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Annexin A5 Inhibits Myocardial Inflammation and Improves Cardiac Function during Endotoxemia in Mice

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Annexin A5 Inhibits Myocardial Inflammation and Improves Cardiac Function during Endotoxemia in Mice

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Abstract

Sepsis is a complex clinical syndrome that results from a harmful or damaging host response to infection. Sepsis is characterized by the production of proinflammatory cytokines like TNF-α and IL-1β, which have been shown to contribute to cardiovascular dysfunction. Annexin A5 is a phospholipid binding protein that has been shown to have anti-inflammatory, anti-apoptotic and anti-coagulant properties. However, its effect on cardiac function during sepsis has not been established. This thesis investigated the effect of annexin A5 on myocardial cytokine production and cardiac function during endotoxemia in mice. It was found that annexin A5 treatment at the onset of endotoxemia abrogated TNF-α and IL-1β production and prevented cardiac dysfunction. Annexin A5 likely interacts with cell-surface receptor TLR4 to inhibit myocardial cytokine expression to protect against cardiac dysfunction during endotoxemia. These results suggest that annexin A5 provides a novel protective role in the heart during endotoxemia.

Keywords: Annexin A5, tumor necrosis factor-α, toll-like receptor-4, cardiac function, sepsis

Statement of Co-Authorship

The experiments outlined in this thesis were performed by Paul Arnold in the laboratory of Dr. Qingping Feng, with the assistance of co-authors as listed below.

Dr. Qingping Feng was instrumental in experimental design, data interpretation, and manuscript preparation for all experimentation. My advisory committee consisting of Dr. Doug Jones, Dr. Frank Beier and Dr. Morris Karmazyn provided guidance and scrutiny to my studies.

Dr. Sharon Lu provided training in experimental techniques and performed the *in vivo* and *ex vivo* cardiac function procedures (**Figure 12 and 13, Table 1**). Dr. Houxiang Hu and Ms. Murong Liu performed adult cardiomyocyte isolation (**Figure 17**), and Ms. Murong Liu performed neonatal cardiomyocyte isolation (**Figure 16**). As a 480 student, Yin Liu performed and assisted in many experiments that are included in this thesis. Dr. Dylan Burger, Dr. Lamis Hammoud, Ting Zhang, and Fuli Xiang provided valuable technical assistance.

Dedication

I dedicate this work to my family who have shaped me into the person I am today. Mom and Dad, you have never failed to give me encouragement and support. You have always challenged me to be my best, and you have taught me to pursue God above all else. Thank you for your love.

Dave and Mike, all I am is a reflection of you. It is debatable whether that is a good thing or a bad thing, but I wouldn't have it any other way.

To my friends who have challenged me, stretched me, encouraged me, and supported me. I am forever indebted.

To my beautiful fiancée Krista: I can only hope to give you the same love and support you have given me, but I am excited to have the rest of my life to try.

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List of Abbreviations Symbols and Nomenclature

ANOVA	analysis of variance
ATP	adenosine triphosphate
Bcl-2	B-cell lymphoma 2
BPM	beats per minute
BSA	bovine serum albumin
Ca^{2+}	calcium
CD-14	cluster of differentiation 14
cDNA	complementary deoxyribonucleic acid
CI	cardiac index
CO_2	carbon dioxide
CO	cardiac output
COOH	carboxyl group
cNOS	constitutive nitric oxide synthase
DEPC	diethylpyrocarbonate
$+dF/dt_{max}$	maximal rate of force development during contraction
$-dF/dt_{min}$	minimal rate of force reduction during relaxation
DNA	deoxyribonucleic acid
dNTP	deoxyribonucleotide triphosphate
$+dP/dt_{max}$	maximal rate of left ventricular pressure development during contraction
$-dP/dt_{min}$	maximal rate of left ventricular pressure reduction during relaxation
DTT	dithiothreitol
EDTA	ethylenediaminetetraacetic acid
EDVI	end diastolic volume indexes
EF	ejection fraction
ELISA	enzyme-linked immunosorbent assay
eNOS	endothelial nitric oxide synthase
ERK	extracellular signal-related kinase

ESVI	end systolic volume indexes
FADD	fas-associated protein with death domain
FBS	fetal-bovine serum
FCS	fetal-calf serum
H_2O	water
HCl	hydrochloric acid
HRP	horse-raddish peroxidase
IL-1β	interleukin-1
IL-1R	interleukin-1 receptor
i.m.	intramuscular
iNOS	inducible nitric oxide synthase
i.p.	intraperitoneal
i.v.	intravenous
JNK	c-Jun NH_2-terminal kinase
kDa	kilodalton
LPS	lipopolysaccharide
LV	left ventricle
LVEDP	left ventricular end diastolic pressure
LVEF	left ventricular ejection fraction
LVESP	left ventricular end systolic pressure
LVEDV	left ventricular end diastolic volume
LVSP	left ventricular systolic pressure
MAP	mean arterial pressure
MAPK	mitogen activated protein kinase
MI	myocardial infarction
M-MLV	Moloney murine leukemia virus
mRNA	messenger ribonucleic acid
NADPH	nicotinamide adenine dinucleotide phosphate- reduced form
NF-κB	nuclear factor kappa B
NH_2	amine group
NO	nitric oxide

NOX2	nicotinamide adenine dinucleotide phosphate oxidase
O_2	oxygen
P38	protein-38
PAMP	pathogen associated molecular patterns
PBS	phosphate buffered saline
PCR	polymerase chain reaction
P-ERK	phosphorylated extracellular signal-related kinase
P-p38	phosphorylated protein-38
PRR	pattern recognition receptors
PS	phosphatidylserine
RNA	ribonucleic acid
rRNA	ribosomal ribonucleic acid
RT	reverse transcriptase
RT-PCR	reverse transcriptase polymerase chain reaction
SDS	sodium dodecyl sulfate
SEM	standard error of the mean
SIRS	systemic inflammatory response syndrome
SVR	systemic vascular resistance
TACE	tumor necrosis factor alpha converting enzyme
Tau	time constant of isovolumic relaxation
TLR	toll-like receptors
TNF-α	tumor necrosis factor alpha
TNFR1	tumor necrosis factor receptor 1
TNFR2	tumor necrosis factor receptor 2
TIRAP	toll-interleukin 1 receptor domain containing adaptor protein
TRADD	tumor necrosis factor receptor type 1-associated death domain protein
TRIF	TIR-domain-containing adapter-inducing interferon-β
USP	United States Pharmacopeia
WT	wild-type

Chapter 1 - INTRODUCTION

1.1 Sepsis

1.1.1 Overview of Sepsis

Sepsis is a complex clinical syndrome that is characterized by a systemic inflammatory response to infection. Amplification and regulation of the inflammatory response is mediated by the innate immune system in an effort to control and remove the foreign pathogen(s). Various cell types including monocytes, leukocytes, endothelial cells, and even tissue cells work together in a concerted effort to prevent host damage caused by the foreign pathogen. However, ironically sepsis is not a result of the primary infection, but rather the amplified and deregulated host immune response (reviewed by Cohen 2002).

In humans, sepsis is clinically identified when a patient has met 2 of the stated criteria for systemic inflammatory response syndrome (SIRS) and there is evidence of an infection or suspected infection (reviewed by King 2007). These criteria include body temperature greater than 38°C or less than 36°C, heart rate greater than 90 beats/min, respiratory rate greater than 20 breaths/min, and a white blood cell count greater than 12000/mm^3, less than 4000/mm^3, or greater than 10% immature forms (Bone et al. 1992). The majority of infections that lead to sepsis originate in the lungs, but infections can commonly originate in the gastrointestinal tract, urinary tract, and in wounds (reviewed by Sriskandan and Cohen 1995).

Septic shock is a subset of severe sepsis and is currently the 2nd leading cause of death in intensive care units and the 10th leading cause of death in the United States

(Angus et al. 2001; Martin et al. 2003). In Canada, just over 30% of patients hospitalized with sepsis died, compared to 18% for stroke patients and 9.1% for heart attack patients (CIHI 2007; CIHI 2009). Septic shock is accompanied by multiple organ dysfunction, hypotension, and tissue hypoperfusion which can lead to death if the host is unable to compensate for the inflammatory response (reviewed by Kumar et al. 2001). Current therapies include anti-microbial treatments and aggressive fluid resuscitation to modulate the host response and maintain adequate blood pressure for organ perfusion (reviewed by Rivers et al. 2001). Yet, despite a decrease in mortality rate over the last decade, the total number of sepsis-associated deaths continued to increase as the incidence of sepsis rose (Martin et al. 2003). The increasing incidence will continue to put a substantial burden on health care that reportedly spends $17 billion annually for approximately 750,000 cases in the United States (Angus et al. 2001; Martin et al. 2003).

1.1.2 Myocardial Dysfunction During Sepsis

The cardiovascular system is involved in many of the sepsis-associated mortalities resulting from refractory hypotension or cardiovascular collapse (reviewed by Krishnagopalan et al. 2002). The majority of these deaths reflect an inability of the cardiovascular system to adapt to the hyperdynamic state manifested during sepsis and not a direct result of the pathology (Parker et al. 1984). Consequently, survivors have reversible compensatory mechanisms that return cardiovascular function to normal range within 7-10 days, while non-survivors fail to dilate the left ventricle (LV) and are unable to maintain stroke volume and cardiac output (CO) (Parker et al. 1984).

Early understanding of cardiovascular manifestations of septic shock was hindered by limitations of available technology. With the introduction of the pulmonary artery catheter, it was shown that patients with septic shock exhibited a volume resuscitation-dependent hyperdynamic circulatory state, consisting of high cardiac index (CI) and reduced systemic vascular resistance (SVR) (reviewed by Krishnagopalan et al. 2002). The strong correlation between elevated CI and survival highlights the ability of survivors to demonstrate compensatory mechanisms (Nishijima et al. 1973; Parker et al. 1990). It has been well established that myocardial contractility is compromised during sepsis leading to decreased ejection fraction (EF) (Parker et al. 1984; Natanson et al. 1986). As a result, the LV is dilated in an effort to maintain stroke volume and CO in light of decreased cardiac contractility, which increases end diastolic and end systolic volume indexes (EDVI/ESVI) (reviewed byParrillo et al. 1990). Therefore, there is a strong negative correlation in survivors between EDVI and EF that is not found in non-survivors (reviewed byParrillo et al. 1990).

The cause of the apparent myocardial dysfunction remained elusive for many years. The predominant theory was that during sepsis the heart suffered from hypoperfusion similar to other organs. Decreased SVR and an increased hypercoagulatory state which leads to enhanced deposition of fibrin clots in small blood vessels, contribute to inadequate tissue perfusion and subsequent organ failure (reviewed by Cohen 2002). However, two findings by Cunnion et al. 1986 dispelled this theory for the heart during sepsis. They showed that there was no change in coronary blood flow and no myocardial lactate extraction, evidence that the heart's metabolic energy supply was not compromised (Cunnion et al. 1986). The latter was later

confirmed by the preservation of myocardial oxygen metabolism and high-energy phosphates supporting the suggestion that myocardial ischemia is not present during sepsis (Hotchkiss et al. 1991; Solomon et al. 1994).

Two more theories for sepsis-induced cardiac dysfunction have also proven to be flawed. First, although the activities of proapoptotic caspases are increased in hearts during endotoxemia, the rate of apoptosis is too low and disproportionate to the severity of cardiac dysfunction for apoptosis to be responsible (Carlson et al. 2005; reviewed by Crouser 2005). Second, during sepsis neutrophil accumulation is not prevalent in the heart like it is in other organs and it has been shown that neutrophil accumulation is not required for cardiac dysfunction (Raeburn et al. 2002; Peng et al. 2003; Tavener and Kubes 2006).

However, it was shown that compromised ventricular contractility, and not structural damage, was the major cause of depression in early septic shock (Ognibene et al. 1988). Evidence suggested the presence of humoral factors contributing to shock and cardiac dysfunction after neonatal rat myocyte contractility was depressed when exposed to sera from acute phase septic shock patients (Parrillo et al. 1985). Further studies confirmed that endogenous cytokines, like tumour necrosis factor-α (TNF-α), fit the biochemical profile of a 'myocardial depressant substance' that can produce many of the same hemodynamic abnormalities seen during sepsis (Natanson et al. 1989). It has been shown that other cytokines, like interleukin-1β (IL-1β), act synergistically with TNF-α to directly depress cardiac function (Cain et al. 1999).

There appear to be two distinct stages in which cardiac depression occurs after exposure to foreign pathogens or cytokines. The early stage, which can occur within minutes, appears to be nitric oxide (NO) independent (Yokoyama et al. 1993), while the later stage, influential after several hours, is believed to be NO-dependent (reviewed by Meldrum 1998). While studies have linked early myocardial depression and TNF-α expression to NO production from constitutive nitric oxide synthase (cNOS) or endothelial nitric oxide synthase (eNOS) (Finkel et al. 1992; Kinugawa et al. 1994; Peng et al. 2003), it is believed that NO does not play a major role until the induction of inducible NOS (iNOS), which produces NO in much larger amounts and doesn't occur until approximately 5-12 hr after induction of sepsis (Wolfard et al. 2000; reviewed by Kumar et al. 2001).

Septic myocardial depression involves reversible attenuation of the strength of myocyte contraction. Only 2 mechanisms can account for this change: 1) decreased cytosolic Ca^{2+} release and 2) decreased myofilament sensitivity to Ca^{2+} (reviewed by Kumar et al. 2001). The early stage of myocardial depression is a result of decreased Ca^{2+} transient during systole through the sphingosine-dependent inhibition of ryanodine receptors (Yokoyama et al. 1993; Oral et al. 1997). In the later stage, the induction of iNOS by TNF-α results in increased NO levels which can react with oxygen radicals in the cell and create peroxynitrite. Increased NO and peroxynitrite levels desensitize the myofilament to Ca^{2+} leading to sustained contractile dysfunction (Goldhaber et al. 1996). Yet, the precise mechanism of cardiac depression during sepsis remains somewhat elusive. Studies have also emphasized the importance of innate cell receptors, adhesion molecules, and components of the complement system in regulating sepsis-

associated cardiac depression, making a potential therapeutic method difficult to establish (Raeburn et al. 2002; Tavener et al. 2004; reviewed by Rittirsch et al. 2008).

1.2 TNF-α and IL-1β during Sepsis

1.2.1 Overview

The defining characteristic of sepsis is the excessive production of pro-inflammatory cytokines that contribute to systemic inflammation. Cytokines such as TNF-α and IL-1β have been implicated in the pathogenesis of myocardial dysfunction and cardiomyocyte death in ischemia/reperfusion injury, sepsis, and chronic heart failure (Levine et al. 1990; reviewed by Meldrum 1998).

Cytokines are a family of peptides that include peptide growth factors, erythropoietin, transforming growth factor-β, and inflammatory cytokines (eg. TNF-α, interleukins, interferons). Therefore, not surprisingly, they display significant functional diversity in which TNF-α alone can activate growth-promoting effects or initiate programmed cell death.

TNF-α is a 17 kDa pyrogen that was initially discovered as the molecule associated with necrotizing effects in mouse tumors (Carswell et al. 1975), but it is well established that TNF-α is an important inflammatory cytokine that contributes to a wide range of organ pathologies (reviewed by Meldrum 1998). TNF-α remains inactive until it is inserted into the membrane where it undergoes post-translational modification by TNF-α converting enzyme (TACE) prior to its release into the extracellular space (McGeehan et al. 1994). Through TNF-α receptors 1 and 2 (TNFR1, TNFR2), TNF-α can activate receptor-associated domains like FADD and TRADD, the 'death domain',

and TRAF to initiate the activation of caspases and nuclear factor-kappaB (NF-κB) (Chinnaiyan et al. 1995; Hsu et al. 1995).

Proinflammatory cytokines act to increase their own production through activation of transcription factors like NF-κB. IL-1β is produced in response to TNF-α, but was the first cytokine shown to have a wide range of biological properties (reviewed by Dinarello 2005). Increased expression of IL-1β has been implicated in sepsis and rheumatoid arthritis, where treatment with a receptor antagonist decreased inflammation (Bresnihan et al. 1998).

1.2.2 TNF-α and IL-1β Production in the Heart During Sepsis

Traditionally, the liver and spleen are viewed as the primary sources of TNF-α because they have large populations of macrophages, a major source TNF-α production. In the heart however, cardiomyocytes can produce substantial amounts of TNF-α in response to ischemia or lipopolysaccharide (LPS) (Giroir et al. 1992; Kapadia et al. 1995; Gurevitch et al. 1996; Grandel et al. 2000). Although it has been proposed that resident macrophages and cardiomyocytes produce equal amounts of TNF-α in the myocardium, our lab has shown cardiomyocytes to be the major source of TNF-α after LPS exposure (Peng et al. 2003).

Cytokine production during sepsis is adaptive in the short term to fight and clear bacteria, but becomes maladaptive after excessive or long term exposure (reviewed by Elahi et al. 2007). TNF-α and IL-1β levels increase during sepsis and septic shock with elevated TNF-α plasma levels being associated with increased mortality in septic patients (Casey et al. 1993). However, inhibition of TNF-α has failed to demonstrate an

improvement in survival of septic patients (Abraham et al. 1995; Fisher et al. 1996). This is likely due to multiple redundant pathways and is evidence to the complexity of the disease.

1.2.3 TNF-α and IL-1β in Myocardial Dysfunction During Sepsis

The negative impact of TNF-α on cardiovascular function was first observed during immunomodulatory therapy for cancer (Blick et al. 1987; Steinmetz et al. 1988). Since then it was identified as a 'myocardial depressant substance' responsible for many of the cardiac abnormalities observed during sepsis (Natanson et al. 1989). Additionally, many of the biological effects of TNF-α are mirrored by IL-1β (Last-Barney et al. 1988). Synergy of actions between the cytokines can cause myocyte depression at concentrations 50-100 times lower than required individually (Gulick et al. 1989).

Administration of TNF-α or IL-1β into animals or *in vitro* and *ex vivo* tissue results in a concentration-dependent depression of contractility (Finkel et al. 1992; Cain et al. 1999). TNF-α challenge in dogs simulates cardiac depression during human septic shock (Natanson et al. 1989) and cardiac specific TNF-α over expression in mice induces LV dilatation, reduced EF, and premature mortality (Kubota et al. 1997; Bryant et al. 1998). Inhibition of excessive cytokine action through IL-1β receptor antagonists or TNF-α production pathway inhibitors improves myocardial function in response to septic shock (Wakabayashi et al. 1991; Peng et al. 2003).

1.3 Innate Immune Response

1.3.1 Overview of the Innate Immune Response Following Infection

The innate immune system is the body's first line of defense against infection. Macrophages, neutrophils, mast cells, and effector cells trigger the inflammatory response observed during sepsis in an effort to hold the potential infection in check (Figure 1) (reviewed by Warren 2005). Increased vascular permeability, vasodilation, inflammation, and coagulation are initiated in a concerted attempt to isolate and remove foreign pathogen(s) without compromising oxygen and nutrient delivery throughout the body (Clark et al. 2007). Sepsis results when the inflammatory response is exaggerated and becomes damaging to the host. Although deficiency of the innate immune response is associated with an increased susceptibility to infection (reviewed by Rosenzweig and Holland 2004), a greater threat is posed by an excessive inflammatory response (reviewed by Smith 1994).

Innate immunity requires recognition of evolutionarily conserved pathogen associated molecular patterns (PAMPs) associated with particular classes of pathogens. Toll, a *Drosophila* receptor essential for the production of antimicrobial peptides in response to fungal pathogens, led to the discovery of a family of mammalian transmembrane Toll-like receptors (TLR) (reviewed by Miller et al. 2005). TLRs are genome-encoded pattern recognition receptors (PRR) responsible for the identification of foreign pathogens and initiation of the immune response. TLR4, the human homologue of *Drosophila* Toll, is responsible for recognition of gram-negative bacteria, which accounts for approximately 37% of sepsis-associated infections (Angus et al.

2001; Martin et al. 2003). Gram-positive bacteria and fungi are responsible for the remainder.

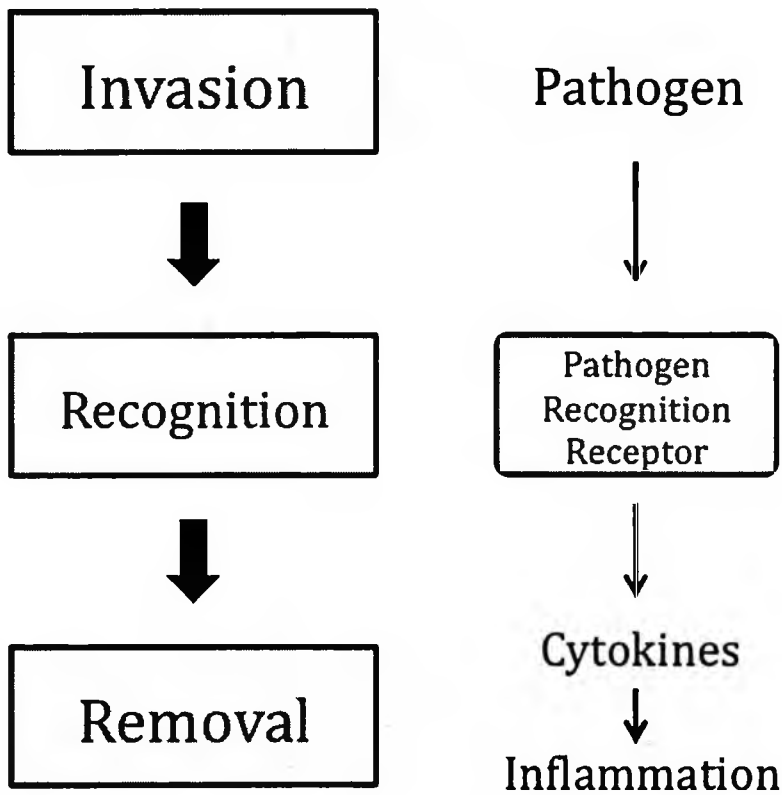


Figure 1. The innate immune system is the body's first line of defense against infection. Innate immunity requires recognition of foreign pathogens by pathogen recognition receptors (PRR) to initiate an immune response that involves pro-inflammatory cytokine production in an effort to control and remove foreign pathogens from the body.

1.3.2 Lipopolysaccharide

Lipopolysaccharide (LPS), also referred to as endotoxin, is a major element of gram-negative bacterial cell walls and is essential for membrane structure and integrity. The lipid A portion of LPS serves to anchor LPS in the bacterial cell wall and is the component recognized by PRRs of the innate immune system. The presence of endotoxins has been correlated with increased mortality and shown to qualitatively mimic many hemodynamic, hematologic, and metabolic manifestations of sepsis (Natanson et al. 1986; Casey et al. 1993).

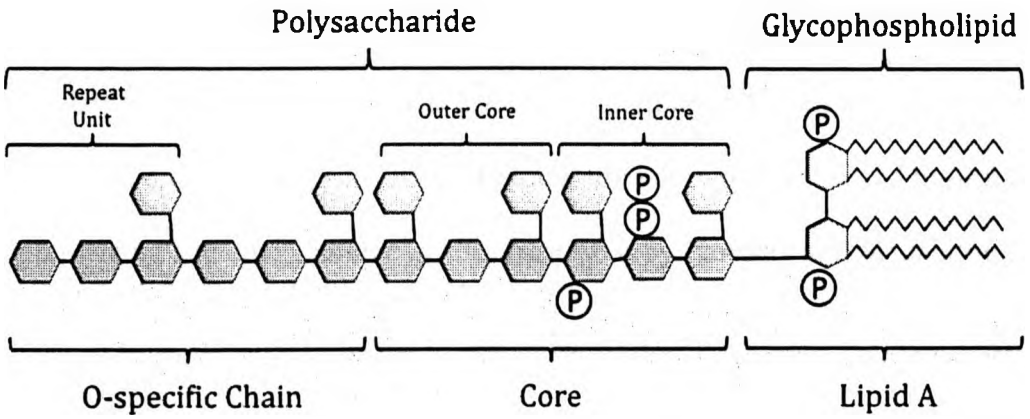


Figure 2. Diagram of lipopolysaccharide structure. Adapted from Caroff et al., 2002.

LPS is amphiphilic and consists of polysaccharide and glycophospholipid portions (Figure 2) (reviewed by Caroff et al. 2002; reviewed by Jerala 2007). The polysaccharide segment is hydrophilic and composed of three sections: the inner core, outer core, and O-specific chain. Bacterial species stereotype specificity and protection from host defence is conferred to the O-specific chain which consists of 1-8 sugar

repeating units (reviewed by Caroff et al. 2002). The glycophospholipid segment is the hydrophobic and negatively charged portion called lipid A. Lipid A is highly diverse and defines the function of LPS through the varied number and length of fatty-acid side chains (reviewed by Miller et al. 2005; reviewed by Jerala 2007). It is stabilized by divalent cations and because of its hydrophobic nature, it serves as the anchor to the bacterial cell wall (reviewed by Caroff et al. 2002). LPS is recognized in mammals by TLR4, which upon activation is responsible for the production of pro-inflammatory cytokines that characterizes sepsis and septic shock (Baumgarten et al. 2001).

1.3.3 TLR4 Signalling and LPS Recognition

TLR homologues are found in all multicellular organisms. They are traditionally expressed on the cell surface but are also found in intracellular compartments and in the bloodstream and tissue fluids. The principal functions of TLRs include induction of apoptosis, opsonization, activation of proinflammatory pathways, and initiation of complement and coagulation cascades (reviewed by Janeway and Medzhitov 2002).

Drosophila Toll was the first member of the 10-member Toll family known to have functional similarities to human IL-1R (ie. TIR domain) because of it's ability to induce activation of the transcription factor NF-κB during the immune response (reviewed by Janeway and Medzhitov 2002). NF-κB is the most important transcription factor that regulates cytokine expression and has been shown to be activated by gram-positive and –negative bacteria, viruses, cytokines, free radicals, and oxidants (reviewed by Zingarelli 2005). Activation of NF-κB, which involves translocation to the nucleus,

can occur within 30 minutes of TLR activation in the myocardium (Baumgarten et al. 2001).

Macrophages are the most studied cell type in understanding LPS signalling through TLR4. Recognition of LPS requires the help of accessory proteins like LPS binding protein (LBP) and CD-14 to transfer LPS to the membrane and enable interactions with TLR4, respectively (reviewed by Janeway and Medzhitov 2002). MD-2 is another small extracellular protein tightly bound to TLR4, which binds directly to LPS and is essential for LPS signalling (Shimazu et al. 1999). The precise role of these accessory proteins remains unclear but mice deficient in any of the LPS receptor components are more susceptible to gram-negative bacterial infection and are hyporesponsive to LPS administration (Haziot et al. 1996; Shimazu et al. 1999; Arbour et al. 2000).

Upon activation by LPS, TLR4 functions as a homodimer to allow its intracellular TIR domain to interact with the adaptor molecules MyD88, TIRAP/MAL, TRAM, and TRIF to initiate signalling (reviewed by Warren 2005). The eventual activation of NF-κB is regulated by various signalling molecules like NOX2 (NADPH Oxidase) and the mitogen-activated protein kinases (MAPK). Our lab has shown previously that NOX2 and the MAPKs p38 and extracellular signal-regulated kinase (ERK) play a pivotal role in regulating TLR4 signalling, and thus the production of proinflammatory cytokines in cardiomyocytes (Peng et al. 2003; Peng et al. 2005).

Activation of TLR4 in cardiomyocytes correlates with increased cytokine production (Baumgarten et al. 2001) and decreased cardiomyocyte contractility (Boyd et

al. 2006). In addition, studies that inhibit TLR4 function through competitive inhibition or point mutations have decreased TNF-α and IL-1β levels and improved myocardial function in response to LPS (Baumgarten et al. 2001; Nemoto et al. 2002; Baumgarten et al. 2006). Therefore TLR4-dependent cytokine production is necessary for myocardial depression during sepsis.

1.3.5 Phosphatidylserine

Phosphatidylserine (PS) is a negatively charged phospholipid normally asymmetrically distributed by an ATP-driven phospholipid translocase (flippase) on the cytosolic surface of cell membranes (reviewed by Vance and Steenbergen 2005). It is believed that PS is involved in regulating membrane charge and protein localization at cytosol facing membrane leaflets. However during periods of stress such as apoptosis, coagulation or anoxia, inhibition of translocase and the Ca^{2+}-dependent activation of scramblase result in PS translocation to the outer membrane leaflet, where it serves as a signal to the extracellular environment (Kuypers et al. 2007).

During coagulation, PS becomes exposed after activation of the endothelium and platelets to serve as the docking site for prothrombinase complex assembly (reviewed by Vance and Steenbergen 2005). During apoptosis, exposed PS is known as an 'eat me' signal for phagocytosis by monocytes. However, while PS exposure is often used as a marker for apoptosis (Koopman et al. 1994), it is not a determined path to apoptosis. Exposure of PS can occur independent of apoptotic mechanisms (Balasubramanian et al. 2007) and has been shown in T-lymphocytes and myoblasts to modulate signal

transduction and mediate cell-cell interactions without committing to apoptosis (van den Eijnde et al. 2001; Elliott et al. 2005).

1.4 Annexin A5

1.4.1 Annexin A5 Overview

Annexin A5 is a 37kDa protein and part of a 13-member family of ubiquitously and constitutively expressed proteins; however, no single annexin is expressed in all cells. All members of the annexin family must satisfy two major criteria: 1) they must be capable of reversibly binding in a Ca^{2+}-dependent manner to negatively charged phospholipids, and 2) they must contain as a conserved structural element, the 'annexin repeat', a segment of some 70 amino acids (reviewed by Gerke and Moss 2002).

Annexins are soluble intracellular proteins that are freely distributed in the cytoplasm at resting Ca^{2+} levels and move to the membranes when Ca^{2+} levels are elevated (reviewed by Gerke et al. 2005). Interestingly, some annexins including annexin A5 have been found to have extracellular functions even though they lack a secretory sequence (reviewed byGerke and Moss 2002). The approximate circulating concentration of annexin A5 is less than 5 ng/ml (Flaherty et al. 1990), and because of its relatively small size, it is readily filtered by the kidney and only has a half-life of roughly 15 minutes (Kuypers et al. 2007).

Although the annexin family lacks functional unity, all of the known functions involve phospholipid membrane interactions because of their ability to bind to

negatively charged phospholipids. Annexin A5 has a unique ability to form a two dimensional crystal shield on planar lipid bilayers containing negatively charged phospholipids, in particular phosphatidylserine (PS) (Voges et al. 1994; Oling et al. 2000). This two dimensional shield is what gives annexin A5 its trade mark anti-coagulant and anti-apoptotic functions (Figure 3) (van Heerde et al. 1994; London et al. 1996; Kenis et al. 2006). In addition, annexin A5 has also been shown to be involved in membrane scaffolding, Ca^{2+} signalling, and membrane-associated internalization (reviewed by van Genderen et al. 2008). Annexin A5's apparent ability to internalize PS expressing membrane patches has even caused it to be proposed as a target for intracellular drug delivery into living tumors (reviewed by Kenis et al. 2007). The mechanism of such a phenomenon is still relatively unknown as it is independent of traditional endocytotic pathways like those of macropinocytosis and receptor-mediated internalization.

1.4.2 Annexin A5 Structure and Binding

Each annexin is made up of 2 principal domains: a conserved COOH-terminal protein core, and a divergent NH_2-terminal head. The COOH-terminal core consists of 4 homologous α-helices forming a slight curvature with 2 principle sides. The convex side contains the Ca^{2+} and phospholipid binding sites that face the membrane when bound. The concave side points away from the membrane and appears accessible for interactions with the NH_2-terminal domain or other binding partners (reviewed by Gerke and Moss 2002). The variable NH_2-terminal head confers the functional specificity for each annexin as it extends along the concave side of the protein core (reviewed by Swairjo and Seaton 1994). The protein core forms a hydrophilic pore between the repeat

1/4 and 2/3 modules where it is believed the NH_2-terminal head may be involved in the regulation of Ca^{2+} conductance through the central pore (reviewed by Gerke and Moss 1997). However, the *in vivo* evidence for Ca^{2+} conductance is scarce, making annexin

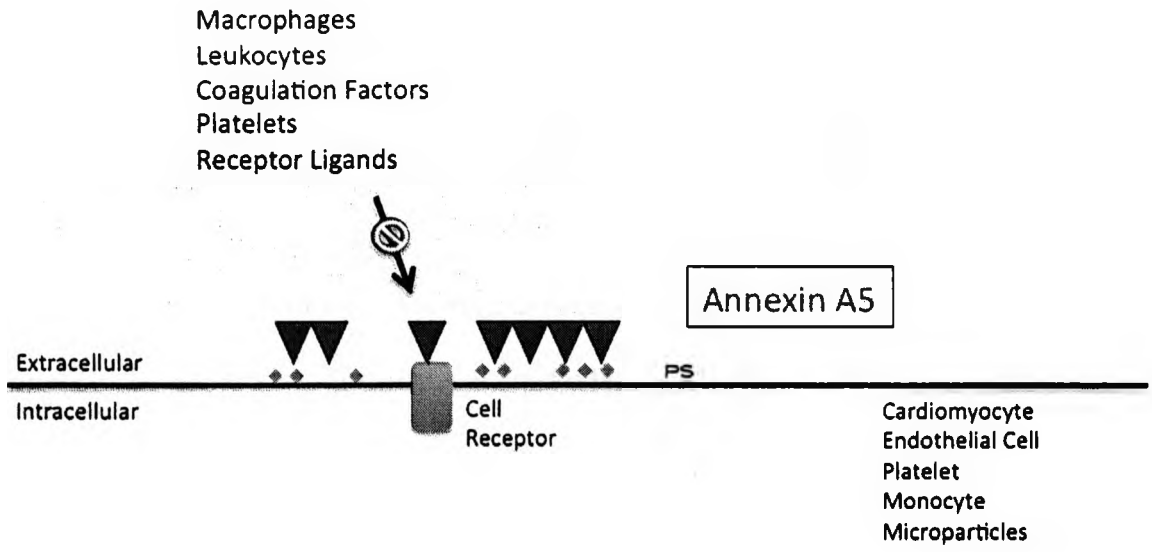
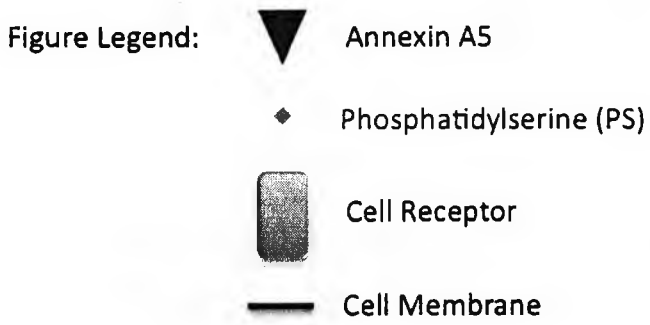


Figure 3. Schematic illustrating the known functions of annexin A5. Annexin A5 binds to cell receptors or exposed phosphatidylserine (PS) on the surface of various cell types like cardiomyocytes, endothelial cells, and platelets. Annexin A5 has the unique ability to form a two-dimensional shield on cell membranes to sterically hinder cell receptor function and PS interaction with various extracellular factors to inhibit coagulation, apoptosis, and inflammation.



A5's ability to form Ca^{2+} channels inconclusive. The diameter of the central pore is inconsistent with the observed conductance values and annexins lack the obvious

exposed hydrophobic regions typical of membrane spanning proteins (reviewed by Swairjo and Seaton 1994; reviewed by Gerke et al. 2005). Therefore it is difficult to assign traditional ion channel properties to the annexins.

Among the annexins, annexin A5 requires the highest Ca^{2+} concentration of 10 μM – 100 μM for half-maximal binding (reviewed by Gerke and Moss 1997). With Ca^{2+} concentration of approximately 1 mM in the circulation, any expressed PS on the cell surface favors the rapid binding of annexin A5 (reviewed by van Genderen et al. 2008). In solution, annexin A5 molecules exist as monomers, but upon binding, they form trimers to assemble a two-dimensional crystal lattice over the surface of PS-expressing membranes (Voges et al. 1994; Oling et al. 2000). It has not yet been demonstrated whether annexin A5 can crystallize on PS-expressing cell surfaces, but *in vitro* studies using constructed phospholipid bilayers demonstrate that crystal lattice density is dependent on PS content and Ca^{2+} concentration. Annexin A5's associate with each other through amino acid mediated protein-protein interactions (reviewed by van Genderen et al. 2008).

The asymmetrical structure of annexin A5 may lead to membrane indentation that could be a necessary mechanism for both Ca^{2+} channel signalling and PS-dependent membrane internalization (reviewed byKenis et al. 2004; reviewed by Gerke et al. 2005). Membrane internalization has been associated with annexin A5's ability to regulate cell-surface receptor expression (Ravassa et al. 2005) and interestingly, annexin A5 has also been shown to bind to and inhibit the function of cell-surface membrane receptors (Leon et al. 2006). In a study investigating the regulation of epithelial

junctions in the kidney, it was found that a conserved sequence in the NH_2-terminal of annexin A5 interacts with a leucine-rich repeat of polycystin-1 to delay the recruitment of E-cadherin at cell-cell epithelial junctions (Markoff et al. 2007).

1.4.3 Annexin A5 Function

Annexin A5 was originally identified as an anti-coagulant in the human placenta to protect against vascular thromboembolism and recurrent pregnancy loss (Reutelingsperger et al. 1985). Annexin A5 has been localized to the apical surface of syncytiotrophoblasts (placentas) where removal or inhibition of annexin A5 leads to loss of placental integrity and pregnancy loss (Wang et al. 1999). Annexin A5 has the ability to interact with exposed PS on platelets and endothelial cells and inhibit prothrombinase complex formation and platelet adhesion through competitive inhibition (van Heerde et al. 1994; Galan et al. 2006; Ghosh et al. 2007). Furthermore, because of its anti-coagulant functions, connections have been made between annexin A5 expression and the incidence of early myocardial infarction (MI) in individuals under 45 years old. A decrease of annexin A5 was associated with early MI patients (Roldan et al. 2002) while an increase in expression through a common polymorphism decreases the risk of early MI two-fold (Gonzalez-Conejero et al. 2002).

However, the most commonly used function of annexin A5 is as a marker of apoptosis. Annexin A5 can be easily conjugated with a wide range of markers to identify exposed PS on the surface of apoptotic cells (Koopman et al. 1994; Vermes et al. 1995). Annexin A5 has also been shown to have anti-apoptotic properties by

inhibiting macrophage or neutrophil phagocytosis that identifies exposed PS as an 'eat-me' signal (Krahling et al. 1999; Kenis et al. 2006; Maugeri et al. 2009).

Some annexins also have the ability to moderate inflammation by controlling leukocyte rolling and adhesion along inflamed vascular beds (Damazo et al. 2005). Diannexin, a recombinant human annexin A5 homodimer, provided profound anti-inflammatory protection against liver ischemia/reperfusion injury (Shen et al. 2007; Teoh et al. 2007). Inhibition of pro-inflammatory molecule expression and recruitment maintained microvascular circulation that dramatically improved survival in rat liver transplants (Shen et al. 2007).

1.5 Hypothesis and Research Objectives

The role of annexin A5 in sepsis-associated inflammation has not been previously investigated. This thesis proposes a novel function of annexin A5 as an inhibitor of inflammation through cell-receptor signalling. The objectives of the results presented in this thesis are to 1) identify annexin A5's interaction with the TLR4 signalling pathway, and to 2) determine if annexin A5 provides protection against sepsis-mediated cardiac dysfunction.

We hypothesized that annexin A5 inhibits TLR4-depedent myocardial TNF-α expression and improves cardiac function in endotoxemic mice (Figure 4).

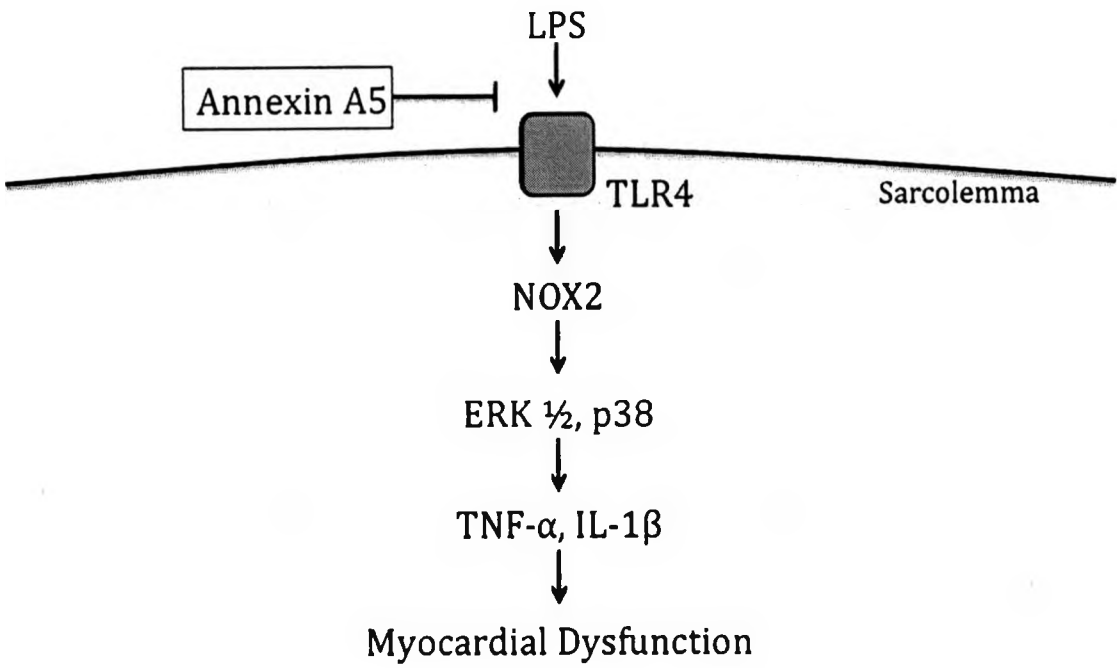


Figure 4. Proposed mechanism of annexin A5 on the LPS-induced TLR4-signalling pathway leading to TNF-α expression and myocardial dysfunction.

Chapter 2 - METHODS

2.1 Experimental Protocol

Animals

The mice used in these studies were wild-type (WT) C57BL/6 (Jackson Laboratories, Bar Harbor, ME). A breeding program was carried out at the Lawson Health Research Institute animal care facility to produce offspring. A total of 103 adult (3-4 months old) male mice weighing 21-26 g were studied. Eighty-seven mice were randomly assigned to the following groups: saline (sham, n=23), recombinant human annexin A5 (n=20), lipopolysaccharide (LPS, n=22), and LPS plus recombinant human annexin A5 treatment group (n=22). In addition, 16 mice were used for macrophage (n=12) and adult cardiomyocyte (n=4) studies. All animals were used in accordance with the guidelines of the Canadian Council on Animal Care and experimental protocols were approved by the Animal Use Subcommittee at the University of Western Ontario, Canada.

Treatments

LPS and annexin A5 treatment time was 4 hr for all measured parameters except for western blot samples, which were collected after 30 min (Figure 5). Western blot samples were taken after 30 min because LPS-induced MAPK phosphorylation was not detectable after 4 hr. The control group received intraperitoneal (i.p.) and intravenous (i.v.) saline injections. The annexin A5 alone group received 5 μg/kg i.v. injections of human recombinant annexin A5 (Biovision, Mountain View, CA) and i.p. saline injections. The LPS alone group received 4 mg/kg i.p. injections of *Salmonella typhosa*

LPS (Sigma-Aldrich, St. Louis, MO). The LPS+annexin A5 group received sequential injections at the same concentrations used in the individual treatment groups (Figure 5).

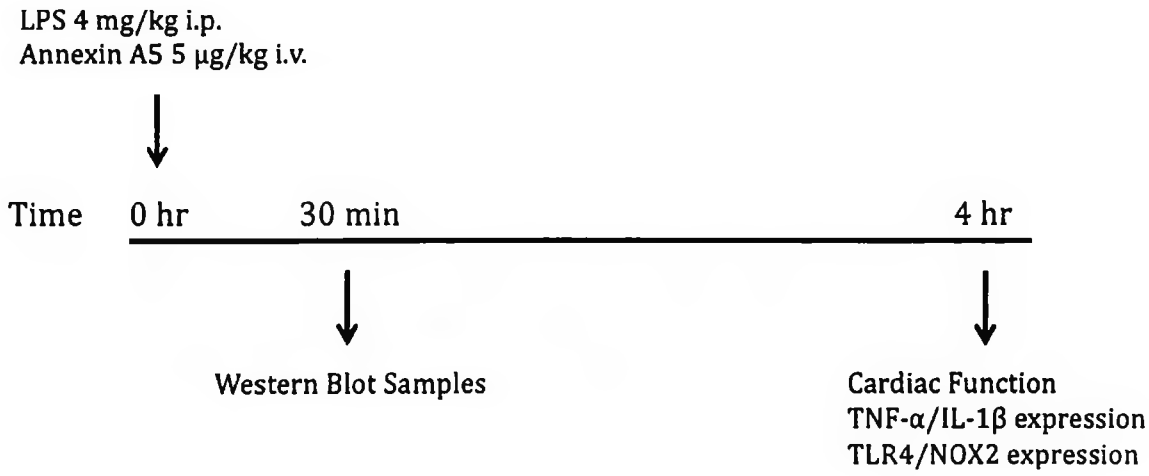


Figure 5. In vivo experimental protocol. Mice were separated into 4 treatment groups of control, annexin A5, LPS, and LPS+annexin A5. The LV was collected for western blot samples after 30 min treatment. After 4hr treatment, the LV was isolated for TLR4, NOX2, TNF-α, and IL-1β mRNA analysis, and TNF-α and IL-1β protein expression after 4 hr treatment. Blood samples were also collected after 4 hr for TNF-α and IL-1β protein expression. Cardiac function was assessed after 4 hr treatment using *in vivo* and *ex vivo* techniques.

Sample Collection

After the treatment period, the mice were sacrificed and the hearts were then excised and the LVs were isolated and stored at -80°C. For hemodynamic assessment, mice were anesthetized with an intramuscular (i.m.) injection of a ketamine (50 mg/kg) and xylazine (12.5 mg/kg) mixture. Blood samples were collected by cardiac puncture into 1.5 mL eppendorf tubes containing 10 µl of heparin (1,000 USP units/mL, Pharmaceutical Partners of Canada Inc., Richmond Hill, ON). Plasma was obtained after centrifugation of the blood for 10 min at 5,000 rpm.

2.2 Western Blot Analysis

Protein Isolation and Measurement

Heart tissues were homogenized in cold lysis buffer (0.5% sodium dodecyl sulfate (SDS), 1 mM EDTA, 10 mM Tris-HCl pH 7.5) and sonicated for 10 seconds at 2 output watts. Protein concentrations were measured using a Lowry protein assay (Bio-Rad, Mississauga, ON) with bovine serum albumin (BSA) as the standard. Concentrations were measured to allow equal loading of 60 μg protein per well.

Gel Electrophoresis

Samples were run on 10% polyacrylamide gels containing SDS. The proteins were separated by gel electrophoresis in a blotting cell (Bio-Rad, Mississauga, ON) for 90-120 min at 100 V using a Power Pac 300 (Bio-Rad, Mississauga, ON).

Western Blot

Samples were transferred from the gel onto nitrocellulose membranes overnight at 25 V in 4°C or for 1 hr transfer at 100 V in ice. Membranes were blocked in tris-buffered saline tween-20 (TBS-T) containing 5% non-fat dehydrated milk at room temperature for 1hr. After several washes with TBS-T, the blots were probed with antibodies against α-actinin (anti-mouse; 1:2000, Cell Signaling, Danvers, MA) p38 (phospho-p38 - anti-rabbit; total-p38 - anti-rabbit; 1:1000, Cell Signaling, Danvers, MA) and ERK (phospho-ERK - anti-rabbit; total-ERK - anti-rabbit; 1:2000, Cell Signaling, Danvers, MA) for overnight at 4°C. Blots were then washed with TBS-T and probed with horseradish peroxidase-conjugated secondary antibodies (1:2000, BioRad, Mississauga, ON) for 1 hr at room temperature according to animal specificity of

primary antibodies. Detection was performed using Luminol Enhancer Solution (Thermo Scientific, Rockford, IL) detection method and signals were determined by densitometry (FluorChem 8000, Alpha Innotech Corp., San Leandro, CA).

2.3 RNA Isolation and RT-PCR

Tissue RNA Isolation and Measurement

Total RNA was isolated from the LV by lysing the tissue with TRIzol reagent (Invitrogen, Burlington, ON) as previously described (Song et al. 2000; Hammoud et al. 2007). The lysate was vigorously mixed with chloroform before centrifugation at 12,000 g for 15 min at 4°C to separate the RNA in the aqueous phase of the solution. Isopropanol was added and the sample was centrifuged at 12,000 g for 10min at room temperature to precipitate the RNA samples. The supernatant was decanted and the precipitated RNA pellet was washed with 75% ethanol. Ethanol was removed and the pellet was vacuum-dried for ~5 min. The pellet was re-suspended in diethylpyrocarbonate (DEPC)-H_2O and kept at 70°C for 10 min. RNA concentration was then quantified by measuring the absorbance at 260/280 nm using a RNA/DNA calculator (Pharmacia Biotech, Gene Quant II, Baie d'Urfe, QC).

cDNA Synthesis

cDNA was synthesized from the isolated RNA using M-MLV reverse transcriptase (RT) and random primers (Invitrogen, Burlington, ON). One μg of RNA and 1 μl of random primers were mixed in DEPC-H_2O. Samples were then combined with a reverse transcriptase mix consisting of 1st strand buffer, 0.1 M DTT, 10 mM dNTP, RNAse inhbitor, and M-MLV RT (Invitrogen, Burlington, ON). The final

mixture was then incubated at 37°C for 2 hr. Samples were incubated at 65°C for 10 min to inactivate the reverse transcriptase enzyme, and the cDNA was diluted with 40 μL of sterilized H_2O.

RT-PCR

Real-time PCR was conducted using SYBR Green PCR Master Mix as per manufacturer's instructions (ABM Inc., Richmond, BC). The oligonucleotide primers for TNF-α were sense 5' CCGATGGGTTGTACCTTGTC 3' and antisense 5' GGGCTGGGTAGAGAATGGAT 3'. The primers for IL-1β were sense 5' ACAAGGAGAACCAAGCAACGAC 3' and antisense 5' GCTGATGTACCAGTTGGGGAAC 3'. The primers for TLR4 were sense 5' CCTGGAATGGGAGGACAATCC 3' and antisense 5' CCATGTGTTCCATGGGCTCTC 3'. The primers for NOX2 were sense 5' GCTGGAAACCCTCCTATGACTTG 3' and antisense 5' TGTACTGTCCCACCTCCATCTTG 3'. Twenty eight S rRNA was used as a loading control using oligonucleotide primers for sense 5' TTGAAAATCCGGGGGAGAG 3' and antisense 5' ACATTGTTCCAACATGCCAG 3'. All primers were purchased from Sigma-Aldrich (St. Louis, MO). Samples were amplified for 35 cycles at T_m's appropriate for each primer set using MJ Research Opticon Real-Time PCR machine (South San Francisco, CA). mRNA levels were compared to that of 28S rRNA, and the relative amount of mRNA was obtained.

2.4 ELISA

TNF-α and IL-1β cytokine levels were measured using separate enzyme-linked immunosorbent assay (ELISA) Ready-Set-Go! Kits from eBioscience Inc (San Diego, CA). Briefly, 96-well plates were incubated with anti-mouse TNF-α and IL-1β primary capture antibody in ELISA Coating Buffer overnight at 4°C. Between each step, wells were washed for 1-2 min ~5 times with wash buffer (PBS + 0.05% Tween-20). Wells were blocked with assay diluent for 1 hr at room temperature. Standards and samples were added to the wells at a total volume of 100 µl/well. TNF-α and IL-1β standards provided by the kits ranged from 15.6-500 pg/mL after serial dilutions. Culture media from primary cultures and plasma from isolated blood samples were added to the wells at a volume of 100 µl/well. Tissue samples were homogenized in PBS with proteinase inhibitors (1:1000) and added to the wells at a concentration of 200 µg protein/well. Assay diluent was added to reach the total well volume of 100 µl. Standards and samples were incubated overnight at 4°C or at room temperature for 2 hr. After removal of standards and samples, the wells were incubated with the detection antibody for 1 hr at room temperature. Then the wells were incubated with the active enzyme Avidin-HRP for 30 min at room temperature. The working substrate solution was then added to the wells for 10-15 min at room temperature before addition of the stop solution (1M H_3PO_4) to end the reaction. The plate was read at 450nm using SpectraMax M5 plate reader and SoftMax Pro software (Molecular Devices, Sunnyvale, CA).

2.5 Langendorff Perfusion System

Langendorff perfusion preparations were prepared according to the methods previously described (Peng et al. 2005). After treatment, mice were given heparin (50

USP units/mouse) before sacrifice. Hearts were retrieved and the ascending aorta was cannulated and attached to the Langendorff perfusion system. Hearts were perfused with bubbled (mixture of 95% O_2 and 5% CO_2) Krebs solution at a rate of 2 mL/min (~100 mmHg). Myocardial function was assessed by securing a 6-0 silk suture approximately 2mm from the apex of the LV which was also attached to a lightweight coupling rod. The coupling rod was connected to a force-displacement transducer (model FTO3, Grass Instruments, West Warwick, RI) to record tension and heart rate. Tension was maintained at 2 grams during recordings. The heart work was calculated by multiplying the force (grams) by the heart rate (bpm). Maximal and minimal first derivatives of force ($+dF/dt_{max}$ and $-dF/dt_{min}$) used to approximate the rate of contraction and relaxation were analyzed by PowerLab Chart 4 program (AD Instruments, Colorado Springs, CO).

2.6 Hemodynamic Analysis

Hemodynamic measurements were performed as previously described (Feng et al. 2001; Peng et al. 2003). Mice treated with LPS were given subcutaneous saline injections (0.5 mL) every ½ hr for the 4 hr treatment time. Volume resuscitation was not necessary in control mice. Mice were then anesthetized with a ketamine (50 mg/kg) and xylazine (12.5 mg/kg) mixture for catheter placements. Body temperature was monitored by a rectal temperature probe (TC-1000 Temperature Controller, CWE Inc., Ardmore, PA) and maintained between 36 and 37.5°C by heating pads and lamps on the procedure bench. The right carotid artery was cannulated with a pressure-conductance catheter (Model SPR-839, Size 1.4F, Millar Instruments, Houston, TX) to record mean arterial pressure (MAP) in the right carotid artery. The catheter was then advanced

retrogradly into the LV for recording of heart rate, LV ejection fraction (LVEF), CO, derivatives of LV pressures ($+dP/dt_{max}$ and $-dP/dt_{min}$), LV end systolic pressure (LVESP), LV end diastolic pressure (LVEDP), LV end diastolic volume (LVEDV), time constant of isovolumic relaxation (Tau), maximal power, and LV conductance. Both pressure and conductance signals were fed to an analog-digital converter and analyzed by PowerLab Chart 4 software (AD Instruments Inc., Colorado Springs, CO). Catheter pressure baselines were recorded before and after each experiment. After LV pressure measurements, occlusion of the inferior vena cava was performed by direct pressure on the inferior vena cava in an open abdominal cavity to test volume dependent pressure loading in the heart. Ten µl of isotonic saline (0.9% NaCl) bolus injections were given in the jugular vein to obtain parallel conductance for the calculation of LV volume.

2.7 Macrophage Isolation

Isolation Procedure

Peritoneal macrophages were isolated from mice 4 days after i.p. injection (1.5 mL/mouse) of 3% thioglycollate medium (Sigma-Aldrich, St. Louis, MO) to induce peritonitis as previously described (Zhao et al. 2006). Briefly, a lavage (4 mL 3x) of the peritoneum was performed with RPMI 1640 media (Invitrogen, Burlington, ON). The cells were collected after centrifugation at 1000 g for 3 min and counted using a hemocytometer. The cells were then plated at a concentration of $1.5x10^5$ cells/well on 48 well Costar plates (Corning Inc., Corning, NY) and incubated for 2 hr at 37°C. Cells were then washed 4x with PBS to remove any non-adherent, and thus non-macrophage,

cells. Cells were incubated in RMPI 1640 media with 5% FBS overnight at 37°C in a humidified chamber containing 5% CO_2.

Treatments

The control group contained RPMI 1640 media (Invitrogen, Burlington, ON) with 5% FBS, which served as the media for each treatment group. The media was supplemented with 2 mM Ca^{2+} to reflect physiological conditions, unless stated otherwise. Annexin A5 treatment at 1 μg/mL was used (Biovision, CA). LPS treatment had *Salmonella typhosa* (Sigma-Aldrich, St. Louis, MO) concentrations of 0.01, 0.1, 0.4, and 1 ng/mL. Cells treated with LPS+annexin A5 received combinations of A5 and LPS concentrations used in the individual treatment groups. After the 4 hr treatment, the culture medium and plate was collected and stored separately at -80°C.

2.8 Isolation of Neonatal Cardiomyocytes

Isolation Procedure

Neonatal mouse cardiomyocyte cultures were prepared by methods previously described (Song et al. 2000; Peng et al. 2003; Peng et al. 2005). Briefly, ventricles from C57BL/6 WT mice born within 24 h were minced in a nominally Ca^{2+} and Mg^{2+} free Hanks balance solution. Cardiac myocytes were dispersed by 22.5 μg/mL liberase blendzyme IV (Roche, Laval, QC) in D-Hanks solution at 37°C for 40 min. The isolated cells were pre-plated for 90 min to remove adherent non-cardiomyocytes. Purity of cardiomyocytes determined by flow cytometry using an α-actinin antibody was 98.7% after 72 hours of cell culture (Hammoud et al. 2009). The cardiomyocytes were plated at a density of $0.7x10^6$ cells/well in M199 medium containing 10% FCS in 24 well Costar

plates (Corning Inc., Corning, NY) pre-coated with 1% gelatin. Cells were incubated at 37°C in a humidified chamber containing 5% CO_2. After culturing for 48 hr to allow confluence, cardiomyocytes were treated.

Treatments

The control group contained M199 with 10% FCS, which served as the media for each treatment group. Annexin A5 treatment was 1 μg/mL (Biovision, Mountain View, CA). LPS treatment contained *Salmonella typhosa* (Sigma-Aldrich, St. Louis, MO) concentrations of 0.1, 0.2, and 1 μg/mL. Cells treated with LPS+A5 received combinations of A5 and LPS concentrations used in the individual treatment groups. After 4 hr of treatment the culture medium and plate was collected and stored separately at -80°C.

2.9 Isolation of Adult Cardiomyocytes

Isolation Procedure

Cardiomyocytes were isolated from the hearts of adult C57BL/6 WT mice as previously described (Zhou et al. 2000; Burger et al. 2009). Mice were given heparin (50 USP units/mouse) before sacrifice. Hearts were retrieved and the extended aorta was cannulated and attached to the Langendorff perfusion system. The heart was perfused with buffer (NaCl 113mM, KCl 4.7 mM, $MgSO_4$-$7H_20$ 1.2 mM, NaH_2PO_4 0.6 mM, $NaHCO_3$ 12 mM, Glucose 5.5 mM, 2,3-butanedione monoxime 10 mM, taurine 30 mM) containing 45 μg/mL of liberase blendzyme IV (Roche, Laval, QC) for 4 min to flush away blood and extracellular Ca^{2+} before perfusion with myocyte digestion buffer (perfusion buffer pH 7.46, Ca^{2+} 12.5 μM, 45 μg/mL liberase blendzyme IV (Roche,

Laval, QC)) for approximately 8-10 min. The heart was then removed from the cannula and placed in a 60 mm dish containing the cell suspension. The heart was cut and teased into several small pieces and transferred to a 15 mL tube containing myocyte-stopping buffer (perfusion buffer pH 7.46, bovine calf serum 10%, Ca^{2+} 10 mM). The cells were counted with a hemacytometer to check viability.

The cells were then re-suspended and exposed to a series of sedimentation and resuspension steps in buffers containing increasing concentrations of Ca^{2+} (12.5 μM-1.0 mM) in a 5-step process allowing 4 min at each step. The cells were transferred to a 15 mL tube and resuspended in myocyte plating medium (minimum essential medium 0.9x, bovine calf serum 5%, 2,3-butanedione monoxime 10 mM, penicillin 100 U/mL, L-glutamine 2 mM, Ca^{2+} 1.2 mM). The myocyte plating medium was equilibrated for 2-3 hr at 37°C in 2% CO_2 incubator to temperature and pH balance to approximately 7. Each heart routinely yields approximately $1x10^7$ rod-shaped myocytes and the cells were counted with a hemocytometer. The cell concentration was adjusted to 25,000 rod-shaped cells/mL and plated on laminin-coated 35 mm dishes at a density of 50 rod-shaped cells/mm^2.

Treatment

Isolated adult cardiomyocytes were incubated in equilibrated myocyte culture medium (minimum essential medium 1x, penicillin 100 U/mL, L-glutamine 2 mM, FBS 5%) for 6 hr at 37°C in a 2% CO_2 incubator before treatment. The control group contained myocyte culture medium, which served as the media for each treatment group. The culture medium was equilibrated for 2-3 hr 37°C in a 2% CO_2 prior to use to adjust

temperature and pH balance to approximately 7. Annexin A5 treatment was 1 μg/mL (Biovision, Mountain View, CA) and LPS treatment was 2.5 μg/mL of *Salmonella typhosa* (Sigma-Aldrich, St. Louis, MO). After 4 hr of treatment, the culture medium and plate were collected and stored separately at -80°C.

Adult cell RNA Isolation

Before freezing samples at -80°C, the cells were washed with PBS to remove residual media. RNA isolation was performed using an RNeasy kit (Qiagen, Mississauga, ON). Briefly, the duplicate dishes were combined to increase mRNA concentration and cells were lysed in RLT buffer and mixed with 1 volume of 70% ethanol. The solution was transferred to RNeasy columns to capture RNA. The columns were washed with kit buffers and RNA was collected in RNase free DEPC-H_2O. RNA concentration was quantified by measuring the absorbance at 260/280 nm using a RNA/DNA calculator (Pharmacia Biotech, Gene Quant II, Baie d'Urfe, QC). cDNA synthesis was performed as previously described.

2.10 Co-Immunoprecipitation

Co-immunoprecipitation of TLR4 and annexin A5 was performed using the Dynabead Protein G Immunprecipitation kits (Invitrogen, Burlington, ON) as per manufacturer instructions, with minor revisions. Briefly, the magnetic dynabeads were suspended in an antibody-binding buffer with 20 μg of TLR4 antibody (Santa Cruz, Santa Cruz, CA) for 1 hr at room temperature. To immunoprecipitate annexin A5 from the sample, heart tissues were homogenized in NP40 cell lysis buffer and sonicated for 10 seconds at 2 output watts. Protein concentrations were measured using a Lowry

protein assay (Bio-Rad, Mississauga, ON) using bovine serum albumin (BSA) as the standard. Concentrations were measured to allow for equal loading of 1 mg protein per tube. Human recombinant annexin A5 (0.5 μg) (Biovision, Mountain View, CA) was added to the tissue samples and incubated with the TLR4-coated beads for 2 hr at room temperature. The samples were eluted from the dynabeads by boiling for 8 min in elution buffer and reducing protein-loading dye. The samples were then equally loaded on a 10% polyacrylamide gel and a western blot was performed for annexin A5 detection (anti-rabbit, 1:2000, Biovision, Mountain View, CA). The membrane was stripped using stripping buffer and rotating for 20 min at 50°C, and then reprobed for TLR4 detection (anti-rabbit, 1:500, Santa Cruz, Santa Cruz, CA) as a control.

2.11 Statistical Analysis

All results are expressed as mean ± SEM. Two-way analysis of variance (ANOVA) followed by unpaired Student's t test with Bonferroni corrections was performed to detect differences between treatment groups using Prism 4.0 (GraphPad Software, La Jolla, CA). Statistical significance was assigned when a P value was less than 0.05.

Chapter 3 - RESULTS

3.1 Annexin A5 treatment abrogates LPS-induced myocardial p38 and ERK MAPK phosphorylation

The LPS-induced TLR4 signalling pathway is regulated by MAPKs and can result in NF-κB activation within 30 min of LPS exposure (Baumgarten et al. 2001). Therefore, to determine if annexin A5 affects the TLR4 signalling pathway, myocardial p38 and ERK MAPK phosphorylation (P-p38, P-ERK) was measured after 30 min treatment of LPS (4 mg/kg i.p.). Phosphorylation MAPK activation was measured as a ratio of protein phosphorylation levels compared to total protein levels by western blot analysis. LPS treatment significantly increased phosphorylation of p38 ($P<0.05$, Figure 6A and 6B) and ERK ($P<0.05$, Figures 7A and 7B). The responses were inhibited with annexin A5 treatment (5 μg/kg i.v.) to similar levels as control ($P<0.05$, Figure 6 and 7).

3.2 Annexin A5 treatment abrogates LPS-induced myocardial TLR4 and NOX2 mRNA expression

To determine if annexin A5 affected LPS-induced gene transcription through the MAPK-dependent pathway, we measured the mRNA expression of TLR4 and NOX2, two proteins shown to be essential for cardiomyocyte LPS-induced gene expression (Baumgarten et al. 2001; Peng et al. 2005). mRNA expression was measured in isolated LVs after 4 hr of LPS (4 mg/kg i.p.) treatment using real-time RT-PCR with 28S mRNA expression as a loading control. LPS increased myocardial TLR4 and NOX2 mRNA expression by 2-fold and 3-fold respectively ($P<0.05$) and the response was inhibited with annexin A5 treatment (5 μg/kg i.v.)($P<0.01$, Figures 8 and 9).

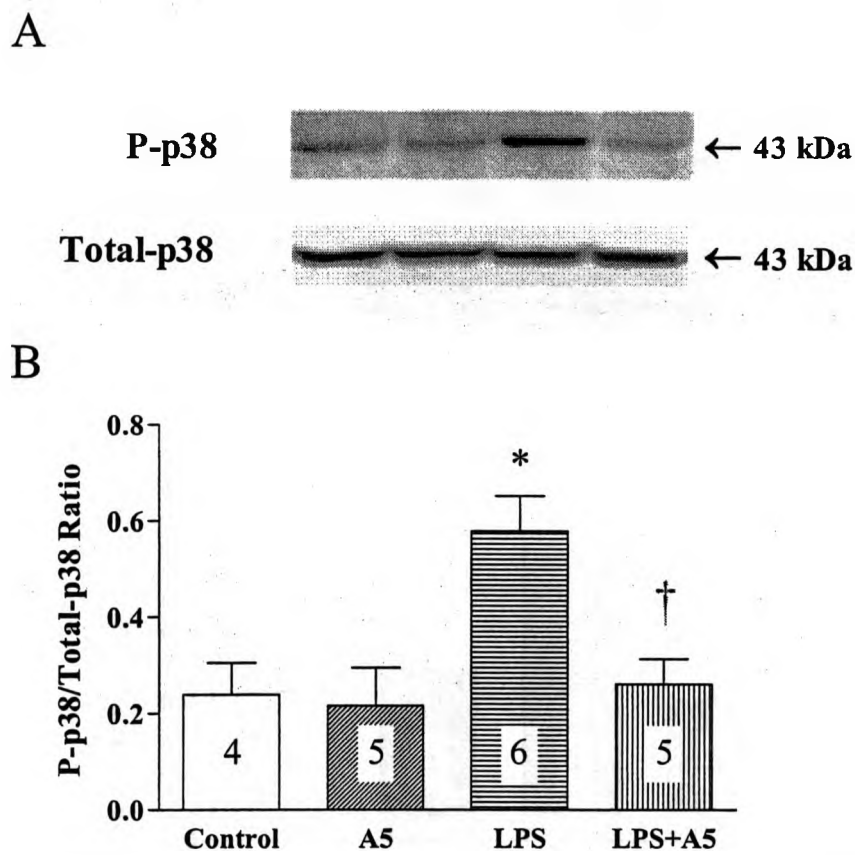


Figure 6. Annexin A5 abrogates LPS-induced myocardial p38 MAPK phosphorylation. Mice were treated with LPS (4 mg/kg i.p.) with and without annexin A5 (5 μg/kg i.v.) for 30 min. Phosphorylation of p38 was analyzed by western blot analysis and expressed as a ratio of P-p38/Total-p38 band intensity. A: Representative western blot. B: Graph representing densitometry of Western blot analysis. N numbers are given in each column above. * $P<0.05$ vs. Control and A5, † $P<0.05$ vs. LPS.

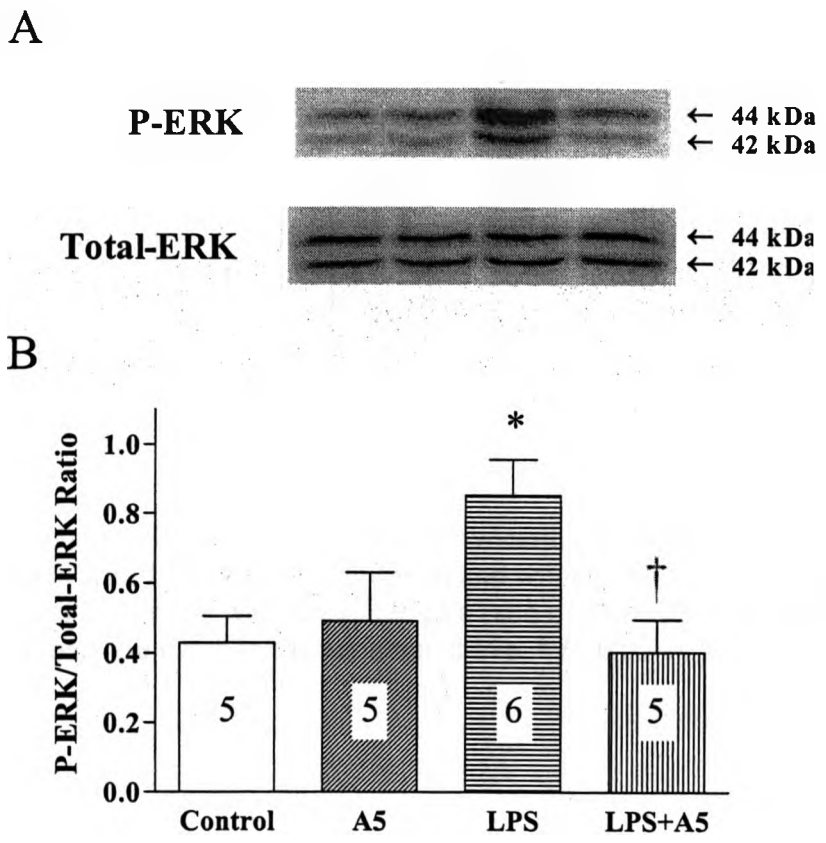


Figure 7. Annexin A5 abrogates LPS-induced myocardial ERK1/2 MAPK phosphorylation. Mice were treated with LPS (4 mg/kg i.p.) with and without annexin A5 (5 μg/kg i.v.) for 30 min. Phosphorylation of ERK was analyzed by western blot analysis and expressed as a ratio of P-ERK/Total-ERK band intensity. A: Representative western blot. B: Graph representing densitometry of Western blot analysis. N numbers are given in each column above. * $P<0.05$ vs. Control and A5, † $P<0.05$ vs. LPS.

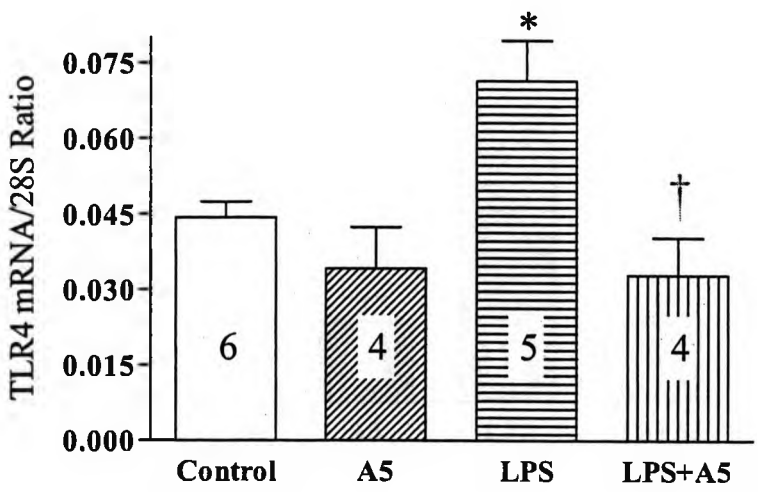


Figure 8. Annexin A5 abrogates LPS-induced myocardial TLR4 mRNA expression. Mice were treated with LPS (4 mg/kg i.p.) with and without annexin A5 (5 μg/kg i.v.) for 4 hr. TLR4 mRNA levels were measured as a ratio of 28S using real time RT-PCR analysis. N numbers are given in each column above. * $P<0.05$ vs. Control and A5, † $P<0.01$ vs. LPS.

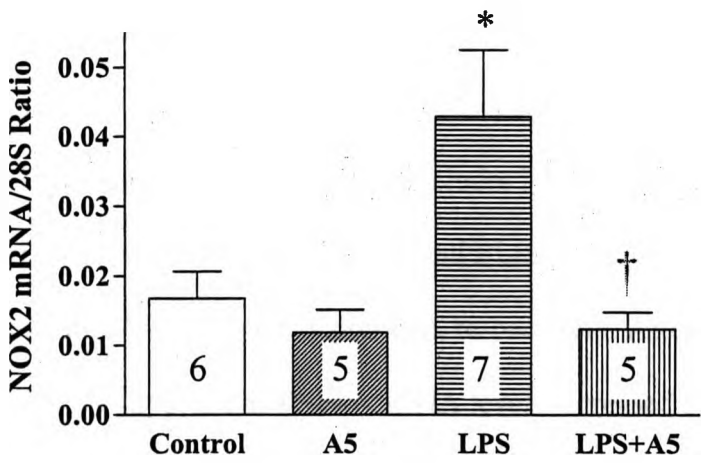


Figure 9. Annexin A5 abrogates LPS-induced myocardial NOX2 mRNA expression. Mice were treated with LPS (4 mg/kg i.p.) with and without annexin A5 (5 μg/kg i.v.) for 4 hr. NOX2 mRNA levels were measured as a ratio of 28S using real time RT-PCR analysis. N numbers are given in each column above. * $P<0.05$ vs. Control and A5, † $P<0.01$ vs. LPS.

3.3 Annexin A5 treatment attenuates myocardial TNF-α and IL-1β expression

Our lab has shown previously that LPS-induced myocardial cytokine expression depends on the TLR4 signalling pathway that includes NOX2, p38, and ERK (Peng et al. 2003; Peng et al. 2005). Real time RT-PCR and ELISA analyses were used to measure cytokine mRNA and protein expression, respectively. After 4 hr treatment of LPS (4 mg/kg i.p.), LV myocardial TNF-α mRNA levels increased approximately 10-fold, which was associated with a 2-fold increase in protein levels ($P<0.05$, Figure 10A and 10B). Conversely, LV myocardial IL-1β mRNA levels increased after LPS treatment ($P<0.05$) but protein levels did not change (Figure 11A and 11B). Annexin A5 treatment (5 μg/kg i.v.) inhibited TNF-α and IL-1β mRNA expression but did not fully decrease TNF-α protein levels to control values ($P<0.05$, Figure 10 and 11).

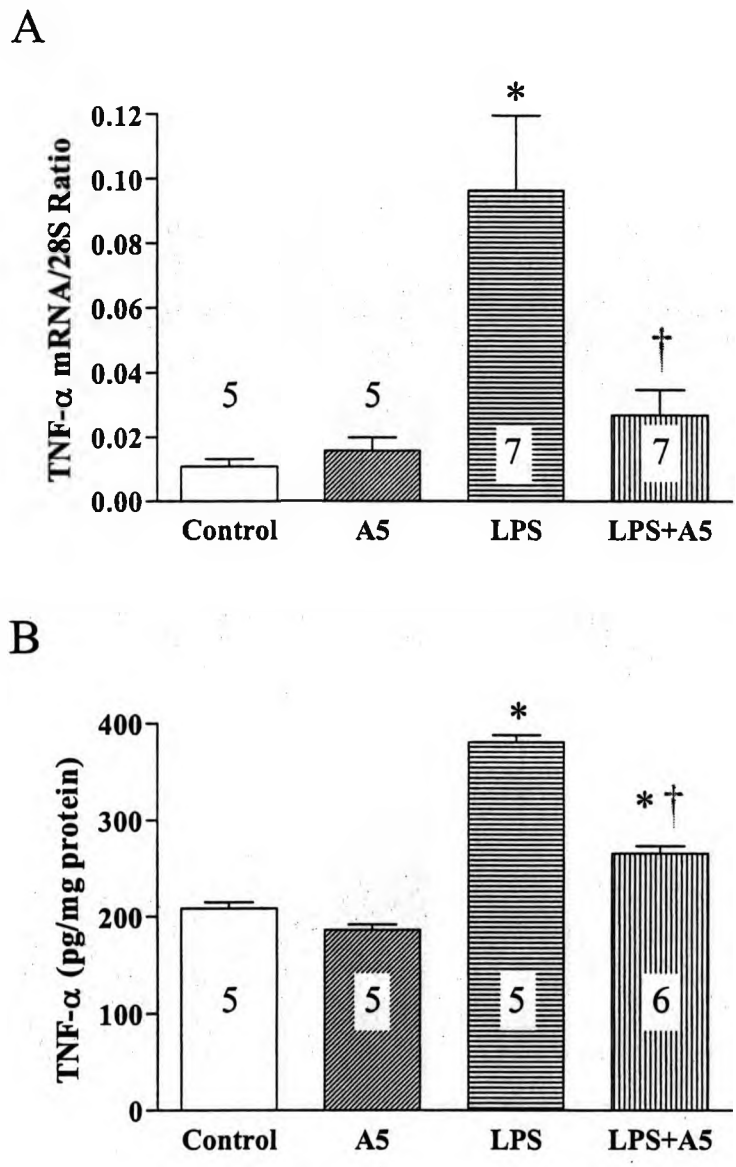


Figure 10. Annexin A5 attenuates LPS-induced myocardial TNF-α expression. Mice were treated with LPS (4 mg/kg i.p.) with and without annexin A5 (5 μg/kg i.v.) for 4hr. A: TNF-α mRNA levels were measured as a ratio of 28S using real time RT-PCR analysis. B: TNF-α protein levels measured by ELISA analysis. N numbers are given in each column above. * $P<0.05$ vs. Control and A5, † $P<0.05$ vs. LPS.

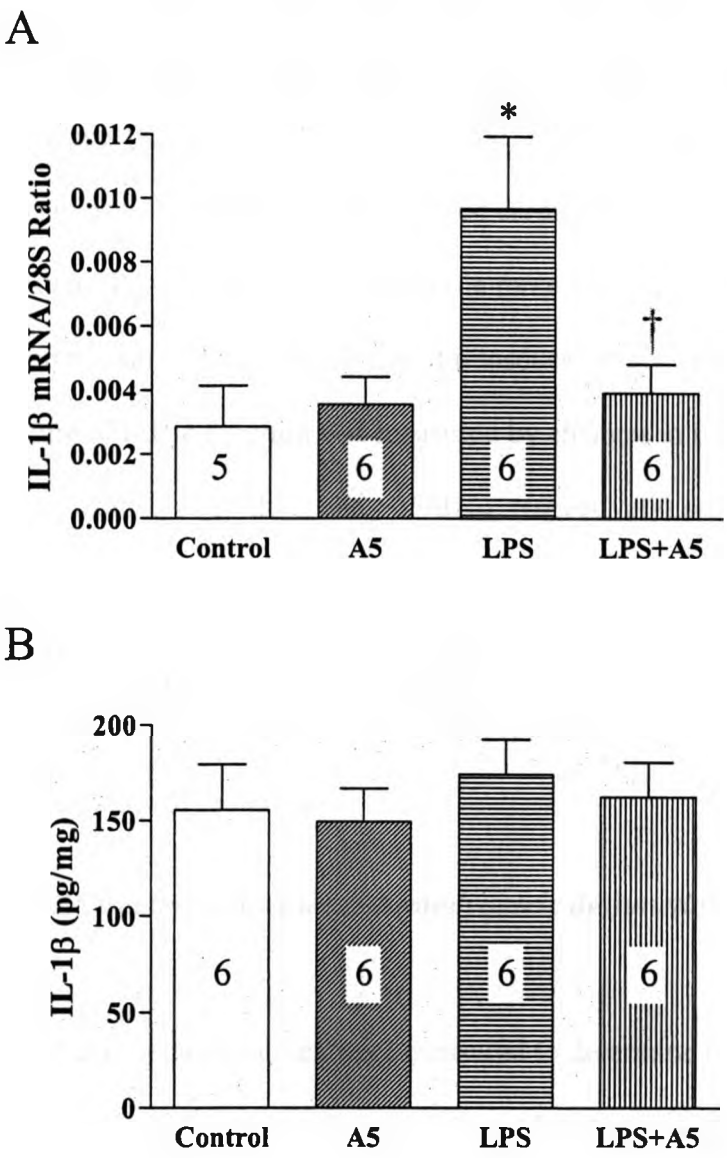


Figure 11. Annexin A5 abrogates LPS-induced myocardial IL-1β mRNA expression. Mice were treated with LPS (4 mg/kg i.p.) with and without annexin A5 (5 μg/kg i.v.) for 4hr. A: IL-1β and mRNA levels were measured as a ratio of 28S using real time RT-PCR analysis. B: IL-1β protein levels measured by ELISA analysis. N numbers are given in each column above. * $P<0.05$ vs. Control and A5, † $P<0.05$ vs. LPS.

3.4 Annexin A5 treatment attenuates cardiac dysfunction during endotoxemia

Increased levels of TNF-α and IL-1β have been shown to directly inhibit cardiac function during sepsis (Kumar et al. 1996; Cain et al. 1999; Grandel et al. 2000). Previously, our lab had demonstrated LPS-induced cardiac dysfunction after 4 hr using a Langendorff system (Peng et al. 2005). Contractile force was measured in the Langendorff system using a force-displacement transducer attached to the apex of the LV by a silk suture. Cardiac function was measured by changes in the force of contraction and relaxation ($+dF/dt_{max}$ and $-dF/dt_{min}$, respectively) and heart work. After 4 hr of LPS treatment (4 mg/kg i.p.), $\pm dF/dt_{max/min}$ and heart work were decreased when compared to control mice ($P<0.01$, Figure 12). Annexin A5 treatment (5 μg/kg i.v.) prevented LPS-induced cardiac dysfunction ($P<0.01$, Figure 12). There was no change in heart rate between any of the groups.

3.5 Annexin A5 treatment attenuates hemodynamic dysfunction during endotoxemia

Cardiovascular hemodynamics were measured to determine the extent of annexin A5 protection on cardiac function *in vivo*. A Millar pressure-conductance catheter was used to measure arterial pressure and changes in LV pressure and volume. Hemodynamic parameters obtained include MAP, heart rate, LVEF, CO, $+dP/dt_{max}$ and $-dP/dt_{min}$, LVESP, LVEDP, LVEDV, Tau and maximal power (see Table 1 and Figure 13). Following LPS treatment (4 mg/kg i.p.), MAP, $+dP/dt_{max}$ and $-dP/dt_{min}$, LVESP, LVEDP and maximal power were significantly decreased ($P<0.01$) while Tau was significantly higher ($P<0.01$). Treatment with annexin A5 (5 μg/kg i.v.) resulted in

significantly higher MAP and lower Tau when compared to endotoxemic mice ($P<0.05$, Table 1). Importantly, $+dP/dt_{max}$ and $-dP/dt_{min}$ function was significantly higher by approximately 35% and 33% respectively in LPS with annexin A5 treatment compared to the LPS group ($P<0.05$, Figure 13).

3.6 Annexin A5 treatment attenuates LPS-induced plasma TNF-α and IL-1β levels

Systemic inflammation with excessive cytokine production is characteristic of sepsis. Blood was isolated from heart puncture and the plasma was separated by centrifugation to measure plasma cytokine levels by ELISA analysis. TNF-α and IL-1β levels were significantly higher in response to 4 hr LPS (4mg/kg i.p.) treatment ($P<0.01$), with TNF-α levels almost 5 times greater than IL-1β levels (Figure 14A and 14B). In contrast to myocardial cytokine expression where annexin A5 eradicated TNF-α and IL-1β expression after 4 hr of LPS (Figure 10 and 11), annexin A5 treatment (5 μg/kg i.v.) only attenuated TNF-α and IL-1β plasma levels by 34% and 51% respectively ($P<0.05$, Figure 14A and 14B). Although IL-1β levels with LPS and annexin A5 treatment were not significantly different from control values, they show a strong trend towards higher IL-1β levels ($P=0.06$).

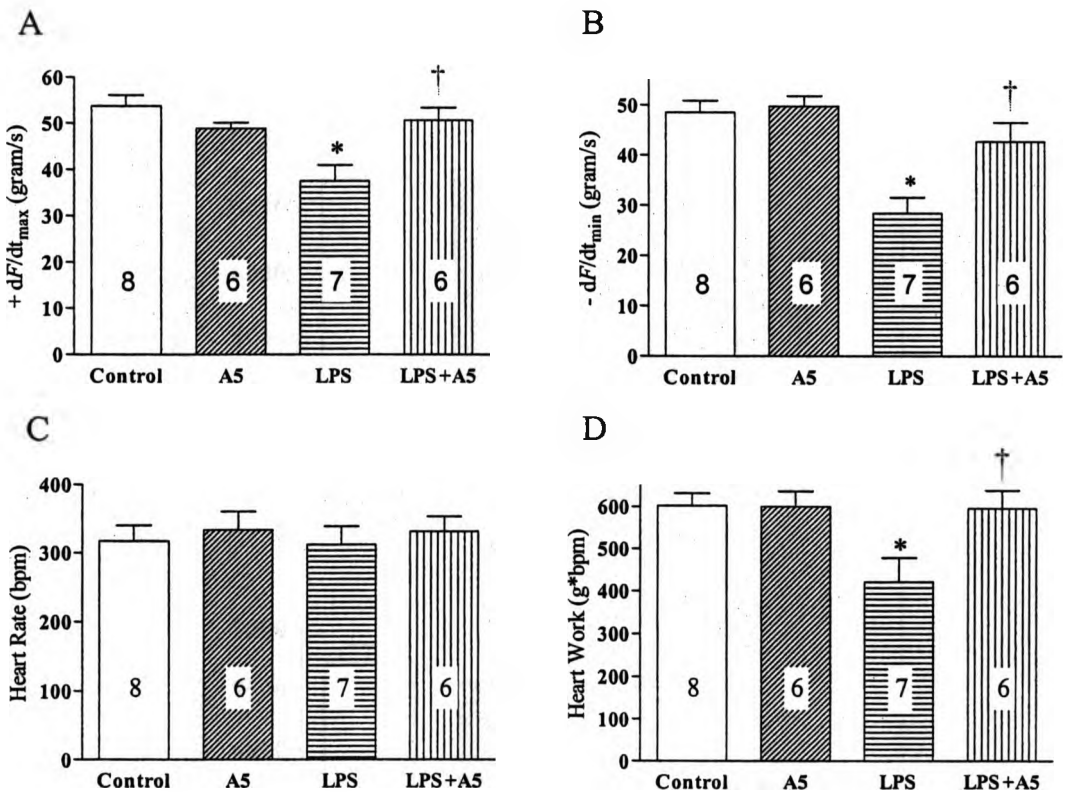


Figure 12. Annexin A5 attenuates cardiac dysfunction during endotoxemia. Hearts were isolated and perfused in a Langendorff system after mice were treated with LPS (4 mg/kg i.p.) with and without annexin A5 (5 μg/kg i.v.) for 4hr. A: Maximal and B: minimal first derivatives of force ($+dF/dt_{max}$ and $-dF/dt_{min}$) were measured to reflect the rate of contraction and relaxation. D: Heart work was calculated as the product of force (gram) and C: heart rate (bpm). Data was analyzed by PowerLab Chart 4 program. N numbers are given in each column above. * $P<0.01$ vs. Control and A5, † $P<0.01$ vs. LPS.

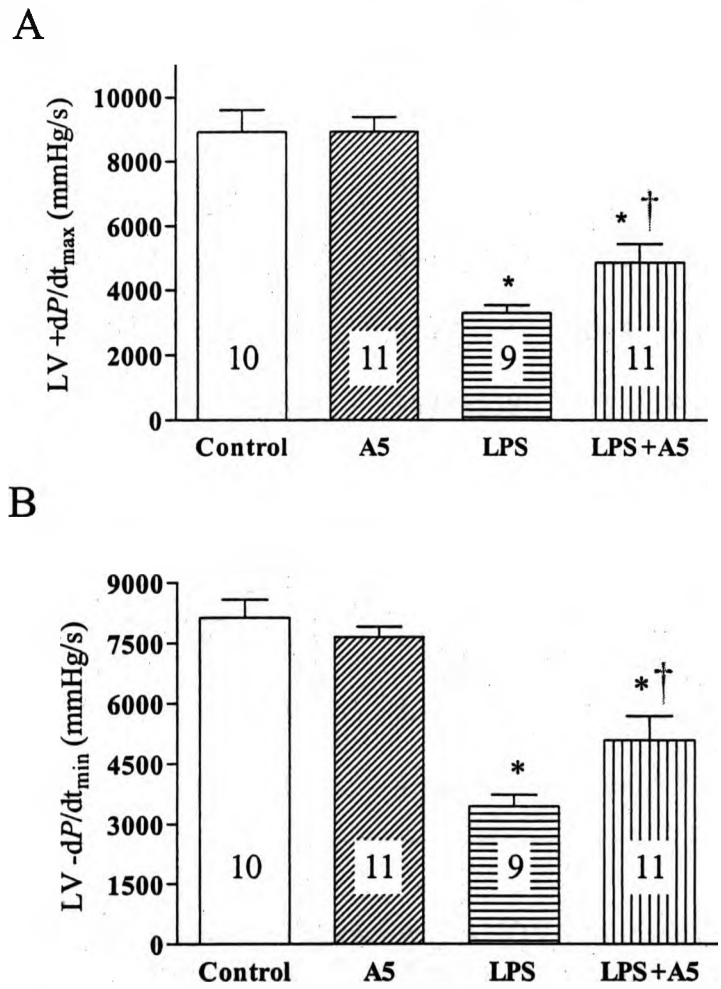


Figure 13. Annexin A5 attenuates hemodynamic dysfunction during endotoxemia. Mice were treated with LPS (4 mg/kg i.p.) with and without annexin A5 (5 μg/kg i.v.) for 4 hr. Mice were anesthetized and measurements of LV pressures were obtained using a Millar pressure-conductance catheter. N numbers are given in each column above. * $P<0.05$ vs. Control and A5, † $P<0.05$ vs. LPS.

Table 1: *In vivo* hemodynamic measurements in mice with endotoxemia

Parameters	Control	A5	LPS	LPS+A5
n	10	11	9	11
Heart rate, beats/min	412±23	395±9	464±19	486±19
MAP, mmHg	86±3	83±5	49±3 **	59±4 ** †
LVEF, %	63±6	67±7	50±4	54±7
CO, μL/min	5188±834	3698±558	3153±786	4386±928
LVESP, mmHg	102±5	95±8	66±5 **	72±6 **
LVEDP, mmHg	6.9±0.9	8.3±2.1	3.6±0.1 **	4.5±0.5
LVEDV, μL	20±4	14±3	13±4	17±4
Tau, ms	7.9±0.3	8.1±0.5	10.2±0.4 **	8.6±0.6 †
Maximal Power, mWatts	6.1±1.1	4.4±0.8	2.0±0.5 **	3.7±0.9

Data are mean ± SEM and analyzed by two-way ANOVA followed by unpaired Student's t-test with Bonferroni corrections. ** $P<0.01$ vs. control, † $P<0.05$ vs. LPS. MAP, mean artery pressure; LV ejection fraction (LVEF); CO, cardiac output; LVESP, LV end systolic pressure; LVEDP, LV end diastolic pressure; LVEDV, LV end diastolic volume; Tau, time constant of isovolumic relaxation.

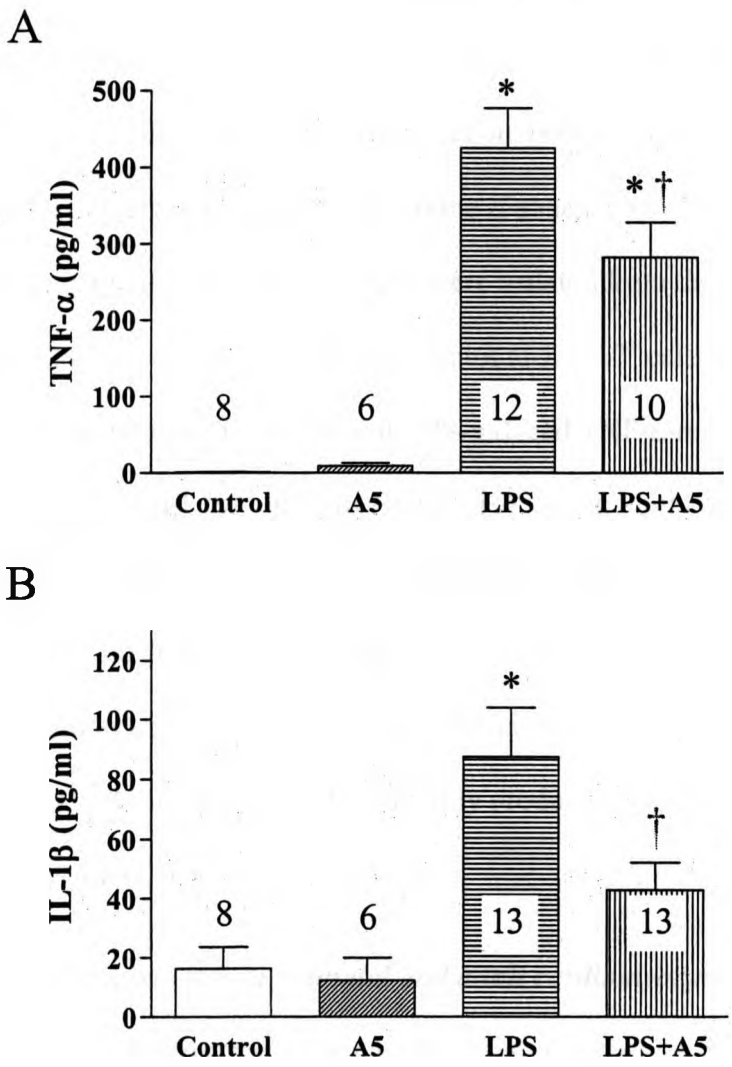


Figure 14. Annexin A5 attenuates LPS-induced plasma TNF-α and IL-1β levels. Mice were treated with LPS (4 mg/kg i.p.) with and without annexin A5 (5 μg/kg i.v.) for 4 hr. The blood was isolated from heart puncture and the plasma was separated by centrifugation for 10 min at 5,000 rpm. A: TNF-α and B: IL-1β levels were determined by ELISA analysis. N numbers are given in each column above. * $P<0.01$ vs. Control and A5, † $P<0.05$ vs. LPS.

3.7 Annexin A5 does not affect LPS-induced TNF-α production in macrophages

Macrophages are an abundant source of TNF-α in the body (reviewed by Meldrum 1998). Since annexin A5 had an impact on TNF-α plasma levels (Figure 14), we isolated and treated macrophages to determine if annexin A5 was inhibiting TNF-α production at the cellular level. Macrophages were isolated from the peritoneum after induction of peritonitis and plated on 48 well plates at $1.5x10^5$ cells/well. Treatment with 1 μg/mL of annexin A5 did not inhibit LPS-induced TNF-α release into the media after 4hr as measured by ELISA analysis (Figure 15). Surprisingly, annexin A5 treatment alone induced significant levels of TNF-α protein. This response was independent of Ca^{2+} concentration. We observed that medium supplemented with 2 mM Ca^{2+} produced similar amounts of TNF-α after exposure to annexin A5 as Ca^{2+}-free medium (Figure 15). Macrophages did not readily produce IL-1β so it could not be observed by ELISA analysis.

3.8 Effects of annexin A5 on neonatal and adult cardiomyocyte TNF-α production

Since annexin A5 treatment during endotoxemia decreases myocardial cytokine levels, we isolated and treated neonatal and adult cardiomyocytes to see if annexin A5 could interact directly with the myocardium. Neonatal cardiomyocytes were isolated in liberase-IV containing D-Hanks solution and treated after 48 hr of cell culture to allow confluence. Conversely, adult cardiomyocytes were treated after 6 hr of culture.

Neonatal cardiomyocytes produce substantial amounts of TNF-α in response to LPS (Peng et al. 2003) but do not produce detectable levels of IL-1β protein in the

supernatant (Westphal et al. 2007). We found that annexin A5 treatment of 1 μg/mL did not affect TNF-α production and release into the supernatant after 4 hr LPS treatment of 0.1, 0.2, and 1 μg/mL, as measured by ELISA analysis (Figure 16).

In contrast, adult cardiomyocytes had decreased TNF-α and IL-1β mRNA expression levels after 4 hr LPS (2.5 μg/mL) and annexin A5 (1 μg/mL) treatment, as determined by real-time RT-PCR ($P<0.05$, Figure 17A and 17B). Expression of TNF-α and IL-1β proteins in the media was not detectable because of the low plating density (50 myocytes/mm^2).

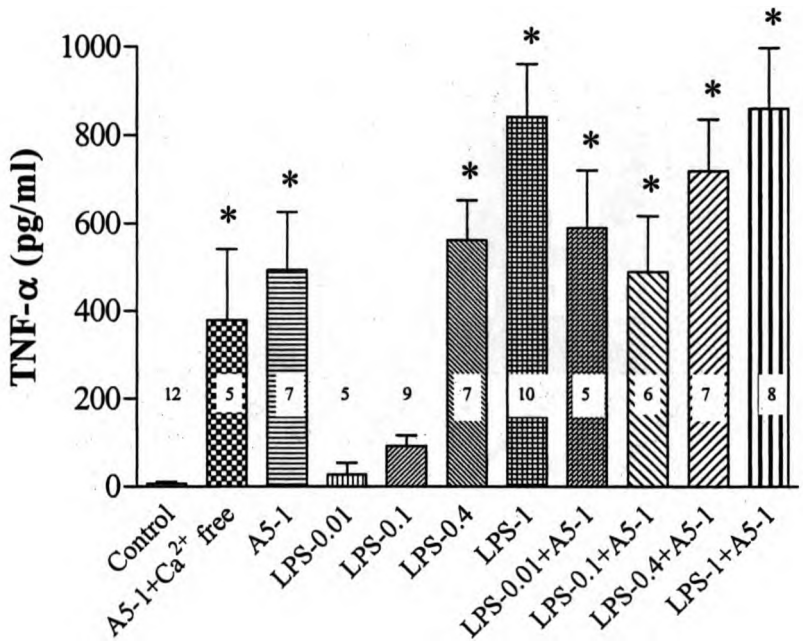


Figure 15. Annexin A5 does not affect LPS-induced TNF-α production in isolated macrophages. Peritoneal macrophages were isolated and plated on 48 well Costar plates at a concentration of $1.5x10^5$ cells/well. Cells were treated for 4 hr one day after isolation and TNF-α levels were measured by ELISA analysis of cell media. The control group contained RPMI 1640 media with 5% FBS, which served as the media for each treatment group. LPS concentrations used were 0.01, 0.1, 0.4, 1 ng/mL, and annexin A5 concentration used was 1 μg/mL. The Ca^{2+} concentraion of the media was 2 mM unless otherwise noted. N numbers are given in each column above. * $P<0.01$ vs. Control, LPS-0.01 and LPS-0.1.

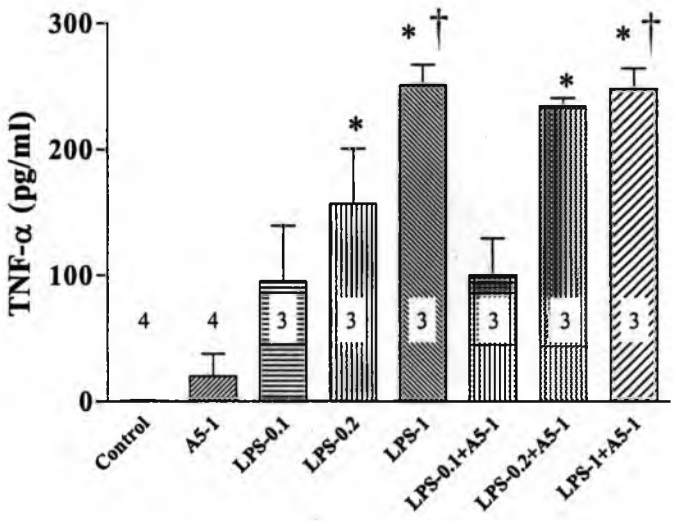


Figure 16. Annexin A5 does not affect LPS-induced TNF-α production in neonatal cardiomyocytes. Neonatal cardiomyocytes were plated on 24 well Costar plates at a concentration of $0.7x10^6$ cells/well and treated 2 days after isolation. The control group contained M199 media with 10% FCS, which served as the media for each treatment group. Cells were treated for 4 hr with LPS concentrations of 0.1, 0.2, and 1 μg/mL and with an annexin A5 concentration of 1 μg/mL. TNF-α protein levels were measured by ELISA analysis of cell media. N numbers are given in each column above. * $P<0.01$ vs. Control and A5, † $P<0.01$ vs. LPS-0.1.

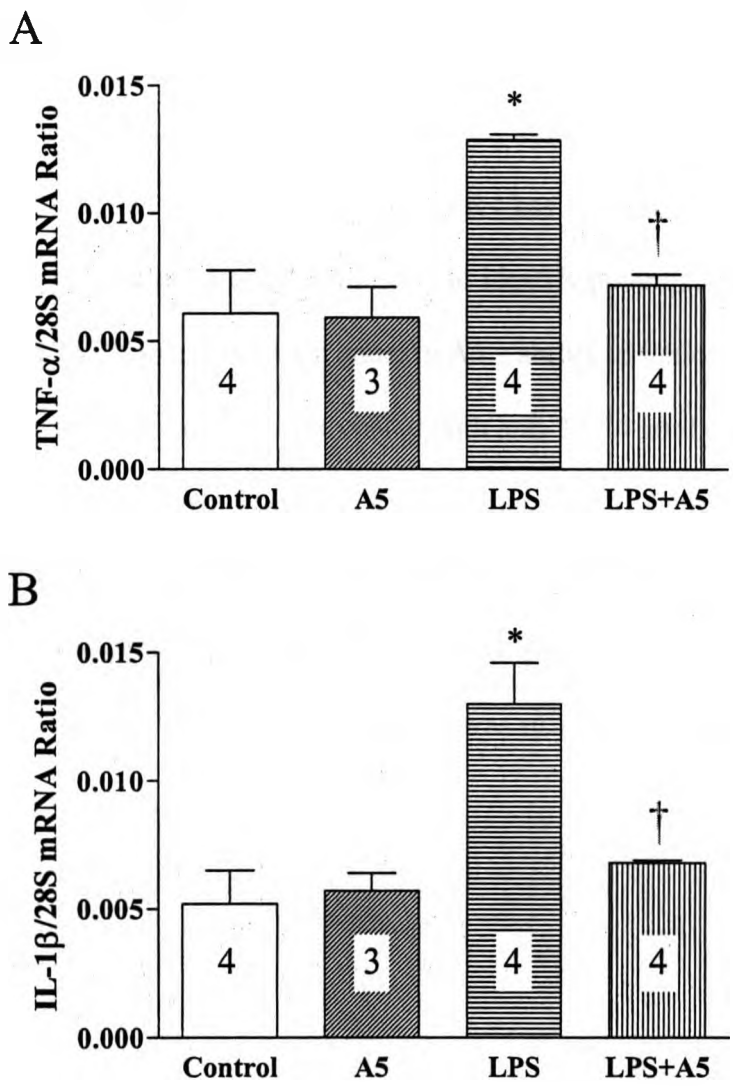


Figure 17. Annexin A5 abrogates LPS-induced TNF-α and IL-1β mRNA expression in adult cardiomyocytes. Adult cardiomyocytes were plated on 35mm dishes. Culture conditions and adult cell viability required cell treatment 6 hr after isolation. Cells were treated with LPS 2.5 μg/mL and annexin A5 1 μg/mL for 4 hr, and A: TNF-α and B: IL-1β mRNA levels were determined by real time RT-PCR analysis with 28S as a control. N numbers are given in each column above. * $P<0.01$ vs. Control and A5, † $P<0.05$ vs. LPS.

3.9 Annexin A5 binds to TLR4

It has been shown previously that annexin A5 can interact with and inhibit cell receptor function by binding to leucine-rich repeats through a conserved NH_2-terminal sequence (Markoff et al. 2007). Since mouse TLR4 has 19 separate leucine-rich repeats, we performed a co-immunoprecipitation assay to identify potential interaction between annexin A5 and TLR4 to help explain annexin A5's ability to inhibit cytokine production. Magnetic dynabeads were coated with anti-TLR4 antibody (Santa Cruz, Santa Cruz, CA) to pull down TLR4 and blot for annexin A5. Annexin A5 human recombinant was used as a positive control and Figure 18 qualitatively shows an annexin A5 specific band in the control and LPS samples, denoting an interaction between TLR4 and annexin A5. The membrane was stripped and reprobed for TLR4 as a loading control.

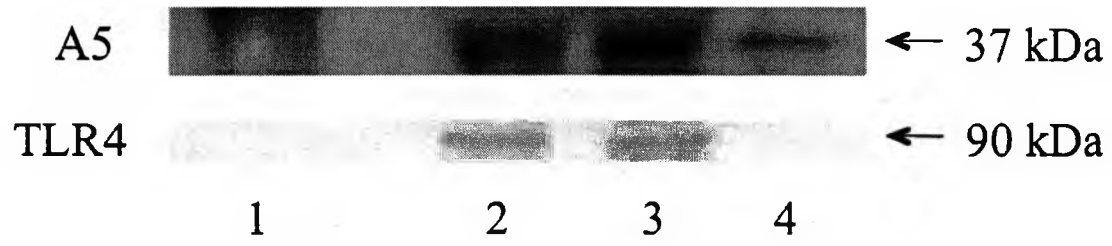


Figure 18. Annexin A5 interacts with TLR4 in myocardial tissue as determined by co-immunoprecipitation analysis. Myocardial tissue was homogenized and incubated with 0.5 μg of annexin A5. TLR4 protein was pulled down using magnetic beads coated with anti-TLR4 antibody. This was followed by a western blot analysis for annexin A5. The membrane was stripped and reprobed for TLR4. Lane 1, a control IgG with no sample. Lane 2, a control myocardial sample from mice treated with saline. Lane 3, myocardial sample from LPS-treated mice (4 mg/kg i.p. for 4 hours). Lane 4, recombinant annexin A5 positive control. The blot shown is a representative from 4 experiments.

Chapter 4 - DISCUSSION

The protective effect of annexin A5 on heart function during sepsis was examined in mice with endotoxemia. It was found that administration of annexin A5 at the same time as LPS decreased myocardial TLR4 signalling through p38 and ERK pathways (Figure 6 and 7), leading to decreased levels of TNF-α and IL-1β in the heart (Figure 10 and 11). The lower LPS-induced myocardial TNF-α and IL-1β levels after annexin A5 treatment was reflected in the plasma cytokine levels (Figure 14) and improved cardiac function during endotoxemia as measured using the Langendorff perfusion system (Figure 12) and the Millar pressure-conductance catheter system (Figure 13). Treatment of isolated adult mouse cardiomyocytes demonstrated a cardiomyocyte-specific effect of annexin A5 in inhibiting LPS-induced TNF-α and IL-1β mRNA expression (Figure 17). Co-immunoprecipitation revealed annexin A5's ability to interact with TLR4 (Figure 18). These results suggest that annexin A5 provides a protective role in the heart during endotoxemia. The effects of annexin A5 may be due to annexin A5's interaction with cell-surface receptor TLR4 and inhibition of inflammatory signalling that leads to cardiac dysfunction (Figure 4).

4.1 Findings

The p38 and ERK MAPKs are intimately involved in the TLR4-signalling pathway after LPS stimulation (reviewed by Guha and Mackman 2001). TLR4 activation can lead to myocardial NF-κB nuclear translocation within 30 min of LPS exposure *in vivo* (Baumgarten et al. 2001) and result in gene expression that can last for hours. In this study we found that annexin A5 treatment abrogated p38 and ERK phosphorylation 30 min after LPS treatment (Figure 6 and 7). The lower p38 and ERK phosphorylation with annexin A5 treatment was reflected in lower mRNA transcription of TLR4 and NOX2 after 4 hr (Figure 8 and 9), both components that are essential in cardiomyocyte's response to LPS (Peng et al. 2005; Boyd et al. 2006).

It has been demonstrated that cardiomyocytes are the predominant source of inflammatory cytokines in the heart (Kapadia et al. 1995; Peng et al. 2003). In this study we established that annexin A5 treatment resulted in lower myocardial TNF-α mRNA and protein and IL-1β mRNA levels 4 hr after LPS treatment (Figure 10 and 11). This is consistent with a previous study in our lab that showed inhibition of p38 and ERK blocked myocardial TNF-α expression (Peng et al. 2005).

Biologically active TNF-α protein is produced in large amounts and appears as early as 1 hr after LPS exposure (Kapadia et al. 1995) while IL-1β is less prominent and is produced several hours later as a result of TNF-α induction (reviewed by Dinarello 1996; Cain et al. 1999). This could be one explanation of why IL-1β protein levels did not increase in the myocardium similar to TNF-α 4hr after LPS treatment. In addition,

the cytokines produced by the myocardium undergo post-transcriptional modification and are readily released into the circulation or surrounding tissue, an explanation likely responsible for the difference in fold change between TNF-α mRNA and protein production.

TNF-α and IL-1β have been shown to act synergistically to induce a shock-like state in animals (Okusawa et al. 1988) and inhibit myocyte contractility (Cain et al. 1999). Since cardiac depression is associated with increased TNF-α levels (Grandel et al. 2000), this thesis demonstrated that lower TNF-α levels after annexin A5 treatment correlated with better cardiac function during endotoxemia (Figure 12 and 13). We observed full protection of cardiac function using the *ex vivo* Langendorff perfusion model (Figure 12). The Langendorff model provided a constant flow rate that allowed the heart to function independent of fluid loss, preload limitations, and neurohumoral influences to achieve normal cardiac function that was not present in the whole animal hemodynamics analysis. In the hemodynamic analyses, annexin A5 attenuated cardiac dysfunction by approximately 35% and 33% in $+dP/dt_{max}$ and $-dP/dt_{min}$ respectively, which is similar to the 34% lower TNF-α plasma levels in the presence of annexin A5 (Figure 14A). IL-1β plasma levels were lowered by approximately 51% with annexin A5 treatment (Figure 14B), but circulating IL-1β levels were almost 5 times lower than TNF-α and would likely have a more modest role in cardiac dysfunction compared to TNF-α.

The lower MAP observed in the LPS treated mice reflects a decrease in SVR. Low blood pressure can activate the baroreceptor reflex and initiate a compensatory

mechanism to increase heart rate and maintain CO. There was a strong trend towards higher heart rates in LPS and LPS+annexin A5 treated mice (Table 1). Surprisingly, there was a trend toward a lower heart rate in the annexin A5-alone treated mice that did not reach significance (Table 1). Currently there is no evidence of annexin A5's involvement in autonomic regulation or adrenergic signalling to affect heart rate. Conversely, annexin A6 is involved in Ca^{2+} regulation in the heart because functional studies reveal that knockout of annexin A6 led to an increase in Ca^{2+} cycling efficiency from the sarcoplasm (Song et al. 2002). Ca^{2+} cycling efficiency is increased when heart rate is increased to boost contractility of the cardiomyocytes. However, it was observed that the reduction or over-expression of annexin A6 had no effect on heart rate (Gunteski-Hamblin et al. 1996; Song et al. 2002).

It has also been shown that annexin A6 is reduced in the failing heart which increases contraction-relaxation dynamics of the heart to compensate for increased cardiovascular demand (reviewed by Kaetzel and Dedman 2004). In contrast, annexin A5 is upregulated in the failing heart (Matteo and Moravec 2000) and therefore probably has a different role, if any, in Ca^{2+} cycling in the heart compared to annexin A6.

No research has been done to investigate annexin A5's interaction with the central nervous system or autonomic regulation of heart function. Given the evidence that annexin A5 did not affect heart rate in the Langendorff perfusion system (Figure 12), annexin A5's influence on heart rate could be a result of experimental procedures and sample sizes, or it may have an indirect effect not yet investigated.

This thesis has demonstrated that annexin A5 treatment prior to endotoxemia abrogates myocardial cytokine production and lessens cardiac dysfunction. In order to further elucidate the mechanism of annexin A5 function, we isolated peritoneal macrophages and neonatal and adult cardiomyocytes to determine if annexin A5 was interacting with the myocardium at the cellular level. In our study, we did not see annexin A5 inhibit the release of TNF-α from the macrophage or neonatal cell's into the supernatant (Figure 15 and 16). In contrast, we saw that annexin A5 treatment alone significantly increased TNF-α production in isolated macrophages (Figure 15). This response was independent of the Ca^{2+} concentration used. The Ca^{2+} concentration needed for half-maximal binding of annexin A5 to negatively charged phospholipids is approximately 10 - 100 μM (reviewed by Gerke and Moss 1997) and we observed that medium with or without Ca^{2+} produced similar amounts of TNF-α after exposure to annexin A5. Therefore, annexin A5 was able to interact with isolated macrophages in a Ca^{2+}-independent manner. This is consistent with previous studies that demonstrate potential immunomodulatory effects of annexin A2 and A5 on macrophages (Munoz et al. 2007; Swisher et al. 2007; Swisher et al. 2009). Annexin-induced activation of inflammatory mediators in macrophages has been proposed to 'prime' macrophages for efficient microbial clearance and to increase the immunogenicity of apoptotic cells (Munoz et al. 2007; Swisher et al. 2007). Interestingly, we did not see any inflammatory response in the plasma (Figure 14) or myocardial tissue (Figure 10 and 11) after annexin A5 treatment alone, suggesting that macrophages may only respond to annexins in situations of stress, injury, or invasion.

While neonatal cardiomyocytes produce substantial amounts of TNF-α in response to LPS treatment (Figure 16), IL-1β was not detectable in the supernatant. This is consistent with a recent study that found rat neonatal cardiomyocytes produce IL-1β mRNA but did not release mature protein 5 hr after LPS treatment (Westphal et al. 2007) because a significant amount of pro-IL-1β remained in the cell (Mosley et al. 1987). This is also consistent with our results of IL-1β production in the myocardium where we did not see an increase in myocardial IL-1β protein expression after 4 hr LPS treatment (Figure 11). Similarly, macrophages are unable to process and release IL-1β solely by TLR ligands (Netea et al. 2009), which is the primary mode of activation by LPS.

In contrast to annexin A5's influence on macrophage and neonatal cardiomyocyte TNF-α expression, annexin A5 inhibited TNF-α and IL-1β mRNA expression after LPS treatment in adult cardiomyocytes (Figure 17). Moreover, a co-immunoprecipitation of annexin A5 and TLR4 displayed a possible interaction that could be attributed to annexin A5's ability to control myocardial inflammation through inhibition of TLR4 signalling (Figure 18). It is likely that annexin A5's conserved NH_2-terminal sequence interacts with one of TLR4's 19 leucine-rich repeats (Markoff et al. 2007). Annexin A5 has also been shown to interact with and regulate surface receptor expression (Ravassa et al. 2005). It's ability to interact with various cell surface receptors may remove it from the circulation and could help to explain its lasting impact hours after treatment given its short plasma half-life of approximately 15 min (Teoh et al. 2007).

While we were able to measure cytokine mRNA levels in isolated adult cardiomyocytes, our inability to obtain TNF-α and IL-1β protein measurements is reflective of the difficulty to work with adult cardiomyocytes. Adult cardiomyocytes are less viable than their neonatal counter parts and therefore must be plated at a much lower density and treated on the same day as isolation. Given these culture conditions, mRNA measurements were the only achievable measurement for cytokine activity in adult cells. Also, under certain circumstances, adult cardiomyocytes may respond differently than their neonatal counterparts. For example, adult cardiomyocytes have been shown to have longer action potential durations than neonates (Nuss and Marban 1994) and they also undergo apoptosis at doses of LPS that do not initiate similar apoptotic responses in neonatal cells (Hickson-Bick et al. 2006).

Furthermore, it has been shown that neonatal cardiomyocytes express low levels of CD14 and LBP and are activated by LPS in a CD-14-independent manner (Cowan et al. 2000). Conversely, adult cardiomyocytes are hyposensitive to LPS exposure when CD-14 function is inhibited (Comstock et al. 1998). The differing expression and utilization of TLR4 binding components in adults and neonates may affect annexin A5's ability to bind to TLR4 via leucine-rich repeats and inhibit TLR4 signalling. Neonatal cardiomyocytes also show more similarity with macrophages in NF-κB pathway activation in response to LPS than with adult cardiomyocytes (Liu et al. 2001; Cuenca et al. 2007). This may shed light on the disparity of annexin A5 impact on macrophage and neonatal cells compared with adult cardiomyocyte TNF-α production.

Data from this thesis suggest that annexin A5 may interact with TLR4 to inhibit myocardial cytokine production, but it is possible that annexin A5 may also be interacting with other cell membrane receptors. In 2006, Tavener demonstrated through knockout studies that both TNF-α receptors 1 and 2 (TNFR1/2) are necessary for myocyte dysfunction after LPS treatment (Tavener and Kubes 2006). In addition, Tavener also reported that leukocyte TLR4 expression and not cardiomyocyte TLR4 expression was responsible for contractile dysfunction after LPS treatment (Tavener et al. 2004). However, while leukocytes are vital for the inflammatory response to infection, there is currently no direct evidence for leukocyte or neutrophil infiltration being responsible for cardiac dysfunction (Raeburn et al. 2002). The evidence that annexin A5 is able to inhibit pro-inflammatory cytokine mRNA expression in adult cardiomyocytes (Figure 17) is consistent with our observations that annexin A5 protects the heart from LPS-induced cardiac dysfunction (Figure 12 and 13), but not from systemic inflammation (Figure 14). So while the impact of other cell receptors cannot be ruled out and must be further investigated, we believe that cardiomyocyte TLR4 is the prominent cell receptor responsible for mediating sepsis-associated cardiac dysfunction through which annexin A5 principally acts (Baumgarten et al. 2001; Baumgarten et al. 2006; Boyd et al. 2006).

This thesis gives evidence for annexin A5 to be protective in endotoxemia-induced cardiac dysfunction by inhibiting myocardial cytokine production through direct interaction with TLR4.

4.2 Limitations

Mouse Model of Human Disease Conditions

The use of mice to study human disease is necessary in the evaluation of potential therapeutics for both ethical and practical reasons. Animal models allow for control of experimental conditions and collection of tissue samples that are otherwise not possible in humans. Mice also provide an economic benefit with short gestation and maturity periods that allow increased sample sizes to make the study of adult related diseases much more accessible.

This thesis utilized LPS to simulate human sepsis. LPS, a gram-negative endotoxin, is effective in inducing a sepsis-like state in animal models (Natanson et al. 1986) but it is not exhaustive in simulating all sepsis-associated infections. In a study cataloging sepsis cases from 1979 through 2000, it was found that only 37.6% of the cases were a result of gram-negative bacteria infection, while the rest was made up of gram-positive bacteria, polymicrobial, and fungi infections (Martin et al. 2003). Moreover, in a recent study, it was determined that 67% of infections originated in the lungs while only 22% were in the abdomen (Vincent et al. 2006). Nonetheless, the model of LPS induced endotoxemia through intraperitoneal injection continues to be one of the most prominently accepted sepsis-models as it is easily reproduced and provides an appropriate model for studying many manifestations of sepsis.

Dosing

Our lab has shown previously that an LPS dose of 4 mg/kg i.p. is appropriate to induce cardiac dysfunction after several hours in mice (Peng et al. 2005; Peng et al.

2008; Peng et al. 2009). In contrast, the effect of annexin A5 treatment during endotoxemia has not been examined. An annexin A5 dose of 5 μg/kg i.v. was used because it provided cardiac protection during endotoxemia and there was no previous evidence of a more optimal dose of annexin A5 for sepsis-associated organ dysfunction. Previous studies investigating the role of diannexin, a recombinant annexin A5 homodimer, in liver ischemia/reperfusion injury used doses ranging from 100-1000 μg/kg (Shen et al. 2007; Teoh et al. 2007). This dose is much greater than the dose of 5μg/kg used in this thesis because ischemia/reperfusion injury is a greater risk to tissue and organ damage.

However, we did not investigate the efficacy of annexin A5 over a range of doses, thus limiting our knowledge of its potential clinical usefulness and impact on other organ systems during sepsis.

Timing

Although the total number of sepsis-associated deaths continue to increase, the total in-hospital mortality rate fell approximately 10% from the early 1980's to the late 1990's (Martin et al. 2003). Improved characterization and understanding of sepsis has no doubt had a beneficial effect on patient prognosis, but the major caveat in sepsis therapy continues to be the time at which identification of sepsis can occur. Early identification and therapy directly reduces mortality rates (Rivers et al. 2001).

In this thesis, annexin A5 was administered at the same time as LPS and may act as a preventative treatment rather than a restorative treatment. However, annexin A5 has been shown to inhibit inflammatory mechanisms and decrease tissue damage up to 1 hr

after ischemia/reperfusion liver injury (Teoh et al. 2007). Since sepsis is a self-perpetuating disease, it is encouraging that annexin A5 may be able to inhibit the progression of inflammation.

In addition, it would be beneficial to have a more comprehensive time course detailing the inflammatory response in the presence of annexin A5. For example, MAPK phosphorylation was analyzed after 30 min to demonstrate its activity after LPS treatment and its involvement in the TLR4-signalling pathway (Figure 6 and 7). However, since cytokine production and cardiac function were assessed after 4 hr, we cannot conclude that there is a direct correlation between MAPK phosphorylation and cytokine production and cardiac function. Therefore, we are limited to a snapshot of the model we have established.

4.3 Suggestions for Future Research

Annexin A5 was originally identified as an anti-coagulant with antithrombotic activity (Reutelingsperger et al. 1985). More recently it has been shown to be a potent anticoagulant through inhibition of platelet adhesion, prothrombinase complex formation, and fibrin deposition, thus maintaining microvascular circulation (van Heerde et al. 1994; London et al. 1996; Kuypers et al. 2007; Shen et al. 2007; Teoh et al. 2007). Interestingly, the anticoagulant drotrecogin alfa (activated), a recombinant human activated protein C, has displayed improved survival and respiratory and cardiovascular function in sepsis in phase III clinical trials (Ely et al. 2003; Kalil et al. 2004). Activated protein C has also been demonstrated to have anti-inflammatory properties through inhibition of NF-κB activation, cytokine production, and leukocyte cell rolling and

adhesion (Grey et al. 1994; Joyce and Grinnell 2002; Favory et al. 2006; Lehmann et al. 2008). Therefore, it would be beneficial to investigate the role of annexin A5 in the microvascular circulation and coagulatory systems during sepsis.

It is also possible that annexin A5 may improve cardiac function during endotoxemia by additional mechanisms not explored in this thesis. Despite the fact that apoptosis is very low in the myocardium and believed not to be responsible for cardiac dysfunction in endotoxemic mice, LPS and TNF-α induce caspase activation and apoptotic pathways in the heart (reviewed by Carlson et al. 2005; Crouser 2005). Moreover, inhibition of caspase activation or overexpression of Bcl-2 attenuated myocardial dysfunction in mice (Fauvel et al. 2001; Lancel et al. 2005). While there is currently no evidence for annexin A5 to have anti-apoptotic mechanisms beyond the inhibition of phagocytosis at the cell membrane level, it has been shown that uptake of annexin A5 in rat cardiomyocytes during endotoxemia could be caspase-dependent (Petillot et al. 2007). Therefore, further study needs to be done in order to determine if annexin A5 internalization is related to or simply a result of caspase activation and if it has any affect on caspase activity and apoptosis.

4.4 Conclusion

In conclusion, the results of this thesis demonstrate that annexin A5 treatment at the onset of endotoxemia attenuates TNF-α and IL-1β production and prevents cardiac dysfunction. It was found that annexin A5 might interact with TLR4 to inhibit an excessive LPS-induced inflammatory response. However, annexin A5 also forms a two dimensional shield on cell membranes and has anticoagulant effects. Whether these mechanisms contribute to the beneficial effects observed in this study cannot be excluded. This thesis provides encouraging evidence for annexin A5 to be a potential new therapeutic treatment in the management of sepsis and septic shock.

Chapter 5 - REFERENCES

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Appendix: UWO Animal Use Sub-Committee Protocol Approvals

Western

03.31.08
*This is the 1st Renewal of this protocol
*A Full Protocol submission will be required in 2011

Dear Dr. Feng

Your Animal Use Protocol form entitled:

Modulation of cardiac function in myocardial infarction, sepsis and diabetes

has been approved by the Animal Use Subcommittee.
This approval is valid from **04.01.08 to 03.31.09**
The protocol number for this project remains as **2007-011-03**

1. This number must be indicated when ordering animals for this project.
2. Animals for other projects may not be ordered under this number.
3. If no number appears please contact this office when grant approval is received.
   If the application for funding is not successful and you wish to proceed with the project, request that an internal scientific peer review be performed by the Animal Use Subcommittee office.
4. Purchases of animals other than through this system must be cleared through the ACVS office. Health certificates will be required.

ANIMALS APPROVED FOR 1 YEAR **Highest Pain Level: D**

Species	Other Detail	Housing/Use Locations	Animal # Total for 1 Year
Rat	Sprague-Dawley	LHSC-VRL	200
Mouse	C57BL/6	HSACF / M249	1000
Mouse	Various KO Strains (as outlined in protocol renewal)	HSACF / M249	700
Mouse	Various other Transgenic Strains (as outlined in protocol renewal)	HSACF / M249	500

REQUIREMENTS/COMMENTS
Please ensure that individual(s) performing procedures on live animals, as described in this protocol, are familiar with the contents of this document.

c.c. Approved Protocol - Q Feng, T Kirkpatrick, W Lagerwerf
Approval Letter - T Kirkpatrick, W Lagerwerf

[redacted]

The University *of* Western Ontario
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