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Endothelial HSPA12B is a Novel Protein for the Preservation of Cardiovascular Function in Polymicrobial Sepsis via Exosome MiR-126

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ABSTRACT

Endothelial HSPA12B is a Novel Protein for the Preservation of Cardiovascular Function in Polymicrobial Sepsis via Exosome MiR-126

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Sepsis is the most frequent cause of mortality in most intensive care units. Cardiovascular dysfunction is a major complication associated with sepsis, with high mortality rates up to 70%. Currently, there is no effective treatment approach for sepsis.

The integrity of the endothelium is fundamental for the homeostasis of the cardiovascular system. Sepsis induces endothelial cell injury which is the key factor for multiple organ failure. The increased expression of adhesion molecules and chemokines in endothelial cell promotes leukocytes infiltration into the tissue. The loss of tight junction proteins and increased permeability of the endothelial cells will provoke tissue hypoxia and subsequent organ failure. Therefore, preservation of endothelial function is a critical approach for improving sepsis-induced outcome.

In this study, endothelial specific protein HSPA12B has been found to play a critical role in the preservation of cardiovascular function in polymicrobial sepsis. HSPA12B is a newest member of HSP70 family which predominantly expresses in endothelial cells.

HSPA12B deficiency (HSPA12B$^{-/-}$) was found to exaggerated polymicrobial sepsis-induced endothelial dysfunction, leading to worse cardiac dysfunction. HSPA12B$^{-/-}$ significantly increases the expression of adhesion molecules, decreases tight junction protein levels and enhances vascular permeability. HSPA12B$^{-/-}$ also markedly promotes the infiltration of inflammatory cells into the myocardium and inflammatory cytokine production.

The cardioprotective mechanisms of HSPA12B was investigated in sepsis induced cardiovascular dysfunction. Exosomes play a critical role in cell-cell communication. Exosome is a natural vehicle of microRNAs. We found that exosomes isolated from HSPA12B$^{-/-}$ septic mice induced more expression of adhesion molecules in endothelial cells and inflammatory responses in macrophages. Interestingly, the levels of miR-126 in serum exosomes isolated from HSPA12B$^{-/-}$ septic mice were significantly lowers than in WT septic mice. Importantly, delivery of miR-126 carried exosomes significantly improved cardiac function, suppressed the expression of adhesion molecules, reduced immune cell infiltration in the myocardium, and improved vascular permeability in HSPA12B$^{-/-}$ septic mice. The data suggests that HSPA12B is essential for endothelial function in sepsis and that miR-126 containing exosomes plays a critical role in cardiovascular-protective mechanisms of endothelial HSPA12B in polymicrobial sepsis.

DEDICATION

With pride and affection, I dedicated this dissertation to my loving and unwavering supportive husband, Zhibo, April, our sweet little girl, my encouraging little brother Rui and Sister Qin, and to my always inspiring parents.

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CHAPTER1

INTRODUCTION

Sepsis and Septic Cardiomyopathy

Sepsis is a Major Healthcare Problem.

The Definition of Sepsis. Sepsis is systemic immune and inflammatory response to pathologic infection. The symptom ranges from minor symptom systemic inflammatory response syndrome (SIRS), sepsis, severe sepsis and septic shock [1]. Bacteremia means the presence of detectable bacteria in the blood. However bacteremia is only found in half of severe sepsis and septic shock patients.

The Epidemiology of Sepsis. In the United States, the incidence of sepsis increased to 1,141,000 in 2008 (http://www.cdc.gov/sepsis/datareports/index.html). The number is predicted to continuously increase due to a population aging, making this disease a major healthcare problem [2,3].

Sepsis and Multiple Organ Failure. Severe sepsis is sepsis with organ dysfunction. Manifestation of organ dysfunction varies from acute heart failure; lung injury; oliguria and increased creatinine; coagulopathy; thrombocytopenia; unexplained mental changes; hyperbilirubinaemia; or hyperlactatemia. Septic shock is sepsis with persistent hypotension despite fluid resuscitation. The yearly incidence of severe sepsis has been reported as 132 per 100 000 population [4]. Severe sepsis and septic shock are

common causes of death in critically ill patients in non-cardiac Intensive Care Units. The mortality rate of severe sepsis is 27%, while septic shock with higher rate at 50% [5,2]. So far, the treatment of sepsis still primarily relies on removal of infectious resource, antibiotics and supportive care [6,7].

Septic Cardiomyopathy Plays a Major Role in the Mortality of Septic patients.

The Epidemiology of Myocardial Depression in Sepsis. Multi-organ failure is a major problem in sepsis/septic shock [8,9,10]. Cardiovascular dysfunction is a well-recognized manifestation in severe sepsis [11,12,13,4,14,15]. Recently cardiac dysfunction has been listed as one of criteria for the diagnosis of severe sepsis [4]. Studies suggest that 40% to 50% of septic shock patients demonstrate myocardial depression characterized by ventricular dilation and decreased left ventricle ejection fraction (LVEF < 45%) [16]. Myocardial depression has been shown to be global but reversible in septic patients.

Septic Cardiomyopathy Contributes to High Mortality in Sepsis. Cardiac dysfunction in sepsis has been referred as “septic cardiomyopathy”. Septic cardiomyopathy is also transient and recovery can occur in 7-10 days in survivors of septic patients [17]. But there is compelling evidence that cardiovascular dysfunction is a major complication associated with sepsis induced morbidity and mortality [11,4,12]. The severity of septic cardiomyopathy predicts worse outcomes [18]. Septic patients with cardiovascular dysfunction are shown to have significantly higher mortality rates of 70% to 90% in contrast to 20% in those without myocardial depression [19,20]. But so far there

is no specific therapy for septic cardiomyopathy; this is due, in part, to the fact that the etiology of cardiac depression is still unclear [21,22,23]. Therefore, understanding the cellular and molecular mechanisms leading to septic cardiomyopathy will promote the development of new and effective therapies that can decrease the morbidity and mortality associated with sepsis/septic shock.

Mechanisms that Contribute to Septic Cardiomyopathy.

The precise mechanism of septic cardiomyopathy is still unknown. Previously, it was assumed cardiac dysfunction may be due to reduced circulating blood volume. But recent studies show that septic patients still develop myocardial depression despite sufficient fluid resuscitation [4,20]. The mechanism of myocardial dysfunction in sepsis is probably multifactorial [16,24,25,26,27,28], such as increased apoptosis [29], mitochondrial dysfunction [30,31,32], changed calcium channels [33] and Toll-like receptors (TLRs) activation [34,35].

Recent evidences have demonstrated that innate immunity and inflammatory responses play a critical role in septic cardiomyopathy [36,37,35,38]. Polymicrobial sepsis activates inflammatory cells, such as macrophages [39,40] and neutrophils [41,42,43,44,45], to release inflammatory mediators [46,47].

Macrophages and neutrophils express TLRs [48]. TLRs, the best characterized pattern recognition receptors (PRRs), play an important role in activation of the innate immune response [49,50]. TLRs have been found both in humans (TLRs 1–10) and in mice (TLRs 1–9 and 11–13). Neutrophils express all human TLRs except TLR3 [48]. TLRs stimulate neutrophils function [48]. TLRs recognize pathogen-associated molecular

patterns (PAMPs), and also damage associated molecular patterns (DAMPs) [51]. It is well-documented that TLRs mediate innate immune and inflammatory response in sepsis [52,53,35]. TLRs signal predominantly through the adaptor protein myeloid differentiation factor 88 (MyD88) into nuclear factor kappa-B (NF-κB) pathway exception for TLR3 [54,55,49,56]. Transcription factor NF-κB comprises two subunits, p50 and p65, which regulate innate immunity and inflammation [57]. Phosphorylation of IκBα controls the activation of NF-κB [58]. NF-κB is activated in sepsis and contributes to organ damage.

Our laboratory has reported that NF-κB activation plays a central role in mediating the development of sepsis/septic shock and septic cardiomyopathy [59,60,61,62,63,64], suggesting that TLR-mediated NF-κB signaling may be a target for prevention/management of septic cardiomyopathy.

Pro-inflammatory cytokines, such as interleukin-6 (IL-6) and tumor necrosis factor (TNF-α), are produced upon NF-κB activation. IL-6 plays a critical role in the development and mortality of sepsis [65]. Several studies also suggest IL-6 may serve as diagnostic and/or prognostic biomarker of sepsis severity and mortality [66,67,68,69,65]. Anti-IL6 antibody treatment has been demonstrated to significantly improve survival rate [70,69]. The pro-inflammatory cytokine TNF-α level is also suggested to predict the risk of sepsis [71,72]. Pretreatment with TNF-α antibody has been shown to improve survival rate in response to Lipopolysaccharides (LPS) [73].

Except for cytokines, other molecules in serum have also shown to depress cardiac function during septic shock, i.e. increased nitric oxide, endothelin-1 and complement [74].

Endothelial Activation/Dysfunction in Sepsis

The Function of Endothelial Cells

The endothelial monolayer, constituting the vessel wall, is the first line of defense against inflammation, hypoxia and other stresses. The integrity of the endothelium is essential for the homeostasis of the cardiovascular system. Under physiological conditions, the endothelium dynamically maintains normal coagulator state, blood fluidity regulates, barrier function and modulates vasomotor tone. The endothelium is not merely an inactive barrier between the blood and the surrounding tissues; it orchestrates the innate immune and inflammatory responses in sepsis [75,76].

The Manifestation of Endothelial Activation/Dysfunction

Dysregulated Endothelial Inflammation. Similar to leukocytes, endothelial cells also express PRRs [77], such as TLRs in relatively low levels [78,77,79], which activates inflammation through NF-κB and MAP Kinase. DAMPs released from damaged or dying cells and were found in the circulation of patients with stroke, trauma, and autoimmune diseases [51]. During sepsis, PAMPs, such as LPS, as well as DAMPs bind to PRRs and initiate innate immune and inflammatory responses, which cause tissue damage. Extracellular histones [80], HMGB1 [81] and DNA [51] are released during sepsis and induce cytokine production by primarily activating TLRs. Dysregulated endothelial inflammation stimulates coagulation pathways, increases vascular permeability and leukocyte infiltration into tissue.

Upregulation of Adhesion Molecules in Endothelial cells. In physiologic conditions, endothelial cells express very low levels of adhesion molecules. After stimulation, activated endothelial cells show dramatically upregulated expression of P-selectin, E-selectin, plasminogen activator inhibitor 1 (PAI-1), fms-like tyrosine kinase 1 (sFlt-1), intercellular adhesion molecule 1 (ICAM-1) [82,83], and vascular cell adhesion molecule 1 (VCAM-1) [84,85]. The augmented adhesion molecules interact with leukocytes and promote their adhesion and infiltration into tissues [86,87].

Disrupted Tight Junction Proteins and Increased Permeability. Endothelial Intercellular junction incorporates adherens junctions, gap junctions and tight junctions (TJ) [88]. Tight junctions play a critical role in endothelial barrier function. The integrity of TJs is maintained by appropriate expression level and cellular localization TJ proteins. Transmembrane components, Occludin and Claudin, link to intracellular partner zonula occludens, i.e. ZO-1, ZO-2 and ZO-3 [88]. ZO-1 was the first discovered in 1986. Occludin was discovered in 1993 [89,90]. Endothelial highly express Claudin-5 [91,92]. Sepsis promotes the disruption and intracellular translocation of endothelial cell TJ proteins [93]. The loss of tight junction proteins will promote tissue hypoxia and subsequent organ failure, correlated with adverse consequences during sepsis.

Abnormal Nitric Oxide (NO) Production. Under physiological conditions, endothelial cells constitutively express endothelial NO synthase (eNOS) and produce small amount of NO. After stimulation, inducible NO synthase (iNOS) increases in endothelium and generates abundant NO. Elevated NO production contributes to

vasodilation and hypotension. Excessive NO promotes reactive oxygen species (ROS) production in endothelial cells. The upregulation of iNOS contributes to systemic endothelial dysfunction and inhibition of iNOS attenuates endothelial dysfunction and organ injury induced by LPS. Dysregulated NO production is also an early event of various cardiovascular diseases, such as hypertension, diabetes, and atherosclerosis.

The endothelium is a key organ involved in the pathogenesis of sepsis [75,76]. It has been demonstrated that sepsis induces endothelial dysfunction [94,84,95]. Sepsis induces endothelial cell activation/injury and increased endothelial permeability [96]. Endothelial cell dysfunction triggers secondary injuries or subsequent events of sepsis [97]. The increased expression of adhesion molecules and chemokines by endothelial cell promotes leukocytes infiltration into tissue [98]. The loss of tight junction protein and the increased permeability promotes tissue hypoxia and subsequent organ failure. It is well known that endothelial cells play key roles in sepsis-induced multiple organ dysfunction and high mobidity and mortality [75,99]. Therefore, preservation of endothelial function is an important approach for improving sepsis-induced outcome.

Endothelial Function and Cardiac Function

The cascade responses mediated by both bacterial toxins and inflammatory cytokines cause endothelial permeability, resulting in leak of fluid into the extravascular space that leads to life-threatening edema in multiple organs such as lung, kidney, brain and heart of septic patients. The expression of ICAM-1 and VCAM-1 has been implicated in neutrophil and macrophage infiltration in septic heart [100]. We have previously reported that polymicrobial sepsis [35] and endotoxemia [101] significantly

induced increased expression of adhesion molecules in the myocardium, resulting in accumulation of neutrophils and macrophages in the myocardium, which contribute to sepsis-induced cardiac dysfunction [102]. ICAM-1 and VCAM-1 also mediate septic myocardial dysfunction through as yet unknown mechanisms [103]. A multicenter study demonstrated that the level of endothelial activation markers positively correlated with sepsis severity, organ dysfunction, and mortality [84,85].

Endothelial Heat Shock Protein A12B

Heat Shock Proteins

Heat shock proteins (HSPs) are a family of conserved proteins that are produced by cells in response to a wide variety of stress. They were first discovered in 1962 and was named because of their relation to heat shock in 1974 [104]. HSPs are classified into several groups according to molecular mass or structure. They include high-molecular-mass HSPs (≥100 kD), HSP90 (81 to 99 kD), HSP70 (65 to 80 kD), HSP60 (55 to 64 kD), HSP40 (35 to 54 kD), and small HSPs (≤34 kD) [105].

The Function of HSPs

HSPs facilitate protein folding as chaperones [105]. HSPs are constitutively expressed or induced by various stimuli. The location of HSPs is either intracellular or secreted into the extracellular matrix [106]. Intracellular HSPs protect against diverse stresses, including hypoxia, ischemia, inflammation, and oxidative stress. Hsp27 have been shown to protect against LPS induced cardiac dysfunction. Extracellular HSPs are found to stimulate the immune system [106]. HSPs have been reported to be a ligand of TLRs [107].

As an example, circulating Hsp60 has been shown to promote apoptosis in cardiomyocytes [107] by binding to TLR4.

HSPA12B is a Member of Hsp70 Family

Hsp70 family is the largest and most conserved family of heat shock proteins. In addition to the classical properties of HSPs as chaperones or stress proteins, Hsp70s also act as signal molecules. Recently, Hsp70 has been demonstrated to increase intracellularly and extracellularly in the lung after hyperoxia and confer protective effects in endothelial cells as a TLR4 ligand [108].

The newest member of the HSP70 protein, HSPA12 was been discovered and characterized by Han *et al* in 2003 [109]. HSP70 family members have two distinct domains, a conserved N-terminal ATPase domain and a diverse C- terminal substrate–binding domain. HSPA12 has atypical ATPase domains which are separated into two parts by spacer amino acids. There are two forms of HSPA12, HSPA12A and HSPA12B. Both of them significantly increase in atherosclerotic aortic lesions, especially HSPA12A [109]. Traditionally HSP70 proteins are thought to express ubiquitously and not in one unique cell type. However, HSPA12A was found to be highly express in the human brain in 2004 [110] and HSPA12B was shown to be predominantly expressed in endothelial cells in 2006 [111].

The Role of HSPA12B in Endothelia Function.

The role of HSPA12B in endothelial cell function have not been intensively investigated. However, Han *et al* has also demonstrated that endothelial HSPA12B is

involved in angiogenesis through turnover of a known angiogenesis regulator, Kinase anchoring protein 12 (AKAP12), resulting in upregulation of vascular endothelial growth factor (VEGF) expression [111]. Hu *et al* have shown that the endothelial specific HSPA12B plays a significant role in endothelial cell function [112]. These authors have shown that knockdown of HSPA12B by small interfering RNAs in human umbilical vein endothelial cells (HUVECs) blocked wound healing, migration and tube formation. In contrast, overexpression of HSPA12B enhanced migration and accelerated wound healing [112]. The data indicates that HSPA12B is required for endothelial cell proliferation and migration in wound healing.

We have previously reported that transgenic mice with endothelial specific expression of HSPA12B significantly attenuated the expression of adhesion molecules and cardiac dysfunction induced by endotoxin [101], suggesting that HSPA12B may play an important role in regulation of endothelial function in sepsis. However, the role of endothelial HSPA12B in cardiovascular function in polymicrobial sepsis has not been investigated precisely. The present study aimed to elucidate the mechanisms by which endothelial specific expression of HSPA12B protects against polymicrobial sepsis-induced cardiovascular dysfunction.

MicroRNAs

The microRNAs Processing

MicroRNAs (MiRNAs) are small non-protein-coding RNA molecules which are 21 to 23 nucleotides in length. MiRNAs originate from precursor RNAs, also named primary miRNAs, which are hundreds to thousands of nucleotides long. After transcription,

primary miRNAs are then processed by the enzyme Drosha and RNA binding protein DGCR8 into precursor miRNA which contain 70 to 100 nucleotides with a hairpin. Exportin 5 facilitates the transportation of precursor miRNA into the cytoplasm. Precursor miRNA is further processed by the enzyme Dicer into a shorter double-stranded RNA and then incorporated into the RNA-induced silencing complex. One strand becomes the mature miRNA and binds with targeted mRNAs, while another one is usually degraded soon after.

MiRNA typically binds to the 3′ untranslated region (UTR) of an mRNA with a complementary sequence. Nucleotides 2 to 8 of the miRNA, termed the “seed” region, are essential for the binding. However, miRNAs are also associated with 5′ UTRs or exons. More than 1000 miRNAs have been predicted to exist in humans. MiRNAs are believed to regulate up to 90% of human genes [113,114,115,116]. Several miRNA target prediction resources are available online, such as TargetScan, DIANA-microT, miRanda, PicTar, MicroInspector, miTarget and miRecords.

The Function of MiRNA

MiRNAs have been identified as novel regulators of gene expression at the post-transcriptional level, either promoting mRNA degradation or inhibiting translation [117,118]. MiRNAs are new players in regulating innate immunity and inflammation [119,120,118,121,122,123,124,125]. Several MiRNAs (miR-146a, miR-155, and miR-125b, etc.) have been reported to regulate NF-κB-mediated inflammatory responses [126,127,119,116,128,129,130].

The Role of MiRNAs in Sepsis. MiRNAs have been reported to play a critical role in sepsis/septic shock induced innate immune and inflammatory responses [118,117]. Some miRNAs have been shown to significantly increase in septic patients compared with healthy people, such as miR-133a [131], miR-223 [132], miR-16 [133], miR-574-5p [134] and miR-150 [135]. However, some miRNAs are downregulated in sepsis patients, including miR-146a [136], miR-181b [137], and miR-4772 [135].

MiR-146a directly targets IL-1 receptor associated kinase 1 (IRAK1) and tumor necrosis factor receptor associated factor 6 (TRAF6) [138,139,140]. IRAK1 and TRAF6 are critical adaptor proteins in NF-κB signaling pathway [129]. The induction of miR-146a is NF-κB dependent, but miR-146 can negatively regulate NF-κB signaling pathway through a feedback mechanism [141,138] by directly targeting and suppressing IRAK1 and TRAF6 expression [138]. MiR-146a also silences the transcription and translation of TNF-α [142]. MiR-146a has been reported to protect organ function against ischemia/reperfusion (I/R) injury [143,144].

MiR125b-5p increases in macrophages after TLR activation. MiR125b-5p downregulates the expression of TNF-α and iNOs induced by LPS [129,145] as a negative regulator of inflammatory responses. Anti-inflammatory macrophages (M2) have higher miR-125b levels than pro-inflammatory macrophages (M1) [146]. Also miR-125b negatively regulates apoptotic signaling p53 [147], p38 [148] and Bak-1.

The Role of MiRNAs in Cardiac Function. MiRNAs have emerged as new players in cardiac pathophysiology [149,150,151]. Specific MiRNAs are enriched in heart, such as miR-1, let-7, miR-30, miR-29, miR-26, miR379 and miR-143. MiRNA expression profiling has

been shown to be altered in cardiac diseases, such as miR-125, miR-146, miR-27 and miR-181b.

Modulating miRNAs levels has been shown to improve cardiac function. Lin et al have shown that miR-23a is upregulated in cardiac hypertrophy and suppression of miR-23a by a specific antagomir alleviated cardiac hypertrophy induced by isoproterenol [152]. We have reported that miR-146a level are significantly increased after sepsis and enhanced miR-146a level via delivery of lentiviral miR-146a protects cardiac function against septic injury [138,153] by down-regulation of IRAK1 and TRAF6. Also we have found that upregulation of either miR-125b or miR-146a levels attenuate cardiac ischemia/reperfusion injury [144].

The Role of MiRNAs in Endothelial Cell. MiRNAs also have been shown to play an important role in endothelial cell activation, proliferation, angiogenesis and inflammation [154]. MiR-181b has been reported to negatively regulate NF-κB mediated endothelial inflammation and upregulation of miR-181b suppresses NF-κB signaling in endothelium [137]. As we mentioned previously, miR-146a and miR-125b also attenuates inflammation. MiR-17-3p and miR-31 induced by TNF-α negatively regulates ICAM-1 and E-selectin respectively in TNF-α treated endothelial cells [155].

In this study we investigated the role of miR-126 on controlling endothelial cells inflammation in *vivo* and *in vitro*. MiR-126 is predominantly expressed in endothelial cells [156] and regulates the progression of angiogenesis, proliferation, and migration [157]. Endothelial inflammation is critically regulated by miRNAs such as miR-126 *in vitro* and *in vivo*. Endothelial miR-126 level has been reported to decrease in type 2 diabetes [158].

MiR-126 also plays a critical role in vascular dysfunction and modulates the expression of vascular cell adhesion molecule-1 induced by TNF-α [159] and chemokine stromal cell-derived factor-1 (SDF-1/CXCL-12) [160]. The levels of miR-126 have been reported to be downregulated in murine and human serum during the course of experimental chronic kidney disease [161] and in human diabetic patients [158]. Interestingly, a recent study has demonstrated that the survival and function of plasmacytoid dendritic cells are modulated by miR-126 via the VEGFR2 pathway [162], suggesting that miR-126 may regulate innate immune responses by targeting VEGF signaling. However, the role of miR-126 in ploymicrobial sepsis-induced cardiac dysfunction has not been investigated.

The Diagnotic and Therapeutic Potential of MiRNAs

MiRNAs are novel potential diagnostic markers and therapeutic targets for various cardiovascular diseases [163,164,165,166,167]. MiR-133a has been reported to prominently upregulate in sepsis patients and is associated with disease severity and organ dysfunction [131]. The elevation of miR-133a may serve as diagnostic marker and predictor of severity in sepsis patients. MiR-150 in peripheral blood leukocytes and plasma is significantly reduced in sepsis patients and correlated with the prognosis of sepsis [135]. Restoration of MiR-150 may provide therapeutic potential for sepsis patients. Other miRNAs have been demonstrated to associate with the diagnosis and severity of sepsis, such as miR-146a [136] and miR-4772-5p-iso [135]. They also provide potential therapeutic targets for sepsis.

The beauty of miRNAs as a therapy is that miRNA coordinately regulates multiple targets at different levels of a process. One miRNA has the potential to modulate

hundreds of mRNA post-transcriptionally. 3′ UTR of mRNA also have redundant binding sites of varies miRNAs. All these potentials allow the possibility of complicated but fine-tuned coordinated negative or positive regulation.

However, miRNA therapy has several potential obstacles. Effective development of MiRNAs therapeutics depends on the delivery methods, including neutral lipid emulsion, nanoparticles and exosomes. Naturally existing RNA shuttles, such as exosomes, have triggered increasing interest as a drug carrier [168].

Exosomes

Intercellular communication coordinates cells to accomplish specific cellular functions. Gap junctions allow cells to exchange information by direct contact. Soluble factors can transmit information short distances in paracrine fashion, or enter the blood to target distant organs as in the case of hormones. Three decades ago, another method for intercellular communication appeared, involving the production and secretion of extracellular vesicles, including macrovesicles (MVs) and exosomes. Macrovesicles are heterogeneous vesicles with relatively larger sizes of 100-1000 nm in diameter. They originate via shedding from plasma membranes [169]. MVs contain proteins, such as Annexin 2 and caspases, but no specific markers. Exosomes are homogenous nanovesicles with a size range of 30-100nm. Exosomes originate from the end of endocytic-lisosomal system and are released from cells when multivesicluar bodies fuse with plasma membrane. Exosomes have specific markers, such as HSP70

and tetraspanins CD63, CD81 and CD9 [170]. In this study we will mainly focus on the function of exosomes in our model.

The Biogenesis of Exosomes

It is well known that eukaryotic cells release vesicles into the extracellular environment. The most widely studied and best known types of vesicles are microparticles (MPs) and exosomes [171]. The term exosomes was initially described in 1983 by Harding *et al* and named by Johnstone *et al* in 1987 when researchers investigated vesicle formation in reticulocytes. Exosomes attracted more interest in 1996 for their role of antigen presentation. Recent evidence indicates that exosomes can carry and transfer genetic information. Exosomes have been secreted *in vivo* or *in vitro* by most cell types, including cardiomyocytes [172], cancer cells [173], neutrophils, platelets, endothelial cells [174], dendritic cells [175], macrophages [176] and mesenchymal stem cells [177].

The Composition of Exosomes

Exosomes as lipid-rich vesicles also contain cellular proteins, such as tetraspanins CD9, CD63, and CD81, heat shock proteins, major histocompatibility complex (MHC) and the endosomal-sorting complex required for transport (ESCRT) related proteins [178,179,180]. Despite proteins and lipids, exosomes also carry a variety of RNAs, including miRNAs, mRNAs, transfer RNA and lncRNAs, among them miRNAs are most abundant. The five most abundant miRNAs have been reported to account for almost half of the miRNAs in human plasma exosome, including miR-99a-5p, miR-128, miR-

124-3p, miR-22-3p, and miR-99b-5p [181]. Information regarding exosome content is summarized in Exocarta (http://www.exocarta.org). The lipid composition of exosomes is still less known.

Exosomes from different origins have unique signatures of their parental cells, i.e.CD31 for endothelial cells, CD41 for platelet, F4/80 for macrophage, NIMP-R14 for neutrophil and CD45 for leukocyte. Exosomes can be constitutively released from cells, or they can be induced by various stimuli [182]. The unique profiles of exosomal components indicate proteins and MiRNAs maybe selectively incorporated into exosomes.

The Purification and Characterization of Exosomes

The most common procedure to isolate exosomes is a series of centrifugations, first to remove dead cells and cell debris, discriminate larger particles through 0.22μm filters and then pellet exosomes by ultracentrifugation. To further eliminate large protein aggregation and other small vesicles, exosomes will float on sucrose gradients (density 1.13g/ml to 1.19g/ml). Some commercial precipitation reagents are also been used to isolate exosomes, especially for low sample volumes. When isolating exosomes from cell culture medium, it is necessary to exclude exosomes from serum by overnight high-speed ultracentrifugation because serum has large amounts of exosomes.

Transmission electron microscopy is commonly performed to validate the existence of exosomes. Exosomes are 30-100nm in diameter and have characteristic saucer-shape morphology [178,179]. Western blot and fluorescence-activated cell sorting (FACS)

analysis are also routinely used to confirm the presence of exosome by detecting specific exosome markers [183,184].

The Biological Function of Exosomes

When firstly discovered, researchers thought that exosomes may serve as a degradation mechanism to discard cellular "garbage". Recently, exosomes as extracellular organelles have also been demonstrated to play a critical role in cell-to-cell communication [185,186,175,179,168]. Exosomes are novel vehicles for transferring proteins and/or MiRNAs from one cell to another [187,188,189,190]. Exosomes can function in a paracrine fashion, targeting proximally located cells/ tissue, or as hormones, travelling to distant cells/tissues. Exosomes release their contents into the target cells via membrane fusion with the target cells or via binding with specific receptors on target cells. Exosomes are also uptaken via endocytotic internalization.

Exosomes are novel vehicle for delivering mRNAs and microRNAs to exchange genetic information between cells [185]. MiRNAs transferred by exosomes are functional [191,175] and induce target gene repression in recipient cells [175,192]. The functions of exosomes from antigen-presenting cells have been investigated intensively. MiRNAs in exosomes have been shown to modulate immune responses [193,192]. MiR-155 and miR-146a have been shown to be in exosomes released by dendritic cell (DC). Those miRNAs can be transferred into recipient DC *in vitro and in vivo* and suppress target gene expression [192].

The Role of Exosomes in Sepsis. The functions of exosomes produced *in vitro* and *in vivo* have been investigated in sepsis. Recent evidence suggests that exosomes may play an important role in sepsis [194,195,192]. Azevedo et al reported that exosomes from septic shock patients induce myocardial dysfunction in isolated rabbit hearts [194]. The main source of septic exosomes is platelets. Exosomes from septic patients increase reactive oxygen species production and cause apoptosis in endothelial cells [194]. Gambim et al have shown that platelet derived exosomes induce endothelial cell apoptosis and endothelial dysfunction [194,195]. Immature dendritic cell-derived exosomes dampen inflammatory response and reduce mortality in septic animals. Exosomal miR-155 released by DC enhances while miR-146a suppresses inflammatory response in endotoxin-treated mice [192].

The Role of Exosomes in Endothelial Cell Function. Endothelial cells have been reported to secrete exosomes and also can be targeted by exosomes [174]. Endothelial cells communicate with other cells through exosomes [196]. It have been reported miRNA incorporated in exosome can be transferred into endothelial cell and are functional in recipient cells [197].

The Role of Exosome MiRNAs in Sepsis. The profile of exosomal miRNAs in mouse serum have been shown to be altered after polymicrobial sepsis induced by cecum ligation and puncture (CLP). Some miRNAs significantly increased in exosomes after CLP for eight hours, including miR-16, miR-17, miR-20a/b and miR-26a/b. Exosomal

miR-155 released by DC enhances while miR-146a suppresses inflammatory responses in endotoxin-treated mice [192].

The conventional biomarkers for sepsis that are currently used clinically have low specificity and sensitivity, including procalcitonin, C-reactive protein and IL-6. Recently the differences in exosomal miRNAs (miR-671-5p, miR-16-5p, and miR-150-3p) in patient serum have been shown to provide specific and sensitive information for diagnosis and categorization in virus infectious disease.

The Diagnostic and Therapeutic Potentials of Exosomes

Studies have demonstrated that exosomes can be identified in cell culture medium and many bodily fluids, such as breast milk [198], blood [199], amniotic fluid [200], synovia [184], bronchoalveolar lavage fluid [201] and urine [183] all of which are accessible in the clinic. The composition of exosomes reflects the cellular origins and status. It has been reported that the secretion and composition of exosomes changes during cancer, sepsis and hypoxia [202,182]. Especially the components of exosomal miRNAs is demonstrated to change in diseases. Therefore exosomes have significant diagnostic potential in patients [203,204].

Exosomes, as naturally existing RNA shuttles, have stimulated increasing interest as a drug carrier [168,205]. They have been utilized as shuttles to package and deliver drugs [206] because of their low immunogenicity and bio-distribution. As a novel drug delivery strategy, exosome may enhance anti-inflammatory activity of drugs. However, the biodistribution of systemically delivered exosomes has been challenged by a recent finding that unmodified exosomes injected intravenously will be mostly uptaken by liver

and spleen [207]. Despite miRNAs, exosomes also carry proteins, such as a well characterized transmembrane protein, lysosomal associated membrane protein 2b (Lamp2b). Recently, researchers have successfully engineered exosomes by fusing exosomal protein Lamp2b with CNS-specific rabies viral glycoprotein (RVG) peptide [206].The targeted exosomes boost short interfering RNA delivery efficacy into neural cells and inhibit gene expression post-transcriptionally [206].Therefore, modified exosomes are a potential ideal shuttle for in vivo delivery of miRNAs to target tissue [168].

Exosomes secreted by mesenchymal stromal cells (MSCs) have been shown to have cytoprotective effects and can attenuate myocardial ischemia/reperfusion injury [208] and hypoxia/induced pulmonary hypertension [209]. MSCs secrete a huge amount of extracellular microvesicles [210] and are efficient manufacturer of exosomes for drug delivery [211,205]. MSCs exosomes as a novel therapy promotes cardiac regeneration and recovery in several cardiovascular diseases [212,213,214].

In this study, efforts were directed at understanding the role of exosomes, especially exosomal miRNAs level, in a cardiac phenotype of HSPA12B deficiency after sepsis. Herein we investigated the changes in exosomal miRNAs after sepsis in our mouse model. We also detected whether the miRNA message of exosomes isolated from septic mice could be functionally transferred into macrophages and endothelial cells *in vitro*. Finally we also tested the therapeutic potential of miRNA incorporated by exosome in septic mice.

CHAPTER 2

CONSTRUCTON OF ENDOTHELIAL SPECIFIC HSPA12B DEFICIENT MICE

Introduction

The critically ill patient frequently develops a complex disease spectrum that may include acute respiratory distress syndrome (ARDS), systemic inflammatory response syndrome (SIRS), sepsis syndrome, septic shock and multiple organ dysfunction syndromes (MODS). There is compelling evidence that cardiovascular dysfunction is a major complication associated with sepsis induced morbidity and mortality [11,4,12]. Therefore, understanding the cellular and molecular mechanisms leading to septic cardiomyopathy will promote development of new and effective therapies.

As mentioned above, endothelial cell function is a critical factor contributing to cardiovascular dysfunction in sepsis/septic shock [94]. Preservation of endothelial function is critically important for protection against sepsis/septic shock induced multiple organ failure. We have previously reported that increased expression of HSPA12B attenuated endotoxin-induced cardiac dysfunction through activation of PI3K/Akt dependent signaling. The data suggests that HSPA12B is required for the preservation of cardiovascular function. HSPA12B is specifically expressed by endothelial cells [111] and is an essential for endothelial function. It is of interest to understand the role of HSPA12B in endothelial function in sepsis. To accomplish this goal, endothelial specific HSPA12B deficient ($HSPA12B^{-/-}$) mice were produced. $HSPA12B^{-/-}$ flox/flox mice were

designed and developed by Dr. Zhihua Han who was a faculty in the Department of Biomedical Science at ETSU.

Materials and Methods

Constructing Endothelial Specific HSAP12B Knockout Mice.

The knockout targeting strategy is outlined in Figure 1A. The targeting construct contained a PGK-driven Neo cassette and MC1 promoter-driven HSV-TK cassette, allowing for positive and negative selection. The right and the left arm loxP knockin were confirmed by genomic Southern with (EcoRI and probe A) and SalI digestion of PCR product by external and internal primer (5'-TCTGTGTCTGCCTGTGTTCTGT-3' and 5'-TAGTCTGCATTCGGAGGCAAGT-3'). The successful homologous recombination clones were subsequently transfected with pCre-Pac for excision by Cre to generate targeted alleles.

Endothelial specific HSPA12B knockout mice were generated by cross-breeding the conditionally targeted HSPA12B mice with C57BL/6.Cg-Tg (Tek-cre) strain which carries Cre recombinase under the control of the Tek promoter. Genotypes for the tissue specific deletions were confirmed by PCR of tail genomic DNA analysis with specific primers of floxed allele (HSPA12B-cko-1: 5'-GAAGCAAGCATATTCATCTCATTACTATTC-3'; HSPA12B-cko-2: 5'-GCTTGCTCAAAAGTGATGGTTGCTC-3'. 191 bp for knockout and 151 bp for wild type mice), HSPA12B deletion (HSPA12B-cko-1: GAAGCAAGCATATTCATCTCATTACTATTC; HSPA12B-cko-4: TAAAGCCTACACTCAGATGAGAGCAG. 240 bp product for deletion; >2kB or no

product for wild type control) and for Cre gene expression (5'-GTGAAACAGCATTGCTGTCACTT-3' and 5'-GCGGTCTGGCAGTAAAAACTATC-3'). Western blot and immunohistochemistry were also performed to identify endothelial specific deficiency of HSPA12B.

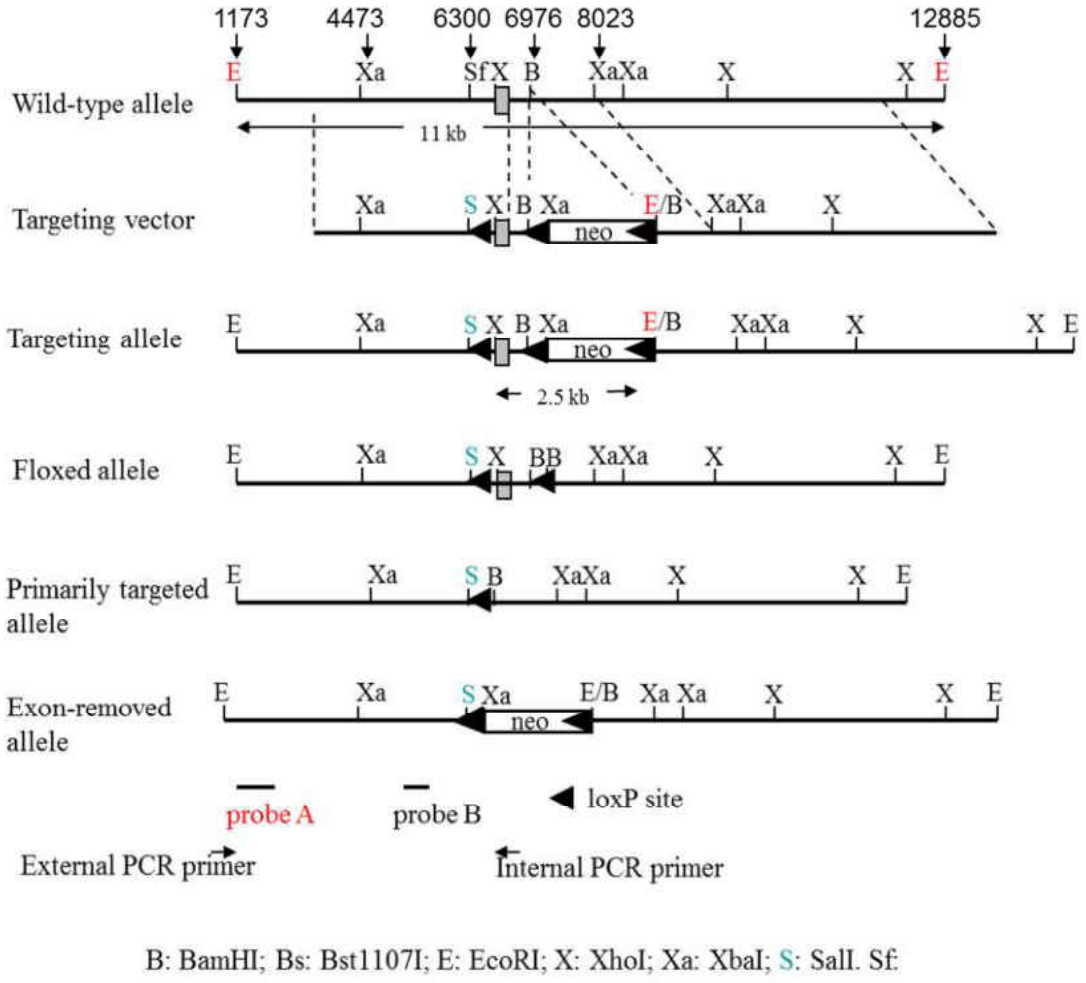

Figure1. The knockout targeting strategy. The knockout targeting strategy is outlined in Figure 1A. LoxP sites flanking exon 2 were introduced by recombineering and the linearized targeting construct.

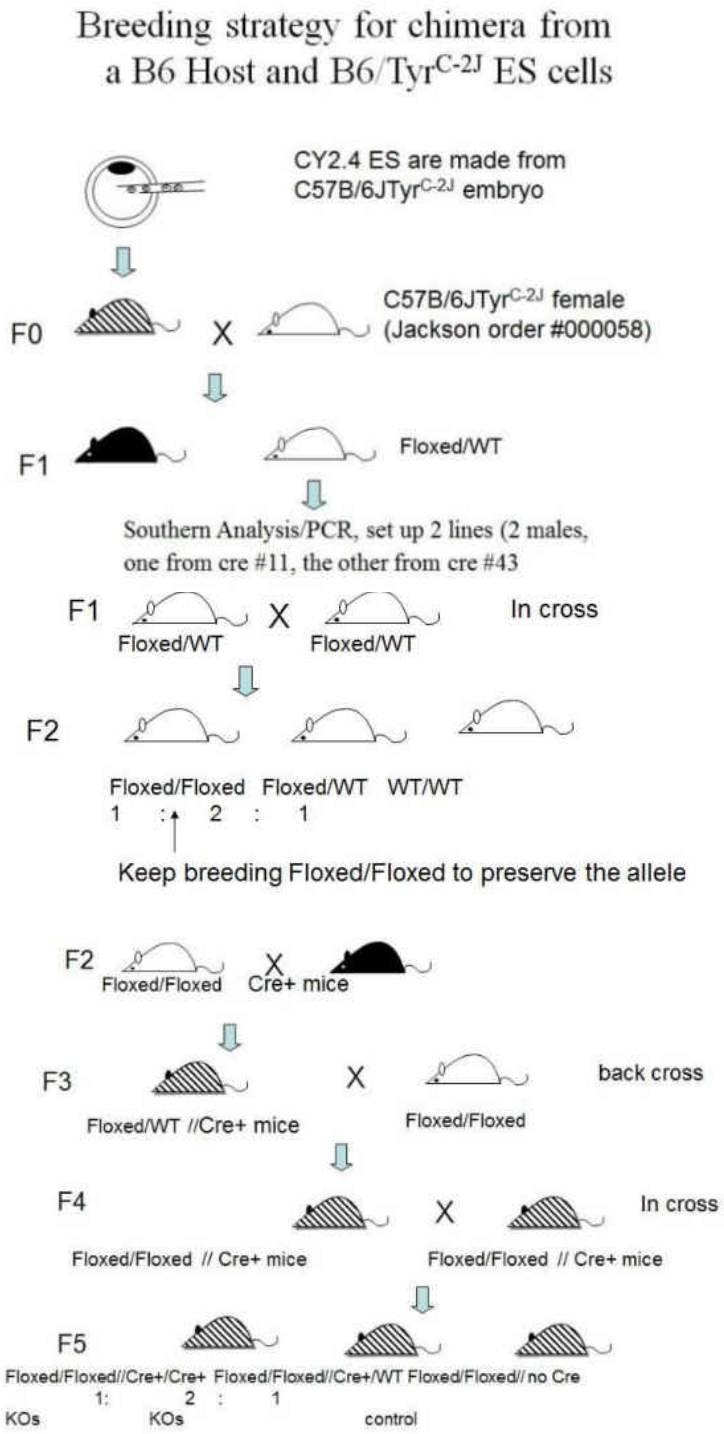

Figure2. Strategy for constructing the endothelial specific HSPA12B knockout mice. Endothelial specific HSPA12B knockout mice were generated by cross-breeding the conditionally targeted HSPA12B mice with C57BL/6.Cg-Tg (Tek-cre) strain which carries Cre recombinase under the control of the Tek promoter.

DNA Extraction from Mice Tails, PCR and Gel Electrophoresis.

Mice tails were removed into Eppendorf tube and added with10 µl of Protein K (10mg/mL) and DNA digestion buffer (50 µl) for each sample. The samples were incubated at 55°C overnight until complete dissolve of the tail. TE buffer (pH 8.0) 500µl was added and then Phenol Chloroform bottom layer (~ 600ml) by 1:1. Mix the contents of the tube for approximately 15 minutes until an emulsion forms. Centrifuge at 14,000 rpm, 4°C for 15 minutes. Remove the aqueous phase (400µl) to a new tube. Add 1/10 amount of 3M NaAc (PH 7.0) approximately 40 µl. Mix well. Add 1:1 concentration of isopropanol solution (~440µl). Mix until DNA appears as white floccules. Centrifuge for 15 minutes at 14,000 rpm, 4°C. Discard aqueous layer and add two times~700 µl 70% ethanol (-20 °C) to the sediment and invert several times. Centrifuge for 5 minutes and discard supernatant. Carefully remove last drop of ethanol. Let the sample stand and air dry at room temperature for 10-15mins. Add 100µl TE buffer to resuspend DNA. Vortex and centrifuge at short speed (~30 sec). Dilute DNA with TE buffer: For each sample adds 12µl primer and 2µl diluted DNA sample. Run PCR. PCR products by agarose gel at 80 volts and 124 milliamps, then take picture by G:BOX.

Western Blot.

Briefly, the cellular proteins were isolated by using RIPA buffer and separated by SDS-polyacrylamide gel electrophoresis and transferred onto Hybond ECL membranes (Amersham Pharmacia, Piscataway, NJ). The ECL membranes were incubated with primary anti-HSPA12B antibody (a kind gift from Dr. Han Zhihua) followed by incubation with peroxidase-conjugated secondary antibodies (Cell Signaling Technology, Inc.) and

analysis by the ECL system (Amersham Pharmacia, Piscataway). The signals were quantified using the G: Box gel imaging system by Syngene (Syngene, USA, Fredrick, MD). GAPDH (Meridian Life Science, Inc, TN) was detected as loading control.

Immunofluorescence Staining.

Briefly, the heart sections were stained with specific first antibodies rabbit anti-HSPA12B and rat-anti-CD31 (PECAM-1) (1:100) for overnight at 4 ˚C. Then secondary antibody Alexa Fluor-488 goat-anti-rabbit IgG (H+L) (green, Thermo Fisher Scientific) and Alexa Fluor-555 goat-anti-rat IgG (H+L) (red) (Thermo Fisher Scientific) were added to the section for 1 hour at room temperature. Then the slides were examined with fluorescent microscope at a magnification of 400×.

Statistical Analysis

The data are expressed as mean ± SE. Comparisons of data between two groups were made using t-test. Probability levels of 0.05 or smaller were used to indicate statistical significance.

Results and Discussion

HSPA12B specifically expresses in endothelial cells [112]. To investigate the role of HSPA12B in polymicrobial sepsis, we constructed endothelial specific HSPA12B deficient mice. The strategy we employed is as shown in Figure 1 and Figure 2. We obtained two independent knockout lines (lines 11 and 43). In this study, we employed

line 43 knockout mice for all the experiments. PCR was performed to examine the successful deletion of HSPA12B. As shown in Fig.3a, there is a 100bp band for Cre, a 190bp band for Flox, and a 240 bp band for HSPA12B deletion in knockout mice. In contrast, WT mice show no band for Cre, a 150bp band for Flox and no band for HSPA12B deletion). Figure 3b shows that the protein levels in the myocardium of HSPA12B knockout mice were undetectable, when compared with WT mice. Immunohistochemistry staining shows that HSPA12B (green) was co-localized with CD31 (red) which is a marker of endothelial cells (Fig. 3c) in WT myocardial tissue. However, there is undetectable of HSPA12B in the myocardium of HSPA12B deficient mice, suggesting that HSPA12B was specifically knocked out from endothelial cells. We observed that endothelial specific knockout of HSPA12B does not compromise normal reproduction.

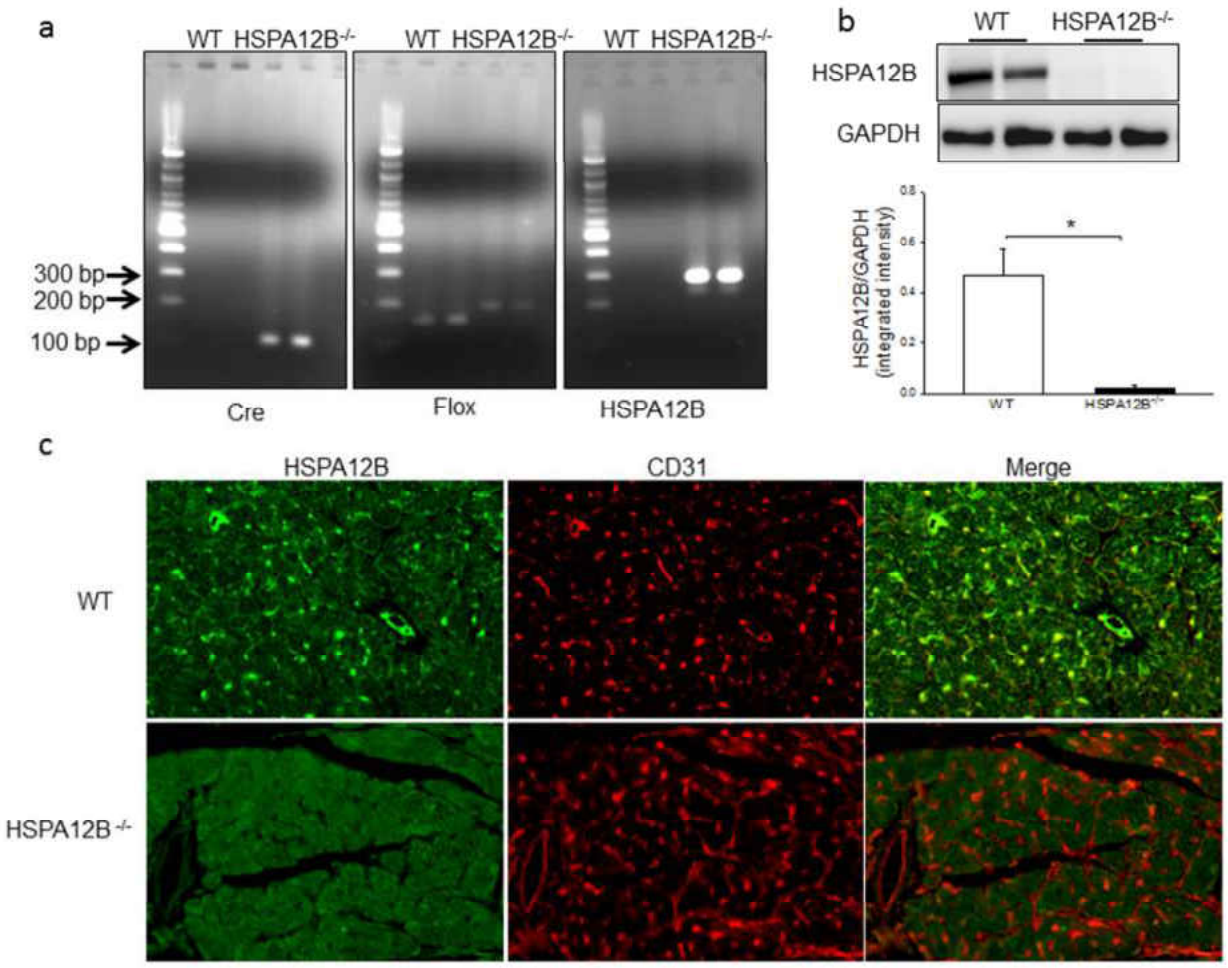

Figure 3 HSPA12B knockout mice have no expression of HSPA12B protein in endothelial cells. a. PCR was performed to examine the successful deletion of HSPA12B, by using specific primers of Cre, flox, and HSPA12B. b. The expression level of HSPA12B in the cytoplasm of myocardium was detected by WB (Fig. 3B, N=12/group). GAPDH serves as loading control. * indicates $P<0.05$. c. The expression level and localization of HSPA12B was also detected by immunofluorescent staining. HSPA12B (green) was co-localized with CD31 (red) which is a marker of endothelial cells in WT myocardial tissue (n=4/group).

CHAPTER 3

ENDOTHELIA HEAT SHOCK PROTEIN A12B (HSPA12B) IS REQUIRED FOR MAINTENANCE OF CARDIOVASCULAR FUNCTION IN POLYMICROBIAL SEPSIS

Introduction

The endothelium is a major target of sepsis-induced injury and endothelial cells are responsible for much of the pathophysiology of sepsis [75,94]. Vascular endothelial cells are the first cell type in the body that contact with circulating bacterial molecules. Endothelial cells possess mechanisms that recognize structural pathogen associated molecular patterns (PAMPs) and subsequently initiate the expression of inflammatory mediators [215]. In general, the cellular response to bacterial toxins normally provides protection against pathogen invasion. However, over activated cellular responses could lead to critical injury of organs. Under normal conditions, our body contains the stringent control mechanisms that avoid overresponses induced by danger signals. However, during sepsis, this balance is disrupted and the disturbance is manifested by profound changes in the relative production of different mediators.

Both endotoxin from Gram negative bacteria and PAMPs from Gram positive bacteria play a role in the initiation of sepsis/septic shock [216,34,24,217,24]. These bacterial toxins will be recognized by TLRs, resulting in activation of TLR-mediated NF-κB signaling pathways [218,34,35] which stimulates inflammatory cytokine expression. In addition, activation of integrins on the leukocyte membranes promotes the adhesion of leukocytes to cytokine-activated adhesion molecules on endothelial cells, resulting in the infiltration of leukocytes into the tissues and stimulation of inflammatory responses.

In addition, inflammatory cytokines, such as TNF-α and IL-1β, act synergistically in the initiation of the inflammatory responses of sepsis, leading to the further activation of other important mediators in the cascade of sepsis. Collectively, the cascade responses mediated by both bacterial toxins and inflammatory cytokines cause endothelial permeability, resulting in loss of fluid into the extravascular space that leads to life-threatening edema in the multiple organs such as lung, kidney, brain and heart of septic patients. Therefore, preservation of endothelia function is one important target for treatment and management of sepsis/septic patients.

It is well known that endothelial cells play key roles in sepsis-induced multiple organ dysfunction and high mobidity and mortality [75,99]. Therefore, preservation of endothelial function is an important approach for improving sepsis-induced outcome. HSPA12B is predominately expressed on endothelial cells and has been demonstrated to play an important role in endothelial cell function. In this study, we examined whether HSPA12B is essential for maintenance endothelial function in polymicrobial sepsis. Also we evaluated the role of HSPA12B in sepsis induced cardiac dysfunction and mortality.

A multicenter study has demonstrated that the level of endothelial activation markers is positively correlated with sepsis severity, organ dysfunction, and mortality [84,85]. We have previously reported that polymicrobial sepsis [35] and endotoxemia [101] induced expression of adhesion molecules in the myocardium, resulting in accumulation of neutrophils and macrophages in the myocardium, which contribute to sepsis-induced cardiac dysfunction [102]. The expression of ICAM-1 and VCAM-1 has been implicated in neutrophil and macrophage infiltration in septic heart [100]. In addition to leukocyte adhesion, ICAM-1 and VCAM-1 also mediate septic myocardial dysfunction through

other unknown mechanisms [103]. In this study, we investigated the changes in adhesion molecules as well as neutrophils and macrophages infiltration in HSPA12B deficient mice after sepsis.

Materials and Methods

CLP Polymicrobial Sepsis Model.

Cecal ligation and puncture (CLP) was performed to induce polymicrobial sepsis in mice as previously described [63,35,219]. Briefly, the mice were anesthetized by Isoflurane (Induced by 5.0% and maintained by 3%). A midline incision was made on the anterior abdomen and the cecum was exposed and ligated with a 4-0 suture. Two punctures were made through the cecum with an 18-gauge needle and feces were extruded from the holes. The abdomen was then closed in two layers. Sham surgically operated mice served as the surgery control group. Immediately following surgery, a single dose of resuscitative fluid (lactated Ringer's solution, 50 ml/kg body weight) was administered by subcutaneous injection [63,35,219].

Echocardiography.

Transthoracic two-dimensional M-mode echocardiogram was obtained using a Toshiba Aplio 80 Imaging System (Toshiba Medical Systems, Tochigi, Japan) equipped with a 12-MHz linear transducer as described previously [35]. M-mode tracings were used to measure LV end-systolic diameter (LVESD), and LV end-diastolic diameter (LVEDD). Percent fractional shortening (%FS) and ejection fraction (EF%) were calculated as described previously [37,35,220].

Tissue Accumulation of Neutrophils and Macrophages.

Inflammatory cells accumulation in heart tissues was examined with neutrophil specific antibody and macrophage specific antibody F4/80 (1:50 dilution, Santa Cruz, CA), separately [35,219]. Three samples from each group were evaluated, counterstained with hematoxylin, and examined with brightfield microscopy. Four different areas of each section were evaluated. The results are expressed as the numbers of neutrophils or macrophages per field (400x).

Myeloperoxidase (MPO) Activity Assay.

MPO activity was measured using a MPO fluorometric Detection kit (Assay Designs Inc., Ann Arbor, MI) according to the manufacturers' instructions.

Immunohistochemistry Staining.

Immunohistochemistry was performed as described previously [35,219]. Briefly, heart tissues were immersion-fixed in 4% buffered paraformaldehyde, embedded in paraffin, and cut at 5 μm sections. The sections were stained with specific goat anti-intercellular adhesion molecule 1 (ICAM-1, 1:50 dilution, Santa Cruz Biotechnology) and rabbit anti-vascular cell adhesion molecule (VCAM-1, 1:50 dilution, Santa Cruz Biotechnology), respectively, and treated with the ABC staining system (Santa Cruz Biotechnology) according to the instructions of the manufacturer. Three slides from each block were evaluated, counterstained with hematoxylin, and examined with brightfield microscopy. Four different areas of each section were evaluated.

Electrophoretic Mobility Shift Assay (EMSA).

Nuclear proteins were isolated from heart samples as previously described [221,35,219]. NF-κB binding activity was performed using a LightShift Chemiluminescent EMSA kit (Thermo Fisher Scientific, Waltham, MA) as described previously [62,222,35] in a 20 µl binding reaction mixture containing 1 x binding buffer, 50 ng poly dI:dC, 20 fmol of double stranded NF-κB consensus oligonucleotide which was end-labelled with Biotin, 15µg nuclear proteins. The binding reaction mixture was incubated at room temperature for 20 min and analyzed by electrophoresis, transferred to a nylon membrane. The biotin end-labeled DNA was detected using the Streptavidin-Horseradish peroxidase conjugate and the chemiluminescent substrate [62,222,35].

ELISA for Cytokine Assay.

The levels of cytokines (TNF-α, IL-6) in cell-free supernatants were measured by ELISA development kits (Peprotech) according to manufacturers' instructions. Briefly, ELISA plate was incubated overnight at room temperature (R.T.) with 100µl capturing antibody. After washing for four times, 300µl blocking buffer was added for 1 hour at R.T. After washing, serial 100 µl dilute standard or sample was added and incubated at room temperature for at least 2 hours. 100µl diluted detection antibody was immediately added after washing and incubate at R.T. for 2 hours. Plates were aspirated and washed plate 4 times. Diluted Avidin-HRP conjugate was added and incubated 30 min. Plates were aspirated and washed plate 4 times. Add 100µl of ABTS liquid substrate solution to each well. Monitor color development with an ELISA plate reader at 405 nm.

Wet/dry Lung Weight Ratio.

The water content of lungs of HSPA12B$^{-/-}$ and WT mice was measured by calculating the wet/dry ratios. After CLP surgery for 6 h, the lung of each mouse was removed and weighed before drying (wet weight) and then dried in an oven for 72 h at 50°C (dry weight). Wet/dry weight ratio was calculated by dividing the wet weight by the dry weight.

Western Blot.

Western blot (WB) was performed as described previously [221,35,219]. Briefly, the cellular proteins were separated by SDS-polyacrylamide gel electrophoresis and transferred onto Hybond ECL membranes (Amersham Pharmacia, Piscataway, NJ). The ECL membranes were incubated with primary antibodies and followed by incubation with peroxidase-conjugated secondary antibodies (Cell Signaling Technology, Inc.) and analysis by the ECL system (Amersham Pharmacia, Piscataway). The signals were quantified using the G: Box gel imaging system by Syngene (Syngene, USA, Fredrick, MD). For the source of antibodies, anti-HSPA12B is a kind gift from Dr. Han Zhihua. Zo-1 and occludin were bought from Invitrogen, iNOs from BD, anti-GAPDH from Meridian Life Science, Inc, anti-VCAM1 and anti-ICAM1 from Santa Cruz Biotechnology, TN, respectively,

Statistical Analysis

The data are expressed as mean ± SE. Comparisons of data between groups were made using one-way analysis of variance (ANOVA), and Tukey's procedure for multiple-range tests was performed. The log-rank test was used to compare group

survival trends. Probability levels of 0.05 or smaller were used to indicate statistical significance.

Results

HSPA12B Significantly Increases after Polymicrobial Sepsis

HSPA12B is specifically expressed in endothelial cells [112] and has been demonstrated to play a critical role in the regulation of angiogenesis and endothelial function. We observed that expression level of HSPA12B significantly increases after polymicrobial sepsis (Figure 4).

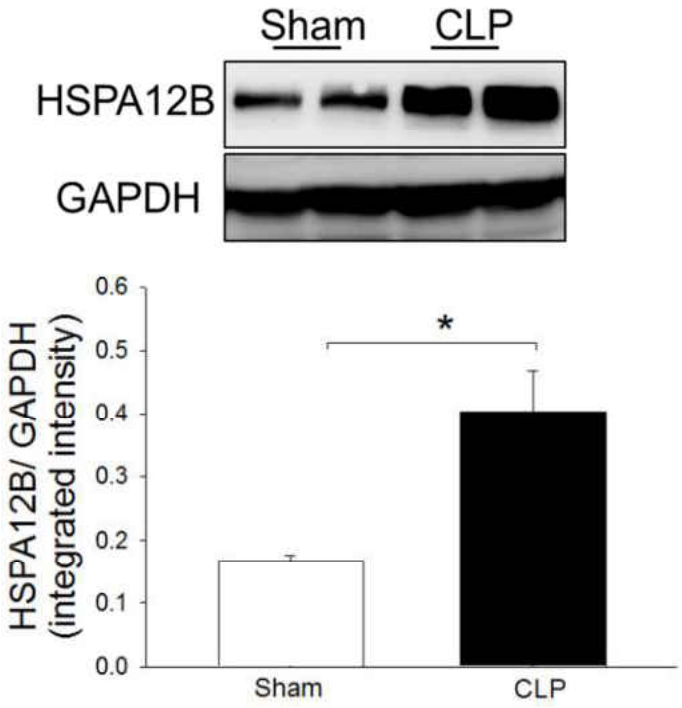

Figure 4 HSPA12B significantly increases after polymicrobial sepsis. WT mice were subjected polymicrobial sepsis induced by CLP. Sham surgical operation served as sham control. Heart tissue was harvested six hours after CLP and proteins were isolated. The level of HSPA12B was evaluated by WB (N=7-8/group).

Endothelial Specific Deficiency of HSPA12B Results in Severe Cardiac Dysfunction Following Polymicrobial Sepsis

The endothelium is a key organ involved in the pathogenesis of sepsis [75,76]. We examined whether HSPA12B will be an essential for cardiovascular function in polymicrobial sepsis. As shown in Figure 5a, there is no significant difference in the baseline values of ejection fraction (EF %) and fractional shortening (%FS) between WT and HSPA12B$^{-/-}$ mice. However, the values of EF% and %FS in WT septic mice were markedly reduced by 34.3% and 42.8% respectively, when compared with WT sham control. Interestingly, the EF% and %FS values in HSPA12B$^{-/-}$ septic mice were significantly decreased by 48.2% and 56.5%, respectively compared with HSPA12B$^{-/-}$ sham control which were further decreased by 20.5% and 22.8% as compared with WT septic mice. Echocardiographic images (Fig. 5b) show that movement of the chamber walls of HSPA12B$^{-/-}$ septic mice was worse than that in WT septic mice. These data suggests that endothelial HSPA12B is an essential for cardiac function following polymicrobial sepsis.

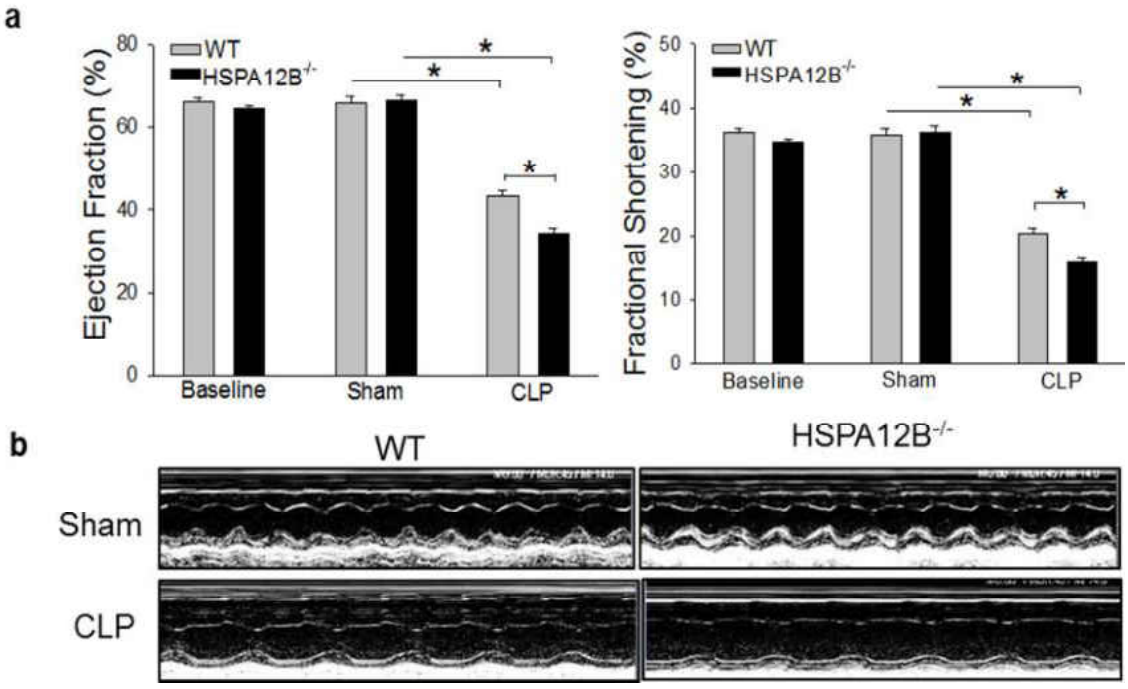

Figure 5 Endothelial cell specific HSPA12B deficiency potentiates sepsis-induced cardiac dysfunction. a. HSPA12B deficient and wild type (WT) mice were subjected to polymicrobial sepsis induced by CLP. Sham surgical operation served as sham control. The cardiac function was examined by echocardiography before and 6 hour after CLP (N=6-13/group). Two cardiac function parameters ejection fraction (EF%) and fractional shortening (%FS) was calculated. b. Typical echocardiographic images show that movement of the chamber walls of heart. * indicates $P<0.05$.

Endothelial Cell Specific HSPA12B Deficient Mice have Shorter Survival Time after Polymicrobial Sepsis

Next, we examined the effect of HSPA12B on survival time following polymicrobial sepsis. Figure 6 shows that the septic mice began to die at 18.9 h after CLP-induced sepsis. The mean survival time for WT septic mice was 53.6 h. In HSPA12B$^{-/-}$ septic mice, however, the mean survival time was 38.1 h. The data indicates that endothelial specific knockout HSPA12B accelerates the polymicrobial sepsis induced death.

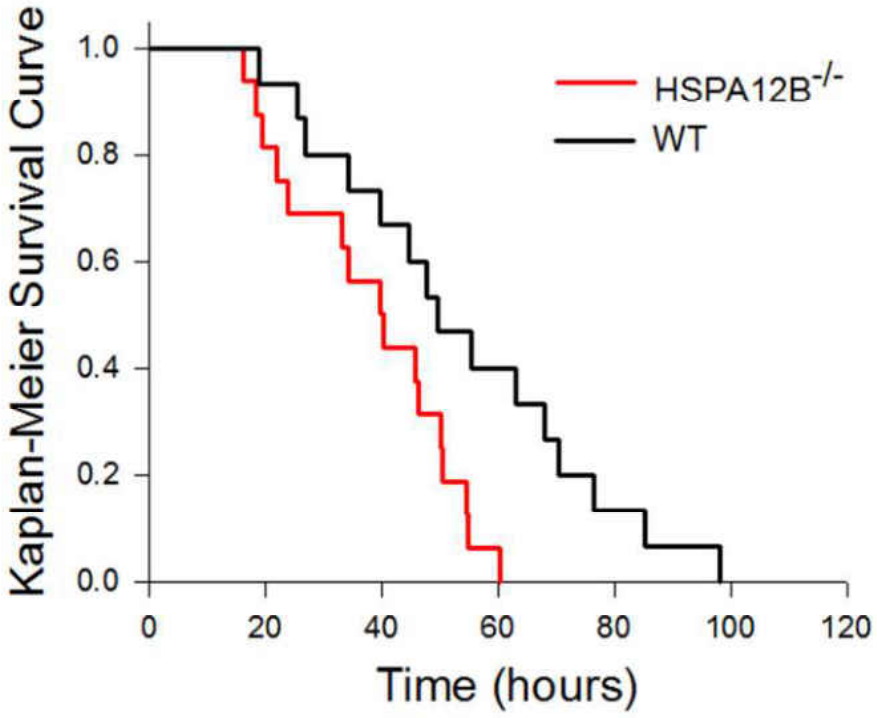

Figure 6 Endothelial cell specific HSPA12B deficient mice have shorter survival time after polymicrobial sepsis. HSPA12B deficient and wild type (WT) mice were subjected to polymicrobial sepsis induced by CLP. Survival rate was closely monitored up to 5 days (N=15-16/group).

Endothelial Specific Deficiency of HSPA12B Decreased Tight Junction Proteins and Increased Endothelial Permeability after Polymicrobial Sepsis.

Sepsis induces endothelial cell activation/injury and increases endothelial permeability [96]. We performed WB to detect the level of tight junction proteins and iNOs in heart. As shown in Fig. 7a, Sepsis causes the loss of tight junction proteins and an increase in iNOs. However, we observed that the levels of tight junction proteins in the myocardium were significantly reduced and iNOs markedly increased in HSPA12B$^{-/-}$ septic mice when compared to WT septic mice. We examined endothelial permeability using wet/dry lung ratio after sepsis. Sepsis significantly induced a severe lung edema

in HSPA12B$^{-/-}$ mice compared to WT mice (Fig. 7c). The ratio of wet/dry lung in WT septic mice was increased by 12% compared with sham control. In HSPA12B$^{-/-}$ septic mice, the ratio of wet/dry lung was increased by 22.6%, when compared with HSPA12B$^{-/-}$ sham control. There is no significant difference in the ratio of wet/dry lung between WT sham and HSPA12B$^{-/-}$ sham mice. The data suggests that HSPA12B plays an important role in endothelial permeability following polymicrobial sepsis and deficiency of endothelial HSPA12B markedly increased endothelial permeability, resulting in lung edema in sepsis.

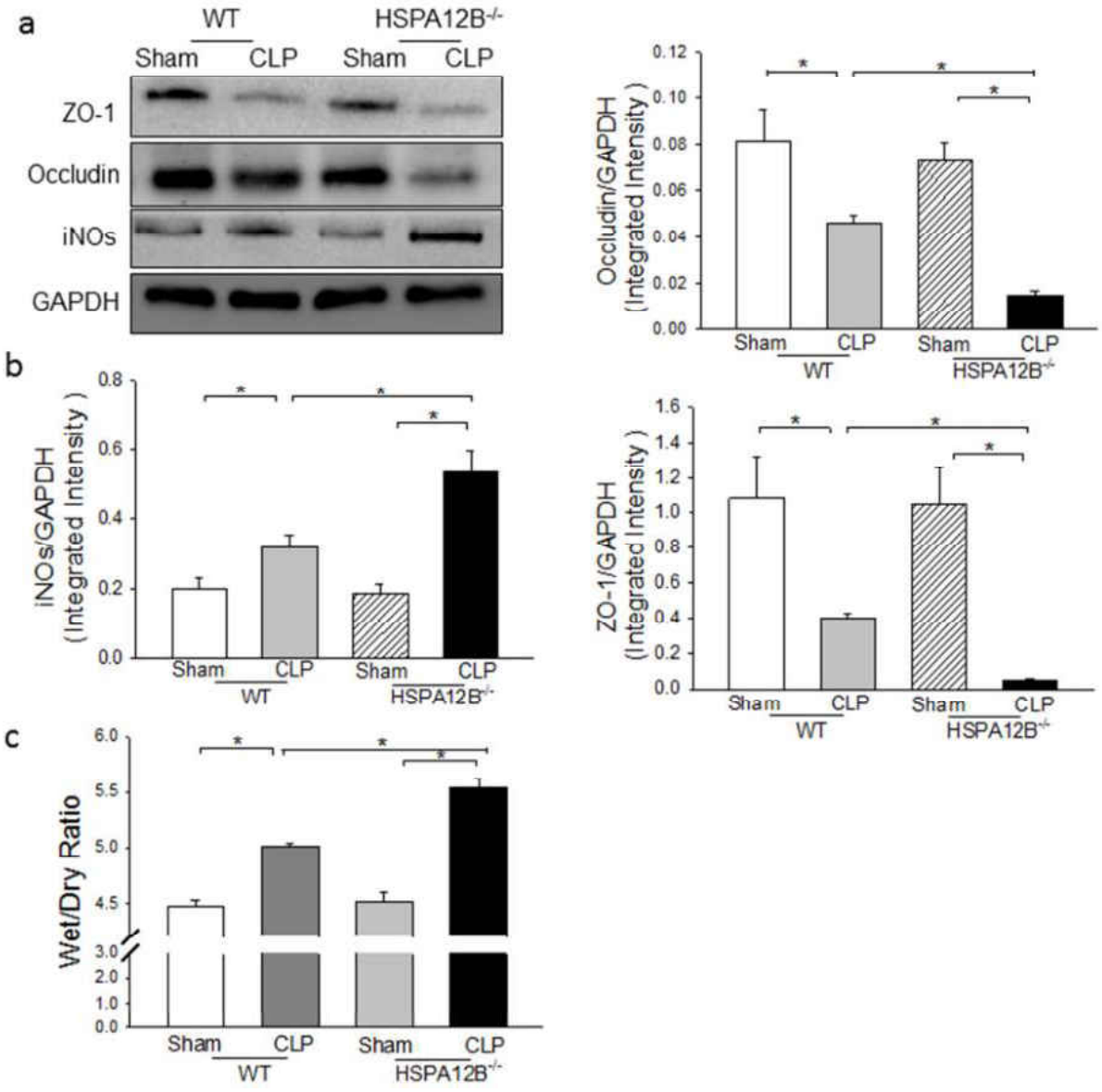

Figure 7 Endothelial cell specific HSPA12B deficiency exaggerated increased endothelial permeability and decreased tight junction proteins in the myocardium in response to polymicrobial sepsis. HSPA12B deficient and WT mice were subjected polymicrobial sepsis induced by CLP. Sham surgical operation served as sham control. The hearts and lungs were then harvested. The levels of tight junction proteins and iNOs in heart were examined with western blot (N=4-6/group). GAPDH served as loading control. The water content of lungs of HSPA12B$^{-/-}$ and WT mice was measured by calculating the wet/dry lung ratios (N=6/group). * indicates P<0.05

Endothelial HSPA12B Deficiency Enhanced the Expression of Adhesion Molecules after Polymicrobial Sepsis.

The infiltration of inflammatory cells into the myocardium is dependent on the expression of adhesion molecules on endothelial cells [86]. We examined the effect of HSPA12B on the expression of VCAM-1 and ICAM-1 in the myocardium following polymicrobial sepsis by WB. As shown in Figures 8a, the levels of VCAM-1 and ICAM-1 in the myocardium were significantly increased in WT septic mice compared with sham control. In HSPA12B$^{-/-}$ septic mice, myocardial VCAM-1 and ICAM-1 were enhanced by 1.91 fold and 1.73 fold, respectively, when compared with WT septic mice. Immunohistochemistry staining shows that CLP sepsis increased the immunostaining of VCAM-1 and ICAM-1 in the myocardium compared with sham controls (Fig. 8b and c). There is more positive staining of VCAM-1 and ICAM-1 in the myocardium from HSPA12B$^{-/-}$ septic mice than that in WT septic mice. The data suggests that HSPA12B plays an important role in the regulation of adhesion molecule expression on endothelial cells which will facilitate the infiltration of inflammatory cells into the myocardium following polymicrobial sepsis.

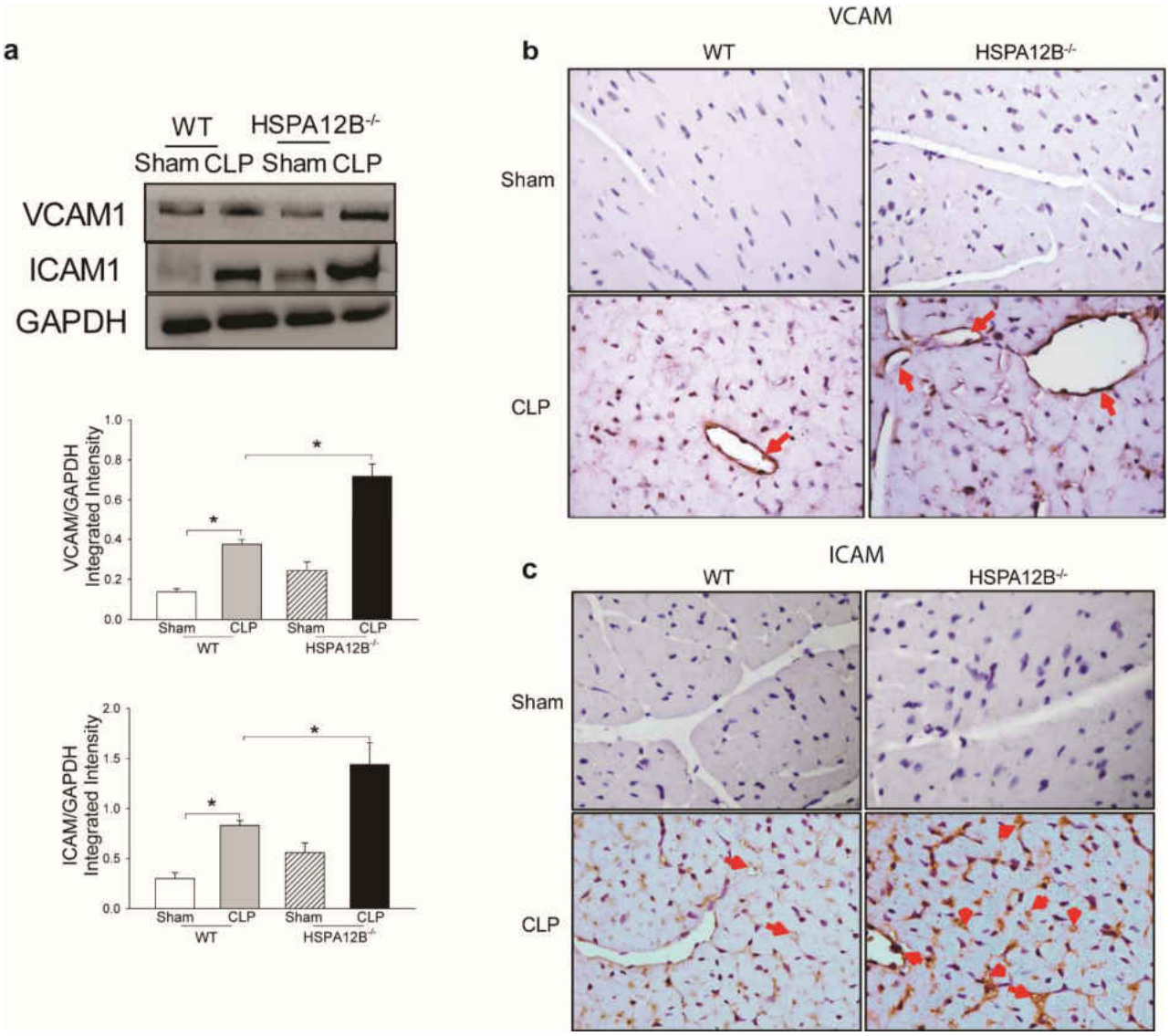

Figure 8 Endothelial cell deficiency of HSPA12B increases expression of adhesion molecules after sepsis. Sham surgical operation served as sham control. Heart was harvested 6 hour after CLP. The expression level of adhesion molecules VCAM (N=7-8/group) and ICAM (N=5-7/group) were examined with WB (Fig. 8a). The levels of GAPDH served as loading control in WB. * indicates $P<0.05$. Immunohistochemistry was also performed to evaluate the level of VCAM and ICAM in heart tissue as shown in Figure 8b&c. The dark brown color (red arrow) indicates VCAM-1 and ICAM-1.

HSPA12B Deficiency Exacerbates Inflammatory Cell Infiltration in the Myocardium following Polymicrobial Sepsis.

The infiltration of inflammatory cells such as macrophages and neutrophils into the myocardium contributes to septic cardiomyopathy [35]. We examined the effect of HSPA12B on the infiltration of macrophages and neutrophils in the myocardial tissues following polymicrobial sepsis. As shown in Figure 9a, CLP sepsis significantly increased the accumulation of neutrophils in WT septic mice compared with WT sham (9.3±0.66 vs 1.8±0.22). In HSPA12B$^{-/-}$ septic mice, the neutrophil accumulation in the myocardium markedly increased by 170% compared with WT septic mice. The data from myeloperoxidase activity (MPO) measurement shows that CLP sepsis increased myocardial MPO activity by 89.9% in WT septic mice and by 169.6% in HSPA12B$^{-/-}$ septic mice respectively, when compared with the respective controls (Fig. 9b). CLP sepsis also markedly increased the infiltration of macrophages into the myocardium (Fig. 9c). The numbers of macrophages in the myocardium were increased in WT septic mice compared with WT sham (15.6±1.01 vs 2.6±0.0.36). In HSPA12B$^{-/-}$ septic mice, the macrophage accumulation in the myocardium significantly increased by 57.9% compared with WT septic mice. The data indicates that endothelial HSPA12B plays a role in the infiltration of inflammatory cells into the myocardium following polymicrobial sepsis possibly through regulation of the expression of adhesion molecules.

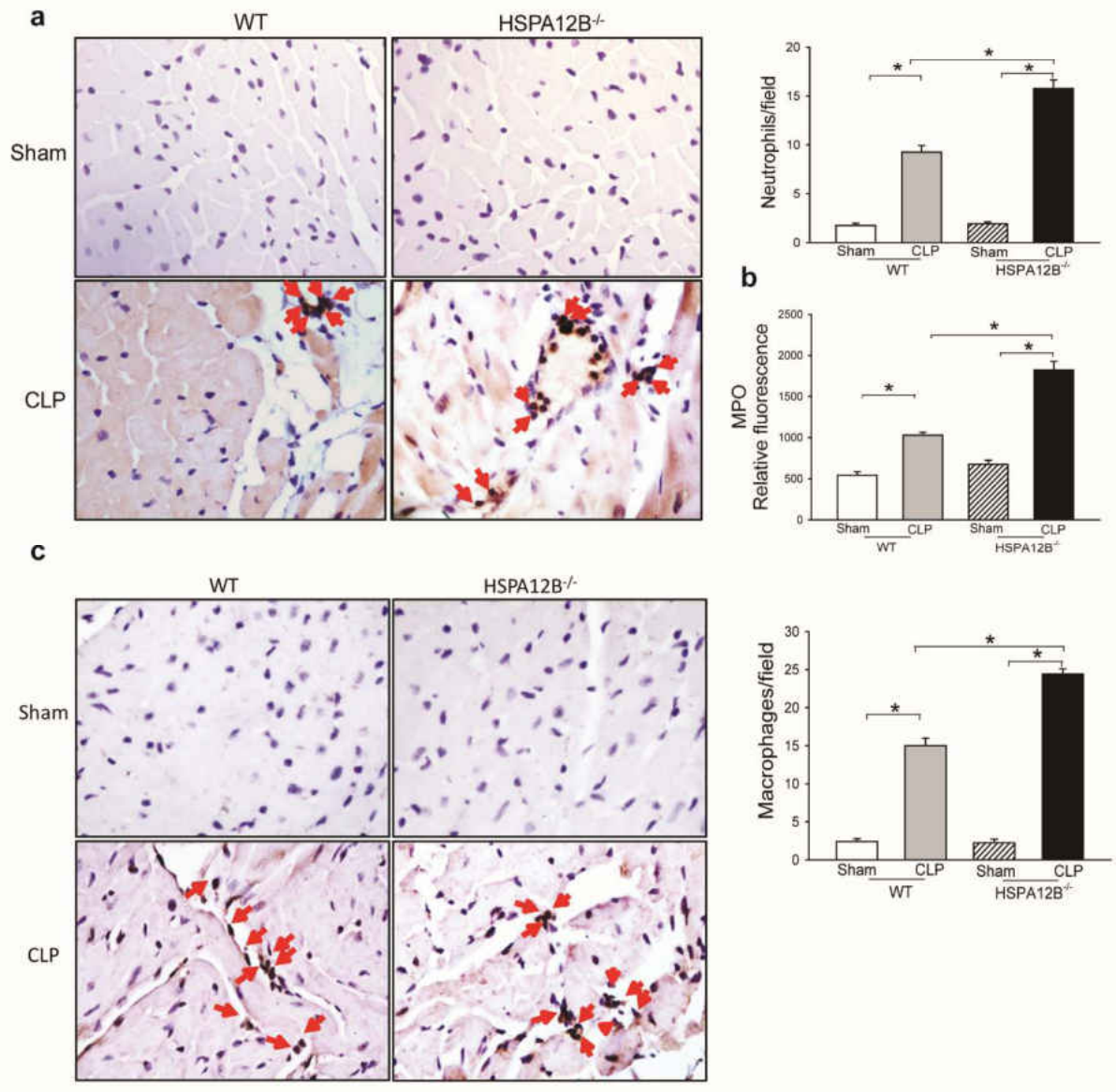

Figure 9. Endothelial cell deficiency of HSPA12B exacerbates inflammatory cell infiltration in the myocardium following polymicrobial sepsis. HSPA12B deficient and WT mice were subjected polymicrobial sepsis induced by CLP. Sham surgical operation served as sham control. Heart tissue was harvested six hours after CLP. Inflammatory cells accumulation in heart tissues was examined with neutrophil specific antibody (a) and macrophage specific antibody F4/80 (c), separately. Three samples from each group were evaluated. Four different areas of each section were evaluated. Red arrows point at the infiltrated inflammatory cells. The results are expressed as the numbers of neutrophils or macrophages per field (400x). Expression of myeloperoxidase (MPO) by neutrophils is necessary for their activation by neutrophil, so MPO activity was measured by kit (b, N=6-8/group). * indicates $P<0.05$.

HSPA12B Deficiency Increased Myocardial NF-κB Binding Activity and Promoted the Production of Inflammatory Cytokines in Serum.

It is well known that pro-inflammatory cytokines contribute to cardiovascular dysfunction during sepsis/septic shock [38]. NF-κB is an important transcription factor that regulates inflammatory cytokine production [57]. HSPA12B deficiency promoted the infiltration of inflammatory cells into the myocardium (Fig. 9). Therefore, we examined myocardial NF-κB binding activity and serum levels of pro-inflammatory cytokines in CLP septic mice. Figure 10a shows that CLP sepsis markedly increased myocardial NF-κB binding activity by 36.8% compared with WT sham control. However, the NF-κB binding activity in HSPA12B$^{-/-}$ septic mice was significantly increased by 82.3%, as compared with HSPA12B$^{-/-}$ sham mice. Figure 10b shows that CLP-sepsis markedly increased the levels of TNF-α and IL-6 in WT septic mice, when compared with WT sham control. However, the serum levels of TNF-α and IL-6 in HSPA12B$^{-/-}$ septic mice were further increased by 243% and 223% respectively, as compared with WT septic mice. The data suggests that endothelial HSPA12B deficiency resulted in activation of pro-inflammatory signaling and pro-inflammatory cytokine production following polymicrobial sepsis.

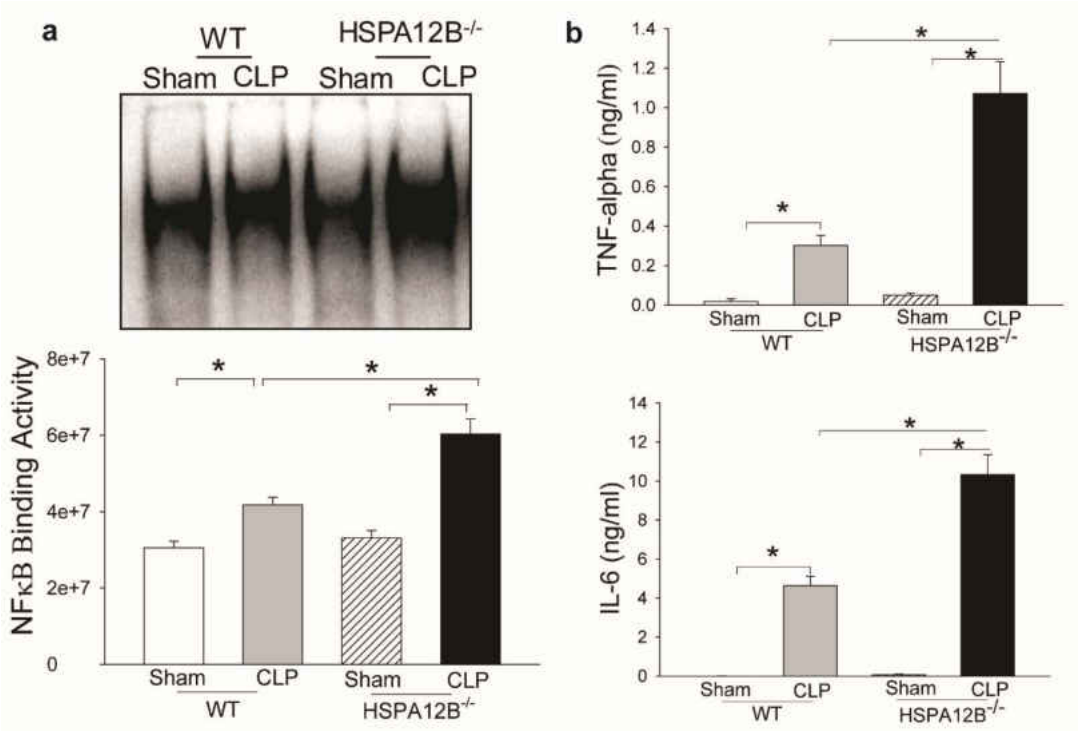

Figure 10 Endothelial cell deficiency of HSPA12B increased myocardial NF-κB binding activity and serum levels of pro-inflammatory cytokine production. HSPA12B deficient and WT mice were subjected to polymicrobial sepsis induced by CLP. Sham surgical operation served as sham control. Heart and serum were harvested six hours after CLP. Nucleoproteins of heart were isolated and NF-κB activity was measured by EMSA (N=8-10/group). The levels of TNF-α (N=5-11/group) and IL-6 (N=8/group) in serum were assessed by ELISA kits. * indicates $P<0.05$.

Discussion

HSPA12B was discovered by Han *et al* in 2003 [109] and characterized as a new family of Hsp70 proteins. HSPA12B has been shown to predominantly expressed in vascular endothelium [111]. Han *et al* has also demonstrated that endothelial HSPA12B is involved in angiogenesis through turnover of a known angiogenesis regulator a Kinase

anchoring protein 12 (AKAP12), resulting in upregulation of vascular endothelial growth factor (VEGF) expression [111]. Hu *et al* have shown that zebrafish orthologue of mammalian HSPA12B plays an important role in endothelial cell function [112]. These authors have also shown that knockout HSPA12B in human umbilical vein endothelial cells (HUVECs) prevent wound healing; while overexpression of HSPA12B promote the process [112]. The mechanism is indicated to involve Akt phosphorylation. These data indicates that HSPA12B is required for endothelial cell proliferation and migration in wound healing.

The role of endothelial HSPA12B in cardiovascular function during polymicrobial sepsis has not been investigated previously. It is well known that endothelial dysfunction plays a major role in the pathophysiology of septic shock and multiple organ dysfunctions [94]. We have previously reported that polymicrobial sepsis [35] or endotoxemia [101] induced increased expression of adhesion molecules in the myocardium,with a concomitant accumulation of neutrophils and macrophages, which contribute to sepsis-induced cardiac dysfunction [102]. Therefore, preservation of endothelial function is important for prevention and protection against sepsis induced multiple organ failure. We have observed that endothelial cell specific deficiency of HSPA12B resulted in more severe cardiac dysfunction in polymicrobial sepsis. Our observation is consistent with our previous report showing that endothelial specific overexpression of HSPA12B in transgenic mice exhibited against endotoxin induced cardiac dysfunction. Our observations suggest that endothelial HSPA12B serves a protective role in cardiovascular function in sepsis.

It is well known that the endothelium is an essential membrane barrier that regulates the hemostasis of macromolecules and separates the cellular elements of the blood providing from tissue compartment. In the septic condition, however, the endothelial barrier function is impaired which may be a central pathophysiological process in septic shock [96,93]. We have observed that the ratio of wet/dry lung in HSPA12B$^{-/-}$ septic mice was significantly higher than that in WT septic mice. The levels of tight junction proteins (ZO-1 and Occludin) were markedly lower compared with WT septic mice. The data indicates that endothelial barrier function in HSPA12B$^{-/-}$ septic mice was worsen compared with WT septic mice. The mechanisms of endothelial barrier dysfunction include the activation of innate immune and inflammatory responses and the interaction between monocyte/macrophage and endothelial cells [86,87]. Indeed, we observed that the infiltration of macrophages and neutrophils into the myocardium were significantly enhanced in the myocardium of HSPA12B$^{-/-}$ septic mice (Fig. 9). Macrophages and neutrophils have been observed to rolling, polarizing and recruiting to ed reported that recruitment of circulating activates inducible nitric oxide synthase with rapid efflux of nitric oxide, leading to vasodilatation, opening of endothelial gaps and loss of endothelial barrier function [97,86]. Our data shows that the levels of myocardial iNOS in HSPA12B$^{-/-}$ septic mice were significant greater than in WT septic mice (Fig. 7a).

Since increased expression of adhesion molecule expression on endothelial cells contributes to cardiovascular dysfunction in sepsis [75], we examined the effect of HSPA12B on sepsis-induced expression of VCAM-1 and ICAM-1 in the myocardium following polymicrobial sepsis. We observed that endothelial specific deficiency of

HSPA12B markedly enhanced expression of myocardial VCAM-1 and ICAM-1 relative WT septic mice. The data is consistent with our previous observation showing that endothelial overexpression of HSPA12B markedly suppresses endotoxin-increased adhesion molecule expression in the myocardium [101]. Collectively, our data suggest that endothelial HSPA12B plays a critical role in the regulation of adhesion molecule expression in polymicrobial sepsis. To elucidate the mechanisms by which endothelial HSPA12B control adhesion molecule expression in sepsis, we performed the following experiments described in chapters 4 and 5.

It is well known that increased expression of adhesion molecules, such as ICAM-1 and VCAM-1 plays a critical role in recruitment of macrophages and neutrophils into the myocardium which contributes to cardiac dysfunction in sepsis [103,40,42]. Activated macrophages release chemokines that attract neutrophils into the myocardium [103]. Thus, the inflammatory cytokines released by infiltrated macrophages and neutrophils are thought to be important suppressors of cardiac function [42]. We observed that endothelial specific deficiency of HSPA12B results in increased accumulation of macrophages and neutrophils in the myocardium in polymicrobial sepsis, when compared with WT septic mice. Increased infiltration of inflammatory cells into the myocardium is associated with enhanced inflammatory response in HSPA12B$^{-/-}$ septic mice. Myocardial NF-κB binding activity and serum inflammatory cytokine levels, i.e. TNF-α and IL-6, in HSPA12B$^{-/-}$ septic mice were markedly greater than that in WT septic mice. Our findings suggest that endothelial HSPA12B regulates inflammatory responses through controlling adhesion molecule expression and accumulation of inflammatory cells in the myocardium in polymicrobial sepsis. In the following experiments, we will

investigate the mechanisms of endothelial HSPA12B regulation of inflammatory response in sepsis.

Collectively, our observations demonstrated that endothelial HSPA12B is essential for the regulation of endothelial barrier function in polymicrobial sepsis via controlling the expression of adhesion molecules and innate immune and inflammatory responses.

CHAPTER 4

INVESTIGATE THE MECHANSIMS BY WHICH ENDOTHELIAL SPECIFIC HSPA12B$^{-/-}$ CAUSES ENDOTHELIAL DYSFUNCTIN IN POLYMICROBIAL SEPISIS

Introduction

The data generated from Chapter 3 suggests that endothelial specific expression of HSPA12B serves a protective role in preservation of cardiovascular function in polymicrobial sepsis. Specifically, we observed that endothelial HSPA12B deficiency results in severe cardiovascular dysfunction in polymicrobial sepsis. We also observed that endothelial HSPA12B deficiency significantly enhances inflammatory responses by activation of NF-κB mediated signaling. It is well known that endothelial cells are a major target of sepsis-induced cardiovascular dysfunction [75,76] and multiple organ failure. To better understand the role of HSPA12B in endothelial function during polymicrobial sepsis, we performed the following experiments focusing on the role of exosomes in mediating endothelial cell function and inflammatory response.

Exosomes have been demonstrated to play a critical role in mediating cell-cell and organ-organ communication [179,173,223]. Exosomes release their contents into the target cells via membrane fusion with the target cells or via binding with specific receptors on target cells or endocytotic internalization. The functions of exosomes produced *in vitro* and *in vivo* have been investigated in sepsis area. Recent evidence suggests that exosomes may play an important role in sepsis [194,195,192]. Azevedo et al reported that exosomes from septic shock patients induce myocardial dysfunction in isolated rabbit hearts [194]. The main source of septic exosomes is the platelet. Exosomes from septic

patients increase reactive oxygen species production and cause apoptosis in endothelial cells [194]. Gambim et al have shown that platelet derived exosomes induce endothelial cell apoptosis and endothelial dysfunction [194,195]. Immature dendritic cell-derived exosomes dampen inflammatory response and reduce mortality in septic animals.

Exosomes are novel vehicle for delivering mRNAs and microRNAs to exchange genetic information between cells [185]. MiRNAs transferred by exosomes are functional [191,175] and induce target gene repression in recipient cells [175,192]. MiRNAs in exosomes have been shown to modulate immune responses [193,192]. MiR-155 and miR-146a have been shown to present in exosomes released by dendritic cell (DC). Those miRNAs can be transferred into recipient DC in vitro and in vivo and suppress target gene expression [192]. Herein we will investigate whether serum exosome could deliver information to endothelial cells in vitro.

The profile of exosomal miRNAs in mouse serum have been shown to be altered after polymicrobial sepsis induced by cecum ligation and puncture. Some miRNAs significantly increased in exosomes after CLP for eight hours, including miR-16, miR-17, miR-20a/b and miR-26a/b. Exosomal miR-155 released by DC enhances while miR-146a suppresses inflammatory response in endotoxin-treated mice [192].

In addition, exosomes contain microRNAs which have been shown to play an important role in regulation of cardiovascular function and inflammatory processes during sepsis. Exosomal miR-155 released by DC enhances while miR-146a suppresses inflammatory response in endotoxin-treated mice [192]. Endothelial cells have been reported to secrete exosomes and also can be targeted by exosomes [174].

Endothelial cells communicate with other cells through exosomes [196]. It have been reported miRNA incorporated into exosome can be transferred into endothelial cells and function in the recipient cells [197]. However, the relationship of endothelial cell function and exosomes in sepsis is still poorly understood. Here we will determine the miRNA composition in serum exosomes after sepsis in HSPA12B$^{-/-}$. We hypothesize that that circulating exosomes from septic mice (septic exosomes) are involved in endothelial dysfunction and inflammatory responses in polymicrobial sepsis.

In this study, we observed that the level of serum exosome miRNAs changed in HSPA12B deficient mice compared with wild type after sepsis. We demonstrated that exosomes isolated from serum of septic mice significantly increased the expression of adhesion molecules and decreased the levels of tight junction proteins of endothelial cells. Importantly, exosomes isolated from HSPA12B$^{-/-}$ septic mice resulted in more severe endothelial dysfunction. The data suggests that exosomes plays a critical role in mediating endothelial dysfunction during polymicrobial sepsis.

Materials and Methods

Isolation of Exosomes.

Ten hours after CLP, blood was collected from the experimental mice followed by centrifugation at 1,500 g for 15 min at 18˚C. The supernatant was collected and added to ExoQuickTM exosome precipitation solution (63 µl/ 250µl plasma, ExoQ5A-1, SBI) according to manufacturer's instruction. The mixture was incubated at room temperature for 30 min followed by centrifugation at 1,500g for 30min. The supernatants were removed and the pellets were exosomes.

Isolation of MiRNAs from Exosomes.

Total RNA was extracted from the exosome pellets using Trizol (RN190, Molecular Research Center, Cincinnati, USA) according to manufacturer's instructions. Approximately 10 ng of isolated total RNA was applied to examination of microRNA levels as described previously [153].

qPCR Assay of MiRNAs.

MiRNAs were isolated from heart tissues or exosomes using the mirVanaTM miR isolation kit (Ambion) as described previously[224]. MiRNAs levels were quantified by qPCR using specific Taqman assays (Applied Biosystems, USA) and specific primers (Applied Biosystems, Primer identification numbers: 002228 for hsa-miR-126-3p and 001973 for snRU6). The levels of miRNAs were quantified with the 2(-ΔΔct) relative quantification method that was normalized to the U6 small nucleolar RNA (snRU6).

Delivering Septic Exosomes into Endothelial Cells *in vitro*.

Mice were subjected to CLP and then serum was collected at 10 hours after surgery. Blood was collected and exosomes in serum was isolated with ExoQuickTCTM exosome precipitation solution (ExoQ5A-1, SBI). Human umbilical vein endothelial cells (HUVECs) were cultured and treated with exosomes (5μg/ml) diluted in conditional medium. The conditional medium was exosome free medium, i.e. centrifuged at 120,000 rpm, 4˚C for 18 hours. Cells were collected and cellular proteins were isolated. The level of adhesion molecules (ICAM and VCAM) were analyzed by WB.

Western Blot.

Briefly, the cellular proteins were isolated by using RIPA buffer and separated by SDS-polyacrylamide gel electrophoresis and transferred onto Hybond ECL membranes (Amersham Pharmacia, Piscataway, NJ). The ECL membranes were incubated with primary antibody followed by incubation with peroxidase-conjugated secondary antibodies (Cell Signaling Technology, Inc.) and analysis by the ECL system (Amersham Pharmacia, Piscataway). The signals were quantified using the G: Box gel imaging system by Syngene (Syngene, USA, Fredrick, MD). GAPDH (Meridian Life Science, Inc, TN) was detected as loading control. For the source of antibodies, CD63 and CD 81 were bought from SBI, zo-1 and occludin from Invitrogen, VCAM1 and ICAM1 from Santa Cruz.

Immunofluorescence Staining.

Briefly, the heart sections were stained with specific first antibodies rabbit anti-tight junction protein ZO-1 (1:100) for overnight at 4 ˚C. Then secondary antibody Alexa Fluor-555 goat-anti-rabbit IgG (H+L) (red) (Thermo Fisher Scientific) were added to the section for 1 hour at room temperature. Then the slides were examined with fluorescent microscope at a magnification of 400×.

Statistical Analysis

The data are expressed as mean ± SE. Comparisons of data between groups were made using one-way analysis of variance (ANOVA), and Tukey's procedure for multiple-range tests was performed. The log-rank test was used to compare group

survival trends. Probability levels of 0.05 or smaller were used to indicate statistical significance.

Results

Exosomes Isolated from HSPA12B$^{-/-}$ Septic Mice Decreased the Levels of Tight Junction Proteins in Endothelial Cells

Exosomes have been demonstrated to play an important role in the communication between cells and organs [179]. Exosomes can also carry microRNAs which regulate gene expression at the post transcription levels [185,191]. We observed that the levels of tight junction proteins in the myocardium were significantly reduced and the ratio of wet/dry of lung markedly increased in HSPA12B$^{-/-}$ septic mice than in WT septic mice (Fig. 7). We examined the effect of exosomes isolated from septic mouse serum on tight junction proteins in endothelial cells. Serum exosomes were isolated from serum of WT sham, septic mice and HSPA12B$^{-/-}$ sham and septic mice, respectively. Figure 11a shows that CD63 and CD81 which are specific markers for exosomes are present in isolated exosomes. Endothelial cells were treated with the exosomes isolated from sham and septic mice. Endotoxin was used as positive control. As shown in Figure 11b, LPS or WT septic exosomes markedly decreased the levels of ZO-1 and Occludin in the endothelial cells compared with sham exosomes. However, treatment of HUVECs with HSPA12B$^{-/-}$ septic exosomes resulted in more decreases in the levels of ZO-1 and Occludin, when compared with WT septic exosomes treated group. Fluorescent images show that WT exosomes treatment caused breaks of tight junction of endothelial cells

(Fig. 11c). However, treatment of endothelial cells with HSPA12B$^{-/-}$ septic exosomes resulted in lost and breakdown of tight junction protein on the endothelial cells.

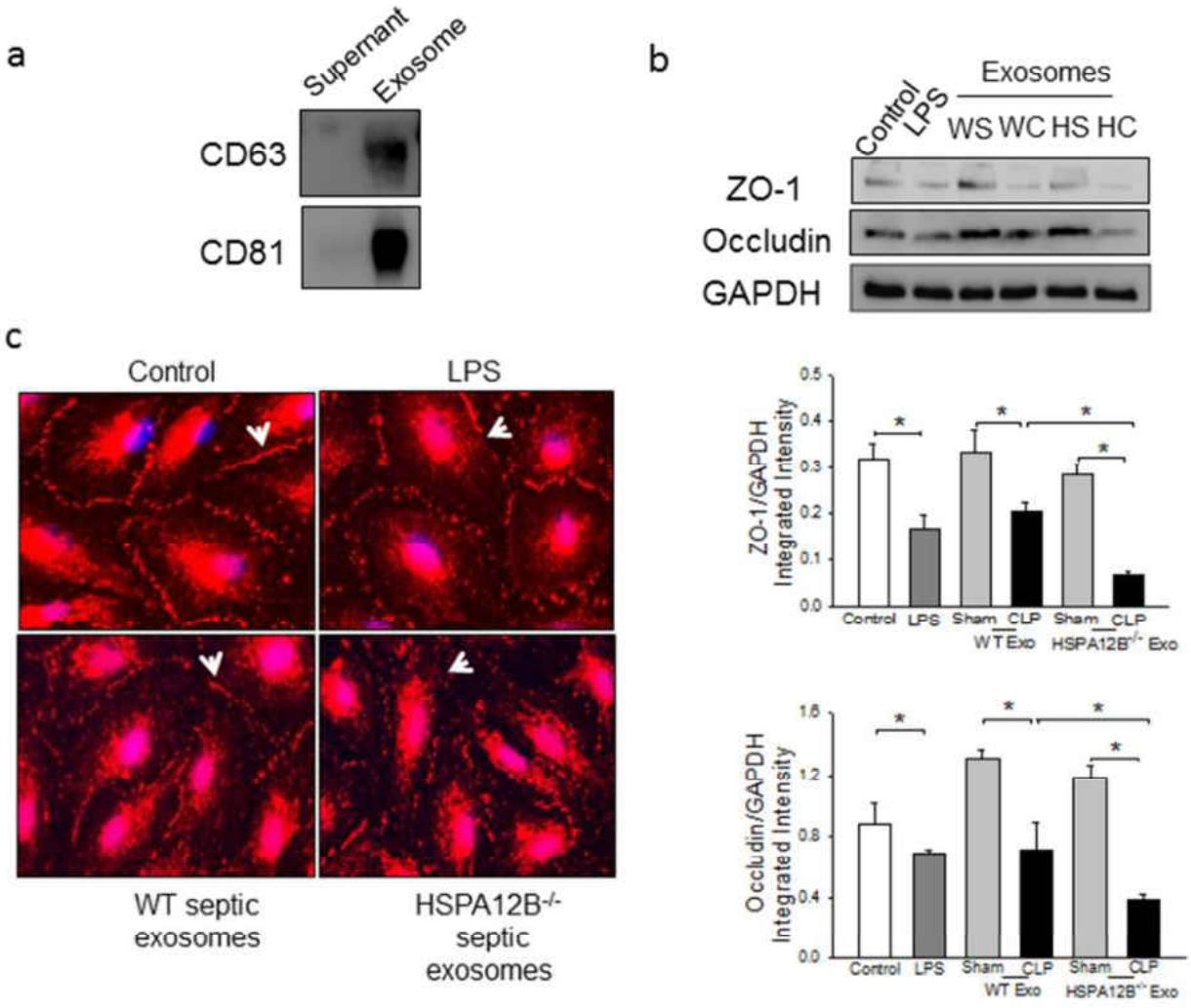

Figure 11 Exosomes isolated from septic HSPA12B$^{-/-}$ mice decreased the levels of tight junction proteins in endothelial cells. HSPA12B deficient and WT mice were subjected CLP-induced sepsis. Sham surgical operation served as sham control. Ten hours after CLP, blood was collected and serum exosomes were isolated by ExoQuickTM exosome precipitation solution. (a) Exosome markers CD63 and CD81 were detected in supernatant and exosomes by WB. (b) Endothelial cells (HUVEC) were treated with exosomes diluted in conditional medium (5 mg/ml) for 12 hours. The cells were harvested and the level of tight junction proteins ZO-1 and Occludin in cytoplasm were analyzed by WB (N=5-12/group). GAPDH served as loading control. WS = WT sham; WC = WT CLP; HS= HSPA12B$^{-/-}$ sham; HC= HSPA12B$^{-/-}$ CLP. * indicates P<0.05. (c) The expression level and location of tight junction protein ZO-1 was detected by immunofluorescence. N=3/group.

Exosomes from HSPA12B$^{-/-}$ Septic Mice Enhances the Expression of Adhesion Molecules on Endothelial Cells

We observed that endothelial specific deficiency of HSPA12B results in significantly increased expression of VCAM-1 and ICAM-1 in the myocardium following polymicrobial sepsis. We examined the effect of exosomes isolated from WT and HSPA12B$^{-/-}$ mice on the expression of VCAM-1 and ICAM-1 in endothelial cells. HUVECs were treated with exosomes isolated from sham and septic mice respectively. LPS treatment served as positive control. Figure 12 shows that exosomes isolated from WT septic mice markedly increased the levels of VCAM-1 by 87.2% and ICAM-1 by 157.3%, respectively, compared with sham exosome treated group. However, treatment of HUVECs with HSPA12B$^{-/-}$ septic exosomes resulted in more expression of VCAM-1 and ICAM-1 than in WT septic exosome treatment. The levels of VCAM-1 and ICAM-1 in HSPA12B$^{-/-}$ septic exosomes group were greater by 75.1% and 78.9% respectively, compared with the group treated with WT septic exosomes. The data indicates that septic exosomes may play a critical role in up regulation of adhesion molecule expression in the endothelial cells and increased expression of adhesion molecules could be responsible for increased infiltration of inflammatory cells into the myocardium in polymicrobial sepsis.

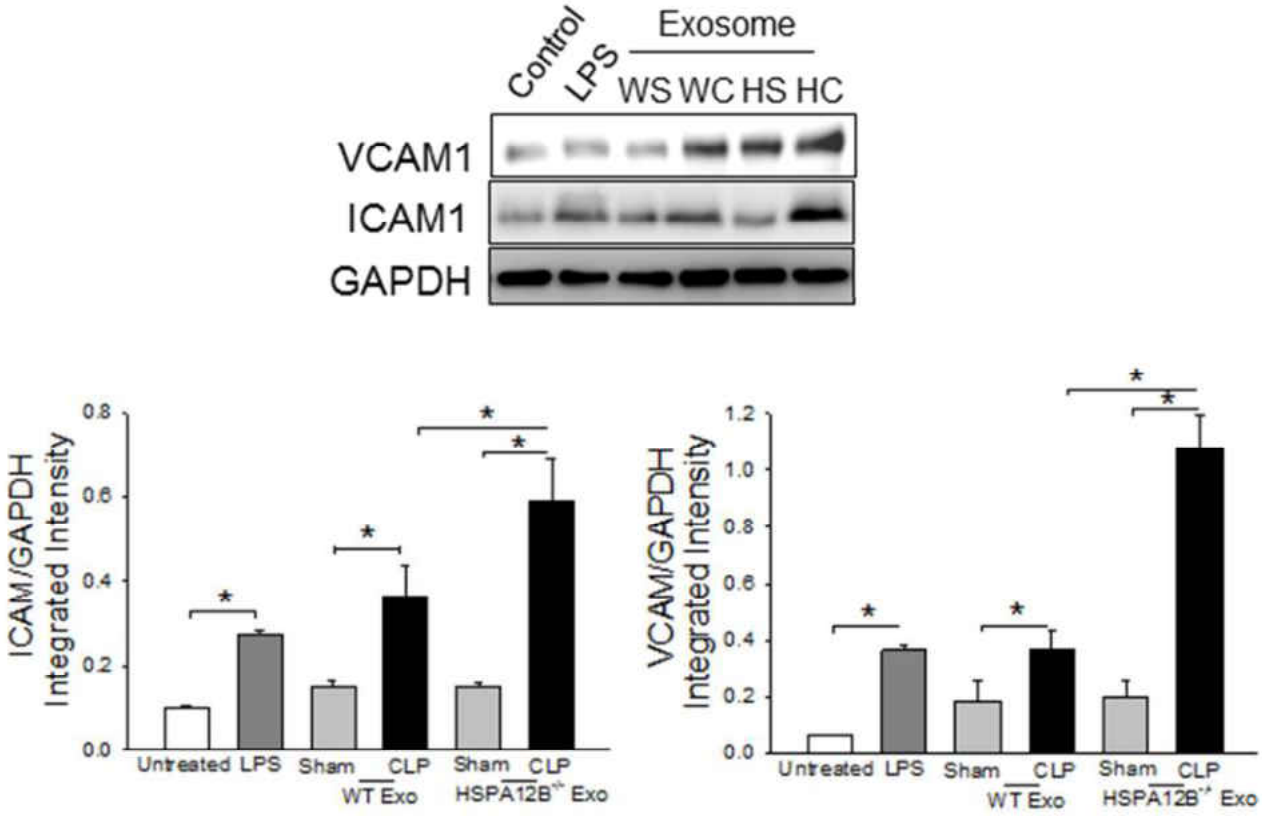

Figure 12 Exosomes isolated from HSPA12B$^{-/-}$ septic mice decreased the levels of tight junction proteins in endothelial cells. HSPA12B deficient and WT mice were subjected CLP-induced sepsis. Sham surgical operation served as sham control. Ten hours after CLP, blood was collected and serum exosomes were isolated by ExoQuickTM exosome precipitation solution. Endothelial cells (HUVEC) were treated with exosomes diluted in conditional medium (5 mg/ml) for 12 hours. The cells were harvested and the level of adhesion molecules VCAM and ICAM in cytoplasm were analyzed by WB (N=8-10/group). GAPDH served as loading control. WS = WT sham; WC = WT CLP; HS= HSPA12B$^{-/-}$ sham; HC= HSPA12B$^{-/-}$ CLP. * indicates $P<0.05$.

Decreased Levels of MiRNA-126 in Endothelial Cells Treated with HSPA12B$^{-/-}$ Exosomes from Septic Mice.

Recent studies have demonstrated that microRNAs play critical roles in regulation of endothelial cell function [157,159]. MiR-126 has been reported to suppress endothelial cell adhesion molecule expression [159]. Therefore, we examined the levels of miR-126 in endothelial cells treated with septic exosomes. HUVECs were treated with WT and

HSPA12B$^{-/-}$ exosomes, respectively. MiR-126 levels were measured by qPCR (Fig. 13). The data shows that treatment of HUVECs with exosomes from WT and HSPA12B$^{-/-}$ sham mice slightly reduced the levels of miR-126 compared with untreated control. WT septic exosome treatment decreased the expression of miR-126 but the decrement was not statistical different, when compared with the WT sham exosome group. However, the levels of miR-126 in HSPA12B$^{-/-}$ treated cells were significantly reduced, when compared with the groups treated with HSPA12B$^{-/-}$ sham or WT septic exosomes, respectively.

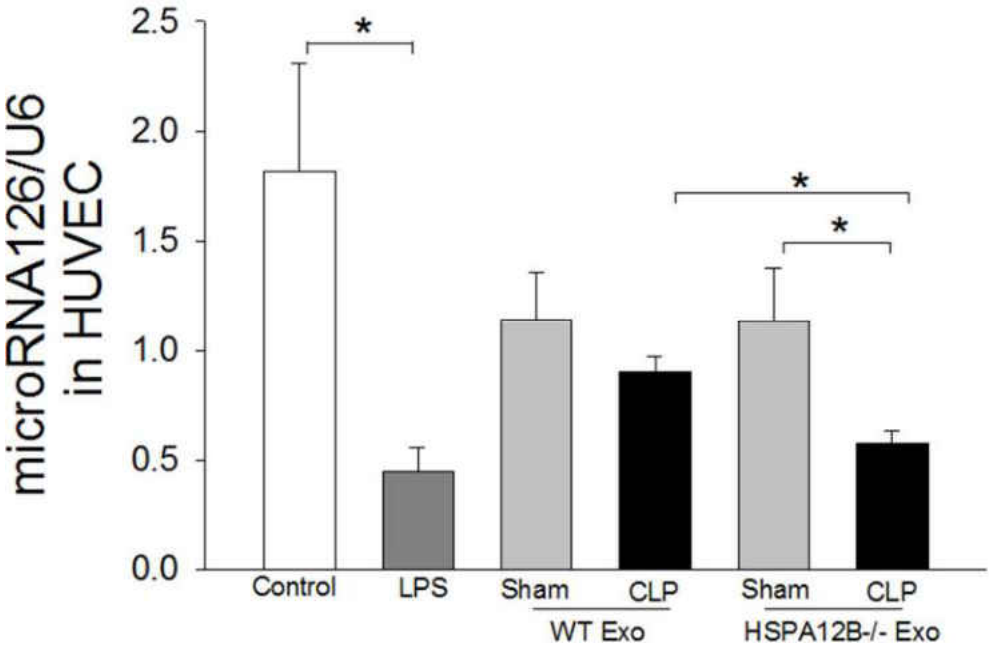

Figure. 13 Decreased levels of miR-126 in endothelial cells treated with HSPA12B$^{-/-}$ exosomes from septic mice. HSPA12B deficient and WT mice were subjected CLP induced polymicrobial sepsis. Sham surgical operation served as sham control. Ten hours after CLP, blood was collected for isolation of serum exosomes. Endothelial cells (HUVEC) were treated with exosomes for 12 hours. The cells were harvested. Total miRNAs was extracted from the endothelial cells. MiR-126a level in endothelial cell were measured by RT-PCR after the exosomes treatment (N=6/group). * indicates $P<0.05$.

Decreased Expression of MiR-126 in Serum Exosomes from HSPA12B$^{-/-}$ Septic Mice

We also examined the levels of exosomes isolated from WT and HSPA12B$^{-/-}$ mice. The data shows that there was no significant difference in the levels of miR-126 between WT sham and HSPA12B$^{-/-}$ sham (Fig.14). The miR-126 levels were markedly increased in WT septic exosomes, when compared with WT sham exosomes. However, in HSPA12B$^{-/-}$ septic exosomes, the miR-126 levels were comparable with HSPA12B-/- sham exosomes but significantly lower than in WT septic exosomes. The data suggests that lower levels of miR-126 in HSPA12B$^{-/-}$ septic exosomes and their treated endothelial cells may be responsible for increased expression of adhesion molecules in HSPA12B$^{-/-}$ septic exosome treated cells.

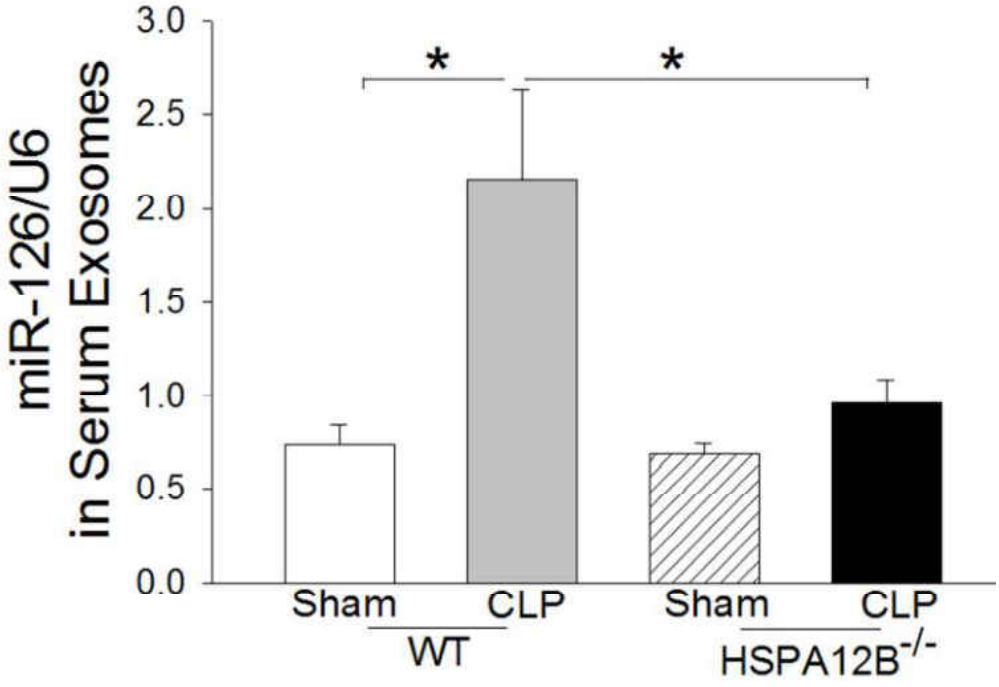

Figure 14 The expression of miR-126 decreased in serum exosomes from HSPA12B$^{-/-}$ septic mice. Ten hours after CLP, the blood was collected and serum exosomes were isolated by exosome precipitation solution. Total RNA was extracted from the exosome pellets using Trizol. MiR-126 levels in serum exosomes were quantified by qPCR (N=6-9/group). The levels of examined MiRNAs were normalized to the U6 small nucleolar RNA (snRU6). * indicates $P<0.05$.

Discussion

The data shows that exosomes isolated from septic mice may play a critical role in mediating endothelial dysfunction, including decreased tight junction proteins and increased adhesion molecules. MiRNAs incorporated in exosomes may play an important roles in these effects.

It is well known that eukaryotic cells secrete vesicles into outside environment. Those vesicles mainly incorporate three categories, i.e. apoptotic bodies, exosomes and microvesicles or microparticles, based on their biogenesis. All three vesicles are confined by a lipid bilayer, but the composition and the size vary (from 30 to 2,000 nm in diameters). The most widely studied and best known types of vesicles are microparticles and exosomes [171]. Microvesicles are budded directly from plasma membrane. In contrast, exosomes are generated inside from endosomal compartments called multivesicular bodies (MVBs). Exosomes are released via the integration of MVBs with the plasma membrane. Exosomes have been intensively investigated in infection [194,195,192]. However, the role of exosomes in sepsis-induced cardiovascular dysfunction has not been investigated previously.

Azevedo *et al* reported that exosomes from septic shock patients induce myocardial dysfunction in isolated rabbit hearts [194]. Gambim *et al* have shown that platelet derived exosomes induce endothelial cell apoptosis and endothelial dysfunction [195]. We have observed in the present study that the exosomes isolated from the serum of septic mice significantly increased the expression of adhesion molecules (VCAM-1 and ICAM-1) and decreased the levels of tight junction proteins (ZO-1 and Occludin) in endothelial cells. Interestingly, treatment of endothelial cells with HSPA12B $^{-/-}$ septic

exosomes resulted in further increases in the expression of levels of adhesion molecules and decreases in the levels of tight junction proteins. Our data suggest that exosomes from septic mice could contain compounds that markedly regulate the function of targeted cells through cell-cell communication. At present, we do not know which components in the septic exosomes cause endothelial dysfunction. However, we do know that exosomes play a critical role in regulating cellular function of targeted cells during polymicrobial sepsis. Indeed, recent studies have shown that exosomes can act locally or circulate through various bodily fluids, including blood and lymph and play a critical role in cell-to-cell communication [185,175,189,190] by transferring proteins and/or nucleic acids from one cell to another [185,175,189,190]. Therefore, the exosomes contain microRNAs which may play an important role in regulation of cardiovascular function in polymicrobial sepsis.

MicroRNAs (miRNAs) are 21 to 23 nucleotide non-protein-coding RNA molecules and have been identified as novel regulators of gene expression at the post-transcriptional level by binding to target messenger RNAs (mRNAs) [118,121,225]. MiR-126 is predominately expressed in endothelial cells [156]. MiR-126 has been reported to suppress endothelial adhesion molecule expression [159], and it is involved in the regulation of angiogenesis [157]. We have observed that the levels of miRNA-126 in septic exosomes were markedly decreased in HSPA12B$^{-/-}$ septic exosomes (Fig. 14). Our data indicates that lower levels of miR-126 in the exosomes may be responsible for the higher levels of adhesion molecules in the myocardium of HSPA12B$^{-/-}$ septic mice. Although we do not understand the mechanisms by which specific deficiency of endothelial HSPA12B resulted in lower levels of miR-126 in circulation exosomes,

recent evidence has shown that specific miRNAs play a critical role in the negative regulation of TLR/NF-κB mediated innate immune and inflammatory responses [117,121,116,226]. Several miRNAs (miR-146a, miR-155, and miR-125, etc.) have been reported to regulate TLR-mediated NF-κB activation [119,116,226]. Thus those miRNAs play an important role in the pathophysiology of sepsis/septic shock [227,137]. We have previously reported that increased expression of miR-146a in the myocardium significantly attenuates septic cardiomyopathy [144]. We have also found that transfection of lentivirus expressing miR-125b protects against sepsis-induced cardiomyopathy [228]. Our previous studies indicate that MiRNAs carried by exosomes could differentially regulate detrimental signaling pathways, thus dictating the outcome of septic cardiomyopathy and survival in sepsis. We hypothesize that modulation of exosomes containing miR-126 may be a new and novel approach for the treatment of cardiomyopathy in HSPA12B$^{-/-}$ septic mice. The experiments presented in the part V were performed to evaluate this hypothesis.

CHAPTER 5

INVESTIGATE THE MECHANISMS BY WHICH ENDOTHELIAL SPECIFIC HSPA12B$^{-/-}$ DEFICIENCY ENHANCES THE INFLAMMATORY RESPONSE

Introduction

In the chapter 3 studies, we observed that inflammatory responses in HSPA12B$^{-/-}$ septic mice were significantly greater when compared with WT septic mice, suggesting that HSPA12B may serve as a negative regulator of the pro-inflammatory responses during sepsis. However, we still do not understand how HSPA12B negatively regulates inflammatory responses during sepsis/septic shock.

It has well been demonstrated that TLRs play a critical role in mediating the innate immune and inflammatory responses which contribute to septic cardiomyopathy [229,50]. TLR mediated signaling predominately activates NF-κB that is a critical transcription factor regulating gene expression including inflammatory cytokines, cell death and survival [230,231]. We have previously demonstrated that that NF-κB activation plays a central role in mediating the development of sepsis/septic shock and septic cardiomyopathy [35]. However, the mechanisms by which activation of TLR/NF-κB signaling results in septic sequelae have not been elucidated.

The data generated from the Chapter 4 studies show that exosomes isolated from septic mice play a critical role in mediating endothelial dysfunction. It is possible that septic exosomes could be responsible for sepsis-induced inflammatory responses. Exosomes contain microRNAs which have been reported to regulate cardiovascular function and inflammatory processes in several models of diseases [144,156,228]. Indeed,

microRNAs play a critical role in sepsis/septic shock-induced innate immune and inflammatory responses [192]. Several microRNAs such as miR-146a, miR-155, and miR-125, etc. have been reported to regulate activated NF-κB-mediated inflammatory responses [138,232,119,116,119,116]. Thus, these microRNAs play an important role in the pathophysiology of sepsis/septic shock [227,137]. We have previously reported that miR-146a plays a protective role in attenuation of sepsis induced cardiomyopathy by targeting the NF-κB activation pathway [144]. We have also found that miR-125b protects against sepsis-induced cardiac dysfunction in polymicribial sepsis by targeting TNF-α and sepsis-induced apoptosis [228].

Our previous studies indicate that microRNAs carried by exosomes could differentially regulate detrimental signaling pathways. In the Chapter 4 experiments, we demonstrated that septic exosomes stimulate inflammatory responses in macrophages. Interestingly, the exosomes isolated from HSPA12B$^{-/-}$ septic mice, which are associated with the lower levels of miRNA-146a and miRNA-125b, resulted in more inflammatory cytokine production in macrophages. The data suggests that the lower levels of mR-146a and miR-125b may be responsible for the losing of negative feedback regulation of inflammatory response in HSPA12B$^{-/-}$ septic mice.

Materials and Methods

Isolation of Exosomes.

Ten hours after CLP, the blood was collected from the experimental mice followed by centrifugation at 1,500 g for 15 min at 18˚C. The supernatant was collected and added to ExoQuickTM exosome precipitation solution (63 µl/ 250µl plasma,

ExoQ5A-1, SBI) according to manufacturer's instruction. The mixture was incubated at room temperature for 30 min followed by centrifugation at 1,500g for 30min. The supernatants were removed and the pellets were exosomes.

Isolation of MiRNAs from Exosomes.

Total RNA was extracted from the exosome pellets using Trizol (RN190, Molecular Research Center, Cincinnati, USA) according to manufacturer's instructions. Approximately 10 ng of isolated total RNA was applied to examination of microRNA levels as described previously [153].

qPCR Assay of MiRNAs.

MiRNAs were isolated from heart tissues or exosomes using the mirVanaTM miR isolation kit (Ambion) as described previously[224]. MiRNAs levels were quantified by qPCR using specific Taqman assays (Applied Biosystems, USA) and specific primers (Applied Biosystems, Primer identification numbers: 000468 for hsa-miR-146a, 000449 for miR-125b and 001973 for snRU6). The levels of examined miRNAs were quantified with the 2(-ΔΔct) relative quantification method that was normalized to the U6 small nucleolar RNA (snRU6).

Delivering of Septic Exosomes into Macrophage *in vitro*.

Mice were subjected to CLP and then serum was collected at 10 hours after surgery. Blood was collected and exosomes in serum was isolated with ExoQuickTCTM exosome precipitation solution (ExoQ5A-1, SBI). Murine macrophages J774.1A were

treated with exosomes 5μg/ml diluted in conditional medium for different times. The conditional medium is exosome free medium, prepared by centrifuged at 120,000 rpm, 4˚C for 18 hours. The medium was collected. The production of TNF-α and IL-6 by macrophages were determined by ELISA. Cells were collected and cellular proteins were isolated.

ELISA for Cytokine Assay.

The levels of cytokines (TNF-α, IL-6) in cell-free supernatants were measured by ELISA development kits (Peprotech) according to manufacturers' instructions. For cytokines release from cells, macrophages (50.000/50 μl/well) were seeded into wells of 96-well microtiter plates. After 12 h treatment cell-free supernatants were collected, centrifuged at 1000g for 10 min at 4 °C and aliquots were frozen at −20 °C until measurement. Supernatants were analyzed. Briefly, ELISA plate was incubated overnight at room temperature (R.T.) with 100μl capturing antibody. After washing for four times, 300μl blocking buffer was added for 1 hour at R.T. After washing, serial 100 μl dilute standard or sample was added and incubated at room temperature for at least 2 hours. 100μl diluted detection antibody was immediately added after washing and incubate at R.T. for 2 hours. Plates were aspirated and washed plate 4 times. Diluted Avidin-HRP conjugate was added and incubated 30 min. Plates were aspirated and washed plate 4 times. Add 100μl of ABTS liquid substrate solution to each well. Monitor color development with an ELISA plate reader at 405 nm.

Western Blot.

Briefly, the cellular proteins were isolated by using RIPA buffer and separated by SDS-polyacrylamide gel electrophoresis and transferred onto Hybond ECL membranes (Amersham Pharmacia, Piscataway, NJ). The ECL membranes were incubated with primary anti-phosphor-IκB or IκB antibody (Cell Signaling Technology) followed by incubation with peroxidase-conjugated secondary antibodies (Cell Signaling Technology, Inc.) and analysis by the ECL system (Amersham Pharmacia, Piscataway). The signals were quantified using the G: Box gel imaging system by Syngene (Syngene, USA, Fredrick, MD). GAPDH (Meridian Life Science, Inc, TN) was detected as loading control.

Statistical Analysis

The data are expressed as mean ± SE. Comparisons of data between groups were made using one-way analysis of variance (ANOVA), and Tukey's procedure for multiple-range tests was performed. The log-rank test was used to compare group survival trends. Probability levels of 0.05 or smaller were used to indicate statistical significance.

Results

HSPA12B$^{-/-}$ Septic Exosomes Stimulated Inflammatory Cytokine Production in Macrophages

We found that endothelial specific deficiency of HSPA12B results in enhanced inflammatory cytokine production in serum (Fig. 10). We also observed that septic

exosomes induces dysfunction of endothelial cells (Figure 7 and 8). We hypothesize that septic exosomes could stimulate inflammatory response in macrophages. To evaluate our hypothesis, we isolated serum exosomes from WT and HSPA12B$^{-/-}$ mice, treated macrophages with exosomes, followed by analysis of inflammatory cytokine production. As shown in Figure 15, treatment of macrophages with WT septic exosomes markedly increased TNF-α production by 133.9% and IL-6 by 427.9% respectively, compared to the WT sham exosome group. However, HSPA12B$^{-/-}$ septic exosomes stimulated even more inflammatory cytokine production in macrophages. The levels of TNF-α and IL-6 in HSPA12B$^{-/-}$ septic exosomes treated cells were significantly enhanced by more 184% and 360% respectively than that in WT septic exosome treated group. The data suggest that exosomes play an important role in the induction of the inflammatory response in macrophages.

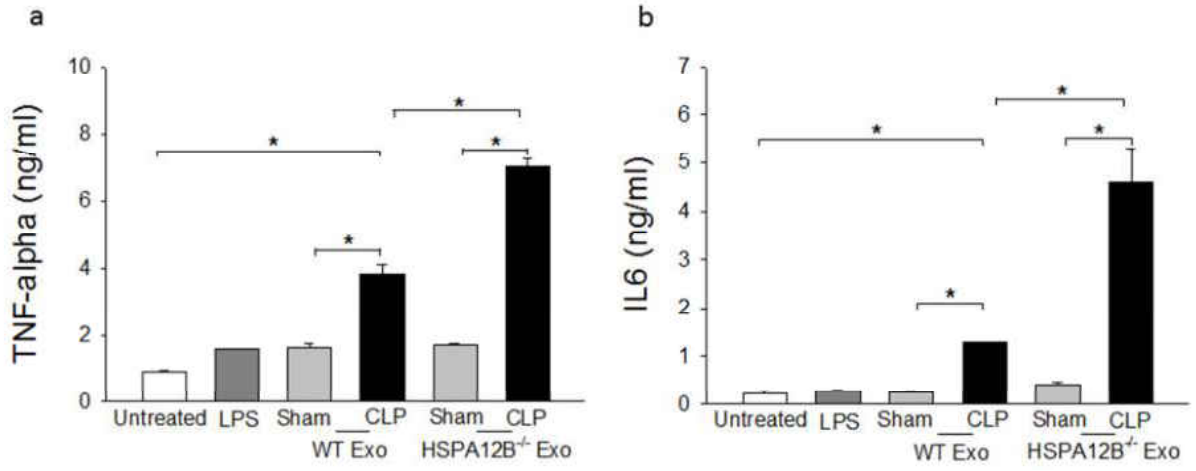

Figure 15 HSPA12B$^{-/-}$ septic exosomes stimulate inflammatory cytokine production in macrophages. HSPA12B deficient and WT mice were subjected to polymicrobial sepsis induced by CLP. Sham surgical operation served as control. Ten hours after CLP, blood was collected for isolation of serum exosomes. The murine macrophage J774.1A cells were treated with exosomes (5 mg/ml) for 12 hours. The medium was collected and cytokine production, TNF-α (a) and IL-6 (b) was measured by ELISA. (N=4-8/group). * indicates $P<0.05$.

Exosomes from Septic Mice Increase NF-κB Binding Activity in Macrophages

We observed that septic exosome stimulation significantly increased inflammatory cytokine production in macrophages (Figure 15). Activation of NF-κB plays a critical role in the regulation of inflammatory cytokine production [218]. Therefore, we examined whether septic exosome-induced increases in the inflammatory cytokine production are mediated through activation of NF-κB binding activity. IκBα phosphorylation and degradation are crucial steps for NF-κB translocation and binding activity [58]. Macrophages (J774.1A cells) were treated with exosomes isolated from WT and HSPA12B$^{-/-}$ mice. Cellular proteins were isolated for measurement of IκBα phosphorylation. As shown in Figure 16, sham exosomes from WT and HSPA12B$^{-/-}$ mice did not alter the levels of IκBα phosphorylation or total IκBα in macrophages. However, WT septic exosome stimulation markedly increased the levels of phosphorylated IκBα by 154.1% and decreased total IκBα levels by 102.3% compared with WT sham exosome treated group. Importantly, HSPA12B$^{-/-}$ septic exosome increased the level of phosphorylated IκBα even further by 89.3% and decreased total IκBα levels by 90.2%, when compared with WT septic exosome group. The data suggests that septic exosomes induce IκBα phosphorylation, which is critical for NF-κB translocation and binding activity. These data indicate that endothelial specific deficiency of HSPA12B amplified the effect of septic exosomes on IκBα phosphorylation in macrophages.

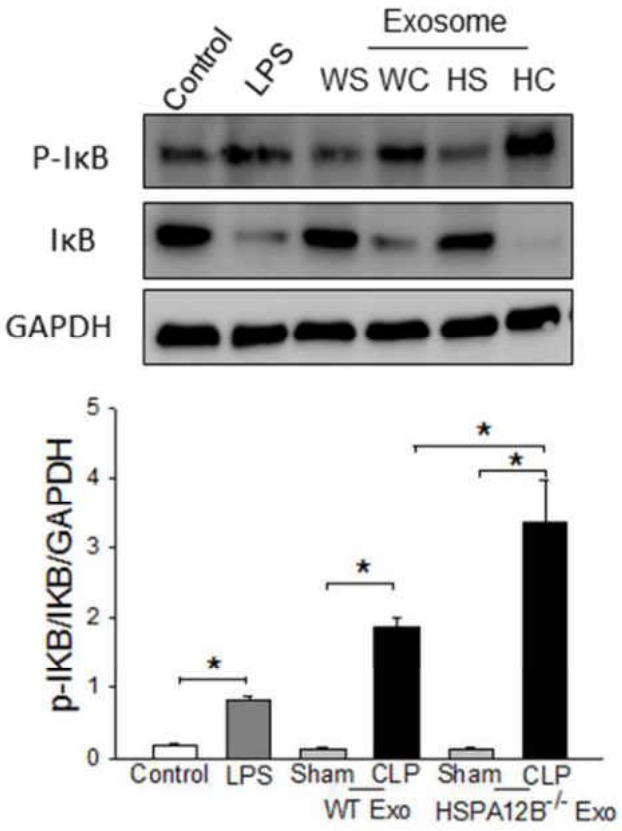

Figure 16 HSPA12B$^{-/-}$ exosomes from septic mice increased NF-κB binding activity in macrophages. HSPA12B deficient and WT mice were subjected to polymicrobial sepsis induced by CLP. Sham surgical operation served as control. Ten hours after CLP, blood was collected for preparation of serum exosomes. The murine macrophage J774.1A cells were treated with exosomes (5 mg/ml) for 45 mins. Protein levels of phosphor-IκB and IκB in macrophage cytoplasm were detected by Western blot. GAPDH served as loading control (N=4/group). Exosomes isolated from septic HSPA12B-/- mice promotes cytokine release from macrophages. WS = WT sham; WC = WT CLP; HS= HSPA12B$^{-/-}$ sham; HC= HSPA12B$^{-/-}$ CLP. * indicates $P<0.05$.

Decreased Levels of miR-146a in Macrophages Treated with HSPA12B$^{-/-}$ Exosomes from Septic Mice

MicroRNA-146a has been reported to serve as a negative regulator for NF-κB activation [138,143]. We and others have previously shown that miR-146a suppresses NF-κB binding activity by targeting TRAF6 and IRAK1 [153,143]. We examined whether NF-κB activation induced by septic exosomes is due to downregulation of miR-146a. The

levels of miR-146a in the macrophages treated with WT and HSPA12B$^{-/-}$ exosomes were analyzed by qPCR. Figure 17 shows that there was no significant alteration in the levels of miR-146a following exosomes treatment for 4 hours. Interestingly, treatment of macrophages with WT or HSPA12B$^{-/-}$ sham exosomes markedly enhanced the levels of miR-146a, when compared with untreated control. In contrast, WT septic exosome treatment decreased expression of miR-146a as compared with WT sham exosomes. However, HSPA12B$^{-/-}$ exosomes resulted in even lower levels of miR-146a, when compared with WT septic exosomes. The data indicates that decreased levels of miR-146a in HSPA12B$^{-/-}$ septic exosomes and their treated macrophages could be responsible for stimulation of NF-κB mediated inflammatory cytokine production.

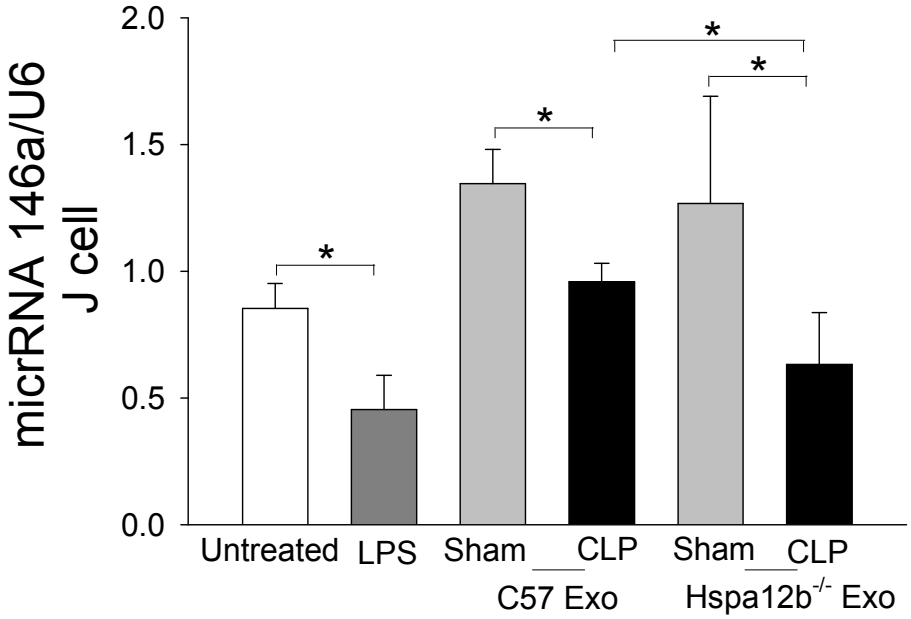

Figure 17 HSPA12B$^{-/-}$ exosomes from septic mice decreased miR-146 in macrophages. HSPA12B deficient and WT mice were subjected to polymicrobial sepsis induced by CLP. Sham surgical operation served as control. Ten hours after CLP, blood was collected for preparation of serum exosomes. Murine macrophage cell line J774.1A was treated with 5μg/ml exosomes for 12 hours. MiR-146a level in macrophage were measured by RT-PCR (N=6/group). * indicates $P<0.05$.

Decreased Expression of MiR-146a and MiR-125b in Serum Exosomes from HSPA12B$^{-/-}$ Septic Mice

We also examined the levels of miR-146a in the exosomes isolated from WT and HSPA12B$^{-/-}$ sham and septic mice. MiR-146a was markedly increased in WT septic exosomes compared with WT sham control. However, there were significantly lower levels of miR-146a in HSPA12B$^{-/-}$ septic exosomes when compared with WT septic exosomes. CLP sepsis markedly increased the levels of miR-146a in exosomes compared with WT sham exosomes, while there was no significant difference in the levels of miR-146a between HSPA12B$^{-/-}$ septic exosomes and HSPA12B$^{-/-}$ sham exosomes. MiR-125b has been reported to suppress TNF-α expression [232]. We analyzed the levels of miR-125b in serum exosomes from WT and HSPA12B$^{-/-}$ mice. CLP sepsis markedly decreased miR-125b (b). In contrast, HSPA12B$^{-/-}$ septic exosomes have significantly lower level of miR-125b compared with WT septic exosomes. These data indicate that the decreased MiRNAs in serum exosomes from HSPA12B$^{-/-}$ septic mice may be responsible for losing negative feedback regulation mechanisms for sepsis-induced inflammatory responses in HSPA12B$^{-/-}$ mice.

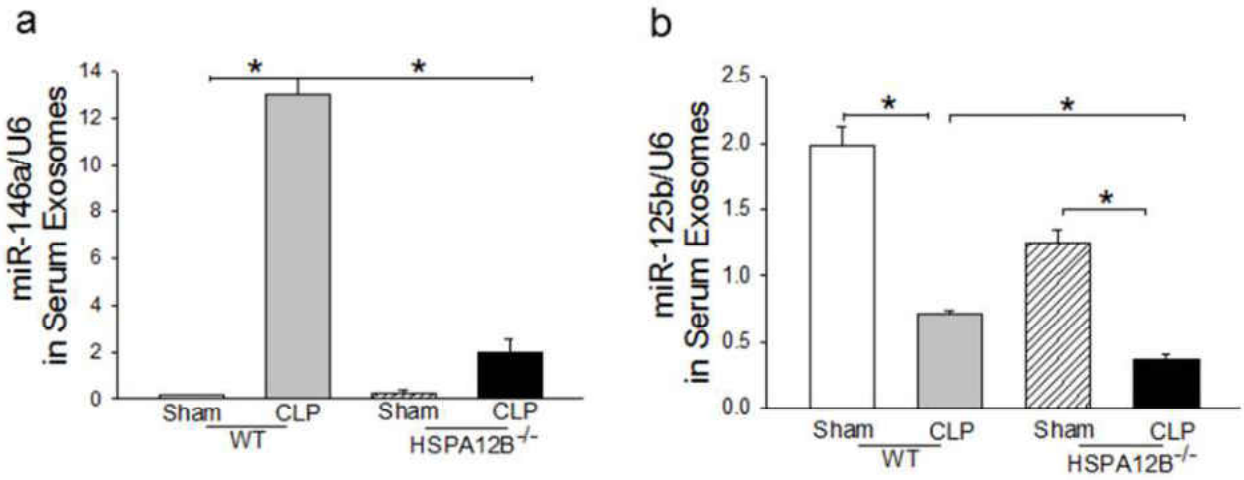

Figure 18 The expression of miR-146a and miR-125b decreased in serum exosomes from HSPA12B$^{-/-}$ septic mice. Ten hours after CLP, the blood was collected and serum exosomes were isolated by exosome precipitation solution. Total RNA was extracted from the exosome pellets using Trizol. MiR-146a (N=13/group) and miR-125b (N=10-12/group), levels in serum exosomes were quantified by qPCR. The levels of examined MiRNAs were normalized to the U6 small nucleolar RNA (snRU6). * indicates $P<0.05$.

Discussion

Innate immune and inflammatory responses have been demonstrated to play a critical role in cardiovascular dysfunction in sepsis/septic shock [36,37,35,38]. TLRs are PRRs that recognize PAMPs to activate innate and adaptive immune responses [49,50]. TLR mediated cellular signaling predominately activates NF-κB which is an important transcription factor regulating gene expression including inflammatory cytokines and chemokines as well as cell survival and death [54,55,49,233,56,231,218]. Pro-inflammatory cytokines, such as IL-6 and TNF-α, are produced upon NF-κB activation. We have previously demonstrated that TLR-mediated NF-κB activation pathway plays a detrimental role in sepsis-induced cardiovascular dysfunction [59,60,61,62,63,64]. Either deficiency of TLR4 or targeting TLR-mediated NF-κB activation significantly improved outcome and cardiac function in polymicrobial sepsis. Recently, we found that targeting

TLR mediated NF-κB activation with miR-146a or miR-125b markedly attenuated polymicrobial sepsis-induced cardiac dysfunction [153,228]. Our data and other reports clearly demonstrated that activation of innate immune and inflammatory responses mediated by TLR/NFκB signaling contribute to cardiovascular dysfunction in sepsis/septic shock. However, mechanisms by which TLR mediated NF-κB activation during sepsis/septic shock are unclear.

The present study demonstrated that circulating exosomes in septic mice play a critical role in the induction of inflammatory responses in macrophages. Stimulation of macrophages with septic exosomes significantly increased the production of inflammatory cytokines (TNF-α and IL-6). Our finding suggests that septic exosomes may play an important role in mediated inflammatory responses either by carrying endogenous ligands that activate the TLR-mediated NF-κB pathway or by loss of some substances in septic exosomes that will play a role in negative feedback regulation of sepsis. Indeed, we observed that stimulation of macrophages with septic exosomes significantly enhanced phosphorylation of IκBα which controls the activation of NF-κB [58].

The data suggests that septic exosomes may contain endogenous ligands that activate TLR-mediated NF-κB activation pathway. In our pilot studies, we observed that the levels of high mobility group box 1 protein (HMGB1) in septic exosomes were markedly greater compared with the exosomes isolated from sham control mice. It has well been demonstrated that HMGB1 is a critical factor causing organ failure in sepsis/septic shock [234]. It is possible that HMGB1 in septic exosomes may be, in part, responsible for the inflammatory response induced by septic exosomes in macrophages. We observed HSPA12B$^{-/-}$ septic exosomes induced more inflammatory

cytokine production in macrophages than that in WT septic exosomes. Interestingly, the levels of HMGB1 in HSPA12B$^{-/-}$ septic exosomes were markedly greater compared with WT septic exosomes, indicating that septic exosomes may carry endogenous ligands, such as HMGB1, that activate innate immune and inflammatory responses. At present, we do not understand the mechanisms by which endothelial cell specific deficiency of HSPA12B resulted in high levels of endogenous ligand levels in exosomes. Recent studies have shown that activation of glycolysis regulates HMGB1 release from inflammatory cells. Both clinical and basic studies have demonstrated that sepsis/septic shock significantly increase glycolytic metabolism. In endothelial cells, there is more than 60% of metabolism via glycolysis. Therefore, it is possible that HSPA12B regulates septic exosomes containing endogenous ligands via glycolytic dependent mechanisms.

Exosomes are novel vehicles for carrying and delivering microRNAs to the target cells, resulting in regulation of signaling pathways in the recepient cells [197,235]. MiR-146a has been shown to attenuate small intestine ischemia/reperfusion injury by downregulating IRAK1 [143]. We observed that the levels of miR-146a and miR-125b in HSPA12B$^{-/-}$ septic exosomes were markedly lower than in WT septic exosomes. We have reported that increased expression of miR-146a in the myocardium via delivery of lentivirus expressing miR-146a protects the myocardium from polymicrobial sepsis-induced cardiac dysfunction [153]. We demonstrated that miR-146a targets TRAF6 and IRAK1, resulting in downregulation of NF-κB binding activity in polymicrobial sepsis [153]. We also observed that increased expression of miR-125b in the myocardium significantly attenuates sepsis-induced cardiac dysfunction via targeting TNF-α and apoptotic factors (Bim and Bak) [228]. In the present studies, we observed that the levels

of both miR-146a and miR-125b in the HSPA12B$^{-/-}$ septic exosomes were lower when with WT septic exosomes. Therefore, lower levels of these MiRNAs in HSPA12B$^{-/-}$ septic exosomes may be, in part, responsible for HSPA12B$^{-/-}$ septic exosomes induced more inflammatory cytokine production in macrophages. We will determine in our future studies whether increased levels of miR-146a and/or miR-125b in HSPA12B$^{-/-}$ septic exosomes will markedly attenuate inflammatory response in macrophages and improve cardiovascular function in HSPA12B$^{-/-}$ septic mice.

CHAPTER 6

IN VIVO DELIVERY OF EXOSOMAL MIR-126 ATTENUATED SEPSIS-INDUCED CARDIAC DYSFUNCTION

Introduction

We have found that the exosomes isolated from HSPA12B$^{-/-}$ septic mice stimulated the expression of adhesion molecules and decreased the levels of tight junction proteins in endothelial cells. Interestingly, we observed that the levels of miRNA-126 in HSPA12B$^{-/-}$ septic exosomes were much lower than that in WT septic exosomes. The importance of miR-126 in the regulation of endothelial cell function has been documented. MiRNA-126 predominantly expresses in endothelial cells [111] and regulates the progression of angiogenesis, proliferation, and migration of endothelial cells [157], and the expression of vascular cell adhesion molecule-1[159] as well as chemokine stromal dell-derived factor-1 (SDF-1/CXCL-12) [160]. Interestingly, miR-126 has also been reported to regulate the survival and function of plasmacytoid dendritic cells via modulation of the VEGFR2 pathway [38], indicating that miR-126 may regulate innate immune responses by targeting VEGF signaling. In our studies, we found that the lower levels of miR-126 in septic exosomes are associated with worst outcome and cardiovascular dysfunction as well as over-production of inflammatory cytokines in HSPA12B$^{-/-}$ septic mice.

Based on our findings that endothelial cell specific deficiency of HSPA12B significantly increases the expression of adhesion molecules which promote the

infiltration of inflammatory cells into the myocardium and myocardial inflammatory responses as well as loss of tight junction proteins, it is possible that decreased levels of miR-126 in septic exosomes may be the important factor causing the responses in polymicrobial sepsis. We hypothesize that restoration of miR-126 levels in exosomes could significantly improve cardiac function in HSPA12B$^{-/-}$ septic mice.

Exosomes are membranous nanovesicles (30-100 nm) which arise inside many cells from endosomal compartments called multivesicular bodies (MVBs) [171,236]. Exosomes are novel vehicle for delivering mRNAs and microRNAs to exchange genetic information between cells [185,186,175,179,168]. MiRNAs transferred by exosomes are functional [191,175] and induce target gene repression in recipient cells [175,192]. Exosomes, as natural RNA shuttles, have triggered increasing interest as a drug carrier [168,205]. They have been utilized as shuttles to package and deliver drugs [206] because of their low immunogenicity and bio-distribution. Mesenchymal stromal cells secrete a huge amount of extracellular microvesicles [210] and are efficient manufacturer of exosomes for drug delivery [211,205]. We tested the therapeutic potential of miRNA-126 incorporated by exosome on septic mice.

Materials and Methods

Transfection of MiRNA Mimics *in vitro*.

HUVEC (1×10^6) in 6 well plates were transfected with 40pmol microRNA (scrambled, anti–miR-126 [Exiqon] or miR-mimic-126 [Ambion] by lipofectamine 2000 (Thermo Fisher Scientific). Twenty-four hours later, endothelial cells were treated with

LPS (5ug/ml). Four hours later, endothelial cell adhesion molecules (VCAM and ICAM) were evaluated by WB. Endothelial cell injury was evaluated by LDH release.

Measurement of LDH Release.

The cell impermeable enzyme, lactate dehydrogenase (LDH), leaks through damaged cell membranes. Cell injury was assessed by measurement of LDH activity in culture medium using a commercial kit (Cytotoxicity Detection Kit, Sigma) according to the manufacturers' instructions. Briefly, cells (10^5/ well) were seeded into 96-well plates. After treatment, 50 µL medium was transferred into a new plate and same volume reaction mixtures were added. After incubation for 30 mins at room temperature, reactions are stopped by adding Stop Solution. Absorbance at 490 nm and 680 nm was measured using a plate reader.

Isolation of Exosomes.

Ten hours after CLP, blood was collected from the experimental mice followed by centrifugation at 1,500 g for 15 min at 18˚C. The supernatant was collected and added with ExoQuickTM exosome precipitation solution (63 µl/ 250µl plasma, ExoQ5A-1, SBI) according to manufacturer's instruction. The mixture was incubated at room temperature for 30 min followed by centrifugation at 1,500 rpm for 30min. The supernatants were removed and the pellets were exosomes.

Isolation of MiRNAs from Exosomes

Total RNA was extracted from the exosome pellets using Trizol (RN190, Molecular Research Center, Cincinnati, USA) according to manufacturer's instructions. Approximately 10 ng of isolated total RNA was applied to examination of microRNA levels as described previously [153].

qPCR Assay of MiRNAs.

MiRNAs were isolated from heart tissues or exosomes using the mirVanaTM miR isolation kit (Ambion) as described previously[224]. MiRNAs levels were quantified by qPCR using specific Taqman assays (Applied Biosystems, USA) and specific primers (Applied Biosystems, Primer identification numbers: 002228 for hsa-miR-126-3p and 001973 for snRU6). The levels of examined MiRNAs were quantified with the 2(-ΔΔct) relative quantification method that was normalized to the U6 small nucleolar RNA (snRU6).

Preparation of Exosomes Containing MiR-126.

Bone marrow stromal cells (BMSCs) were isolated from HSPA12B$^{-/-}$ and WT mice as described previously [237]. Briefly, mice were euthanized and bone marrow was isolated by flushing the femur and tibia with Dulbecco's modified Eagle's medium (DMEM) using a 25G 0.5-inch needle (BD). The bone marrow was dissociated by syringe. The cell mixture was cultured in DMEM supplemented with 10% fetal bovine serum (FBS) (HyClone, Thermo Fisher Scientific Waltham, MA), glutamine (2 mM) and penicillin/streptomycin (50 U/ml and 50 mg/ml, Sigma). After incubation in a 37 °C with

5% CO_2 for 3 h, non-adherent cells were removed carefully by two washes with PBS and fresh medium was replaced. The medium was changed every other day. Cells at the 4^{th} -7^{th} generation were transfected with 40 nmol/L hsa-miR-126 mimics (MC12841, Ambion), hsa-miR-126 inhibitor (MH12841, Ambion) or Cy3TM dye labeled miR-scrambled control (AM17010, Ambion), using Lipofectamine 2000 transfection reagent (Thermo Fisher Scientific Inc.) according to the manufacturer's protocol. Twenty-four hours after transfection, supernatants were harvested for exosomes isolation using Exoquick-TC™ Exosome Precipitation Solution (SBI) according to the manufacturer's protocol.

In vivo Delivery of Exosomes Loaded with MiR-126 into Mice Hearts.

Mice were transfected with exosomes loaded with miR-126 or exosomes loaded with miR-control through the right carotid artery as described previously [224,228]. Briefly, mice were intubated and mechanically ventilated. The anesthesia was induced by 5% isoflurane and maintained by 1.5% isoflurane driven by 100% oxygen. Body temperature was maintained at 37°C by surface water heating. An incision was made in the middle of the neck and the right common carotid artery was carefully exposed. A micro-catheter was introduced into the isolated common carotid artery and positioned into the aortic root. Exosomes (10μg diluted in 100μl PBS) loaded with miR-126 or loaded with miR-Con were injected through the micro-catheter immediately after the induction of polymicrobial sepsis. The micro-catheter was gently removed and the common carotid artery was tightened before the skin was closed [63,35,219].

Western Blot.

Western blot (WB) was performed as described previously [221,35,219]. Briefly, the cellular proteins were separated by SDS-polyacrylamide gel electrophoresis and transferred onto Hybond ECL membranes (Amersham Pharmacia, Piscataway, NJ). The ECL membranes were incubated with primary antibodies anti-VCAM1, anti-ICAM1 (Santa Cruz Biotechnology), and anti-GAPDH (Meridian Life Science, Inc, TN), respectively, followed by incubation with peroxidase-conjugated secondary antibodies (Cell Signaling Technology, Inc.) and analysis by the ECL system (Amersham Pharmacia, Piscataway). The signals were quantified using the G: Box gel imaging system by Syngene (Syngene, USA, Fredrick, MD).

Immunohistochemistry Staining.

Immunohistochemistry was performed as described previously [35,219]. Briefly, heart tissues were immersion-fixed in 4% buffered paraformaldehyde, embedded in paraffin, and cut at 5 μm sections. The sections were stained with specific goat anti-intercellular adhesion molecule 1 (ICAM-1, 1:50 dilution, Santa Cruz Biotechnology) and rabbit anti-vascular cell adhesion molecule (VCAM-1, 1:50 dilution, Santa Cruz Biotechnology), respectively, and treated with the ABC staining system (Santa Cruz Biotechnology) according to the instructions of the manufacturer. Three slides from each block were evaluated, counterstained with hematoxylin, and examined with brightfield microscopy. Four different areas of each section were evaluated.

Tissue Accumulation of Neutrophils and Macrophages.

Inflammatory cells accumulation in heart tissues was examined with neutrophil specific antibody and macrophage specific antibody F4/80 (1:50 dilution, Santa Cruz, CA), separately [35,219]. Three samples from each group were evaluated, counterstained with hematoxylin, and examined with brightfield microscopy. Four different areas of each section were evaluated. The results are expressed as the numbers of neutrophils or macrophages per field (400x).

Wet/dry Lung Weight Ratio.

The water content of lungs of HSPA12B$^{-/-}$ and WT mice was measured by calculating the wet/dry ratios. After CLP surgery for 6 h, the lung of mice was removed and weighed before drying (wet weight) and then dried in an oven for 72 h at 50°C (dry weight). Wet/dry weight ratio was calculated by dividing the wet weight by the dry weight.

Echocardiography.

Transthoracic two-dimensional M-mode echocardiogram was obtained using a Toshiba Aplio 80 Imaging System (Toshiba Medical Systems, Tochigi, Japan) equipped with a 12-MHz linear transducer as described previously [35]. M-mode tracings were used to measure LV end-systolic diameter (LVESD), and LV end-diastolic diameter (LVEDD). Percent fractional shortening (%FS) and ejection fraction (EF%) were calculated as described previously [37,35,220].

Statistical Analysis

The data are expressed as mean ± SE. Comparisons of data between groups were made using one-way analysis of variance (ANOVA), and Tukey's procedure for multiple-range tests was performed. The log-rank test was used to compare group survival trends. Probability levels of 0.05 or smaller were used to indicate statistical significance.

Results

Transfection of MiR-126 Mimics Attenuated LPS-induced Adhesion Molecules in Endothelial Cells.

MiR-126 predominantly expresses in endothelial cells [156] and targets adhesion molecule expression [159]. We observed decreased levels of miR-126 in HSPA12B$^{-/-}$ septic exosomes which may be responsible for increased expression of adhesion molecules in the myocardium and septic exosomes treated endothelial cells. We examined whether increased miR-126 levels will suppress adhesion molecule expression in endotoxin treated endothelial cells. HUVECs were transfected with miR-126 mimics or scrambled miR which served as control (miR-control). Twenty-four hours after transfection, the cells were treated with LPS for 4 hours. The cellular proteins were isolated for analysis of VCAM-1 and ICAM-1 expression. Figure19a shows the transfection efficiency for microRNA delivery into the cells reached 86.3%. LPS treatment significantly increases expression of adhesion molecules (Figure 19b). Importantly, LPS-induced increases in the expression of VCAM-1 and ICAM-1 were

prevented by transfection of miR-126 mimics. Transfection of miR-control or antagomir-126 mimics did not markedly alter LPS-induced increases in the expression of adhesion molecules. The data suggests that miR-126 is essential for regulation of adhesion molecule expression in endothelial cells stimulated with endotoxin.

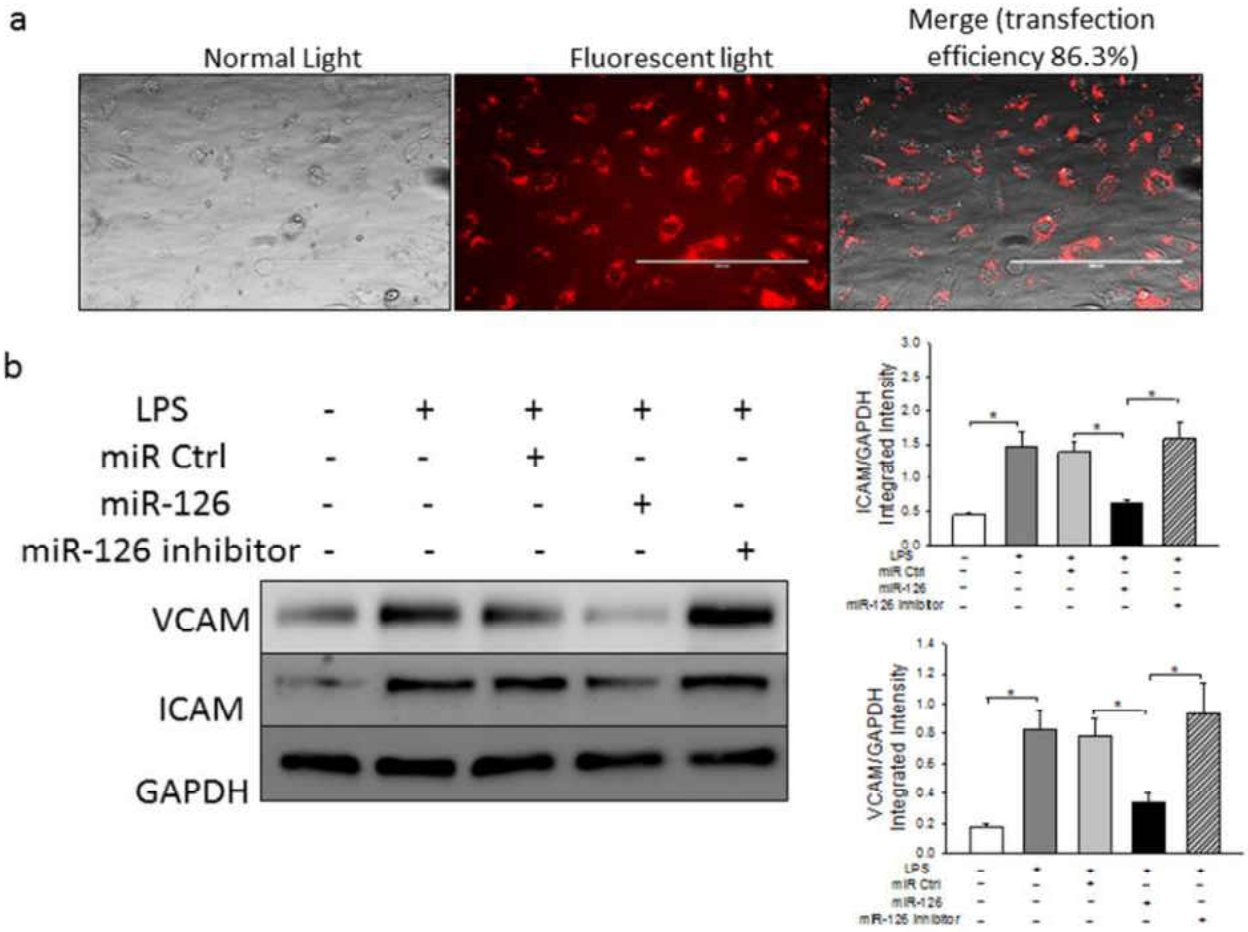

Figure 19 Transfection of miR-126 mimics attenuated LPS-induced expression of adhesion molecules in endothelial cells. Endothelial cells (HUVECs) were transfected with 40pmol microRNA (scramble, miR-126 mimics or inhibitors by lipofectamine 2000 (Thermo Fisher Scientific). The transfection efficiency was evaluated by RT-PCR (a). Twenty-four hours later, endothelial cells were treated with LPS (1ug/ml). Four hours later, adhesion molecules (VCAM and ICAM) in endothelial cells were evaluated by WB (b, N=6-9/group). * indicates $P<0.05$.

Transfection of MiR-126 Mimics Reduced the Release of LDH from Endothelial Cells after LPS treatment

We also examined the effect of transfection of miR-126 mimics on LPS induced damage of endothelial cells by releasing LDH. As shown in Figure 20, LPS treatment significantly increased LDH activity by 40.8% compared with the untreated control. Transfection of miR-control mimics did not significantly alter LPS-induced LDH release. However, miR-126 mimic transfection prevented LDH release in LPS-treated endothelial cells. Inhibition of miR-126 by transfection of anta-miR-126 mimics slightly enhanced LPS-induced LDH release but no significant difference. The data suggests that miR-126 plays a protective effect on LPS-induced cell damage and adhesion molecule expression.

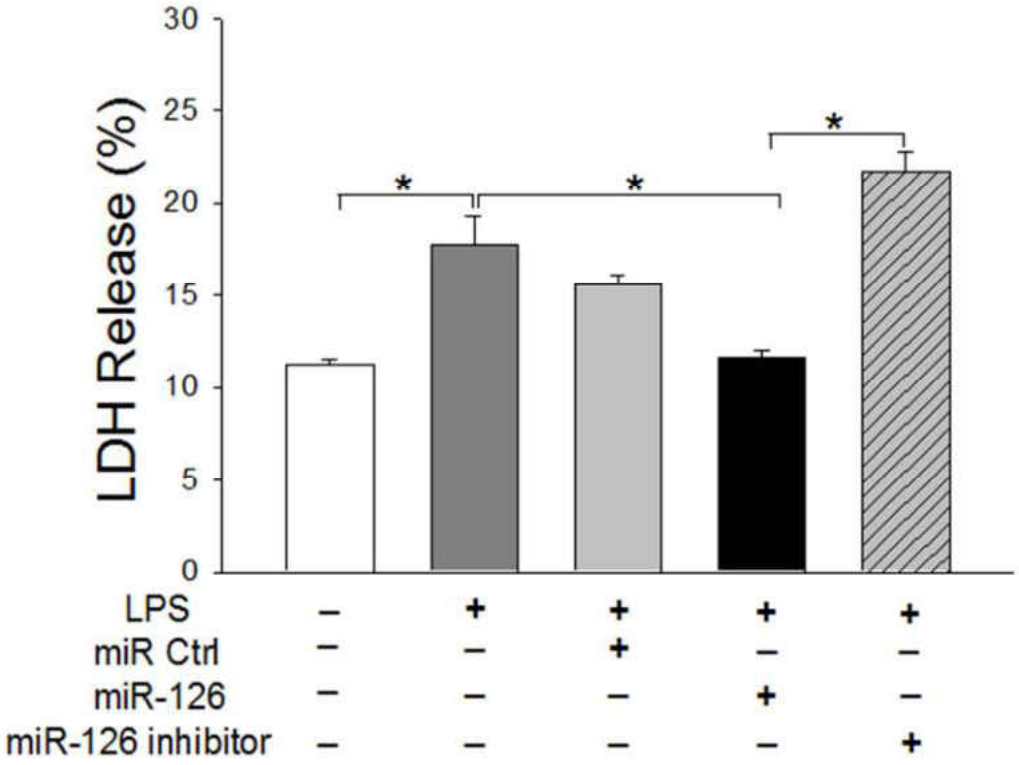

Figure 20 Transfection of miR-126 mimics prevented LPS-induced release of LDH from endothelial cells. Endothelial cells (HUVECs) were transfected with 40pmol microRNA (scramble, miR-126 mimics or inhibitors by lipofectamine 2000 (Thermo Fisher Scientific). Twenty-four hours later, endothelial cells were treated with LPS (1ug/ml). Twenty four hours later, endothelial injury was evaluated by LDH release (N=8/group). * indicates $P<0.05$.

Delivery of MiR-126 in Exosomes Prevented Sepsis-induced Expression of Adhesion Molecules in the Myocardium of HSPA12B$^{-/-}$ Septic Mice

In vitro data suggests that increased miR-126 levels significantly attenuated LPS-induced adhesion molecule expression and injury of endothelial cells. We examined whether increased miR-126 levels in exosomes will suppress sepsis-induced cardiovascular dysfunction. Exosomes are excellent vectors for carrying and delivery of MiRNAs into cells and tissues [173]. We prepared exosomes that were loaded with miR-126 mimics from bone marrow stromal cells. Bone marrow stromal cells (BMSCs) were

collected by flushing the femurs and tibias from HSPA12B$^{-/-}$ deficient mice. The cultured BMSCs were then transfected with miR-126 mimics or miR-control mimics which served as miR-control. Forty-eight hours after transfection, the exosomes were isolated from cultured medium. The levels of miR-126 in the exosomes were measured by qPCR. As shown in Figure 21a, the levels of miR-126 were significantly increased by more than thousand times (>14547 folds) in the exosomes that were loaded with miR-126 mimics, when compared with the exosomes loaded with miR-control mimics.

To examine whether delivery of exosomes that were loaded with miR-126 would suppress adhesion molecule expression in the myocardium, we delivered the exosomes carrying miR-126 or miR-control into the myocardium through the right carotid artery immediately after induction of CLP in HSPA12B$^{-/-}$ mice. As shown in Figures 21b, delivery of exosomes loaded with miR-126 prevented sepsis-induced increases in the expression of VCAM-1 and ICAM-1 in the myocardium. Immunohistochemistry staining of heart tissues (Fig. 21c) show that sepsis-induced strong immunostaining of ICAM-1 and VCAM1 were attenuated by delivery of exosomes loaded with miR-126 mimics. Delivery of exosomes loaded with miR-control did not alter sepsis-induced immunostaining of ICAM-1 and VCAM-1. Western blot data show that delivery of exosomes loaded with miR-126 prevented sepsis induced ICAM-1 and VCAM-1 expression in the myocardium. In contrast, delivery of miR-control carried by exosomes did not significantly alter sepsis-increased expression of ICAM-1 and VCAM-1 in the myocardium. The data clearly suggest that miR-126 plays a critical role in controlling the expression of adhesion molecules in the myocardium in polymicrobial sepsis.

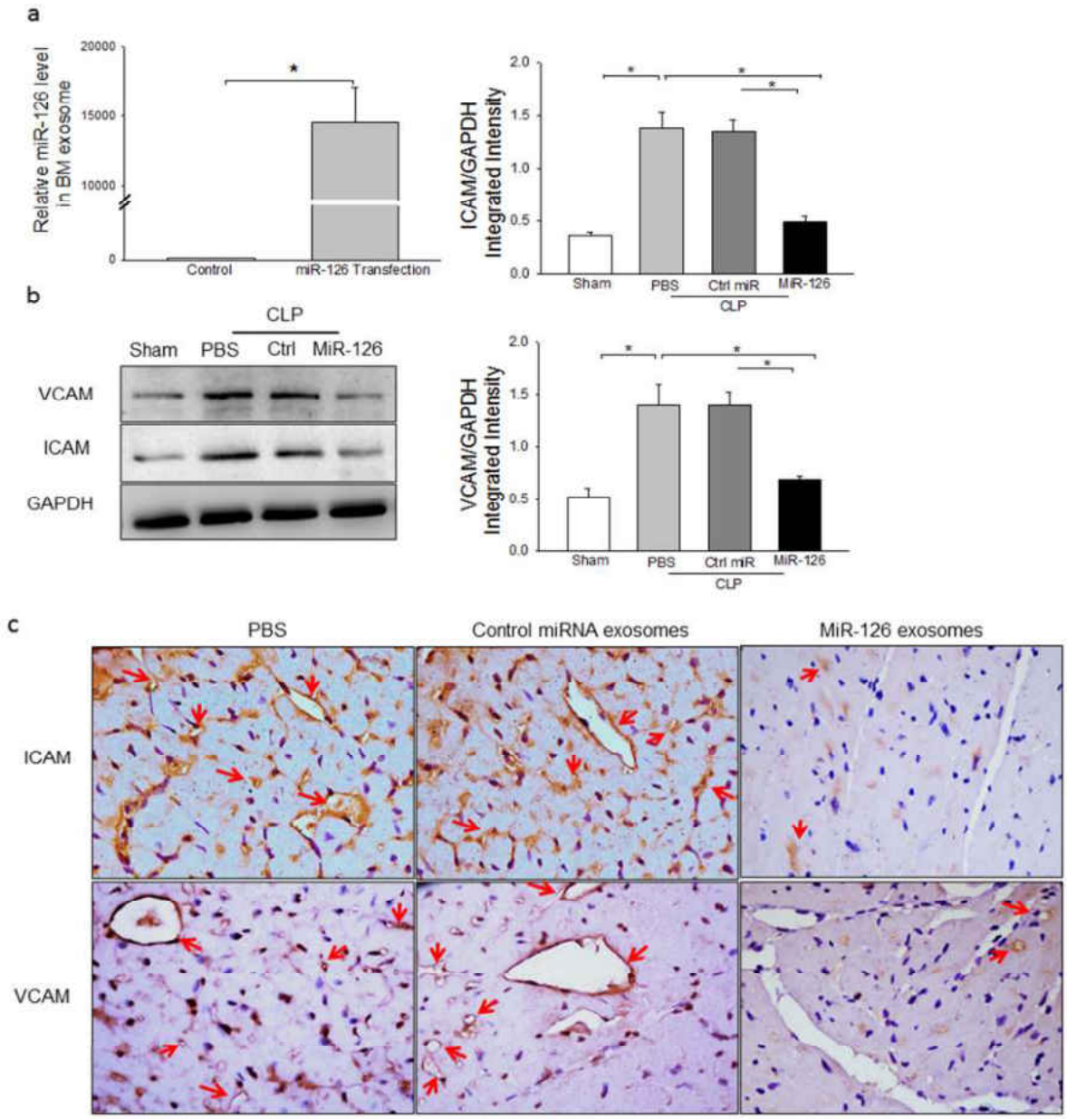

Figure 21 Delivery of miR-126 in exosomes prevented sepsis-induced expression of adhesion molecules in the myocardium of HSPA12B$^{-/-}$ septic mice. Bone marrow stromal cells (BMSCs) were isolated from HSPA12B$^{-/-}$ mice. Cells at the 4th -7th generation were transfected with 40 nmol/L scramble or miR-126 mimics. Twenty-four hours after transfection, supernatants were harvested for exosome isolation. The level of miR-126 in exosomes was detected by RT-PCR (a, N=4-6/group). Mice were transfected with exosomes, loaded either with miR-126 or miR-control through the right carotid artery, immediately after the induction of polymicrobial sepsis. Six hours later, the hearts were harvested. The level of adhesion molecules (ICAM and VCAM) in myocardium were evaluated by WB (b, N=8-11/group) and immunohistochemistry (c, N=3/group). * indicates P<0.05.

Delivery of MiR-126 by Exosomes Decreased the Infiltration of Inflammatory Cells into the Myocardium of HSPA12B$^{-/-}$ Septic Mice

Delivery of miR-126 via exosomes significantly suppresses sepsis induced increases in the expression of adhesion molecules in the myocardium (Figure 22), therefore, we examined the effect of delivery of exosomes loaded with miR-126 on sepsis-induced increases in the filtration of inflammatory cells into the myocardium. As shown in Figure 22, sepsis-induced infiltration of neutrophils and macrophages into the myocardium were suppressed by delivery of exosomes loaded with miR-126 mimics as evidenced by significant decreases in the numbers of immunostained neutrophils and macrophages in the heart tissues. The data suggests that miR-126 could suppress sepsis induced infiltration of macrophages and neutrophils into the myocardium by targeting the expression of adhesion molecules in polymicrobial sepsis.

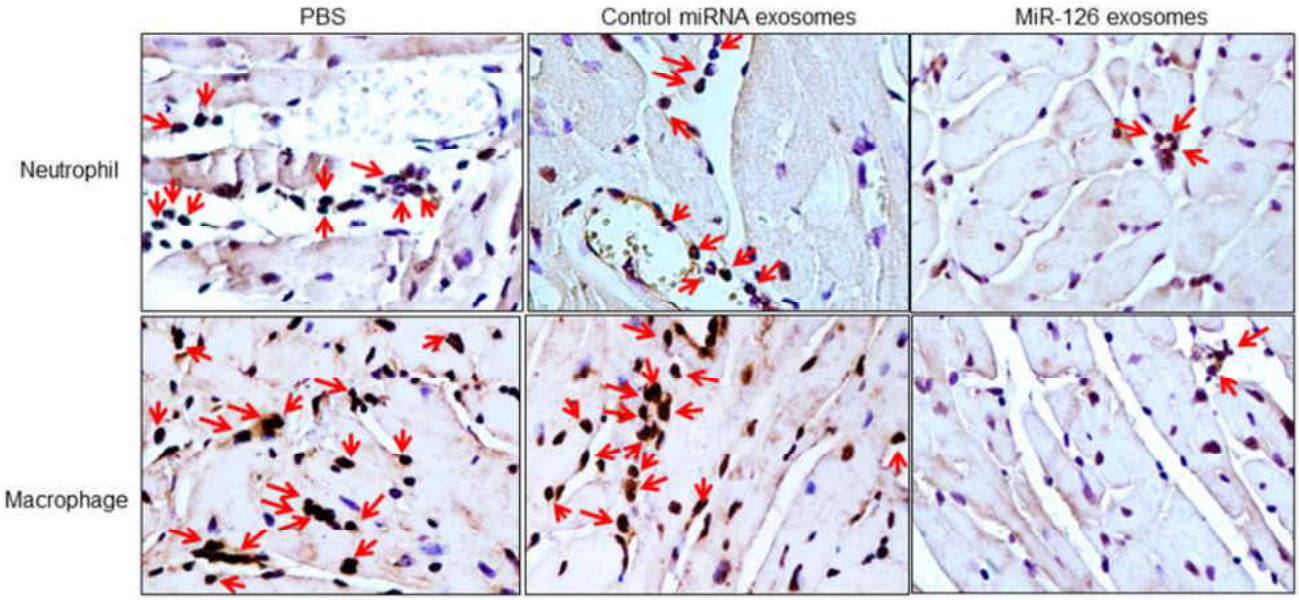

Figure 22 Delivery of exosomal miR-126 by exosomes decreased the infiltration of inflammatory cells into the myocardium of HSPA12B$^{-/-}$ septic mice. Bone marrow stromal cells (BMSCs) were isolated from HSPA12B$^{-/-}$ mice. Cells at the 4th -7th generation were transfected with 40 nmol/L scrambled or miR-126 mimics. Twenty-four hours after transfection, supernatants were harvested for exosome isolation. Mice were transfected with exosomes, loaded either with miR-126 or miR-control, through the right carotid artery, immediately after the induction of polymicrobial sepsis. Six hours later, the hearts were harvested. Macrophage and neutrophil infiltration were evaluated by immunohistochemistry (N=3/group).

In vivo Delivery of MiR-126 by Exosomes Improved Cardiac Dysfunction in HSPA12B$^{-/-}$ Septic Mice

It has been demonstrated that infiltration of inflammatory cells into the myocardium contributes to cardiac dysfunction in polymicrobial sepsis [40,45]. We examined whether delivery of miR-126 carried by exosomes will improve sepsis induced cardiac dysfunction in HSPA12B$^{-/-}$ mice. We delivered exosomes loaded with miR-126 or miR-control into the myocardium through the right carotid artery immediately after induction of CLP sepsis. First, we examined whether delivery of miR-126 will enhance the levels of miR-126 in circulation. Figure 23a shows that the serum miR-126 levels

after delivery of exosomes loaded with miR-126 were markedly increased (↑178%), when compared with HSAP12B$^{-/-}$ septic mice that were delivered by exosomes loaded with miR-control. The data from echocardiographic measurements show that delivery of miR-126 carried by exosomes into the myocardium significantly increased the values of EF% by 47.8% and %FS by 61.2% respectively, when compared with HSPA12B$^{-/-}$ septic mice that received exosomes loaded with miR-control (Fig. 23b). In addition, delivery of exosomal miR-126 markedly improved endothelial cell permeability in the HSPA12B$^{-/-}$ septic mice that received miR-126 carried with exosomes (Fig. 23c). The data suggests that miR-126 is required for preservation of cardiac function and maintenance of endothelial cell integrity in HSPA12B$^{-/-}$ septic mice.

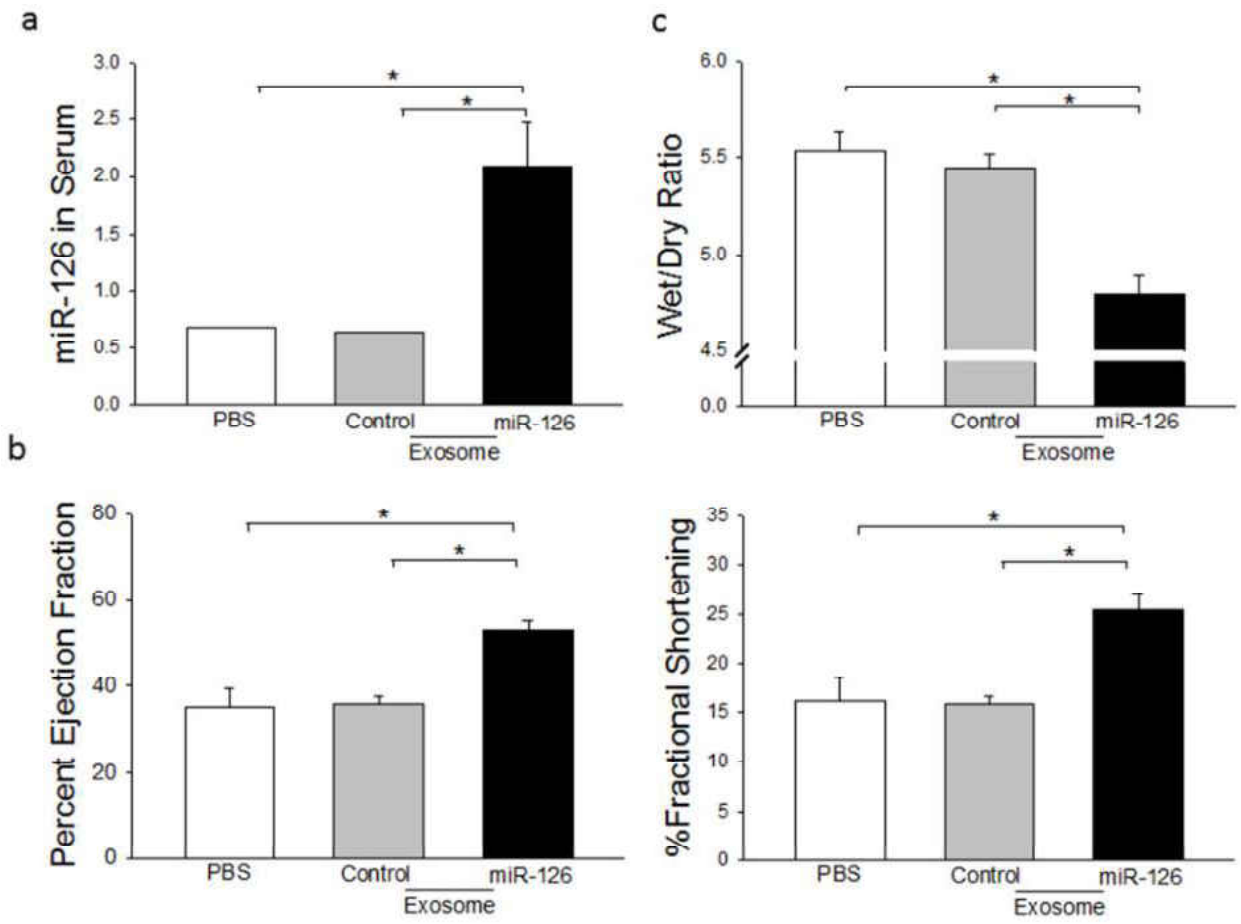

Figure 23 *In vivo* delivery of miR-126 by exosomes improved cardiac dysfunction in HSPA12B-/- septic mice. Bone marrow stromal cells (BMSCs) were isolated from HSPA12B$^{-/-}$ mice. Cells at the 4th -7th generation were transfected with 40 nmol/L scramble or miR-126 mimics. Twenty-four hours after transfection, supernatants were harvested for exosome isolation. Mice were transfected with exosomes, loaded either with miR-126 or miR-control through the right carotid artery, immediately after the induction of polymicrobial sepsis. Six hours later, cardiac function was evaluated by echo (b, N=5-9/group). The blood was collected and the level of miR-126 in mice serum was detected by RT-PCR (a, N=5-7/group). Endothelial permeability was evaluated by wet/dry lung ratio (c, N=6/group). * indicates P<0.05.

Discussion

Endothelial dysfunction is one of major important factors causing multiple organ failure in polymicrobial sepsis/septic shock [75]. MiR-126 predominately expresses in endothelial cells [156] and regulates adhesion molecule expression [159]. We have made a

novel observation that the levels of miR-126 in HSPA12B$^{-/-}$ septic exosomes are significantly lower than that in WT septic exosomes. It is possible, therefore, lower miR-126 in septic exosome may be responsible for server endothelial dysfunction in HSPA12B$^{-/-}$ septic mice by losing the negative feedback regulation of adhesion molecule expression. It is well known that endothelial cell dysfunction during sepsis/septic shock will cause more accumulation of inflammatory cells in organs, resulting in significant inflammatory response in both local and systemic. We hypothesized that increased levels of miR-126 will attenuate sepsis-induced endothelial dysfunction *in vivo*.

First, we performed *in vitro* experiments to examine whether increased levels of miR-126 will attenuated endotoxin induced the expression of adhesion molecules in endothelial cells. The data shows that transfection of endothelial cells with miR-126 mimics prevented endotoxin-induced adhesion molecule expression. Our observation is consistent with the reports showing miR-126 targets VCAM-1 [159]. Interestingly, we observed that increased levels of miR-126 significantly attenuated endotoxin induced endothelial injury and death. Our observation indicates that miR-126 may function as anti-cell injury and death, in addition to target adhesion molecules. At present, we do not know whether the protection against endothelial cell injury by miR-126 has a direct effect or indirect effect that through regulation of adhesion molecule expression. Maybe miR-126 will regulate cellular signaling pathways that involve regulation of cell survival.

Exosomes, as naturally existing RNA shuttles, have triggered increasing interest as a drug carrier [168,205]. They have been utilized as shuttles to package and deliver drugs [206]. Exosomes secreted by Mesenchymal stromal cells (MSCs) have been shown to

have cytoprotective effect and can attenuate myocardial ischemia/reperfusion injury [208] and hypoxia/induced pulmonary hypertension [209]. MSCs secrete a huge amount of extracellular microvesicles [210] and are efficient manufacturer of exosomes for drug delivery [211,205]. MSCs Exosomes as a novel therapy promotes cardiac regeneration and recovery in several cardiovascular diseases [212,213,214].

We have previously shown that exosomes prepared from bone marrow stromal cells are the excellent vehicle for delivering microRNA to cardiac myocytes [228]. In the present study, we observed that the levels of miR-126 in exosomes prepared from bone marrow stromal cells (BMSCs) by transfection of BMSCs with miR126 mimics were significantly increased by thousand fold compared with control exosome. The data suggests that exosomes derived from BMSCs are excellent vehicle for carrying microRNAs. We then delivered BMSC derived exosomes loaded with miR126 into the myocardium via the right carotid artery immediately induction of CLP sepsis in HSPA12B$^{-/-}$ mice. We observed that the transfection of miR-126 into the myocardium by delivering exosomes loaded with miR-126 was high. Importantly, we observed that transfection of miR-126 by exosomes significantly attenuated sepsis-induced increases in the expression of adhesion molecules in the myocardium of HSPA12B$^{-/-}$ septic mice. The exosomes containing miR-control did not alter the expression of adhesion molecules in the myocardium of HSPA12B$^{-/-}$ septic mice.

In our studies, we employed minimal mounts of exosomes that did not significantly alter sepsis-induced cardiac dysfunction, suggesting that attenuation of sepsis-induced increases in the expression of adhesion molecules in HSPA12B$^{-/-}$ mice was mediated by miR-126 but not by exosomes. In consistent with this observation, the infiltrated

numbers of neutrophils and macrophages in the myocardium have been significantly reduced in HSPA12B$^{-/-}$ septic mice by delivery of exosomes loaded with miR-126, when compared with HSPA12B$^{-/-}$ septic mice treated with exosomes loaded with miR-control. More importantly, delivery of exosomes carrying on miR-126 to the myocardium of HSPA12B$^{-/-}$ septic mice significantly improved cardiac function, when compared with HSPA12B$^{-/-}$ septic mice treated with exosomes loaded with miR-control. Our finding clearly demonstrated that miR-126 plays a critical role in the regulation of endothelial function by targeting adhesion molecule expression in polymicrobial sepsis.

Although we do not know the mechanisms by which endothelial specific deficiency of HSPA12B resulted in decreased levels of miR-126 in the exosomes following polymicrobial sepsis, delivery of exosomes loaded with miR-126 could be a novel approach for improvement of cardiovascular dysfunction in sepsis. In the future studies, we will determine whether exosomes loaded with combined miR126 and miR146a will result in even more beneficial to improve survival outcome and cardiovascular function in polymicrobial sepsis.

In summary, the levels of miR-126 in HSPA12B$^{-/-}$ septic exosomes are significantly lower than that in WT septic exosomes. Transfection of endothelial cells with miR-126 mimics prevented endotoxin-induced adhesion molecule expression and cell injury. Restoration of miR-126 levels in exosomes could significantly improve cardiac function in HSPA12B$^{-/-}$ septic mice. Decreased levels of miR-126 in septic exosomes may be the important factor causing the serial responses in polymicrobial sepsis.

CHAPTER 7

CONCLUSION

Sepsis is the host inflammatory response to severe, life-threatening infection with the presence of organ dysfunction, and is the most frequent cause of mortality in most intensive care units. Cardiovascular dysfunction is a major complication associated with sepsis, with high mortality rates up to 70%. Currently, no drugs are approved to be effective for the treatment of sepsis.

The integrity of the endothelium is fundamental for the homeostasis of the cardiovascular system. Sepsis practically affects all aspects of endothelial cell function which is the key factor for sepsis induced multiple organ failure. The increased inflammatory response and expression of adhesion molecules as well as chemokines on endothelial cells markedly promotes the infiltration of inflammatory cells, such as macrophages and neutrophils, into the tissues. The loss of tight junction proteins and the increased permeability of the endothelial cells will provoke tissue hypoxia and subsequent organ failure. Therefore, preservation of endothelial function is a critical approach for the protection against sepsis induced multiple organ failure.

In this study, we demonstrated, for the first time to our knowledge, that endothelial cell specific protein called HSPA12B plays a critical role in the preservation of cardiovascular function in polymicrobial sepsis. HSPA12B is a newly discovered member of HSP70 family which predominantly expresses in endothelial cells. We observed that HSPA12B deficiency exaggerated sepsis-induced endothelial dysfunction, leading to cardiac dysfunction in polymicrobial sepsis. The mechanisms

involve: 1) increased expression of adhesion molecules, loss of tight junction proteins and increased vascular permeability; 2) promoted the infiltration of inflammatory cells into the myocardium and inflammatory cytokine production.

Further, to investigate the precise mechanisms HSPA12B deficiency induced endothelial cell dysfunction and enhanced inflammatory response, we examined the role of exosomes in sepsis-induced cardiovascular dysfunction in HSPA12B deficient mice. Exosomes are cell-derived vesicles that are present in many biological fluids, including blood and urine. The diameter of exosomes is between 30 and 100 nm. Exosomes have been demonstrated to play a critical role in cell-cell communication. Exosome-mediated transfer of microRNAs (MiRNAs) is a novel mechanism of genetic exchange between cells. We found that HSPA12B exosomes isolated from septic mice induced more expression of adhesion molecules on endothelial cells and inflammatory responses in macrophages compared with wild type exosomes from septic mice. We also found that the levels of miR-126 in serum exosomes were significantly lower in HSPA12B deficient septic mice than that in wild type septic mice. MiR-126 has been reported to target the expression of adhesion molecules. We demonstrated that delivery of miR-126 in exosomes significantly improved cardiac function in sepsis via suppression of adhesion molecule expression, reduction of the infiltration of inflammatory cells into the myocardium, and preservation of endothelial function in HSPA12B deficient septic mice. Our finding suggests that HSPA12B is essential for endothelial function in sepsis. MiR-126 in exosomes plays a critical role for the cardiovascular protective effect of endothelial HSAP12B in sepsis.

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