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Involvement of Interleukin-33/ST2 in Myocardial Dysfunction in Murine Model of Sepsis

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A thesis submitted in partial fulfillment of the requirements for the degree in Master of Science

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By

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ABSTRACT

The disruption of myocardial extracellular matrix (ECM) has been implicated in myocardial dysfunction during sepsis. However, the underlying mechanism(s) are not clear. Interleukin-33 (IL-33) is a cytokine which regulates collagen synthesis in various cardiac pathologies. The purpose of the present study is to test whether IL-33 contributes to sepsis-induced myocardial dysfunction through regulation of matrix metalloproteinase-9 (MMP-9). The in vivo, feces-induced peritonitis (FIP) in mice and in vitro lipopolysaccharide (LPS) treatments to isolated cardiomyocytes were used. In FIP mice, myocardial IL-33 and MMP-9 expression were increased and myocardial contractility was decreased. Myocardial dysfunction in FIP mice was prevented when treated with soluble ST2 (sST2), a decoy receptor of IL-33. This study showed for the first time that inhibiting IL-33 prevented cardiac dysfunction and MMP-9 expression in septic mice. In vitro studies produced congruent results. Our results support that IL-33 plays an important role in mediating sepsis-induced myocardial dysfunction.

Keywords: sepsis; myocardial dysfunction; interleukin-33; ST2; matrix metalloproteinase-9; feces-induced peritonitis; lipopolysaccharide; cardiomyocytes

DEDICATION

I dedicate this thesis to my loving family who has been my constant source of inspiration and comfort. My mother's persistent faith in me keeps me going every day and my father's respect for education pushes me to be better. Without them I would not be here.

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ANOVA	Analysis of variance
AP-1	Activator protein-1
CD-14	Cluster of differentiation-14
CLP	Cecal ligation puncture
CO	Cardiac output
CRP	C-reactive protein
dP/dt max	Maximal rate of pressure rise
dP/dt min	Maximal rate of pressure decline
ECM	Extracellular matrix
EF	Ejection fraction
ERK	Extracellular signal-regulated kinase
ESPVR	End systolic pressure volume relationship
FIP	Feces –induced peritonitis
HR	Heart rate
IFN	Interferon
IKK	Inhibitor of NF-κB kinase
IL-1β	Interleukin-1β
IL-1RAcP	IL-1 receptor accessory protein
IL-33	Interleukin-33
IRAK	Interleuin-1 receptor-associated kinase
IRF3	IFN-regulatory factor 3
JNK	c-Jun N-terminal kinases
LAL	*Limulus* amebocyte lysate
LBP	LPS binding protein
LPS	Lipopolysaccharide
LV	Left ventricular
LVEDP	Left ventricular end diastolic pressure
LVEF	Left ventricular ejection fraction
LVESP	Left ventricular end systolic pressure
MAL	MyD88 adaptor-like
MAP	Mean arterial pressure
MAPK	Mitogen-activated protein kinase
MD-2	Myeloid differentiation factor-2
MMP-9	Matrix metalloproteinase-9
MRI	Magnetic resonance imaging
MyD88	Myeloid differentiation factor 88
NF-HEV	Nuclear factor from high endothelial venules

NF-kB	Nuclear factor κB
PAGE	Polyacrylamide gel electrophoresis
PAMP	Pathogen-associated molecular patterns
PBS	Phosphate buffered saline
PV	Pressure-volume
PVDF	Polyvinylidene difluoride
SDS	Sodium dodecyl sulfate
SEM	Standard error of mean
sST2	Soluble ST2
ST2	Suppression of tumorigenicity 2
ST2L	Membrane bound ST2
SV	Stroke volume
SVR	Systemic vascular resistance
SW	Stroke work
TIMP	Tissue inhibitors of metalloproteinases
TIR	Toll/IL-1 receptor
TIRAP	TIR domain-containing adaptor protein
TLR	Toll-like receptors
TNF- α	Tumor necrosis factor-α
TRAF6	TNF receptor-associated factor 6
TRAM	TRIF-related adaptor molecule
TRIF	TIR-domain-containing adapter-inducing interferon-β

CHAPTER 1: INTRODUCTION

Section 1.1: Sepsis

Sepsis is the leading cause of mortality in the critically ill, with mortality rate as high as ~50% [1]. Despite continued advances in modern medicine, the incidence of sepsis and its mortality continue to rise. Epidemiological reports indicate that the incidence is increasing at rates between 1.5% to 8% every year [2].

Sepsis is a syndrome defined as a systemic inflammatory response to an infection. Infection can be triggered by bacteria, virus or fungi and its damage depends on the pathogen's virulence and the resistance of the host to that pathogen. In the case of gram-negative bacteria, various constituents of the cell wall determine its virulence including lipopolysaccharides (LPS), peptidoglycans, lipoproteins and fragellin. These molecules are known as pathogen-associated molecular patterns (PAMPs) and they are recognized by the host's immune system [3]. The host's initial reaction to an infection is local inflammation. First, the innate immune system attempts to eradicate the pathogen using physical barriers, inflammatory cells, proteases, cytokines and chemokines in co-ordination with other components. Inability of the innate immune system to eradicate the pathogen can result in bacteria entering the bloodstream, thus becoming a systemic infection. The host's exaggerated response to the inflammation is called sepsis. In the event of organ failure the term severe sepsis is used. As the disease progresses it may develop into septic shock where hypotension persists despite adequate fluid resuscitation. Gram-negative infections are responsible for a large proportion (38%) of septic cases [4].

The majority of cases of mortality in septic patients are from multiple-organ dysfunction rather than the initial infection [2]. Septic patients experiencing myocardial dysfunction have a much poorer prognosis than those without cardiac complications [5].

A better understanding of causes and mechanism behind sepsis pathology is essential in developing effective intervention to improve patient prognosis.

Section 1.2: Sepsis-induced Cardiac Dysfunction

Cardiac dysfunction is observed in 40% to 50% of patients with sepsis where the ability of the heart to pump blood, or ejection fraction, is attenuated [6]. This reduction of ejection fraction is referred to as myocardial depression. Myocardial dysfunction involves both left and right ventricles, which greatly increases the mortality of septic patients [7].

Initially cardiac dysfunction in sepsis was thought to exist in two phases. Patients undergoing "warm shock" exhibited hyperdynamic circulation with peripheral vasodilation and high cardiac output (CO) [8]. This was followed by "cold shock" with the characteristics of increased vascular tone and low CO [8]. With the introduction of pulmonary artery catheters which allowed accurate measurements of CO, it was found that septic patients who were adequately resuscitated, consistently demonstrated those characteristics of warm shock – low systemic vascular resistance and high CO [9], leading to the understanding that the haemodynamic profile of patients in "cold shock" was due to inadequate resuscitation. Despite high CO seen in sepsis patients, many suffer from myocardial dysfunction. Common features include biventricular dilation, decreased contraction, reduced ventricular compliance, and reduced ejection fraction [7]. This dysfunction is reported to peak after a few days of sepsis onset and resolve within 7 to 10 days in patients that survive [7].

It has been well documented that cardiomyocytes experience contractile dysfunction under septic stimuli [10, 11]. Cardiomyocytes express Toll-like receptors

(TLRs) like those expressed on innate immune dendritic cells, which enables them to respond to danger signals and mediate local inflammatory responses [12]. Previous studies have shown a correlation between sepsis and alterations in myocardial structure [13, 14] but the underlying molecular mechanisms involving cardiomyocytes remain to be elucidated.

Section 1.3: Mechanisms of sepsis-induced cardiac dysfunction

One of the earliest theories behind myocardial dysfunction observed in sepsis was global myocardial ischemia. This has been disproven by several studies. Investigators explored this idea by using thermodilution coronary sinus catheters to measure coronary blood flows in septic patients. Their measurements showed normal or even elevated coronary blood flow in septic hearts compared to healthy controls [15]. There was also no elevation in myocardial lactate production, a key byproduct of anaerobic respiration [15]. Another group of investigators also verified these finding using the same methods [16]. These studies provide strong evidence that myocardial perfusion is not decreased during sepsis and thus is not a contributing factor to myocardial depression.

The idea of circulating substance causing cardiac dysfunction in sepsis originates back to 1947 in Wiggers' report on myocardial depressant factors present in hemorrhagic shock [17]. In 1985 it was shown, for the first time, that the serum of septic patients with myocardial dysfunction exhibited myocardial depressant effects on isolated cardiomyocytes [18]. When cultured cardiomyocytes were treated with septic patients' serum, it caused a significant depression in myocardial cell shortening in a concentration-

dependent manner. The presence of myocardial depressant factors also correlated with higher mortality in sepsis patients [19].

Many different cytokines have been identified to make up the myocardial depressant substance which has been characterized as a water-soluble, heat stable, 10-25 kDa protein. Out of the exhaustive list of potential mediators of myocardial depression, tumor necrosis factor-α (TNF-α) and Interleukin (IL)-1β have been shown to have a pivotal role in sepsis-induced myocardial dysfunction. Increased TNF-α levels in dogs produced effects similar to clinical septic shock such as hypotension, increased CO, peripheral vasodilation [20] and a reduction in ejection fraction [21]. Furthermore, in vitro systems using mammalian cardiomyocytes also exhibited decreased contractility when exposed to TNF-α [22]. Many promising results that pointed towards TNF-α being at the core of sepsis-induced myocardial dysfunction led to a hopeful, yet unsatisfactory, pilot study of administering anti-TNF-α monoclonal antibody to septic patients. Although the antibody improved left ventricular function, it did not alter the patients' outcome [23].

IL-1β studies have produced similar results as TNF-α where it directly depressed cardiac contractility. Increased levels of IL-1β have been detected in human and animal models of sepsis [24]. Just as seen with TNF-α, in vitro treatment of IL-1β to cultured cardiomyocytes caused attenuation of the maximum extent and peak velocity of myocyte shortening [25]. However, using IL-1β antagonist in human septic shock patients failed to alter the overall mortality despite its beneficial effects on hemodynamic and metabolic parameters [26].

Section 1.4: Lipopolysaccharide

Gram-negative bacteria infection accounts for large proportion of septic cases [4]. An important component of the gram-negative bacteria cell wall that is responsible for eliciting strong host immune response is LPS. Along with TNF-α and IL-1β, it is an important contributing factor of myocardial depression. The bacterial endotoxin, LPS, stimulates a strong inflammatory response by upregulating and promoting the release of pro-inflammatory cytokines. Many studies have indicated that LPS negatively influences cardiovascular function. In order to deduce the direct correlation between LPS and cardiovascular function, healthy human subjects were injected with a bolus dose of endotoxin. The investigators were able to observe similar hemodynamic characteristics of sepsis in the volunteers 3 hours after the injection, such as reduced systemic vascular resistance (SVR) [27]. They also observed in an in vitro system that LPS treatment directly decreased the extent of myocyte shortening [11].

LPS is a glycolipid molecule that is comprised of three subregions: lipid A, the core, and the O-polysaccharide. Lipid A is the representative toxic element of LPS and is conserved across various strains of gram-negative bacteria. It is composed of β-D-glucosaminyl-(1-6)-α-D-glucosamine disaccharide with two phosphoryl groups and acylated by four R-3-hydroxy fatty acid residues. The core consists of hetero-oligosaccharide that is less variable between different bacterial species, as opposed to highly variable O-polysaccharide region. It is typically composed of heptose and 2-keto-3-deoxyoctonic acid. The O-polysaccharide region has variable sugar composition with different number of repeating oligosaccharide units. Due to its variability, this region is responsible for serological specificity between different bacterial strains [28].

The Toll receptor was first discovered in *Drosophila melanogaster* and its role in innate immunity was recognized when those without the receptor were highly susceptible to fungi [29]. The mammal counterpart of Toll receptors, Toll-like receptors (TLR), was discovered in 1997 [30]. Since then, ten different TLR have been identified in humans [31] and twelve in mice [32]. TLRs can be classified based on their subcellular localization and their agonist. TLR-3, TLR-7, TLR-8 and TLR-9 are located in intracellular compartments and are sensitive to nucleic acid recognition, while TLR-1, TLR-2, TLR-4, TLR-5 and TLR-6 are typically found on the cell surface and specialize in detecting a variety of products present in bacteria, parasites and fungi such as glycolipids, lipopeptides and flagellin [33]. TLRs are classified as type-1 transmembrane receptors with extracellular leucin-rich repeat motifs and a conserved cytoplasmic Toll/IL-1 receptor (TIR) domain [34]. It was shown in 1998 that TLR-4 was the signaling receptor for LPS in a study that showed mice without TLR-4 lack responses to LPS [35].

Circulating LPS is bound to soluble LPS binding protein (LBP) which acts as an opsonin [36]. LPS-LBP is then accepted by cluster of differentiation (CD)-14 on the cell membrane, CD-14 mediates LPS binding to the TLR-4 and myeloid differentiation factor 2 (MD-2) and initiate the cell signaling cascade. MD-2 is a co-receptor of TLR-4 and crucial in LPS induced signaling [37]. The lipid A portion of LPS interacts directly with the hydrophobic pocket in MD-2 and induces dimerization of TLR-4 receptors [38]. Subsequent to LPS binding to TLR-4-MD-2, two pathways are activated (early vs. delayed) depending on which TIR domain-containing adaptor molecules are recruited to TLR-4 through interaction with the TIR domain of TLR-4. The early response is

mediated by myeloid differentiation factor 88 (MyD88) and TIR domain-containing adaptor protein (TIRAP), also called MyD88 adaptor-like (MAL). This pathway leads to early activation of nuclear factor κB (NF-κB) which induces production of proinflammatory cytokines such as TNF-α. The delayed response is independent of MyD88 and signals via TIR-domain containing adaptor, which induces interferon β (TRIF) and TRIF-related adaptor molecule (TRAM). This pathway leads to activation of interferon (IFN)-regulatory factor 3 (IRF3) and subsequently delayed activation of NF-κB. This leads to production of IFN-β and IFN-inducible genes [39]. The NF-κB pathway has been reported in the past to be a key participant in decreased cardiomyocyte contractility and inflammatory response after TLR activation [11].

LPS initiates numerous responses in the mammalian system including activation of the innate immune system, complement cascade and characteristic shock syndrome [40]. Its effects are secondary to LPS inducing the upregulation and release of inflammatory cytokines. Though macrophages are the primary targets of LPS [41], cardiomyocytes have also been reported to express TLR-4 receptor on their cell surface, thus they are capable of responding to LPS [11]. Numerous reports provide strong evidence that LPS has a detrimental effect on the heart including mediating vascular inflammation in atherosclerosis [42]. In chronic heart failure patients, increased LPS levels were detected in patient plasma [43]. Increased level of plasma LPS has also been measured from septic patients and thus has been implicated to be a crucial instigator of sepsis pathology [44]. Also when LPS was injected in low doses to healthy human volunteers, they exhibited similar hyperdynamic response seen in septic patients [44].

Section 1.5: Myocardial Extracellular Matrix

The collagen network is a crucial component of the heart's extracellular matrix (ECM) where it works to secure and support cardiomyocytes in proper alignment required for coordinated contraction and it helps determine heart compliance. The ECM is mainly composed of collagen with smaller proportion contributed by elastin, laminin and fibronectin [45]. The major collagen type accounting for >50% is collagen type I, followed by collagen type III which comprises around 10% of the heart's ECM [45]. The architectural complexity of the ECM was identified through the use of scanning election microscopy [46] where the highly organized network provided structural integrity between adjacent cardiomyocytes and coordination to overall myocyte shortening. The interstitial collagen in the myocardium is dependent upon a balance between synthesis and degradation. In normal myocardium, only about 2-4% of the myocardium is reported to be comprised of collagen. Yet, even small changes in collagen concentration have been shown to mediate drastic effects on the heart's mechanical properties [45]. In addition to the quantity, the relative proportion, the diameter and the spatial alignment of collagens can all lead to alterations to this network, which can exert marked effects on the functions of the heart.

The aberrant balance of collagen production and degradation was observed in patients with dilated cardiomyopathy where a 30-fold increase in collagenase activity has been reported [47]. Another study looked at isolated papillary muscle as well as felines with pressure overload-induced hypertrophy that were treated with plasmin to disrupt the ECM. Decreased systolic function, as well as decreased collagen content in the papillary muscles was observed [48]. In a landmark paper published by Parker showed that septic

patients exhibited decreased left ventricular ejection fraction (LVEF) and ventricular dilation which was reversible in 7 to 10 days in survivors [6]. This lead to subsequent studies that looked at myocardial ECM changes in sepsis and its potential mediators [49].

Section 1.6: Interleukin-33 and ST2

A relatively new cytokine implicated in heart collagen biology is interleukin (IL)-33. IL-33 (also known as IL-1F11) was identified as a newest member of the IL-1 family in 2005 while looking for proteins with similar structure [50]. It has been identified in the past by different researchers as DVS27, a gene up-regulated in canine cerebral vasoplasm [51] and NF-HEV, nuclear factor from high endothelial venules [52]. The sequence of IL-33 has been mapped in both human chromosome 9 and mouse chromosome 19 which encodes for proteins consisting of 270 and 266 amino acids respectively [53]. IL-33 shares a close amino acid homology to IL-18 and a β-sheet trefoil fold structure, characteristic of all IL-1 family members [53]. Functionally IL-33 appears to have dual actions as a traditional cytokine and also as an intracellular nuclear factor [53].

IL-33 has been shown to be expressed in various tissue types including the heart. Initially it was thought that IL-33 needed to be cleaved by caspase-1 to be active, like IL-1β [50]. However, recent papers have demonstrated that IL-33 is not a physiological substrate of caspase-1, as it lacks a classical caspase-1 cleavage site [54-56]. Furthermore, it was shown that full length IL-33 is able to exert its biological effects via its receptor without proteolytic processing [55].

ST2 (suppression of tumorigenicity 2) is a member of the Toll-like/IL-1 family of receptors [56]. This receptor for IL-33 has two forms: a membrane bound form (ST2L)

and a soluble form (sST2). The sST2, released alongside elevated IL-33 expression, acts as a negative feedback mechanism by acting as a decoy receptor. Upon binding of IL-33 to sST2, the signaling via ST2L is inhibited [57]. Elevated serum sST2 has been detected in patients with asthma [58], acute heart failure [59], ulcerative colitis [59] and sepsis [60]. This elevated level of serum sST2 showed a positive correlation with disease severity and mortality in patients with severe sepsis. When soluble ST2-Fc was administered to mice, it effectively attenuated inflammation and lethality after intestinal ischemia and reperfusion injury [61]. It is suggested ST2 signaling is involved in regulating the inflammatory response.

IL-33 may act in an autocrine or paracrine manner. IL-33 binds to its membrane receptor ST2L which forms a complex with IL-1 receptor accessory protein (IL-1RAcP) [62]. This leads to sequestering MyD88 and MAL. This results in activation of TNF receptor-associated factor 6 (TRAF6) mediated by Interleuin-1 receptor-associated kinase (IRAK), with subsequent activation of mitogen-activated protein kinase (MAPK) and inhibition of IκB kinase (IKK) and NF-κB [63, 64]. General events downstream of IL-33/ST2 signaling may include activation of NF-κB [64], activator protein-1 (AP-1) [65] and, phosphorylation of p38 MAPK, extracellular signal-regulated kinase (ERK) 1/2, and c-Jun N-terminal kinases (JNK) [50].

Cardiomyocytes, as a response to biomechanical strain, will become fibrotic by laying down more collagen. Exposure to IL-33 has been shown to attenuate this response by reducing fibrosis [56] suggesting that IL-33 could be a potential mediator of cardiac collagen organization. IL-33 has been observed to participate in various inflammatory conditions as well such as in inflammatory bowel disease, ulcerative colitis [66].

Section 1.7: Matrix Metalloproteinases

MMPs are a family of zinc-dependent endopeptidases that are able to degrade various components of the ECM and play a role in both physiological and pathological processes such as uterine involution, embryogenesis, metastasis and arthritis [67-70]. The first MMP to be purified by Gross and Lapiere in 1962, came from tadpole tails. It was initially characterized by its ability to degrade collagen. Hence it was named collagenase-1 (now referred to as MMP-1) [71]. Since its discovery, over 20 MMP species have been identified. It was quickly observed that their substrates ranged anywhere from structural proteins to non-ECM substrates. Because of their wide range of substrates, MMP can actively participate in many cellular function including cell growth, apoptosis, cell migration, cell-cell communication and tumour progression [72]. Of particular interest, MMPs have been implicated in various cardiac pathologies including heart failure, myocardial remodeling and dilated cardiomyopathy [73-75].

MMPs are initially secreted as a latent zymogen or pro-MMP and tightly controlled at the level of transcription, translation and activation by proteolytic cleavage [76]. In pro-MMPs, the cysteine residue in the propeptide domain creates a bond with the Zn^{2+} site in the catalytic domain, inactivating the proenzyme [77]. When this bond is replaced by a water molecule in a step called “cysteine switch”, the MMP’s catalytic domain is released and which produces an intermediate active enzyme [77]. To fully activate the MMP, the pro-domain needs to be cleaved by proteases or autolytic cleavage [77]. Their activity is also regulated by different inhibitors, most important of which is tissue inhibitors of metalloproteinases (TIMPs) [78]. MMP-9 can degrade wide range of

substrates including gelatin (a denatured fibrillar collagen), collagen type IV, fibronectin and laminin [74].

Section 1.7.1: MMP-9 and Sepsis

MMP-2 and MMP-9 are capable of degrading different components of the ECM and both have been previously demonstrated to be highly expressed in the heart [79]. In addition, it has been shown that cardiomyocytes are capable of expressing these MMPs [75]. LPS has been shown to induce transcription of MMP-9 in cell culture experiments [80, 81]. Rats injected with LPS as a model of sepsis, have increased MMP-9 activity in heart perfusates, whereas MMP-2 activity was decreased with LPS. Administering MMP inhibitor reduced MMP-9 activity and significantly improved cardiac function [82]. Metalloproteinase inhibition was also shown to improve survival after cecal ligation and puncture (CLP) in rats [83]. In a clinical study, higher plasma MMP-9 [84] and ST2 levels [60] in septic patients directly correlated with mortality.

A novel observation was made in the rat sepsis model which had decreased collagen content in the myocardium [13]. However, the detailed relationship between collagen remodeling and sepsis is not fully understood. It is speculated that MMPs play an important role in septic myocardial collagen remodeling.

Elevated MMP expression was also associated with septic patients [85]. TNF-α, an important cytokine mediating septic pathophysiology and classified as a myocardial depressant substance, has also been identified to induce MMP gene transcription. [86].

Section 1.8: Animal Sepsis Models

There are several different animal models of sepsis that researchers have used. Each have their own advantages and disadvantages in representing clinical sepsis. The simplest is the LPS model. This model of sepsis assumes that sepsis is not caused by the intact pathogen but relies on host's response to it. A set dose of LPS is administered intravenously or into the abdominal cavity of the animal, which causes sepsis-like symptoms such as hematological changes [88] and increased release of proinflammatory cytokines [89]. Cytokines, TNF-α and IL-1 mediate LPS-induced response in the animal. With this promising result clinical trials attempted to reproduce this by treating septic patients with TNF-α antibodies, but it failed [90]. The LPS infusion model and clinical sepsis have major differences, especially characteristics of cytokine release. In human sepsis, cytokine levels were lower and peaked later than those of LPS models [88, 91]. Also blocking LPS with antibodies in septic patients did not alter their outcome (33). Overall, although the model allows accurate doses of LPS to be administered and easily stored, it may not effectively simulate human sepsis.

Another sepsis model commonly used is cecal ligation and puncture (CLP). Like the name implies the animal is ligated just below the ileocecal valve and the cecum is punctured with a needle. This provides a polymicrobial infection focus as the contents of cecum slowly leaks out into the peritoneal cavity. The animals have similar characteristics of sepsis with high mortality [92]. Like in human sepsis, high levels of IL-6 in CLP animals correlated with decreased survival [93]. Although the CLP model shows clinical relevance, it is difficult to achieve consistent data. Several steps in the

procedure can alter the outcome including the length of the cecum ligated and individual composition of bacteria.

In our study we chose to use the FIP model. FIP involves making a fecal slurry with feces taken from a donor animal and injecting it into the peritoneal cavity of the experimental animal. Similar to the CLP model, feces inoculation provides a mixture of all different bacteria present in the feces, thus creating a polymicrobial infection. Although it does not reproduce a continual release of infectious focus like CLP, it is advantageous over CLP in that the investigator is able to better control how much feces is injected into the peritoneal cavity. Also since all the animals in the experimental group are getting treated with the fecal slurry prepared from a single donor, the amount of bacterium in the feces can be controlled.

Section 1.9: Purpose of Study

Etiologies of sepsis-induced cardiac dysfunction remain unclear. Previous studies have reported that septic hearts go through various structural changes. Studies report involvement of both MMP-9 and IL-33 in various cardiomyopathies by altering the myocardial structure. IL-33 has been implicated in regulating collagen turnover where it reduced fibrosis in pressure overloaded heart [87]. Increased MMP-9 activity has been measured in plasma of sepsis patients [85] and MMP-9 inhibitor reduced the mortality rate of septic rats [83]. It is speculated that changes in myocardial collagen during sepsis are mediated by IL-33 via regulation of myocardial MMP-9. Purpose of this study was to deduce pathological mechanisms of sepsis which lead to cardiac dysfunction.

Section 1.10: Hypothesis

IL-33 will contribute to myocardial dysfunction observed in a mouse sepsis model which may be carried out partly through regulation of MMP-9 expression in cardiomyocytes (Figure 1.1).

Section 1.11 Objectives of Thesis

1. Generate a mouse sepsis model. Inject fecal slurry into mice peritoneum (FIP model) to induce systemic inflammation. Compare hematological parameters and lactate levels to clinical observations.
2. Assess heart tissues for changes in cardiac collagen content. Determine if FIP treated mice has differential collagen staining compared to control.
3. Evaluate myocardial expression of IL-33 and MMP-9 and how they correlate with cardiac function in FIP mice.
4. Investigate whether IL-33 inhibition affects myocardial function and MMP-9 expression in FIP mice.
5. Use an in vitro model to study how cardiomyocytes respond to LPS.

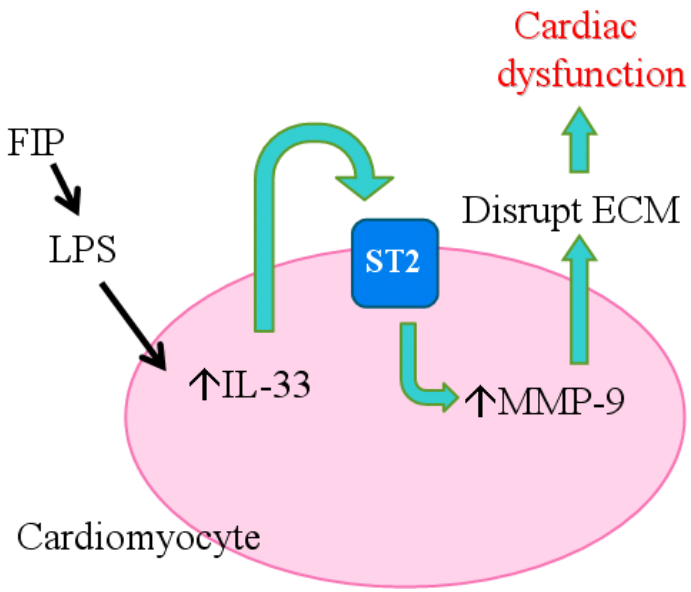

Figure 1.1 Schematic diagram of proposed hypothesis

IL-33 will contribute to myocardial dysfunction observed in mice sepsis model which may be carried out through regulation of MMP-9 expression in cardiomyocytes.

CHAPTER 2: MATERIALS AND METHODS

Section 2.1: Animals

C57BL/6 mice were obtained from Charles River Laboratories Canada (St. Constant, Quebec, Canada). The mice were housed in Victoria Research Labs Vivarium Service with a 12-hour light/dark cycle with access to rodent chow and water *ad libitum*. The mice were used for in vivo experiments as well as a source for neonatal cardiomyocytes for in vitro experiments. The experimental protocols were approved by the University of Western Ontario Animal Care and Use Committee (protocol no. 2006-111-11).

Section 2.2: Feces-induced peritonitis

10 week-old mice were divided into three groups of intraperitoneal injections: Sham; FIP or FIP+ST2. The sham group received normal saline, the FIP group received fecal slurry (32 mg/ 100 g body weight), and the FIP+ST2 group received fecal slurry (32 mg/ 100 g) and sST2 (0.2 mg/ 100 g). The fecal slurry was prepared from collecting the bowel contents of a mouse from same litter. It was suspended in normal saline so that equal volume of 800 µl was injected per mice.

Section 2.3: Measurement of Cardiac Function

There are numerous ways to assess cardiovascular parameters. There are non-invasive techniques such as echocardiography and magnetic resonance imaging (MRI). Echocardiography is an inexpensive method to observe structures in vivo using sound waves. The CO and shortening fraction of the heart can be estimated but it is limited by subjective interpretation. MRI provides a better estimation as it provides higher

resolution. There are also in vitro techniques that utilize a whole heart preparation or isolated myocytes. Isolated heart system allows the investigator to examine the effect of specific compounds without any contribution from other organs. Similarly the contractility for cell systems can also be measured and potential cellular mechanisms can be deduced. However in vitro systems are limited in their interpretation with regards to how it translates to clinical situations. In our study we chose to do invasive hemodynamic monitoring via arterial catheterization. This tool is used in clinical settings to directly measure patient's CO, stroke volume and even venous oxygen saturations. It measures parameters such as intracardiac pressures, believed to reflect the current myocardial condition.

In our study, mice were anesthetized with ketamine (150 mg/kg) and xylazine (5 mg/kg) subcutaneous 48 hours after fecal inoculation. A Millar tip transducer catheter (Model SPR-893, 1.4 Fr.) was inserted into the left ventricle via the right carotid artery. Various cardiac parameters were measured using PowerLab system (AD Instruments) connected to Millar catheter, including left ventricular (LV) pressure-volume (PV) loops which were generated by occluding the inferior vena cava. The LV end-systolic pressure-volume relationship (ESPVR) was used, along with other parameters such as stroke work, as a measurement of myocardial contractile function. Plotting pressure versus volume has been used not only in cardiology but also in respiratory systems to measure the amount and efficiency of the work done. When LV pressure and volume is plotted in real time, one closed loop is generated per single cardiac cycle. From this loop, various parameters such as stroke volume, CO, ejection fraction can be calculated. ESPVR provides a more accurate measurement of cardiac function because it does not depend on ventricular load.

For example, dP/dt max represent the maximum rate of pressure change in the left ventricle and is used as an index of ventricular performance. Though it does provide valuable information it is dependent on preload, after load, heart rate and myocardial hypertrophy. ESPVR depicts maximum pressure that is generated at any LV volume. During animal surgery the inferior vena cava is occluded reducing the volume and slowly allowed to flow back into left ventricle. While this is happening, a series of PV loops are generated and a line is drawn connecting the point where systole ends on every loop. This generates a straight line and the slope of this line is used as an index of myocardial contractility. Steeper ESPVR represents stronger contractility.

Section 2.4: Hematological analysis

Approximately 1 mL of whole blood (treated with heparin) was drawn via cardiac puncture of the left ventricle from animals at certain time points after treatment. Whole blood samples were analyzed with a UniCel DxH 800 Coulter Cellular Analysis System (Beckman Coulter, Fullerton, CA) for complete blood counts. Blood smears were made and stained with Wright-Giemsa for white blood cell differentials which were analyzed by a specialized technologist. The technologist was not told which samples belonged to which group. Wright-Giemsa stain is a modified combination of Wright's and Giemsa's stain and is commonly used to stain peripheral blood and bone marrow smears. The important components of this dye include methylene blue, azure B and eosin Y. The acidic DNA of nucleus will be stained shades ranging from blue to purple by the basic methylene blue and azure B dyes while acidic eosin Y stains the basic cytoplasm varying degrees of orange and pink [94]. This allows for accurate interpretation of cell

morphology in the blood smear. The other types of stains, Romanowsky, Wright's and Giemsa, mainly vary in proportion of agents. The Wright-Giemsa stain was chosen because it is a widely used stain that has been optimized to produce accurate histological staining for identification of different blood cell types.

Section 2.5 Measurement of circulating factors

Full-strength plasma samples were used for LPS (ToxinSensor™ Chromogenic Limulus Amebocyte Lysate Endotoxin Assay Kit, GenScript, NJ), IL-33 enzyme-linked immunosorbent assay (ELISA) (Quantikine Mouse IL-33 Immunoassay, R&D Systems, MN) and lactate measurement (YSI 2300 STAT Plus™ Glucose & Lactate Analyzer, YSI Life Science, OH).

The *Limulus* amebocyte lysate (LAL) assay is a popular quantitative method used to detect endotoxin in biological samples. It provides several advantages such as sensitivity and specificity. The assay depends on the clotting of a protein from amebocytes of horseshoe crab (*Limulus polyphemus*) as it reacts to endotoxin. The lysate from amebocytes were very sensitive to endotoxin [95] and thus utilized as a diagnostic tool in the laboratory. For a long time, the LAL assay was the only practical method to measure endotoxin as it was difficult developing antibodies for such heterogeneous substance. It is only recently that companies have developed an ELISA kit for detecting LPS.

The commercially-available ELISA kit was purchased for detection of plasma IL-33. ELISA is used in the lab measure an antigen or antibody in a sample by using antibodies or antigen coupled to an easily-assayed enzyme. Accurate results can be obtained with ELISA because it combines specificity of antibodies and sensitivity of enzymes. It is

much faster and cheaper than a western blot. Also it is advantageous if you have many samples to run at once.

Section 2.6: Picrosirius staining of the heart tissue

The picrosirius red stain is a strong anionic dye which was first described in 1979 as a novel method to detect collagen by its ability to bind basic amino acids of collagen fibers [96, 97]. Picrosirius red staining is an excellent way to detect and analyze collagen in tissue sections without the concerns of the stain fading over time. Specifically, picrosirius red is able to consistently stain collagen fibers including thin collagen fibers that other traditional stains (e.g. van Gieson, trichrome) fail to detect which can lead to underestimation of total collagen [98, 99].

For this study, the heart was excised from the mouse and was fixed in 10% formalin before being embedded in paraffin. The tissue was cut in sections of 5 µm and transferred to glass slides. Slides were de-paraffinized in two changes of xylene for 10 minutes and hydrated following a series of washes: two changes of 100% ethanol for 10 minutes, two changes of 95% ethanol for 5 minutes, 80% ethanol for 2 minutes and 70% ethanol for 2 minutes. The slides were washed in water for 3 minutes before staining with picrosirius red (0.8%) for 1 hour at room temperature. The stained slides were dehydrated in two changes of 100% ethanol and cleared in xylene. They were then mounted with a glass cover slip in Eukitt quick-hardening mounting medium (Sigma Aldrich, St. Louis, MO). When viewed under brightfield microscope (20X objective lens) the collagen appeared red on a yellow background. The pictures taken of the slide (5 fields per slide) were analyzed using Sigma-Scan Pro software. The percentage of collagen staining was

calculated as a percentage of the whole tissue. The staining solution consisted of 0.5 g Sirius red (Sigma-Aldrich, Cat#365548) and 500 mL picric acid solution (Sigma-Aldrich, Cat#P6744-1GA).

Section 2.7: Primary neonatal mouse cardiomyocyte culture

Neonatal mice (C57BL/6) were anaesthetized and the chest was cut open with sterilized pair of scissors. Hearts were excised and immediately transferred to ice-cold D-Hanks solution in a 50 mL tube. The hearts were washed and minced into ~1mm^3 small pieces. The heart pieces were resuspended into 2 mL of pre-warmed 37 ℃ Liberase working digestion solution (Liberase TM Research Grade, Roche, IN). Tissue pieces were dispersed and cells were released with a transfer pipette until the supernatant became cloudy. The supernatant was collected in a 15mL tube. This digestion procedure was repeated two more times with remaining heart pieces and the supernatant collected. The collected supernatant was centrifuged at 200 g for 5 minutes. The supernatant was discarded and the pellet was resuspended in medium 199 (M199) containing 10% fetal bovine serum (FBS). The resuspended cells were preplated in a petri dish for 1 hour for fibroblast adhering to enrich for cardiomyocytes. The cardiomyocyte-rich supernatant was collected and seeded into tissue culture plates (Costar) pre-coated with 0.1% gelatin. After 48 hours, the conditions of cardiomyocytes were checked under the microscope to see that they formed a confluent monolayer and were beating spontaneously infrequently. The isolated cardiomyocyte culture was subjected to LPS administration (LPS-EK, InvivoGen). Cardiomyocytes were treated with LPS (1 μg/mL) for 4, 8 or 24 hours.

Section 2.8: SDS-PAGE and western blot

Western blot is a popular analytical technique used to quantify specific proteins by using antibodies. The denatured protein sample from source of interest is separated by size with polyacrylamide gel electrophoresis (PAGE) and transferred to a carrier-membrane (e.g. polyvinylidene difluoride, PVDF or nitrocellulose) which is incubated in a specific antibody to your protein of interest. A secondary antibody will bear the reporter which will luminesce when exposed to its substrate. This chemiluminescent signal is then detected by a camera or X-ray film and the quantity of protein is determined by optical density. Western blot is useful in detecting several proteins from a single sample. Since the carrier-membrane contains all proteins from your sample, different proteins can be detected by using different antibodies. In our study, cardiomyocytes were washed with ice-cold phosphate buffered saline (PBS) three times and sodium dodecyl sulfate (SDS) loading buffer was added. After sonication using 4710 Series Ultrasonic Homogenizer (Cole Parmer Instrument Co.) and heating on VWR Digital Heatblock, protein quantification was done using the Bio-Rad protein assay. Equal quantity of each protein samples were loaded into SDS-PAGE gel (12%) for electrophoresis. Then the protein was transferred to PVDF membranes. Nonspecific binding to the blot was blocked with 3% milk blocking solution made from Blotting Grade Blocker Non-Fat Dry Milk (Bio-Rad) then incubated with a primary antibody against IL-33 (R&D; 1:1000), ST2 (Abcam Inc; 1:500) or MMP-9 (Cell Signaling; 1:1000). An internal control antibody anti-housekeeping protein, tubulin (Abcam Inc; 1:500), was used as protein loading control.

Section 2.9: Measurement of MMP-9 activity

Expression of MMP-9 was examined by gelatin zymography. This technique utilizes the gelatinolytic activity of proteases to quantitatively analyze the concentration of protein. A gel was made just as regular SDS-PAGE gel except it was co-polymerized with gelatin. After loaded protein sample was separated according to size, the SDS is washed away from gel with Triton-X, which allows MMPs to renature. Any MMPs present is able to degrade gelatin while incubating in calcium-containing developing buffer. Since there are two species of MMPs that degrade gelatin (MMP-2, 72kDa and MMP-9, 92 kDa) the predicted molecular weight of the MMP allows identification of which gelatin clearing is attributed to which MMP.

Gelatin zymography is a qualitative method to detect gelatinolytic activity, and from that, the expression level of MMP expressed is analyzed [100]. Zymography offers many advantages over other methods such as ELISA. It is relatively inexpensive as it does not require antibodies and one gel can detect multiple proteases which share the same substrate. Gelatin zymography is commonly used to study gelatinases MMP-2 (72 kDa) and MMP-9 (92 kDa) which can be differentiated on the gel according to size.

Gelatin (Gelatin from porcine skin, Type A, Sigma) was copolymerized to 10% polyacrylamide gels at 0.1% concentration. The cardiomyocyte conditioned media was concentrated using a centrifugal filter before analyzing with gelatin zymography. Non-heated concentrated media, cell lysate or tissue homogenate samples plus 5x loading dye were loaded along with a positive control, recombinant MMP-9 (R&D Systems, Minneapolis, MN). After 2 hours of eletrophoresis, the gels were washed in 2.5% Triton X-100 (Sigma) for 30 minutes with gentle agitation at room temperature to remove SDS.

After, the gels were incubated in zymogram developing buffer (50 mM Tris-HCl, 0.2M NaCl, 5 mM $CaCl_2$, and 0.02% Brij 35) for 30 minutes at room temperature with gentle agitation. The buffer was replaced with fresh developing buffer and gels were incubated at 37 ℃ overnight. After the incubation the gels were stained with Coomassie Blue R-250 (Sigma) in a mixture of 5% methanol:10% acetic acid until the gels were stained uniformly dark blue. The gels were destained in aqueous 10% methanol: 5% acetic acid. Areas of gelatinolytic activity were detected as clear bands against the dark blue background. Zymograms were scanned and band intensities were quantified.

Section 2.10: Statistical analysis

All values are presented as mean ± standard error of the mean (SEM). Statistical analysis was performed using Student's t-test or analysis of variance (ANOVA) with a Tukey's post-hoc test to compare multiple groups. Student's t-test was used to compare the means of 2 groups and ANOVA was used to compare the means of more than 2 groups. Differences between groups were considered significant if the $P < 0.05$.

CHAPTER 3: RESULTS

Section 3.1: Establishing murine sepsis model

To characterize the established mouse sepsis model, white blood cell differential and platelet count was completed for FIP mice at different time points (Table 3.1) Also plasma lactate was measured using STAT Plus™ Glucose & Lactate Analyzer (Table 3.2). A similar pattern was observed in FIP mice – decreased platelet count, increased neutrophils and decreased lymphocyte differentials – as seen in other animal sepsis models [101]. There was no difference in plasma lactate concentration, in contrast to observations made in past studies [102].

Table 3.1. White blood cell differential and platelet count

	Neutrophils (%)	Lymphocytes (%)	Platelets ($10^3/\mu L$)
Sham	16.00±1.92	81.80±1.7	1046.1±61.74
FIP 6h	60.80±7.02***	36.80±6.39***	767.64±30.26**
FIP 24h	45.67±2.73*	48.67±0.88**	721.4±71.63**
FIP 48h	57.67±8.01**	37.00±7.81***	868.83±24.35

After onset of sepsis % lymphocytes and platelet count are decreased, while % neutrophils is increased. FIP was induced by injecting a fecal slurry into the mouse peritoneal cavity and a blood sample was collected at various time points. Values are mean ± SEM, n=3-5/group, *p<0.05, **p<0.01, ***p<0.001 vs. sham.

Table 3.2. Plasma concentration of lactate

Plasma Lactate (mmol/L)	
Sham	1.022 ± 0.297
FIP 6h	1.538 ± 0.358
FIP 24h	0.940 ± 0.147
FIP 48h	1.071 ± 0.176

There was no difference in lactate measured in FIP mice compared to sham. FIP was induced by injecting a fecal slurry into the mouse peritoneal cavity and a blood sample was collected at various time points. Values are mean ± SEM, n=4/group. There were no significant differences between time points.

Section 3.2: ST2 prevents cardiac dysfunction observed in FIP mice

In order to compare the hemodynamic patterns of different treatment groups: sham, FIP and FIP + ST2-treatment, left ventricle function was measured with a Millar catheter-tip pressure transducer 48 hours after individual treatment. Mice with FIP had cardiac dysfunction compared to saline controls (Table 3.3). The dysfunction induced by FIP was prevented upon sST2 injection, a decoy receptor of IL-33 (Table 3.3). The left ventricular end-systolic pressure (LVESP) (93.4 $\pm$ 2.1 vs. sham 109.2 $\pm$ 0.8), velocity of contraction (dP/dt max; 5243 $\pm$ 429 vs. sham 7291 $\pm$ 279), stroke work (759.5 $\pm$ 107.4 vs. sham 1208.8 $\pm$ 110.1) and end systolic pressure-volume relationship (ESPVR) (3.6 $\pm$ 0.1 vs. sham 4.7 $\pm$ 0.3) all were significantly impaired in FIP mice compared to sham control mice (Figure 3.1). sST2 treatment had statistically significant prevention of dysfunctions (LVESP, 106.1 $\pm$ 5.5 vs. FIP 93.4 $\pm$ 2.1; dP/dt max, 7105.5 $\pm$ 118.8 vs. FIP 5243 $\pm$ 429; stroke work, 1176 $\pm$ 162.8 vs. FIP 759.5 $\pm$ 107.4; ESPVR, 4.6 $\pm$ 0.2 vs. FIP 3.6 $\pm$ 0.1). It appears that sST2 has a protective role against the deteriorative effects FIP has on mouse cardiac contractility.

Table 3.3. In vivo hemodynamic measurements

Parameters	**Sham (n=5)**	**FIP (n=6)**	**FIP+ST2 (n=4)**
HR (b.p.m)	304 ± 18	305 ± 14	308 ± 18
MAP (mmHg)	39.4 ± 0.4	35.1 ± 0.9	39.2 ± 2.1
LVESP (mmHg)	109.2 ± 0.8	93.4 ± 2.1*	106.1 ± 5.5$^{\#}$
LVEDP (mmHg)	15.4 ± 1.9	14.5 ± 3.6	10.5 ± 2.2
SV (µL)	15.4 ± 0.9	11.9 ± 1.6	14.4 ± 1.1
EF (%)	20.5 ± 1.0	18.0 ± 1.7	18.5 ± 2.0
CO (µL/min)	4722.7 ± 521.1	3595.0 ± 425.4	4401.4 ± 296.5
SW (mmHg*µL)	1208.8 ± 110.1	759.5 ± 107.4*	1176 ± 162.8$^{\#}$
dP/dt max (mmHg/s)	7291 ± 279	5243 ± 429*	7105.5 ± 118.8$^{\#}$
dP/dt min (mmHg/s)	-7024 ± 698	-5842 ± 564	-5793 ± 720
ESPVR (mmHg/µL)	4.7 ± 0.3	3.6 ± 0.1*	4.6 ± 0.2$^{\#}$

HR, heart rate; MAP, mean arterial pressure; LVESP, left ventricular end systolic pressure; LVEDP, left ventricular end diastolic pressure; SV, stroke volume; EF, ejection fraction; CO, cardiac output; SW, stroke work; dP/dt max, maximal rate of pressure rise; dP/dt min, maximal rate of pressure decline; ESPVR, end systolic pressure-volume relationship. Data are mean ± SEM. *P<0.05 vs. sham, #P<0.05 vs. FIP.

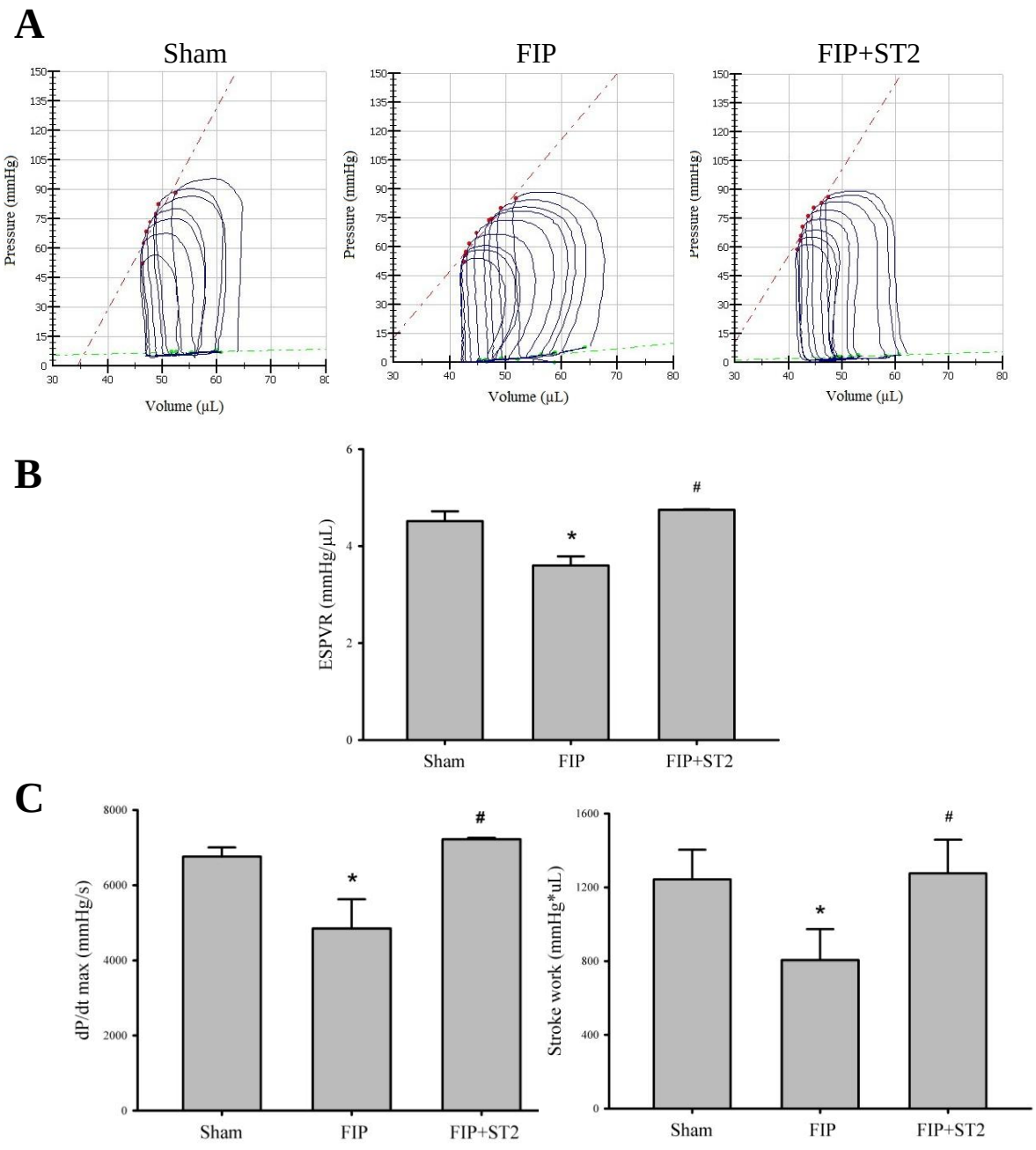

Figure 3.1. Soluble ST2 prevents cardiac dysfunction caused by feces-induced peritonitis.

Cardiac function of mice subjected to FIP in vivo. A) Representative left ventricular pressure-volume loops. B) Quantitative analysis of ESPVR. C) Analysis of dP/dt max and stroke work. FIP treatment decreased various cardiac parameters while sST2 treatment prevented these effects. Data are mean ± SEM, n=3-6/group, *p<0.05 vs. sham, #p<0.05 vs. FIP.

Section 3.3: Collagen staining

Hearts were collected from FIP/sham mice 48 hours after treatment and stained using picrosirius red solution. There was no difference in cardiac collagen staining between sham control and FIP mice (Figure 3.2).

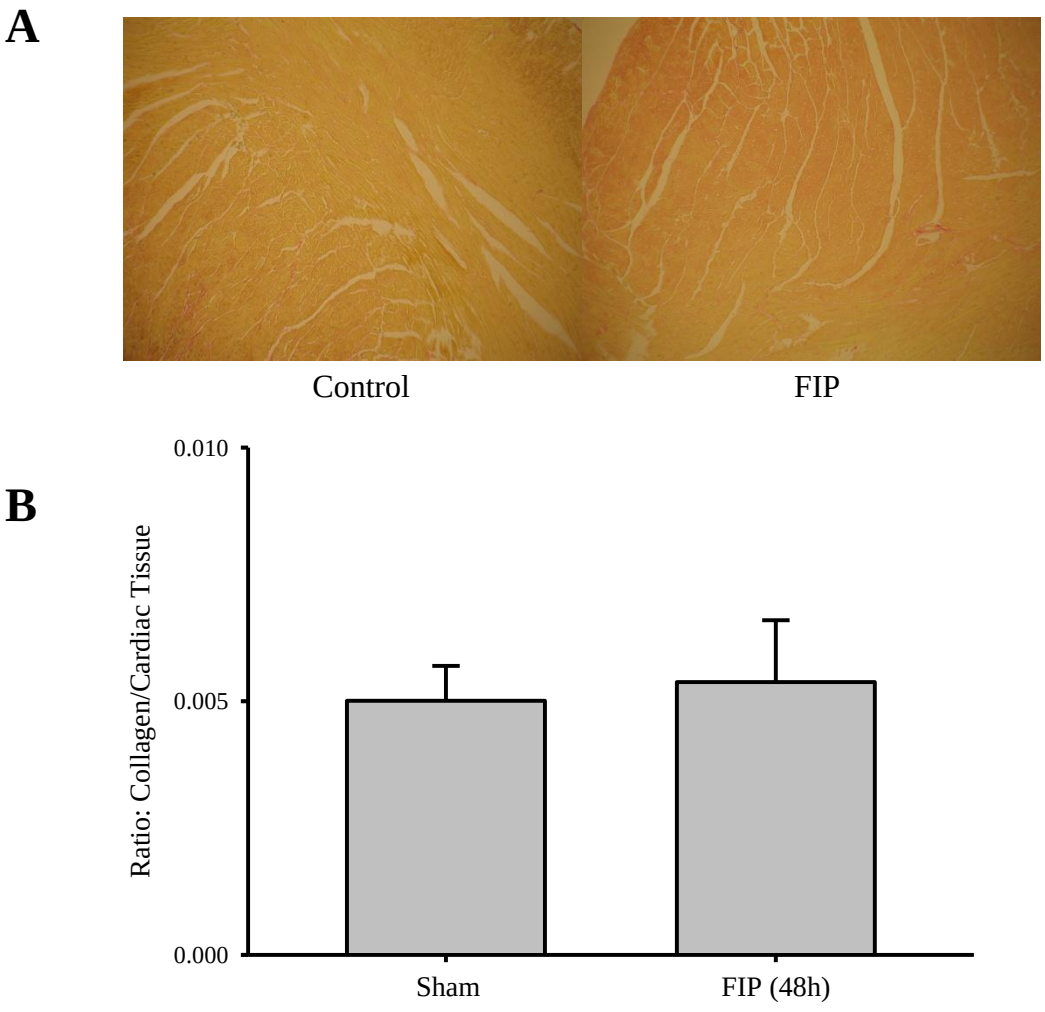

Figure 3.2. Collagen staining of FIP myocardium

A) Representative micro-pictures. Collagen stains red against a yellow tissue background. B) Quantification of collagen content. The numbers are reported as ratio between collagen staining and area of tissue. There was no difference in collagen content between sham and FIP mice. Data are mean ± SEM, n=3/group.

Section 3.4: Expression of IL-33 and MMP-9 in FIP mice

Hearts were harvested from FIP/sham mice after 24 or 48 hours. Cardiac tissue protein expression of IL-33 and MMP-9 were detected using western blot. A significant increase in IL-33 expression was observed in the myocardium from FIP mice that peaked at 24 hours after fecal injection. It returned to basal levels after 48 hours post-FIP (Figure 3.3A). The expression of MMP-9 showed a different time-course than that of IL-33. MMP-9 expression also increased with time however it was seen to be continually increasing well over the 24 h time-point, at which expression of IL-33 peaked. MMP-9 expression in cardiac tissue was seen to be significantly increased 48 hours after fecal injection (Figure 3.3B).

Mouse plasma was also collected to measure circulating IL-33 levels using ELISA. The circulating IL-33 was not detected within the range of sensitivity offered by the ELISA kit (data not shown).

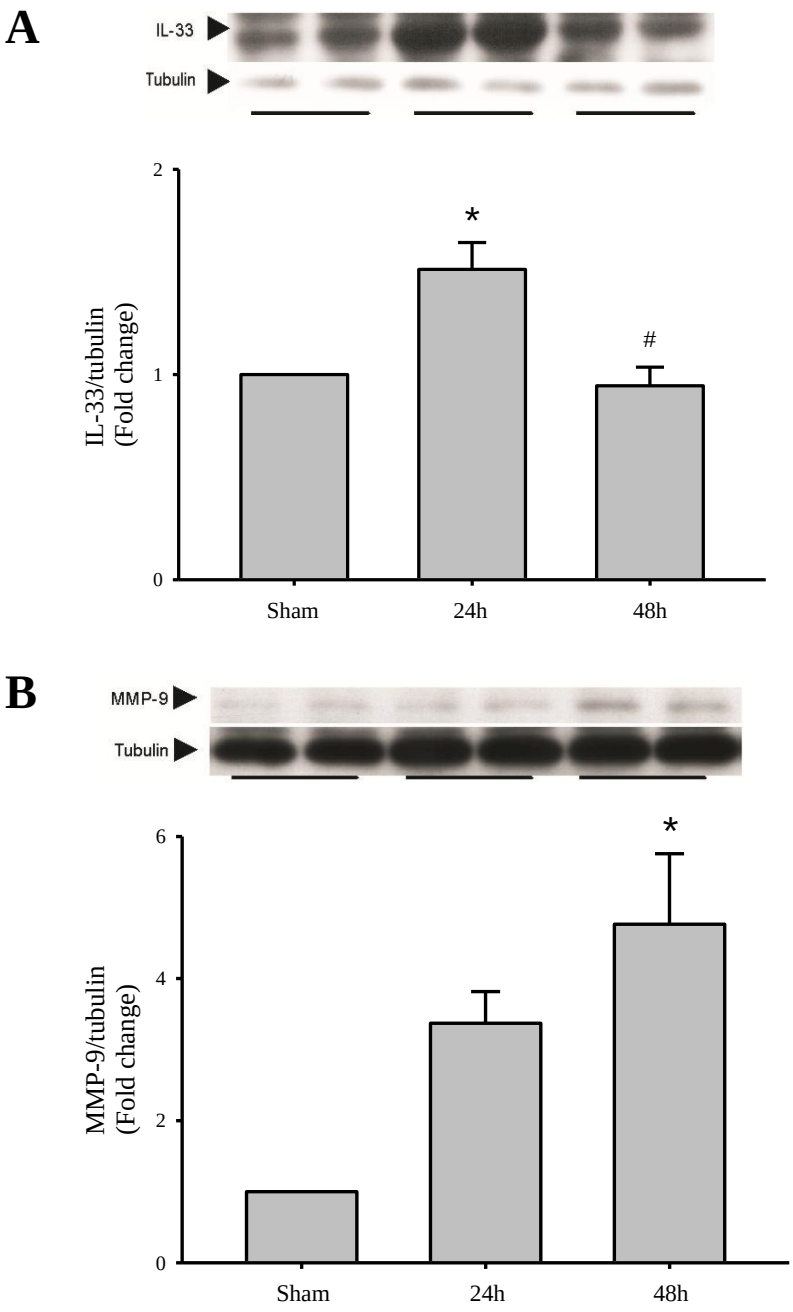

Figure 3.3: FIP leads to increased expression of myocardial IL-33 and MMP-9.

Densitometric analysis of A) IL-33 and B) MMP-9. Representative western blots are shown above. IL-33 and MMP-9 levels increased in FIP mice myocardium compared to sham controls. IL-33 levels peaked at 24 hours while MMP-9 levels were slower to rise. Data are mean ± SEM, n=3-4/group, *$p<0.05$ vs. sham, #$p<0.05$ vs. 24h.

Section 3.5: Myocardial MMP-9 in FIP mice using gelatin zymography

Level of MMP-9 in heart tissue was also measured by utilizing the protein's ability to degrade gelatin. There was significant gelatin clearing at 92kDa region in sample lanes loaded with heart lysate from FIP mice 48 hours after treatment (Figure 3.4). The increased MMP-9 activity indicates higher protein concentration in those heart samples.

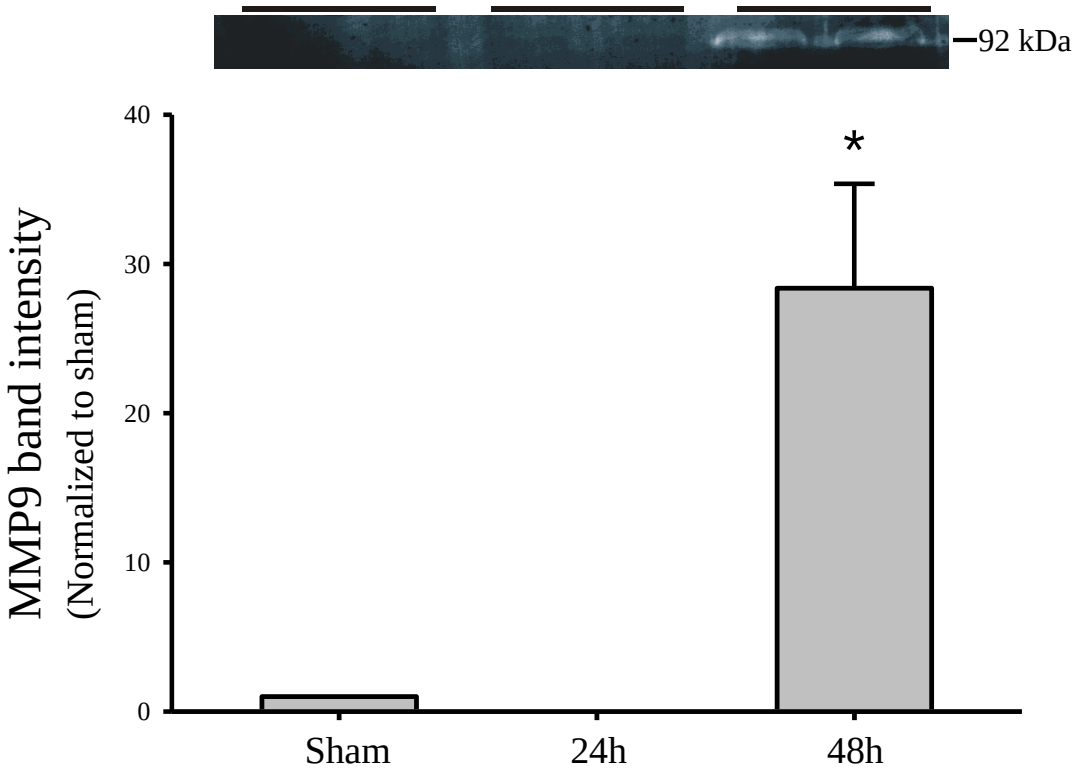

Figure 3.4: FIP leads to increased expression of myocardial MMP-9

Representative zymogram reveals presence of MMP-9 activity in heart tissue samples from FIP mice, 48 hours-post inoculation. Densitometric analysis of specific 92 kDa gelatinolytic activity is shown below. Data are mean ± SEM, n=3/group, *$p<0.05$ vs. sham.

Section 3.6: Soluble ST2 prevents augmentation of MMP-9 expression in FIP mice myocardium

To elucidate the relationship between IL-33 pathway and regulation of MMP-9 in mice myocardium, FIP mice were treated with sST2, a decoy receptor of IL-33, 8 hours post fecal injection. 48 hours after FIP initiation the heart was excised out and gelatin zymography was performed with tissue lysate. As observed previously, FIP treated mice have increased activity of MMP-9 in its myocardium lysate. In FIP mice treated with sST2 the induction of MMP-9 in myocardium lysate is inhibited (Figure 3.5).

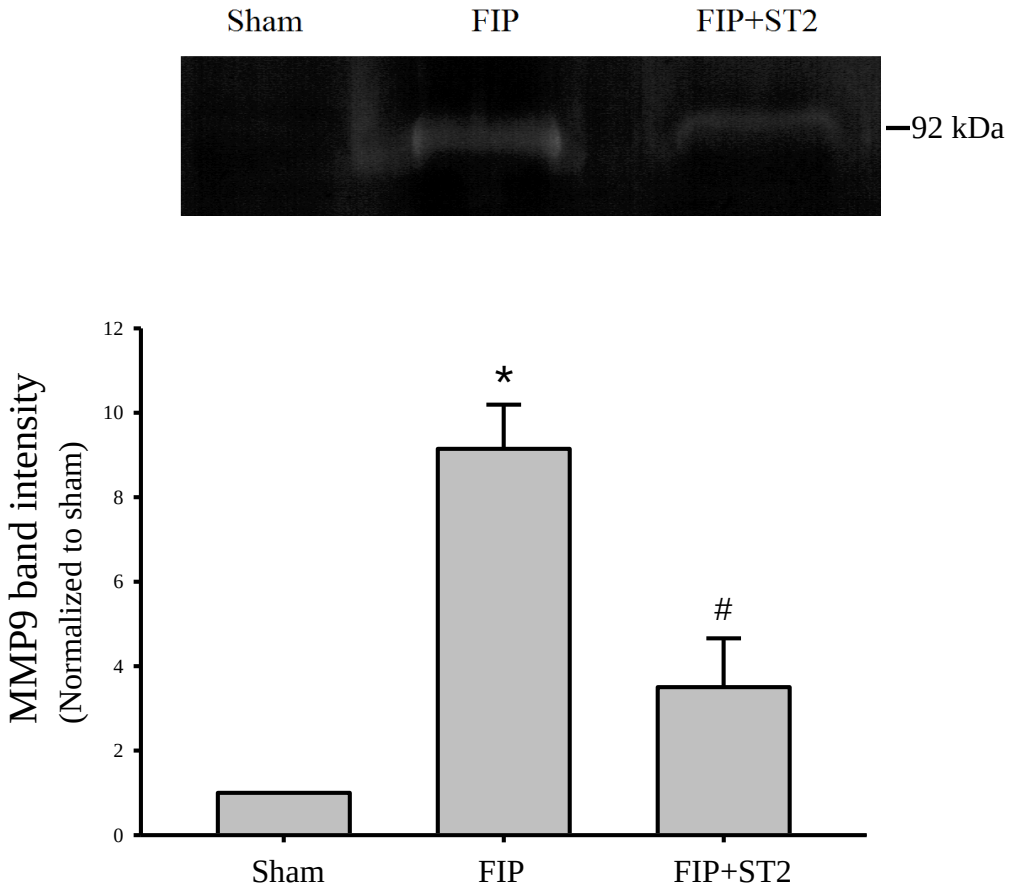

Figure 3.5. ST2 prevents MMP-9 induction in FIP mice heart

Representative zymogram reveals presence of MMP-9 activity in heart tissue samples prepared 48 hours after treatment. The increased gelatinolytic activity in FIP mice heart was prevented by sST2 treatment. Densitometric analysis of specific 92 kDa gelatinolytic activity is shown below. Data are mean ± SEM, n=3/group, *$p<0.05$ vs. sham, #$p<0.05$ vs FIP.

Section 3.7: Endotoxin levels, comparison between in vivo and in vitro system

In order to determine if fecal inoculation released significant amount of endotoxin into the circulation, the plasma samples were collected at various time points after the initiation of peritonitis. Endotoxin in the circulation was significantly increased 1 hour after fecal inoculation and rapidly cleared out of the system (Figure 3.6A). The LPS in regular growth cell culture media and LPS-treatment cell culture media were also quantified (Figure 3.6B). All samples were analyzed with commercially available Chromogenic Limulus Amoebocyte Lysate Endotoxin Assay kit from GenScript (ToxinSensor™). This kit measures endotoxin based on its biological activity. It was found that 1 μg of LPS does not translate to full biological activity; only 643.2 ± 1.3 pg of active LPS was measured (Figure 3.6B).

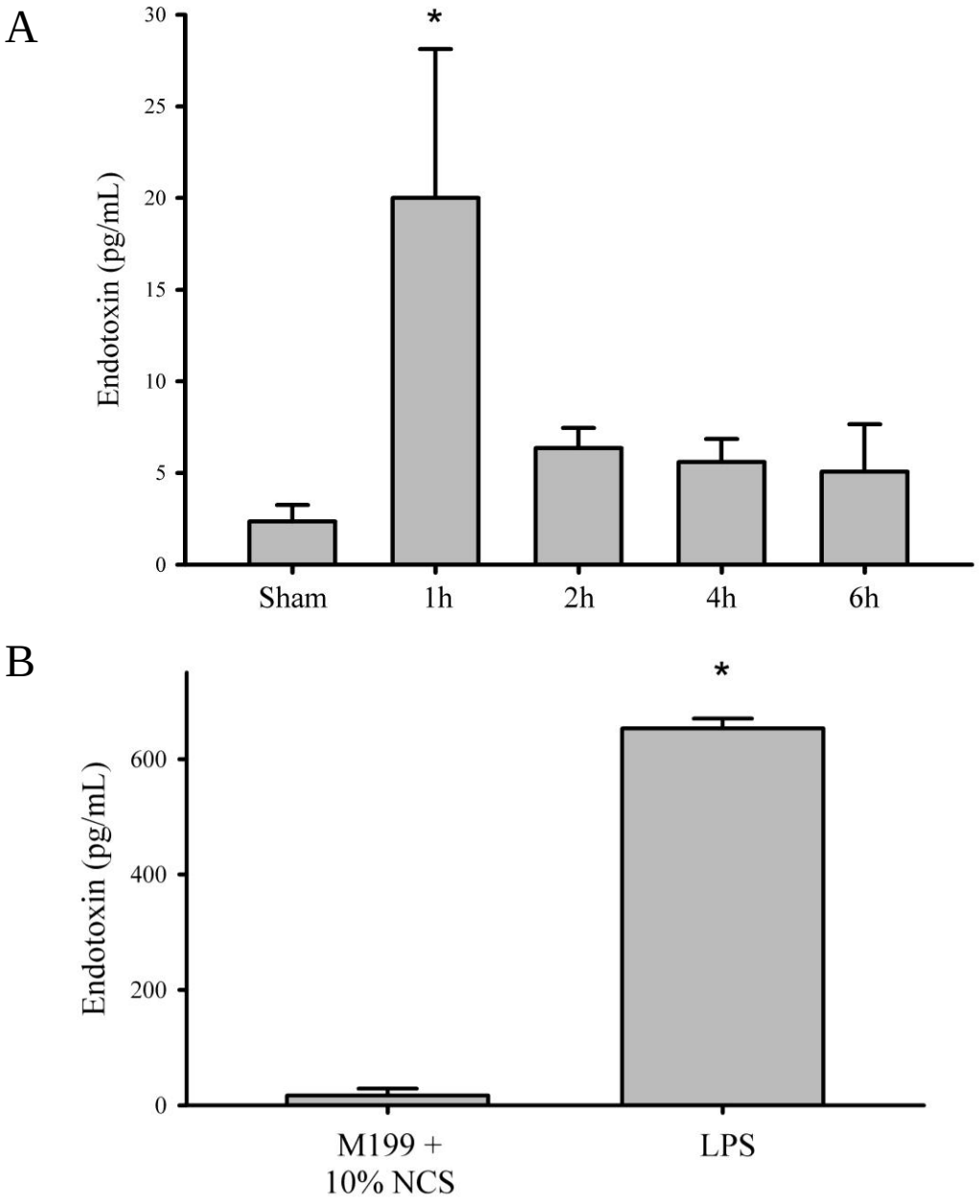

Figure 3.6: Measurement of endotoxin in in vivo and in vitro model.

A commercial endotoxin detection kit was used to measure A) endotoxin present in full-strength plasma from FIP mice and B) cell culture treatment media with LPS (1 µg/mL in M199). FIP treatment increased circulating LPS which was significant after 1 hour. It was seen with cell culture treatment media that not all of LPS present exhibit endotoxic activity. Data are mean ± SEM, n=6/group, *$p<0.05$ vs. control.

Section 3.8: LPS induces IL-33 expression in cultured cardiomyocytes

To determine the expression profile of IL-33 induced by LPS, primary cardiomyocyte culture isolated from neonatal mice was treated with LPS (1 µg/ml) in M199 supplemented with FBS. After 4, 8, or 24 hours the cell lysate was collected to be analyzed by western blot. IL-33 expression was significantly increased 4 hours after LPS administration and peaked at 8 hours. LPS-induced IL-33 expression started to decline at 24 hours back to basal levels (Figure 3.7).

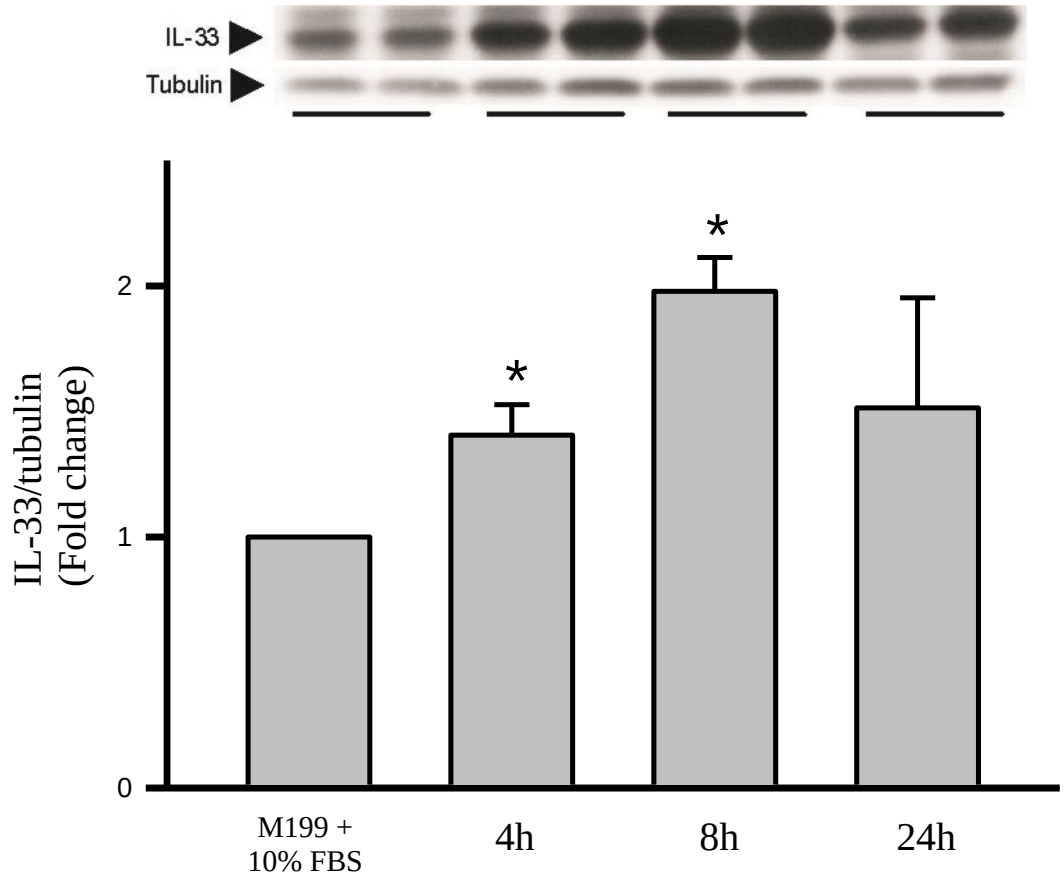

Figure 3.7: LPS treatment increases IL-33 expression in cardiomyocytes.

Representative western blots are shown above and densitometric analysis below. LPS treatment increased IL-33 expression in cardiomyocytes compared to control, which peaked around 8 hours. Data are mean ± SEM, n=3/group, *$p<0.05$ vs. control (M199+10% FBS).

Section 3.9: Cardiomyocytes secrete MMP-9 in response to LPS

To determine if LPS can induce release of MMP-9, the cardiomyocytes were treated with LPS (1µg/ml) in M199 without serum. MMP-9 expression in the conditioned media was significantly increased 8 hours after LPS treatment (Figure 3.8). There was no detectable level of intracellular MMP-9 (data not shown).

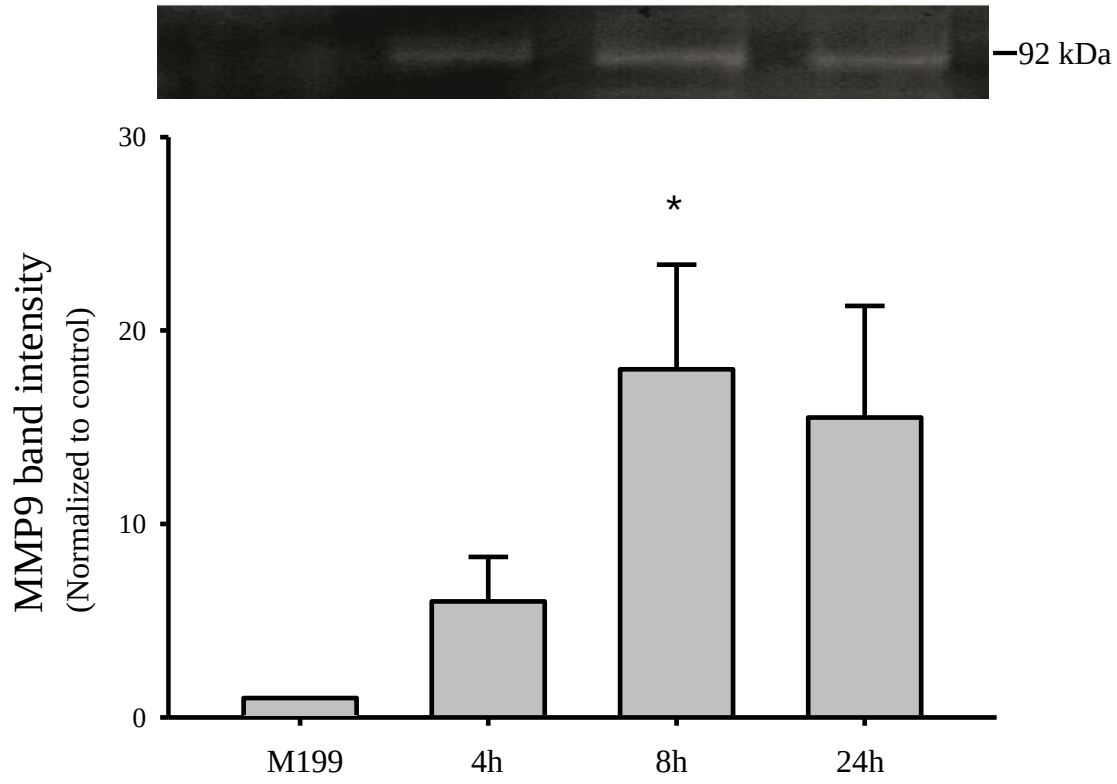

Figure 3.8: LPS treatment increases MMP-9 release from cardiomyocytes.

Representative zymogram of MMP-9 in cardiomyocyte culture media is shown above and densitometric analysis below. The 92 kDa gelatinolytic activity in the media is significantly increased 8 hours after LPS treatment. Data are mean ± SEM, n=3/group, *$p<0.05$ vs. M199.

Section 3.10: Soluble ST2 inhibits induction of MMP-9 in cardiomyocytes

Similar to what was done in vivo where FIP mice were treated with sST2, the cardiomyocytes were treated with LPS (1 µg/mL) or LPS+ST2 (100 µg/mL) or M199 alone to look at the IL-33/ST2 and MMP-9 relationship. As we observed before, LPS induced cardiomyocytes released significant amount of MMP-9 into the cell culture media. This increase was inhibited in cell culture treated with LPS and sST2 (Figure 3.9).

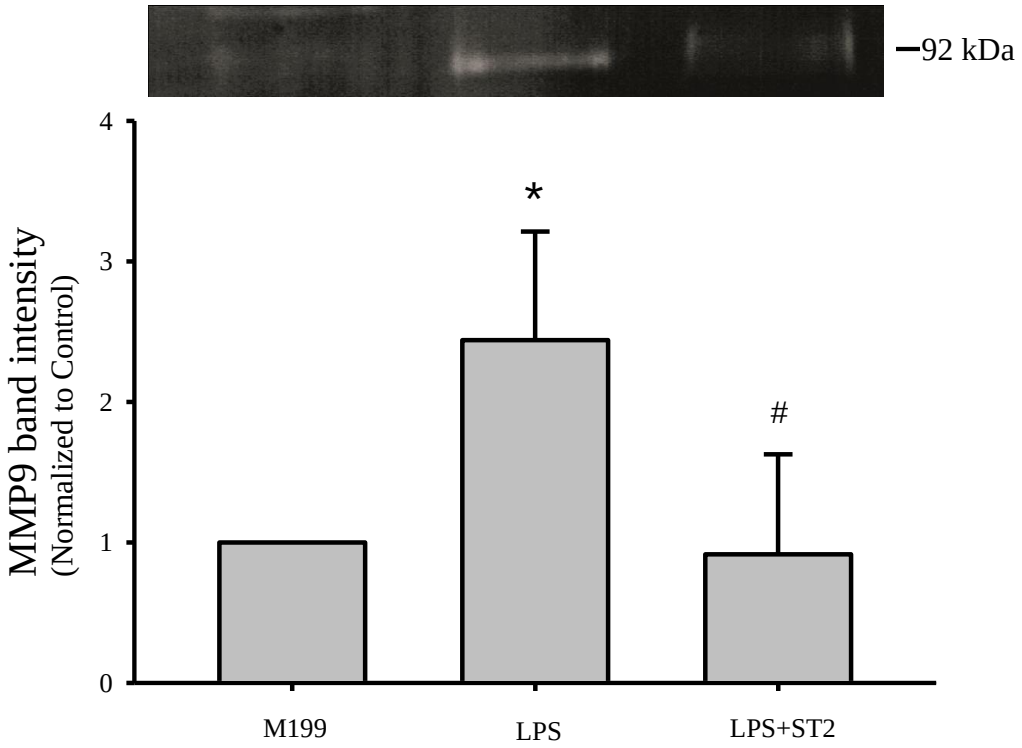

Figure 3.9: Soluble ST2 inhibits LPS-induced MMP-9 release from cardiomyocytes.

Representative zymogram of MMP-9 activity in cardiomyocyte culture media is shown above and densitometric analysis below. LPS-induced 92 kDa gelatinolytic activity is prevented by sST2 treatment. Data are mean ± SEM, n=3/group, *$p<0.05$ vs. M199, #$p<0.05$ vs LPS.

CHAPTER 4: DISCUSSION

Cardiac dysfunction is a major contributing factor to increased morbidity and mortality in septic patients. However, the exact cellular mechanisms underlying the maladaptive changes are not yet fully elucidated. This study demonstrates, for the first time, that IL-33 may contribute to the myocardial depression observed in the mouse sepsis model. Feces inoculation in the mice peritoneal cavity successfully reproduced the hematological patterns commonly observed in clinical sepsis. Using this model, the results showed increased expression of IL-33 and MMP-9 in mouse myocardium which was reproduced in cell culture studies where cardiomyocytes were treated with LPS. Also, inhibition of IL-33 using a decoy receptor, sST2, prevented decreases in cardiac function observed in FIP treated mice. This was reproduced in cell culture studies where LPS-induced MMP-9 expression in cardiomyocytes was inhibited by sST2 treatment.

Previous studies have reproduced cardiac dysfunction in animal models of sepsis [103] and endotoxemia [104]. To establish a comparable sepsis animal model, hemodynamic and hematological characteristics were compared and observed to be similar to previous animal models [101] and clinical studies. However the plasma lactate levels were opposite to what is most commonly observed [105]. This may be attributed to a variety of things. Serum lactate level is a balance of lactate production and utilization. Hence, it will not only depend on processes that increase lactate production, but also on how much is used. Hyperlactatemia is prevalent in many septic patients, and lactate levels serve as a good diagnostic index [102]. The common understanding of hyperlactatemia in shock is that it's a secondary response to tissue hypoxia brought upon by tissue hypoperfusion. This indeed is the case for some septic situations but certainly not all. Previous studies have reported hypoxia-induced hyperlactatemia in septic patients

with low cardiac output, where high lactate levels predominantly reflect poor oxygen delivery [106, 107]. There are also cases where hyperlactatemia in septic shock cannot be solely attributed to anaerobic metabolism. If lactate production came from hypoperfused organs, we would expect to see decreased ATP levels. This was not the case when they measured ATP levels from muscle, which turned out to be normal [108]. Hence, it appears that elevated lactate levels observed in septic conditions may not always reflect tissue hypoxia and may occur from a variety of etiologies. Though we did not observe a difference in lactate levels in our model of sepsis, it cannot be concluded this was solely due to lack of cellular hypoxia. Also, previous reports have indicated lactate improve heart function by acting as substrate for additional energy [109].

Generally, there are four criteria that need to be satisfied to confirm a causal relationship. First, the presence and elevation of causative factor must be observed. Then, the response caused by factor must be attenuated by inhibiting the factor. This is then confirmed by reproducing the response by providing the factor. And finally, appropriate temporal relations are compared between the elevation of the factor and the observed response. In our study, three of these criteria were confirmed in in vivo and in vitro systems.

This study demonstrated that the inflammatory stimulus induced by feces inoculation increased myocardial IL-33 and MMP-9 expression. This was demonstrated via western blotting and again using gelatin zymography for MMP-9. Although there appears to be some MMP-9 expressed at 24 hour time point as analyzed with western blotting (Figure 3.3B) opposed to none in gelatin zymography (Figure 3.4), this increase at 24 hours was not statistically significant. However, for both western blot and gelatin

zymography, the MMP-9 expression increased significantly at 48 hours post-feces inoculation. Thus, MMP-9 results from two quantitative methods were congruent, providing stronger evidence of MMP-9 induction. Also, comparing the time course for induction of IL-33 and MMP-9, IL-33 expression peaked at 24 hours, ahead of MMP-9 induction, which peaked at 48 hours. There was no evidence of elevated IL-33 in the circulation when plasma samples were analyzed with ELISA. It appears that not enough IL-33 was produced in the myocardium and was released into the circulation and thus could only be measured locally (i.e. in the heart tissue). Though we did not look at circulating MMP-9 in our animal sepsis model, previous studies have reported elevated MMP-9 in the blood of septic patients which correlated with disease severity [110].

Our sepsis model reproduced cardiac dysfunction observed in septic patients with decreased cardiac function after FIP injection. These decreased hemodynamic parameters were prevented when IL-33 was inhibited by injecting the mice with sST2. This supports our hypothesis that IL-33 is involved in sepsis-induced cardiac dysfunction. In an attempt to better understand the process, the heart tissue from sST2-injected FIP mice were further analyzed. Myocardial MMP-9 was measured using gelatin zymography. We observed that FIP mice injected with sST2 did not have increased expression of myocardial MMP-9. Taking the observation that IL-33 is involved in aberrant cardiac function and controls expression of MMP-9, we looked at a possible target of MMP-9 expression.

A novel finding by Yu et al. showed there was reduction in collagen content in a rat model of sepsis [13]. Our animal sepsis model could not be used to analyze the impact of FIP on the quantity of myocardial collagen. Both the control sham and FIP groups had

very little collagen staining that it was difficult to accurately determine if MMP-9 altered the absolute collagen content, if at all, since it was hard to see any changes at a small scale. However, actions of MMPs are not only limited to collagen degradation. It can disrupt the organization of ECM matrix which alone can have aberrant effect on the cardiac contractility [111].

Taking together the results that: 1) the time course of IL-33 and MMP-9 induction differ, with increased expression of IL-33 ahead of MMP-9 expression; 2) inhibition of IL-33 prevents induction of MMP-9 seems to suggest that signaling of IL-33 and MMP-9 expression is a cause and effect relationship. The results demonstrate that the IL-33/ST2 pathway plays a role in regulating MMP-9 expression in the myocardium and cardiac function of FIP treated mice.

Although IL-33 could not be measured in the circulation in our study, it was significantly elevated in the heart. It is a strong indication that IL-33 mediates its effects at to the myocardium as many growth factors and cytokines have been known to get trapped in the tightly knitted ECM [112]. However, it should not be immediately assumed that there is a link between IL-33 and MMP-9 expression based only on time profile. To address this, we followed up with in vitro studies which provide a simpler system to study signaling mechanisms.

We cultured primary cardiomyocytes and subjected them to LPS treatments. LPS is a well known myocardial depressant as seen after animals have been injected with LPS [113]. We have demonstrated that LPS levels are detectable in the circulation in our animal model and thus acceptable to use as a reasonable agent to use to treat cardiomyocytes. Although it was only elevated significantly for a short period of time (1

hour; Figure 3.6), it may be able to initiate the release of other mediators such as cytokines that relay the myocardial depressant effects on to effector proteins. LPS treatment has already been reported to induce expression of MMP-9 in various cellular systems [80, 81]. Our study aimed to identify if LPS-induced MMP-9 expression is regulated by IL-33 as observed in in vivo. Results showed significant induction of IL-33 and MMP-9 expression in cardiomyocytes after LPS treatment. IL-33 was significantly increased 4 hours after LPS treatment and started returning back to basal level after 24 hours. MMP-9 expression was significantly increased 8 hours after LPS treatment and was observed to be maintained past this time point. Similar to in vivo results, IL-33 induction preceded MMP-9. The induction of IL-33 and MMP-9 appears to be quicker in vitro than in vivo and this may be due to the extra time required for gram-negative bacteria to shed its cell wall components into the circulation and for LPS to reach the myocardium. Also confirming results from sST2 injection in mice, when LPS treated cardiomyocytes were incubated with sST2, induction of MMP-9 was prevented.

This study presents a novel finding where inhibition of IL-33 via sST2 treatment prevented LPS-induced MMP-9 expression. We demonstrated similar results where FIP-induced MMP-9 expression in the myocardium was inhibited by sST2 inoculation. We have also shown a differential time induction of IL-33 and MMP-9 expression after LPS and FIP treatment. Taken together, the inhibition of IL-33 prevents MMP-9 induction and the delayed induction of MMP-9 compared to IL-33, provide support for the concept that IL-33 regulates the expression of MMP-9 in LPS treated cardiomyocytes.

Potential pathways involved between LPS, IL-33 and MMP-9 need to be discussed. It is well documented that LPS binds to the TLR4/MD-2/CD14 complex [114]

and initiates subsequent MyD88-dependent signaling which will eventually activate NF-κB via activation of MAPKs. NF-κB is a powerful inducer of innate immunity and triggers expression of many pro-inflammatory cytokines to mediate the inflammatory response [115]. This study demonstrates that LPS induces IL-33 expression in cardiomyocytes. It remains to be confirmed whether LPS-induced IL-33 expression is mediated via NF-κB. Since in our study sST2 treatment inhibited MMP-9 expression in cardiomyocytes, it provides a strong indication that IL-33/ST2 signaling is involved in LPS-induced MMP-9 expression. Cheng and colleagues also reported LPS treatment lead to induction of MMP-9 in cardiomyoblast cells and this was dependent upon ERK1/2 [116]. IL-33/ST2 pathway activates several MAPK pathways and ERK1/2 is one of them [50]. Perhaps LPS-induced MMP-9 expression in cardiomyocytes is mediated by ERK1/2, activated by IL-33/ST2 signaling cascade (Figure 4.1).

This study has demonstrated IL-33 plays a crucial role in mediating cardiac dysfunction in septic mice. It has been shown for the first time that myocardial expression of IL-33 and MMP-9 are increased after septic insult and sST2 treatment prevented the resulting myocardial depression. This suggests that downstream effectors of IL-33 have a deleterious role in altering the cardiac function after inflammatory stimuli. One of the effectors involved in mediating myocardial depression may be MMP-9. This study's strength relies on confirming in vivo results with in vitro results. This strengthens the hypothesis and opens possibilities for further research to answer novel questions this study poses. This study has not addressed details of the signaling mechanism between LPS, IL-33 and MMP-9 (highlighted in dashed arrows in Figure 4.1). Also, though we have a functional endpoint to our animal study, we have not been able to establish an

anatomical or mechanistic endpoint. We observed that sST2 prevent cardiac dysfunction but could not answer how after picrosirius heart staining produced non-significant results.

Despite substantial research completed with animal sepsis models with breakthrough finding, it has produced disappointing results in clinical trials [23, 26]. Mice cannot accurately simulate clinical sepsis for various reasons. For one, mice of the same age and weight are inbred and do not have the varying comorbidities of people, which contribute significantly to patient outcome. In addition, clinical sepsis arises from various infectious sources such as pneumonia and abdominal infection which may have an impact on the magnitude of response to infection. There are also differences at the molecular level since mice and human are different species. For example, in humans, C-reactive protein (CRP) is known activate the complement system in inflammatory situations whereas in the rat, it does not [117, 118]. However, the use of animal model still allows investigators to look into fundamental pathological processes that may help us understand clinical cases. Also, it allows for much larger sample size, control of confounding variables and is relatively inexpensive.

In conclusion, this study demonstrated IL-33 inhibition reduced the myocardial dysfunction in FIP treated mice. Combined results from cellular and animal studies point to induction of IL-33 expression in the myocardium and that IL-33 regulates MMP-9 response in cardiomyocytes.

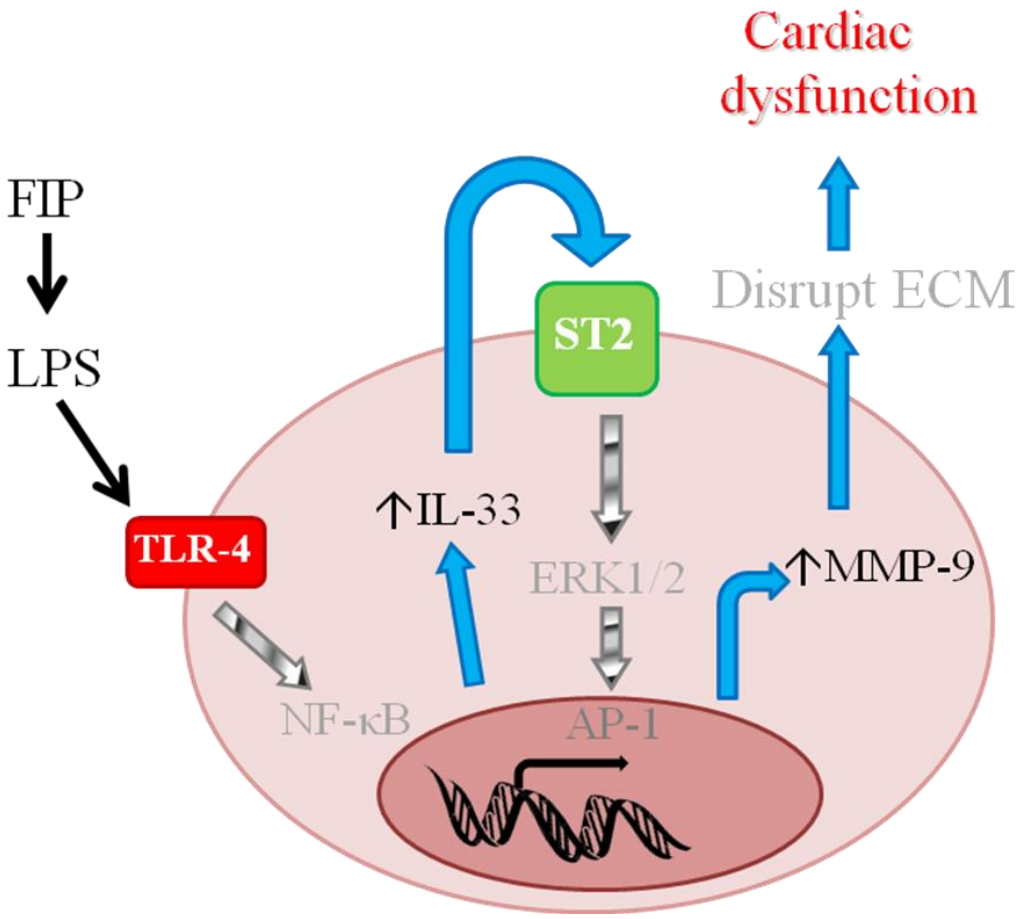

Figure 4.1. Schematic diagram of the hypothetical mechanism in the LPS/IL-33 signaling pathway in cardiomyocyte.

This study demonstrated IL-33 inhibition reduced the myocardial dysfunction in FIP treated mice. FIP induces IL-33 expression in the myocardium and IL-33 regulates the MMP-9 response in cardiomyocytes. The signaling pathways indicated by dashed arrows have not been confirmed in this study but a proposed mechanism that remains to be investigated in future studies.

CHAPTER 5: FUTURE STUDIES

Although cardiac dysfunction was observed using a functional study, the morphology of the diseased heart could not be observed due to limited resources. Septic heart has been reported to go through reversible dilation of ventricular walls [6, 119]. It would be interesting to see if IL-33 levels correlate with ventricular dilation and if it can be prevented by sST2 treatment.

Also, though we could not observe quantitative changes in myocardial collagen, various cardiac pathologies where MMPs are involved have disorganization of the collagen network rather than an absolute change in content [120]. It would be interesting to study the structural organization of the collagen network of the myocardium using scanning electron microscopy.

To further confirm our experimental findings it would be interesting to repeat the experiments with a CLP sepsis model. The scope of our study was limited to looking at cardiac function; however, the next step will require addressing the signaling pathways between LPS, IL-33 and MMP-9 and also, look at other organs commonly affected during sepsis.

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