APOPTOTIC T-CELL DERIVED MICROPARTICLES CONTAINING SONIC HEDGEHOG MODULATE ENDOTHELIAL CELL FUNCTIONS IN INFLAMMATION VIA RHO KINASE PATHWAY

by

Neha Attal

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Approved by:

_____

Dr. Mark G. Clemens

_____

Dr. Didier A. Dréau

_____

Dr. Ian Marriott

_____

Dr. Kenneth Bost

_____

Dr. Charles Y. Lee

ABSTRACT

NEHA ATTAL. Apoptotic T-cell derived microparticles containing sonic hedgehog modulate endothelial cell functions in inflammation via rho kinase pathway. (Under the direction of DR. MARK G. CLEMENS)

Impaired vascular regulation as a result of endothelial dysfunction is a major determinant of sepsis-associated liver failure. We have previously shown that inhibition of one signaling molecule rho-kinase (ROCK) predominantly restores endothelial function impaired by endotoxin lipopolysaccharide. In the present study, we determined a ligand that activates ROCK mediated endothelial dysfunction. A potential candidate is sonic hedgehog (SHH) as it activates RhoA/ROCK non-canonically. More relevantly, T-lymphocytes that undergo massive apoptosis in septic patients release SHH in vesicles called microparticles (MPs). However, the role and mechanisms of $^{SHH+}$MPs in endothelial dysfunction is not completely delineated. In this study, MPs were derived by inducing apoptosis of human T-lymphoblastoid cell line and contained SHH (80%). These $^{SHH+}$MPs induced stress fibers, hyperpermeability (+71.5%), mitochondrial depolarization (-61.5%), increased oxidative stress (+242%) and decreased nitric oxide production (-46%) in HMECs and impaired endothelium-dependent vasodilation (-61.82%) in ex-vivo porcine mesenteric rings, by activating RhoA/ROCK pathway. SHH agonist also caused the same endothelial dysfunction as $^{SHH+}$MPs. Inhibition of the SHH pathway or ROCK before addition of MPs abrogated endothelial dysfunction, indicating that activation of ROCK through the SHH pathway is required for MP-induced endothelial dysfunction. In

summary, this study elucidated a novel mechanism in which apoptotic T-cell derived MPs containing SHH induce endothelial dysfunction via RhoA/ROCK pathway and thereby can contribute to vascular dysfunction of liver during sepsis.

DEDICATION

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ABBREVIATIONS

Ach	Acetylcholine
act D	Actinomycin D
Akt	Protein Kinase B
APCs	Antigen presenting cells
ATP	Adenosine triphosphate
BD	Bile ductule
BH_4	Tetrahydrobiopterin
BSA	Bovine serum albumin
Ca^{+2}	Calcium
CaM	Calmodulin
CARD	Caspase recruitment domain containing protein
Caspase	Cysteine-dependent-aspartate directed proteases
Cav-1	Caveolin-1
$CD4^+$	Helper T-lymphocytes
$CD8^+$/CTL	Cytotoxic T-lymphocytes
CEM-T cells	Human T-lymphoblastoid cell line
cGMP	3', 5'-cyclic guanosine monophosphate
CLP	Cecal ligation and puncture
cNOS	Constitutive nitric oxide synthase
COX	Cytochrome C oxidase

CRD	Carboxyl-terminal cysteine rich domain
CV	Central venule
Cyt c	Cytochrome C
DAF-FM	4-Amino-5-methylamino-2’7’-difluorofluorescein, Diaminofluorescein-FM
DAG	Diacylglycerol
DAPI	4’,6-Diamidino-2 Phenylindole, Dihydrochloride
DAPI	4’, 6-Diamidino-2-Phenylindole dihydrochloride
DHE	Dihydroethidium
DHH	Desert hedgehog
DIC	Disseminated intravascular coagulopathy
DMEM	Dulbecco’s Modified Eagle Medium
DPBS	Dulbecco’s Phosphate Buffered Saline
DRAL	Adaptor protein downregulated in rhabdomyosarcoma domain protein
DYRK1	Dual specificity Yak-1 related kinase
ECE	Endothelin converting enzymes
EDRF	Endothelium derived relaxing factor
EMPs	Endothelial derived microparticles
eNOS	Endothelial nitric oxide synthase
ER	Endoplasmic reticulum
ERM	Ezrin-radixin-moesin

ET	Endothelin
ET_AR	Endothelin-A receptor
ET_BR	Endothelin-B receptor
ETC	Electron transport chain
ETRs	Endothelin receptors
EV	Extracellular vesicles
FAD	Flavin adenine dinucleotide
FITC	Fluorescein isothiocyanate
FMN	Flavin mononucleotide
GAP	GTPase activating protein
GDP	Guanosine diphosphate
GEF	Guanine nucleotide exchange factor
Gli	Glioma transcription factor
GPCR	Guanine nucleotide binding protein (G-protein) coupled receptors
GRK2	Guanine nucleotide binding protein coupled receptors (GPCR)-kinase 2
GTP	Guanosine triphosphate
H_20	Water
H_20_2	Hydrogen peroxide
HA	Hepatic arteriole
HBSS	Hanks balanced salt solution
HH	Hedgehog

HHC	C-terminal fragment
HHN	N-terminal fragment
HMECs	Human microvascular endothelial cells
HO-	Hydroxyl radical
Hop	Heterogeneity of perfusion
HSCs	Hepatic stellate cells
HUVEC	Human umbilical vein endothelial cells
ICAM-1	Intercellular adhesion molecule-1
ICU	Intensive care unit
IHH	Indian hedgehog
IL-12	Interleukin-12
IL-1β	Interleukin-1β
IL-4	Interleukin-4
IL-6	Interleukin-6
INF-γ	Interferon gamma
iNOS	Inducible nitric oxide synthase
IP3	Inositol 1, 4, 5,-triphosphate
KCs	Kupffer cells
LAMP1	Lysosomal associated membrane protein-1
LIMK	Lim-kinase
LPS	Lipopolysaccharide
LSECs	Liver sinusoidal endothelial cells

LTB4	Leukotriene B4
MBS	Myosin-binding subunit
MeK1	Mitogen activated protein kinase kinase
MHC I and II	Major histocompatibility I and II
MLC	Myosin light chain
MLCK	Myosin light chain kinase
MLCP	Myosin light chain phosphatase
MMP	Mitochondrial membrane potential
mNOS	Mitochondrial nitric oxide synthase
MOF	Multiple organ failure
MPs	Microparticles
MVB	Multivesicular bodies
NADPH	Nicotinamide adenine dinucleotide phosphate
NALP1	NLR family pyrin domain containing 1
NETs	Neutrophil extracellular traps
NF-κB	Nuclear factor kappa B
NKT	Natural killer cells
nNOS	Nitric oxide synthase
NO	Nitric oxide
O_2	Oxygen
O_2^-	Superoxide
$ONOO^-$	Peroxynitrite

PAMPS	Pathogen associated molecular pattern
PE	Phosphatidyl ethanolamine
PH	Pleckstrin homology domain
PHA	Phytohemagglutinin
PI	Propidium Iodide
PI3K	Phosphoinositide 3-kinase
PIP2	Phosphatidylinositol 4, 5,-bisphosphate
PIP3	Phosphatidylinositol 3, 4, 5,-trisphosphate
PKC	Protein kinase C
PKG	Protein kinase G
PLC	Phospholipase C
PMA	Phorbol-12-myristate-13-acetate
PRR	Pathogen recognition receptors
PS	Phosphatidyl serine
Ptch1	Patched-1 receptor
Ptch2	Patched-2 receptor
PTX	Pertussis toxin
Pur	Purmorphamine
PV	Portal venule
RBD	Rho binding domain
Rh123	Rhodamine 123
ROCK	Rho associated kinase

ROS	Reactive oxygen species
RT	Room temperature
RTKs	Receptor tyrosine kinase
SANT-1	4-Benzyl-piperazin-1-yl-(3, 5-dimethyl-1phenyl-1H-pyrazol-4ylmethylene)-amine
sGC	Soluble guanylate cyclase
SHH	Sonic hedgehog
SLV	Terminal sub-lobular hepatic venule
SMO	Smoothened
SOD	Superoxide dismutase
SOFA	Sepsis-related Organ Failure Assessment
SUFU	Suppressor of Fused
T-cells	T-lymphocytes
TF	Tissue factor
TGF-1β	Transforming growth factor Beta 1
TIAM-1	Rac guanine nucleotide exchange factor T-lymphoma invasion and metastasis-1
TLR-4	Toll like receptor 4
TNF-α	Tumor necrosis factor alpha
TSG101	Tumor susceptibility gene 101 protein
VASP	Vasodilator stimulated phosphoprotein
VCAM	Vascular adhesion molecule

VSMCs	Vascular smooth muscle cells
Y-27632	[R-(+)-trans-N-(4-pyridyl)-4-(1-Aminoethyl)-cyclohexanecarboxamide dihydrochloride monohydrate

CHAPTER 1: INTRODUCTION

1.1. Liver-Guardian and Target of Sepsis

Despite extensive research and widespread use of standardized care, sepsis remains as a leading cause of mortality and morbidity in intensive care unit (ICU) for decades now [1-3]. This is largely due to complex alterations in immune, metabolic and microvascular function. There are several key steps in pathophysiology of sepsis [4]. First, host response to an infection that primarily determines patient outcome rather than type of pathogen. Second, central role of endothelial and immune cells and in initiating and perpetuating the host response. Third, sepsis associated concomitant activation of inflammatory and coagulation cascades. Eventually, in a concerted effort to eliminate the pathogens, there is dysregulation of body's own immune response that ensues in protracted and unabated systemic inflammation [5]. This excessive inflammation inflicts collateral damage on cells and tissues that subsequently leads to subsequent failure of multiple organs (MOF) and eventually death. A major hurdle in devising therapeutics for sepsis is our incomplete understanding of the mechanism involved in manifestation of MOF. One of the most important organs to fail is liver. In sepsis, liver plays crucial role in defense by scavenging bacteria and in mediating inflammation [6, 7]. But, liver also becomes a potential target by virtue of overwhelming inflammatory response and is shown to fail early in sepsis [8, 9]. Also, liver injury can further exacerbate the severity of sepsis by contributing to detrimental inflammatory response in other organs [10]. Liver dysfunction has an exceptional prognostic relevance for the course of sepsis and is a powerful independent predictor of mortality [11, 12]. Serum bilirubin levels, as a key marker of hepatic

dysfunction, are a vital component of prognostic scores at ICU, such as the Sepsis-related Organ Failure Assessment (SOFA) score [13]. Attenuation of sepsis associated liver injury may lower the morbidity of sepsis. However, the mechanism of inflammatory pathogenesis in the liver is not well understood.

1.1.1.Liver as a Guardian of Sepsis

Liver is the largest gland in human body and plays a central role in immunological and metabolic homeostasis. Liver is responsible for many vital life functions including blood detoxification, storage, energy production, metabolism of macromolecules, nutrient conversion, synthesis of plasma proteins, hormone balance, coagulation and inflammatory response [14]. These important physiological functions make the liver a critical organ for host survival following severe injury. In sepsis, liver plays crucial role in clearing bacteria, in mediating inflammatory responses, and in coagulation, which regulate renal failure, acute lung injury, acute respiratory distress syndrome, coagulopathy, and hepatic encephalopathy [15]. These critical functions are accomplished by liver's unique microcirculation system and the various cells that compose liver.

1.1.2.Hepatic Microcirculation and its Associated Cells

Hepatic microcirculation is of utmost importance for physiology and function of the whole organism. It supplies the parenchymal tissue with nutrients and oxygen, serves as a gate for immune cells entrance in hepatic inflammation and is responsible for detoxification and clearance of foreign bodies from the blood stream [16]. Liver microcirculation comprises of portal venules (PV), hepatic arterioles (HA), sinusoids and central venules (CV). This vascular network delivers oxygenated blood and removes de-oxygenated blood and waste to and from hepatic parenchyma. The portal venules, hepatic arterioles and bile ducts

together form the structural unit called portal triad; and then a hexagonally shaped structure with one triad located at each of its exterior ventrices plus a centrally located central venule forms the functional unit of the hepatic microcirculation called as the hepatic lobule. In each lobule, the parenchymal cells of the liver; hepatocytes are connected with each other and are visible as plates. These hepatocytes face blood channels called as sinusoids. The sinusoids are wrapped with fenestrated endothelial cells (LSECs) and are also populated with resident macrophages, the kupffer cells (KCs). There is a space between the endothelial cells linings and the apical membrane of hepatocytes called the Space of Disse where the hepatic stellate cells (HSCs) reside (Figure 1).

Liver is unique organ supplied by both arterial and venous blood. The majority of blood (75-80%) enters the hepatic microvasculature from portal vein. The portal vein is a valve-less afferent vessel that collects deoxygenated, nutrient-rich, endotoxin-rich blood supply from splenic vein (spleen), superior mesenteric vein (small intestine), and also receives blood from inferior mesenteric (large intestine), left and right gastric veins (stomach) and cystic vein (gall bladder). The inlets of these portal venules are guarded by sphincters that compose of sinusoidal lining termed as the inlet sphincters (Figure 1). In contrast, the oxygenated blood represents 20 to 25% of the total blood that enter the liver. This blood supply originates from the celiac artery; the first major branch of the abdominal aorta and is delivered to the hepatic microvascular system through branches of hepatic arterioles called arterio-sinus twigs (Figure 1). In addition, occasional direct connections between the portal venules and hepatic arterioles known as the arterio-portal anastomoses are present (Figure 1). After the blood flows through the sinusoids, it exits the hepatic lobule through the central venule located in the middle of the hepatic

lobule and is then drained to the terminal hepatic venules through which the blood is returned to the inferior vena cava (Figure 1). Together it comprises of 25% cardiac output.

The sinusoidal network is the principal regulatory site of the hepatic microcirculation. In addition to the upstream resistance vessels in the splanchnic viscera, the hepatic microcirculation can be regionally regulated by vasoconstriction and vasodilation of the sinusoids [17-20]. The cellular component of hepatic sinusoids i.e. hepatocytes, liver, LSECs, HSCs, and KCs can actively regulate the diameter of the vessels in response to gut derived toxins and regional vasoactive factors. This locally regulated distribution of hepatic microcirculation results in heterogeneity of perfusion (Hop), in which flow can be highly variable ranging from 25 to 300 μm/sec in individual sinusoids with an average volumetric flow of 6.0 μl/sec. The organization of sinusoids is functionally and structurally heterogeneous. Near the portal vein and hepatic arterioles, the sinusoids are arranged in an interconnecting polygonal network while near the central venules, they are organized as parallel vessels that terminate in terminal hepatic venules. Short inter-sinusoidal sinusoids are present to connect these parallel vessels, and the sinusoids lining cells form inter-sinusoidal sphincters to regulate blood flow in short segment of sinusoids (Figure 1).

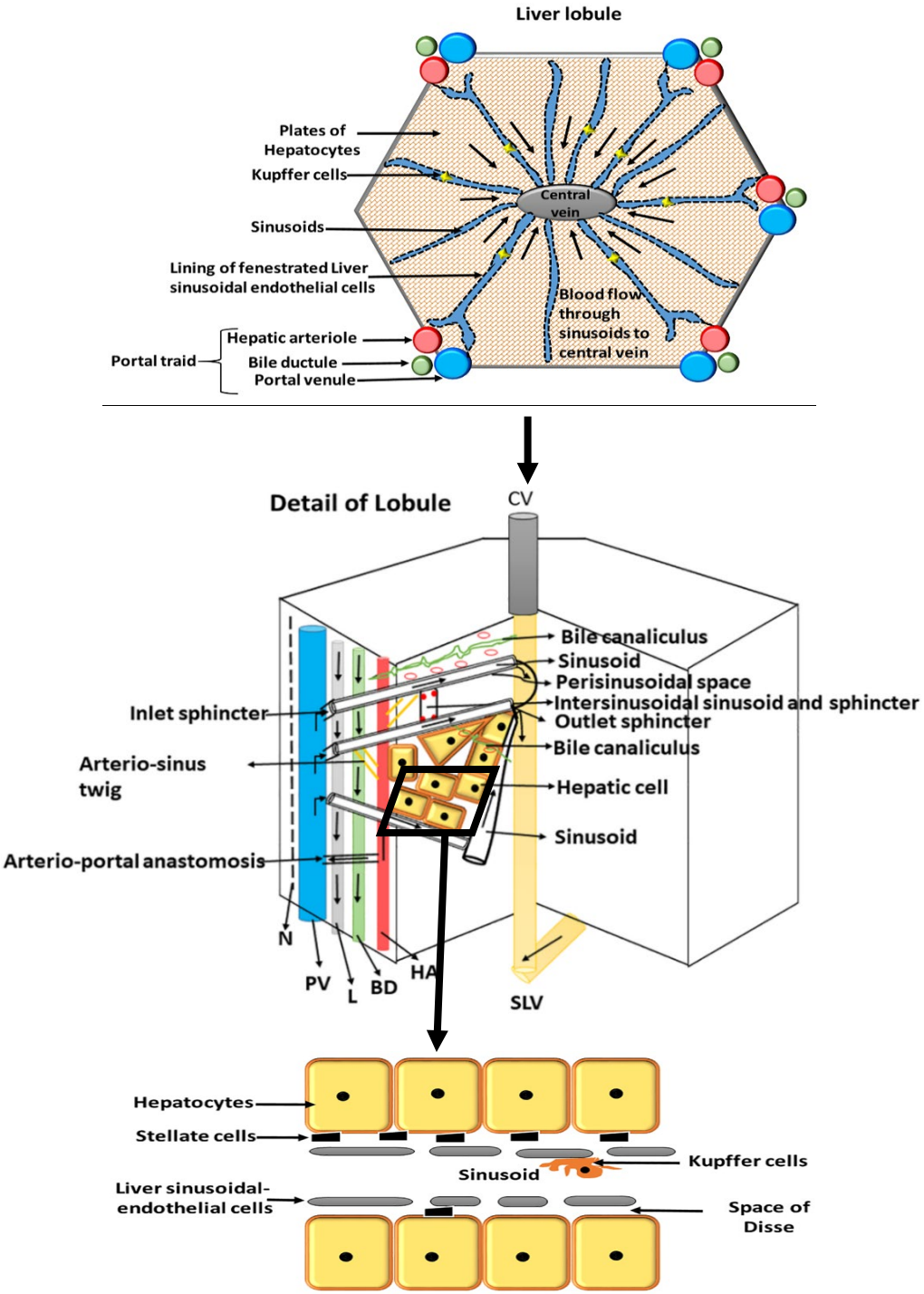

Figure 1: Schematic representation of hepatic microvasculature

The liver is divided into hexagonal-shaped lobules, which have portal triad of vessels at each corner consisting of portal venule (PV), hepatic arteriole (HA) and bile ductule (BD).

Each lobule is comprised of plates of hepatocytes, lined by sinusoids. Sinusoids are liver-specific capillaries that are lined by discontinuous fenestrated endothelial cells (LSECs), while the hepatic stellate cells (HSCs) are present the sub-endothelial Space of Disse and resident macrophages kupffer cells (KCs) are found within or below the endothelium. Blood from HA and (PV) enters the hepatic lobule, flows through the sinusoids, and exist the lobule through the central venule (CV) and terminal sub-lobular hepatic venule (SLV). Arrows indicate direction of flow. L: Lymphatic, N: Nerve, BD: Bile ductule (Original drawing by author). This figure has been modified from diagram in reference [21]

1.1.3.Liver Immune Surveillance Mechanism

Liver is located in the upper right-hand portion of the abdominal cavity beneath the diaphragm and on top of the stomach, right kidney and intestines. Owing its unique anatomical location, the portal vein coming from gastrointestinal tract brings de-oxygenated blood full of circulating antigens, endotoxins, danger signals and microorganism to the liver for detoxification before entering the heart. Microbes in sepsis frequently translocate from the gut lumen into liver through portal vein [22]. The gut lumen harbors normal flora bacteria that facilitate digestion and use of nutrients [23]. The gut and the associated local immune system work together to prevent translocation of intestinal bacteria into the portal vein. After the gut intestinal epithelial barrier, the liver along with spleen constitutes the second line of defense that detect and eliminate the invading bacteria and bacterial products, inhibiting spread of bacteria into the body and therefore sepsis [22]. In animal models of intravenous bacteria injection, >60% of the injected bacteria can be trapped in the liver within 10 min [24].

Majority of mechanisms related to immune surveillance by liver takes place in hepatic sinusoids [25]. This is because the capillary-like sinusoids structure allows the flow of blood to reduce more than 50 folds, maximizing the detection of pathogens and their capture by liver-resident immune cells. The cellular component of hepatic sinusoids that play role in bacterial phagocytosis and clearance include hepatocytes, LSECs, HSCs , KCs and manifold of immune subsets such as polymorphonuclear leukocytes, monocytes, natural killer T-cells (NKT) and lymphocytes [26]. KCs, the largest population (80-90% of total fixed macrophages in body) of resident macrophages in liver are the first immune cells that come in contact with the gut bacteria, endotoxins and microbial debris derived from gastrointestinal tract [27]. These KCs are equipped array of pathogen recognition receptors (PRRs) such as toll like receptor-4 (TLR-4) that allow them recognize a pathogen associated molecular patterns (PAMPs) such as lipopolysaccharide (LPS) found in cell wall of gram negative bacteria and conduct phagocytosis [28-30].This initiates specific signaling pathways activating transcription factors such as nuclear factor kappa B (NF-κB) among others. NF-κB induces transcription of specific genes involved in pro-inflammation such as interleukin-6 (IL-6), interleukin-12 (IL-12), interleukin-1β (IL-1β), tumor necrosis factor alpha (TNF-α) and secondary metabolites such as nitric oxide (NO) and reactive oxygen species (ROS). These chemokines and cytokines inhibit proliferation of microorganisms and allow KCs to function as immune sentinel, alerting other components of the immune system to the presence of harmful pathogens including antigen specific adaptive immune responses [15]. KCs allow rapid clearance of bacteria while the bulk of bacteria in liver is taken by the bactericidal neutrophils. KCs produced cytokines such as leukotriene B4 (LTB4), TNF-α [31, 32] that recruits neutrophils into the liver to control

bacterial infection by phagocytosis or by releasing anti-microbial granule proteins [33, 34]. KCs also interacts with other blood cells such as platelets, leukocytes, erythrocytes in promoting neutrophil recruitment [35]. Platelets actively patrol the liver vasculature via touch- and-go interactions with KCs, scanning for pathogen. When a bacteria is trapped on KCs, platelets firmly adhere to the cells and interact with neutrophil [26]. This interaction promotes neutrophils to release of neutrophil extracellular traps (NETs) composed of released DNA and proteolytic activity that traps the bacteria and kills it extracellularly [35]. These NETs are found in the liver vasculature during sepsis [36]. In a mouse model of *Staphylococcus aureus* induced sepsis, NETs formed in the liver efficiently cleared the bacteria and were protected against sepsis [37]. Hepatocytes contributes to endotoxin clearance such as LPS by primarily using lipoprotein. Lipoprotein is an LPS-binding protein that transfers LPS to cluster of differentiation 14 (CD14); a co-receptor along with TLR-4 to detect LPS. This facilitates interaction between LPS and TLR-4 on the surface of phagocytes such as neutrophils to remove LPS and initiate pro-inflammatory cascade [38]. LSECs and HSCs also recognize PAMPs via PRR and induce phagocytosis. Apart from pathogen capture and clearance, LSECs, HSCs, KCs and hepatocytes act as liver antigen-presenting cells (APCs), expressing major histocompatibility complex (MHC) I, II and co-stimulatory molecules needed for activation of NKT and classic T cells- helper $CD4^+$ and cytotoxic $CD8^+$ T-cells and induce local and systemic inflammatory responses in sepsis [39]. $CD4^+$ T-cells support $CD8^+$ T-cells to proliferate and differentiate into licensed cytotoxic T-lymphocytes (CTL) that kill intracellular microorganisms through secretion of cytokines such as interferon-gamma (IFN-γ), TNF-α, interleukin-4 (IL-4) or attack infective cells via cytosolic granules containing perforins and granzymes [40].

In sepsis, liver shows a rapid switch from tolerogenic towards immunogenic response. Under basal conditions, low amounts of microbial product such as LPS entering the liver from the gut via portal vein induce tolerogenic response. A tolerogenic environment is characterized by decreased antigen presentation of KCs, hepatocytes to the T-cells, absence of co-stimulatory molecules and increased expression of inhibitory molecules such as PD-L1 by LSECs. This resulting condition supports proliferation of non-licensed T-lymphocytes that are unable to kill the targets cells in antigen specific manner, thus suppressing adaptive immune response. Normally, the liver mediated immune hyporesponsiveness is the default program to make sure no unwanted inflammatory responses are raised against harmless food antigens or normal levels of microbe-derived molecules that enter blood stream on daily basis from the gut [41]. However; if the level or context of microbial product changes or during concomitant experimentally induced injury, the same antigens are processed in immunogenic fashion, and induce cytotoxic T-cell responses [42]. This process triggers robust inflammatory response that contribute to efficient elimination of the pathogen. However, in sepsis where inappropriate immune responses are initiated and inflammation is sustained, this protective response results in liver function impairment and multiple organ dysfunction syndrome. Notably, liver induced immunogenic response represents a double-edged sword in sepsis that contribute to clearing bacteria and toxins but also causes inflammation, immunosuppression and organ damage [15].

1.1.4.Liver as Target in Sepsis

In sepsis, the liver is injured by pathogens, inflammatory mediators or microvascular alterations. The injury progresses from hepatocellular dysfunction to liver damage and

subsequently to liver failure. Liver dysfunction is associated with subtle changes in the hepatocellular functions such as decreased metabolic function and or decreased immune function. Liver dysfunction often occurs early in sepsis, usually 1.5hrs after cecal ligation and puncture in animals (CLP-a procedure where cecum is ligated to produce polymicrobial infection), or less than 24hrs post onset of sepsis in patients due to inflammation [8, 15]. Clinical and experimental data suggests that liver dysfunction is an early sign of sepsis [9]. In liver damage, there is irreversible injury to hepatocytes. Liver failure is defined as severe damage to the liver with 80-90% loss of function in liver cells. Ongoing inflammation and microvascular dysfunction can cause liver damage and failure [15]. Liver being a critical organ for survival of sepsis, liver injury before or after the onset of sepsis has grave consequences on severity and outcome of sepsis patients [1]. However, the mechanism of pathophysiology of liver failure are complex and not well comprehended.

1.2. Microvascular/Endothelial dysfunction- a Major Determinant of Liver Failure

The pathogenesis of sepsis associated liver dysfunction is complex process that is mainly attributed to metabolic, inflammatory and microvascular alterations in the liver. Previous research from our lab and others [43-56] have shown that a major determinant of liver failure in sepsis and trauma patients is failure of liver microcirculation. The impaired hepatic microcirculation leads to areas of ischemia locally in the liver triggered by inadequate oxygen (O_2) concentration in blood/ reduced blood flow due to decreased arterial/ or increased venous pressures or lack of O_2 carriers [57]. This is also known as shock liver which not only causes hepatocellular injury, but also provide the first element that can trigger the progression of MOF [58].

A critical factor that contributes to impaired vascular regulation of liver in sepsis is dysfunction of endothelial cells [59-62]. Endothelial cells are inner cell linings of blood vessels that form an interface between blood and tissue. Initially thought to be as an inert cellophane like single layered membrane, endothelium is now identified as dynamic heterogeneous, disseminated organ that possess vital secretory, synthetic, metabolic and immunological functions [63]. Under quiescent conditions, endothelial cell exhibit a number of important physiological functions that allow for normal homeostasis. Acting as a semi-selective gate keeper between blood and tissues, these endothelial cell regulate flow of diverse molecules such as proteins, lipids, metabolites, hormones and blood cells themselves through membrane bound receptors [64]. The endothelium also plays pivotal role in regulating blood flow and pressure by producing number of vasodilator such as NO, prostacyclin and vasoconstrictor substances such as endothelin-1 (ET-1) which regulate vasomotor tone by increasing blood flow or decreasing the blood flow respectively [65]. Endothelial cells maintain an active non-thrombic (non-clotting) surface by inhibiting platelet aggregation, coagulation and thus facilitating transit of plasma and cellular constituents throughout the vasculature [66]. When perturbed, the endothelium is capable of responding rapidly to diverse stimuli such as microbial components, shear stress coagulation proteins, cytokines and growth factors by interacting with inflammatory mediators and generating inflammatory mediators themselves [64]. These activation responses include controlling transmigration of leukocytes into the tissue by a carefully regulated process involving adhesion molecules that mediate the adhesion of immune cells and platelets to the endothelium by binding to specific ligands on these cells [67]. Endothelial cells can themselves induce production pro-inflammatory mediators such as

TNF-α, IL-1β by recognizing PAMPS via PRR on their cell surface resulting in pro-inflammatory and pro-thrombin phenotype and play role in host defense against microorganisms and for repair of tissue injury [65]. All these responses are generally localized and beneficial. However, under some circumstances such as sepsis these endothelial functions are deteriorated i.e. there is endothelial dysfunction.

Endothelial dysfunction is a general term used to describe a diminished capacity to produce vasodilators importantly NO release [68] and a tendency toward a pro-thrombotic and pro-inflammatory state. The pathological changes associated with sepsis induced endothelial dysfunction include a loss of vascular integrity and permeability functioning, platelet aggregation, thrombosis, leukocyte infiltration into the surrounding tissue and increased cytokine production [69]. This endothelial activation and dysfunction culminate in the clinical manifestations of inflammation as seen in sepsis. Microscopically visible shape changes or injury to the ECs has been assessed in several studies [70-72]. A single injection of LPS has long been demonstrated to be a non-mechanical technique for removing endothelium [70]. As early as 15 min after LPS injection [73], cellular injuries are apparent with cytoplasmic swelling and protrusions, nuclear vacuolization, cytoplasmic fragmentation and detachment of endothelial cells from the internal elastic laminae. This can also be observed in CLP rat model 10 hours after onset of sepsis [74]. Endothelial injury results in increased leukocyte infiltration into tissue and increased production of pro-inflammatory cytokines which is manifested in about 6 hours after the inflammation has been triggered and reached maximal potential at 12-14 hours as the combination of cytokines exert potentiating effects [74, 75]. Endothelial physical disruption allows inflammatory fluid and cells to shift from the blood into the interstitial space. Recently,

indirect immunofluorescence was devised to detect circulating endothelial cells to provide direct evidence of endothelium shedding in human sepsis. They demonstrated that the number endothelial cells per mL was significantly higher in patients who died of septic shock than in survivors [76]. Endothelial injury is sustained over time. In endotoxin rabbits, the endothelium denudation is present at the level of abdominal aorta as early as several hours following injury and persisted for at least 5 days while, the recovery took total of 21 day [72]. Similarly in 12 human volunteer, receiving 4ng/Kg *Escherichia coli* LPS by intravenous injection showed two stage response. An immediate symptomatic inflammatory stage for 8 hours involving phagocytosis and endothelial injury. This was followed by 12 hour long asymptomatic non-inflammatory stage where they showed second peak activation inflammatory and coagulation response along with secondary endothelial cell injury [77]. Thus, vascular endothelium plays a central role in control of microvascular flow and widespread vascular activation, dysfunction and eventually injury occurs in sepsis and contribute to MOF [78].

1.3.Imbalance in Endothelial Vasoactive Molecules as Contributor to Hepatic Failure.

In liver, the most important constrictor is endothelin-1 (ET-1) that causes blood vessels to constrict, but also stimulates production of essential vasodilator nitric oxide (NO) moderating that constriction [79]. During sepsis, there is a shift towards decreased NO mediated dilation due to inactivation of NO producing enzyme endothelial nitric oxide synthase (eNOS), resulting in uncompensated constriction [45, 80] and thereby liver injury.

1.3.1.Endothelin

The vasoconstrictor ET is produced by endothelial cells, with marked effects on vascular tone. There are three types of ET; ET-1, ET-2 and ET-3 but vascular endothelial cells

produce only ET-1 [81]. In liver, ET-1 is predominantly produced in LSECS [82, 83] but minimally in HSCs [84] and KCs [85]. In addition to the cellular level, ET-1 production is regulated at molecular level especially during transcription and by proteolytic cleavage. At the transcription level, the production of pre-pro-ET-1(inactive form) can be induced by interleukins [86] as well as other vasoactive factors including epinephrine, angiotensin II and vasopressin [87]. Once the pre-pro-ET-1 peptide (203-212 amino acids) are formed, they are inactive until they are cleaved into an intermediate form called as big ET-1 (38-41 amino acids). Ultimately, the big ET-1 peptides are further cleaved by endothelin converting enzyme (ECE) in its 21 amino acids active form as ET-1 (Figure 2).

ET-1 was first discovered in 1988 as a potent vasoconstrictor in endothelium. It plays a significant role in regulation of hepatic microcirculation by primarily mediating sinusoidal vasoconstriction [17, 18, 88, 89]. However, the vasoactive properties of ET-1 in liver are far more complex that simple induction of HSC contraction (Figure 2). Although it primarily acts as vasoconstrictor, there is considerable evidence to indicate that ET-1 also induces vasodilation [90]. Moreover, the vasoactive role of ET-1 in the liver microcirculation is even more important under inflammatory and oxidative stress conditions. ET-1 expression and response increases following liver injuries induced by LPS [50, 91], Ischemia/ reperfusion injury [91] and chronic alcohol consumption [92]. Besides hepatic microcirculation, ET-1 is also involved in the regulation of wide spectrum of other biological functions in the liver, including the induction of calcium mobilization, glycogenolysis, bile acid secretion, phospholipase A2 production and eicosanoid release [93].

1.3.2. Endothelin Receptors

In the liver, ET bind to two major ET receptor subtypes: the endothelin-A (ET_A) and endothelin-B (ET_B) receptors (Figure 2). Endothelin receptors (ETRs) are guanine nucleotide-binding protein (G-protein)-coupled receptors (GPCRs). In general, agonist binding to GPCRs leads to the dissociation of the heterotrimeric G-protein on the cytoplasmic side of the receptors into Gβγ and Gα subunits. G-protein signaling is complex and can mediate through many possible permutations, including more than 20 Gα, 6Gβ and 12Gγ isoforms [94-96] . In addition, GPCR's are classified as either pertussis toxin (PTX)-sensitive that is coupled to $G\alpha_{i/o}$ and PTX-insensitive that is coupled to $G\alpha_{q/11}$, $G\alpha_s$, and $G\alpha_{12/13}$. PTX inhibits GPCR-couple signaling by catalyzing ADP ribosylation of $G\alpha_{i/o}$ subunits that maintains the $G\alpha_{i/o}$ subunit in its inactive ADP-bound form in order to prevent its interaction with the GPCRs. In general, ET_A receptors are coupled to G-protein of $G\alpha_q$ and $G\alpha_i$ families which activate PLC but at the same time inhibit adenylyl cyclase [94, 97]. In contrast, the stimulation of the ET_B receptor is coupled to the G proteins of $G\alpha_q$ and $G\alpha_s$ families which activate phospholipase C (PLC) and inositol 1,4,5-triphosphate (IP3) to induce the release of calcium (Ca^{+2}) from endoplasmic reticulum (ER) stores [98]. The increase in cytosolic Ca^{+2} then stimulate calmodulin (CaM) binding to eNOS, which facilitates the displacement of eNOS from caveolin-1 (Cav-1) to increase eNOS activity (Figure 2). In the LSECs, ET_B receptors are also sensitive to PTX inhibition and the ET-1 mediated NO production is linked to $G\alpha_{i/o}$ signaling pathways. This ET-1 mediated NO production in the LSECs can be inhibited by GPCR-kinase 2 (GRK2) which is upregulated following liver injury to inhibit protein kinase B (Akt) activity and results in portal hypertension [99].

The ETRs are heterogeneously expressed among the liver cell population [100]. HSCs and hepatocytes express both ET_A and ET_B receptors. Binding to ET_A receptors on HSC stimulates the contraction of the cytoplasmic processes of the HSCs (Figure 2) that mediates vasoconstriction of the sinusoids [101, 102]. All cells in the sinusoids express ET_B receptors. The LSECs and KCs do not express ET_A receptors, so these cells only have ET_B receptors on the plasma membrane. Stimulation of the ET_B receptors induces NO production in LSECs (Figure 2) that mediates vasodilation of the sinusoids [103-105]. However, the vasoactive effects of ET-1 binding to the ET_B receptor are much more complex than just straight forward induction of vasodilation of the hepatic sinusoids; it has been shown that binding the ET_B receptors also mediates vasoconstriction [106-108]. This observation leads to the suggestion of classifying ET_B receptors into two putative subtype ET_{B1} receptor for vasodilation and ET_{B2} receptors for vasoconstriction [109, 110].

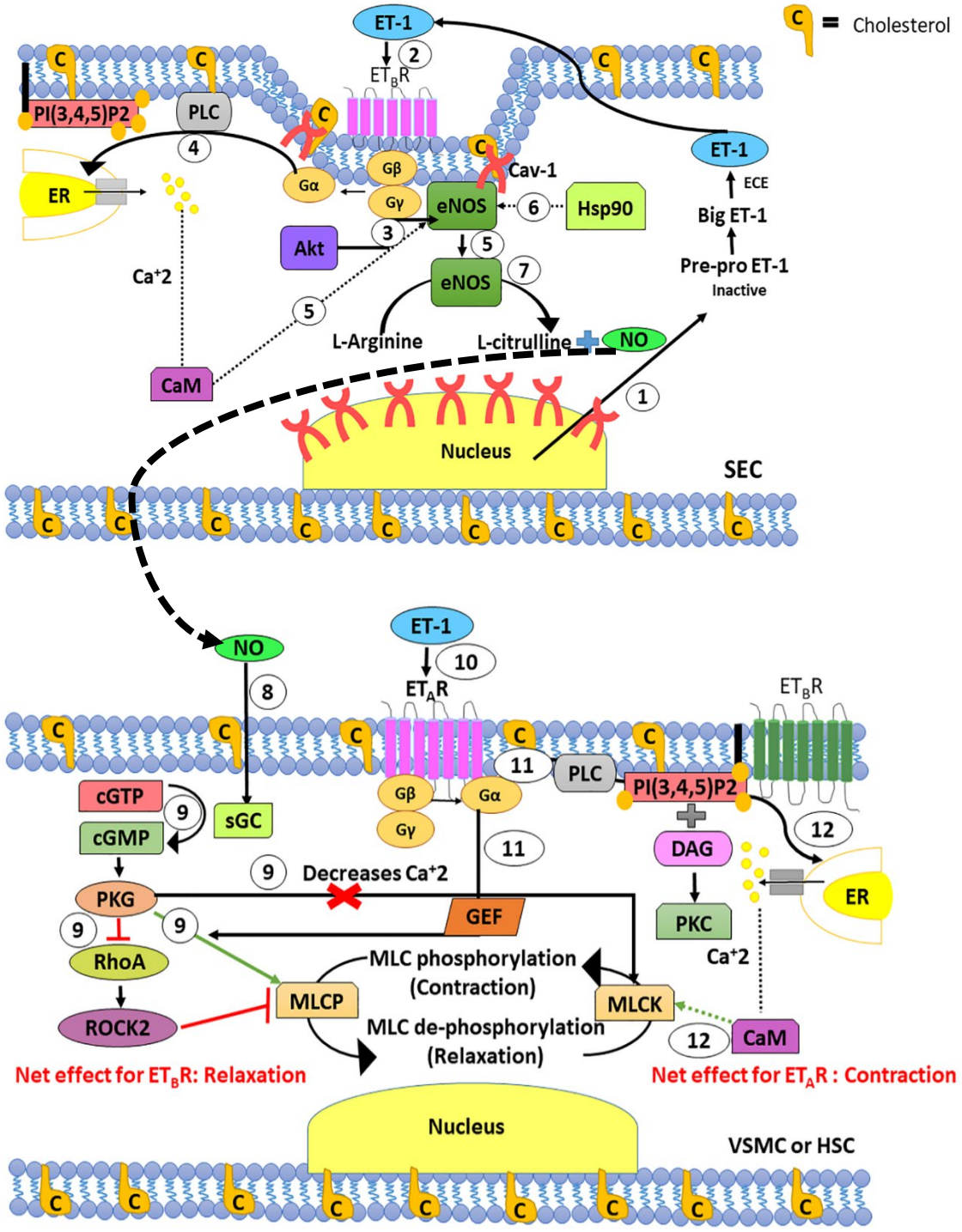

Figure 2: Endothelin and nitric oxide signaling pathways.

(1) In the LSECs, the production of the active form of ET-1 is regulated at levels of transcription as well as proteolytic cleavage by endothelin converting enzymes (ECE). (2)

The binding of ET-1 to the ET_B receptor (ET_BR) induces the translocation of eNOS to the plasma membrane and also stimulates the dissociation of heterotrimeric G-protein. (3) The G-protein βγ subunits activates Akt. (4) The G-protein α subunit ($G\alpha_{q/11}$) stimulates phospholipase C (PLC) to produce inositol 1,4,5-triphosphate (IP3) that subsequently induces calcium (Ca^{+2}) release from the endoplasmic reticulum (ER). (5) This Ca^{+2} flux induces calmodulin (CaM) binding to eNOS, which facilitates the dissociation of eNOS from caveolin-1 (Cav-1). (6) Hsp90 further increases eNOS activity by facilitating displacement of eNOS from Cav-1. (7) As a result, ET-1 stimulates eNOS to produce NO and L-citrulline from L-Arginine. (8) NO diffuses to the neighboring VSMCs or HSCs, and binds to the heme group of soluble guanylate cyclase (sGC), which leads to cGMP production and activation of cGMP-dependent protein kinase G (PKG). (9) Activated PKG interacts with myosin light chain phosphatase (MLCP) and stimulates MLCP activity, by not only phosphorylating MLCP at ser695 ser852 residues but also by preventing ROCK-2 mediated phosphorylation of MLCP at Thr696 and Thr853 residues and inhibition of myosin light chain kinase (MLCK) by decreasing Ca^{+2} through inhibition of voltage gated Ca^{+2} channels. The serine phosphorylations of MLCP, the suppression of RhoA/ROCK-2 mediated threonine phosphorylations and inhibition of MLCK facilitate MLCP activity in the de-phosphorylation of myosin light chain (MLC), which results in relaxation of the MLC. (10) In the VSMCs or HSCs, ET-1 binding to the ET_A receptor (ET_AR) stimulates the dissociation of heterotrimeric G-protein. (11) The G-proteins of the $G\alpha_{12/13}$ family stimulate RhoGEF, which activates RhoA by promoting release of GDP and facilitating the subsequent binding of GTP. In addition, the G-proteins of $G\alpha_q$ and $G\alpha_s$ families activate PLC, which then cleaves phosphatidylinositol 4, 5-bisphosphate (PIP2) to release IP3 and

diacylglycerol (DAG). (12) IP3 stimulates the release of Ca^{+2} from ER stores, which in turn leads to the activation of Ca^{+2}/CaM-dependent MLCK and ultimately results in vasoconstriction. (13) (Original drawing by author).

1.3.3. Nitric Oxide

NO is a gaseous free radical molecule involved in many physiological and pathological processes. For years, the true identity of NO was unknown, and it had been referred to as endothelium-derived relaxing factor (EDRF). Furchgott, Ignarro, and Murad were awarded a Nobel Prize in 1998 for their contribution in identification of NO as EDRF and in the classification of NO as biological mediator [111, 112]. NO has been recognized as key determinant of vascular homeostasis, regulating several physiological properties of blood vessels including vasodilation, vascular permeability and antithrombic properties [113].

Low levels of NO produced by the endothelial cells play a key role maintaining vasorelaxation by exerting its effects on vascular smooth muscle cells (VSMCs), the contractile state of which defines vascular tone. NO dependent vasodilation is initiated when agonist such as acetylcholine (Ach), adenosine triphosphate (ATP), shear stress, ET-1 activate the endothelial cells phosphoinositol pathway and increase cytosolic Ca^{+2}. In the endothelial cells, Ca^{+2} binds to CaM, which then activates eNOS to form NO from its precursor substrate, L-arginine. NO diffuses to the adjacent SMC where it activates soluble guanylate cyclase (sGC) to increase 3', 5'-cyclic guanosine monophosphate (cGMP) levels. cGMP relaxes SMC by decreasing Ca^{+2} levels through inhibition of voltage-gated Ca^{+2} channels and activates protein kinases that phosphorylate proteins in the sarcoplasmic reticulum, as well as Ca^{+2}-dependent potassium channels. Reduction in cytosolic Ca^{+2} concentration results in inhibition of Ca^{+2}/CaM mediated myosin light chain

kinase (MLCK) complex formation in the VSMC, promoting vasorelaxation (Figure 2). Production of NO is found in virtually every tissue of the body; however, the exact role of NO in regulating biological functions is complex.

1.3.4.Nitric Oxide Synthase

There are four isoforms of nitric oxide synthases (NOSs): Inducible (iNOS), endothelial (eNOS), and neuronal (nNOS) and mitochondrial (mtNOS). All the isoforms are homodimer and each monomer contain an oxygenase domain and a reductase domain (Figure 3). Although these isozymes are encoded by different genes located on different chromosomes, there is remarkable sequence homology among them. Most notably, several regions necessary for NOS activity are highly conserved. These include the binding sites for reduced nicotinamide adenine dinucleotide phosphate (NADPH), flavin adenine dinucleotide (FAD), flavin mononucleotide (FMN), CaM and tetrahydrobiopterin (BH_4). All four isozymes catalyze the same reaction, which involves consumption of O_2 and acceptance of electrons from NADPH to convert L-Arginine to NO and L-Citrulline:

$$\text{L-Arginine} + 1.5\ \text{NADPH}/H^+ + 2O_2 \longrightarrow \text{L-Citrulline} + NO + 1.5NADP^+ + H_2O$$

This reaction involves formation of several intermediates resulting from a series of electron transfer steps from NADPH to O_2 (Figure 3). Although the transfer of electrons to O_2 in the oxygenase domain is independent of the availability of BH_4 and L-arginine, the presence of BH_4 and L-arginine is critical for the proper functioning of NOSs. If L-arginine or BH_4 is deficient, the electron is accepted from NADPH will be transferred directly to O_2 to produce superoxide (O_2^-) rather than NO (Figure 3). Consequently, at low levels of BH_4

or L-arginine, both NO and O_2^- are produced, which subsequently react to form peroxynitrite ($ONOO^{--}$).

Unlike the absolute requirement for BH_4 as cofactor for proper functioning in all NOSs, the functional requirement of Ca^{+2} is different among the isoforms. Constitutive NOSs (cNOSs) including nNOS and eNOS and mtNOS are more dependent on Ca^{+2} than iNOS. In the absence of Ca^{+2}, cNOSs are inactive. The binding of Ca^{+2}/CaM to cNOSs stimulates their enzymatic activity [114] to produce low level of NO (<1μM) by inducing conformational change of cNOSs to facilitate the rate-limiting electron transfer step between FMN and BH_4 [115, 116]. In contrast, iNOS is less dependent on Ca^{+2} binding. In response to immunological stimuli such as TNF-α, IL-1β, IFN-γ and endotoxin [114] iNOS produces large amount of NO (>1μM). Since the reaction of NO and O_2^- is much faster than with superoxide dismutase (SOD) [117], at high concentration, NO combines with O_2^- to form $ONOO^-$, which is often associated with tissue injury and causes detrimental effects such as hypotension and shock [118].

All four NOSs can be purified from the liver; however iNOS and eNOS appear to be most important for hepatic microcirculation. In the liver, iNOS was identified in the KCs, HSCs, and hepatocytes as well as in endothelial cells and SMC. However eNOS is only expressed in LSECs [119]. In normal liver, iNOS is virtually absent, but its expression is significantly upregulated in response to various inflammatory and oxidative stress stimuli. This led to the assumption that NO production from iNOSs, but not eNOS was crucial for the maintenance of hepatic blood flow following inflammatory and oxidative stress conditions [120] . However, the treatment with iNOS antagonist did not improve but rather increased mortality of sepsis patients, although hypotension was

improved [118, 121]. Strikingly, the treatment with specific eNOS inhibitor or non-specific NOS inhibitors worsens the stress induced liver injury [122]. The exacerbation of injury is associated with failure of local microcirculation [120] and the formation of patchy necrosis [122]. The detrimental effects of eNOS inhibition in the liver is confirmed in eNOS gene knock out mice [123, 124] and were completely reversed by endogenous and exogenous NO-donor but not by iNOS inhibition [125]. These results suggest that relative impaired NO produced by eNOS and excessive NO generation followed by stress-induced iNOS gene expression due to $ONOO^-$ exacerbate liver injury [118].

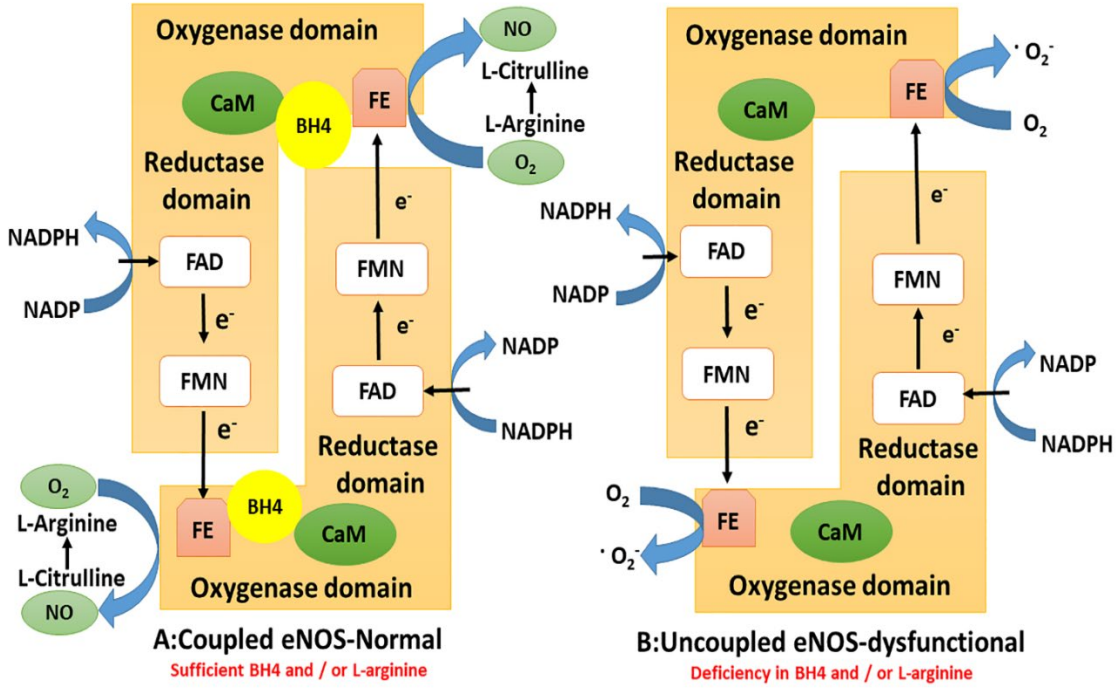

Figure 3: Schematic diagram of mechanism of nitric oxide synthase action

Nitric oxide synthase (NOS) is a homo-dimer and each monomer units consists of an oxygenase domain in the N-terminus and a reductase domain in the C-terminus. The catalytic production of nitric oxide (NO) involves a sequential electron transfer steps. An electron is accepted from a reduced nicotinamide adenine dinucleotide phosphate

(NADPH), which is subsequently transferred to flavin adenine dinucleotide (FAD) and then flavin adenine mononucleotide (FMN) in the reductase domain. The electron transfer from the reductase domain to the oxygenase domain occurs in trans (from one monomer to another) and is a rate-limiting step in constitutive NOSs (CNOSs). Calcium/calmodulin (Ca^{+2}/CaM) triggers a conformational change of cNOSs to enhance kinetic energy. In coupled eNOS (A), sufficient tetrahydrobiopterin (BH_4) and L-arginine yield L-citrulline and NO in the oxygenase domain. BH_4 in conjunction with iron protoporphyrin 1X (heme) groups (Fe) hold the subunits tightly bound and keep the dimer stabilized. However, when there is deficiency of BH_4 or L-arginine, electrons flow from reductase domain get uncoupled from L-arginine oxidation (B) leading to direct transfer of electrons to oxygen (O_2) in the oxygenase domain, to yield superoxide (O_2^-) instead of NO. Note that in actual conformation the heme groups from two subunits lie in proximity. The reaction of NO and O_2^- form peroxynitrite ($OONO^-$) which may cause oxidative damage and have detrimental effects. Also, the binding of caveolin-1 (Cav-1) to the eNOS reductase domain prevents the initiation of the electron transfer steps by blocking eNOS from accepting electrons from NADPH. This figured is referred from [126]

1.4.Activation of Rho Kinase Pathway Mediates Endothelial Dysfunction

The mechanisms involved in inactivation of eNOS leading to decreased NO mediated dilation that significantly contribute to sepsis associated liver injury, are complex and incompletely understood. However; we found that inhibition of one intracellular signaling molecule rho kinase (ROCK) largely restores ET-1 induced NO production impaired by LPS via inactivation of eNOS [127].

RhoA is a member of the small G-protein superfamily, which contains over 100 members that cycle between an inactive guanosine diphosphate (GDP)-bound form and active guanosine triphosphate (GTP)-bound form. The rate limiting step of the GDP/GTP exchange is the dissociation of GDP, which is facilitated by guanine nucleotide-exchange factors (GEFs). For example, the $G\alpha_{12/13}$ and $G\alpha_{q/11}$ mediated RhoA activation involves RhoGEF; PDZ-RhoGEF, LARG, p115-RhoGEF [128, 129]. The active GTP-bound form of the small G-protein is converted to the inactive GDP-bound form due to its intrinsic GTPase activity, which can be stimulated by GTPase-activating proteins (GAPs) (Figure 4).

The small G-protein superfamily is classified into five subfamilies: Rho family GTPases (Rho, Rac and cdc42), Ras family GTPases (Ras, Rap, Ral), Arf family GTPases (Arf1-6, Arl1-7, Sar), Rab family GTPases (>60 members) and Ran family GTPases [130]. In general, the Rho-GTPases regulate the assembly of actin cytoskeleton, the Ras-GTPases control gene transcription, the Rab and Arf-GTPases regulate the trafficking of vesicles between cellular compartments, and the Ran-GTPases regulate both microtubule organization and nucleo-cytoplasmic protein transport [130].

RhoA can associate with the caveolae through its direct interaction with Cav-1 in the endothelial cells [131]. This caveolar translocation of RhoA is mediated by its post-translation modification; geranylgeranylation and lipid attachment to the plasma membrane and is necessary for its activation in the endothelial cells [132]. PKA phosphorylates RhoA on Ser188 and causes RhoA to disassociate from the membrane without affecting the ability of RhoA to bind GTP and without modifying its intrinsic GTPase activity [133].The ROCKs are the downstream targets of RhoA [134]. Following

activation, RhoA stimulates the activity ROCKs. In the mammalian system, two isoforms of ROCKs exist and are termed as ROCK-1 and ROCK-2. ROCK-1 is also known as ROKβ and p160ROCK, whereas ROCK-2 is also known as ROKα and together referred to as Rho-kinase. ROCKs consist of amino-terminal kinase domain, followed by a coiled-coil Rho-binding domain (RBD) and a carboxyl-terminal cysteine-rich domain (CRD) located within the pleckstrin homology (PH) motif. The RBD and CRD domains serve as auto regulatory inhibitor of the amino-terminal kinase domain [135]. The binding of GTP-bound RhoA to the RBD of ROCKs increases ROCK activity by minimizing the auto-inhibition from RBD and CRD domains (Figure 4). PKC is also required for the RhoA activation of the ROCKs [136]. Y-27632 and fasudil inhibit ROCK by targeting the ATP-dependent kinase domains of ROCKs (Figure 4).

RhoA/ROCK pathway has been reported to be involved in angiogenesis [137], atherosclerosis [138], cerebral ischemia [139], erectile dysfunction [140], glomerulosclerosis [141], hypertension [142], myocardial hypertrophy [143], myocardial ischemia-reperfusion injury [174], pulmonary hypertension [144] and vascular remodeling [145]. In many of these studies, inhibition of ROCKs upregulates eNOS and mediated beneficial and protective effects in ECs.

Accumulating evidence indicates that RhoA and ROCK-2 are involved in mediating endothelial dysfunction by inhibition of eNOS activity [146, 147]. For instance, inhibition of RhoA geranylgeranylation by statins decreases ROCK activity and leads to eNOS activation. Similarly, direct activation of the Rho/ROCK signaling pathway by ROCK inhibitors or dominant negative mutant of RhoA increase eNOS expression [147, 148]. RhoA and ROCK-2 also inhibits eNOS activity by inhibiting direct phosphorylation

of eNOS at its stimulatory site Ser1117 [149], increase phosphorylation at its inhibitory site Thr495 [150], through inhibition of protein kinase B [149] which mediates phosphorylation ser1117 and by decreasing eNOS mRNA half-life [132] (Figure 4).

RhoA/ROCK instigate endothelial barrier dysfunction by modulating actin cytoskeleton [151, 152]. Broken endothelial barriers are one of the potential contributors in pathogenesis of sepsis [153]. In the regulation of the assembly of actin cytoskeleton and cell contractility, ROCKs phosphorylate a number of downstream target including myosin light chain (MLC), myosin-binding subunit (MBS) on MLC phosphatase (MLCP), ezrin-radixin-moesin (ERM) proteins, LIM-kinase (LIMK) and adducin [154]. The consensus sequence of ROCK phosphorylation are R/KXS/T or R/KXXS/T, where R is arginine, K is lysine, X is any amino acids, S is serine and T is threonine [155, 156]. Phosphorylation of MBS inactivates MLCP that leads to the phosphorylation of MLC and ultimately the contraction of VSMCs [135, 157] (Figure 4). Phosphorylation of the ERM proteins activate ERM and increases the cross-linking between the plasma membrane and actin filaments. Phosphorylation of LIMK-1 at Thr508 and LIMK-2 at Thr505 leads to the phosphorylation of cofilin at Ser3, which inhibits the cofilin-mediated disassembly of actin filaments [158] (Figure 4). This results in maintenance of contractile F–actin stress fibers in the cytoskeleton rather than cortical arrangement of actin [155, 159, 160] These stress fibers can retract cells from their margins making them hyperpermeable [151] (Figure 4). Phosphorylation of adducin increased its association at cell-cell contact sites to enhance contractile response [161, 162]. Globular (G)-actin can also directly interact with eNOS and increases its activity [163]. Actin depolymerization and actin polymerization are regulated by cofilin and vasodilator-stimulated phosphoprotein (VASP) respectively.

RhoA induces VASP-ser239 [164] phosphorylation and ROCK-2 mediates cofilin-Ser3 phosphorylation [165] to promote excess actin polymerization over depolymerization. Our lab showed that LPS stimulates RhoA and ROCK-2 to increase VASP activity and decrease cofilin activity to not only lower G-actin formation but also to inhibit of ET-1 mediated eNOS activation [127].

RhoA/ROCK increase oxidative stress in endothelial cells. Sepsis associated hypoxia and inflammation have been linked to increased production of ROS and reactive nitrogen species (RNS) including O_2^-, hydrogen peroxide (H_20_2), hydroxyl radicals (HO^-) and NO [166]. Under physiological conditions, there is balance between the formation of oxidant substances and their removal by antioxidant scavenging compounds. While in sepsis patients, oxidative imbalance with increased ROS and decreased antioxidants have been demonstrated [167, 168]. Harmful mechanisms include modification of proteins, lipids and nucleic acids contributing to cellular injury and endothelial dysfunction [166]. In addition, they impair cellular junctions between the endothelial cells that lead to increased vascular permeability, a corner stone of sepsis development [169]. Recently, ROCK has been shown to increase mitochondrial ROS production by modulating the interaction between Rac1b and cytochrome c (cyt c) [170] (Figure 4). Rac1b is an alternatively spliced variant of G-protein Rac1 that comprises of a self-activated GTPase unit [171] and plays role in cytoskeletal reorganization and ROS production [172]. Cyt c is a mitochondrial redox carrier that is part of complex III in electron transport chain (ETC) of mitochondria. Cyt c have heme as prosthetic group that accepts electrons from complex III and transfers them to complex IV cytochrome c oxidase ; COX of ETC cycle [173]. In turn, reduced COX conducts the final electron transfer to O_2

allowing its complete reduction into water (H_20), which ultimately drives proton pump across the inner mitochondrial membrane for ATP production [174]. RhoA/ROCK activation induces phosphorylation of Rac1b at ser71 and facilitate its interaction with cyt c. This interaction results in electron transfer from Cyt c to Rac1 at Cys-178 instead of their transfer to COX [175]. Therefore, COX is unable to completely reduce O_2 to H_20 leading to production of ROS [170] (Figure 4).

RhoA/ROCK has been shown to induce mitochondrial dysfunction. Mitochondrial dysfunction is a critical contributor of endothelial dysfunction. The primary function of mitochondria is production of ATP to facilitate cellular energy demand. To meet this, mitochondrial respiratory complex I, III and IV generate mitochondrial membrane potential (MMP) by transferring protons from the matrix into the intermembrane space [170]. Oxidative stress, inflammation, ischemia /reperfusion injury can cause drop in MMP leading to mitochondrial depolarization [176, 177] . Decreased MMP leads to energy depletion, release of cytochrome C and apoptosis [178]. A recent study showed that deterioration of COX activity leads to mitochondrial dysfunction through decreasing MMP [179] and this effect is mediated by RhoA/ROCK [170]. Y-27632 treatment recovered MMP and increased COX production [170]. Also, RhoA upregulates pro-apoptotic protein Bax, which undergoes oligomerization, translocate to mitochondria, where it induces release of cyt c, caspase activation, DNA fragmentation and activate mitochondrial death pathway (intrinsic) [180] (Figure 4).

In summary, RhoA/ROCK pathway has been shown to mediate endothelial dysfunction by inducing F-actin stress fibers, mitochondrial depolarization, hyper-contraction, increased production of ROS and decreased NO mediated vasodilation (Figure

4). Yet, little is known about how this pathway gets activated. In present study, our aim is to determine the signaling molecule/s responsible for activating RhoA/ROCK and its mediated endothelial dysfunction in sepsis. A very good candidate is protein sonic hedgehog (SHH).

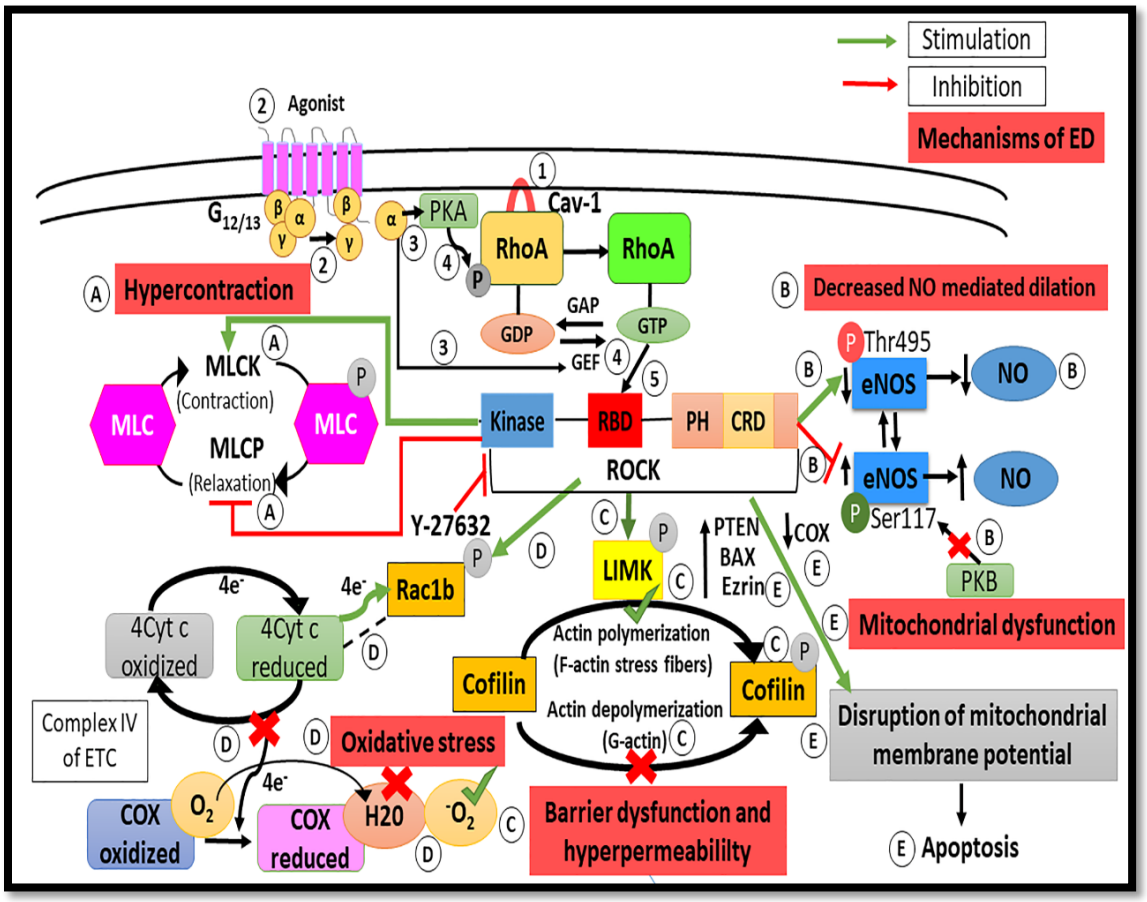

Figure 4: Rho signaling pathway and its role in endothelial dysfunction

Rho A is a member of small G-protein superfamily. (1) Rho proteins are associated with caveolae in plasma membrane through direct interactions with caveolin-1 (Cav-1). (2) Binding of agonist to G-protein coupled receptor such as $G_{12/13}$ stimulates heterotrimeric G-protein dissociation into G-protein βγ and G-protein α. (3) G-protein α activate guanine nucleotide exchange factors (GEF) and protein kinase A (PKA). (4) PKA phosphorylates

RhoA and causes RhoA to dissociate from the membrane. While, GEF activates RhoA by facilitating conversion of RhoA inactive-GDP bound form into RhoA active-GTP bound form. GTPases-activating proteins (GAP) can convert them back into the inactive-GDP bound form. (5) Active Rho-GTP stimulates activity of Rho-associated kinases (ROCKs) by binding to its Rho-binding domain (RBD). ROCK mediates endothelial dysfunction via different mechanisms. (A) ROCKs regulate phosphorylation of myosin light chain (MLC) by direct phosphorylation of MLC via activation of myosin light chain kinase (MLCK) and by inactivation of myosin phosphatase (MLCP) through phosphorylation of its myosin-binding subunit (MBS). This induces smooth muscle contraction and stress fiber formation in non-muscle cells. (B) RhoA can decrease nitric oxide (NO) production and its associated vasodilation by impairing endothelial nitric oxide synthase (eNOS) activity via enhanced inhibitory phosphorylation at Thr495 and reduced stimulatory phosphorylation at Ser1117. (C) RhoA/ROCK instigate endothelial barrier dysfunction by activating LIM-kinases (LIMK) that in turn phosphorylate and inactivate an actin de-polymerization factor cofilin, leading to maintenance of contractile F–actin stress fibers in the cytoskeleton. These stress fibers can retract cells from their margins making them hyper-permeable. (D) ROCK increases interaction between Racb1 and cytochrome c (cyt c) leading to transfer of electrons between Rac1 cys178 and cyt c rather than transfer of electrons to complex IV cytochrome c oxidase (COX) in electron transport chain (ETC) of mitochondria. This results in partial reduction of molecular oxygen (O_2) into superoxide (O_2^-) instead of water (H_20) and therefore increase in oxidative stress. (E) ROCK can also induce mitochondrial depolarization in endothelial cells by increasing pro-apoptotic BAX and decreasing COX, ultimately leading to apoptosis of cells via release of cyt c.

1.5.Sonic Hedgehog Pathway.

The hedgehog gene (HH) was identified in a now classic screen for genes that specify the formation of embryonic pattern in fruit fly, *Drosophila melanogaster*, and named for an abnormal bristle pattern in HH mutant larvae [181]. Isolation of HH gene revealed that it encodes a secreted protein that forms segmentally repeated stripes, consistent with its role in specifying segmental patterns [182-185]. Once protein is expressed, HH protein undergoes auto processing to form a 45-kDa precursor that cleaves itself into an N-terminal fragment of 19kDa (HHN) retaining all signaling activity and a C-terminal fragment (HHC) of 25kDa with a cholesterol attached to it [182, 186, 187]. The HHN is lipid modified with palmitoylation [188]. Cholesterol modification allow HH trafficking [189] and movement whereas palmitoylation enables HH signaling. HHN is released from its site of synthesis by protein dispatched, which is a novel sterol-sensing domain protein [190] that is dedicated to the release of cholesterol-modified HH from signaling cells .

In the early 1990s, three HH gene homologs were discovered in vertebrate; Sonic hedgehog (SHH), Indian Hedgehog (IHH) and Desert Hedgehog (DHH) [191, 192]. DHH and IHH have been shown to play role in normal tissue development including pancreas and testis organogenesis, bone formation and cartilage development respectively [193, 194]. SHH is the most potent of these ligand as it is a key morphogenetic factor that organizes centers including the zone of polarizing activity in the limb bud, formation of notochord , pre-chordal and floor plate and ventral forebrain within neural tube [191, 195-197]. Loss of *SHH* function causes cyclopia , midline face and brain defects, as well as loss of the axial skeleton and severe limb patterning defects, [198].

HH signaling pathway is critical for cell fate decisions, including proliferation, apoptosis, migration and differentiation. Recently, it has become evident that HH more specifically SHH is also re-activated during adult life where it regulates homeostasis and repair in tissues undergoing constant renewal such as blood, skin, colon, liver. Further, SHH pathway is tightly regulated in most adult tissue but hyper activation of this pathway is found in most solid tumors [199]. The core components that mediate HH signaling are Patched-1 (Ptch1), a 12-pass integral membrane protein [200, 201], and smoothened (SMO), a seven-pass integral membrane protein with homology to GPCR [202, 203]. Patched-2 (Ptch2) is another receptor for SHH that shares approximately 54% homology with Ptch1. However, the expression and signaling of Ptch2 is different from Ptch1, having decreased ability to inhibit SMO in absence of SHH ligand [204]. Ptch1 normally inhibits the activity of SMO and is a positive regulator of hedgehog pathway activation [205, 206]. Activation of SHH pathway can happen in two majors pathways; canonical signaling (Figure 5) that is dependent upon ligand interaction or through receptor induced signaling and the non-canonical pathway (Figure 6) that involves activation downstream of SMO [207-209].

1.5.1. Canonical Sonic Hedgehog Pathway

The canonical SHH pathway is triggered by binding of endogenously or exogenously produced SHH glycoproteins to Ptch1 on target cells (Figure 5). This leads of inhibition of the Ptch1 [210] via cellular internalization and SMO localization on the cell surface, both by cilium mediated mechanism [211, 212] (Figure 5). How signals are transmitted from HH to Ptch1 to SMO remains unclear. SMO activation leads to nuclear translocation of Glioma (Gli); zinc finger transcription factors (Figure 5). In vertebrates, there are three Gli

(Gli1, Gli2, and Gli3) [213, 214]. Gli1 is the only full-length transcriptional activator while Gli2 and Gli3 acts as either positive or negative regulator as determined by post-transcriptional and post-translational processing [215, 216]. Gli transcription factors can activate target genes that includes targets involved in HH pathway feedback (e.g.; Gli1, Ptch1), proliferation (e.g.; Cyclin-D, MYC), inhibition of apoptosis (e.g.; Bcl-2), angiogenesis (e.g.; ANG1/2), epithelial to mesenchymal transition (e.g.; SNAIL) and stem cell self-renewal (e.g.; NANOG, SOX2) [217-219] (Figure 5). The biological effect is cell proliferation with deregulation contributing to tumorigenesis. In the absence of ligand, Suppressor of Fused (SUFU) negatively regulates the pathway by directly binding to Gli target genes [220, 221]. SUFU sequesters Gli and facilitates its degradation (Figure 5). Several protein kinases, such as PKA and protein kinase C (PKC), CK1, mitogen activated protein kinase kinase (MeK1), GSK3, Phosphoinositide-3-kinase (PI3K), or dual specificity Yak1-related kinase (DYRK1) can also modulate this pathway at several levels [222, 223] Abnormal HH pathway activation occurs not only through canonical HH signaling but also genetic alterations or ciliary protein overexpression leading to functional redundancy of Gli transcription factors, crosstalk between HH signaling and unrelated pathways, are all causes of non-canonical HH signaling activation [207].

1.5.2. Type I Non-Canonical Sonic Hedgehog Pathway

The non-canonical SHH signaling usually occurs through Gli-independent mechanisms. The Gli-independent mechanisms include two types: Type I is independent of SMO and plays role in modulating cell proliferation and survival. While, Type II is downstream of SMO, which modulates Ca^{+2} and actin cytoskeleton [208, 224]. Ptch1 displays dual signaling by functioning as a dependence receptor whose activity is dependent on the

availability of HH ligand. In the presence of HH ligand, Ptch1 transduce normal canonical-SHH pathway survival signals but in the absence of HH ligand as in type I non-canonical signaling, Ptch1 is not inactive but rather actively trigger apoptosis (Figure 6A), thus generating a state of cellular dependence on HH ligand for survival [225, 226]. Ptch1 contains a dependence associated receptor C-terminal motif that is cleaved by caspase-3 at conserved aspartic acid in the absence of SHH, to expose a pro-apoptotic domain [225, 227]. This autonomous apoptosis activation is not dependent on SMO [225, 228, 229]. Caspase-cleaved Ptch1 can now associate with cyclin-B and pro-apoptotic complex that includes adaptor protein downregulated in rhabdomyosarcoma LIM-domain protein (DRAL), Caspase recruitment (CARD)-domain containing protein TUCAN or NLR family pyrin domain containing 1 (NALP1) and apical caspase-9 [228] (Figure 6A). Cyclin-B is a mitotic cyclin that is necessary for the progression of cells into and out of mitotic phase of cell cycle, allowing cell division. Interaction with cyclin-B inhibit cyclin-B1 nuclear accumulation and cyclin-B mediated proliferation [230, 231]. The C-terminal of Ptch1 activates caspase-9, that in turn speeds up formation of this complex by promoting activation of caspase-3 and therefore leading to apoptosis by both intrinsic and extrinsic pathways [232] (Figure 6A). HH binding disrupts the interaction of Ptch1 with cyclin-B and the pro-apoptotic complex, likely through conformational change in Ptch1 that allows its sequestration via cellular internalization. This leads to increased proliferation and survival [224].

1.5.3. Type II Non-Canonical Sonic Hedgehog Pathway

Many studies have implicated type II pathway in regulating actin cytoskeleton through activation of Rho family such RhoA and Rac1 in a SMO dependent manner [224, 233].

Activation of RhoA promotes formation of actin stress fibers in cultured endothelial cells [229, 234]. In fibroblast, stimulation of RhoA and Rac1 by SMO mediates migration of fibroblasts [235]. Type II non-canonical pathway (Figure 6B) is activated when SMO localizes on the cell surface [211, 212] due to SHH mediated inhibition of Ptch1 [210] via cellular internalization and interacts with inhibitory G protein. SMO-GPCR interaction with G_i lead to activation of phosphoinositide-3-kinase (PI3K) via non-receptor proto-oncogene tyrosine kinase Src [208, 224]. There are three classes of PI3K while class I is most relevant for Rho kinase activation. Class I PI3K are heterodimers composed of a regulatory subunit (p85) and a catalytic subunit (p110) [236]. When a ligand is sensed by receptor tyrosine kinases (RTKs) or G protein-coupled receptors (GPCRs), PI3K phosphorylates the 3-hydroxyl group of the inositol ring of membrane bound phosphatidylinositol 4,5-biphosphate (PIP2), generating phosphatidylinositol-3,4,5-trisphosphates (PIP_3) [236] (Figure 6B). PIP3 serves as docking stations for proteins that harbor lipid binding pleckstrin homology domains (PH). One such protein that interacts with PIP3 via PH domain is Rho-GEF [237] (Figure 6B) which allow Rho-GTPases to convert from inactive GDP-bound state to active GTP-bound state. Active Rho-GTP stimulates activity RhoA downstream effector protein Rho-associated kinases (ROCKs). ROCK activates LIMK that in turn phosphorylate and inactivate an actin de-polymerization factor cofilin, leading to maintenance of contractile F–actin stress fibers in the cytoskeleton [158] (Figure 6B). These stress fibers can retract cells from their margins making them hyper-permeable [155, 159, 160]. Also, SMO mediates Rac1 activation by allowing interaction with Rac guanine nucleotide exchange factor (GEF) T-lymphoma invasion and metastasis-1 (Tiam1) [224, 238]. Rac1 in turn can activate RhoA and mediate

migration in fibroblast by a mechanism that is still not clearly understood [238] (Figure 6B). Apart from actin cytoskeleton modulation in ECs, SHH also promotes capillary morphogenesis, EC angiogenic activity via non-classical RhoA/ROCK pathway [229, 234]. Thus, SHH is a good candidate to induce RhoA/ROCK pathway SHH ligands acting as paracrine or autocrine mediators can potentially modify phenotypes of endothelial cells during sepsis and cause blood flow dysregulation in the liver. This is because, biologically active SHH were demonstrated to be released in sub-micron sized vesicles called microparticles (MPs) generated from blebbing plasma membrane of various cells after stimulation with inflammatory mediators or apoptosis [239, 240].

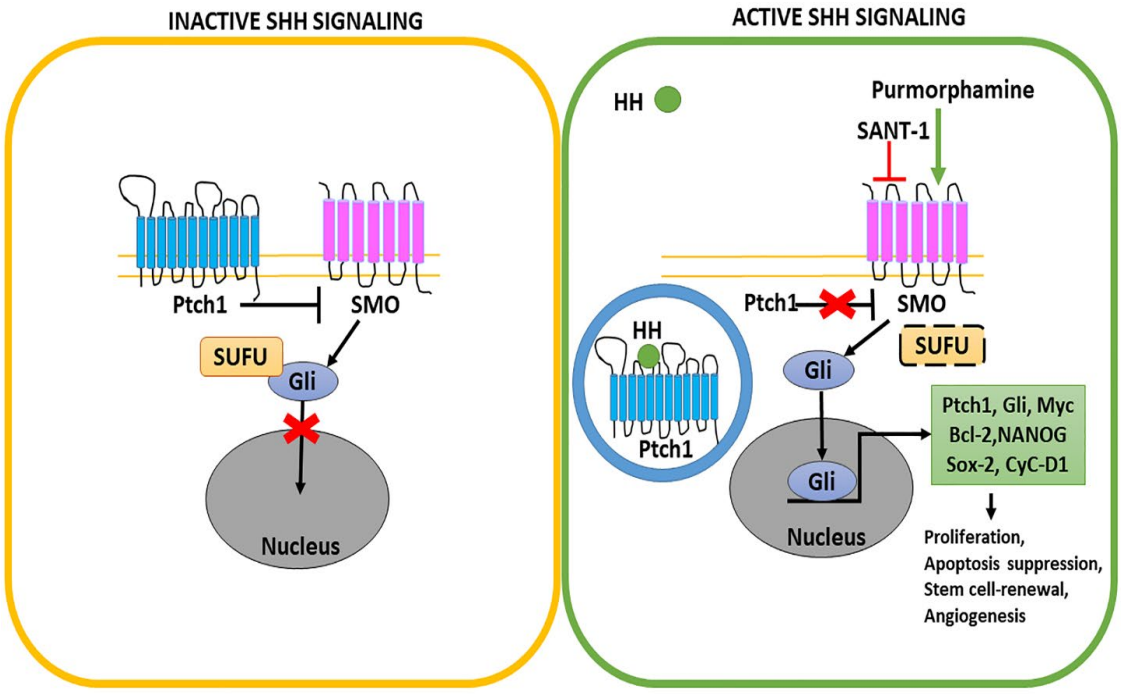

Figure 5: Canonical Hedgehog signaling pathway

Inactive signaling occurs (left) in the absence of hedgehog (HH) ligand wherein patched-1 receptor (Ptch1) inhibits smoothened (SMO) transmembrane protein resulting in Glioma (Gli) transcription factor being sequestered in cytoplasm by Suppressor of Fused (SUFU).

In the presence of HH ligand, Ptch1 suppression of SMO is abrogated resulting in nuclear accumulation of Gli and activation of genes that includes targets involved in HH pathway feedback (e.g.; Gli1, Ptch1), proliferation (e.g.; Cyclin-D, MYC), inhibition of apoptosis (e.g.; Bcl-2), angiogenesis (e.g.; ANG1/2), epithelial to mesenchymal transition (e.g.; SNAIL) and stem cell self-renewal (e.g.;NANOG,SOX2). 4-Benzyl-piperazin-1-yl-(3, 5-dimethyl-1-phenyl-1H-pyrazol-4ylmethylene)-amine (SANT-1) is antagonist of SMO while purmorphamine (Pur) acts as SMO agonist.

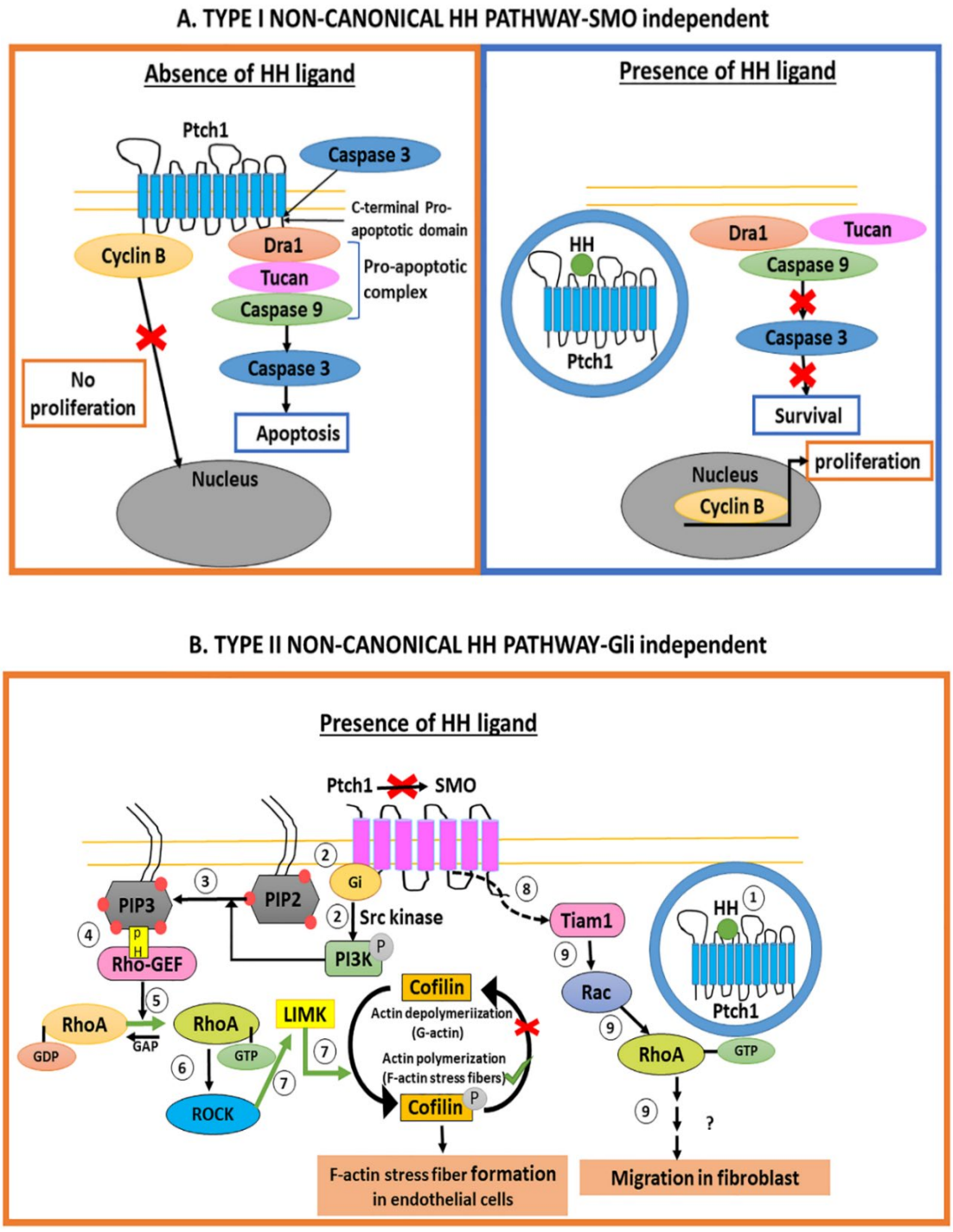

Figure 6: Type I and Type II Non-canonical Hedgehog pathway.

In Type I non-canonical HH pathway (A), the absence of Hedgehog (HH) ligand (Left) allow caspase-3 to cleave dependence associated receptor C-terminal motif of receptor

Patched1 (Ptch1), exposing the pro-apoptotic domain. Cleaved-caspase Ptch1 with its pro-apoptotic domain interacts with cyclin-B and a pro-apoptotic complex that includes adaptor protein downregulated in rhabdomyosarcoma LIM-domain protein (DRAL), Caspase recruitment (CARD)-domain containing protein TUCAN or NLR family pyrin domain containing 1 (NALP1) and apical caspase-9. This pathway is SMO independent. The interaction with cyclin-B inhibits proliferation by sequestering cyclin-B outside the nucleus. C-terminal domain of Ptch1 activates capase-9 that in turn speeds up the formation of this complex by promoting activation of caspase-3, leading to apoptosis. HH binding (right) disrupts the interaction of Ptch1 and cyclin-B and pro-apoptotic complex, likely through a conformational change in Ptch1 that allows its cellular internalization, leading to increased survival and proliferation. (B). In Type II non-canonical HH pathway, SMO regulates the actin cytoskeleton through small GTPases RhoA and Rac1. (1) HH ligand binds to Ptch1 leading to its inhibition via cellular internalization. (2) Relieve from Ptch-1 mediated inhibition allows SMO to re-localize on cell surface and interact with inhibitory G protein; G_i to activate phosphoinositide 3-kinase (PI3K) via non-receptor proto-oncogene tyrosine kinase Src. (3) When HH binds to SMO, PI3K phosphorylate the 3-hydroxyl group of the inositol ring of membrane bound phosphatidylinositol-4,5-bisphosphate (PIP2), generating phosphatidylinositol-3,4,5-triphosphate (PIP3). (4) PIP3 serves as docking stations for proteins that harbor lipid binding pleckstrin homology domains (PH) such as Rho-GEF. (5) Rho-GEF activates RhoA by facilitating conversion of RhoA inactive-GDP bound form into RhoA active-GTP bound form. GTPases-activating proteins (GAP) can convert them back into the inactive-GDP bound form. (6) Active Rho-GTP stimulates activity of its downstream target Rho-associated

kinases (ROCKs). (7) ROCKS mediate stress fiber formation by activating LIM-kinase (LIMK) which in turn phosphorylate and inactivate actin de-polymerization factor cofilin, leading to maintenance of contractile F–actin stress fibers in the cytoskeleton of endothelial cells. (8) Activated SMO also mediate Rac1 activation by allowing interaction with Rac guanine nucleotide exchange factor (GEF) T-lymphoma invasion and metastasis-1 (Tiam1). (9) Rac1 in turn can activate RhoA and mediate migration in fibroblast by a mechanism that is still not clearly understood. This figure is a modified diagram in reference [224]

1.6.Sonic Hedgehog is released in Apoptotic T-cell Derived Microparticles during Sepsis

SHH has been shown to be contained in sub-micron sized vesicles called microparticles (MPs) [240-243] that are released from blebbing plasma membrane of various cells types such as platelets, T and B lymphocytes, monocytes and endothelial cells when undergoing apoptosis or stimulated with inflammatory mediators. In sepsis, there is massive apoptosis of T-lymphocytes within first 24 hours of the insult [244] producing MPs from their cell surface. This is relevant as numerous studies have demonstrated decrease in lymphocyte numbers [245, 246] and increase in circulating MPs [247, 248] including T-cell derived MPs in animal models and sepsis patients. Our hypothesis is that these apoptotic T-cell derived MPs containing SHH induce endothelial dysfunction by activation of type-II non-canonical SHH-SMO-ROCK pathway. This highly novel signaling pathway is likely be a major contributor to the development of altered vascular regulation in liver during sepsis.

1.6.1. Apoptosis of T-Lymphocytes during Sepsis

In sepsis, systemic inflammatory response results from failure of initial pathogenic clearance and / or susceptibility to secondary infections. The innate and adaptive immune

response mediate the pathogen clearance and inflammation responses. Innate arm involves an antigen non-specific response using majorly polymorphonuclear cells and macrophages to control the infection whereas the adaptive arm involving T and B lymphocytes among others bear antigen specific receptors [244]. Many studies in literature shows that aberrant innate immune response early in sepsis leads to MOF [249-251]. Classically, the onset of adaptive immune responses was thought to occur after the innate immune response has subsided [252, 253]. The adaptive cell role was then to maintain surveillance and prevent new infections from gaining a foothold in the already compromised host. However, there is an emerging view that adaptive responses are engaged much earlier in sepsis than previously thought. Interestingly, a large degree of lymphocyte apoptosis also occurs within the first 24 hour of septic insult, suggesting an earlier role in immune response [244]. Therefore, we chose our treatment time with apoptotic T-cell derived MPs to be 24hrs or less.

Lymphocyte apoptosis has been recognized as an important step in the pathogenesis of sepsis [254, 255]. Several studies have demonstrated profound decrease in peripheral and splenic lymphocyte number during sepsis in both animals and humans [246, 256]. Intraperitoneal injection of Gram-negative bacteria to mice was followed by apoptosis of $CD4^+$ and $CD8^+$ lymphocytes in the thymus [257] while in CLP model, lymphocyte apoptosis also involved lymphocytes from the spleen and other organs [256, 258]. Accelerated apoptosis in $CD4^+$, $CD8^+$, CD19 lymphocyte was demonstrated in sepsis patients but not in non-septic, critically ill patients [255]. In sepsis, the immune response follows a biphasic pattern with an initial hyperinflammatory state characterized by high levels of pro-inflammatory cytokines and a second immunoparalysis phase characterized

by decreased responses of immune cells to the inflammatory stimuli. Lymphocytes play critical role in both the phases. On one hand, as the lymphocytes produce pro-inflammatory cytokines activating macrophages, so loss of lymphocytes may seem beneficial to survival as there will be down-regulation of the excessive inflammatory response during sepsis [245, 259]. Alternatively, loss of lymphocytes may be detrimental as the ability of the immune system to combat the pathogens is impaired [245, 259]. A number of studies show that lymphocytes apoptosis is detrimental as sepsis patients develop immunological impairment [256]. Sepsis patients have no response to skin testing with antigens derived from microbes to which they have been previously exposed [260]. There is decrease in circulating lymphocytes in trauma patients who developed infection or died [246]. Furthermore, ICU patients who develop decreased lymphocyte count for more than 3 days are great risk for nosocomial sepsis [261]. The degree of circulating lymphocyte apoptosis correlated with sepsis severity [262], with prevention of apoptosis improved host responses to sepsis [245, 263]. T-cell sepsis induced apoptosis is subset specific. Spleen analyzed from a mice subjected to CLP for 24 hours showed 47% reduction in $CD4^+$ T-cells and 66.7% reduction in $CD8^+$ T-cells [244].

Studies using loss-of-function approaches suggested that the mechanisms of lymphocyte apoptosis in sepsis involve both receptor mediated and mitochondrial pathways of apoptosis with latter playing the pre-dominant role [264]. The death receptor pathway is mediated by number of death receptors including Fas, TNF receptor type I, death receptor 3, TRAIL receptor type I etc. that results in activation of caspase-8. An alternative to death receptor pathways involves release of pro-apoptotic factors from mitochondrial such cyt c resulting in activation of caspase-9. Both active caspase-8,

caspase-9 induce activation of caspase-3 resulting in DNA fragmentation, degradation of cytoskeletal and nuclear proteins, crosslinking of proteins, formation of apoptotic body, expression of ligands for phagocytic cell receptors and finally uptake by phagocytic cells such as macrophages [265]. Once T-cells undergo apoptosis, they can regulate immune response in sepsis via different mechanisms. One proposed theory is modulation in macrophage produced cytokine profile as the level of T-cell apoptosis occurs along gradient during sepsis [262]. Initially with little to no phagocytosis of apoptotic T-cells, macrophages in response to IFN-γ continue produce pro-inflammatory cytokines TNF-α and IL-12 as well as maintain MHC- II expression, resulting in increased inflammation and decreased bacterial load clearance. As the number of apoptotic T-cells ingested by macrophages increases, the macrophages phenotype shifts to production of transforming growth factor beta-1(TGF-β) and IL-10 along with down regulation of MHC II. This phenotypic change can result in decreased inflammation, increased bacterial load and profound immune suppression as seen in later stages of sepsis. Also, when T-cells undergo apoptosis, they produce MPs from their cell surface which are considered to be another critical mediator of inflammation and ED in sepsis [266, 267] . Venous peripheral blood isolated from septic patients showed 1.5-fold increase in T- lymphocyte derived MPs along with MPs derived from platelets, neutrophils and monocytes as compared to healthy subjects [268]. However, to date, little is known about the effects of these T-cell derived MPs on the functions of endothelial cells and more importantly the mechanisms involved. Therefore, we specifically chose to study T-cell derived MPs and elucidate the mechanisms by which they mediate endothelial dysfunction and thereby contribute to liver failure in sepsis.

1.7. Microparticles

MPs were first reported in 1967, in association with platelets in human plasma and were considered “platelet” dust with pro-coagulant activity [269]. MPs are sub-micron sized (0.1-1 μm) vesicles shed from plasma membrane of cells undergoing activation by agonist, shear stress or apoptosis [270, 271]. In vitro, the release of MPs has been shown from cells such as platelets, T and B lymphocytes, leukocytes, monocytes and endothelial cells. In the past decade, it has become evident that these MPs are just not dormant particles but are actively involved in physiology and pathophysiology. MPs occur in blood of healthy individual in low quantity while their number, cellular origin, composition significantly changes in patients suffering from disease. Under normal physiological conditions, MPs are involved in tightly controlled biological activities [272] including inflammation and hemostasis [273], transfer of surface proteins [274] and angiogenesis [275]. While in disease state, they are emerging as biomarkers of vascular injury and inflammation in atherosclerosis, endothelial dysfunction, acute myocardial infarction, type 2 diabetes, acute ischemic stroke, hyperglyceridemia, cancers and metabolic syndrome [276, 277]. The severity of disease is often correlated with both concentration and relative activity of MPs in circulation [278, 279].

MPs are just one type of extracellular vesicles (EV). The other major vesicles are exosomes and apoptotic bodies (Figure 7). These EVs have been classified based on their size and origin. Exosomes are very small sized vesicles ranging from 0.03 to 0.10μm in diameter (Figure 7). They are secreted from endosomal compartments and play role in carrying the information by delivering various effector or signaling molecules between specific cells [280]. While, the apoptotic bodies are much larger with 1-5μm in

diameter. Apoptotic bodies are released upon cell fragmentation during late phase of apoptosis. They can be identified by detection of broken DNA and histones [281] (Figure 7). MPs are released into the external milieu after selective incorporation of proteins, nuclei acids, lipids and miRNA. They are more heterogeneous than exosomes containing various surface markers such as integrin, selectins and immunoglobulins whose expression level reflects the properties of the parental cells [282] (Figure 7). These adhesion molecules allow them to interact with their counter-receptors on the surface of the target cells. This can activate intracellular signaling cascades and cause responses such as inflammation, endothelial dysfunction, thrombosis, extracellular matrix degradation and vascular remodeling. MPs can also transfer proteins, bioactive lipids, and genetic material: DNA, RNA, miRNA to target cells by either fusion or internalization and bring about structural and/ or functional change in target cell [283].

1.7.1.Microparticles Formation

MPs formation can take place via two well-known processes; cell activation and apoptosis. At present, the difference in MPs produced by these methods in terms of size, lipid and protein composition, (patho-) physiological effects are not clearly defined [284, 285]. However, they differ in mechanisms resulting in their formation. Many agonist such as thrombin for platelets [286, 287], LPS, cytokines such as TNF-α, IL-1, complement, hydroperoxide, hypoxia, oxidative damage and shear stress stimulation of monocytes, endothelial cells, lymphocytes, arterial smooth muscle and hepatocytes can induce release of MPs via cell activation or apoptosis [288-290]. In general, this release of MPs either by activation or apoptosis is time and Ca^{+2} dependent [290-292] and requires reorganization of cytoskeleton. The shedding starts within minutes after addition of agonist [293-295].

Although the exact mechanism governing the shedding of MPs is not yet fully understood, but there are different mechanisms proposed. In cell activation, increase in cytosolic Ca^{+2} activates kinases, inhibits phosphatases and activate actin regulatory proteins such as proteolytic calpain and lipid binding gelsolins. Also, it leads to conversion of membrane lipid PIP2 into IP3 and DAG. PIP2 acts as binding sites for calpain and gelsolins. Calpain is a Ca^{+2} dependent, non-lysosomal cysteine proteolytic enzyme. Calpain breaks down talin, a protein that is part of membrane skeleton along with actin and vinculin providing membrane stability [296]. This leads to disruption of membrane skeleton stability [296]. Gelsolins sever and cap actin filaments to generate smaller fragments that are associated with apoptosis, cell differentiation and cancer metastasis [297]. While upon induction of apoptosis, rise in intracellular Ca^{+2} leads to activation of caspase-3 that in turn promotes activation of ROCK in a RhoA independent manner by cleaving the putative auto-phosphorylation/ auto-inhibitory domain of ROCK. Cleaved ROCK-1 in turn activates MLCK [298]. Thereafter, MLCKs phosphorylate MLC, [299] and triggers the interaction of myosin head with actin and enable myosin ATPases to create movement between actin and myosin [300, 301] and stimulate the contractile activity of myosin This movement cause force on the plasma membrane to cause detachment of the cytoskeleton from the membrane [299, 301]. Apart from activation of these signaling pathways, the rise of Ca^{+2} also mediate rapid changes in the expression of phospholipids in the membrane layer to disrupt the membrane cytoskeleton [302]. Normally, the aminophospholipids such as phosphatidylserine (PS), and phosphatidylethanolamine (PE) are sequestered to the inner membrane leaflet, whereas the choline-containing phosphatidylcholine, and sphingomyelin are arranged on the outer membrane [303].The maintenance of this

asymmetry is mediated through modulation of ATP dependent transporters flippase, floppase and scramblase [304]. Flippase/floppase govern inward and outward movement of phospholipids respectively while, scramblase does in both directions. Flippase and scramblase are usually inactive [279, 304]. During activation/apoptosis, the sudden surge of Ca^{+2} decreases flippase activity while increasing the floppase and scramblase activation, resulting in loss of normal phospholipid asymmetry [305-307]. This loss of phospholipid asymmetry along with activation of signaling pathways leads to cytoskeleton disruption, local bulges form, finally leading to release of MPs [304]. Generated MPs have increased expression of PE on the external surface [308].

1.7.2. Role of Microparticles in Sepsis

MPs have a key role in the endothelial and hemostatic responses to sepsis. MPs can be detected in circulation of healthy individual [276, 309] but their numbers are greatly increased after sepsis [247, 310]. MPs have been implicated in multiple organ dysfunction that characterizes sepsis since they circulate systemically and can acts as pathogenic autocrine disseminators [309]. Injection of MPs isolated from septic rats into healthy rats reproduces hemodynamic, septic inflammatory pattern associated with oxidative and nitrosative stresses [310]. Similarly, MPs extracted from the whole blood of septic rat exert pleiotropic and differential effects depending on the target tissue with regard to expression of pro-inflammatory proteins related with nitrative and oxidative stresses [248]

1.7.2.1.MPs induce Inflammation in Sepsis

MPs induce deleterious effects on vascular function through increased synthesis of pro-inflammatory cytokines and chemokines and increased expression of endothelial adhesion molecules. Platelet derived MPs induce platelet aggregation through changes in the

transcellular metabolism in endothelial cells [311]. MPs deliver concentrated bioactive lipids such as arachidonic acid, cyclooxygenase-2 that induce interactions between monocytes and endothelial cells and increase chemotaxis of the monocytes [312]. Leukocyte derived MPs are upregulated in inflammation [313]. They activate ECs to stimulate to release of pro-inflammatory cytokines such as IL-6, IL-8 and upregulate Intracellular adhesion molecule-1 (ICAM-1), vascular adhesion molecule-1 (VCAM-1) and E selectin [274]. Platelet derived MPs can also bind to neutrophils and increase neutrophil aggregation and phagocytic activity [314]. Monocyte derived MPs can induce O_2^-, cytokine release and NF-κB activation in monocytes [315]. Taken together, these MPs from various cells are elevated and amplify inflammation and vascular injury [316].

1.7.2.2.MPs Promote Thrombosis

MPs have been shown to contribute to the prothrombic state in sepsis by initiating disseminated intravascular coagulopathy (DIC) [247, 317, 318], a known contributor to MOF. DIC is a serious disorder in which proteins that control blood clotting become overactive. The proteins that are usually over express include prothrombin, tissue factor (TF) etc. that plays key role in initiating blood clotting. TF has been found on the surface of MPs and its activity was shown to correspond with the clinical disease severity [319]. MPs contain other pro-coagulant molecules as well. A cohort study involving 100 patients with septic shock demonstrated increased endothelial and leukocyte MPs was strongly associated with DIC and might predict DIC occurrence and early vascular injury among septic patients [320, 321]. Increase in calpain that is involved in MPs formation can also cause platelet activation. Calpastatin is a specific endogenous inhibitor of calpain. In CLP model of sepsis, transgenic mice over expressing calpastatin had better survival, less

number of MPs, decreased inflammation and DIC. Furthermore, MPs transferred from septic wild type mice worsened the survival and increased coagulopathy of septic calpastatin overexpressing mice [247, 267].

1.7.2.3.MPs Induce Endothelial Dysfunction

MPs may play a paracrine role in promoting endothelial dysfunction. Elevated levels of MPs have been associated with vascular dysfunction including decrease of endothelium dependent vasodilation and / or alteration of responsiveness of vascular smooth muscle to vasoconstriction stimuli. Elevated levels of endothelium derived MPs have been shown to directly alter endothelial function by impairing acetylcholine (Ach) induced vasorelaxation and NO production in aortic rings from rat. This effect was accompanied by increased O_2^- production by aortic rings and endothelial cells [322]. These lymphocytic MPs have been implicated in mediating endothelial dysfunction by reducing phosphatidylinsoitol-3 kinase (PI3K) mediated eNOS activation and subsequent NO production and by increasing reactive oxygen species (ROS) via pathway involving xanthine oxidase [266]. Furthermore, same MPs inhibit angiogenesis in-vitro and in-vivo by enhancing ROS production which lead to suppression of endothelial cell survival, proliferation and migration [323]. On the other hand, when SHH is associated with these activated/apoptotic T-cell derived MPs, they induced cell differentiation [324]. Also, these SHH carrying activated/ apoptotic T-lymphocyte derived MPs have been shown to correct angiotensin-II mediated hypertension and endothelial dysfunction in mice aorta by increasing expression of enzymes linked with NO production and by decreasing ROS production [325]. Moreover, these SHH containing MPs play role in regulating multiple pathway of angiogenesis through production of pro-angiogenic factors and upregulation of cell

adhesion proteins and NO pathways [243, 326]. Since SHH can activate RhoA/ROCK pathway, which has been implicated in mediating endothelial dysfunction, potentially, these SHH containing MPs may mediate endothelial dysfunction via RhoA/ROCK pathway, which has not been explored yet. However, the role and mechanism of action of these SHH containing apoptotic T-cell derived MPs in mediating endothelial dysfunction via RhoA/ROCK pathway has not been explored. Contrasting effects of these apoptotic T-cell derived MPs display their complex behavior, which are probably due to differences in the signaling molecule they express for example presence and absence of SHH and different stimulation for their formation.

Therefore, the aim of the study was to first isolate and determine if the SHH ligands are expressed by MPs derived from apoptosis of human T-lymphoblastoid cell line (CEM-CM3) and second to test if these apoptotic T-cell derived MPs containing SHH mediate endothelial dysfunction by activating RhoA/ROCK pathway. This would help underpinning a new molecular mechanism governing the MP-induced endothelial dysfunction, which has been associated with many diseases that show vascular dysfunction including liver failure in sepsis.

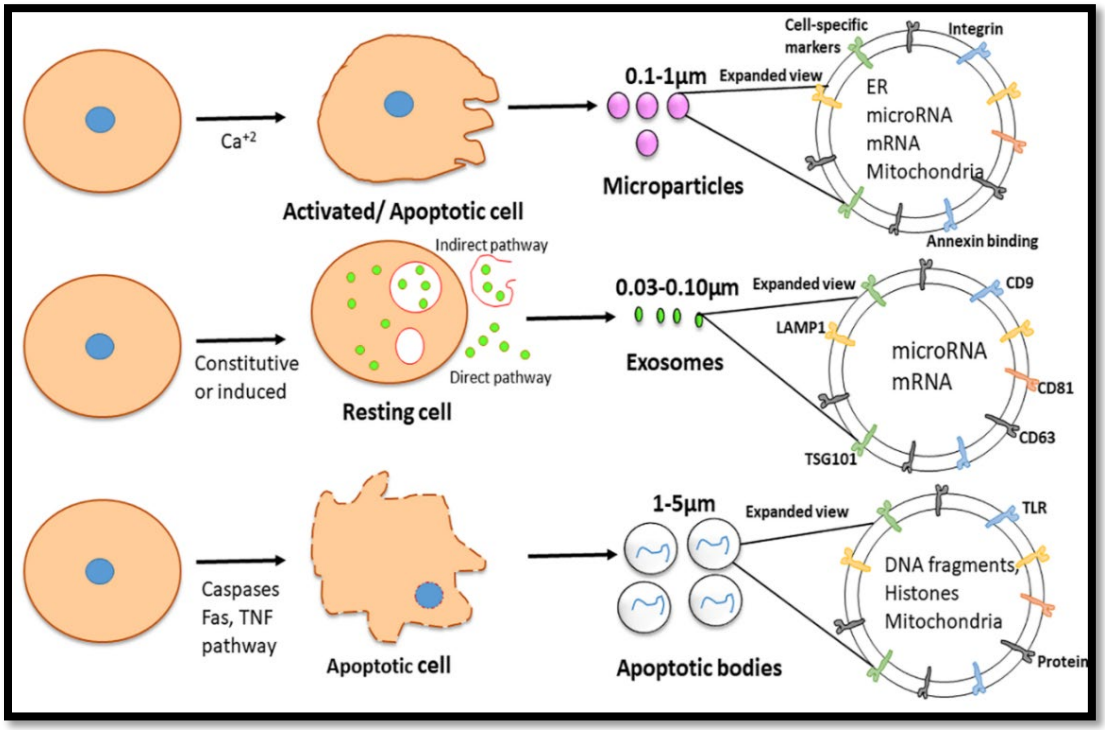

Figure 7: Schematic comparison of different types of extracellular vesicles: microparticles, exosomes and apoptotic bodies.

Microparticles (MPs) are 0.1-1µm in size. They are released from the outer surface of activated or apoptotic cell after selective incorporation of proteins, microRNA, lipids and mRNA. They contain various surface markers such as integrin, selectins and immunoglobulins whose expression level reflects the properties of the parental cells. Exosomes are sized in the approximate range of 0.03-0.10µm and are thus smaller than MPs and express surface markers such as Lysosomal associated membrane protein-1 (LAMP1), Tumor susceptibility gene 101 protein (TSG101) that play role in sorting of endocytic vesicles. Exosome can either be generated from direct pathway from the membrane or mature in the cytosol into late endosome and collect in multivesicular bodies (MVB) and become release via exocytosis. Apoptotic bodies are 1-5µm in size and are

released as cellular blebs as the cell undergoes apoptosis. Apoptotic bodies can be differentiated according to the size and the presence of fragmented DNA and histones.

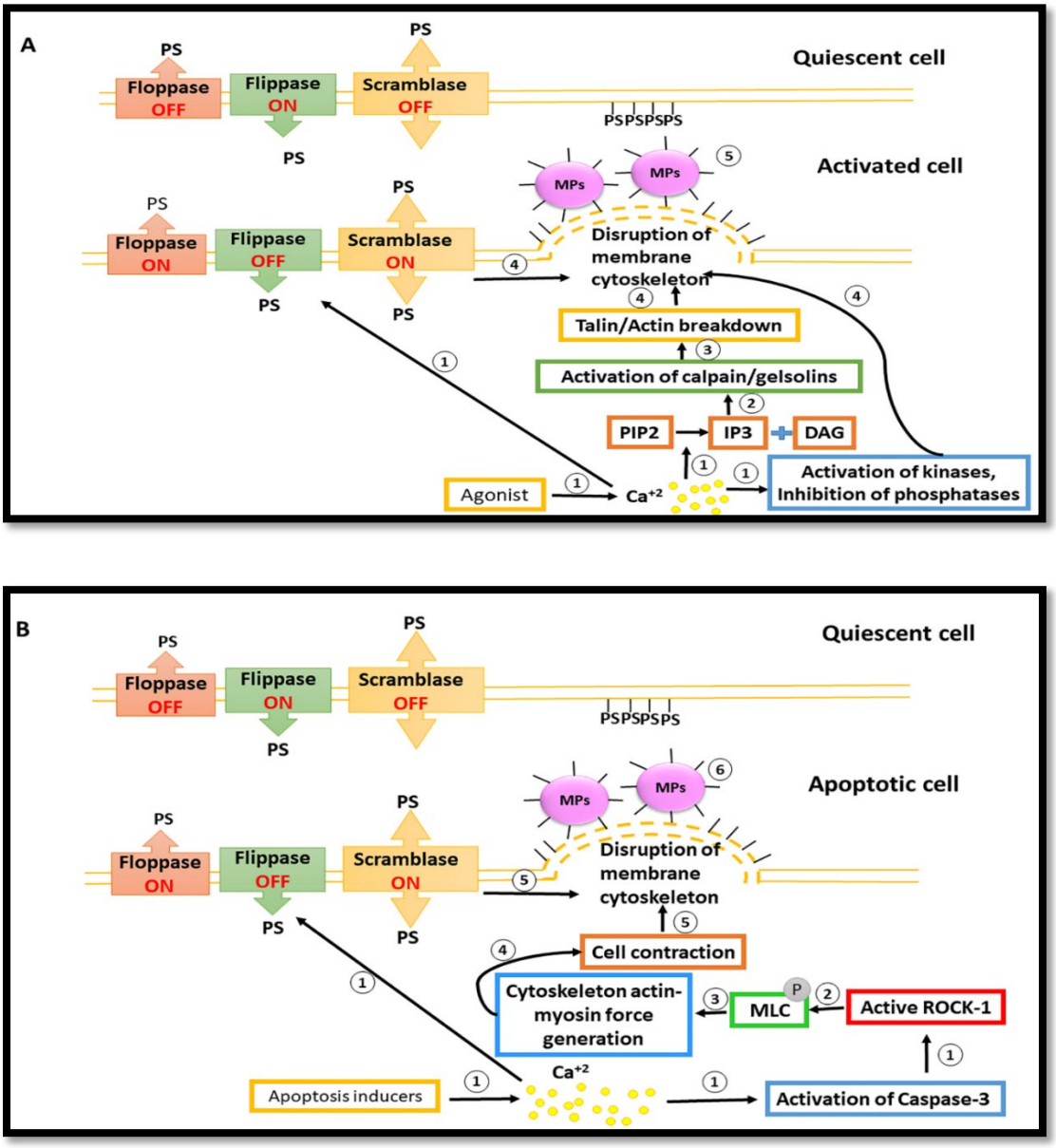

Figure 8: Schematic representation of general mechanisms involved in microparticles formation

Microparticles (MPs) formation requires increase in calcium (Ca^{+2}), changes in membrane lipid asymmetry and cytoskeletal protein reorganization. MPs are formed from plasma

membrane blebbing of various cells during activation and /or apoptosis. In a quiescent cell, only flippase is active, allowing phosphatidyl serine (PS) localization in the inner leaflet. (A) During cell activation (1) the rise in intracellular Ca^{+2} activate floppase and scramblase and inactivate flippase. This leads to loss of phospholipids asymmetry and PS localization on outer surface of membrane. Also, Ca^{+2} inhibits phosphatases, activates kinases and converts membrane lipid phosphoinositol 4, 5 bisphosphate (PIP2) into inositol 1, 4, 5-triphosphate (IP3) and diacylglycerol (DAG). (2) PIP2 acts as binding sites for calpain and gelsolins proteases such as calpain and gelsolins. (3) Calpain breakdowns talin, a membrane protein that along with actin and vinculin providing membrane stability while gelsolin sever actin into fragments (4) Loss in asymmetry and breakdown of membrane stability proteins leads to disruption of membrane cytoskeleton. (5) This remodeling of cytoskeleton leads to formation of membrane blebs and release of sub-micron MPs with PS exposed on their outer membrane. (B) During apoptosis, the rise in intracellular Ca^{+2} activate floppase and scramblase and inactivate flippase. This leads to loss of phospholipids asymmetry and PS localization on outer surface of membrane. Also, increased Ca^{+2} during apoptosis activates caspase-3 which in turn activate rho kinase 1 (ROCK-1) in a RhoA independent manner by cleaving the putative auto-phosphorylation/ auto-inhibitory domain of ROCK-1. (2) Cleaved ROCK-1 in turn activates myosin light chain kinase (MLCK) by phosphorylation. (3) MLCKs phosphorylate myosin light-chains (MLC) triggering interaction of myosin head with actin and enable myosin ATPases to generate force and stimulate the contractile activity of myosin. (5) This movement of actin-myosin and loss of phospholipid asymmetry causes detachment of the cytoskeleton from the

membrane. (6) Reorganization of the cytoskeleton leads to formation of membrane blebs and release of sub-micron MPs with PS exposed on their outer membrane.

2.1.Overall Hypothesis.

Our overall hypothesis that early in development of the inflammatory response to infection, both circulating immune cells (T-lymphocytes) and vascular endothelial cells are either stimulated by inflammatory mediators or are subjected to programmed cell death (apoptosis) to release SHH in sub-micron sized vesicles called microparticles. These MPs bind to their respective receptor on the hepatic sinusoidal endothelial cells lining the blood vessels of the liver and activate RhoA/ROCK pathway. Activation of ROCK pathway leads to impairment of endothelial cells functions and disruption of blood flow regulation. Some cells will get too much blood flow while others receive too little. The result is loss of liver function and eventual liver failure.

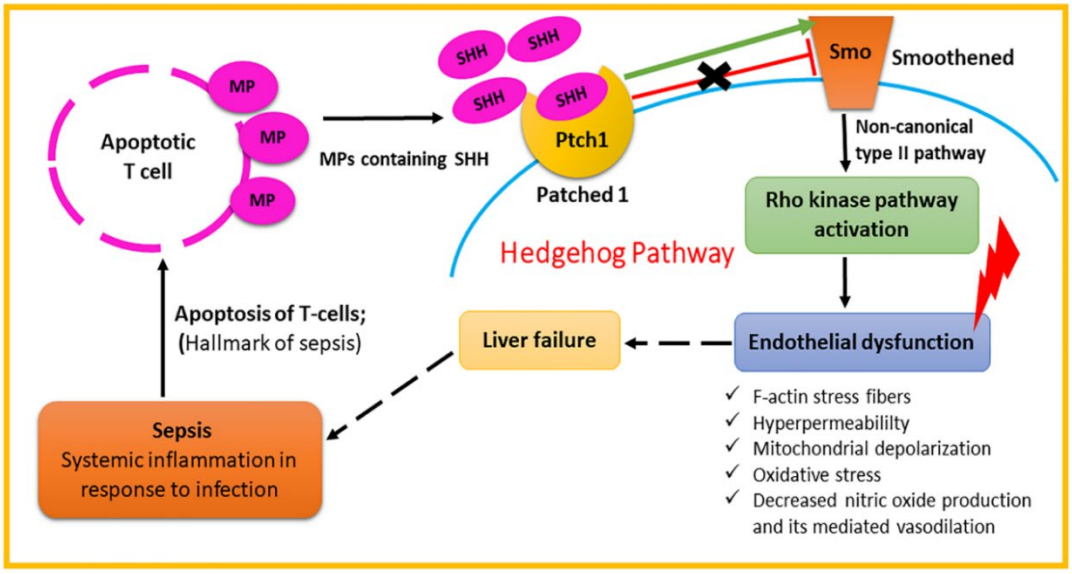

Figure 9: Overall rationale of the study.

Sepsis associated inflammation induces massive apoptosis of T-lymphocytes, a hallmark of sepsis. When a cell undergoes apoptosis, it releases sub-micron MPs from their cell

surface. These apoptotic T-cell derived MPs contain a protein called sonic hedgehog (SHH) that can bind to receptor patched 1 (Ptch1) of the hedgehog pathway and relieve Ptch1 mediated inhibition of smoothened (SMO). SMO is a downstream protein in this pathway that can activate rho kinase pathway non-canonically (RhoA/ROCK) to induce endothelial dysfunction (ED). RhoA/ROCK pathway mediate endothelial dysfunction by inducing F-actin stress fibers, mitochondrial depolarization, hypercontraction, increased production of ROS and decreased NO mediated vasodilation.

2.2.Experimental Design

Human microvascular endothelial cells (HMECs) were used as a study model to assess the effects and mechanism of action of apoptotic T-cell derived MPs on endothelial cell function. HMECs are immortalized cells obtained from human foreskins. The cells were maintained in MCDB-131 media without L-glutamine (Life technologies-Gibco; Grand Island, NY) supplemented 10ng/mL epidermal growth factor (EGF), 1µg/mL hydrocortisone, 10mM Glutamax and 10% heat inactivated fetal bovine serum (FBS). All the cultures were maintained at 37°C under an atmosphere of 5% $C0_2$ and 95% air. They were passaged and harvested using 0.25% Trypsin-0.53mM EDTA solution. The culture medium was replaced every two days. Most of the experiment consists of five treatment groups. (1) Untreated HMECs (negative control), (2) HMECs treated with 50µg protein/mL $^{SHH+}$MPs, (3) HMECs treated with 10µM Purmorphamine (Pur). Pur activates the HH signaling pathway by directly binding to smoothened (SMO), a downstream protein in this pathway. If we can duplicate the effect of MPs with Pur, this would suggest that SHH pathway does contribute to the MPs mediated ED (positive control). (4) HMECs treated with 10µM SANT-1. SANT-1 is potent SHH pathway antagonist that directly

inhibits by binding to SMO. If the effect of Pur on endothelial cells is abated by SANT-1, this would further ensure involvement of SHH pathway in mediating ED. (5) HMECs treated with 10µM ROCK inhibitor Y-27632 one hour prior to addition of MPs. Y-27632 is cell permeable, highly potent and selective inhibitor of ROCK. Y-27632 inhibits both ROCK-1 and ROCK-2 by competing with ATP for binding to the catalytic site. If inhibition of RhoA/ROCK abrogates or reduces the effect of $^{SHH+}$MPs on ECs, this would indicate that indeed these SHH containing microparticles derived from apoptotic T-cell induce endothelial dysfunction through activation of RhoA/ROCK pathway. The treatments were done for a period of 24 hours to simulate acute inflammation during which large degree of sepsis induced T-cell apoptosis is documented to occur.

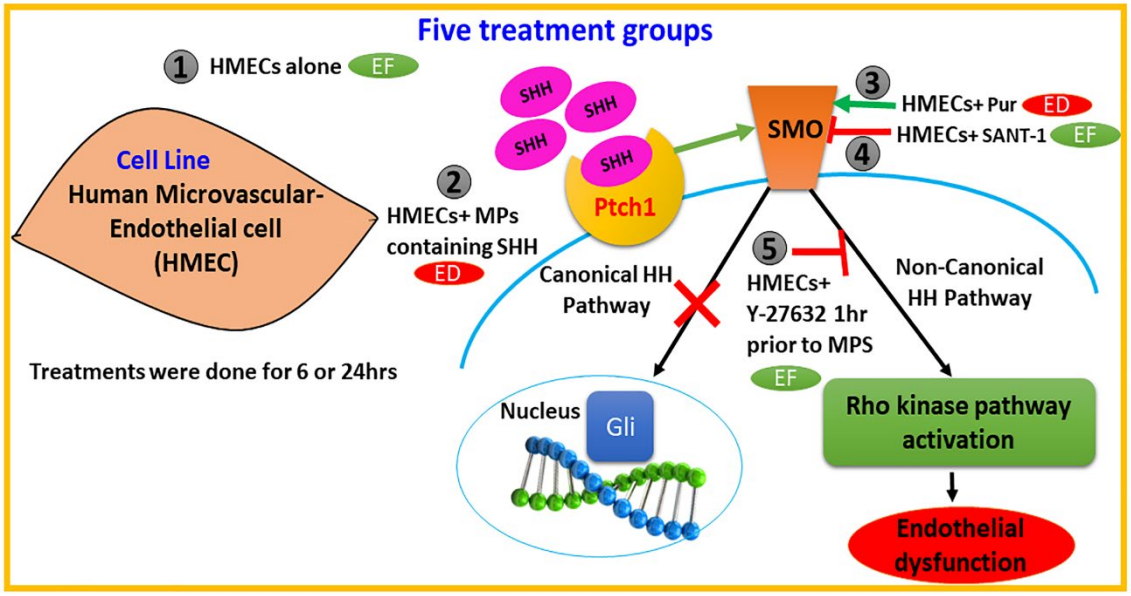

Figure 10: Schematic representation of experimental treatments

Human microvascular endothelial cells (HMECs) were used as study model. Overall hypothesis is that MPs containing sonic hedgehog protein (SHH) bind to patched receptor 1 (Ptch1) and relieve Ptch1 mediated inhibition of smoothened (SMO), a downstream

protein in this pathway. SMO can transduce signals via canonical Gli pathway and non-canonical RhoA/ROCK pathway. In this case, as RhoA/ROCK is known to induce endothelial dysfunction (ED), we hypothesize that SHH contained in MPs activate non-canonical RhoA/ROCK pathway to induce endothelial dysfunction. Most experiments have five treatment groups. (1) HMECs alone is the negative control group that is expected to show normal endothelial function (EF). (2) HMECs treated with MPs is expected to display ED as these MPs contain SHH which can non-canonically activate RhoA/ROCK pathway to induce ED. (3) HMECs treated with Pur will serve as positive control group since Pur can activate SHH pathway by inducing SMO activity. Pur treatment is also expected to induce ED as activated SMO can transduce signals via non-canonical RhoA/ROCK similar to that of MPs. (4) HMECs treated with ROCK inhibitor Y-27632 one hour prior to treatment with MPs is expected to display normal EF as ROCK pathway has already been inhibited in these cells so MPs containing SHH would unable to induce ED via RhoA/ROCK pathway. The treatments were done for either 6 or 24hrs.

2.3.Preliminary data

Human T-lymphoblastoid cell line (CEM-CM3) (American Type Culture Collection, Manassas, VA) were used for MPs isolation. After isolation, Morphological verification of MPs, expression of SHH in MPs and the percentage of MPs that express SHH was determined.

2.4.Methodology

2.4.1.Apoptotic T-cell derived Microparticles Isolation

MPs were isolated based on the protocol as described previously [323, 327]. T-cells were seeded at density of $3X10^6$ cells/mL and cultured in RPMI-1640 modified media

(American type culture collection, Manassas, VA) till a minimum of 200mL culture was obtained. Cells were then treated with phytohemagglutinin (PHA; 3µg/mL; Sigma-Aldrich, St. Louis, MO) for 72 hours to allow proliferation followed by treatment with phorbol-12-myristate-13-acetate (PMA; 20ng/mL; Sigma-Aldrich, St. Louis, MO) and actinomycin D (act D; 0.5µg/mL; Sigma-Aldrich, St. Louis, MO) to induce apoptosis. After 24hrs, the cells were centrifuged at 750g for 15 minutes at 4ºC to remove cell debris. The supernatant obtained was subjected to centrifugation at 1500g for 5 minutes at 4ºC to remove large debris. The latter supernatant was re-centrifuged at 14000g for 45 minutes at 4ºC. The pelleted MPs were re-suspended in 15mL phosphate buffer saline (PBS) and centrifuged again at 14000g for 45 minutes at 4ºC. The pellet was recovered in 2mL PBS. The µg protein/mL of MPs was determined by measuring MPs associated proteins using the Bradford assay (Micro BCA protein assay kit; Pierce Biotechnology; Rockford IL) with bovine serum albumin (BSA) as standard.

2.4.2.Microparticles Visualization

Morphology of MPs was detected by staining them with fluorescent Vybrant® DiI Cell labeling Solution (Molecular probes Inc; Eugene, OR). DiI is a lipophilic membrane stain that diffuses laterally to stain entire cell. It is weakly fluorescent until incorporated into membranes. Briefly, 1µl of Vybrant® DiI Cell-Labeling solution was added to 100µl of MPs suspension. The mixture was incubated at 37ºC for 10 minutes. Images were taken using a 60X objective of Olympus IX70 microscope in phase contrast mode.

2.4.3.Western blotting

SHH expression in MPs was determined by western blotting. Protein concentration of MPs was determined by Micro BCA protein assay kit (Pierce Biotechnology; Rockford IL) and

the volume of protein loading in western blot was adjusted accordingly. Zero, seven point five, fifteen, thirty and sixty micrograms of MPs protein sample were boiled for 7 minutes at 95°C in a 1:1 mixture of 2-mercaptoethanol and Laemmli loading buffer (1:20 ratio respectively) prior to separation on a 10% SDS PAGE gel. Standard western blotting procedures were then followed as previously described. Increasing concentration of MPs protein separated on SDS PAGE were transferred to PVDF membrane (Immubilon-FL membrane, Millipore Sigma) by electro blotting. Blots were then probed with primary mouse monoclonal antibodies for sonic hedgehog (Sigma-Aldrich St. Louis, MO) overnight at 4°C followed by secondary goat anti-mouse IgG HRP (Thermo Fischer Scientific; Rockford, IL) for one hour at room temperature (RT), at a dilution of 1:200 and 1:2000 respectively. A monoclonal mouse anti-human βactin antibody (Sigma-Aldrich; St. Louis, MO) was used at 1:5000 dilution for visualization of protein gel loading**.**

2.4.4.Flow cytometry

SHH expression in MPs was quantified by using BD fortessa flow cytometer. 1X 10^6 /mL MPs were fixed in 2% formaldehyde solution followed by permeabilization using 0.1% saponin. Permeabilized MPs were labelled with 100µL of 1:200 dilution of primary mouse anti-SHH antibody (Sigma-Aldrich, St. Louis, MO) in 0.5% BSA for 1 hour at RT followed by centrifugation at 400g for 5 minutes. The supernatant was replaced by 0.5% Bovine serum albumin (BSA) and washed by centrifugation at 400g for 5 minutes. The pelleted MPs were then labelled with 100µL of 1:5000 dilution of secondary fluorescein affnipure Donkey-anti-mouse IgG (Jackson Immune Research Laboratories; West grove, PA) in 0.5% BSA for 30 minutes at RT followed by wash with 0.5% BSA using centrifugation at 400g for 5 minutes. The labelled MPs were re-suspended in 0.5mL 1X DPBS for analysis

on flow cytometer. To locate MPs, a gate was established by setting parameters of forward and side scatter channels using size calibrated 0.2µm and 1µm yellow and red fluorescent latex beads respectively (Figure 13A). The vesicles within this gate ranging from 0.2-1µm were identified as MPs (Figure 13B). Further, SHH expression was analyzed by measuring FITC fluorescence. The data is represented as histogram that plots the intensity of FITC expression on X-axis versus number of events on the Y-axis. 10,000 events were acquired for each run with n=7.

2.4.5.Apoptotic T-cell derived Microparticles Dosage Determination

MP dose was determined using induction of nitric oxide production in RAW 264.7 cells. Macrophages under unstressed conditions do not express nitric oxide synthase; however, in response to inflammatory stresses upregulate iNOS/ NOS2 making them a sensitive indicator of proinflammatory signals. Briefly, cells were grown to 80-90% confluence in 24-wells plates. Following addition of MP (1µg/ml-500µg/ml), cells were incubated for 24 hours at 37ºC. A nitrite standard curve was produced using 0.1M sodium nitrite. Then, 100µl of standard curve samples and supernatant from each well of MP-treated cells was pipetted into a 96-well plate in duplicate. Next, 100µl of Griess Reagent (0.5g sulfanilamide + 0.05g N-ethyl-maleimide in 6% (v/v) phosphoric acid) was added to each well followed by a 30-minute RT incubation period in the absence of light. The absorbance at 540nm was read using a plate reader. The concentration of MP that provided a consistent increase in nitrite production was marked as the optimal MP dose to use in all HH pathway experiments. This dose was 50ug protein/mL (data not shown).

2.5.Results

2.5.1.Detection of Apoptotic T-cell derived Microparticles

MP generated from apoptotic CEM T-lymphocytes are shown below in Figure 11. These image shows many small, highly concentrated spots, each being either single MP/ multiple MPs.

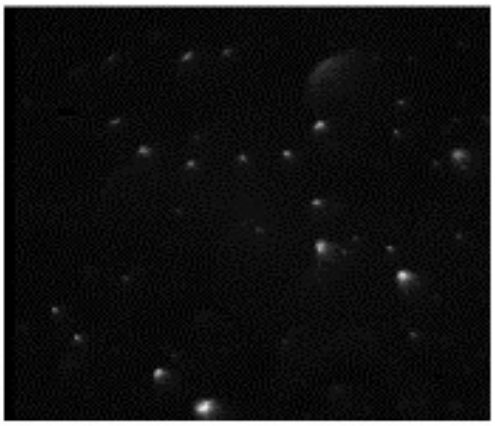

Figure 11: Visualization of apoptotic T-cell derived MPs using DiI fluorescent stain 100µL MPs purified from apoptotic T-cell supernatant were labelled with 5µM DiI fluorescent stain and observed by phase contrast microscope. Each dot in the picture represent MPs.

2.5.2.Apoptotic T-Cell derived Microparticles express Sonic Hedgehog.

As a first step toward determining if SHH protein is expressed in apoptotic T-cell derived MPs, we performed western blot. Increasing concentrations of MPs from 0 to 60µg protein/mL (Fig.12) showed a consequent increase in 45kDa SHH protein, indicating these MPs do express SHH. β-actin was used as a load control.

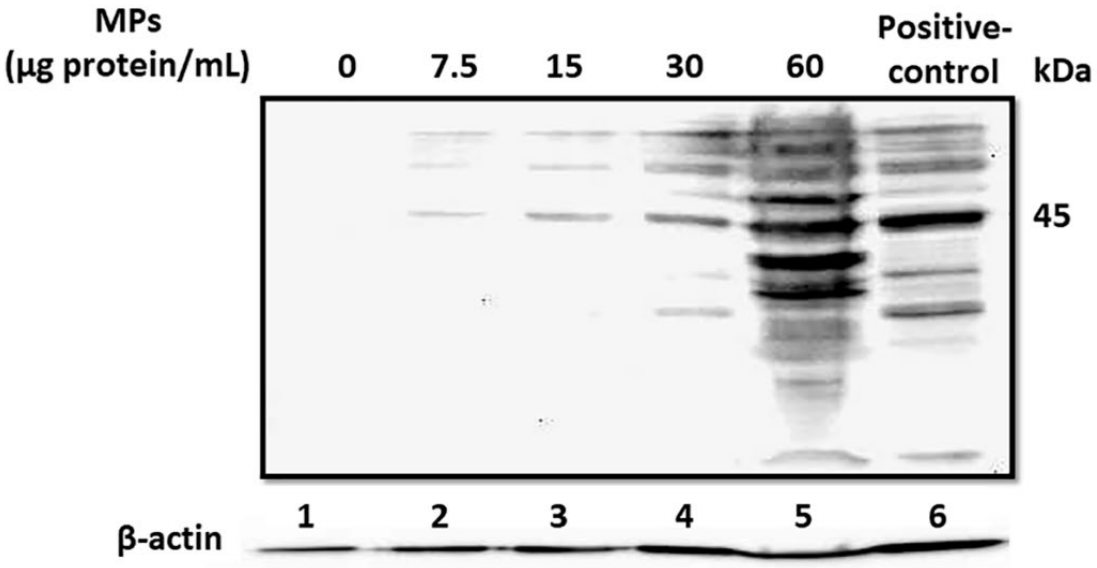

Figure 12: Western blot depicting sonic hedgehog expression by apoptotic T-cell derived microparticles.

MPs generated by PHA/PMA/act D induced apoptosis of CEM T-cells were loaded at concentrations of 7.5µg protein/mL (lane 2), 15µg protein /mL (lane 3) 30µg protein /mL (lane 4) 60µg protein /mL (lane 5). Human endothelial lysate was used as positive control (lane 6). β-actin control was included. There was increase in 45kDa SHH protein with increase in concentration MPs.

2.5.3. 80% Apoptotic T-cells derived Microparticles express Sonic Hedgehog.

FACS analysis using primary SHH antibody and secondary FITC antibody revealed that 80.8% MPs do express SHH (Figure 13D) as compared to control MPs (Figure 13C) that accounted for 19.2 % of non-specific fluorescence. The optimal MP dose was determined to be 50µg/mL protein.

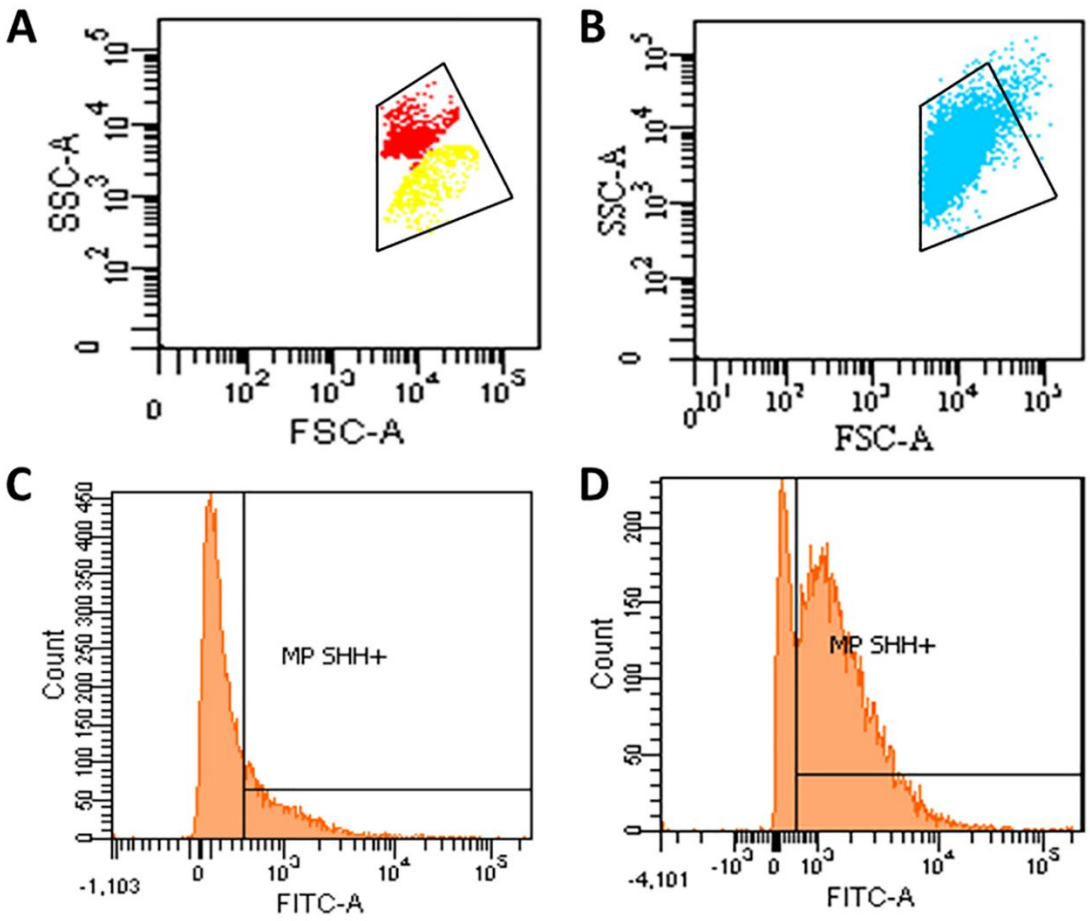

Figure 13: Flow cytometry analysis depicting percentage of apoptotic T-cell derived microparticles that express sonic hedgehog protein.

(A) Sizing of MPs was based on fluorescent latex beads of 0.2µm yellow and 1µm Red. (B). Representative forward scatter (FSC-A) vs side scatter (SSC-A) dot blot showing MPs (blue) that were identified based on the gate ranging from 0.2µm to 1µm. (C) Representative flow cytometry fluorescence histogram of control MPs labelled with only secondary donkey anti-mouse FITC antibody. 19.2% MPs showed non-specific fluorescence. (D) Representative flow cytometry fluorescence histogram of MPs labelled

with primary mouse anti-SHH antibody and secondary donkey anti-mouse FITC antibody**.** 80.8% MPs showed SHH specific fluorescence.

2.6.Discussion

Our preliminary result confirm that MPs obtained from apoptosis of CEM T-cells do contain SHH protein. Indeed 80% of these apoptotic T-cell derived MPs express SHH. This confirmation allowed us to test the effect of SHH containing MPs on functions of endothelial cells.

CHAPTER 3: EFFECT OF SONIC HEDGEHOG CONTAINING APOPTOTIC T-CELL DERIVED MICROPARTICLES ON ENDOTHELIAL BARRIER FUNCTION MEDIATED BY ACTIVATION OF RHO KINASE PATHWAY

3.1.Background

Endothelial cells lining the inner surface of the blood vessel partitions the underlying tissue from blood components. In a healthy state, the close apposition and alignment of endothelial cells allow it to function as a semi-permeable barrier that controls blood-tissue exchange of plasma fluids, nutrients, proteins and metabolic byproducts while preventing the pathogens or harmful materials in the circulation from entering into the tissues [328]. This restrictive barrier establishes the trans-endothelial oncotic pressure gradient that is required to maintain tissue fluid homeostasis and host defense [329]. Appropriate regulation of the microvascular fluid hydrodynamics and endothelial barrier function is vital to support normal tissue viability and physiological functions of various organ [330].

Cell-cell junctional complexes play important role in preserving and regulating endothelial barrier function. Endothelial cells have several types of junctions including adherens junctions, gap junctions and tight junctions. Adherens junctions control paracellular permeability to leukocytes and solutes, gap junctions allow passage of small molecular weight solutes between neighboring cells while, the tight junctions provide barrier within membranes by regulating paracellular permeability and cell polarity [331] . But, these cell- cell junctional complexes do not work in isolation. They are linked to cytoskeleton that ensures that the junctional forces and cell shape are coordinated. Furthermore, remodeling of cytoskeleton itself leads to rapid changes in the cell shape which in turn introduce gaps in the endothelial monolayer, causing microvascular leak

[153]. Actin filaments are critical components of the cytoskeleton whose tethering to the adhesion complexes is essential in maintaining a functional endothelial barrier [332]. In quiescent endothelium, actin forms a cortical rim just underneath the membrane skeleton that in association with several proteins regulate polymerization of actin filaments and tethering of actin to membrane proteins. But, agents known to increase microvascular leak such as thrombin, histamine, inflammatory cytokines disrupt the cortical actin and instigate formation of contractile actin-containing stress fibers [153]. Stress fibers are short cross-linked bundles of F-actin that are typically divided into ventral stress fibers, dorsal stress fibers and transverse arc and play role in cell contraction. These actin stress fibers distribute throughout the interior of cell creating a pulling centripetal tension which along with the reorganization of the adhesion complex architecture, mediate retraction of cell-cell borders into discernible gaps [333]. This actin reorganization from its cortical organization into stress fibers is a principal component of endothelial response to inflammation [334]. But, ongoing inflammation as in sepsis shifts the balance of actin dynamics from cortical actin cytoskeleton to destabilizing contractile stress fibers, thus disrupting endothelial barrier integrity and increased vascular permeability [335]. Aberrant actin dynamics also contribute to excessive immune cell recruitment into the sites of inflamed tissue area [336].

The pathophysiology of endothelial barrier dysfunction is characterized by hyperpermeability of blood vessels that is clinically manifested as protein rich edema into tissues, a hallmark of inflammation [337]. This vascular leakage disturbs the fluid homeostasis, increases migration of blood cells and impairs tissue oxygenation. Also, there is large production of ROS, proteases, amines and lipid mediators, rendering an exaggerated host defense that attacks normal cells and tissues [338]. The pathological

consequence of these otherwise bona fide events is tissue injury and eventually organ failure [338]. Endothelial barrier dysfunction and microvascular leak have been shown to critically contribute to the pathogenesis of organ failure in sepsis and of sepsis related complications including liver injury [339-341]. Sepsis patients show microvascular leak that is manifested as tissue and organ edema and hypotension [342].

Increased vascular permeability is essential for inflammatory responses but can also contribute to development of pathological conditions such as sepsis [343]. There is strong evidence for involvement of RhoA/ROCK pathway in regulation of actin dynamics and vascular hyperpermeability after cecal ligation and puncture (CLP) induced sepsis and liposaccharide (LPS)-induced inflammation [344]. ROCK destabilizes the endothelial barrier by inactivating actin depolymerization factor. This induces actin remodeling in a way that it favors formation of contractile stress fibers over cortical actin arrangement leading to compromise in endothelial barrier and microvascular leak. [151, 345]. However; which signaling molecule/s activate RhoA/ROCK to mediate endothelial barrier dysfunction in sepsis is not known. In this study, we hypothesized that the apoptotic T-cell derived MPs containing SHH activate RhoA/ROCK pathway to mediate endothelial barrier dysfunction by inducing F-actin stress fibers in cytoskeleton and by making endothelial monolayer leaky.

3.2.Materials and Methods

3.2.1.HMEC Cell Culture

Human microvascular endothelial cells (HMECs) were a kind gift from Dr. Vijay Kumar Kalra (University of Southern California, Los Angeles, California). HMECs are immortalized cells obtained from human foreskins. The cells were maintained in MCDB-

131 growth media without L-glutamine (Thermo Fisher Scientific; Rockford, IL) supplemented 10ng/mL endothelial growth factor (EGF), 1µg/mL hydrocortisone, 10mM GlutaMAX (Life Technologies, Carlsbad, CA), 10% heat-inactivated fetal bovine serum (FBS) (Serum Source, Charlotte, North Carolina) and 10% penicillin-streptomycin (Thermo Fisher, Waltham, MA). Cells were maintained in T-75 flask at 37°C under an atmosphere of 5% $C0_2$ and 95% air. Cells were harvested using 0.25% Trypsin-0.53mM EDTA solution and seeded on collagen-coated wells, allowing them to attach overnight. Cells were quiesced the following day in 0.1% FBS medium overnight. The next day, medium was refreshed with 1% FBS medium and treatments were added for either 6 or 24hrs.

3.2.2.Reagents

Texas Red ™-X Phalloidin, 4', 6-Diamidino-2-Phenylindole dihydrochloride; DAPI were obtained from Thermo Fisher Scientific (Rockford, IL). Purmorphamine (Pur), Fluorescein-isothiocyanate (FITC) labelled dextran-70kDa and Dulbecco's phosphate buffered saline (DPBS) was obtained from Sigma-Aldrich (St. Louis, MO), Y-27632 (R)-(+)-trans-N-(4-Pyridyl)-4-(1-Aminoethyl) cyclohexanecarboxamide dihydrochloride monohydrate was acquired from Cayman chemical (Ann harbor, MI).

3.2.3.Treatments

Each experiment consists of four treatment groups. (1) Untreated HMECs (negative control), (2) HMECs treated with 50µg protein/mL $^{SHH+}$MPs, (3) HMECs treated with 10µM Pur. Pur activates the hedgehog signaling pathway by directly binding to smoothened (SMO), a downstream protein in this pathway. If we can duplicate the effect of MPs with Pur, this would suggest that SHH pathway does contribute to the MPs

mediated endothelial dysfunction (positive control). (4) HMECs treated with 10μM Y-27632 one hour prior to treatment addition of MPs. Y-27632 is cell permeable, highly potent and selective inhibitor of ROCK. Y-27632 inhibits both ROCK-1 and ROCK-2 by competing with ATP for binding to the catalytic site. If inhibition of ROCK abrogates or reduces the effect of $^{SHH+}$MPs on endothelial cells, this would indicate that indeed these SHH containing microparticles derived from apoptotic T-cell induce endothelial dysfunction through activation of RhoA/ROCK pathway. The treatments were done for a period of 24 hours to simulate acute inflammation during which large degree of sepsis induced T-cell apoptosis is documented to occur.

3.2.4 Determination of Changes in Cell Morphology.

HMECs were grown on collagen coated 12 well plate for a day. Then, cells were quiesced overnight in 0.1% FBS MCBD-131 media. The next day, media was refreshed with 1% FBS MCDB-131 media and the cells were treated with 50μg protein/mL MPs or 10μM Pur and /or 10μM ROCK inhibitor Y-27632 one hour prior to addition of MPs. HMECs alone was used as control. After 24hrs, the cells were imaged using 20X objective lens of an inverted microscope (Olympus1X70) in a phase contrast mode for n=3 experiments.

3.2.5. Determination of Changes in Cytoskeleton Morphology

To detect the F-actin stress fibers, Texas Red-X phalloidin fluorescent staining was used. Phalloidin is F-actin specific bicyclic peptide that belongs to family of toxins isolated form deadly *Amanita phalloides*. HMEC monolayers were prepared on collagen coated covered glass chamber slide (Lab-Tek). HMECs were treated with 50μg protein/mL MPs or 10μM Pur and /or 10μM ROCK inhibitor Y-27632 one hour prior to addition of MPs for 24hrs. HMECs alone was used as control. After treatments, the HMECs monolayer were fixed

with 4% formaldehyde followed by permeabilization using 0.25% triton-X 100. Both fixing and permeabilization were done for 10 minutes at room temperature. Cells were washed three times with ice-cold Dulbecco's phosphate-buffered saline 1X (DPBS 1X) between fixing and permeabilizing steps. Cells were then loaded with 200µL of 1µM solution per coverslip Texas red-X phalloidin and stained for 20 minutes at 37°C under an atmosphere of 5% $C0_2$ and 95% air**.** Excess phalloidin was removed by washing with PBS twice followed by treatment with DAPI (300nM for 1min) for nuclear counterstaining. Excess DAPI was removed from slides by washing with 1X DPBS and air dried. Microscopic slides were viewed by using the 40X UPlanApo 0.85 objective lens of Olympus FV500 laser scanning confocal microscope. Laser excitation of 405nm and 610nm were used in rapid succession and F-actin in red (Texas-Red X Phalloidin staining) and nucleus in blue (DAPI staining) were visualized.

3.2.6. Determination of Endothelial Monolayer Permeability with Transwell FITC-Dextran Assay.

Increased permeability across the endothelial monolayer was measured using polyethylene terephthalate (PET) track-etched transparent, low pore density transwell inserts (12mm diameter, 0.4µm pore size; Corning Costar; Pittston, PA). Subsequently, the lower chamber of the 12 well transwell chambers was filled with 1.5mL MCDB-131 media. HMECs were seeded in the upper chamber of the insert at density of $1X10^5$ cells in 500µL MCDB-131 media per well. They were grown to confluence for 24-48 h. MCDB-131 media in upper chamber was replaced with 500µL Dulbecco's Modified Eagle Medium (DMEM) media containing FITC labelled dextran (70kDa, 1mg/mL; Sigma- Aldrich, St. Louis, MO). Cells were treated as indicated with MPs, or Pur or Y-27632 with MPs in DMEM media with

FITC labelled dextran. At 6 and 24hrs, 100µL samples were taken from lower compartment for each treatment and control. The sample taken at 6hrs was replaced by 100µL DMEM in the lower chamber. FITC-fluorescence was measured with multi-mode microplate reader (Synergy^{TM} HT x; BioTek instruments; Winooski, VT) equipped with excitation filter 485nm and emission filter at 535nm. The data is expressed FITC-dextran fluorescence units.

3.2.7.Statistical Analysis

All data are presented as means ± standard error of the mean (SEM). Statistical analysis was performed using GraphPad Prism software (San Diego, California). Statistical significance was assessed by one-way analysis of variance (ANOVA) with independent Dunnett's *post hoc* test was used when statistical differences were detected. Statistical significance was set at $p < 0.05$.

3.3.Results

3.3.1. Apoptotic T-Cell Derived Microparticles Containing Sonic Hedgehog Modulate Morphology of Endothelial Cells via Rho Kinase Pathway.

HMECs alone (Figure 14 A) demonstrated the typical cobble stone appearance. Treatment of HMECs with MPs (Figure 14 B) or Pur (Figure 14 C) altered cell shape from cobblestone to round, causing cells to detach from surface. Rho kinase inhibition by Y-27632 before addition of MPs (Figure 14 D) prevented rounding of the cells. Collectively, this result shows the SHH containing apoptotic T-cell derived MPs alter shape of the endothelial cells via activation of rho kinase pathway.

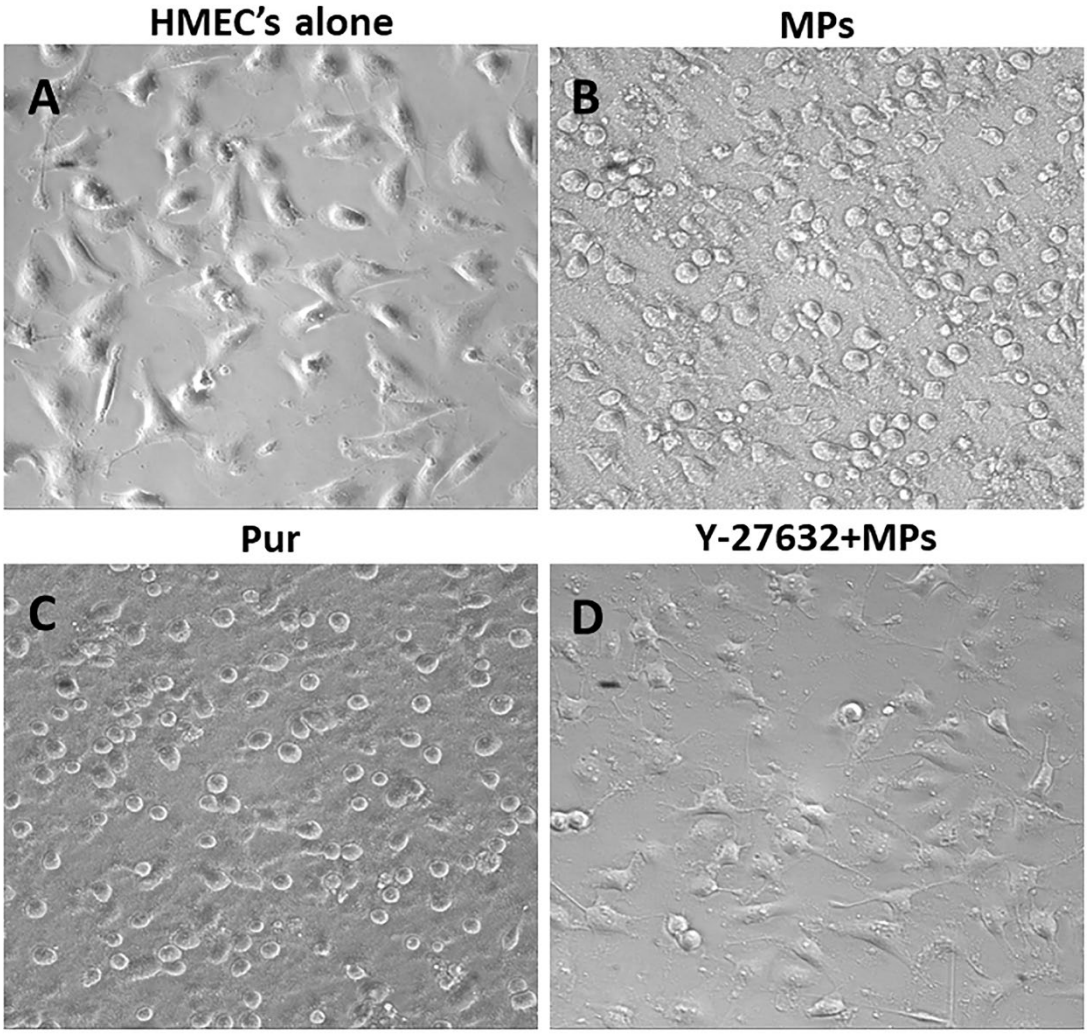

Figure 14: Apoptotic T-cell derived MPs containing sonic hedgehog modulate morphology of endothelial cells by rho kinase pathway

HMECs were treated with 50µg protein/mL MPs (B) or 10µM Pur (C) and or 10µM Y-27632 one hour prior to addition of MPs (D) for 24hrs. HMECs alone were the control (A). After 24hrs, the cells were imaged using 20X objective of Olympus 1X70 inverted microscope with phase contrast. HMECs alone demonstrated the typical cobble stone appearance of endothelial cells. While treatment with MPs or Pur, lead to rounding of cells. Inhibition of rho kinase pathway before addition of MPs prevented the rounding of cells as

indicated by cobble stone appearance. The white speckles in MPs and Y-27632 +MPs groups are the MPs. The images are representative of n=3 experiments.

3.3.2. Apoptotic T-cell derived microparticles containing sonic hedgehog modulate endothelial cytoskeleton by inducing contractile F-actin stress fibers via rho kinase pathway.

Inflammatory stress, such as sepsis activate Rho and its associated kinase ROCK to induce endothelial barrier dysfunction by increasing F-actin stress fiber related contractile tension [151-153, 346]. Therefore, we assessed F-actin stress fiber formation in endothelial cytoskeleton induced by SHH containing apoptotic T-cell derived MPs via activation of RhoA/ROCK. To locate these stress fibers, we loaded the cells with F-actin specific phalloidin Texas red stain (1μM) and DAPI (300nM). F-actin (red), nucleus (blue) was imaged using 60X objective lens of Olympus FV500 laser scanning confocal microscope. In untreated HMECs (Figure 15A), actin was mainly located at the periphery of the cells, displaying the principal cortical rim arrangement indicated by white arrow. Treatment of HMECs with MPs (Figure 15B) or Pur (Figure 15C) respectively for 24hrs caused the actin network to reorganize into F-actin stress fibers as indicated by yellow arrows. Rho kinase pathway activation was assessed by using ROCK inhibitor Y-27632 one hour prior to addition of MPs (Figure 15D). Importantly, MPs-evoked stress fibers were prevented in presence of Y-27632 as indicated by white arrow pointing the cortical rim arrangement. Taken together, this data demonstrates that SHH containing MPs derived from apoptotic T-cells induce F-actin stress fibers in endothelial cytoskeleton via activation of rho kinase pathway.

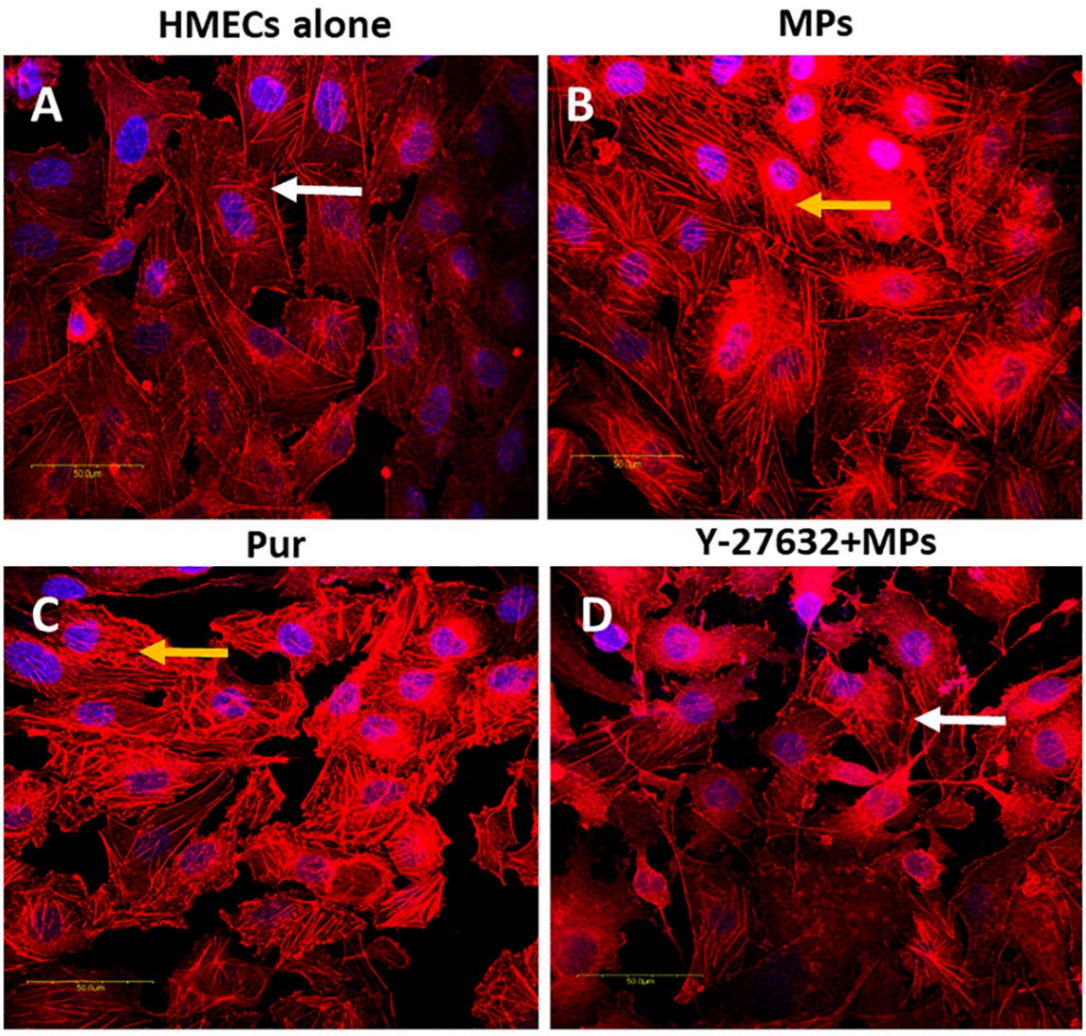

Figure 15: Apoptotic T-cells derived microparticles containing sonic hedgehog induce contractile f-actin stress fibers in endothelial cells via rho kinase pathway

HMECs were treated with 50µg protein/mL MPs (B) or 10µM Pur (C) and or 10µM Y-27632 one hour prior to addition of MPs (D) for 24hrs. HMECs alone were the control (A). Cells were then fixed, permeablized and loaded with F-actin specific Texas Red™-X Phalloidin and DAPI. F-actin stress fibers and nucleus were localization by visualizing fluorescence at red (Texas Red™-X Phalloidin) and blue (DAPI) channels.HMECs alone displayed typical cortical rim arrangement of actin (white arrow). Treatment of HMECs

with MPs for 24hrs caused the actin network to reorganize into stress fibers (yellow arrow). Treatment of HMECs with Pur for 24hrs also reorganized cortical actin network into F-actin stress fibers (yellow arrow). Inhibition of ROCK with Y-27632 one hour prior to addition of MPs restored the cortical rim arrangement of actin (white arrow). Images are representative of three separate experiments.

3.3.3. Apoptotic T-Cell Derived Microparticles Containing Sonic Hedgehog Increase Permeability across Endothelial Monolayer via Rho Kinase Pathway

Increased endothelial permeability is a hall mark of liver injury in sepsis [78]. To examine if the SHH containing MPs increase permeability of endothelial cells via rho kinase pathway, we assessed the flux of 70kDa FITC labelled dextran across the endothelial monolayer. HMECs were cultured on upper chamber of transwell inserts for 2 days to form a monolayer. The media in the upper chamber was replaced by DMEM media containing 1mg/mL FITC-labelled dextran followed by addition of respective treatments. Thereafter, the permeability was determined by measuring the fluorescence of FITC-dextran that percolated into lower chamber after 24hrs, using a plate reader. HMEC monolayers treated with MPs displayed a significantly higher fluorescence (flux) of FITC-dextran (+71.5%; ***=$p<0.001$) as compared to control, indicating MPs increase permeability across endothelial monolayers. Stimulation of SHH pathway by addition of Pur to HMEC monolayer also significantly increased permeability (+86.81%; ***=$p<0.001$) similar to that observed with $^{SHH+}$MPs. The MPs-induced hyperpermeability was almost abolished when HMECs were incubated in presence of ROCK inhibitor Y-27632 one hour prior to addition of MPs, as indicated by relative flux of FITC-dextran that was not significantly different from control. Altogether, this result shows that SHH containing apoptotic T-cell

derived MPs augment endothelial monolayer permeability via activation of rho kinase pathway (Figure 16).

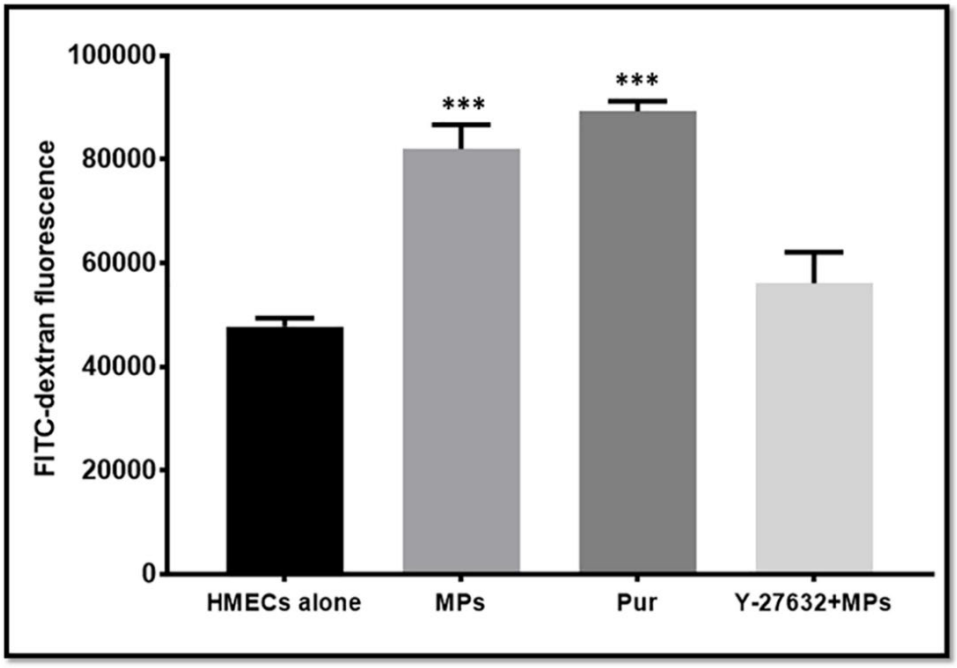

Figure 16: Apoptotic T-cell derived MPs containing SHH increase permeability across endothelial monolayer via activation of rho kinase pathway.

HMECs monolayer were grown on upper chamber of transwell insert for 2 days. MCDB-131 media was replaced with DMEM media containing 1mg/mL FITC-dextran (70kDA) followed by treatment with 50µg protein/mL MPs or 10µM Pur and or 10µM Y-27632 one hour prior to addition of MPs for 24hrs. Thereafter, permeability was assessed based on amount of FITC -dextran that percolated through endothelial monolayer into lower chamber. After 24hrs, 100uL samples were drawn from lower chamber and FITC-dextran fluorescence was detected using a plate reader. FITC-dextran fluorescence was significantly higher in HMEC's treated with MPs as compared to HMECs alone. Treatment with Pur to HMECs duplicated the effect shown by MPs. Addition of MPs to HMECs after

inhibition of rho kinase with Y-27632 displayed FITC dextran flux that is not significantly different from control and therefore prevented MPs induced vascular leakage. Statistical significance was obtained by performing one-way ANOVA with post hoc Dunnett's test. ***=P<0.001; MPs, Pur vs control. Control vs Y-27632+ MPs is not significantly different. All data are mean ± s.e.m. of three experiments.

3.4.Discussion

Appropriate regulation of the microvascular fluid hydrodynamics and endothelial barrier function is vital to support normal tissue viability and physiological functions of various organ as its establishes gradients between tissues [330]. A hallmark of impaired homeostasis in sepsis is barrier incompetency. Sepsis is associated with decreased flow velocity in the microcirculation and reduced density of perfused capillaries [340, 347]. One of the key events that induces alteration in microcirculation is the breakdown of endothelial barrier and its associated leakage that manifest as protein edema. Severe tissue edema increases the diffusion distance for oxygen and compresses the post capillary venules which further impairs the microcirculatory flow [153]. Alteration in microcirculation is characterized by some capillaries being hypoperfused while other exhibit normal or even abnormally high flow [348, 349]. This heterogeneous perfusion of microcirculatory units disturbs the tissue oxygenation and leads to hypoxic areas even in the presence of preserved total blood flow to organ [350]. Altered microvascular perfusion as a result of barrier failure is seen in patients with sepsis and has been linked to organ failure and mortality [53].

The endothelial cells are highly exposed to circulating MPs and are among first cells to respond to MP-mediated signaling. Platelet derived MPs isolated from patients

suffering from multiple sclerosis have been shown to strongly disrupt the endothelial barrier of human umbilical vein endothelial cell (HUVEC) in vitro than those derived from healthy controls [351]. Microvascular endothelial cells derived MPs produced under glucose deprivation also contribute to blood brain barrier disruption in vitro and in vivo. However; the potential role of apoptotic T-cell derived MPs in mediating endothelial barrier dysfunction in sepsis or other diseases has not been explored yet. Since T-cell undergo massive apoptosis in sepsis and produce MPs from their surface, we hypothesized that these MPs containing SHH mediate endothelial barrier dysfunction by inducing F-actin stress fibers and by increasing permeability across the endothelial monolayer. To visualize F-actin stress fibers, we imaged phalloidin staining fluorescence using confocal microscopy. We, for the first time demonstrate that apoptotic T-cell derived MPs containing SHH induce F-actin stress fibers. So, F-actin specific phalloidin stained all the polymerized actin filaments in the endothelial cells. The resulting image was bright red phalloidin fluorescence staining the dorsal, transverse and ventral stress fibers filaments throughout the interior of the endothelial cells along with the cortical actin around the rim. To test if apoptotic T-cell derived MPs increased endothelial permeability, we measured the flux of FITC labelled dextran across the endothelial monolayer using plate reader. Our results show that apoptotic T-cell derived MPs induce endothelial hyperpermeability. So, more FITC-dextran percolated into the lower chamber of the transwell insert. Therefore, 71.5% more fluorescence of FITC-dextran was measured in HMECs treated with MPs than in control HMECs. We also elucidated the mechanism by which these SHH containing apoptotic T-cell derived MPs induce endothelial barrier dysfunction.

Imbalance between the adhesive force of intracellular junctions and contractile force of actomyosin results in endothelial barrier dysfunction [352]. Actin is dynamically regulated by the members of Rho family GTPase notably Rac, cell division control protein 42 homolog (Cdc42) and RhoA. Signaling through Rac1 and cdc42 stabilizes cortical actin and enhances barrier stability. In contrast, over-activation of RhoA by inflammatory stimuli induces F-actin stress fibers and enhance endothelial barrier dysfunction. Activated ROCK indirectly initiates polymerization of actin cytoskeleton by two mechanisms. First, ROCK inhibits myosin light chain phosphatase (MLCP). The contractile machinery of endothelial cells is mainly driven by mechano-chemical interaction between actin and myosin. Myosin being the primary force generator is controlled by phosphorylation status of its regulatory light chain (MLC). Myosin light chain kinase (MLCK) phosphorylates MLC and allows its sliding against actin. As a counter regulator, myosin associated protein phosphatase (MLCP) dephosphorylates MLC thereby terminating the contraction. RhoA/ ROCK pathway promotes actomyosin contraction by inactivating MLCP, directly phosphorylating MLC and indirectly activating MLCK. This increase in actomyosin contractility breaks down intercellular junction leading to endothelial barrier dysfunction [353, 354]. Mice with genetic deletion of MLCK subjected to IP injection of LPS had less effects on permeability than WT mice [355]. Second, RhoA/ROCK inhibit activity of cofilin, an actin-severing protein. Under physiological conditions, the actin cytoskeleton maintains a dynamic equilibrium between the polymerization of globular actin (G-actin) at the barbed end and de-polymerization of F-actin at the pointed end [356]. These actin filaments are randomly distributed throughout the cells as short, diffuse actin monomers and at cell periphery as cortical actin [357]. Upon

activation of RhoA pathway, ROCK phosphorylates LIMK which in turn phosphorylate and inactivate actin de-polymerization factor cofilin leading to organization of actin filaments into linear, parallel bundles stress fibers all across the cell interior [160]. Stress fiber formation is often accompanied by a contractile cell morphology and formation of gaps between adjacent endothelial cells and therefore hyperpermeability [353]. LPS-induced inflammation in LIMK1-deficient animals results in less edema formation and mortality compared to control mice [358]. On the other hand, over expression of LIMK1 in HUVEC leads to more intercellular gap formation due to LIMK1-dependent phosphorylation of cofilin [358].

CLP induced sepsis and intravenous injection of LPS results in over-activation of RhoA/ROCK followed by cytoskeletal rearrangements, contraction and enhanced vascular permeability in murine models of sepsis [359-361]. Rats when pre-treated with ROCK inhibitor Y-27632 prior to CLP show lower wet to dry ratio of septic organs, lower histopathological scores as compared with non-treated CLP animals [362]. Also, thrombin-a serine protease that normally controls endothelial permeability is over expressed in sepsis and mediates endothelial hyperpermeability by inducing RhoA activation [363]. Clearly, RhoA/ROCK pathway is critical for early events during sepsis progression leading to endothelial barrier dysfunction and organ damage. But, how does this RhoA/ROCK pathway get activated to induce endothelial barrier dysfunction in sepsis is still not elucidated. In this study, we demonstrate that apoptotic T-cell derived MPs containing SHH induce endothelial cytoskeletal rearrangement and barrier hyperpermeability by activating of RhoA/ROCK pathway.

3.5.Summary and conclusions

In summary, we provide evidence that SHH containing apoptotic T-cell derived MPs disrupt endothelial barrier function by remodeling cortical actin into F-actin stress fibers and by increasing permeability across endothelial monolayer (+71.5%). Direct activation of SHH by SMO agonist Pur also induced F-actin stress fibers and increased percolation of FITC dextran across endothelial monolayer (+86.81%) similar to SHH containing MPs. This indicates that SHH pathway mediates MPs induced endothelial barrier dysfunction. Inhibition of rho kinase pathway in endothelial cells before addition of MPs prevented F-actin stress fibers formation and allowed flux of FITC-dextran that is not significantly different from control. This indicates that SHH in MPs activates rho kinase pathway to induce endothelial barrier dysfunction. Overall, this result indicates that one potential mediator that can activate ROCK to mediate endothelial barrier dysfunction via induction of stress fiber formation and hyperpermeability is SHH containing MPs derived from apoptotic T-cell.

CHAPTER 4: EFFECT OF SONIC HEDGEHOG CONTAINING APOPTOTIC T-CELL DERIVED MICROPARTICLES ON REACTIVE OXYGEN SPECIES PRODUCTION IN ENDOTHELIAL CELLS VIA ACTIVATION OF RHO KINASE PATHWAY

4.1.Introduction

Reactive oxygen species (ROS) such as superoxide (O_2^-), hydrogen peroxide (H_20_2) and hydroxyl radical ($HO^·$) are free radicals/reactive molecules formed by partial reduction of oxygen [364]. ROS is endogenously produced as byproduct during mitochondrial oxidative metabolism as well as in cellular response to xenobiotic, cytokines and bacterial invasion. Enzymes contributing to increased ROS production includes protein complexes of mitochondrial electron transport chain (ETC), Nicotinamide adenine dinucleotide phosphate (NADPH) oxidase, xanthine oxidase, and uncoupled endothelial nitric oxide synthase (eNOS) [365]. Major contribution of ROS takes place at ETC cycle located on the inner mitochondrial membrane during the process of oxidative phosphorylation. In ETC cycle, electrons are passed through series of proteins via oxidation-reduction, with last acceptor molecule being oxygen (O_2) molecule. Leakage of electron from this chain leads to partially reduction of O_2 to form O_2^-. Under physiological conditions, low levels of ROS exert several vital and beneficial physiological cellular functions. ROS interacts with critical signaling molecules to initiate signaling in broad variety of cellular processes such as proliferation and survival, antioxidant regulation, mitochondrial oxidative stress, apoptosis, aging, iron homeostasis and DNA damage response [364]. ROS and reactive nitrogen species (RNS) act as an important defense system, are produced by activated phagocytes neutrophils and monocytes/macrophages as antimicrobial agents to directly destroy the microbial pathogens [366]. Several cytokines, growth factors, hormones and

neurotransmitter use ROS as secondary messenger in intracellular signal transduction [367]. These free radicals are neutralized by the cellular anti-oxidant defense system. In healthy individuals, a delicate balance between free radicals and antioxidants exist [368].

Oxidative stress occurs when balance between production of ROS, reactive RNS and antioxidant protection is disrupted. Oxidative stress results in direct or indirect ROS mediated damages to nucleic acids, proteins and lipids. This injury mechanism includes oxidation of polyunsaturated fatty acids of the phospholipids present in plasma membrane, mitochondrial membrane damage, DNA damage and decrease in nitric oxide (NO) availability [369]. Thus, oxidative stress has been associated with several pathophysiological condition and diseases including sepsis. In sepsis, decreased O_2 utilization in tissues is observed in both animal models and sepsis patients. The end result is low levels of ATP being produced and more electron leak from the ETC cycle, leading to over production of free radicals and oxidative stress [370]. Apart from ETC cycle, xanthine oxidation activation as a result of ischemic reperfusion, respiratory burst by neutrophils and macrophages and arachidonic acid metabolism can also produce ROS [371]. Among various ROS, O_2^- ion plays key role in pathogenesis of sepsis [372]. The pro-inflammatory effects of ROS include endothelial damage, increased microvascular permeability, formation of chemotactic factors, neutrophil recruitment, mitochondrial damage, lipid per oxidation, and DNA single strand damage [373]. In model of lethal sepsis, excess free radical production and oxidative damage occurs within 12 hours in the course of disease [374]. Many human studies have reported increased oxidative stress markers, direct evidence of free radical production, xanthine oxidase activation and low concentrations of antioxidants in septic patients as compared to healthy volunteer [375].

Oxidative stress also plays critical role in mediating endothelial dysfunction. ROS can directly attack endothelial cells, promoting deterioration of the endothelium and enhancing vascular permeability and thereby aggravating hypotension and decreasing colloid osmotic pressure of the plasma [376]. ROS are also important modulators of vascular tone. Endothelial cells produce vasodilator such as ·NO in balance with vasoconstrictors to regulate vascular tone. Many studies have shown that ROS increase the tone of arteries by reducing NO bioavailability and endothelium-dependent relaxation [377]. NO is a highly reactive radical that can interact with O_2^- and also with other ROS and lipid radicals , forming peroxynitrite ($ONOO^-$) [378]. Under physiological conditions, ROS production is low and endogenous antioxidants systems make sure to balance the production of O_2^- and elimination, thus preventing the formation of $ONOO^-$ and ensuring availability of NO for relaxation of vessels. While, in diseases where ROS production is increased or the antioxidant capacity is decreased, NO is transformed into $ONOO^-$ resulting in inhibition of endothelial dependent relaxation and therefore endothelial dysfunction [379]. $ONOO^-$ is also a potent oxidant that mediates lipid peroxidation and eNOS uncoupling. This eNOS uncoupling contributes to further increase in ROS production and decreased NO formation and consequent endothelial dysfunction [380]. Increased ROS bioavailability and deregulated redox signaling (oxidative stress) together with decreased NO due to reduced eNOS activity and increased NO consumption by ROS contribute significantly to molecular events underlying endothelial injury [380]. Moreover, they affect oxygen consumption by cells, which accelerates endothelial dysfunction causing multiple organ failure [376]

ROS in addition to mediating decreased NO and increased $ONOO^-$ production via modulation of intracellular Ca^{+2} concentration, can significantly upregulate Ca^{+2} independent RhoA/ROCK pathway in animal models to mediate endothelial dysfunction [354]. Studies in aortic endothelial cells show that high glucose or $ONOO^-$ increases RhoA activity which in turn decrease NO production by suppressing eNOS expression and hence cause endothelial dysfunction. While, NO production is largely restored by blocking $ONOO^-$ or RhoA/ROCK pathway [381]. Also, ROS can stimulate Rho translocation and activate ROCK which in turn inhibits MLC phosphatase, resulting in smooth muscle contraction in Ca^{+2} independent mechanism [382]. Recently, ROCK-1 has been demonstrated to in turn induce ROS production, indicating there may be positive feedback loop in play. This increase in ROS production may be one of the potential mechanisms by which Rho/ROCK decrease NO levels to induce endothelial dysfunction. However; which signaling molecule/s actuate RhoA/ROCK to induce oxidative stress in sepsis is not known. In this study, we hypothesized that apoptotic T-cell derived MPs containing SHH induce oxidative stress in endothelial cells by enhancing O_2^-production via activation of RhoA/ROCK pathway.

4.2. Materials and Methods.

4.2.1.HMEC Cell Culture

Human microvascular endothelial cells (HMECs) were a kind gift from Dr. Vijay Kumar Kalra (University of Southern California, Los Angeles, California). HMECs are immortalized cells obtained from human foreskins. The cells were maintained in MCDB-131 growth media without L-glutamine (Thermo Fisher Scientific; Rockford, IL) supplemented 10ng/mL endothelial growth factor (EGF), 1µg/mL hydrocortisone, 10mM

GlutaMAX (Life Technologies, Carlsbad, CA), 10% heat-inactivated fetal bovine serum (FBS) (Serum Source, Charlotte, North Carolina) and 10% penicillin-streptomycin (Thermo Fisher, Waltham, MA). Cell were maintained in T-75 flask at 37°C under an atmosphere of 5% $C0_2$ and 95% air. Cell were harvested using 0.25% Trypsin-0.53mM EDTA solution and seeded on collage-coated wells, allowing them to attach overnight. Cells were quiesced the following day in 0.1% FBS medium overnight. The next day, medium was refreshed with 1% FBS medium and treatments were added for either 6 or 24hrs.

4.2.2.Reagents

Dihydroethidium (DHE) was obtained from Thermo Fisher Scientific (Rockford, IL). Purmorphamine (Pur), Hanks balanced salt solution (HBSS), Dulbecco's phosphate buffered saline (DPBS) was obtained from Sigma- Aldrich, (St. Louis, MO), Y-27632 (R)-(+)-trans-N-(4-Pyridyl)-4-(1-Aminoethyl) cyclohexanecarboxamide dihydrochloride monohydrate was acquired from Cayman chemical (Ann harbor, MI).

4.2.3.Treatments

Each experiment consists five treatment groups. (1) Untreated HMECs (negative control), (2) HMECs treated with 50µg protein/mL $^{SHH+}$MPs, (3) HMECs treated with 10µM Purmorphamine (Pur). Purmorphamine activates the hedgehog signaling pathway by directly binding to smoothened (SMO), a downstream protein in this pathway. If we can duplicate the effect of MPs with Pur, this would suggest that SHH pathway does contribute to the MPs mediated endothelial dysfunction (positive control). (4) HMECs treated with 10µM SANT-1, an antagonist of SMO. If the effect of Pur on endothelial cells is abated by SANT-1, this would further ensure involvement of SHH pathway in mediating endothelial

dysfunction. (5) HMECs treated with 10μM Y-27632 one hour prior to treatment addition of MPs. Y-27632 is cell permeable, highly potent and selective inhibitor of ROCK. Y-27632 inhibits both ROCK-1 and ROCK-2 by competing with ATP for binding to the catalytic site. If inhibition of ROCK abrogates or reduces the effect of $^{SHH+}$MPs on endothelial cells, this would indicate that indeed these SHH containing microparticles derived from apoptotic T-cell induce endothelial dysfunction through activation of RhoA/ROCK pathway. The treatments were done for a period of 24 hours to simulate acute inflammation during which large degree of sepsis induced T-cell apoptosis is documented to occur.

4.2.4.Dihydroethidium Staining to Detect Superoxide Production

To evaluate if apoptotic T-cell derived MPs containing SHH induce oxidative stress in endothelial cells via rho kinase pathway, we used redox indicator dihydroethidium (DHE). DHE is cell permeable dye that exhibits blue fluorescence in cytosol until oxidized by O_2^- to 2-hydroxy ethidium (2-0H-E), which intercalates with DNA, staining its nucleus a bright fluorescent red. HMECs were plated on 25mm glass coverslip in a 6-well plate at a density of 1.5X10^{5}cells /mL in MCDB-131 media supplemented with 10% FBS at 37°C under an atmosphere of 5% C02. After indicated treatments for either 6 or 24hrs, coverslips were washed with PBS twice followed by staining with DHE prepared in HBSS (5μM; Thermo Fischer Scientific; Rockford, IL) for 20 minutes at 37°C. Excess stain was removed by washing with PBS twice. Coverslip was mounted on a chamber with 1mL warm Hank's balanced salt solution (HBSS) and imaged using 60X objective of laser reflected confocal scanning fluorescence microscope (Olympus FV 500) with a Fluoview operating software.

DHE fluorescence was examined by laser excitation at 535nm and emission at 595nm with rapid succession.

4.2.5.Offline Image Analysis

Offline image analysis was performed by using FIJI software. 60X images recorded with Texas red filters were used. Ten areas of interest were randomly selected per image with total of n=3 per group. To quantify the extent of superoxide production, the mean of red fluorescence intensity was measured. Greater is the ROS production, brighter is the red color and hence higher is the mean of fluorescence intensity.

4.2.6.Statistical Analysis

All data are presented as means ± standard error of the mean (SEM). Statistical analysis was performed using GraphPad Prism software (San Diego, California). Statistical significance was assessed by one-way analysis of variance (ANOVA) with independent Dunnett's *post hoc* test was used when statistical differences were detected. Statistical significance was set at $p < 0.05$.

4.3.Results

4.3.1.Apoptotic T-Cell Derived Microparticles Containing Sonic Hedgehog Induce Superoxide Production In Endothelial Cell Via Rho Kinase Pathway.

Marked oxidative stress as a result of inflammatory responses is a characteristic of endothelial dysfunction [168, 383-385]. To test whether SHH containing MPs induce oxidative stress in HMECs via RhoA/ROCK, we assessed O_2^- formation by measuring the DHE fluorescence. At 6hrs, HMECs alone (Figure 17A) showed very little O_2^- production while significantly enhanced DHE fluorescence was observed with MPs (Figure 17B;

****=P<0.0001vs control). Extensive O_2^- production was also observed in HMECs treated with SHH agonist Pur (Figure 17C; ****= P<0.0001vs control) while, inhibition of SHH by SANT-1 (Figure 19D) resulted in very little O_2^- production. Treatment with ROCK inhibitor Y-27632, one hour prior to MPs (Figure 17E) prevented excess O_2^- production (not different from control). Similar results were obtained at 24hrs (Figure 19). SANT-1 treatment was not included at 6hrs. Collectively, this result suggests that SHH containing apoptotic T-cell derived MPs induce oxidative stress in endothelial cell by enhancing O_2^- production via rho kinase pathway. To quantify the extent of O_2^- production, we determined the mean of red DHE fluorescence displayed by the confocal images taken after 6 (Figure 18) and 24hrs (Figure 20) treatment respectively using FIJI software.

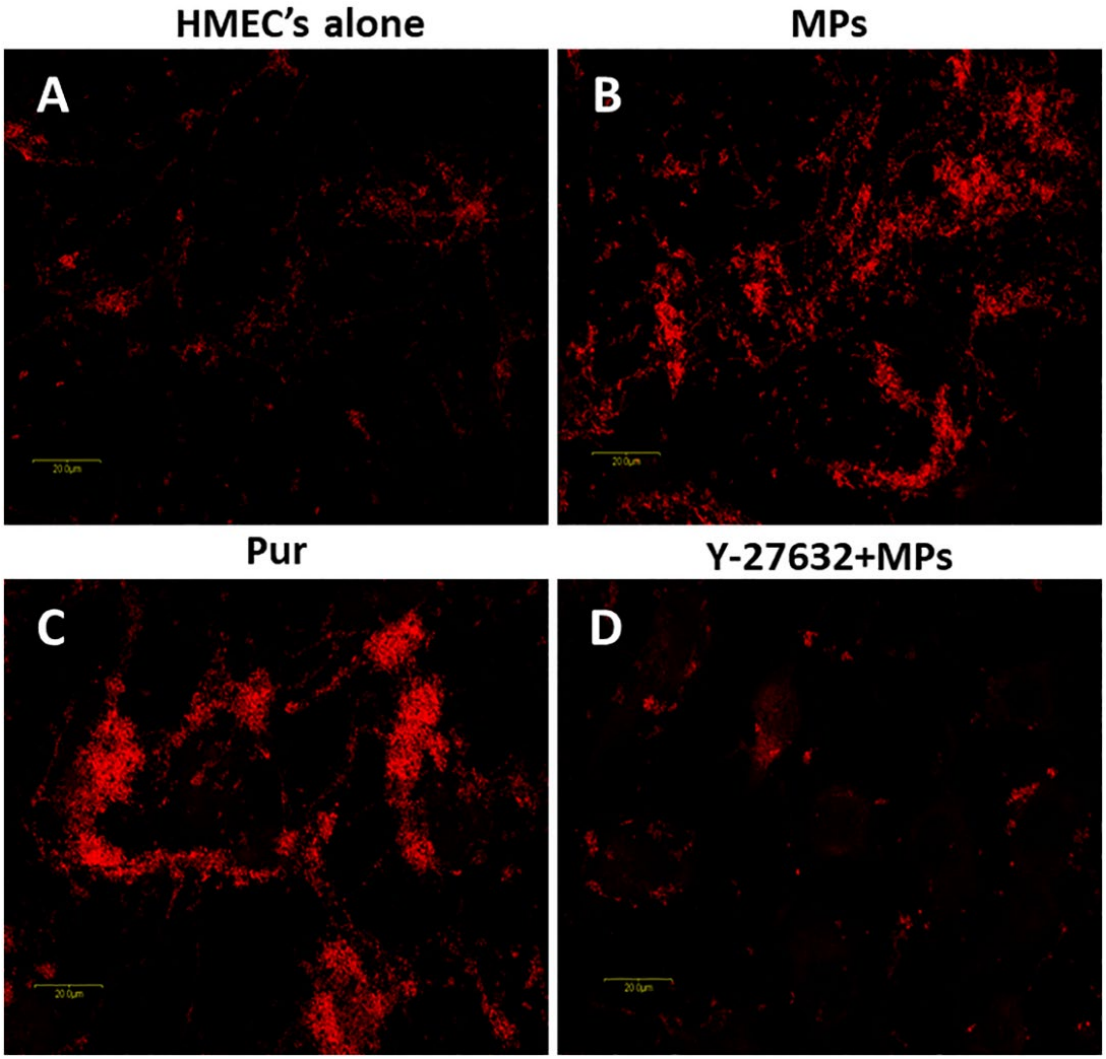

Figure 17: Apoptotic T-cell derived microparticles containing sonic hedgehog induce superoxide production in endothelial cells via rho kinase pathway.

HMECs grown on 25mm glass coverslips were incubated with 50µg protein/mL MPs (B) or with 10µM Pur (C) or or with 10µM Y-27632 one hour prior to addition of MPs (D) for 6hrs. Control was HMEC's alone (A). Cells were stained with 2.5µM DHE in HBSS for 20 minutes. O_2^- production was localized by visualizing fluorescence of DHE (red) on a confocal microscope at 60x magnification and scale bar =20µm. HMECs alone showed very little DHE staining while, extensive DHE staining was observed with addition of MPs.

Treatment of HMECs with SHH agonist Pur displayed extensive O_2^- production. Inhibition of ROCK with Y-27632 one hour prior to addition of MPs prevented excess O_2^- production as indicated by less DHE staining that is not significantly different from control. Representative images from three experiments are shown.

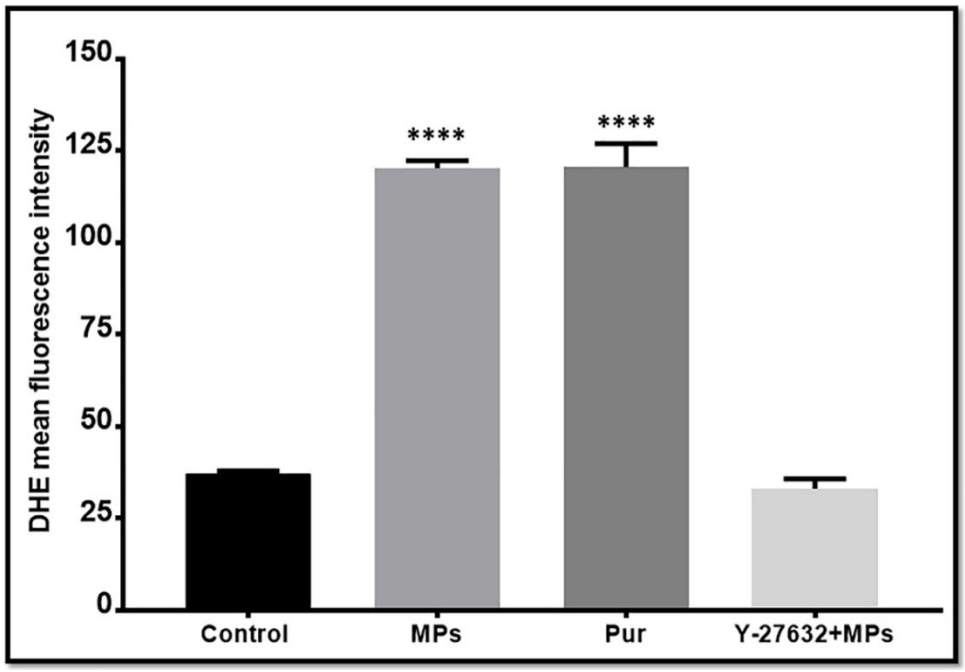

Figure 18: Quantification of Dihydroethidium fluorescence in confocal images taken after 6hrs of treatment

HMECs were treated with MPs (B) or with Pur (C) and or with Y-27632 one hour prior to addition of MPs (D) for 6hrs. Cell were loaded with 2.5µM DHE in HBSS and confocal images were taken 20 minutes later with 60x magnification and scale bar 20µm. To quantify the degree of O_2^- production, average mean intensities of DHE fluorescence was measured for 10 spots per picture using FIJI software. Statistical significance was analyzed by one-way ANOVA with post hoc Dunnett's test. ****$P \leq 0.0001$ for MPs, Pur compared to control. All data are represented as the mean ± s.e.m. of three experiments.

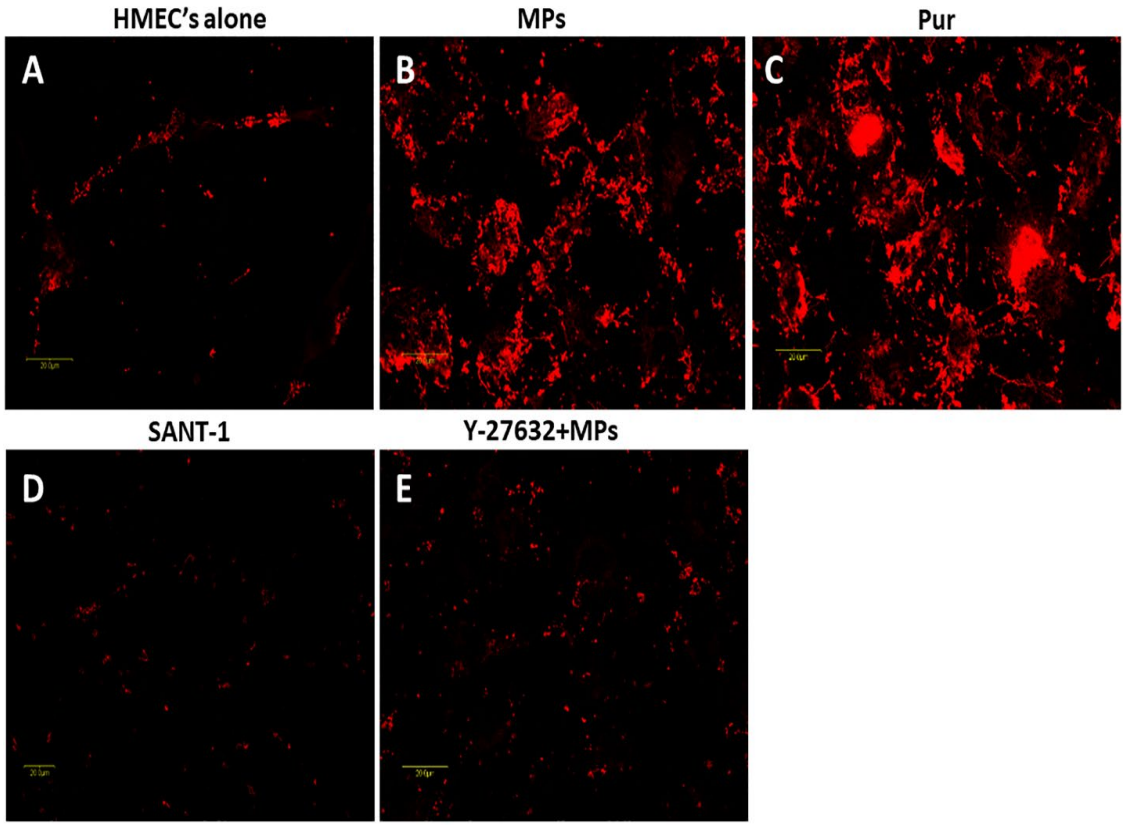

Figure 19: Apoptotic T-cell derived microparticles containing sonic hedgehog induce superoxide production in endothelial cells via rho kinase pathway.

HMECs grown on 25mm glass coverslips were incubated with 50µg protein/mL MPs (B) or with 10µM Pur (C) or with 10µM SANT-1 (D) and or with 10µM Y-27632 one hour prior to addition of MPs (E) for 24hrs. Control was HMEC's alone (A). Cells were stained with 2.5µM DHE in HBSS for 20 minutes. O_2^- production was localization by visualizing fluorescence of DHE (red) on a confocal microscope at 60x magnification and scale bar =20µm. HMECs alone showed very little DHE staining while, extensive DHE staining was observed with addition of MPs. Treatment of HMECs with SHH agonist Pur displayed extensive O_2^- production while, the SHH antagonist SANT-1 abrogated it. Inhibition of ROCK with Y-27632 one hour prior to addition of MPs prevented excess O_2^- production

as indicated by less DHE staining that is not significantly different from control. Representative images from three experiments are shown.

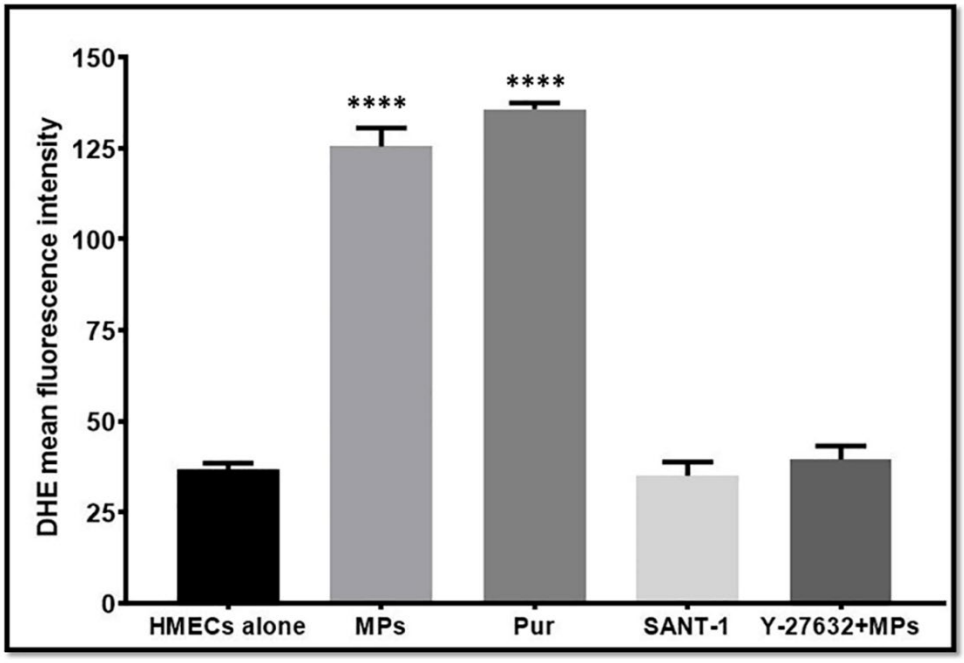

Figure 20: Quantification of Dihydroethidium fluorescence in confocal images taken after 24hrs of treatment.

HMECs were treated with MPs (B) or with Pur (C) or and or with Y-27632 (D) one hour prior to addition of MPs for 24hrs. Cell were loaded with 2.5µM DHE in HBSS and confocal images were taken 20 minutes later with 60x magnification and scale bar 20µm. The extent of O_2^- production was quantified by analyzing average mean intensities of DHE fluorescence for 10 spots per picture using FIJI software. Statistical significance was analyzed by one-way ANOVA with post hoc Dunnett's test. ****=P<0.0001 for MPs, Pur compared to control. All data are represented as the mean ± s.e.m. of three experiments.

4.4. Discussion

The pathological conditions in sepsis caused by extensive inflammatory response is characterized by massive production of ROS, RNS and inflammatory cytokines [166]. Sepsis patients show overwhelming production of ROS and/ or deficit in antioxidant system which correlates with the mortality [385]. Increase of ROS after LPS challenge has been demonstrated in different models of sepsis [386]. LPS is capable of inducing ROS by interacting with myriad of immune cells, can be beneficial or detrimental to the host. Early in sepsis, phagocytic cells macrophages and neutrophils get activated to engulf and destroy the bacteria through LPS recognition by producing antimicrobial ROS and RNS, a mechanism that is certainly required and advantageous to the host [387]. Mice deficient in producing O_2^-, is associated with decreased bacterial clearance [388]. However; a continued exposure of high doses of LPS triggers prolonged production of ROS which can be detriment, including significant cytotoxicity to organs and contribution to sepsis associated MOF. Among various ROS, O_2^- plays a key role in pathogenesis of hemodynamic instability and organ dysfunction during sepsis. LPS also increases expression of iNOS and NF-κB, and consequently promote production of large amounts of NO for longer periods, leading to hypotension. This is evidenced by increased levels of nitrite and nitrate; stable metabolites of NO measured in the plasma of septic patients [389] and by the ability of iNOS selective inhibitors to restore blood pressure in experimental models of sepsis and reverse hypotension in human endotoxemia [390]. Excessive NO can react with O_2^- to form $ONOO^-$, a strong oxidant [391] that inhibit mitochondrial respiration, resulting in increased lactate and decreased ATP production [392]. This leads to opening of potassium (K^+) channels, allowing K^+ to move out the cells, causing a decrease in

membrane potential and hyperpolarization, closing voltage gated calcium channels in the cell membranes and vascular muscle relaxation. Dysfunction of these K^+ channels closure has been seen in vascular diseases including sepsis leading to abnormal vasodilation and hypotension, a hall mark of sepsis, which also contributes to MOF [393].

MPs are potent autocrine/paracrine signals for oxidative stress. Endothelial derived microparticles (EMPs) have been shown to induce ROS in microvascular endothelial cells and in ex-vivo aortic rings [322]. In addition, ROS produced by EMPs promotes anti-angiogenic effect in endothelial cells [394]. Similarly, ROS production is also induced in HUVECs exposed to EMPs. This exogenous ROS production is mediated by both mitochondrial and NADPH oxidase mediated [395]. MPs derived from apoptotic T-cell derived have also been reported to induce endothelial dysfunction by increasing ROS by a mechanism sensitive to xanthine oxidase [396]. However, role and mechanism of action of SHH containing apoptotic T-cell derived MPs in mediating oxidative stress in sepsis has not been investigated yet. Since T-cell undergoes massive apoptosis in sepsis producing MPs, we hypothesized that these MPs mediate oxidative stress in endothelial cells by inducing production of excess O_2^-, a potent mediator of sepsis pathophysiology that can injure liver. To visualize and quantify O_2^-, we imaged DHE fluorescence using confocal laser scanning microscopy. Apoptotic T-cell derived MPs induced significantly high amount of O_2^- in endothelial cells. So, DHE was oxidized by O_2^- to 2-hydroxy ethidium (2-0H-E), which intercalated with DNA. The resulting image was bright red fluorescence staining the nucleus of the endothelial cells and the measured mean of fluorescence intensity for the area of interest was very high in numerical value (+242%).

We also elucidated the mechanism by which these apoptotic T-cell derived MPs mediate oxidative stress in endothelial cells.

Impaired vascular regulation in sepsis rats is mediated by increased oxidative stress and RhoA/ROCK pathway [152]. ROS has been shown to activate ROCK mediated endothelial dysfunction [382]. Recently, ROCK in turn has been shown to increase mitochondrial ROS production by modulating the interaction between Rac1b and cytochrome c (cyt c) [170]. Rac1b is an alternatively spliced variant of G-protein Rac1 that comprises of a self-activated GTPase unit [171] and plays role in cytoskeletal reorganization and ROS production [172]. Cyt c is a mitochondrial redox carrier that is part of complex III in electron transport chain (ETC) of mitochondria. Cyt c have heme as prosthetic group that accepts electrons from complex III and transfers them to complex IV cytochrome c oxidase ; COX of ETC cycle [173]. In turn, reduced COX conducts the final electron transfer to O_2 allowing its complete reduction into H_20, which ultimately drives proton pump across the inner mitochondrial membrane for ATP production [174]. RhoA/ROCK activation induces phosphorylation of Rac1b at ser71 and facilitate its interaction with cyt c. This interaction results in electron transfer from cyt c to Rac1 at Cys-178 instead of their transfer to COX [175]. Therefore, COX is unable to completely reduce O_2 to H_20 leading to production of ROS [170]. However, which signaling molecule/s activate RhoA/ROCK to induce oxidative stress in sepsis is not known. In our study, we demonstrate that apoptotic T-cell derived MPs containing SHH increase O_2^- production in endothelial cells by activation of RhoA/ROCK pathway.

4.5.Summary and Conclusions

In summary, we demonstrate that SHH containing apoptotic T-cell derived MPs induce oxidative stress in endothelial cells by increasing O_2^- production as indicated by extensive DHE fluorescence and high mean value (+242%). Stimulation of SHH pathway by SMO agonist also induces excessive production of O_2^-as indicated by high mean value of DHE fluorescence (+270%) While inhibition of SHH pathway by SANT-1 abrogates it, as evident by low DHE fluorescence. This suggest that MPs induced oxidative stress is mediated by SHH pathway. Inhibition of ROCK by Y-27632 one hour prior to addition of MPs prevented excessive O_2^- as indicated by low DHE fluorescence and low mean value that is not significantly different from control. Therefore, SHH in MPs activate rho kinase pathway to induce excessive O_2^- production in endothelial cells. Overall, this results indicates that SHH containing MPs derived from apoptotic T-cell derived MPs are one potential agonist that can activate rho kinase pathway to induce endothelial cell dysfunction.

CHAPTER 5: EFFECT OF SONIC HEDGEHOG CONTAINING APOPTOTIC T-CELL DERIVED MICROPARTICLES ON MITOCHONDRIA OF ENDOTHELIAL CELLS MEDIATED BY ACTIVATION OF RHO KINASE PATHWAY

5.1.Introduction

A growing body of evidence suggests that inability of cells to consume oxygen (O_2) may play crucial role in pathophysiology of sepsis [166]. For example, studies where supranormal level of O_2 was available failed to decrease the morbidity and mortality of critically ill patients [397]. Since mitochondrial respiratory chain utilizes 90% of the total body O_2 consumed, impaired O_2 utilization and dysfunctional mitochondria are implicated in pathogenesis of sepsis. Mitochondrial respiratory chain is located in the inner mitochondrial membrane and is composed of five individual complexes I to V that bring about oxidative phosphorylation. These complexes transfer electrons from NADH, and succinate produced by the Krebs cycle down a redox gradient, finally reducing O_2 to water. The transfer of electrons allows the complexes I, III and IV to translocate protons from the mitochondrial matrix to the intermembrane space, generating a mitochondrial membrane potential (MMP). MMP is a transmembrane potential of hydrogen ions that serves an intermediate source of energy generating an electric potential due of charge separation i.e. negative on inside and positive on outside and a proton gradient. This proton gradient is used by ATP synthase (complex V) to generate ATP from ADP and inorganic phosphate [370]. Both ATP generation and MMP are essential for functioning of mitochondria and host cell, where prolonged depolarization (loss of charge separation across the membrane) leads to point of no return and mitochondrion as functional entity dies and has fatal consequence for the cell due to bioenergetics failure [398]. Apart from ATP production,

mitochondria are involved in heat generation, production of reactive oxygen species (ROS), thermoregulation, maintenance of intracellular Ca^{+2} and intracellular redox potential. Sepsis induces structural and functional damages to mitochondria, conversely mitochondrial dysfunction aggravates sepsis as there is failure of ATP production [177]. Alterations in mitochondria have been described in skeletal muscle and liver obtained from septic rats, pigs and primates [399, 400] where decreased mitochondrial respiratory rate and swelling have been identified along with microcirculatory blood flow changes.

The pathogenesis of mitochondrial damage as a result of sepsis is a complex series of events. Multiple stressors have been shown to influence mitochondrial functions including oxidative phosphorylation, biosynthetic, regulatory and signaling function. In order to carry out this complex array of activities, mitochondria must maintain a normal global MMP [401]. In sepsis, there is uncoupling of O_2 consumption from the oxidative phosphorylation reflecting proton leak back into the inner mitochondrial matrix without any ATP production [402] and therefore loss of MMP. Excessive amount of endogenous gaseous mediator nitric oxide (NO), carbon monoxide (C0) and hydrogen sulphide (H_2S) produced during sepsis also plays pivotal role in mediating mitochondrial dysfunction by inhibiting the function of complex IV in ETC cycle [403] and has been associated with severity of sepsis [404]. Inhibition of complex IV prevents the electron transfer to O_2, resulting in enhanced production of superoxide (O_2^-) and decreased ATP production. O_2^- is considered as most potent in inducing mitochondrial dysfunction, can directly inhibit mitochondrial respiratory chain, and cause direct damage to the mitochondrial protein and lipid membrane. When produced in huge amounts, O_2^- coupled with increased NO favors formation of peroxynitrite ($ONOO^-$) which further leads to respiratory inhibition,

decreased cellular energy, and loss of cell function by inhibiting complex I , as observed in heart and skeletal muscle of rodent model of sepsis [176]. ATP depletion, loss of MMP i.e. mitochondrial depolarization, and oxidative stress can damage mitochondria releasing cytochrome (cyt c), the mediator in electron flow between complex III and IV into the cytosol. The next step is formation of apoptosome which reacts with caspases to induce apoptosis [405].

Endothelial dysfunction is associated with mitochondrial alterations [406]. Apart from regulating proliferation, ATP production, apoptosis as in any cells, endothelial cell mitochondria play critical in regulating response to environmental cues [407]. Endothelial mitochondria mainly regulate signaling by maintaining Ca^{+2} concentration in the cytosol, an important aspect for many endothelial functions. For example acetylcholine (Ach) activate eNOS expression by increasing cytosolic Ca^{+2} [408]. There is increasing evidence that mitochondrial morphological and functional changes is implicated in vascular endothelial dysfunction. Enhanced mitochondrial fission or attenuated fusion, disturbance in mitochondrial autophagy, morphological changes in mitochondria, and increase in accumulation of irreversibly depolarized mitochondria have been implicated in disrupting endothelial physiological function [409]. Extensive damage of sustained mitochondrial depolarization facilitates the opening of mitochondrial permeability transition pore (mPTP), increases mitochondrial membrane permeability and releases pro-apoptotic molecules, resulting in endothelial cell apoptosis [410].

Mitochondrial alteration as a result of loss of MMP has been documented in immune cells i.e. monocytes, platelets, T-lymphocytes of septic patients [411]. Loss of MMP was only present in patients that died from sepsis and not in the patients that survived

sepsis, suggesting loss of MMP is a characteristic of sepsis disease [411]. RhoA/ROCK pathway have been implicated in mediating mitochondrial dysfunction by decreasing MMP via inhibition of complex IV [170] and by increasing expression of pro-apoptotic proteins [180] to induce mitochondrial death pathway. However, how is RhoA/ROCK stimulated to induce mitochondrial alteration in endothelial cells during sepsis has not been elucidated. In this study, we hypothesized that apoptotic T-cell derived MPs containing SHH mediate mitochondrial dysfunction in endothelial cells by inducing mitochondrial depolarization via activation of RhoA/ROCK pathway.

5.2.Materials and Methods

5.2.1.HMEC Cell Culture

Human microvascular endothelial cells (HMECs) were a kind gift from Dr. Vijay Kumar Kalra (University of Southern California, Los Angeles, California). HMECs are immortalized cells obtained from human foreskins. The cells were maintained in MCDB-131 growth media without L-glutamine (Thermo Fisher Scientific; Rockford, IL) supplemented 10ng/mL endothelial growth factor (EGF), 1µg/mL hydrocortisone, 10mM GlutaMAX (Life Technologies, Carlsbad, CA), 10% heat-inactivated fetal bovine serum (FBS) (Serum Source, Charlotte, North Carolina) and 10% penicillin-streptomycin (Thermo Fisher, Waltham, MA). Cell were maintained in T-75 flask at 37°C under an atmosphere of 5% $C0_2$ and 95% air. Cell were harvested using 0.25% Trypsin-0.53mM EDTA solution and seeded on collagen-coated wells, allowing them to attach overnight. Cells were quiesced the following day in 0.1% FBS medium overnight. The next day, medium was refreshed with 1% FBS medium and treatments were added for either 6 or 24hrs.

5.2.2.Reagents

Rhodamine 123 (Rh123) was obtained from Thermo Fisher Scientific (Rockford, IL). 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide (MTT), Purmorphamine (Pur), Hanks balanced salt solution (HBSS), Dulbecco's phosphate buffered saline (DPBS) was obtained from Sigma-Aldrich (St. Louis; MO), Y-27632 (R)-(+)-trans-N-(4-Pyridyl)-4-(1-Aminoethyl) cyclohexanecarboxamide dihydrochloride monohydrate was acquired from Cayman chemical (Ann harbor, MI).

5.2.3.Treatments

Each experiment consists of five treatment groups. (1) Untreated HMECs (negative control), (2) HMECs treated with 50µg protein/mL $^{SHH+}$MPs, (3) HMECs treated with 10µM Purmorphamine (Pur). Purmorphamine activates the hedgehog signaling pathway by directly binding to smoothened (SMO), a downstream protein in this pathway. If we can duplicate the effect of MPs with Pur, this would suggest that SHH pathway does contribute to the MPs mediated endothelial dysfunction (positive control). (4) HMECs treated with 10µM SANT-1, an antagonist of SMO. If the effect of Pur on endothelial cells is abated by SANT-1, this would further ensure involvement of SHH pathway in mediating endothelial dysfunction. (5) HMECs treated with 10µM Y-27632 one hour prior to treatment addition of MPs. Y-27632 is cell permeable, highly potent and selective inhibitor of ROCK. Y-27632 inhibits both ROCK-1 and ROCK-2 by competing with ATP for binding to the catalytic site. If inhibition of ROCK abrogates or reduces the effect of $^{SHH+}$MPs on endothelial cells, this would indicate that indeed these SHH containing microparticles derived from apoptotic T-cell induce endothelial dysfunction through activation of RhoA/ROCK pathway. The treatments were done for a period of 24 hours to

simulate acute inflammation during which large degree of sepsis induced T-cell apoptosis is documented to occur.

5.2.4.Rhodamine 123 Staining to Determine Intact Mitochondrial Membrane Potential

To test if apoptotic T-cell derived MPs containing induce mitochondrial depolarization in HMECs via RhoA/ROCK pathway, we used cationic green fluorescent Rhodamine 123 dye (Rh123). Rh123 preferentially enters mitochondria by distributing according to the high negative membrane potential across the mitochondrial inner membrane. Imbalance in mitochondrial membrane potential results in loss of dye into the cytoplasm and greater overall bright green fluorescence. Propidium iodide (PI) was utilized to check cell death. The red fluorescence from PI and green fluorescence from Rh123 appears yellow if there is concomitant mitochondrial depolarization and cell death. HMECs were seeded at density of $1.5X10^5$cells /mL on 25mm glass coverslip in MCDB-131 media supplemented with 10% FBS at 37°C under an atmosphere of 5% C02 and 95% air. After indicated treatments for either 6 or 24hrs, coverslips were washed with PBS twice followed by staining with Rh123 (100nM; Thermo Fischer Scientific; Rockford, IL) and PI (10μM; Thermo Fischer Scientific; Rockford, IL) for 20 minutes at 37°C. The coverslips were washed with PBS twice to remove excess stain. Confocal images were acquired at excitation/emission wavelengths of 488/610nmusing 60X objective of laser reflected confocal scanning fluorescence microscope (Olympus FV 500) with a Fluoview operating software. HMECs with polarized or depolarized mitochondria were identified based on punctuate or diffused bright green fluorescent staining respectively.

5.2.5.Offline Image Analysis

Offline image analysis was performed by using FIJI software. 60X images recorded with FITC green filters were used. Ten areas of interest were randomly selected per image with total of n=3 per group. To quantify the degree of mitochondrial depolarization, we measured the standard deviation (SD) of Rh123 fluorescence intensity. A polarized mitochondrion shows high SD as most of the Rh123 remains inside mitochondria, providing sharp contrast between mitochondria and cytoplasm. Following depolarization Rh123 equilibrates between mitochondria and cytoplasm yielding a more homogeneous intensity, i.e. low SD.

5.2.6.Cell Proliferation by MTT Assay

MTT assay measures the reduction of yellow tetrazolium salt MTT by succinate dehydrogenase found mainly in respiring mitochondria into dark purple formazan. HMECs were seeded in a 96 well plate (tissue culture grade, flat bottom) at concentration of 12000 cells per well in 200µL MCDB-131 media along with indicated treatments (except SANT-1) and incubated at 37°C under an atmosphere of 5% CO_2. After 24hrs, 20uL of MTT reagent (final concentration 3mg/mL) was added to each well and the microplate was incubated for 3hrs (37°C; 5% CO_2). The formazan crystal formed were solubilized by adding 150uL of MTT solvent (4mM HCL, 0.1% Nonidet P-40 in isopropanol) after the media was carefully aspirated. The optical density of purple color of solubilized formazan was read at 590nm with a reference filter of 620nm in µQuant spectrophotometer microplate reader (BioTek instruments; Winooski, VT).

5.2.7.Cell Viability by MTT Assay

HMECs were cultured in 96 well plates at concentration of 3000 cells per well in 200µL MCDB-131 media containing 10% FBS for 24hrs at 37°C under an atmosphere of 5% CO_2. HMECs were then starved overnight in 0.1% FBS MCDB-131 media. The media was replaced with 1% FBS and indicated treatments (except SANT-1) were added. After 24hrs, 20uL of MTT reagent (final concentration 3mg/mL) was added to each well and the microplate was incubated for 3hrs (37°C; 5% CO_2). The formazan crystal formed were solubilized by adding 150uL of MTT solvent (4mM HCL, 0.1% Nonidet P-40 in isopropanol) after the media was carefully aspirated. The optical density of purple color of solubilized formazan was read at 590nm with a reference filter of 620nm in a µQuant spectrophotometer microplate reader (BioTek instruments; Winooski, VT). Higher the absorbance, more is MTT was converted into formazan by respiring mitochondria and therefore more are the viable cells.

5.2.8.Statistical Analysis

All data are presented as means ± standard error of the mean (SEM). Statistical analysis was performed using GraphPad Prism software (San Diego, California). Statistical significance was assessed by one-way analysis of variance (ANOVA) with independent Dunnett's *post hoc* test was used when statistical differences were detected. Statistical significance was set at $p < 0.05$.

5.3.Results

5.3.1.Apoptotic T-Cell Derived Microparticles Carrying Sonic Hedgehog Induce Mitochondrial Depolarization in Endothelial Cells via Rho Kinase Pathway.

Decreased MMP leads to energy depletion, release of cytochrome C and apoptosis [176, 177, 412-414]. To assess if MPs containing SHH alter MMP and induce cell death of endothelial cells via activation of RhoA/ROCK pathway, we visualized fluorescence of Rh123 (green) and PI (red), using confocal microscope**.** Under control conditions, HMECs alone **(**6hrs**:** Figure 21A, 24hrs: Figure 23A) demonstrate punctate staining indicative of well-polarized mitochondria. Treatment with MPs (6hrs: Figure 21B; ****=P<0.0001, 24hrs: Figure 23B; ****=P<0.0001) induced mitochondrial depolarization and cell death as indicated by diffuse cytoplasmic fluorescence due to loss of Rh123 from mitochondria into cytoplasm and yellow patches in nucleus (green and red mixed) respectively**.** Activation of SHH pathway with SMO agonist Pur (6hrs: Figure 21C; ****=P<0.0001, 24hrs: Figure 23C; ****=P<0.0001) also induced mitochondrial depolarization and cell death as seen with MPs. While, addition of SHH antagonist SANT-1 (Fig.23D) impeded depolarization as indicated by punctuate staining of Rh123. Inhibition of Rho kinase pathway by ROCK inhibitor Y-27632 one hour prior to MPs (6hrs: Figure 21D, 24hrs: Figure 23F; not significantly different from control) prevented MPs induced mitochondrial depolarization as indicated by punctate staining of Rh123 like HMECs alone. Collectively, this indicates that SHH containing apoptotic T-cell derived MPs induce depolarization of mitochondria in endothelial cells and injure them via activation of rho kinase pathway. To quantify extent of mitochondrial depolarization, we determined the average standard

deviation of Rh123 fluorescence intensity displayed by the confocal images taken after 6 (Figure 22) and 24hrs (Figure 24) treatment respectively using image master software.

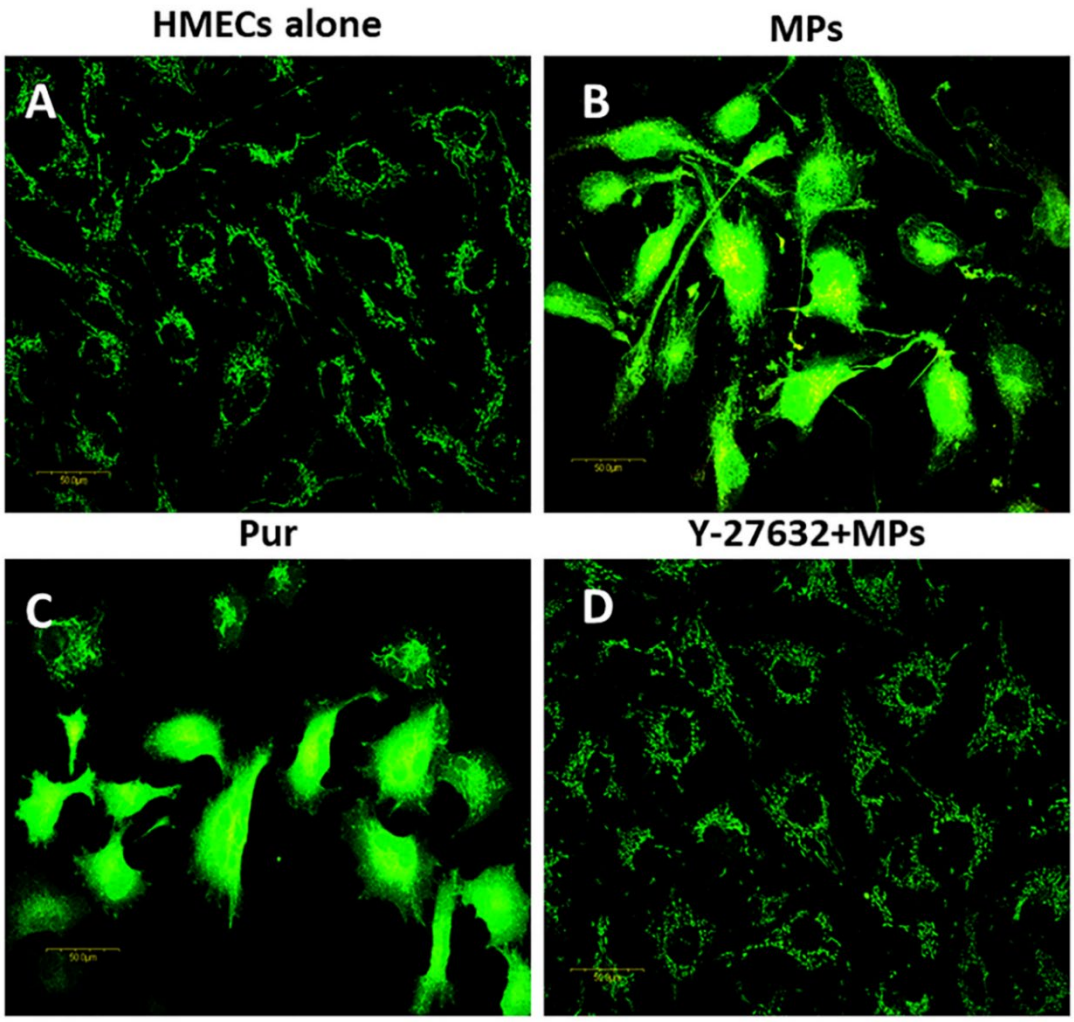

Figure 21: Apoptotic T-cell derived microparticles containing sonic hedgehog promote depolarization of mitochondria in endothelial cells via rho kinase pathway**.**

HMECs were grown on 25mm glass coverslips. Following 24hrs, HMECs were treated with MPs (B) or with Pur (C) or and or with Y-27632 one hour prior to addition of MPs (D) for 6hrs respectively. Control was HMEC's alone. Media was replaced by 100nM of Rh123 and 10μM PI in HBSS and imaged on confocal microscope after 20 minutes with

60x magnification and scale bar 50µm. HMECs alone showed punctuate staining of Rh123 while, treatment with MPs or Pur displayed loss of Rh123 into the cytoplasm and cell death as indicated by bright green color and yellow patches in nucleus respectively. Inhibition of ROCK with Y-27632 and then addition of MPs prevented the loss of Rh123 as seen with MPs alone treatment and displayed punctate staining. The images are representative of three separate experiments.

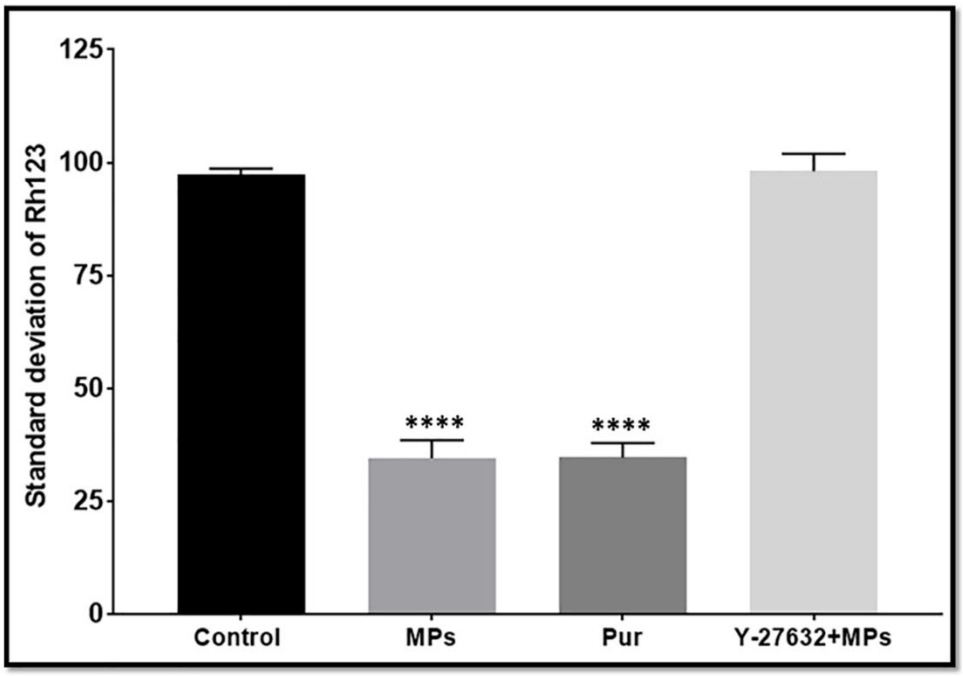

Figure 22: Quantification of Rhodamine 123 fluorescence in confocal images taken after 6hrs of treatment

HMECs were treated with MPs (B) or with Pur (C) and or with Y-27632 one hour prior to addition of MPs (D) for 6hrs. Cell were loaded with 100nM Rh123 and 10µM PI in HBSS and confocal images were taken 20 minutes later with 60x magnification and scale bar 50µm. To quantify the degree of mitochondrial depolarization, average standard deviation of Rh123 fluorescence was quantified for 10 spots per picture using FIJI software.

Statistical significance was analyzed by one-way ANOVA with post hoc Dunnett's test. ****P≤0.0001 for MPs, Pur compared to control. All data are represented as the mean ± s.e.m. of three experiments. A polarized mitochondrion shows high SD as most of the Rh123 remains inside mitochondria, providing sharp contrast between mitochondria and cytoplasm. Following depolarization, Rh123 equilibrates between mitochondria and cytoplasm yielding a more homogeneous intensity, i.e. low SD.

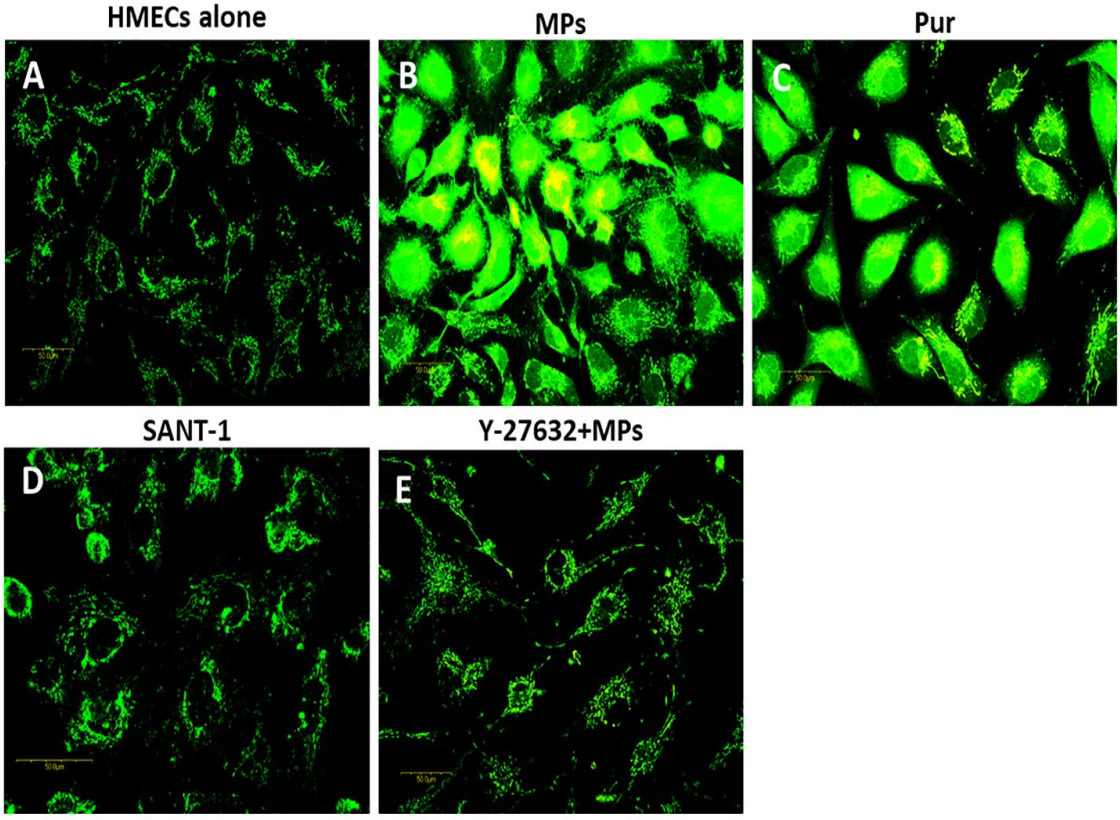

Figure 23: Apoptotic T-cell derived microparticles containing sonic hedgehog induce mitochondrial depolarization in endothelial cells via rho kinase pathway**.**

HMECs were grown on 25mm glass coverslips were treated with MPs (B) or with Pur (C) or SANT-1 (D) and or with Y-27632 one hour prior to addition of MPs (E) for 24hrs respectively. Control was HMEC's alone. After 24hrs, media was replaced by 100nM of

Rh123 in HBSS and 10μM PI and cells were imaged on confocal microscope after 20 minutes with 60x magnification and scale bar 50μm. HMECs alone showed punctuate staining of Rh123 while, treatment with MPs or Pur displayed loss of Rh123 into the cytoplasm and cell death as indicated by bright green color and yellow patches localized at nucleus respectively. Inhibition of SHH pathway by SANT-1 displayed punctuate staining as opposed to treatment with SHH agonist Pur. Inhibition of ROCK with Y-27632 and then addition of MPs prevented MPs induced mitochondrial depolarization as displayed punctate staining of mitochondria. The images are representative of three separate experiments.

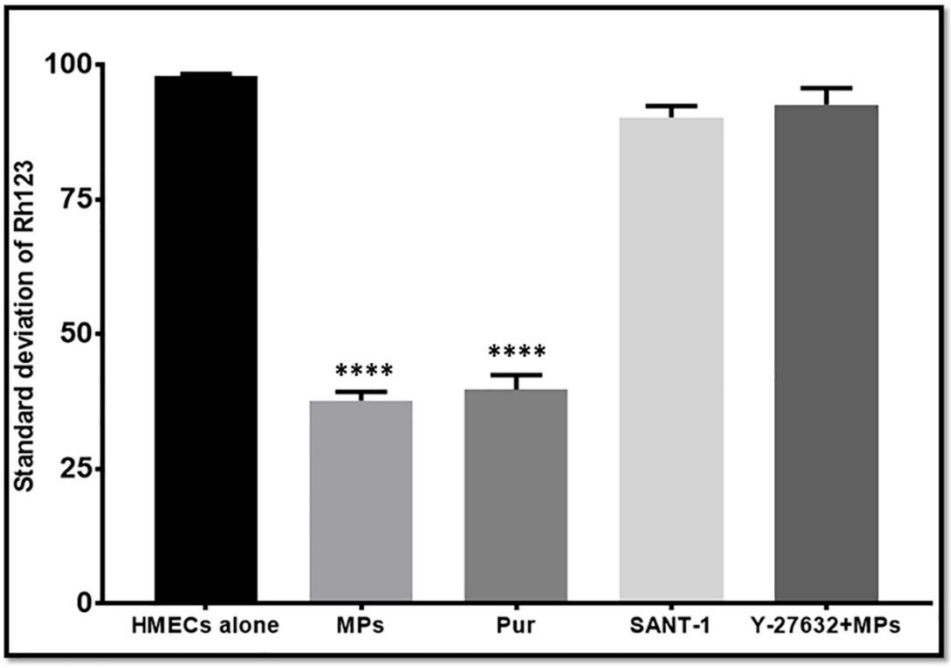

Figure 24: Quantification of Rhodamine 123 fluorescence in confocal images taken after 24hrs of treatment

HMECs were treated with MPs (B) or with Pur (C) and or with Y-27632 one hour prior to addition of MPs (D) for 24hrs. Cell were loaded with 100nM Rh123 and 10μM PI in HBSS

and confocal images were taken 20 minutes later with 60x magnification and scale bar 50μm. Degree of mitochondrial depolarization was quantified by analyzing average standard deviation of Rh123 fluorescence for 10 spots per picture using FIJI software. Statistical significance was analyzed by one-way ANOVA with post hoc Dunnett's test. ****P≤0.0001 for MPs, Pur compared to control. All data are represented as the mean ± s.e.m. of three experiments. A polarized mitochondrion shows high SD as most of the rh123 remains inside mitochondria, providing sharp contrast between mitochondria and cytoplasm. Following depolarization, Rh123 equilibrates between mitochondria and cytoplasm yielding a more homogeneous intensity, i.e. low SD.

5.3.2.Apoptotic T-Cell Derived Microparticles Carrying Sonic Hedgehog Impair Proliferation of Endothelial Cells by A Mechanism that is Independent of Rho Kinase Pathway.

Cell proliferation and migration of endothelial cells is required for endothelial barrier maintenance to prevent loss of its integrity [415]. To assess if MPs containing SHH impair endothelial cell proliferation by rho kinase pathway, MTT assay was performed. Addition of MPs to HMECs resulted in 1.5 folds reduction (-35.5%) in absorbance of MTT and thus caused significant reduction in proliferation of HMECs as compared to control (**=P<0.01). In contrast, Pur produced no significant effect on proliferation compared to control implying SHH is not involved in reducing proliferation as seen with MPs alone. Inhibition of ROCK with Y-27632 followed by addition of MPs also resulted in 1.4 folds reduction (-30%) in absorbance of MTT and therefore did not prevent MPs mediated impairment of endothelial proliferation (*=P<0.05). Together, this indicates reduced

endothelial proliferation induced by SHH containing MPs derived from apoptosis of T-cells is not RhoA/ROCK dependent (Figure 25).

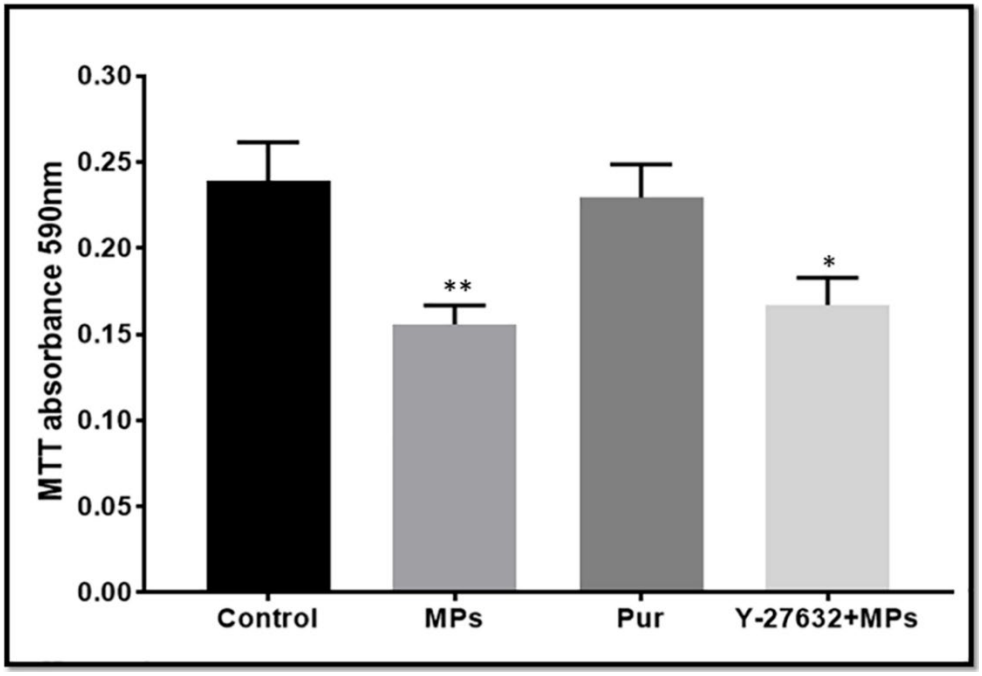

Figure 25: Apoptotic T-cell derived microparticles containing sonic hedgehog reduce proliferation of endothelial cells in a rho kinase independent manner**.**

Cell proliferation was performed with HMECs seeded in 96 well plate alone or treated with MPs or with Pur and or with Y-27632 one hour prior to addition of MPs. After 24hrs, MTT assay was performed. Treatment with MPs reduced proliferation of endothelial cells 1.5 folds as compared to control. Addition of Pur produced no significant (ns) effect on proliferation of endothelial cells. Inhibition of ROCK one hour prior to addition of MPs also reduced the proliferation 1.4 folds and therefore did not prevent MPs mediated reduction in endothelial cell proliferation. Statistical significance was analyzed by one-way ANOVA with post hoc Dunnett's test. **P<0.01; for MPs and *P<0.05 Y-27632+MPs

compared to control. All data are represented as the mean ± s.e.m. with n=9 (each performed in triplicates). MTT; 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide.

5.3.3 Apoptotic T-Cell Derived Microparticles Containing Sonic Hedgehog Significantly Decrease Endothelial Cell Viability by A Mechanism that is Not Dependent on Rho Kinase Pathway.

Endothelial cells undergo apoptosis in sepsis [416, 417]. MTT assay was performed to assess the effect of MPs containing SHH decreased viability of endothelial cells via rho kinase pathway. As shown in Figure 26, compared to HMECs alone, addition of MPs resulted in 1.8 folds reduction (-45%) in absorbance of MTT and thus caused significant reduction in viability of endothelial cells (***=P<.001vs control). In contrast, SHH agonist Pur did not show any significant effect on viability of endothelial cells implying SHH is not involved in reducing proliferation as seen with MPs alone. Inhibition of ROCK with Y-27632 one hour prior to addition of MPs also resulted in 1.8 folds reduction (-45%) in the absorbance of MTT and therefore did not rescue cell death in endothelial cells caused by MPs (***=P<0.001 compared to control). This suggests that SHH containing apoptotic T-cell derived MPs mediated reduction in survival of endothelial cell is not RhoA/ROCK dependent.

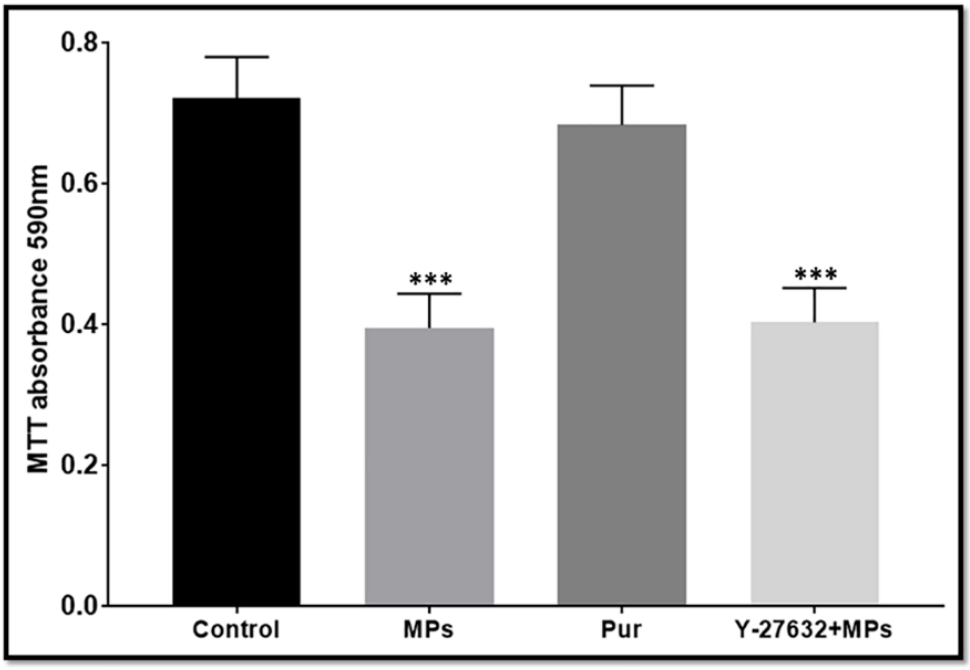

Figure 26: Apoptotic T-cell derived microparticles containing sonic hedgehog reduce viability of endothelial cells in a rho kinase independent manner.

HMECs were seeded on 96 well plates in MCDB-131 media and maintained for 24hrs. The cells were then subjected to treatment with MPs, or with Pur and or with Y-27632 one hour prior to addition of MPs. Control was HMECs alone. After 24hrs, MTT assay was performed. Treatment with MPs reduced endothelial cell viability 1.8 folds as compared to control. Addition of Pur produced no significant effect (ns) on viability of endothelial cells. Inhibition of ROCK by Y-27632 one hour prior to addition of MPs also reduced the viability 1.8 folds and therefore did not prevent MPs mediated reduction in endothelial cell viability. Statistical significance was analyzed by one-way ANOVA with post hoc Dunnett's test. ***$P<0.001$; for MPs and Y-27632+MPs compared to control. All data are represented as the mean ± s.e.m. with n=9 (each performed in triplicates). MTT; 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide.

5.4.Discussion

Organ dysfunction is a hallmark of severe sepsis despite normal or high systemic oxygen [418]. Hepatosplanchnic organs are susceptible to impaired perfusion as a result of intrinsic and extrinsic fluid losses and decreased intake, endothelial dysfunction leading to microcirculatory redistribution of blood flow, loss of vascular tone, plugging of capillaries by sequestered platelets and leukocytes, deformability of erythrocytes and hyperpermeability, all of which leads to tissue hypoxia [412] . Vascular hyporeactivity and microcirculation flow abnormities with tissue hypoperfusion have been well-recognized as a contributor of experimental and human sepsis. But, improved O_2 perfusion and delivery were ineffective in improving survival/ organ dysfunction [397] or at worst deleterious [419] and patients in early sepsis exhibit hyperdynamic state [420]. This lead to the theory that organ dysfunction in sepsis not only because of inadequate oxygen delivery but perhaps a concomitant inefficiency of cells to utilize O_2-cytopathic hypoxia [421]. Because mitochondria utilize more than 90% of total body oxygen consumption for generation of ATP via oxidative phosphorylation, dysfunction of mitochondria and decreased ATP is implicated in pathogenesis of sepsis. Microcirculatory and mitochondrial dysfunction likely co-exist in sepsis. Both the mechanisms impair oxygen delivery and utilization, but the relative contribution of either mechanism is difficult to discern and may vary considerably.

Several studies have reported alterations in oxygen utilization at the mitochondrial level during experimental sepsis. LPS has been shown to inhibit mitochondrial respiratory chain enzymes complexes [422]. Furthermore, LPS induce mitochondrial structural alterations, leading to oxygen waste through inner mitochondrial

membrane [423]. In cultured hepatocytes, the maximum oxygen consumption increased markedly following 6 hours of LPS stimulus but decreased significantly by 24hrs [424]. In humans, skeletal muscle mitochondrial dysfunction with progressive decreased oxygen consumption has been related to severity of sepsis and poor outcome [404]. Apart from compromised bioenergetics capacity, mitochondrial damage has also been deemed to contribute to mitochondrial pathology during sepsis [425]. Ultra-damage was seen in mitochondria of liver obtained from patients who died from sepsis [426]. A key feature of mitochondria that allows that to participate in cell survival is proton pumping across the impermeable inner membrane. This generates an electrochemical gradient, composed of MMP and pH, which is used for generation of ATP, transport of anions and cations to regulate volume and regulation of protons to control heat production [427]. Also, mitochondrial protects cells from death by buffering cells against high concentration of Ca^{+2} and sequestering pro-apoptotic agents such as cyt C. But in sepsis, excess ROS, NO, CO and H2S production can lead to profound depolarization of mitochondrial membrane resulting from inhibition of mitochondrial respiratory chain, alterations in intracellular calcium dynamics and opening of mitochondrial permeability transition pore [403, 428]. Loss of depolarization may lead to apoptosis, but these bring large and possibly catastrophic changes in the mitochondrial function.

Pathogenesis of mitochondrial dysfunction has been seen in different cells during severe sepsis [429]. In patient with septic shock, human peripheral blood monocytes show bioenergetics failure due to reduced activity of complex V ATP synthase. Mitochondrial membrane potential alterations and subsequent cell death were observed ex vivo in monocytes from septic patients [430]. In a pilot study consisting of 26 septic

patients, platelets displayed mitochondrial depolarization that correlated significantly with the simultaneously assessed clinical disease severity by Acute Physiological Assessment and Chronic Health Evaluation (APACHE II) score during the disease. Alterations in MMP were more pronounced in non-survivor septic patients that in survivor patients, indicating change in MMP could be used as marker for the disease outcome [177]. Also, the number of MPs released from endothelial cells decreases when their mitochondria is intact and undergoes biogenesis [431]. The change in MMP of the mitochondria in endothelial cells during sepsis and the effect of MPs on mitochondria of endothelial cells have not been examined so far. Here in this study, we hypothesized that mitochondria of endothelial cells would show alterations in MMP and this change would be mediated by apoptotic T-cell derived MPs containing SHH. To visualize and quantify the changes in MMP, we imaged Rh123 fluorescence using confocal laser scanning microscopy. Apoptotic T-cell derived MPs caused mitochondria of endothelial cells to lose their electronegativity i.e. depolarizing them. So, the Rh123 was no longer sequestered in the mitochondria and diffused into the cytosol. The resulting image was diffuse green stain throughout the cell and the measured standard deviation of the area of interest was low in numerical value (-61.5%). Also, since change in MMP can lead to cell death, we also hypothesized that apoptotic T-cell derived MPs will significantly reduce viability and proliferation of endothelial cells. To assess cell viability and proliferation, we measured the absorbance of MTT. Apoptotic T cell derived MPs significantly reduced the viability and proliferation of endothelial cells. So, the reduction of MTT to purple insoluble formazan crystal was less. The resulting absorbance of MTT at 590nm with a reference filter of 620nm was lower by 45% and 35% respectively as compared to control. We also elucidated the mechanism that

drives apoptotic T-cell mediated mitochondrial dysfunction and decreased viability and proliferation of endothelial cells.

RhoA/ROCK pathway has been implicated in mediating mitochondrial dysfunction. Activation of RhoA/ROCK decreases activity of complex IV cytochrome C oxidase (COX) [170]. COX plays a critical role in transferring electrons to O_2 in the final step of ETC cycle, allowing its complete reduction into water and contribute to generation of MMP across inner mitochondrial membrane essential for ATP generation. Decreased Cox activity by RhoA/ROCK leads to compromised MMP which in turn decreased the efficiency of oxidative phosphorylation and therefore decreased ATP production and increased ROS production [170, 179]. Also, Rho kinase up-regulates 3-4-fold increase in pro-apoptotic Bcl-2 family protein BAX mRNA and its localization on the mitochondria to activate mitochondrial death pathway. However, which signaling molecule/s activate RhoA/ROCK to induce mitochondrial depolarization and cell death of endothelial cells in sepsis is not known. Our results show that apoptotic T-cell derived MPs containing SHH activate RhoA/ROCK pathway to mediate mitochondrial depolarization in endothelial cells. However, the apoptotic T-cell derived MPs mediated decrease in viability and proliferation was rho kinase independent.

5.5.Summary and Conclusions

In summary, the result of this study provide evidence that SHH containing apoptotic T-cell derived MPs mediate endothelial mitochondrial dysfunction by depolarizing them, as indicated by diffused bright green Rh123 fluorescence and low value of SD (-61.5%).These apoptotic T-cell derived MPs also significantly decrease viability and proliferation of endothelial cells as indicated by 45% and 35% decrease in MTT absorbance respectively.

Direct stimulation of SHH pathway by Pur also induced endothelial mitochondrial depolarization, as indicated by loss of Rh123 into cytoplasm and low value of SD (-59.5%). While inhibition of SHH pathway prevented depolarization of endothelial mitochondria, as indicated by high SD value of Rh123. This indicates that SHH pathway mediates MPs induced endothelial mitochondrial depolarization. However, SHH pathway does not contribute to reduced proliferation and viability of endothelial cells. Addition of ROCK inhibitor one hour before addition of MPs prevented depolarization of endothelial cell mitochondria, as indicated by punctuate staining of Rh123 and high SD value that is not significantly different from control. This indicates that SHH in MPs activates rho kinase pathway to induce endothelial mitochondrial depolarization. Therefore, one potential molecule that can activate rho kinase pathway to induce endothelial mitochondrial depolarization is SHH containing MPs derived from apoptotic T-cell derived MPs.

CHAPTER 6: EFFECT OF SONIC HEDGEHOG CONTAINING APOPTOTIC T-CELL DERIVED MICROPARTICLES ON NITRIC OXIDE PRODUCTION AND ENDOTHELIUM-DEPENDENT VASODILATION IN ARTERIES VIA ACTIVATION OF RHO KINASE PATHWAY

6.1.Introduction

In present study we tested if apoptotic T-cell derived microparticles (MPs) containing sonic hedgehog (SHH) induce endothelial dysfunction by decreasing nitric oxide (NO) production and ATP mediated endothelium dependent dilation in porcine mesenteric arteries via activation of rho kinase pathway (RhoA/ROCK). NO is a highly diffusible, labile and reactive free radical gas mediator. Endogenous NO is generated largely from enzymatic pathways involving nitric oxide synthase (NOS) by oxidation of guanidine nitrogen atoms in L-arginine to form NO and L-citrulline but a non-enzymatic pathway also exist [432]. There are four different isoforms of NOS in mammals: Endothelial NOS (eNOS) found in endothelial cells, epithelial cells and cardiac myocytes, Neuronal NOS (nNOS) found in neuronal cells and skeletal muscle, inducible NOS (iNOS) that is induced by inflammatory mediators in macrophages, hepatocytes, smooth muscle and other tissue and mitochondrial NOS (mtNOS) whose expression is still under debate. eNOS and nNOS are expressed constitutively producing low burst of NO and are activated by increase in calcium (Ca^{+2}) to regulate vascular tone [433] and neurotransmission [434] respectively. Diverse extracellular stimuli including vascular endothelial growth factor (VEGF), ATP, acetylcholine (Ach), shear stress and other agents that increase intracellular Ca^{+2} can activate eNOS and nNOS to produce NO constitutively [435]. Whereas, iNOS is functionally Ca^{+2} independent and activated in immune cell following endotoxin or pro-

inflammatory cytokine stimulation for sustained production of large quantities of NO to allow NO-mediated cytotoxic destruction of microorganisms after phagocytosis [436].

Nitric oxide (NO) is a highly reactive molecule that is widely utilized as signaling molecule in cells throughout the body. In vasculature, NO regulates vascular tone and blood flow by mediating vasodilation of blood vessels. Constriction and dilation of blood vessels in response to environmental stimuli depends on the changes in intracellular Ca^{+2}. Vessels constrict by increase in Ca^{+2} through influx of Ca^{+2} via ion channels or release from sarcoplasmic reticulum (SR) Ca^{+2} stores. Increased Ca^{+2} along with calmodulin (CaM) leads to phosphorylation of myosin and subsequent sliding of myosin over actin and thereby contraction [437]. Dilation of vessels is initiated in endothelial cells by Ca^{+2} mediated stimulation of eNOS that produces NO constitutively in low bursts. NO diffuses into the SMC and stimulates SMCs to relax by either direct or indirect removal of elevated Ca^{+2} and/ or by decreasing sensitivity of the contractile apparatus to Ca^{+2}. Direct effects of NO include re-uptake of Ca^{+2} into intercellular stores by stimulation of sarcoplasm reticulum Ca^{+2} ATPase (SERCA) [438], inhibition of SMC Ca^{+2} release by inositol 1,4,5, triphosphate (IP3) [439] and opening of Ca^{+2} activated potassium channels [440]. Indirect effects of NO include activation of soluble guanylate cyclase (sGC) and guanosine 3', 5'-cyclic monophosphate (cGMP) which in turn effects Ca^{+2} influx or efflux via stimulation of myosin phosphatase and dephosphorylation of myosin [441], leading to relaxation of SMC.

Apart from maintenance of vascular tone and permeability, NO keeps the platelet adhesion and activation in check. In acute damage, platelet aggregation is essential to initiate clotting and stop bleeding by forming a physical plug to heal the hemorrhaging

vessel. But when the injury to the endothelium is mild, platelet aggregation initiates inflammatory response leading to vascular complications including thrombosis, stroke [442]. Low amount of NO produced in normal homeostasis by eNOS and nNOS inhibits platelet inhibition mediated by activation of cGMP dependent protein kinases, a known platelet inhibitor [443]. While, high concentrations of NO produced by activation of iNOS mediate platelet aggregation as seen in many disease conditions including sepsis patients [444].

Nanomolar concentrations of NO produced by eNOS inhibit cytochrome oxidase (complex IV in electron transport chain of mitochondrial) in competition with O_2, a means by which rate of cellular respiration in mitochondria is regulated and is completely reversible when NO is removed [445]. However, high concentrations of NO can inhibit other complexes of the mitochondrial respiratory chain by nitrosylating them or oxidizing protein thiols and removing irons from the iron-sulphur centers [446]. Peroxynitrite ($ONOO^-$) formed by reaction between high concentrations of NO with superoxide (O_2^-) also causes irreversible inhibition of mitochondrial components via oxidizing reactions [447].

Leukocyte recruitment is a dynamic cellular process in inflammation that requires leukocyte tethering, rolling, adhesion and ultimately emigration from microvasculature into the injury site [448]. NO produced by eNOS modulate the expression of selectins which mediate tethering and rolling during leukocyte recruitment and therefore prevent leukocyte adhesion and production of pro-inflammatory mediators [449]. While, iNOS mediated prolonged periods of NO and ROS produced by cells of innate immunity play dual role of an immunotoxin as well as an immunomodulatory. High amounts of NO

and ROS not only intercept and kill pathogens but also activates downstream signaling pathways that lead to full expression of immune response [450]. But, excess production of NO and ROS due to over activation of immune cells can also lead to damage of body's own cells and has been implicated in diseases including sepsis [451].

Lack of constitutive low NO produced by eNOS and nNOS and continuous production of excess NO by iNOS contribute significantly to pathophysiology of sepsis [452]. Large amount of iNOS induced NO contributes to cardiac depression, impaired vasoconstriction, hypotension and $ONOO^-$ mediated tissue damage, all characteristic of sepsis. But, selective inhibitors of iNOS does not improve rather increase mortality, although, they were able to restore blood pressure in experimental models of sepsis and reverse hypotension in human endotoxemia. However, non-selective inhibition of NOSs is detrimental with patients showing increased systemic vascular resistance, elevated pulmonary artery pressure, reduced cardiac output and oxygen delivery, increased platelet accumulation and survival is not improved. Some of these detrimental effects were due to inhibition of eNOS that play critical role in many physiological processes. Selective inhibitors that can elevate the expression of down-regulated eNOS have been shown to increase survival of rodent model of sepsis [434].

Endothelium dysfunction is characterized by reduction of the bioavailability of vasodilators, in particular , nitric oxide (NO), whereas endothelium derived contracting factors are increased [453]. This imbalance leads to impairment of endothelium dependent vasodilation, which represent the functional characteristic of endothelial dysfunction. This decreased NO production is mainly due to reduced expression/activity of eNOS. Accumulating evidence suggest that RhoA/ROCK activation

mediates endothelial dysfunction by preventing activation of eNOS. Also, T-cell derived MPs have been shown to decrease NO by reducing eNOS expression. But, how RhoA/ ROCK pathway gets activated to decrease NO and mediated vasodilation in sepsis is unknown. In present study, we hypothesized that apoptotic T-cell derived microparticles containing SHH reduce NO production and NO-mediated vasodilation in porcine mesenteric arteries by activating rho kinase pathway.

6.2.Materials and Methods

6.2.1.HMEC Cell Culture

Human microvascular endothelial cells (HMECs) were a kind gift from Dr. Vijay Kumar Kalra (University of Southern California, Los Angeles, California). HMECs are immortalized cells obtained from human foreskins. The cells were maintained in MCDB-131 growth media without L-glutamine (Thermo Fisher Scientific; Rockford, IL) supplemented 10ng/mL endothelial growth factor (EGF), 1µg/mL hydrocortisone, 10mM GlutaMAX (Life Technologies, Carlsbad, CA), 10% heat-inactivated fetal bovine serum (FBS) (Serum Source, Charlotte, North Carolina) and 10% penicillin-streptomycin (Thermo Fisher, Waltham, MA). Cell were maintained in T-75 flask at 37°C under an atmosphere of 5% $C0_2$ and 95% air. Cell were harvested using 0.25% Trypsin-0.53mM EDTA solution and seeded on collage-coated wells, allowing them to attach overnight. Cells were quiesced the following day in 0.1% FBS medium overnight. The next day, medium was refreshed with 1% FBS medium and treatments were added for either 6 or 24hrs.

6.2.2.Isolation of Porcine Second Order Mesenteric Arteries

Once all the internal organs were out of the pig abdominal cavity, the greater omentum was incised. With the splenic artery as a landmark, the celiac trunk was located and dissected to completely expose the upper part of the abdominal aorta. The dissection was continued along the downside of abdomen, until the beginning of superior mesenteric artery was found. At this point, 50mL of cold Krebs buffer was injected into the 1st order branch of the superior mesenteric artery to remove blood. The second and third order branches of superior mesenteric artery were then carefully excised with the lengths as long as possible.

6.2.3.Reagents

4-Amino-5-methylamino-2’7’-difluorofluorescein, Diaminofluorescein-FM (DAF-FM Diacetate) was obtained from Thermo Fisher Scientific (Rockford, IL). Endothelin-1 was obtained from American Peptide Company (Palo Alto, Calif). Adenosine 5’-triphosphate disodium salt hydrate (ATP), Purmorphamine (Pur), Hanks balanced salt solution (HBSS), Dulbecco’s phosphate buffered saline (DPBS) was obtained from Sigma-Aldrich, (St. Louis, MO), Y-(R)-(+)-trans-N-(4-Pyridyl)-4-(1-Aminoethyl) cyclohexanecarboxamide dihydrochloride monohydrate was acquired from Cayman chemical (Ann harbor, MI).

6.2.4.Treatments

Each experiment consists of five treatment groups. (1) Untreated HMECs (negative control), (2) HMECs treated with 50µg protein/mL $^{\mathbf{SHH+}}$MPs, (3) HMECs treated with 10µM Purmorphamine (Pur). Purmorphamine activates the HH signaling pathway by directly binding to SMO, a downstream protein in this pathway. If we can duplicate the effect of MPs with Pur, this would suggest that SHH pathway does contribute to the MPs

mediated endothelial dysfunction (positive control). (4) HMECs treated with 10μM SANT-1, an antagonist of SMO. If the effect of Pur on endothelial cells is abated by SANT-1, this would further ensure involvement of SHH pathway in mediating ED. (5) HMECs treated with 10μM Y-27632 one hour prior to treatment addition of MPs. Y-27632 is cell permeable, highly potent and selective inhibitor of ROCK. Y-27632 inhibits both ROCK-1 and ROCK-2 by competing with ATP for binding to the catalytic site. If inhibition of ROCK abrogates or reduces the effect of $^{SHH+}$MPs on endothelial cells this would indicate that indeed these SHH containing microparticles derived from apoptotic T-cell induce endothelial dysfunction through activation of RhoA/ROCK pathway. The treatments were done for a period of 24 hours to simulate acute inflammation during which large degree of sepsis induced T-cell apoptosis is documented to occur.

6.2.5. DAF-FM Staining to Detect Nitric Oxide Production

DAF-FM staining was done to determine if apoptotic T-cell derived MPs containing SHH impair intracellular NO production in endothelial cells via RhoA/ROCK pathway. DAF-FM diacetate (Molecular probes INC.) is a cell permeant dye that is essentially non-fluorescent until it reacts with NO and other auto-oxidation derivatives of NO such as N_20_3 to yield highly fluorescent benzotriazole. HMECs were plated on 25mm glass coverslip in a 6 well plate at a density of $1.5X10^5$cells /mL in MCDB-131 media supplemented with 10% FBS at 37°C under an atmosphere of 5% C02. After indicated treatments for either 6 or 24hrs, cells were washed with PBS twice and then treated with DAF-FM diacetate (2.5μM; Thermo Fischer Scientific; Rockford, IL) for 20 minutes at 37°C. After excess probe was removed, cells were incubated for additional 20 minutes to allow complete de-esterification of the intracellular diacetates. Coverslip was mounted on a chamber with

1mL warm HBSS and imaged using 60x objective of laser reflected confocal scanning fluorescence microscope (Olympus FV 500) with a Fluoview operating software. After recording the unstimulated fluorescence, cells were stimulated with 10nM endothelin-1 (ET-1) to activate eNOS. The confocal images were captured every 30 secs for a period of 30 minutes using XYT function at emission/excitation wavelength of 495/515nm and fluorescence in green channel (DAF-FM) fluorescence was visualized.

6.2.6. Offline Image Analysis

Offline image analysis was performed by using FIJI software. 60X images recorded with FITC green filters were used. Ten areas of interest were randomly selected per image with total of n=3 per group. Average mean fluorescence intensity of DAF-FM fluorescence was measured, to quantify the extent of NO production. Greater is the NO production, brighter is the green fluorescence and hence higher is the mean of fluorescence intensity.

6.2.7. Isolated Organ Chamber Experiments to Test Ex-Vivo Endothelium Dependent Vasodilation

To test if apoptotic T-cell derived MPs containing impair ATP induced ex-vivo endothelium dependent vasodilation via RhoA/ROCK pathway, second order mesenteric arteries were excised from pigs. They were placed in ice cold Krebs buffered solution (119mM Nacl, 7.97µM $MgSO_4$, 7.95µM $MgSO_4.7H_20$, 1.2mM KH_2PO_4, 4.7mM KCL, 25mM $NaHC0_3$ 0.1mM EDTA, 1.5mM $CaCl_2$, 5.5mM glucose, adjusted to pH 7.4, gassed with 95% O_2 and 5% $C0_2$) after isolation. Then, the arteries were carefully cleaned of fat and excess connective tissue and cut into 5mm rings with a blade by using measuring scale as a guide. The rings were then placed one per well in a 96 well plate containing 200µL of MCBD-131 media with indicated treatments and incubated for 6hrs at 37°C under an

atmosphere of 5% C02. One aortic ring per treatment group was mounted on two pins in four respective modular tissue bath chambers (TISSUEBATH4; BIOPAC systems; Goleta, CA) and attached with nylon ties. Each chamber was filled with Krebs buffer that was continuously maintained at 37°C and gassed with 95% O_2 and 5% CO_2 at pH 7.4. Arterial rings were progressively stretched to 1g equivalent force passive tension in 0.1g steps and allowed to equilibrate for 30 minutes. Cumulative ATP (10^{-8} M to 10^{-3} M, 5 minutes between each concentration) concentration- response curves were obtained after pre-contraction of the rings with 40mM KCL (80% of the maximal contractile response). The presence of functional endothelium was assessed by ability of ACH (10^{-3}M) to induce more than 50% relaxation of vessels pre-contracted with KCL 40mM. Isometric tension was collected by force transducers and recorded using BIOPAC MP-150A-CE AcqKnowledge software.

6.2.8. Statistical Analysis

All data are presented as means ± standard error of the mean (SEM). Statistical analysis was performed using GraphPad Prism software (San Diego, California). Statistical significance was assessed by one-way analysis of variance (ANOVA) with independent Dunnett's *post hoc* test was used when statistical differences were detected. Statistical significance was set at $p < 0.05$.

6.3.Results

6.3.1.Apoptotic T-Cell Derived Microparticles Containing Sonic Hedgehog Impair Nitric Oxide Production in Endothelial Cell by Activation of Rho Kinase Pathway.

Decrease in vasodilator NO bioavailability is a key event resulting from endothelial dysfunction [78, 454, 455]. We have previously shown that Y-27632 mediated ROCK

inhibition restores ET-1 mediated NO production in liver sinusoidal endothelial cells after LPS pre-treatment. To determine the molecule that activate rho kinase pathway, we tested if MPs derived from apoptotic T-cell containing SHH decrease NO production via activation of rho kinase pathway in endothelial cells. HMECs were loaded with DAF-FM (5uM), a dye that specifically reacts with NO to yield highly fluorescent benzotriazole. After recording the unstimulated fluorescence in confocal microscope, cells were stimulated with 10nM endothelin-1 (ET-1) to activate eNOS mediated NO production. The confocal pictures were captured every 30 secs for a period of 30 minutes. The images shown here are for 15 minutes as the change in fluorescence was constant after this. Control HMECs (6hrs: Figure 27A, 24hrs: Figure 29A) responded to ET-1 stimulation with robust DAF-FM fluorescence indicative of significant NO production. Addition of MPs (6hrs: Figure 27B, 24hrs: Figure 29B) to HMECs for 6 or 24hrs resulted in significantly lower DAF-FM fluorescence (****=P<0.0001 vs control at 20 minutes), indicating reduced ET-1 stimulated NO production. Direct activation of the SHH pathway via Pur (6hrs: Figure 27C, 24hrs: Figure 29C) also showed significantly reduced ET-1 stimulated NO production similar to effect of MPs (****=P<0.0001 vs control at 20 minutes) while SHH antagonist SANT-1 (24hrs: Fig.29D) impeded this decrease in NO production. Rho kinase pathway activation was assessed by inhibiting ROCK using Y-27632 one hour before addition of MPs. MPs mediated reduction in NO production was largely restored in presence of Y-27632 (6hrs: Figure 27D, 24hrs: Figure 29E) as indicated by bright DAF-FM fluorescence that is not significantly different from control. Collectively, these results demonstrate that SHH containing apoptotic T-cell derived MPs significantly impair NO production via activation of rho kinase pathway. To quantify NO production, we determined average mean

fluorescence intensity of DAF-FM displayed by the confocal images taken after 6hrs (Figure 28) and 24hrs (Figure 30) were measured using FIJI software.

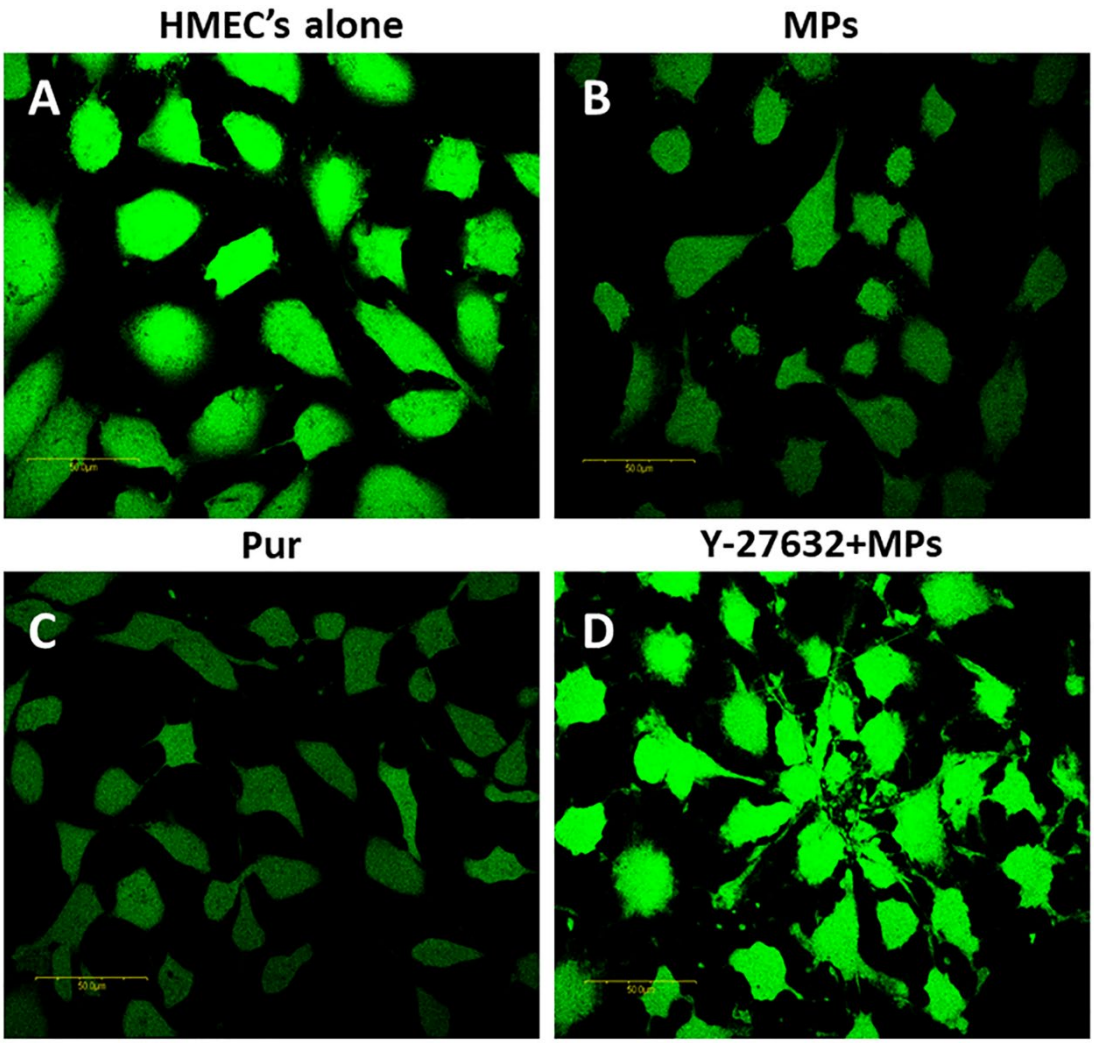

Figure 27: Apoptotic T-cell derived microparticles containing sonic hedgehog significantly diminish NO production in endothelial cells via rho kinase pathway.

HMECs were plated on 25mm glass coverslips were treated for 6hrs with either MPs (B) or Pur (C) and or ROCK inhibitor Y-27632 (D) one hour prior to treatment with MPs. HMECs alone are control (A). HMEC's were loaded with DAF-FM (5uM) and then imaged on a confocal microscope. NO production was localized by visualizing

fluorescence in green channel (DAF-FM). After recording the unstimulated fluorescence, cells were stimulated with 10nM endothelin-1 to activate eNOS mediated NO production. The confocal pictures were captured every 30 secs for a period of 30 minutes with 60x magnification and scale bar=50μm. The images shown here are for 15 minutes as the change in fluorescence was constant after this. HMECs alone displayed robust production of NO as indicated by bright green DAF-FM fluorescence. Treatment of HMECs with MPs significantly reduced NO production as indicated by diminished fluorescence in comparison with control. Addition of SHH agonist Pur also showed dim DAF-FM fluorescence as $^{SHH+}$MPs. Inhibition of ROCK one hour before addition of MPs (D) mostly abolished MPs induced impairment in the NO production as indicated by bright green DAF-FM fluorescence that is like control. Representative images from three experiments are shown.

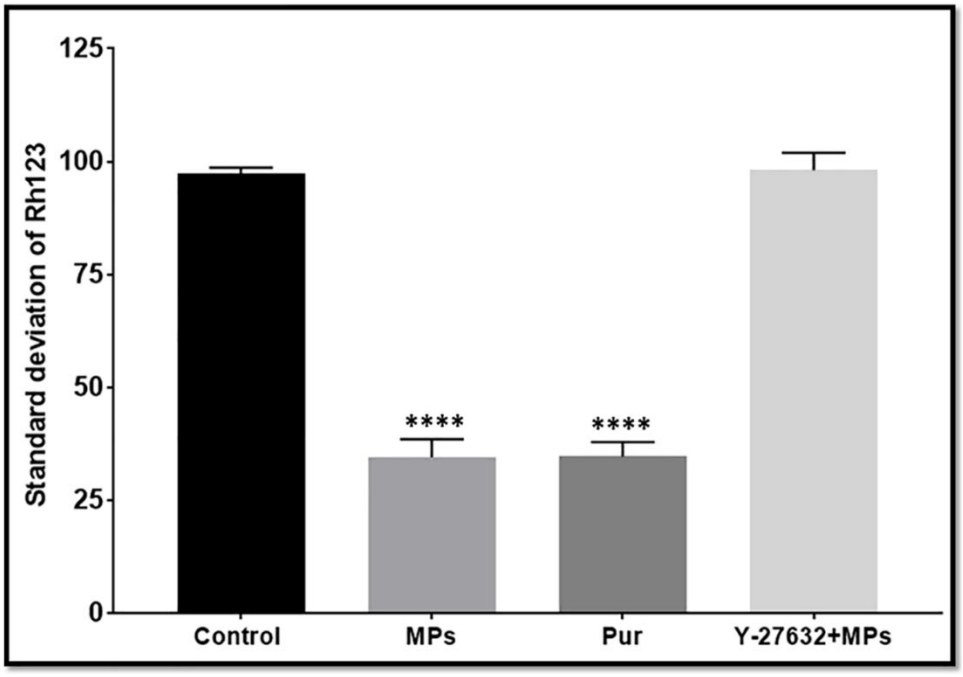

Figure 28: Quantification of DAF-FM fluorescence in confocal images taken after 6hrs of treatment

HMECs were treated with MPs (B) or with Pur (C) and or with Y-27632 one hour prior to addition of MPs (D) for 6hrs. Cell were loaded with 5uM DAF-FM in HBSS and confocal images were taken 20 minutes later with 60x magnification and scale bar 50µm. To quantify the degree of NO production, average mean of fluorescence intensity of DAF-FM fluorescence was measured for 10 spots per picture using image master software. Statistical significance was analyzed by one-way ANOVA with post hoc Dunnett's test. ****$P \leq 0.0001$ for MPs, Pur compared to control. All data are represented as the mean ± s.e.m. of three experiments.

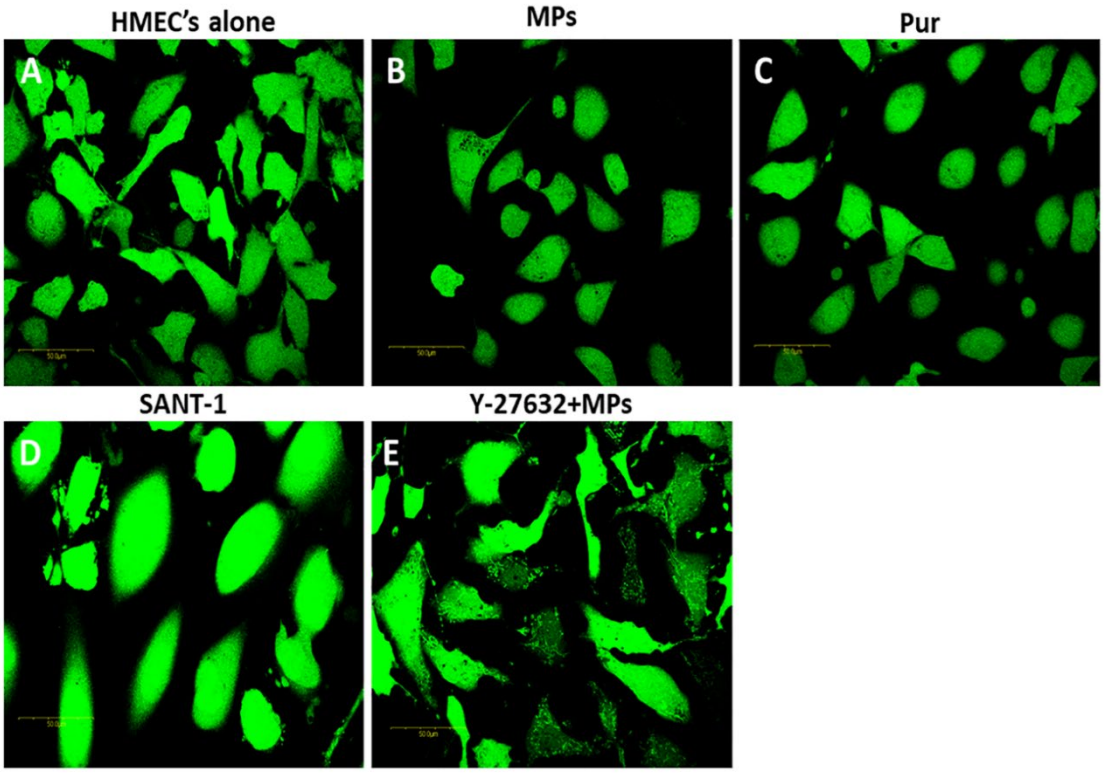

Figure 29: Apoptotic T-cell derived microparticles containing sonic hedgehog significantly diminish NO production in endothelial cells via rho kinase pathway.

HMECs were plated on 25mm glass coverslips. After 24hrs, HMEC's were treated for 24hrs with either MPs or Pur or SANT-1 and or Y-27632 rho kinase inhibitor one hour prior to treatment with MPs. HMEC's were loaded with DAF-FM (5uM) and then imaged on a confocal microscope. NO production was localized by visualizing fluorescence in green channel (DAF-FM). After recording the unstimulated fluorescence, cells were stimulated with 10nM endothelin-1 to activate eNOS mediated NO production. The confocal pictures were captured every 30 secs for a period of 30 minutes with 60x magnification and scale bar=50µm. The images shown here are for 15 minutes as the change in fluorescence was constant after this. HMECs alone displayed robust production

of NO as indicated by bright green DAF-FM fluorescence. Treatment of HMECs with MPs significantly reduced NO production as indicated by diminished fluorescence in comparison with control. Addition of SHH agonist Pur also showed dim DAF-FM fluorescence as $^{SHH+}$MPs. Inhibition of SHH pathway by SANT-1 (D) largely restored NO production as opposed to decrease seen with SHH activator Pur. Inhibition of ROCK one hour before addition of MPs (E) mostly abolished MPs induced impairment in the NO production as indicated by bright green DAF-FM fluorescence that is similar to control. Representative images from three experiments are shown**.**

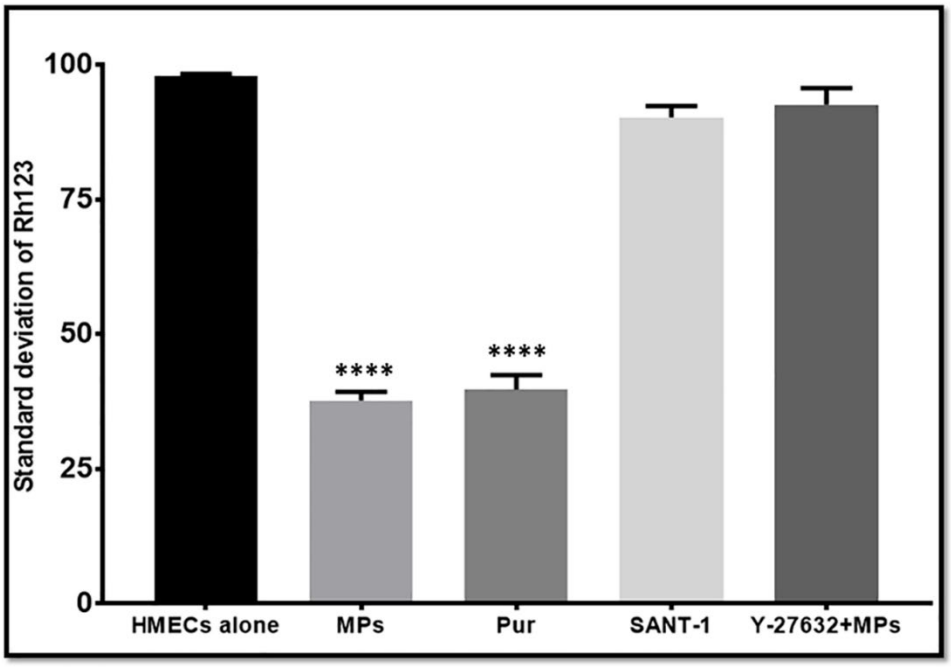

Figure 30: Quantification of DAF-FM fluorescence in confocal images taken after 24hrs of treatment

HMECs were treated with MPs (B) or with Pur (C) and or with Y-27632 one hour prior to addition of MPs (D) for 24hrs. Cell were loaded with 5uM DAF-FM in HBSS and confocal images were taken 20 minutes later with 60x magnification and scale bar 50µm. Average

mean of fluorescence intensity for 10 spots per was measured using FIJI software to quantitate the extent of NO production. Statistical significance was analyzed by one-way ANOVA with post hoc Dunnett's test. ****P≤0.0001 for MPs, Pur compared to control. All data are represented as the mean ± s.e.m. of three experiments.

6.3.2.Apoptotic T-Cell Derived Microparticles Containing Sonic Hedgehog Impair Ex-Vivo Endothelium Dependent Relaxation in Porcine Second Order Mesenteric Arteries via Rho Kinase Pathway

To establish pathophysiological relevance of MPs, we evaluated if MPs containing SHH impair the ex-vivo endothelium-dependent relaxation in response to ATP via activation of rho kinase pathway. Isolated 5mm vessel rings were obtained from porcine second order mesenteric artery. Vessel rings were treated for 6hrs with either vehicle or MPs or Pur and or with ROCK inhibitor Y-27632 one hour prior to treatment with MPs, in a 96 well plate with MCDB-131 media. Exposure of MPs or direct stimulation of SHH with Pur to porcine mesenteric arteries significantly reduced the mean maximal ATP-evoked relaxation by 10.93 ± 1.1 and 4.48 ± 3.11 percent respectively vs 27.16 ± 2.65 percent in control arteries. Inhibition of ROCK with Y-27632 one hour prior to treatment with MPs showed an average mean relaxation of 32.93± 4.47 vs 27.16± 2.65 that was not significantly different than in control arteries and thereby prevented MPs mediated impairment in endothelium-dependent relaxation response to ATP. Altogether, this data indicates that SHH containing apoptotic T-cell derived MPs impair ex-vivo endothelium dependent vasodilation of arteries via activation of rho kinase pathway (Fig. 31).

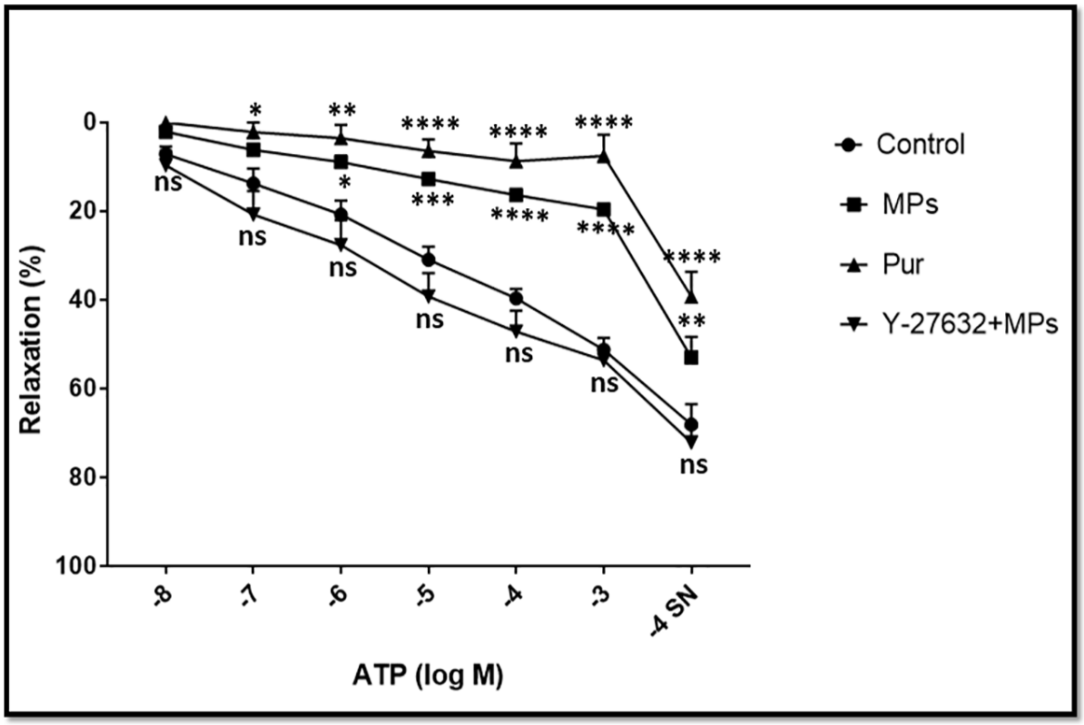

Figure 31: Apoptotic T-cell derived MPs containing SHH impair endothelium-dependent relaxation in porcine second order mesenteric arteries via rho kinase pathway.

Endothelium dependent relaxation to different concentration of ATP is shown in 5mm vessel rings exposed for 6hrs to vehicle (●; n=6), 50µg protein/mL MPs (■ ; n=6), 10µM Pur (▲ ; n=6) and 10µM ROCK inhibitor Y-27632 one hour prior to addition of MPs (▼; n=6) in 96 well plate with MCDB-131 media. Results are expressed as percentage of relaxation to different concentrations of ATP after 40mM KCL induced pre-contraction. Statistical significance was obtained by conducting two-way ANOVA with post hoc Dunnett's multiple comparison test. ****=$P<0.0001$; MPs, Pur at 10^{-4}M ATP, 10^{-3}M ATP compared with control at 10^{-4}M ATP, 10^{-3}M ATP respectively. ***=$P<0.001$, Pur at 10^{-5}M ATP compared with control at 10^{-} M ATP. **=$P<0.01$, Pur at 10^{-6}M ATP compared to control at 10^{-6}M ATP. *=$P<0.05$, Pur at 10^{-7}M, compared with control at 10^{-7}M ATP.

*=P<0.05, MPs at 10^{-6}M ATP compared to control at 10^{-6}M ATP. All data are represented as the mean ± s.e.m. with 6 experimental repeats.

6.4.Discussion

The homeostatic balance between of iNOS and eNOS alters during progression of sepsis, contributing to systemic hypotension resulting in impaired tissue perfusion and oxygen extraction, multiple organ system failure and eventually mortality [456]. In sepsis, various stimulators such as LPS, pro-inflammatory cytokines induce initial activation of eNOS followed by induction of iNOS. Interestingly, during the late phase of sepsis, there is an impairment in biosynthesis of constitutive NO production by downregulation of eNOS in vascular endothelium, as the inducible form is expressed. NO overproduction for prolonged period (up to 10hrs) due to activation of iNOS, despite presence of negative feedback mechanism has been implicated in pathophysiology of microcirculatory failure and organ dysfunction in sepsis. Induction of iNOS in various immune cells in necessary for cytostatic and cytotoxic effects of the NO that facilitate host defense [457]. But, sustained over production of NO is deleterious to host as it can induce platelet aggregation, impair mitochondrial respiration, cell cycle arrest, apoptosis, and production of strong potent oxidant peroxynitrite ONOO-, hypotension, decreased responsiveness to vasoconstrictors [458, 459]. This is evident by the increased levels of nitrite and nitrate measured in plasma of septic patients and inflammation induced iNOS expression [460]. Reducing the overproduction of NO by inhibition of iNOS was postulated to be beneficial intervention in the treatment of sepsis. Selective iNOS inhibitors were able to restore blood pressure in experimental models of sepsis and reverse hypotension in human endotoxemia [461]. However, non-specific NOS inhibitor N^G-methyl-L-arginine hydrochloride did not

improve rather increased mortality in a multicenter, randomized, double-blind, placebo-controlled trial of patients with septic shock although hypotension was improved [462]. Also, treatment with NOS inhibitor N^{w}-methyl-L-arginine hydrochloride raised vascular resistance but increased mortality in awake canines challenged with endotoxin [463]. This suggests that a low basal NO synthesis is critical for organ perfusion and survival in endotoxin shock and the deleterious effects of NOS inhibitors in endotoxin shock even though improve hypotension might be related to blockade of eNOS.

A potential role of eNOS in the pathophysiology of sepsis is unsubstantiated. Initial studies suggested that eNOS knock out mice still have high blood pressure [464] and it has been shown recently that eNOS-derived NO is vital for facilitating iNOS expression in sepsis [465]. However, chronic overexpression of eNOS generated similar levels of plasma nitrite and nitrate levels but displayed resistance to LPS-induced hypotension, lung injury and death [466]. Moreover, eNOS expression was diminished in blood vessels from rabbits [467] and in lung tissue from mice [468] following induction of sepsis with LPS. Also, 3-hydroxyl-3-methyl-glutaryl-coenzyme A (HMG-CoA) reductase inhibitor such as simvastatin and other statins have been shown to upregulate GTP cyclohydrolase I, a key enzyme of tetrahydrobiopterin (BH4), a cofactor for eNOS activation and thereby reverse down-regulation of eNOS expression and profoundly increase survival in murine model of sepsis [469]. As the low concentration of NO produced by eNOS under physiological conditions provides important regulations including vascular tone, bronchial tone, neurotransmission or immune defense [434], impairment and expression of activity of eNOS, if any may contribute to tissue injury in sepsis by hindrance of physiological regulatory events with involvement of eNOS-derived

NO [470]. Thus, we specifically chose to study if apoptotic T- cell derived MPs induce endothelial dysfunction by decreasing eNOS mediated NO production in vitro and endothelium dependent dilation in pig second order mesenteric arteries ex-vivo.

T-lymphocyte derived MPs have been shown to decrease protein expression of eNOS, altered levels of NO and prostacyclin and impair endothelial mediated dilation in response to agonist and flow in both conductance and resistance MP-treated arteries [327]. But another study reported that the SHH carried by T-lymphocyte derived microparticles corrects angiotensin II mediated hypertension and endothelial injury by inducing NO production [242]. In our study, we hypothesized that SHH carried by apoptotic T-cell derived MPs will diminish NO production in endothelial cells. To visualize and quantify the changes in NO production, we imaged DAF-FM diacetate fluorescence using laser scanning confocal microscopy. Apoptotic T-cell derived MPs significantly diminished NO production in endothelial cells. So, the conversion of DAF-FM into highly fluorescent benzotriazole was low. The resulting image showed dim green fluorescence throughout the cell and the measured mean of the area of interest was low in numerical value.

We also tested if apoptotic T-cell derived MPs impair ATP mediated endothelium dependent vasodilation in porcine mesenteric arteries. Extracellular ATP levels function as autocrine or paracrine mediators via activation of purinergic P2 receptors that facilitate release of Ca^{+2} from intracellular stores [471]. Ca^{+2} in turn activates eNOS to produce NO, which mediates dilation of SMC via indirect and direct mechanisms. To assess vasodilation, 5mm aortic rings were placed in isolated organ bath equipped with an isometric transducer to measure force/tension when tissue responds to stimuli by

contracting or relaxing. The aortic rings were pre-contracted with KCL and then endothelium dependent relaxation response to different doses of ATP (10^{-8}M to 10^{-3}M) was measured. The contraction and relaxation response were determined based on initial increase in isometric tension (80% of the maximum contractile response) followed by decrease in tension (more than 50% from initial pre-contracted tension for highest dose of ATP) respectively. Decrease in tension i.e. vasodilation will only be seen if a functional endothelium is present. Apoptotic T-cell derived MPs significantly impaired endothelium dependent vasodilation in ex-vivo porcine mesenteric arteries. The ATP induced concentration-dependent relaxation with a maximum of 60% at to 10^{-3}M was severely limited by MPS to relaxation of about 13%.

A hall mark of endothelial dysfunction is reduced availability of NO, which may be caused by reduced expression of eNOS, inactivation of eNOS or utilization of NO for production of reactive oxygen species (ROS). Activation of RhoA/ROCK pathway have been implicated in reducing the expression and activity of eNOS via different mechanisms [132, 146, 147]. In the mesenteric arteries of hypertensive profilin1 transgenic mice, RhoA/ROCK pathway activation significantly reduced eNOS expression by preventing stimulatory phosphorylation at ser1117 [149]. Thrombin is reported to decrease eNOS mRNA level by shortening the half-life of eNOS mRNA via activation of RhoA and ROCK in human endothelial cells [472]. RhoA and ROCK-2 also inhibits eNOS activity by inhibiting increase in phosphorylation at its inhibitory site Thr495 [150], through inhibition of protein kinase B [149] which mediates phosphorylation ser1117. Consequently, inhibition of RhoA geranylgeranylation by statins decreases ROCK activity can increase eNOS mRNA half-life and upregulate eNOS expression in animal and human

vascular disease. Similarly, direct activation of the Rho/ROCK signaling pathway by ROCK inhibitors or dominant negative mutant of RhoA increase eNOS expression [147, 148]. and by decreasing eNOS mRNA half-life [132] **.** However, which molecule/s stimulate rho kinase pathway to diminish NO production in endothelial cells during sepsis is not known**.** In this study, we demonstrate that SHH containing apoptotic T-cell derived MPs decreases NO production and impair endothelium dependent dilation in arteries ex vivo via activation of RhoA/ROCK pathway.

6.5.Summary and Conclusions

In summary, the result of this study provide strong evidence that SHH containing apoptotic T-cell derived MPs induce endothelial dysfunction by reducing ET-1 induced NO production as indicated by dim DAF-FM fluorescence and low mean value (-46%). These apoptotic T-cell derived MPs also significantly impair endothelium dependent dilation in porcine mesenteric arteries, as indicated by attenuated ATP induced reduction in the tension (-61.82%). Direct activation of SHH by SMO agonist Pur also significantly reduced ET-1 induced NO production, as indicated by low average mean value of DAF-FM (-63%). While inhibition of SHH by SMO antagonist SANT-1 largely restored NO production, as indicated by bright green fluorescence of DAF-FM. Treatment of porcine aortic rings with Pur impaired endothelium dependent dilation similar to MPs (-65%). This indicates that MPs induced decreased NO production in vitro and impaired ex vivo endothelium dependent vasodilation is mediated by SHH pathway Therefore, demonstrating the pathophysiological relevance of MPs in mediating endothelial dysfunction. Inhibition of rho kinase pathway one hour before addition of MPs largely restored NO production as indicated by high DAF-FM fluorescence and high mean value that is not significantly

different from control. This indicates that SHH in MPs activates rho kinase pathway to decrease ET-1mediated NO production. Also aortic rings in which ROCK was inhibited before addition of MPS, showed maximum relaxation of 60% with to 10^{-3}M ATP like that of control. Overall, this result indicates that one potential agonist that can activate rho kinase pathway to mediate in vitro and ex vivo endothelial dysfunction is SHH containing apoptotic-T cell derived MPs.

CHAPTER 7: CONCLUSIONS AND FUTURE DIRECTIONS

7.1. Conclusion and Future directions

The complex functions of the liver in biosynthesis, metabolism, clearance and host defense are tightly dependent on adequate microcirculation. Deterioration of liver microcirculation is a common event not only in sepsis but in other liver injuries and pathologies including, ischemia and reperfusion injury [473], trauma mediated injury [474], cirrhosis [475], hepatic steatosis and liver tumors [476]. To date, there are no effective treatments to restore normal blood flow in severe injuries. A barrier to developing such therapies is our incomplete understanding of the mechanisms leading to loss of normal regulation. In the present study, we demonstrate that apoptotic T-cell derived MPs containing SHH induce endothelial dysfunction through activation of RhoA/ROCK pathway and thereby can contribute to disruption of blood flow regulation. Our overall rationale is that the documented apoptosis of T-lymphocytes in sepsis leads to release of SHH containing MPs from their cell surface. These SHH containing MPs bind to receptors of endothelial cells and impact endothelial functions by activating ROCK. The end result is inadequate blood supply to cells and liver injury. This study provides proof of concept for a highly novel signaling pathway that is likely be a major contributor to the development of altered vascular regulation in liver during sepsis.

In summary, these apoptotic T –cell derived MPs containing SHH seem to have pleotropic effects in impairing endothelial functions ranging from modulation of their cytoskeleton, depolarization of mitochondria, significant reduction in viability and proliferation, diminished NO production and enhanced production of reactive oxygen species. Pathophysiological relevance of these MPs in mediating endothelial dysfunction

was evident by significantly impaired endothelium dependent vasodilation in porcine mesenteric arteries after addition of MPs. Direct stimulation of SHH pathway by Pur also caused endothelial dysfunction while inhibition of SHH pathway by SANT-1, abrogated it. This indicates that SHH signaling pathway mediates MPs induced endothelial dysfunction. Inhibition of ROCK before addition of $^{SHH+}$MPs abolished endothelial dysfunction. This indicates SHH in MPs activate rho kinase pathway to induce endothelial dysfunction Together, this data strongly suggest that activation of rho kinase pathway by SHH is required for MPs induced endothelial dysfunction. This study shed new light on a novel mechanism by which MPs may be contribute to pathophysiology of various disease characterized by impaired vascular regulation including liver failure in sepsis. Inhibition of RhoA/ROCK pathway may therefore constitute a novel target for therapeutic intervention to improve microcirculation and have beneficial effects after injuries.

Future directions: To establish clinical relevance of our hypothesis , we would like to test if the blood of critically ill patient with varying degree of severity of sepsis also have elevated levels of circulating MPs containing SHH. There is substantial support in the literature showing that ICU patients do have increased numbers of circulating microparticles; however, there is no information in the literature regarding SHH in these microparticles. Once we have established if SHH is released in MPs in blood if ICU patients, the effect of these MPs will be evaluated on the functions of human endothelial cell in vitro. Also, endothelium function in mice after intravenous injection of MPs will be assessed to establish pathophysiological relevance of these SHH containing MPs in mediating endothelial dysfunction.

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