon cardiac myocytes, NRVMs were harvested 10 minutes, 30 minutes, 60 minutes, or 24 hours after agonist stimulation. 2 cell cultures were left untreated to serve as controls. NRVM nuclear and cytosolic fractions were separated using Digitonin (D) buffer, 1% JNK buffer, and RIPA buffer. Whole cell protein lysates or nuclear fractionations were harvested, protein concentrations were determined by BCA assay and run on Western blots. Blots were exposed to primary antibodies for molecules in the JAK/STAT, NF-κB, and MAP kinase pro-inflammatory pathways. Western blots were run with n=3-9 biological samples with only one sample containing a technical replicate. Western blots were quantified using AlphaView, background color was subtracted, and fold change was calculated over untreated control cells. Data with inconsistent loading controls were eliminated and any remaining outliers were determined with GraphPad PRISM8 software and removed. An unpaired one-tailed t-test was used to determine the statistical significance of increased protein phosphorylation in treated NRVMs compared to control cells. Error was calculated among biological samples and represented as standard error of the mean.

Quantitative polymerase chain reaction (qPCR) was used to examine the expression of inflammatory genes in NRVMs after treatment with IL-1β or IL-18. RNA was extracted from NRVMs 60 minutes, 4 hours, 8 hours, or 24 hours after stimulation with 1ng/mL IL-1β or 10ng/mL IL-18. The 260/280 purity and concentration of RNA was determined and cDNA was

generated from RNA using High-Capacity cDNA Reverse Transcription Kit. cDNA was diluted 1:25 and loaded into 96 well qPCR plates along with TaqMan Rat primers. GAPDH was used as a loading control. cT values were obtained and fold change was calculated over GAPDH control using double delta Ct analysis. An unpaired one-tailed t-test was used to determine the statistical significance of increased mRNA expression in treated NRVMs compared to untreated control cells. qPCR was run with n=4 or 5 biological samples with each sample containing 2-6 technical replicates. After data with inconsistent loading controls and outliers were removed, experimental values were shown as averages of n=2-5 per time point. Error was calculated among biological samples and represented as standard error of the mean.

Results

To determine whether IL-1β or IL-18 activate NF-κB, nuclear fractions were prepared from cells exposed to cytokine agonists for 60 minutes and levels of p65 subunit in nuclear fractions were examined by Western blot. Nuclear p65 shows an increasing trend in response to both IL-1β and IL-18 (Fig. 1A), although only IL-1β was determined to significantly increase the presence of NF-κB p65 subunit in the nucleus of NRVMs (Fig. 1B). This is important because p65 acts as a transcription factor for pro-inflammatory genes including chemokines and cytokines.

The MAP kinase pathway is another stress pathway that can parallel to the NF-κB pathway as well as regulate NF-κB activation in cardiomyocytes, as both are activated by similar stress responses and cytokines. To determine if IL-1β or IL-18 also activate molecules along this kinase cascade in cardiomyocytes, NRVM whole cell lysates were immunoblotted for the presence of two phosphorylated MAPKs, P38 and JNK. The data shown in Fig. 2B and 3B suggest that both P-JNK and P-P38 are significantly activated 10 minutes after exposure to IL-1β. The phospho-P38 Western blots indicate a possible decrease in P38 phosphorylation over

time after treatment with both IL-1β and IL-18 (Fig. 2A), whereas JNK phosphorylation potentially increases by IL-18 at 30 minutes of exposure before decreasing (Fig. 3A).

Since IL-1β influences both NF-κB and MAPK pathways, I went on to investigate the phosphorylation of TAK1, a MAPKKK that is upstream of NF-κB and MAPK and has been shown in non-cardiomyocytes to be downstream of an IL-1 receptor and regulate these two cascades. Western blots of NRVM whole cell lysates were exposed to P-TAK1 Thr184/187 primary antibodies to observe the effect of IL-1β or IL-18 on this enzyme. The greatest activation of TAK1 by IL-1β appears to occur at 24 hours with a 1.3-fold increase in phosphorylation (Fig. 4A). These findings suggest that IL-18 has a minimal effect on TAK1 activation as phosphorylation is only slightly increased at 30 minutes at 1.05-fold compared to the control (Fig. 4B).

In addition to NF-κB and MAPK, a third cascade involved in the regulation of pro-inflammatory genes is the JAK/STAT pathway. To determine whether IL-1β or IL-18 additionally affect JAK/STAT molecules, immunoblotting was performed on NRVM whole cell lysates treated up to 24 hours with cytokines. In conjunction with previous findings that IL-1β activates molecules along pro-inflammatory signaling pathways, STAT3 is significantly phosphorylated by IL-1β treatment after 60 minutes and activation continues to increase at 24 hours after exposure (Fig. 5A and B). This suggests that STAT3 is phosphorylated by JAK and activated in response to IL-1β in cardiomyocytes.

Previous studies have implicated the role of CaMKII in mediating NF-κB nuclear translocation and NLRP3 inflammasome activation to induce transcription and release of pro-inflammatory chemokines and cytokines. To test the hypothesis that IL-1β or IL-18 stimulate a second wave of CaMKII pro-inflammatory cascades and gene expression in cardiomyocytes,

CaMKII activation was measured by examining increases in CaMKII auto-phosphorylation, a marker for its activation, using Western blots. The data suggest that IL-1β has a steady effect on CaMKII for the first hour until a potential increase in phosphorylation at 24 hours (Fig. 6A and B). IL-18 has a potentially consistent impact on CaMKII as phosphorylation remains at a similar level until 30 minutes when it decreases only 0.5-fold from the control (Fig 6A). However, the P-CaMKII primary antibody is inconsistent and it can be difficult to distinguish the correct bands on Western blots. Therefore, phosphorylation of phospholamban (PLB) was also measured because PLB is a substrate for CaMKII and can provide an additional indication of CaMKII activation. PLB responds to IL-1β stimulation with the maximum phosphorylation at a 1.3-fold increase from the control (Fig. 7A and B). IL-18 initiates similar trends in both P-CaMKII and P-PLB with a likely increase in P-PLB at 10 minutes (Fig. 6 and 7A). These differences in the kinetics of the effects of IL-1β on P-CaMKII and P-PLB may be attributed to the irregular efficacy of the P-CaMKII primary antibody.

In order to evaluate whether IL-1β or IL-18 activate signaling pathways in adult cardiomyocytes as observed in NRVMs, cardiomyocytes were isolated from adult mice and treated with 1ng/mL IL-1β for 10 minutes. Whole cell lysates were immunoblotted and probed for changes in P-TAK1 and P-CaMKII. Only a small increase in P-TAK1 was observed. On the other hand, P-CaMKII appears to be greatly augmented by IL-1β with phosphorylation increased 1.8-fold compared to the control (Fig. 8).

I showed by Western blots that cardiomyocytes respond to IL-1β and IL-18 by initiating signaling pathways. These signals can lead to transcriptional activation of genes for cytokines and chemokines, small proteins that are secreted by the cardiomyocyte and are involved in cell-to-cell communication. Since activation of these aforementioned pathways can lead to

transcriptional regulation of pro-inflammatory genes, I investigated whether canonical pro-inflammatory genes, that could contribute to a propagated inflammatory response in cardiomyocytes, were upregulated. qPCR was used to assess if IL-1β and IL-18 treatment results in transcriptional upregulation of cytokine gene expression. Since gene transcription occurs after phosphorylation events, RNA was isolated from NRVMs at longer time points of 60 minutes, 4 hours, 8 hours and 24 hours after IL-1β and IL-18 treatment.

TGF-β is a cytokine shown to be involved in cardiac fibrosis, apoptosis, and hypertrophy. TGF-β mRNA was significantly upregulated by 1ng/mL IL-1β 4 hours after exposure (Fig. 9B). While IL-1β lead to a peak in TFG-β mRNA expression at 4 hours, IL-18 likely has its greatest effect at 60 minutes after treatment, with 8 hours being the lowest point in TGF-β mRNA expression (Fig. 9A).

Cytokines TNFα and IL-6 are both regulated by NF-κB activation. TNFα has been implicated in cardiomyocyte necrosis and apoptosis and increased levels have been seen in patients with various forms of heart disease. Similarly, research has shown the involvement of IL-6 in apoptosis of heart cells and cardiac dysfunction. The quantification of TNFα and IL-6 mRNA expression suggested that IL-1β upregulates these genes 60 minutes after treatment (Fig. 10 and 11). However, no statistical significance was determined for TNFα or IL-6 as N=1 and N=2, respectively.

Next, I examined the upregulation of chemokine mRNA in response to IL-1β and IL-18 because an increase in chemokine mRNA means the possibility of the immune response being enhanced by a greater infiltration of immune cells to the heart. One subfamily of chemokines that recruit leukocytes, monocytes, and neutrophils to the heart contain the nomenclature “C-X-C” due to their amino acid structure.

To investigate the effects of IL-1β and IL-18 on cardiomyocyte expression of chemokine CXCL1 mRNA, NRVMs were treated with 1ng/mL IL-1β and 10ng/mL IL-18 at multiple time points up to 24 hours. One hour after treatment with recombinant IL-1β, expression of chemokine CXCL1 mRNA increased 100-fold compared to the CXCL1 mRNA in untreated control cells (Fig. 12A). This increase in gene expression was statistically significant (Fig. 12B). CXCL2 mRNA levels following IL-1β and IL-18 treatment increased in a very similar pattern to those of CXCL1. The greatest increase in CXCL2 mRNA was stimulated by 60 minutes of IL-1β treatment. IL-18 did not cause increases in mRNA expression for either chemokine when compared to effects of IL-1β. The fold change in CXCL1 and CXCL2 mRNA remained at a consistent level in response to IL-18 (Fig. 12A and 13A, B). Likewise, the effect of IL-18 on CXCL9 mRNA levels remain seemingly unchanging with a slight decrease between 4 and 8 hours (Fig. 14A). Meanwhile, IL-1β may upregulate CXCL9 at earlier timepoints as 4 hours after treatment, the chemokine mRNA expression increased 5-fold compared to the control (Fig. 14A and B). Lastly, IL-1β and IL-18 produce visibly alike trends in the transcription of CXCL10 mRNA. The fold increase in CXCL10 is the greatest at 60 minutes and lowest at 8 hours after treatment with each cytokine (Fig. 15A and B).

Another subfamily of chemokines follows the nomenclature C-C for the structure of their cysteine residues. MCP-1/CCL2 and CCL3 both function to recruit macrophages to the heart. The results indicate that MCP-1/CCL2 mRNA is upregulated by IL-1β stimulation with a 5 to 10-fold increase in mRNA expression. On the other hand, NRVMs treated with IL-18 express mRNA at levels similar to the control (Fig. 16). Notably, IL-18 potentially has a greater effect than IL-1β on the transcription of CCL3 with mRNA levels increased over 2-fold compared to the control at 4 hours after treatment (Fig. 17A). 4 hours is also when IL-1β

appeared to stimulate the greatest increase in CCL3 mRNA expression at approximately 1.5-fold compared to untreated control cells (Fig. 17A and B). No statistical significance was determined for MCP-1/CCL2 IL-1β 1ng/mL 60 minutes or CCL3 IL-18 10ng/mL 4 hours because N=2 for each time point.

Lastly, to provide further evidence that IL-1β and IL-18 can re-activate the NLRP3 inflammasome to produce pro-inflammatory cytokines, qPCR was used to assess gene expression of endogenous NLRP3, IL-1β, and IL-18 in NRVMs. Analysis of mRNA expression revealed that both IL-1β and IL-18 influence NLRP3 transcription with IL-18 increasing NLRP3 mRNA to similar levels at 4 and 24 hours (Fig. 18A and 18B). Exogenous IL-1β significantly upregulated mRNA of endogenous IL-1β 1.7-fold, 4 hours after exposure to the cytokine (Fig. 19B). Transcription of endogenous IL-1β mRNA in cardiomyocytes also responded to exposure to IL-18 but mRNA levels remained less than the control except for the 4-hour time point at 1.3-fold (Fig. 19A and 19B). No statistical analysis was performed for NLRP3 or IL-1β 8 hours after IL-1β treatment because N=2. Likewise, there was a nearly 2-fold increase in endogenous IL-18 mRNA levels in after to both exogenous IL-1β and IL-18 after 1 hour and 4 hours of treatment, respectively (Fig. 20A and 10B).

Discussion

When the heart experiences stress or injury, it undergoes hypertrophy, fibrosis, and other compensatory mechanisms to maintain homeostasis and continual function of the heart. However, protective cardiac remodeling eventually becomes adverse and the heart progresses to failure. Inflammation is a key component that contributes to this transition from adaptive to maladaptive hypertrophy and heart failure. My data indicate that IL-1β and IL-18, cytokines produced during the initial inflammatory response that accompanies the adaptive phase

of the stress response, have a direct effect on cardiomyocytes that could contribute to prolonged inflammation and perpetuate cardiac remodeling past the protective state. More specifically, IL-1β and IL-18 re-activate pro-inflammatory cascades and expression of genes involved in sustained immune cell recruitment and adverse cardiac remodeling.

The results of this study demonstrate that both kinase cascades and consequent protein phosphorylation pathways, as well as pro-inflammatory gene expression are triggered in cardiomyocytes by IL-1β or IL-18. Nearly all of my data indicates that IL-1β has greater and more significant direct effects on inflammatory signaling and genes in cardiomyocytes than IL-18. Quantitative analysis indicates that IL-1β significantly activates molecules along the NF-κB, MAPK, and JAK/STAT kinase cascades involved in cardiac inflammation. Specifically, IL-1β induces the translocation of the NF-κB p65 subunit to the nucleus, phosphorylates P38 and JNK, and activates STAT-3 (Fig. 1, 2, 3, 5). Likewise, IL-1β increases the transcription of inflammatory cytokines, chemokines, and NLRP3 inflammasome products. The mRNA of TGF-β, CXCL-1, and IL-1β are significantly upregulated in NRVMs after 4 hours of exposure to IL-1β (Fig. 9, 12, 19).

It is notable that IL-1β and IL-18 can affect and activate the NF-κB, MAPK, and JAK/STAT signaling cascades in NRVMs because this is evidence that they could perpetuate the immune response through effects on the myocyte. Positive feedback is created as the released cytokines and chemokines re-initiate signaling pathways and enhance immune cell recruitment to the heart, respectively. Once the NF-κB p65 subunit translocates to the NRVM nucleus, p65 acts as a transcription factor for pro-inflammatory genes, such as TNFα, IL-6, MCP-1/CCL2. The data from Figures 1, 10, 11, and 16 support this sequence of events in response to 1ng/mL IL-1β. The NF-κB-mediated upregulation of TNFα is important because TNFα is a cytokine that

binds to receptors on the cardiomyocyte surface and phosphorylates IKB, a protein that inhibits NF-κB [23]. Thus, TNFα re-activates the NF-κB pathway by removing its inhibitor and allows for enhanced expression and production of C-C chemokines such as MCP-1/CCL2, a key protein in the recruitment of macrophages to the heart. NF-κB also upregulates IL-6, a cytokine which activates the JAK/STAT pathway. Upon activation, STAT3 functions as a transcription factor for genes involved in tissue remodeling and cell growth [24]. Interestingly, IL-1β also phosphorylates the MAP kinase P38, which has been implicated as a regulator of NF-κB (Fig. 2) [25]. Therefore, IL-1β may serve to further connect these two pro-inflammatory pathways. TAK1 is a similar link between MAP kinases and NF-κB and TAK1 is activated when cytokine TGF- β binds its receptor on the cell surface [26]. This is another example of a reaction that positively contributes to pro-inflammatory signaling as TGF-β is involved in attracting monocytes and activating fibroblasts [27]. Thus, elevated levels of TGF-β in the heart, as a result of IL-1β activated pathways, can further enhance cardiac remodeling.

In the heart, NF-κB signaling is critical to activation of the NLRP3 inflammasome, which ultimately leads to the production and release of cytokines IL-1β and IL-18. NF-κB regulates the transcription of inactive precursors pro-IL-1β and pro-IL-18 as well as the transcription of NLRP3 [28]. When the NLRP3 complex is activated it cleaves Caspase-1, which in turn cleaves pro IL-1β and pro-IL-18 into their biologically active forms to be released [19]. Interestingly, the qPCR data from this study demonstrates that the NLRP3 inflammasome product IL-1β is significantly upregulated in NRVMs by treatment with exogenous IL-1β (Fig. 19). This suggests that endogenous, NRVM produced IL-1β will continue to be released past the initial inflammatory response. Thus, cardiac inflammation can be sustained by IL-1β at multiple stages of phosphorylation and gene expression. It remains to be determined if upregulation of the IL-1β

precursor in response to exogenous IL-1β is mediated through P-CaMKII activation of NF-κB or activation of NF-κB or through an alternate pathway.

IL-1β and IL-18 are both members of the Interleukin-1 (IL-1) cytokine family [22]. Many studies have established the role of IL-1 in the inflammatory response and have used blockade of IL-1 receptors to attenuate cardiac remodeling after injury. IL-1 antagonists, such as the drug anakinra, have been used to block IL-1 action in patients with acute myocardial infarction (AMI). More specifically, anakinra inhibits the binding of IL-1 to receptors located on immune cells from which the cytokine was released. The data from this clinical trial indicated that inhibiting IL-1 was also successful in reducing left ventricular remodeling post AMI [29]. The conceptual basis for trials such as this is to target IL-1 produced by inflammatory cells, considered the primary site of NLRP3 inflammasome activation, rather than cardiomyocytes. Thus, the data in the present study is important because it suggests that the cardiomyocyte may be a target of IL-1 action, as well as production, and thus a mediator of prolonged inflammation.

Although IL-1β and IL-18 belong to the same cytokine family, each relies on its own subset of IL-1 receptor. IL-1β binds to IL-1 type 1 receptors (IL-1R1) while IL-18 is the ligand for the IL-18 receptor (IL-18R). Rabbit models have been used to study the expression of IL-18R in hypertrophied and failing hearts and have found that baseline IL-18R levels are low until a heart fails, in which IL-18R expression increases [30]. Expression of IL-1R1 depends on cell type, however, IL-1R1 is responsible for the phosphorylation and transcriptional regulatory functions of IL-1. Furthermore, the downstream effects of IL-1 binding to IL-1R1 can be increased despite low receptor expression [22]. The varying expression and functions of IL-1 receptors may explain the differential effects of IL-1β and IL-18 in triggering kinase cascades

and regulation transcription of pro-inflammatory genes in NRVMs. This study examined the effects of IL-1β and IL-18 in cells isolated from healthy, unstressed neonatal rats. It may be of interest to compare IL-1R and IL-18R expression levels in NRVMs under basal and stress conditions. The important question that needs to be addressed, is whether IL-1β and IL-18 regulate expression of these receptors, providing a feed-forward loop between the inflammasome and its products, as suggested by this study.

Ultimately, the results of this study elucidate mechanisms by which endogenous IL-18 and IL-1β can feedback on the cardiomyocyte to enhance inflammation and contribute to our earlier studies suggesting that the myocyte itself may be a target for early intervention and prevention of cardiac inflammation that contributes to heart disease after injury. Further research is needed to identify the direct effects of IL-1β and IL-18 on pro-inflammatory pathways in adult ventricular myocytes, endothelial cells, and fibroblasts in the heart. Studies using cardiomyocyte specific deletion of NLRP3 and its products are underway to determine the extent to which cardiomyocytes contribute to the generation of IL-1β and IL-18 that perpetuates inflammation by enhancing immune cell recruitment to the heart.

FIGURES

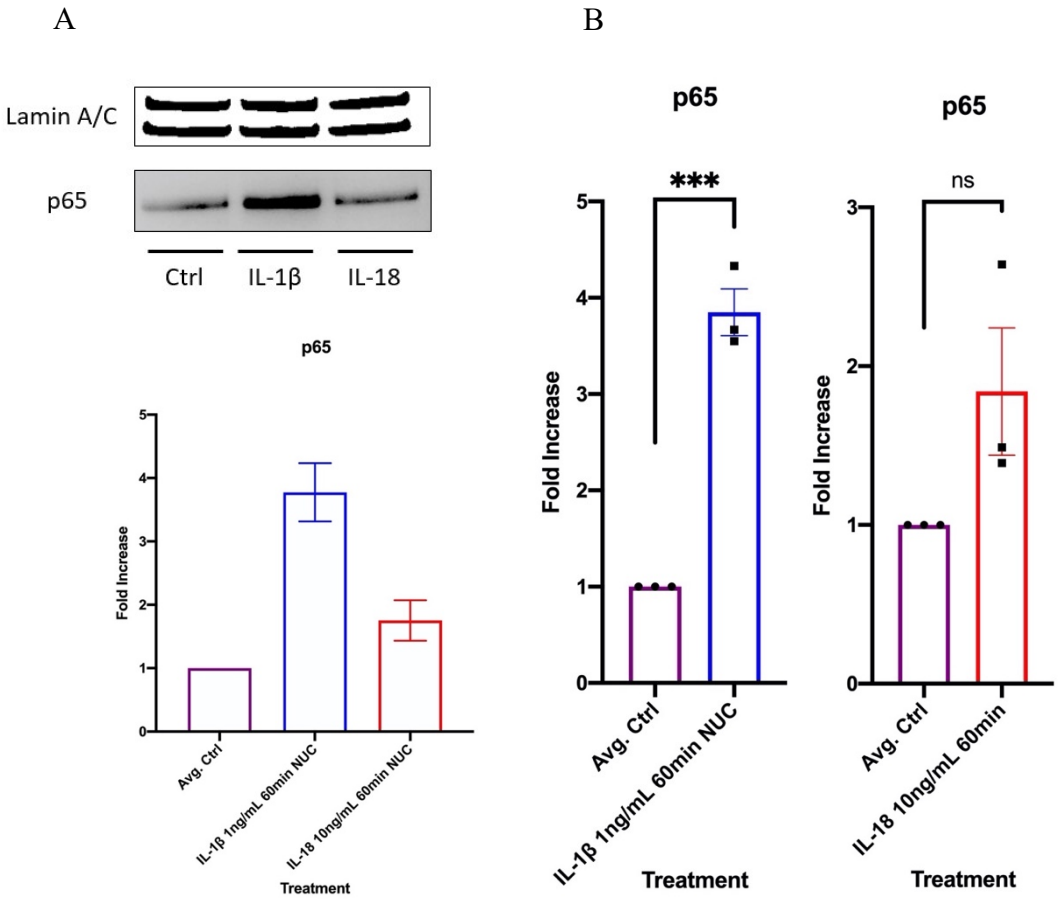


Figure 1: Presence of total NF-κB p65 subunit in the nucleus of NRVMs increases in response to IL-1β and IL-18. A. Western blot and quantification of p65 determined by immunoblotting NRVMs isolated nuclear fractionations treated with 1ng/ml IL-1β and 10ng/mL IL-18 60 minutes before harvesting. Lamin A/C was used as nuclear control. N=3 with 1 sample containing a technical replicate. Fold change normalized over untreated control NRVMs. **B.** Unpaired one-tailed t-test of IL-1β 1ng/mL 60 minutes and IL-18 10ng/mL 60 minutes compared to untreated control cells. Significance depicted by *** $p \leq 0.001$. Error represented by ± SEM. NF-κB indicates nuclear factor kappa-light-chain-enhancer of activated B cells; NRVM, neonatal rat ventricular myocytes; IL-1β, interleukin 1 beta; IL-18, interleukin 18.

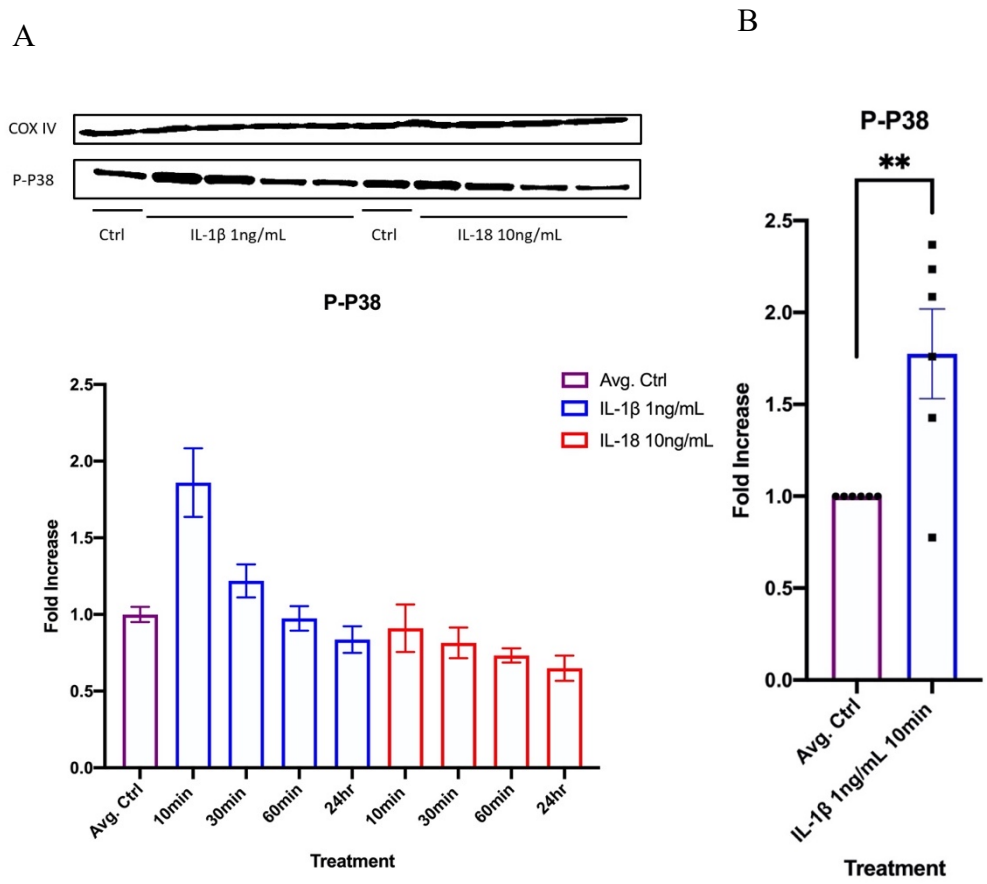


Figure 2: Effect of IL-1β and IL-18 on the phosphorylation of P38 MAPK in NRVMs. A. Western blot and quantification of P38 phosphorylation determined by immunoblotting NRVM whole cell lysates treated with 1ng/mL IL-1β and 10ng/mL IL-18 at 10 minutes, 30 minutes, 60 minutes, and 24 hours before harvesting. COX IV was used as loading control. N=6 with 1 sample containing a technical replicate. Fold change normalized over untreated control NRVMs. **B.** Unpaired one-tailed t-test of IL-1β 1ng/mL 10 minutes compared to untreated control cells. Significance depicted by ** $p \leq 0.01$. Error represented by ± SEM. IL-1β indicates interleukin 1 beta; IL-18, interleukin 18; MAPK, mitogen-activated protein kinase; NRVMS, neonatal rat ventricular myocytes; COX IV, cytochrome c oxidase subunit 4.

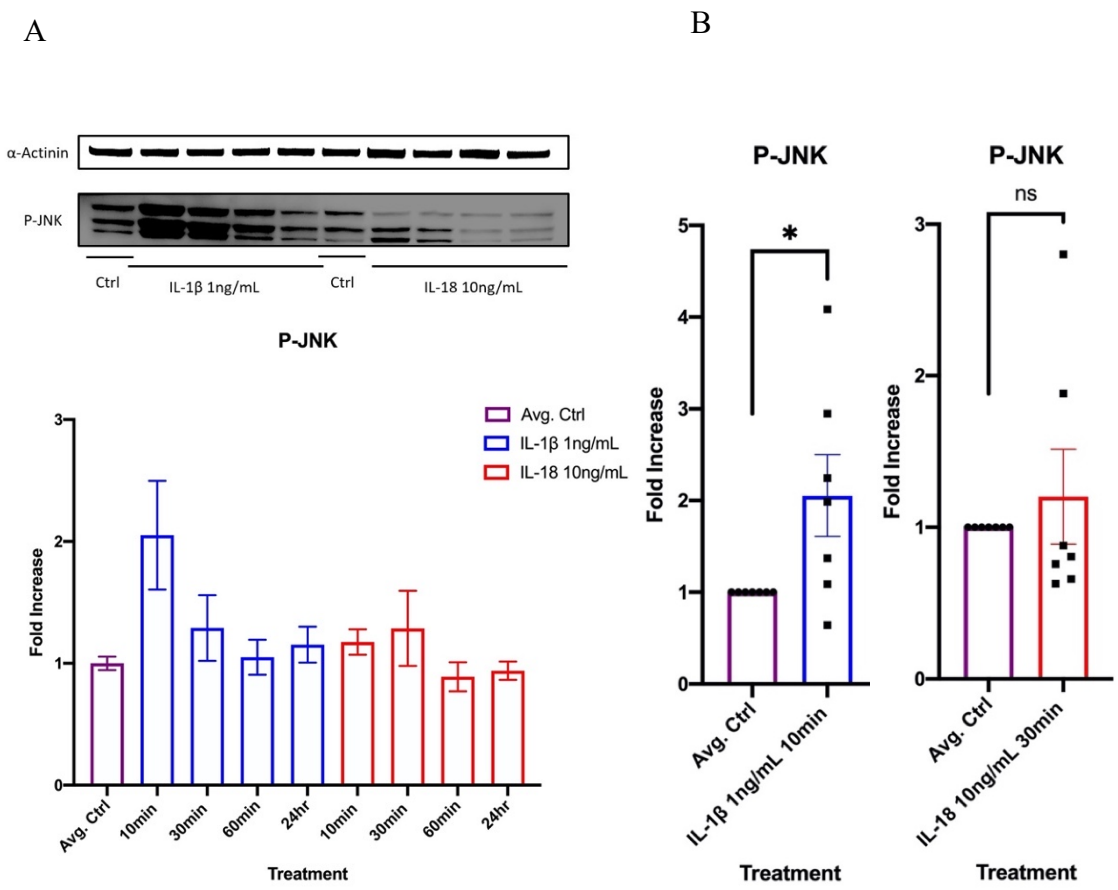


Figure 3: Effect of IL-1β and IL-18 on the phosphorylation of JNK MAPK in NRVMs. A. Western blot and quantification of JNK phosphorylation determined by immunoblotting NRVM whole cell lysates treated with 1ng/mL IL-1β and 10ng/mL IL-18 at 10 minutes, 30 minutes, 60 minutes, and 24 hours before harvesting. α-Actinin was used as loading control. Fold change normalized over untreated control NRVMs N=7. **B.** Unpaired one-tailed t-test of IL-1β 1ng/mL 10 minutes and IL-18 10ng/mL 30 minutes compared to untreated control cells. Significance depicted by ns $p>0.05$. Error represented by ± SEM. IL-1β indicates interleukin 1 beta; IL-18, interleukin 18; JNK, c-Jun N-terminal kinase; MAPK, mitogen-activated protein kinase; NRVM, neonatal rat ventricular myocytes.

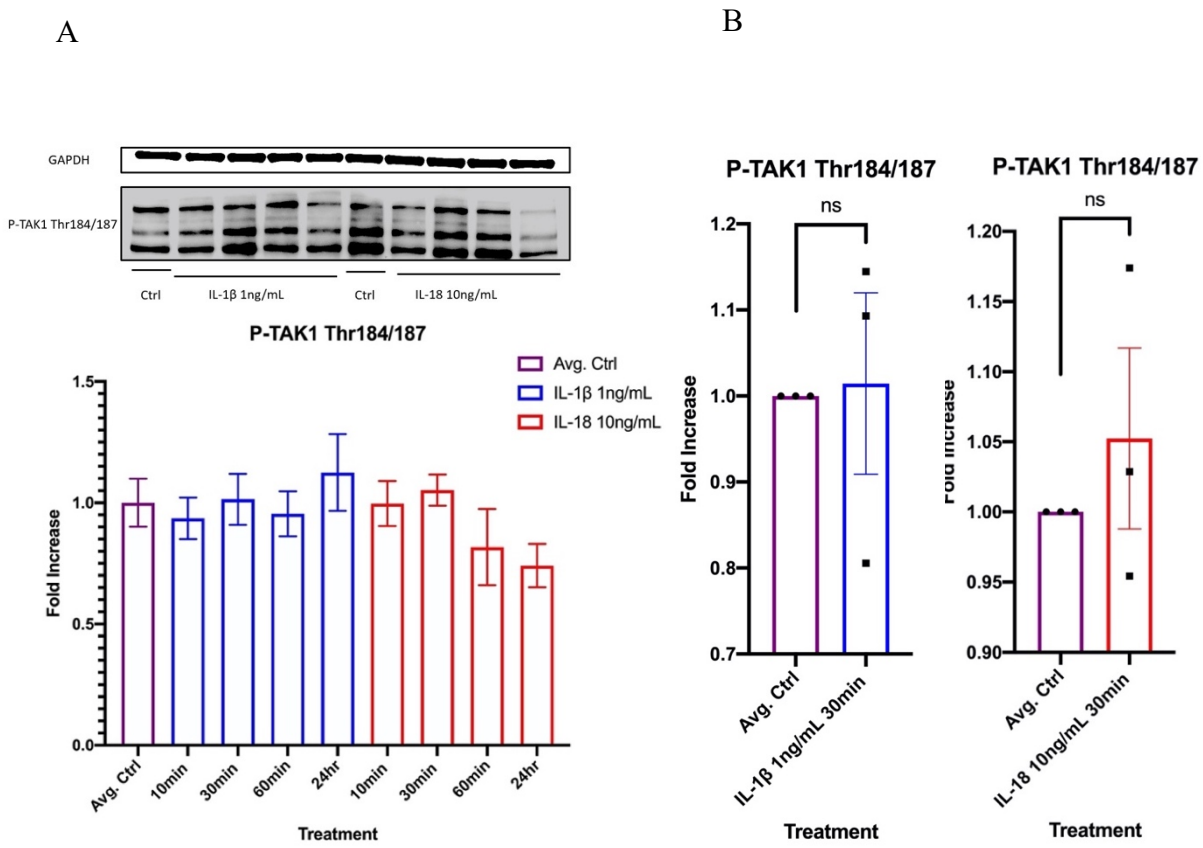


Figure 4: Effect of IL-1β and IL-18 on the phosphorylation of MAPKKK TAK1 at Thr184/187 in NRVMs. A. Western blot and quantification of TAK1 phosphorylation at Thr184/187 determined by immunoblotting NRVM whole cell lysates treated with 1ng/mL IL-1β and 10ng/mL IL-18 at 10 minutes, 30 minutes, 60 minutes, and 24 hours before harvesting. GAPDH was used as loading control. Fold change normalized over untreated control NRVMs N=3. **B.** Unpaired one-tailed t-test of significance IL-1β 1ng/mL 30 minutes compared to untreated control cells. Significance depicted by ns $p>0.05$. Error represented by ± SEM. IL-1β indicates interleukin 1 beta; IL-18, interleukin 18; MAPKKK, mitogen-activated protein kinase kinase kinase; TAK1, transforming growth factor- β-activated kinase 1; NRVM, neonatal rat ventricular myocyte; GAPDH, glyceraldehyde-phosphate dehydrogenase.

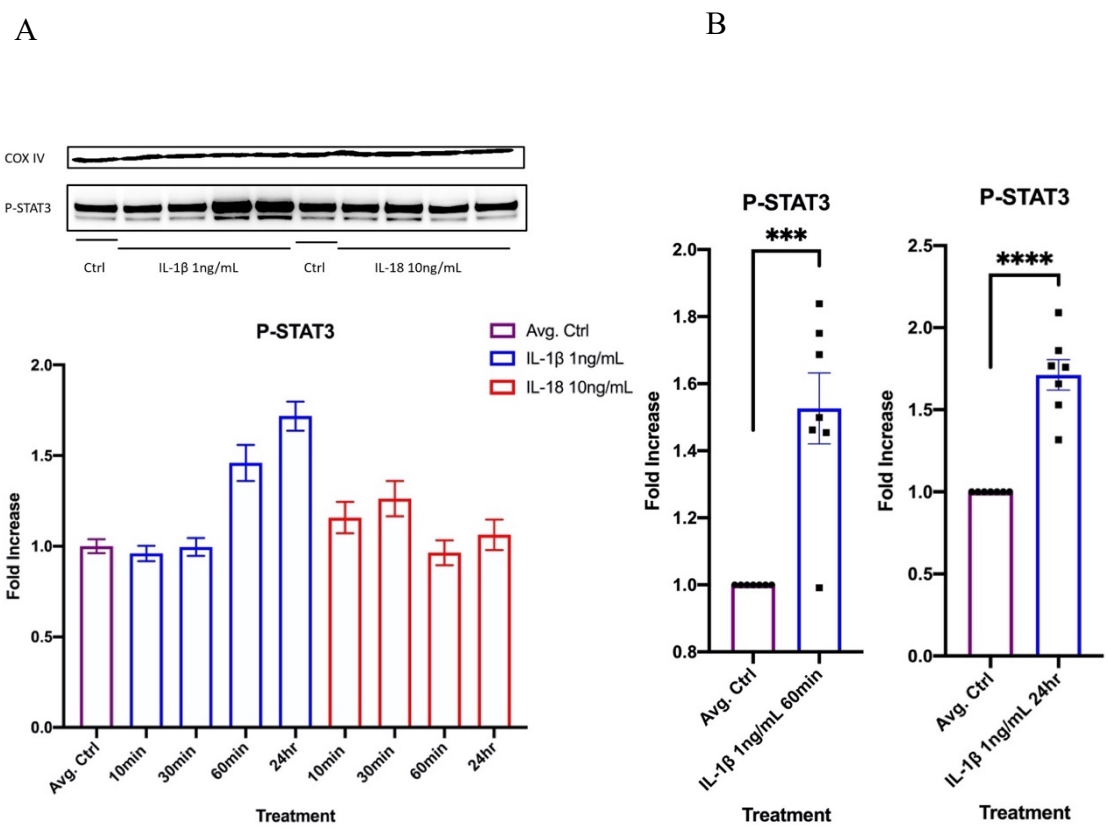


Figure 5: Effect of IL-1β and IL-18 on the phosphorylation of STAT3 in NRVMs. A. Western blot and quantification of STAT3 phosphorylation determined by immunoblotting NRVM whole cell lysates treated with 1ng/mL IL-1β and 10ng/mL IL-18 at 10 minutes, 30 minutes, 60 minutes, and 24 hours before harvesting. COX IV was used as loading control. N=7 with 1 sample containing a technical replicate. Fold change normalized over untreated control NRVMs. **B.** Unpaired one-tailed t-test of IL-1β 1ng/mL 60 minutes and 24 hours compared to untreated control cells. Significance depicted by *** $p \leq 0.001$ and **** $p \leq 0.0001$. Error represented by ± SEM. IL-1β indicates interleukin 1 beta; IL-18, interleukin 18; STAT3, signal transducer and activators of transcription 3; NRVM, neonatal rat ventricular myocyte; COX IV, cytochrome c oxidase subunit 4.

.

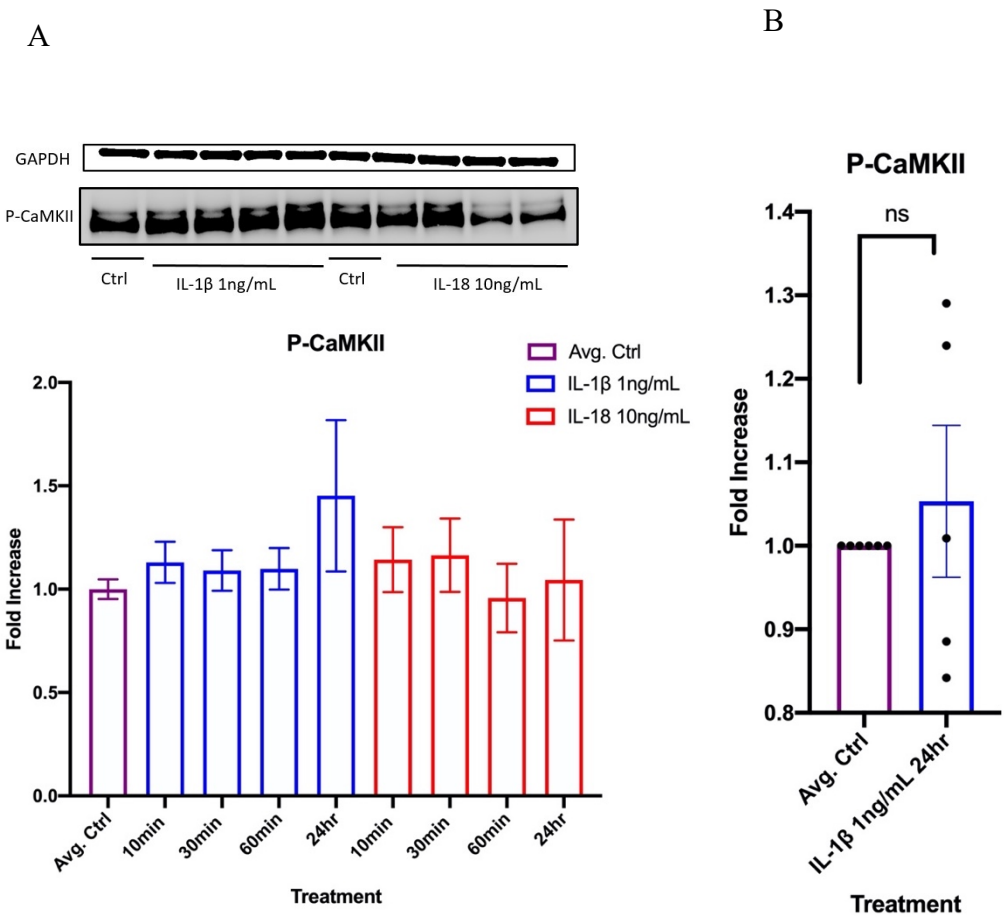


Figure 6: Effect of IL-1β and IL-18 on the phosphorylation of CaMKII in NRVMs. A. Western blot and quantification of CaMKII phosphorylation determined by immunoblotting NRVM whole cell lysates treated with 1ng/mL IL-1β and 10ng/mL IL-18 at 10 minutes, 30 minutes, 60 minutes, and 24 hours before harvesting. GAPDH was used as loading control. N=6 with 1 sample containing a technical replicate. Fold change normalized over untreated control NRVMs. **B.** Unpaired one-tailed t-test of IL-1β 1ng/mL 24 hours compared to untreated control cells. Significance depicted by ns $p>0.05$. Error represented by ± SEM. IL-1β indicates interleukin 1 beta; IL-18, interleukin 18; CaMKII, calmodulin-dependent protein kinase II; GAPDH, glyceraldehyde-phosphate dehydrogenase.

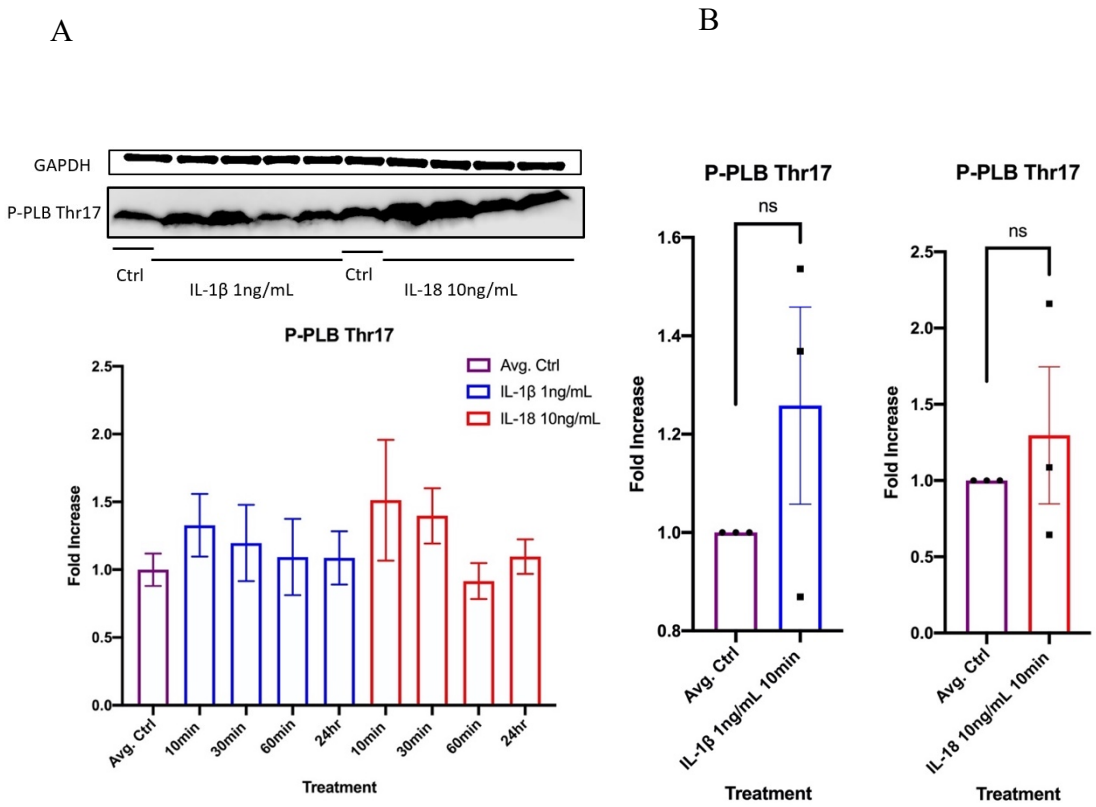


Figure 7: Effect of IL-1β and IL-18 on the phosphorylation of PLB at Thr17 in NRVMs. A. Western blot and quantification of PLB phosphorylation at Thr17 determined by immunoblotting NRVM whole cell lysates treated with 1ng/mL IL-1β and 10ng/mL IL-18 at 10 minutes, 30 minutes, 60 minutes, and 24 hours before harvesting. GAPDH was used as loading control. N=3 with 1 sample containing a technical replicate. Fold change normalized over untreated control NRVMs. **B.** Unpaired one-tailed t-test of IL-1β 1ng/mL 10 minutes and IL-18 ng/mL 10 minutes compared to untreated control cells. Significance depicted by ns $p>0.05$. Error represented by ± SEM. IL-1β indicates interleukin 1 beta; IL-18, interleukin 18; PLB, phospholamban; NRVM, neonatal rat ventricular myocyte; GAPDH, glyceraldehyde-phosphate dehydrogenase.

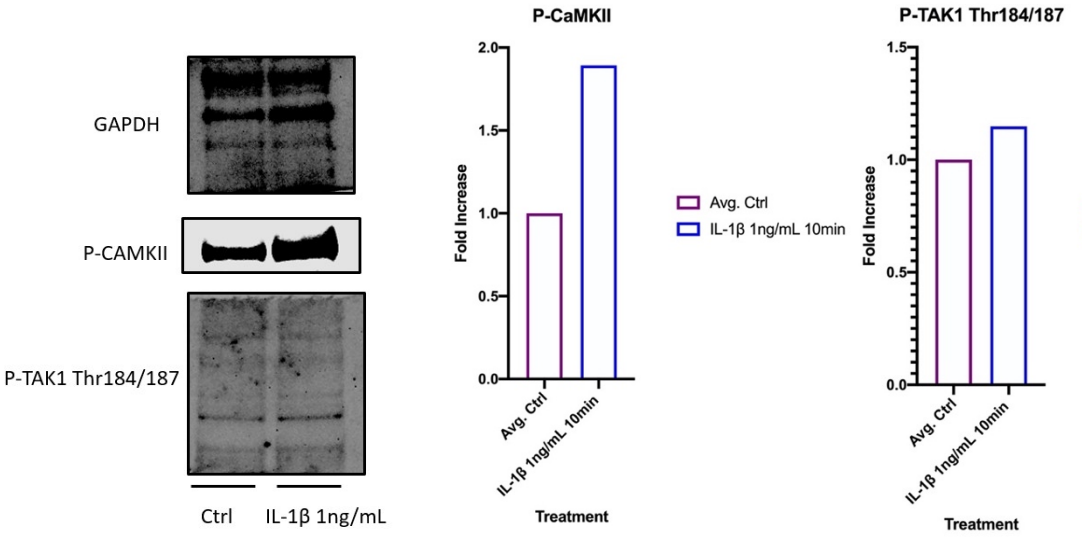


Figure 8: Effect of IL-1β on the phosphorylation of CaMKII and MAPKKK TAK1 at Thr184/187 in adult rat cardiomyocytes (CM). Phosphorylation of CaMKII and TAK1 at Thr184/187 determined by immunoblotting CM whole cell lysates treated with 1ng/mL IL-1β 10 minutes before harvesting. GAPDH was used as loading control. Fold change normalized over untreated control NRVMs N=1. IL-1β indicates interleukin 1 beta; MAPKKK, mitogen-activated protein kinase kinase kinase; TAK1, transforming growth factor- β-activated kinase 1; CaMKII, calmodulin-dependent protein kinase II; GAPDH, glyceraldehyde-phosphate dehydrogenase.

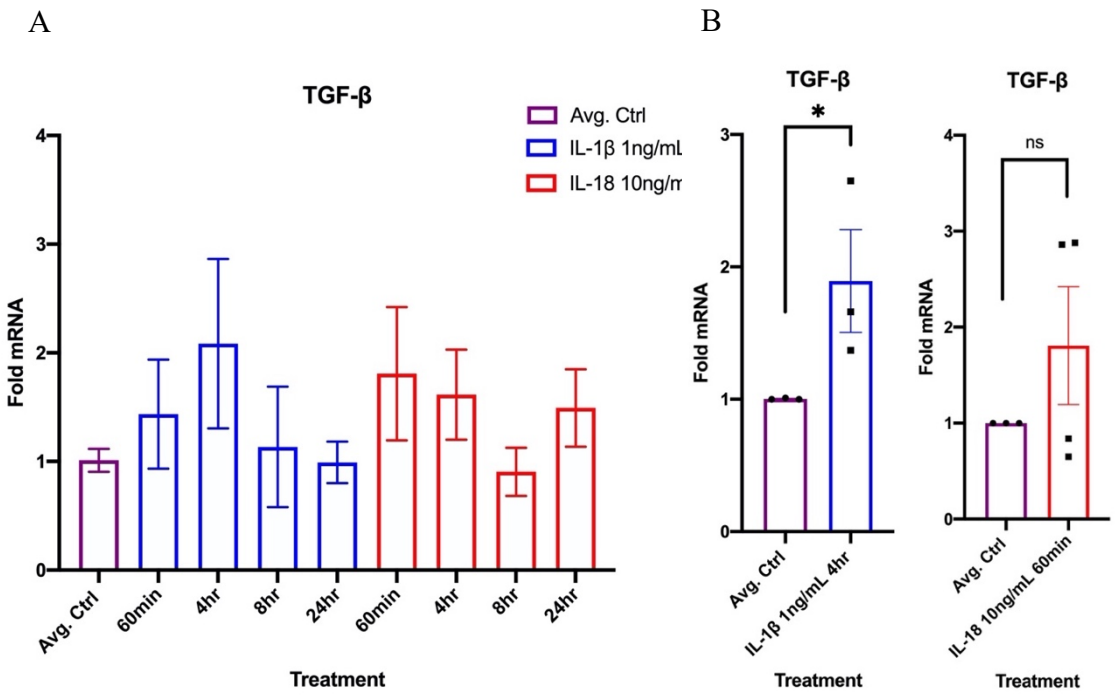


Figure 9: TGF-β mRNA expression in NRVMs in response to IL-1β and IL-18. A. NRVMs treated with 1ng/mL IL-1β and 10ng/mL IL-18 at 60 minutes, 4 hours, 8 hours and 24 hours before harvesting. TGF-β mRNA in whole cell lysates measured by qPCR, normalized over GPADH loading control and expressed as mRNA fold increase over untreated control cells. mRNA fold change averaged among N=5 with 2 samples containing 1 technical replicate each. **B.** Unpaired one-tailed t-test of IL-1β 1ng/mL 4 hours and IL-18 10ng/mL 60 minutes compared to untreated control cells. Significance depicted by * $p \leq 0.05$ and ns $p > 0.05$. Error represented by ± SEM. TGF- β indicates transforming growth factor beta; mRNA, messenger ribonucleic acid; NRVM, neonatal rat ventricular myocyte; IL-1β, interleukin 1 beta; IL-18, interleukin 18; qPCR, quantitative polymerase chain reaction; GAPDH, glyceraldehyde-phosphate dehydrogenase.

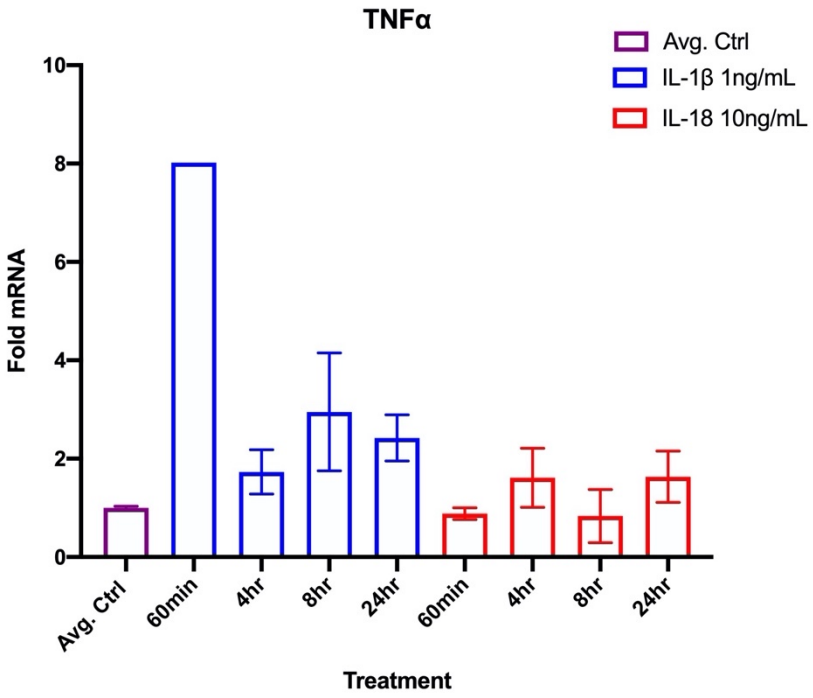


Figure 10: TNFα mRNA expression in NRVMs in response to IL-1β and IL-18. NRVMs treated with 1ng/mL IL-1β and 10ng/mL IL-18 at 60 minutes, 4 hours, 8 hours and 24 hours before harvesting. TNFα mRNA in whole cell lysates measured by qPCR, normalized over GPADH loading control and expressed as mRNA fold increase over untreated control cells. mRNA fold change averaged among N=1-4 with 3 samples containing 1 technical replicate each. Error represented by ± SEM. TNFα indicates tumor necrosis factor alpha; mRNA, messenger ribonucleic acid; NRVM, neonatal rat ventricular myocyte; IL-1β, interleukin 1 beta; IL-18, interleukin 18; qPCR, quantitative polymerase chain reaction; GAPDH, glyceraldehyde-phosphate dehydrogenase.

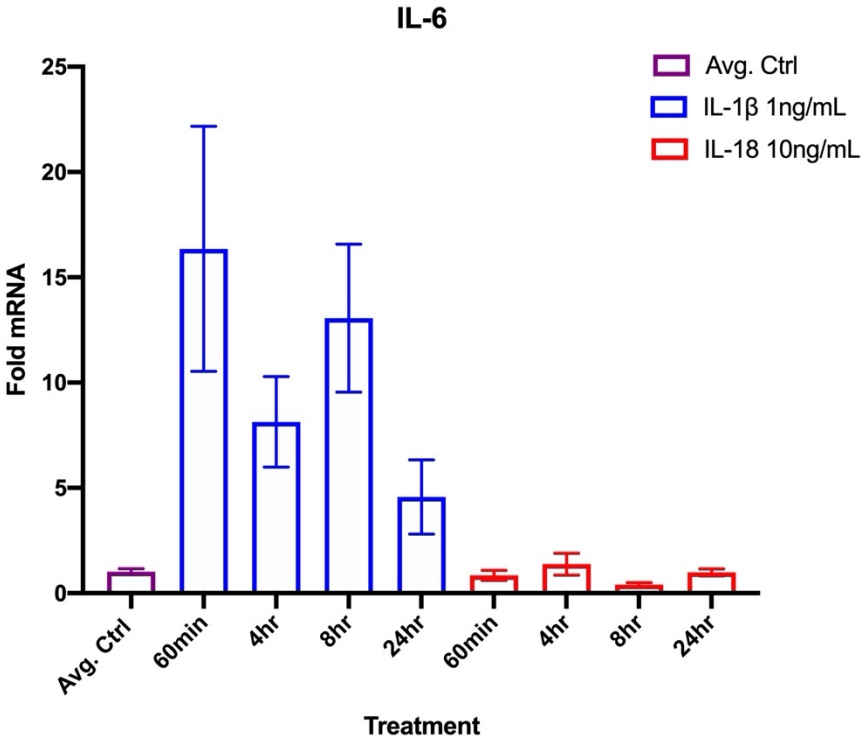


Figure 11: IL-6 mRNA expression in NRVMs in response to IL-1β and IL-18. NRVMs treated with 1ng/mL IL-1β and 10ng/mL IL-18 at 60 minutes, 4 hours, 8 hours and 24 hours before harvesting. IL-6 mRNA in whole cell lysates measured by qPCR, normalized over GPADH loading control and expressed as mRNA fold increase over untreated control cells. mRNA fold change averaged among N=2-4 with 3 samples containing 1 technical replicate each. Error represented by ± SEM. IL-6 indicates interleukin 6; mRNA, messenger ribonucleic acid; NRVM, neonatal rat ventricular myocyte; IL-1β, interleukin 1 beta; IL-18, interleukin 18; qPCR, quantitative polymerase chain reaction; GAPDH, glyceraldehyde-phosphate dehydrogenase.

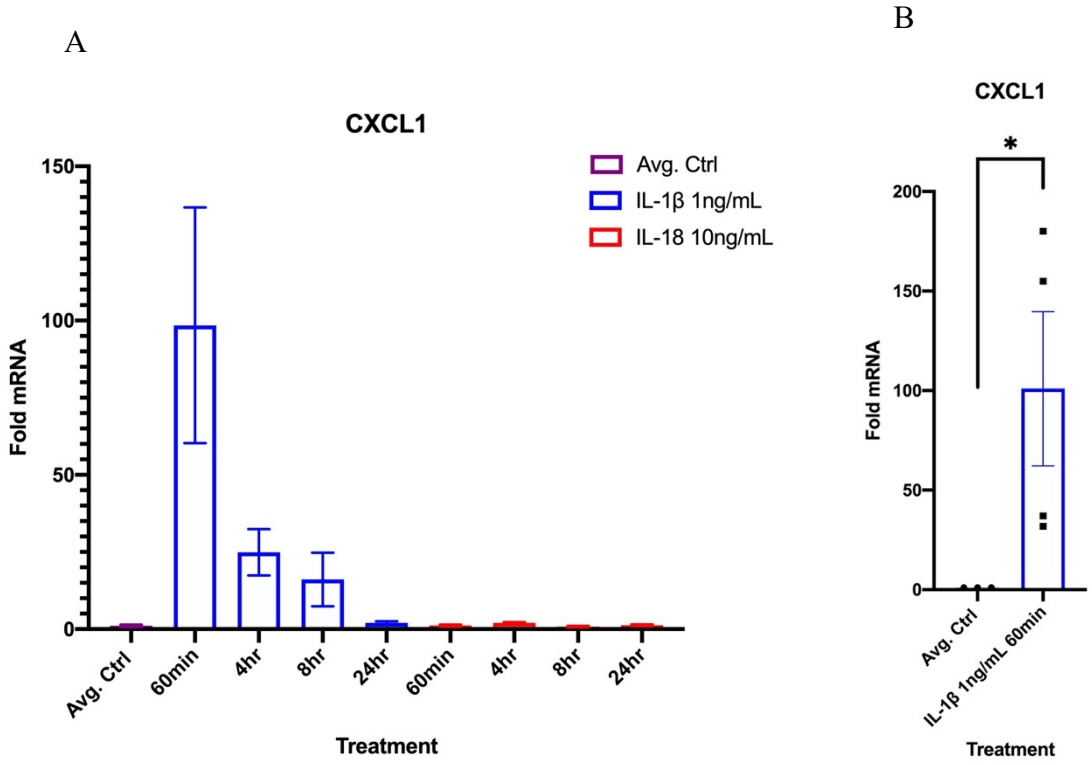


Figure 12: NRVM CXCL1 mRNA expression increases in response to IL-1β. A. NRVMs treated with 1ng/mL IL-1β and 10ng/mL IL-18 at 60 minutes, 4 hours, 8 hours and 24 hours before harvesting. CXCL1 mRNA in whole cell lysates measured by qPCR, normalized over GPADH loading control and expressed as mRNA fold increase over untreated control cells. mRNA fold change averaged among N=5. **B.** Unpaired one-tailed t-test of IL-1β 1ng/mL 60 minutes compared to untreated control cells. Significance depicted by * $p \leq 0.05$. Error represented by ± SEM. CXCL1 indicates chemokine (C-X-C motif) ligand 1; mRNA, messenger ribonucleic acid; NRVM, neonatal rat ventricular myocyte; IL-1β, interleukin 1 beta; IL-18, interleukin 18; qPCR, quantitative polymerase chain reaction; GAPDH, glyceraldehyde-phosphate dehydrogenase.

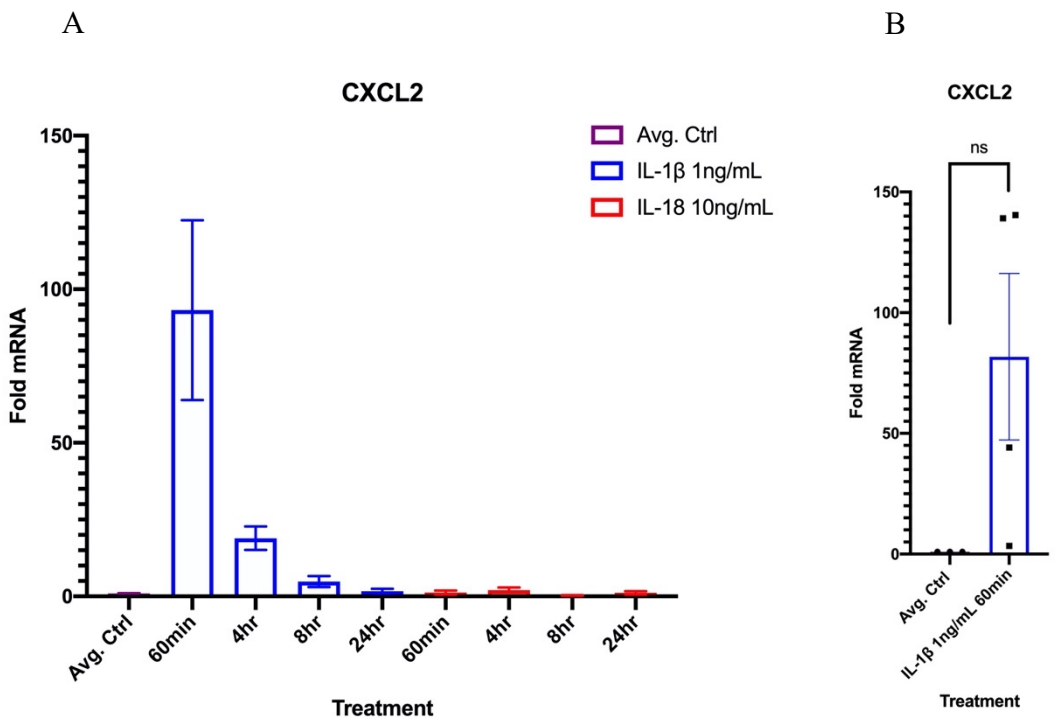


Figure 13: CXCL2 mRNA expression in NRVMs in response to IL-1β and IL-18. A. NRVMs treated with 1ng/mL IL-1β and 10ng/mL IL-18 at 60 minutes, 4 hours, 8 hours and 24 hours before harvesting. CXCL2 mRNA in whole cell lysates measured by qPCR, normalized over GPADH loading control and expressed as mRNA fold increase over untreated control cells. mRNA fold change averaged among N=4-5 with 3 samples containing 1 technical replicate each. **B.** Unpaired one-tailed t-test of IL-1β 1ng/mL 60 minutes compared to untreated control cells. Significance depicted by ns $p>0.05$. Error represented by ± SEM. CXCL2 indicates chemokine (C-X-C motif) ligand 2; mRNA, messenger ribonucleic acid; NRVM, neonatal rat ventricular myocyte; IL-1β, interleukin 1 beta; IL-18, interleukin 18; qPCR, quantitative polymerase chain reaction; GAPDH, glyceraldehyde-phosphate dehydrogenase.

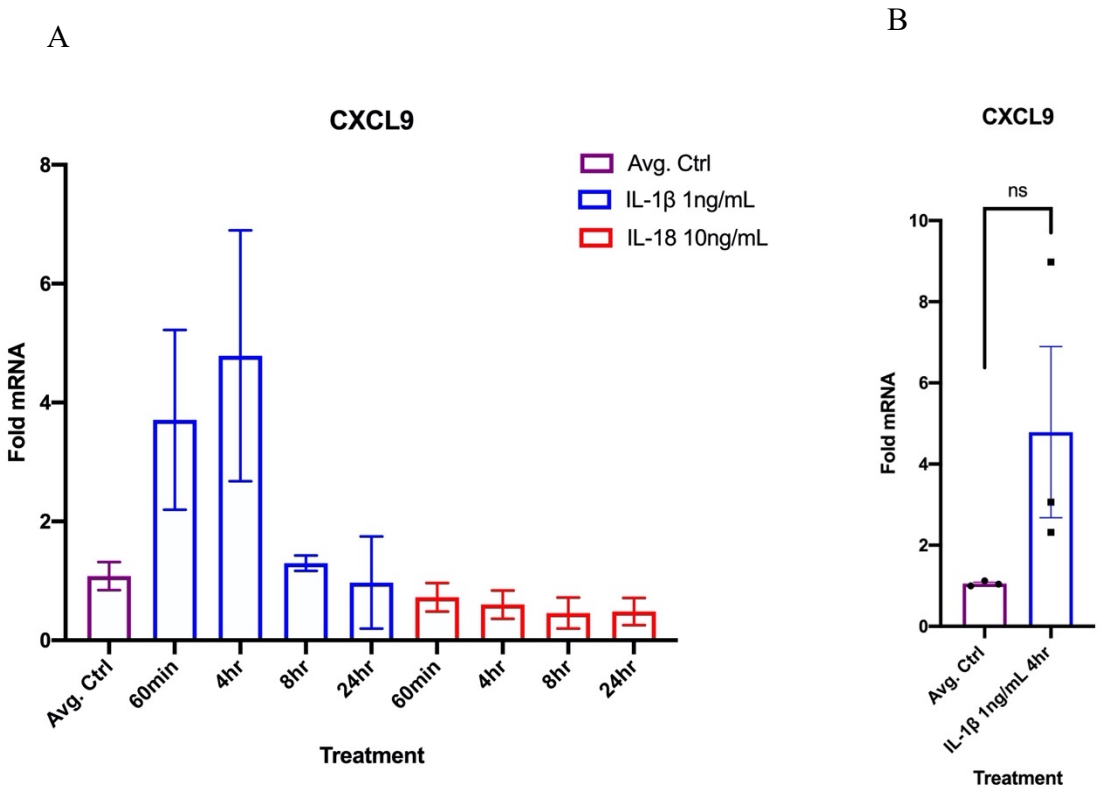


Figure 14: CXCL9 mRNA expression in NRVMs in response to IL-1β and IL-18. A. NRVMs treated with 1ng/mL IL-1β and 10ng/mL IL-18 at 60 minutes, 4 hours, 8 hours and 24 hours before harvesting. CXCL9 mRNA in whole cell lysates measured by qPCR, normalized over GPADH loading control and expressed as mRNA fold increase over untreated control cells. mRNA fold change averaged among N=3-5 with 1 sample containing 1 technical replicate. **B.** Unpaired one-tailed t-test of IL-1β 1ng/mL 4 hours compared to untreated control cells. Significance depicted by ns $p>0.05$. Error represented by ± SEM. CXCL9 indicates chemokine (C-X-C motif) ligand 9; mRNA, messenger ribonucleic acid; NRVM, neonatal rat ventricular myocyte; IL-1β, interleukin 1 beta; IL-18, interleukin 18; qPCR, quantitative polymerase chain reaction; GAPDH, glyceraldehyde-phosphate dehydrogenase.

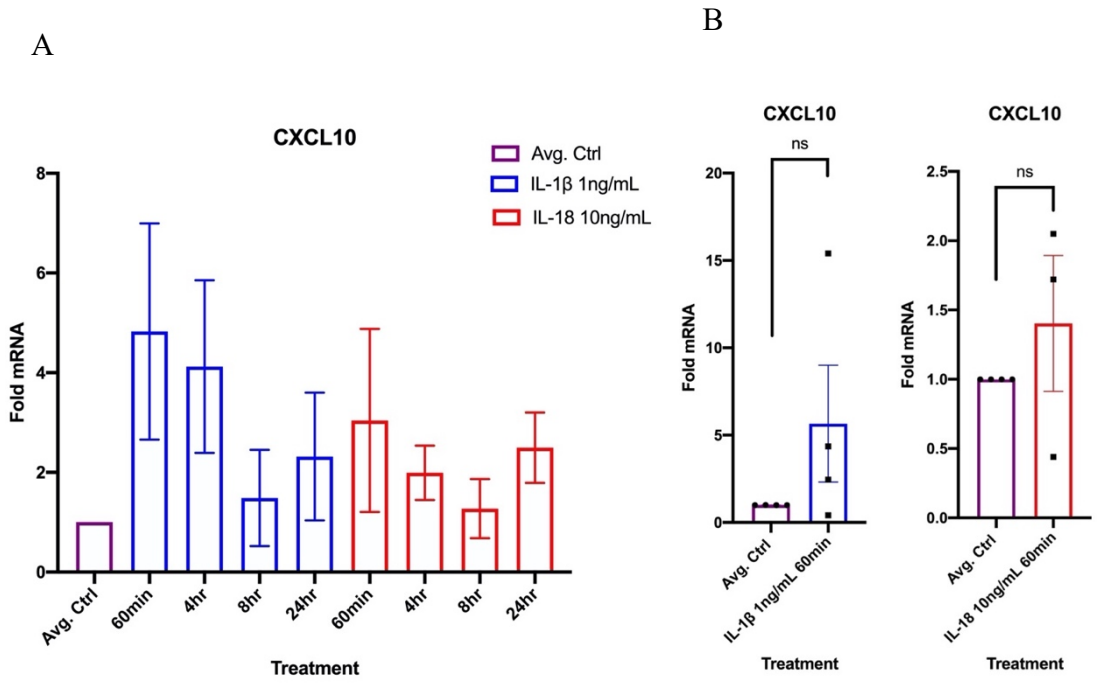


Figure 15: CXCL10 mRNA expression in NRVMs in response to IL-1β and IL-18. A. NRVMs treated with 1ng/mL IL-1β and 10ng/mL IL-18 at 60 minutes, 4 hours, 8 hours and 24 hours before harvesting. CXCL10 mRNA in whole cell lysates measured by qPCR, normalized over GPADH loading control and expressed as mRNA fold increase over untreated control cells. mRNA fold change averaged among N=1-5 with 3 samples containing 1-2 technical replicates. **B.** Unpaired one-tailed t-test of IL-1β 1ng/mL 60 minutes and IL-18 10ng/mL 60 minutes compared to untreated control cells. Significance depicted by ns $p>0.05$. Error represented by ± SEM. CXCL10 indicates chemokine (C-X-C motif) ligand 10; mRNA, messenger ribonucleic acid; NRVM, neonatal rat ventricular myocyte; IL-1β, interleukin 1 beta; IL-18, interleukin 18; qPCR, quantitative polymerase chain reaction; GAPDH, glyceraldehyde-phosphate dehydrogenase.

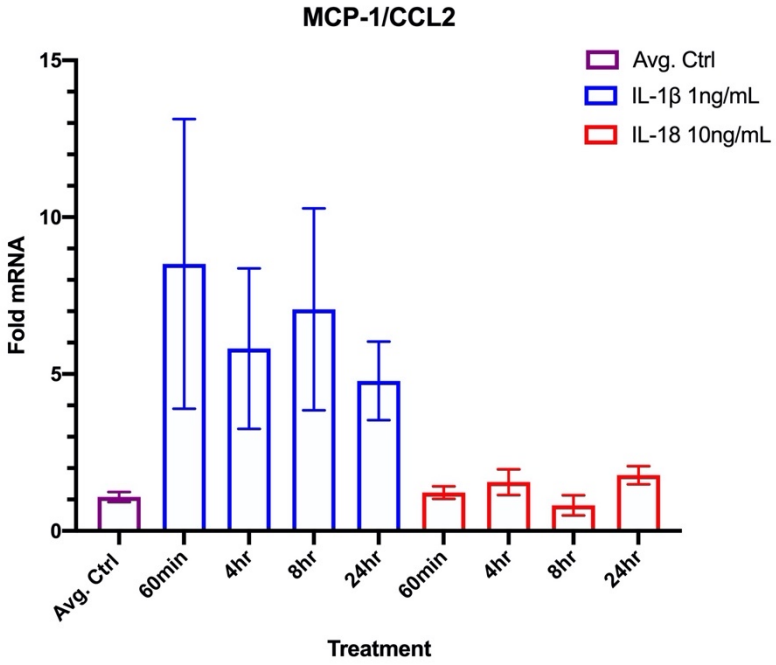


Figure 16: MCP-1/CCL2 mRNA expression in NRVMs in response to IL-1β and IL-18. NRVMs treated with 1ng/mL IL-1β and 10ng/mL IL-18 at 60 minutes, 4 hours, 8 hours and 24 hours before harvesting. MCP-1/CCL2 mRNA in whole cell lysates measured by qPCR, normalized over GPADH loading control and expressed as mRNA fold increase over untreated control cells. mRNA fold change averaged among N=2-4 with 3 samples containing 1 technical replicate. Error represented by ± SEM. MCP-1/CCL2 indicates monocyte chemoattractant protein-1/ chemokine (C-C motif) ligand 2; mRNA, messenger ribonucleic acid; NRVM, neonatal rat ventricular myocyte; IL-1β, interleukin 1 beta; IL-1s8, interleukin 18; qPCR, quantitative polymerase chain reaction; GAPDH, glyceraldehyde-phosphate dehydrogenase.

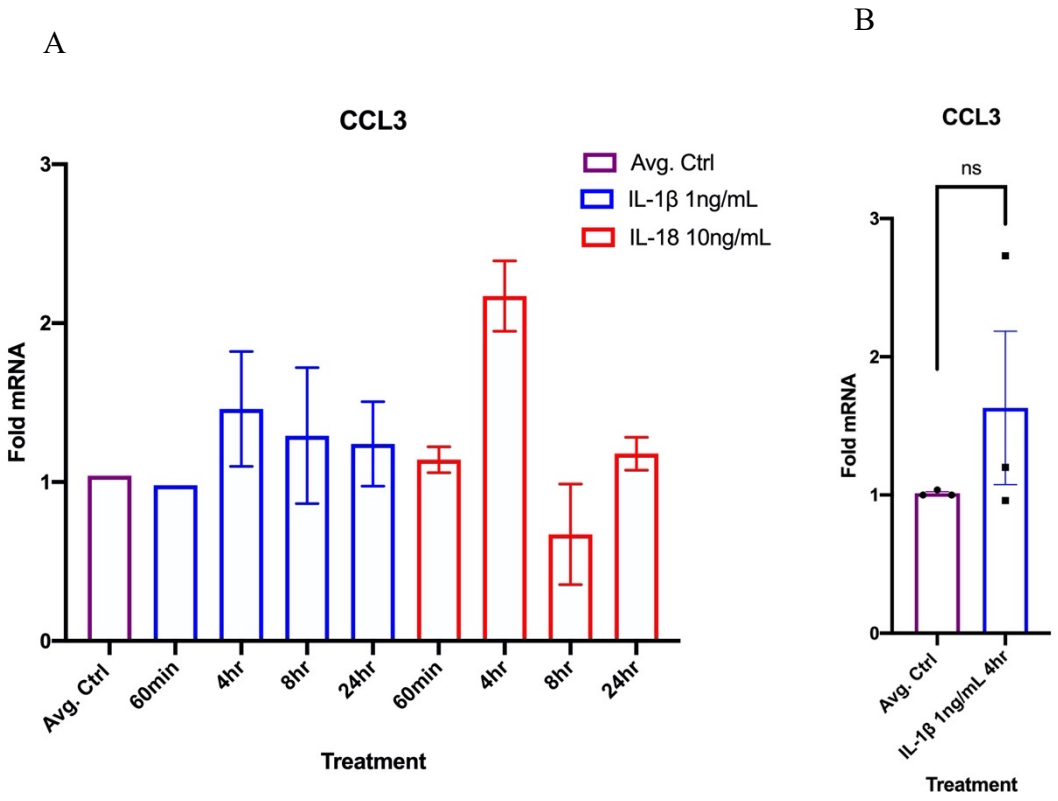


Figure 17: CCL3 mRNA expression in NRVMs in response to IL-1β and IL-18. A. NRVMs treated with 1ng/mL IL-1β and 10ng/mL IL-18 at 60 minutes, 4 hours, 8 hours and 24 hours before harvesting. CCL3 mRNA in whole cell lysates measured by qPCR, normalized over GPADH loading control and expressed as mRNA fold increase over untreated control cells. mRNA fold change averaged among N=1-5 with 1 sample containing 1 technical replicate. **B.** Unpaired one-tailed t-test of IL-1β 1ng/mL 4 hours compared to untreated control cells. Significance depicted by ns $p>0.05$. Error represented by ± SEM. CCL3 indicates chemokine (C-C motif) ligand 3; mRNA, messenger ribonucleic acid; NRVM, neonatal rat ventricular myocyte; IL-1β, interleukin 1 beta; IL-18, interleukin 18; qPCR, quantitative polymerase chain reaction; GAPDH, glyceraldehyde-phosphate dehydrogenase.

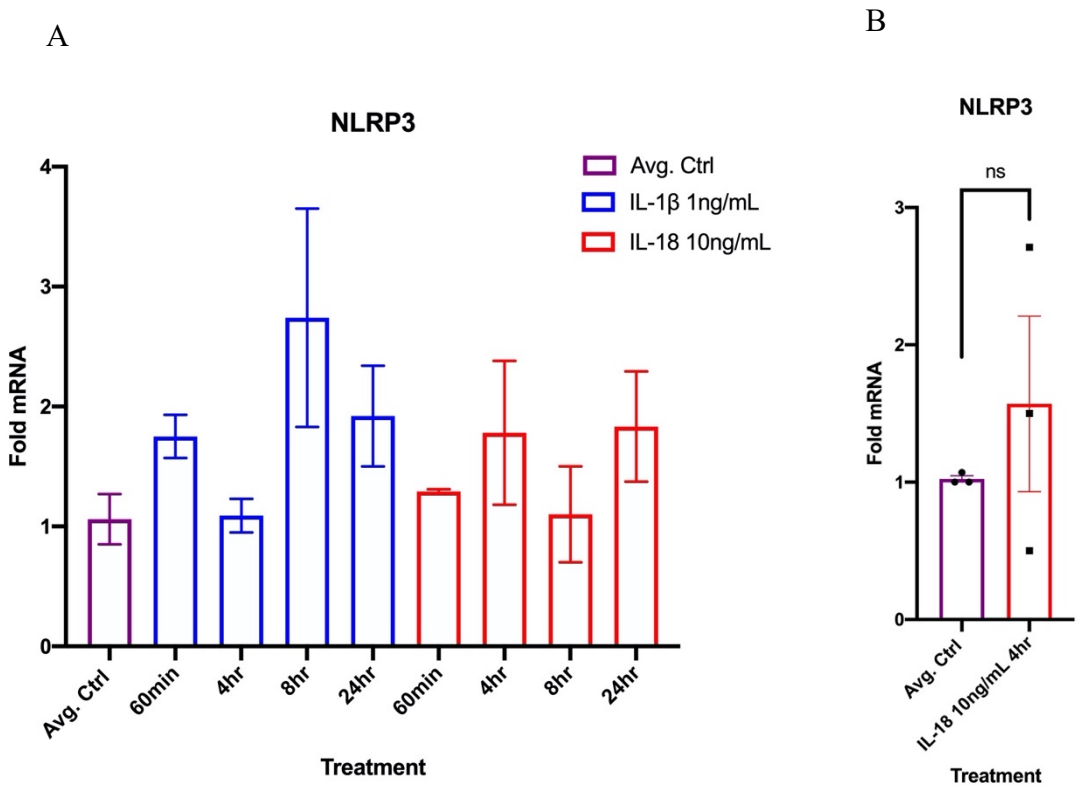


Figure 18: NLRP3 inflammasome mRNA expression in NRVMs in response to IL-1β and IL-18. A. NRVMs treated with 1ng/mL IL-1β and 10ng/mL IL-18 at 60 minutes, 4 hours, 8 hours and 24 hours before harvesting. NLRP3 mRNA in whole cell lysates measured by qPCR, normalized over GPADH loading control and expressed as mRNA fold increase over untreated control cells. mRNA fold change averaged among N=2-4 with 3 samples containing 1 technical replicate. **B.** Unpaired one-tailed t-test of IL-18 10ng/mL 4 hours compared to untreated control cells. Significance depicted by ns $p>0.05$. Error represented by ± SEM. NLRP3 indicates NOD-like pyrin domain-containing protein 3; mRNA, messenger ribonucleic acid; NRVM, neonatal rat ventricular myocyte; IL-1β, interleukin 1 beta; IL-18, interleukin 18; qPCR, quantitative polymerase chain reaction; GAPDH, glyceraldehyde-phosphate dehydrogenase.

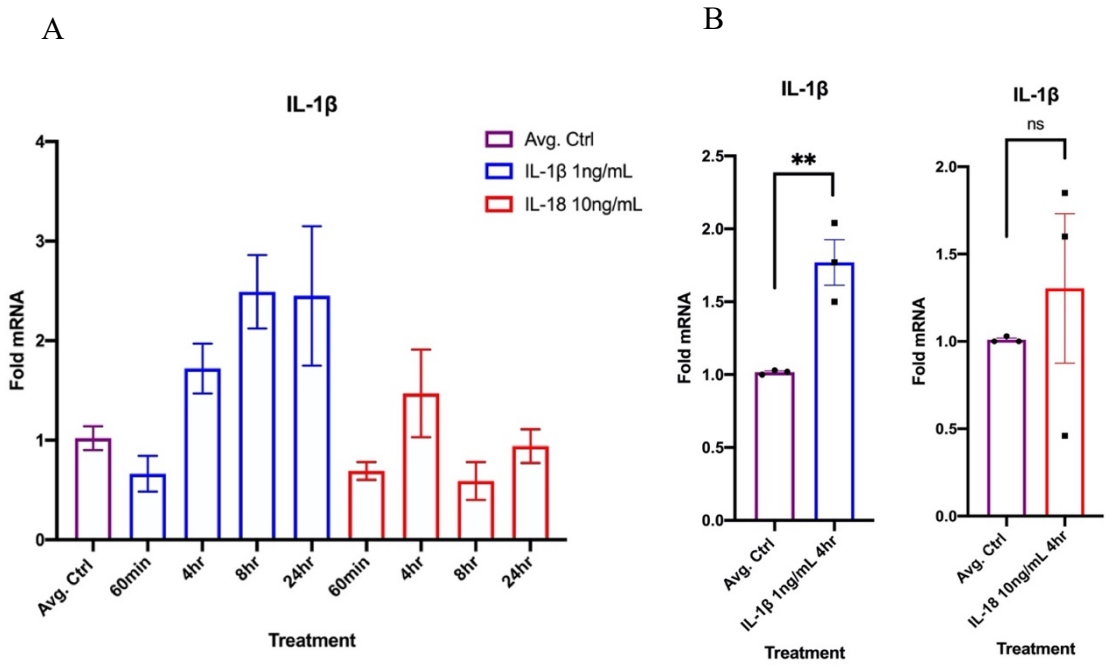


Figure 19: mRNA expression of NLRP3 inflammasome substrate IL-1β increases in response to exogenous IL-1β. A. NRVMs treated with 1ng/mL IL-1β and 10ng/mL IL-18 at 60 minutes, 4 hours, 8 hours and 24 hours before harvesting. IL-1β mRNA in whole cell lysates measured by qPCR, normalized over GPADH loading control and expressed as mRNA fold increase over untreated control cells. mRNA fold change averaged among N=2-4 with 3 samples containing 1 technical replicate. **B.** Unpaired one-tailed t-test of IL-1β 1ng/mL 4 hours and IL-18 10ng/mL 4 hours compared to untreated control cells. Significance depicted by ** $p \leq 0.01$ and ns $p > 0.05$. Error represented by ± SEM. mRNA indicates messenger ribonucleic acid; NLRP3, NOD-like pyrin domain-containing protein 3; IL-1β, interleukin 1 beta; NRVM, neonatal rat ventricular myocyte; IL-18, interleukin 18; qPCR, quantitative polymerase chain reaction; GAPDH, glyceraldehyde-phosphate dehydrogenase.

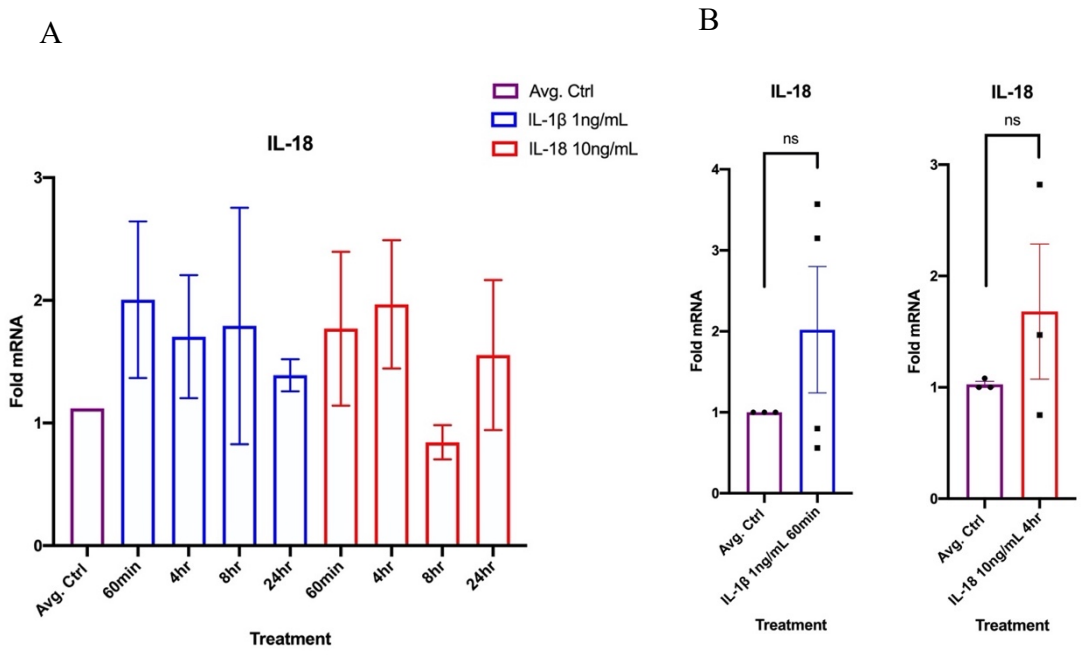
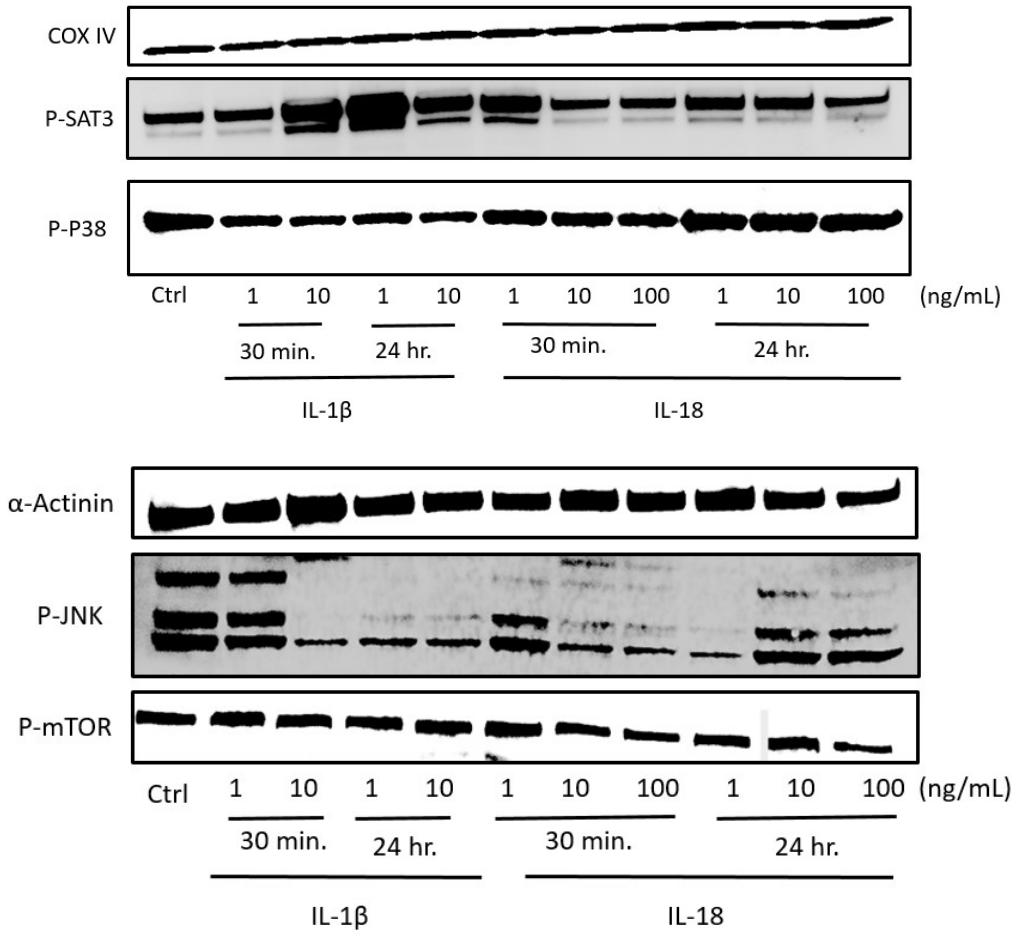
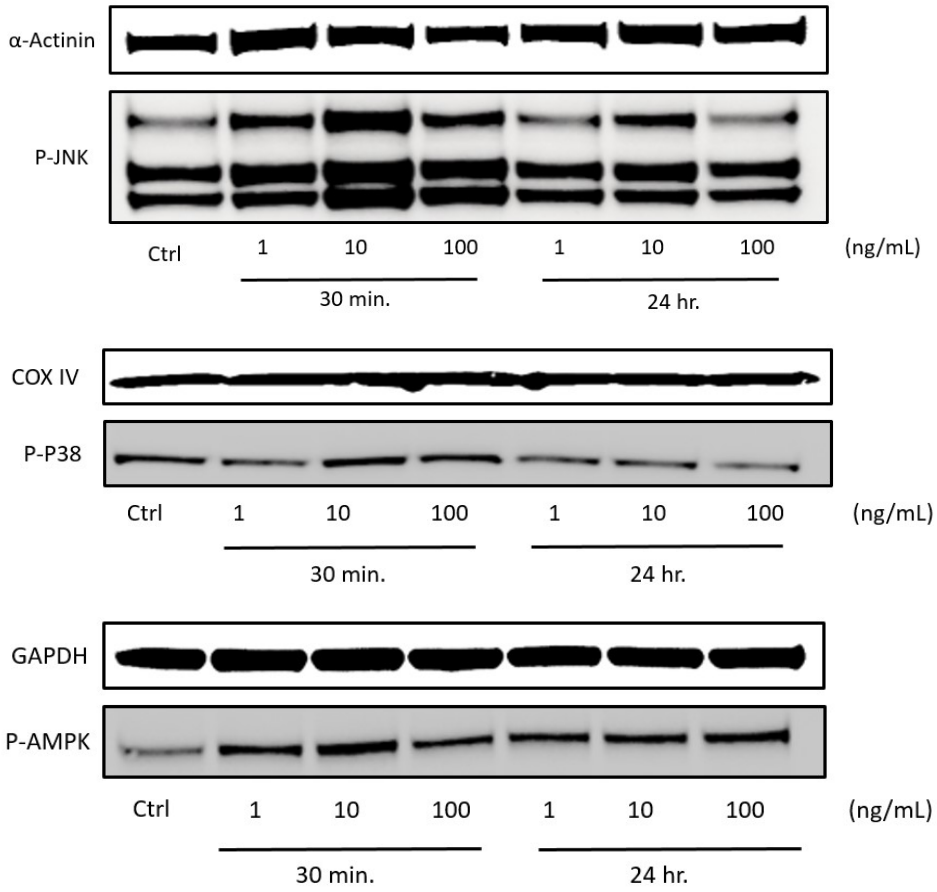


Figure 20: mRNA expression of NLRP3 inflammasome substrate IL-18 in response to exogenous IL-1β and IL-18. **A**. NRVMs treated with 1ng/mL IL-1β and 10ng/mL IL-18 at 60 minutes, 4 hours, 8 hours and 24 hours before harvesting. IL-18 mRNA in whole cell lysates measured by qPCR, normalized over GPADH loading control and expressed as mRNA fold increase over untreated control cells. mRNA fold change averaged among N=3-5 with 3 samples containing 1 technical replicate. **B.** Unpaired one-tailed t-test of IL-1β 1ng/mL 60 minutes and IL-18 10ng/mL 4 hours compared to untreated control cells. Significance depicted by ns $p>0.05$. Error represented by ± SEM. mRNA indicates messenger ribonucleic acid; NLRP3, NOD-like pyrin domain-containing protein 3; IL-18, interleukin 18; IL-1β, interleukin 1 beta; NRVM, neonatal rat ventricular myocyte; qPCR, quantitative polymerase chain reaction; GAPDH, glyceraldehyde-phosphate dehydrogenase.

SUPPLEMENTARY FIGURES



Supplementary Figure 1: Protein phosphorylation in response to increasing concentrations of recombinant IL-1β and IL-18. Immunoblots of NRVM whole cell lysates treated with 1ng/mL and 10ng/mL IL-1β or 1ng/mL, 10ng/mL, and 100ng/mL IL-18 30 minutes or 24 hours before harvesting. COX IV and α-Actinin were used as loading controls. IL-1β indicates interleukin 1 beta; IL-18, interleukin 18; NRVM, neonatal rat ventricular myocyte; COX IV, cytochrome c oxidase subunit 4; STAT3, signal transducer and activators of transcription 3; JNK, c-Jun N-terminal kinase; mTOR, mammalian target of rapamycin.



Supplementary Figure 2: Protein phosphorylation in response to increasing concentrations of recombinant IL-18. Immunoblots of NRVM whole cell lysates treated with 1ng/mL, 10ng/mL and 100ng/mL IL-18 30 minutes or 24 hours before harvesting. α-Actinin, COX IV, and GAPDH were used as loading controls. IL-18 indicates interleukin 18; NRVM, neonatal rat ventricular myocyte; COX IV, cytochrome c oxidase subunit 4; GAPDH, glyceraldehyde-phosphate dehydrogenase; JNK, c-Jun N-terminal kinase; AMP-activated protein kinase.

REFERENCES

1. Dalen, J., Alpert, J., Goldberg R., Weinstein, R. The Epidemic of the 20th Century: Coronary Heart Disease. *The American Journal of Medicine.* 2014; 127(9), 807-812. doi: 10.1016/j.amjmed.2014.04.015

2. Heron, M. Deaths: Leading Causes for 2017. *National Vital Statistics Reports*. 2019; 68(6), 1-76

3. Heart Disease. (2018). Retrieved from https://www.mayoclinic.org/diseases-conditions/heart-disease/symptoms-causes/syc-20353118

4. American Heart Association. What is Heart Failure? (n.d.). Retrieved from https://www.heart.org/en/health-topics-heart-failure/what-is-heart-failure

5. American Heart Association News. Heart failure projected to increase dramatically, according to new statistics (2017). Retrieved from https://www.heart.org/en/news/2018/05/01/heart-failure-projected-to-increase-dramatically-according-to-new-statistics

6. Fiordelisi, A., Iaccarino, G., Morisco, C., Coscioni, E., Sorriento, D. NFkappaB is a Key Player in the Crosstalk between Inflammation and Cardiovascular Diseases. *International Journal of Molecular Sciences*. 2019; 20(7), 1599. doi: 10.3390/ijms20071599

7. Heinzel, F., Hohendanner, F., Jin, G., Sedej, S., Edelmann, F. Myocardial hypertrophy and its role in heart failure with preserved ejection fraction. *J Appl Physiol (1985)*. 2015; 119(10), 1233–1242. doi:10.1152/japplphysiol.00374.2015

8. Ivey, J., Tallquist, D. Defining the Cardiac Fibroblast. *Circ J*. 2016; 80(11), 269-2276. doi:10.1253/circj.CJ-16-1003

9. Institute of Medicine (US) Committee on Social Security Cardiovascular Disability Criteria. Cardiovascular Disability: Updating the Social Security Listings. Washington (DC): National Academies Press (US); 2010. 7, Ischemic Heart Disease. Available from: https://www.ncbi.nlm.nih.gov/books/NBK209964/

10. Ghigo, A., Franco, I., Morello, F., Hirsch, E., Myocyte Signaling in Leucocyte Recruitment to the Heart. *Cardiovascular Research*. 2014; 102(2), 270-280. doi:10.1093/cvr/cvu030

11. Toldo, S., Mezzaroma, E., Mauro, A., Salloum, F., Van Tassell, B., Abbate, A. The Inflammasome in Myocardial Injury and Cardiac Remodeling. *Antioxidants and Redox Signaling.* 2015; 22(13), 1446-1461. doi:10.1089/ars.2014.5989

12. Maguire, O., Collins, C., O'Loughlin, K., Miecznikowski, J., & Minderman, H. Quantifying nuclear p65 as a parameter for NF-κB activation: Correlation between ImageStream cytometry, microscopy, and Western blot. *Cytometry. Part A: the journal of the International Society for Analytical Cytology*. 2011; *79*(6), 461–469. https://doi.org/10.1002/cyto.a.21068

13. Napetschnig, J., & Wu, H. Molecular basis of NF-κB signaling. *Annual review of biophysics*. 2013; 42, 443–468. doi.org/10.1146/annurev-biophys-083012-130338

14. Zhang, Y., Bauersachs, J., Langer, H., Immune mechanisms in heart failure. *European Journal of Heart Failure.* 2017; 19(11), 1379-1389. doi.org/10.1002/ejhf.942

15. An, N., Gao, Y., Si, Z., Zhang, H., Wang, L., Tian, C., Yuan, M., Yang, X., Li, X., Shang, H., Xiong, X., Xing, Y. Regulatory Mechanisms of the NLRP3 Inflammasome, a Novel Immune-Inflammatory Marker in Cardiovascular Diseases. *Front Immuno. 2019*; 10, 1592. doi:10.3389/fimmu.2019.01592

16. Mehta, J., Li, D. Inflammation in ischemic heart disease: Response to tissue injury or a pathogenetic villain?, *Cardiovascular Research*. 1999; 43(2), 291–299. doi.org/10.1016/S0008-6363(99)00132-7

17. Bluemke, A. MRI of nonischemic cardiomyopathy. *AJR Am J Roentgenol*. 2010; 195(4), 935-940. doi:10.2214/AJR.10.4222

18. "Causes of Heart Failure." *The Society for Cardiovascular Angiography and Interventions*. (2016). Retrieved from www.secondscount.org/heart-condition-centers/info-detail-2/causes-of-heart-failure#.XrMi5RNKgnU

19. Willeford, A., Suetomi, T., Nickle, A., Hoffman, H., Miyamoto, S., Heller Brown, J. CaMKIIδ-mediated Inflammatory Gene Expression and Inflammasome Activation in Cardiomyocytes Initiate Inflammation and Induce Fibrosis. *JCI Insight.* 2018; *3*(12). doi:10.1172/jci.insight.97054

20. Suetomi, T., Willeford, A., Brand, C., Cho, Y., Ross, R., Miyamoto, S., Heller Brown, J. Inflammation and NLRP3 Inflammasome Activation Initiated in Response to Pressure Overload by Ca 2 /Calmodulin-Dependent Protein Kinase II δ Signaling in Cardiomyocytes Are Essential for Adverse Cardiac Remodeling. *Circulation.* 2018; 138(22), 2530-2544, doi:10.1161/circulationaha.118.034621

21. Ling, H., Zhang, T., Pereira, L., Means, C., Cheng, H., Gu, Y., Dalton, N., Peterson, K., Chen, J., Bers, D., Heller Brown, J. Requirement for Ca2+/calmodulin-dependent kinase II in the transition from pressure-overload induced cardiac hypertrophy to heart failure in mice. *J Clin Invest.* 2009; 119(5), 1230-1240. doi.org/10.1172/JCI38022.

22. Bujak, M., Frangogiannis, G. The Role of IL-1 in the Pathogenesis of Heart Disease. *Archivum Immunologiae et Therapiae Experimentalis*. 2009; 57(3), 165-176. doi.org/10.1007/s00005-009-0024-y

23. Gordon, J., Shaw, J., Kirshenbaum., L. Multiple Facets of NF-κB in the Heart To Be or Not to NF-κB. *Circulation Research.* 2011; 108(9), 1122-1132. doi.org/10.1161/CIRCRESAHA.110.226928

24. Brasier, A. The nuclear factor-kappaB-interleukin-6 signalling pathway mediating vascular inflammation. *Cardiovasc Res*. 2010; 86(2), 211-218. doi:10.1093/cvr/cvq076

25. Olson, C., Hedrick, M., Izadi, H., Bates, T., Olivera, E., Anguita, J. p38 Mitogen-Activated Protein Kinase Controls NF-κB Transcriptional Activation and Tumor Necrosis Factor Alpha Production through RelA Phosphorylation Mediated by Mitogen- and Stress-Activated Protein Kinase 1 in Response to *Borrelia. Infection and Immunity.* 2006; 75(1), 270-277. doi**:**10.1128/IAI.01412-06

26. Bujak, M., Frangogiannis, G. The role of TGF-beta signaling in myocardial infarction and cardiac remodeling. *Cardiovasc Res*. 2007; 74(2), 184-195. doi:10.1016/j.cardiores.2006.10.002

27. Hanna, A., Frangogiannis, G. The Role of the TGF-β Superfamily in Myocardial Infarction. *Front Cardiovasc Med*. 2019; (6)140. doi:10.3389/fcvm.2019.00140

28. Liu, T., Zhang, L., Joo, .D, Sun, C. NF-κB signaling in inflammation. *Signal Transduct Target Ther*. 2017; 2, 17023. doi:10.1038/sigtrans.2017.23

29. Abbate, A., Kontos, M., Grizzard, J., Biondi-Zoccai, G., Van Tassel, B., Roshanak, R., Roach, L., Arena, R., Roberts, C., Varma, A., Gelwix, C., Salloum, F., Hastillo, A., Dinarello, C., Vetrovec, G. Effects of interleukin-1 blockade with anakinra on adverse cardiac remodeling and heart failure after acute myocardial infarction [from the Virginia Commonwealth University-Anakinra Remodeling Trial (2) (VCU-ART2) pilot study]. *Am J Cardiol*. 2013; 111(10), 1394-1400. doi:10.1016/j.amjcard.2013.01.287

30. Yoshida, T., Friehs, I., Mummidi, S., del Nido, P., Addulnour-Nakhoul, S., Delafontaine, P., Valente, A., Chandrasekar, B. Pressure overload induces IL-18 and IL-18R expression, but markedly suppresses IL-18BP expression in a rabbit model. IL-18 potentiates TNF-α-induced cardiomyocyte death. *J Mol Cell Cardiol*. 2014; 75, 141-151. doi:10.1016/j.yjmcc.2014.07.007