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Circulating Complexes of the Vitamin D Binding Protein with G-Actin Induce Lung Injury by Targeting Endothelial Cells

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Abstract of Dissertation

Circulating Complexes of the Vitamin D Binding Protein with G-Actin Induce Lung Injury by Targeting Endothelial Cells

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Actin is the most abundant and highly conserved intracellular protein in all eukaryotic cells. Cell necrosis can release actin into the extracellular environment where conditions favor spontaneous generation of F-actin filaments. A robust extracellular actin scavenger system consisting of gelsolin and the vitamin D binding protein (DBP) has evolved to sever F-actin filaments (gelsolin) then bind G-actin monomers (DBP) for subsequent clearance. This study investigated the role of DBP in this process using the DBP null mouse that has a systemic deficiency of DBP. Intravenous injection of G-actin into wild-type (DBP+/+) and DBP null (DBP-/-) mice showed that, contrary to expectations, DBP+/+ mice developed much more severe acute lung injury. Tissue injury was restricted to the , and pathological changes were clearly evident at 1.5 and 4 hours post-injection but were largely resolved by 24 hours. In the DBP+/+ mice there was noticeably more vascular leakage, immune cell infiltration, thickening of the alveolar

wall, and formation of hyaline membranes. Flow cytometry analysis of whole lung homogenates showed increased neutrophil infiltration into DBP+/+ mouse lungs. Increased amounts of protein and leukocytes were also noted in the airspaces of DBP+/+ mice 4 hours after actin injection, indicating permeability changes in the lung microvasculature. The effect of DBP-actin complexes on cultured human lung microvascular endothelial cells (HLMVEC) and human umbilical vein endothelial cells (HUVEC) was investigated next. Cells treated in vitro with purified DBP-actin complexes showed a significant reduction in cell viability at 4 hours, and this effect was reversible if cells were then cultured in fresh medium for another 24 hours. However, cells cultured in the presence of DBP-actin for 24 hours showed a significant increase in cell death (95% for HLMVEC, 45% for HUVEC). The mechanism of endothelial cell death was shown to be via the caspase 3 mediated apoptosis pathway since cells treated with DBP-actin complexes had a dramatic increase in the amount of cleaved Poly ADP ribose polymerase (PARP), a specific substrate and marker of this pathway. These results demonstrate that elevated levels and/or prolonged exposure to DBP-actin complexes may induce endothelial cell injury and death, particularly in the lung microvasculature.

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List of abbreviations

ALI – Acute lung injury

ANOVA – Analysis of variance

AO – Acridine orange

ARDS – Acute respiratory distress syndrome

ATP - Adenosine triphosphate

BAL – Bronchoalveolar lavage

BSA – Bovine serum albumin

BSL-2 – Biosafety level-2

CLRs – C-type lectin receptors

CTB – Cell titer blue

CVF – Cobra venom factor

DAMPs – Danger associated molecular patterns

DBP – Vitamin D binding protein

DCFDA – 2',7' –dichlorofluorescein diacetate

DMSO – Dimethyl sulfoxide

DNGR-1 – C-type lectin domain family 9

DPBS – Dulbecco's phosphate buffered saline

E. coli – *Escherichia coli*

EASS – Extracellular actin scavenging system

EB – Ethidium bromide

EDTA – Ethylenediaminetetraacetic acid

EGM-2 – Endothelial growth medium-2

FBS – Fetal bovine serum

FSC – Forward scatter

Gc-globulin – Group-specific component globulin

H&E – Hemotoxylin and eosin

HBSS – Hank's Balanced Salt Solution

HLMVEC – Human lung microvascular endothelial cell

HMGB1 – High mobility group box 1

HRP – Horseradish peroxidase

HUVECs – Human umbilical vein endothelial cells

i.p. – Intraperitoneal

ICAM-1 – Intercellular adhesion molecules-1

IKK – IκB kinase

IL-6 – Interleukin 6

IL1-β – Interleukin 1- β

KO – Knock out

LPS – Lipopolysaccharide

MAF – Macrophage activating factor

MAPK – Mitogen-activated protein kinases

MCP-1 – Monocyte chemoattactant protein-1

MODS – Multiple organ dysfunction syndrome

MOF – Multiple organ failure

NAIP5 – neuronal apoptosis inhibitory protein 5

NALP1 – neuronal apoptosis inhibitory protein leucine-rich-repeat protein 1

NALP3 – neuronal apoptosis inhibitory protein leucine-rich-repeat protein 1

NLRs – Nod like receptors

PAMPs – Pathogen associated molecular patterns

PARP – Poly ADP ribose polymerase

PCR – Polymerase chain reaction

PMA – phorbol 12-myristate 13-acetate

PRR – Pattern recognition receptor

PTH – Parathyroid hormone

RAGE – Receptor for advanced glycation end product

RBC – Red blood cell

RLR – Rig like receptors

ROS – Reactive oxygen species

RPMI – Roswell Park Memorial Institute medium

SDS – Sodium dodecyl sulfate

SDS-PAGE – Sodium dodecyl sulfate polyacrylamide gel electrophoresis

SEM – Standard error of the mean

SIRS – Systemic inflammatory response syndrome

SRBC – Sheep red blood cell

SSC – Side scatter

TBHP – tert-butyl Hydrogen Peroxide

TLRs – Toll like receptors

TNF-α – Tumor necrosis factor-α

TRIF - Toll/IL-1R domain-containing adaptor inducing interferon

UVB **–** Ultraviolet B

VCAM-1 – Vascular cell adhesion molecule-1

WT – Wild type

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1. INTRODUCTION

1.1 Tissue Injury and Inflammation

Inflammation is a complex physiological response that has evolved to rapidly neutralize or contain the inciting agent. This very effective homeostatic mechanism utilizes cells and molecules of the innate immune system to provide immediate (seconds to minutes) responses to infections and tissue damage [1]. In addition, there are other elements such as platelets and the coagulation system, and the autonomic nervous system that play an essential role during inflammation but are peripheral components of innate immunity [2-4]. Blood flow changes in the affected tissue microvasculature result in the classic signs of an inflammatory response, redness and heat due to hyperemia (increased blood flow), swelling due to edema fluid caused by increased vascular permeability. These microvascular alterations can be triggered very quickly due to the release of preformed mediators from several cell types, but most prominently tissue mast cells. The autonomic nervous system can also contribute to altered blood flow by relaxing smooth muscle sphincters on arterioles. The increased permeability of the endothelium allows the leakage of plasma proteins and promotes extravasation of innate immune effector cells (neutrophils and monocytes) from the blood into the tissue spaces where they will attempt to neutralize the infection or tissue damage [5]. The duration of the inflammatory response and the type of immune cells found in tissue lesions can categorize the inflammation as either acute or chronic. Acute inflammation is short duration (minutes to a few days) and is characterized by

neutrophils, while the chronic form persists for weeks to years and has lymphocytes and macrophages as the primary cell types infiltrating the tissue.

The primary innate immune effector cell during an acute inflammatory response is the neutrophil [5]. Neutrophils are the fastest migrating mammalian cells and are the first responders to inflammatory injury. In humans, neutrophils are the most abundant leukocytes in circulation and in the bone marrow. Approximately 100 billion neutrophils are produced daily in the bone marrow and cleared from the blood. It is estimated that 60% of the total cellular output of the bone marrow, which includes all red and white blood cells and platelets, is dedicated to producing just neutrophils. They also make up about 50-60% of all leukocytes in the blood, and their average life span in circulation is up to 5 days [6]. Neutrophils are not usually found in healthy tissue, and these cells are directed to the site of inflammation by multiple diverse chemotactic factors [7]. However, before neutrophils can exit the microvasculature they first must interact with “activated” endothelial cells in a loose (selectin-mediated) followed by a tight binding (integrin-mediated) reaction. This adherence to the endothelium is essential for leukocytes to exit the vasculature as evidenced by the lack of tissue neutrophils in individuals with a genetic deficiency of certain of these adhesion molecules [8]. In addition to neutrophils, resident tissue macrophages and recruited monocytes (that mature into macrophages) also play a significant role in the acute phase of inflammation but their numbers are dwarfed by a massive recruitment of neutrophils. Many of the same chemoattactants that recruit neutrophils also recruit blood monocytes to the site of injury [9].

The complement system is the primary humoral component of innate immunity and functions as a molecular triage system to assess injury and signal the appropriate cells to respond. Complement is a large and complex system of over 40 plasma and cell surface proteins that interact in a specific and sequential cascade to neutralize invading microbes and help clear damaged tissue [10]. Complement activation products have potent pro-inflammatory functions that are primarily mediated through the recruitment and activation of innate immune effector cells. However, excessive or dysregulated complement activation can induce significant tissue injury and has been strongly linked with the pathogenesis of numerous diseases [11, 12]. The role of complement in certain forms of acute trauma (burns, systemic inflammatory response syndrome, sepsis) is well established [13-15]. In general, the degree of complement activation (measured systemically in plasma) correlates with the severity of injury. Animal models have clearly shown that inhibition of complement significantly attenuates injury and improves survival in these conditions [15, 16]. Complement is rapidly activated by most microbes, damaged tissue and altered self-molecules (i.e., amyloid). Since complement is ubiquitous in all fluid compartments, it is often the first part of innate immunity to interact with an inflammatory stimulus. Activated complement proteins can signal immune cells to synthesize and secrete numerous proinflammatory cytokines that direct and continue the inflammatory response.

1.2 Danger Signals and Pattern Recognition Receptors

It is widely believed that innate immunity and the inflammatory response evolved to provide immediate protection against microbial pathogens. Indeed, as “first responders” to infections, the cellular and humoral components of innate immunity can recognize and destroy pathogens without prior immune exposure. Innate immune effector cells utilize pattern recognition receptors (PRR) to recognize the conserved structural elements common among different microbial species [17-19]. These conserved structures are called pathogen associated molecular patterns (PAMPs). There are several classes of PRRs: Toll like receptors (TLRs), RIG-I-like receptors, Nod-like receptors (NLRs), and C-type lectin receptors (CLRs). TLRs are the best-characterized PRR family, and there are ten TLRs in human and twelve in mice. TLRs play an important role in sensing both extracellular and intracellular pathogens, but TLRs can also recognize self-components. Ligation of TLRs on immune cells stimulates phagocytosis, activates microbicidal functions and induces gene expression [17-19]. Interestingly, proteins of the complement system recognize many of the same PAMPs as PPRs, and complement components have been referred to as soluble PPRs.

It has long been known that inflammation can be triggered without any apparent infection. This form is known as sterile inflammation, and recent studies have provided the mechanism by which self components induce an inflammatory response [20]. Molecules released from dead or dying cells can be recognized by PRRs and elicit an innate immune response; these self-molecules were named danger associated

molecular patterns (DAMPs) [21, 22]. Another term “alarmin” has also been used to describe endogenous molecules that signal cell and tissue damage. The number of DAMPs that have been identified continues to grow, as well as the PRRs that recognize them. Certain criteria have been proposed for a molecule to be a DAMP: 1) It is not present normally in the extracellular space but is released by necrotic cell death. It is thought that apoptotic cell death will not release these molecules because they are compartmentalized. 2) Healthy immune cells can be induced to produce DAMPs and secrete them using a specialized secretion pathway. 3) DAMPs can be recognized by PRRs on innate immune cells. DAMPs can stimulate dendritic cells and trigger adaptive immunity. 4) DAMPs should promote the repair of damaged tissue that results from primary damage or is secondary to inflammation [21, 22].

One of the earliest DAMPs identified was High Mobility Group Box 1 (HMGB1). HMGB1 is a chromatin architectural protein that normally binds to nucleosomes and promotes DNA bending inside of the nucleus [23, 24]. But upon necrotic cell death, HMGB1 is released into the extracellular space where it has a completely different function [24, 25]. HMGB1 is a proinflammatory cytokine in sepsis [26-28], hemorrhagic shock and ischemia/reperfusion injury [29]. HMGB1 mediates its activity through TLR2, and TLR4 is thought to be essential for HMGB1 induced macrophage activation and the development of secondary damage in ischemia/reperfusion injury [30-32]. In addition to TLRs, HMGB1 also interacts with the receptor for advanced glycation end products (RAGE) on various cell types, and this interaction is involved in multiple inflammatory responses [33, 34].

A protein complex that has DAMP-like activity and is released from activated neutrophils is the S100A8/A9 dimer [35]. S100A8 and S100A9 are two proteins of the large S100 protein family (also known as calgranulins) that form calcium dependent heterodimers and heterotetramers [36]. S100A8/A9 dimer is also called calprotectin, L1 antigen, calgranulin A and B, or myeloid-related protein 8 and 14 (MRP8/14). S100A8/A9 is largely expressed by mature neutrophils and the proteins comprise almost 40% of the total cytosolic protein in neutrophils. Upon neutrophil stimulation and calcium influx, S100A8/A9 dimer can form a heterotetramer and translocate to plasma membrane. Neutrophils actively secrete S100A8/A9 into the extracellular space via a non-classical secretion pathway in response to chemotactic stimuli such as C5a and formylated peptides [37]. Once secreted, low concentrations of S100A8/A9 (1 to 100 pM) have been shown to induce chemotaxis of mouse neutrophils, while a higher concentration of S100A8/A9 increases the expression of neutrophil surface adhesion molecules that facilitate transendothelial migration [38]. S100A8/A9 can also increase vascular permeability, and it even cause endothelial cell death by both caspase-dependent and -independent pathways [39]. Endothelial cells treated with recombinant S100A8/A9 up-regulate the expression and secretion of proinflammatory cytokine interleukin-6 (IL-6), the chemokine CCL2, also known as monocyte chemoattractant protein-1 (MCP-1) and the adhesion molecules intercellular adhesion molecular-1 (ICAM-1) and vascular adhesion molecular-1 (VCAM-1). In endothelial cells, S100A8/A9 induces its cellular effects largely by binding to TLR4 and RAGE [40].

1.3 Acute Lung Injury

The lungs are unique in that they are very susceptible to both air-borne and blood-borne inflammatory stimuli. Lungs can be directly injured by conditions such as pneumonia, acid aspiration, hypoxia, high pressure ventilation, pulmonary contusion or reperfusion [41, 42]. These conditions can induce a severe form of acute inflammation known as acute lung injury (ALI). The lung is one of two organs with a dual circulatory system (the other being the liver). The blood supply for the lung parenchyma comes from the bronchial circulation, while the pulmonary circulation is utilized for systemic oxygen exchange. The first microvasculature that any blood-borne pathogen, embolus or inflammatory stimulus in venous blood will encounter is the pulmonary capillary bed in the pulmonary circulation. This anatomical feature of the lung can produce a secondary exacerbation of other inflammatory conditions such as sepsis, trauma, pancreatitis, or transfusion. ALI, and its more severe form acute respiratory distress syndrome (ARDS), are characterized by the increased permeability of the alveolar-capillary barrier resulting in lung edema with protein rich fluid, which is observed as diffuse bilateral pulmonary infiltrates on a chest x-ray [43]. The edema in the lungs causes impairment of arterial oxygen exchange. The Neutrophils is the major immune cell that infiltrates the lungs during acute injury and is a hallmark for ALI and ARDS [42]. Neutrophils are recruited after the change in endothelial permeability and generation of lung edema. Activation of neutrophils in the lung can lead to significant tissue damage and impairment of lung function [42]. Whereas most patients can eliminate the initial insult and resolve the inflammation, about 1/3 will progress to ARDS, and the mortality

in ARDS patients is very high, approaching 50%. The chance of a person surviving ARDS is determined by the severity of the lung injury, the extent of other organ dysfunction, preexisting medical conditions and the quality of the supportive care. Resolution of ARDS requires reabsorption of the lung edema, repair of the endothelial and epithelial barriers, and removal of the inflammatory cells and fluid in the alveoli [44]. This is a delicate balance between the inflammatory and healing phases and many survivors of ARDS will develop secondary lung fibrosis due to a dysregulated healing process.

1.4 Systemic Inflammatory Response Syndrome

Although inflammation is necessary for clearing the infection and initiating the healing process, poorly regulated inflammation can become systemic and cause an overproduction of cytokines that can enter the circulation. This condition is known as a cytokine storm. This surge of proinflammatory cytokines can cause a condition known as the systemic inflammatory response syndrome (SIRS) [20, 45]. In the case of sepsis and multiple trauma, a systemic inflammation can result in subsequent multiple organ dysfunction syndrome (MODS) and multiple organ failure (MOF), which often leads to death [46, 47]. The role of cytokines in systemic inflammation is the subject of ongoing investigations, but the levels of tumor necrosis factor-α (TNF-α), interlukin-1β (IL-1β) and IL-6 are most often elevated [47, 48]. HMGB1 also has been shown to be a late stage mediator of lethal systemic inflammation [49, 50]. Attempts have been made to

block cytokines and/or cytokine receptors to improve the clinical outcome of the systemic inflammation, but these trials have not been successful. NF-κB is usually activated by the inflammatory stimuli and results in the production of cytokines. However, attempts to utilize IKK and NF-κB inhibitors blunted the systemic inflammation but exacerbated the local injury in an intestinal ischemia-reperfusion model [51]. It is currently thought that the balance of starting tissue repair and preventing systemic inflammation cannot be achieved simply by inhibiting one pathway in SIRS [51, 52]. The complexity of systemic inflammation and the range of the diseases it may be associated with require treatment tailored to the specific condition.

1.5 PRRs and DAMPs in Acute Lung Injury

PRRs and DAMPs have been implicated extensively in the development of acute lung injury. TLRs are one of the most important PRRs that mediate acute lung injury [53]. Once PAMPs and DAMPs are recognized by TLRs, the NF-κB pathway is activated to up-regulate the expression and secretion of proinflammatory cytokines. The MAP kinase pathway is also activated to produce inflammatory cytokines as well as promoting cell survival [54]. However, TLRs may also have a protective role in acute lung injury [55]. TLR4 deficient mice develop more severe vascular leakage and alveolar epithelial cell apoptosis in Gram-negative bacteria pneumonia [56]. In the bleomycin-induced lung injury model, TLR2/TLR4 double deficient mice have less acute lung inflammation but higher mortality, as well as more vascular leakage and alveolar

epithelial cell apoptosis [57]. These results suggest that role of TLRs in acute lung injury is highly complex and must be considered in the context of the genetic background and the insulting agents. There are various DAMPs that are reported to induce ALI. Components of the extracellular matrix such as fibronectin, hyaluronan, and heparin sulfate signal through TLRs and have been shown to induce ALI/ARDS. Stress response molecules such as heat shock proteins and HMGB1 also contribute to ALI/ARDS development through TLR2/TLR4. Moreover, HMGB1 increases the proinflammatory cytokine release via a RAGE mediated pathway.

1.6 Evaluation of Acute Lung Injury in Animal Models

Although ALI is well defined and studied in humans, a standard for evaluating acute lung injury in animal models has not been developed. There are several reasons why it is not possible to employ the ALI physiological parameters used for humans to measure experimental lung injury in animals. Besides, none of the existing animal models of ALI can produce all the pathological changes of human ALI. Accordingly, the American Thoracic Society recently sponsored a workshop to standardize the physiological and pathological features of ALI in animal models [58]. The recommendations were 1) histological evidence of lung injury, 2) alteration of the alveolar capillary barrier, 3) an inflammatory response, and 4) evidence of physiological dysfunction; of these main features, tissue injury and alveolar capillary barrier change were considered the most important. The histological evidence of tissue injury is the single most important aspect

of ALI in experimental animals. The most relevant features are: neutrophil infiltration into the alveolar or the interstitial space, formation of hyaline membrane, presence of proteinaceous debris in the alveolar space, and thickening of the alveolar wall. These pathological changes can be quantified by a histology score. Other relevant features include evidence of hemorrhage, areas of atelectasis and discoloration of the lungs by gross examination. The alteration in the lung endothelium-epithelium permeability in small animals like rodents can be determined by measuring total protein in the bronchoalveolar lavage (BAL) fluid. Intravascular injected Evans blue dye can also be used to trace the vascular leakage. To measure the inflammation of the lung, the most relevant standard is the absolute number of neutrophils that infiltrated the lungs obtained by counting the neutrophil number in the BAL. The increase of lung myeloperoxidase activity is also a relevant finding for neutrophil infiltration. Moreover, measurement of proinflammatory cytokines in the BAL or lung tissue can help determine the extent of inflammation. Lastly, the physiological dysfunction can be measured by assessing hypoxemia and increased alveolar-arterial oxygen difference. Hypoxemia can be measured using the arterial partial pressure of oxygen (Pa_{O2}) from arterial blood gases and hypoxemia is defined as Pa_{O2} < ~ 60 mm Hg. However, measuring arterial blood gases may be extremely difficult in small animals like mice. Most of these standards have been employed to evaluate murine lung injury in this dissertation [58].

1.7 Extracellular Actin Scavenger System

Actin is the most abundant and highly conserved protein inside all eukaryotic cells and is the central molecule of the cytoskeleton. Inside cells actin exists in two forms, monomeric globular actin (G-actin) and polymerized filamentous actin (F-actin) [59]. The polymerization and depolymerization of actin filaments is a continuous process that is controlled both by ionic conditions and numerous actin regulatory proteins [59]. This dynamic process plays a very important role in maintaining cell shape, as well as regulating cell motility [60, 61] and even transcription [62]. During tissue injury and necrotic cell death both G-actin and F-actin will be released into the extracellular fluids where the ionic conditions and lack of regulators favor spontaneous actin polymerization. F-actin has deleterious effects in the circulation where it can alter blood flow, obstruct the microcirculation and cause localized ischemia [63, 64]. Evidence has shown that actin can induce platelet aggregation [65, 66], interact with fibrin during clot formation and slow fibrinolysis by directly binding to plasmin [67-69]. It was noted for many years that actin polymerizes spontaneously in physiological buffers, but when it is added to serum depolymerization is observed, suggesting that a serum protein prevents formation of actin filaments [70, 71]. Two plasma proteins, vitamin D binding protein and gelsolin, were identified as essential for this process and were referred to as the extracellular actin scavenger system (EASS) [72, 73].

1.8 Gelsolin

Plasma gelsolin is a splice variant of cytosolic gelsolin and is mainly synthesized and secreted by skeletal muscle cells [74, 75]. The physiological concentration of gelsolin in plasma is 190 to 300 μg/ml, and it functions as an EASS protein by binding to the barbed end of F-actin. This binding caps the filaments from growing by preventing the addition of G-actin monomers, but gelsolin also severs G-actin from the filaments and binds two G-actin monomers simultaneously in a Ca^{2+} dependent fashion [76, 77]. This G-actin binding is transient since the vitamin D binding protein (DBP) has a much higher affinity for G-actin than gelsolin [78]. DBP binds G-actin tightly (K_d of 10^{-9} M) in a 1:1 molar ratio for subsequent clearance from extracellular fluids. Previous in vivo experiments injected purified skeletal muscle actin intravenously into rats or rabbits and then evaluated the ability of EASS proteins to clear actin from the blood [72, 73, 79, 80]. These studies showed that 10 mg or more of actin i.v. can kill a rat almost instantly due to acute cardiac failure, while 3, 5 or 7 mg of actin cause the formation of microthrombi in the pulmonary circulation, acute lung injury and damage to the pulmonary capillary endothelium [63]. These studies also showed that DBP-actin complexes have a half-life of 30 minutes and are cleared primarily via the reticuloendothelial system in the liver, but the lungs, spleen and skeletal muscle also were minor sites of DBP-actin complex clearance [72, 73, 79, 80]. These animal studies are consistent with observations in humans, where significantly elevated actin-scavenger protein complexes were detected in the blood of patients with fulminant hepatic necrosis, septic shock, acute respiratory distress syndrome and pregnancy complications [81-86]. It is proposed that the EASS

protects organs from the pathological consequences caused by actin filaments, but massive tissue injury can saturate and/or deplete the EASS and cause the pathological changes described above. Conditions such as severe fulminant hepatic necrosis and sepsis can also lead to the systemic inflammatory response syndrome and multiple organ failure. However, it is very difficult to determine if there is a cause and effect relationship between these other disease sequelae and massive actin release with the subsequent generation of DBP-actin complexes and F-actin filaments in the circulation. These correlations could be investigated using animals with a systemic deficiency of DBP, such as the DBP null (-/-) mouse.

1.9 Vitamin D Binding Protein

Vitamin D binding protein (DBP; in some papers it is abbreviated as VDBP) is also known as Gc-globulin, and it is a 56 kDa plasma protein that is largely synthesized by liver hepatocytes. DBP is one of four members of the albumin gene family and is linked with albumin, α-fetoprotein and afamin on human chromosome 4 [87, 88]. DBP shares the structural elements of this protein family including very little glycosylation (one O-linked tri-saccharide), and multiple disulfide bonds (DBP has 14 cystine bridges) that form a triple domain structure. The third (C-terminal) domain of DBP is truncated compared to others members of this family [88-91]. The plasma concentration of human DBP ranges from 4-8 μM (mean of 6 μM), and DBP has a rapid turnover in blood with a half-life of about 2 to 2.5 days [87]. The plasma concentration of DBP can rise modestly

(25-50%) during the acute phase inflammatory response, whereas blood levels typically double in women during the third trimester of pregnancy [92, 93]. The increased DBP in pregnant women is thought provide additional EASS capacity for childbirth and placental detachment. There are three primary alleles of DBP that are referred to as Gc1f, Gc1s and Gc2 (using an abbreviation of the alternate name Gc-globulin). There are also numerous rare alleles that account for about 1% of the DBP genotypes in the human gene pool. Gc1f has an aspartic acid at amino acid position 416 while Gc1s has a glutamic acid at that position. Both Gc1 forms have a threonine at position 420 that is glycosylated with an O-linked tri-saccharide. Gc2 has a lysine at position 420 and is not glycosylated. All vertebrates are thought to express a DBP-like protein, and there are no known natural DBP deficiencies in any vertebrate suggesting a crucial physiological function [87]. The protein is well conserved among different mammalian species since mouse DBP is 75% identical to human DBP, and almost 90% with conserved amino acid substitutions [94]. Although there are no known deficiencies, a DBP null (-/-) mouse has been generated and is viable and healthy when fed a standard vitamin D sufficient mouse chow diet [95].

The protein was originally described in 1959 as Gc-globulin (short for group-specific component because of its polymorphisms), until several studies published in 1975 and 1976 discovered that this protein was the major carrier for all vitamin D metabolites in plasma [96]. Thus, it was proposed to rename it as the vitamin D binding protein, although the name Gc-globulin is still used today but less frequently than DBP. DBP has different binding affinities for the various vitamin D metabolites, binding most tightly to

the hydroxylated forms of vitamin D_3. The order of binding affinity is: 25 OH-D_3 (K_d of 5 x 10^{-8} M) > 1,25-$(OH)_2D_3$ (K_d of 5 x 10^{-7} M) > vitamin D_3 [87]. Vitamin D_3 is synthesized from the precursor 7-dehydrocholesterol in the dermis of the skin when exposed to wavelengths of light in the UVB range. Vitamin D_3 binds to DBP in the skin and is transported to the liver where the enzyme 25-hydroxylase converts it to 25 OH-D_3, the major stable form of the vitamin in plasma. This form of vitamin D then can be converted to the hormonally active vitamin by the enzyme 1α-hydroxylase in proximal tubule cells of the kidney, where a second hydroxylation reaction produces 1,25-$(OH)_2D_3$. DBP (both with and without vitamin D bound) is filtered from the blood by kidney glomeruli but is reabsorbed into plasma via a megalin/cubulin mediated endocytosis pathway in proximal tubule cells [97]. Mouse studies have shown that megalin deficient animals have severely depleted levels of plasma DBP and are vitamin D deficient [97]. Moreover, DBP null mice need to maintain adequate dietary intake of vitamin D; otherwise, they will rapidly show signs of vitamin D deficiency due to loss in the urine [95]. These animal studies have clarified the vitamin D transporter role of DBP in vivo and have shown that its primary role is to maintain vitamin D levels within a physiological range to protect against transient deficiencies. Therefore, DBP essentially "buffers" vitamin D levels in the blood. Interestingly, DBP null mice have no detectable vitamin D in plasma yet animals have normal blood calcium and parathyroid hormone (PTH) levels indicating that they are vitamin D sufficient [95]. It is assumed that all dietary vitamin D in these animals is transported directly to target tissues by a less efficient mechanism (albumin or lipoproteins), since there is no buffering effect of DBP.

Interestingly, the concentration of DBP in plasma is much higher than vitamin D. Normally only 1-2% of the total circulating DBP pool has vitamin D bound, and this percentage never rises above 5% [92]. This is in contrast with other vitamin transport proteins in blood where typically 50% of the protein is bound with vitamin [98, 99]. This fact prompted many to speculate that DBP must have another essential function. The other major function of DBP, which was discovered shortly after reports of vitamin D binding, is the binding to G-actin monomers. As discussed above, DBP binds actin with high affinity (K_d of 10^{-9} M) for subsequent rapid clearance from the circulation (DBP-actin complexes have a plasma half-life of 30 minutes in rats) [100]. The vitamin D sterol and actin binding sites in DBP have been mapped to distinct regions, a vitamin D binding pocket in the N-terminal domain and a large actin binding region largely in the C-terminal domain [101, 102]. DBP can simultaneously bind both ligands without altering their individual affinities [102]. Although less than 5% of total plasma DBP ever has vitamin D bound, a very large percentage of DBP can be bound to actin. A study of humans with fulminant hepatic necrosis has shown almost 75% of plasma DBP is complexed with actin [82]. The observations that large amounts of plasma DBP can be bound to actin, coupled with the recent evidence that DBP null mice are vitamin D sufficient, have caused investigators to re-evaluate the physiological roles of DBP and conclude that actin binding may be its primary biological function.

There are two other well-documented functions of DBP, a chemotactic cofactor for leukocytes and a role as a macrophage activating factor (MAF), and these are often referred to as immune functions of DBP. A partially deglycosylated form of DBP has

been shown to augment macrophage phagocytic and tumoricidal activity in vitro, and it is known as DBP-MAF [103]. However, this activity of DBP has many skeptics since the effects of DBP-MAF could be due to lipopolysaccharide (LPS) contamination. The chemotactic cofactor function of DBP can enhance neutrophil, monocyte, and fibroblast migration towards the complement activation peptide C5a in vitro [104-107]. Several laboratories, including ours, have clearly demonstrated using in vitro chemotaxis assays that DBP is absolutely specific for C5a, but this function had not been confirmed in vivo. Very recently, our lab utilized DBP null (-/-) mice to investigate how a systemic absence of this plasma protein affects leukocyte recruitment in alveolitis models of lung inflammation [108]. DBP-/- mice had significantly reduced (~50%) neutrophil recruitment to the lungs compared to their wild-type DBP+/+ counterparts in three different alveolitis models, two acute and one chronic. The histology of DBP-/- mouse lungs also shows significantly less injury than wild-type animals. In contrast to previous in vitro findings, the chemotactic cofactor function of DBP in vivo appears to be selective for neutrophil recruitment and can enhance the activity of other chemoattractants including CXCL1. The reduced neutrophil response in DBP-/- mice can be rescued to wild-type levels by administering exogenous DBP. The bronchoalveolar lavage (BAL) from wild-type mice showed extensive DBP-actin complexes (~75%) 4 hours after induction of acute alveolitis. Moreover, an in vitro chemotaxis assay demonstrated that DBP-actin enhances neutrophil migration to C5a significantly more than does unbound DBP. These results suggest that, mechanistically, the chemotactic cofactor function may be associated with the actin binding capacity of DBP.

All of the known functions of DBP require it to bind to the surface of a target cell. Numerous investigators have reported a cell-associated form of DBP in many cell types including all leukocytes [109-120]. Cell-associated DBP is not a novel cellular form but rather plasma-derived DBP bound to the cell surface [121]. Our laboratory has shown that DBP first must bind to the leukocyte surface before chemotactic cofactor activity is observed [122, 123]. DBP appears to be promiscuous in its preference of a cell surface ligand, utilizing many low affinity (μM range) scavenger-type molecules such as megalin and cubulin on epithelial cells, and chondroitin sulfate proteoglycans and CD44 on leukocytes [124]. However, several studies have noted that DBP can bind very tightly to the plasma membrane of most cell types and can only be dissociated by harsh (high or low pH) or denaturing conditions. DBP has two distinct cell binding sequences, one in the N-terminal domain adjacent to the chemotactic cofactor sequence (previously identified in our laboratory), the other in the C-terminal domain contained within the larger G-actin binding sequence. Although generally not widely appreciated, numerous studies have demonstrated actin on the surface of many diverse cell types including monocytes, lymphocytes, endothelial cells, neurons, and a number of cancer cell lines [109, 125-130]. Recent unpublished evidence from our laboratory has shown that DBP binds to actin on the surface of activated and migrating neutrophils but not to unstimulated quiescent cells. The function of actin on the external face of the plasma membrane of activated neutrophils is not clear and is the subject of future investigations. It is interesting to note that cell bound DBP is not internalized by neutrophils but is shed by the action of the enzyme elastase on the binding site, and DBP is not modified during this process [131]. Both DBP binding, and shedding of the

binding site, are constitutive and occur in the absence of a chemotactic stimulus [124, 131]. This constitutive activity may reflect other unknown cellular functions of the protein.

Synthesis of DBP and its clearance from the circulation are both mediated by cells in the liver. Hepatocytes are clearly the primary source of systemic DBP synthesis, and accordingly, reduced plasma DBP levels are a consequence of liver failure. The hepatic cell type that removes DBP and/or DBP-actin from the blood has not been definitely identified, and there is evidence that Kupffer cells, hepatic stellate cells and hepatocytes can all endocytose DBP [132]. The half-life of DBP (not bound to actin) in human blood has been estimated to be 2 - 2.5 days, considerably shorter than most other plasma proteins, particularly albumin whose in vivo half-life is almost 10-fold longer [133]. The reason for this rapid turnover is not known but could be correlated with its function as an EASS protein.

In summary, DBP is an important multifunctional plasma protein that may play an essential role in tissue injury and inflammation by binding G-actin released from cells during tissue necrosis. The current paradigm is that DBP is required to prevent actin filament formation. An alternative hypothesis is that DBP-actin complexes function in a DAMP-like capacity to signal ongoing tissue injury. The role of DBP in actin binding and clearance in vivo has not been adequately addressed. However, our laboratory has the unique resource to investigate this question vivo: the DBP null strain of mice.

2. MATERIALS AND METHODS

2.1 Reagents

Purified human DBP was obtained from Athens Research & Technology (Athens, GA). The IgG fraction of goat polyclonal anti-human DBP was purchased from DiaSorin (Stillwater, MN) and then affinity-purified in our laboratory using immobilized DBP. Chicken anti-human DBP was purchased from Gallus Immunotech (Cary, NC). Highly purified rabbit skeletal muscle actin was obtained from Cytoskeleton, Inc. (Denver, CO). Goat anti-actin (I-19) was purchased from Santa Cruz Biotechnology (Santa Cruz, CA) and pan anti-actin mAb was purchased from Thermo Scientific/Lab Vision (Kalamazoo, MI). Polyclonal anti-gelsolin (ab74420) was obtained from Abcam (Cambridge, MA). Monoclonal antibody specific for the S100A8/A9 dimer (clone 27E10) was purchased from Hycult Biotech (Plymouth Meeting, PA). Sterile, pyrogen-free solutions of Hank's balanced salt solution (HBSS), Dulbecco's phosphate buffered saline (DPBS) and Roswell Park Memorial Institute medium 1640 (RPMI 1640) were purchased from Mediatech, Inc. (Manassas, VA). EDTA solution was purchased from Life Technologies-Gibco (Grand Island, NY). Collagenase and DNase I was purchased from Roche (Indianapolis, IN). Rat anti-mouse monoclonal antibodies for flow cytometry and their corresponding labeled isotype controls, were all purchased from Biolegend (San Diego, CA): PE-labeled anti-Gr-1 (RB6-8C5), and FITC-labeled anti-F4/80 (BM8). Purified cobra venom factor (CVF) and sheep erythrocytes were obtained from Complement Technology, Inc. (Tyler, TX). Mouse monoclonal anti-human factor B

(clone D33/3) was obtained from Santa Cruz Biotechnology (Santa Cruz, CA). Purified human TNF-α and IL-1β were purchased from R&D Systems (Minneapolis, MN). Bio-Plex 23-mouse cytokine panel was obtained from BioRad (Hercules, CA).

2.2 DBP-/- Mice

The DBP-/- mouse line has been fully backcrossed on a C57BL/6 background for 11 generations. Frozen sperm from DBP-/- mice was used to revive the line at the University of Pennsylvania's Transgenic and Chimeric Mouse Facility. DBP+/- hemizygotes were generated by in vitro fertilization of eggs from wild-type C57BL/6J mice (Jackson Labs, Bar Harbor, ME) with DBP-/- sperm. The DBP +/- hemizygotes were bred to produce the DBP-/- (null) and DBP+/+ (wild type) homozygotes mouse colonies at the Stony Brook University animal facility. Tail tissue from mice was used to genotype animals by PCR using specific DBP primers [95]. The DBP null phenotype of -/- mice was also confirmed by western blot analysis of sera using chicken anti-human DBP that cross-reacts strongly with mouse DBP. In some experiments, wild type C57BL6/J mice were purchased from Jackson Labs. Only mature male mice (age ranged from 10 weeks to one year) were utilized, and all animals were housed in a maximum isolation facility at Stony Brook University. DBP+/+ and -/- mice were always age-matched for each experiment, and the age of the mice made no difference in the response to injected actin. Animal experiments were performed using protocols approved by the Institutional Animal Care and Use Committee at Stony Brook University.

2.3 Intravenous Injection of G-actin into Mice

Highly purified rabbit skeletal muscle actin was reconstituted using sterile pyrogen-free DPBS at 1 mg/ml under aseptic conditions. DBP+/+ and -/- mice were injected with 100 µl (100 µg actin) solution via the tail vein to introduce an actin bolus into the circulation. The control group received 100 µl of DBPS through the tail vein. The mice were observed during the course of incubation for signs of respiratory distress. After 1.5, 4 and 24 hours, mice were anesthetized by an i.p. injection (0.3 ml) of ketamine (90 mg/kg) and xylazine (10 mg/kg) solution. EDTA plasma was obtained by retro-orbital bleeding after anesthesia and the mice were then euthanized by cervical dislocation and secured to a small animal surgery board (Kent Scientific, Torrington, CT). A small midline incision was made in the suprasternal region and the trachea was exposed by blunt dissection. A sterile BD angiocath (18 gauge needle stylet in a 1.3 x 44 mm long I.V. catheter) was inserted into the anterior portion of the exposed trachea between cartilage rings and the lungs were lavaged once with 1 ml of DPBS + 5 mM EDTA and the BAL fluid was stored on ice until analyzed for total protein and cell count. BAL fluid was centrifuged at 200 x g for 5 min at 4°C to pellet the cells. BAL cell pellets were resuspended in 1 ml of PBS-1% BSA, and the presence of leukocytes were determined by evaluating stained cytospin slides. Duplicate counts of total cell number were made using a BioRad TC10 automated cell counter. The protein concentration of the BAL supernatant was measured using a Lowry protein assay.

2.4 Preparation of Organs for Histology

In a separate group of mice that were not lavaged, mouse lungs were inflated with 10% PBS-buffered formalin, pH 7.2 for 15 min at 25 cm water pressure to ensure the proper degree of lung inflation. The lungs were removed from the thoracic cavity en bloc with the heart and submerged in 10% PBS-buffered formalin for 24 hours at 4°C. The kidneys and livers were also isolated and submerged into the same solution. The fixed organs were transferred to the Stony Brook University Hospital Histology Laboratory for preparation of thin-sectioned slides stained with hematoxylin and eosin (H&E). Digital photos were taken using a Nikon Eclipse Ti inverted microscope with an Insight Spot2 digital camera using 200x and 1000x objectives.

2.5 Preparation of Lung Homogenates

Single cell suspensions were prepared from whole lung homogenates at each time point after actin injection. Lungs were isolated and placed into a well of a 24-well plate containing 1 ml of complete culture medium (RPMI 1640 + 10% FBS) and thoroughly minced using a small surgical scissor. Minced tissue was transferred to a 15 ml conical tube containing 5 ml of fresh collagenase solution (1 mg/ml type IV collagenase, 25 U/ml DNase I, 5% FBS and the volume was brought up to 5 ml with RPMI). The samples were then incubated at 37°C for 45 minutes with shaking. After the incubation, the tissue was broken up into cellular suspension by passing it through an 18-gauge needle 20 times. Then the samples were centrifuged at 500 x g for 5 minutes. The

supernatant was aspirated and the pellet was resuspended in RBC lysis buffer and immediately centrifuged at 50 x g for 5 minutes. The supernatant was saved and the pellet, containing large tissue aggregates, was discarded. The supernatant was then again centrifuged at 500 x g for 5 minutes and the cells were resuspended in 500 µl of flow cytometry staining buffer. The samples were counted using an automated cell counter and then stained for Gr-1 and F4/80 and analyzed by flow cytometry.

2.6 Nondenaturing PAGE and Immunoblot Analysis of EASS-Actin Complexes

Mouse serum or EDTA plasma was diluted 1:2 in DPBS and 50 µl of the diluted sample was loaded onto a 10% native polyacrylamide gel. After separation the proteins were transferred to PVDF membranes (Millipore, MA). The membranes were probed for DBP-Actin or gelsolin-actin complexes using primary antibodies against human proteins that strongly cross react with mouse DBP or gelsolin. Both proteins were detected using a 1:1000 dilution of primary antibody followed by a 1:5000 dilution of HRP-conjugated secondary antibody. The blot was developed with HyGlo Quick spray chemiluminescent HRP antibody detection reagent (Denville Scientific Inc, South Plainfield, NJ) and visualized by autoradiography film.

2.7 Measurement of Serum Complement Activation

The effect of DBP-actin complexes on serum complement was evaluated using a hemolytic assay to test for classical or lectin pathway activation, and an immunoblot for

factor B cleavage to assess alternative pathway activation. Sheep erythrocytes were obtained from Complement Technology, Inc. (Tyler, TX). Normal human serum with a defined hemolytic value was purchased as lyophilized powder from Sigma-Aldrich (St. Louis, MO). Rabbit anti-sheep erythrocyte antibody was purchased from Dako (Accurate Scientific Westbury, NY). Sheep red blood cells (SRBC) were washed and resuspended in GVB^{2+} buffer (0.1% Gelatin, 8.3 g/liter NaCl, 1.02 g/liter sodium barbital, 150 μM $CaCl_2$, 500 μM $MgCl_2$, pH = 7.27) at 10^9 cells/ml. SRBCs were treated with rabbit anti sheep red blood cell antibody in the presence of EDTA for 30 min at 37°C to coat the cells with antibody, referred to as EA. After washing the EA were resuspended at 10^9 cells/ml in GVB^{2+} buffer. Normal human serum was treated with from 10 nM to 1500 nM of actin or DBP-actin complexes for 1 hour at 37°C. An aliquot (10 μl) of the treated serum was mixed with EA and incubated for 30 min at 37°C. The samples were centrifuged and the red blood cell lysis was measured by reading the absorbance at 412 nm for the release hemoglobin. Water lysed EA were used as a positive control. The data were presented as percentage of hemolysis.

Factor B cleavage was evaluated in serum samples treated with DBP-actin as above. Diluted serum was separated using 10% SDS-PAGE and blotted onto a PVDF membrane. The blot was probed with mouse monoclonal anti-human factor B (clone D33/3) primary antibody followed by a goat anti-mouse HRP-conjugated secondary antibody. The blot was developed using HyGlo Quick spray chemiluminescent HRP antibody detection reagent and visualized by autoradiography film. The cleavage of factor B is readily observed by the disappearance of the native 100 kDa factor B band

and the appearance of the cleavage product, 33 kDa Ba fragment. The positive control for alternative pathway activation was CVF-treated serum.

2.8 Migration Assay

Cell movement was measured with a 48 well microchemotaxis chamber (Neuroprobe, Cabin John, MD) using a 5.0 μm pore size cellulose nitrate filters (purchased from Neuroprobe) as previously described [106]. Human neutrophils were isolated from venous blood of healthy, medication-free donors who gave informed consent [106]. Briefly, blood was drawn into a BD Vacutainer tube with EDTA and neutrophils were isolated using a standard three-step procedure. First, blood was treated with an equal volume of 3% dextran T-500 (GE Healthcare, Piscataway, NJ), prepared in sterile, pyrogen-free DPBS, for 30 minutes to sediment the RBCs. After washing the cells in DPBS, leukocyte-rich pellet was centrifuged through an endotoxin-free Lymphoprep (Accurate Scientific, Westbury, NY) gradient (400 x g for 30 min at 22°C) to separate the mononuclear leukocytes from the neutrophils. The neutrophil-rich pellet was resuspended in DPBS and exposed to hypotonic conditions (75 mosM) using sterile, pyrogen-free water for 1 minute to lyse any contaminating RBCs. Neutrophils were then washed twice in DPBS, counted, and placed on ice. Typical cell preparations contained 98% neutrophils, 1% eosinophils, and 1% mononuclear cells, and were 98 to 99% viable by trypan blue dye exclusion [106]. The human promyelocytic cell line HL-60 was also used and differentiated for 4 days using 1.3% DMSO to obtain a neutrophil-like phenotype [134]. In each assay, the migration of 200,000 (50 μl of $4x10^6$/ml)

neutrophils or differentiated HL-60 cells was evaluated. Cell movement was quantitated microscopically by measuring the distance in microns (μm) that the leading front of cells had migrated into the filter according to the method described by Zigmond and Hirsch [135]. In each experiment, five fields per duplicate filter were measured at 400 X magnification.

2.9 Endothelial Cell Culture

Human lung microvascular endothelial cells (HLMVEC) and endothelial cell growth media kits (CC-3156 & CC-4176) were obtained from Lonza (Walkersville, MD). HLMVEC were obtained at passage 3 and immediately thawed and cultured in EGM-2 Bulletkit at 5000 cells/cm^2 in T25 flask. The medium was changed the day after and subsequently every other day until cells were 70% to 80% confluent. HLMVECs were detached using Lonza sub-culturing reagents and the viability was determined by trypan blue assay. Human umbilical cord endothelial cells (HUVECs) were freshly isolated and generously provided by Dr. Martha Furie, Stony Brook University. These cells were grown in the same media as HLMVEC to 70 - 80% confluence.

2.10 Cell Viability Assay

Cell titer blue (CTB) viability assay kit was obtained from Promega (Madison, WI). Endothelial cells were cultured to 70% to 80% confluence and harvested as described above. 5000 cells in 100 μl of EGM2 complete medium were seeded to 96 well plates

and incubated at 37°C, 5% CO_2 incubator overnight for the cells to attach. The cells were treated for 4 hours or 24 hours and then CTB reagent was added after the treatment period. Cells with CTB reagent were incubated for 2 hours and the fluorescence intensity was then measured at 560 nm excitation and 590 nm emission.

2.11 Nuclear Staining to Evaluate Cell Death

Acridine orange (AO) and ethidium bromide (EB) were purchased from Fisher Scientific (Fairlawn, NJ). Endothelial cells were harvested at 70% to 80% confluence and seeded in 48 well plates at 2 x 10^5 cells/well and incubated overnight to allow the cells to attach. AO/EB solution was prepared with 100 µg/ml of each reagent. Cells were then treated for 24 hours with DBP-actin then each sample was stained with 100 µl of AO/EB solution just prior to microscopy and quantification. At least 300 cells were counted for each treatment and the percent of dead (red-orange nucleus) and live (green nucleus) cells was calculated.

2.12 Immunoblot for cleaved PARP

Rabbit anti-PARP antibody (Asp214) was purchased from Cell Signaling Technology (Danvers, MA). Endothelial cells were cultured to 70 - 80% confluence then harvested and seeded into 6 well plates at 10^6 cells/ml. Cells were incubated overnight to allow attachment and then were treated for 24 hours. After the treatment period cells were washed with DBPS 3 times and then detached. Whole cell lysates were obtained using

lysis buffer containing 100 mM Tris pH 7.4, 150 mM NaCl, 1.0 mM EDTA pH 8.0, 1% Triton X-100, 10% glycerol and a complete protease inhibitor cocktail tablet (Millipore). The protein concentration in the cell lysate was determined by a protein assay and 50 µg total protein was loaded on 10% SDS-PAGE. After separation the proteins were transferred to PVDF membranes (Millipore, MA). The PVDF membrane was probed with 1:500 rabbit anti-human cleaved PARP antibody. The blot was developed with HyGlo Quick spray chemiluminescent HRP antibody detection reagent (Denville Scientific Inc, South Plainfield, NJ) and visualized by autoradiography film.

2.13 Treatment of Endothelial Cells with Conditioned Supernatant from DBP-Actin Treated Neutrophils

Purified human neutrophils were resuspended at a concentration of 5 million cells per ml in HBSS. One million neutrophils in a total volume of 225 µl of HBSS containing 1 µM DBP, Actin and DBP-actin complex were transferred into microfuge tubes and incubated at 37°C and 5% CO_2 for 30 minutes. After 30 minutes, the cells were centrifuged at 1200 rpm (100 x g) for 5 minutes at 4°C, and the conditioned supernatant transferred into new microfuge tubes. The supernatants were blotted for S100A8/A9 complex and 100 µl of the supernatant were used to treat the endothelial cells for 24 hours, then cell viability was measured as described in the CTB viability assay.

2.14 Assay for Cellular Reactive Oxygen Species

Detection of cellular reactive oxygen species (ROS) in neutrophils and endothelial cells was performed using the DCFDA reagent as part of a kit purchased from Abcam (ab112851, Cambridge, MA). Primary human neutrophils were freshly purified as above and 4,000,000 cells were used per sample. Endothelial cells were grown to 70 - 80% confluence and 150,000 cells per well were seeded into 96 well plates and allowed to adhere for 24 hours before treatment. Cells were stained with 20 μM of DCFDA for 30 min at 37°C. Neutrophils were then treated with 50 μM TBHP (positive control), 1 μM PMA, 1 μM DBP, 1 μM actin, and 1 μM DBP-actin for 1 hour at 37°C. Endothelial cells were treated with 50 μM TBHP, EGM-2 media, 1 μM DBP, 1 μM actin or 1 μM DBP-actin for 24 hours. The fluorescence intensity was then measured at 485 nm excitation and 535 nm emission.

2.15 Statistical Analysis of Data

A minimum of 5 animals were used in each animal experiment and at least 3 experiments were performed for each in vitro analysis. Statistical differences between the mean values of the treatment groups were evaluated using either an unpaired t-test (comparing two groups) or analysis of variance (ANOVA) followed by a multiple comparisons post-test (comparing three or more groups). Statistical testing was performed using the software program InStat (GraphPad Software, San Diego, CA).

3. CHAPTER 1: DBP-ACTIN COMPLEXES INDUCE ACUTE LUNG INJURY IN VIVO

3.1 Actin-EASS Protein Complex Formation in Mouse Serum ex vivo

Once actin is released from dead or damaged cells, polymerized F-actin quickly will be severed to G-actin and then sequestered by DBP. As a prelude to in vivo experiments, the effect of adding purified actin to DBP+/+ and DBP-/- serum was tested ex vivo. To the best of our knowledge, the consequence of adding actin to DBP-/- (or DBP+/+) serum has never been examined. Serum from DBP+/+ (n = 3) and DBP-/- (n = 3) mice was spiked with 5 μM purified actin and analyzed by non-denaturing native gels to preserve the non-covalent complexes. Actin-EASS protein complexes were then detected by immunoblotting to verify the actin binding capacity of gelsolin and DBP (Figure 1). The results showed that actin complexes only with DBP in DBP+/+ mouse serum and there are little to no gelsolin-actin complexes. In contrast, as predicted there are no DBP-actin complexes in DBP-/- serum and all the gelsolin is bound to actin. This experiment verifies that actin-EASS protein complexes form in serum from these mice. Furthermore, it shows that in DBP+/+ serum all actin is bound to DBP, confirming that DBP has 100-fold higher affinity than gelsolin for binding actin [73].

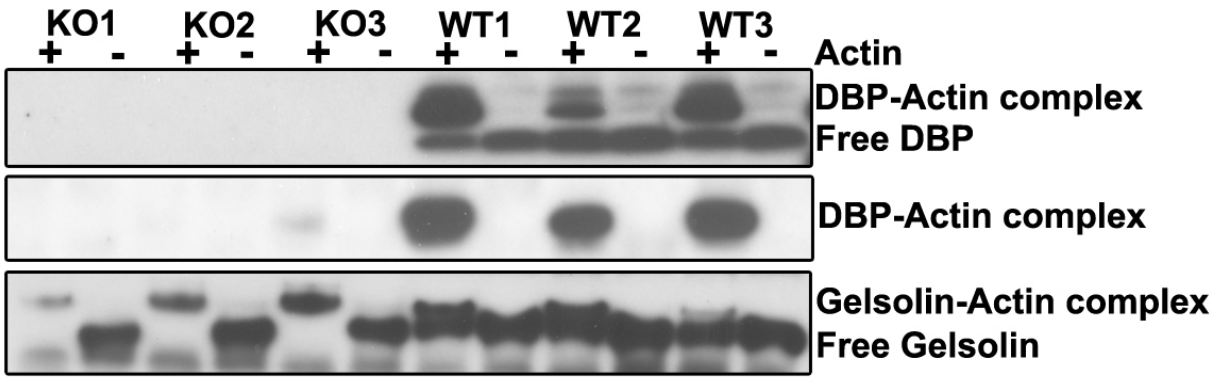

Figure 1: Actin added to mouse serum ex vivo forms complexes with EASS proteins.

Top panel: Blotted for DBP. Middle panel: Blotted for actin. *Bottom panel*: Blotted for gelsolin. Individual serum samples from three DBP+/+ (WT) and DBP-/- (KO) mice are shown. Each sample was analyzed after 5 μM purified actin was added and incubated for 30 minutes at 37°C, as indicated by a plus sign (+) at the top of the gel, or without actin (-). The positions of free and actin bound EASS proteins are shown.

3.2 Intravenous Injection of Actin into DBP+/+ and DBP-/- Mice

The actin scavenger function of DBP was investigated in vivo by intravenous injection of purified actin. The precise concentration of DBP in mouse plasma has not been reported but it is thought to be similar to rabbits and rats (approximately 5 μM). Thus, we initially injected 100 μg (~2.5 μM) or 200 μg (~5 μM) actin into wild-type (DBP+/+) mice to determine how much would be needed to elicit an effect. Wild-type mice were used for this pilot experiment, since published reports would suggest that large amounts of actin may cause rapid death in DBP-/- mice due to massive formation of actin filaments [63]. However, the two DBP+/+ mice that were injected with 200 μg of actin died almost immediately, possibly due to acute heart failure [63], while the DBP+/+ mice that were injected with 100 μg survived the procedure. Therefore, 100 μg of actin per mouse was used in all subsequent experiments.

Figure 2 shows the experimental approach employed to investigate the physiological effects of intravenous injected actin in wild-type (DBP+/+) and DBP null (DBP-/-) mice. Both DBP+/+ and -/- mice were injected with 100 μg of purified rabbit skeletal actin via the tail vein and then sacrificed after either 1.5, 4 or 24 hours. None of the mice in either group showed signs of overt respiratory distress and all displayed normal behavior following actin injection. At the indicated time points, mice were anesthetized and blood collected by retro-orbital bleeding. Mice were then euthanized, and the lungs, heart, liver and kidneys were collected for histological analysis to assess the effect of circulating DBP-actin complexes. In another parallel group of mice, the lungs were

isolated and homogenized to obtain single cell suspension that were analyzed by flow cytometry to assess the number of infiltrating neutrophils and macrophages. At 4 hour time point (generally the peak of acute inflammation after a single stimulus with actin in animal models), lungs were lavaged and BAL fluid was collected for protein concentration and total number of leukocytes. EDTA plasma samples were analyzed using non-denaturing (native) gels to verify formation of DBP-actin complexes. Figure 3A demonstrates that, as expected, DBP-actin complexes are observed only in wild-type (DBP+/+) mice following actin injection. Analysis of the blots by densitometry revealed that the greatest amount of complexes were observed after 1.5 hours (78% of total DBP) and then declined to about 50% at 4 hours and 30% at 24 hours (Fig. 3B). This observation is consistent with previous studies showing that DBP-actin complexes have a very short half-life and are quickly cleared from circulation [100]. Figure 4A shows that there are little to no gelsolin-actin complexes formed in DBP+/+ mouse plasma whereas almost all the gelsolin is bound to actin in DBP-/- mice. Furthermore, analysis of DBP+/+ and -/- mouse plasma by SDS-PAGE indicated that both strains had approximately the same amount of total gelsolin in blood (Fig. 4B), indicating that DBP-/- mice did not have a compensatory increase in the other plasma EASS protein.

Previous published reports indicate that F-actin in the circulation has pathological effects if it is not sequestered and cleared promptly [63]. Therefore, we initially hypothesized that DBP-/- mice will develop much more severe tissue injury due to the systemic lack of DBP. However, the DBP+/+ mice showed more extensive lung inflammation than DBP-/- mice. Figure 5 shows that DBP +/+ mice had extensive

interstitial edema, thickening of the alveolar wall, infiltration of neutrophils, hemorrhage, formation of hyaline membranes and the presence of proteinaceous debris in large regions of the lung 1.5 hours after actin injection. The inflammation was diminished at 4 hours and largely resolved 24 hours following actin injection. In contrast, DBP-/- mice showed almost no acute lung inflammation at 1.5 hours, mild inflammation at 4 hours that was considerably less than the DBP+/+ group (Fig. 5). Histological evidence of acute lung inflammation in both DBP+/+ and -/- mice was noticeably decreased at 24 hours (Fig. 5). The amount of circulating DBP-actin complexes (Fig. 3A) correlated with the degree of inflammation (Fig. 5) suggesting that DBP-actin complexes could mediate lung inflammation. Interestingly, the lung was the only organ that showed inflammatory injury after intravenous actin injection; the heart, liver and kidneys all appeared essentially identical to the PBS controls (data not shown). The degree of lung inflammation was quantitated by measuring the number of Gr1+ neutrophils and F4/80+/Gr1- macrophages by flow cytometry (flow gating shown in Fig. 6A) in lung homogenates 1.5, 4 or 24 hours after actin injection (Fig. 6B). Both groups showed the largest number of neutrophils after 4 hours that decreased by 24 hours (Fig. 6B). There were also significantly reduced numbers of lung neutrophils in the DBP-/- group versus DBP+/+ animals at the 1.5 and 4 hour time points (Fig. 6B). Neither of the groups showed significant macrophage infiltration at any of the time points and there were no difference of macrophage percentage between DBP+/+ and DBP-/- mice.

The pulmonary capillary bed is the first microvasculature that any intravenously injected substance would encounter. Injury to the capillary endothelium can induce

permeability changes and provoke inflammation of the airspaces (alveolitis). To determine if circulating DBP-actin complexes can also cause an alveolitis, bronchoalveolar lavage (BAL) was performed using DBP+/+ and -/- mice 4 hours after intravenous injection of actin. Figure 7A shows that actin injection induced significant protein leakage into the airspaces of DBP+/+ mice that was not observed in DBP-/- animals. In addition, DBP+/+ mice also had a significant increase in the number of BAL leukocytes compared to the DBP-/- group (Fig. 7B). Thus, the preceding experiments demonstrate that DBP-actin complexes are associated with acute lung inflammation that is largely resolved by 24 hours.

Finally, we tested the inflammatory response 4 hours after actin injection by measuring the level of 23 cytokines (Bio-Plex 23 panel mouse cytokine assay) in the plasma (n = 1) and the BAL (n = 4). There were no significant differences between DBP+/+ and -/- mice in the levels of 23 cytokines measured in either plasma (Fig. 8) or the BAL (Fig. 9) 4 hours after injection of DBP-actin complexes. Although for some cytokines it seems that DBP-/- mice have elevated amounts, particularly in the BAL (Fig. 9), these were not significantly different. The amount of cytokines in both mouse strains was at the lowest limit of detection and considerably lower than levels reported in other murine models of lung injury [136], thus these variations could be “background noise”. The chemokine KC (CXCL1 in mice), which is a potent neutrophil chemoattractant, is increased dramatically in the DBP+/+ mouse plasma comparing to their DBP-/- counterparts (Fig. 8). However, this one sample could be an outlier and result needs to be validated by repeating the experiments to include more samples for statistical

significance. Based on the difference in DBP+/+ and -/- with respect to histology and protein leakage, we propose that it is DBP-actin complexes, and not F-actin, that is responsible for the actin-induced acute lung inflammation.

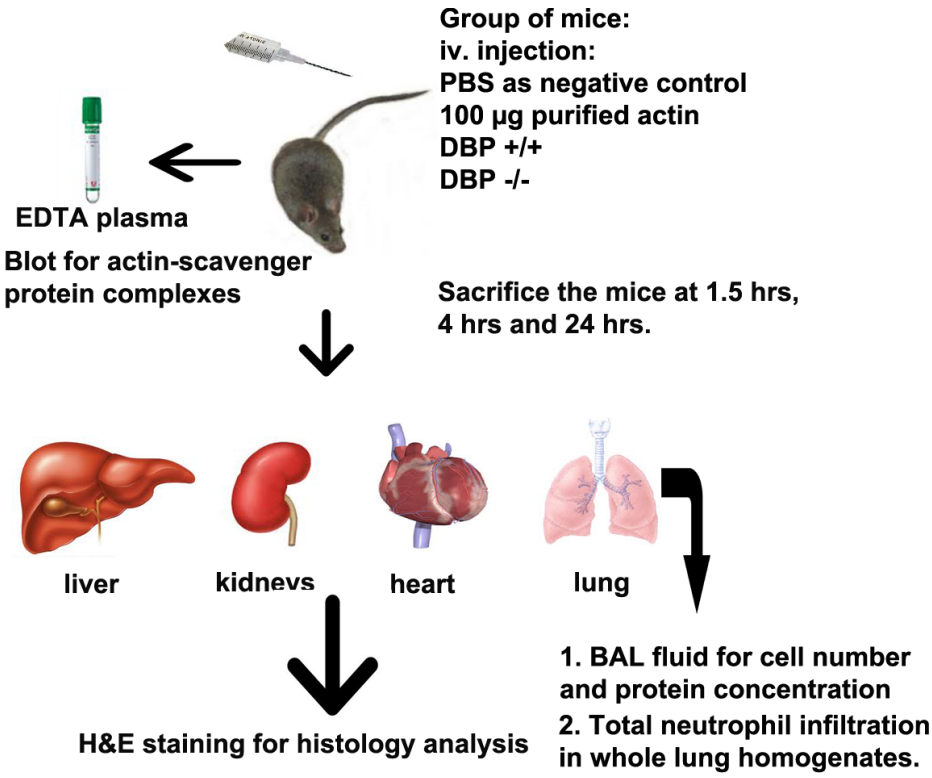

Figure 2: Experimental design of in vivo actin injection in DBP+/+ and DBP-/- mice.

Age matched DBP+/+ and DBP-/- mice were injected with 100 µg of purified skeletal muscle actin via tail vein. After 1.5, 4, and 24 hours, mice were anesthetized. EDTA plasma was collected by retro-orbital eye bleed. Liver, kidneys, heart and lungs were collected for histological analysis. Lungs were inflated with 10% neutral buffered formalin prior to removal, in other mice, lungs were lavaged and BAL fluid was collected. In selected mice, lungs were removed and processed to obtain whole lung homogenates to quantify the percentage of neutrophils by flow cytometry.

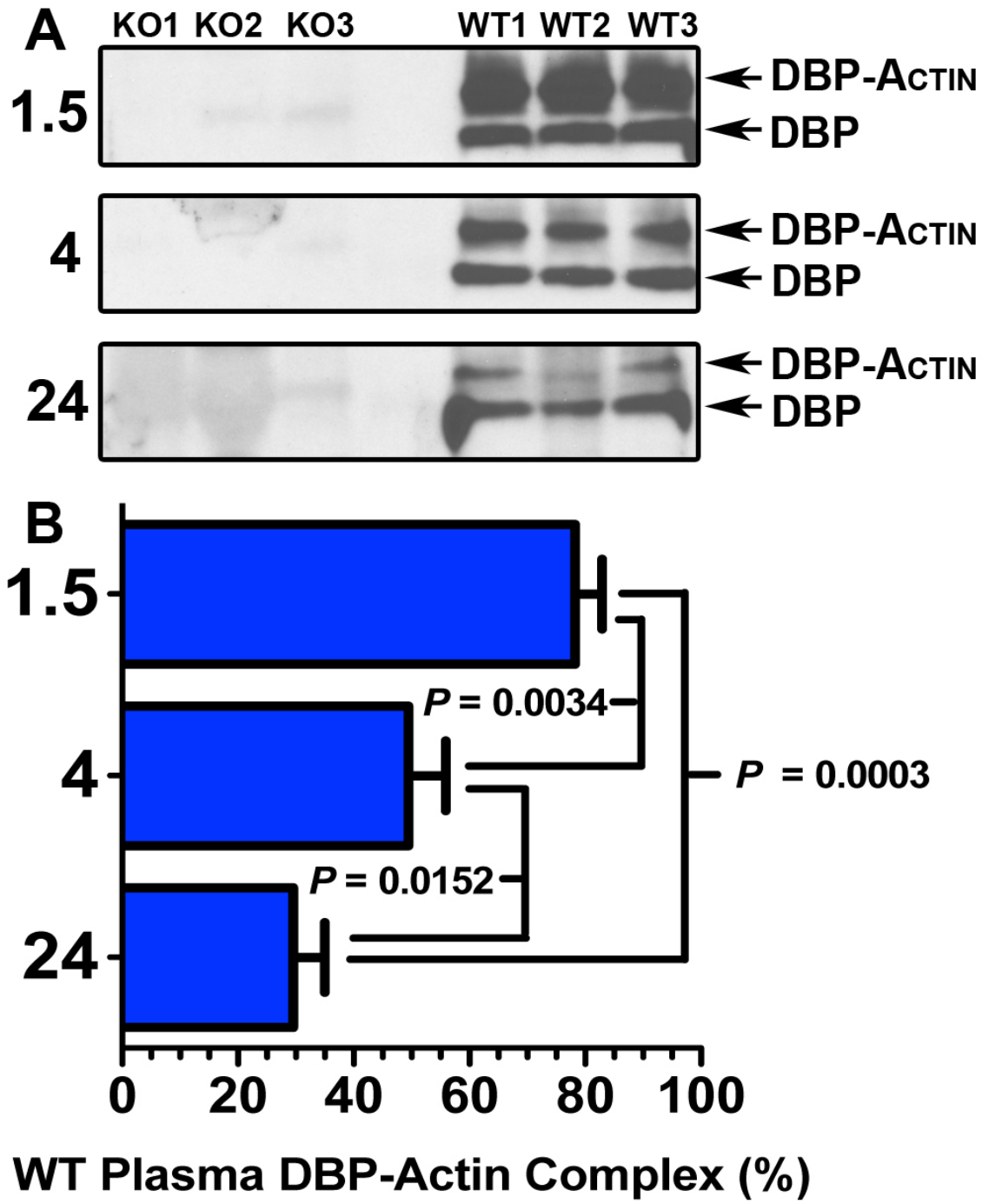
A
KO1 KO2 KO3
WT1 WT2 WT3
1.5
DBP-ACTIN
DBP
4
DBP-ACTIN
DBP
24
DBP-ACTIN
DBP
B
1.5
4
24
P = 0.0034
P = 0.0003
P = 0.0152
0
20
40
60
80
100
WT Plasma DBP-Actin Complex (%)

Figure 3: DBP-actin complexes level in DBP+/+ and DBP-/- mice after intravenous actin injection.

(A) DBP immunoblot of EDTA plasma samples from three DBP+/+ wild-type (WT) mice and three DBP-/- (KO) mice each obtained 1.5, 4 and 24 hours after actin injection. Plasma aliquots were separated using a 10% native (non-denaturing) gel then blotted for DBP. The electrophoretic separation of unbound DBP and DBP-actin complexes is shown. **(B)** Densitometry measurement of DBP-actin complexes in DBP+/+ mice in panel A. Data is presented as mean ± SEM (n = 3) of DBP-actin as a percent of total DBP (unbound + DBP-actin) in WT plasma. Statistical significance is indicated.

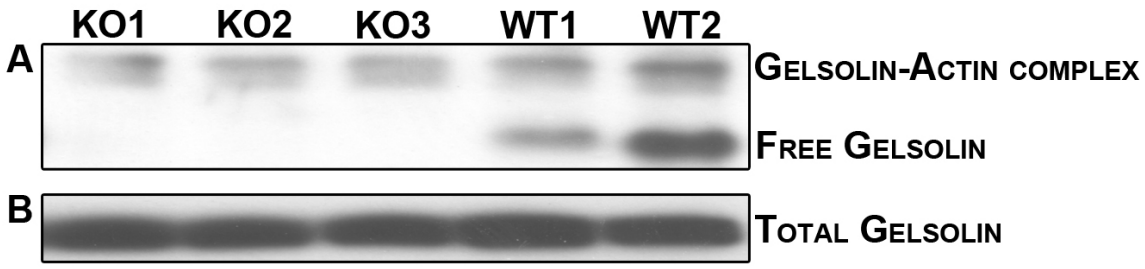

Figure 4: Plasma total gelsolin and gelsolin -actin complexes in DBP+/+ and DBP-/- mice after actin injection.

(A) Gelsolin immunoblot of plasma separated by non-denaturing PAGE from three DBP-/- (KO) and two DBP+/+ (WT) mice. **(B)** Gelsolin immunoblot of plasma separated by SDS-PAGE from three DBP-/- (KO) and two DBP+/+ (WT) mice.

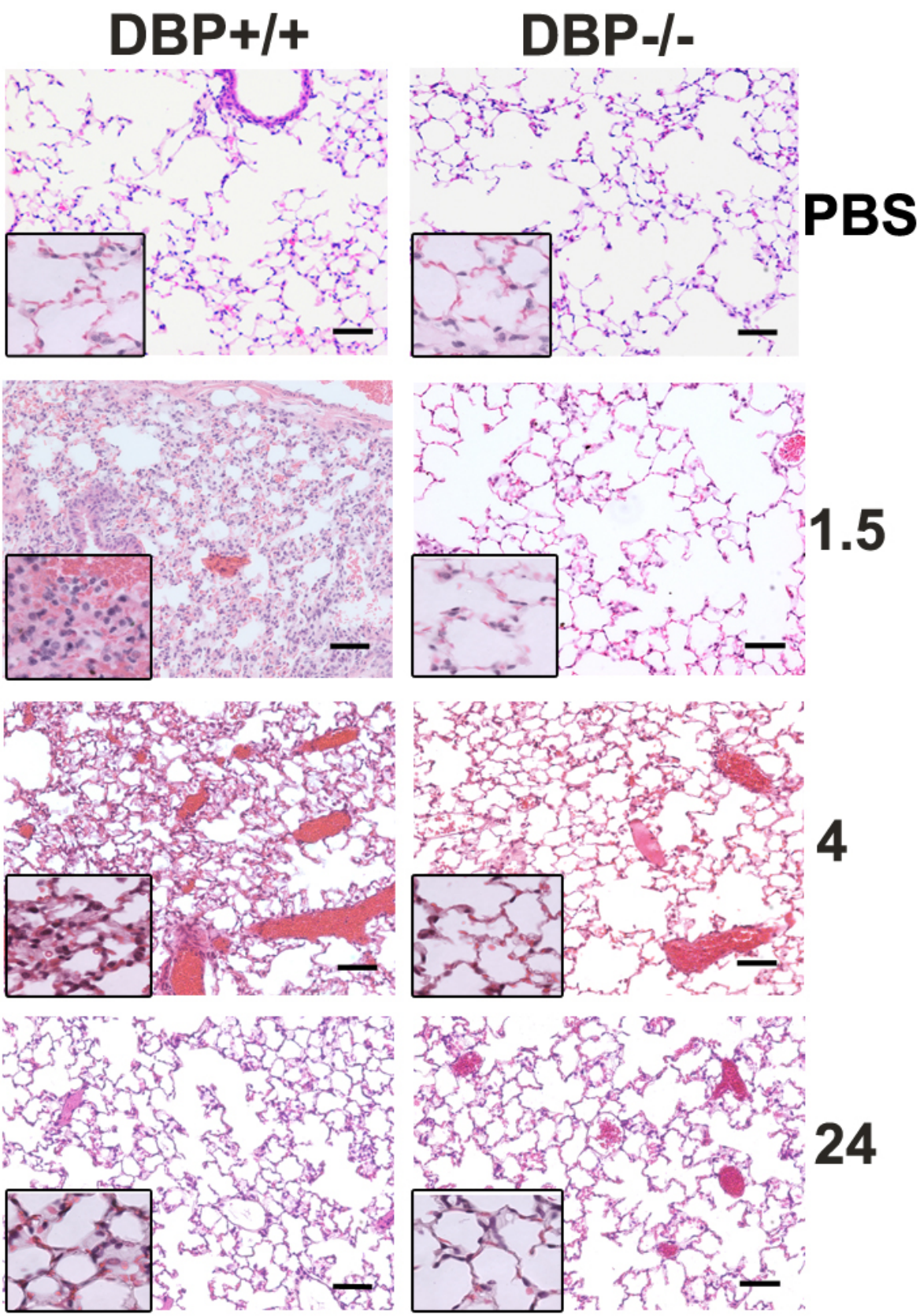
DBP+/+
DBP-/-
PBS
1.5
4
24

Figure 5: Histology of DBP+/+ and DBP-/- mouse lungs after actin injection.

Histology of acute lung inflammation in DBP+/+ and DBP-/- mice 1.5, 4 and 24 hours after intravenous injection of 100 µg purified rabbit skeletal muscle actin in a total volume of 100 µl. H&E stained lung sections were examined microscopically. The image in each main panel was at 200x magnification, bar = 100 µm. The inset image was obtained at 1000x magnification

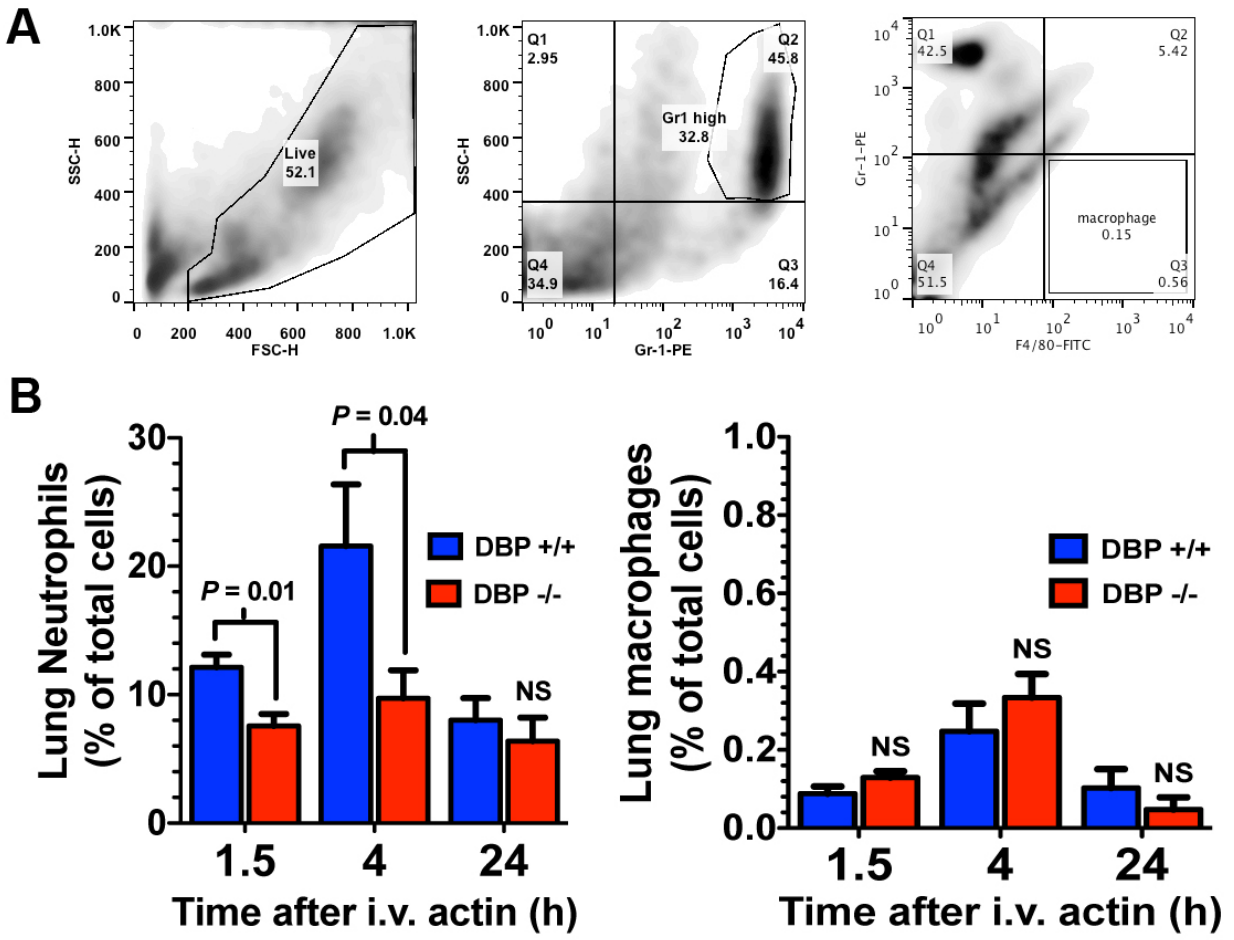

Figure 6: Analysis of total neutrophils and macrophages in whole lung homogenates 1.5, 4, and 24 hours after actin injection.

(A) Flow cytometry gating for analyzing neutrophils and macrophages in whole lung homogenates**.** A total of 10^4 cells were collected and the forward scatter (FSC) and side scatter (SSC) gates set to exclude dead cells and debris (left panel). Neutrophils were gated as the Gr-1-PE high, side scatter high cell population (center panel). Macrophages were gated as F4/80-FITC high, Gr-1-PE low population. **(B)** The percent of Gr-1+ neutrophils and F4/80 +/ Gr-1- macrophages in lung homogenates measured by flow cytometry. Numbers represent mean percent of total lung cells ± SEM (n= 5); statistical significance is indicated.

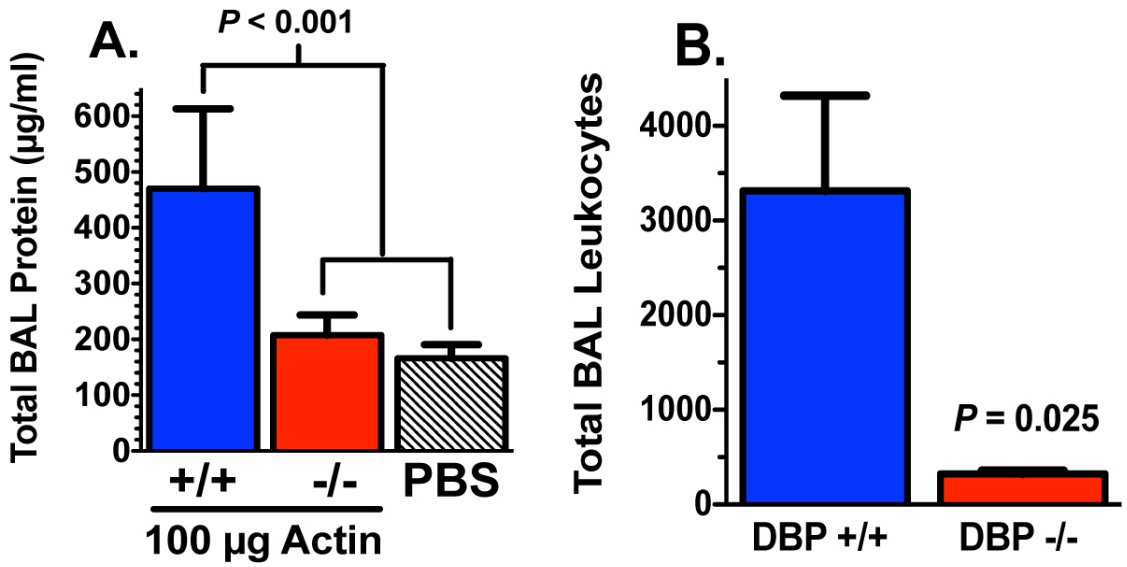

Figure 7: Analysis of total protein and leukocytes in BAL 4 hours after actin injection.

(A) Total protein content of cell-free BAL fluid from actin injected DBP+/+ mice (n = 6), actin injected DBP-/- mice (n = 7) and PBS injected mice (n = 4). Statistical significance is indicated. **(B)** Total BAL leukocytes from actin injected DBP+/+ and DBP-/- mice (n = 5) as measured from stained cytospin slides. Numbers represent mean ± SEM; statistical significance is indicated.

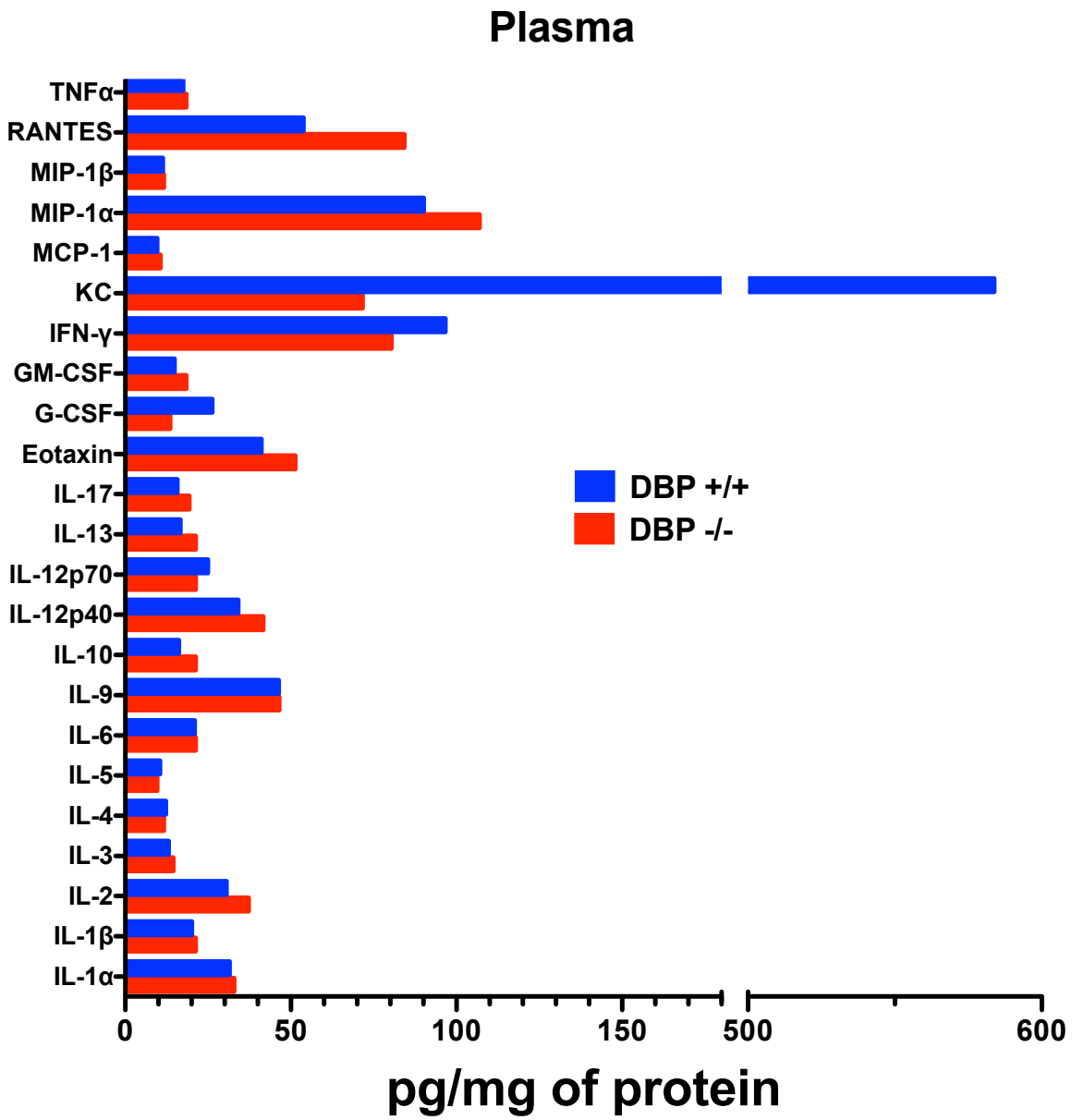

Figure 8: Plasma cytokine level of DBP+/+ and DBP-/- mice 4 hours after actin injection.

Bio-Plex 23 panel mouse cytokine assay in DBP+/+ and DBP-/- mouse plasma collected 4 hours after actin injection. Results are from one DBP+/+ and one DBP-/- mouse.

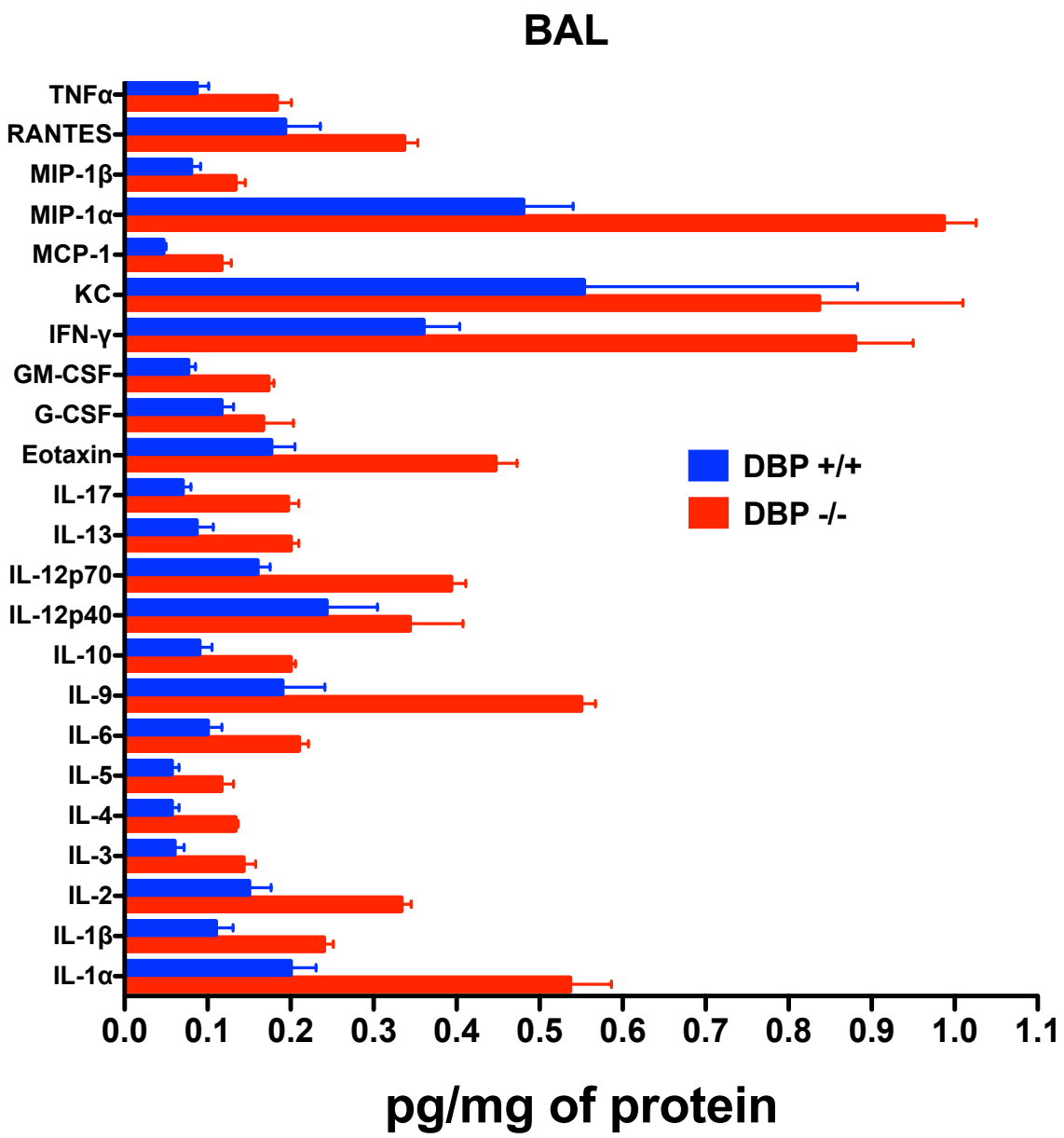

Figure 9: BAL cytokine level of DBP+/+ and DBP-/- mice 4 hours after actin injection.

Bio-Plex 23 panel mouse cytokine assay in DBP+/+ and DBP-/- mouse BAL fluid (n = 4) obtained 4 hours after actin injection.

4. CHAPTER 2. DBP-ACTIN COMPLEXES CAN DIRECTLY CAUSE ENDOTHELIAL CELL INJURY AND DEATH

4.1 DBP-Actin Complexes Do Not Activate Complement

The possible effects of DBP-actin complexes on triggering immediate inflammatory mechanisms were investigated next. The activation of complement system is often involved in infection-induced alveolitis due to the production of C5a and the subsequent neutrophil infiltration into the lungs. Endogenous molecules such as DAMPs have been shown to activate complement in ischemia-reperfusion injury models. We investigated if DBP-actin complexes can active serum complement in vitro. A hemolytic assay was utilized to test if DBP-actin complexes can activate complement through the classical and/or lectin pathways. The classical pathway is typically activated by IgG or IgM immune complexes, while the lectin pathway is activated by microbial polysaccharides. These two pathways share a common enzyme activation step and this assay detects activation of either pathway. Human serum was incubated with various concentrations of actin or DBP-actin complexes for 30 min at 37°C. The treated serum samples were then mixed with antibody coated sheep red blood cells to test if the pretreatment activated, and thus depleted, complement resulting in an inhibition of hemolysis. Red blood cells lysed in water were used as positive control (100% lysis). All serum samples treated with actin or DBP-actin showed close to 100% hemolysis (Fig. 10), indicating that DBP-actin complexes do not active the classical/lectin pathway of the complement.

The possible effect of DBP-actin complexes on the alternative pathway was examined next. The activation of the alternative pathway will result in cleavage of factor B (92 kDa) to produce factor Ba (33 kDa). Treated serum samples were separated by SDS-PAGE and then immunoblotted using an anti-factor B antibody. CVF-activated serum was used as a positive control. Figure 11 shows that there is no increase in Ba fragment with DBP-actin treatment, indicating that DBP-actin does not activate the alternative pathway of complement.

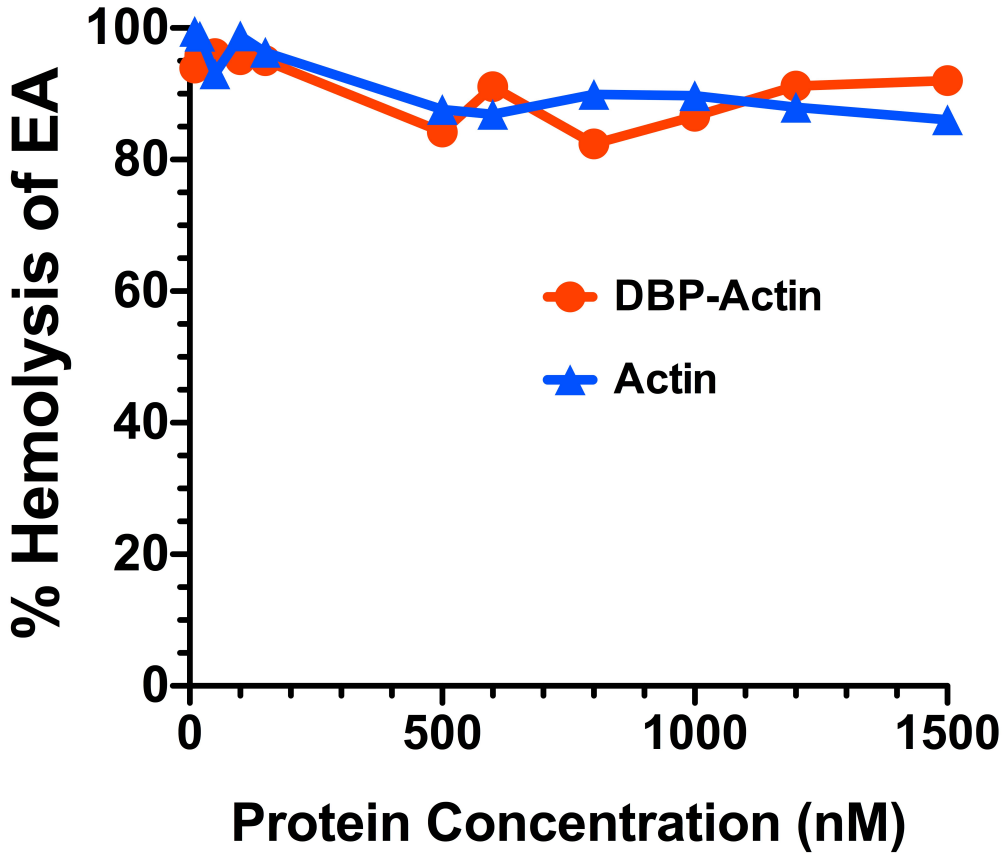

Figure 10: DBP-actin complexes do not activate the classical/lectin pathway of the complement.

Hemolytic assay using normal human serum treated with purified actin or DBP-actin complexes (10 nM to 1500 nM) for 1 hour at 37°C. After treatment, an aliquot of serum was added to antibody coated sheep red blood cells to determine if the pretreatment of serum activated (depleted) complement, producing an inhibition of hemolysis.

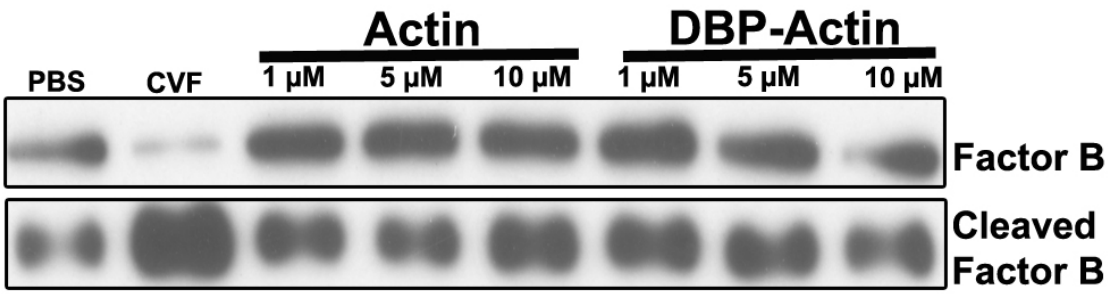

Figure 11: DBP-actin complexes do not activate the alternative pathway of the complement.

Purified actin or DBP-actin complexes were added to pooled normal human serum and incubated for 1 hour at 37°C. Samples were analyzed by SDS-PAGE and blotted for factor B to determine cleavage. Lane 1: sham-treated serum; Lane 2: CVF-treated serum; Lane 3: 1 μM actin; Lane 4: 5 μM actin; Lane 5: 10 μM actin; Lane 6: 1 μM DBP-actin; Lane 7: 5 μM DBP-actin; Lane 8: 10 μM DBP-actin. Cleavage of 92 kDa factor B and appearance of the 33 kDa Ba fragment indicates alternative pathway activation as observed in the CVF lane.

4.2 DBP-Actin Complexes are not Chemotactic for Neutrophils

Figures 5 and 6 shows that there were a large number of neutrophils infiltrating into the lungs of DBP+/+ mice 1.5 hours and 4 hours after actin injection. The possibility that DBP-actin complexes directly recruited these neutrophils was tested next. Both normal human neutrophils from a healthy donor and DMSO-differentiated HL-60 cells were used to test if DBP-actin complexes are chemotactic in vitro using a filter-based migration assay. Cells were exposed to several concentrations of DBP-actin complexes and results show that cells did no migrate towards DBP-actin complexes (Fig. 12).

4.3 DBP-Actin Complexes do not Induce Neutrophils to Generate Reactive Oxygen Species in vitro

The production of ROS by neutrophils is a mechanism that these cells use to kill pathogens but also can induce collateral tissue injury. The possibility that DBP-actin complexes can trigger neutrophils to generate ROS, and thus contribute to the acute lung injury we observe in vivo, was investigated. Figure 13 showed that there was no cellular ROS generation after DBP-actin complex treatment, discounting another potential mechanism by which DBP-actin complexes might induce acute lung inflammation.

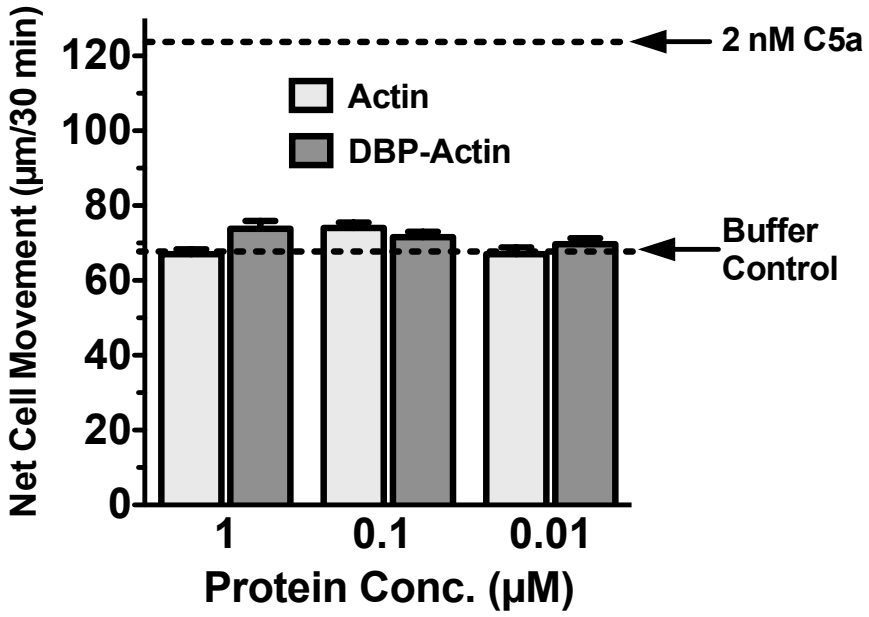

Figure 12: DBP-actin complexes are not chemotactic for neutrophils.

Indicated concentrations of purified actin or DBP-actin complexes were diluted in chemotaxis buffer and added to the lower wells of the chemotaxis chamber. Neutrophils or differentiated HL-60 cells (2 x 10^5) were added to the upper chambers and cells were allowed to migrate into a 5 µm pore size cellulose nitrate filter for 30 min at 37°C. The positive control was 2 nM purified human C5a, and the negative control was the assay buffer, these values are shown as dotted lines.

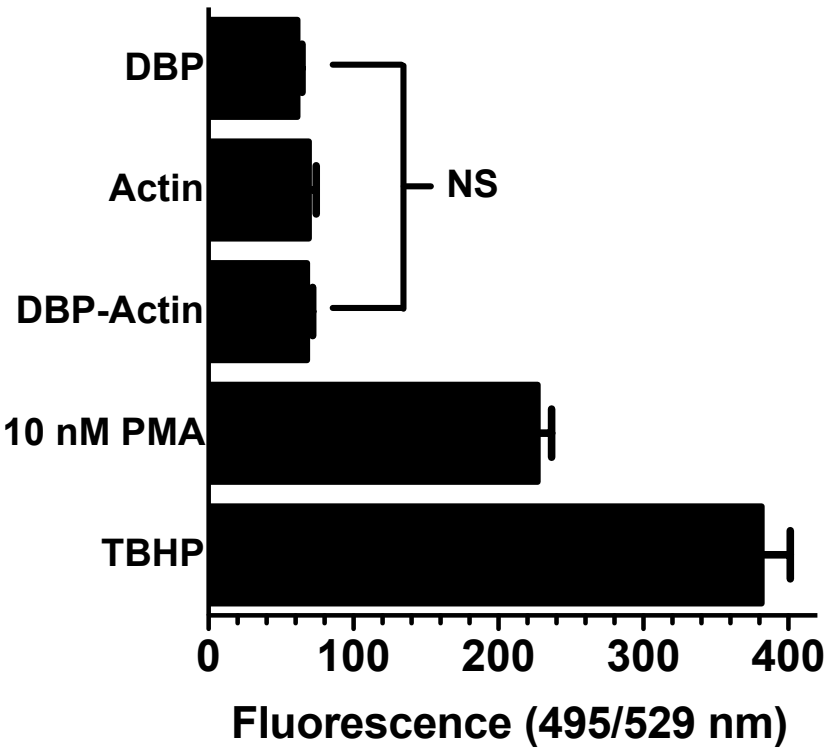

Figure 13: DBP-actin complexes do not induce ROS generation in neutrophils. Neutrophils preloaded with DCFDA were treated with 1 μM of DBP, actin or DBP-actin complex; 10 nM PMA and TBHP were used as positive controls. The generation of ROS was determined by reading the fluorescence at 485 nm excitation and 535 nm emission.

4.4 DBP-Actin May Causes Acute Lung Inflammation by Targeting Endothelial Cells

The increased vascular leakage and damage to pulmonary capillary endothelium are some of the most important characteristics of acute lung inflammation. The observed lung inflammation presented above (Figs. 5-7) would be consistent with damage to lung microvascular endothelial cells. Thus, the possibility that DBP-actin complexes have a direct effect on endothelial cells was investigated next in vitro.

4.4a DBP-actin Complex Decreases Cell Viability in HLMVECs and HUVECs and the Effect is Reversible.

Primary human endothelial cells from the microvasculature of an adult lung (HLMVEC) and from an umbilical cord (HUVEC) were treated with DBP alone, actin alone or DBP-actin complexes for 4 hours at 37°C and the effect was assessed using the cell-titer blue (CTB) viability assay. HLMVEC treated with DBP-actin complexes showed a significant reduction in the ability to metabolize the fluorescent CTB substrate compared to untreated cells, DBP treated or actin treated cells (Fig. 14). The effect of serum starvation is shown as the positive control (Fig. 14). HUVECs are more robust cells and showed increased viability in response to the treatments as compared to HLMVECs, but there was still a significant reduction in viability with DBP-actin treatment (Fig. 15). A similar reduction in cell viability with DBP-actin treatment was also observed using primary human neonatal dermal endothelial cells (data not shown). The morphology of DBP-actin treated endothelial cells by phase contrast microscopy shows

that HLMVECs are retracted and partially rounded-up (Figure 16), similar results were also noted with HUVECs (data not shown). To determine if damage is reversible, cells were treated with DBP-actin complexes for 4 hours then either washed and placed in fresh medium for a 24 hour recovery period or tested immediately using the CTB assay. Figures 17 and 18 show that the injurious effects of a 4 hour DBP-actin treatment were fully reversible in both HLMVEC and HUVECs. These results show that DBP-actin complexes can directly alter the viability of primary endothelial cells from large (HUVEC) and small (HLMVEC) vessels from different organs.

4.4b Prolonged Exposure to DBP-actin Complexes Induces Endothelial Cell Death

The previous cell viability and morphology data would indicate that DBP-actin complexes directly induce endothelial cell injury (Figs. 14-16). To determine if prolonged treatment can cause cell death, primary endothelial cells were treated for 24 hours with DBP-actin complexes and then stained with acridine orange and ethidium bromide to determine the percent live/dead cells. The nucleus of live cells stains green with acridine orange whereas dead cells take up ethidium bromide and display a red or orange nucleus. This assay is quantified by counting the number of dead and live cells in multiple fields. Figure 19 demonstrates that DBP-actin complexes induce the same degree of HLMVEC cell death as the positive control (serum starvation), and significantly less than untreated or single treated (DBP alone, actin alone) cells. Figure 20 shows that HUVECs are more resistant to the injurious effects of DBP-actin

complexes or serum starvation, but still display a significant decrease in the number of live cells following a 24 hour treatment.

The kinetics of endothelial cell injury and death following treatment with DBP-actin complexes would be consistent with an apoptotic mechanism. Therefore, potential mechanisms of cell death were next investigated by examining the cleavage of poly ADP ribose polymerase (PARP), a key substrate in the caspase-3 dependent apoptosis pathway. Cell lysates of HUVECs were treated with controls such as H_2O_2, TNF-α, IL-1β that can cause endothelial cell injury or death [137-139], as well as DBP-actin complexes, and samples were blotted for cleaved PARP. In HLMVECs, serum starvation was used as positive control (Fig. 21). There is an increased cleaved PARP in HUVECs 24 hours after DBP-actin treatment, as well as in IL-1 treated HUVECs indicating that DBP-actin induces cell death in HUVECs through caspase-3 dependent apoptosis pathway (Fig. 21A). In contrast, although HLMVECs are more sensitive to DBP-actin induced injury and death, there was no cleaved PARP detected in these cells (Fig. 21B). This may indicate that an unknown caspase-3 independent pathway triggers cells death in HLMVECs.

One mechanism to trigger an apoptotic pathway in endothelial cells is by generation of intracellular reactive oxygen species. Figure 22 shows that DBP-actin complexes did not increase the amount of intracellular oxidants over untreated control cells. These results indicate that DBP-actin complexes can directly trigger HLMVEC cell death by inducing an apoptosis pathway via an unknown mechanism.

Finally, because endothelial cells are very sensitive to the effects of LPS, we considered the possibility that the pure actin and DBP we used in the above in vitro assays may be contaminated with LPS. Both DBP and actin were purchased as lyophilized powders that were reconstituted with endotoxin-free sterile reagents using aseptic technique in a laminar flow BSL-2 cabinet. All subsequent experiments were performed with endotoxin-free reagents in the sterile hood. In addition, we have performed experiments where polymyxin B was added to DBP and this treatment did not reduce the effect of DBP-actin on endothelial cells. Purified actin was not tested, but since cells treated with actin alone did not show an increase in cell injury or death, we conclude that actin is also not contaminated with LPS.

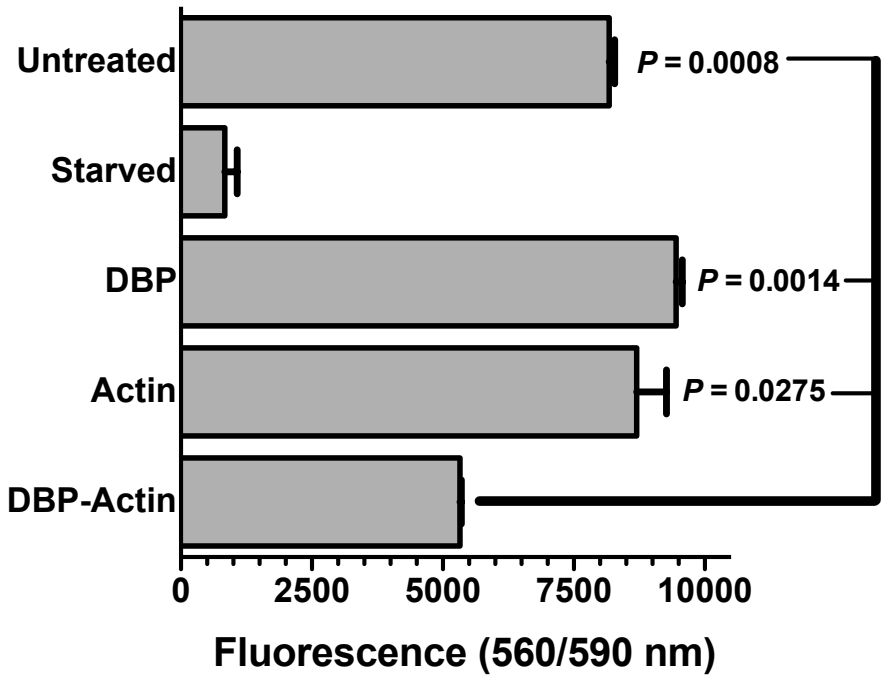

Figure 14: CTB viability assay of HLMVEC treated with DBP-actin complexes.

HLMVECs were treated for 4 hours with: untreated (cultured with EGM-2 growth medium as negative control), Starved (cultured with EGM-2 base medium without serum as positive control), or 1 μM of the indicated purified proteins in EGM-2 growth medium. CTB reagent was added to the wells at 20 μl/well. Cells were incubated for 1 hour with CTB reagent and the fluorescent intensity was measured at 560 nm excitation and 590 nm emission. Numbers represent mean ± SEM (n = 3), statistical significance is indicated.

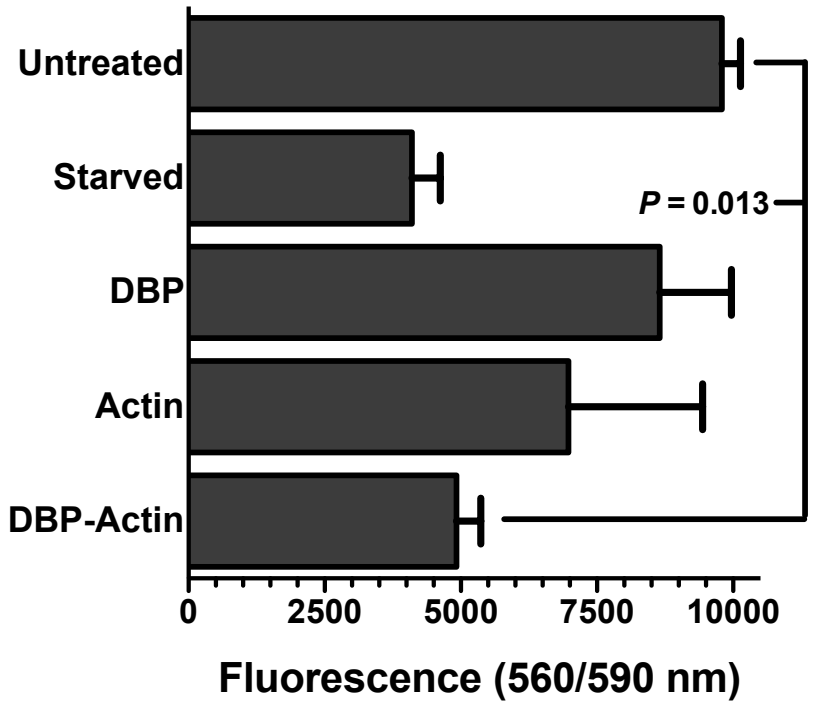

Figure 15: CTB viability assay of HUVEC treated with DBP-actin complexes.

HUVECs were treated for 4 hours with: untreated (cultured with EGM-2 growth medium as negative control), Starved (cultured with EGM-2 base medium without serum as positive control), or 1 μM of the indicated purified proteins in EGM-2 growth medium. CTB reagent was added to the wells at 20 μl/well. Cells were incubated for 1 hour with CTB reagent and the fluorescent intensity was measured at 560 nm excitation and 590 nm emission. Numbers represent mean ± SEM (n = 3), statistical significance is indicated.

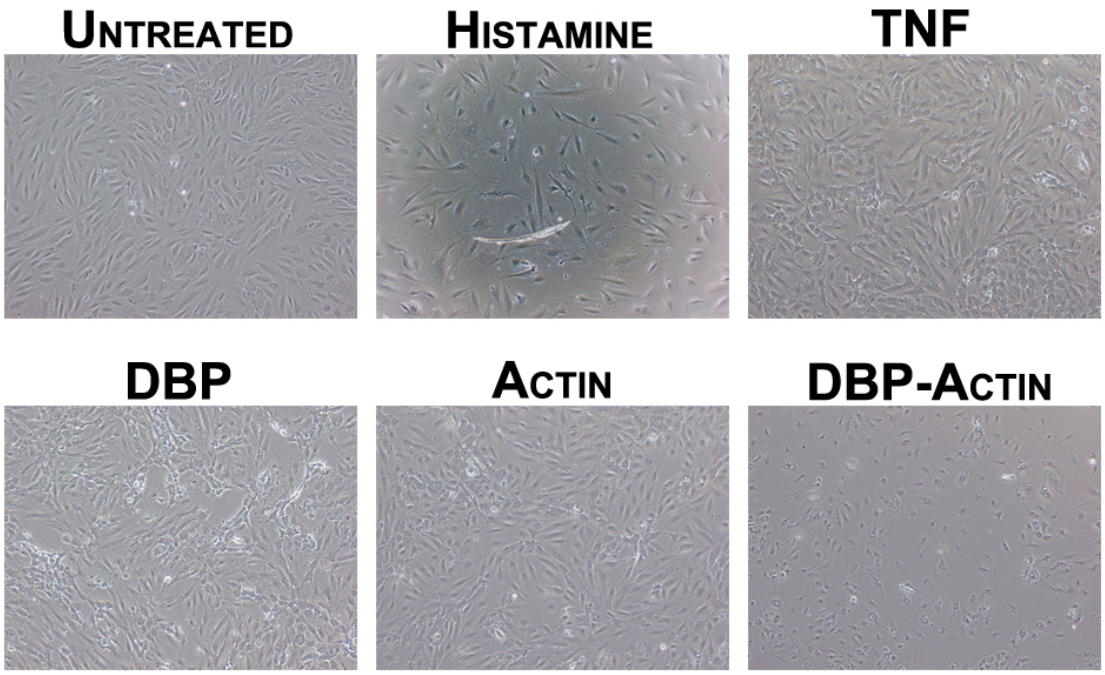

Figure 16: Morphology of HLMVEC treated with DBP-actin complexes.

HLMVECs were treated with: untreated (cultured with EGM-2 growth medium as negative control), 1 μM histamine, 10 nM TNFα, 1 μM DBP, 1 μM actin and 1 μM DBP-actin complex. Phase contrast images were taken at 200x magnification.

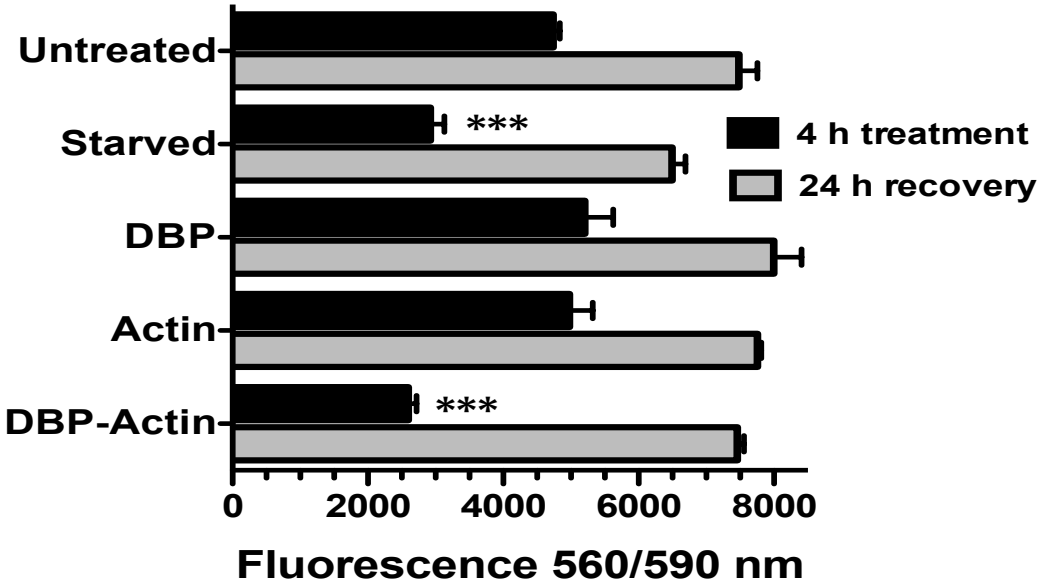

Figure 17: CTB viability assay to test reversible injury in HLMVEC treated with DBP-actin complexes.

HLMVECs were treated for 4 hours with: untreated (cultured with EGM-2 growth medium as negative control), Starved (cultured with EGM-2 base medium without serum as positive control) or 1 μM of the indicated purified proteins in EGM-2 growth medium (black bars). A duplicate set of cells were washed after the 4 hour treatment and allowed to recover in EGM-2 growth medium for an additional 24 hours. CTB reagent was added to the wells at 20 μl/well. Cells were incubated for 1 hour with CTB reagent and the fluorescent intensity was measured at 560 nm excitation and 590 nm emission. Numbers represent mean ± SEM (n = 3). Triple asterisk indicates that the values of DBP-actin treated cells and serum starved cells were significantly lower ($P < 0.001$) than all other groups.

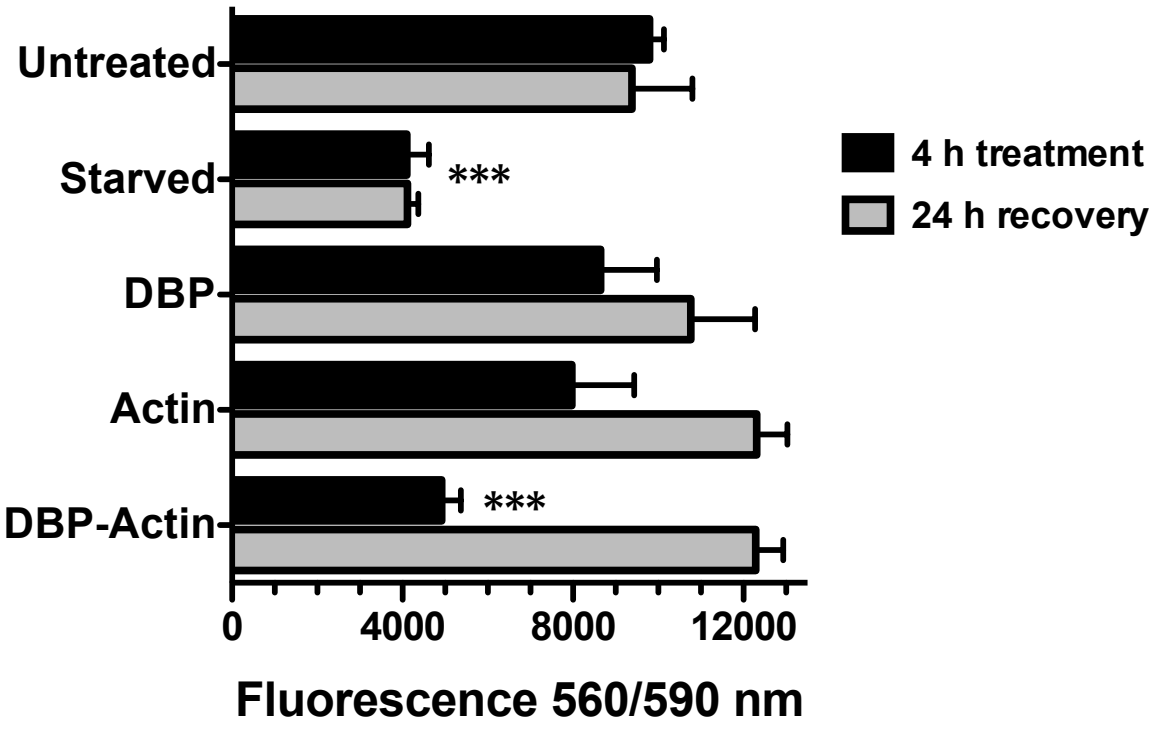

Figure 18: CTB viabiligy assay to test reversible injury in HUVEC treated with DBP-actin complexes.

HUVECs were treated for 4 hours with: untreated (cultured with EGM-2 growth medium as negative control), Starved (cultured with EGM-2 base medium without serum as positive control) or 1 μM of the indicated purified proteins in EGM-2 growth medium (black bars). A duplicate set of cells were washed after the 4 hour treatment and allowed to recover in EGM-2 growth medium for an additional 24 hours. CTB reagent was added to the wells at 20 μl/well. Cells were incubated for 1 hour with CTB reagent and the fluorescent intensity was measured at 560 nm excitation and 590 nm emission. Numbers represent mean ± SEM (n = 3). Triple asterisk indicates that the values of DBP-actin treated cells and serum starved cells were significantly lower ($P < 0.001$)

than all other groups.

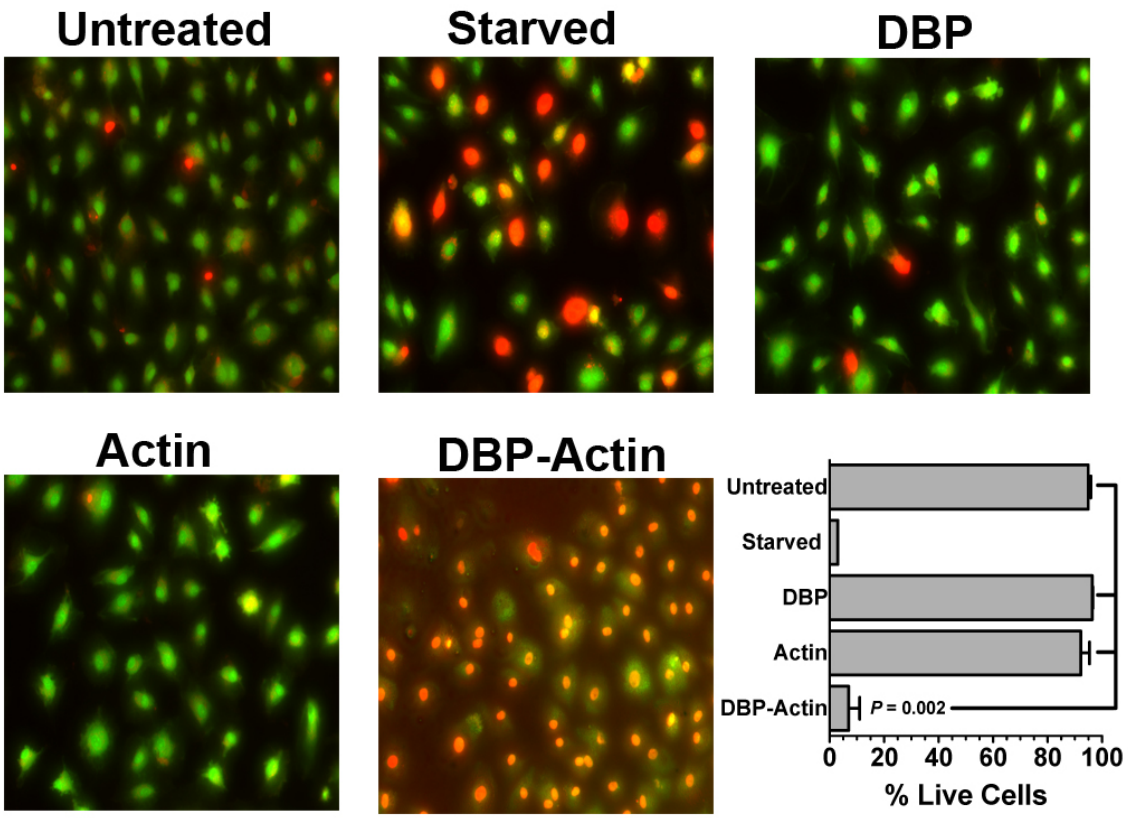

Figure 19: Acridine orange and ethidium bromide nuclear staining of HLMVEC treated with DBP-actin complexes for 24 hours.

Green nuclei indicate live cells while red/orange/yellow nuclei indicate dead cells. HLMVEC were treated with: untreated (cultured with EGM-2 growth medium as negative control), Starved (cultured with EGM-2 base medium without serum as positive control), 1 μM DBP, 1 μM actin or 1 μM DBP-actin complex for 24 hours. Cells were

stained with AO/EB and the percentage of live cells was quantified by counting at least 300 cells per treatment.

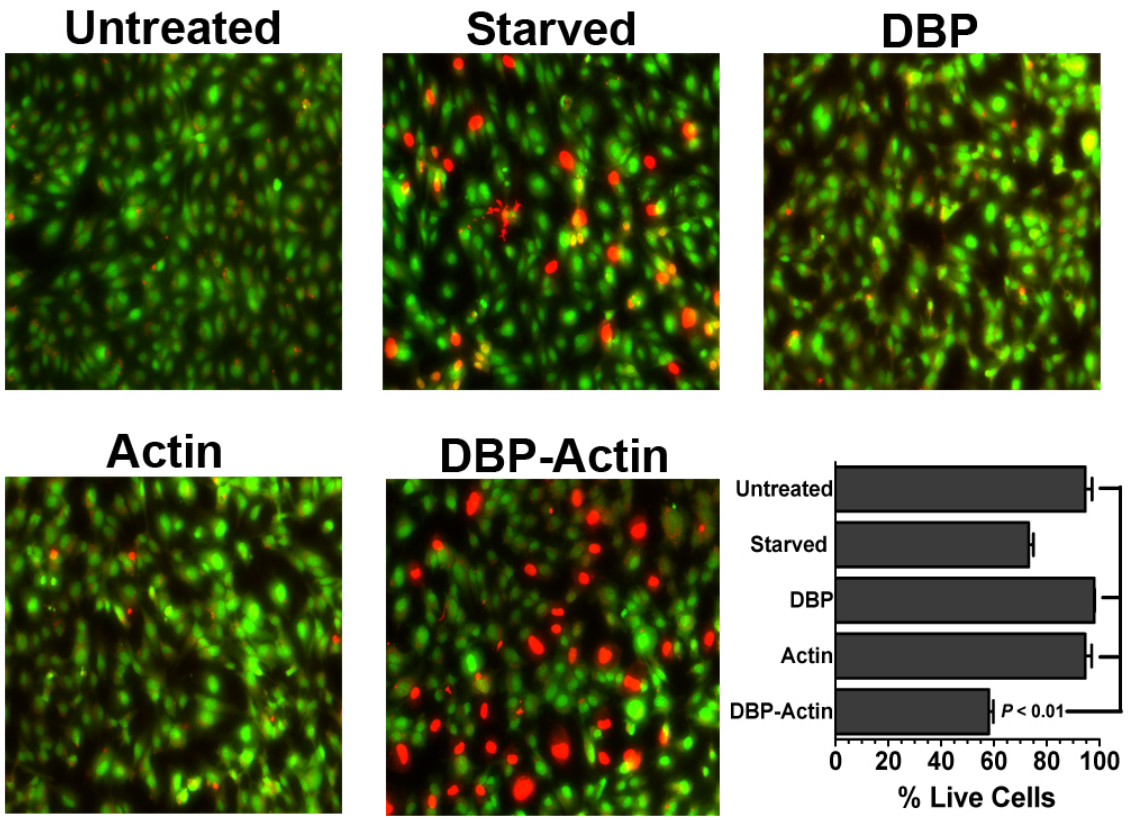

Figure 20: Acridine orange and ethidium bromide nuclear staining of HUVEC treated with DBP-actin complexes for 24 hours.

Green nuclei indicate live cells while red/orange/yellow nuclei indicate dead cells. HUVEC were treated with: untreated (cultured with EGM-2 growth medium as negative control), Starved (cultured with EGM-2 base medium without serum as positive control), 1 μM DBP, 1 μM actin or 1 μM DBP-actin complex for 24 hours. Cells were stained with AO/EB and the percentage of live cells was quantified by counting at least 300 cells per treatment.

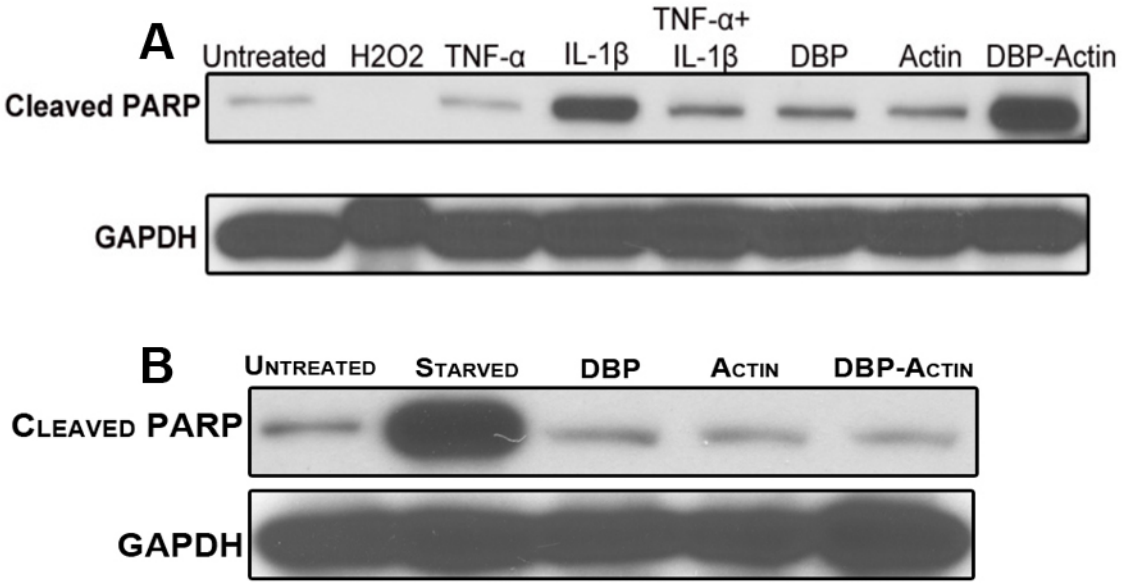

Figure 21: Immunoblot of PARP in HUVEC and HLMVEC treated with DBP-actin complexes for 24 hours.

(A) HUVECs treated with EGM-2 growth medium (untreated), hydrogen peroxide (H_2O_2), 10 nM TNF-α, 10 nM IL-1β, 10 nM TNF-α+IL-1β, 1 μM DBP, 1 μM Actin, and 1 μM DBP-actin complex. **(B)** HLMVECs treated with EGM-2 growth medium (untreated), 1 μM DBP, 1 μM Actin, and 1 μM DBP-actin complex. GAPDH is shown as a loading control.

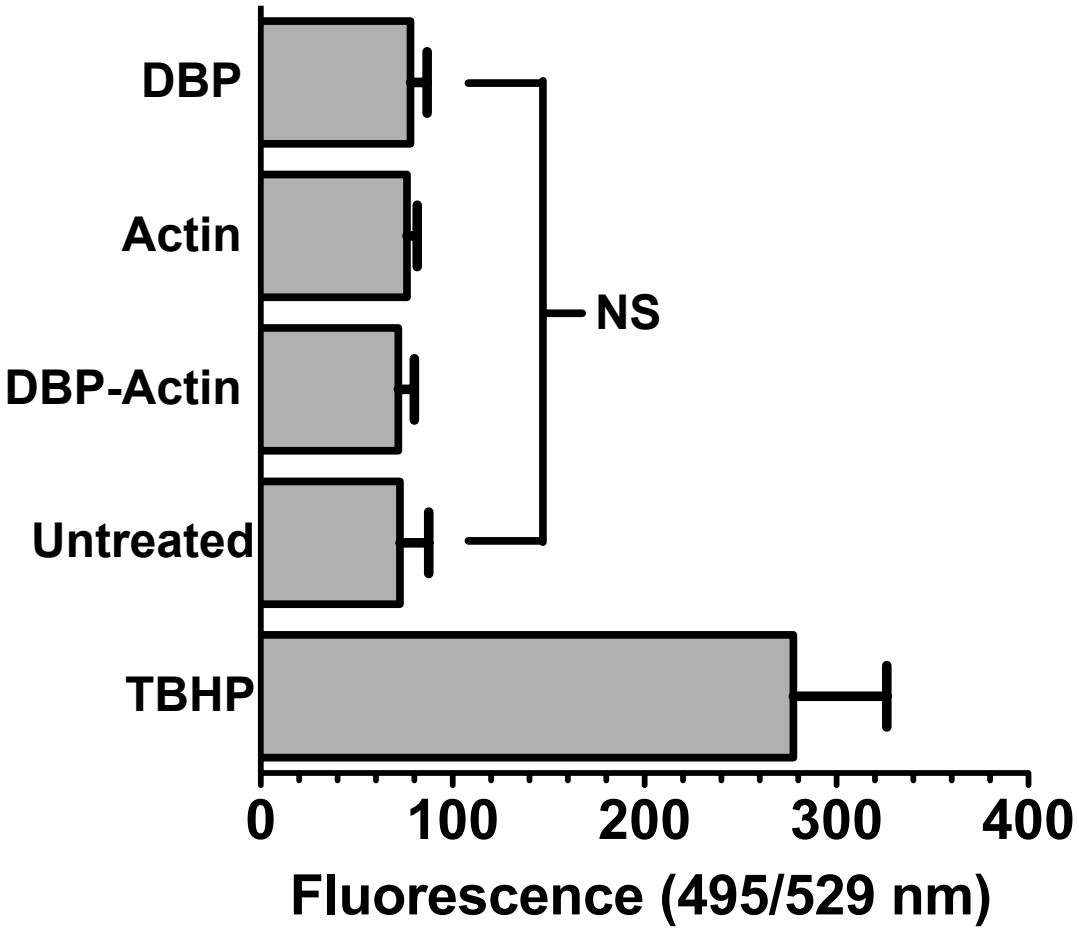

Figure 22: DBP-actin complexes do not induce ROS generation in HLMVEC.

DCFDA cellular ROS detection assay in DBP-Actin complex treated HLMVECs. HLMVECs were treated with 1 μM of DBP, Actin or DBP-Actin complex, 10 nM PMA and TBHP were used as positive control. The generation of ROS was determined by measuring the fluorescence at 485 nm excitation and 535 nm emission.

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4.5 DBP-Actin Complexes may Have an Indirect Effect on Endothelial Cells by Inducing S100A8/A9 Release from Neutrophils

The onset of acute lung inflammation after actin injection happens very fast once the DBP-actin complex form in the circulation. We observed severe acute lung inflammation 1.5 hours after actin injection. It is possible that DBP-actin can also have an indirect effect on endothelial cells via activation of immune cells in the blood. Recent work in our lab by myself and a previous graduate student (David Habiel) has shown that DBP-actin complexes can trigger dramatic S100A8/A9 release from neutrophils. Neutrophils can respond to various stimuli such as fMLP, C5a, LPS, PMA, ATP and ionomycin [37, 140, 141] and release S100A8/A9 heterodimer from cytosol into the extracellular environment. We isolated fresh human neutrophils and treated them with DBP-actin complexes as well as the above stimuli and collected the conditioned supernatant to blot for S100A8/A9 heterodimer. Interestingly, DBP-actin complexes induce noticeably more S100A8/A9 release than the known stimuli such as chemoattractants, ionomycin or ATP (Fig. 23). S100A8/A9 is involved in multiple inflammatory diseases and it can enhance chemotaxis of murine neutrophils [38]. Purified S100A8/A9 also is toxic to endothelial cells and can increase proinflammatory cytokine release from endothelial cells by a RAGE dependent mechanism [39, 40]. To test this possibility, primary human neutrophils were treated with DBP-actin complexes and the conditioned supernatant was subsequently used to treat HLMVECs and HUVECs for 4 hours. Cell viability was measured. Although S100A8/A9 was detected in the conditioned supernatants, these treatments did not alter endothelial cell viability in either cell type. The level of

S100A8/A9 complexes was not measured in the conditioned media, just detected by a native gel, so the concentration of S100A8/A9 may be too low to cause an effect. This potential mechanism needs to be investigated further.

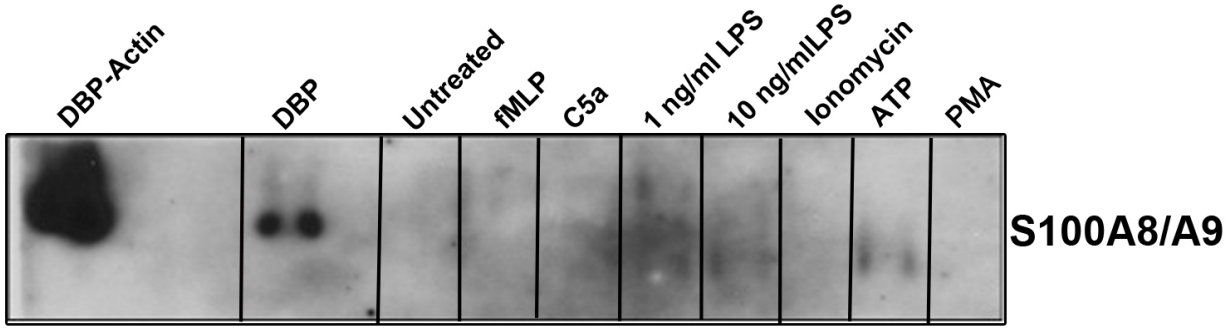

Figure 23: DBP-actin complexes are potent inducers of S100A8/A9 release from human neutrophils.

Neutrophils were purified from whole blood and then resuspended in HBSS. The cells were treated with 1 μM DBP-actin complexes, 1 μM DBP, DPBS as a control (untreated), 1 nM M fMLP, 1 nM C5a, 1 ng/ml LPS, 10 ng/ml LPS, 1 μM Ionomycin, 2 μM ATP and 10 ng/ml PMA for 30 minutes at 37ºC. The cells were then centrifuged and the conditioned supernatants were separated using a native gel, transferred to a PVDF membrane and blotted for the S100A8/A9 dimer. The monoclonal antibody (clone 27E10) recognizes a unique neoepitope on the complexed dimer and does not react with A8 or A9 monomers, thus the reason for analyzing these samples using a non-denaturing gel.

5. DISCUSSION

5.1 DBP-actin Complexes Induce Acute Lung Inflammation

5.1a DBP-Actin complexes, an Unrecognized Mediator in Traumatic Tissue Injury,

Massive tissue injury triggered by burns, sepsis or fulminant liver failure will result in the release of large amounts of actin into extracellular fluids [64, 81-86]. Although G-actin polymerizes into F-actin in physiological buffers, it does not polymerize in plasma or serum [70, 71]. Two plasma proteins, gelsolin and the vitamin D binding protein, comprise the extracellular actin scavenging system (EASS) to sever F-actin filaments and then sequester G-actin monomers [64]. The role of actin-free DBP has been the focus of several studies in both humans and animals where low plasma DBP levels have been shown to significantly correlate with poor prognosis in human cases of sepsis, multiple trauma and acetaminophen-induced liver failure [81-83]. Intravenous injection of actin into rabbit and rats has confirmed the in vivo EASS function of plasma gelsolin and DBP. Microthrombi and endothelial cell injury were observed in the lungs of these animals [63, 72, 73]. The conclusion from these studies is that when a large percentage of the circulating DBP pool is consumed (i.e., bound to G-actin and cleared from the blood) there is very little remaining DBP to neutralize actin released from ongoing cell injury. Thus, a reduced level of unbound DBP facilitates formation of actin filaments that then induce microvascular damage and tissue ischemia. However, there

is an increased level of DBP-actin complexes that mirrors the decreased level of actin-free DBP, but the role of these complexes as potential mediators of tissue injury has received little attention, presumably because they are cleared rapidly from the circulation largely by the liver [80]. The possibility that generation of large amounts of DBP-actin complexes can exacerbate tissue injury is an alternative hypothesis that had not been investigated. This dissertation investigated this possibility and determined that DBP-actin can induce lung inflammation in vivo, perhaps by directly injuring endothelial cells.

The present study was possible because of the development of the DBP null mouse strain that enabled us to evaluate the role of DBP-actin complexes in vivo. The prevailing hypothesis is that maintaining a critical concentration of free DBP in blood is essential to protect against the damaging effects of extracellular actin. If this premise is correct then a systemic deficiency of DBP should be lethal when actin is injected intravenously. However, the opposite was observed when actin was injected into mice, DBP+/+ animals developed more severe acute lung inflammation. We confirmed that DBP-actin complexes only form in DBP+/+ mice while actin forms complexes with gelsolin in DBP-/- animals. Moreover, the DBP-/- mice do not have an increase in plasma gelsolin, the other EASS protein in blood, to compensate for the lack of DBP (Fig. 1C). Previous studies have shown that plasma gelsolin, much like DBP, gets depleted during traumatic tissue injury [142]. Nevertheless, plasma gelsolin in DBP-/- mice may limit actin filament formation by capping/binding F-actin and possibly weakly binding of G-actin monomers [143], thus preventing obstruction of the lung

microvasculature. But the EASS role of gelsolin in DBP-/- mice remains to be investigated.

5.1b DBP-/- Mice Show Less Actin-Induced Acute Lung Inflammation

In contrast to the prevailing hypothesis, the DBP-/- mice were largely refractory to lung inflammation induced by intravenous injection of actin. The DBP+/+ mice developed severe acute inflammation. It is highly unlikely that the differences in actin-induced lung inflammation observed between DBP+/+ and -/- mice are due to strain variations, other than a systemic lack of DBP, since the DBP-/- mice were backcrossed for 11 generations on a C57BL/6J background and both the DBP+/+ and -/- colonies were bred from the same founder hemizygotes (DBP+/-) [108]. Additional in vivo experiments need to be performed in order to confirm that DBP-actin complexes are indeed a mediator that causes acute lung inflammation. First, DBP-actin complexes could be generated in vitro using purified proteins and then injected into both strains to determine if DBP-/- mice will develop lung injury to the same extent as DBP+/+ mice. However, purified mouse DBP is not commercially available and it would take massive amount of mouse plasma to purify mouse DBP in the lab. Previously, we attempted to generate and purify *E. coli* expressed recombinant mouse DBP but with little success. Another approach would be to add purified actin to DBP+/+ plasma and then inject the spiked plasma into mice. If DBP-/- mice develop a similar degree of acute lung injury as DBP+/+ animals, this will be further convincing evidence that DBP-actin is the mediator of acute lung injury.

5.1c Inflammatory Cytokines in DBP+/+ and DBP-/- Plasma and BAL Fluid.

Cytokine levels in BAL and plasma were determined but results were generally negative. All 23 cytokines tested were at the lowest level of detection in the BAL (Fig. 9) and the small variations observed probably reflect “background noise” [144]. However, these BAL samples were collected 4 hours after actin injection, and during inflammation de novo transcription and translation of proinflammatory cytokines most likely requires more than 4 hours for adequate amounts to be released and accumulate in fluids. BAL samples would need to be collected at other time points such as 8, 12 or 24 hours after actin injection. In the plasma samples, a DBP+/+ mouse had a much higher level of one chemokine, KC (CXCL1), than the DBP-/- mouse (Fig. 8). However, this data needs to be validated by testing the plasma samples from multiple mice. KC is the mouse counterpart of human IL-8 (CXCL8), which is a potent neutrophil chemoattractant [145]. The chemokine KC is synthesized by various cell involved with the inflammatory response including mast cells, endothelial cells, macrophages, neutrophils and epithelial cells [146-149]. This chemokine also has been reported to be preformed in mast cell granules and can be released very quickly in response to TLR4 activation [146]. The de novo synthesis of KC can be induced by cytokines such as IL-1β, IL-6 and TNF-α and LPS ligation and activation of TLR4 [146, 150-152]. There are numerous mast cells in the lung interstitium in close proximity to the microvasculature, so mast cell-derived KC could have been released almost immediately following the initial interaction of DBP-actin with the pulmonary capillary endothelium. This rapid release of KC may help

explain the neutrophil infiltration before other chemokines could be released. However, additional in vivo experiments at different time points with a larger sample size need to be performed before the role of KC in this injury model can be validated.

5.1d DBP-actin Complexes may Mediate the Inflammation by Directly Targeting Endothelial Cells in a Concentration Threshold and Time-dependent Manner

Due to the fast clearance of DBP-actin complexes in vivo (DBP-actin complexes in blood have a half-life of 30 minutes in rats), the acute lung injury in DBP+/+ mice is largely resolved 24 hours after a single intravenous injection of actin. The reduced level of lung injury at 24 hours directly correlates with the decreased amount of DBP-actin complexes in mouse plasma (Figs. 3 and 5). In vitro, short-term exposure (4 hours) of primary endothelial cells to purified DBP-actin complexes induced reversible injury (Figs. 14 to 18) whereas longer exposure (24 hours) resulted in cell death (Figs. 19 and 20). These results are consistent with a mechanism that would require either a critical threshold concentration and/or exposure time to induce cell injury. Varying levels of DBP-actin complexes are continually formed in vivo as a result of minor tissue trauma, menstrual cycles, infections or surgery. Therefore, low levels of circulating DBP-actin would be inconsequential. On the other hand, high concentrations of DBP-actin in blood could act as a danger signal of ongoing and significant tissue injury. Indeed, a large percentage (78%) of the total plasma DBP pool was bound to actin 1.5 hours after injection in DBP+/+ mice (Fig. 1A). This percentage is similar to a previous study examining fulminant hepatic necrosis (FHN) in humans where 72% of plasma DBP

complexes with actin in patients who died of the disease whereas only 22% of total DBP was bound to actin in FHN survivors [153]. The current mouse model demonstrated that injury after actin injection was limited to the lungs, probably due to the anatomical feature that the lung microvasculature is exposed to the entire venous return. No injury was observed in the liver, kidneys or heart after actin injection. It is possible that prolonged exposure may cause injury in these organs as well and thus contribute to multiple organ dysfunction that follows conditions such as ARDS and SIRS. A major limitation of the current study is the method of inducing injury in mice, i.e., a single intravenous bolus of purified actin. A more physiological approach would be to employ a model that has continuous injury over a period of hours to days, such as the cardiotoxin-induced muscle necrosis model [154]. Necrotic skeletal myocytes will release massive amounts of actin over a period of hours to days before the injury is resolved. Consequently, there will be high levels of circulating DBP-actin complexes that persist. This model may be ideal to evaluate both the acute lung injury and the potential effects on other organs, and may validate the hypothesis that DBP-actin complexes are mediators of tissue injury. In addition, we could also utilize an Alzet osmotic pump to deliver actin into the circulation over a period of hours to days.

The in vitro results clearly showed that DBP-actin complexes injure endothelial cells, and possible mechanisms of endothelial injury and death were investigated. Results demonstrate that a caspase-3 dependent apoptotic pathway is activated in HUVECs treated with DBP-actin (Fig. 21A). In contrast, HLMVECs showed no cleaved PARP in response to DBP-actin treatment, perhaps indicating that an alternative apoptosis

pathway is triggered in these cells (Fig. 21B). HLMVECs also did not show increased cellular ROS production in response to DBP-actin treatment, thereby eliminating another possible mechanism for inducing cell death. Nevertheless, studies have shown that endothelial cells from different organs can respond differently to the same stimulant. For example, large vessel endothelial cells such as HUVECs are more sensitive to ionophore (A2318) induced calcium toxicity and apoptotic death than microvascular endothelial cells [155]. This could explain the differences in PARP cleavage between HUVECs and HLMVECs. Regardless of these differences and lack of a defined mechanism, the fact remains that both of these primary endothelial cells are sensitive to injury and death when treated with DBP-actin in vitro. Perhaps this could suggest that endothelial cells in any organ may be sensitive to the effects of DBP-actin, but the lung may be particularly vulnerable due to its anatomical features and blood flow. Alternatively, it is possible that DBP-actin complexes can induce low level of injury to a large number of lung microvascular endothelial cells, since the endothelial surface area of the lung capillary bed is very large. Nevertheless, this report clearly demonstrates that DBP-actin is a mediator of tissue injury and this knowledge may change the current paradigm that focuses on the concentration of actin-free DBP in plasma as the important prognostic indicator in traumatic injury. The information from this study also may be consequential for the proposed efforts to infuse purified DBP intravenously into trauma patients to augment their actin-free DBP levels as a potential supportive therapy [156]. Results from the mouse experiments reported herein would suggest that approach could actually enhance tissue injury.

5.2 Possible Targets of DBP-Actin Complexes

5.2a Potential Indirect Effects of DBP-actin Complexes in vivo

Since the complement system is the first line of defense when there is infection or injury and can be activated in the matter of seconds, we tested the possibility that DBP-actin complexes can activate serum complement. Our results showed that in the purified system, DBP-actin complexes do not trigger any of the three activating pathways of the complement system (Figs. 10 and 11). However, often in vivo the primary injury or infection activates the complement system and produces the potent proinflammatory peptide C5a, which may synergize with DBP-actin to exacerbate acute lung injury. Products of activated complement have been shown to synergize and/or cross talk with several other components of innate immunity [157-159]. The cross talk between PRRs and complement system has been extensively reported in systemic inflammation [160]. If DBP-actin interacts directly or indirectly via a cell surface PRR, then cross talk may also affect complement. Potential activation of complement in DBP-actin induced acute lung injury could be tested by measuring C5a levels in the BAL fluid. To determine if DBP-actin complexes can directly induce chemotaxis of neutrophils, and thus help explain the neutrophil infiltration into the lungs in DBP+/+ mice after actin injection, an in vitro chemotaxis assay was performed. The results showed that DBP-actin complexes are not chemotactic in the purified system. However, DBP-actin complexes may indirectly facilitate chemotaxis induced by other chemoattractants in the acute lung

injury model. To confirm this premise, the production of primary chemoattractants can be measured in BAL fluids at various time points after actin injection.

5.2b DBP-actin Complexes and Neutrophil Release of S100A8/A9 Dimers

We have shown that DBP-actin complexes can trigger S100A8/A9 heterodimer release from primary human neutrophils (Fig. 23). It has been demonstrated that upon neutrophil stimulation and calcium influx, S100A8/A9 dimer can form a heterotetramer and translocate to plasma membrane. Neutrophils actively secrete S100A8/A9 into the extracellular space via a non-classical secretion pathway in response to chemotactic stimuli such as C5a and formylated peptides [37]. Figure 23 shows that DBP-actin can trigger robust release of S100A8/A9, much more than the previously reported stimuli. This S100A8/A9 heterodimer has been reported to have multiple functions including neutrophil chemotaxis and increased expression of adhesion molecules that facilitates transendothelial migration [38]. S100A8/A9 can also increase vascular permeability, and even cause endothelial cell death by both caspase-dependent and independent pathways [39]. Endothelial cells treated with recombinant S100A8/A9 up-regulate the expression and secretion of proinflammatory cytokine IL-6, the chemokine MCP-1 and adhesion molecules ICAM-1 and VCAM-1. In endothelial cells, S100A8/A9 induces its cellular effects largely by binding to TLR4 and RAGE [40]. We hypothesize that the large amounts of S100A8/A9 released by neutrophils after contact with DBP-actin complexes may serve to: 1) enhance neutrophil migration into the lungs in an autocrine fashion, 2) increase proinflammatory cytokine production from endothelial cells through

RAGE dependent pathway, 3) cause endothelial cell injury and cell death, all of which can contribute to the acute lung injury we observed in vivo.

5.3 Actin and Danger Associated Molecular Patterns

Danger associated molecular patterns are groups of protein that are released into the extracellular environment during necrotic cell death and can be recognized by the PRRs and elicit an innate immune response. The previous paradigm to define DAMPs requires that it be actively released from immune cells during infection or injury and the DAMP must be able to initiate tissue repair [21]. One of the earliest described DAMPs, HMGB1, meets all these criteria. However, more and more self components such as uric acid, hyaluronan, mitochondrial transcription factor, ATP, S100 family proteins and tissue acidosis have been identified as DAMPs but do not meet all the criteria to be called DAMPs [161-170]. The most important features of a DAMP are released during necrotic cell death and the ability to be recognized by PRRs to trigger inflammation. Both G-actin and F-actin are released from necrotic cells, and the detrimental effect of F-actin filaments in circulation has been documented. However, G-actin once released from the cells will not remain as a monomer; it will either bind to EASS proteins or polymerize into F-actin filaments once the EASS is saturated. Thus, the G-actin monomer is transient and may not be significant.

5.3a F-actin as a DAMP

The previous paradigm was that the F-actin filaments formed after tissue damage were responsible for the angiopathic effects observed in humans and experimental animals [63]. However, the mechanism of how F-actin mediates this effect was never investigated, and the presence of DBP-actin complexes was largely overlooked. Ever since the concept of DAMPs was proposed, the biological significance of extracellular actin re-emerged since its presence in circulation after tissue injury is usually accompanied by inflammation. However, it is difficult to dissect if the acute inflammation is triggered from the initial insult or from the released actin. Two back-to back papers published in the journal *Immunity* in 2012 proposed that F-actin is a conserved DAMP and is recognized by DNGR-1, a receptor on dendritic cells [171, 172]. DNGR-1 recognizes F-actin by direct binding and it also recognizes damaged cells via exposed actin filaments. Both papers did not consider how the presence of EASS will affect the state of actin in circulation. Until the EASS is saturated, most of the actin will be bound to DBP and/or gelsolin but it is entirely possible that at the site of injury, the actin released may temporarily saturate the local EASS and polymerization may occur under these transient conditions. The downstream effect of F-actin after being recognized by DNGR-1 will trigger the clearance of the damaged cells and even trigger a T cell response [171, 172]. The inflammatory response or cytokine profile of this interaction was not evaluated. We believe that these results actually provide support for our hypothesis since we focused on issues they did not address, actin bound to scavenger

proteins and if the DBP-actin complex can serve as DAMP to trigger innate immune response.

5.3b DBP-Actin Complexes as a DAMP

In our model, we showed that the presence of DBP-actin complexes in the circulation causes acute lung inflammation and our in vitro experiments with endothelial cells suggest that DBP-actin complexes can cause direct endothelial cell injury and death. It is possible that DBP-actin can also act on endothelial cells indirectly through S100A8/A9. However, the cell surface ligand for DBP-actin complex remains unknown. Previous reports from our lab and others have shown that unbound DBP binds with low avidity to multiple cell surface macromolecules such as chondroitin sulfate proteoglycans [173], CD44 [174], megalin [97], and cubulin [175]. However, it is not known if actin alters the binding of DBP to the cell surface and/or its preference for a cellular ligand.

It is possible that DBP-actin signals through one of the known PRRs. For TLRs, it is not likely that DBP-actin will be recognized by TLR3, TLR7 or TLR9 since these are the TLRs that recognize nucleic acid PAMPs [18]. TLR signaling generally is divided into two distinct pathways through two different adaptor proteins: MyD88 and TRIF. The TRIF pathway is activated by TLR3 ligation to dsRNA and is not likely the pathway DBP-actin complexes will signal through. MyD88 pathway will lead to the activation of NF-κB [176]. To determine if DBP-actin complexes signal through TLRs, we could treat

endothelial cells or immune cells and then test for NF-κB activation. The Rig-like-receptors (RLR) family generally recognizes virus PAMPs and is also not likely to be the candidate receptors for DBP-actin complexes. Nod-like-receptors (NLRs), NALP1, NALP3, IPAF and NAIP5 are the components of inflammasome. The inflammasome complex is comprised of one or some of the NLRs and caspase-1. NLRs are involved in recognizing various types of self-molecules. NALP3 inflammasome is not activated by direct binding of ligands but rather by sensing the changes caused by the DAMPs. De-stabilization of lysosomes, potassium efflux, and generation of ROS can trigger the activation of NALP3 inflammasome. NALP5 inflammasome is activated by bacterial flagellin [18]. If DBP-actin complexes can activate any of the inflammasomes, this can be tested by IL-1β secretion that is common product of activating this pathway. However, further work is required to identify the receptors for, and signaling by, DBP-actin complexes.

6. SUMMARY AND CONCLUSION

To summarize, by injecting purified actin into DBP+/+ and DBP-/- mice, the formation of DBP-actin complexes leads to more severe acute lung injury in DBP+/+ mice. This effect is reversible when DBP-actin is quickly cleared from circulation. We identified endothelial cells as one of the direct targets of DBP-actin complexes. Short-term exposure to DBP-actin decreases endothelial cell viability and this effect is reversible. However, long-term exposure to DBP-actin complexes will eventually lead to endothelial cell death, perhaps via a caspase-3 dependent pathway. But the mechanism of how DBP-actin complexes induce lung microvascular endothelial cell death remains unknown. DBP-actin complexes may act on endothelial cells by inducing S100A8/A9 release from neutrophils. The release of S100A8/A9 may induce other inflammatory responses, but this hypothesis also needs to be investigated further. The receptor for DBP-actin, and if DBP-actin complexes induce inflammatory responses through this receptor, requires further investigation. We believe that dissecting the targets of DBP-actin complexes and identifying the receptors and signaling pathways can help identify new therapeutic targets to block the inflammation and may improve outcomes in traumatic injury.

There are many factors involved in DBP-actin complex induced inflammation that need to be identified and investigated. To evaluate the effect of prolong exposure to DBP-actin complexes in vivo, a more physiological approach would be to employ a model that has continuous injury over a period of hours to days, such as the cardiotoxin-

induced muscle necrosis model [154]. Necrotic skeletal myocytes will release massive amounts of actin over a period of hours to days before the injury is resolved. Consequently, there will be high levels of circulating DBP-actin complexes that persist. This model may be ideal to evaluate both the acute lung injury and the potential effects on other organs, and may validate the hypothesis that DBP-actin complexes are mediators of tissue injury. To determine if DBP-actin complexes have DAMP-like activities, the cell surface receptor that mediates DBP-actin's function needs to be identified. The main focus will be to investigate if any of the known PRRs can recognize DBP-actin complexes. Besides the direct effect that DBP-actin complexes have on endothelial cells, they may also mediate the lung inflammation by inducing S100A8/A9 release from neutrophils, and S100A8/A9 can further exacerbate lung inflammation. Additional experiments need to be performed to assess the role of S100A8/A9 in DBP-actin complexes induced acute inflammation.

Identifying how DBP-actin complexes mediate inflammation may have important clinical implications. In conditions like sepsis, liver necrosis or trauma, there are very high concentrations of DBP-actin complexes in the circulation. Some of these patients develop acute lung injury even when the initial insult is not in the lungs. They also often developed a condition called systemic inflammatory response syndrome (SIRS) due to the over production of inflammatory cytokines. DBP-actin complex induced acute lung inflammation may serve to initiate the acute lung injury in these patients. If the initial injury is not repaired promptly, the prolonged exposure of DBP-actin complexes may eventually lead to the development of acute respiratory distress syndrome, multiple

organ failure, and eventually the death of the patients. By determining the role of DBP-actin complexes in these conditions, these investigations may provide insight that will allow for the development of new therapeutic methods or drugs.

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