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Roles of Endothelial Cell Heat Shock Protein A12B and β-glucan, a reagent for trained Immunity in the Regulation of Inflammation in Sepsis

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by

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ABSTRACT

Roles of Endothelial Cell Heat Shock Protein A12B and β-glucan, a Reagent for Trained Immunity, in the Regulation of Inflammation in Sepsis

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Sepsis is dysregulated host immune response to infection causing life-threatening organ dysfunction. Endothelial cell dysfunction and uncontrolled inflammatory responses are two contributors for sepsis-induced mortality. The crosstalk between endothelial and immune cells plays a critical role in the pathophysiology of sepsis. Therefore, understanding the mechanism of interaction between endothelial and immune cells will provide novel information to develop therapeutic strategies for sepsis.

Pathogen associated moleculear patterns (PAMPs) and/or damage associated molecular patterns (DAMPs) produced during sepsis, activate endothelial cells to increase the expression of adhesion molecules, attracting immune cell infiltration into the tissues. Uncontrolled inflammatory responses during the early phase of sepsis contribute to organ failure and lethality. Over 100 clinical trials, targeting inflammatory responses in sepsis, have failed in the past three decades. Thereby, developing novel therapeutic strategies for sepsis are urgent.

Heat shock protein A12B (HSPA12B), as one member of HSP70 family, predominately expressed in the endothelial cells, plays important roles in many pathophysiological processes. Currently, we observed endothelial cell specific

HSPA12B deficiency (HSPA12B$^{-/-}$) exacerbates mortality in sepsis induced by cecal ligation puncture (CLP). HSPA12B$^{-/-}$ septic mice exhibits increased expressions of adhesion molecule and infiltrated macrophages in the myocardium and activated macrophages in the peritoneal cavity. *In vitro* studies show that HSPA12B could be secreted from endothelial cells via exosome. HSPA12B carried by exosomes can be uptaken by macrophages to downregulate macrophage NF-κB activation and pro-inflammatory cytokine production.

Trained immunity, induced by β-glucan, causes immune memory in innate immune cells, with an altered response towards another challenge. We have found that mice received β-glucan seven days before CLP sepsis exhibit attenuated mortality with decreased pro-inflammatory responses. We found that β-glucan significantly increased the levels of HSPA12B in endothelial cells and endothelial exosomes. β-glucan induced endothelial exosomes markedly suppress macrophage NF-κB activation and pro-inflammatory responses.

The current data suggests that HSPA12B plays a novel role in the regulation of immune and inflammatory responses and that HSPA12B could be an important mediator for the crosstalk between endothelial cells and macrophages during sepsis. β-glucan regulates endothelial cell functions and immune/inflammatory responses, thus improving survival outcome in CLP sepsis.

DEDICATION

I dedicate my dissertation to my family members and friends, who support, encourage and assist me to overcome obstacles during Ph.D study. I express sincere gratitude to my loving wife, Jie Yin, whose words of encouragement ring in my ears. As for my son Andy Tu, he brings a lot of joy to assist me to get over troubles in work. I also appreciate my mother Guixiang Ye and my father Xuebin Tu for bringing me up with consistent caring.

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TABLE OF CONTENTS

LIST OF FIGURES

CHAPTER1. INTRODUCTION

Sepsis and Septic Cardiomyopathy

The History of Sepsis

The Previous Recognition of Sepsis. The word sepsis is originated from "decomposition" or "decay" in the Greek, which was first recorded in Homer's poems dated at around 2700 years ago.[1] In the following centuries, it also appeared in the works of Hippocrates (circa 400 BC) and Galen (129–199 AD).[2] In 1878, the germ theory of diseases was formally established by Pasteur, which resulted in the recognition that sepsis is related to detrimental microorganisms.[2] In 1914, a German Scientist, Hugo Schottmüller, proposed the first modern sepsis definition, writing that sepsis is present if a focus has developed from which pathogenic bacteria, constantly or periodically, invade the blood stream in such a way that this causes subjective and objective symptom.[3] During the majority of the 20th century, there were still tremendous challenges in diagnosing, treating and investigating sepsis due to no widely acceptable definition of sepsis worldwide.[4]

The Modern Definition of Sepsis. In 1991, an international conference hosted by the Society of Critical Care Medicine (SCCM) and The American College of Chest Physicians (ACCP) developed a consensus definition of sepsis that defined it as: systemic immune responses to infection, which was categorized as systemic inflammatory response syndrome (SIRS), severe sepsis and septic shock based on degree of severity.[5] The definition of sepsis was further refined to emphasis the role of organ dysfunction at the 2001 consensus

conference.[6] With advances in diagnostic approaches and a deeper understanding of basic science, the latest concept of sepsis was proposed by Merinoff Symposium in 2010, whereby sepsis is defined as the life-threatening organ dysfunction caused by host responses to infection. The new definition mainly focused on the role of organ dysfunction in sepsis. It was widely accepted by the Third International Consensus Conference in 2016.[7]

The Burden of Sepsis

Incidence of Sepsis Globally. Although the global burden of sepsis is hard to determine due to the various circumstance in global development, data from the World Health Organization (WHO) show that over 30 million people are affected by sepsis annually. Data from Europe presented around 41% mortality in septic patients. The data from a multicenter study in Australia and New Zealand reported that the mortality rates were still close to 20%.[8,9]. In Spain, the percentage of septic hospital admissions was markedly increased from 3.6% in 2000 to 5.8% in 2013. The percentage of sepsis-related deaths was significantly increased from 18.7% in 2000 to 29% in 2013 respectively.[10] A study from China reported that the incidence rate of sepsis in major cities was increased from 0.38% in 2003 to 0.49% in 2007 in males and from 0.40% in 2003 to 0.45% in 2007 in females respectively.[11] Thus, it is a worthy and urgent assignment for scientific community to investigate how to effectively diagnose and cure septic patients and substantially reduce mortality and the cost of sepsis worldwide.

The Burden of Sepsis in United States. A retrospective analysis of over 2.9 million adults admitted to US hospitals in 2014 indicated that there was no

significant reduction in the septic incidence and the survival outcome between 2009 and 2014.[12] Moreover, the current situation of sepsis in the United States is also a huge burden for the healthcare system. More than 1.6 million adult septic cases occurred each year, and 258,000 septic patients died annually, contributing to 1 out of 3 hospital deaths with more than $20 billion for the treatment of sepsis in 2011 alone.[13]

Pathophysiology and Sepsis

Organ Dysfunction in Sepsis. Clinical observations indicate multiple organ dysfunction syndrome (MODS) is more positively related with septic mortality, where mortality could range from 11% to 54% in septic patients with MODS.[14] Most recent studies have demonstrated that six major dysfunctions, including the cardiovascular, respiratory, renal, neurological, hematological and hepatic systems, are affected by sepsis.[15–20] However, the effect of sepsis on organs is not limited to a single organ, instead one organ dysfunction often causes another organ failure.[21] Consider the central role of the heart in the circulatory system that is able to provide the whole body with oxygen and nutrients and remove the metabolic waste. Cardiovascular failure caused by sepsis is capable of exacerbating other organ dysfunctions by causing tissue hypoxia, mitochondria dysfunction, dysregulated metabolism, and unbalanced physiological equilibrium. Thereby, there is no doubt that cardiovascular dysfunction is an important indicator for septic death, deserving close attention. [22]

Cardiomyopathy in Sepsis. Cardiomyopathy is defined as a myocardial disorder characterized by heart muscles that are an abnormal structurally and

functionally. The first report about septic cardiomyopathy in a patient was published in 1984, it has been regarded as a predictor or indicator for septic death, which was observed in over 40% of septic patients and in nearly 70% of septic mortality.[22,23] Although there is no diagnostic criteria for septic cardiomyopathy currently, three characteristics have been observed, increased end-diastolic volume, decreased ejection fraction and then a returned to normality by 7~10 days in the patients who survive.[24]

Mechanism of Cardiomyopathy in Sepsis

In the past, scientists have proposed two possible causes for the induction of septic cardiomyopathy. The first explanation was a result of animal experiments, which showed hypoxia in the myocardium caused less coronary blood flow.[25] However, Cunnion *et al* investigated this possibility using coronary sinus catheterization in patients, where the coronary flow was the same or greater than in the normal control.[26] This data excludes less coronary blood flow as the cause of septic cardiomyopathy. With a deeper understanding of septic cardiomyopathy, many myocardial depression factors have been discovered. Anthony *et al* found the depressant effects of endotoxin on the myocardium, and observed the reduction of left ventricular function in humans with endotoxin administration. [26,27] Following these studies, scientists have also demonstrated cytokines, including TNF-α and IL-1β, could mimic the poor cardiac functions caused by sepsis.[28–31] Other studies unveiled other mediators for myocardium depression in sepsis, such as energetic starvation[32], damage associated molecular patterns (DAMPs)[33], cell apoptosis[34], and calcium transport[35].

DAMPs are released from host cells as danger signals that activate immune cells, while pathogen associated molecular patterns (PAMPs) are expressed in the pathogens but not in the host cells. Although DAMPs and PAMPs are different, they are both recognized by shared pattern recognition receptors (PRRs), which are proteins located on the cell membrane or cell cytoplasm in different types of cells.[36] Although researchers have already realized that DAMPs/PAMPs could be the triggers for activation of innate immune and inflammatory responses, resulting in septic cardiomyopathy, the discovery of Toll-like receptors (TLRs) that could recognize DAMPs/PAMPs enabled scientists to understand those processes in sepsis.[37] Now, at least 10/11 different TLRs have been identified in the human and mouse.[38] TLR1, TLR2, TLR4, TLR5 and TLR6 are located on the cell surface, recognizing and binding various components of cell walls or membranes of pathogens, whereas TLR3, TLR7, TLR8, TLR9 and TLR13 are on the endosomes of cells and target self or no-self nucleic acid.[37] TLR4 is the receptor of endotoxin/LPS, which not only is expressed on immune cells, including macrophages[39], dendritic cells[40], and neutrophils[41], but is also identified on non-immune cells, such as endothelial cells[42], adipocytes[43] and myocytes[44]. It is widely accepted that infiltration of immune cells, such as the macrophage and the neutrophil, into the myocardium, resulted in cardiac dysfunction during sepsis.[45,46] The previous studies by our lab demonstrated that TLR3 exerts a deleterious role in sepsis-induced cardiac dysfunction, which was associated with activation of TLR4-medicated NF-κB and TRIF-dependent IFN signaling pathways as well as the upregulation of adhesion

molecules, such as VCAM1 and ICAM1, in the myocardium.[47] However, TLR9 activated by CpG-ODN attenuated cardiac dysfunction by activation of both PI3K/Akt and ERK signaling.[47] Besides other members of the TLR family, TLR3 and TLR4 areexpressed at a high level in the human umbilical vascular endothelial cells (HUVECs) and human microvascular endothelial cells (HMVECs).[48] LPS/endotoxin can also activate TLR4 on the endothelial cells to induce endothelial permeability, promoting the progression to cardiac dysfunction/failure.[49] Moreover, endothelial activation by TLR ligands leads to the upregulation of ICAM1 and VCAM1 expressions, which contributes to monocyte and neutrophil infiltration into the myocardium during sepsis.[50] Thus, it is necessary to understand well the roles of TLRs, immune cells, and endothelial cells in the cardiomyopathy caused by sepsis.

Role of Endothelial Cells in Sepsis

Functions of Endothelium

Endothelium composed of endothelial cells forms barriers by cell-cell junctions, such as adhesion junctions (AJ), gap junction (GJ) and tight junctions (TJ), between the circulation system and other tissues.[51] The endothelium also senses hemodynamic forces, regulate coagulation and hemostasis, and modulate immune cell patrolling on the surface of endothelium or infiltration through endothelium.[51] Endothelial cells widely distribute among organs. Therefore, the homeostasis of endothelium is crucial to maintain organ physiological function. As a first line of defense against internal and external

stimulators, including cytokines, chemokines, DAMPs/PAMPs, growth factor and others, the responses of endothelial cells are critical to the following responses.

Dysregulated Endothelial Cell Function during Sepsis

Glycocalyx shedding in sepsis. Since the latest definition of sepsis released in 2016, the contributions of endothelial dysfunction to multiple organ dysfunctions in sepsis have received more and more attention. The integrity of the endothelial cell lining is tightly regulated by the glycocalyx and the endothelial cytoskeleton, both of which can be disrupted during sepsis.[49] The glycocalyx consists of proteoglycans and glycoproteins, lining the luminal side of endothelium as a thick gel-like layer.[52] Glycocalyx has been shown to be shed from endothelium in the presence of oxidants, cytokines, and endotoxin in the circulation.[49] This shedding is highly related to the occurrence of many diseases, including sepsis. Glycocalyx has been found to modulate a variety of processes during sepsis, such as endothelial barrier function, immune cell adhesion, and inflammatory responses.[49] Eric et al found that glycocalyx shedding was caused by TNF-α in sepsis-induced acute lung injury, which was accompanied by neutrophil adhesion and inflammation.[53] With glycocalyx shedding, the endothelium is exposed to the circulating leukocytes and leukocyte released cytokines, which activate endothelial cells to express adhesion molecules, such as VCAM1, ICAM1 and selectins, to assist immune cell adhesion following transendothelial migration into the tissues.[49,54] In addition, shedding of glycocalyx also resulted in edema formation, which is a serious result of the loss of the barriers of endothelium and a predictor for organ failure in sepsis.[55]

Hemostasis and Endothelium in Sepsis. The initiation of sepsis not only induces dysregulated immune responses, but also uncontrolled hemostasis at different stages of sepsis.[56] The complicated hemostatic system is made of the endothelium, platelets, coagulation factors and leukocytes.[49] Tissue factor (TF) is a procoagulant transmembrane glycoprotein produced by endothelial cells under normal conditions.[49] It is induced by endotoxins, and cytokines, which form a complex with factor VIIa and ultimately promote final fibrin clot formation.[57–59] Moreover, endothelium regulates anticoagulation by the endothelial protein C receptor, by activating protein C to reduce factor V, factor VIII and PAI-1, which involves the regulation of fibrinolysis in the normal situation, and could be activated by inflammation. In the early stages of sepsis, pro-inflammatory responses induce hypocoagulation, resulting in vascular occlusion, which is a key inducer for organ failure.[60]However, with the development of sepsis, the situation of hypo-coagulation accompanies the consumption of platelets and coagulation factors in the later phase of sepsis.[61,62]

Inflamed Endothelium in Sepsis. Although endothelial cells are not considered as subsets of immune cells, PRRs have been observed to express on the endothelial cells, including TLRs, NOD-like receptors (NLRs) and RIG-I-like receptors (RLRs). These PRRS recognize PAMPs/DAMPs from different sources.[63,64] This information explains why endothelial cells could secret pro-inflammatory cytokines, such as TNF-α, IL-6 and IL-8, in the presence of microbial components.[65,66] Moreover, endothelial cells have also expressed MHC class I and class II.[67] Only antigen presenting cells (APCs) present MHC II

expression, indicating the capability of endothelial cells to initiate adaptive immune responses.[68] Due to all of this, endothelial cells have been regarded as conditional innate immune cells. PAMPs/DAMPs, during sterile or microbial sepsis, are capable of activating endothelial cells to express adhesion molecules, such as ICAM1, VCAM1 and E-selectin, and chemokines on the luminal side to assist leukocyte migration into the tissues. The cytokines released from endothelial cells not only recruit more monocytes and neutrophil infiltration to destroy invading microbes, but also induce ROS production by activating NADPH oxidases to modulate immune cell activation and kill pathogens.[69] If not quickly and effectively resolved, excess inflammation induced by endothelial cells can result in irreversible organ damage by loss of endothelial barrier function, increased ROS production and severe hypercoagulation.[69] The NF-κB signaling cascade in endothelial cells plays a critical role in the regulation of adhesion molecule expression, inflammatory responses, coagulation processes and endothelial barrier function.[70]

Role of Endothelial Cells in Cardiac Function. The heart is a complex organ consisting of several cell types, including cardiomyocytes, endothelial cells, fibroblasts, and diverse immune cells.[71] Among them, endothelial cells and cardiomyocytes represent the majority of cardiac cell types.[72] Considering the limited proliferation of cardiomyocytes, recent evidence highlights the key role of endothelial cells in cardiac repair/cardiac regeneration.[73] Endothelial related inflammation, coagulation and barrier function are critical to normal cardiac function.[33] The contribution of endothelial cells in neutrophil/monocyte infiltration

into the myocardium during sepsis is via the production of pro-inflammatory cytokines, such as TNF-α and IL-1β, to attract neutrophils/monocytes, as well as the increase of the expression of adhesion molecules, such as ICAM1 and VCAM1, to assist neutrophil/monocyte migration.[74,46] Our lab's previous studies have demonstrated that accumulated neutrophil/monocyte infiltration in the myocardium plays an important role in sepsis-induced cardiac dysfunction.[75] In addition, increased ROS from endothelium has been found to induce cardiac dysfunction through dysfunctional isometric contractions of cardiac trabeculae in sepsis.[76] Due to the significant role of endothelial activation during sepsis, multi-center validation studies have demonstrated the positive associations between biomarkers of endothelial cell activation and sepsis severity, organ dysfunction and septic mortality.[77,78]

Heat Shock Proteins

Heat Shock proteins (HSPs) were evolutionarily conservative proteins in both prokaryotes and eukaryotes, whose induced expression were found in temperature-shocked Drosophila in 1964.[79,80] HSPs are a large group of proteins, consisting of several subfamilies, which are classified by their molecule weight. They are small HSPs (≤ 34KDa), HSP40 (35~ 45KDa), HSP70 (65~80KDa), HSP90 (81~99KDa) and HSP110 (≥ 100KDa).[81] The basic contribution of HSPs, in stressed and unstressed conditions, is based on their chaperone function, which maintains native protein structure, facilitates deformed protein refolding, and aids in protein translocation.[82] Apart from their responses to heat shock, HSPs can be induced by various stimulations, such as starvation[83], hypoxia[84],

infection[85], and inflammation[86]. Due to their widespread distribution in the body, they have been found to play significant roles in many physiological pathological processes, such as neurodevelopment[87], cell proliferation[88], metabolism[89], tumorigenesis[90], cardiovascular diseases[91], and sepsis[92].

Roles of HSPs in Inflammation and Sepsis

HSPs play protective roles within the intracellular environment, but present as immunomodulators in extracellular settings.[93] Intracellular HSP72[94], HSP70[95] and HSP90[36] are able to protect cells from stress-induced apoptosis by blocking caspase activation, and activating PI3K/Akt signal pathways. Among the HSP family, HSP70 and HSP90, as both intracellular and extracellular HSPs, modulate immune responses in immune cells with or without stimulations.[96–98] Increasing evidence indicates both the anti-inflammatory role of intracellular HSP70[99,100] and HSP90[101,102] by inhibiting NF-κB signal pathways, and the pro-inflammatory function of extracellular HSP70[103,104] and HSP90[101,105]. Schroeder *et al* discovered that endotoxin reduced HSP70 expression in peripheral blood mononuclear cells from severe septic patients with increased IL-6 expression, indicating the protective role of HSP70 in sepsis.[106] In addition, HSP70 has also been reported to ameliorate ROS, inflammatory responses in myeloid cells after lipoteichoic acid stimulation.[107] However, Silva's study found that reduced serum HSP70 levels are positively related with improved survival outcome in septic animals.[108] This clinical observation showed significantly increased serum HSP70 in non-survival septic patients compared with control and septic survival patients.[109] This conflicted function of HSP70 in sepsis indicates the complexity

of septic occurrence, such as the distinct responses in animals and humans under sepsis, different pathophysiological conditions among septic patients, and the diverse approaches for the treatments of sepsis.

Discovery of HSPA12B

In a study of the development of atherosclerotic lesions, Han *et al* discovered, for the first time, two new HSP70 family members by gene expression profiling, named HSPA12A and HSPA12B, which have an atypical HSP70 ATPase domain separated by spacer amino acids.[110] Both proteins are highly conserved in between human and mice with distinct tissue-specific expression patterns. In the following study, Han and his colleagues have demonstrated HSPA12B is predominantly expressed in the endothelial cells *in vivo* and *in vitro*, which is necessary for angiogenesis and endothelial cell migration.[111] Meanwhile, another group identified the HSPA12B orthologue in zebrafish also highly expresses in vasculature and *in vitro* endothelial cells, which contributes to zebrafish vascular development and endothelial cell migration required Akt phosphorylation.[112]

The Contributions of HSPA12B in Pathophysiology

Zhu *et al*[113], discovered, for the first time, HSPA12B plays a protective role in endotoxin-induced cardiac dysfunction. The mechanisms by which HSPA12B protected the myocardium from endotoxemia involve decreased the expression of adhesion molecules, such as ICAM1 and VCAM1, resulting in reducing leucocyte infiltration into the myocardium during endotoxemia. Moreover, HSPA12B attenuated endotoxin-induced vascular inflammation, by attenuating

increased iNOS and decreased eNOS expression, and NF-κB signaling in the myocardium. The activation of the PI3K/Akt signaling pathway is necessary for the protective role of HSPA12B in endotoxin-induced cardiac dysfunction. Ma *et al*[114] reported the protective role of HSPA12B in cerebral ischemia/reperfusion (I/R) injury, which is through preventing I/R-induced blood–brain-barrier (BBB) permeability and attenuating I/R-induced apoptosis. Other studies have also demonstrated the protective roles of HSPA12B in myocardium infarction by alleviating cardiac dysfunction and remodeling[115], in sepsis-induced lung injury by reducing neutrophil infiltration and apoptosis[116] and in recovery after ischemic stroke through an eNOS-dependent mechanism[117]. Wu *et al*[118] found HSPA12B in endothelial cells is able to prevent LPS-induced increase in pro-inflammatory cytokine production, such as TNF-α and IL-6, which accompany by reduction of adhesion molecules, such as ICAM1, VCAM1 and E-selectin. HSPA12B was surprisingly observed in the plasma of septic mice and septic patients. The level of HSPA12B in the plasma of septic patients is positively correlated with the outcome of severe septic patients, which indicates the potential biomarker of HSPA12B to predict the poor outcome of patients with severe sepsis. [119] In our current study, we investigated the mechanisms by which HSPA12B protect against sepsis induced endothelial dysfunction, which is a major cause of organ dysfunction during sepsis.

Immune Response/Inflammation in Sepsis

Immune Responses in Sepsis

In the last three decades, there have seen huge advances in the understanding of sepsis and in sepsis management clinically. However, the mortality induced by sepsis is still around 30% in the ICUs.[120,121] This is due to no FDA-approved drug for treatment of sepsis at the present. Although initially cytokine storm was thought to be responsible for sepsis induced mortality, this has now been disproven and it is not a viable therapeutic approach[122]. In addition, surviving patients over the initial pro-inflammatory stage develop a prolonged immunosuppressive stage, which causes poor responses to secondary challenges.[123,124] This biphasic effect provides a novel perspective on how the immune system responds during sepsis.

Hyperinflammation in Sepsis. Once sepsis occurs, the stimulators from pathogens, such as bacteria, fungi and virus, are recognized by PPRs on the innate immune cells and initiate immune responses. If there is no effective immune resolution in the first three days, hyperinflammation causes more than 70% of septic deaths.[123] In this hyperinflammatory phase, several intracellular signaling that regulates pro-inflammatory cytokine and chemokine productions in innate immune cells include mitogen activated protein kinases (MAPKs); signal transducers and activators of transcription (STAT); Janus Kinases (JAK) and nuclear factor κ-light-chain-enhancer of activated B cells (NF-κB).[125] Such extensive and sustaining inflammatory cytokine productions result in tissue

damage, cellular compromise, and molecular dysregulation, which induce multiple organ failure and its associated subsequent mortality.[3]

Immunosuppression in Sepsis. While hyperinflammation contributes to septic mortality, a host of recent scientific evidence and clinical observations indicate that immunosuppression plays a key role in sepsis mortality.[126–129] Moreover, epidemiological surveys display the highest morbidity of sepsis in older patients, who do not respond to immune challenges as strongly as the young.[130,131] Increased age in patients is also associated with raised septic mortality, which makes up the highest mortality in patients over 85 years old.[132–135] Previous studies have shown that resolution of the hyperinflammatory phase starts several days after the initial cytokine storm. However, recent evidence unveils the co-existence of anti-inflammatory responses and pro-inflammatory responses after sepsis initiation.[136] Clinical observation has discovered that there is a substantial loss of cells, including innate immune cells and adaptive cells, induced by apoptosis.[127,128] Also, research in pediatric patients dying of sepsis indicates apoptosis-induced loss of immune cells.[137,138] However, regulatory immune cells, such as T regulatory cells (Tregs)[139–141], and myeloid derived suppressor cells (MDSCs)[142], increase their populations during sepsis. Thus, the combination of decreased immune effort cells and increased regulatory immune cells results in the immunosuppressive situation in sepsis. The anti-apoptotic approach to halt the death of immune effector cells has been reported to result in increased survival outcome in the septic animal models.[143–145]

Cellular Defects in Sepsis

Neutrophils. are the primary innate immune cells in the first-time response to infection[146]. Neutrophils are rapidly activated and possess the ability to eradicate invading pathogens by phagocytosis[147]. In addition, ROS released from neutrophils boosts microorganism eradication upon reaching the infected areas.[148] It is evident that neutrophil extracellular traps (NETs)[149] released from neutrophils could eliminate a wide range of pathogens, which consist of DNA, cytoplasmic proteins and histones[150]. During sepsis, the short half-life of neutrophils is expanded with neutrophil dysfunction, which results from increased anti-apoptotic protein expression.[151] Several signal pathways have been reported to be involved in the resistance of apoptosis by increasing anti-apoptotic protein expression, such as MAPK pathways, and decreasing pro-apoptotic proteins in neutrophils, such as ERK pathways and PI3K pathways, which provide survival signaling in neutrophils with persistent inflammation.[152,153]

From clinical research, scientists discovered an increased percent of immature band-like neutrophils from bone marrow in the circulation of septic patients.[154] Further studies have demonstrated the deficits of immature band-like neutrophils in ROS production[155], cell migration[156,157], and bacterial clearance[158]. These observations of deficits in TLR2 signaling pathway[159], chemotaxis reduction[160] and immune senescence[161] contribute to neutrophil dysfunctions during sepsis. Multiple studies have indicated that combined use of immature neutrophil count with other biomarkers could be useful approach to diagnose the occurrence of sepsis.[162,163]

Monocyte/Macrophages. The diversity of macrophages in the tissues display a vital role in physiologic homeostasis throughout life.[164] Macrophages in the tissues were recognized predominately from monocytes that move into the tissues and then differentiated into macrophages, which have various names, such as kupffer cells in the liver, intestinal macrophages in the gut, osteoclasts in the bone, and Langerhans cells in the skin.[165] This concept of monocytes as precursors of macrophages in various tissues has been challenged by the recent discovery that tissue macrophages originate from precursors during early embryogenesis and have an ability to self-renew to maintain cell population.[166,167] This novel concept explains rather well how microglia cells in the brain originate from yolk sac precursors, but not from bone marrow derived monocytes. The reason why macrophages are important to sustain normal physiology is their contributions in the phagocytic activity of removing dead cells, as a major role of innate immunity, and in antigen-presentation in adaptive immunity. In addition, macrophages in different tissues are involved in distinct physiological processes, such as promoting bone generation by osteoblasts[168]; modulating synaptic maturation by microglia cells[169]; maintaining lung homeostasis by alveolar macrophages[170]; and orchestrating cardiac repair by cardiac macrophages[171].

The functions of macrophages in different tissues result from their plasticity during physiological or pathological progresses. Macrophages have been classified into classically activated or M1 macrophages and alternatively activated or M2 macrophages.[172] M1 macrophages preferentially express iNOS in the presence of either IFN-γ or IFN-γ associated with LPS and produce pro-

inflammatory cytokines, such as IL-1β, TNF-α, IL-6 and IL-23; while M2 macrophages markedly present arginase in the present of IL-4 and produce anti-inflammatory cytokines and growth factors, such as IL-10 and TGF-β.[173] Multiple transcription factors regulate M1/M2 polarization, such as interferon regulator factor (IRF), STATs, and suppressor of cytokine signaling (SOCS) proteins.[174] Macrophages are capable of switching towards M1 states via activation of the IRF/STAT1 pathway with the presence of IFNs and TLR agonists, or by skewing towards M2 states via activation of the IRF/STAT6 pathway with the presence of IL-4 and IL-13.[175] Macrophage polarization could also be induced by low levels of oxygen (hypoxia).[176] Hypoxia is involved in the regulation of macrophage polarization by two subsets of hypoxia-induced factor (HIF), HIF-1α and HIF-2α, whereby HIF-1α promotes M1 macrophage polarization and HIF-2α enhances M2 macrophage polarization.[177,178]

In the early stage of sepsis, macrophages, which are a major source of cytokines, are activated by PAMPs from invading pathogens, and differentiated into M1 macrophages to assist bacterial eradication or damaged tissue clearance and produce a mass of pro-inflammatory cytokines, such as IL-1β, TNF-α, IL-6 and IL-8.[179] Massive amounts of apoptosis factors produced by overactivation of macrophages in the early phase of sepsis, inversely promote macrophage apoptosis, which has been observed in human autopsies and animal septic models.[180,181] Survivors of the early phase of sepsis display an immunosuppressive phenotype with characteristics of M2 macrophages by simultaneously displaying both downregulated pro-inflammatory cytokine

productions, and upregulated anti-inflammatory cytokine productions.[182] Clinical observation found a deficit in producing proinflammatory cytokines in LPS-stimulated septic monocytes, described as “endotoxin tolerance”.[183] Chiara et al has shown that monocytes during endotoxin tolerance display an M2 skewed inflammation via inhibiting the NF-κB signal pathway by p50.[184] The immunosuppression of monocytes is associated with decreased HLA-DR expression on the monocytes, which correlates with septic mortality in patients.[185,186] Until now, no clear explanation of monocyte-associated endotoxin tolerance or immunosuppression in sepsis existed. However, current research in epigenetics highlight sepsis induced epigenetic modification in immune cells, which may provide potential targets to predict or diagnose the occurrence of sepsis.[187]

MDSCs. Myeloid-derived-suppressor cells (MDSCs) are immature myeloid cells that regulate the immune responses in many pathological processes, such as cancers[188,189], autoimmunity[190], and infectious diseases[191]. The major characteristic of MDSCs is derived from their immunosuppressive functions whereby they suppress the production of L-arginine, promote the secretion of anti-inflammatory mediators (IL-10, and TGF-β) and induce Tregs activation.[192] L-arginine, as the product of ROS and RNS, is a semi-essential amino acid to maintain the function of T cells[193] and natural killer (NK) cells[194]. IL-10 and TGF-β from MDSCs could polarize monocytes/macrophages and T cells into an M2 state and a Th2 state with immunosuppressive situations.[142,195] Myeloid differentiation primary response 88 (MyD88), nuclear factor I A (NFIA) and

glycoprotein 130 (GP130) have all been reported to modulate immunosuppressive functions of MDSCs by promoting MDSCs expansion during pathological conditions.[142,196,197]

Due to the immunosuppressive role of MDSCs, they are regarded to have dual roles in the different stage of sepsis. The protective role of MDSCs might be involved on the early stage of sepsis to dampen cytokine storm-induced mortality, but MDSCs operate negatively to sustain or prolong the immunosuppressive situation, then cause sequential persistent inflammation-immunosuppression and catabolism syndrome (PICS). Animal experiments exhibit different functions of MDSCs between early phase (3~5 days) and late phase (10 days) of the spleen of septic mice.[198] Early MDSCs stimulated with GM-CSF produce more inflammatory cytokines, while late MDSCs express more anti-inflammatory cytokines.[199] However, clinical studies highlight the deadly effects of MDSCs.[192] Clinical observations reveal that the level of MDSCs in the blood is positively related with nosocomial infections and mortality of septic patients.[200,201] Due to undetectable levels of MDSCs in healthy blood, MDSCs have been regarded as a promising biomarker to predict the outcome of septic patients. Inhibiting MDSC-related immunosuppression is an attractive approach for the treatment of sepsis.

Gamma delta T cells (γδ T cells). Based on the subsets of antigen receptor expression in the lymphocyte membrane, there are two types of T cells, αβ T cells and γδ T cells. Although γδ T cells are make up only a tenth of the total T cells in circulation, the majority of them locate in the mucosa to play a role in the first line of defense.[202,203] Different from αβ T cells, the antigen recognition

of γδ T cells does not depend on interaction between receptors and the major histocompatibility complex (MHC).[204] Additionally, γδ T cells were first obseved to recognize phosphorylated microbial metabolites and lipid-peptides.[205,206] Activated γδ T cells produce pro-inflammatory cytokines and chemokines, such as IFN-γ, IL-17, TNF-α, CCL3, and CCL-5.[207]

In animal models of sepsis, deficiency of γδ T cells correlates with increased mortality, which results from poor bacteria clearance, and increased proinflammatory cytokine production.[208,209] Clinical studies have also demonstrated the positive relationship between septic mortality and a diminished population of circulating γδ T cells.[210,211] The reduction of γδ T cells during sepsis is probably caused by apoptosis-induced cell death in the intestinal mucosa with a subsequent higher risk to secondary infections.[212]

Tregs. Regular T cells (Tregs) are a distinctive subset of helper T cells, which are involved in the suppression of immune responses to sustain homeostasis. Markers used to recognize Tregs are combinations of CD4, CD25, and FOXP3 in mice, though this same combination is hard to characterize Tregs in humans. However, the discovery of the methylation status of the Treg-specific demethylated region (TSDR) assists the identification of the “real” Tregs in humans[213], which is not an ideal tool to isolate Tregs. In the clinical study, the combination of $CD4^+CD25^+CD127^-$ is used to purify Tregs in humans.[214] Adoptive transfer of ex vivo $CD4^+CD25^+CD127^-$ Tregs in humans display protective roles in chronic graft versus host disease (GvHD) with their immunosuppressive functions.[215] There are two types of Tregs, natural Tregs

($CD4^+CD25^+$) and adaptive Tregs ($CD4^+D25^-$), natural Tregs being derived from thymus and adaptive Tregs being induced from antigen in the periphery. [216]

The suppressive function of Tregs is through a direct or an indirect way to regulate various immune cell subsets. Tregs have been found to suppress adaptive cell immunity by inhibiting T cell activation by cell interaction[217] and by dampening B cells, functioning as APC[218]. In addition, Tregs are able to polarize monocyte into M2 states, inhibit functions of dendritic cells by CTLA-4 and LAG-3 and suppress neutrophil inflammatory responses.[218] In a septic patient study, a mounting percentage of $CD4^+CD25^+CD127^-$ Tregs present at the early time of sepsis with decreased proliferative responses of blood mononuclear cells after stimulation.[140] Moreover, increased levels of Treg cells are also observed in septic animal experiments. Increased levels of biomarkers in Tregs from septic patients, such as FOXP3 and CTLA-4, indicate a higher possibility of death by a prolonged immunoparalysis.[219] An animal experiment to block Tregs with antibodies against CD4 and CD25 do not change mortality in the septic mice[220], and a similar result is also observed in another group by depleting CD25+ cells before CLP[221]. However, Heuer et al discovered the adaptive transfer of CD4+CD25+ cells in septic mice significantly improves survival.[222] This controversial result indicates the difficulty of consistent identification of Tregs, which would aid our understanding of the characteristics of Tregs during sepsis.

B cells. B cells are traditionally recognized by their ability to produce antibodies.[223] Current research has revealed the diverse functions of B cells, such as antigen presentation[224], and cytokine production[225]. It is evident that B

cells contribute to both innate and adaptive immunities. [226] Murine B cells have been observed to express all TLRs (TLR1-9), and TLR agonists could activate B cells to produce cytokines, including IL-6, IL-10, IL-12 and IFN-γ.[227] B1 cells, as a specific subset of B cells, is one of the major sources of IL-10, which could inhibit the phagocytic function of macrophages in vitro.[228]

From clinical observation, we see distinct patterns shown in the circulating B cells between septic survival and septic death, wherein a high percentage of CD80+ and CD95+ and a low percentage of CD23+ on B cells are effective indicators for mortality in septic patients.[229] Additionally, innate response activator (IRA) B cells, derived from B1a cells, are surprisingly observed to produce GM-CSF, which is thought to be produced in vivo by macrophages and T cells in some cases.[230] In mice, a deficiency of IRA B cells dampens bacterial eradication, elicits a cytokine storm, and causes septic shock.[230] In the following study, IRA B cells are reported to produce IL-3, promoting the production of monocytes and neutrophils for cytokine storms during sepsis. Moreover, the levels of IL-3 are positively related to mortality in the septic patients.[231] Considering both the higher morbidity of sepsis and reduction of IgM production in the elderly, it is necessary to better understand the role of B cells during sepsis.

Exosomes

Cell communication among cells in multi-cellular organisms depends on junctions connecting neighboring cells, or is performed through paracrine signaling which releases messenger molecules to affect neighboring cells in the short distance, while hormones are the main regulators to modulate the functions

of targeting cells in the long distance in both plants and animals. Extracellular vesicles (EVs) that are secreted from all cells are involved in cell communication, which are evolutionally conserved from bacteria and to human. [232–234] Based on the vesicle size, EVs have been classified as exosomes (50-150nm in diameter) and macrovesicles (50-1000nm, up to 10μm in some situations in diameter). [235]

The Discovery of Exosomes

In 1987, Johnstone *et al* discovered that exosomes, sized at ~50nm, could be harvested from reticulocytes with centrifugation at 100, 000x g for 90min. Exosomes were first thought to carry cellular waste during the maturation of reticulocytes to erythrocytes.[236] In 1996, Raposo *et al* discovered exosomes secreted from B lymphocytes contain MIIC (major histocompatibility complex [MHC] class II-enriched compartment) and could stimulate T cell responses.[237] This result indicates the biological function of exosomes among cell communications. In the following study, exosomes are observed to be secreted from dendritic cells that induce anti-tumour immune responses[238]. To date, exosome secretions have been found in most cell types, such as tumor cells[239], immune cells[240], stem cells[241], endothelial cells[242], epithelial cells[243] and neurons[244]. It is evident that the components of exosomes are various, including 4400 proteins[245], 194 lipids[246], 1639 mRNAs, and 764 miRNAs[247,248]. Considering the complex cargos and communicative roles of exosomes, research on exosomes provides valuable insights and therapeutic approaches for a variety of diseases.

Methods to Extract and Identify Exosomes

Due to our current knowledge of exosomes, they are identified based on size, morphology, flotation density and protein markers. Thus, approaches to extract and identify exosomes are derived from those mentioned characteristics.

Extractions of Exosomes

Ultracentrifugation. Ultracentrifugation is a conventional method to harvest exosomes, which are hard to acquire in a pure state due to the existence of macrovesicles, apoptotic vesicles and others.[249–251] Thus, density gradient ultracentrifugation is introduced to overcome partially the disadvantages of ultracentrifugation. The centrifugation begins at 230x g to remove cellular debris, then process to remove macrovesicles at 10,000x g, and finally end at 100,000x g to pellet exosomes.[252,253] Although the purest exosomes are harvested from density gradient ultracentrifugation, it is both time-consuming and labor-intensive as well as requiring expensive equipment.[254] Thereby, this method is not suitable for clinical samples.

Size-Based Filtration and Polymer Precipitation. Ultrafiltration is a sized based method to isolate exosomes, which does not need special equipment and is faster than ultracentrifugation. A large volume of cell medium is concentrated into a low volume of medium with the Centricon Plus-70 Centriifugal Filter device centrifuging at 3500x g at 4°C.[255] However, the limit of ultrafiltration is to separate contaminating proteins from exosomes.

Polymer precipitation is a simple, and rapid way to precipitate exosomes in the “polymer nets” by low-speed centrifugation. The polyethylene glycol (PEG) based method inherits the same mechanism to harvest exosomes.[256] Cell medium or biological fluid is centrifuged at 2000x g for 30min to remove cellular debris, followed by the medium supernatant mixed with PEG reagent overnight prior to being centrifuged 3000x g for 30min. Finally, pellets are suspended with PBS. The limit of the PEG-based method is co-precipitation of non-vesicular contaminants, such as lipoproteins and PEG.

Immune-Affinity Purification. This method is based on the protein-protein interaction that a large quantities of proteins expressed on the exosome membrane, as antigens, bind with specific antibodies.[257] Immuno-affinity based techniques take advantage of membrane-bound biomarkers, not mixed with soluble counterparts, to purify exosomes from a complex source.[258] Since there are different levels of various markers on the exosomes, individual antibody is used to isolate exosomes from a specific cell type.[259] Chang et al developed $CD34^+$-coating magnetic microbeads to isolate only $CD34^+$ exosomes from acute myeloid leukemia, which contains unique marker, CD34.[260] The ELISA-based method is another useful approach to harvest exosomes by different antibodies. The quality and quantity of exosomes captured by the ELISA-based method are comparable to the production of the ultracentrifugation. To enhance the yields of exosomes, submicron-sized magnetic particles are created to produce 10 to 15 times higher levels of exosomes than ultracentrifugation.[261] The advantage of the immune-affinity approach that it is without volume limitations. However,

properties of exosomes are not consistent for the different markers used in the exosome isolation.

Microfluidics-Based Isolation. To overcome the current challenge of isolating exosomes in the clinical practice, microfluidics-based technologies have been developed to isolate, detect and analyze exosomes, based on both physical and biochemical characteristics. This method not only takes advantage of the size, density and immunoaffinity of exosomes, but also incorporates other novel sorting mechanisms, such as acoustic, electrophoretic and electromagnetic settings; nano-based traps; and viscoelastic flow.[262–264] Wang et al developed a microfluidic structure to separate exosomes from proteins, other extracellular vesicles and cellular debris, using ciliated nanowires to trap exosomes with 40~100nm diameters.[265] Kanwar et al created a simple and low-cost platform, called “Exochip”, to capture specific exosomes in the circulation, which could be regarded as biomarkers to diagnose the occurrence of cancers.[266] These cutting-edge techniques provide promising approaches to exosome isolation for point-of-care and clinical applications.

Characterizations of Exosomes

Transmission Electron Microscopy. As the gold standard to measure exosomes, transmission electron microscopy (TEM) is used to qualify and count exosomes. There are still several deficits in counting exosomes by TEM, which is time-consuming, labor-intensive, and low-yield.[267] For the complex processes of sample preparation, the electron beam could damage the exosome biological features and cause damage in shape.[268] Modifications on standard TEM, cyro-

EM has been developed to the damage during the sample preparation without influencing dehydration and fixation for TEM.[269,270]

Nanoparticle Tracking Analysis. Based on examining the size of exosomes, nanoparticle tracking analysis (NTA) was created to scale the concentration and size distribution of EVs ranging from 10nm to 2000nm in diameter.[271] NTA utilizes Brownian motion of nanoparticles in a fluid to track the movement of exosomes. The outcomes of NTA are the size, size distribution, concentration, and phenotype of EVs. In addition, NTA combing with fluorescence could be used to analyze conjugated quantum dots. The advantage of utilizing NTA is timesaving within minutes and to maintain the native situation of EVs.

Dynamic Light Scattering. Dynamic light scattering (DLS) is a technique to measure the size of exosomes based on the scattering of a laser beam.[272] DLS is able to record the intensity of the scattered light as a function of time by observing the movement of particles in a liquid suspension. The advantage of DLS is that it is capable of measuring EVs sized from 1nm to 6000nm. However, there are some shortcomings by DLS as well.[272] First, DLS could not provide visualized results of exosomes. Second, due to the characteristics of scattering light, even a small amount of large particles could influence the accuracy of detection.[273,274] Third, DLS is not able to display any biochemical information of EVs. The application of DLS has been used to access EVs from red cells and cancer cells.[275,276]

Flow Cytometry. The basic mechanism of conventional flow cytometry is to measure particles over 300nm in diameter, and is without the ability to detect exosomes due to the size limitation.[277] However, a modified flow cytometer with higher-sensitivity forward scatter detection could detect exosomes labelled with fluorescent dyes from background signalings.[278,279] The new generation of flow cytometers is able to detect exosomes with a higher EV resolution by multiple angles by forward scatter light detection.[280] Due to the wide availability of flow cytometers in clinical settings, they possess the capability of quick and reproducible detection of exosomes for diagnosis.

The Biogenesis of Exosomes

To date, two distinct mechanisms are involved in the biogenesis of exosomes. The first one depends on the endosomal sorting complex required for transport (ESCRT) function.[281] Different subsets of ESCRTs, such as ESCRT-0, ESCRT-I, ESCRT-II, and ESCRT-III are involved in the process of exosome formation.[282] ESCRT-0 recognizes and leads the cytoplasmic proteins to the specific domain of the endosomal membrane before interacting with ESCRT-I/ESCRT-II complex to initiate the multi-vesicular bodies (MVB) formation with ESCRT-III that promote the development of budding. Finally, ATPase VPS4 protein promotes the membrane scission to release exosomes.[282] However, several studies with depletion of subsets of ESCRTs could not totally block the production of exosomes.[283,284] Alternative mechanism has been proposed, which is based on lipid raft-related exosome segregation from the endosomal membrane.[285] Researchers observe that the depletion of sphingomyelinase,

which regulates formation of ceramide, results in the reduction of proteolipid protein via exosomes. However, replenishment of sphingomyelinase recovers the production of exosomes. Additionally, the presence of ceramide is necessary for the secretion of miRNAs in exosomes, which are ESCRT-independent.[286]

The Roles of Exosomes in the Endothelial Function

Exosomes targeting Endothelial Cells.

Exosomes from other cells could be uptaken by endothelial cells, which regulate the endothelial functions. It is evident that miRNAs, one subset of non-coding RNAs, are involved in the regulation of cellular processes.[287] miRNAs, as key components of exosomes, have been reported to modulate the various functions of endothelial cells. MiR-92a secreted from leukemia cells, which decreases integrin α5 expression, promotes cell migration and tube formation, but not angiogenesis in endothelial cells via exosomes[288], while miR-210 from hypoxic leukemia cells is also observed to improve the tube formation of endothelial cells by downregulating target gene (EFNA3).[289] However, miR-320 in exosomes from diabetic or higher-glucose treated cardiomyocytes impairs endothelial angiogenesis by downregulating the target genes(IGF-1, Hsp20 and Ets2) in mouse cardiac endothelial cells [290], and miR-125a in exosomes of mesenchymal stem cells is capable of inhibiting the angiogenesis of endothelial cells by suppressing the production of angiogenic inhibitor delta-like 4 (DLL4)[291]. Recently, long non-coding RNAs (lncRNAs), another subset of non-coding RNAs, have been discovered to play roles in the physiological conditions[292,293], and were observed in exosomes[294]. CD90+ liver cancer cells secret exosomes with lncRNA H19 to

affect VEGF and ICAM1 expression in endothelial cell and tube formation to promote interaction between cancer cells and endothelial cells.[295]

Moreover, exosomes modulate endothelial inflammatory responses too. Macrophages under hypertensive condition could secret exosomes with increased levels of CD68, a macrophage marker, and induce inflammatory responses in endothelial cells with increased productions of ICAM and plasminogen activator inhibitor-1 (PAI-1).[296] Mast cell derived exosomes are also able to activate endothelial inflammation by promoting PAI-1 expression in the endothelial cells.[297] Mature dendritic cell derived exosomes are reported to activate NF-κB related inflammation in endothelial cells, triggered by TNF-α on the membrane of exosomes.[298]

Exosomes from Endothelial Cells. Since there is wide distribution of endothelial cells in the body, EVs from endothelial cells contribute to a large part of circulating microparticles. [299] Exosomes from endothelial cells could target both other cells and themselves to alter cell functions. In a co-culture system, researchers found exosomes from endothelial cells, incubating with cardiomyocytes for 45mins followed by ischemia/reperfusion stimulation, induced cardioprotective function through the ERK1/2 MAPK signaling pathway, which may partly explain the benefits of ischemic preconditioning (short, non-lethal periods of ischemia and reperfusion to protect heart from subsequent ischemia and reperfusion injury) induced higher level of exosomes from rat heart to cardiomyocytes in vivo.[242] MiR-214 in endothelial exosomes promotes angiogenesis and migration of endothelial cells in vivo and in vitro with

suppression of ataxia telangiectasia mutated (ATM) expression in recipient cells nearby.[300] Halkein et al reported 16-kDa prolactin increased miR-146a expression in endothelial cells, which could be exported into exosomes captured by cardiomyocytes and modify the metabolic situation of cardiomyocytes with decreased expressions of Erbb4, Notch1, and Irak1.[301] Endothelial cell derived exosomes are involved in the regulation of functions of immune cells. Song et al observed that exosomes carrying integrin αvβ6 from cardiac endothelial cells are capable of stimulating active TGF-β expression in B cells, which display a suppressive effect on the T cell proliferation.[302] The immunomodulation by exosomes from endothelial cells is discovered in monocytes, where miR-10a translocases from endothelial cells into monocytes targeting several members of the NF-κB signaling pathway to reduce inflammatory responses of monocytes in vivo and in vitro.[303] Furthermore, another group demonstrated endothelial cell derived exosomes have ability to polarize macrophages into M2 state with anti-inflammatory responses.[304] The above information reminds us of the important roles of exosomes from endothelial cells in the regulation of functions in other cells.

The Roles of Exosomes in Inflammation

The contributions of exosomes in the regulation of inflammation are diverse for their different sources and settings.

Anti-Inflammatory Roles of Exosomes. Exosomes from various cancer cells have been reported to downregulate inflammatory responses of immune cells. Clayton et al presented exosomes from different cancers, such as bladder,

colorectal and prostate, present inhibitory functions on T cells, which are CD73 or CD39 dependent.[305] Exosomes from nasopharyngeal carcinoma (NPC) display the capability to recruit Treg cells in mouse model and promote the conversion of $CD4^+CD25^-$ T cells into $CD4^+CD25^+$ cells with immunosuppressive functions to bolster immune evasion.[305] In addition, reduced cytotoxicity of NK cells are induced by exosomes loading MICA*008 from Hela cells.[306] Due to the contributions of tumor-associated macrophages (TAMs) in tumor development, recent study unveiled exosomes, derived from gastric cancer, educate a generation of TAMs, which produce anti-inflammatory cytokines and dampen function of CD8+ T cells with a result of gastric cancer progression and low survival outcome of patients.[307]

Pro-Inflammatory Roles of Exosomes. Tumor derived HSP70/HSP90 have been observed in the circulation of cancer cachexia in mice. Both HSPs could be released into exosomes from tumors and work as DAMPs to induce the NF-κB signaling pathway by activating TLR4 on muscle cells.[308] Increased levels of HSP70 in exosomes from bronchoalveolar lavage fluid (BALF) of patients with sarcoidosis could activate peripheral blood mononuclear cells (PBMCs), with elevated IFN-γ and IL-13 production, and induce epithelial cells to produce more IL-8, which indicate the pro-inflammatory roles of BALF exosomes in sarcoidosis.[307] Additionally, immunomodulatory miRs in the exosomes are reported to modulate inflammation. MiR-21 and miR-29a in tumor-secreted exosomes display pro-inflammatory regulation on the recipient immune cells by binding TLR, such as human TLR8, to activate NF-κB signaling to produce

prometastatic inflammatory cytokines.[309] Induced production of exosomes from hepatocytes treated with alcohol are reported in humans and in mice; and increased levels of miR-122 in exosomes of alcohol-treated hepatocytes sensitize monocytes to LPS stimulation with increased pro-inflammatory cytokine production.[310]

The Roles of Exosomes in the Sepsis

Clinical observations show increased populations of plasma exosomes[311,312], and plasma exosomes during CLP sepsis, present smaller sizes but higher numbers, compared with exosomes from sham mice[313]. Both clinical research and basic studies indicate the important roles of exosomes in the progress of sepsis. However, the specific contribution of exosomes in sepsis is still debated. Exosomes from platelets in septic patients play important roles in the development of cardiomyopathy dysfunction, where platelet-derived exosomes contain NOS to boost production of NO in the myocardium.[314,315] Moreover, Essandoh et al reported that suppression of exosome generation by its inhibitor, GW4869, could significantly improve survival outcome in CLP sepsis mice and endotoxin mice with reduced inflammatory responses and alleviated cardiac dysfunction caused by sepsis.[316]

On the contrary, Gao et al observed that exogenous exosomes from septic mice improve survival outcome of septic mice with reduced immune responses.[317] Wang et al discovered the cardioprotective role of exosomes from mesenchymal stem cells (MSCs) administrated in septic mice exhibiting improved survival outcome requires miR-233 that is exported from MSCs

downregulating expression of Sema3A and Stat3 in cardiomyocytes.[318] MiR-146a released from MSCs by exosomes has been reported to polarize macrophages into an M2 state with attenuated septic death in mice.[319] Miksa et al pointed out that milk fat globule epidermal growth factor-factor VIII (MFG-E8) in exosomes from bone marrow derived dendritic cells (BMDCs) alleviates sepsis-induced death by reducing cell apoptosis and inflammatory responses.[320]

The inverse functions of exosomes in sepsis indicate the complicated mechanism of exosomes in the development of sepsis. One reasonable explanation is that exogenous and endogenous sources of exosomes hold different roles during sepsis.

The Potential of exosomes for the diagnosis and treatment of Sepsis

To date, there is no ideal biomarker for diagnosis of sepsis in the clinical practice.[321] Considering the alteration of components in exosomes from septic patients, they have been regarded as potential biomarkers to diagnose and predict the progress of sepsis. Septic animal experiments show increased levels of serum miRNAs, such as miR-16, miR-17, miR-20a, miR-20b and miR-26a, which present a time-dependent mode.[322] Plus, there is a highly positive relationship between specific miRNAs, such as miR-15a and miR-27a, and the development of endothelial dysfunction in septic patients.[323] However, further studies are required to determine various miRNAs in exosomes to indicate the different stages of sepsis. Aside from exosomes as biomarkers for sepsis, exosomes, as drug carriers, are potential tools for the treatment of sepsis.

Trained Immunity

Traditionally, the immune system consists of innate and adaptive immunity. The specific characteristics of adaptive immunity are its immunological memory and specificity for quick response to secondary stimulation, which is not found in the innate immunity. However, mounting evidence indicates innate immune cells can be trained such that they will exhibit immunological memory to secondary stimulation, which is called trained immunity.

Discovery of Trained Immunity

Studies in Plants. Studies in the natural protection of plants revealed an interesting phenomenon that plants possess resistance against a broad spectrum of microorganisms after inoculating with weaken pathogens. This observation called “systemic acquired resistance” (SAR), was published in 1933.[324] Following studies in the past decades lead us to conclude SAR in plants is the counterpart of immunization in vertebrate and indicate plants have innate immune memory, which is well summarized in several reviews.[325,326]

Studies in Invertebrates. Because of the deficit of the counterparts of adaptive cells found in vertebrates in the invertebrate organisms, researchers are interested in how they respond quickly to secondary stimulation without immune memory. A reasonable explanation for this phenomenon is that there are other mechanisms to execute immunological function. Thus, organisms in the invertebrate kingdom provide ideal animal models to investigate whether there is immunological memory in the innate immune responses for the secondary challenge.[327] Studies in Drosophila reveal a prolonged antimicrobial pattern after

a pathogenic stimulation, and a broad spectrum of resistance to other secondary infections present in several conditions.[328–330] Pham et al showed that drosophila with a sublethal dose of *S. pneumoniae* could resist to a following lethal challenge, which is required for activation of the TLR signal pathway.[330] Apart from Drosophila, other invertebrates, such as shrimps and copepods, are also found to possess immunological memory for secondary and heterogeneous stimulations.[331,332]

Studies in Vertebrates. The existence of an immunological memory of innate immunity in plants and invertebrates hints the possibility of innate immune memory in vertebrates. In 1931, a report in Sweden was published that showns the vaccines, Bacillus Calmette-Guerin (BCG), not only protect infants against Mycobacterium tuberculosis (*M. tuberculosis*) but also produce unintended results of decreasing death in infants.[333] This reduction of mortality in infants with BCG was also reported in the following years.[334] Besides the off-targets of BCG on infection, smallpox vaccines offer protection against leprosy.[335] In animal experiments, mice with BCG display resistance to secondary challenges with *Candida albicans* (*C. albicans*) or *Schistosoma mansoni*. This resistance is through a T cell–independent mechanism,[336] but requires activation of tissue macrophages[337]. Further experiments within athymic mice show that protection against *Staphylococcus aureus* is induced in mice injected with attenuated strains of *C. albicans*, which indicate this cross-protection is not a T cell-dependent mode.[338] An unexpected experimental result in the Netea Lab records monocytes, from donators vaccinated with BCG, against *C. albicans* infections by

increasing expression of IL-1β and TNF. Further study demonstrates that β-1, 3-(D)-glucan (β-glucan), a major cell wall component of *C. albicans*, contributes to the non-specific protection of monocytes against secondary stimulation.[339] Combining these results with other published researches about non-specific adaptation of innate cells, Dr. Netea proposed a novel concept called “trained immunity”, where innate immune cells could be trained with specific agents to produce non-specific memory against secondary infections. This novel concept has prompted scientists around the world to rethink the role of innate immunity.

Trained Immunity in Immune Cells

NK cells. NK cells are grouped as members of innate immunity, although they work like CD8+ cytotoxic T cells, in adaptive immunity, and share a similar feature in cytokine production.[340] During an infection, T cells, classic adaptive cells, undergo four phases: expansion phase[341], contraction phase[342], memory maintenance phase[343] and recall phase[344], to complete a whole cycle of immune response. There were only first two phases recognized in NK cells. However, in 2009, Sun et al reported that NK cells also have the ability to shape memory about infection for a quick response to secondary challenge.[345] In this study, “memory” NK cells could even be detected at 70 days after infection, the cells being activated by higher levels of IFN-γ transcripts than those found in naïve NK cells. Neonatal mice without mature NK cells are transferred with naïve and “memory” NK cells prior to infection. Neonates with “memory” NK cells present significant improvement in survival compared to that in neonates given by naïve NK cells. The following study by Dr. Netea group identifies BCG vaccination

could induce trained immunity in NK cells with increased IL-1β, IL-6 and TNF under *M. tuberculosis* stimulation. BCG vaccination significantly improves survival outcome of SCID mice, which are partially NK cell dependent.[346] An excellent review by Cerwenka and colleagues summarizes the immunological memory of NK cells in various pathological conditions.[347]

Innate Lymphoid Cells (ILCs). ILCs share the same lymphoid progenitors with B cells and T cells, working analogous to CD4+ T helper cells, but respond quickly to infection like innate immune cells and do not express antigen receptors on T cells and B cells.[348] Thus, ILCs have been regarded as intermediates or bridges between innate immunity and adaptive immunity.[349] When Halim et al investigated the differentiation of Th2 cells under allergen stimulation, they surprisingly found that ILC2s located in the lung produce cytokines, such as IL-5 and IL-13, and parts of the ILC2s, after allergy, stay on the lung and lymph node.[350] In 2016, further study from the same group demonstrates that allergen-experienced ILC2s are capable of memorizing the allergen to respond quickly to a second one. Moreover, *Aspergillus* stimulated ILC2s with another papain challenge 3.5 months later, displayed an increased population of IL-5^{+}IL-13^{+} ILC2s in the lungs with mounting levels of IL-5 and IL-13, which indicate that non-specific memory is built in ILC2s after stimulation.[351]

Monocytes/Macrophages. Although several studies about the role of macrophages in acquired immunological memory have been published 30 years ago[337,338], this interesting feature of macrophages has not attracted enough attention from other scientists. Not until Netea and colleagues published a series

of papers about monocytes with non-specific memory, more related studies emerge.[352-353] Dr. Netea in 2011, for the first time, named immunological memory of innate cells as “trained immunity”.[333] His group was also the first to propose an in-vitro model to test trained immunity in monocytes, where monocytes are incubated with unlethal *C. albicans* for 24 hours, after resting for 6 days, prior to any other stimulations, such as PRRs, ligands or bacteria, for 24 hours. This study indicates that monocytes incubated with *C. albicans* or β-glucan could markedly express pro-inflammatory cytokines, such as TNF-α and IL-6, in monocytes with stimulation of TLR ligands, such as LPS, P3C and Pam3, later. Training of monocytes is requiring activation of the dectin-1/ Raf-1 pathway, whereby dectin-1 is the receptor of β-glucan on the monocytes.[339] In the following publication, research from Netea’s lab presents that BCG vaccination-induced non-specific protection, in newborns facing other infections, is macrophage-dependent.[352] Apart from increased pro-inflammatory cytokine productions, from peripheral blood mononuclear cells (PBMCs) vaccinated with BCG after various pathogen challenges, these increased cytokine productions could be detected in the PBMCs of volunteers with BCG vaccination after 3 months. Regulation of monocytes by BCG is through NOD2, but not through TLRs or dectin-1.[354] Further study indicates that BCG-induced metabolic, skewed towards glycolysis, is essential for trained immunity in monocytes, which is regulated by the Akt-mTOR signaling pathway.[353] Besides BCG primed trained immunity in monocytes, β-glucan has been reported to contribute to induction of trained immunity in monocytes. β-glucan pre-treated macrophages exhibit the protective function

against *L. braziliensis* infection, with increased IL-6 and IL-10 productions, and β-glucan-induced trained immunity is IL-32 dependent.[355] Additionally, β-glucan primed monocytes undergo a metabolic switch, where glycolysis, glutaminolysis and cholesterol synthesis are crucial for the induction of trained immunity in monocytes.[356] β-glucan related glycolysis in monocytes is modulated by the dectin1-Akt-mTOR-HIF-α signaling pathway, which is demonstrated by abrogated trained immunity in monocytes from dectin-1 deficient patients.[357] Moreover, LPS-priming induced immunological tolerance could be partly reversed by β-glucan in vivo and in vitro, whereby β-glucan-induced tolerant reversion in monocytes is through modulation of transcriptional responses at epigenetic levels.[358] Further study in Netea and colleagues showed that β-glucan reverses the reduction of succinate dehydrogenase (SDH) in LPS-induced tolerance, which contributes to trained immunity in monocytes. β-glucan induced trained immunity enhances histone acetylation, to promote SDH production, by maintaining metabolic status and boosting pro-inflammatory cytokine production in monocytes.[359] This demonstrates β-glucan in the regulation of immunometabolism is essential for trained immunity in monocytes against LPS-induced tolerance.

Trained Immunity in Non-Immune Cells

Stem Cells. Mesenchymal stem cells(MSCs) could work like immune cells, which express TLRs[360] and produce pro-inflammatory cytokines, or anti-inflammatory cytokines, in distinct settings [361]. Liu et al, for the first time, report trained immunity in MSCs.[362] In their study, adipose-derived MSCs (AD-MSCs),

stimulated with LPS or TNF-α, display increased expression of MCP-1, IL-8 and IL-6, as well as quick cytokine expression, in the secondary stimulation. Interestingly, LPS primed AD-MSCs show better therapeutic effect on skin flap survival in diabetic rats than the counterpart in rats with unprimed AD-MSCs. However, the time range of immunological memory shown in AD-MSCs is just 7 days, while the trained immunity in monocytes could persist for several months and even years.[362,363] Moreover, Kanfmann et al observed an interesting phenomena, that BCG vaccine could arrive in bone marrow and persist for several months, and that BCG trained hematopoietic stem cells (HSCs) prefer to polarize towards myeloid lineage, to generate trained monocytes/macrophages, with increased pro-inflammatory cytokine production against *M. tuberculosis* infection.[364] Since the life span of circulating monocytes is relatively short, and monocytes are differentiated from bone marrow, Kanfmann's findings well may explain why immunological memory in monocytes could persist for months and even years. Additionally, Mitroulis et al reported that β-glucan, a trained immunity agonist, could promote myelopoiesis with increased populations of hematopoietic progenitors (LSKs) and multipotent progenitors (MPPs) in bone marrow. Increased MPPs stimulated by β-glucan exhibit a switch toward the myeloid subset. Mice challenged with LPS 28 days prior to β-glucan administration display markedly increased hematopoietic progenitor expansion, including LSKs and MPPs with reducing LPS-induced DNA damage. β-glucan related protection also relieves chemotherapy-induced myelosuppression with increased numbers of white blood cells and granulocytes in the circulation.[365]

Fibroblasts. Fibroblasts are widely distributed in all tissues and exhibit various morphologies with different functions, such as contractility[366], locomotion[367], collagen production[368], and regulation of the extracellular matrix[368]. Interestingly, fibroblasts are also reported to be involved in the regulation of inflammation, whereby they serve as sentinels, to modulate leucocyte extravasation by releasing cytokines in pathological conditions.[369,370] In addition, fibroblasts express all TLRs and produce pro-inflammatory cytokines and chemokines.[371] Fibroblasts can be considered as immune-like cells, to some extent. Several publications point out that fibroblasts possess immunological-like memory. Ara et al, while investigating periodontal disease, found that gingival fibroblasts could persist to produce pro-inflammatory cytokines with successive LPS challenges for 7 days, whereas macrophages undergo LPS tolerance.[372] Moreover, fibroblasts in tendinopathy exhibit a feature of persistent activation 2-4 years after treatments, and produce more activated markers under IL-1β re-stimulation, with increased IL-6 and IL-8 productions, compared with the counterpart from fibroblasts of healthy donators.[373] In condition of rheumatoid arthritis (RA), Fibroblast-like synoviocytes (FLS) exposed to TNF-α pretreatment for 3 days are more sensitive to secondary IFN-β or IFN-γ stimulation with 1 day gap with increased cxcl-10 expression. This inflammatory activation could be sustained even the gap time is extended to 3 days.[374] The same group also found that the memory feature of FLS from joints in both RA and healthy patients, which indicate the immunological memory in FLS is induced and may play an important role in the development of chronic joint diseases.[375]

Epithelial Cells. Like fibroblasts, epithelial cells express most TLRs, and work as an interface between the external and internal milieu and fight as a first line to external invading pathogens or other stimulations[376]. The important role of epithelial cells in the regulation of immune responses have been reviewed in these publications.[377–380] Currently, bronchial epithelial cells (BECs) display the memory feature against non-specific infections. Bigot et al performed experiments by utilizing BECs incubating with different PAMPs, such as flagellin, Pam3 and lipopeptide for 2 days followed 4 day resting prior to other PAMPs, including LPS, *A. fumigatus* and *S. maltophilia* respectively. BECs pre-stimulated with flagellin present increased levels in IL-6 and IL-8 expression with an *A. fumigatus* challenge, which exhibits a broad protection of BECs under different stimulation. However, BECs pre-stimulated with flagellin could display a tolerant situation, when re-stimulated by LPS. The immunological characteristics of BECs are related with epigenetic modification, which could be suppressed by inhibitors of histone acetyltransferase or histone methylation.[381] In addition, epithelial stem cells (EpSCs), exhibit inflammatory memory to respond quickly to later challenge. Naik and colleagues investigating how inflammation affect EpSCs, unexpectedly observed that EpSCs triggered with NALP3 agonist (imiquimod, IMQ) display features of faster wound healing after a secondary assault, whereas enhanced wound healing could also be obtained by EpSCs with other pre-stimulations, such as *C. albicans* (to induce infection), vitamin D analog MC903 (to mimic atopic dermatitis) and 12-o-tetradecanoylphorbol 12- acetate (TPA) (to induce hyperplasia). Further study demonstrates improved wound healing is

macrophage-independent and T cell-independent, and inflammatory memory of EpSCs is only restricted in the treatment area, not transmitted to other sites of body[382].

Mechanisms of Trained Immunity

Mechanism of Epigenetic Regulation.

Epigenetic dynamics influence gene expression and modify cellular functions without altering the DNA sequence, such as DNA methylation, and histone modification. Environmental factors (nutrients, toxins, infections and hypoxia) and pathological conditions contribute to epigenetic modification, which is reversible and inheritable for multiple generations of cells[383,384]. Gene expression is tightly controlled by the cooperation among transcription factors (TFs) and several regulatory elements in genes, such as promoters and enhancers.[385] Epigenetics is an important factor in the development of inflammatory responses in immune cells.[386]

DNA Methylation.

DNA methylation occurs at a cytosine nucleotide, forming 5-methylcytosine (5mc) immediately followed by a guanine nucleotide, which is referred as 5'-cytosine-phosphate-guanine-3' dinucleotide (CpGs). CpGs forms CpG islands, mostly located in the promoter of a gene transcription start site, which is associated with gene suppression.[387] On the one hand, 5mc is identified by CpG-binding proteins, which recruit other transcriptional repressors on the CpG in the promoters to silence gene expression at transcriptional levels.[388] On the other hand, 5mc could occur at other regulatory elements, such as enhancers and insulators, also be involved in the transcription elongation and

alternative slicing.[389] Current studies indicate that DNA methylation in different pathological conditions influence macrophage polarization.[390,391]

Histone Acetylation. Nucleosome, as the basic unit of chromatin, consists of 146 bp DNA sequence and octameric core histones that are composed of two H3 and H4 homodimers and two H2A/H2B heterodimers.[392] Unpackaged tails of H3 and H4 histones from the nucleosome could be modified to affect the interaction between DNA and histone proteins for transcriptional activation/inactivation.[393] Among the variety of histone modifications, acetylation at lysines (K), methylation at lysines (K) and arginines (R), have been widely investigated.[394] Histone acetylation is regulated by two distinct groups of enzymes, with histone acetyltransferases (HATs) contributing to the transfer of an acetyl group to histone lysine tails, and the histone deacetylases (HDACs), which lead to the removal of acetyl groups from histone lysine tails.[393] The detailed functions of HATs and HDACs are summarized in these reviews.[395,396] The acetyl groups added on lysine residues of histone tails, neutralize the positive charges of histones, which results in weak binding between histone and DNA and also the subsequent allowance of transcription.[393] Current studies have identified that K 9, 14, 18, 23 and 56 on histone H3 could be acetylated, and acetylation of K9[397,398] and K27[399] (H3K9ac and H3K27ac) are usually related with enhancers and promotors of active genes.

Histone Methylation. Compared with acetylation on histones, the occurrences of methylation on histones are more complicated. Although histone methylation does not influence the state of charge of the histone proteins, the

different residues that are added by methyl groups, and the numbers of transferred methyl groups, could determine the status of transcriptional suppression or activation.[400] For example, mono-methylation at K 20 of histone H4 (H4K20me) is associated with gene activation[401], while tri-methylation at K 20 of histone H4 (H4K20me3) at the promoters of gene represses gene activation[402]. H3K4me and H3K4me3 are primarily located in enhancers and promoters separately.[403] H3K9me3 are reported with, either gene activation in cancers,[404] or gene suppression in stem cells[405]. Current studies indicate the important role of histone methylation in inflammation.[386,406,407] Histone methylation is regulated by two distinct groups of enzymes, whereby histone methyltransferases (KMTs) contribute to the transfer of methyl groups to histone lysine tails and histone demethylases (KDMs) lead to the removal of methyl groups from histone lysine tails. Detailed information about KMTs and KDMs is summarized by these publications.[408,409]

Mechanism of Epigenetic Regulation in Trained Immunity. Although induction of trained immunity in different cell types is associated with persistent epigenetic modifications, epigenetic profiling in the trained immunity of macrophages/monocytes has been relatively more investigated.[354,381,358] Here, we just focus on the introduction of macrophages/monocytes related epigenetic regulation in trained immunity. Chip-seq assay indicates a globally increased level of H3K4me3 in peritoneal macrophages 7 days after *C. albicans* primed mice, but β-glucan induced the change of H3K4me3, observed after 7 days, appears after 24 hours of β-glucan training human monocytes. Moreover,

enhanced H3K4me3 at the promoters of proinflammatory cytokines, such as TNF-α, IL-6 and IL-8, displays in the β-glucan training monocytes.[339] Plus, BCG-induced nonspecific protection from secondary infection is monocyte-dependent, and evaluated H3K4me3 is still maintained at promoters of TLR4, IL-1β and TNF-α, in peripheral blood mononuclear cells (PBMCs), 3 months after BCG vaccination.[354] BCG priming BMDMs also present significantly gains in H3K27me3 and H3K27ac at promoters of immunological genes against M. tuberculosis infection.[364]

LPS-induced tolerance in monocytes (refractory state) displays distinct functions within β-glucan-induced trained immunity in monocytes (sensitive responsive state), which possess different epigenetic profiles. β-glucan priming monocytes show higher marked H3K27ac at all promoters and enhancers, whereas LPS induces persistent H3K4me but not HEK27ac marking enhancers, which accounts for the tolerant feature.[410] LPS priming macrophages present marked H3K4me enhancers, different from β-glucan priming macrophages. RNA-seq assay indicates that β-glucan contributes to phagocytosis and the cytokine release of monocytes, but that LPS exposure suppresses their activation. Further study unveils that re-exposure of β-glucan could reverse LPS-induced tolerance in monocytes by restoring H3K27ac marking promoters and enhancers of genes related to metabolism and lipid biosynthesis.[358]

Unlike the wide investigation of histone epigenetic regulation for trained immunity, much less has been reported of the contribution of DNA methylation in trained immunity. Although DNA methylation has been reported in monocyte-to-

macrophage differentiation with the loss of DNA methylation, no change in DNA methylation presents in trained macrophages. However, specific stable DNA methylation patterns occur in LPS priming macrophages, which provide a potential biomarker for immune-tolerance caused by LPS.[358]

Role of Trained Immunity in Sepsis

Sepsis-induced tolerance of innate immune cells results in an immunosuppressive state in patients, which puts them at tremendous risk of any opportunistic infections and increases the chance of mortality.[411] Sepsis-induced immunosuppression is positively related with activity of HDACs[412], and autopsies of septic patient's brains present increased expression of the HDAC6[413]. Current studies unveil the role of β-glucan in the activation of histone acetylation.[414,415] β-glucan-induced trained immunity in monocytes comes with increased H3K27ac modifications, which are also broadly distributed in inflammatory genes.[357,356,416] β-glucan not only induces memory in monocytes for a quick response to secondary non-specific challenges by histone acetylation[357], but also reverses LPS-caused tolerance in monocytes, by restoring H3K27ac deposition[358], both of which indicate the potential role of trained immunity in the treatment of sepsis-induced tolerance through the regulation of the epigenetics of histones. Indeed, research from Dr. Netea's lab has demonstrated that β-glucan trained septic mice display significantly decreased mortality compared with normal septic mice.[357]

In our current research, efforts are directed at understanding the role of endothelial exosomes, carrying HSPA12B, in cardiac function after sepsis. We

are also interested in the mechanism by which β-glucan, as a stimulator of trained immunity, induce protection in CLP sepsis. Herein, we test the role of endothelial HSPA12B in β-glucan induced trained immunity in CLP sepsis.

CHAPTER 2. HSPA12B ATTNEUATES CARDIAC DYSFUNCTION BY REGUALTION OF NF-κB SIGNALING PATHWAY IN ENDOTHELIAL CELLS IN POLYMICROBIAL SEPSIS

Introduction

Sepsis is recently defined as the life-threatening organ dysfunction caused by a dysregulated host response to infection.[7] Since the first defense line of endothelium against infection, PRRs on endothelial cells could recognize PAMPs to activate endothelial cells for recruitment of immune cells, eradicating invading microorganisms, which regain physiological equilibrium in the normal situation.[68]However, endothelial cell activation in sepsis plays an important role in cardiac dysfunction, which contributes largely to the mortality of severe septic patients.[417,418] Activation of endothelial cells during the pathogenesis of sepsis could express adhesion molecules, such as ICAM1 and VCAM1, to assist immune cells, including innate cells and adaptive cells, to anchor themselves and ultimately transmigrating through endothelium into tissues, the result of which is tissue damage by cytokine storm from immune cells.[419,50]

Dr. Han and his colleagues discovered and demonstrated that HSPA12B is a member of HSP70, which predominately expresses in endothelial cells.[110,111] This specific expression pattern indicates the role of HSPA12B in regulating the functions of endothelial cells. In the following studies, Dr. Liu's lab has done excellent work in unveiling the contributions of HSPA12B in different pathophysiological conditions. Their studies have proven that HSPA12B could attenuate MI, or endotoxin-induced cardiac dysfunction, by downregulating the

infiltration of immune cells, with reduced adhesion molecule expression in myocardium.[420,421] Moreover, their studies found that HSPA12B could prevent LPS-induced inflammatory responses in the endothelial cells by inhibiting activation of PI3K/Akt signaling pathway. However, the mechanism of HSPA12B regulating adhesion molecule expression that modulates immune cell infiltration in CLP sepsis is still unclear.

The NF-κB signaling pathway is widely known to regulate the activation of innate immune cells. For instance, TLR4 on the members of immune cells binds with LPS/endotoxin activating several kinases to induce phosphorylation of I-κB for degradation and then releases NF-κB dimers in the cytoplasm, then translocating into the nucleus, finally activating down-stream gene expression.[422] However, the functions of The NF-κB signaling pathway are not just restricted to the regulation of inflammatory responses. Past research has demonstrated the role of the NF-κB signal pathway in the regulation of adhesion molecule expression, such as ICAM1 and VCAM1, in endothelial cells.[423,424] Furthermore, the protective role of HSPA12B requires activation of the PI3K/Akt signaling pathway[425,113,114], which has been reported to negatively regulate NF-κB binding activity.[426–428]

In this study, we hypothesize that endothelial HSPA12B plays a role in the downregulation of NF-κB signal pathway, contributing to reduced adhesion molecule expression in endothelial cells. Our results show that HSP12B deficiency significantly induced server septic mortality with worse cardiac function

and increased adhesion molecule expression in the myocardium. And HSPA12B can downregulate NF-κB signal pathway with reduced adhesion molecule in the endothelial cells.

Materials and Methods

Animals

Endothelial specific HSPA12B knockout mice ($HSPA12B^{-/-}$) 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. $HSPA12B^{-/-}$ mice and age matched wild type (WT) C57BL/6 mice were used for experiments. The mice were maintained in the Division of Laboratory Animal Resources at East Tennessee State University. The experiments outlined in this manuscript conform to the Guide for the Care and Use of Laboratory Animals published by the National Institutes of Health (8th edition, 2011). The animal care and experimental protocols were approved by the Eastern Tennessee State University Committee on Animal Care.

Cecal Ligation Sepsis Model

Murine polymicrobial sepsis was induced by cecal ligation and puncture as described previously[429,365,47,75]. To induce a septic model, mice were anesthetized by 5.0% isoflurane. A midline incision was made on the anterior abdomen and the cecum was exposed and 1/3 of cecum was ligated with a 4-0 suture. One puncture was made at the end of cecum with a 23-gauge needle and feces were extruded from the hole. The abdomen was then closed in 2 layers. Sham surgically operated mice served as sham control. Immediately following

surgery, a single dose of resuscitative fluid (lactated Ringer's solution, 50 mL/kg body weight) was administered by subcutaneous injection.

Echocardiography

Transthoracic 2-dimensional M-mode echocardiogram and pulsed wave Doppler spectral tracings (Toshiba Aplio 80 Imaging System, Tochigi, Japan) were used to measure left ventricular (LV) wall thickness, LV end-systolic diameter, and LV end-diastolic diameter. Percentage of fractional shortening (%FS) and ejection fraction (EF%) were calculated as described previously[47,75,429].

Immunofluorescent Staining

The procedure of the immunofluorescent staining was modified based on the protocol provided by Cell Signaling Technology. Briefly, heart tissue was harvested from septic mice for paraffin embedding. Paraffin embedded heart blocks were sectioned at 5μm. Paraffin embedded sections were deparaffinized and rehydrated by xylene three times for 5 min each, 100% ethanol twice for 5 min each, 95% ethanol twice for 5 min each, 75% ethanol once for 5 mins, 50% ethanol once for 5 min and ddH2O once for 5 min. 10% goat serum was used to block sections for 60 min at room temperature followed primary antibodies at 4 ^{o}C overnight. At the second day, sections were washed by wash buffer (1xPBS) for 5 min three time each followed by fluorochrome-conjugated secondary antibody for 60 min at room temperature in the dark. Sections were washed by wash buffer for 5 min three times each followed nuclear staining by DAPI. Sections

were finally washed by wash buffer for 5 mins three times each followed anti-fade reagent for microscope examination.

Immunohistochemistry Staining

The procedure of the immunofluorescent staining was modified based on the protocol provided by Cell Signaling Technology. Briefly, heart tissue was harvested from septic mice for paraffin embedding. Paraffin embedded heart blocks were sectioned at 5μm. Paraffin embedded sections were deparaffinized and rehydrated by xylene three times for 5 min each, 100% ethanol twice for 5 min each, 95% ethanol twice for 5 min each, 75% ethanol once for 5 mins, 50% ethanol once for 5 min and ddH2O once for 5 min. Antigen unmasking worked with 10mM sodium citrate buffer with sections for 10mins at 95 ^{o}C and then cooled sections for 30 min. 3% hydrogen peroxide was incubated with sections for 10mins followed wash buffer (1x PBS) 5 min three times each. 10% goat serum was used to block sections for 60 min at room temperature followed primary antibodies (HSPA12B,1:100,Santa Cruz; CD31, 1:100, BD Pharmingen) at 4 ^{o}C overnight. At the second day, sections were washed by wash buffer for 5 min three time each followed by biotinylated secondary antibody for 90 min. Sections were washed by wash buffer 5 mins three times each by AB enzyme reagent (avidin mixed with biotinylated HRP) for 30 mins. Sections were washed by wash buffer 5 min three times each by peroxidase substrate for 30s to 10 min and were checked staining under a microscope.

ELISA for Organ Injury Assay

The serum levels of aspartate aminotransferase (AST, hepatic injury marker) and creatinine (kidney injury marker) were measured with commercially available kits according to the instructions provided by the manufacturer (Millipore Sigma).

Genotyping

The procedure of genotyping was based on the HOTSHOT method.[430] Briefly, 1 to 2 mm mice tail was removed and placed in a 500ul Eppendorf tube and digested with 50ul Alkaline Lysis Reagent (25mM NaOH, 0.2mM EDTA, pH 12.0) at 95 ^{o}C for 30mins and chilled on ice followed by 50ul Neutralization Reagent (40 mM Tris-HCl at pH 5.0) for DNA analysis. Genotypes of tissue specific deletion in mice were analyzed with primers of floxd allele (HSPA12B-cko-1: 5'-GAAGCAAGCATATTCATCTCATTACTATTC-3'; HSPA12B-cko-2: 5'-GCTTGCTCAAAAGTGATGGTTGCTC-3'. 191bp for flox mice; 151bp for wild type mice), HSPA12B$^{-/-}$ (HSPA12B-cko-1: 5'-GAAGCAAGCATATTCATCTCATTACTATTC-3'; HSPA12B-cko-4: 5'-TAAAGCCTACACTCAGATGAGAGCAG-3'. 240bp for HSPA12B-/-, >2kb or no band for wild type control) and Cre expression (5' GTGAAACAGCATTGCTGTCACTT-3' and 5'-GCGGTCTGGCAGTAAAAACTATC-3') for PCR, which was run in agarose gel at 60 volts and 110 milliamps followed taking picture with G-box.

Real-time PCR

Cell medium were removed, and cells were lysed with RNAzol@ RT by pipetting several times to ensure complete lysis. Add 0.4ml DEPC water into homogenate/lysate per 1ml RNAzol@ RT for homogenization followed by shaking well and standing for 10mins. Centrifuge at 12000g for 15mins to move 1ml supernatant to a new tube and precipitate RNA by adding 0.4ml 75% ethanol, waiting 10min followed 12000g for 8mins. Wash pellets (mRNAs) by 75% ethanol twice at 8000g for 3 mins each. DEPC water dissolved pellets for cDNA reverse transcription. cDNA was used for real-time PCR.

In vitro Experiments

Human umbilical vein endothelial cells (HUVECs) were cultured in vascular cell basal medium supplemented with endothelial cell growth kits (ATCC). After reaching 70%–80% confluence, HUVECs were transfected with adenovirus expressing HSPA12B (Ad-HSPA12B-GFP) or adenovirus expressing GFP. Six hours after transfection, the cells were washed and incubated with fresh medium overnight followed by LPS stimulation for four hours. The cells were harvested and the cellular proteins were prepared for Western blot[431,118].

Western Blot

Western blot was performed as described previously[47,75,432,433]. In brief, cellular proteins were separated by sodium dodecyl sulphate–polyacrylamide gel electrophoresis and transferred onto Hybond enhanced chemiluminescence (ECL) membranes (Amersham Pharmacia, Piscataway, New Jersey). The ECL membranes were incubated with the appropriate primary antibodies followed by

peroxidase-conjugated secondary antibody, which was purchased from Cell Signaling Technology, Inc. The signals were quantified using the G: Box gel imaging system by Syngene (Frederick, Maryland).

Statistical Analysis

The data are expressed as mean ± standard error. Comparisons of data between groups were made using one-way analysis of variance (ANOVA), and Tukey procedure for multiple-range tests was performed. The log-rank test was used to compare group survival trends. Probability levels of ≤.05 were used to indicate statistical significance.

Results

Construction of Endothelial Specific HPSA12B Knockout Mice

The current evidence indicates the protective role of HSPA12B in many pathophysiological processes, including endotoxin-induced sepsis[113], myocardial infarction[420], and stroke[117]. Thus, in our project, we investigated the function of HSPA12B in polymicrobial sepsis. First, endothelial specifically deficient HSPA12B mice were constructed by crossbreeding between Tek-Cre mice and flox-HSPA12B mice shown in Figure1-3.

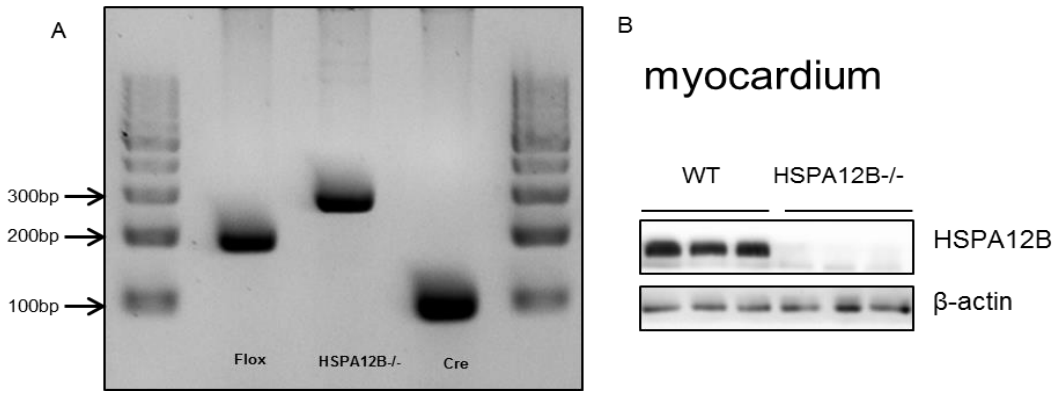


Figure 1. Development of endothelial HSPA12B specific deficient mice. A) The depletion of HSPA12B is detected by PCR, by using three primers of Tek-Cre, flox(HSPA12B-cko-1 and HSPA12B-cko-2) and HSPA12B$^{-/-}$(HSPA12B-cko-1 and HSPA12B-cko-4). B) The expression of HSPA12B in the myocardium is examined by WB. β-actin serves as loading control. No band in HSPA12B-/- group indicates the successful depletion of HSPA12B.

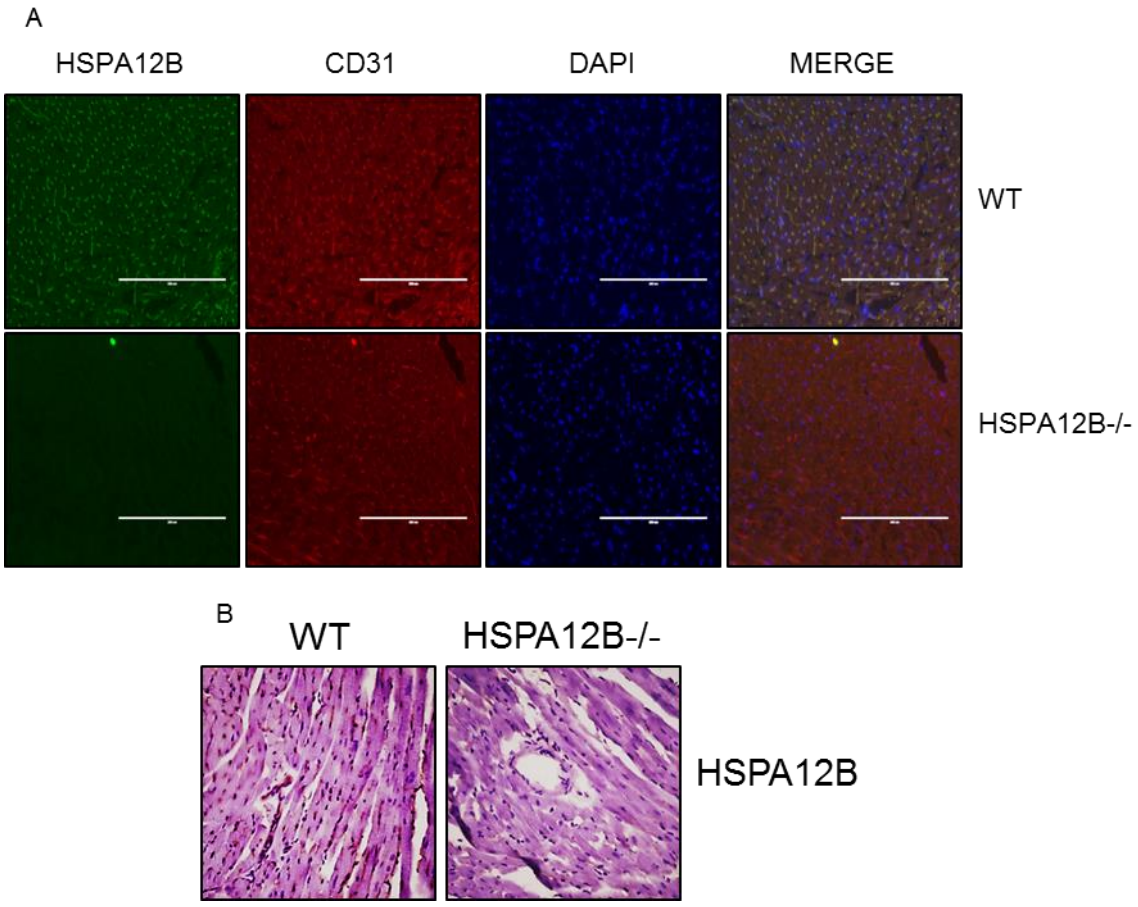


Figure 2. The successful depletion of HSPA12B in endothelial cells of myocardium. A) The distribution of HSPA12B is tested by immunofluorescent staining. HSPA12B (green) are overlapped with CD31 (red), which is a marker of endothelial cells in the myocardium of WT and HSPA12B-/- mice. (n=3/group); *B*) The distribution of HSPA12B in the myocardium is also tested by immunohistochemistry staining, which brown colors indicating HSPA12B are in the endothelial cells of cardiac capillaries. n=3/group

Endothelial HSPA12B Deficiency Exacerbate Survival Outcome with Severe Cardiac Dysfunction in Polymicrobial Sepsis

Due to the critical role of endothelium in the development of sepsis, we investigated the effect of endothelial HSPA12B on the survival outcome in a CLP septic model in mice. As shown in Figure 3, HSPA12B$^{-/-}$ septic mice exhibited an

exacerbation of mortality compared with WT septic mice. The time to 100% mortality of HSPA12B$^{-/-}$ septic mice was 163 hours, while the mortality to 62% of WT septic mice was 200 hours after induction of CLP sepsis. The data suggests that endothelial cell HSPA12B could play a protective role in limiting mortality of CLP sepsis. Sepsis induced cardiomyopathy contributes to mortality and mobility of sepsis[434]. We measured cardiac function during CLP sepsis. As shown in Figures 4, cardiac function was not reduced at 6 hours after induction of CLP sepsis. However, ejection fraction (EF%) and fractional shortening (FS%) were both markedly reduced in both WT and HSPA12B$^{-/-}$ septic mice 24 and 36 hours after CLP sepsis, when compared with their respective sham controls. Importantly, the values of EF% and FS% in HSPA12B$^{-/-}$ septic mice were markedly lower (12.1% and 19% at 24 hours, 6.1% and 15.1% at 36 hours) than in WT septic mice. In addition, the serum levels of AST and creatinine kinase in the HSPA12B$^{-/-}$ septic mice were significantly greater by 17.5% and 22.2% than in WT septic mice (Fig.5). The data suggests that endothelial HSPA12B could play an important role in the protection against sepsis induced organ injury.

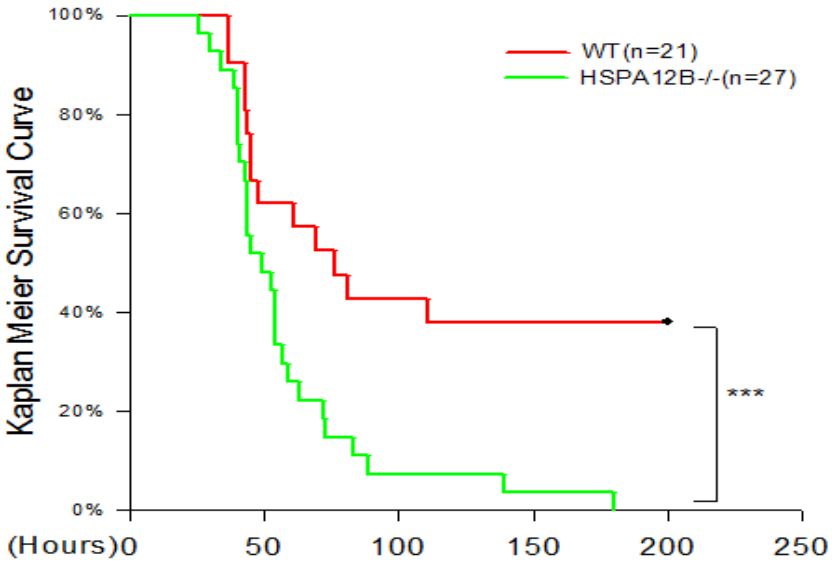


Figure 3. Increased mortality in HSPA12B$^{-/-}$ septic mice. WT and HSPA12B$^{-/-}$ mice were subjected to CLP sepsis. The survival outcome was monitored for 200 hours following induction of CLP (n=21~27/group). All HSPA12B$^{-/-}$ septic mice were died, however, which nearly 40% WT septic mice still survived at the observing end point. ***P < 0.001 compared with indicated groups.

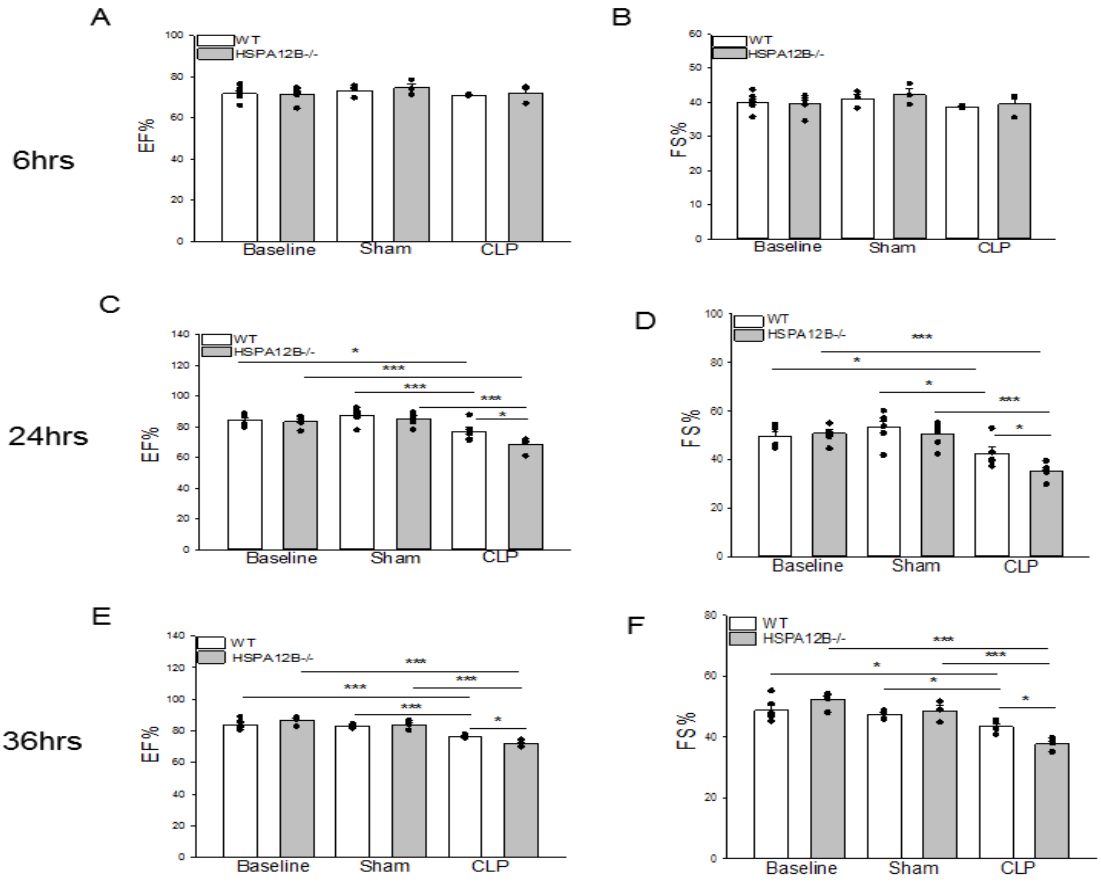


Figure 4. More severe cardiac dysfunction in HSPA12B-/- septic mice. A-F) Cardiac function was examined by echocardiography at 6, 24 and 36 hours after CLP. EF%: Ejection fraction (%EF). %FS: Fractional shortening. (n=3~6/group) *P < .05, **P < 0.01, and ***P < 0.001 compared with indicated groups.

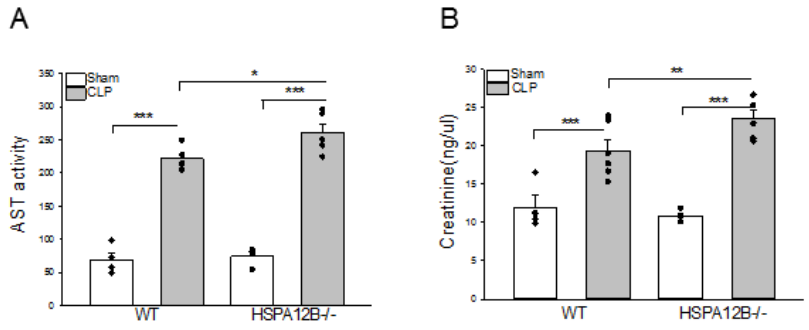


Figure 5. Worse organ injuries in HSPA12B-/- septic mice. A-B) Serum levels of aspartate aminotransferase (AST; A) indicating liver injury, creatinine indicating kidney injury (B) were measured by commercially available enzyme-linked immunosorbent assay kits. n=3~6/group. *P < .05, **P < 0.01, and ***P < 0.001 compared with indicated groups.

Endothelial HSPA12B Deficiency Increases Adhesion Molecule Expression in Polymicrobial Sepsis

An increased population of immune cells in the myocardium contributes to septic cardiomyopathy[435,46], whereby adhesion molecules expressed by endothelial cells facilitate immune cell accumulation in the heart tissue.[436] As shown in Figure 6, the levels of VCAM-1 and ICAM-1 in the myocardium of HSPA12B$^{-/-}$ septic mice were significantly greater than in WT septic mice. The data indicates that endothelial HSPA12B could suppress myocardial adhesion molecule expression, contributing to attenuation of immune cells (including macrophages, and neutrophils) infiltration into the myocardium during sepsis.

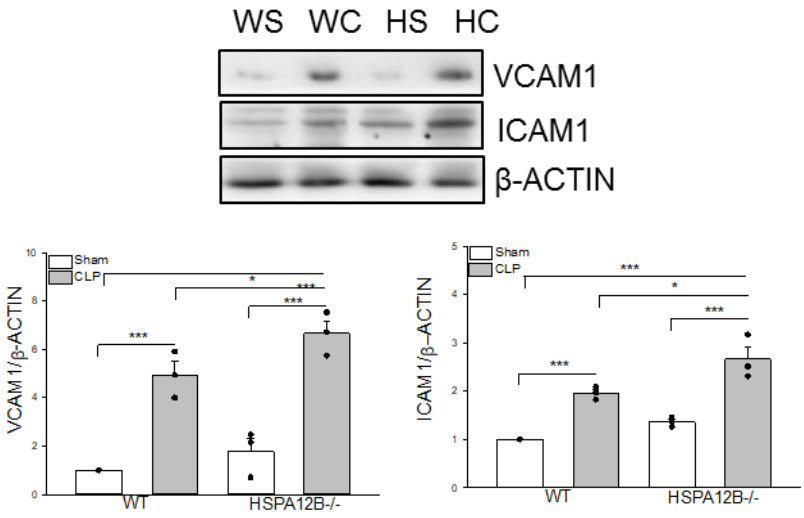


Figure 6. Worse organ injuries in HSPA12B-/- septic mice. Western blot analysis presented markedly increased levels of VCAM-1 and ICAM-1 in the myocardium of HSPA12B-/- mice 36 hours after CLP sepsis, compared with WT septic mice. (n=3/group). *P < .05, **P < 0.01, and ***P < 0.001 compared with indicated groups. WS, WC, HS and HC indicate WT/SHAM, WT/CLP, HSP12B$^{-/-}$/SHAM, and HSP12B$^{-/-}$/CLP separately.

HSPA12B Overexpression in Endothelial Cells Decreases LPS-Induced Adhesion Molecule Expression *in vitro*

To define how HSPA12B regulates adhesion molecule expression in endothelial cells, we transfected endothelial cells (HUVECs) with adenovirus expressing HSPA12B (Ad-HSPA12B-GFP) or Ad-GFP which served as vector control. As shown in *Figure 7A*, HUVECs are successfully transfected with Ad-HSPA12B-GFP or Ad-GFP, indicated by GFP expression. The qPCR result (*Fig.7B*) presents significantly higher expression in HUVECs transfected with Ad-HSPA12B-GFP, but not in control groups. *Figure8* shows that HSPA12B markedly suppresses ICAM1 (*Fig.8A*) and VCAM1 (*Fig.8B*) expression in LPS-stimulated endothelial cells, which further demonstrate the role of HSPA12B in the regulation of adhesion molecule expression in endothelial cells.

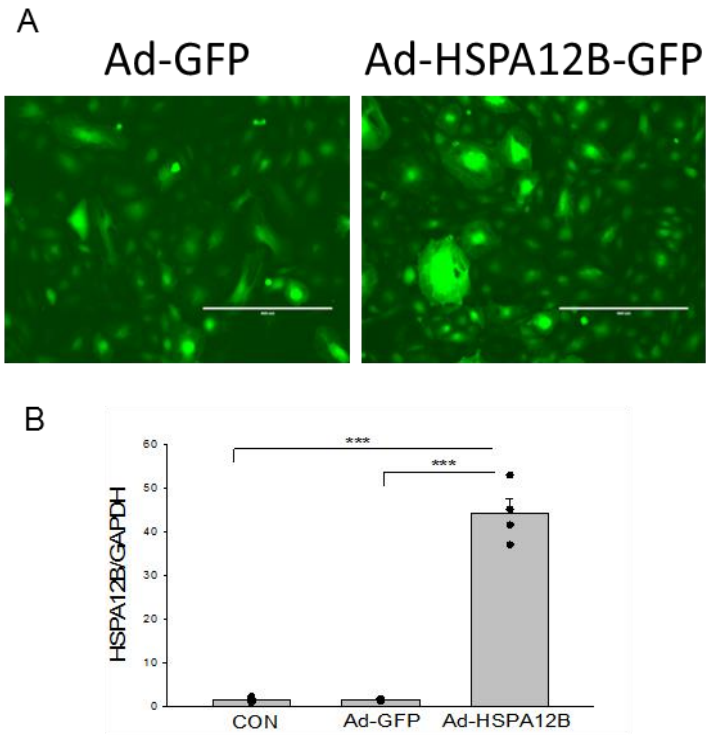


Figure 7. Overexpression of HSPA12B in endothelial cells in vitro. *A*) Immunofluorescent analysis of HSPA12B overexpression in HUVECs labeled by GFP, and Ad-GFP which served as vector control. *B*) qPCR analysis displayed significantly higher expression of HSPA12B in HUVECs transfected with Ad-HSPA12B-GFP, but not in control groups. (n=4/group) **P* < .05, ***P* < 0.01, and ****P* < 0.001 compared with indicated groups.

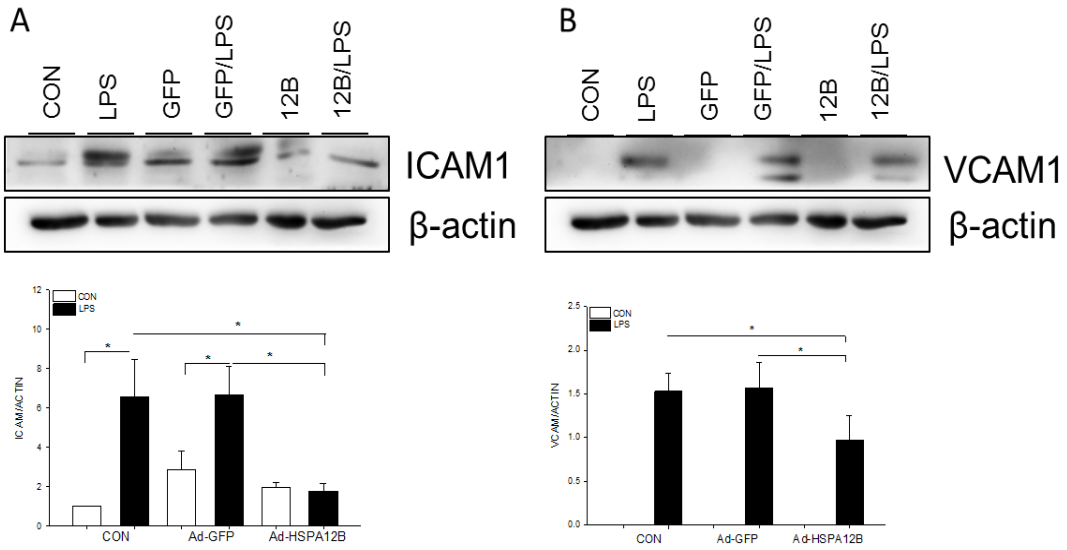


Figure 8. Downregulation of HSPA12B in adhesion molecule expression in LPS-stimulated endothelial cells *in vitro*. *A, B*) HUVECs were transfected with Ad-HSPA12B-GFP and Ad-GFP separately for 6 hours followed with fresh medium change, resting overnight. On the second day, LPS (0.1ug/ml) were used to stimulate HUVECs for 4 hours, then the levels of ICAM1 and VCAM1 were evaluated by western blot. (n=3/group) *$P < .05$, compared with indicated groups.

HSPA12B Overexpression Downregulates NF-κB Signaling Pathway in LPS-Stimulated Endothelial Cells

Due to the contribution of the NF-κB signaling pathway in the regulation of adhesion molecule expression in endothelial cells[423,424], we further investigated whether HSPA12B modulates the NF-κB signaling pathway in endothelial cells. As shown in *Figure 9*, HPSA12B could prevent NF-κB P65 subunit, nuclear translocation in LPS-stimulated endothelial cells. Moreover, HPSA12B significantly suppressed phosphorylation of I-κB in the cytoplasm (*Fig.10*), which inhibits P65 nuclear translocation to activate gene expression. The data suggests that endothelial HSPA12B could play an important role in the protection against sepsis induced increased levels of adhesion molecules.

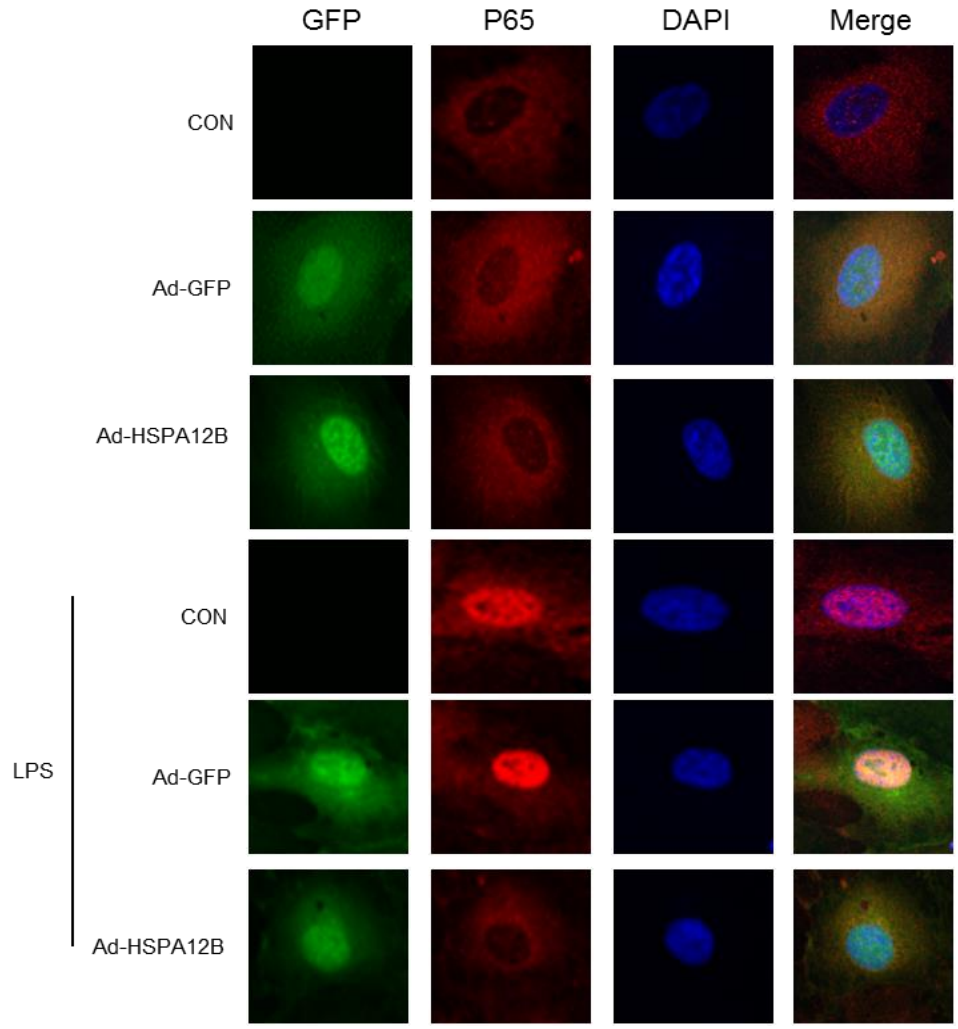


Figure 9. Suppression of HSPA12B in P65 nuclear translocation in LPS-stimulated endothelial cells in vitro. HUVECs were transfected with Ad-HSPA12B-GFP and Ad-GFP separately for 6 hours followed with fresh medium change, resting overnight. At second day, LPS (0.1ug/ml) were used to stimulate HUVECs for 0.5 hours, then P65 nuclear translocation was evaluated by immunofluorescent staining. Note: P65 nuclear translocation was inhibited by HSPA12B in endothelial cells.

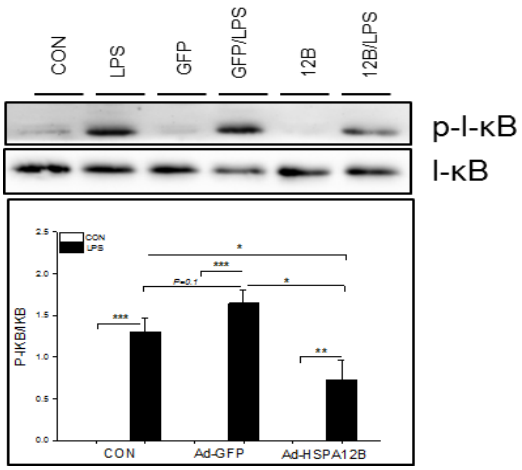


Figure 10. Suppression of HSPA12B in phosphorylation of I-κB in the endothelial cytoplasm LPS-stimulated cells in vitro. HUVECs were transfected with Ad-HSPA12B-GFP and Ad-GFP separately for 6 hours followed with fresh medium change, resting overnight. At second day, LPS (0.1ug/ml) were used to stimulate HUVECs for 0.5 hours, then phosphorylation of I-κB was evaluated by immunofluorescent staining. Note: phosphorylation of I-κB was inhibited by HSPA12B in endothelial cells. (n=3/group) *P < .05, **P < 0.01, and ***P < 0.001 compared with indicated groups.

Discussion

The present data demonstrates that endothelial HSPA12B exerts a protective role on sepsis induced cardiac cardiomyopathy and mortality. The mechanisms involve in the regulation of adhesion molecules in endothelial cells, which are the key regulators to assist immune cell infiltration. Our data suggests HSPA12B may downregulate the NF-κB signaling pathway, contributing to decreased levels of adhesion molecules on endothelial cells.

Endothelial HSPA12B has been reported to prevent multiple organ damage in different pathophysiological conditions, such as endotoxemia[437], cardiac injury[425] and stroke[114].The mechanism is involved in the activation of PI3K/Akt signaling.[114,425] Overexpression of endothelial HSPA12B has been

reported to prevent LPS-induced increased levels of adhesion molecules with less neutrophil infiltration, in the lung of LPS-challenged mice.[437] In our present study, we found the deficiency of HSPA12B in the endothelial cells results in more severe cardiac dysfunction with increased mortality, in polymicribial sepsis. These findings provide evidence that endothelial HSPA12B may play an important role in the regulation of expressions of adhesion molecule in the myocardium, contributing to immune cell infiltration during sepsis.

To elucidate the mechanisms by which endothelial HSPA12B attenuates sepsis induced cardiomyopathy and organ injury, we focused on the role of endothelial HSPA12B in the regulation of adhesion molecule expression to sepsis. Past research has demonstrated the contribution of adhesion molecule expression in sepsis induced organ dysfunction in the clinical settings.[438,78] We found that endothelial HSPA12B deficiency results in increased expression of ICAM1 and VCAM1, in the myocardium, during CLP sepsis. Interestingly, overexpressing HSPA12B in the endothelial cells can significantly decrease LPS-induced upregulation of ICAM1 and VCAM1 in vitro. The in vitro and in vivo data directly demonstrated the regulation of endothelial HSPA12B on adhesion molecule expression.

Activation of the NF-κB signaling pathway has been reported to contribute to sepsis-induced mortality and organ dysfuntions[439]. In our previous studies, we have demonstrated that TLR-associated the NF-κB signaling pathway plays a significant role in sepsis-related mortality[440] and cardiac dysfunction[47]. To elucidate the mechanisms by which endothelial HSPA12B modulates adhesion

molecule expression in endothelial cells, we measured the role of the NF-κB signaling pathway on adhesion molecule expressions in endothelial cells. We found that HSPA12B significantly suppresses IκBα phosphorylation in LPS-induced endothelial cells, assisting NF-κB P65 subunit nuclear translocation in LPS stimulated endothelial cells, which is also confirmed by the immunostaining results. Our data suggests that endothelial HSPA12B regulates the NF-κB activation in endothelial cells. Further studies are needed to investigate the specific mechanisms by which HSPA12B suppresses IκBα phosphorylation in the NF-κB signaling pathway in endothelial cells.

Our results have indicated that HSPA12B prevents CLP-induced cardiac dysfunction, partly through suppression of NF-κB activation in endothelial cells. Targeting HPSA12B could be an effective approach for treatment of cardiac dysfunction in septic patients.

CHAPTER 3. ENDOTHELIAL HSPA12B REGULATES MACROPHAGE INFLAMMATORY RESPONSES VIA EXOSOMES SECRETION IN POLYMICROBIAL SEPSIS

Introduction

In the United States, over 166,500 cases of sepsis occur yearly with a mortality rate up to 50%[441]. Sepsis induced dysregulated immune responses play a critical role in cardiovascular dysfunction, which is a major complication associated with increased mortality in sepsis.[442] Cardiac dysfunction in sepsis/septic shock is associated with mortality rates as high as 70%.[443]

Endothelial cell dysfunction contributes to sepsis induced mortality and organ dysfunction[49]. Heat shock protein A12B (HSPA12B) is a member of HSP70 family and is predominately expressed in endothelial cells[110]. Previous studies have shown that transgenic mice with endothelial cell specific overexpression of HSPA12B exhibits a protection against endotoxemia or myocardial ischemia induced cardiac dysfunction[115]. Increased expressing of HSPA12B in endothelial cells attenuates LPS-induced adhesion molecules expression and pro-inflammatory cytokine production via activation of the PI3K/Akt signaling[118]. These data indicate that endothelial cell HSPA12B may be an endogenous mechanism that serves to protect the host during sepsis.

Macrophages play a critical role in mediating innate immune and inflammatory responses to sepsis challenge[444,445]. Increasing evidence demonstrates that macrophages play different roles during different stages of sepsis via the regulation of innate immune and inflammatory responses[445]. In

general, macrophage polarization has been divided into two groups, *i.e*. M1 pro-inflammatory and M2 anti-inflammatory phenotypes[446]. In the early stage of sepsis, macrophages polarize toward the M1 phenotype, resulting in production of pro-inflammatory cytokines[446]. In the late stage of sepsis, however, macrophages frequently exhibit an M2 phenotype that regulates anti-inflammatory mediators[447]. However, these heterogeneous functions of macrophages are highly dependent on the microenvironment conditions[165]. The role of endothelial HSPA12B in the regulation of macrophage inflammatory responses during sepsis has not been fully elucidated.

Recent evidence suggests that there is a crosstalk between endothelial cells and immune cells during sepsis[448]. Exosomes have been demonstrated to play an important role in cell-cell communication[449]. Exosomes are one subset of extracellular vehicles (EVs) that can be secreted from most cells[450]. Recent studies have shown that exosomes are involved in the regulation of immune and inflammatory responses[451]. Macrophage exosomes have been reported to alter endothelial cell migration and inflammation[452,296]. However, whether endothelial exosomes can regulate macrophage inflammatory responses during sepsis have not been investigated.

In the present study, we hypothesized that endothelial HSPA12B regulate macrophage inflammatory responses. To investigate this hypothesis, we did *in vitro* and *in vivo* experiments to demonstrate that endothelial HSPA12B are released via exosome secretion during sepsis and that macrophages can uptake endothelial HSPA12B containing exosomes in both *in vivo* and *in vitro*. HSPA12B

carried by exosomes attenuated macrophage inflammatory response to LPS stimulation via downregulation of NF-κB activation.

Materials and Methods

Animals

Endothelial specific HSPA12B knockout mice ($HSPA12B^{-/-}$) 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. $HSPA12B^{-/-}$ mice and age matched wild type (WT) C57BL/6 mice were used for experiments. The mice were maintained in the Division of Laboratory Animal Resources at East Tennessee State University. The experiments outlined in this manuscript conform to the Guide for the Care and Use of Laboratory Animals published by the National Institutes of Health (8th edition, 2011). The animal care and experimental protocols were approved by the Eastern Tennessee State University Committee on Animal Care.

Cecal Ligation Sepsis Model

Murine polymicrobial sepsis was induced by cecal ligation and puncture as described previously[432,440,433,47,75]. To induce of a septic model, mice were anesthetized by 5.0% isoflurane. A midline incision was made on the anterior abdomen and the cecum was exposed and 1/3 of cecum was ligated with a 4-0 suture. One puncture was made at the end of cecum with a 23-gauge needle and feces were extruded from the hole. The abdomen was then closed in 2 layers. Sham surgically operated mice served as sham control. Immediately following

surgery, a single dose of resuscitative fluid (lactated Ringer's solution, 50 mL/kg body weight) was administered by subcutaneous injection.

ELISA for Serum Lactate and Cytokine Assay

The serum levels of serum lactate were measured with commercially available kits according the instructions provided by the manufacturer (Millipore Sigma). And Serum inflammatory cytokines (TNF-α, IL-1β and IL-10) were measured with commercially available kits according the instructions provided by the manufacturer (Peprotech).

Tissue Accumulation of Macrophages

Accumulation of macrophages in heart tissues were examined using immunostaining with anti F4/80 antibody (Cell Signaling Technology). Three slides from each block were evaluated, counterstained with hematoxylin, and examined with confocal microscopy. The results are expressed as the numbers of immune cells/field (×40)[432,433,47].

Immunofluorescent Staining

The procedure of the immunofluorescent staining was modified based on the protocol provided by Cell Signaling Technology. Briefly, cells were grown on the coverslips in a multiwell plate, fixed with 4% formaldehyde for 15 min in room temperature and washed for three times with PBS for 5 minutes each. Cells were permeabilized with ice-cold 100% methanol for 10 min at -20^{o}C followed by rinsing in PBS for 5 min. The cells were incubated with 10% goat serum for 60 min at room temperature followed by incubation with the primary antibody (F4/80, 1:100, Cell signal technology; p65, 1:400, Cell signal technology) that was diluted

with 10% goat serum, at 4^{o}C overnight according to the instruction provided by manufacturer. After washing for three times with PBS for 5 min each, the cells were incubated with a fluorochrome-conjugated secondary antibody for 60 min at room temperature in dark. The cells were washed with PBS for three times, covered by antifade reagent with DAPI and examined with confocal microscope.

Flow Cytometric Analysis

The procedure of flow cytometry was modified based on the protocol from BD Biosciences. Briefly, the cells were harvested from blood and spleen tissues and prepared for a single cell suspension. Red blood cells were lysed using BD Pharmingen's PharM LyseTM(Cat. No.555899) solution. The mixture of cell suspension was incubated with LyseTM buffer at room temperature for 15 min in the dark and centrifuged at 200g for 5 minutes at 10^{o}C. The supernatants were carefully removed. The pellets were suspended with 1x cold wash buffer (PBS/0.1% NaN3/1.0%FBS) followed by centrifugation at 350g for 5 min. Cells were counted, and reconstituted in staining buffer (BD Biosciences) to a concentration of 1x10^{6} cells/ml. Subsequently, cells will be incubated with LIVE/DEAD Fixable Dead Cell Stain single-color dyes (Invitrogen) for 30 min at room temperature. After one rinse with washing buffer, cells will be incubated with anti- Fc_{γ} III/II (clone 2.4G2) antibody (BD Pharmingen) for 15 min and labeled at 4°C overnight followed by incubation with rat anti-mouse primary antibodies: F4/80-Alexa 647, CD11b-PE-CY7 to label monocytes/macrophages, and Gr-1-PerCP-Cy5.5, Ly-6B.2-FITC to label neutrophils. Finally, cells will be washed twice, resuspended in staining buffer and immediately analyzed with a

Becton Dickinson LSRII flow cytometer (BD Biosciences). Monocytes/macrophages were identified using the gating strategy as CD11b+ F4/80+ cells, while neutrophils were identified using the gating strategy as Gr-1^{+}ly6b.2^{+} cells. Data analysis and quantification were performed using FlowJo. Antibodies dilution will be adjusted for specific experiment according to manufacture manual.

In vitro Experiments

Human umbilical vein endothelial cells (HUVECs) were cultured in vascular cell basal medium supplemented with endothelial cell growth kits (ATCC). After reaching 70%–80% confluence, HUVECs were transfected with adenovirus expressing HSPA12B (Ad-HSPA12B) or adenovirus expressing GFP. Six hours after transfection, the cells were washed and incubated with fresh medium overnight followed by LPS stimulation for four hours. The cells were harvested, and the cellular proteins were prepared for Western blot[118,431].

Transfection of Raw 264.7 Macrophages with Adenovirus Expressing HSPA12B (Ad-HSPA12B)

The procedure for transfection of macrophages with Ad-HSPA12B was modified according to K2 transfection system (Biontex). Briefly, when macrophages reached to 80%–90% confluence, the cells were pre-treated with K2 multiplier for two hours. K2 transfection reagent and Ad-HSPA12B or Ad-GFP were mixed at 1:2 ratio and incubated at room temperature for 30 min. The mixed transfection reagent was added into cultured macrophages. Six hours after transfection, the cells were washed and incubated with fresh culture medium

overnight. The transfection efficiency was examined by viewing green fluorescence in the cells.

Isolation of Exosomes

HUVECs were incubated with FBS-free medium. The medium was collected and centrifuged at 2000g for 30 min to remove debris. The supernatants were transferred into a new tube. Thirty percent (30%) PEG6000 (Sigma-Aldrich) reagent was mixed with the supernatant at ratio of 1:2 by votexing until there is a homogenous solution. The mixture was incubated at 2~8^{o}C overnight and centrifuged at 3000g for 30 min. The supernatant was removed. Exosomes in the pellet were resuspended in cold PBS and the exosome markers were examined by Western blot.

Western Blot

Western blot was performed as described previously[47,75,432,433]. In brief, cellular proteins were separated by sodium dodecyl sulphate–polyacrylamide gel electrophoresis and transferred onto Hybond enhanced chemiluminescence (ECL) membranes (Amersham Pharmacia, Piscataway, New Jersey). The ECL membranes were incubated with the appropriate primary antibodies followed by peroxidase-conjugated secondary antibody, which was purchased from Cell Signaling Technology, Inc. The signals were quantified using the G: Box gel imaging system by Syngene (Frederick, Maryland).

Statistical Analysis

The data are expressed as mean ± standard error. Comparisons of data between groups were made using one-way analysis of variance (ANOVA), and Tukey procedure for multiple-range tests was performed. The log-rank test was used to compare group survival trends. Probability levels of ≤.05 were used to indicate statistical significance.

Results

High levels of serum lactate and inflammatory cytokines in HSPA12B$^{-/-}$ septic mice

The serum lactate levels serve as a biomarker for severity and mortality of sepsis.[453] *Figure 11* shows that CLP sepsis significantly increased the serum lactate levels in both WT and HSPA12B$^{-/-}$ mice compared with sham controls. However, the levels of serum lactate in HSPA12B$^{-/-}$ septic mice were markedly greater by 27.5% than in WT septic mice. Although CLP sepsis also markedly increased the levels of serum TNF-α, IL-1β and IL-10 in both WT and HSPA12B$^{-/-}$ mice 36 hours after induction of CLP sepsis (*Figs. 12A-C*). Interestingly, the serum TNF-α and IL-1 β levels in HSPA12B$^{-/-}$ septic mice were markedly increased by 53.5% and 38.9% while IL-10 decreased by 52.7% at 36 hours after CLP, when compared with WT septic mice. These data indicate that endothelial HSPA12B could participate in the regulation of inflammatory cytokine production during CLP sepsis.

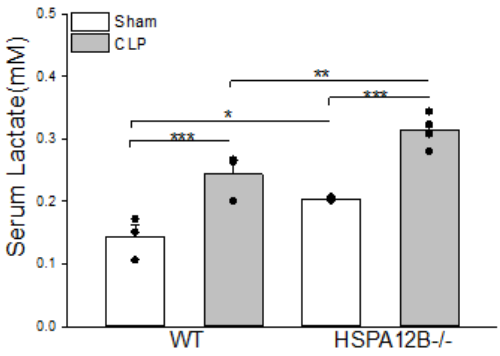


Figure 11. Endothelial cell specific HSPA12B deficiency exacerbated sepsis-induced production of lactate following polymicrobial sepsis. WT and HSPA12B$^{-/-}$ mice were subjected to CLP sepsis. Serum was collected at 36 hours after CLP sepsis. Serum lactate levels were evaluated by enzyme-linked immunosorbent assay. HSPA12B deficiency resulted in remarkable increased serum lactate compared with WT septic mice. (n=3~6/group) *P < 0.05, **P < 0.01, and ***P < 0.001 compared with indicated groups.

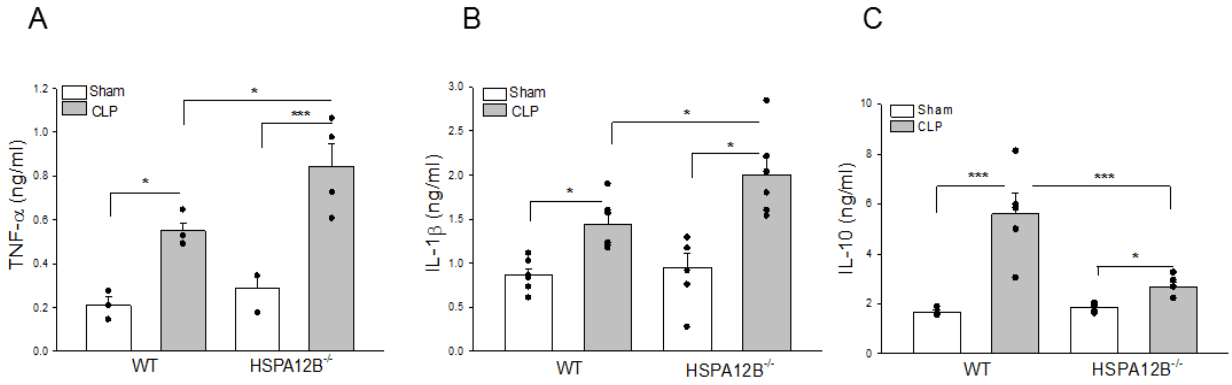


Figure 12. Endothelial cell specific HSPA12B deficiency exacerbated sepsis-induced inflammatory cytokine production following polymicrobial sepsis. WT and HSPA12B$^{-/-}$ mice were subjected to CLP sepsis. Serum was collected at 36 hours after CLP sepsis. The levels of tumor necrosis factor alpha (TNF-α; *A*), interleukin 1 beta (IL-1β; *B*), and interleukin 10 (IL-10; *C*) were analyzed with enzyme-linked immunosorbent assay. CLP sepsis significantly alter serum TNF-α, IL-1β and IL-10 at 36 hours compared with those in the sham groups. However, HSPA12B deficiency resulted in remarkable increased serum TNF-α and IL-1β and decreased serum IL-10 compared with WT septic mice. (n=3~6/group) *P < .05, **P < .01, and ***P < .001 compared with indicated groups.

Increased accumulation of macrophages in the myocardium of HSPA12B$^{-/-}$ septic mice

Increased immune cells in the myocardium contributes to septic cardiomyopathy[435,46]. *Figure 13* shows that the numbers of F4/80^{+} macrophages in the myocardium from HSPA12B$^{-/-}$ septic mice were significantly greater than in WT septic mice. Increased expression of adhesion molecules facilitates immune cell accumulation in the heart tissues[436]. The result shown in the chapter 3 has demonstrated increased levels of ICAM1 and VCAM1 in the myocardium of HSPA12B-/- septic mice were significantly greater than in WT septic mice. The data indicates that endothelial cell HSPA12B could attenuate macrophage infiltration into the myocardium through suppressing myocardial adhesion molecule expression during sepsis.

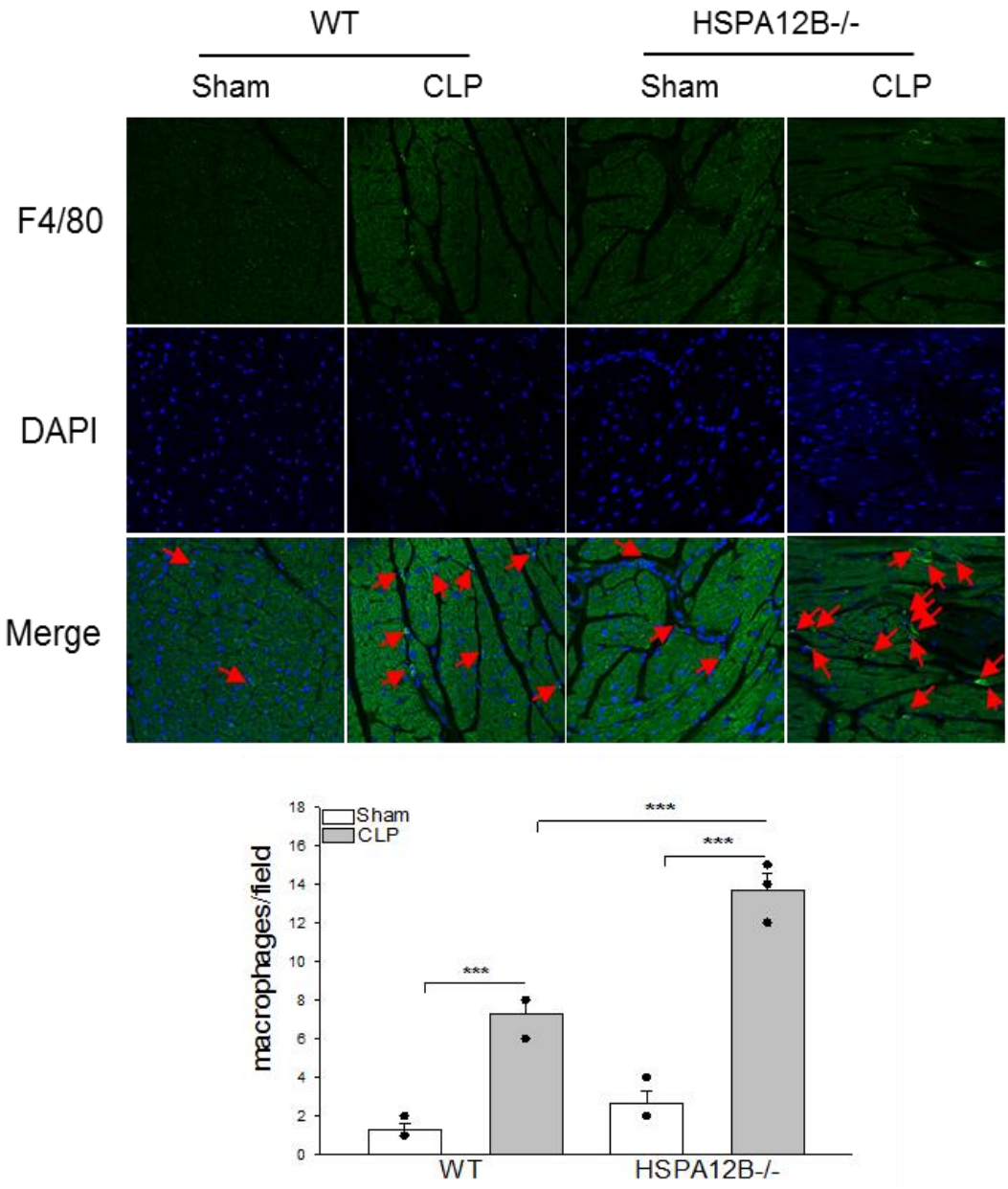


Figure 13. Endothelial cell specific HSPA12B deficiency increased macrophage population in myocardium following polymicrobial sepsis. Heart was harvested at 36 hours after CLP sepsis for immunofluorescent staining. Heart tissues were sectioned and stained with anti-F4/80 antibody (green). Nucleus were stained with DAPI (blue). HSPA12B deficiency increased macrophage (marked by F4/80+) infiltration into myocardium of septic mice at 36 hours. n=3/group. *P < 0.05, **P < 0.01, and ***P < 0.001 compared with indicated groups.

Increased monocytes/macrophages population in circulation and spleen in HSPA12B$^{-/-}$ septic mice

Activation of neutrophils and monocytes/macrophages plays a critical role in sepsis induced organ injury[212,454]. We examined the effect of endothelial HSPA12B on the immune cell populations in circulation and spleen tissue after induction of CLP sepsis. Figure 14A shows that the numbers of circulation monocytes were markedly increased in HSPA12B$^{-/-}$ septic mice, but not in WT septic mice. However, the numbers of circulating and splenic neutrophils in HSPA12B$^{-/-}$ septic mice were greater than in WT septic mice (Figs. 14B, C). Flow cytometry analysis shows that there are also more CD11b^{+}/F4/80^{+} macrophages populations in the spleen from HSPA12B$^{-/-}$ septic mice, when compared with WT septic mice (Figs. 15A, B). Further studies on the subsets of CD11b^{+}/F4/80^{+} macrophages present more proinflammatory macrophages (labeled by LY6C^{+}/CCR2^{+}) in the spleen of HSPA12B-/- septic mice.(Figure 15C). To investigate whether septic macrophages contain activated NF-κB, we isolated peritoneal macrophages from WT and HSP12B$^{-/-}$ mice and examined NF-κB subunit p65 nuclear translocation with immunostaining. As shown in Figure 16, macrophages that were isolated from peritoneal cavity of HSPA12B$^{-/-}$ septic mice exhibit greater NF-κB p65 subunit nuclear translocation than in WT septic mice. These data indicate that endothelial HSPA12B could regulate macrophage response to CLP sepsis challenge and regulate macrophage inflammatory response.

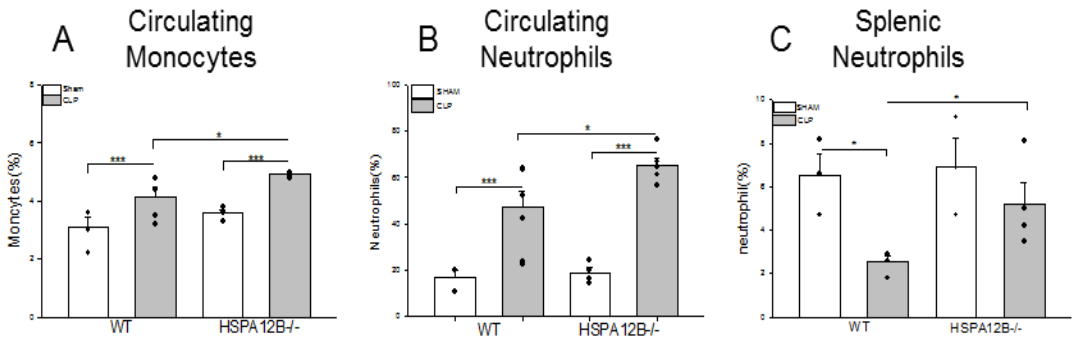


Figure 14. Increased macrophage populations in circulation and altered neutrophils populations in circulation and spleen in HSPA12B-/- septic mice. WT and HSPA12B$^{-/-}$ mice were subjected to CLP sepsis. Blood, and spleen were harvested at 36 hours after CLP for analysis of macrophage and neutrophil populations. (*A*) Flow cytometry analysis of the monocytes in blood with anti-CD11b+antibodies, and the neutrophils (Gr-1+ly6b.2+) in blood (B) and spleen (C) with anti-Gr-1+Ly6b.2+ antibodies. n=3~5/group *P < 0.05, **P < 0.01, and ***P < 0.001 compared with indicated groups.

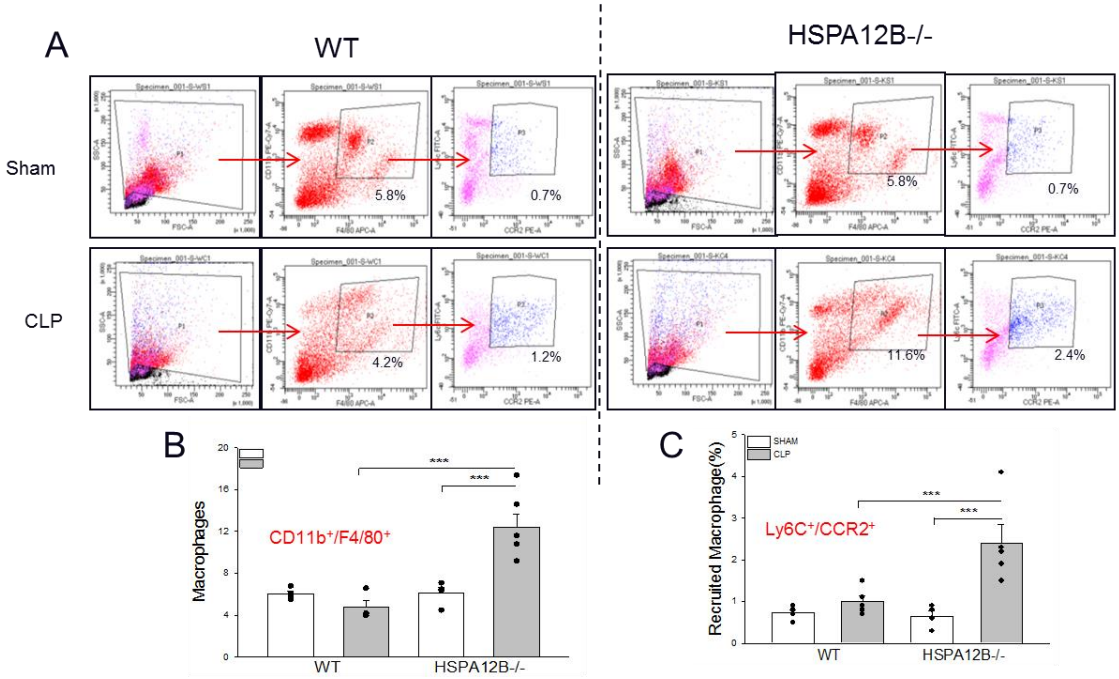


Figure 15. Increased pro-inflammatory macrophage populations in spleen in HSPA12B-/- septic mice. *A*) Gating strategy in Flow cytometry analysis of the macrophage population in spleen. Macrophages in spleen were identified based on the combination with anti-CD11b^{+}F4/80^{+} antibodies (*B*) and pro-inflammatory macrophages were identified based on combination with anti-Ly6C+/CCR2+ antibodies (*C*) from CD11b^{+}F4/80^{+} macrophages. n=3~5/group. *P < 0.05, **P < 0.01, and ***P < 0.001 compared with indicated groups.

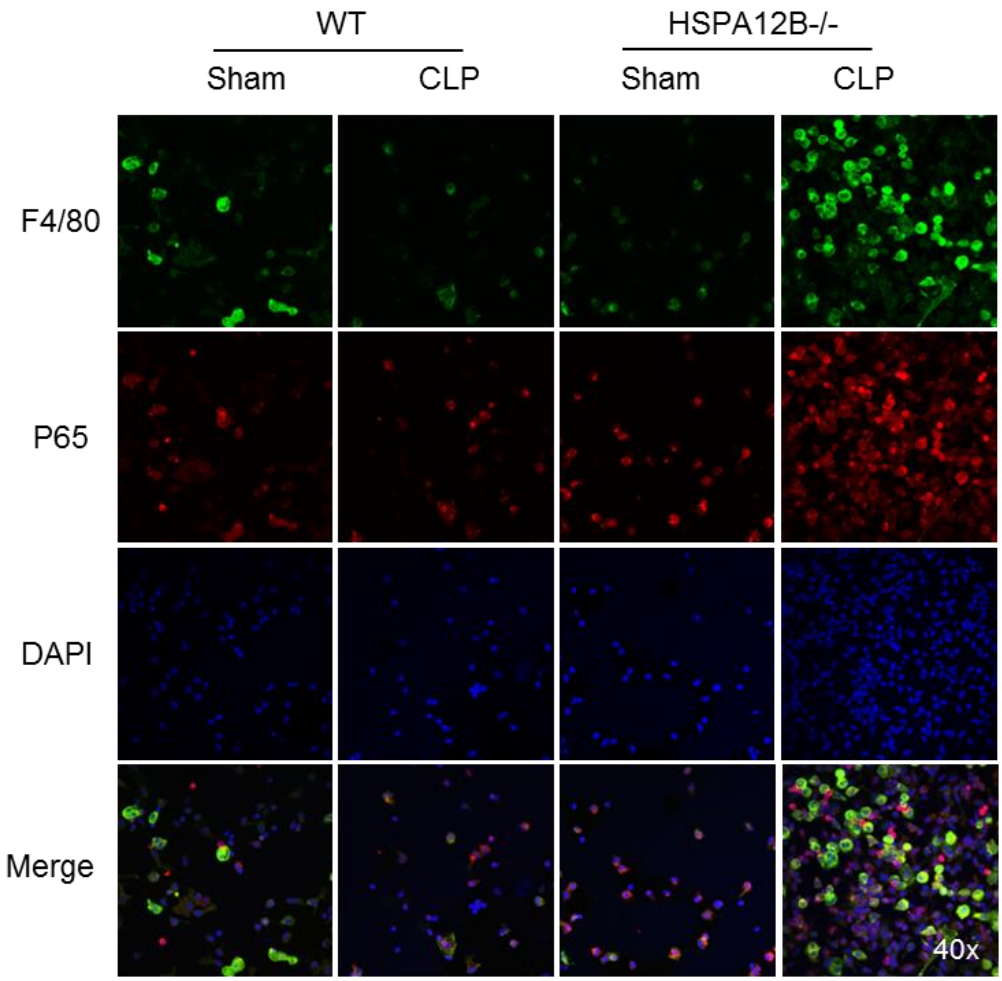


Figure 16. Activation of macrophages from peritoneal cavity in HSPA12B$^{-/-}$ septic mice. WT and HSPA12B$^{-/-}$ mice were subjected to CLP sepsis. Peritoneal macrophages were collected at 36 hours after CLP sepsis, which were plated for 2 hours, then adherent cells (macrophages) were stained for P65 (red), F4/80 (green) and DAPI (blue) to indicate activation of peritoneal macrophages.

Tissue Exosomes Contain Endothelial HSPA12B in WT Mice

Exosomes have been demonstrated to play a critical role in mediated cell-cell and tissue-tissue communication[454,236]. We examined whether endothelial HSPA12B could be released via exosome secretion. Exosomes were isolated from tissues for examination of HSPA12B by Western blot. Figure 17 shows that

the exosomes isolated from the heart, lung, kidney and spleen tissues from WT, but not HSPA12B$^{-/-}$ septic mice contain HSPA12B. Since HSPA12B is mainly expressed in endothelial cells[111], the data indicates HSPA12B could be released from endothelial cells via exosome secretion.

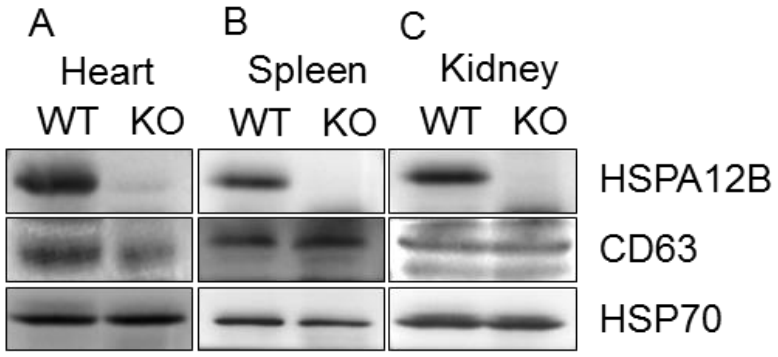


Figure 17. Tissue exosomes contain endothelial HSPA12B in WT mice. (*A-C*) exosomes were isolated from heart (*A*), Spleen (*B*), and Kidney (*C*) of WT and HSPA12B$^{-/-}$ mice.

Endothelial HSPA12B can Be Uptaken by Macrophages

To define whether HSPA12B containing exosomes can be up taken by macrophages, we transfected endothelial cells (HUVECs) with adenovirus expressing HSPA12B (Ad-HSPA12B) or Ad-GFP served as vector control. Twenty-four hours after transfection, we harvested the medium as an endothelial conditioned medium (ECM), incubated the macrophages (Raw 246.7) with the ECM, and examined whether endothelial HSPA12B could be up taken by macrophages. As shown in Figure 18, green fluorescence that indicates endothelial HSPA12B appeared in the macrophages that were incubated with ECM from the HUVECs transfected with Ad-HSPA12B (containing green

fluorescence), but not from Ad-GFP transfected HUVECs. Interestingly, stimulation of HUVECs with LPS increased HSPA12B in the ECM as evidenced showing that macrophages contained more fluorescence than control group. The data indicates that LPS stimulation could promote endothelial cells to release HSPA12B via exosome secretion. To confirm our finding, we transfected HUVECs with Ad-HSPA12B or Ad-GFP for 24 hours, isolated exosomes from cultured medium, and incubated macrophages with the isolated exosomes. Figure 19 shows that endothelial HSPA12B containing exosomes were up taken by macrophages. The data demonstrated that endothelial cell HSPA12B could be transmitted into macrophages via uptake of exosomes.

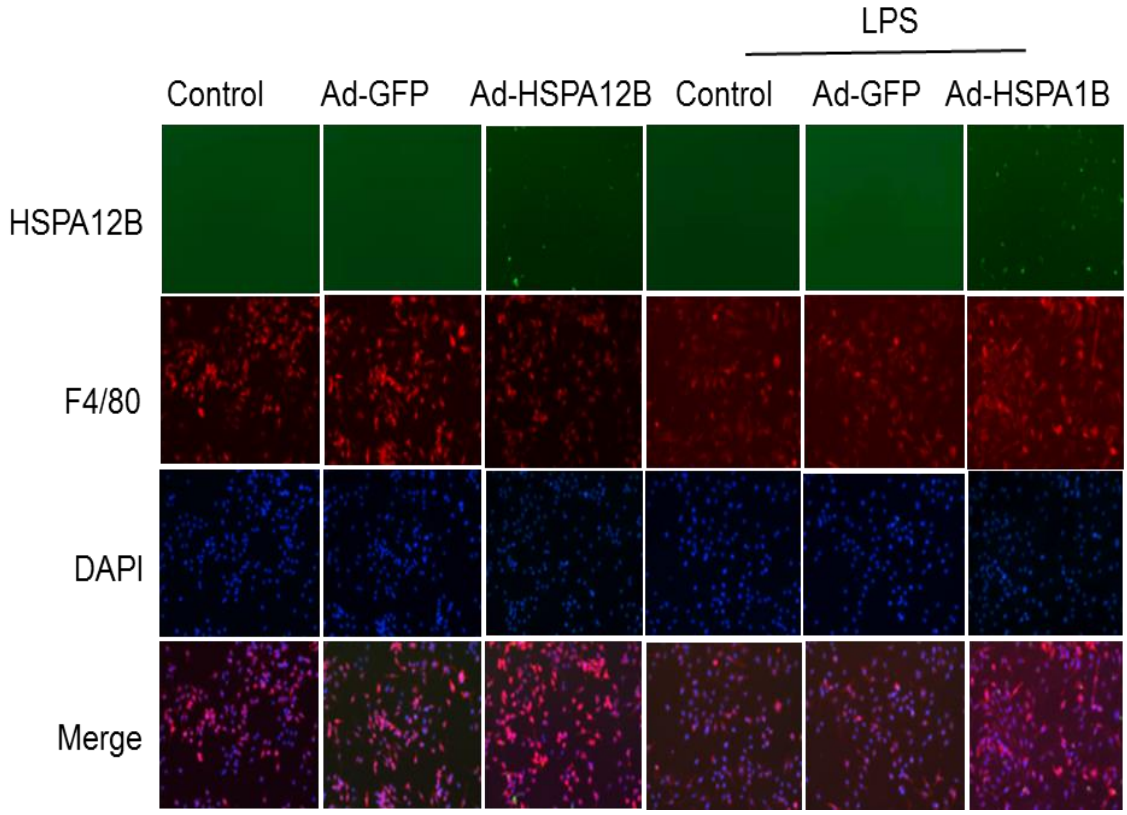


Figure 18.Macrophages uptake endothelial exosomes that contain HSPA12B. Macrophages uptake endothelial HSPA12B from endothelial cell conditioned medium. HUVECs were transfected with Ad-HSPA12B or Ad-GFP. Twenty-four

hours after transfection, the cultured medium was collected as the endothelial conditioned medium (ECM). Macrophages (Raw 264.7) were incubated with ECM. Endothelial HSPA12B in macrophages were examined with fluorescent microscope. HSPA12B exhibits green. Macrophages were strained with anti-F4/80 antibody. Nucleus was stained with DAPI.

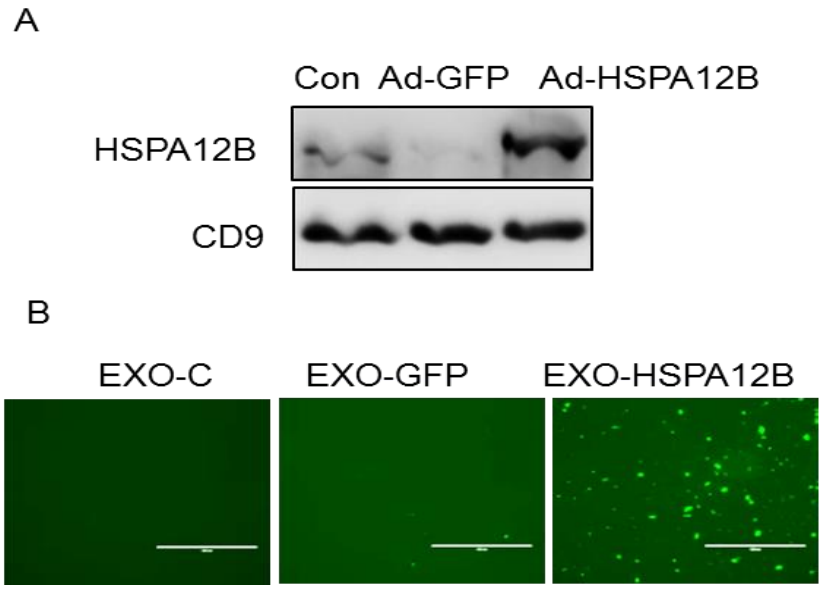


Figure 19. Macrophages uptake endothelial exosomes that contain high levels of HSPA12B. *A*) The levels of HSPA12B in Exosomes from ECM were evaluated by western blot. CD9 serves as a loading control for exosomes. *B*) Macrophages were incubated with endothelial exosomes that were isolated from ECM for one hour. Endothelial HSPA12B (green) in macrophages was viewed with fluorescent microscope.

Endothelial Conditioned Medium (ECM) Attenuated LPS Stimulated Inflammatory Cytokine Production in Macrophages

To investigate that HSPA12B released from endothelial cells could regulate the inflammatory cytokine production in macrophages, we first incubated macrophages with ECM before the cells were stimulated with LPS. Figures 20A-C show that LPS stimulation markedly increased the levels of TNFα (A), IL-1β (B) and IL-10 (C) compared with unstimulated control. However, ECM harvested

from Ad-HSPA12B transfected HUVECs, but not from Ad-GFP transfected HUVECs significantly attenuated LPS stimulated production of TNFα by 20.4% and IL-1β by 60.6% in macrophages. Interestingly, ECM harvested from Ad-HSPA12B transfected endothelial cells further enhanced LPS stimulated IL-10 production (by 27.3%) in macrophages. Similarly, incubation of bone marrow derived macrophages (BMDMs) with ECM collected from Ad-HSPA12B transfected HUVECs markedly suppressed LPS stimulated TNFα and IL-1β production and enhanced IL-10 production (Figs. 21A-C). The data suggests that endothelial HSPA12B could regulate macrophage response to LPS stimulation.

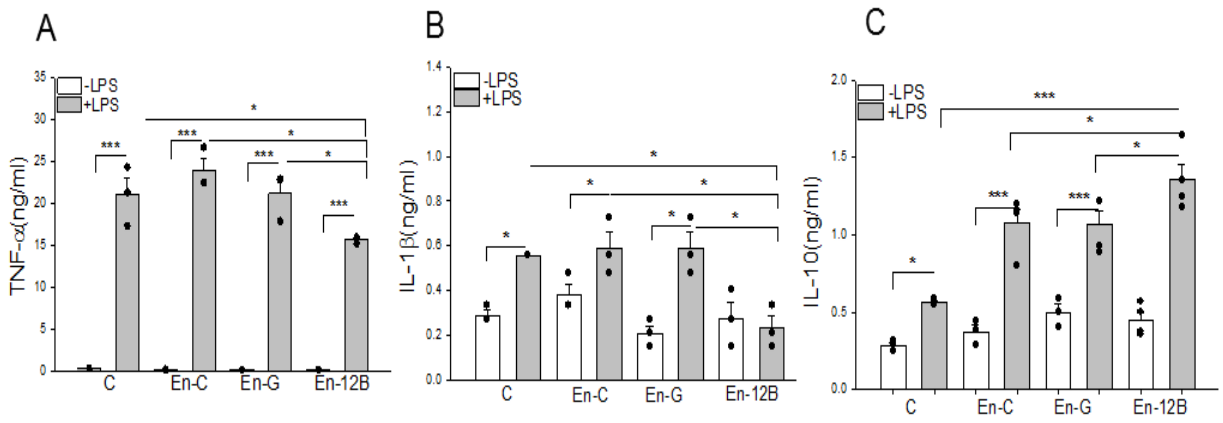


Figure 20. Endothelial conditioned medium (ECM) altered macrophage response to LPS stimulation. Raw 264.7 macrophages were incubated with ECM followed by LPS (1 μg/ml) stimulation for 24 hours. The medium was collected for the measurement of TNFα (*A*), IL-1β (*B*) and IL-10 (*C*) by ELISA. **P* < 0.05, ***P* < 0.01, and ****P* < 0.001 compared with indicated groups. C, En-C, En-G and En-12B indicate Control, endothelial-control, endothelial-Ad-GFP, and endothelial-Ad-HSPA12B separately.

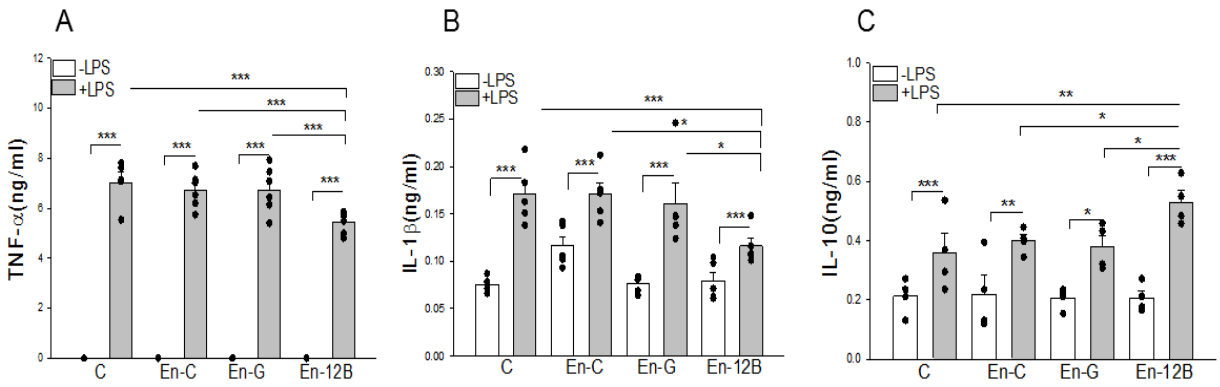


Figure 21. Endothelial conditioned medium (ECM) altered macrophage response to LPS stimulation. (A-C) Bone marrow derived macrophages (BMDM) were incubated with ECM followed by LPS (1 μg/ml) stimulation for 24 hours. The medium was collected for the measurement of TNF-α (*A*), IL-1β (*B*) and IL-10 (*C*) by ELISA. n= 3~4/group.

Endothelial HSPA12B Containing Exosomes Regulate Inflammatory Responses in Macrophages

We have shown that HSPA12B can be released from endothelial cells via exosomes (Fig. 19). We then investigated whether HSPA12B released by endothelial cells could regulate macrophage response to LPS stimulation. Endothelial exosomes were isolated from ECM that was collected from cultured endothelial cells (HUVECs) transfected with or without Ad-HSPA12B or Ad-GFP (defined as exosomes/control, exosomes/GFP and exosomes/HSPA12B). Macrophages were incubated with the isolated endothelial exosomes followed by LPS stimulation. Figures 22A-C show that LPS stimulation markedly increased TNFα (A), IL-1β (B) and IL-10 (C) production in macrophages. Importantly, exosomes/HSPA12B, but not exosomes/GFP or exosomes/control significantly attenuated LPS induced increases in TNFα and IL-1β levels and enhanced LPS

stimulated IL-10 production in macrophages. The data suggests that exosomes with high levels of HSPA12B could attenuate the response of macrophages to LPS challenge.

To confirm the role of HSPA12B in exosomes/HSPA12B regulating macrophage response to LPS stimulation, we directly transfected macrophages with Ad-HSPA12B or Ad-GFP 24 hours before the cells were stimulated with LPS. Figure 23A shows that high levels of HSPA12B were observed in the macrophages that were directly transfected with Ad-HSPA12B. Figures 23B-D show that transfection of Ad-HSPA12B, but not Ad-GFP attenuated LPS stimulated TNFα and IL-1β production and enhanced LPS stimulated IL-10 in macrophages. The data demonstrated that endothelial HSPA12B can regulate the response of macrophages to LPS stimulation.

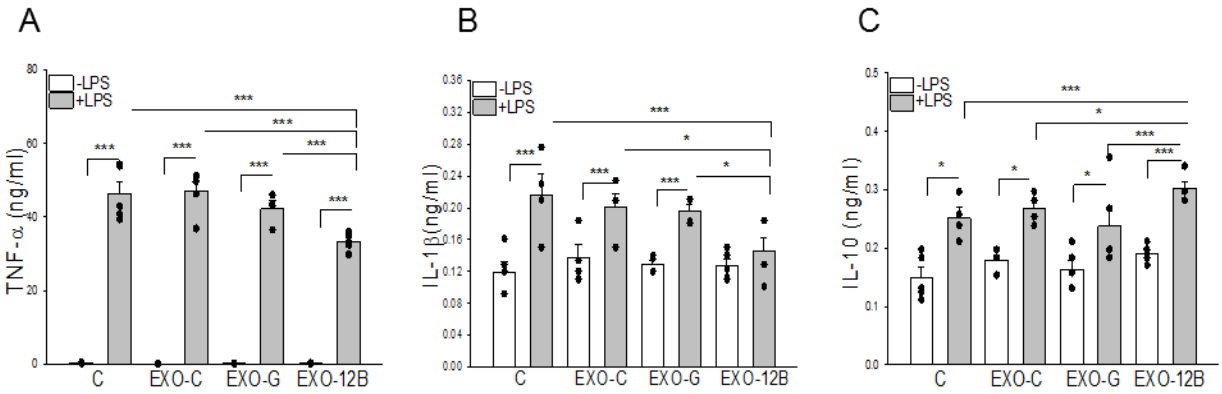


Figure 22. Endothelial exosomes altered macrophage response to LPS stimulation. Exosomes were isolated from HUVECs that were transfected with Ad-HSPA12B or Ad-GFP followed by LPS stimulation for 24 hours. Macrophages (RAW 264.7) were incubated with the isolated exosomes before LPS (1 μg/ml) stimulation for 24 hours. The medium was collected for measurement of TNF-α (*A*), IL-1β (*B*) and IL-10 (*C*) with ELISA. n= 4/group.

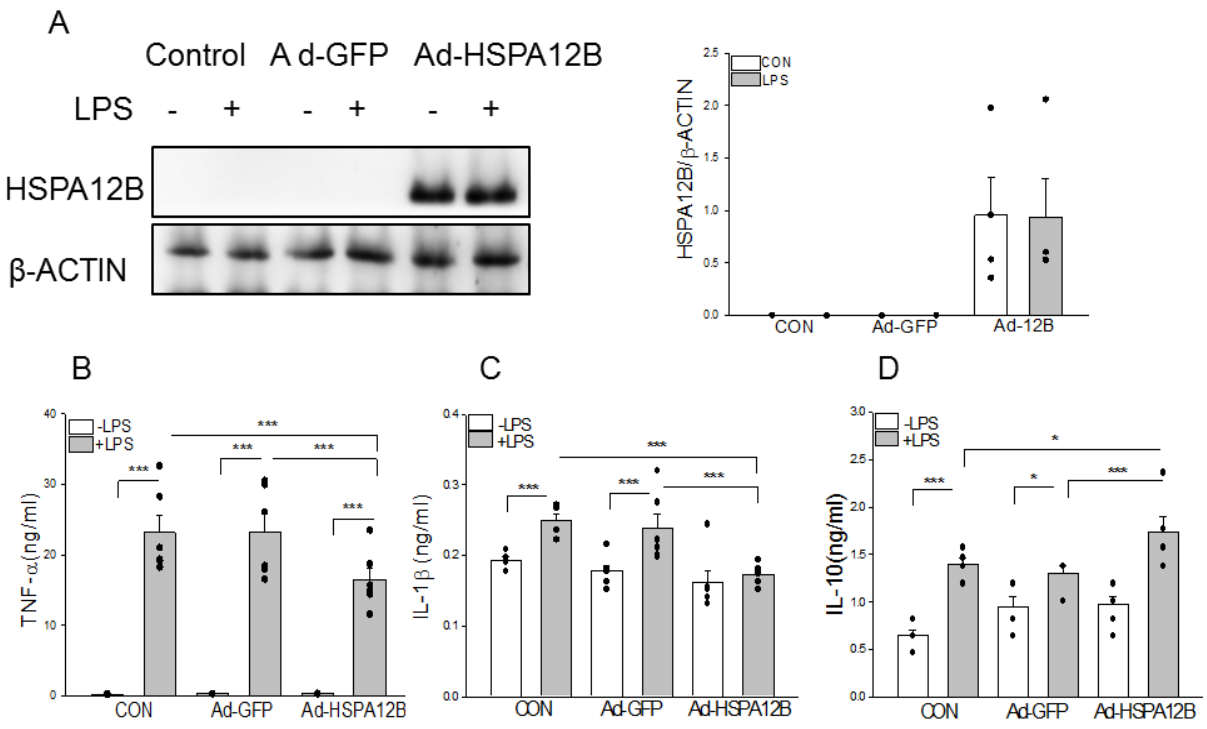
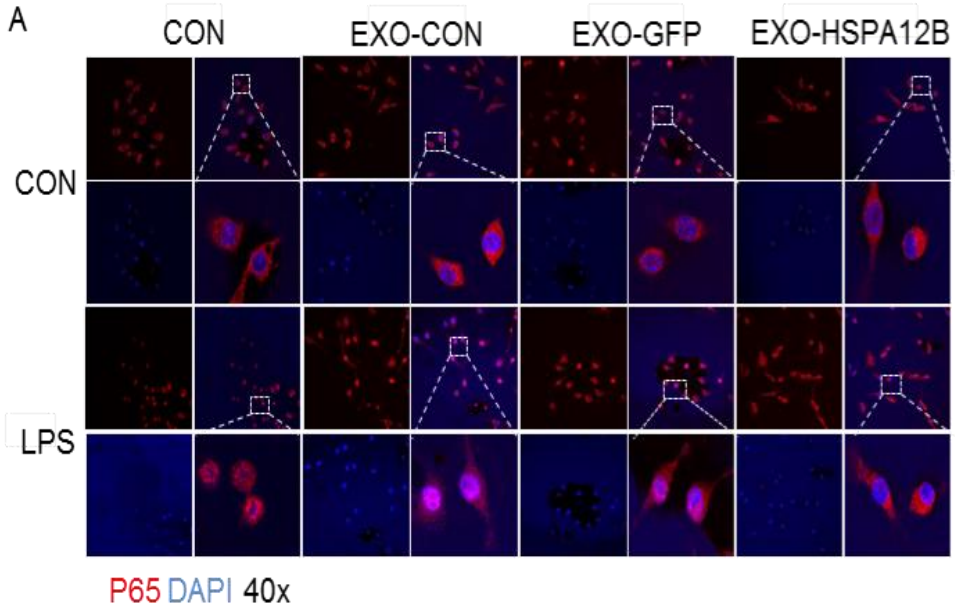


Figure 23. HSPA12B directly alters macrophage response to LPS stimulation. (*A*) Western blot analysis of HSPA12B levels in macrophages. The cultured medium was collected for the measurement of TNF-α (*B*), IL-1β (*C*) and increased IL-10 (*D*) with ELISA. N= 3~4/group. **P* <0.05, ***P* <0.01, and ****P* <0.001 compared with indicated groups.

Endothelial HSPA12B Containing Exosomes Downregulate NF-κB Activation in LPS Stimulated Macrophages

To investigate the mechanisms by which endothelial HSPA12B regulates macrophage response to LPS stimulation, we examined NF-κB activation in macrophages. Confocal microscopy examination shows that LPS stimulation induced NF-κB subunit p65 nuclear translocation (Fig. 24A). Western blot analysis shows that LPS stimulation increased the levels of phosphorylated IκBα (Fig. 24B) in macrophages. However, exosomes/HSPA12B administration

markedly attenuated LPS induced NF-κB subunit p65 nuclear translocation (A), IκBα phosphorylation (B). In addition, directly transfection of macrophages with ad-HSPA12B, but not ad-GFP significantly attenuated LPS induced IκBα phosphorylation (Fig.25A) and NF-κB subunit p65 nuclear translocation (Fig.25B). These data suggest that endothelial HSPA12B can directly regulate macrophage response to LPS stimulation via downregulation of NF-κB activation.



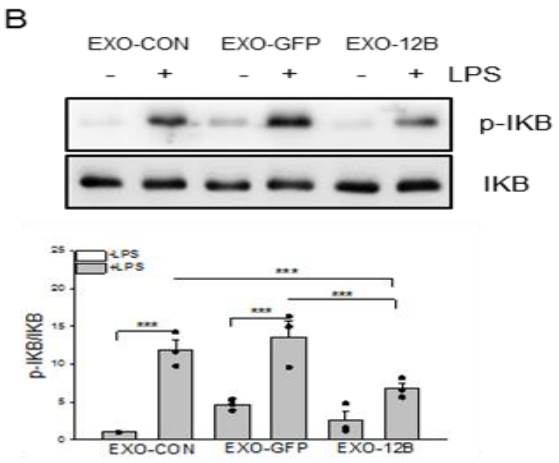


Figure 24. Endothelial exosomes that contain high levels of HSPA12B altered NF-κB activation in LPS stimulated macrophages. Exosomes were isolated from HEVECs that were transfected with Ad-HSPA12B or Ad-GFP followed by LPS stimulation for 24 hours. Macrophages (RAW 264.7) were incubated with the isolated exosomes for two hours prior to LPS (1 μg/ml) stimulation for one hour. (*A*) Immunostaining of NF-κB subunit p65 (red) nuclear translocation. The nucleus was stained with DAPI (blue). (*B*) Macrophages (RAW 264.7) were incubated with the endothelial exosomes (5 μg/ml) for 8 hours followed by LPS stimulation for one hour. Phosphorylated IκBα and total IκBα were assessed with Western blot. N=3~4/group. **P* < 0.05, and ****P* < 0.001 compared with indicated groups.

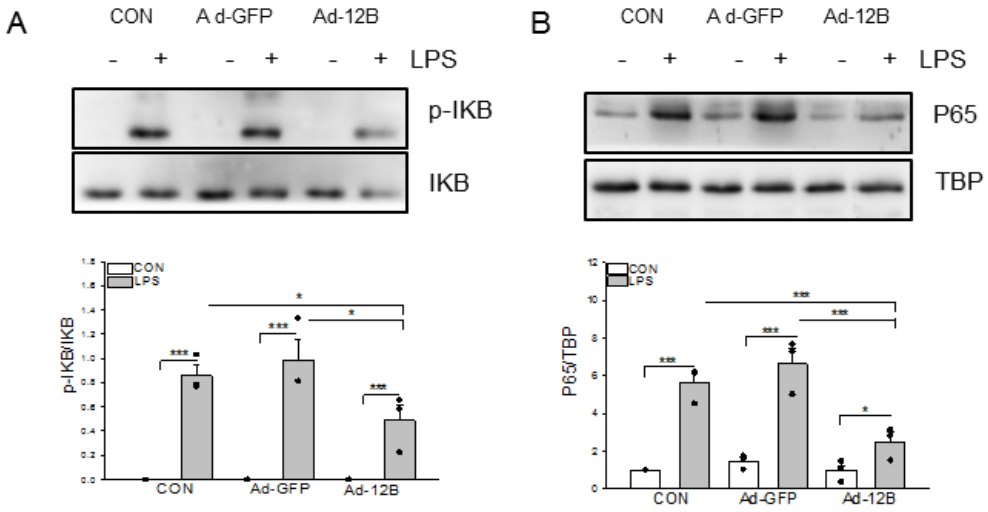


Figure 25. HSPA12B directly alters NF-κB activation in LPS stimulated macrophages. Macrophages (RAW 264.7) were transfected with the Ad-

HSPA12B-GFP or Ad-GFP for 6 hours by K2 transfection reagents separately for 8 hours, then cultured with fresh medium followed by LPS stimulation for half hour. Phosphorylated IκBα (*A*) and total IκBα (*A*) in the cytoplasm and the levels of nuclear NF-κB subunit p65 (*B*), were assessed with Western blot. N=3~4/group. **P* < 0.05, and ****P* < 0.001 compared with indicated groups.

Discussion

The present study demonstrated that endothelial HSPA12B exerts a protective role in sepsis by limiting cardiac dysfunction and maintaining survival, shown in the chapter 2. We also found that endothelial HSPA12B is released via exosome secretion. Recent evidence has shown that exosomes play a critical role in mediating cells-to-cell and organ-to-organ communication[236,455]. Interestingly, we found that the endothelial exosomes containing HSPA12B can be uptaken by macrophages, where it downregulates macrophage pro-inflammatory cytokine production and enhances IL-10 levels in response to LPS. Our data provides compelling evidence that there is crosstalk between endothelial cells and macrophages during sepsis and that endothelial cells can regulate macrophage function through exosomal HSPA12B.

Endothelial HSPA12B has been reported to attenuate endotoxemia[113] and cardiac injury due to myocardial infarction[115]. The mechanisms involve activation of PI3K/Akt signaling and suppression of NF-κB activation[113,115]. Endothelial HSPA12B also protects vascular endothelial cells from sepsis induced acute lung injury[437]. We observed in the present study that deficiency of HSPA12B in endothelial cells results in severe cardiac dysfunction, decreased survival outcome, and increased pro-inflammatory cytokine production in polymicrobial sepsis. These findings provide further evidence that endothelial cell HSPA12B

plays an important role in limiting pro-inflammatory responses and protecting the host against sepsis induced cardiomyopathy and mortality.

To elucidate the mechanisms by which endothelial HSPA12B attenuates sepsis induced cardiomyopathy and organ injury, we focused on the role of endothelial HSPA12B in the regulation of macrophage inflammatory response to sepsis. Recent evidence highlighted the role of macrophage function in sepsis induce organ dysfunction[46,47]. We found that endothelial HSPA12B deficiency results in higher levels of adhesion molecules in the myocardium, which are associated with increased macrophage infiltration into the heart during CLP sepsis. Our observation suggests that endothelial HSPA12B not only regulates the expression of adhesion molecules on endothelial cells, but it also regulates macrophage inflammatory response during sepsis. It is well known that endothelial cell dysfunction contributes to sepsis induced multiple organ dysfunction[456]. Increased expression of HSPA12B prevents LPS-induced increases in the expression of adhesion molecules (ICAM1 and VCAM1) and attenuates LPS-induced inflammatory responses in endothelial cells[113,118]. We have previously demonstrated that endothelial HSPA12B downregulates adhesion molecule expression via microRNA-126 (unpublished data). In the present study, we demonstrated that endothelial cells can release HSPA12B in exosomes and that exosomal HSPA12B can be uptaken by macrophages. This observation indicates that endothelial HSPA12B mediate crosstalk between endothelial cells and macrophages in the response to septic challenge. Of

greater importance, endothelial HSPA12B exerts an anti-inflammatory effect on macrophages.

Previous studies have shown that there is crosstalk between endothelial cells and macrophages during sepsis and/or inflammatory diseases[303,457]. Increasing evidence suggests that endothelial cells and macrophages engage via specific interactions to regulate vascular function[458,459]. Adult endothelial cells provide critical signals for the selective growth and differentiation of macrophages from several hematopoietic progenitors[460,461]. Thus, endothelial cells may serve as endogenous regulators for the activation or suppression of macrophage mediated innate immune and inflammatory responses. Indeed, we observed that the endothelial cell conditioned medium (ECM) generated from Ad-HSPA12B transfected endothelial cells markedly downregulated LPS stimulated inflammatory cytokine production by macrophages, suggesting that endothelial HSPA12B could regulate macrophage response to LPS stimulation.

Exosomes have been demonstrated to play a critical role in the cellular communication in both physiological and pathological conditions[455,236]. Exosomes carry on components, including proteins, lipids, mRNA, and miRNAs from parent cells, which could be taken up by and affect the functions of recipient cells[462]. Njock *et al* reported endothelial vehicles (EVs) loaded with miRNAs suppress inflammatory response in monocytes/macrophages[303]. He *et al* reported the endothelial EVs modulated macrophage polarization toward M2 anti-inflammatory phenotype[304]. Interestingly, we observed that exosomes isolated

from tissues contain HSPA12B. *In vitro* data shows that HSPA12B can be transmitted from endothelial cells to macrophages via exosomes.

We also found that endothelial HSPA12B is uptaken by macrophages and that it markedly downregulated LPS stimulated pro-inflammatory cytokine production and enhanced IL-10 levels. To confirm our observation, we performed direct transfection of macrophages with Ad-HSPA12B and observed that transfected HSPA12B suppressed LPS increased the levels of pro-inflammatory cytokines and enhanced anti-inflammatory cytokine production. The data strongly supports the concept that endothelial HSPA12B can regulate macrophage response to LPS stimulation.

Activation of NF-κB pathway contributes to sepsis induced mortality and organ dysfunction[463]. Our previous studies have demonstrated that targeting Toll-like receptors (TLRs) mediated NF-κB activation pathway increased survival outcome in ploymicriobial sepsis[440,47]. The present study demonstrated that endothelial HSPA12B significantly suppresses IκBα phosphorylation and NF-κB subunit P65 nuclear translocation in LPS stimulated macrophages, suggesting that endothelial HSPA12B regulates NF-κB activation in macrophages. Future studies are needed to investigate the mechanisms by which endothelial HSPA12B suppresses NF-κB activation in LPS challenged macrophages.

CHAPTER 4. β-GLUCAN DOWNREGULATES INFLAMMATORY RESPONSES IN PLOYMICROBIAL SEPSIS BY A CROSSTALK BETWEEN MACROPHAGES AND ENDOTHELIAL CELLS THROUGH EXOSOMAL HSPA12B

Introduction

Traditionally, immune systems in vertebrate animals consist of innate immunity and adaptive immunity.[464] It is immunological memory that distinguishes adaptive immunity from innate immunity. Innate immune cells lack the immune memory to respond quickly to secondary stimulations.[465] However, a series of exciting studies led by Netea and colleagues have challenged this conventional notion. It was Netea who coined the term “trained immunity”, when he discovered that myeloid cells hold immune memory to respond to secondary stimulation quickly and non-specifically.[333,356,357,410,352]

β-1,3-(D)-glucan (β-glucan), a major component of the Candida cell wall, has been recognized as an immune-stimulator, to modulate innate immunity, and the proinflammatory signaling pathway.[75] It does this by binding with receptor, Dectin-1, expressed on the membrane of immune cells, to activate them.[466,467] The previous studies by our lab have reported that β-glucan administration before induction of sepsis protects against sepsis-induced mortality, by downregulating the NF-κB signaling pathway to suppress proinflammatory responses in a murine of CLP sepsis.[468,440] In previous experiments in septic mice , β-glucan was administered by intraperitoneal injection 1 hour before surgery. Therefore, the protection of β-glucan is not associated with immune memory in innate immune cells, due to the short gap between β-glucan

administration and surgery .[440,468,469] Recent studies have shown that β-glucan administration 7 days before *S. aureus* infection significantly increased the survival rate from 40% to 90% in *S. aureus* induced sepsis. Increased survival outcome is mediated by hypoxia-induced factor 1α (HIF-1α) regulating aerobic glycolysis in myeloid cells[357]. The data indicates the critical role of β-glucan-induced trained immunity against bacterial stimulation. However, the mechanism by which β-glucan-induced trained immunity in CLP sepsis is still unclear.

Endothelium, formed by endothelial cells, has been reported to regulate macrophage inflammatory responses during sepsis.[49] In the previous chapter, we have demonstrated that endothelial HSPA12B, released via exosomes, could be captured by macrophages and subsequently decrease inflammatory responses. Meanwhile, barely derived β-glucan plays a role in the regulation of endothelial cell functions.[414,415]. Moreover, β-glucan trained monocytes/macrophages exhibit sensitive and strong responses to secondary stimulations in vitro.[339] However, the contribution of β-glucan for the interaction between macrophages and endothelial cells remain unknown.

In our current study, we hypothesized that β-glucan induced trained immunity benefits the survival outcome of septic mice through a crosstalk between endothelial cells and macrophages. To test this hypothesis, we did *in vitro* and *in vivo* experiments that demonstrate β-glucan can induce trained endothelial cells that regulate innate immunity to improve survival outcome of CLP sepsis. The mechanisms involve increased endothelial cell HSPA12B expression and release via exosome secretion. The endothelial exosomal

HSPA12B downregulates activated NF-κB mediated inflammatory response in macrophages.

Materials and methods

Animals

Endothelial specific HSPA12B knockout mice ($HSPA12B^{-/-}$) 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. $HSPA12B^{-/-}$ mice and age matched wild type (WT) C57BL/6 mice were used for experiments. The mice were maintained in the Division of Laboratory Animal Resources at East Tennessee State University. The experiments outlined in this manuscript conform to the Guide for the Care and Use of Laboratory Animals published by the National Institutes of Health (8th edition, 2011). The animal care and experimental protocols were approved by the Eastern Tennessee State University Committee on Animal Care.

Cecal Ligation Sepsis Model

Murine polymicrobial sepsis was induced by cecal ligation and puncture as described previously[432,440,433,47,75]. To induce of a septic model, mice were anesthetized by 5.0% isoflurane. A midline incision was made on the anterior abdomen and the cecum was exposed and 1/3 of cecum was ligated with a 4-0 suture. One puncture was made at the end of cecum with a 23-gauge needle and feces were extruded from the hole. The abdomen was then closed in 2 layers. Sham surgically operated mice served as sham control. Immediately following

surgery, a single dose of resuscitative fluid (lactated Ringer's solution, 50 mL/kg body weight) was administered by subcutaneous injection.

Immunofluorescent Staining

The procedure of the immunofluorescent staining was modified based on the protocol provided by Cell Signaling Technology. Briefly, cells were grown on the coverslips in a multiwell plate, fixed with 4% formaldehyde for 15 min in room temperature and washed for three times with PBS for 5 minutes each. Cells were permeabilized with ice-cold 100% methanol for 10 min at -20oC followed by rinsing in PBS for 5 min. The cells were incubated with 10% goat serum for 60 min at room temperature followed by incubation with the primary antibody (p65, 1:400, Cell signal technology) that was diluted with 10% goat serum, at 4oC overnight according to the instruction provided by manufacturer. After washing for three times with PBS for 5 min each, the cells were incubated with a fluorochrome-conjugated secondary antibody for 60 min at room temperature in dark. The cells were washed with PBS for three times, covered by antifade reagent with DAPI and examined with confocal microscope.

Isolation of Exosomes

HUVECs were incubated with FBS-free medium. The medium was collected and centrifuged at 2000g for 30 min to remove debris. The supernatants were transferred into a new tube. 30% PEG6000 (Sigma-Aldrich) reagent was mixed with the supernatant at ratio of 1:2 by votexing until there is a homogenous solution. The mixture was incubated at 2~8^{o}C overnight and centrifuged at 3000g for 30 min. The supernatant was removed. Exosomes in the

pellet were resuspended in cold PBS and the exosome markers were examined by Western blot.

Western Blot

Western blot was performed as described previously[47,75,432,433]. In brief, cellular proteins were separated by sodium dodecyl sulphate–polyacrylamide gel electrophoresis and transferred onto Hybond enhanced chemiluminescence (ECL) membranes (Amersham Pharmacia, Piscataway, New Jersey). The ECL membranes were incubated with the appropriate primary antibodies followed by peroxidase-conjugated secondary antibody, which was purchased from Cell Signaling Technology, Inc. The signals were quantified using the G: Box gel imaging system by Syngene (Frederick, Maryland).

ELISA for Cytokine Assay

The serum levels of aspartate aminotransferase (AST, hepatic injury marker) and creatinine (kidney injury marker) were measured with commercially available kits according the instructions provided by the manufacturer (Millipore Sigma). And serum inflammatory cytokines (TNF-α, IL-1β and IL-6) were measured with commercially available kits according the instructions provided by the manufacturer (Peprotech).

Flow Cytometric Analysis

Seed HUVECs at a density of $2.0x10^5$ cells in 6-well plate until the cells are ~90% confluent. Abandon the medium followed with 1ml warm PBS wash and 0.5ml warm PBS incubate cells at incubator for 5min~10min. Pellet the cells

at 350g for 10 min and suspended with 50ul blocking buffer (0.5% NRS, 0.5% BSA, 5mM EDTA, 0.1% NaN_3) for 10^6 cells, and incubate on ice for 5 min. Antibody incubation (Dectin-1,10ul) at 0.5~1h on ice in the dark. Pellet the cells at 350g for 5 min and suspend with 100ul wash buffer (PBS/0.1% NaN_3/1% FBS). Transfer the 100ul cell mixture into tubes with 250ul wash buffer prior to test by flow cytometry.

Statistical Analysis

The data are expressed as mean ± standard error. Comparisons of data between groups were made using one-way analysis of variance (ANOVA), and Tukey procedure for multiple-range tests was performed. The log-rank test was used to compare group survival trends. Probability levels of ≤.05 were used to indicate statistical significance.

Results

β-Glucan Increased Survival Outcome and Attenuated Immune and Inflammatory Responses in Polymicrobial Sepsis

To investigate whether β-glucan could improve survival outcome of sepsis, WT mice were trained with 1 mg β-glucan intraperitoneally (i.p.) 7 days prior to CLP sepsis. As shown in Figure 26, β-glucan trained septic mice exhibited an increased survival outcome by 18% compared with septic mice. The time to reach a 62% mortality of WT septic mice was ~110 hours, while a mortality of 44% of β-glucan trained septic mice was 200 hours, after induction of CLP sepsis. However, the increased survival outcome wasn't statistically significant, which may be due to the limited number of β-glucan trained septic mice.

Moreover, although CLP sepsis significantly increased serum TNF-α and IL-6 levels at 36 hours, β-glucan markedly attenuated the levels of serum TNF-α (Fig.27A) and IL-6(Fig.27B) by 22.99% and 51.91% separately, compared with the counterparts in septic mice. The data suggests that β-glucan, a reagent for trained immunity could improve survival outcome of CLP sepsis with attenuated immune responses.

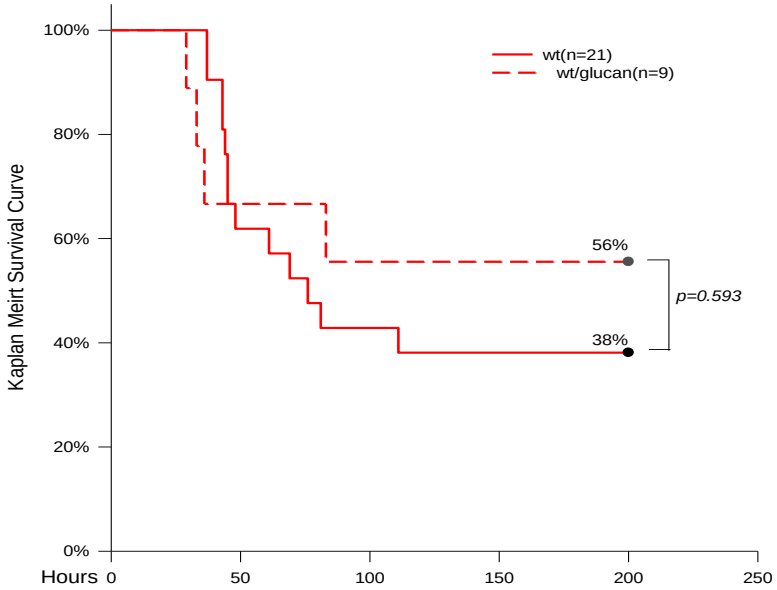


Figure 26. β-glucan, a reagent for trained immunity is involved in the regulation of mortality in polymicrobial sepsis. 1mg fresh β-glucan solution was injected into WT mice by i.p. 7 days prior to CLP sepsis. The survival outcome was monitored for last to 200 hours following induction of CLP. n=9~21/group.

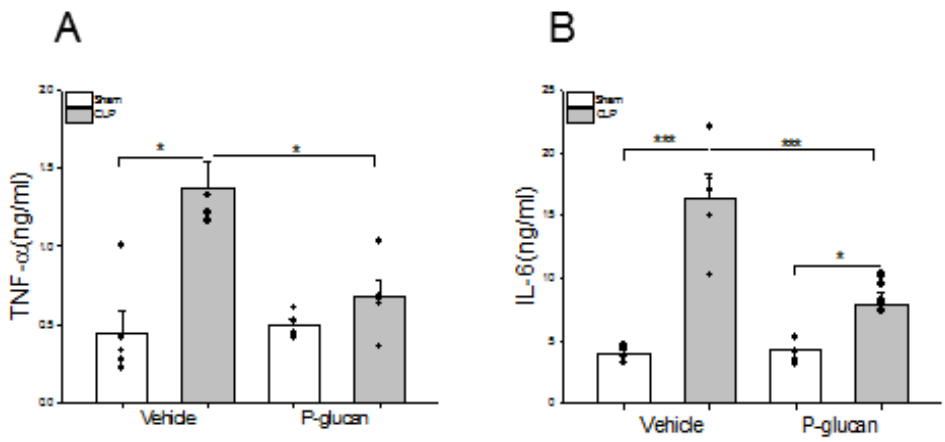


Figure 27. β-glucan, a reagent for trained immunity is involved in the regulation of immune responses in polymicrobial sepsis. 1mg fresh β-glucan solution was injected into WT mice by i.p. 7 days prior to CLP sepsis. Serum was collected at 36 hours after CLP sepsis. The levels of TNF-α and IL-6 was analyzed with enzyme-linked immunosorbent assay. CLP sepsis significantly altered serum TNF-α (*A*) and IL-6(*B*) levels at 36 hours septic mice. However, β-glucan markedly attenuated the levels of serum TNF-α and IL-6, compared with the counterparts in septic mice. n= 4~6/group. **P* < .05, ***P* < .01, and ****P* < .001 compared with indicated groups.

β-Glucan Increases the Levels of HSPA12B in Endothelial Cells and the Exosomes Derived from Endothelial Cells *in vitro*

Due to the protective role of HSPA12B in sepsis, shown in the previous chapters, we tested the role of β-glucan in the regulation of HSPA12B expression in endothelial cells. β-glucan was incubated with HUVECs for 24 hours. As shown in Figure 27A, β-glucan can markedly enhance the expression of HSPA12B in endothelial cells, which indicates the role of β-glucan in the regulation of endothelial cell function. Furthermore, β-glucan remarkably augmented the levels of HSPA12B in exosomes secreted from endothelial cells. Moreover, β-glucan treatment induced increased expression of Dectin-1 on

endothelial cells. The data suggests the regulation of β-glucan in HSPA12B expression may be through Dectin-1 in endothelial cells.

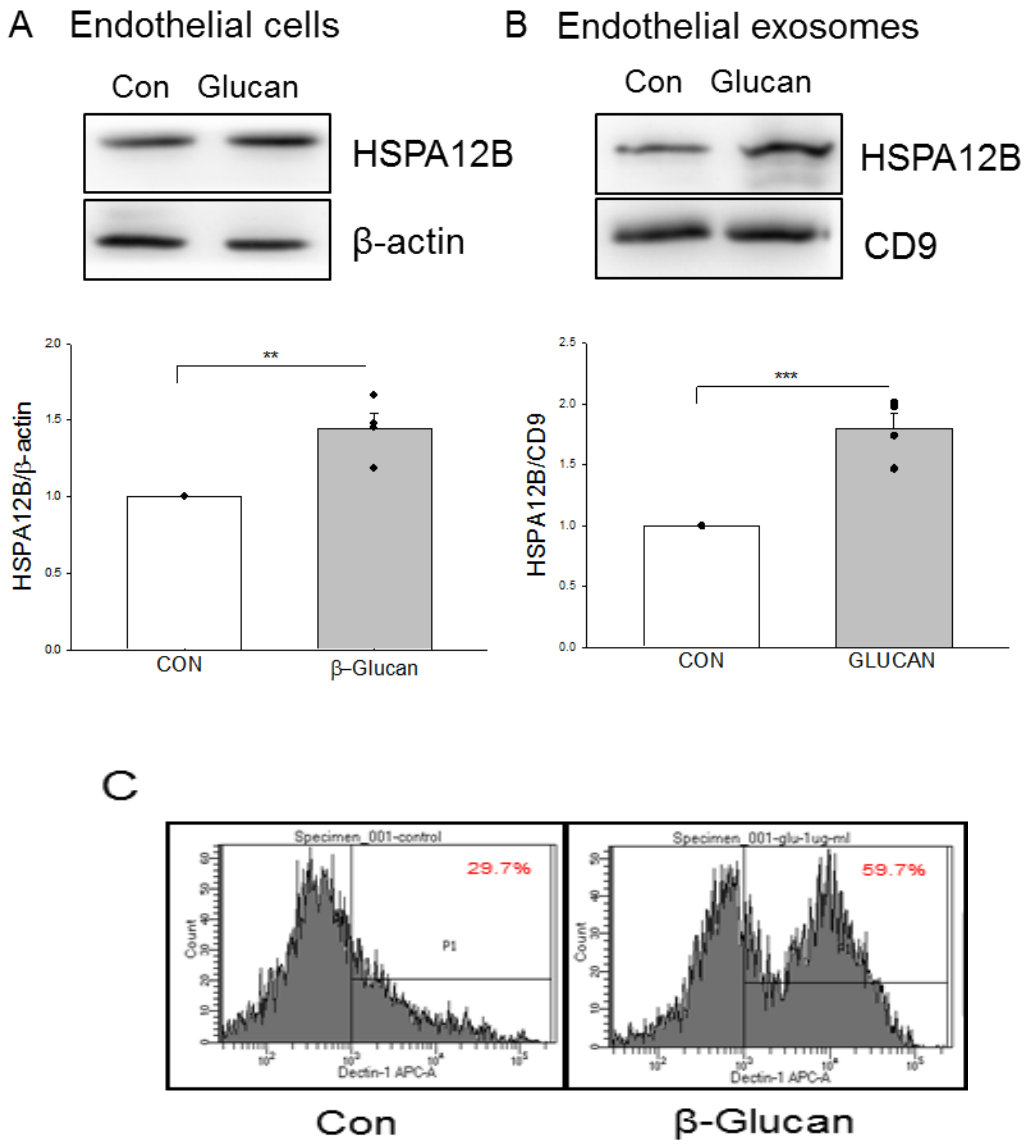


Figure 28. β-glucan regulates levels of HSPA12B in endothelial cells and endothelial cell derived exosomes. (1ug/ml) fresh β-glucan in endothelial medium was incubated with endothelial cells for 24 hours. The levels of HSPA12B in endothelial cells (*A*) and endothelial exosomes (*B*) were evaluated by western blot. n=4/group. (*C*) (1ug/ml) fresh β-glucan in endothelial medium was incubated with endothelial cells for 5 hours. Cells were harvested to detect expression of dectin-1 by flow cytometry. **P* < 0.05, ***P* < 0.01, and ****P* < 0.001 compared with indicated groups.

Exosomes from β-glucan Treated Endothelial Cells Downregulate Inflammatory Responses in LPS-Stimulated Macrophages

Due to the suppression of HSPA12B in inflammatory responses in LPS-stimulated macrophages, we further investigated the roles of exosomes from β-glucan treated endothelial cells (Glucan-exosomes). Figure29A-B show that LPS stimulation markedly increased the levels of TNFα (A), and IL-6(B) compared with unstimulated control. However, Glucan-exosomes significantly attenuated LPS stimulated production of TNFα by 13.44%, and IL-6 by 25.77% in macrophages. The data indicates that β-glucan significantly increased levels of HSPA12B in endothelial exosomes which may contribute to the attenuation of macrophage inflammatory response to LPS challenge.

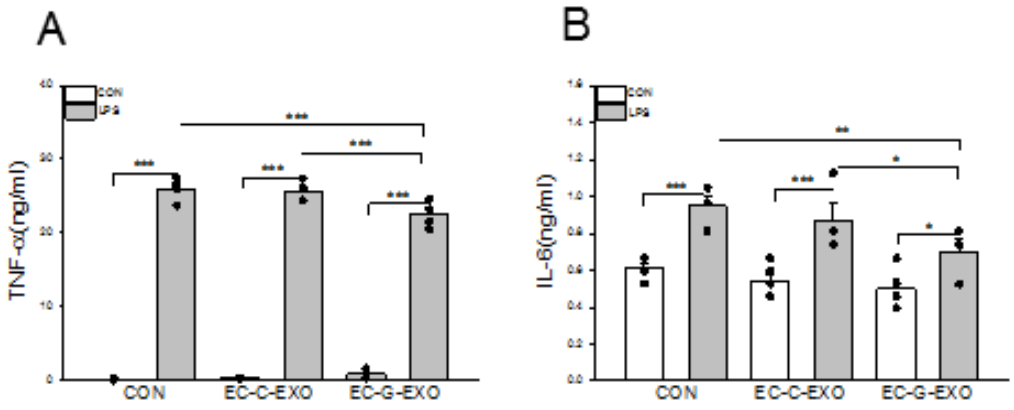


Figure 29. Exosomes from β-glucan treated endothelial cells downregulate inflammatory responses in LPS-stimulated macrophages. Exosomes were collected from (1ug/ml) treated endothelial cells and were used to incubate with macrophages for 2 hours followed by LPS (1ug/ml) stimulation for 24 hours. The medium was collected for the measurement of TNF-α (*A*), and IL-6 (*B*) by ELISA. n= 4~5/group. **P* < .05, ***P* < .01, and ****P* < .001 compared with indicated groups.

Exosomes from β-Glucan Treated Endothelial Cells Suppress the Translocation of Nuclear NF-κB Subunit p65 in LPS-Stimulated Macrophages

To investigate the mechanisms by which Glucan-exosomes regulate macrophage response to LPS stimulation, we examined NF-κB activation in macrophages. Confocal microscopy examination shows that LPS stimulation induces NF-κB subunit p65 nuclear translocation. As shown in figure 29, Glucan-exosomes incubating with macrophages markedly attenuated LPS induced NF-κB subunit p65 nuclear translocation. This data suggests that glucan-exosomes could directly regulate macrophage responses to LPS stimulation via the downregulation of NF-κB activation.

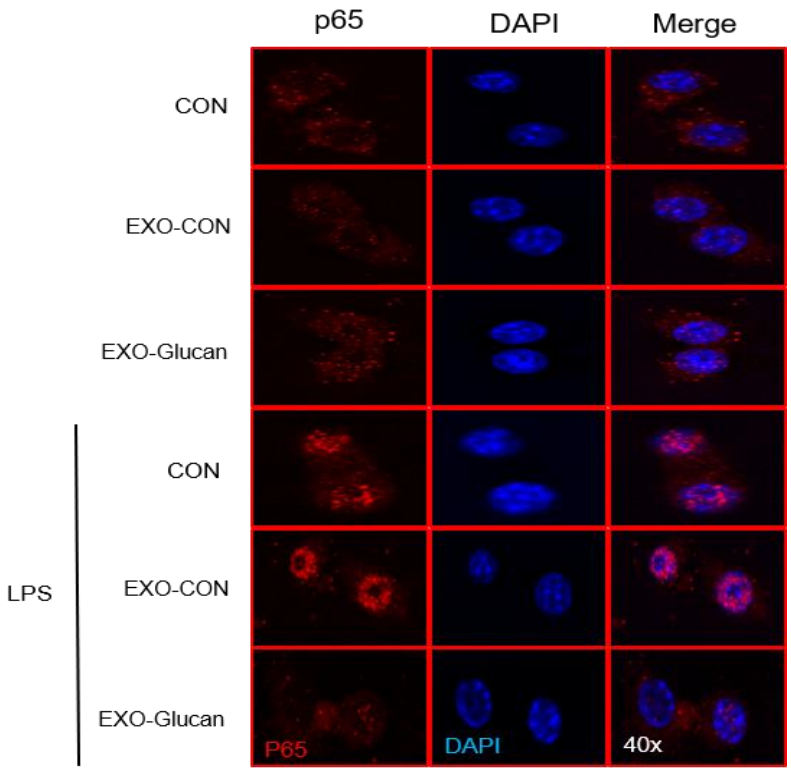


Figure 30. Exosomes from β-glucan treated endothelial cells downregulate inflammatory responses in LPS-stimulated macrophages. Exosomes were collected from the medium of β-glucan (1ug/ml) treated endothelial cells and were used to incubate with macrophages for 2 hours followed by LPS(1ug/ml) stimulation for one hour.) Immunostaining of NF-κB subunit p65 (red) nuclear translocation. The nucleus was stained with DAPI (blue).

β-Glucan Induced Trained Immunity Partially Rescues HSPA12B Deficiency Induced Mortality in Polymicrobial Sepsis

To investigate whether β-glucan induced trained immunity could reduce the severe mortality induced by HSPA12B deficiency, we examine the effect of β-glucan induced trained immunity on the survival outcome in CLP septic mice. HSPA12B$^{-/-}$ mice were trained with 1 mg β-glucan i.p. 7 days prior to CLP sepsis. As shown in Figure 30, compared with 100% mortality in HSPA12B$^{-/-}$ septic mice, β-glucan trained HSPA12B$^{-/-}$ septic mice exhibited an increased survival outcome

by 17% until the end of the observation. The data suggests that β-glucan induced trained immunity plays an important role in the protection against mortality in sepsis.

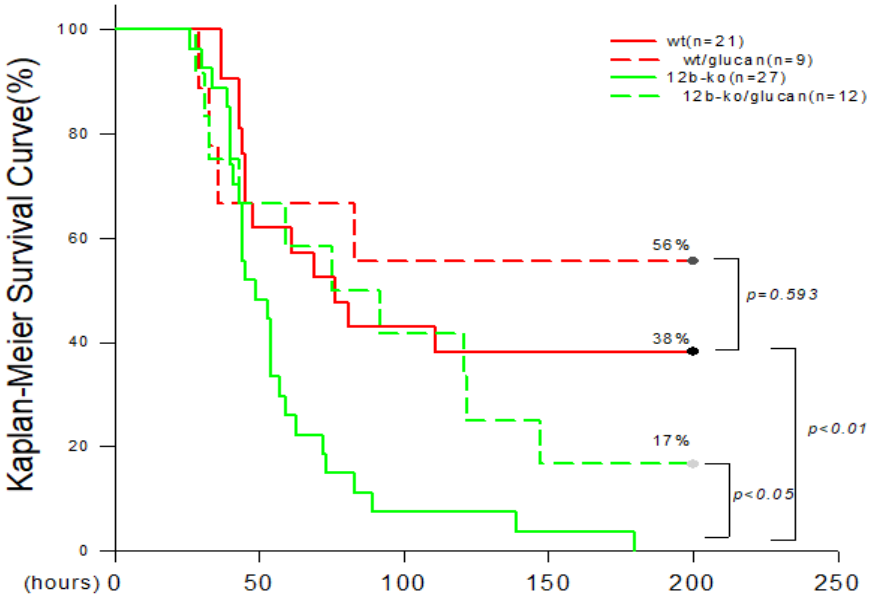


Figure 31. β-glucan-induced trained immunity partially rescues HSPA12B deficiency induced mortality in polymicrobial sepsis.1mg fresh β-glucan solution was injected into WT and HSPA12B$^{-/-}$ mice by i.p. 7 days prior to CLP sepsis. The survival outcome was monitored for last to 200 hours following induction of CLP (n=9~27/group). group *P < .05, **P < .01, and ***P < .001 compared with indicated groups.

β-Glucan Induced Trained Immunity Rescues HSPA12B$^{-/-}$ Induced Increased Inflammatory Cytokine in Polymicrobial Sepsis

Due to the non-specific protective immunological memory, induced by β-glucan induced trained immunity, we further tested whether β-glucan induced trained immunity regulates immune responses in CLP sepsis. Figure32 shows that although sepsis markedly increases serum TNF-α levels in both septic

groups, higher levels of serum TNF-α, caused by HSPA12B deficiency, was attenuated by β-glucan induced trained immunity. This is remarkably close to the counterpart in the serum of WT septic mice. The data suggests that β-glucan induced trained immunity may play a protective role against the upregulation of immune responses caused by HSPA12B deficiency in CLP sepsis.

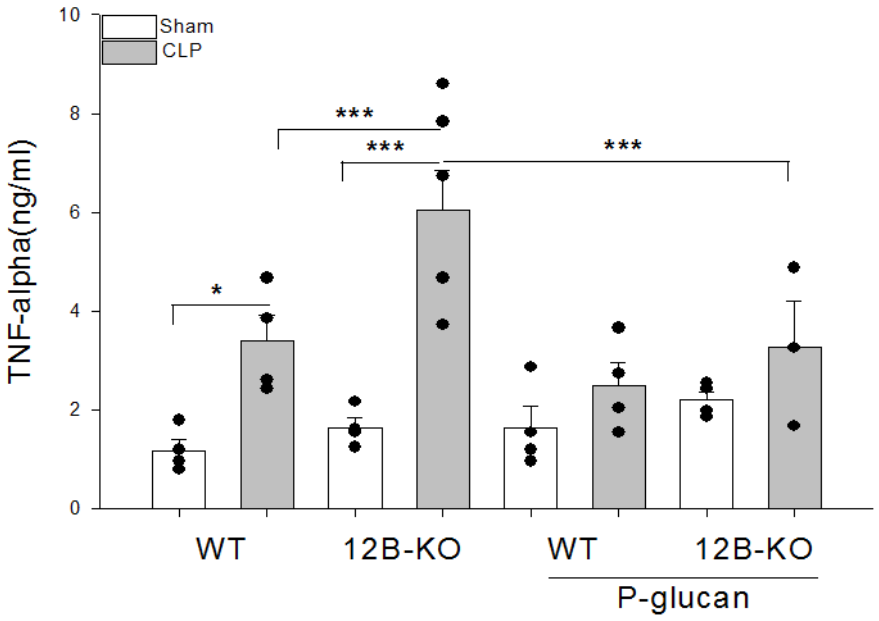


Figure 32. β-glucan-induced trained immunity attenuates HSPA12B deficiency induced increased inflammatory cytokine in polymicrobial sepsis. Serum was collected at 36 hours after CLP sepsis. The levels of TNF-α was analyzed with enzyme-linked immunosorbent assay. CLP sepsis significantly altered serum TNF-α at 36 hours between WT septic mice and HSPA12B$^{-/-}$ septic mice. However, HSPA12B deficiency resulted in remarkable increased serum TNF-α compared with WT septic mice, which was attenuated by β-glucan. n=4~5/group *P < .05, **P < .01, and ***P < .001 compared with indicated groups.

Discussion

The present study demonstrates that β-glucan induced trained immunity plays a protective role in CLP sepsis-induced mortality. The mechanisms of β-glucan induced trained immunity may involve the regulation of endothelial cell function to modulate macrophage inflammatory responses. Our data suggests that there is crosstalk between endothelial cells and macrophages during sepsis, and that endothelial cells can regulate macrophage pro-inflammatory function through endothelial exosomal HSPA12B with β-glucan stimulation.

Trained immunity has been reported to modulate innate immune cells to acquire immune memory, which protects the host with quick and broad responses to re-infection and benefits survival outcome in animal pathophysiological settings and vaccinated humans[470]. β-glucan, as a major component of fungal cell walls, is used to trained monocytes/macrophages to obtain immune memory against non-specific secondary infection. β-glucan can alter epigenetics in innate cells and these alterations can even last from weeks to months.[339,355] In the present study, we found β-glucan trained mice display much less mortality with decreased pro-inflammatory cytokine production in CLP sepsis. This indicates the protective role of β-glucan-induced trained immunity in limiting pro-inflammatory responses and protecting the host against CLP sepsis induced mortality. Further studies are required to investigate the mechanism by which reduced pro-inflammatory responses caused by β-glucan-induced trained immunity is related with the epigenetic modification in the macrophages.

To elucidate the mechanisms by which β-glucan induced trained immunity attenuates sepsis-induced inflammatory responses, we focused on the role of β-glucan in the regulation of functions of endothelial cells, and its contribution to the regulation of macrophage inflammatory response to CLP sepsis. Recent studies reported that barley β-glucan can contribute to angiogenesis .[414,415] Interestingly, our present study indicated that β-glucan promotes the levels of HSPA12B in endothelial cells, and even in endothelial exosomes. Dectin-1, as a receptor of β-glucan, is mainly expressed on the immune cells.[467,466] Current study has demonstrated the presence of Dectin1 in non-immune cells[471], like endothelial cells, and our research discovered the increased levels of Dectin-1 in endothelial cells treated by β-glucan. The data indicates the increased expression of HSPA12B in endothelial cells may by via the Dectin1-related signal pathway, but further studies are needed to confirm Which Dectin-1 related signal pathway is involved in the regulation of increased endothelial HSPA12B exrepssion.

Additionally, our study found that an increase of HSPA12B in endothelial exosomes, by β-glucan treatment, can markedly attenuate pro-inflammatory cytokine production in LPS-stimulated macrophages. This decreased pro-inflammatory cytokine production is a result of the suppression of NF-κB subset p65 nuclear translocation by endothelial exosomal HSPA12B in macrophages. To elucidate whether β-glucan induced trained immunity decreases the worse survival outcome caused by HSPA12B deficiency during CLP sepsis, HSPA12B$^{-/-}$ mice were administrated with β-glucan 7 days before CLP sepsis for survival monitering. Interestingly, β-glucan-induced trained immunity significantly

enhanced survival outcome by 17% in β-glucan trained HSPA12B$^{-/-}$ septic mice, compared with untrained HSPA12B$^{-/-}$ septic mice. Moreover, β-glucan treatment significantly reduces the increased level of TNF-α induced by HSPA12B deficiency. This data suggests that β-glucan-induced trained immunity can partly rescue HSAP12B deficiency induced mortality in CLP sepsis, which More work is needed to figure out how β-glucan produces an increase of HSPA12B expression in endothelial cells and whether decreased macrophage inflammatory responses, induced by endothelial HSPA12B, is associated with epigenetic change.

To the best of our knowledge, this is the first report that an increase of endothelial HSPA12B can be released through exosome secretion by β-glucan treatment. Moreover, the endothelial exosomal HSPA12B mediates crosstalk between endothelial cells and macrophages during CLP sepsis. This crosstalk is a result of endothelial exosomal HSPA12B, regulating macrophage pro-inflammatory cytokine production, via the attenuation of NF-κB activation.

CHAPTER 5. CONCLUSIONS

Although there have been advances in the clinical management and scientific understanding of sepsis, it is still one of the leading contributors of death in hospitals, costs the United States around 20 billion annually, and is currently incurable. Sepsis is regarded as a pathophysiological condition, induced by dysregulated immune responses, which in turn cause multiple organ failure, a great contributor to mortality in the clinic. Cardiovascular dysfunction is a common complication of sepsis and severe sepsis and leads to a higher likelihood of death. Sadly, there are no drugs approved by the FDA available for the treatment of sepsis.

Endothelium, which is widely distributed in the body, is regarded as an important regulator for the development of sepsis. Besides the essential role in maintaining the vascular permeability in the cardiovascular system, activated endothelial cells are also involved in recruiting and facilitating immune cell migration and transmigration. They do this by expressing adhesion molecules on the endothelial cells, into myocardium to clear invading pathogens. Uncontrolled inflammatory responses in myocardium can induce irreversible damage and subsequent cardiac dysfunction with severe mortality. The NF-κB signal pathway has been regarded as a critical regulator of inflammatory responses in immune cells. However, the NF-κB signal pathway plays a key role in the regulation of adhesion molecule expression in the endothelial cells.

HSPA12B, as a protein predominately expressed in the endothelial cells, has been reported to be involved in the regulation of angiogenesis of endothelial cells. Mounting studies have revealed the protective role of HSPA12B in different pathophysiological situations. In our current study, we found that HSPA12B deficiency resulted in severe cardiac dysfunction with increased mortality in CLP sepsis. Our study also shows that HSPA12B deficiency contributes to higher levels of adhesion molecules (ICAM1 and VAM1) in the myocardium in CLP sepsis. To understand how HSPA12B regulates adhesion molecule expression in endothelial cells during CLP sepsis, we performed in-vitro experiments by overexpressing HSPA12B in endothelial cells stimulated with LPS stimulation, which show the downregulation of adhesion molecule expression by HSPA12B. This downregulation results from suppression of NF-κB subset p65 nuclear translocation.

Recent evidence highlighted the role of macrophage function in sepsis induced organ dysfunction. In the present study, we found that HSPA12B deficiency resulted in increased pro-inflammatory cytokine (TNF-α, and IL-1β) production and decreased anti-inflammatory cytokine (IL-10) production. The data suggests the important role of endothelial HSPA12B in regulating immune responses in CLP sepsis. Other groups have reported that endothelial cells could modulate macrophage polarization into an M2 state through cell-cell communication by endothelial exosomes. Interestingly, our previous study has observed the presence of HSPA12B in serum exosomes from WT mice, and current studies found that there is no HSPA12B expression in exosomes from the

organs of HSPA12B deficient mice. In-vitro experiments also confirm the presence of HSPA12B in endothelial exosomes, which could be uptaken by macrophages. Moreover, endothelial exosomal HSPA12B can downregulate inflammatory responses by decreased pro-inflammatory cytokine (TNF-α, and IL-1β) production, and increased anti-inflammatory cytokine (IL-10) production. These decreased inflammatory responses are due to the suppression of NF-κB subset p65 nuclear translocation in LPS-stimulated macrophages, by endothelial exosomal HSPA12B. Expressing HSPA12B in macrophages by adenovirus further confirmed the direct regulation of HSPA12B in LPS-stimulated macrophage inflammatory responses by preventing NF-κB subset p65 nuclear translocation.

To investigate how endothelial HSPA12B regulates the infiltration of monocytes/macrophages into the tissues, we analyzed monocytes/macrophages in the circulation and spleen by flow cytometry. Interestingly, there are no changes in the numbers of circulation and splenic monocytes/macrophages in $HSPA12B^{-/-}$ sham mice, when compared with WT sham control. However, the numbers of circulating monocytes marked with $F4/80^{+}/CD11b^{+}$ in $HSPA12B^{-/-}$ septic mice were markedly lower than in WT septic mice, while splenic $Ly6C^{+}/CCR2^{+}$ macrophages from $F4/80^{+}/CD11b^{+}$ macrophage populations in $HSPA12B^{-/-}$ septic mice were greater than in WT septic mice. The data indicates that endothelial HSPA12B could play an important role in the regulation of monocyte/macrophage infiltration into the tissues. Although we do not fully understand how endothelial HSPA12B regulates the infiltration of

monocytes/macrophages into the tissues during CLP sepsis at present, we observed that the levels of adhesion molecules in the myocardium were markedly increased in HSPA12B$^{-/-}$ septic mice compared with WT septic mice. Increased adhesion molecule expression in HSPA12B could be responsible for increased infiltration of monocytes/macrophages into the tissues. In our previous study, we demonstrated that HSPA12B regulates endothelial cell miR-126 expression which targets adhesion molecule expression.

To investigate how to reduce HSPA12B deficiency induced mortality, we focus on the β-glucan induced trained immunity. Trained immunity contributes to the formation of immune memory in the innate immune cells, such as monocyte/macrophages, which protect the host against re-infection with quick and non-specific immune responses. Moreover, β-glucan is also involved in the regulation of endothelial cell function. In our present study, we found that β-glucan enhanced the levels of HSPA12B in endothelial cells, and even in the endothelial exosomes. Plus, exosomes from β-glucan treated endothelial cells can significantly decrease LPS-stimulated macrophage inflammatory responses by suppression of NF-κB subset p65 nuclear translocation. Furthermore, β-glucan trained HSPA12B deficient mice display markedly enhanced survival outcomes with decreased pro-inflammatory cytokine production in the serum.

To the best of our knowledge, this is the first report that endothelial HSPA12B can be involved in the communication between endothelial cells and macrophages by endothelial exosomes, and that endothelial HSPA12B can be enhanced by β-glucan stimulation. The endothelial exosomal HSPA12B

regulates macrophage pro-inflammatory cytokine production via attenuation of NF-κB activation.

In the future work, we will investigate the mechanisms by β-glucan regulates of HSPA12B expression in endothelial cells. Considering the role of β-glucan in epigenetic modifications in the trained macrophages, we will examine whether β-glucan could induce trained endothelial HSPA12B via epigenetic modification. Moreover, we will test whether β-glucan could induce trained endothelial cells that will regulate macrophage/monocyte function in CLP sepsis. Finally, we will define detailed mechanisms by which endothelial HSPA12B modulates inflammatory cytokine gene expressions in macrophages.

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