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The Effects of Interleukin-1β on Cardiac Reserve and Exercise Capacity in the Mouse

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The Effects of Interleukin-1β on Cardiac Reserve and Exercise Capacity in the Mouse

A Thesis submitted in partial fulfillment of the requirements for the degree of Master of Science in Physiology and Biophysics at Virginia Commonwealth University.

By:
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B. S. Biomedical Engineering, Virginia Commonwealth University, 2019

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Abstract

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By Habeebah Z. Vohra, B.S.

A thesis submitted in partial fulfillment of the requirements for the degree of Master of Science at Virginia Commonwealth University.

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Major Director: Stefano Toldo, Ph.D., Department of Internal Medicine

Background: Heart failure (HF) is characterized by dyspnea, fatigue, and exercise intolerance. Clinical evidence points to increased interleukin-1β (IL-1β) activity in patients with HF, with an IL-1 blockade improving the exercise capacity in HF patients. In healthy mice, recombinant-mouse IL-1β (rmIL-1β) induces acute systolic dysfunction, peaking 4 hours after administration. However, the direct effects of rmIL-1β on exercise capacity are unknown.

Hypothesis: rmIL-1β diminishes the exercise capacity in the mouse.

Methods: Adult mice were trained to run on a treadmill and exercise capacity was assessed before, 4 hours, and 96 hours after intraperitoneal administration of rmIL-1β (3 μg/kg) or vehicle (0.9% NaCl) (N=7-10/group). In separate groups of mice, left ventricular ejection fraction (LVEF) was assessed before, 4 hours, and 96 hours after intraperitoneal administration of rmIL-1β (3 μg/kg) or vehicle (0.9% NaCl) using transthoracic echocardiography. The ultrasound operator was blinded to treatments. The cardiac reserve was measured by calculating the difference in LVEF before and after β-adrenergic stimulation using isoproterenol (10 ng/mouse) in IL-1β and vehicle-treated mice (N=5-11/group).

Results: Treatment with rmIL-1β significantly reduced exercise capacity measured at 4 hours and 96 hours (32% and 33% reduction, respectively, $P<0.01$ vs baseline), whereas no effect was observed in the vehicle-treated group ($P<0.05$ vs IL-1β-treated group). LVEF was significantly

reduced 4 hours after treatment with rmIL-1β (29% reduction, $P<0.01$ vs baseline), without any significant changes after treatment with vehicle. After 96 hours, LVEF had recovered to pre-treatment values. Cardiac reserve was significantly reduced 4 hours and 96 hours after IL-1β administration (7% and 2% LVEF change, respectively, $P<0.05$ vs baseline) but not after vehicle administration ($P<0.05$ vs IL-1β-treated group).

Conclusion: Administration of exogenous IL-1β reduces exercise capacity through an impairment in cardiac reserve.

Introduction

Heart Failure

The prevalence of heart failure (HF) is increasing overtime along with the aging of the population. An estimated 6.2 million Americans over the age of 20 years had HF between 2013 and 2016 which was an increase from an estimated 5.7 million between 2009 and 2012. Approximately half of hospitalized HF events are characterized by reduced left ventricular ejection fraction (LVEF) and the other half by preserved ejection fraction.[1] For patients under the age of 65, the most common type of HF is with a reduced ejection fraction (HFrEF)[2] and for patients over the age of 70, the most common type of HF is with a preserved ejection fraction (HFpEF).[3] While contemporary HF treatments have slowed disease progression and improved survival, the overall incidences of HF morbidity and mortality continue to rise.[4,5]

HF is a complex clinical syndrome that can result from any structural or functional cardiac disorder that impairs the ability of the ventricle to fill with or eject blood.[6] It is the inability to provide sufficient blood flow to meet the needs of the body for oxygenation during regular activity or to do so only with elevated pressures in the heart.[7] HF is characterized by dyspnea, fatigue, and peripheral fluid retention which alone or in combination may limit a person's ability to perform activities.[4,5] It represents the final phenotype for a variety of cardiovascular diseases culminating in impaired cardiac systolic and/or diastolic function.[8]

As cardiac output decreases due to stresses placed on the myocardium, activation of the sympathetic nervous and renin-angiotensin-aldosterone systems increases blood pressure and volume. The activation of these compensatory mechanisms can lead to further myocardial deterioration and worsening contractility. In HFrEF, cardiac output is decreased directly through a reduced LVEF and is characterized by systolic dysfunction. HFpEF is characterized by

diastolic dysfunction in which cardiac output is compromised by poor ventricular compliance, impaired relaxation, and high end-diastolic pressure.[6,9]

Risk Factors for HF

Coronary artery disease (CAD) is the most common underlying etiology in patients with HFrEF[10,11] and a predictor for the progression from asymptomatic to symptomatic left ventricular systolic dysfunction. Hypertension and valvular heart disease are significant risk factors for HF as well.[11] Cardiomyopathy is a disease that causes the heart to ineffectively pump blood which can lead to HF. The main types are dilated, hypertrophic, restrictive, and diabetic cardiomyopathy. Dilated cardiomyopathy is the enlargement of the left ventricular cavity while hypertrophic cardiomyopathy involves abnormal thickening of the left ventricular myocardium and restrictive cardiomyopathy is the stiffening of the myocardium without an increase of cardiac mass.[12] Diabetes mellitus increases the risk of HF twofold by directly leading to cardiomyopathy and significantly contributing to CAD, and is the strongest risk factor for HF in women with CAD.[13] Common to CAD, hypertension, valvular heart disease, and cardiomyopathies is an elevation of proteins involved in inflammatory processes[14–17] which continue to be present in HF.

All cardiomyopathies are associated with a compensatory activation of the sympathetic nervous system (SNS), the renin–angiotensin–aldosterone system (RAAS), and natriuretic peptides. Activation of these pathways is referred to as “neurohormonal activation”, which acts to restore and improve the function of the cardiovascular system and prevent a decrease in the cardiac output. However, overtime, a sustained neurohormonal activation drives the progression of HF through the deleterious effects exerted on the circulation and the myocardium. Classes of medications commonly prescribed to patients with HF target these pathways or aim to reduce the

energy consumption in cardiomyocytes. These include angiotensin-converting enzyme (ACE) inhibitors, angiotensin receptor blockers (ARBs), angiotensin receptor-neprilysin inhibitors (ARNI), aldosterone antagonists, diuretics, β-adrenergic blocking agents, I_f channel blockers, digoxin, the nitric oxide donor hydralazine and isosorbide dinitrate (specifically benefiting African Americans with HF).[18] These pharmaceutical strategies developed over the course of the past decades have been successful in reducing mortality from HF, yet the burden of disease remains unacceptably high.[8,19,20] This phenomenon highlights the need to identify new pathophysiological pathways that participate in HF development and progression.

HF and Functional Capacity

Patients diagnosed with HF, regardless of the cause, demonstrate a compromised functional capacity. Functional capacity describes a person's ability to perform daily activities that require physical exertion. The ability to perform aerobic activities is a central, but not complete, determinant of functional capacity. It is well established that HF patients demonstrate attenuated muscular strength and endurance because of the progression of their disease. Typically, their ability to perform daily activities that are either aerobic or resistive in nature is compromised and contribute to the decline in functional capacity.[21]

One of the hallmarks of HF is exercise intolerance which is accompanied by symptoms of fatigue and shortness of breath.[22,23] As the disease progresses, patients experience a downward spiral as these symptoms usually result in reduced physical activity leading to progressively worse exercise intolerance.[24,25] Typically, patients with HF are faced with what is termed functional disability[26–28] which is their inability to carry out daily activities leading them to suffer from an impaired quality of life.[29–31]

Inflammation and HF

The immune system works to restore tissue homeostasis through an integrated response that involves inflammation. The activation of inflammation within the heart is tightly regulated and can either be beneficial or lead to harm.[32] Inflammation is an important process activated following the perturbation of cellular or organ homeostasis. It can modify the cellular or organ function and as a physiological response, it coordinates the return to homeostasis. There are two different types of inflammation based on the triggers of immune response: inflammatory response initiated by exogenous or endogenous (also called sterile) inducers.[32]

Pathogen-associated molecular patterns (PAMPs) activate the innate immune system involving monocytes-macrophages, dendritic cells, and natural killer cells that interact with each other through a wide variety of cytokines including type I and type II interferons, which in turn have potent anti-viral effects.[33] Persistent viral antigens elicit the adaptive immune system with clonal expression of antigen-specific T and B lymphocytes, ensuing cellular and antibody-mediated immune responses, respectively. Although innate and adaptive actions are fundamental to resolve cardiac infection, when unopposed, they elicit cardiac injury, cardiomyocyte death, and, eventually, loss of myocardial contractility.[32] Myocarditis is the prototype of cardiac inflammation initiated by exogenous sources and is most often second to viral infection.[34] Often self-limiting, it can debut with or progress to HFrEF.

Endogenous inducers of inflammation have been called danger-associated molecular patterns (DAMPs).[33] Similar to PAMPs, DAMPs activate the innate immune response and can modify the activity of T and B lymphocytes during the course of heart disease development.

In the cardiovascular system, inflammation is a central pathway that is often linked to the pathogenesis of cardiovascular disease. The infiltration of leukocytes, cytokine production, and

phagocytic removal of cellular debris are essential for cardiac repair after myocardial infarction.[35] The persistence of cardiac inflammatory signals, however, is implicated in maladaptive cardiac remodeling that can lead to HF following ischemic or non-ischemic injury. Inflammation has long been known to be associated with HF and to predict the outcome of affected patients. In recent years it is increasingly recognized as one of the factors promoting HF initiation and progression. Patients with HF have elevated levels of pro-inflammatory markers, and animal models show a causative role of inflammation in the pathophysiology of CVD and HF.[32,36]

After myocardial infarction, cell, tissue, plasma, and extracellular matrix-derived products trigger an immune response like the one induced by infections, including the recruitment of leukocytes and secretion of cytokines.[32] Moreover, increased production of cytokines such as interleukin-1β (IL-1β), IL-6, and tumor necrosis factor-α (TNF-α) by cardiac fibroblasts has been associated with reduction in collagen synthesis and expansion of the infarcted area.[35] (Fig. 1) Elevated local and systemic inflammatory markers correlate with the deterioration of cardiac function and predict worse outcomes in both HFrEF and HFpEF.[37–39] Paradoxically, sustained inflammation within the myocardium causes extracellular matrix degradation via matrix metalloproteinases (MMPs) promoting a maladaptive ventricular remodeling that promotes the development of HF.[40]

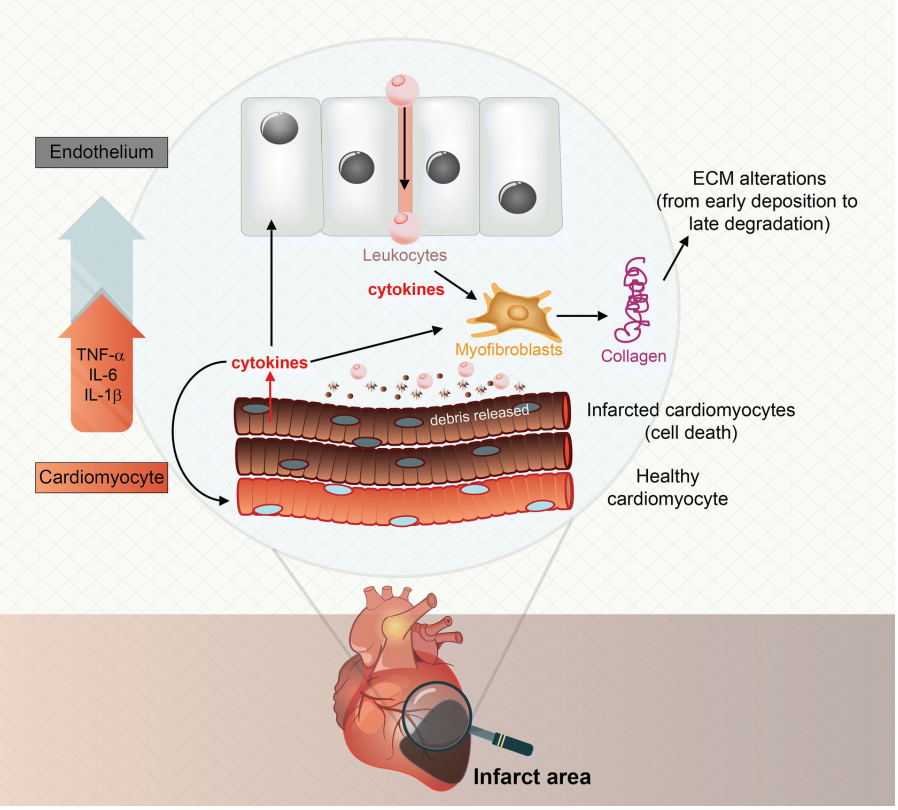

Figure 1: Inflammatory pathways in HFrEF [32]

Inflammation also favors the decrease in sarcoplasmic reticulum (SR) calcium load in cardiomyocytes, which characterizes HFrEF.[41] Two mechanisms are, at least in part, responsible for this phenomenon: decreased expression and activity of the SR calcium ATPase (SERCA), which is responsible for ~70% of cytosolic calcium reuptake in humans,[42] and increased diastolic calcium leakage from the SR via ryanodine receptors (RyRs).[41] Because of the reduced SR calcium availability, systolic contraction is impaired; on the other hand, calcium accumulation in diastole slows myocardial relaxation and enhances cardiomyocyte tension. In addition, the elevated calcium in diastole increases the open probability of the RyRs, causing spontaneous SR calcium release, which is a predisposition to early afterdepolarizations triggering ventricular arrhythmias.[43] Pro-inflammatory cytokines, such as IL-1β, IL-6, IL-18 and TNF-α, affect SERCA function.[38,44]

Conversely, HFpEF is thought to be caused by systemic inflammation resulting in injury of cardiac cells, in a way that identifies this type of HF as an "inflammatory" disease, similarly to what has been observed in atherosclerosis.[32] The current paradigm of HFpEF pathophysiology recognizes comorbidity-initiated subtle, chronic, systemic inflammation the origin of cardiac alterations. (Fig. 2)

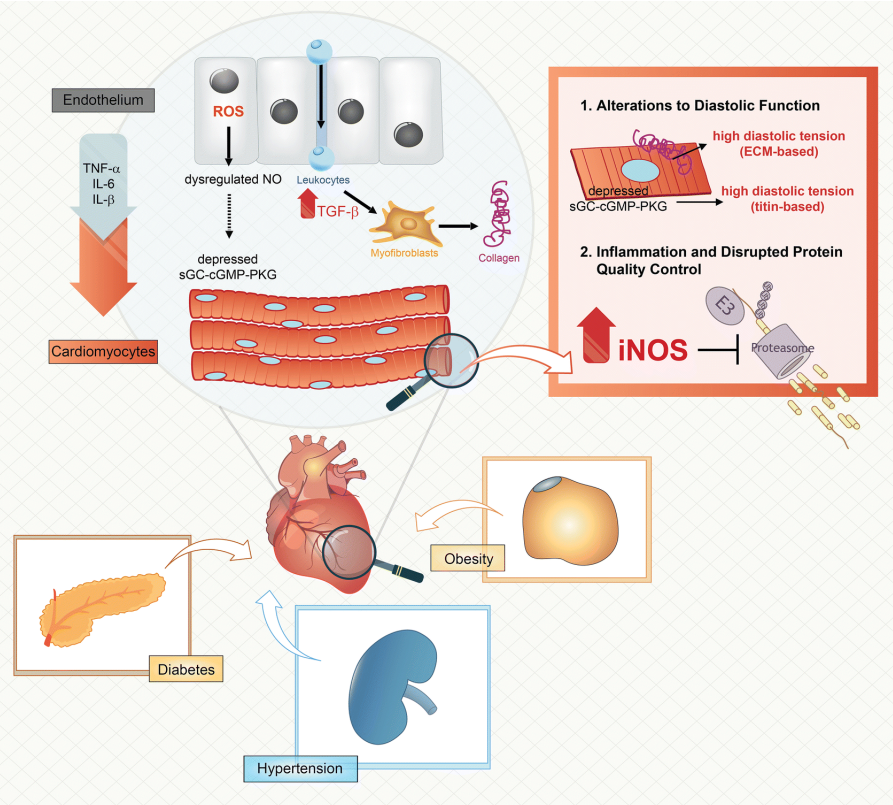

Figure 2: Inflammatory pathways in HFpEF[32]

According to this model, coronary microvascular inflammation and dysfunction lead to cardiomyocyte hypertrophy, stiffness, and myocardial fibrosis.[45] Comorbidities accompanying HFpEF, such as, in particular, metabolic syndrome and diabetes, are associated with low-grade chronic inflammation.[46–48] HFpEF patients have increased circulating markers of inflammation.[49] It has been postulated that the primary cardiac alterations in HFpEF are coronary microvascular endothelial dysfunction, oxidative stress, and inflammation. (Fig. 2) Local release of cytokines,

including transforming growth factor-β (TGF-β), stimulate the differentiation of fibroblasts into myofibroblasts, which secrete collagen, in turn, making the ECM stiffer.[45,50]

Interleukin-1β Production and Signaling

The IL-1 family has 11 cytokines and 10 receptors; two related genes code for the isoforms IL-1α and IL-1β that are part of this family. IL-1α is synthesized as a fully active peptide that remains membrane-bound or may be released from the cytoplasm during cell death. IL-1α thereby participates more prominently in the local response to injury and less in the systemic inflammatory response.[51,52] IL-1β, the main form of circulating IL-1, is initially synthesized as a precursor (proIL-1β) that becomes activated by caspase-1 cleavage in the setting of a macromolecular structure known as the inflammasome, a multiprotein complex that contributes to the recognition of harmful intracellular substances.[51,53] Sterile cellular debris activate sensing pathways[33] and elicit the assembly of inflammasomes. Among the five different types of inflammasomes, the nucleotide oligomerization domain (NOD)-like receptor pyrin domain-containing 3 (NLRP3) inflammasome has been identified in the myocardium as a potential player in maladaptive post-ischemic cardiac remodeling.[54] The scaffolding proteins ASC and procaspase-1 are the core inflammasomes. In response to stimulation, the assembly of the complex of NLRP3, ASC, and procaspase-1 autoactivates caspase-1, which is then responsible for the activation of pro-inflammatory cytokines such as IL-1β or IL-18.[32] IL-1α is an inflammasome-independent cytokine that binds to the same receptor as IL-1β, the IL-1 receptor type 1 (IL-1R1). However, the inflammasome formation can promote the release of IL-1α from the cytoplasm.[55] Caspase-1 also participates in the secretion of active IL-1β that can partake in autocrine, paracrine, and endocrine signaling.[51,52] Both IL-1 isoforms induce proIL-1β synthesis, as do other proinflammatory stimuli like numerous Toll-like receptor agonists.[51,56]

Many other potential triggers of the inflammasome have been identified, including microbial antigens, cell debris, ATP, ischemia, cholesterol crystals, and other Toll-like receptor ligands such as DAMPs or PAMPs.[53,57–60] Activation of the inflammasome after tissue injury induces a local surge of IL-1β that significantly amplifies the inflammatory response, recruiting more inflammatory cells, stimulating metalloproteinase activities, and ultimately inducing inflammatory cell death (pyroptosis) in leukocytes and resident cells.[61–63]

IL-1 mediated inflammation is initiated when IL-1 binds its two receptors, IL-1R1, the ligand binding chain, and the IL-1 receptor accessory protein (IL-Rap or IL-1R3), the coreceptor. Both IL-1α and IL-1β bind to IL-1R1 to cause a structural change that allows IL-1R3 to bind and form a heterotrimeric complex. The intracellular domains of each of the two receptors contain the Toll-IL-1-receptor (TIR), which is found in all members of the IL-1 family of receptors. Because this domain is nearly identical to the TIR domain of TLRs (Toll-like receptors) associated with microbial pathogens, the IL-1 family and the TLR family share the same proinflammatory mechanisms for inducing inflammation. When the two intracellular complexes come together, the adaptor protein, MyD88 (myeloid differentiation factor 88) binds to the TIR domain. The binding of MyD88 leads to a rapid cascade of phosphorylation resulting in the activation of NF-kB (nuclear factor kappa-light-chain-enhancer of activated B cells).[55]

Interleukin-1 and Inflammation

There is a significant correlation between declining functional class and increasing levels of inflammatory cytokines in HF patients.[37] Clinical evidence points to increased IL-1β activity in patients with HF, with IL-1 blockade improving the exercise capacity in HF patients. IL-1 is an apical proinflammatory mediator in acute and chronic inflammation and an inducer of the innate immune response.[51,52] Its production and activity are finely regulated as very small levels

of exogenous IL-1 can induce sepsis-like syndrome and shock.[51,52] IL-1 induces synthesis of many secondary inflammatory mediators as well as inducing its own production and processing, which is a key process in the pathogenesis of many autoinflammatory diseases.[51,52,64]

IL–1β is a prototypal inflammatory cytokine that causes an acute phase reaction following tissue injury and is persistently elevated in patients with chronic HF.[65–67] In early investigations of septic cardiomyopathy, IL-1β was identified as a soluble "depressant factor" in the sera, producing a concentration-dependent depression of myocyte contractility in vitro.[67] Further studies identify a pathologic role for IL-1β in ventricular remodeling, systolic dysfunction, and cardiomyocyte death in both ischemic and non-ischemic models of HF.[67–72] IL-1β has also been shown to affect β-adrenergic receptor sensitivity in vitro, which may be a key determinant of exercise tolerance in HF patients.[73,74] IL-1β contributes to the pathogenesis of HF by inducing both systolic and diastolic dysfunction. (Fig. 3)

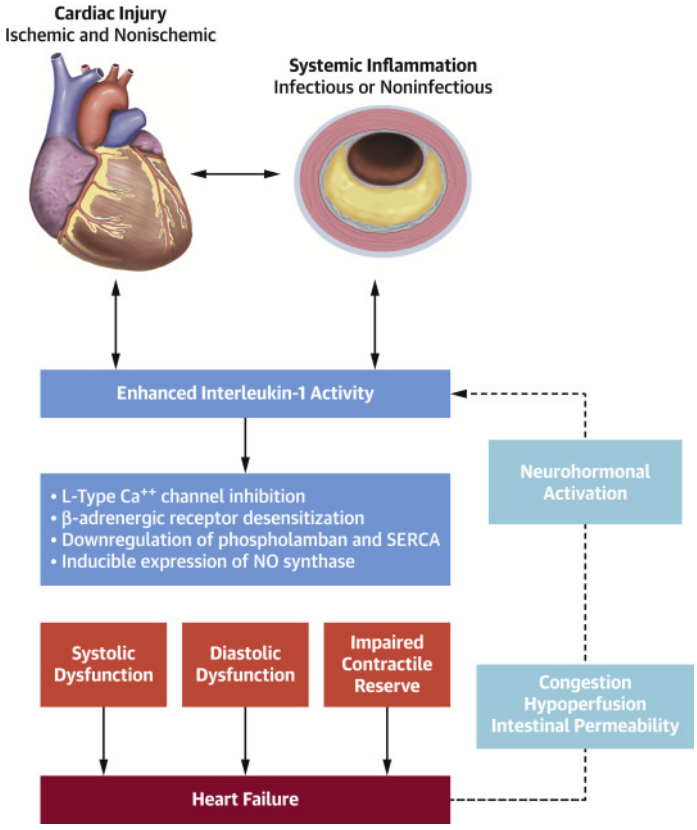

Figure 3: IL-1 signaling in HF[36]

Systolic function is impaired through the uncoupling of both L-type calcium channels and adenylyl cyclase to β-adrenergic receptors, resulting in desensitization to endogenous or exogenous β-adrenergic agonists. Diastolic dysfunction occurs due to impaired calcium reuptake by the SR through down-regulation of phospholamban and SERCA.[36]

Although IL-1β is identified in HF, the direct effects of IL-1β on exercise capacity are unknown. In healthy mice, administration of recombinant-mouse IL-1β (rmIL-1β) induces acute systolic dysfunction, peaking 4 hours after administration. Continuous injections of rmIL-1β for 15 days produce a continuous dysfunction that resolves within 5 days. (Fig. 4)

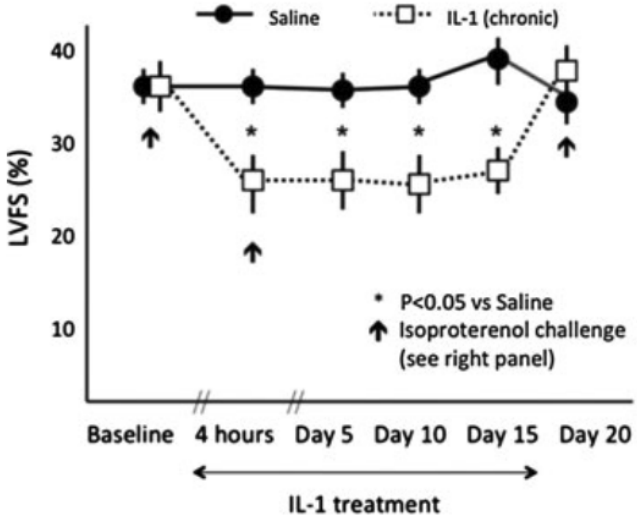

Figure 4: Effects of recombinant IL-1β on left ventricular function in the mouse. IL-1β induces a reversible reduction in left ventricular function that was maintained throughout chronic dosing at day 15. By day 20, 5 days after stopping IL-1β, LVFS had returned to values close to baseline.[75]

The effects of IL-1β and IL-18 on contractility are transient and reversible,[75] supporting the hypothesis that blocking these cytokines could improve or restore cardiac function in humans. The link between IL-1β and HF is also supported by elevated circulating levels of IL-1β as well as surrogate biomarkers such as IL-1Ra (IL-1 receptor antagonist), IL-6, or CRP, each of which correlates with worsening HF symptoms and outcomes.[37,76–79]

The definitive data from the CANTOS (Canakinumab Anti-inflammatory Thrombosis Outcomes Study)[80] established that IL-1β blockade with canakinumab, an IL-1β antibody, in

patients with stable atherosclerotic CVD prevents recurrent cardiovascular events and, thus, provides compelling proof for the IL-1β atherothrombosis concept.[81] The benefit of canakinumab on clinical events was independent of any effect on lipoproteins or blood pressure, and it was closely related with the inflammatory response, as patients showing the greatest reduction in C-reactive protein (CRP) had improved survival with canakinumab.[82] This preclinical data shows that IL-1 and the NLRP3 inflammasome are involved in the formation and progression of the atherosclerotic plaques, and IL-1β blockade with canakinumab reduces cardiovascular events in patients with previous coronary atherothrombotic events.[55]

In a study of 23 patients with rheumatoid arthritis—a condition with elevated IL-1β levels—a single dose of 150 mg of anakinra, a recombinant IL-1Ra, resulted in a significant improvement in cardiac and vascular function.[83] In another study, anakinra administered to patients with stable chronic systolic HF resulted in a significant reduction in circulating levels of CRP and IL-6 levels; these patients experienced a significant improvement in cardiorespiratory function (measured as peak oxygen consumption) and quality of life related to cardiac fitness.[84] In a study with patients hospitalized for acute decompensated systolic HF, anakinra initiated within 24 hours of admission had significantly reduced acute inflammatory responses compared with placebo and after 14 days of treatment, LVEF had improved.[85] In another study with patients hospitalized for acute decompensated systolic HF, anakinra was initiated at discharge and continued for 12 weeks which resulted in improved cardiorespiratory function, reduced levels of NT-proBNP (N-terminal pro-B-type natriuretic peptide), and increased quality of life.[86] A study with 31 HFpEF patients showed significant increases in treadmill exercise time, lower NT-proBNP levels, and improved quality of life measures without significant change in peak oxygen consumption.[87] This preclinical data shows that the IL-1 contributes to cardiac

dysfunction, and IL-1 blockade with anakinra or canakinumab in patients with HF improves cardiorespiratory fitness and prevents HF hospitalizations.[55]

Contractile Reserve

Myocardial contractile reserve refers to the difference between myocardial contractility at rest and during stress either through physiological or pharmacological means. The assessment of contractile reserve provides an indication of cardiac reserve, which is the maximum amount that cardiac output can increase from during a state of rest to during a state of stress. Assessment of the presence of contractile reserve relies on the assessment of change in systolic function or left ventricular.[88] Contractile reserve is measured as the difference in left ventricular function at rest and under stress. Exercise and inotropic stress have been used as stress protocols for the assessment of contractile reserve. Both stresses provoke a generalized increase of regional wall motion as an increment of ejection fraction.[89]

Myocardial contractile reserve is associated with prognostic biomarkers and molecular expression in cardiomyocytes. Left ventricular inotropic reserve is associated with exercise capacity and correlates with peak oxygen consumption (peak VO_2) in cardiopulmonary exercise testing.[90–92] Patients with a greater increase in myocardial contractile reserve achieve a greater peak VO_2.[92] Impaired left ventricular contractile reserve has been shown to be associated with cardiac sympathetic dysfunction.[93] Reduced adrenergic myocardial contractile reserve is related to myocardial expression of contractile regulatory protein genes, such as β_1 adrenergic receptor, sarcoplasmic reticulum calcium-adrenergic triphosphatase, and phospholamban.[94]

Patients with markedly reduced contractility at rest, but a healthy residual contractile reserve, have a favorable exercise capacity, while patients with mildly reduced contractility at rest, but significantly reduced contractile reserve, have poor exercise capacity.[95] Studies have

indicated that evaluating the myocardial contractile reserve in pharmacological stress testing and peak VO_2 in cardiopulmonary exercise testing may be complementary approaches to predict a prognosis of non-ischemic dilated cardiomyopathy. Furthermore, impaired contractile reserve by pharmacological stress testing may be associated with molecular remodeling caused by overactivation of the SNS.[96]

The use of stress echocardiography to assess contractile reserve has shown promise as a prognostic indicator in the assessment of systolic HF.[97] There are reported indications that the presence of residual contractile reserve is associated with a good prognosis and impaired contractile reserve is affected by multiple factors including, but not limited to, exercise intolerance, cardiac sympathetic dysfunction, reduced myocardial blood flow and histopathological changes. Additionally, there is a suggestion that myocardial contractile reserve would predict a reversibility of left ventricular dysfunction after initiation of cardioprotective therapy.[96] Previous studies have shown that repeated administrations of IL-1β induces reversible, acute and chronic, contractile dysfunction and impaired β_1 adrenergic responsiveness. (Fig. 5)

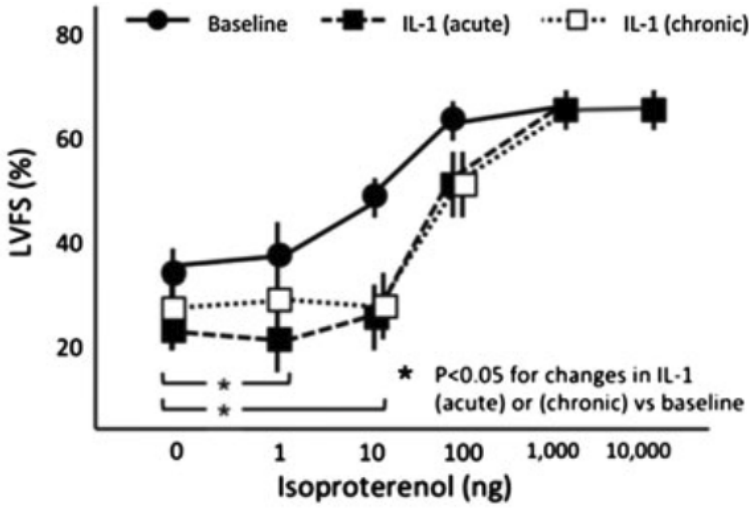

Figure 5: Effects of recombinant IL-1β on left ventricular function in the mouse and contractile reserve signaling in HF. IL-1β-treated mice showed impaired contractile reserve with a right shift of the dose–response curve to isoproterenol at 4 hours (acute) and 15 days (chronic).[75]

Goal of the Study

The central hypothesis of the study was that IL-1β diminishes the exercise capacity in the mouse. The first aim of the study was to determine whether IL-1β decreases exercise tolerance in the mouse. The second aim of the study was to determine whether changes in the systolic function and contractile reserve correlate with changes in exercise tolerance.

Methods and Materials

Ethical Approval

All experimental procedures were conducted in accordance with the "Guide for the Care and Use of Laboratory Animals" published by the National Institutes of Health (8th ed. revised 2011). The study protocol was approved by the Institutional Animal Care and Use Committee (IACUC) of Virginia Commonwealth University.

Study Design

This study tested changes that occur to exercise tolerance, left ventricular ejection fraction, and contractile reserve, 4 hours and 96 hours after administration of IL-1β to mice. The 4-hour time point was chosen because it has been previously reported that IL-1β induces a significant reduction in LVEF 4 hours after administration, with no changes seen in vehicle-treated mice at 4 hours. (Fig. 6)

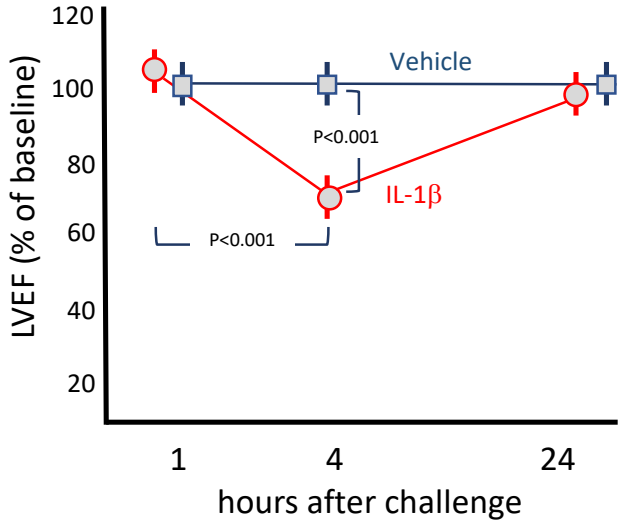

Figure 6: IL-1β-induced left ventricular systolic dysfunction in mice. Mice either treated with IL-1β 3 μg/kg or vehicle. LVEF assessed at multiple time points as a percentage of the baseline (hour 0) LVEF from hour 0 to hour 24.

The 96-hour time point was chosen to provide confidence of normalization of LVEF. In a previous study, mice were injected with increasing doses of IL-1β, which 4 hours after

administration, produced significant reductions in LVFS at all doses ≥ 0.3 μg/kg. IL-1β 3 μg/kg was characterized as a standard dose in subsequent experiments as this dose was the minimum required to significantly impair contractile function and appeared to give the greatest numerical reduction in LVEF.[84]

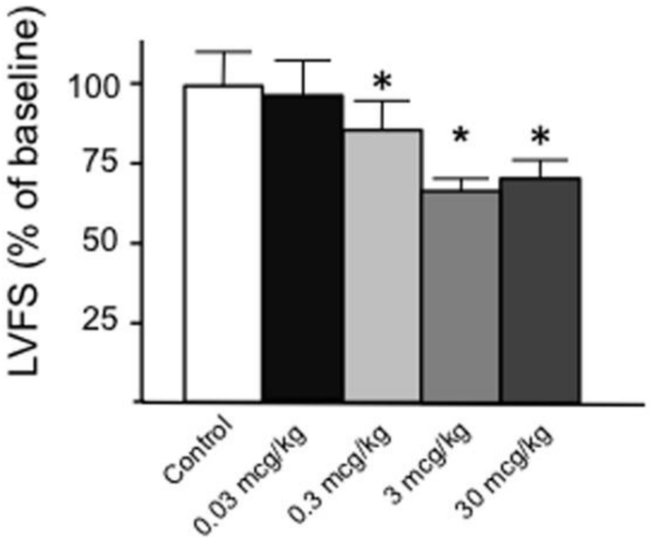

Figure 7: Healthy, adult, mice underwent baseline echocardiography followed by a single intraperitoneal injection of recombinant human IL-1β (0, 0.03, 0.3, 3, or 30 μg/kg) and subsequent echocardiography at 4 hours. All doses of IL-1β ≥0.3 μg/kg produced a significant 28 – 32% reduction in left ventricular fractional shortening (LVFS) at 4 hours.[84]

In this study 52 male Institute of Cancer research (ICR) mice, 8-10 weeks old and weighing 35-45g, supplied by Envigo (Indianapolis, IN), underwent exercise training and testing. Based on their baseline exercise capacity, 17 mice were chosen and treated with either IL-1β (n=10) or a vehicle (n=7). The mice were then tested again 4 hours and 96 hours after administration to assess the change in their exercise tolerance. A separate group of 25 mice were assessed for their baseline LVEF via transthoracic echocardiography (TTE). Of these, 13 were treated with IL-1β and 12 were treated with a vehicle, and their LVEF was assessed 4 hours and 96 hours after administration. The IL-1β-treated mice that did not experience a reduction in LVEF 4 hours after administration were eliminated from the study. The remaining mice underwent an isoproterenol challenge to test the heart's contractile reserve.

Exercise Testing Protocol

Clinical manifestations of HF include shortness of breath including exercise-induced dyspnea, exercise intolerance, and fatigue. These symptoms are prominent in numerous other diseases such as diabetes, which also plays a role in perpetuating HF. A preclinical assay of fatigue and exercise capacity for rodents is the treadmill.[98] Fatigue testing via the rodent treadmill was a useful testing method in this study where a significant change was seen following administration of IL-1β.

The exercise training and testing protocol used in this study was adapted from Dougherty et al., J Vis, 2016.[98] The treadmill used was the Exer 3/6 animal treadmill from Columbus Instruments. It has the capability of exercising up to three rats or six mice, simultaneously, in individual lanes (5.7 x 41.3 cm for mice, 11.8 x 41.3 cm for rats). The belt speed can be adjusted from 0.0 to 102.3 m/min, and the running surface can be inclined from 0° to 25° above horizontal in 5° increments. A stimulus can be created using the electrical shock grid system composed of six shock grids, each with an individual on/off switch. The intensity and repetition rate of the stimulus is user controlled (current 0.34 – 1.60 mA, duration 200 msec, repetition rate 1, 2 or 3 Hz). For this study, the treadmill was fixed at a 20° incline and configured to allow six mice to run at a given time. The shock grid was fixed at 1.22 mA and 2 Hz.

Treadmill exercise testing was used to measure fatigue/exercise intolerance as an effect of administration of IL-1β. Animals were forced to run to exhaustion on a conveyor belt with gradually increasing speeds. The animals were motivated to run until they were unable or unwilling to continue running as a means of escaping electrical shocks. Testing ended when the animals reached exhaustion, which was defined as at least 5 seconds in the fatigue zone. The

fatigue zone was defined as the region of the conveyor belt that is within approximately 1 body length of the shock grid and includes the shock grid itself.

Mice were properly acclimated to the treadmill prior to any experimentation. The training consisted of three days of subsequently increasing speeds. On day 1, the mice were placed directly on the treadmill belt in each lane, with the speed at 0 m/min and the shock grids on. The mice were allowed to explore the treadmill for 5 minutes. The treadmill was turned on and the speed was slowly increased to 8 m/min. All the mice were monitored to ensure they were walking against the direction of the belt. If a mouse did not begin walking or was walking toward the shock grid, they were readjusted by tapping them with a brush or by tail tickling. If a mouse refused to walk it was excluded from the study. The mice ran for 5 minutes, then the speed was increased to 9 m/min for 7 minutes, and finally to 10 m/min for 10 minutes. On day 2 of training, the mice were placed on the belt and the treadmill was turned on at a speed of 10 m/min. After 5 minutes, the speed was increased to 11 m/min for 10 minutes, and finally to 12 m/min for 15 minutes. On day 3 of training, the mice were placed on the belt and the treadmill was turned on at a speed of 12 m/min. After 5 minutes, the speed was increased to 13 m/min for 10 minutes, and finally to 14 m/min for 15 minutes. At the end of each session, the shock grid was turned off and the mice were allowed to rest for 2 minutes before being removed from their lanes. The mice were rested for 1 day and then underwent a preliminary exercise test to establish their baseline capacity.

Before starting the treadmill test, the treadmill was set to a speed of 5 m/min. Mice were individually placed in each lane and the corresponding shock grid was turned on. The mice ran at a speed of 5 m/min for 5 minutes. The speed was then increased to 14 m/min and the speed was increased by 2 m/min at 3 minute intervals. The mice were forced to run until they reached a

state of exhaustion at which point their individual shock grid was turned off, allowing them to rest. When all the mice reached exhaustion, the treadmill was turned off and they were allowed to rest for 2 minutes before being removed. A stopwatch was used to monitor the amount of time each mouse ran. The speed and time were used to determine the distance each mouse ran, which was representative of their exercise capacity.

Treatment and Testing

The mice were rested for 2 days following the baseline test before they were injected intraperitoneally. The recombinant IL-1β was administered at a dose of 3 μg/kg in 200 μL, while the 0.9% normal saline was administered in the same volume. Exercise capacity was assessed as a measure of distance run on a treadmill at baseline, and 4 hours and 96 hours after injection.

Transthoracic Echocardiography

Transthoracic echocardiography (TTE) was performed to assess the changes in LV systolic function in IL-1β-treated mice compared to vehicle-treated mice. TTE is a noninvasive imaging technique that uses ultrasound technology to allow visualization of the heart in real-time and is an important diagnostic tool for a variety of cardiac pathologies. The high-frequency sound waves are produced by a piezoelectric crystal contained in a transducer.[99] The transducer emits and then detects ultrasound waves that are reflected from the tissue. These waves are converted into electrical signals and converted to images based on the density of the tissue.

The echocardiography system used in the study was the Prospect T1 Scintica Instrumentation Inc. with a transducer probe with a high transmit frequency of 30 MHz. Before the TTE was performed, each mouse was sedated with ketamine/xylazine (100/10 mg/kg), and their upper and lower chest was shaved. The mice were then placed and secured in a supine position on a warmed platform. The TTE operator applied warm ultrasound transmission gel to

the animal's chest and then placed the transducer probe on the thoracic region to visualize the heart from the parasternal short-axis view.

For this study, the animal's hearts were visualized using B-mode and M-mode in the echocardiography system. When the transducer was placed in view of the parasternal short-axis, the B-mode produced images of the heart that allowed visualization of ventricular wall motion and ventricular size. Using these images, the probe was placed to allow visualization of the left ventricular wall and, using the M-mode, the left ventricular end-diastolic diameter (LVEDD) and left ventricular end-systolic diameter (LVESD) were measured. These left ventricular structural parameters were utilized to calculate LVEF.

LVEF is a measure of the contractile function of the heart and was calculated using the Teichholz formula. The measured LVEDD and LVESD were converted to volumes using the following formulas:

$$\textbf{LVEDV} = (7*LVEDD^3)/(2.4+LVEDD)$$

$$\textbf{LVESV} = (7*LVESD^3)/(2.4+LVESD)$$

Using the values for left ventricular end-diastolic volume (LVEDV) and left ventricular end-systolic volume (LVESV), the LVEF was calculated with the following formula:

$$\textbf{LVEF} = (LVEDV - LVESV)/LVEDV*100$$

The LVEF was assessed at baseline to ensure that the mice met the inclusion criteria. The acceptable LVEF for inclusion was >58% at baseline. The selected mice were randomized into two groups: IL-1β-treated and vehicle-treated groups. The LVEF was assessed again 4 hours and 96 hours after intraperitoneal administration of 200 μL IL-1β 3 μg/kg for the IL-1β-treated group or 200 μL 0.9% normal saline for the vehicle-treated group. The echocardiography operator was blinded to the treatments.

Isoproterenol Challenge and Calculation of the Contractile Reserve

Immediately following the 4-hour and 96-hour echocardiogram, isoproterenol (10 ng) was injected intraperitoneally to each sedated mouse while in the supine position on the platform. The isoproterenol dose was chosen from a dose-response study in which 10 ng/mouse was the highest dose possible before there was a saturated response as seen in Figure 7.

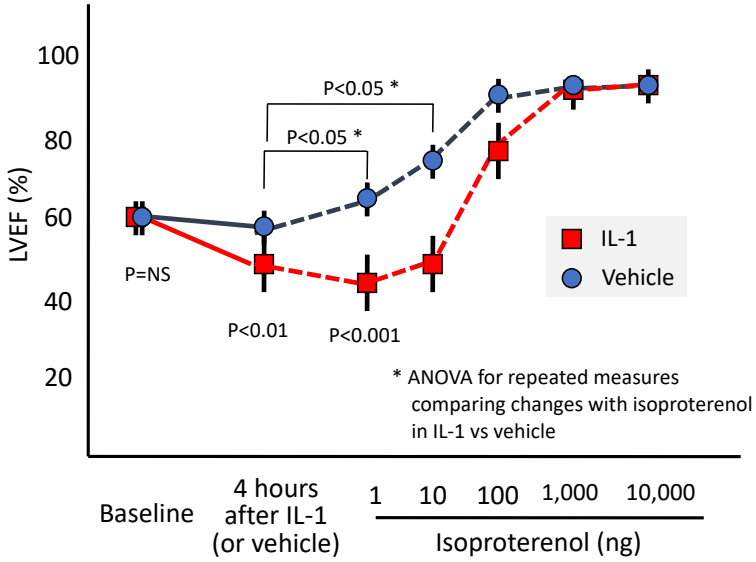

Figure 8: IL-1β induces β-adrenergic desensitization in the mouse as demonstrated by rightward curve shift during the isoproterenol challenge. Absolute and relative changes in LVEF are shown with increasing doses of isoproterenol: a right-ward shift of the dose-response curve reflects desensitization (N=8 per group).

Additional mice were used to measure the contractile reserve in the absence of treatment and were used as reference controls (N=6/group). The purpose of the challenge was to assess myocardial contractile reserve. Isoproterenol is a potent, synthetic, β-adrenergic receptor agonist that has positive chronotropic and inotropic properties through β-adrenergic stimulation. The effect of IL-1β on systolic function, contractile reserve, and β-adrenergic sympathetic responses was characterized using the isoproterenol challenge. Using TTE, the LVEF was measured 3-5 minutes after administration of isoproterenol, and the percent increase in LVEF was assessed. The difference from rest in these parameters, expressed as percentage change, indicates the

cardiac reserve. The cardiac reserve was calculated using the following formula: ((LVEF during stress - LVEF at rest)/ LVEF at rest) * 100 = Cardiac Reserve

Statistical Analysis

All data presented utilized values derived from the procedures explained above. Additionally, the values from these procedures are presented as the mean, standard error of the mean, and absolute values. For the exercise tolerance data, a One-way ANOVA was used to compare changes over time for the vehicle and IL-1β groups. An unpaired T-test was used for comparison between the vehicle and IL-1β groups at each time point. For the echocardiography data, a paired T-test was used for comparison from baseline to each time point for both the vehicle and IL-1β groups. For the contractile reserve data, an unpaired T-test was used for all comparisons. Unadjusted p-values are reported throughout, with the statistical significance set at a 2-tailed distribution and a significance level of 0.05 and 0.01.

Results

Effects of IL-1β on Exercise Tolerance

There were three rounds of exercise training and testing culminating in the use of 52 mice total. After each round of exercise training and baseline testing, mice were chosen for the study based on the distance they ran, which represented their exercise capacity. The mice that ran less than 220 meters and more than 280 meters were excluded. This represented the lowest third and highest third of performers. To ensure the vehicle and IL-1β groups had similar averages, the middle performing group was ordered from least to greatest and every other mouse was assigned to one group while the rest were assigned to the other. Ultimately, there were 7 vehicle-treated mice and 10 IL-1β-treated mice.

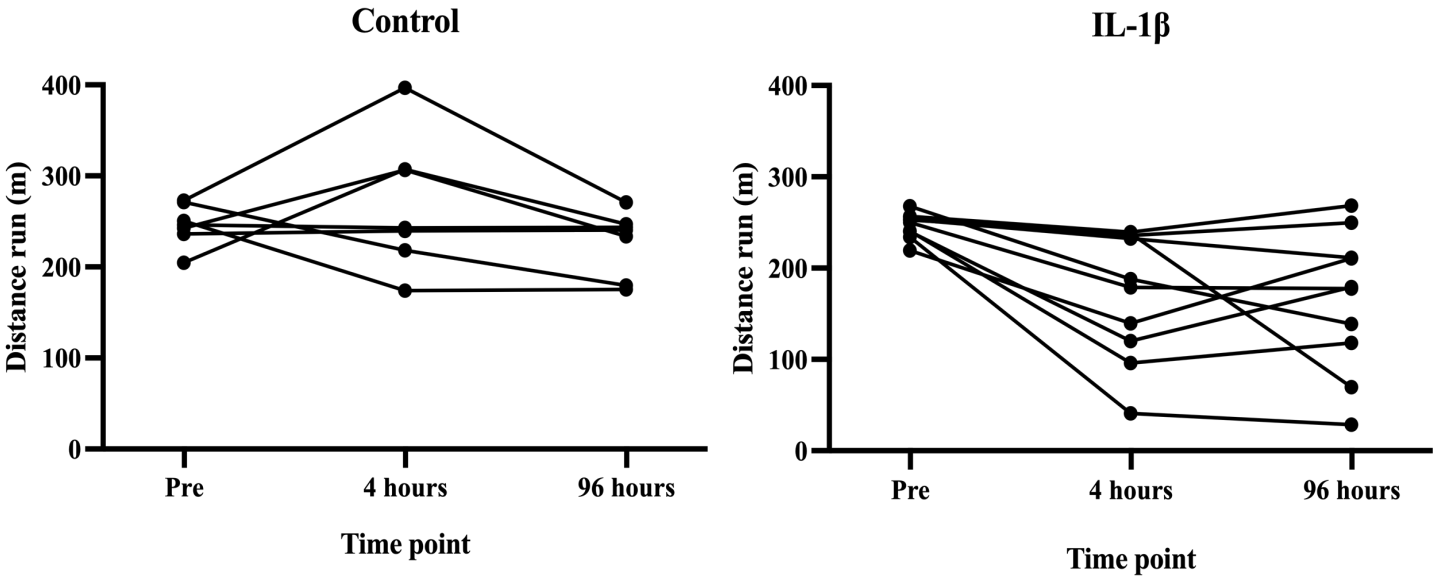

Figure 9: Absolute values of distance run at each time point. Left panel shows the values for the vehicle-treated group and right panel shows the values for the IL-1β-treated mice at before, 4 hours, and 96 hours after administration.

The baseline averages for the vehicle-treated and IL-1β-treated groups in the exercise capacity test were 246 meters and 247 meters, respectively. Administration of IL-1β significantly reduced exercise capacity after 4 hours, by 32%, and after 96 hours, by 33%, compared to baseline capacity. The P value for the comparison from each of the time points to baseline for the

IL-1β-treated group was less than 0.05. There was no significant effect observed in the vehicle-treated group after 4 hours or after 96 hours. The exercise capacity for the vehicle-treated group was significantly greater than the capacity of the IL-1β-treated group at both time points. The P value comparing the vehicle-treated and IL-1β-treated groups at each time point was less than 0.05.

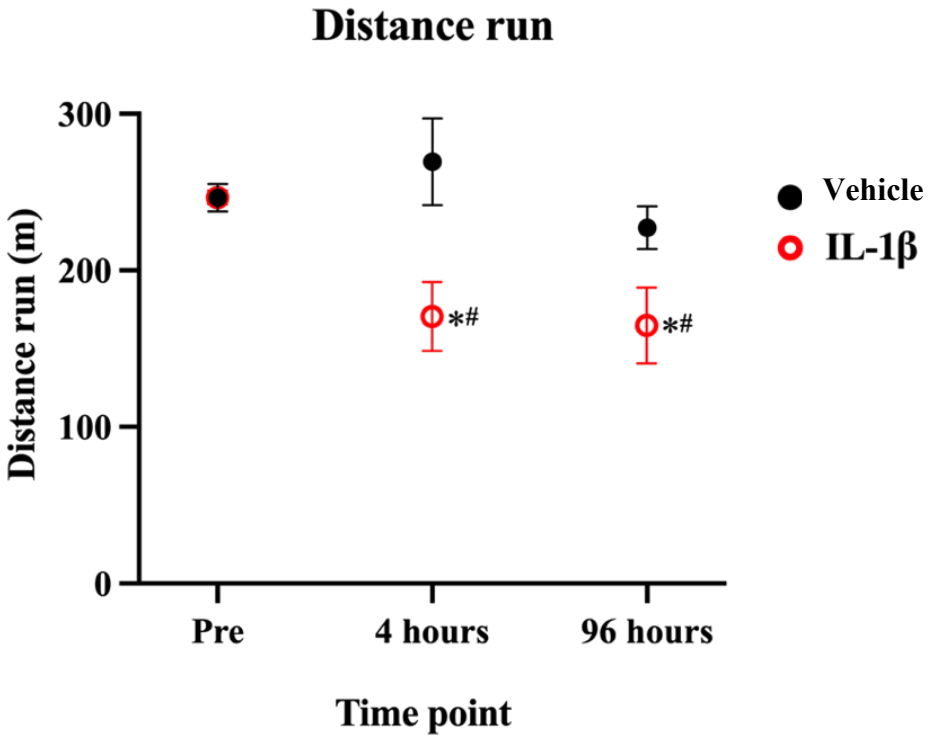

*Figure 10: Averages of distance run by both control and experimental groups during exercise testing before, 4 hours, and 96 hours after administration of a vehicle or IL-1β treatment (*P<0.05 vs Pre; #P<0.05 vs Vehicle).*

Effects of IL-1β on Systolic Function

The resting LVEF was used as an evaluation of systolic function in the mice. The baseline averages for the vehicle-treated and IL-1β-treated groups were 61% and 59%, respectively. Resting LVEF was measured before, 4 hours, and 96 hours after administration of vehicle or IL-1β. The LVEF for the IL-1β-treated group was significantly reduced 4 hours after administration. After 4 hours, there was a 29% reduction with a P value less than 0.01 compared to the baseline LVEF. After 96 hours, LVEF had recovered to the pre-treatment values seen at

baseline. The vehicle-treated group showed no significant change in LVEF after administration of the vehicle at each of the time points. The average LVEF at 96 hours for the vehicle-treated and the IL-1β-treated groups were 62% and 59%, respectively.

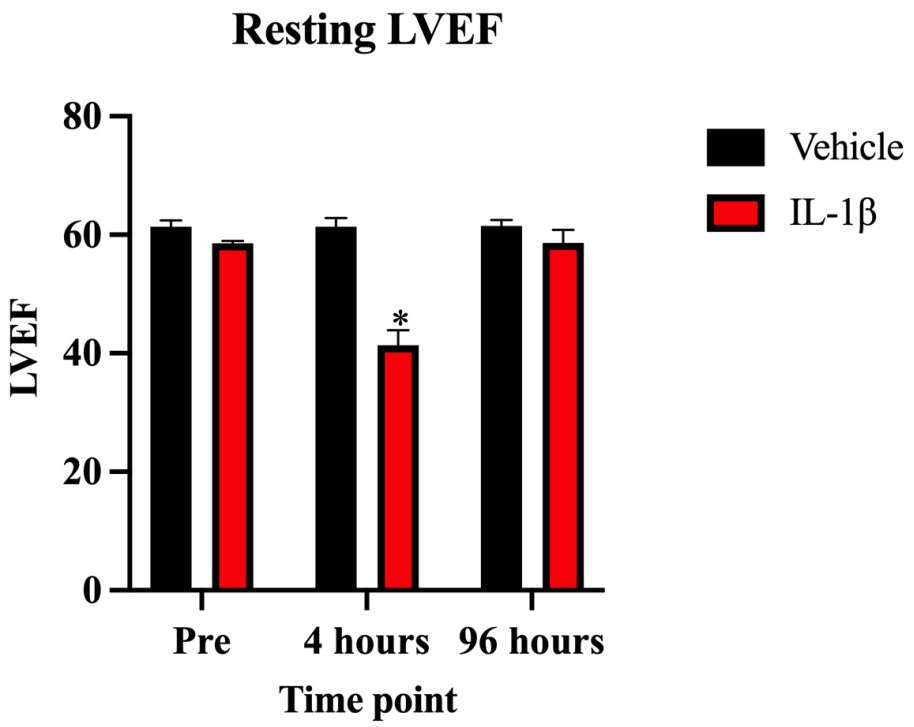

*Figure 11: Average resting LVEF for mice before, 4 hours, and 96 hours after administration of a vehicle or IL-1β treatment (*P<0.01 vs Pre)*

Effects of IL-1β on Cardiac Reserve

Cardiac reserve was demonstrated as the change in LVEF after treatment with isoproterenol, a β-adrenergic stimulator. It was calculated as the percent change from rest to stress of the LVEF. The change in LVEF at baseline for the vehicle-treated and IL-1β-treated group were 23% and 26% increase, respectively. There was a diminished cardiac reserve for the IL-1β-treated group 4 hours after administration of IL-1β. The increase in LVEF was 7% after 4 hours and 2% after 96 hours. The change in LVEF was significantly reduced at both time points with a P value of 0.01 compared to baseline. The change in LVEF in the vehicle-treated group was not significantly different at the either of the time points. The P value for the comparison

between the IL-1β-treated and the vehicle-treated group was less than 0.05 at the 4-hour time point and less than 0.01 at the 96-hour time point.

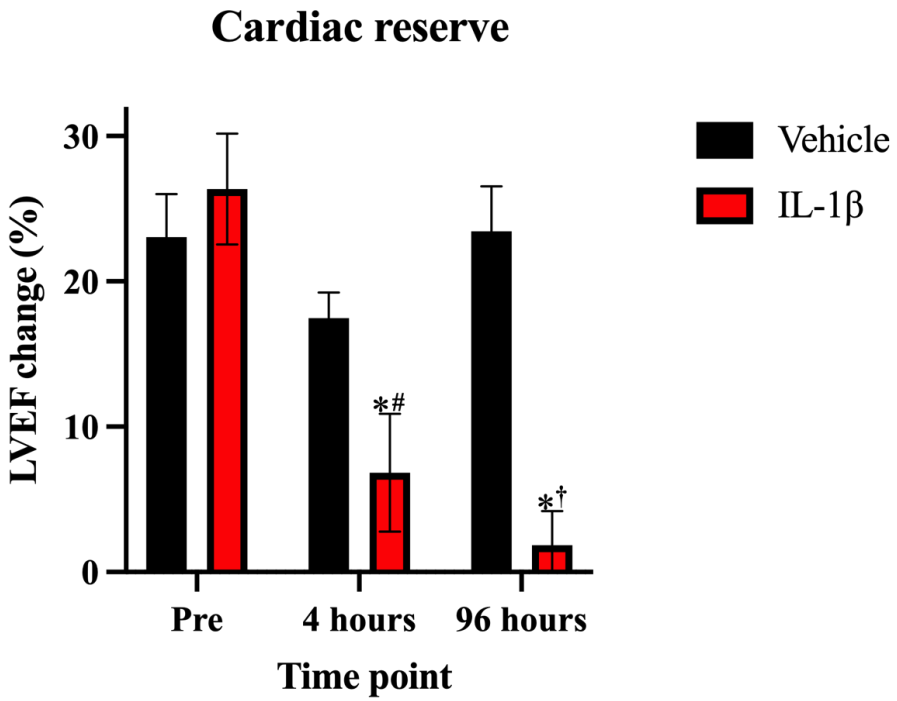

*Figure 12: Cardiac reserve represented as LVEF change from rest to after β-adrenergic stimulation (*P<0.01 vs Pre; #P<0.05 vs Vehicle; †P<0.01 vs Vehicle)*

Discussion

Improving functional capacity, clinical outcomes, and quality of life in patients with HF remains an unmet clinical need. Inflammation promotes and aggravates HF.[100,101] The effects of inflammatory cytokines such as IL-1β in HF are a continuous topic of research. The results of the present study indicate that exogenous IL-1β significantly reduces exercise capacity in mice 4 hours and 96 hours after administration. These results are significant as they are independent of the changes in LVEF caused by administration of IL-1β. LVEF is only impacted 4 hours after IL-1β and normalizes by 96 hours.

There are several reports that show that exercise training and exhaustive exertion increase levels of several cytokines, but there is little knowledge on the effects of select cytokines on the exercise performance. IL-6 is known to increase during exercise, and it increases exercise tolerance.[102] A single injection of IL-1β significantly increases the IL-6 levels within 4 hours.[103] However, in our study, the treatment with recombinant IL-1β produced a reduction in exercise tolerance within 4 hours and, therefore, suggests that the effects of IL-1β and IL-6 on exercise capacity are different. Cavalli et al. demonstrated that in mice, a low dose of lipopolysaccharide (LPS, 10 mg/mouse), a compound from the outer-membrane of Gram-negative bacteria that binds to the TLR-4, reduced their treadmill run time.[104] The administration of IL-37, a cytokine of the IL-1 family that has anti-inflammatory activity, prevented this decrease. Our dose of IL-1β was much smaller compared to the LPS, and despite this, it potently reduced the exercise time of our mice. The IL-1R1 and TLR-4 have similar downstream pathway and it is possible that they similarly impair the exercise tolerance of mice. However, a dose of LPS lower than 60 mg/mouse (equivalent to 2 mg/kg) is insufficient to induce a reduction in LVEF.[105,106] Therefore, we can hypothesize that in the model used by Cavalli and colleagues, the LVEF was likely

unaffected by the LPS treatment. We found that exercise intolerance was not dependent on the LVEF because the LVEF normalized after 96 hours, while the exercise tolerance of the mice was still impaired. Therefore, the resting LVEF does not correlate with the exercise capacity of the mice. However, the cardiac reserve was significantly impaired after 4 and 96 hours compared to the vehicle-treated mice. This suggests that the administration of IL-1β reduces exercise capacity through an impairment in cardiac reserve. The reduction in exercise capacity and cardiac reserve after 96 hours indicates a prolonged effect from IL-1β that was previously not reported without repetitive administration of IL-1β. A study by Van Tassell et al., conducted at Virginia Commonwealth University, has shown that targeting IL-1 using a recombinant human IL-1 receptor antagonist in patients with HF increases the exercise tolerance of the patients.[107] Thus, our study confirms a direct role of IL-1β as a negative regulator of exercise tolerance.

This present study had several potential drawbacks and limitations. Some of the more common limitations seen in preclinical studies relate to the use of murine models and the lack of diverse subjects. Limitations included using only one breed, one sex, using only relatively young healthy male mice, and having a small sample size. As discussed above, cytokines of the IL-1 family are elevated and contribute to several cardiovascular diseases, including atherosclerosis, atherothrombosis, and AMI in obesity, and diabetes, hypertension, and dilated cardiomyopathy.[108–110] In those conditions, IL-1β is not the only cytokine that is elevated, and the levels of several dozens of cytokines are altered. However, our goal was to prove that in healthy mice, a single dose of IL-1β could show a physiological effect in the absence of confounding factors. For future studies, performing experiments in mice of different breeds or performing IL-1β blockade in murine models of HF could be a way to limit these drawbacks.

Limitations more unique to this present study are related to the exercise testing protocol that was used as a preclinical assay of fatigue and exercise capacity.[98] The protocol for testing is subjective and can lead to inter-user error, which can lead to imprecise results. However, a single user was responsible for the conduction of all the experiments, therefore minimizing variability.

Lastly, we did not identify a molecular mechanism regulating the effects of IL-1β on the contractile reserve and exercise tolerance. Several pathways that control the contractility and relaxation of cardiomyocytes have been identified. These include an intracellular increase in nitric oxide that can be associated with a reduction of ATP production and with the alteration of cytoplasmatic calcium influx by affecting the coupling of the calcium channel with the β-adrenergic receptor.[111–114] As discussed above, IL-1 also regulates calcium reuptake in the SR through downregulation of mRNA and protein levels of phospholamban and SERCA.[112,115] A separate report describes the role of IL-1 in ischemia-induced systolic dysfunction. [116] More recently, a role for phosphoinositide-3-kinase-γ (PI3Kγ) in IL-1 signaling has been identified as being potentially linked to impaired contractility, phosphodiesterase-3 activity, and β-adrenergic receptor desensitization.[117–121]

An unconventional signaling of the IL-1 receptor through PI3Kγ has been characterized in inflammation and cancer.[118] PI3Kγ expression and activity have been increased in patients with HF and animal models of HF, negatively regulating cardiac contractility and inhibiting the physiologic response to β-adrenergic stimulation.[121,122] PI3K is a family of kinases that generate PIP3, which is a key second messenger involved in downstream signaling of its receptors.[123] The main catalytic protein for the PI3Kγ isoform, p110γ, has adaptor proteins p87 and p101 that regulate the specificity of its function.[121,124,125] Future studies will be performed to define how IL-1β affects the myocardial expression of PI3Kγ and its adaptor proteins looking at gene and

protein expression, as well at the potential use of PI3Kγ inhibitors to prevent the decrease in exercise tolerance due to IL-1β challenge.

In conclusion, the results presented in this study show that administration of exogenous IL-1β significantly reduces exercise capacity through an impairment in cardiac reserve, while being independent of the LVEF at rest.

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