IN VIVO STUDIES OF CELL-FREE DNA AND DNASE IN A MURINE MODEL OF POLYMICROBIAL SEPSIS

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By:

Safiah H.C. Mai, Hons. B.Sc.

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TITLE: ***In vivo*** **studies of cell-free DNA and DNase in a murine model of polymicrobial sepsis**

AUTHOR: **Safiah H.C. Mai**

Hons. B.Sc. Biology

McMaster University

SUPERVISOR: **Patricia C. Liaw, Ph.D.**

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Abstract

Sepsis is a clinical syndrome characterized by the systemic activation of inflammatory and coagulation pathways in response to microbial infection of normally sterile parts of the body. Despite considerable advances in our understanding of sepsis pathophysiology, sepsis remains the leading cause of death in non-coronary intensive care units (ICU) with a global disease burden between 15 and 19 million cases per year (Dellinger et al., 2008). Severe sepsis, defined as sepsis associated with organ dysfunction is associated with mortality rates of 33% to 45%. The incidence of severe sepsis continues to increase by 1.5% per annum due to the aging population, a rise in the prevalence of comorbidities, and the wider use of immunosuppressive agents and invasive procedures (Angus et al., 2001). Over the past several decades, many potential treatments for sepsis have shown early promise, yet have failed to improve survival in over 100 Phase II and Phase III clinical trials (Marshall, 2014) suggesting that some fundamental knowledge is lacking in our understanding of sepsis pathophysiology.

Emerging studies on cell-free DNA (cfDNA), DNA released extracellularly into the circulation, demonstrate that cfDNA is a crucial link between inflammation and coagulation . In various conditions characterized by excessive inflammatory responses or aberrant prothrombotic responses, cfDNA has been implicated in exacerbating disease pathology (Atamaniuk, Kopecky, Skoupy, Säemann, & Weichhart, 2012; Fuchs, Brill, & Wagner, 2012; Swystun, Mukherjee, & Liaw, 2011). In clinical sepsis, levels of cfDNA upon admission into the ICU have strong prognostic value in predicting mortality (Dwivedi et al., 2012; Saukkonen et al., 2008). However, it is unclear whether these

increases in cfDNA are an epiphenomenon during sepsis progression, or whether cfDNA actively plays a role in sepsis pathophysiology. In this work, *in vivo* studies were conducted to characterize the role of cfDNA in sepsis, the effects of DNase administration, and the potential mechanism by which cfDNA is released during experimental sepsis. In addition, mortality studies were conducted to identify surrogate markers of death to promote the design of humane and ethical animal studies in conducting sepsis research.

Polymicrobial sepsis was induced via a surgical procedure whereby the cecum is exteriorized, ligated and punctured twice to introduce a continuous source of microorganisms, a model termed cecal ligation and puncture (CLP). In our CLP sepsis model, levels of cfDNA increased in a time-dependent manner. These increases accompanied an early pro-inflammatory response marked by increased pro-inflammatory IL-6, a transient increase in anti-inflammatory IL-10, and elevated lung myeloperoxidase (MPO) activity. Septic mice with elevated cfDNA levels also had high bacterial loads in the lungs, blood, and peritoneal cavity fluid. Organ damage was also observed in mice following CLP surgery versus mice subjected to the non-septic sham control surgery marked by increased levels of creatinine and alanine aminotransferase (ALT) indicative of kidney and liver injury, respectively. Histological analyses further confirmed lung and kidney damage following CLP surgery. Changes in coagulation were also observed in septic mice as mice subjected to CLP had sustained increases in thrombin-antithrombin (TAT) complexes. In addition, plasma from CLP-operated mice had increased thrombin generation (i.e. increased endogenous thromin potential, increased peak thrombin,

decreased time to peak, and decreased lag time) mediated by FXIIa and enhanced by platelets. Following CLP-induced sepsis, elevations in cfDNA levels accompanied pro-inflammatory and pro-coagulant responses.

The effects of *in vivo* DNase treatment in septic mice were time-dependent. Early DNase treatment when cfDNA levels were low resulted in an exaggerated pro-inflammatory response marked by increased plasma IL-6 levels and increased lung damage. In contrast, delayed DNase treatment at time-points when cfDNA levels were elevated suppressed inflammation characterized by an increase in anti-inflammatory IL-10 and reductions in cfDNA, IL-6, lung MPO, and ALT activity. Furthermore, delayed DNase administration resulted in decreased bacterial load in the lungs, blood, and peritoneal cavity fluid. Delayed DNase treatment also resulted in blunted pro-coagulant responses as levels of TAT complexes were suppressed and thrombin generation from septic mouse plasma was normalized. Moreover, DNase treatment when cfDNA levels were elevated increased survival in CLP-operated mice by 80% and reduced lung and liver damage. These findings suggest that administration of DNase when cfDNA levels are elevated may reduce pro-inflammatory and pro-coagulant responses and that delayed DNase treatment may infer protection in the CLP model of sepsis.

One mechanism by which cfDNA is released is via the formation of neutrophil extracellular traps (NETs). Upon inflammatory stimulation, some neutrophils release chromatin material and antimicrobial proteins (i.e. neutrophil elastase, MPO, and histones) in an active process termed NETosis. Although NETs ensnare bacteria and exert antimicrobial properties, NETs may also exert harmful effects on the host by activating

inflammation and coagulation. While some *in vitro* evidence suggest that neutrophils are the main source of cfDNA released following inflammatory stimulation, others have reported that neutrophils are not the main source of circulating cfDNA following septic challenge. To determine whether NETs contribute to cfDNA released during CLP sepsis, genetically modified mice that are incapable of forming NETs, PAD4-/- mice, were used. Levels of cfDNA in PAD-/- mice were significantly lower than cfDNA levels in C57Bl/6 mice following CLP surgery, suggesting that NETs were a source of cfDNA in our model. Levels of IL-6, MPO, and bacterial load in the lungs, blood, and peritoneal cavity were significantly reduced, indicating that NETs exert pro-inflammatory effects in CLP sepsis. Thrombin generation was also suppressed in PAD4-/- mice which suggests that NETs contribute to thrombin generation following CLP sepsis. NETs contribute to increases in circulating cfDNA and may exacerbate pathology by driving pro-inflammatory and pro-coagulant responses in CLP-induced sepsis.

Appreciating the implications of conducting research using animals, it is pertinent that researchers ensure the highest ethical standards and design animal studies in the most humane, yet scientifically rigorous manner. Using mortality studies, we validated the utility of physiological and phenotypic markers to assess disease severity and predict death in murine sepsis. Temperature via a rectal probe monitor and sepsis scoring systems which assess components such as orbital tightening, level of consciousness, and activity were effective surrogate markers of death. These tools offer a non-invasive assessment of disease progression which do not artificially exacerbate sepsis pathology and immediate information regarding any changes in the health status. Surrogate markers of death also

provide reliable monitoring to meet increasing standards of ethical, humane animal research and a feasible and cost-efficient means to obtain vital signs in small rodents. We have proposed a scoring system which can be used for assessing disease severity, endpoint monitoring, and predicting death to obviate inhumane methods of using death as an endpoint in sepsis studies.

In summary, cfDNA levels are elevated in CLP-induced sepsis and these elevations accompany pro-inflammatory and pro-coagulant responses. NETosis may be a mechanism by which cfDNA is released and NETs may drive inflammation and coagulation in CLP sepsis. Delayed DNase administration may suppress inflammation and coagulation and may be protective in polymicrobial sepsis. In future animal sepsis studies, surrogate markers of death and a sepsis scoring system can be used in place of death as an endpoint to raise the standards in conducting ethical, humane sepsis research.

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future adventures overseas. I cannot wait for us to take Elon Musk's hoverboard (tuned to Shostakovich), philosophize the feasibility of Danielle Martin's pharmacare or the effects of modern day feminism in a capitalist society, and decide whether that is too many.

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List of Abbreviations

α_2-PI – α_2-antiplasmin
ADP – adenosine diphosphate
ALT – alanine transaminase
ANOVA – analysis of variance
APC – activated protein C
AT – antithrombin III
AUC – area under the curve
BALF – bronchoalveolar lavage fluid
Ca^{2+} – calcium
CAT – calibrated automated thrombin generation
CARS – compensatory anti-inflammatory response syndrome
cfDNA – cell-free DNA
CLP – cecal ligation and puncture
CRP – c-reactive protein
DIC – disseminated intravascular coagulation
DMSO – dimethyl sulfoxide
DNA – deoxyribonucleic acid
DNase – deoxyribonuclease
DVT – deep vein thrombosis
EDTA – Ethylenediaminetetraacetic acid
ELISA – enzyme-linked immunosorbent assay
EPCR – endothelial protein C receptor
EWS – early warning scores
FDPs – fibrin degradation products
FITC – Fluorescein isothiocyanate
H&E – hematoxylin and eosin
HMWK – high molecular weight kininogen
HRP – horseradish peroxidase
HTAB – hexadecyltrimethylammonium bromide
HUVEC – human umbilical vein endothelial cell
IL – interleukin

iNOS – inducible nitric oxide synthase
IVC – inferior vena cava
kDa – kilodalton
LBP – LPS-binding protein
LPS – lipopolysaccharide
LTA – lipoteichoic acid
LMWH – low-molecular-weight heparin
mRNA – messenger ribonucleic acid
MRSA – methicillin-resistant *Staphylococcus aureus*
MPO – myeloperoxidase
NETs – neutrophil extracellular traps
NETosis – NET formation
NO – nitric oxide
OD – optical density
PAD4 **–** peptidylarginine deiminase type 4
PAF **–** platelet activating factor
PAI – plasminogen activator inhibitor
PAMPs – pathogen-associated molecular patterns
PARs – protease-activated receptors
PBS – phosphate buffered saline
PC – protein C
PCPS – phosphatidylcholine:phosphatidylserine
PepG – peptidoglycan
PMA – phorbol 12-myristate 13-acetate
PolyP – polyphosphates
PPP – platelet-poor plasma
PRP – platelet-rich plasma
PRR – pattern recognition receptors
PS – protein S
PTAH – phosphotungstic-acid and hematoxylin
RBC – red blood cell
rhDNaseI – recombinant human DNaseI
RNA – ribonucleic acid
RNase – ribonuclease
ROS – reactive oxygen species
SD – standard deviation
SEM – standard error of the mean
Serpin – serine protease inhibitor

SIRS – systemic inflammatory response syndrome
TM – thrombomodulin
TAFI – thrombin activatable fibronolysis inhibitor
TATs – thrombin-antithrombin complexes
TF – tissue factor
TFPI – tissue factor pathway inhibitor
Th1 – type 1 helper T cells
Th2 – type 2 helper T cells
TLRs – toll-like receptors
TNF – tumor necrosis factor
tPA – tissue-type plasminogen activator
uPA – urokinase-type plasminogen activator
VTE – venous thromboembolism
VWF – von Willebrand Factor

Chapter 1: Introduction

1.1 Overview of hemostasis

Hemostasis involves a complex network of tightly regulated processes to minimize extravasation of blood from the host's vasculature, delicately balancing coagulation and fibrinolysis (Marder et al., 2012). The normal vascular endothelium maintains blood fluidity by producing and secreting inhibitors of platelet aggregation and blood coagulation (Marder et al., 2012). Prostaglandin I_2 (prostacyclin), nitric oxide (NO), and ecto-ADPase/CD39 inhibit platelet aggregation (Marcus et al., 1997; Moncada, Gryglewski, Bunting, & Vane, 1976; Palmer, Ferrige, & Moncada, 1987; Voelkel, Gerber, McMurtry, Nies, & Reeves, 1981) while thrombomodulin, heparan sulfate, and tissue factor pathway inhibitor (TFPI) inhibit blood coagulation in the normal vessel (Bauer & Rosenberg, 1987; Broze, 1995; C. T. Esmon & Owen, 1981; Mann, Jenny, & Krishnaswamy, 1988; Teien, Abildgaard, & Höök, 1976). The endothelium also functions as a protective barrier by containing blood and hemostatic components within the vessel lumen and separating it from highly reactive elements in the subendothelium (Pohl, Holtz, Busse, & Bassenge, 1986). In response to a breach of vascular endothelial integrity, an explosive chain of activation events occur to minimize the extravasation of blood and damage of tissues as well as to initiate repair (Marder et al., 2012). These hemostatic processes are crucial defence mechanisms for the host to maintain sterility and function of a high pressure circulatory system, as exposure to pathogens and microbes renders the host susceptible to infection and further tissue damage.

1.1.1 Platelet adhesion and aggregation

Platelet adhesion occurs upon endothelial disruption due to the affinity between receptors expressed on the surfaces of circulating platelets and matrix proteins exposed in the disrupted endothelium such as collagen fibers, non-collagenous microfibrils, and fibronectin (Packham & Mustard, 1984). Platelets adhere to type IV collagen, type III reticular fiber bundles, as well as type I collagen fibers of the subendothelium (Nieswandt & Watson, 2003; Packham & Mustard, 1984; Pytela, Pierschbacher, Ginsberg, Plow, & Ruoslahti, 1986). In areas of high shear stress, von Willebrand factor (VWF), a large multimeric glycoprotein produced constitutively by the endothelium and megakaryocytes (Jaffe, Hoyer, & Nachman, 1973; Montgomery & Zimmerman, 1978) binds subendothelial collagen types I, III, and VI following endothelial disruption (Reddick et al., 1990; Reddick, Griggs, Lamb, & Brinkhous, 1982; Sakariassen, Bolhuis, & Sixma, 1979; Weiss, 1991). Through the simultaneous binding of VWF to glycoprotein Ib-IX-V and collagen, VWF anchors platelets to the walls of injured vessels, initiating platelet adhesion and activation (Pytela et al., 1986). Anchored platelets also bind VWF by platelet surface integrins, GPIIb-IIIa, thereby facilitating platelet adhesion (Shattil, Hoxie, Cunningham, & Brass, 1985; Shattil, Kashiwagi, & Pampori, 1998). Platelet activation is followed by a change in shape from disc-like to a spherical shape with projections and the constriction of microtubules toward the centre of the platelet. This shape change triggers various reactions (e.g. adenylate cyclase inhibition, mobilization of intracellular calcium, etc. (Hynes, 2002; Shattil et al., 1985, 1998) which further potentiate platelet activation and aggregation.

Activated platelets also undergo degranulation, releasing dense granule contents including Ca^{2+}, adenosine diphosphate (ADP), polyphosphates, and serotonin (Andrews, López, & Berndt, 1997; Ashby, Daniel, & Smith, 1990; Holmsen, 1985; Kroll & Schafer, 1989; Rink & Sage, 1990; Senzel, Gnatenko, & Bahou, 2009) and alpha granule contents such as von Willebrand factor (VWF), fibronectin, vitronectin, thrombospondin, and fibrinogen (Pytela et al., 1986; Senzel et al., 2009; Weiss, 1991). Platelet activating factor (PAF), ADP, thrombin, thromboxane A_2, and collagen induce platelet aggregation (Berndt, 2003; Coppinger et al., 2004; Heijnen et al., 1998; Senzel et al., 2009), promoting the formation of a platelet plug at the site of injury.

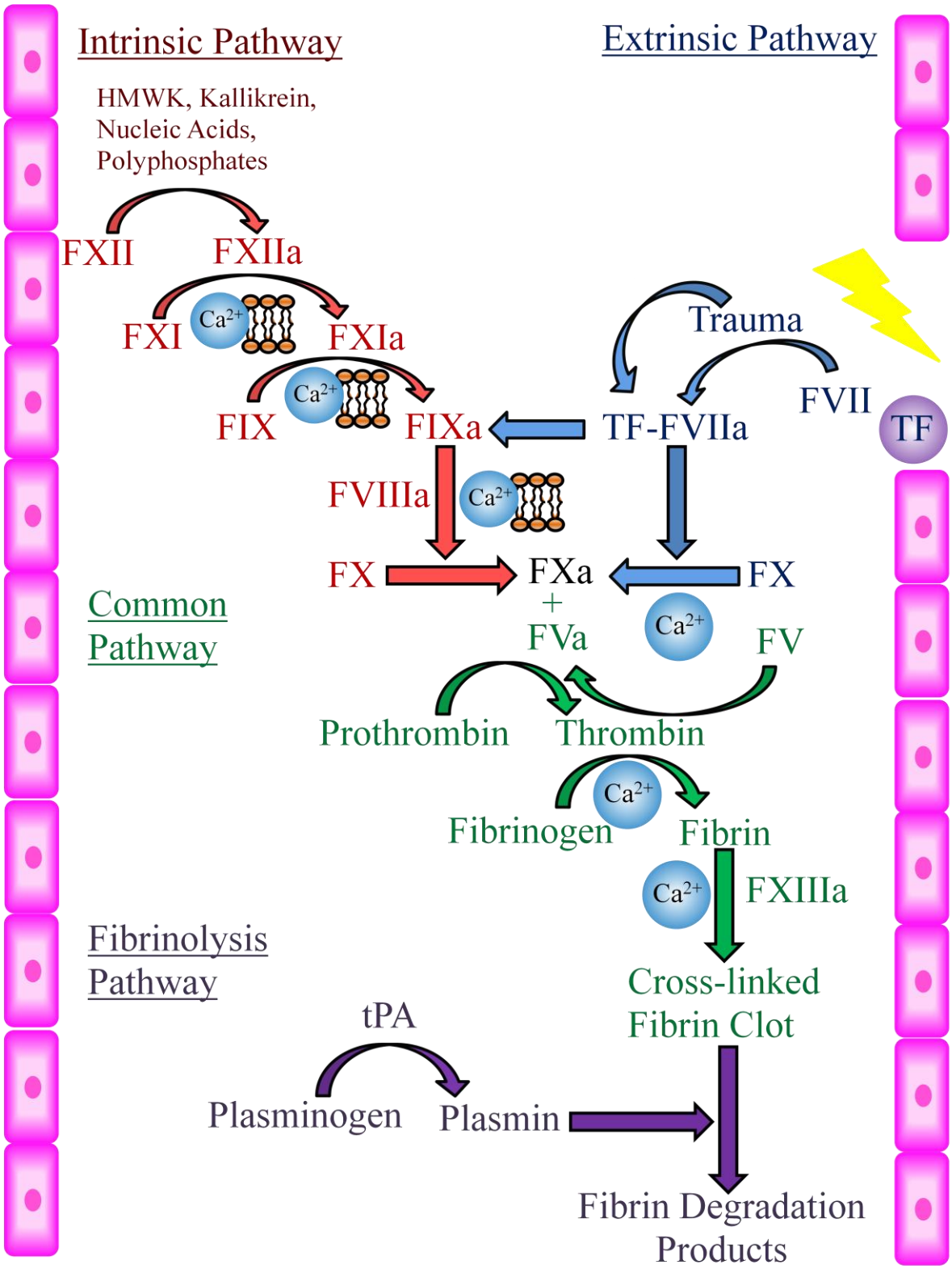
1.2 Blood coagulation

Although formation of a primary platelet plug is a vital hemostatic process for minimizing extravasation immediately following vessel injury, it alone is insufficient to completely prevent blood loss through the damaged endothelium. The generation of thrombin (required for the cleavage of fibrinogen to fibrin) and formation of blood clots through the activation of a tightly regulated coagulation cascade are crucial for hemostasis following endothelial disruption (Favaloro & Lippi, 2011; Schenone, Furie, & Furie, 2004; Versteeg, Heemskerk, Levi, & Reitsma, 2013). Although it was previously believed that formation of the platelet plug (primary hemostasis) preceded fibrin generation (secondary hemostasis), studies of arterial thrombus formation utilizing real-time *in vivo* imaging have demonstrated that platelet aggregation and fibrin formation can occur simultaneously (Falati, Gross, Merrill-Skoloff, Furie, & Furie, 2002).

1.2.1 Extrinsic pathway of coagulation

There are two main pathways by which activation of Factor X occurs to generate thrombin (Figure 1.1). The extrinsic pathway of the coagulation cascade is initiated by tissue factor (TF). The TF pathway is triggered by the contact of blood with extravascular TF-expressing cells in the vascular media (smooth muscle cells) and vascular adventitia (neutrophils, monocytes, and fibroblasts) (Maiellaro & Taylor, 2007; Marder et al., 2012). The TF pathway can also be triggered with TF-expressing monocytes and microparticles in the circulation (Berckmans et al., 2001; Falati et al., 2003; Sabatier et al., 2002; Satta et al., 1994; Thiagarajan & Tait, 1991). The Ca^{2+}-dependent binding of extravascular TF to Factor VII is a crucial step in initiating the TF pathway (Nemerson, 1988). TF/FVIIa (extrinsic tenase) is able to activate FX directly (producing FXa) and indirectly through the activation of FIX (Mackman, Tilley, & Key, 2007; Marder et al., 2012). In the presence of Ca^{2+}, FIXa bound to cofactor FVIIIa (intrinsic tenase) on a negatively-charged phospholipid membrane surface activates additional FX. Subsequently, generation of the prothrombinase complex (FXa-FVa-Ca^{2+}) on negatively-charged phospholipids converts prothrombin (FII) to thrombin (FIIa) (Furie & Furie, 1988; Mackman et al., 2007; Mann et al., 1988; Mann, Nesheim, Church, Haley, & Krishnaswamy, 1990; Pryzdial & Mann, 1991; Schenone et al., 2004). The small amount of thrombin generated by the TF pathway is sufficient to activate FXI and thus can initiate the contact activation pathway before the TF pathway of coagulation is inhibited by tissue factor pathway inhibitor (TFPI) (Broze et al., 1988).

Figure 1.1. Simplified schematic of the coagulation cascade. Blood coagulation is initiated by the extrinsic or tissue factor pathway when vascular injury induces TF-bearing cells to activate FVII. TF-FVIIa activates FIX and FX to FIXa and FXa, respectively. FXa and its cofactor FVa form the prothrombinase complex on a negatively-charged phospholipid surface and, in the presence of Ca^{2+}, catalyzes the conversion of prothrombin to thrombin. The small amount of thrombin generated activates platelets, FV, FVIII, FXI, and FXIII. The intrinsic or contact pathway is initiated by nucleic acids, polyphosphates, or a complex with prekallikrein, FXII, FXI, and HMWK on a collagen surface which produces kallikrein and FXIIa. Subsequent steps in the intrinsic pathway require a negatively-charged phospholipid surface and Ca^{2+}. FXIIa activates FXI to FXIa which activates FIX to FIXa. FIXa with its cofactor FVIIIa form the tenase complex which also activates FX to FXa. Prothrombinase (FXa/FVa) activates prothrombin to thrombin which converts soluble fibrinogen into insoluble fibrin strands stabilized by FXIIIa. Breakdown of the fibrin clot occurs via fibrinolysis. Tissue-type plasminogen activator (tPA) activates plasminogen to plasmin which lyses the fibrin mesh into fibrin degradation products. Figure adapted from (Sabir, Khavandi, Brownrigg, & Camm, 2014).


Intrinsic Pathway
Extrinsic Pathway
HMWK, Kallikrein,
Nucleic Acids,
Polyphosphates
FXII
FXIIa
FXI
Ca2+
FXIa
FIX
Ca2+
FIXa
Trauma
FVII
TF
TF-FVIIa
FVIIIa
Ca2+
FX
FXa
+
FVa
FX
Ca2+
FV
Common
Pathway
Prothrombin
Thrombin
Ca2+
Fibrinogen
Fibrin
Ca2+
FXIIIa
Fibrinolysis
Pathway
Cross-linked
Fibrin Clot
tPA
Plasminogen
Plasmin
Fibrin Degradation
Products

In vivo, hemostasis is achieved predominantly via the extrinsic pathway. This is supported by the observations that FVII deficiencies are rare (Tuddenham, Pemberton, & Cooper, 1995) and that TF deficiencies have not been found in humans. Moreover, humans with FVII deficiency experience bleeding symptoms which may vary from soft tissue bleeding to severe intra-cranial or gastrointestinal bleeding (Perry, 2003). Mice deficient in TF die during embryonic development as vascular smooth muscle cells required to support blood vessels fail to migrate (Carmeliet et al., 1996; Pyo, Sato, Mackman, & Taubman, 2004). In contrast, mice deficient in FVII develop normally but die during the perinatal period due to hemorrhaging from normal blood vessels (Rosen et al., 1997).

1.2.2 *In vivo* studies of the extrinsic pathway: blood-borne tissue factor

It was previously believed that activation of the extrinsic pathway occurs exclusively following tissue injury and that TF is only found outside of the vasculature (hence "extrinsic" pathway). However, these notions are challenged by the finding of basal levels of TF in human blood (Giesen et al., 1999) and supported by the discovery of TF-expressing cell-derived microparticles in circulation (Falati et al., 2003). Furthermore, *in vivo* studies utilizing real-time imaging demonstrate that TF-expressing microparticles also promote the initiation of thrombus formation. (Falati et al., 2003). TF delivery via microparticles (derived from hematopoietic cells (Chou et al., 2004) is essential for thrombus formation in instances when the vessel remains intact. In studies using intravital high-speed widefield and confocal microscopy to visualize laser-induced vessel wall injury, it was demonstrated that initial TF delivery is mediated by blood-borne microparticles. The presence of TF was observed in the developing thrombus prior to the

accumulation of leukocytes suggesting that TF delivery in the initial phases of thrombus development occurs in a microparticle-dependent manner (Gross, Furie, Merrill-Skoloff, Chou, & Furie, 2005).

1.2.3 Intrinsic pathway of coagulation

Activation of the intrinsic pathway, or contact pathway of coagulation, requires factors contained exclusively within the vascular system (Gailani & Renné, 2007). The binding of FXII (Hageman factor) to a charged surface allows for FXII autoactivation and initiates the contact pathway. FXIIa cleaves prekallikrein producing kallikrein, a serine protease that induces further FXII activation and releases bradykinin from high molecular weight kininogen (HMWK) (Cochrane, Revak, & Wuepper, 1973; Mandle & Kaplan, 1977; Vroman, Adams, Fischer, & Munoz, 1980). FXIIa also binds and activates FXI in a Ca^{2+}-dependent manner, allowing for the conversion of FIX to FIXa (Furie & Furie, 1988; Macfarlane, 1964; Marder et al., 2012). Consequently, formation of intrinsic tenase on a negatively-charged phospholipid surface (FIXa-VIIIa-Ca^{2+}) results in a greatly amplified activation of FX (Gailani & Renné, 2007). Generation of the prothrombinase complex (FXa-Va-Ca^{2+}) then converts prothrombin to thrombin on phosphatidylserine membrane surfaces.

The intrinsic pathway of coagulation does not appear to play a role in hemostasis. This is supported by observations that individuals with hereditary deficiencies of FXII, HMWK, or prekallikrein do not present with any bleeding disorders (Broze, 1995). A deficiency in FXI results in a mild to moderate hemostatic disorder (Bolton-Maggs, Wan-Yin, McCraw, Slack, & Kernoff, 1988; Seligsohn, 2009). Activation of the intrinsic

pathway has been shown to potentiate pathological coagulation in animal models of thrombosis. Inhibition of FXIa activity (Tucker et al., 2008), FIXa activation by FXIa (Gruber & Hanson, 2003; Tucker et al., 2008), FXI activation by FXIIa (Cheng et al., 2010), or FXIIa activity (Gailani & Renné, 2007; Renné et al., 2005) result in antithrombotic effects in murine and baboon models of thrombosis. Mice deficient in FXII have delayed times to arterial thrombosis and have decreased cerebral ischemia (Kleinschnitz et al., 2006; Renné et al., 2005). Small, unstable platelet thrombi form at sites of injury, suggesting that FXIIa is required for thrombus propagation (Renné et al., 2005; Van Der Meijden et al., 2009). Mice deficient in FXI are protected from pathological fibrin formation and FXI is required for thrombus formation following ferric chloride injury (Rosen, Gailani, & Castellino, 2002). FXII deficiency in humans is not associated with an increased risk in thrombosis (Koster, Rosendaal, Briët, & Vandenbroucke, 1994; Müller, Gailani, & Renné, 2011). Decreased plasma levels of FXII levels in humans are associated with a decreased risk of thrombosis (Zito, Lowe, Rumley, McMahon, & Humphries, 2002) and are protective in acute coronary syndrome (Endler et al., 2001). Severe FXI deficiency in humans results in reduced incidence of DVT and ischemic stroke (Salomon et al., 2011; Salomon, Steinberg, Koren-Morag, Tanne, & Seligsohn, 2008).

1.2.4 Activators of the intrinsic pathway of coagulation: platelet polyphosphates and nucleic acids

Polyphosphates (polyP) are polymers of inorganic phosphate groups stored in platelet dense granules which are released during platelet activation (Ruiz, Lea, Oldfield,

& Docampo, 2004). PolyP exerts pro-coagulant effects via various mechanisms. PolyP triggers contact pathway activation in the presence of FXIIa, decreases clotting time (Smith et al., 2006), and accelerates thrombin generation by accelerating FV activation catalyzed by both thrombin and FXa (Smith et al., 2006). PolyP also increases feedback activation of FXI by thrombin (S. H. Choi, Smith, & Morrissey, 2011) and promotes contact activation via FXIIa, independent of FXIa (Puy et al., 2013). In addition, polyP blocks the anticoagulant effect of tissue factor pathway inhibitor (Smith et al., 2006), incorporates into fibrin clots (Mutch, Engel, Uitte de Willige, Philippou, & Ariëns, 2010; Smith & Morrissey, 2008), increases fibrin thickness (Smith & Morrissey, 2008), and delays fibrinolysis (Mutch et al., 2010; Smith et al., 2006). PolyP of varying sizes exert differential effects on clotting. Shorter polyP chains exert hemostastic effects by providing surfaces for FV activation while longer polyP chains contribute to thrombosis by providing surfaces for FXII activation (Smith et al., 2010).

Nucleic acids also exert pro-coagulant effects by activating the intrinsic pathway of coagulation. In purified systems, RNA accelerates plasma clotting time in a FXI- and FXII-dependent manner (Kannemeier et al., 2007). *In vivo*, extracellular RNA was found in fibrin-rich thrombi and treatment with RNase (but not *in vivo* treatment with DNase) either delayed (Kannemeier et al., 2007) or abrogated occlusive thrombosis (T. T. Vu et al., 2015). Extracellular DNA, or cell-free DNA (cfDNA) stimulates the intrinsic pathway via FXII activation (Swystun, Mukherjee, & Liaw, 2011; von Brühl et al., 2012) and increases thrombin generation in plasma (Swystun et al., 2011). DNA expelled from neutrophils (described in more detail in section 1.5) promotes thrombosis by acting as a

scaffold for platelet activation, red blood cell recruitment, and fibrin deposition (Fuchs et al., 2010). Moreover, cfDNA has also been implicated in the formation of microvascular thrombosis in bacteremia and sepsis (Clark et al., 2007). In septic patients, plasma levels of cfDNA also correlate with thrombin generation (Gould et al., 2014).

1.2.5 Common pathway of coagulation

FXa generated through the extrinsic or intrinsic pathways of coagulation activates the common pathway to ultimately produce thrombin, a 37 kDa two-chain molecule with procoagulant and anti-inflammatory properties. FXa produces small amounts of thrombin by cleaving the N-terminal Gla domain of prothrombin, releasing thrombin from the phospholipid surface (Mann, Elion, Butkowski, Downing, & Nesheim, 1981; Owen, Esmon, & Jackson, 1974). However, the cleavage of prothrombin and generation of thrombin increases 300 000-fold in the presence of the prothrombinase complex in comparison with FXa alone (Nesheim, Taswell, & Mann, 1979). FXa binding to FVa and Ca^{2+} on the phospholipid surface of activated platelets forms the prothrombinase complex which efficiently cleaves prothrombin to generate thrombin (Mann et al., 1988, 1990; Pryzdial & Mann, 1991). Thrombin promotes coagulation by activating FV and FVIII (Dahlbäck, 1980; Hultin & Jesty, 1981), factors required for the formation of prothrombinase and tenase complexes, respectively. Thrombin also promotes platelet aggregation by activating platelet protease-activated receptor-1 (PAR-1) resulting in phosphatidylserine exposure and platelet degranulation (Andersen et al., 1999) and by activating GPIb resulting in the mobilization of intracellular Ca^{2+} (Soslau et al., 2001).

1.2.6 Formation of the fibrin clot

Large amounts of thrombin are also required for the cleavage of fibrinogen into fibrin to form a loose mesh at the site of injury. In the absence of thrombin, fibrinopeptides A and B prevent the linking and polymerization of fibrin monomers. Thrombin cleaves fibrinogen into fibrin, removing the fibrinopeptides A and B to expose fibrin binding sites and allowing for Ca^{2+}-mediated polymerization of fibrin monomers (Blombäck & Blombäck, 1972; Henschen, Lottspeich, Kehl, & Southan, 1983). Polymerized fibrin molecules lengthen to form double-stranded protofibrils which interact to form long, thin chains or broad fibrin sheets. Furthermore, thrombin activates FXIII, an enzyme which catalyzes the formation of covalent crosslinks between fibrin monomers, strengthening fibrin-fibrin interactions and allowing for the formation of a fibrin aggregate or lattice (Blombäck & Blombäck, 1972; Henschen et al., 1983; Loewy, Dahlberg, Dunathan, Kriel, & Wolfinger, 1961; Loewy, Dunathan, Gallant, & Gardner, 1961; Loewy, Dunathan, Kriel, & Wolfinger, 1961; Mosesson, 2005). FXIIIa may also function to protect the fibrin clot from fibrinolysis by linking fibrin to α_2-antiplasmin (Mimuro, Kimura, & Aoki, 1986). The anchored fibrin lattice traps circulating platelets and erythrocytes, resulting in the formation of a blood clot at the site of endothelial disruption (Nemerson, 1988; Schenone et al., 2004).

1.2.7 Fibrinolysis

Fibrinolysis prevents excessive fibrin deposition and clot formation which could potentiate thrombosis and disseminated intravascular coagulation (DIC). The release of tissue plasminogen activator (tPA) from endothelial cells occurs simultaneously with the activation of the coagulation cascade (Holmes et al., 1985; Wu & Diamond, 1995). In the

absence of fibrin, the rate at which tPA converts plasminogen to plasmin is low. In the presence of fibrin, the fibrinolytic activity of tPA is increased 500-1000 fold and fibrin-bound tPA efficiently converts plasminogen into plasmin (Bode, Runge, & Haber, 1990; Holvoet, Lijnen, & Collen, 1985; Tate et al., 1987). Through positive feedback, plasmin cleaves single-chain tPA into the two-chain polypeptide (Cesarman-Maus & Hajjar, 2005). Plasmin also cleaves C-terminal lysine and arginine residues of fibrin and fibrinogen, producing soluble fibrinogen and fibrin degradation products (FDPs) (Ferguson, Fretto, & McKee, 1975; Pizzo, Schwartz, Hill, & McKee, 1973; Southan et al., 1985). Competitive inhibition of thrombin by FDPs slows the conversion of fibrinogen to fibrin and reduces clot formation (Cesarman-Maus & Hajjar, 2005; Pizzo et al., 1973; Weitz, Leslie, & Hudoba, 1998).

FXIa, FXIIa, and kallikrein also activate plasminogen directly (Colman, 1969; Goldsmith, Saito, & Ratnoff, 1978). Bradykinin is a potent stimulator for tPA release, promoting further plasminogen activation and fibrinolysis (Brown, Gainer, Stein, & Vaughan, 1999).

1.2.7.1 Inhibitors of fibrinolysis

Regulation of fibrinolysis is achieved through plasminogen activator inhibitors (PAIs) and plasmin inhibitors (produced by endothelial cells and activated platelets) (Cesarman-Maus & Hajjar, 2005; Holmes, Nelles, Lijnen, & Collen, 1987; Samad, Yamamoto, & Loskutoff, 1996). Type I plasminogen activator inhibitor (PAI-1) rapidly inhibits tPA (Samad et al., 1996) and patients with PAI-1 deficiency experience moderately severe bleeding (Fay et al., 1992). PAI-2 also inhibits tPA with comparable

efficiency but significant levels are only found in humans during pregnancy (Ye, Ahern, Le Beau, Lebo, & Sadler, 1989).

α_2-antiplasmin (α_2-plasmin inhibitor, α_2-PI) is found in circulation and in platelet alpha granules (Holmes et al., 1987). α_2-PI quickly neutralizes circulating plasmin and plasmin near platelet-rich thrombi in a 1:1 inhibitory complex (Erickson et al., 1990; Plow & Collen, 1981). α_2-macroglobulin is also found in platelet alpha granules and binds plasmin with 10% of the efficiency observed with α_2-PI (Aoki, Moroi, & Tachiya, 1978; Mimuro et al., 1986). When activated by thrombin, thrombin-activatable fibrinolysis inhibitor (TAFI) removes the plasmin-binding C-terminal lysine residues of fibrin, preventing co-activation with plasminogen and attenuating plasmin generation (W. Wang, Boffa, Bajzar, Walker, & Nesheim, 1998).

1.3 Inhibitors of coagulation

If left uncontrolled, coagulation and fibrin deposition hinder normal blood flow and can result in thrombosis. Three main inhibitors of the coagulation cascade are antithrombin, tissue factor pathway inhibitor, and protein C (Figure 1.2).

1.3.1 Antithrombin

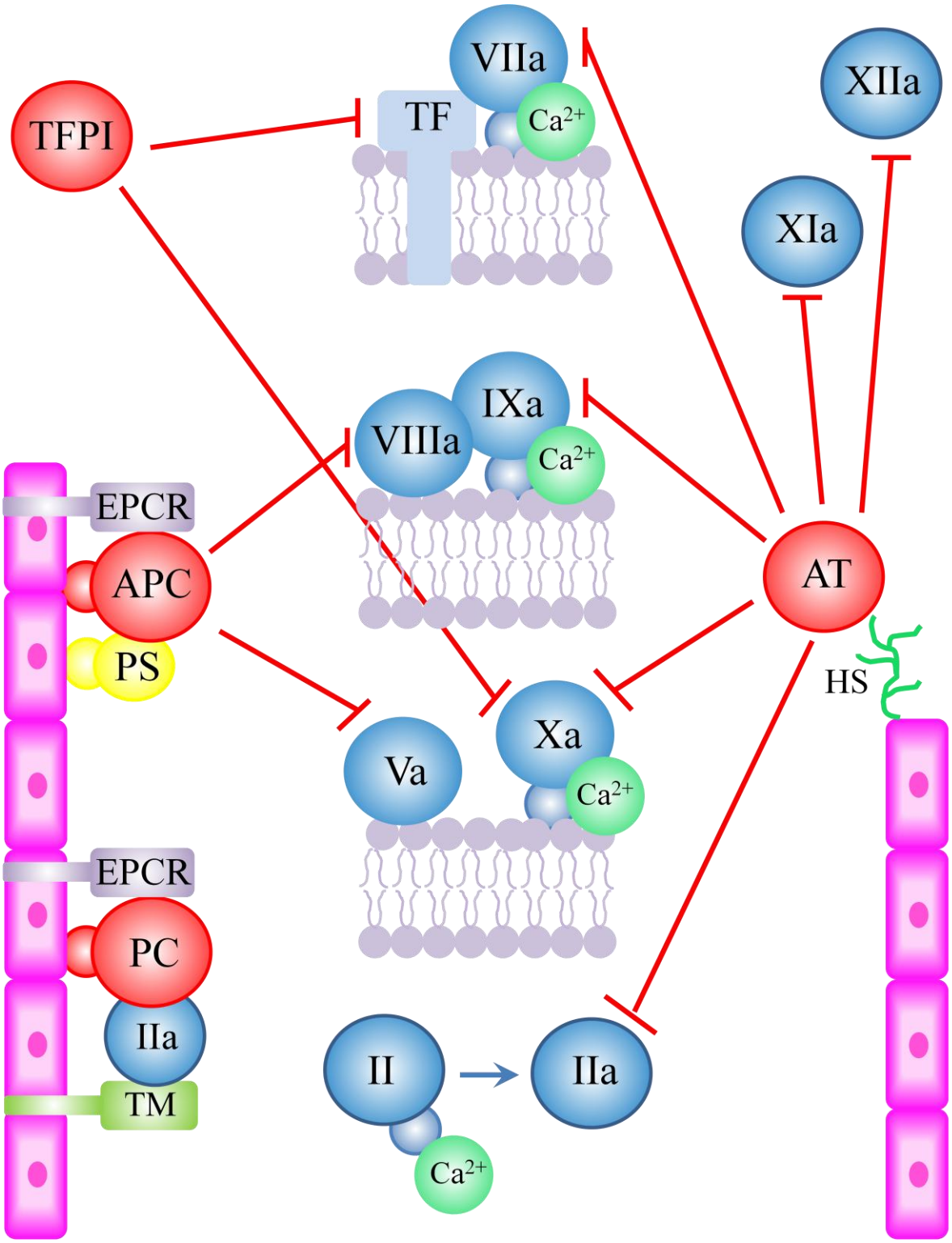
Antithrombin (AT) is an anticoagulant serine protease inhibitor (serpin) that inhibits thrombin, FXa, FIXa, FXIa, FXIIa, and FVIIa (Mauray, 1998; Olson et al., 2004; Rao, Nordfang, Hoang, & Pendurthi, 1995; Reeve, 1980; Yang, Sun, Gailani, & Rezaie, 2009). AT-mediated inhibition occurs through the formation of 1:1 covalent serpin-enzyme complexes (Hensen & Loeliger, 1963; Pixley, Schapira, & Colman, 1985). The inhibition of thrombin by AT increases 2000-4000 fold in the presence of therapeutic

heparin or heparan sulfate, a glycosaminoglycan in the vessel wall (Bray, Lane, Freyssinet, Pejler, & Lindahl, 1989; Carrell, Stein, Fermi, & Wardell, 1994; Griffith, 1982; W. Li, Johnson, Esmon, & Huntington, 2004; Olson et al., 1992, 2004; Schreuder et al., 1994). AT binds heparin at a specific heparin pentasaccharide sequence (W. Li et al., 2004; Lindahl, Bäckström, Thunberg, & Leder, 1980; Weitz, 2003), an interaction which induces a conformational change in the reactive centre loop of AT that facilitates FXa inhibition (Huntington, Olson, Fan, & Gettins, 1996; Langdown, Johnson, Baglin, & Huntington, 2004). Heparin chains that contain this pentasaccharide sequence and are at least 18 saccharide units in length bind thrombin and AT simultaneously, bridging the enzyme and inhibitor through the pentasaccharide chain (Dementiev, Petitou, Herbert, & Gettins, 2004; Olson & Bjork, 1991). Low-molecular-weight heparin (LMWH) chains lack the required polysaccharide length and are unable to mediate the formation of an AT-thrombin-heparin ternary complex (Henry, Connell, Liang, Krishnasamy, & Desai, 2009; Pixley et al., 1985; Yang et al., 2009). However, these shorter heparin chains retain their ability to catalyze AT-mediated inactivation of FXa. Other AT target substrates include kallikrein and plasmin, key mediators in fibrinolysis (Collen, Semeraro, Telesforo, & Verstraete, 1978; Venneröd, Laake, Solberg, & Strömland, 1976).

1.3.2 Tissue factor pathway inhibitor

TFPI is Kunitz-type protease inhibitor which inhibits TF and FXa (Broze, Girard, & Novotny, 1990; Sanders, Bajaj, Zivelin, & Rapaport, 1985). TFPI synthesis occurs primarily in endothelial cells and platelets

Figure 1.2. Inhibitors of coagulation. Antithrombin (AT), tissue factor pathway inhibitor (TFPI), and activated protein C (APC) inhibit blood coagulation. AT inhibits thrombin, FXa, FIXa, FXIa, FXIIa, and FVIIa. The binding of AT to the pentasaccharide sequence of heparan sulfate on the vessel wall facilitates inhibition of FXa. The binding of AT to heparan sulfate chains greater than 18 saccharide units bridges AT to thrombin via the pentasaccharide sequence, inhibiting thrombin. TFPI reversibly inhibits FXa and the TFPI-FXa complex can inhibit TF-FVIIa. Inhibition of coagulation by APC requires a series of steps. Excess thrombin binds cell-surface thrombomodulin (TM) and EPCR presents PC to the thrombin-TM complex. Thrombin cleaves PC to APC and with its cofactor, protein S, APC degrades FVa and FVIIIa. Figure adapted from (Iskander et al., 2013).


VIIa
TF
Ca2+
TFPI
XIIa
XIa
IXa
VIIIa
Ca2+
EPCR
APC
PS
AT
HS
Xa
Va
Ca2+
EPCR
PC
IIa
TM
II
IIa
Ca2+

(Ameri, Kuppuswamy, Basu, & Bajaj, 1992; Bajaj, Birktoft, Steer, & Bajaj, 2001; Bajaj, Kuppuswamy, Saito, Spitzer, & Bajaj, 1990; Osterud, Bajaj, & Bajaj, 1995; Rana, Reimers, Pathikonda, & Bajaj, 1988). TFPI is found in circulation bound by lipoproteins as well as in platelet alpha granules (Bajaj et al., 2001; Wood, Ellery, Maroney, & Mast, 2014). TFPI directly inhibits thrombin generation by inhibiting FXa and forming a TFPI-FXa complex. TFPI inhibits thrombin generation indirectly as TFPI-FXa inhibits TF-FVIIa (Bajaj et al., 2001; Broze, 1995; Broze et al., 1988; Han, Girard, Baum, Abendschein, & Broze, 1999). Recent studies support the association between TFPI and FV (Duckers et al., 2008). TFPI interacts with FV and FVa via its C-terminus (Ndonwi, Girard, & Broze, 2012). TFPI may also modulate bleeding in patients with FV-deficiency as low TFPI levels in FV-deficient plasma decreases the FV requirement for generating thrombin (Duckers et al., 2008, 2010).

1.3.3 Protein C

Under normal physiological conditions, endothelial cells express thrombomodulin (TM), a cell surface receptor that promotes anticoagulation through the binding of circulating thrombin. Upon vascular disruption, exposure to endotoxins, or inflammatory cytokine expression, excess thrombin circulating in the blood complexes with cell-surface TM to convert zymogen protein C (PC) to the anticoagulant enzyme activated protein C (APC) (Comp, Jacocks, Ferrell, & Esmon, 1982; Marciniak, 1970). Endothelial cell protein C receptor (EPCR) aids in this activation by binding and presenting circulating PC to the thrombin-TM complex (Castellino; Davie, Fujikawa, & Kisiel, 1991; C. T. Esmon & Fukudome, 1995; C. T. Esmon & Schwarz, 1995; Fukudome & Esmon, 1994).

The binding of TM to thrombin changes the substrate specificity of thrombin towards PC, increasing the rate at which thrombin cleaves PC to APC by approximately 1000-fold (N. L. Esmon, DeBault, & Esmon, 1983; Rezaie & Esmon, 1992; H. Xu, Bush, Pineda, Caccia, & Di Cera, 2005). APC together with its cofactor, protein S, degrades FVa (Kisiel, Canfield, Ericsson, & Davie, 1977; Marlar, Kleiss, & Griffin, 1982) and FVIIIa (Marlar et al., 1982; Vehar & Davie, 1980), thereby disrupting prothrombinase and tenase formation, respectively.

1.4 Sepsis

Sepsis is a complicated, heterogeneous condition involving the systemic activation of inflammation and coagulation in response to infection of normally sterile parts of the body (Dellinger et al., 2008). Sepsis may progress to severe sepsis if there is dysfunction in one or multiple organs. Severe sepsis is the leading cause of death in non-coronary intensive care units (ICUs) and is associated with mortality rates of 30-50% (Angus & Wax, 2001). In the most severe form of sepsis, hypotension persists despite adequate fluid resuscitation, a condition termed septic shock.

1.4.1 Pro-inflammatory response in sepsis

In early sepsis, the innate immune response to invading pathogens involves the recognition of specific bacterial components as well as the subsequent activation of signal transduction pathways to release key mediators implicated in inflammation, coagulation, and apoptosis. Neutrophils and monocytes act as the primary line of defense against invading microbes through the recognition of unique, pathogenic cell-wall components, specifically pathogen-associated molecular patterns (PAMPs) via pattern recognition

receptors (PRRs) (Møller et al., 2005). Characteristic of gram-negative bacteria, lipopolysaccharide (LPS) is a notable PAMP recognized by the membrane-bound signaling PPR, toll-like receptor-4 (TLR4). LPS, bound by LPS-binding protein (LBP) interacts directly with monocytes via the cell surface CD14-TLR4 complex, consequently activating a TLR signaling cascade and initiating an innate immune response to the invading pathogen (J. E. Wang et al., 2001). Host immune response to gram-positive bacteria occurs similarly with PAMP recognition via PRRs.

Peptidoglycan (PepG), a carbohydrate common to both gram-negative and gram-positive bacterial cell wall, and endotoxic lipoteichoic acid (LTA), a surface adhesion PAMP specific to gram-positive bacteria, are recognized by CD14 and TLR2, respectively (Gupta, Kirkland, Viriyakosol, & Dziarski, 1996; Schröder et al., 2003). The specific structure of LTA determines bacterial pathogenicity and ability to induce shock, while PepG enhances the sepsis-inducing effects of LTA (Thiemermann, 2002).

The interaction between pathogens and cell-surface TLR complexes induces a primary innate immune response through the activation of pro-inflammatory pathways. Activated TLRs result in the nuclear translocation of cytosolic nuclear factor-κB (NF-κB). In the nucleus, NF-κB upregulates cytokine expression by binding to the transcription initiation sites of genes encoding inflammatory cytokines TNF and IL-1β (Russell, 2006). TNF and IL-1β, secreted by type 1 helper T cells (Th1) are crucial pro-inflammatory mediators that initiate an adaptive immune response. These pro-inflammatory cytokines activate additional monocytes, neutrophils, and endothelial cells via the upregulation of cytokines, chemokines, growth factors (Aird, 2005), and cell

surface adhesion molecules P- and E-selectins (essential for leukocyte recruitment and rolling) (Aird, 2003).

However, uncontrolled amplification of inflammatory pathways induced by sepsis, termed systemic inflammatory response syndrome (SIRS), is highly detrimental for the host (Russell, 2006). Therefore, to counteract these pro-inflammatory responses, activated TLR complexes also increase transcription of cytokine IL-10 and IL-1rα, anti-inflammatory mediators produced primarily by monocytes. As part of the adaptive immune response, CD4+ type 2 helper T cells (Th2) secrete IL-10 and neutrophil activation shifts to an anti-inflammatory phenotype (Abbas, Murphy, & Sher, 1996; Gasser & Schifferli, 2004). While neutrophil activation aids in protecting host cells from additional pathogenic invasion, it is also responsible for the increased synthesis and release of the vasodilator nitric oxide (via inducible nitric oxide synthase, iNOS) (Kolaczkowska & Kubes, 2013). Persistent upregulation of anti-inflammatory mediators caused by the compensatory anti-inflammatory response syndrome (CARS) (Bone, 1996) becomes detrimental for the host when it creates an immunosuppressed state that allows for secondary opportunistic infections to further drive sepsis pathology (Hotchkiss, Monneret, & Payen, 2013).

1.4.2 Crosstalk between inflammation and coagulation in sepsis

The procoagulant and pro-inflammatory pathways activated in response to infection are intimately linked. *In vitro,* TNF and IL-1 induce TF expression on endothelial cells (Bevilacqua et al., 1986; Boermeester et al., 1995; Eric Camerer, Anne-Brit Kolst, 1996; van Deventer et al., 1990) while IL-6 (van der Poll et al., 1994) induces

the expression of TF on mononuclear and endothelial cells. *In vivo*, dysregulated coagulation can be characterized by conditions such as the formation of microvascular thrombi (purpura fulminans) and hemorrhaging (Vanderschueren et al., 2000). Increased levels of D-dimers, thrombin-antithrombin complexes (TATs), monocytic TF, microparticles, adhesion receptor P-selectins, and fibrin clots have also been documented in sepsis (Gregory, Morrissey, & Edgington, 1989; R Pawlinski & Mackman, 2004; Rafal Pawlinski & Mackman, 2010; Souza, Yuen, & Star, 2015). The procoagulant state is also indicated by the consumption of anticoagulant mediators AT, protein C, protein S, and TFPI (Kinasewitz et al., 2004).

Activation of coagulation in turn modulates inflammation (Broze, 1982; Levi, 2010). Thrombin, through the activation of protease-activated receptors (PARs), is a key mediator linking coagulation and inflammation (Coughlin, 2000). Thrombin cleaves PAR1 revealing a new N-terminus that functions as a tethered ligand to initiate PAR1-mediated signaling (T. K. Vu, Hung, Wheaton, & Coughlin, 1991). The pro-inflammatory activities of thrombin include inducing endothelial P-selectin expression and neutrophil activation by triggering the production of endothelial platelet activating factor, IL-6, and IL-8 (Gando, Nanzaki, Sasaki, Aoi, & Kemmotsu, 1998; Levi, 2010; Sugama et al., 1992; Vanderschueren et al., 2000).

1.5 Neutrophil Extracellular Traps (NETs)

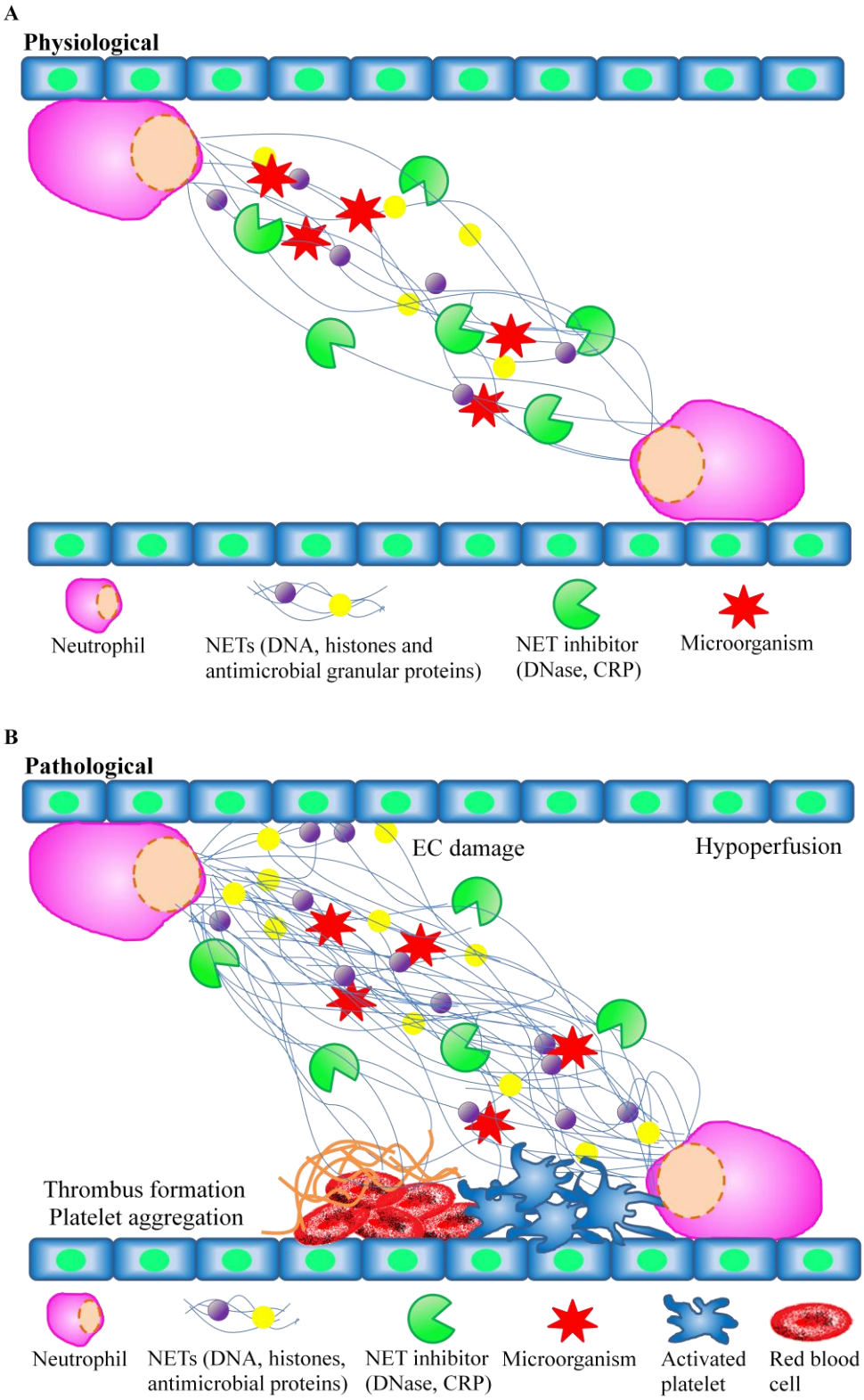
During an exaggerated inflammatory response, cellular injury contributes to the release of DNA from cell types including eosinophils, basophils, neutrophils, and monocytes (J. Choi, Reich, & Pisetsky, 2004; Schorn et al., 2012). In 2004, Brinkmann *et*

al. characterized a novel mechanism of innate immunity whereby cfDNA is actively released (Figure 1.3). Upon exposure to various stimuli such as endotoxins, bacteria, fungi, pro-inflammatory mediators (e.g. IL-8), phorbol myristate acetate (PMA, a protein kinase activator), activated platelets (Bianchi et al., 2009; Bruns et al., 2010; Hakkim et al., 2010; Papayannopoulos & Zychlinsky, 2009; Springer et al., 2010; Urban et al., 2009; Urban, Reichard, Brinkmann, & Zychlinsky, 2006), HMGB1 (Tadie et al., 2013), and monosodium urate crystals (Schorn et al., 2012), neutrophils release neutrophil extracellular traps (NETs) which consist of chromatin (DNA and histones) and neutrophil granular proteins (Brinkmann et al., 2004). NETs support the innate immune response by ensnaring pathogens and preventing the dissemination of microorganisms in the vasculature (Beiter et al., 2006; Brinkmann et al., 2004; Buchanan et al., 2006; Fuchs et al., 2007; Guimarães-Costa et al., 2009; Shapiro et al., 2006). NET-associated proteins such as histones, cathepsin G, neutrophil elastase, myeloperoxidase (MPO), catalase, and bactericidal permeability-increasing protein also have antimicrobial effects on pathogens (Brinkmann et al., 2004; Ramos-Kichik et al., 2009; Shapiro et al., 2006).

1.5.1 NETosis, a unique form of cell death

The formation of NETs, termed NETosis, is an active process. NETosis requires chromatin decondensation (Fuchs et al., 2007), a process mediated by post-translational histone citrullination (Y. Wang, Li, Stadler, & Correll, 2009) whereby peptidylarginine residues are converted into citrulline (Figure 1.4) (Vossenaar, Zendman, van Venrooij, & Pruijn, 2003). Peptidylarginine deiminase type 4 (PAD4) is a nuclear enzyme highly expressed in neutrophils and monocytes (Nakashima, 2002; Vossenaar et al., 2003) that

Figure 1.3. Neutrophil extracellular traps (NETs). Activation by inflammatory mediators and microorganisms induces disintegration of the nuclear membrane and a robust release of neutrophil granules and chromatin material in the form of neutrophil extracellular traps (NETs). NETs are composed of a DNA backbone decorated by histones and antimicrobial granular proteins. NETs ensnare and kill circulating microorganisms, preventing further bacterial dissemination. Endogenous DNases regulate NETs in the microvasculature (A). NETs can also cause collateral host damage as the release of chromatin and cytotoxic enzymes induces endothelial cytotoxicity, potentiates platelet aggregation, and promotes the formation of fibrin rich clots. Improper regulation and clearance of NETs also result in areas of tissue hypoperfusion downstream (B). Figure adapted from (Camicia, Pozner, & de Larrañaga, 2014).

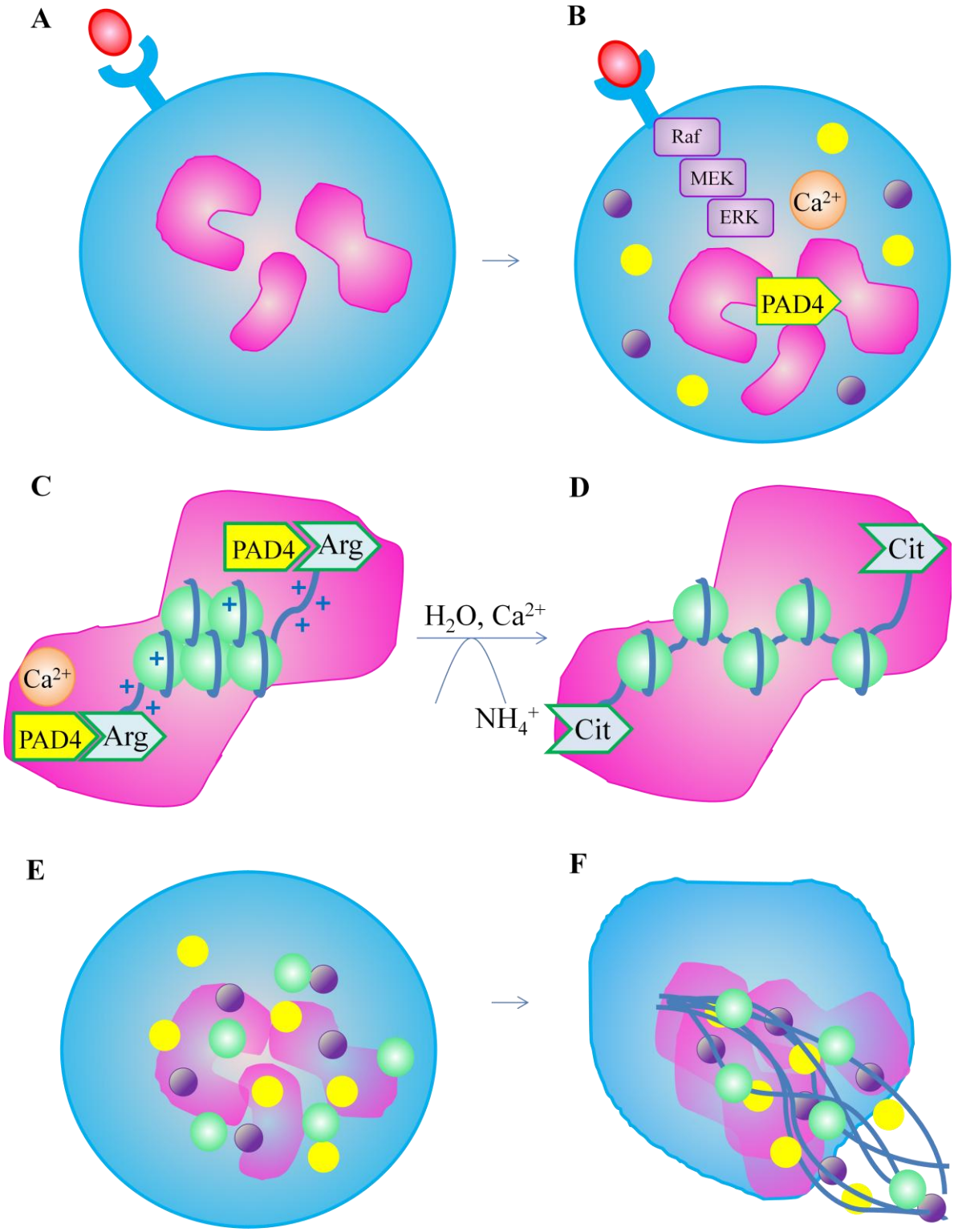

A
Physiological
Neutrophil
NETs (DNA, histones and antimicrobial granular proteins)
NET inhibitor (DNase, CRP)
Microorganism
B
Pathological
EC damage
Hypoperfusion
Thrombus formation
Platelet aggregation
Neutrophil
NETs (DNA, histones, antimicrobial proteins)
NET inhibitor (DNase, CRP)
Microorganism
Activated platelet
Red blood cell

converts arginine to citrulline in linker histone H1 and histones H3 and H4 (P. Li et al., 2010; Y. Wang et al., 2009). The increase in histone citrullination results in chromatin decondensation (Y. Wang et al., 2009). PAD4-/- mice retain other neutrophil functions such as neutrophil recruitment and phagocytosis but cannot produce NETs (Hemmers, Teijaro, Arandjelovic, & Mowen, 2011; P. Li et al., 2010; Martinod et al., 2015). Cleavage of histones by neutrophil elastase will also cause chromatin decondensation (Papayannopoulos, Metzler, Hakkim, & Zychlinsky, 2010) which is followed by the disintegration of nuclear and granule membranes. Chromatin material mixes with neutrophil granular proteins and is expelled extracellularly (Fuchs et al., 2007; Remijsen et al., 2011). The stretches of DNA backbone decorated with granular proteins reach diameters of 15-25 nm and can aggregate to form thicker threads around 50 nm in diameter (Brinkmann et al., 2004). NETosis is distinct from necrosis in that the plasma membrane remains intact while nuclear and cytoplasmic granular components mix during NETosis. NETosis is also distinct from apoptosis since no phosphatidylserine exposure signaling phagocytosis, or nucleosomal cleavage occurs in NETosis (Fuchs et al., 2007; Vandenabeele, Galluzzi, Vanden Berghe, & Kroemer, 2010).

1.5.2 NETosis in infection

NET formation has been shown to occur in the context of infection and sepsis. Clark *et al*. reported that LPS-induced endotoxemia *in vivo* and co-incubation of neutrophils with plasma from severe sepsis patients *in vitro* triggers NET formation. Upon TLR4 stimulation by LPS, circulating platelets bind to neutrophils adhered to the endothelium (Clark et al., 2007). Within minutes of this binding, a robust release of

Figure 1.4. Peptidylarginine deiminase 4 (PAD4) in NET formation. NET formation is an active process which requires chromatin decondensation mediated by PAD4. Pro-inflammatory mediators, microorganisms, and endotoxins stimulate NETosis through the binding of cell surface receptors (e.g. binding of LPS to TLR4) (A). Ligand-receptor binding induces the release of Ca^{2+} from the endoplasmic reticulum and activation of the Raf-MEK-ERK pathway and PAD4 nuclear protein (B). The binding of Ca^{2+} to the C-terminal catalytic domain is required for PAD4 activity (C). PAD4 catalyzes the conversion of positive peptidylarginine residues to peptidylcitrulline residues of histones H1, H3, and H4 leading to chromatin decondensation (D), disintegration of nuclear membranes, and mixing of cytoplasmic and nuclear components (E). DNA, histones, and granule proteins are expelled extracellularly with stretches of DNA backbone decorated with antimicrobial proteins (F). Figure adapted from (Abi Abdallah & Denkers, 2012; Raats, Chirivi, Van Rosmalen, Jenniskens, & Pruijn, 2013).


A
B
Raf
MEK
ERK
Ca2+
PAD4
C
D
PAD4
Arg
Ca2+
PAD4
Arg
H2O, Ca2+
NH4+
Cit
Cit
E
F

neutrophil granules and chromatin material in the form of NETs is observed. The integrity of NETs are maintained under flow conditions which are reflective of physiologic shear forces observed in the microvasculature (0.5 dyne/cm^2). The NETs ensnare bacteria primarily in the liver and lung microvasculature where the capacity to immobilize microorganisms in the small vessel lumen is high (Clark et al., 2007). It was proposed that platelets act as a sensor or barometer via TLR4 signaling for pathogens in the blood, inducing NETosis from neutrophils to ensnare and kill bacteria (Clark et al., 2007).

In experimental sepsis, degradation of NETs have varied effects including increased bacterial load (Meng, Paunel-Görgülü, Flohé, Hoffmann, et al., 2012) and reduced lung damage in septic mice (Luo et al., 2014). One recent study found no change in survival or susceptibility in PAD4-/- mice following CLP-induced sepsis (Martinod et al., 2015). In a model of methicillin-resistant *Staphylococcus aureus* (MRSA), liver injury is significantly reduced in PAD4-deficient mice (Kolaczkowska et al., 2015).

Research investigating the role of NETs in sepsis has progressed significantly over the last 8 years. Since 2007, the research group under Dr. Paul Kubes at the University of Calgary has documented *in vivo*, real-time visualization of NETosis in various sepsis models. These sepsis models include an *E. coli* model of sepsis by McDonald *et al*. (McDonald, Urrutia, Yipp, Jenne, & Kubes, 2012), a CLP model of sepsis by Cools-Lartigue *et al*. (Cools-Lartigue et al., 2013), and most recently an MRSA model of sepsis by Kolaczkowska *et al*. (Kolaczkowska et al., 2015). One other research group, Tanaka *et al*., has characterized various forms of *in vivo* NETs in end organs using an LPS model of sepsis (Tanaka et al., 2014).

1.5.2.1 NETs in sepsis: circulating cf-NETs and anchored NETs

Recently, Tanaka *et al*. described various forms of NETs which appear following LPS challenge *in vivo* and *in vitro,* circulating cell-free NETs and anchored NETs. The presence of cytoplasmic leukocyte vacuoles or circulating cotton-like structures *in vivo* indicates the presence of circulating cell-free NETs (Tanaka et al., 2014). *In vitro,* cfDNA in culture supernatant indicates the presence of circulating cell-free NETs. In contrast, anchored NETs appear linear, reticulated, and/or spot-like *in vivo* and are adherent to culture plates *in vitro* (Tanaka et al., 2014). Tanaka *et al*. also described the association of anchored NETs with reticular and spot-like structures anchored to leukocytes. On the leukocyte surface, membranous NET structures are observed as well as linear structures anchoring some leukocytes and leukocyte-platelet aggregates to the endothelium (Tanaka et al., 2014). This study confirms the presence of two distinct forms of NETs in the post-capillary venules of the cecum, hepatic sinusoids, alveolar space, and pulmonary capillaries. Hypoperfusion in the microvasculature was also documented and likely induced by the presence of platelet-leukocyte aggregates and platelet aggregates causing decreased blood flow downstream. This study further supports the pathogenic role of NETs in CLP sepsis.

Studies on the structure of NETs and the sites at which NETosis occurs show some conflicting results. The widespread formation of circulating, cotton-like NETs in the hepatic sinusoids were observed following CLP surgery (Cools-Lartigue et al., 2013) and following challenge with MRSA (Kolaczkowska et al., 2015), LPS (McDonald et al., 2012), and *E.coli* (McDonald et al., 2012). In contrast, Tanaka *et al*. observed only

reticular, spot-like structures anchored to leukocytes and in platelet aggregates in the liver also following LPS-challenge (Tanaka et al., 2014). In an LPS model, anchored NETs were also observed in the alveolar space and pulmonary capillaries (Tanaka et al., 2014) while widespread, web-like NETs in the pulmonary capillaries were described in CLP sepsis studies (Clark et al., 2007; Cools-Lartigue et al., 2013). Based on these observations, it appears that circulating cf-NETs and anchored NETs are characteristic of the host response to CLP, LPS, *E. coli*, and MRSA challenge, however, there is no consensus on the location and types of NETs formed in various sepsis models.

1.5.3 Effects of NETs and NET components on hemostasis

NETs act as scaffolds for platelet aggregation, leukocyte interactions, and red blood cell (RBC) accumulation, causing microvascular thrombosis and ischemia in affected tissues (Brill et al., 2011, 2012; Clark et al., 2007; Martínez Valle, Balada, Ordi-Ros, & Vilardell-Tarres, 2008). While the effects of either DNA or histones alone are subtle, histones and DNA together bind large fibrin degradation products and delay clot lysis (Longstaff et al., 2013). In deep vein thrombosis, PAD4-/- mice are partially protected from venous thrombus formation following stenosis (Martinod et al., 2013) and PAD-/- mice are protected against vascular injury in carotid artery thrombosis (Knight, Luo, et al., 2014). Ischemic conditions increase the production of IL-8 (Tew, Johnson, Reveille, & Tan, 2001) and reactive oxygen species (Balada et al., 2002) which drive further NET formation (Bodaño et al., 2007). In studies of thrombosis, NETs interact closely with fibrin and platelets via VWF, fibronectin, and fibrinogen, effectively stabilizing platelet-rich clots (Martínez Valle et al., 2008; Napirei, Wulf, & Mannherz,

2004). NETs perfused with blood or platelet-rich plasma stimulate platelet aggregation and promote thrombus formation (Fuchs et al., 2010). NETs promote neutrophil adherence to the microvascular endothelium, fibrin deposition, microvascular rupture with intra-alveolar hemorrhaging, and the formation of platelet-rich micro- and macrovascular thrombi (Ammollo, Semeraro, Xu, Esmon, & Esmon, 2011; Clark et al., 2007; C. T. Esmon, 2011; J. Xu et al., 2009; J. Xu, Zhang, Monestier, Esmon, & Esmon, 2011).

In addition to serving as a scaffold for platelet, RBC, and leukocyte interactions, NET components also exert procoagulant effects. As mentioned previously, cfDNA activates the intrinsic pathway of coagulation. *In vivo* models of thrombosis demonstrated that treatment with RNase or FXIIa inhibitor, but not DNase, delays occlusive thrombus formation (Kannemeier et al., 2007). *In vitro* studies demonstrated that DNA and RNA decrease clotting time by promoting FXI and FXII activation (Kannemeier et al., 2007) and that cfDNA released from whole blood increases thrombin generation via the contact pathway (Gould et al., 2014; Swystun et al., 2011). Moreover, cfDNA isolated from polymorphonuclear cells recruit HMWK and FXII which is followed by prekallikrein activation and the subsequent release of bradykinin (Oehmcke, Morgelin, & Herwald, 2009). In human plasma, hairpin-RNA and -DNA oligomers, but not linear oligomers delay clotting time and mediate PK and FXI activation by binding HMWK (Gansler et al., 2012).

In models of thrombosis, cfDNA levels increase at 6 and 24 hours following induction of deep vein thrombosis and in *in vitro* flow assays, extracellular DNA form

scaffolds on which platelet aggregation, RBC recruitment, and fibrin deposition occurs (Fuchs et al., 2010). *In vitro*, perfusion of DNase degrades extracellular DNA and abolishes platelet aggregation and RBC recruitment (Fuchs et al., 2010). In a mouse model of stenosis, circulating cfDNA levels are elevated 6 hours following IVC stenosis (Brill et al., 2012). DNA was found in the fibrin- and RBC-rich portion of the thrombus, and injection of DNase decreases thrombus weight, thrombus length, and the percentage of mice with thrombi (Brill et al., 2012).

Histones also exert pro-coagulant effects which are pathogenic to the host. Positively-charged histones promote RBC accumulation and platelet aggregation through electrostatic interactions with the negatively-charged surfaces (Semeraro et al., 2011; J. Xu et al., 2011). NET-associated histones impair protein C activation by binding both TM and PC, decreasing TM-dependent PC activation (Ammollo et al., 2011). Histones also promote thrombin generation by inducing platelet activation and the secretion of polyP from platelets (Semeraro et al., 2011; J. Xu et al., 2009). Histones induce prothrombinase activity, increase P-selectin and phosphatidylserine expression, and activate FV (Fuchs et al., 2010; Semeraro et al., 2011). Blocking histones in LPS and CLP models of sepsis rescues mice from death (J. Xu et al., 2009).

1.5.4 Pro-inflammatory effects of NETs and NET components

NETs produce areas of ischemia in downstream tissues (Clark et al., 2007; Martínez Valle et al., 2008) which increase the release of IL-8 (Tew et al., 2001) and reactive oxygen species (Balada et al., 2002) both of which further induce NETosis (Bodaño et al., 2007). *In vitro*, NET formation causes endothelial cell damage and *in vivo*,

NET formation reduces perfusion of liver sinusoids and increases levels of alanine aminotransferase in LPS-challenged mice (Clark et al., 2007). MPO released during NET formation binds the negatively-charged, proteoglycan-rich endothelium, inducing endothelial cell damage and vascular permeability (Baldus et al., 2001; C. J. Marshall et al., 2010). Incubation of NETs with human platelets and THP-1 cells results in the release of pro-inflammatory cytokines including IL-1β, IL-8, and TNFα (Keshari et al., 2012). Platelet-mediated neutrophil interactions cause endothelial cell damage (Clark et al., 2007) and liver damage in LPS- and *E. coli*-induced sepsis (Clark et al., 2007; McDonald et al., 2012; Papayannopoulos & Zychlinsky, 2009) which is prevented by intravenous DNase administration (McDonald et al., 2012). Inhibition of neutrophil recruitment by blocking adhesion molecules reduces lung and liver damage in CLP-induced sepsis (Asaduzzaman, Rahman, Jeppsson, & Thorlacius, 2009; Asaduzzaman, Zhang, Lavasani, Wang, & Thorlacius, 2008; Hasan, Palani, Rahman, & Thorlacius, 2011). Administration of DNase following a 24 hour period of CLP-induced sepsis progression results in reduced lung inflammation and injury (HMGB1 and IL-6) and decreased neutrophil recruitment in the lungs (CXCL1 and CXCL2) by greater than 50% (Luo et al., 2014). These findings suggest that NETs trigger inflammation by promoting neutrophil recruitment and increasing pro-inflammatory mediators in the lung.

PAD4-deficiency (the absence of NETs) in a murine lupus model improves endothelial function (Knight et al., 2013), confers protection against vascular damage, decreases skin inflammation, and is immunomodulatory by downregulating interferon-response genes (Knight, Subramanian, et al., 2014). In MRSA-induced sepsis, PAD4-

deficiency significantly protects mice from liver damage (Kolaczkowska et al., 2015). In our studies, abolishing NETosis reduces the aberrant inflammatory response and organ damage in the lungs in septic mice. These findings suggest that NETs exacerbate inflammation in infection. Studies in cystic fibrosis (Robinson, 2002; Shak, 1995; Suri et al., 2002), systemic lupus erythematosus (SLE) (Villanueva et al., 2011), vasculitis (Kessenbrock et al., 2009), and thrombosis (Brill et al., 2012; Fuchs et al., 2010) also demonstrate that the prolonged exposure of DNA in the circulation drives a pro-inflammatory response.

1.5.5 Inhibitors of NETosis and NET components

NETs can be counter-regulated by the administration of deoxyribonuclease I (DNaseI) (Meng, Paunel-Görgülü, Flohé, Witte, et al., 2012). DNaseI is Ca^{2+}- and Mg^{2+}-dependent enzyme that hydrolyses double-stranded DNA, generating lower molecular weight tri- and/or tetraoligonucleotides with 5′-phosphate and 3′-hydroxyl termini (Counis & Torriglia, 2000; Guéroult, Picot, Abi-Ghanem, Hartmann, & Baaden, 2010). This endonuclease is produced by the pancreas and salivary glands, cleared by the liver (Prince et al., 1998), and is present in urine and plasma at concentrations of around 3 ng/mL (Lacks, 1981; Nadano, Yasuda, & Kishi, 1993; Prince et al., 1998). DNaseI has various physiological functions including the degradation of dietary DNA within the alimentary tract (Grimble, 1994), depolymerization of actin in the regulation of cytoskeletal filaments (dos Remedios et al., 2003), and DNA clearance during apoptosis (Counis & Torriglia, 2000; Peitsch, Polzar, Tschopp, & Mannherz, 1994) and at sites of high cell turnover (Lacks, 1981).

DNaseI degrades cfDNA released during NETosis and prevents anti-DNA autoimmunity (Brinkmann et al., 2004). DNaseI treatment decreases neutrophil recruitment, lung injury, and sputum viscosity and improves pulmonary function in cystic fibrosis and pleural infection by reducing DNA accumulated in the sputum or lungs (Papayannopoulos, Staab, & Zychlinsky, 2011; Rahman et al., 2011; Robinson, 2002; Shak, Capon, Hellmiss, Marsters, & Baker, 1990; Shak, 1995). Studies in DNaseI-deficient mice indicate that NET removal is likely a crucial process in immunity as these mice develop lupus-like symptoms induced by NETs (Napirei et al., 2004). In SLE, mutations in the DNASE1 gene causing low circulating levels of DNase have been implicated in some individuals with SLE (Bodaño et al., 2007; Yasutomo et al., 2001). Low DNase levels in these individuals result in the improper clearance of extracellular DNA from circulation (Leffler et al., 2013; Martínez Valle et al., 2008), the formation of anti-DNA autoantibodies, and aberrant immune responses against the host's DNA (Blatt & Glick, 1999; Shin et al., 2004). In murine studies of SLE, treatment with recombinant murine DNase reduces anti-dsDNA antibodies (Macanovic et al., 1996; Verthelyi, Dybdal, Elias, & Klinman, 1998), delays onset of disease, and suppresses renal damage (Macanovic et al., 1996), but does not rescue mice from death (Verthelyi et al., 1998).

In deep vein thrombosis, the absence of NETs is protective following stenosis (Martinod et al., 2013). In a model of MRSA, liver injury is also significantly reduced in PAD4-/- mice (Kolaczkowska et al., 2015). In experimental sepsis, the effects of DNase are unclear. Repeated injections of DNase in a murine model of CLP lead to increased bacterial load (Meng, Paunel-Görgülü, Flohé, Hoffmann, et al., 2012) in one study and

decreased bacterial load in another study (Luo et al., 2014). In an *E. coli* sepsis model, degradation of NETs via DNase administration decreases liver damage (McDonald et al., 2012) and in a CLP sepsis model, DNase administration ameliorates lung injury (Luo et al., 2014).

NET components can also be neutralized. For example, histones can be cleaved by APC and co-injection of histones and APC reduces histone cytotoxicity and mortality in a murine LPS model and a baboon *E. coli* model of sepsis (J. Xu et al., 2009). Histone toxicity can also be neutralized by c-reactive protein (CRP) (Abrams et al., 2013) and CRP can also rescue mice from histone-induced lethality (Abrams et al., 2013).

1.5.6 Modulation of fibrinolysis by DNA and histones

DNA promotes plasminogen activation by tPA and uPA, independent of fibrin (Komissarov, Florova, & Idell, 2011). DNA and fibrin compete to bind plasminogen and tPA. DNA also inhibits fibrinolysis by accelerating PAI-1 inactivation of tPA (Komissarov et al., 2011). However, Longstaff *et al*. observed that DNA does not cause increased clot lysis, but rather delays clot dissolution. They also observed that DNA binds large FDPs and hold the fibrin network together (Longstaff et al., 2013). The addition of DNA results in clots that were less stable to mechanical shear forces and have reduced stiffness (softer, weaker clots) due to disrupted longitudinal alignment of monomers (Longstaff et al., 2013). Addition of DNA alone results in small changes in clot turbidity with only minor increases in clot lysis times.

A greater effect on clot structure is observed with the addition of histones (with or without DNA) than with the addition of DNA alone. Fibrin clots with added histones are

associated with thicker fibrin fibers and the fibrin clots are more opaque and robust (Longstaff et al., 2013). In the presence of 50 μg/mL of histones, the activity of tPA increases up to 50%. Histones also bind FDPs which may account for inhibition of clot lysis at higher histone concentrations (Longstaff et al., 2013).

Increasing concentrations of DNA and histones results in increased fibrin fiber thickness and a prolonged clot lysis times. Small angle x-ray and neutron scattering studies showed lower protofibril density which correspond to thicker fiber diameters in the presence of both DNA and histones, but not DNA or histones alone (Longstaff et al., 2013). The addition of DNA and histones also result in much more rigid, stable clots which are highly resistant to shear forces (Longstaff et al., 2013). Unfortunately, the potency of tPA in the presence of both DNA and histones was difficult to assess in clot turbidity experiments. In the presence of DNA alone, fibrin holds together longer and in the presence of histones alone, clots are more opaque but lyse more quickly. While DNA impedes clot lysis, histones with or without DNA speeds up the advance of the lysis front. Once initiated, the onset of fibrinolysis is rapid suggesting that plasminogen activation and fibrin breakdown are efficient in the presence of DNA and histones (Longstaff et al., 2013). Clots formed from septic patient plasma containing greater than 5 μg/mL of cfDNA are dense and resistant to fibrinolysis (Gould et al., 2015). DNA forms a ternary complex with plasmin and fibrin, thereby preventing degradation of fibrin clots (Gould et al., 2015).

1.6 Historical development of animal models of sepsis

Animal models are amongst the most effective tools currently available to investigate the progression of sepsis and mechanisms involved in sepsis pathology. While improvements to these models have been made over the last 80 years, animal models of sepsis have their limitations (Deitch, 2005; J. C. Marshall et al., 2005; Wichterman, Baue, & Chaudry, 1980). The isolation of endotoxin in the 1930s were crucial for the development of the earliest animal models of sepsis. Live organisms or endotoxins were infused either intravenously or in the peritoneal cavity and this early animal model dominated sepsis research for the next three decades. In the 1960's, the discovery of new antimicrobial compounds contributed to the switch to injection of live bacteria (Deitch, 2005).

Such models included the injection of *Pseudomonas aeruginosa*, *Escherichia coli*, and *Staphylococcus aureus*. The injection models progressed to inoculation of feces (fecal slurry models) through the intramuscular and intraperitoneal routes and later, subdermal implantation of capsules containing feces (Wichterman et al., 1980). However, a sudden and transient spike of pathogens at supraphysiological levels does not reflect infection in the septic host, where microorganisms are continually released over progression of the disease. Much higher mortality rates were also observed in these models of than those observed clinically, largely due to bacterial overload (Deitch, 2005). Moreover, these models relied heavily on bacterial counts and composition which were highly variable between subjects. To overcome these limitations, defined bacterial inoculum models introduced in the 1970s significantly improved the reliability of animal sepsis models. Defined bacterial inoculum models were pertinent to characterizing the two-stage nature

of intra-abdominal sepsis. In these studies, it was observed that gram-negative enteric bacteria induces peritonitis and anaerobic bacteria are responsible for abscess formation (Wichterman et al., 1980). Antibiotics either improved survival or reduced abscess formation in these models, but were unsuccessful in improving both indicators of disease. The absence of ischemia and necrosis which occur in clinical sepsis were also unaccounted for in the bacteria models.

To address these factors, models of bowel manipulation were developed in dogs separately by the Clowes and Wright groups. The ceca of dogs were ligated at the ileocecal valve, a model which demonstrated an initial hyperdynamic phase and subsequent hypodynamic phase, reminiscent of sepsis (Clowes et al., 1968; Wright, Cornwell, & Thompson, 1971; Wright, Thompson, & Cornwell, 1971). However, neither group reported bacterial counts to confirm bacterial dissemination of bacteria in the vasculature and it is unclear whether these studies modeled abscess formation or true sepsis. Extending these studies, a rat model was developed by Ryan *et al.* which also involved cecal ligation causing devascularizing as well as necrosis (Ryan, Benjamin, & Blumgart, 1974). These models remain the precursors of the gold standard of animal sepsis models frequently used today--the cecal ligation puncture (CLP) model and more recently, the colon ascendens stent peritonitis (CASP) model.

1.6.1 Cecal ligation and puncture (CLP) model of sepsis

In 1980, authors Wichterman, Baue, and Chaudry introduced a novel experimental sepsis model still widely regarded as the gold standard for modelling polymicrobial sepsis—cecal ligation and puncture (CLP) (Wichterman et al., 1980). The cecum is

ligated distal to the ileocecal valve and the antimesenteric surface of the cecum is punctured twice with an 18 G needle. Rats are given saline to account for post-surgical losses. In these studies, blood cultures confirmed that the model was positive for *Escherichia coli, Streptococcus bovis, Proteus mirabilis, Enterococcus,* and *Bacteroides fragilis* (Wichterman et al., 1980). True bacteremia rather than abscess formation was confirmed with cultures of the peritoneal cavity fluid which were also positive for *Streptococcus viridians* and *Clostridium sporogenes*. Rats subjected to CLP in these studies had mortality rates of around 70% (Wichterman et al., 1980). Rats sacrificed at 10 hours post-CLP surgery showed evidence of the early hyperdynamic phase of sepsis indicated by increased blood flow to organs, hyperinsulinemia, and hyperglycemia while rats sacrificed 16-24 hours post-CLP surgery exhibited a hypodynamic state indicated by hypoperfusion, hypoinsulinemia, hypoglycemia, and high serum lactate levels (Wichterman et al., 1980). These states correlate with those observed in the progression of clinical sepsis. Patients initially have increased cardiac output, low peripheral resistance, and increased oxygen consumption, conditions which reverse as sepsis progresses along the disease continuum to septic shock (Dellinger et al., 2008).

The model of CLP used in our studies is modified based on a CLP model proposed by others. Under anaesthesia, the cecum is exposed and trauma is induced via a midline skin laparotomy and blunt dissection of the peritoneum. The cecum is exteriorized and taking care not to damage to the mesenteric vessels, the cecum is ligated with a suture placed distal to the ileocecal valve. The ligated cecum is punctured twice in the mesenteric to anti-mesenteric direction halfway between the ligation and cecal end,

and the cecal end is aspirated for trapped gasses (Wichterman et al., 1980). Fecal content is extruded from both punctures to allow for patency and continuous flow of feces post-operatively. Inadequate expulsion of fecal contents will induce abscess formation around the cecal end, preventing bacterial dissemination into the peritoneum and bloodstream. The cecum is then returned into the peritoneal cavity, taking care not to spread fecal contents on the incision and the peritoneum and skin are closed separately with sutures and staples, respectively (Rittirsch, Huber-Lang, Flierl, & Ward, 2009).

1.6.1.1 Modifying the severity of CLP-induced sepsis

The CLP procedure can be modified to model various stages of the sepsis spectrum from the rapid onset of a robust, hyperinflammatory response to a gradual progression of severe sepsis to immunosuppression observed in septic shock. Increasing the number of punctures, using a smaller gauge needle to puncture the cecum, increasing the length of the cecum, and increasing the amount of fecal matter extruded are methods by which sepsis severity can be increased (Schabbauer, 2012; Wichterman et al., 1980). The flexibility in modeling various severities of disease, ability to recreate hemodynamic, metabolic, and immune changes, the inclusion of surgical trauma, necrosis, and apoptosis of specific cell types (Dellinger et al., 2013) which more closely correlate with clinical sepsis contribute to the acceptance of CLP as the gold standard for modeling polymicrobial, intra-abdominal sepsis.

1.6.1.2 Host response to CLP

The host response to CLP is characterized by changes in hemodynamics, cardiovascular responses, metabolism, cytokines, innate and adaptive immunity, and

abnormalities in mediators of coagulation. These changes have also been observed in the clinical course of sepsis (Dejager, Pinheiro, Dejonckheere, & Libert, 2011). Several factors observed in clinical sepsis are modelled in murine CLP-induced sepsis. Firstly, a localized infection releases microorganisms endogenous to the host into normally sterile parts of the body, subsequently enteric bacteria enter the bloodstream, and organ dysfunction is observed in end organs. These stages are also observed in intra-abdominal clinical sepsis (Wichterman et al., 1980).

The CLP model also incorporates elements which are unaccounted for by earlier models. These elements include trauma, the presence of peritonitis, a necrotic source, bacteremia induced by pathogens from a host-derived flora, and active translocation of live bacteria into the bloodstream (Dejager et al., 2011). The inclusion of these elements as part of the CLP model improves the clinical relevance of outcomes from these preclinical studies.

As observed in clinical sepsis, the initial hyperinflammatory state characterized by a systemic inflammatory response syndrome (SIRS) transitions to a subsequent immunosuppressed state characterized by the compensatory acute response (CARS) (Bone, 1996; Dejager et al., 2011). However, the point at which this transition occurs is not well characterized. The early hyperdynamic stage of sepsis and the later hypodynamic state following the CLP procedure is indicated by changes in response to immune challenge and changes in peripheral blood cells and cytokines (Bone, 1996; Dejager et al., 2011; Dellinger et al., 2008; Wichterman et al., 1980). While the times at which the following changes occur are not the same across CLP studies, overall total white blood

cells, polymorphonuclear leukocytes, and lymphocytes increase rapidly within the first 2 hours, decrease gradually, and plateau until endpoint (Remick, Newcomb, Bolgos, & Call, 2000). The pro-inflammatory response is marked by significant increases in cytokines TNFα and IL-6 and chemokines KC, MIP-2, and MCP-1 and remain elevated for 8-12 hours following surgery (Dejager et al., 2011; Remick et al., 2000). This pro-inflammatory response drives early sepsis progression. Blocking IL-6 (Riedemann et al., 2004; Riedemann, Guo, & Ward, 2003), complement factor C5a or C5a receptor (Czermak et al., 1999; M. S. Huber-Lang et al., 2002; M. Huber-Lang et al., 2001), and depleting neutrophils infer protection and reduce mortality in animals subjected to CLP. The anti-inflammatory response (involving IL-10 and glucocorticoids) are crucial for dampening the pro-inflammatory response (Hotchkiss, Coopersmith, McDunn, & Ferguson, 2009; Hotchkiss & Nicholson, 2006; Muenzer et al., 2010). The hypo-inflammatory phase is characterized by neutrophil paralysis, apoptosis of lymphocytes and dendritic cells, and elevations in anti-inflammatory mediators. The hypoinflammatory phase significantly increases the susceptibility of the septic host to nosocomial infection. Mortality is largely observed in this hypoinflammatory, immunosuppressed state in clinical sepsis (Hotchkiss et al., 2009), however it is unknown if most septic animals die in this state.

1.6.2 Colon ascendens stent peritonitis (CASP)

While sepsis progression in the CLP model is largely driven by the pro-inflammatory response mediated by cytokines, a sepsis model driven by TLR-signalling has more recently been proposed. In the colon ascendens stent peritonitis or CASP model,

the ascending colon is pierced with a stent or cannula which is then inserted and sutured into the colon (Zantl et al., 1998). Fecal contents are expressed through the stent providing a source of enteric microorganisms to translocate from the intestinal lumen to the peritoneum (Zantl et al., 1998). The mortality rate following CASP ranges from 50% to 100% at 48 hours (Traeger et al., 2010; Zantl et al., 1998) depending on the size of the cannulus used (20 G to 14 G) (Buras, Holzmann, & Sitkovsky, 2005; Zantl et al., 1998). One element unique to the CASP model is that it can be used to investigate the host response following surgical interventions where the infectious source is removed by removing the cannulus at a time-point following the CASP procedure (Buras et al., 2005). In this respect, the CASP model may have greater utility for studying a clinically relevant host response following interventions. Given the relatively short duration of time over which the CASP model was proposed in comparison to the CLP model, the number of studies utilizing CLP-induced sepsis far exceeds those using the CASP model (Schabbauer, 2012).

1.6.3 Limitations of animal models of sepsis

Many cardiovascular, respiratory, metabolic, and immune changes in clinical sepsis cannot be sufficiently reproduced by a single injection of endotoxin or bacteria in murine models (Dejager et al., 2011; J. C. Marshall et al., 2005). Injection of endotoxins like LPS, a component of the cell wall of gram negative bacteria, fail to mimic the host response to the many causative agents in clinical sepsis (Deitch, 2005; J. C. Marshall et al., 2005). Endotoxemia models also do not include a source of necrosis or continuous dissemination of microorganisms endogenous to the host. LPS models are largely driven

through TLR4 signalling and a rapid but transient cytokine storm which is much higher than what is observed in human sepsis (Deitch, 2005; Dejager et al., 2011; Remick et al., 2000). Murine animals also exhibit a much higher tolerance to endotoxin than humans (Remick et al., 2000; Wichterman et al., 1980), thus the levels of endotoxins used in animal models are not reflective of human sepsis. LPS models reflect endotoxemic shock and fail to reflect the complex physiological response in clinical sepsis.

Surgical models of sepsis more closely reflect human sepsis in that they include a source of necrosis and an endogenous source of infection, however there are limitations to both CLP and CASP sepsis models. Inter-animal variability can be high if researchers are inexperienced (Dyson & Singer, 2009; Huet et al., 2013). Differences in the length of cecum ligated, amount of fecal matter expressed, the size of the colon or cecal punctures can drastically influence the disease severity between animals (Maier et al., 2004; Schabbauer, 2012). Differences in the amount of surgical manipulation by researchers can also introduce variability in the results (Maier et al., 2004). Inter-study variability is another limitation of surgical sepsis models as various investigators use different CLP or CASP models. The size of the needle, number of punctures, diameter of catheter, and location of stenting and suturing affect disease severity and should be considered when comparing animal sepsis studies from different research groups (Dyson & Singer, 2009; Henderson, Kimmelman, Fergusson, Grimshaw, & Hackam, 2013; J. C. Marshall et al., 2005). To improve the consistency and reproducibility of *in vivo* sepsis studies, standard protocols can be enforced (Dejager et al., 2011; Maier et al., 2004; J. C. Marshall et al., 2005).

1.6.4 Clinical relevance of animal studies in sepsis

Several factors limit the clinical relevance of animal sepsis studies. Young mice around 8 weeks of age which are physiologically equivalent to young adulthood in humans are commonly used in animal studies, despite the septic human population being largely comprised of patients over 65 years of age (Martin, Mannino, Eaton, & Moss, 2003). Age should be considered since a difference in absolute risk reduction of mortality is associated with age in clinical sepsis (Bernard et al., 2001; Drechsler et al., 2012; Gaieski, Edwards, Kallan, & Carr, 2013). Male animals are also almost exclusively used although 40% of the septic human population is female (Wichmann, Inthorn, Andress, & Schildberg, 2000). Male animals are usually chosen to avoid confounders associated with different phases of the estrus cycle which affect the coagulant response, expression of adhesion molecules, and neutrophil levels (Hrekova, Vodianyk, & Chernyshov, 2002; Northern, Rutter, & Peterson, 1994). Adequate fluid resuscitation should also be considered to differentiate pathology due to sepsis from that due to circulatory decline (Rivers et al., 2001) as a result of hypovolemia and lack of hemodynamic support (Natanson et al., 1990; Ondiveeran & Fox-Robichaud, 2004). This is supported by findings demonstrating that the hemodynamic profiles of under-resuscitated animals differ vastly from those of animals given adequate fluid resuscitation and an aggressive fluid regimen was required to replicate the hemodynamic profiles observed in severe sepsis patients (Buras et al., 2005; Natanson et al., 1990). Lastly, the clinical sepsis population is diverse and often presents with various comorbidities such as diabetes, HIV, or renal disease (Melamed & Sorvillo, 2009), which are rarely modelled or considered in

animal sepsis studies. In assessing the clinical relevance of animal sepsis studies, one must consider the relevance of the age and biological sex of the animals used, fluid resuscitation protocols, and the biases in using a relatively homogenous population of animals housed in sterile environments to model sepsis.

1.6.5 Improving the design of animal studies in sepsis

To improve the translational of preclinical studies to the clinical setting, several factors should be considered when designing preclinical sepsis studies. Randomization of animals is an effective method to minimize allocation or selection bias by eliminating known or unknown prognostic variables which may influence study outcomes (Chalmers, Celano, Sacks, & Smith, 1983; Lamontagne et al., 2010; Piper, Cook, Bone, & Sibbald, 1996). Animals should be randomized to sham and CLP or treatment and control groups to ensure that protocols and procedures are conducted in a consistent and systematic manner, regardless of the surgery performed or drug administered.

Experimenter blinding will also minimize the risk of bias in preclinical studies (Bebarta, Luyten, & Heard, 2003). Active steps should be taken where possible to assign subjects in a treatment or surgery group a non-descriptive identifier (numerical or alphabetical).Blinding can occur at many stages of the study including before the procedure (to prevent bias in sepsis induction), during treatment (so that the timing, route, and method of administering potential therapies are consistent), during endpoint monitoring (so that animals will not be euthanized prematurely if given a control versus therapy), and at any point at which the researching may be processing samples taken from animals subjected to either sham or sepsis surgery. Blinding minimizes differential

treatment of animals in study groups (e.g. closer monitoring of one treatment group, prolonging or delaying time to endpoint, etc.) and minimizes unintentional bias in adjudicating subjective outcomes (e.g. histology scores for organ damage, etc.) (Chalmers et al., 1983; Kilkenny, Browne, Cuthill, Emerson, & Altman, 2013; Miller, Colditz, & Mosteller, 1989).

Anaesthetics and analgesics at effective doses as recommended by animal ethics guidelines should be used in animal sepsis studies. While some limit their use over concerns that these drugs will influence or interfere with disease progression, these concerns highlight the importance of considering the effects of anaesthetics and analgesics which are used clinically and incorporating those effects in animal sepsis studies.

Animal sepsis studies should be designed to adequately address scientific questions but also minimize animal suffering whenever possible. Surrogate endpoints, or markers of death should be used in place of death as an endpoint. In clinical sepsis, patients at risk of death may be identified with Early Warning Scores (EWS) (Corfield et al., 2014; Reini, Fredrikson, & Oscarsson, 2012) which document changes in the respiratory rate, heart rate, systolic blood pressure, temperature, and level of consciousness of the patient (Stenhouse, Coates, Tivey, Allsop, & Parker, 2000; Subbe, Kruger, Rutherford, & Gemmel, 2001). This physiological scoring tool facilitates early recognition of a deteriorating patient many of whom may be septic. Similarly, clinically relevant surrogate markers such as temperature, heart rate, respiratory rate, and level of consciousness or activity should be established for animal sepsis studies.

Chapter 2: Hypothesis and aims

Cell-free DNA (cfDNA) levels are elevated in septic patients and levels of cfDNA upon admission into the intensive care unit have strong prognostic value in predicting outcome in severe sepsis patients. It is unclear whether increases in cfDNA are an epiphenomenon or if cfDNA actively contributes to sepsis pathology. Cell-based and animal models of inflammation and pathologic coagulation suggest that cfDNA, potentially released from neutrophils, drives inflammation and clotting and that treatment with DNase may modulate the pro-inflammatory and procoagulant response. Some murine studies suggest that the source of this cfDNA is from neutrophil extracellular trap (NET) formation and that NETs may contribute to sepsis pathology.

We hypothesize that cfDNA is pathogenic in sepsis and DNase administration will improve outcome in a murine model of sepsis. The aims of these studies are to:

Aim 1: Characterize the role of cfDNA in inflammation, coagulation, and outcome in a cecal ligation and puncture (CLP) model of sepsis.

Aim 2: Elucidate the effects of DNase treatment on inflammation, coagulation, and outcome in a CLP model of sepsis.

Aim 3: Determine the contribution of NETs to the inflammatory and coagulant response in CLP model of sepsis.

Aim 4: Evaluate surrogate markers of death and develop a scoring system to assess disease severity and predict outcome in a murine model of sepsis.

Chapter 3: Delayed but not early treatment with DNase reduces organ damage and improves outcome in a murine model of sepsis

Foreword

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3.1 Abstract

Rationale: Sepsis is characterized by systemic activation of coagulation and inflammation in response to microbial infection. Although cell-free DNA (cfDNA) released from activated neutrophils has antimicrobial properties, it may also exert harmful effects by activating coagulation and inflammation. **Objectives**: To determine whether DNase administration reduces cfDNA levels, attenuates coagulation and inflammation, suppresses organ damage, and improves outcome in a cecal ligation and puncture (CLP) model of polymicrobial sepsis. **Methods**: Healthy C57BL/6 mice were subjected to CLP, a surgical procedure involving two punctures of the ligated cecum, or sham surgery (no ligation/puncture). Mice were given DNase or saline by intraperitoneal injection 2, 4, or 6 hours post-surgery. 2 hours after treatment, organs were harvested and plasma levels of cfDNA, IL-6, IL-10, thrombin-antithrombin (TAT) complexes, lung myeloperoxidase (MPO), creatinine, alanine transaminase (ALT), and bacterial load were quantified. Survival studies were also performed. **Results**: CLP-operated mice had rapid, time-dependent elevations in cfDNA which correlated with elevations in IL-6, IL-10, and TAT complexes, and with organ damage in the lungs and kidneys. Administration of DNase at 2 hours post-CLP resulted in increased IL-6 and IL-10 levels and organ damage in the lungs and kidneys. In contrast, DNase administration at 4 or 6 hours post-CLP resulted in reduced cfDNA and IL-6 levels, increased IL-10, and suppressed organ damage, and bacterial dissemination. DNase administration every 6 hours post-CLP also rescued mice from death. **Conclusions**: Our studies are the first to demonstrate that delayed but not early administration of DNase may be protective in experimental sepsis.

3.2 Introduction

Sepsis is characterized by systemic activation of inflammatory and coagulation pathways in response to microbial infection (Dellinger et al., 2013). Severe sepsis, defined as sepsis associated with organ dysfunction, afflicts approximately 3 per 1000 population in the United States annually (Angus et al., 2001) and is associated with mortality rates of 33% to 45% (Dellinger et al., 2013). The incidence of severe sepsis continues to increase by 1.5% per annum due to the aging population, increased prevalence of obesity and diabetes, and wider use of immunosuppressive agents and invasive procedures (H. E. Wang et al., 2012).

Over the past several decades, many potential treatments for sepsis have shown early promise, yet have failed to improve survival in more than 100 Phase II and Phase III clinical trials (J. C. Marshall, 2014). These trials attempted to treat sepsis by dampening inflammation or coagulation. Current strategies for sepsis are largely supportive and include fluid resuscitation, mechanical ventilation, and early administration of broad-spectrum antibiotics (Dellinger et al., 2013). Despite these strategies, the mortality rate from sepsis remains high, suggesting that some fundamental knowledge is lacking in our understanding of sepsis pathophysiology (Angus et al., 2001; Dellinger et al., 2013; J. C. Marshall, 2014).

In recent years, cell-free DNA (cfDNA) has emerged as an important link between innate immunity and coagulation. When activated by microbes, neutrophils release web-like structures termed neutrophil extracellular traps (NETs), which are comprised of cfDNA, histones, proteases, and granular proteins (Brinkmann et al., 2004;

Papayannopoulos et al., 2010). By binding to microorganisms, NETs prevent dissemination of pathogens and ensure a high local concentration of antimicrobial agents. However, the presence of NETs in the microcirculation results in increased coagulation and platelet activation (Ammollo et al., 2011; Fuchs et al., 2010), microvascular thrombosis (Ammollo et al., 2011; Semeraro et al., 2011), and organ damage (Meng, Paunel-Görgülü, Flohé, Hoffmann, et al., 2012). In sepsis, high levels of nucleosomes (DNA bound to histones) are found in blood (Zeerleder et al., 2003) and we have previously reported that elevated levels of circulating cfDNA predict poor clinical outcome in severe sepsis patients (Dwivedi et al., 2012). We have recently reported that neutrophils are the major source of cfDNA released from whole blood upon inflammatory stimulation (Gould et al., 2014).

NETs are counter-regulated by deoxyribonuclease I (DNase I) (Meng, Paunel-Görgülü, Flohé, Witte, et al., 2012), a calcium- and magnesium-dependent endonuclease which hydrolyzes double-stranded DNA (Prince et al., 1998) and degrades chromatin released during necrosis to prevent anti-DNA autoimmunity (Brinkmann et al., 2004). DNaseI has been used to treat patients with various conditions associated with increased cfDNA levels including cystic fibrosis (Shak, 1995) and pleural infection (Rahman et al., 2011).

In experimental sepsis, the protective effects of DNase remain unclear. Meng *et al.* reported an exacerbated inflammatory response and increased bacterial load in septic mice given repeated injections of DNase (Meng, Paunel-Görgülü, Flohé, Hoffmann, et al., 2012). In contrast, Luo *et al.* observed that repeated administration of DNase in septic

mice reduced lung edema and tissue damage (Luo et al., 2014) . We hypothesize that these divergent results may reflect the timing of DNase administration, a crucial element in managing sepsis. To examine this possibility, we compared the effect of early and late DNase administration on levels of cfDNA, markers of coagulation and inflammation, and organ histopathology in mice subjected to a cecal ligation and puncture (CLP) model of polymicrobial sepsis.

3.3 Materials and methods

3.3.1 Experimental sepsis-cecal ligation and puncture

8 week-old (20 to 25 g) male C57Bl/6 mice (*Helicobacter hepaticus*-free) were purchased from Charles River Laboratories (Sherbrooke, Quebec, Canada) and bred at the Thrombosis and Atherosclerosis Research Institute (Hamilton, ON, Canada). The cecal ligation and puncture (CLP) model used in these studies were adapted from well-established protocols by other groups (Baker, Chaudry, Gaines, & Baue, 1983; Hubbard et al., 2005). Under anesthesia, a midline laparotomy was performed and after it was exteriorized, the cecum was ligated distal to the ileocecal valve, 1 cm from the cecal end and perforated through-and-through with an 18G needle. In mice subjected to sham surgery, the cecum was exteriorized and returned to the peritoneal cavity (n = 4-12 per time-point per group). Mice were randomized in the CLP or sham groups according to ARRIVE guidelines (Kilkenny et al., 2013) and methods for randomization and experimenter blinding were in place to reduce allocation, selection, and experimenter biases (Mai, Khan, Liaw, & Fox-Robichaud, 2012). Mice were randomized to both groups on each surgery date. CLP and sham mice were given analgesia (Buprenorphine,

0.1 mg/kg) for pain relief and resuscitation fluids (2 mL, Ringer's lactate) subcutaneously every 4 hours post-operatively to account for surgical losses. DNase (Pulmozyme, Roche Genentech, 20 mg/kg) or saline was administered by intraperitoneal (i.p.) injection at 2, 4, or 6 hours post-operatively (n = 5-18 per time-point per group). 2 hours after DNase or saline administration and blood was collected via the inferior vena cava (IVC) into a one-tenth volume of 3.2% sodium citrate, spun at 2000 x g and plasma was stored at -80ºC.

3.3.2 Isolation and quantification of cell-free DNA

Cell-free DNA (cfDNA) was isolated from 200 µL of mouse plasma using the Qiagen QIAamp® DNA Blood Mini Kit (QIAgen, Valencia, CA, USA) and quantified by measuring absorbance at 260 nm using an Eppendorf BioPhotometer Plus (Hamburg, Germany).

3.3.3 Quantification of interleukin (IL)-6, IL-10, and thrombin-antithrombin (TAT) complexes

IL-6 and IL-10 were quantified using ELISA-based Quantikine® Mouse IL-6 and IL-10 Immunoassay kits (R&D Systems, Minneapolis, MN, USA), whereas TAT complex levels were quantified using the Enzygnost® TAT Micro immunoassay (Siemens Healthcare Diagnostics Products, Marburg, Germany) according to the manufacturer's protocol.

3.3.4 Determination of myeloperoxidase (MPO) activity, alanine aminotransferase (ALT) activity, and creatinine levels

Enzymatic MPO is found in azurophilic granules of polymorphonuclear cells and is indicative of neutrophil accumulation in inflamed tissues. Mice were subjected to sham

or CLP surgery and administered either DNase or saline 6 hours post-surgery. Lung tissues were frozen in liquid nitrogen and stored at -80ºC, and MPO enzymatic activity in the lungs was quantified. 0.050 g of lung tissue was homogenized in ice-cold PBS and centrifuged at 10 000 x g for 10 minutes in 4ºC. The supernatant was discarded and the pellet was resuspended and homogenized in potassium phosphate buffer (K_2HPO_4, 50 mM, pH 6.0) containing hexadecyltrimethylammonium bromide (HTAB, 0.5% w/v). Following centrifugation at 3000 x g for 15 minutes, the supernatant was collected and MPO enzymatic activity was quantified by measuring the H_2O_2-dependent oxidation of *o*-dianisidine. One unit (U) of MPO activity is defined as MPO levels per gram of lung tissue which caused a change in absorbance of 1.0/minute at 450 nm. Plasma levels of creatinine and ALT activity were quantified using commercially available assays (Abcam, Cambridge, MA, USA). Creatinine levels were expressed in mmol/L and ALT activity was expressed in units/L in plasma. One unit (U) of ALT activity was defined as the amount of ALT which generates 1.0 µmol of pyruvate per minute at 37ºC.

3.3.5 Bacterial load, organ histology, and staining procedures

In bacterial culture experiments, lungs were homogenized and peritoneal cavity fluid (PCF) was collected. Fresh blood, lung lysates, and PCF diluted in phosphate-buffered saline (Sigma-Aldrich, St. Louis, MO, USA) were plated on 5% sheep blood agar plates (Teknova, Hollister, CA, USA), and incubated for 48 hours. In all other experiments, mice were perfused with saline and 10% neutral buffered formalin and organs were collected in formalin, embedded in paraffin wax, processed, and sectioned at 5 microns thick. Organ sections were stained with hematoxylin and eosin (H&E) to

visualize overall morphology (nuclei and extracellular DNA appear bluish-purple and cytoplasm, connective tissue, and collagen appear pink). Other sections were stained with phosphotungstic-acid and hematoxylin (PTAH, American MasterTech Scientific, Lodi, CA, USA) to identify fibrin and collagen (RBCs, fibrin, nuclei. and striated fibers appear blue and collagen appear brownish-red). Photomicrographs of stained lung and kidney sections were visualized under 400x magnification. The length of the scale bar represents 25μm. Organ damage was assessed by clinical pathologists blinded to the surgery and treatment allocation. Organ sections were scored by evaluating the level of inflammatory cell infiltration, interstitial edema, and vascular congestion (from 0 representing healthy, absence of abnormal organ pathology up to 3 for severe organ damage indicated by significant inflammatory infiltration, distinct regions of vascular congestion, and cell necrosis).

3.3.6 Mortality studies

Mice were subjected to either sham or CLP surgery and monitored continuously until endpoint or 20 hours post-surgery. A separate cohort of mice were subjected to CLP surgery and given saline control, early DNase treatment (2 hours post-CLP, repeated injections every 2 hours), or late DNase treatment (6 hours post-CLP, repeated injections every 6 hours) and monitored continuously until endpoint of 20 hours post-surgery. All mice were given Ringer's lactate and Buprenorphine (0.1mg/kg) subcutaneously every 4 hours post-operatively.

3.3.7 Statistical analyses

Data are represented as mean ± SEM and results across multiple groups were compared using one-way analysis of variance (ANOVA) and Newman-Keuls post-hoc test. Data were considered significant at $p < 0.05$. Analyses were performed using GraphPad Prism 4.0 (La Jolla, CA, USA) and SigmaPlot 11.0 (San Jose, CA, USA).

3.4 Results

3.4.1 Increases in cfDNA accompany the early pro-inflammatory and procoagulant response in sepsis

To examine the temporal changes in cfDNA in a murine model of sepsis, mice were subjected to CLP or sham surgery and blood was collected 2, 4, 6, or 8 hrs post-operatively. A significant time-dependent increase in cfDNA levels in septic mice from 4 to 8 hours post-CLP surgery was observed (Figure 3.1A). In septic mice, the increases in cfDNA levels were accompanied by significant ($p < 0.001$) time-dependent increase in IL-6 (Figure 3.1B), decrease in IL-10 (Figure 3.1C), and increases in TAT (Figure 3.1D). In contrast, levels of cfDNA, IL-6, IL-10, and TAT complexes remained low in sham-operated mice.

Histological staining was performed in the organs of septic mice harvested 6 hours following CLP surgery (Figure 3.2). Photomicrographs of H&E stained lung sections revealed edema in the interstitium and alveolar border, and neutrophil infiltration. PTAH staining showed intra-alveolar red blood cell (RBC) congestion and thickening of the alveolar septum in the lungs of septic mice. These pathological characteristics were absent in healthy and sham-operated mice. H&E-stained kidney sections exhibited intense staining of tubular brush borders in sham-operated and healthy mice which was lost in the

kidney sections from septic mice. Tubular dilation and cell sloughing were also observed in the kidneys of septic mice, indicative of tubular necrosis and kidney injury. PTAH stains of kidneys of septic mice confirmed intra-glomerular and inter-tubular RBC trapping as well as RBC congestion in the glomeruli (Figure 3.2).

3.4.2 Early administration of DNase 2 hours post-CLP surgery results in increased inflammation and organ damage

In mice subjected to CLP, administration of DNase two hours following surgery resulted in a decrease in plasma levels of cfDNA (Figure 3.3A), an increase in IL-6 (Figure 3.3B) and IL-10 (Figure 3.3C), and no significant changes in TAT levels (Figure 3.3D) compared with CLP-operated mice given saline control. DNase increased infiltration of inflammatory cells trapped within thickened alveolar borders in the lungs (Figure 3.4). Kidneys from septic mice treated with DNase two hours post-operatively showed increased inter-tubular and intra-glomerular RBC congestion, dismantling of tubular structure, vacuolization of tubule centers, and inflammatory infiltration at the periphery of kidney tubules compared with septic mice given saline (Figure 3.5). Organ histology scores for lungs and kidneys of septic mice given DNase 2 hours post-CLP were higher compared to septic mice given saline (Figure 3.6). There were no significant differences in bacterial load in the whole blood, lungs, or peritoneal cavities with DNase or saline administration (Figure 3.7). These findings suggest that early administration of DNase is associated with increased inflammation, RBC congestion, and exacerbated organ pathology compared with septic mice given saline.

Figure 3.1. Time-course of plasma cell-free DNA, IL-6, IL-10, and TAT complexes in sham- and CLP-operated mice. Mice were subjected to CLP or sham surgery. Blood was collected at 2 hour increments following surgery. Plasma levels of cell-free DNA (A), IL-6 (B), IL-10 (C), and TAT complexes (D) were quantified and compared to levels observed in healthy control mice (ANOVA $p < 0.001$, * $p < 0.05$, ** $p < 0.01$, *** $p < 0.005$, n = 4-8 sham, n = 8-12 CLP).

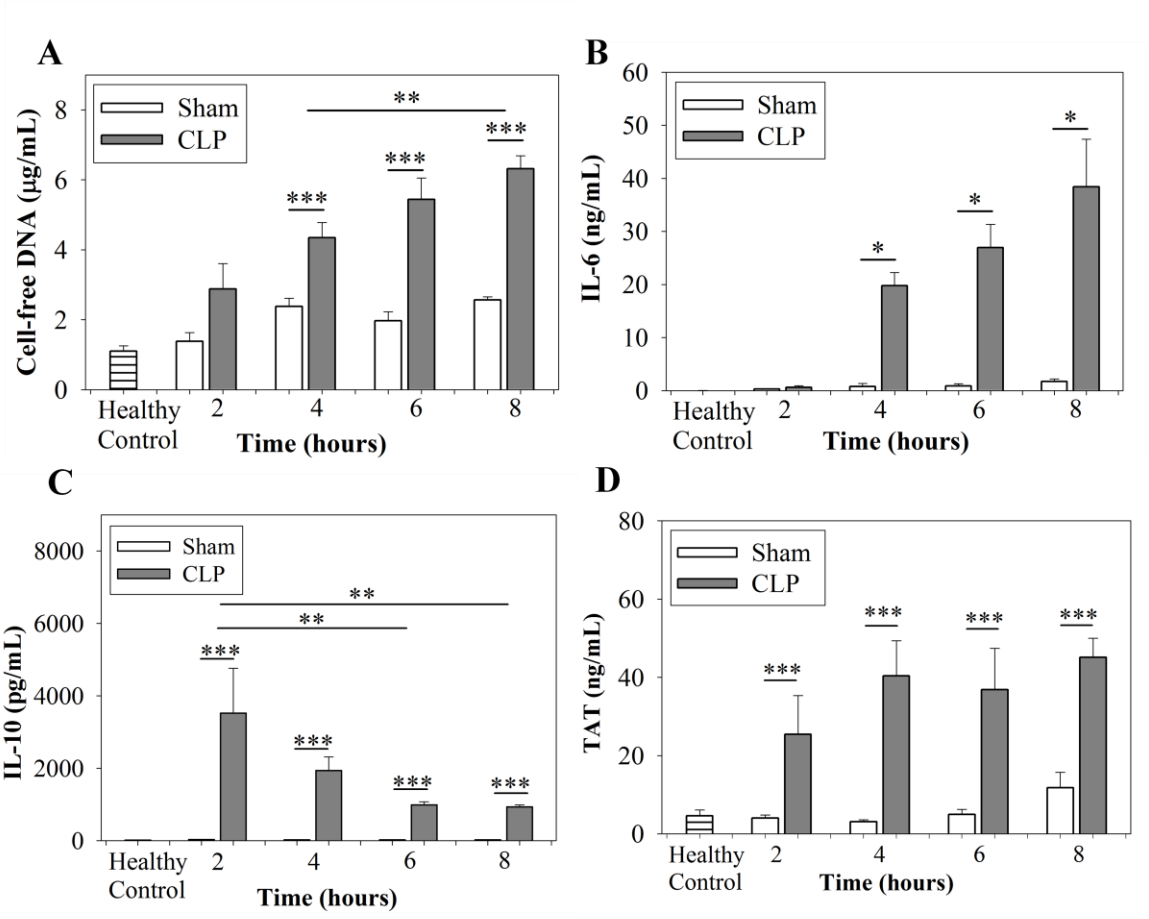

A
Cell-free DNA (μg/mL)
Sham
CLP
Healthy Control
2
4
6
8
Time (hours)
B
IL-6 (ng/mL)
C
IL-10 (pg/mL)
D
TAT (ng/mL)

Figure 3.2. Histological photomicrographs of H&E- and PTAH-stained lungs and kidneys from healthy, sham-operated, and CLP-operated mice. Mice were subjected to sham or CLP surgery and lung and kidney sections were compared to those from healthy control mice. 6 hours post-operatively, mice were perfused with heparinized saline and buffered formalin and lungs and kidneys were harvested. Organs were harvested in formalin and embedded in paraffin wax. Lungs and kidneys were sectioned 5 μm thick and stained with hematoxylin and eosin for overall morphology and phosphotungstic acid and hematoxylin for fibrin. Photomicrographs of stained lung and kidney sections were visualized under 400x magnification. The length of the scale bar represents 25μm.

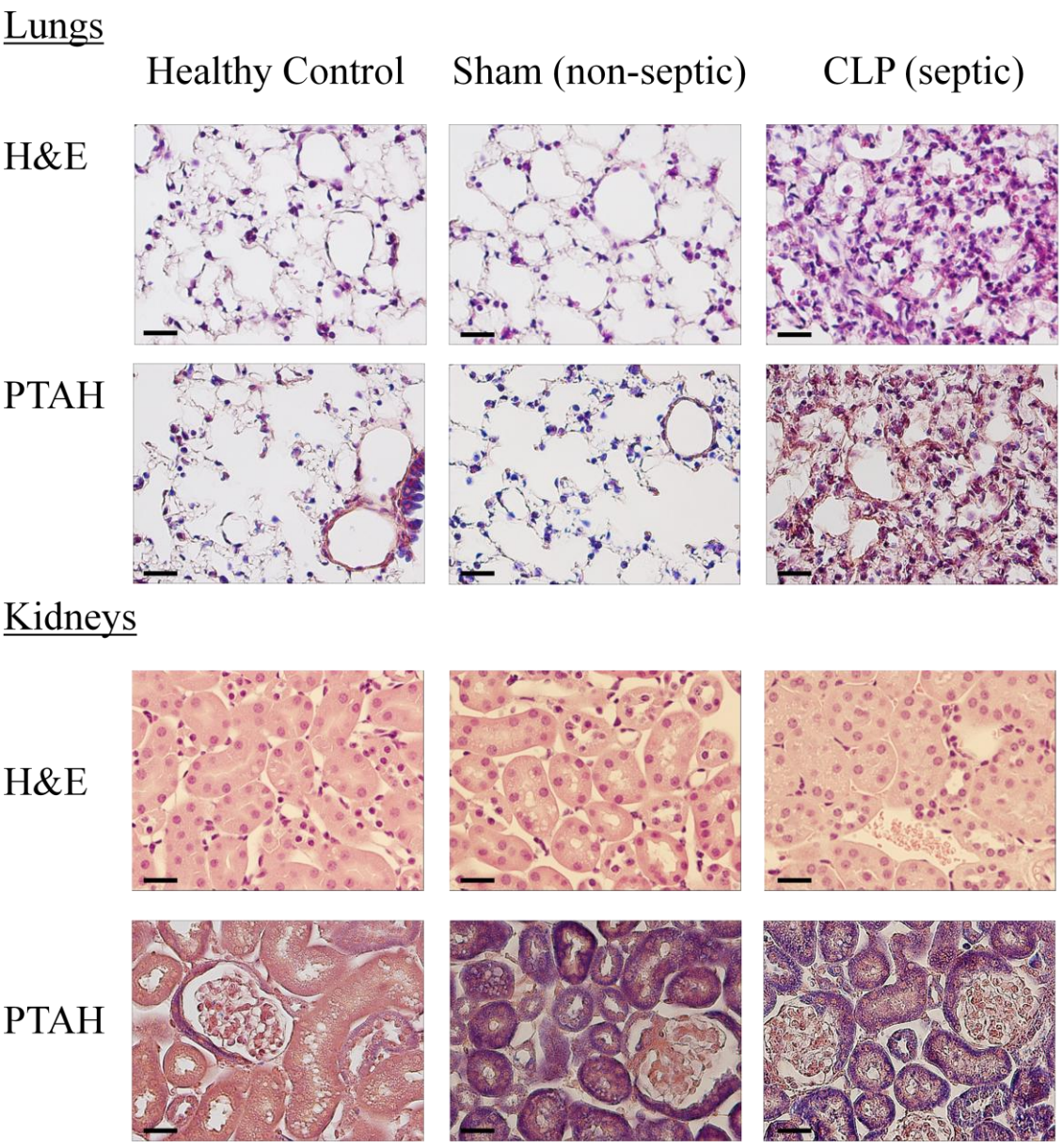
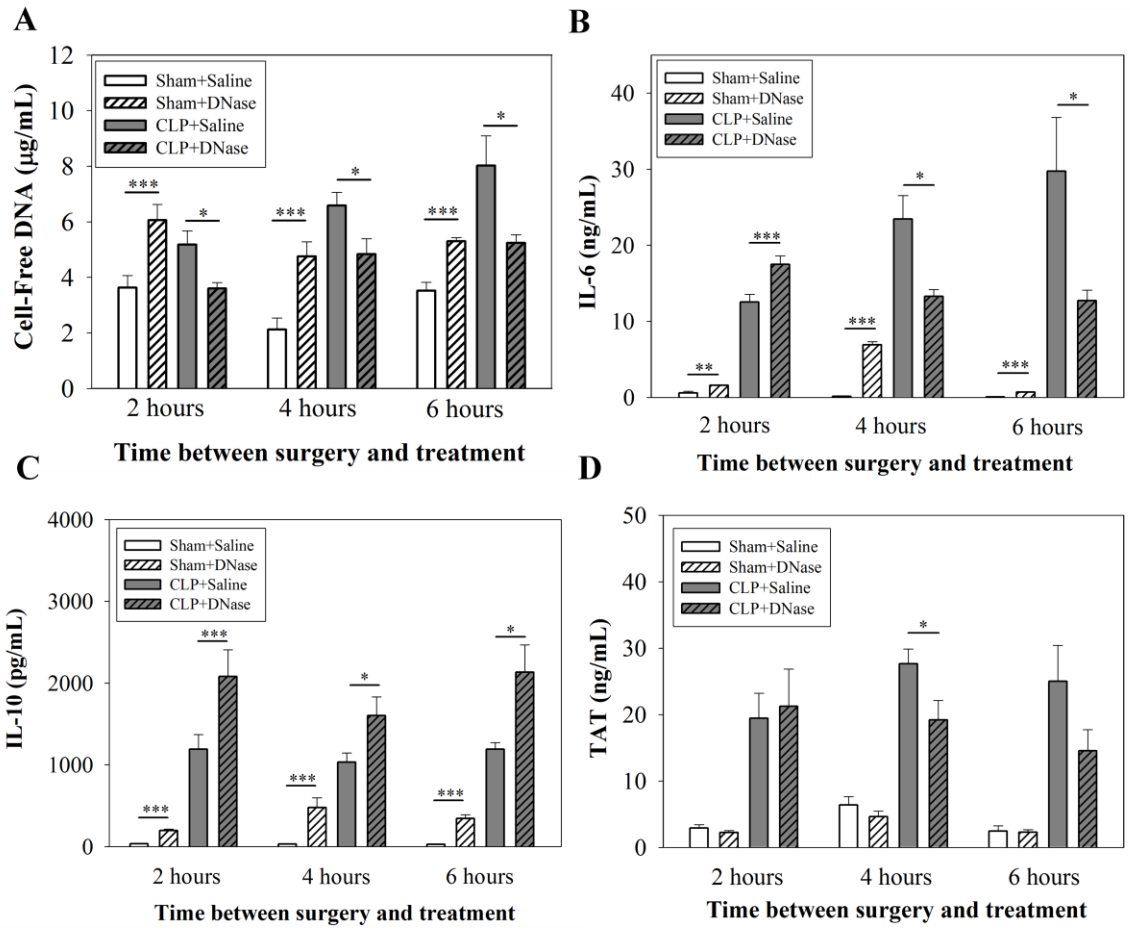

Lungs
Healthy Control
Sham (non-septic)
CLP (septic)
H&E
PTAH
Kidneys
H&E
PTAH

Figure 3.3. Plasma cell-free DNA, IL-6, IL-10, and TAT complexes in sham- and CLP-operated mice administered DNase 2, 4, and 6 hours post-operatively. Mice were subjected to sham or CLP surgery and administered either DNase or saline 2, 4, or 6 hours post-operatively. Blood was collected 2 hours following DNase or saline administration and levels of plasma cfDNA (A), IL-6 (B), IL-10 (C), and TAT complexes (D) were quantified (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.005$, n = 8-12 per sham group, n = 10-14 per CLP group).


A
Cell-Free DNA (μg/mL)
Sham+Saline
Sham+DNase
CLP+Saline
CLP+DNase
2 hours
4 hours
6 hours
Time between surgery and treatment
B
IL-6 (ng/mL)
Time between surgery and treatment
C
IL-10 (pg/mL)
Time between surgery and treatment
D
TAT (ng/mL)
Time between surgery and treatment

3.4.3 Delayed administration of DNase 4 or 6 hours post-CLP surgery reduces inflammation, organ damage, and bacterial dissemination

Administration of DNase four or six hours following CLP surgery resulted in decreases in cfDNA (Figure 3.3A) and IL-6 (Figure 3.3B), increases in IL-10 (Figure 3.3C), and modest decreases in TAT (Figure 3.3D). Changes in plasma biomarkers were accompanied by attenuated organ damage in the lungs and kidneys (Figures 3.4, 3.5). Lungs from septic mice treated with DNase showed decreased neutrophil infiltration, interstitial edema, inter-alveolar RBC congestion, fibrin deposition, and obstruction of pulmonary microvessels compared with septic mice given saline (Figure 3.4). Kidneys of DNase-treated septic mice showed modest improvements in organ damage with a general resolution of tubular structure demarcated by the staining of tubular brush borders and decreases in intra-glomerular and inter-tubular vascular congestion, nuclear dropoff, and tubular necrosis (Figure 3.5). Lung MPO activity in septic mice given delayed DNase treatment was reduced by 38% compared to MPO activity in the lungs of septic mice administered saline (Figure 3.6C). Creatinine levels were elevated in all septic mice versus non-septic mice however, no significant changes in creatinine levels between septic mice given saline versus DNase were observed (Figure 3.6D). ALT activity, a marker of liver injury was increased in septic mice given saline and significantly reduced in septic mice given DNase when DNase was administered at 4 or 6 hours post-CLP surgery (Figure 3.6E). Organ histology scores of lungs and kidneys were lower in septic mice given DNase versus saline when DNase was administered four or six hours post-CLP.

Figure 3.4. Histological photomicrographs of H&E- and PTAH-stained lungs from sham-operated and CLP-operated mice administered saline or DNase. Mice were subjected to sham or CLP surgery and administered saline or DNase at 2, 4 or 6 hours post-operatively. Lungs were harvested in formalin, embedded in paraffin wax, and sectioned 5 μm thick. Lung sections were stained with hematoxylin and eosin for overall morphology and phosphotungstic acid and hematoxylin for fibrin (stains blue). Photomicrographs of stained lung sections were visualized under 400x magnification. The length of the scale bar represents 25μm.

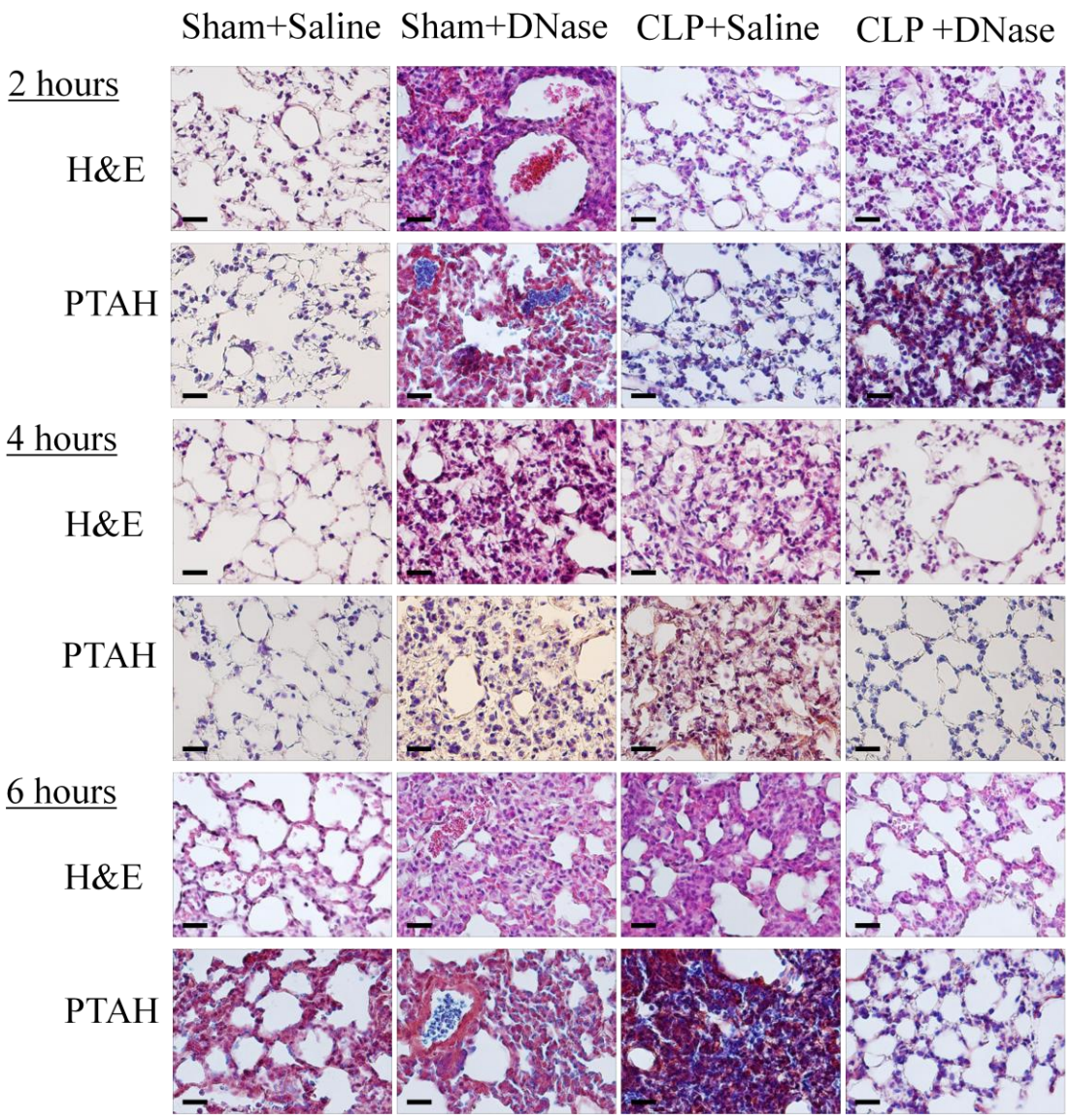

Sham+Saline
Sham+DNase
CLP+Saline
CLP +DNase
2 hours
H&E
PTAH
4 hours
H&E
PTAH
6 hours
H&E
PTAH

Figure 3.5. Histological photomicrographs of H&E- and PTAH-stained kidneys from sham-operated and CLP-operated mice administered saline or DNase. Mice were subjected to sham or CLP surgery and administered saline or DNase at 2, 4 or 6 hours post-operatively. Kidneys were harvested in formalin, embedded in paraffin wax, and sectioned 5 μm thick. Kidney sections were stained with hematoxylin and eosin for overall morphology and phosphotungstic acid and hematoxylin for fibrin (stains blue). Photomicrographs of stained kidney sections were visualized under 400x magnification. The length of the scale bar represents 25μm.

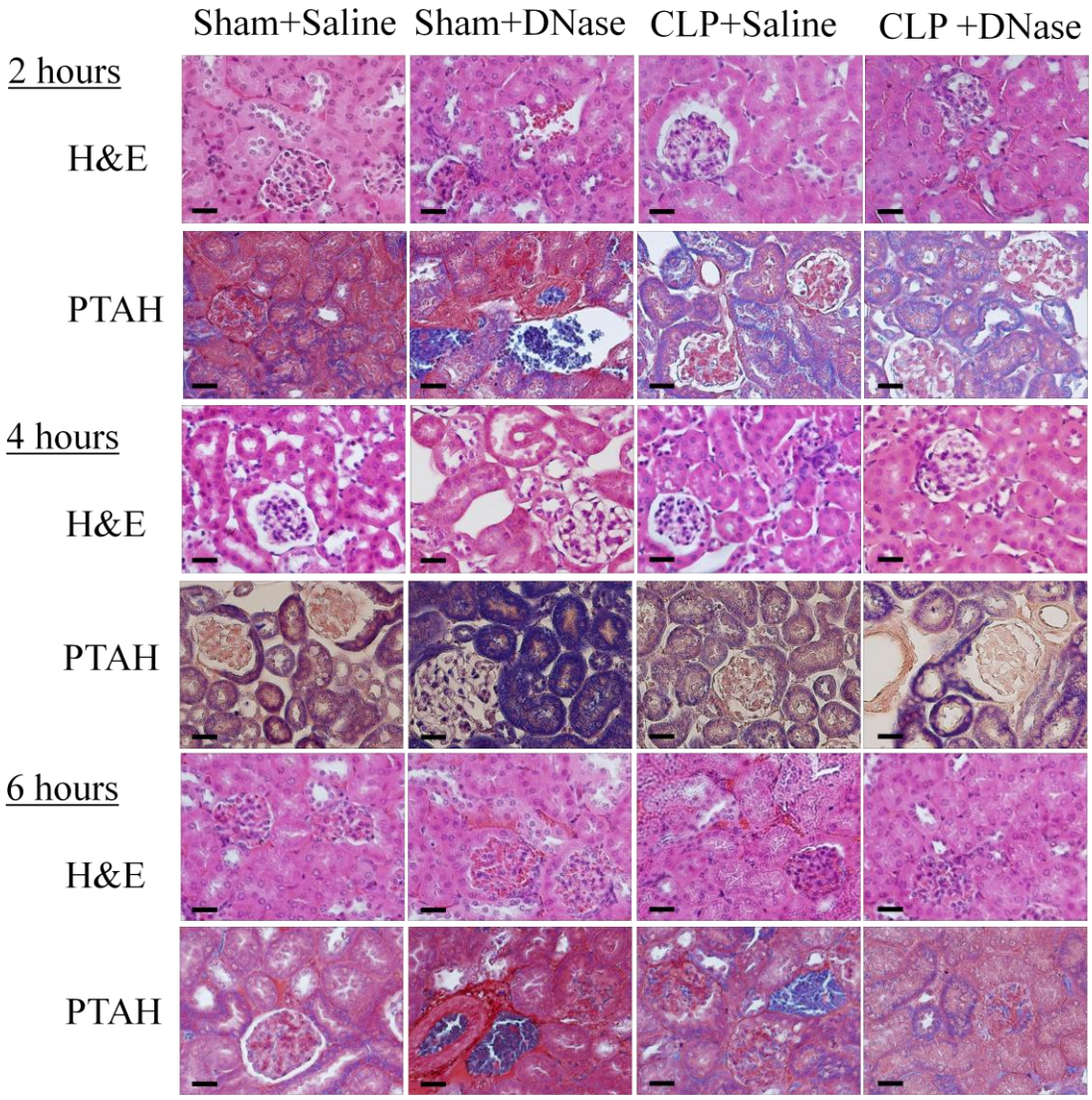
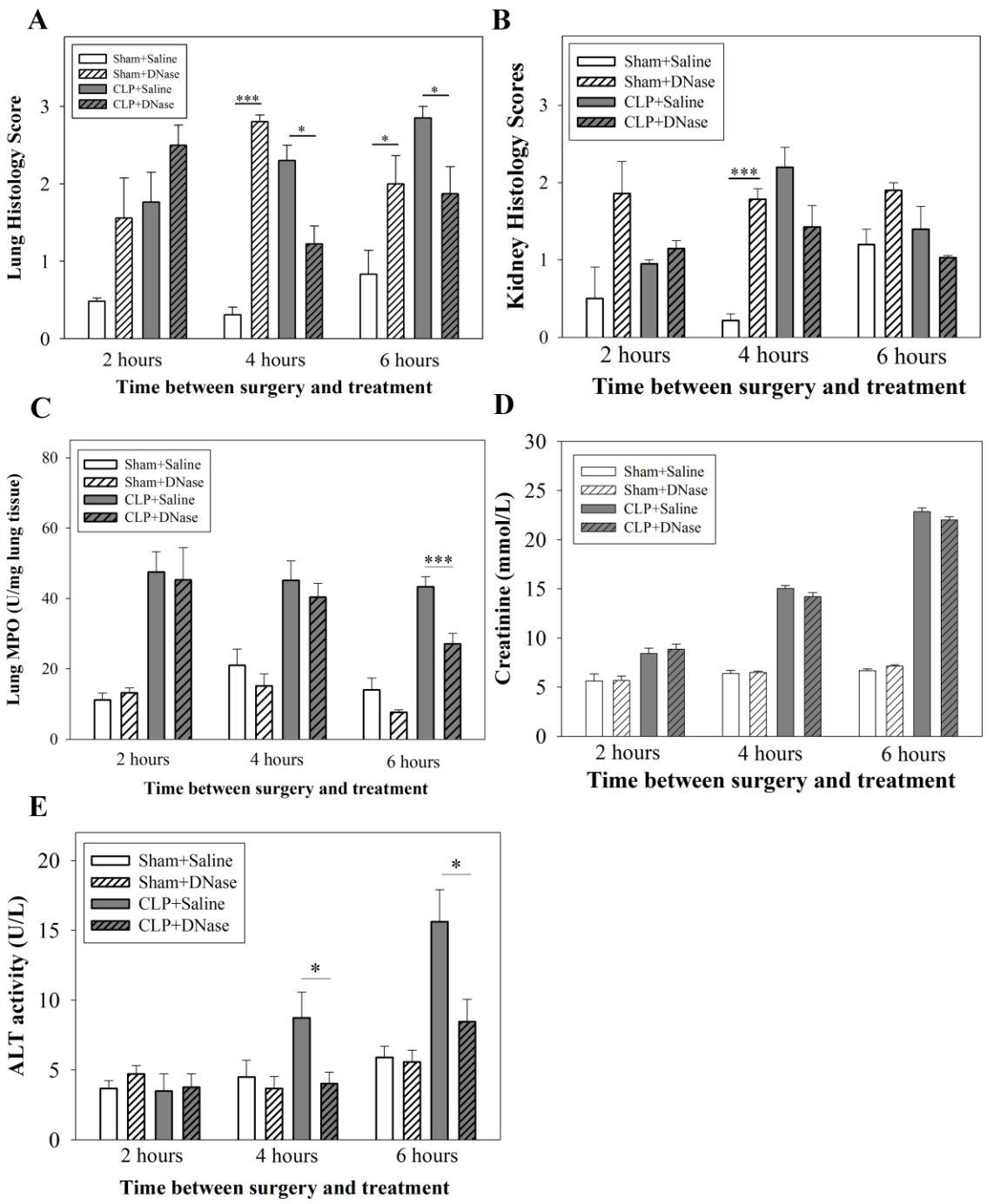

Sham+Saline
Sham+DNase
CLP+Saline
CLP +DNase
2 hours
H&E
PTAH
4 hours
H&E
PTAH
6 hours
H&E
PTAH

Figure 3.6. Histology scores, MPO activity, creatinine levels, and ALT activity of sham- and CLP-operated mice administered DNase or saline. Organs harvested from DNase- or saline-treated mice subjected to sham or CLP surgery were scored for organ damage by a blinded clinical pathologist. Lungs (A) and kidneys (B) were evaluated for inflammatory cell infiltration, interstitial edema, cell necrosis, and vascular congestion and given a score ranging from 0-3 inclusively (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.005$, n = 4-6 per sham group, n = 6-9 per CLP group). Organs from healthy control mice were given histology scores of 0. Mice were subjected to either CLP or sham surgery and given saline or DNase 6 hours post-surgery. Lungs were harvested and snap frozen in liquid nitrogen, and enzyme activity levels of myeloperoxidase (MPO) were quantified (C). Plasma levels of creatinine (D) and enzyme activity of alanine transaminase (ALT) (E), were determined (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.005$, n = 4-6 per sham group, n = 7-8 per CLP group).


A
Lung Histology Score
Sham+Saline
Sham+DNase
CLP+Saline
CLP+DNase
***
*
*
*
0
1
2
3
2 hours
4 hours
6 hours
Time between surgery and treatment
B
Kidney Histology Scores
Sham+Saline
Sham+DNase
CLP+Saline
CLP+DNase
***
0
1
2
3
2 hours
4 hours
6 hours
Time between surgery and treatment
C
Lung MPO (U/mg lung tissue)
Sham+Saline
Sham+DNase
CLP+Saline
CLP+DNase
***
0
20
40
60
80
2 hours
4 hours
6 hours
Time between surgery and treatment
D
Creatinine (mmol/L)
Sham+Saline
Sham+DNase
CLP+Saline
CLP+DNase
0
5
10
15
20
25
30
2 hours
4 hours
6 hours
Time between surgery and treatment
E
ALT activity (U/L)
Sham+Saline
Sham+DNase
CLP+Saline
CLP+DNase
*
*
0
5
10
15
20
2 hours
4 hours
6 hours
Time between surgery and treatment

A decreased bacterial load were also observed in the lungs (Figure 3.7A), blood (Figure 3.7B), and peritoneal cavity fluid (Figure 3.7C) of septic mice given delayed administration of DNase. Taken together, these data suggest that an increased window between sepsis induction (via CLP surgery) and DNase treatment is associated with decreased inflammation, improved organ health, and decreased bacterial dissemination.

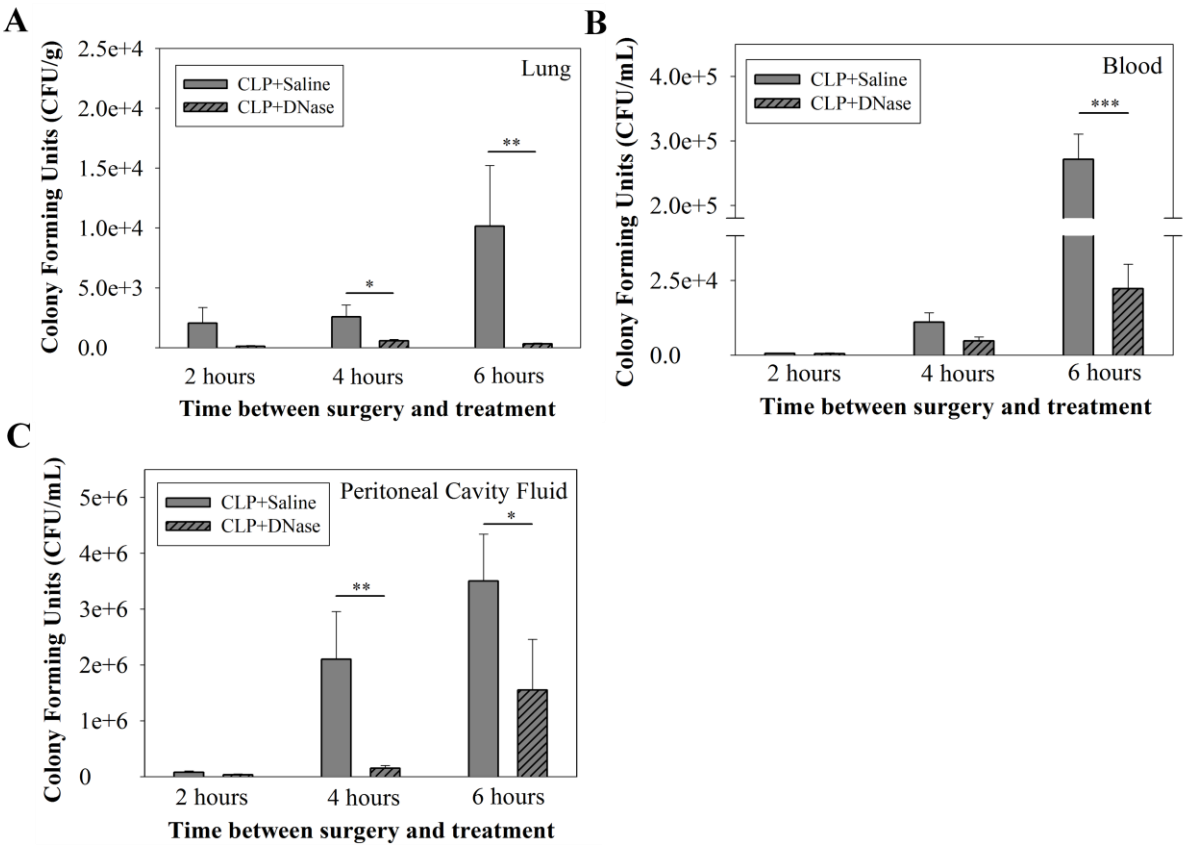
3.4.4 Administration of DNase in sham-operated mice exacerbates inflammation without inducing neutrophil accumulation

To determine the effects of DNase administration in the non-septic condition, DNase was administered to mice 2, 4, or 6 hours following sham surgery and blood was collected 2 hours following DNase injection. DNase administration in mice following sham surgery resulted in increased levels of cfDNA, IL-6 and IL-10 and no changes TAT complexes (Figure 2), MPO activity (Figure 3C), creatinine levels (Figure 3D), and ALT activity (Figure 3E). Changes in organ pathology in these mice include pulmonary edema and vascular congestion in the lungs (Figure 3.4) and microvascular congestion in the kidneys (Figure 3.5) accompanied by increased organ damage scores when DNase was administered 4 hours or 6 hours post-sham surgery (Figure 3.6A). These results suggest that administration of DNase to mice subjected to sham surgery results in increased inflammation in the lungs but no increase in neutrophil accumulation or organ injury.

3.4.5 Late DNase administration rescues septic mice from death

To determine whether DNase administration would improve outcome in our sepsis model, mice were subjected to CLP and given saline, early DNase treatment (every 2 hours post-CLP), or late DNase treatment (every 6 hours post-CLP).

Figure 3.7. Bacterial culture counts from lungs, blood, and peritoneal cavity fluid CLP-operated mice administered DNase or saline. Mice were subjected to sham or CLP surgery and given DNase at 2, 4, or 6 hours post-operatively. 2 hours following DNase or saline administration, homogenized lung lysate (A), blood (B), and peritoneal cavity fluid (C) were collected and cultured on blood agar plates to determine the bacterial load in non-septic and septic mice following administration of DNase or saline (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.005$, n = 5-8 per CLP+saline group, n = 8-12 per CLP+DNase group). No colony forming units were observed in non-septic mice administered DNase or saline (data not shown).


A
Lung
CLP+Saline
CLP+DNase
Colony Forming Units (CFU/g)
2.5e+4
2.0e+4
1.5e+4
1.0e+4
5.0e+3
0.0
*
**
2 hours
4 hours
6 hours
Time between surgery and treatment
B
Blood
CLP+Saline
CLP+DNase
Colony Forming Units (CFU/mL)
4.0e+5
3.0e+5
2.0e+5
2.5e+4
0.0
***
2 hours
4 hours
6 hours
Time between surgery and treatment
C
Peritoneal Cavity Fluid
CLP+Saline
CLP+DNase
Colony Forming Units (CFU/mL)
5e+6
4e+6
3e+6
2e+6
1e+6
0
**
*
2 hours
4 hours
6 hours
Time between surgery and treatment

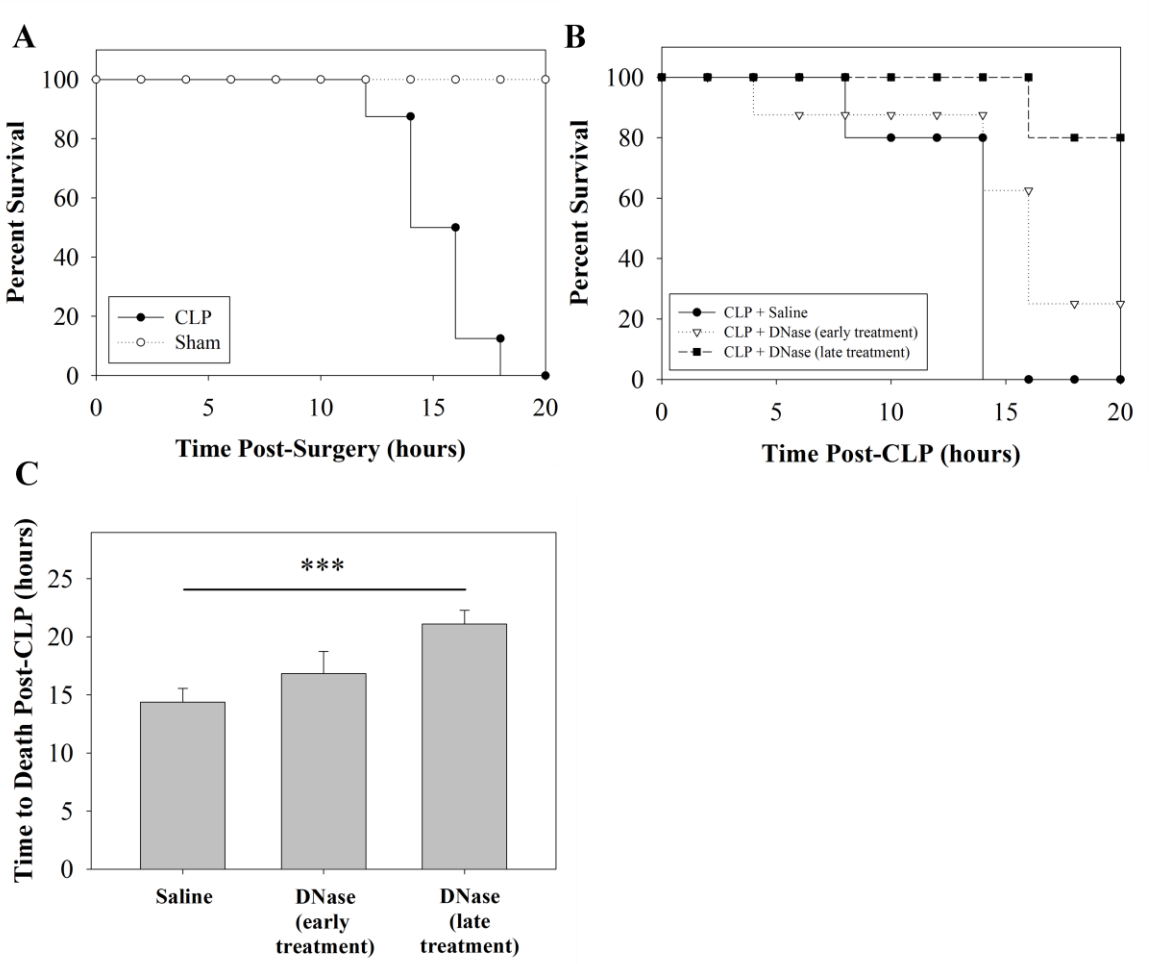
In our model, there is a 100% mortality rate at 20 hours post-CLP (versus 0% in sham-operated mice, Figure 3.8A). 80% of mice given late DNase treatment survived compared to 0% in mice given saline control. In mice administered DNase every 2 hours post-CLP surgery, only 25% of mice survived (Figure 3.8B). There was a significantly prolonged time to death in septic mice given late DNase treatment versus septic mice given saline control (21.10 hrs ± 1.18 hrs DNase-treated vs. 14.39 hrs ±1.17 hrs saline control, Figure 3.8C). Taken together, these findings indicate that repeated DNase administration following a period of sepsis progression significantly reduces mortality of septic mice.

3.5 Discussion

Our studies suggest that delayed DNase treatment to septic mice exerts a protective effect. We have documented elevations in levels of cfDNA and markers of inflammation and coagulation from sepsis induction to 8 hours post-surgery (Figure 3.1). In clinical and experimental sepsis, increases in IL-6 levels and decreases in IL-10 are indicative of the early inflammatory response and sustained increases of IL-6 correlate with poor outcome (Hack et al., 1989; Remick, Bolgos, Copeland, & Siddiqui, 2005). The IL-6 and IL-10 profiles in mice subjected to CLP are consistent with previous reports of an early and gradual rise in IL-6 levels and a transient increase in IL-10 levels over the 8-10 hour period following the onset of sepsis (Remick et al., 2000).

Elevations in cfDNA were associated with elevations in TAT, indicating activation of coagulation. TAT levels increased by 2 hours after sepsis induction and remained elevated for 8 hours (Figure 3.1).

Figure 3.8. Mortality curves of sham-operated mice and CLP-operated mice given saline, early DNase, or late DNase treatment. Mice were subjected to sham or CLP surgery and monitored until endpoint. A separate group of mice were subjected to CLP surgery and given saline, early DNase treatment (2 hours post-CLP), or late DNase treatment (6 hours post-CLP) and monitored until endpoint. The number of hours from CLP surgery to death in each treatment group were documented (*** $p < 0.005$, $n = 5$-8 per group).


A
Percent Survival
100
80
60
40
20
0
0
5
10
15
20
Time Post-Surgery (hours)
CLP
Sham
B
Percent Survival
Time Post-CLP (hours)
CLP + Saline
CLP + DNase (early treatment)
CLP + DNase (late treatment)
C
Time to Death Post-CLP (hours)
25
20
15
10
5
0
***
Saline
DNase (early treatment)
DNase (late treatment)

The time course of these changes is consistent with previous studies in murine sepsis models (Tucker et al., 2012) and with the results of clinical studies, which document increases in TAT levels in early sepsis and severe sepsis patients (Kinasewitz et al., 2004). Using plasma samples from severe sepsis patients, we recently showed that elevations in cfDNA correlate with increases in thrombin generation (Gould et al., 2014), consistent with the procoagulant effects of cfDNA. Using IL-6, IL-10, and TATs as markers of pro-inflammation, anti-inflammation, and coagulation respectively, the current data suggest that increases in cfDNA accompany the early pro-inflammatory and procoagulant response in sepsis.

Increases in cfDNA and the early pro-inflammatory and procoagulant responses are associated with organ damage (Figure 3.2). Septic mice with high cfDNA levels have significant accumulations of inflammatory cells and RBCs as well as intravascular fibrin deposition in the lungs and kidneys. In contrast, these pathological changes are absent in mice subjected to sham surgery with low levels of cfDNA. Clinical observations support a potential association between cfDNA and organ dysfunction in sepsis (Dwivedi et al., 2012; Zeerleder et al., 2003) and we have shown that elevated levels of circulating cfDNA are strongly predictive of mortality in severe sepsis patients (Dwivedi et al., 2012).

We sought to further investigate the relationship between cfDNA and sepsis pathophysiology by modifying levels of cfDNA with DNase. DNase treatment was administered at doses based on pharmacokinetic and toxicology studies investigating the use of DNase in cystic fibrosis (Dayan, 1994). The lowest dose of DNase at which a

decrease in cfDNA levels was observed in mice was chosen for our studies (unpublished data). The half-life of rhDNaseI is short (7-25 minutes) and the rapid clearance of DNase from the circulation into highly perfused tissues (lung, liver, and kidney) occurs within 2-30 minutes (Dayan, 1994). DNase treatment following a prolonged period of sepsis progression (i.e. four or six hours) resulted in reduced levels of circulating cfDNA, reduced pro-inflammatory IL-6, and increased levels of anti-inflammatory IL-10 (Figure 3.3) suggesting that modulating cfDNA may alter the immune response in experimental sepsis. DNase administration at this delayed time-point also resulted in decreased pulmonary edema, fibrin deposition, microvessel obstruction, and vascular congestion in the end organs of septic mice (Figures 3.4, 3.5), reduced neutrophil accumulation in the lungs indicated by decreased MPO activity, reduced ALT (Figure 3.6C, 3.6E), decreased bacterial dissemination in the lungs, blood, and peritoneal cavity (Figure 3.7), and improved outcome (Figure 3.8) suggesting that late DNase treatment may attenuate inflammation and reverse lung and liver damage in sepsis.

The formation and release of NETs and NET-associated proteins in the vasculature have recently been documented in murine models of endotoxemia (Clark et al., 2007) and experimental sepsis (Luo et al., 2014; Meng, Paunel-Görgülü, Flohé, Hoffmann, et al., 2012). It is possible that the degradation of cfDNA by DNase dismantles DNA traps, thereby attenuating NET-mediated inflammation and coagulation in the microvasculature and reducing the hyperinflammatory state and associated organ pathology in sepsis. It is also possible that the protective benefit of DNase is a result of alleviating inflammatory responses such as NET formation and inflammatory infiltration,

which in turn decreases cfDNA levels, although it is not known whether cfDNA promotes inflammation.

While delayed DNase treatment (four or six hours post-CLP) reduced organ damage, DNase treatment at an earlier time-point (two hours post-CLP), resulted in increased inflammation (Figure 3.3), organ damage (Figure 3.6), and decreased survival (Figure 3.8). These findings support the hypothesis that NETs have a protective role in the early immune response to a septic insult but an injurious one when NETs are not cleared in later stages of severe sepsis. Removing NETs during the immediate immune response (within two hours post-operatively) resulted in exacerbated organ pathology and earlier death (starting 5 hours post-CLP, Figure 3.8B) possibly because NETs are required to ensnare and kill pathogens. This concept is supported by findings from groups that demonstrate early DNase administration at just 1 hour following CLP surgery (with subsequent DNase treatments at 4, 7, 10, 21, 24, and 27 hours post-operatively) resulted in increased bacterial dissemination and bacterial colony forming units in the lungs, livers, and peritoneal cavities, increased serum IL-6, increased neutrophil recruitment, and decreased survival (Meng, Paunel-Görgülü, Flohé, Witte, et al., 2012). Other groups have also reported similar findings of increased bacterial dissemination and organ damage resulting from early DNase administration (1-2 hours following CLP surgery (McDonald et al., 2012)). In contrast, Luo *et al*. reported moderate improvements in lung pathology and modest decreases in bacterial load in septic mice given repeated doses of DNase after 24 hours but not at 6 hours post-CLP (Luo et al., 2014). In contrast to our model, Luo *et al*. used a low-grade, less severe model of CLP (punctures with 21G) previously reported

to result in decreased mortality (Baker et al., 1983) and no resuscitation procedure was described in this study (Luo et al., 2014). In our model, mice are resuscitated early following sepsis induction and at high fluid volumes, the benefits of which have been shown in clinical sepsis (Process, 2014; Rivers et al., 2001; Vincent & Gerlach, 2004). Studies from both Meng *et al*. (2012) and Luo *et al*. (2014) report the effects of repeated DNase administration with injections beginning at 1 hour post-CLP, a time-point which precedes any observable pathological changes in our experiments and may not be relevant for investigating the therapeutic potential of DNase in clinical sepsis. However, our studies show that DNase exerts protective effects when administered in mice following a prolonged period of sepsis progression (four or six hours post-CLP), at a time-point which is relevant to the experimental and clinical presentation of severe sepsis, as evidenced by inflammatory biomarkers, changes in organ pathology, increased time to death, and increased survival. Thus, the effects of removing NETs via DNase administration are time-sensitive and appear effective when administered after a prolonged period of sepsis progression.

Interestingly, administration of DNase in sham-operated mice resulted in an increase in cfDNA, IL-6, and IL-10 levels (Figure 3.3), and inflammation in the end organs (Figures 3.4, 3.5). These findings are consistent with previous pharmacological reports investigating the toxicology of rhDNase. Rats administered DNase intravenously exhibited a mild, short-term inflammatory response, with pulmonary changes consistent with injection of a foreign protein (Dayan, 1994). DNase has also been shown to induce an initial pro-inflammatory response when administered in cystic fibrosis (Shah, Scott,

Knight, Marriott, et al., 1996; Suri et al., 2002). DNase administration via the intravenous route induces a mild inflammatory response in highly perfused end organs.

Our studies show that DNase given 2 hours post-operatively (before detectable changes in markers of inflammation and coagulation) increases inflammation and organ injury and that a therapeutic effect is observed when DNase is administered 4 or 6 hours post-operatively. This narrow therapeutic window suggests that while DNase administration may have a therapeutic potential, there are limitations to DNase as an anti-sepsis therapy. The timing and use of DNase administration should be further investigated. Additionally, we recognize that the use of antibiotics is not accounted for in this study and its use in future studies may alter mortality rates of septic mice administered DNase.

In summary, our studies are the first to demonstrate that delayed administration of DNase may be protective in experimental sepsis. The timing of DNase administration may be a crucial element in future investigations of the therapeutic potential of DNase in sepsis.

3.6 Acknowledgements

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Chapter 4: A comparison of scoring systems and surrogate markers of death in a cecal ligation and puncture sepsis model

Foreword

This manuscript was submitted to the journal Shock (May 2015). The authors are as follows: Safiah H. Mai, Momina Khan, Shawn Petrik, Dhruva J. Dwivedi, Alison E. Fox-Robichaud, and Patricia C. Liaw. The corresponding author is Patricia C. Liaw.

The experiments were completed by Safiah H. Mai with assistance from Momina Khan. The studies were designed by Safiah H. Mai, Shawn Petrik, Alison E. Fox-Robichaud, and Patricia C. Liaw. The manuscript was written by Safiah H. Mai with significant input by Patricia C. Liaw.

4.1 Abstract

Rationale: Despite increasing standards for conducting ethical, humane research involving animals, death is still considered the only valid endpoint in murine sepsis studies. Recently, the Mouse Clinical Assessment Score for Sepsis (M-CASS), Murine Sepsis Score (MSS), and Mouse Grimace Scale (MGS) were developed as potential surrogate endpoint tools for assessing disease severity in models of pneumonia, lipopolysaccharide, and fecal slurry injection. **Objectives**: To assess the validity of the M-CASS, MSS, and MGS for assessing disease severity and predicting death in a resuscitated cecal ligation and puncture (CLP) model of sepsis. **Methods**: Healthy C57BL/6 mice were subjected to CLP, a surgical procedure involving two punctures of the ligated cecum to induce sepsis, or a control, non-septic sham surgery (no ligation or puncture). Mice were given Ringer's lactate and buprenorphine every 4 hours post-operatively to account for surgical losses and continuously monitored for potential surrogate markers of death until endpoint or 20 hours post-operatively. M-CASS, MSS, and MGS scores were assigned every 4 hours and the utility of the systems was determined. **Results**: Around 50% of septic mice expired within 16 hours post-CLP and 100% expired within 20 hours post-CLP. M-CASS, MSS, and MGS increased in a time-dependent manner in septic mice and all scores were 0 in non-septic mice over 18 hours. Components of the scoring systems, which assessed the responsiveness of mice to external stimuli, posture, facial grimace markers, and fur condition were informative markers of disease severity. Temperature was a reliable, objective marker that one should consider monitoring to assess disease progression. Vocalizations and chest sounds did not

correlate with disease progression in our CLP model. **Conclusion**: The M-CASS, MSS, and MGS are effective tools for assessing disease severity in the CLP model. Efforts should be made towards using these assessment tools as well as other relevant surrogate markers as valid endpoints rather than mortality.

4.2 Introduction

Experimental animal models of sepsis have been developed and refined for over 80 years to investigate the induction, development, management, and treatment of sepsis (Clowes et al., 1968). Recognizing the ethical implications of using animals in research, the three R's, Replacement, Refinement, and Reduction were proposed in 1959 and to date, form the guiding principles of the standards to which researchers must hold their animal studies (Russell WMS, 1992). These guidelines exclude death as an endpoint and suggest the use of humane endpoints or surrogate markers of death where possible. Various parties involved in different aspects of sepsis research from comparative medicine staff and veterinarians to sepsis researchers, expert working groups, and clinicians have all called for much needed improvements in support of the "3Rs" towards humane, ethical, and clinically relevant research involving animals (Bara & Joffe, 2014; Hooijmans, De Vries, Leenaars, Curfs, & Ritskes-Hoitinga, 2011; Hooijmans, Leenaars, & Ritskes-Hoitinga, 2010; Lilley et al., 2015; Nemzek, Xiao, Minard, Bolgos, & Remick, 2004). While research in other disease areas have identified criteria that can be used to predict death in murine models (Trammell & Toth, 2011), clear endpoint markers have not been established for specific murine sepsis models. Sepsis researchers must aim to find the fine balance between ensuring that treatment of animals are ethical and humane

(preventing unnecessary suffering where possible) and using reliable models with sepsis severities and outcomes which translate to clinical sepsis.

Animal sepsis models have been widely criticized for the failure of animal sepsis data to translate effectively into clinical research (Dyson & Singer, 2009). Failures of therapeutic interventions which appeared promising in animal studies highlight this gap (Buras et al., 2005; Opal & Patrozou, 2009) and efforts to limit this gap in clinical translation must be put forth. One effort towards such improvements are to increase the clinical relevance of how monitoring is achieved in animal sepsis studies. All hospitalized patients are monitored through the use of vital signs such as respiratory rate, heart rate and level of consciousness. In some hospitals, patients at risk of death may be identified with the use of Early Warning Scores (EWS) (Corfield et al., 2014; Reini et al., 2012). These EWS document changes in the respiratory rate, heart rate, systolic blood pressure, temperature, and level of consciousness of the patient (Subbe et al., 2001)(Stenhouse et al., 2000). It is an effective bedside physiological scoring tool that facilitates early recognition of a deteriorating patient many of whom may be septic.

In experimental sepsis, researchers have made progress in designing clinically relevant scoring systems as tools to ethically assess the progression of sepsis in murine animals. The Mouse Clinical Assessment Score for Sepsis (M-CASS) (Huet et al., 2013) was developed in a pneumonia model of septic shock. The Murine Sepsis Score (MSS) was developed from an intraperitoneal fecal slurry injection model of septic shock (Shrum et al., 2014). The Mouse Grimace Scale (MGS) was developed in post-operative pain models to assess post-surgical pain (Langford et al., 2010; Matsumiya et al., 2012).

Symptomatic evaluation using these clinically relevant scoring systems confers significant advantages. These tools offer 1) immediate, non-invasive assessment of disease progression which does not artificially exacerbate sepsis symptoms or sepsis pathology, 2) information regarding the changes in disease state induced by additional insults (in studies investigating sepsis comorbidities) or by therapeutic interventions, 3) techniques for reliable monitoring to meet the needs of ethical, humane animal research, and 4) more feasible and cost-efficient means to obtain vital signs in small rodents.

Despite the development of these validated scoring systems, there is a paucity of animal sepsis research utilizing these tools. Herein, we evaluate the effectiveness of the M-CASS, MSS, and MGS systems and provide recommendations for using components of these scoring systems in predicting death in a cecal ligation and puncture model of sepsis.

4.3 Materials and methods

4.3.1 Experimental sepsis-cecal ligation and puncture

8 week-old (20 to 25 g) male C57Bl/6 mice (*Helicobacter hepaticus*-free) were purchased from Charles River Laboratories (Sherbrooke, Quebec, Canada) and bred at the Thrombosis and Atherosclerosis Research Institute at McMaster University (Hamilton, ON, Canada). Mice were housed in a Helicobacter/MNV-negative clean room in individually ventilated cages (Tecniplast Sealsafe Plus system) under 12 hr:12 hr dark/light cycles. Air was filtrated by prefilters, HEPA filters, and Touch Slimline air handling units which guarantee 75 air changes per hour in each cage. The mice were provided enrichment and allowed unlimited access to sterilized food (Harlan Teklad

Rodent Diet #2018) and water. Mice received humane care in accordance with Canadian Council on Animal Care (CCAC) guidelines. These studies were approved by the Animal Research Ethics Board (AREB, AUP 10-12-79) at McMaster University (Hamilton, ON, Canada).

8 week-old C57Bl/6 male mice were subjected to either cecal ligation and puncture (CLP) to induce sepsis or sham surgery as a non-septic control. Mice were randomized to the CLP or sham groups according to ARRIVE guidelines (Kilkenny et al., 2013) and methods for randomization and experimenter blinding were used to reduce allocation, selection, and experimenter biases (Mai et al., 2012). The CLP model used in these studies was adapted from protocols by others (Baker et al., 1983; Hubbard et al., 2005) and was previously described (Mai, Khan, & Dwivedi, 2015). Briefly, under isoflurane anesthesia, the mice were shaven, the surgical site was cleaned and sterilized with iodine and alcohol, and the cecum was exteriorized. In CLP-operated mice, the cecum was ligated 1 cm proximal of the cecal end and punctured with a double, through-and-through puncture using an 18G needle. Fecal contents were pushed out of the cecal punctures ensuring patency and the ligated and perforated cecum was returned to the peritoneal cavity. In sham-operated mice, the cecum was exteriorized and returned to the peritoneal cavity without further perturbation. Buprenorphine (0.1 mg/kg) and Ringer's lactate (2 mL) were administered pre-operatively and every 4 hours post-operatively for pain relief and resuscitation to account for surgical losses. External heat was provided up to 8 hours post-operatively. Mice were continuously monitored until endpoint (inability to ambulate) or 20 hours post-surgery and organs were collected when mice were no longer

ambulant prior to death. In surviving mice, blood was collected via the inferior vena cava (IVC) into a one-tenth volume of 3.2% sodium citrate, spun at 2000x g and platelet-poor plasma was stored in aliquots at -80ºC. The mice were euthanized by cervical dislocation at various time-points post-operatively.

4.3.2 Organ histology

Organs were collected in formalin, embedded in paraffin wax, processed, and sectioned at 5 microns in thickness. Organ sections were stained with hematoxylin and eosin (H&E) for the analysis of overall morphology (nuclei and extracellular DNA stain bluish-purple and cytoplasm, connective tissue, and collagen stain pink). Photomicrographs of stained lung, liver, and kidney sections were visualized under 400x magnification (scale bars represent 25μm). A clinical pathologist, who was blinded to the surgery and treatment allocation, assessed the extent of organ injury.

4.3.3 Mouse Clinical Assessment Score for Sepsis (M-CASS)

The M-CASS scoring system involves the symptomatic observation of 8 markers: fur aspect, activity, posture, behaviour, chest movements, chest sounds, eyelids, and body weight loss. The established M-CASS score is an average of these 8 components. Body weight loss was excluded in the M-CASS system in our sepsis studies as changes in body weight loss are not observable in the short, 20 hour study period (Huet et al., 2013).

4.3.4 Murine Sepsis Score (MSS)

The MSS system involves observing 7 components: appearance, level of consciousness, activity, response to stimulus, eyes, respiratory rate, and respiratory quality. The established MSS score is the average of these 7 components. Changes in

respiratory quality were not quantifiable by eye and were excluded in our study (Shrum et al., 2014).

4.3.5 Mouse Grimace Scale (MGS)

The MGS scoring system involves the scoring of 5 components: orbital tightening, nose bulge, cheek bulge, ear positioning, and whisker change. The established MGS score is the average of these 5 components (Langford et al., 2010). In this study, nose bulge and cheek bulge were indistinguishable from one another and were grouped as a single score component.

For the purpose of our comparisons, the M-CASS, MSS, and MGS scores were standardized to a 4-point scale ranging from 0 to 3 to make relevant comparisons of the 3 modified scoring systems.

4.3.6 Statistical analyses

Data are represented as mean ± SEM and results across groups were compared using Newman-Keuls post-hoc test. Differences in standardized scores between the two surgery groups were considered significant at $p < 0.05$. Analyses were performed using GraphPad Prism 4.0 (La Jolla, CA, USA) and SigmaPlot 11.0 (San Jose, CA, USA).

4.4 Results

4.4.1 M-CASS, MSS, and MGS increase in a time-dependent manner in septic mice following CLP surgery

Mice were subjected to CLP or sham surgery and various symptomatic parameters, potentially indicative of disease progression, were monitored continuously over a 20-hour post-operative period (Figure 4.1). Around 50% of mice subjected to CLP

died within 16 hours post-CLP and 100% of mice subjected to CLP died within 20 hours post-operatively (Figure 4.2A). 100% of non-septic, sham-operated mice survived past 20 hours post-operatively. M-CASS (Figure 4.2B), MSS (Figure 4.2C), and MGS (Figure 4.2D) scores increased in a time-dependent manner over the 20 hour study period while all scores for sham-operated mice remained at 0 for the duration of the study (Figure 4.3). Temperatures taken via rectal probe monitoring of sham-operated mice did not change significantly over the study duration compared to rectal temperatures taken pre-operatively (35.90 ± 0.06 ºC in sham group vs. 35.15 ± 0.30 ºC before surgery, n = 8). However, rectal temperatures in septic mice dropped significantly from 8 to 18 hours post-CLP (32.71 ± 0.78 ºC at 4 hours post-CLP versus 23.8 ºC at 18 hours post-CLP, Figure 4.2E). Temperatures below 24 ºC were associated with death within 2-3 hours. Representative images of mice at various time-points following CLP surgery are shown (Figure 4.3). Orbital tightening, nose and cheek bulge, erected ears and whiskers are observed with increase in time following CLP surgery. These findings indicate that scoring systems based on visual, symptomatic observations are useful in assessing sepsis progression over time.

4.4.2 Effective indicators of sepsis progression

Symptomatic parameters which consistently changed over the post-operative period and increased in severity score with disease progression in the M-CASS system were: fur aspect (Figure 4.4A), posture (Figure 4.4C), and eyelids (Figure 4.4F). Chest movements varied over time and no chest sounds were detectable in CLP-operated mice (Figure 4.4G). In the MSS system, appearance, level of consciousness, activity, and eye

scores increased over time and were highest near death (Figure 4.5). Using the MGS scoring system, orbital tightening, ear positioning, and whisker change were effective indicators of sepsis progression over time (Figure 4.6).

Parameters which are often used in endpoint monitoring but were uninformative or inconsistent in indicating sepsis progression were: cyanosis, restlessness or tremors, and vocalizations (Figure 4.7). Surrogate markers and components detecting responsiveness of mice to external stimuli, activity, facial grimace markers, and piloerection (appearance and fur aspect) were most informative in indicating sepsis progression while chest sounds and vocalizations were not useful markers of disease progression.

4.4.3 Severe organ damage in septic mice 18 hours post-CLP

The histology of end organs from sham- and CLP-operated mice were examined to confirm the presence of organ damage in septic mice. At 18 hours post-operatively, when the greatest increases in M-CASS, MSS, and MGS scores were detected in mice subjected to CLP, severe organ damage was observable in the lungs, livers, and kidneys of septic mice and not in sham-operated, non-septic mice (Figure 4.8A, 4.8B, and 4.8C). Lungs from septic mice were infiltrated with neutrophils and showed signs of interstitial edema, inter-alveolar red blood cell congestion, and obstruction of pulmonary microvasculature (Figure 4.8D). Livers from septic mice 18 hours post-CLP had degeneration and apoptosis of hepatocytes with congestion of red blood cells in central venules and portal veins (Figure 4.8E). Kidneys from septic mice had destruction of tubular brush borders, tubular necrosis, nuclear dropoff, and intra-glomerular and

Figure 4.1. Endpoint monitoring form. A sample endpoint monitoring form which can be used during post-operative monitoring to assess sepsis progression following cecal ligation and puncture surgery. Components are given a score from 0-3 (0 representing characteristics similar to those in healthy control mice and 3 being the most severe). Temperature measurements taken with a rectal probe can be documented as an objective marker in endpoint monitoring.

Mouse ID:	**M1**	**M2**	**M3**
Time of Surgery:			
Time-Point:			
Scoring Components			
Inability to Ambulate (euthanize if present)			
Orbital Tightening			
Nose and Cheek Bulge			
Ear Positioning			
Whisker Change			
Hunched Posture			
Activity			
Restlessness and Tremor			
Responsiveness			
Fur Aspect/Appearance			
Vocalizations			
Dyspnea or Laboured Breathing			
Temperature (Rectal Probe)			
Cyanosis			

Figure 4.2. Kaplan-Meier mortality curve, mortality scores, and temperature following CLP versus sham surgery. Mice were subjected to CLP or sham surgery and monitored continuously until death (A) or inability to ambulate for components of the M-CASS (B), MSS (C), and MGS (D) scoring systems and for temperature via a rectal probe monitor (E).

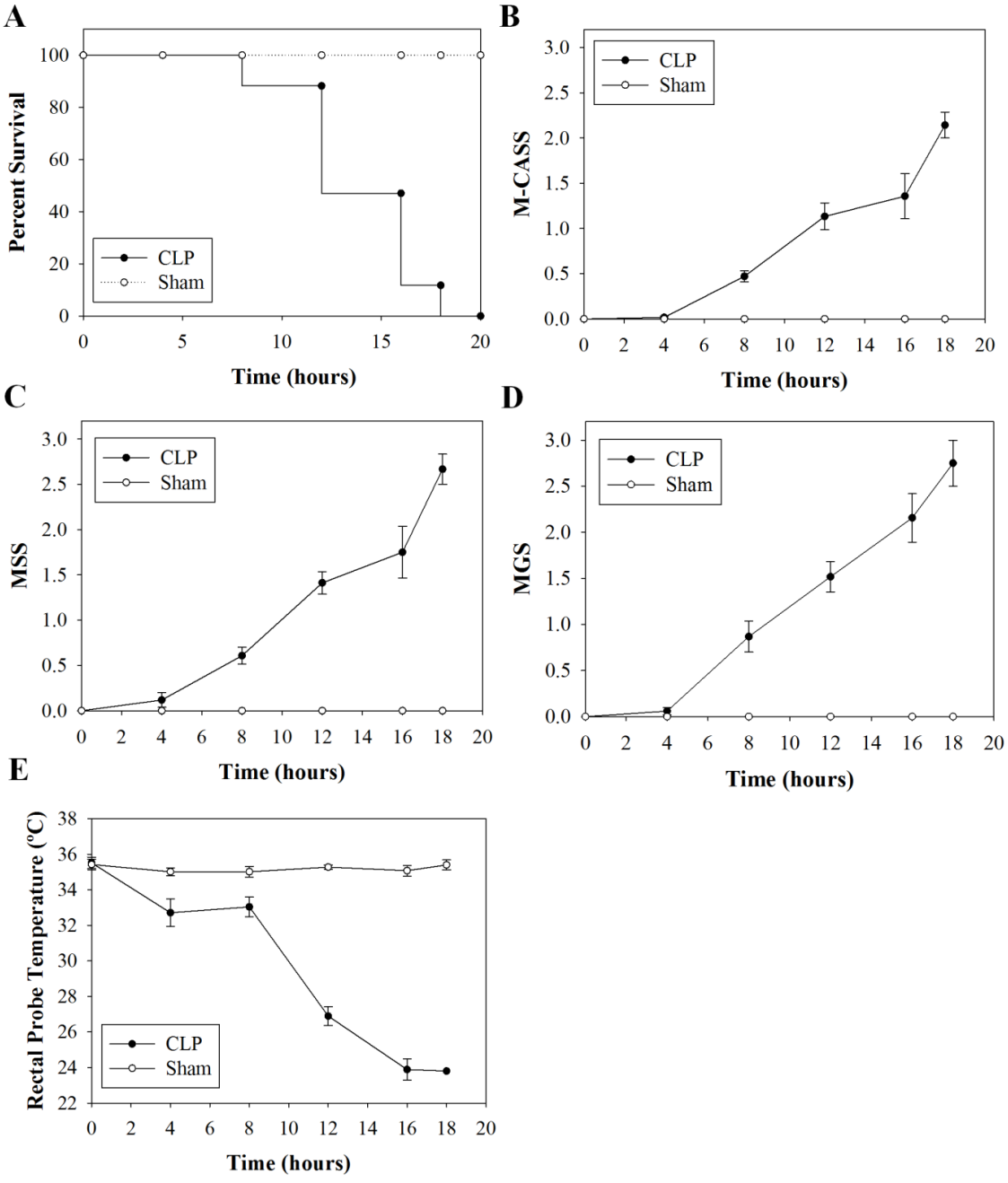

A
Percent Survival
Time (hours)
CLP
Sham
B
M-CASS
Time (hours)
CLP
Sham
C
MSS
Time (hours)
CLP
Sham
D
MGS
Time (hours)
CLP
Sham
E
Rectal Probe Temperature (°C)
Time (hours)
CLP
Sham

Figure 4.3. Representative photographs of mice over progression of sepsis. Photographs of septic mice at 0 hours (A), 12 hours (B), 16 hours (C), and 18 hours (D) post-CLP surgery depicting changes in orbital tightening, nose and cheek bulge, ear positioning, and whisker positioning.

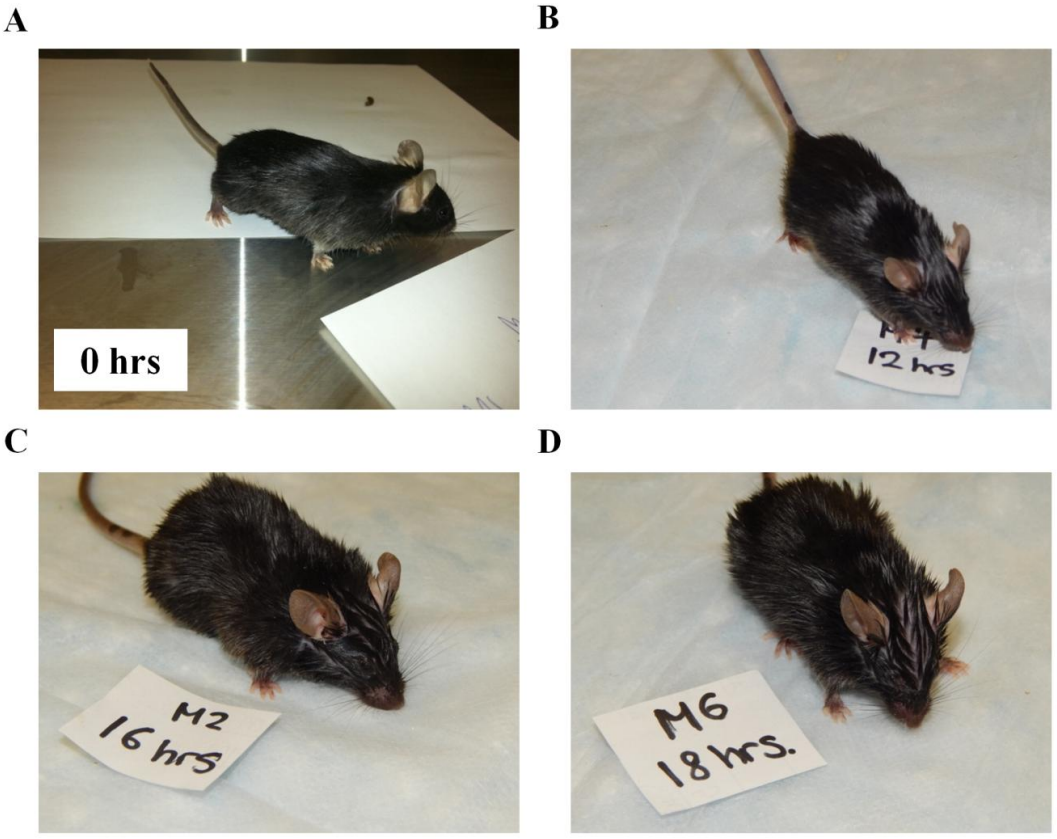

A
B
0 hrs
12 hrs
C
D
M2
16 hrs
M6
18 hrs.

Figure 4.4. Components of the Mouse Clinical Assessment Score for Sepsis (M-CASS). Individual components of the M-CASS comparing mice subjected to CLP vs. sham surgery are shown. Components are the fur aspect (A), activity (B), posture (C), behaviour (D), chest movements (E), eyelids (F), and chest sounds (G).

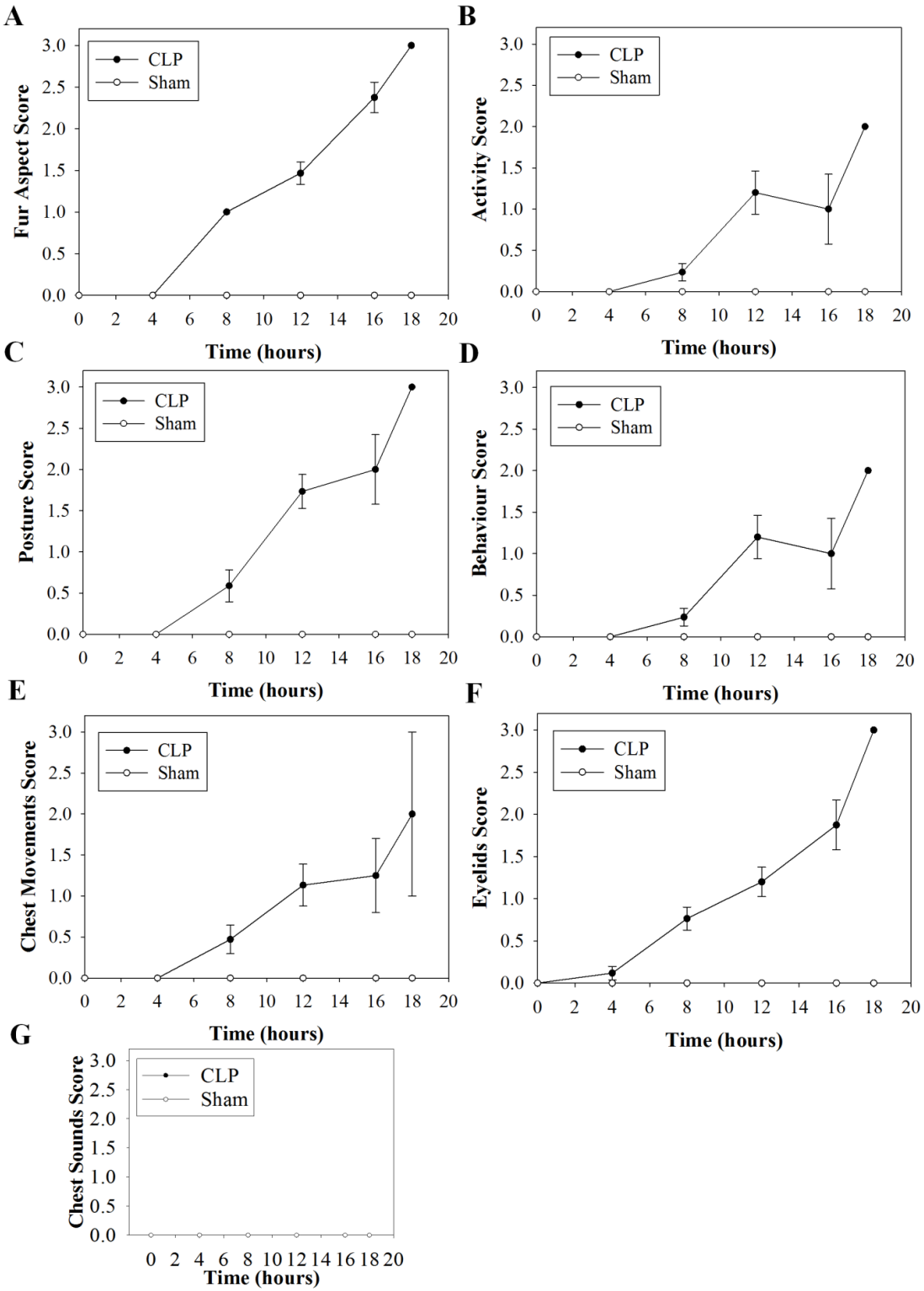

A
Fur Aspect Score
CLP
Sham
Time (hours)
B
Activity Score
CLP
Sham
Time (hours)
C
Posture Score
CLP
Sham
Time (hours)
D
Behaviour Score
CLP
Sham
Time (hours)
E
Chest Movements Score
CLP
Sham
Time (hours)
F
Eyelids Score
CLP
Sham
Time (hours)
G
Chest Sounds Score
CLP
Sham
Time (hours)

Figure 4.5. Components of the Murine Sepsis Score (MSS). Individual components of the MSS comparing mice subjected to CLP vs. sham surgery are shown. Components are appearance (A), level of consciousness (B), activity (C), response to stimulus (D), eyes (E), and respiratory quality (F).

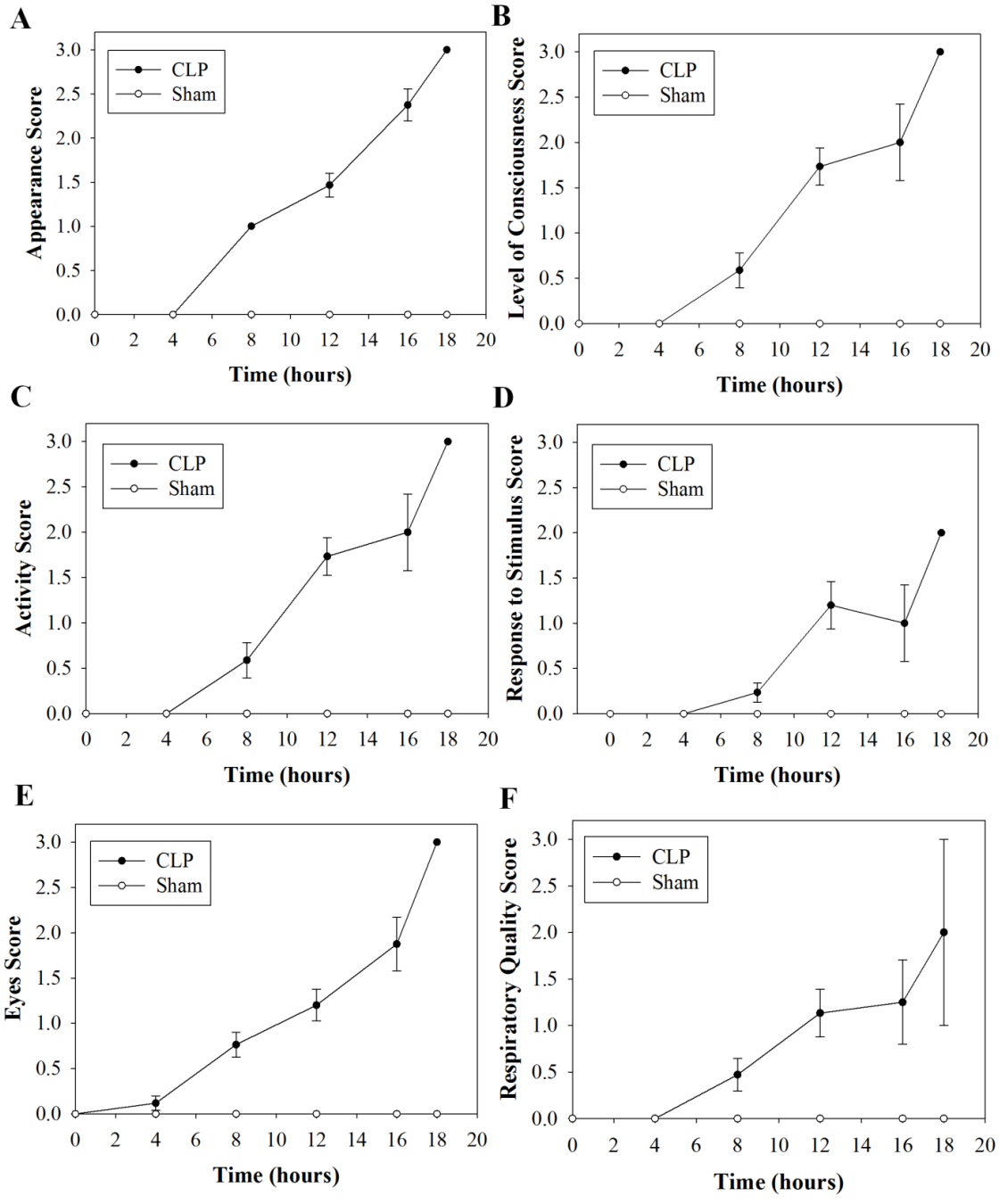

A
Appearance Score
CLP
Sham
Time (hours)
B
Level of Consciousness Score
CLP
Sham
Time (hours)
C
Activity Score
CLP
Sham
Time (hours)
D
Response to Stimulus Score
CLP
Sham
Time (hours)
E
Eyes Score
CLP
Sham
Time (hours)
F
Respiratory Quality Score
CLP
Sham
Time (hours)

Figure 4.6. Components of the Mouse Grimace Scale (MGS). Individual components of the MGS comparing mice subjected to CLP vs. sham surgery are shown. Components are orbital tightening (A), nose and cheek bulge (B), ear positioning (C), and whisker change (D).

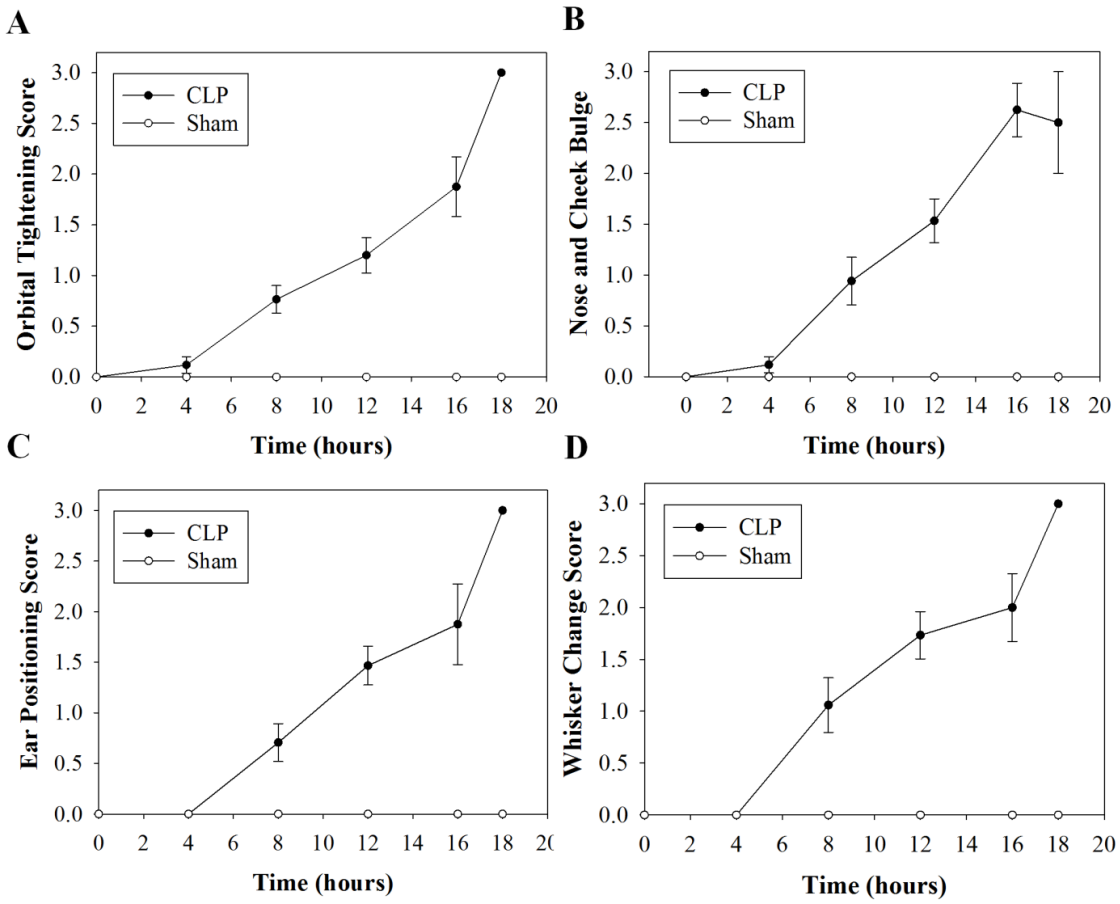

A
Orbital Tightening Score
CLP
Sham
Time (hours)
B
Nose and Cheek Bulge
CLP
Sham
Time (hours)
C
Ear Positioning Score
CLP
Sham
Time (hours)
D
Whisker Change Score
CLP
Sham
Time (hours)

Figure 4.7. Poor surrogate markers of death. Changes in other surrogate markers commonly used in endpoint monitoring were recorded but had low utility in predicting death or assessing sepsis severity in our CLP model. These markers are cyanosis (A), restlessness and tremor (B), and vocalizations (C).

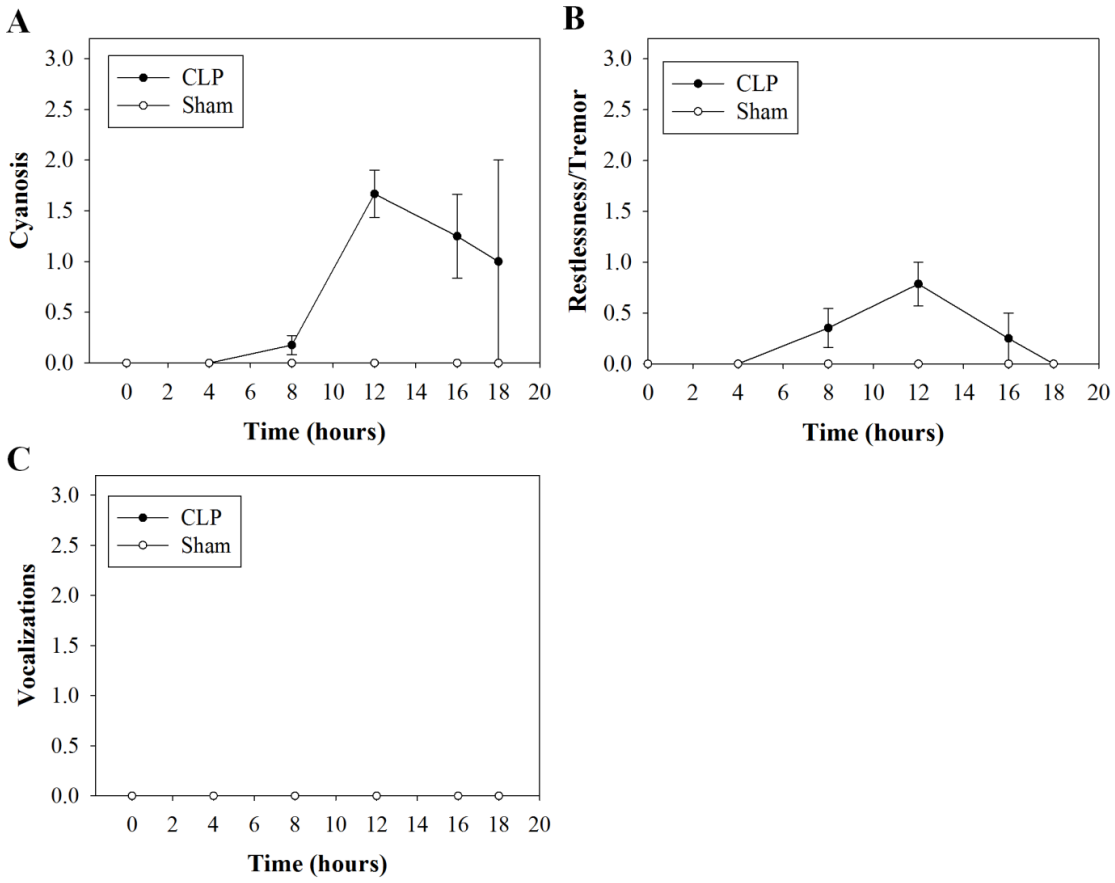
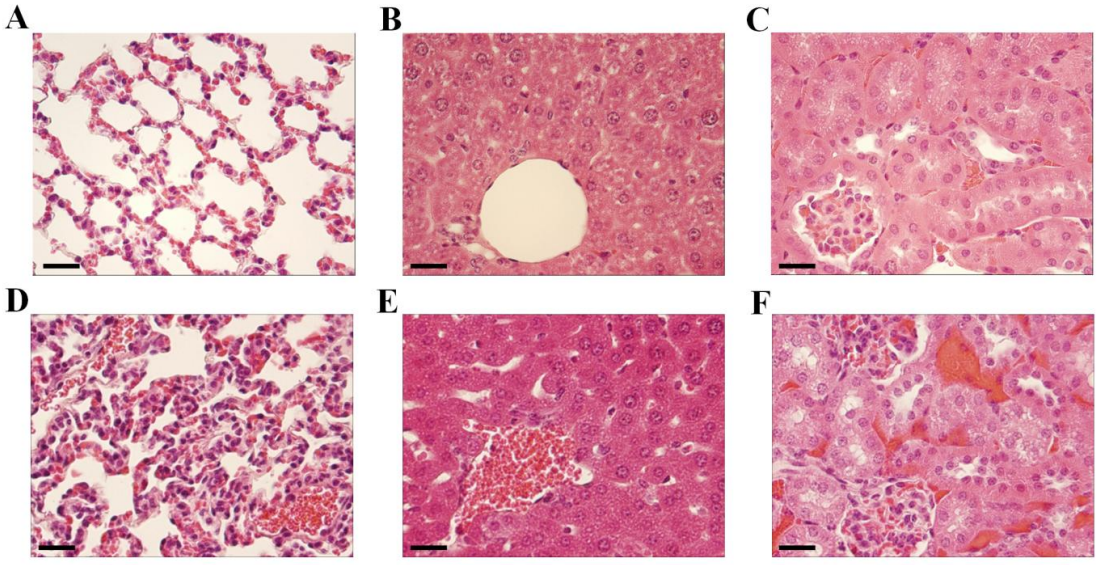

A
Cyanosis
CLP
Sham
Time (hours)
B
Restlessness/Tremor
CLP
Sham
Time (hours)
C
Vocalizations
CLP
Sham
Time (hours)

Figure 4.8. Photomicrographs of end organs of non-septic and septic mice. Photomicrographs were taken of lung (A), liver (B), and kidney (C) sections from sham-operated and lung (D), liver (E), and kidney (F) sections from CLP-operated mice. End Organs were sectioned at 5 μm thick and visualized under 400x magnification. Scale bars represent 25 μm. Organs in CLP-operated mice had severe inflammation, vascular congestion, and necrosis 18 hours post-operatively.


A
B
C
D
E
F

inter-tubular vascular congestion (Figure 4.8F). Increases in severity scores correlate with end organ damage in septic mice.

4.5 Discussion

Humane endpoints or surrogate markers of death involve the use of predetermined criteria (such as severity scores based on visual parameters) to determine the time at which animals should be humanely euthanized (Nemzek et al., 2004). These decisions must be balanced with not terminating animal studies prematurely which may lead to inaccurate data and, potentially, the use of even more animals (Drechsler et al., 2015; Nemzek et al., 2004). Significant advances recently made towards humane, ethical, and clinically relevant sepsis research using animals are the M-CASS scoring system developed by Huet *et al*. in 2013 and the MSS by Shrum *et al*., 2014. Both systems use symptomatic parameters including the level of consciousness, activity, responsiveness to stimuli, and orbital tightening as indicators of disease progression. These scoring systems are of clinical relevance since clinical scoring systems (such as early warning scores) are used to monitor 'at risk' patients. Like clinical scoring systems, the scoring systems proposed in these studies also include clinically relevant parameters such as temperature and level of consciousness (Stenhouse et al., 2000; Subbe et al., 2001). Despite the demonstrated efficacy of these validated scoring systems, the M-CASS and MSS have not been previously tested for the widely used, clinically relevant sepsis model of cecal ligation and puncture. The MGS from Langford *et al*. in 2010 is another scoring tool with high efficacy, the utility of which has not been previously tested in sepsis models.

In our severe, acute, resuscitated model of CLP (18G, double puncture), we observed a mortality rate of 100% within 24 hours, a finding consistent with mortality rates reported by those who developed the CLP procedure (Baker et al., 1983). We also used modified versions of the M-CASS, MSS, and MGS scoring systems to evaluate the use of surrogate endpoints in assessing disease severity and predicting death (Table 4.1).

We observed that M-CASS, MSS, and MGS are all effective scoring systems for predicting death and assessing disease severity. We recommend that these systems be used in animal sepsis studies for several reasons. First, these tools offer a non-invasive assessment of disease progression without interfering or exacerbating sepsis pathology regardless of the frequency of monitoring (i.e. no need for blood draws which may influence blood volume or tissue perfusion). Second, symptomatic evaluation provides immediate information regarding the changes in the animals' disease states, which may be introduced by additional insults or by potential therapeutic interventions. Scoring systems are also more feasible and cost-effective than hemodynamic monitoring to obtain vital signs from small rodents. Moreover, pilot studies with smaller sample sizes can be undertaken to develop a scoring system tailored to specific sepsis studies. For instance, a change in body weight was not relevant in our severe CLP model where death occurs relatively quickly and was removed from the scoring system. Chest sounds were also not observed in any animals in our CLP model. In other CLP models where sepsis develops over 3-10 days (Rittirsch et al., 2009), chest sounds may be an effective surrogate marker. In aggressive models where sepsis develops rapidly, removing the chest sounds

Table 4.1. Modified M-CASS, MSS, and MGS scoring systems for the monitoring of surrogate endpoints and assessment of disease severity. Descriptions of the modified grading scale of M-CASS components adapted from Huet *et al*., 2013 (A), Murine Sepsis Score components adapted from Shrum *et al*., 2014 (B), and Mouse Grimace Scale components adapted from Langford *et al*., 2010 (C).

Mouse Clinical Assessment Score for Sepsis Components. Descriptions of the modified grading scale of M-CASS components adapted from Huet *et al*., 2013. (A**).**

M-CASS	0	1	2	3
Fur Aspect	Normal coat	Slightly ruffled fur	Ruffled fur	Ruffled fur and piloerection
Activity	Normal	Reduced	Only when provoked	Little or none with provocation
Posture	Normal	Hunched, moving freely	Hunched, strained or stiff movement	Hunched, little or no movement
Behaviour	Normal	Slow	Abnormal when disturbed or provoked	Abnormal, no relocation
Chest Movements	Normal	Mild dyspnea	Moderate dyspnea	Severe dyspnea
Chest Sounds	Absent	Occasional chirping	Frequent chirping	Wet chirping
Eyelids	Normal, open	Opened when disturbed	Partially closed, even when disturbed	Mostly or completely closed, even when provoked

Murine Sepsis Score Components. Descriptions of the modified grading scale of MSS components adapted from Shrum *et al*., 2014. (B).

MSS	0	1	2	3
Appearance	Smooth coat	Slightly ruffled fur	Majority of fur on back is ruffled	Piloerection, puffy appearance
Level of Consciousness	Active	Active, avoids standing upright	Active only when provoked	Non-responsive, even when provoked
Activity	Normal	Suppressed, no eating, drinking, or running	Stationary	Stationary, even when provoked
Response to Stimulus	Normal	Slowed response to auditory or touch stimuli	No response to auditory, slowed response to touch	No response to touch stimuli

			stimuli	
Eyes	Open	Not fully open, potentially secretions	Half closed, potential secretions	Mostly or completely closed
Respiration Quality	Normal	Periods of laboured breathing	Consistently laboured breathing	Laboured breathing with gasps

Mouse Grimace Scale Components. Descriptions of the modified grading scale of MGS components adapted from Langford *et al*., 2010. (C).

MGS	0	1	2	3
Orbital Tightening	Eyes open	Eyes slightly closed	Eyes half closed	Eyes closed
Nose and Cheek Bulge	Normal, flat	Slightly rounded extension of skin around nose bridge	Wrinkled nose or cheeks, slight bulge in cheeks	Obvious, rigid appearing nose and cheek bulge
Ear Positioning	Ears flat, back against body	Ears alert, slightly angled from back	Ears partially positioned forward or apart	Ears completely erect, far apart
Whisker Change	Normal	Some whiskers erect	Whiskers mostly erect or clumping	All whiskers standing on end

component may further increase the utility of the M-CASS since no audible chest sounds were heard in our studies. Lastly, we observed that temperature measured via a rectal probe sensor was a quick, objective, and minimally-invasive surrogate marker of death (temperature < 26 ºC indicates death within a few hours in these studies) and recommend that it be considered in future sepsis studies involving animals.

A number of strengths of this study support its clinical relevance. In this CLP model, early and aggressive fluid resuscitation was given to prevent death due to circulatory decline and lack of hemodynamic support rather than due to sepsis progression (Zanotti-Cavazzoni et al., 2009). Additionally, anaesthetics and analgesics at the upper range of the recommended dose were used every 4 hours to minimize pain and to reduce the potential of facial grimacing as a pain response rather than a reflection of disease progression. However, there are limitations. Antibiotics were not used and external heat provided by heating pads was removed at 8 hours post-surgery to ascertain whether temperature was an effective surrogate marker of death. In future studies, the use of antibiotics and prolonged exposure to external heat may very well prolong the survival of mice in this model (Turnbull et al., 2004).

The M-CASS, MSS, and MGS scoring systems and temperature measurement are effective, clinically relevant markers for monitoring endpoint, assessing sepsis progression, and predicting death in animal sepsis. Further efforts to use techniques such as these scoring systems rather than death as an endpoint should be made to meet the increasing standards of ethical, humane animal research in sepsis.

Chapter 5: PAD4-deficiency reduces inflammation and coagulation in a murine model of polymicrobial sepsis

Foreword

This manuscript is in preparation. The authors are as follows: Safiah H. Mai, Momina Khan, Dhruva J. Dwivedi, Kerri A. Mowen, Michelle Willson, Paul Kubes, Alison E. Fox-Robichaud, and Patricia C. Liaw. The corresponding author is Patricia C. Liaw.

The experiments in this manuscript were completed by Safiah H. Mai with assistance from Momina Khan. The mice were generously provided by Kerri A. Mowen, Michelle Willson, and Paul Kubes. The studies were designed by Safiah H. Mai, Alison E. Fox-Robichaud, and Patricia C. Liaw. The manuscript was written by Safiah H. Mai with significant input by Patricia C. Liaw.

5.1 Abstract

Rationale: Sepsis is characterized by systemic activation of coagulation and inflammation in response to microbial infection. Upon inflammatory or microbial stimulation, cell-free DNA (cfDNA) is released from activated neutrophils in the form of neutrophil extracellular traps (NETs). Recent studies show that while NETs have antimicrobial and immunoprotective properties, they may exacerbate the aberrant inflammatory and coagulant response in sepsis. **Objectives**: To determine whether the absence of NETs in PAD4-deficient mice modulates inflammation and coagulation in a cecal ligation and puncture (CLP) model of polymicrobial sepsis. **Methods**: Healthy C57BL/6 mice and PAD4-/- mice were subjected to CLP, a surgical procedure involving two punctures of the ligated cecum, or sham surgery (no ligation/puncture). At 4, 8, and 12 hours or at endpoint in survival studies, organs were harvested and plasma levels of cfDNA, IL-6, lung myeloperoxidase (MPO), bacterial load, and the generation of thrombin were analyzed. **Results**: PAD4-/- mice are partially protected in CLP-induced sepsis. PAD4-/- mice from 10 hours post-CLP are less hypothermic and had less lung damage than C57Bl/6 mice subjected to CLP. Levels of cfDNA, IL-6, MPO, bacterial load, and thrombin generation were elevated in C57Bl/6 and PAD4-/- mice subjected to CLP surgery compared to healthy and sham-operated controls. Levels of cfDNA, IL-6, MPO, bacterial dissemination in the lungs and peritoneal cavity, and thrombin generation were significantly lower in PAD4-/- mice compared to C57Bl/6 mice 12 hours post-CLP surgery. **Conclusions**: The absence of NETs is associated with blunted inflammatory and coagulant responses in polymicrobial sepsis.

5.2 Introduction

Sepsis is a syndrome with substantial clinical burden for which there are currently no directed therapies. In its earliest form, sepsis presents as a systemic inflammatory response to a microbial infection which may develop into organ dysfunction (severe sepsis) and in its most critical form, the septic patient is in septic shock or hypotension refractory to fluid resuscitation (Dellinger et al., 2013). Severe sepsis afflicts approximately 300 per 100 000 population in the US annually (Angus et al., 2001) and is associated with high mortality rates around 30%-45% (Dellinger et al., 2013). The cost of hospital care for sepsis patients is estimated to be $14 billion annually in the United States (Angus & Wax, 2001). The incidence of severe sepsis continues to increase by 1.5% per annum due to the aging population and wider use of immunosuppressive agents and invasive procedures (H. E. Wang et al., 2012).

Neutrophils play a key role in the host immune response to infection through phagocytosis of pathogens, release of antimicrobial proteins, recruitment of immune cells (Ermert, Zychlinsky, & Urban, 2009), and, through the recently characterized formation of neutrophil extracellular traps (NETs) (Brinkmann et al., 2004). Upon stimulation by microbes, microbial components, or inflammatory mediators (Brinkmann et al., 2004; Papayannopoulos et al., 2010), neutrophils can release NETs comprised of a DNA web decorated by antimicrobial proteins including histones, myeloperoxidase, and neutrophil elastase (Brinkmann et al., 2004; Papayannopoulos et al., 2010; Wartha, Beiter, Normark, & Henriques-Normark, 2007). By binding to microorganisms, NETs prevent further pathogen dissemination and ensure a high local concentration of antimicrobial activity.

NETs are released in the vasculature, and their release has been documented in response to animal sepsis induced by cecal ligation and puncture (CLP) (Cools-Lartigue et al., 2013; Meng, Paunel-Görgülü, Flohé, Hoffmann, et al., 2012). The formation of NETs requires chromatin decondensation (Fuchs et al., 2007) mediated by histone citrullination (Y. Wang et al., 2009), the post-translational conversion of peptidylarginine to citrulline (Vossenaar et al., 2003). The citrullination of histones H3 and H4 is catalyzed by peptidylarginine deiminase 4 (PAD4) (P. Li et al., 2010; Y. Wang et al., 2009), a nuclear protein highly expressed in neutrophils and monocytes (Nakashima, 2002; Vossenaar et al., 2003). Neutrophils from mice deficient in PAD4 protein (PAD4-/-) do not participate in chromatin decondensation or NET production (Hemmers et al., 2011; P. Li et al., 2010). While NETs are immunoprotective, the prolonged presence of NETs in the microcirculation can potentiate aberrant inflammation (Kessenbrock et al., 2009; Knight, Luo, et al., 2014; Kolaczkowska et al., 2015), organ damage (Meng, Paunel-Görgülü, Flohé, Hoffmann, et al., 2012), and pathologic coagulation (i.e. platelet activation (Ammollo et al., 2011; Fuchs et al., 2010) and microvascular thrombosis (Ammollo et al., 2011; Semeraro et al., 2011)).

In contrast to a recent study by Martinod *et al*. (Martinod et al., 2015), PAD deficiency was protective in our polymicrobial sepsis model, cecal ligation and puncture (CLP). While Martinod *et al*. observed that PAD4-/- mice subjected to endotoxemia were partially protected (i.e. delayed mortality, decreased hypothermia and thrombocytopenic, and reduced inflammatory and prothrombotic states), they observed no changes in survival or inflammation in PAD4-/- mice subjected to CLP (Martinod et al., 2015).

Studies of other disease states driven by aberrant inflammation also demonstrate that PAD4 deficiency offers immunoprotective effects. In a model of methicillin-resistant *Staphylococcus aureus* (MRSA), liver injury was significantly reduced in PAD4-/- mice (Kolaczkowska et al., 2015). In models of lupus, PAD4-/- mice have improved endothelial function (Knight et al., 2013), decreased vascular damage (Knight, Subramanian, et al., 2014), and decreased skin inflammation (Knight, Subramanian, et al., 2014). PAD4 deficiency was also immunoprotective in models of atherosclerosis (Knight, Luo, et al., 2014) and deep vein thrombosis (Martinod et al., 2013). In this study, we seek to further characterize the role of NETs during the host response to infection. We will investigate the protective effects of abrogated NET formation in PAD4 deficient mice on inflammation and coagulation in a CLP model of sepsis.

5.3 Materials and methods

5.3.1 Experimental sepsis-cecal ligation and puncture

8 week-old (20 to 25 g) male C57Bl/6 mice (*Helicobacter hepaticus*-free) were bred at the Thrombosis and Atherosclerosis Research Institute (Hamilton, ON, Canada). PAD4-/- mice were generously provided by Dr. Paul Kubes (University of Calgary, AB, Canada) and Dr. Kerri Mowen (The Scripps Research Institute, San Diego, CA, USA). The mice were housed in a Helicobacter/MNV-negative clean room in individually ventilated cages (Tecniplast Sealsafe Plus system) under 12 hr:12 hr dark/light cycles. Air was filtrated by prefilters, HEPA filters, and Touch Slimline air handling units. The mice were provided enrichment and allowed unlimited access to sterilized food (Harlan Teklad Rodent Diet #2018) and water. Mice received humane care in accordance with the current

guidelines outlined by the Canadian Council on Animal Care (CCAC). These studies were approved by the Animal Research Ethics Board (AREB) at McMaster University (AUP 10-12-79, Hamilton, ON, Canada).

The cecal ligation and puncture (CLP) model used in these studies were adapted from well-established protocols by others (Baker et al., 1983; Hubbard et al., 2005). Under anesthesia, a midline laparotomy was performed, the cecum was exteriorized, ligated 1 cm from the cecal end, and perforated through-and-through with an 18G needle. In mice subjected to sham surgery, the cecum was exteriorized and returned to the peritoneal cavity. Mice were randomized in the CLP or sham groups according to ARRIVE guidelines (Kilkenny et al., 2013) and methods for randomization and experimenter blinding were in place to reduce allocation, selection, and experimenter biases (Mai et al., 2012). CLP and sham mice were given analgesia (Buprenorphine, 0.1 mg/kg) for pain relief and resuscitation fluids (2 mL, Ringer's lactate) subcutaneously every 4 hours to account for surgical losses. At 4, 8, or 12 hours post-surgery, blood was collected via the inferior vena cava (IVC) into a one-tenth volume of 3.2% sodium citrate, prepared by centrifugation at 2000 x g, and the resulting platelet-poor plasma was stored in aliquots at -80ºC.

5.3.2 Survival studies

Mice were subjected to either sham or CLP surgery and monitored continuously until endpoint (inability to ambulate) or 20 hours post-surgery. Surrogate markers were monitored (Supplemental Digital Content 1) and temperatures were measured every 4 hours using a rectal probe thermometer. Temperature by rectal probe measurement was

found to be a reliable surrogate marker of death in our sepsis model (data not shown). Blood and organs were collected when mice were no longer ambulant prior to death. Blood was collected via the IVC into a one-tenth volume of 3.2% sodium citrate and prepared by centrifugation at 2000 x g. The resulting platelet-poor plasma was stored in aliquots at -80ºC. At endpoint, mice were euthanized by cervical dislocation.

5.3.3 Isolation and quantification of cell-free DNA and interleukin (IL)-6

Cell-free DNA (cfDNA) was isolated from 200 µL of mouse plasma using the Qiagen QIAamp® DNA Blood Mini Kit (QIAgen, Valencia, CA, USA) and quantified by measuring absorbance at 260 nm using an Eppendorf BioPhotometer Plus (Hamburg, Germany). IL-6 levels were quantified using an ELISA-based Duoset Mouse IL-6 Immunoassay kit (R&D Systems, Minneapolis, MN, USA), according to the manufacturer's protocol.

5.3.4 Determination of myeloperoxidase (MPO) activity

Enzymatic MPO is found in azurophilic granules of polymorphonuclear cells and is indicative of neutrophil accumulation in inflamed tissues. Mice were subjected to sham or CLP surgery and 12 hours post-operatively, lungs were collected, frozen in liquid nitrogen and stored at -80ºC. MPO enzymatic activity in the lungs was quantified within 1 week of surgery. 0.050 g of lung tissue was homogenized in ice-cold phosphate-buffered saline (PBS, Sigma-Aldrich, St. Louis, MO, USA) and centrifuged at 10 000 x g for 10 minutes in 4ºC. The supernatant was discarded and the pellet was resuspended and homogenized in potassium phosphate buffer (K_2HPO_4, 50 mM, pH 6.0) containing hexadecyltrimethylammonium bromide (HTAB, 0.5% w/v). Following centrifugation at

3000 x g for 15 minutes, the supernatant was collected and MPO enzymatic activity was quantified by measuring the H_2O_2-dependent oxidation of *o*-dianisidine. One unit (U) of MPO activity is defined as MPO levels per gram of lung tissue which caused a change in absorbance of 1.0/minute at 450 nm.

5.3.5 Thrombin generation assay

Citrated platelet-poor plasma (PPP) collected from healthy mice and mice subjected to sham or CLP surgery for 12 hours was used for thrombin generation experiments. Thrombin generation was run with 50 μL of PPP in the presence of 15 mM $CaCl_2$ and 0.5 mM Z-Gly-Gly-Arg-AMC substrate (Bachem, Bubendorf, Switzerland). Thrombin generation was monitored over 2 hours at 37°C and analyzed using Technothrombin TGA software (Technoclone, Vienna, Austria).

5.3.6 Bacterial load, organ histology, and staining procedures

In bacterial culture experiments, lungs were homogenized and peritoneal cavity fluid (PCF) was collected in ice-cold PBS. Fresh blood, lung lysates, and PCF diluted in PBS were plated on 5% sheep blood agar plates (Teknova, Hollister, CA, USA), and incubated for 24 hours. For experiments investigating organ histology, sham- and CLP-operated mice were perfused with heparinized saline and 10% neutral buffered formalin and organs were collected in formalin, processed, embedded in paraffin wax, and sectioned at 5 μm thick. Organ sections were stained with hematoxylin and eosin (H&E) to visualize overall morphology (nuclei and extracellular DNA appear bluish-purple and cytoplasm, connective tissue, and collagen appear pink). Photomicrographs of stained

lung, liver, and kidney sections were visualized under 200x magnification. The length of the scale bar represents 25μm.

5.3.7 Statistical analyses

Data are represented as mean ± SEM or median where specified and results across multiple groups were compared using one-way analysis of variance (ANOVA) and Newman-Keuls post-hoc test. Data were considered significant at $p < 0.05$. Analyses were performed using GraphPad Prism 4.0 (La Jolla, CA, USA) and SigmaPlot 11.0 (San Jose, CA, USA).

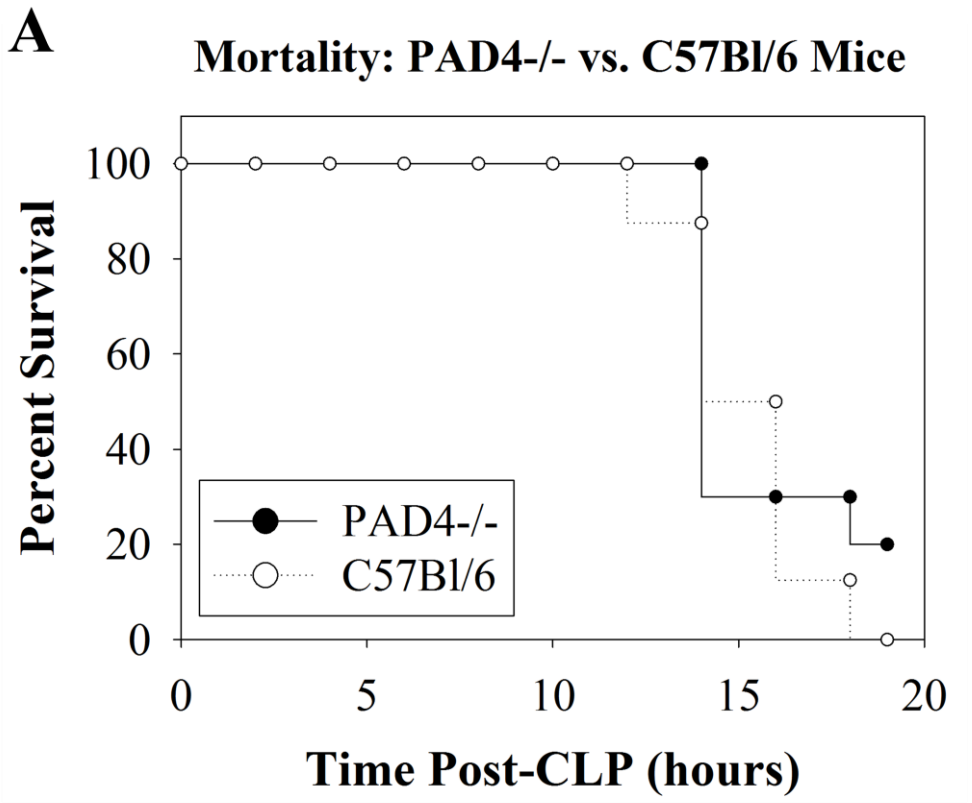
5.4 Results

The survival of PAD4-/- mice versus C57Bl/6 mice was similar although a modest percentage of PAD4-/- mice survived at 19 hours post-CLP surgery (Figures 5.1A, 5.3, 5.3). All sham-operated mice survived in both groups (data not shown). Temperatures in C57Bl/6 mice dropped below those of PAD4-/- mice from 10 hours post-CLP until the end of the study period (19 hours post-CLP, Figure 5.2B).

5.4.1 Levels of cell-free DNA and IL-6 following CLP surgery are suppressed in PAD4-/- mice

Levels of cfDNA were elevated in C57Bl/6 mice subjected to CLP for 8 and 12 hours compared with sham-operated and healthy mice (Figure 5.4A). Levels of cfDNA in PAD4-/- mice did not change significantly over the 12 hour post-operative period in sham- or CLP-operated mice. At 12 hours post-CLP, cfDNA levels in C57Bl/6 mice were

Figure 5.1. Kaplan-Meier curve and temperatures of PAD4-/- vs. C57Bl/6 mice subjected to CLP surgery. PAD4-/- and C57Bl/6 mice were subjected to CLP surgery and monitored continuously for up to 20 hours post-operatively (A). Temperatures (B) were taken via a rectal probe thermometer every 4 hours until endpoint (n = 10-16, ANOVA $p < 0.001$).


A
Mortality: PAD4-/- vs. C57Bl/6 Mice
Percent Survival
100
80
60
40
20
0
0
5
10
15
20
PAD4-/-
C57Bl/6
Time Post-CLP (hours)

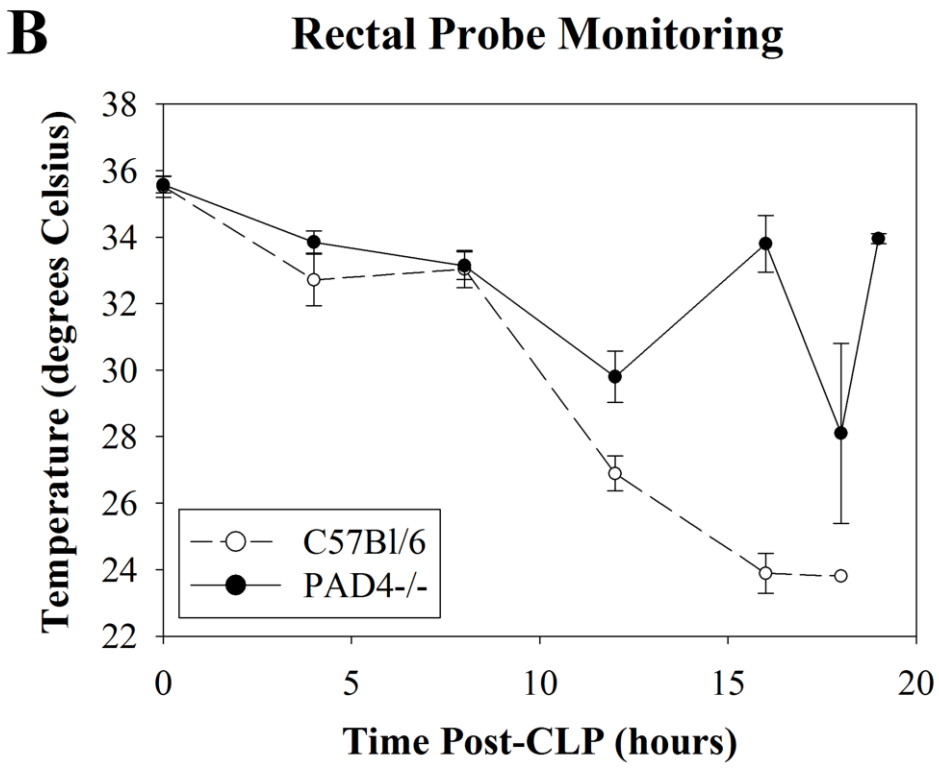

B
Rectal Probe Monitoring
Temperature (degrees Celsius)
38
36
34
32
30
28
26
24
22
0
5
10
15
20
C57Bl/6
PAD4-/-
Time Post-CLP (hours)

Figure 5.2. Mouse Grimace Scores in PAD4-/- and C57Bl/6 mice following CLP surgery. Surrogate endpoints were monitored in survival studies of PAD4-/- and C57B/6 mice subjected to CLP surgery from 0-20 hours post-operatively. The components of the Mouse Grimace Score (MGS) include orbital tightening (A) nose and cheek bulge (B), ear positioning (C), and whisker change (D). The average of these components comprise the MGS (E)

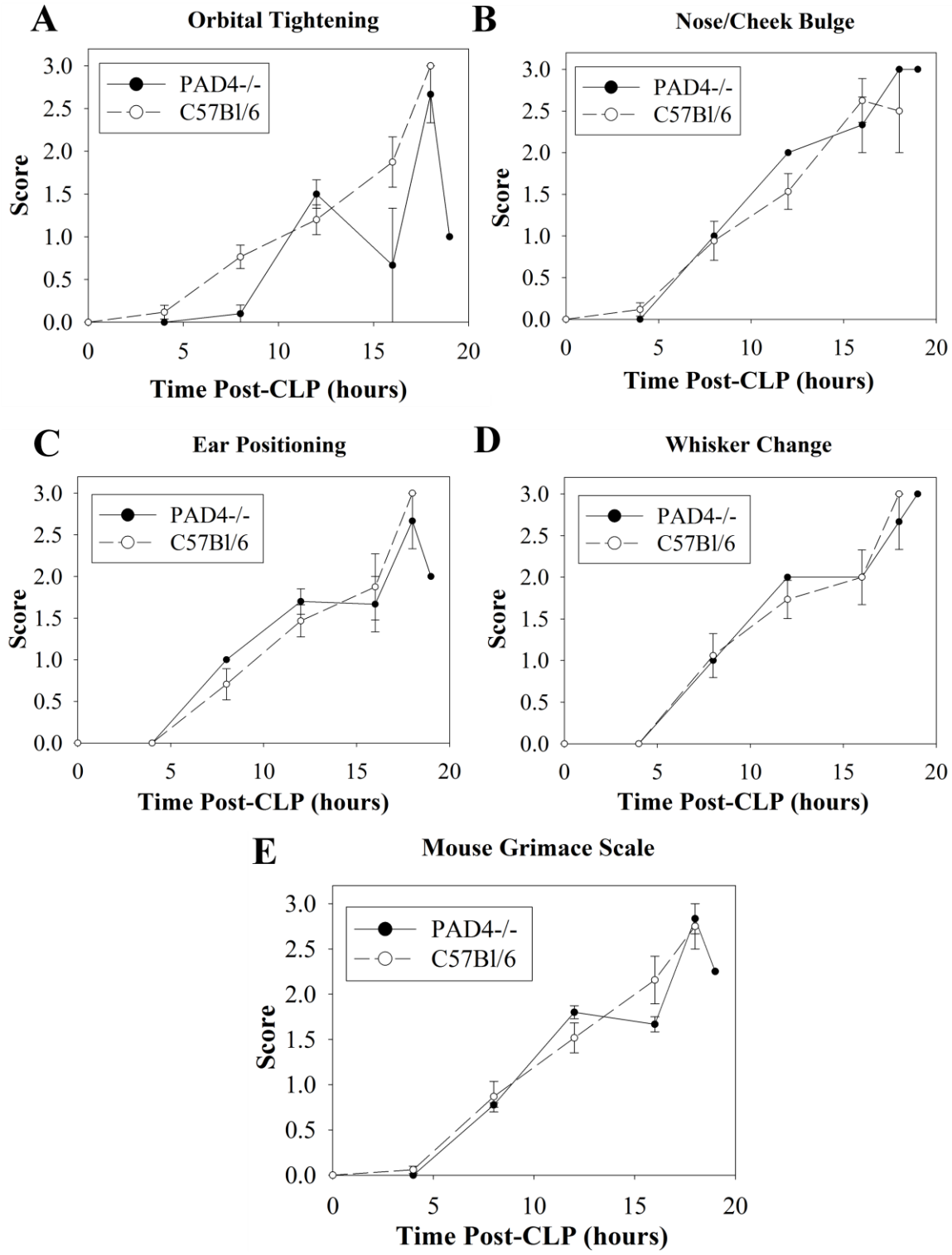

A
Orbital Tightening
B
Nose/Cheek Bulge
C
Ear Positioning
D
Whisker Change
E
Mouse Grimace Scale
PAD4-/-
C57Bl/6
Score
Time Post-CLP (hours)
0.0
0.5
1.0
1.5
2.0
2.5
3.0
0
5
10
15
20

Figure 5.3. Surrogate markers in PAD4-/- and C57Bl/6 mice following CLP surgery. Surrogate endpoints were monitored in survival studies of PAD4-/- and C57B/6 mice subjected to CLP surgery from 0-20 hours post-operatively. The surrogate markers are hunched posture or little movement (A), laboured breathing/dyspnea (B), cold, blue extremities or cyanosis (C), restlessness or tremors (D), responsiveness (E), and vocalizations (E).

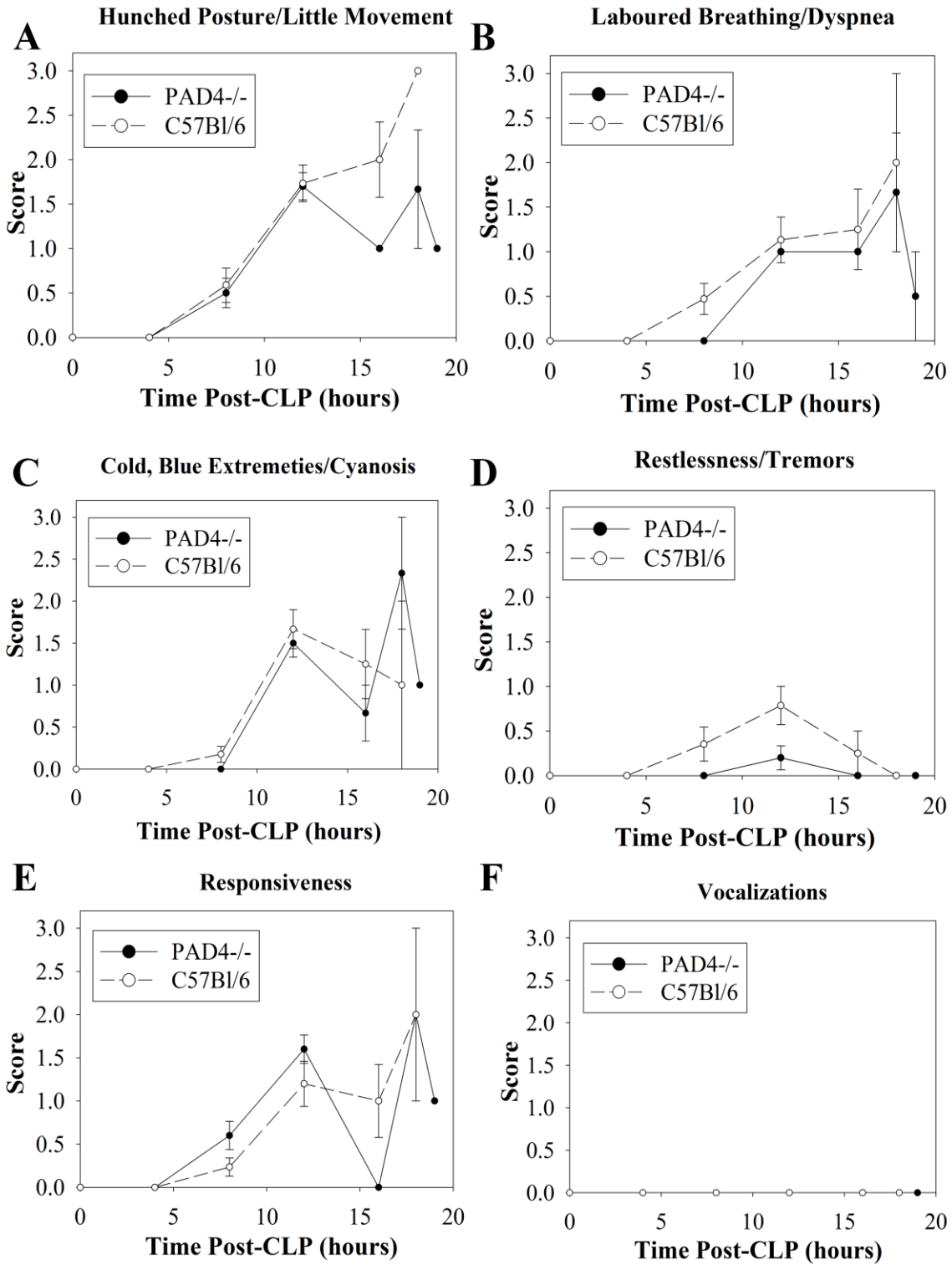
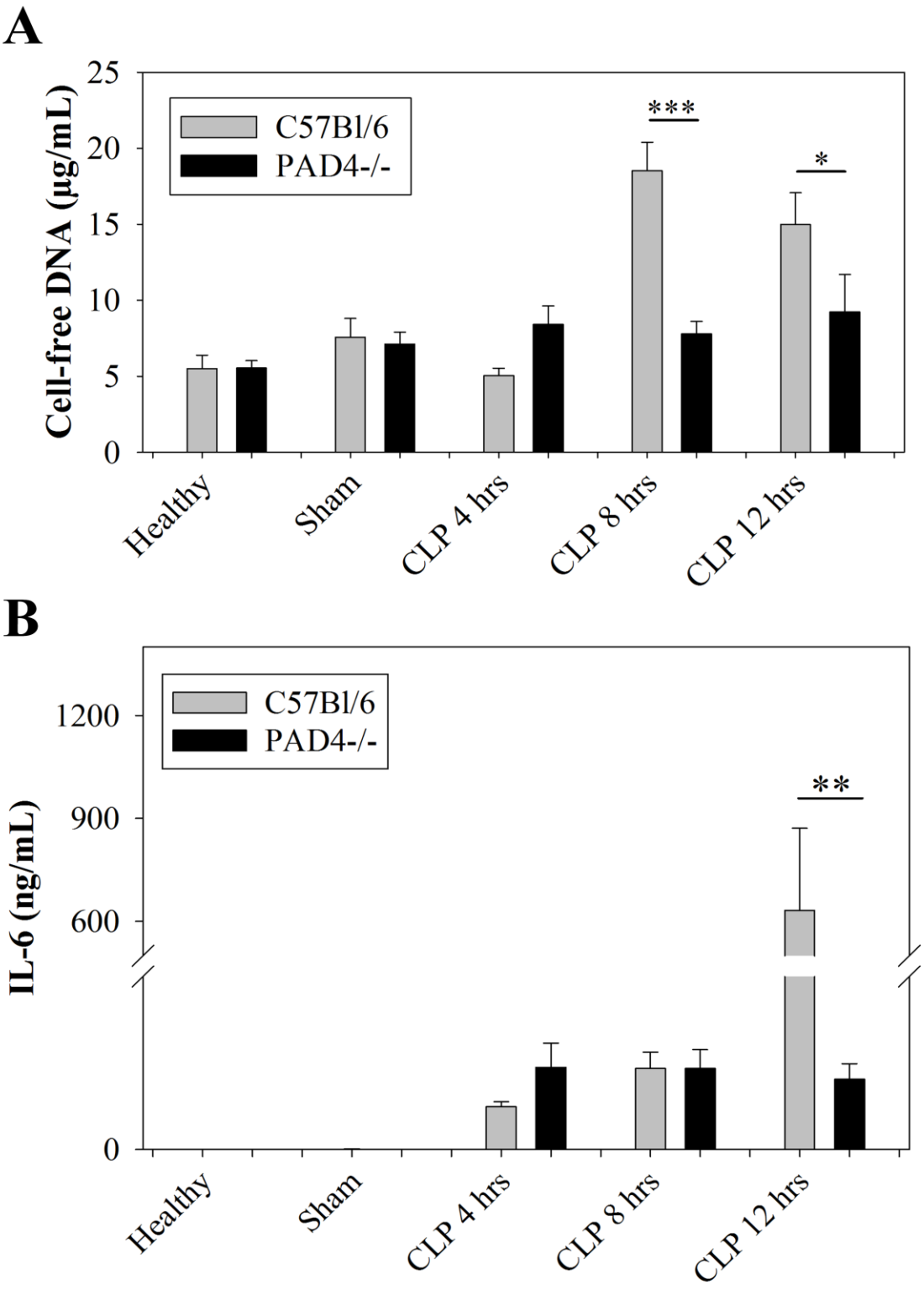

A
Hunched Posture/Little Movement
B
Laboured Breathing/Dyspnea
C
Cold, Blue Extremeties/Cyanosis
D
Restlessness/Tremors
E
Responsiveness
F
Vocalizations
PAD4-/-
C57Bl/6
Score
Time Post-CLP (hours)
0.0
0.5
1.0
1.5
2.0
2.5
3.0
0
5
10
15
20

Figure 5.4. Time-course of cell-free DNA and IL-6 levels in healthy, sham-operated, and CLP-operated PAD4-/- and C57Bl/6 mice. Mice were subjected to sham or CLP surgery for 4, 8, or 12 hours and blood was collected via the inferior vena cava. Plasma levels of cell-free DNA (A) and IL-6 (B) were quantified (ANOVA $p < 0.001$, * $p < 0.05$, ** $p < 0.01$, *** $p < 0.005$, n = 5-8 for cell-free DNA and n = 6-10 for IL-6 per surgery group and time-point).


A
Cell-free DNA (µg/mL)
25
20
15
10
5
0
C57Bl/6
PAD4-/-
***
*
Healthy
Sham
CLP 4 hrs
CLP 8 hrs
CLP 12 hrs
B
IL-6 (ng/mL)
1200
900
600
0
C57Bl/6
PAD4-/-
**
Healthy
Sham
CLP 4 hrs
CLP 8 hrs
CLP 12 hrs

almost double those observed in PAD4-/- mice (12.94 ± 0.60 µg/mL versus 6.84 ± 0.75 µg/mL in C57Bl/6 versus PAD4-/- mice respectively). IL-6 levels in C57Bl/6 mice remained low in healthy sham-operated C57Bl/6 mice (0.012 ± 0.0015 ng/mL in healthy and 0.019 ± 0.0026 ng/mL in sham-operated mice) and increased in a time-dependent manner in CLP-operated mice from 4-12 hours post-CLP surgery (Figure 5.4B). IL-6 levels in CLP-operated PAD4-/- mice were elevated at all time-points compared to healthy and sham-operated PAD4-/- mice but did not change significantly from 4-12 hours post-CLP surgery. At 12 hours post-CLP, IL-6 levels in C57Bl/6 mice were significantly higher than those in PAD4-/- mice (median IL-6 levels of 298.23 ng/mL in C57Bl/6 mice vs. 17.75 ng/mL in PAD4-/- mice, Figure 5.4B).

5.4.2 Neutrophil accumulation following CLP surgery is reduced in PAD4-/- mice

Lung MPO activity was low in healthy mice (6.29 ±1.28 U/g lung tissue and 3.64 ± 1.35 U/g lung tissue, C57Bl/6 versus PAD4-/- mice respectively), slightly elevated in sham-operated mice (12.59 ± 1.88 U/g lung tissue and 11.98 ± 0.93 U/g lung tissue, C57Bl/6 versus PAD4-/- mice respectively), and significantly elevated in CLP-operated mice (Figure 5.5). MPO activity was higher in C57Bl/6 mice compared to PAD4-/- mice however, this difference was not statistically significant (53.47 ± 10.35 U/g lung tissue and 29.44 ± 4.75 U/g lung tissue, C57Bl/6 versus PAD4-/- mice respectively).

5.4.3 Bacterial dissemination following CLP surgery is reduced in the lungs, blood, and peritoneal cavity fluid of PAD4-/-mice

No bacterial colonies were observed in healthy or sham-operated mice of either strain (Figure 5.6) while increased bacterial colonies were observed in CLP-operated

mice of both strains. Bacterial colonies from the lungs (Figure 5.6A) and peritoneal cavity (Figure 5.6C) were significantly higher in C57Bl/6 mice versus PAD4-/- mice. Increased bacterial dissemination was observed in the blood (Figure 5.6B) of C57Bl/6 mice versus PAD4-/- mice, however this difference was not statistically significant. Photomicrographs of lungs, livers, and kidneys from healthy, sham-operated, and CLP-operated mice show increased edema, alveolar thickening, hepatic and tubular vacuolization, and congestion in CLP-operated mice compared to sham-operated and healthy control mice. Thickening of the alveolar border, edema, and pulmonary congestion appear to be reduced in the lungs of CLP-operated PAD4-/- mice (Figure 5.7B) compared to CLP-operated C57Bl/6 mice (Figure 5.7A).

5.4.4 Plasma from septic PAD4-/- mice generates less thrombin than plasma from septic C57Bl/6 mice

Recalcified platelet-poor plasma (PPP) from CLP-operated C57Bl/6 mice generated more thrombin than recalcified PPP from PAD4-/- mice (Figure 5.8). Peak thrombin (Figure 5.9B) and the endogenous thrombin potential or AUC (Figure 5.9D) are reduced in plasma from PAD4-/- mice subjected to CLP compared with plasma from C57Bl/6 mice subjected to CLP.

PAD4-/- mice subjected to CLP surgery have reduced hypothermia, less lung damage, and decreased levels of cfDNA, IL-6, MPO, and bacterial dissemination in the lungs, blood, and peritoneal cavity compared to CLP-operated C57Bl/6 mice. Thrombin generation is also reduced in CLP-operated PAD4-/- mice compared to CLP-operated C57Bl/6 mice.

Figure 5.5. Myeloperoxidase (MPO) levels in the lungs of healthy, sham-operated, and CLP-operated PAD4-/- and C57Bl/6 mice. Mice were subjected to sham or CLP surgery for 12 hours and lungs were collected and frozen in liquid nitrogen. Levels of MPO in the lung were quantified within 1 week of collection (n = 5-6 per group).

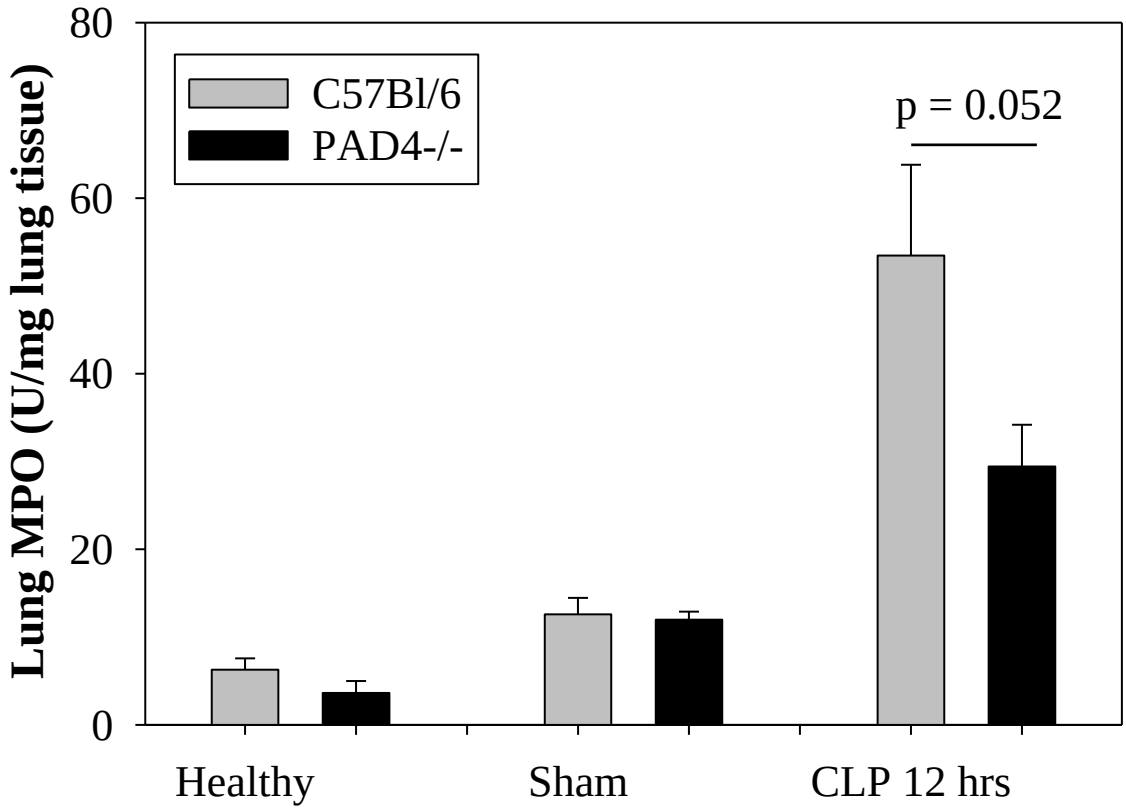

80
60
40
20
0
Lung MPO (U/mg lung tissue)
C57Bl/6
PAD4-/-
p = 0.052
Healthy
Sham
CLP 12 hrs

Figure 5.6. Bacterial cultures from healthy, sham-operated, and CLP-operated PAD4-/- and C57Bl/6 mice. PAD4-/- and C57Bl/6 mice subjected to sham and CLP surgery for 12 hours. Lungs were homogenized in PBS, blood was collected and diluted in PBS, and the peritoneal cavity was flushed with PBS. Lung lysate (A), blood (B), and peritoneal cavity fluid (C) were diluted in PBS and plated on to blood agar plates. Bacterial cultures were kept in 37ºC for 24 hours and the number of colonies were counted ($n = 5$ per group, except blood from PAD4-/- mice where $n = 2$, * $p < 0.05$, ** $p < 0.01$, *** $p < 0.005$).

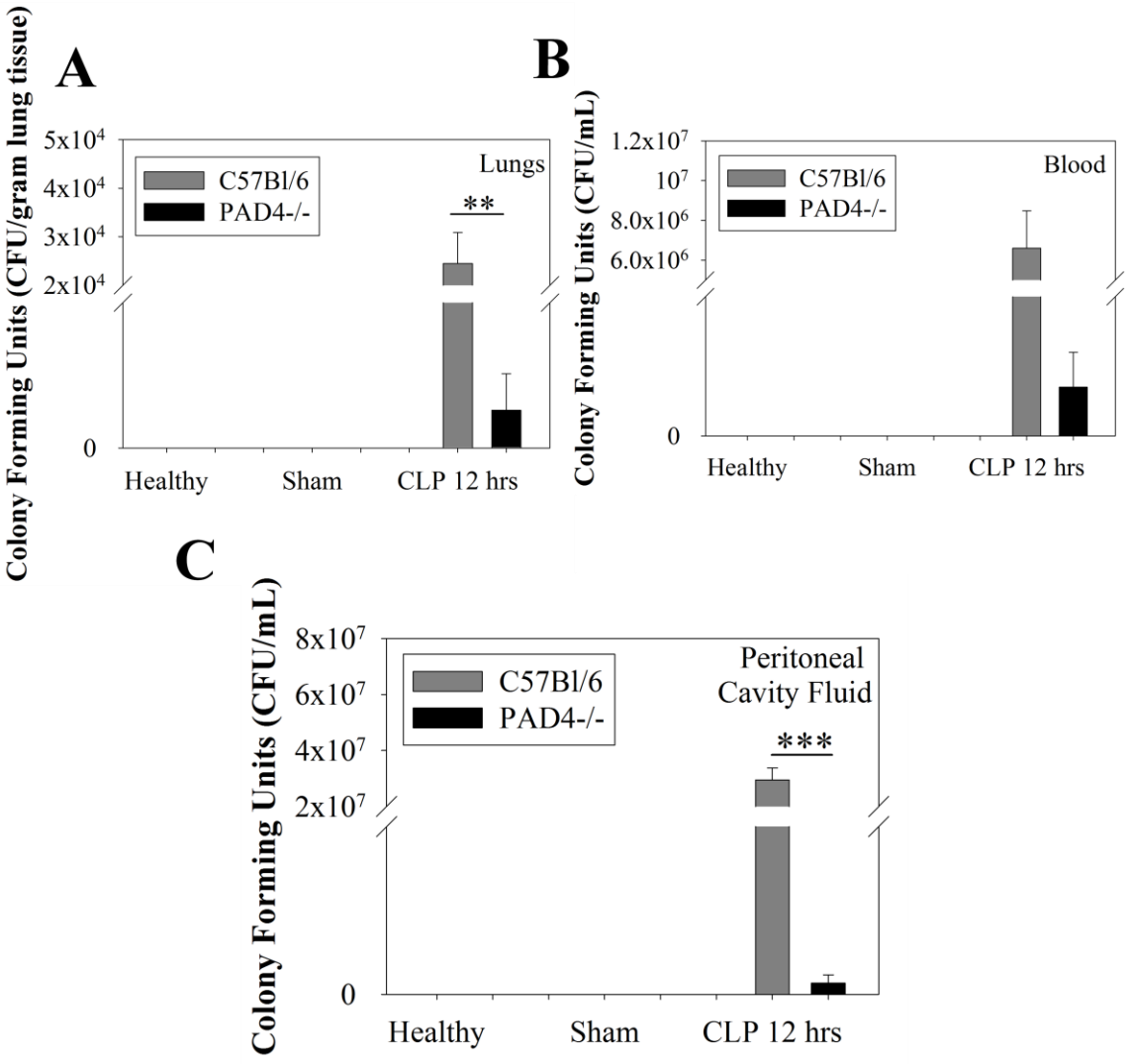

A
Colony Forming Units (CFU/gram lung tissue)
5x10^4
4x10^4
3x10^4
2x10^4
0
C57Bl/6
PAD4-/-
Lungs
**
Healthy
Sham
CLP 12 hrs
B
Colony Forming Units (CFU/mL)
1.2x10^7
10^7
8.0x10^6
6.0x10^6
0
C57Bl/6
PAD4-/-
Blood
Healthy
Sham
CLP 12 hrs
C
Colony Forming Units (CFU/mL)
8x10^7
6x10^7
4x10^7
2x10^7
0
C57Bl/6
PAD4-/-
Peritoneal
Cavity Fluid
***
Healthy
Sham
CLP 12 hrs

Figure 5.7. Photomicrographs of end organs from PAD4-/- and C57Bl/6 mice subjected to sham and CLP surgery and healthy mice. C57Bl/6 (A) and PAD4-/- mice (B) were subjected to sham or CLP surgery and under anaesthesia, mice were perfused with heparinized saline and neutral buffered formalin. Organs were embedded and sectioned at 5 μm in thickness, stained with hematoxylin and eosin, and photomicrographs were taken of the lung, liver, and kidney. Healthy mice were subjected to the same perfusion procedure and organs were harvested in the same manner to serve as healthy controls.

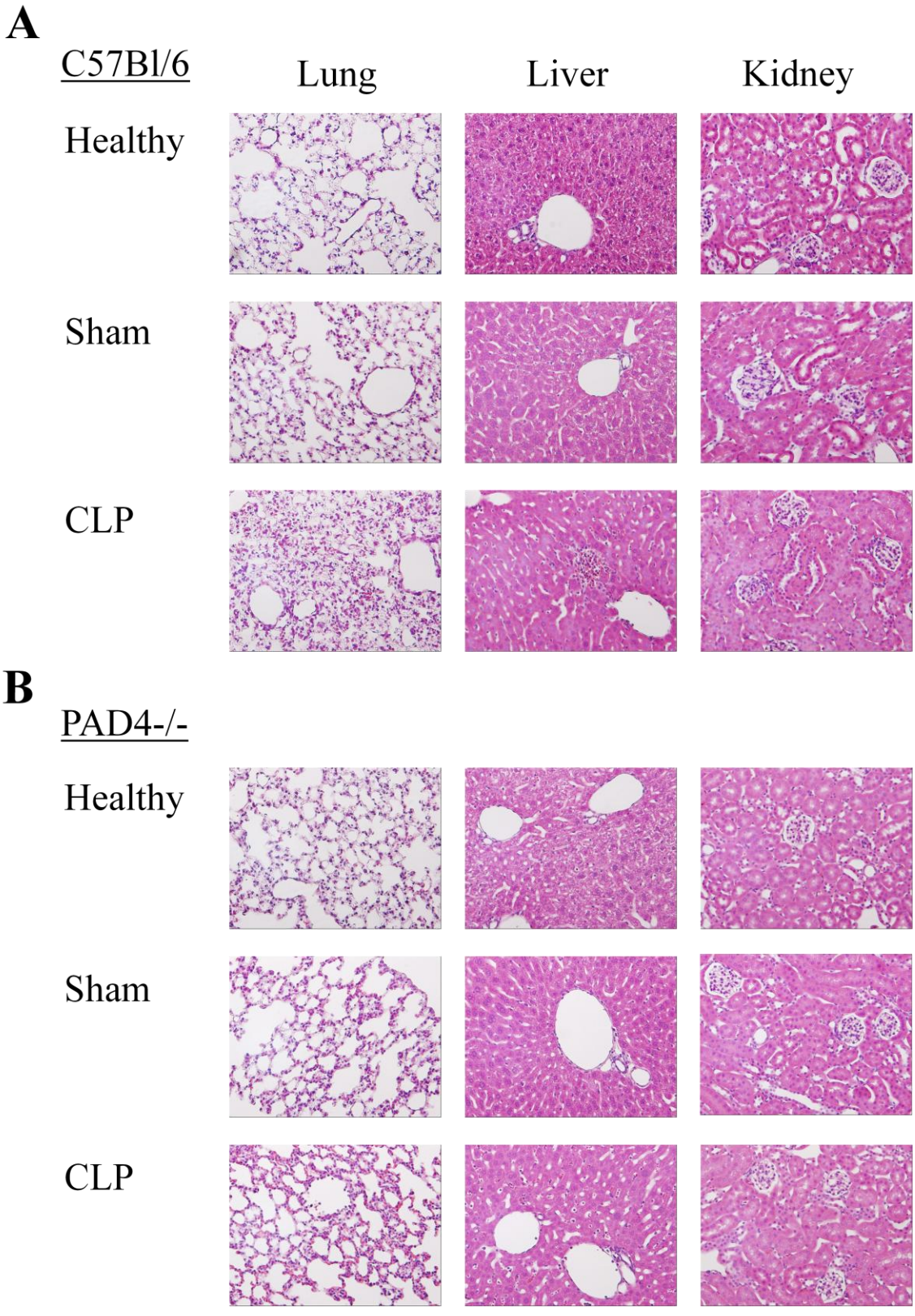

A
C57Bl/6
Lung
Liver
Kidney
Healthy
Sham
CLP
B
PAD4-/-
Healthy
Sham
CLP

Figure 5.8. Thrombin generation in recalcified plasma from healthy, sham-operated, and CLP-operated PAD4-/- and C57Bl/6 mice. Mice were subjected to sham or CLP surgery for 12 hours and blood was collected via the inferior vena cava. Blood was collected in the same manner from healthy control mice. Platelet-poor plasma was isolated and recalcified in 15 mM $CaCl_2$. Thrombin generation at 37ºC was monitored over 2 hours (n = 5-6 per group).

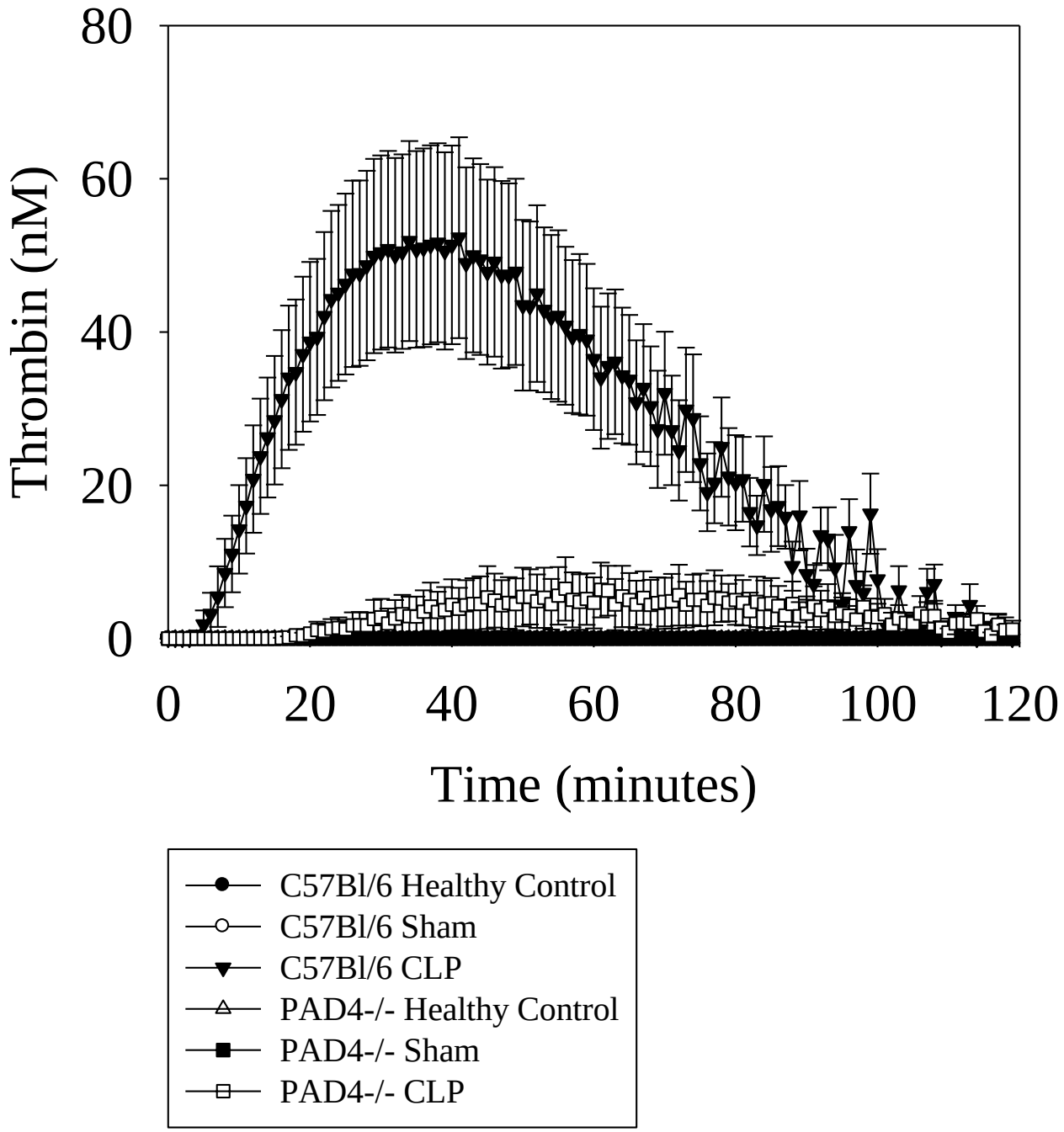
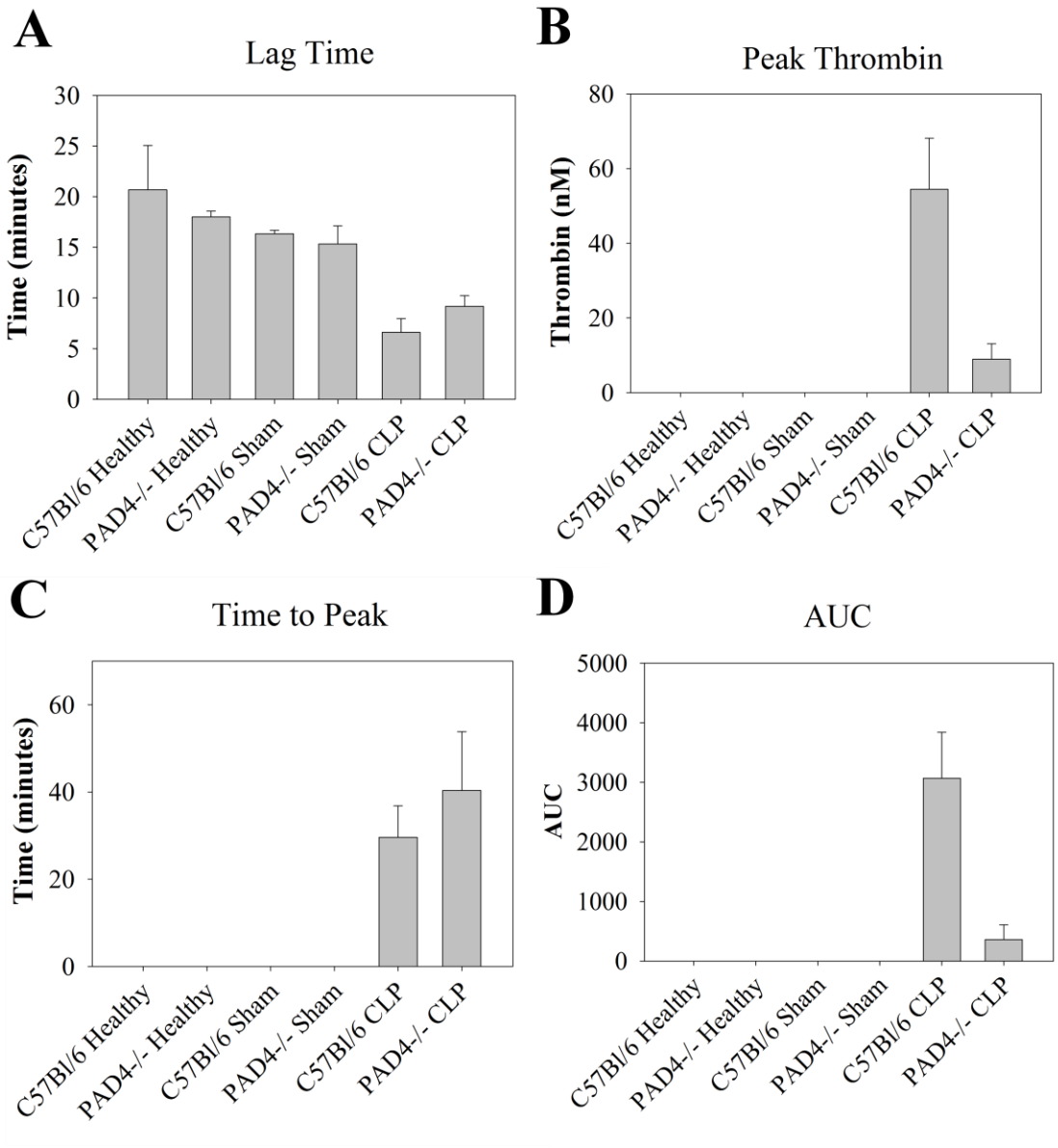

80
60
40
20
0
Thrombin (nM)
0
20
40
60
80
100
120
Time (minutes)
C57Bl/6 Healthy Control
C57Bl/6 Sham
C57Bl/6 CLP
PAD4-/- Healthy Control
PAD4-/- Sham
PAD4-/- CLP

Figure 5.9. Parameters of thrombin generation in plasma from healthy, sham-operated, and CLP-operated PAD4-/- and C57Bl/6 mice. Mice were subjected to sham or CLP surgery for 12 hours and blood was collected via the inferior vena cava. Blood was collected in the same manner from healthy control mice. Platelet-poor plasma was isolated and recalcified in 15 mM $CaCl_2$. Thrombin generation at 37ºC was monitored over 2 hours (n = 5-6 per group). Lag time (A), peak thrombin (B), time to peak (C), and endogenous thrombin potential or area under the curve (D) were documented (n = 5-6).


A
Lag Time
Time (minutes)
0
5
10
15
20
25
30
C57Bl/6 Healthy
PAD4-/- Healthy
C57Bl/6 Sham
PAD4-/- Sham
C57Bl/6 CLP
PAD4-/- CLP
B
Peak Thrombin
Thrombin (nM)
0
20
40
60
80
C57Bl/6 Healthy
PAD4-/- Healthy
C57Bl/6 Sham
PAD4-/- Sham
C57Bl/6 CLP
PAD4-/- CLP
C
Time to Peak
Time (minutes)
0
20
40
60
C57Bl/6 Healthy
PAD4-/- Healthy
C57Bl/6 Sham
PAD4-/- Sham
C57Bl/6 CLP
PAD4-/- CLP
D
AUC
AUC
0
1000
2000
3000
4000
5000
C57Bl/6 Healthy
PAD4-/- Healthy
C57Bl/6 Sham
PAD4-/- Sham
C57Bl/6 CLP
PAD4-/- CLP

5.5 Discussion

In contrast to a previous report (Martinod et al., 2015), our studies demonstrate that PAD4 deficiency is immunoprotective in a CLP sepsis model. PAD4-/- mice subjected to CLP surgery have a suppressed inflammatory response as evidenced by decreased plasma IL-6, neutrophil accumulation in the lung, and bacterial dissemination in the blood, lung, and peritoneal cavity compared with CLP-operated C57Bl/6 mice. In addition, thrombin generation and organ damage are reduced in CLP-operated PAD4-/- mice compared to CLP-operated C57Bl/6 mice.

The reduction in the inflammatory response, decrease in hypothermia, and suppression of the prothrombotic state in PAD4-/- mice following CLP surgery observed in our studies were also observed in a murine model of endotoxemia (Martinod et al., 2015). In contrast to our findings, no changes in inflammation or survival were observed in the CLP sepsis model in the same study (Martinod et al., 2015). Levels of inflammation and bacterial dissemination in the Martinod *et al*. study were much lower than previously reported levels (Andrejko, Chen, & Deutschman, 1998; King, Bauzá, Mella, & Remick, 2014; Lygizos et al., 2013; Mai et al., 2015; Mella, Chiswick, King, & Remick, 2014; Meyer et al., 1995; Remick et al., 2000). For example, levels of circulating IL-6 observed at 18 hours following high-grade CLP in Martinod *et al*., were observed at 2-4 hours post-CLP by other groups (Mella et al., 2014; Remick et al., 2000). Similarly, bacterial counts in the Martinod *et al*. study were also much lower than levels previously reported by others (Deng, 2006; Hotchkiss et al., 2000; Muenzer et al., 2006, 2010). It is possible that in the CLP protocols used by Martinod *et al.*, significantly less fecal matter

is extruded compared to protocols used in other studies. If an inadequate amount of fecal material is extruded, the puncture holes become less patent and fecal matter fails to leak from the punctures post-operatively. This would result in a model closer to abscess formation (Buras et al., 2005; Nemzek, Hugunin, & Opp, 2008) which would address the relatively low levels of inflammatory markers and bacterial load observed by Martinod *et al*. relative to previous CLP studies. It is recognized that interpreting data from various CLP studies has inherent limitations as surgeries can vary greatly between researchers, protocols used, and even the institutions at which surgeries are performed (Buras et al., 2005; Dyson & Singer, 2009; Mai et al., 2012).

Our data support previous studies in which PAD4 deficiency reduces inflammation in disease states where NETs contribute to pathologic inflammation. The inhibition of NET formation via PAD inhibitors (Cl-amidine and BB-Cl-amidine) also reduces the inflammatory response (Knight et al., 2013; Knight, Luo, et al., 2014; Knight, Subramanian, et al., 2014), however no discernible effect is observed in survival. While the pathologic role of NETs has been observed in models of vasculitis (Kessenbrock et al., 2009), MRSA infection (Kolaczkowska et al., 2015), thrombosis (Fuchs et al., 2010; Knight, Luo, et al., 2014), and systemic lupus erythematosus (Garcia-Romo et al., 2011; Hakkim et al., 2010; Knight et al., 2013; Knight, Subramanian, et al., 2014; Villanueva et al., 2011), we have extended these observations to polymicrobial sepsis. Inhibition of NET formation may be protective in diseases where pathology is driven by aberrant inflammation and coagulation.

Chapter 6: General Discussion

Many potential therapies showed early promise in their therapeutic utility in preclinical sepsis studies. From these studies, more than 100 Phase II and Phase III clinical trials have been conducted (J. C. Marshall, 2014). For a great majority, these studies attempt to treat sepsis by either dampening inflammation or reducing aberrant coagulation, yet there is currently no approved biological treatment for sepsis (J. C. Marshall, 2014; Opal & Patrozou, 2009). Current strategies are largely supportive and aim to ensure adequate fluid resuscitation, ventilation, and microbial control with early administration of broad-spectrum antibiotics (Dellinger et al., 2013). Despite these strategies, the mortality rate from sepsis remains high, suggesting that some fundamental knowledge is lacking in our understanding of sepsis pathophysiology.

In recent years, cell-free DNA (cfDNA) has emerged as an important link between innate immunity and coagulation. Studies in various models of inflammation and thrombosis have demonstrated that cfDNA levels increase and that cfDNA potentiates inflammation and clotting (Atamaniuk, Kopecky, Skoupy, Säemann, & Weichhart, 2012; Clark et al., 2007; Cools-Lartigue et al., 2013; Hamaguchi et al., 2015; Kolaczkowska et al., 2015; Luo et al., 2014; Martinod et al., 2015; McDonald et al., 2012; Meng, Paunel-Görgülü, Flohé, Hoffmann, et al., 2012; Tanaka et al., 2014). In septic patients, cell-free DNA levels are elevated and levels of cfDNA upon admission into the ICU have strong prognostic value in predicting outcome in severe sepsis patients (Avriel et al., 2014; Dwivedi et al., 2012; Moreira, Prieto, Rodríguez, & Alvarez, 2010; Rhodes, Wort, Thomas, Collinson, & Bennett, 2006; Saukkonen et al., 2008; Wijeratne et al., 2004). The

source of cfDNA in these septic patients has also been investigated (van der Vaart & Pretorius, 2007). *In vitro* studies have demonstrated that neutrophils are the major source of cfDNA release following inflammatory challenge (Gould et al., 2014). However, it is unclear if the source of cfDNA following septic insult *in vivo* is neutrophils.

One mechanism by which cfDNA is released is via NET formation (Brinkmann et al., 2004). While NETs support innate immunity, prolonged NET formation can induce injury to the host by releasing pro-inflammatory and procoagulant mediators (Brinkmann & Zychlinsky, 2007; Clark et al., 2007; J. Xu et al., 2009) and inducing tissue hypoperfusion, ischemia, and damage in the end organs (Clark et al., 2007; Martinod et al., 2015; McDonald et al., 2012). NETs are counter-regulated by DNaseI *in vivo* however, the protective effects of DNase treatment are not clear (Meng, Paunel-Görgülü, Flohé, Witte, et al., 2012).

The general aims of the studies in this thesis were to investigate the changes in cfDNA in response to CLP-induced sepsis, the role of cfDNA in the pro-inflammatory and procoagulant responses in sepsis, the effect of *in vivo* DNase treatment in sepsis, and the contribution of NET formation to changes in the response in sepsis using PAD4-deficient mice. In addition, methods were developed to increase ethical standards in murine sepsis studies.

6.1 Cell-free DNA in experimental sepsis

In our murine sepsis studies, we observed a sustained increase in cfDNA levels in an LPS model of endotoxemia (Figure 6.1) and a time-dependent, sustained increase in cfDNA levels following sepsis induction via CLP surgery (Figure 3.1) compared to

control mice. Levels of cfDNA in CLP-operated mice increased at 4 hours post-operatively and remained elevated up to the study endpoint at 16 hours post-operatively, at which point 100% lethality was observed in our resuscitated sepsis model (Figure 3.8). These findings support previous studies documenting transient elevations in cfDNA (Meng, Paunel-Görgülü, Flohé, Hoffmann, et al., 2012) and sustained increases in circulating cfDNA in CLP sepsis studies (Hamaguchi et al., 2015; Y. Wang et al., 2014). Increased cfDNA levels in the peritoneal cavity, plasma, and BALF have also been documented suggesting that cfDNA release occurs both locally in the lungs and peritoneum and systemically in circulation (Luo et al., 2014).

While the profile of changes in cfDNA levels were similar in our studies and other reports, the levels of cfDNA reported at each time-point varied greatly across studies, ranging from 400 ng/mL to 6000 ng/mL at 6-hours post-CLP (Hamaguchi et al., 2015; Luo et al., 2014; Mai et al., 2015; Meng, Paunel-Görgülü, Flohé, Hoffmann, et al., 2012). These differences may be due to differences in the CLP model and appear to correspond with the severity of the model. Hamaguchi *et al*. used the least severe model (single puncture, 22G needle) and observed the lowest levels of plasma cfDNA reported in CLP studies. In contrast, our model seems to be the most severe model (double puncture, 18G needle) and corresponded to the highest levels of cfDNA reported. The higher mortality rate at a much earlier time-point in our study (100% by 16 hours) versus other studies (e.g. 80% by 144 hours reported by Meng *et al*.) also support the differences in cfDNA levels as consequences of different severities.

Differences in fluid resuscitation protocols also influence disease severity and outcome in sepsis (Zanotti-Cavazzoni et al., 2009). Mice that were under-resuscitated following CLP surgery (Luo et al., 2014; Martinod et al., 2015; Meng, Paunel-Görgülü, Flohé, Hoffmann, et al., 2012) will have altered hemodynamic responses characterized by decreased cardiac output and stroke volumes (Zanotti-Cavazzoni et al., 2009). In addition, female mice which are more resistant to septic insult compared to their male counterparts were used in some studies (Angele, Pratschke, Hubbard, & Chaudry, 2014; Dejager et al., 2011; Rittirsch et al., 2009). Differences in susceptibility of mice to the model and differences in model severity may explain the differences in survival and cfDNA levels reported in CLP studies.

In addition to the model used, the method of DNA quantification may contribute to the differences in cfDNA levels observed across studies. DNA isolation and quantification by spectrophotometry were used in our studies while other groups used the Quant-iT Pico Green dsDNA fluorescent assay to quantify plasma levels of cfDNA. Our method of DNA quantification includes all DNA fragments greater than 100 bp while the Pico Green system quantifies only double-stranded DNA which may explain the higher levels of cfDNA observed in our studies.

6.1.1 Cell-free DNA and inflammation in sepsis

An association between changes in cfDNA levels and inflammation was also observed. In an LPS model of endotoxemia, a transient increase in IL-6 levels accompanied the sustained increases in levels of cfDNA (Figure 6.2). In the CLP sepsis model, time-dependent elevations in cfDNA were accompanied by a time-dependent

increase in inflammatory IL-6 and an early, transient increase in IL-10, consistent with the cytokine profiles in other murine studies following the onset of sepsis (Ertel et al., 1990; Remick et al., 2000; Walley, Lukacs, Standiford, Strieter, & Kunkel, 1996). The highest levels of cfDNA were observed when plasma biomarkers of inflammation were also the highest in both our study and in previous CLP studies, supporting the link between cfDNA and inflammation in murine sepsis (Luo et al., 2014; Mai et al., 2015; Meng, Paunel-Görgülü, Flohé, Hoffmann, et al., 2012). Clinical and pre-clinical studies in sepsis have demonstrated that high levels of IL-6 and suppressed levels of IL-10 correlate with poor outcome (Hack et al., 1989, 1990; Oda et al., 2005; Remick et al., 2005).

Rapid dissemination of bacteria is characteristic of sepsis progression (Levy et al., 2003). In our studies, increased bacterial burden in the blood, lungs, and peritoneal cavity were observed when cfDNA levels were significantly elevated, suggesting that cfDNA may be involved in sepsis progression. Others have reported that increased bacterial load in the lungs, liver, peritoneal cavity fluid, and blood post-CLP were associated with increasing sepsis severity and poor outcome (Dahlke et al., 2011; Luo et al., 2014; Maier et al., 2004). Increases in cfDNA accompany sepsis progression following CLP surgery.

Organ damage was also correlated with high cfDNA levels. Significant organ damage as evidenced by histological studies and elevated levels of plasma ALT and creatinine (indicators of liver damage and impaired kidney function, respectively) were observed at time-points when cfDNA levels were elevated. It has been proposed that in response to CLP-induced sepsis, DNA deposited in the lungs drives inflammation

Figure 6.1. Time-course of cell-free DNA levels following LPS challenge. Mice were subjected to a model of endotoxemia via intraperitoneal injection of LPS. Mice were euthanized at 2, 4, 6, or 8 hours following LPS injection and blood was collected via the inferior vena cava. Levels of cfDNA were quantified and compared to those in healthy control mice (* $p < 0.010$).

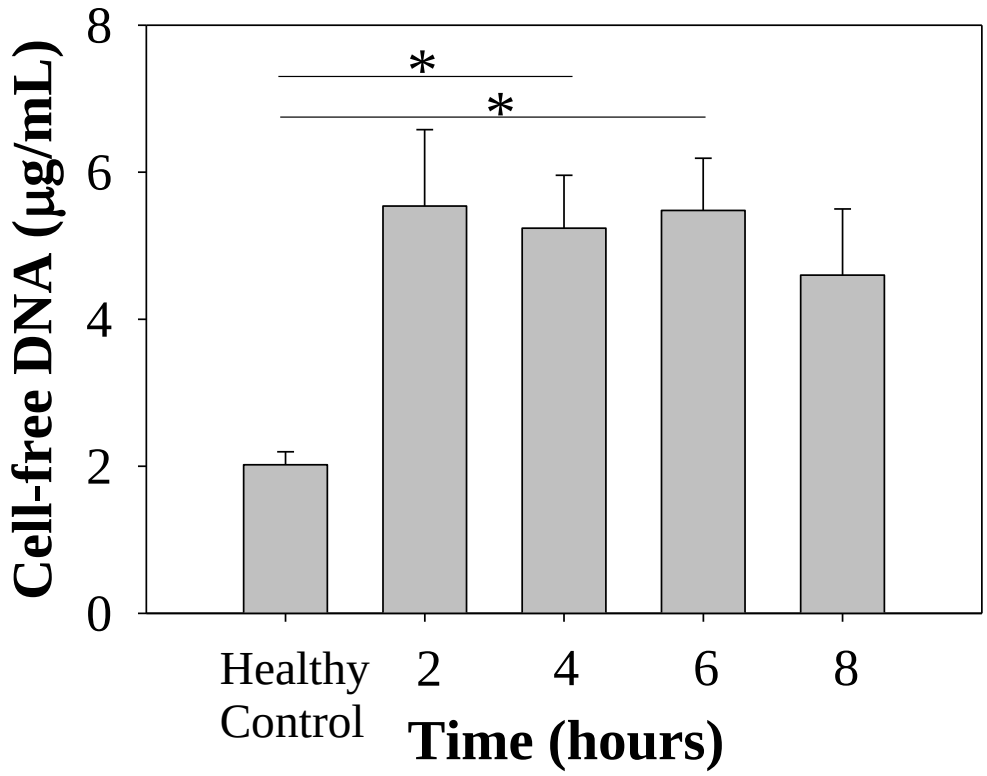
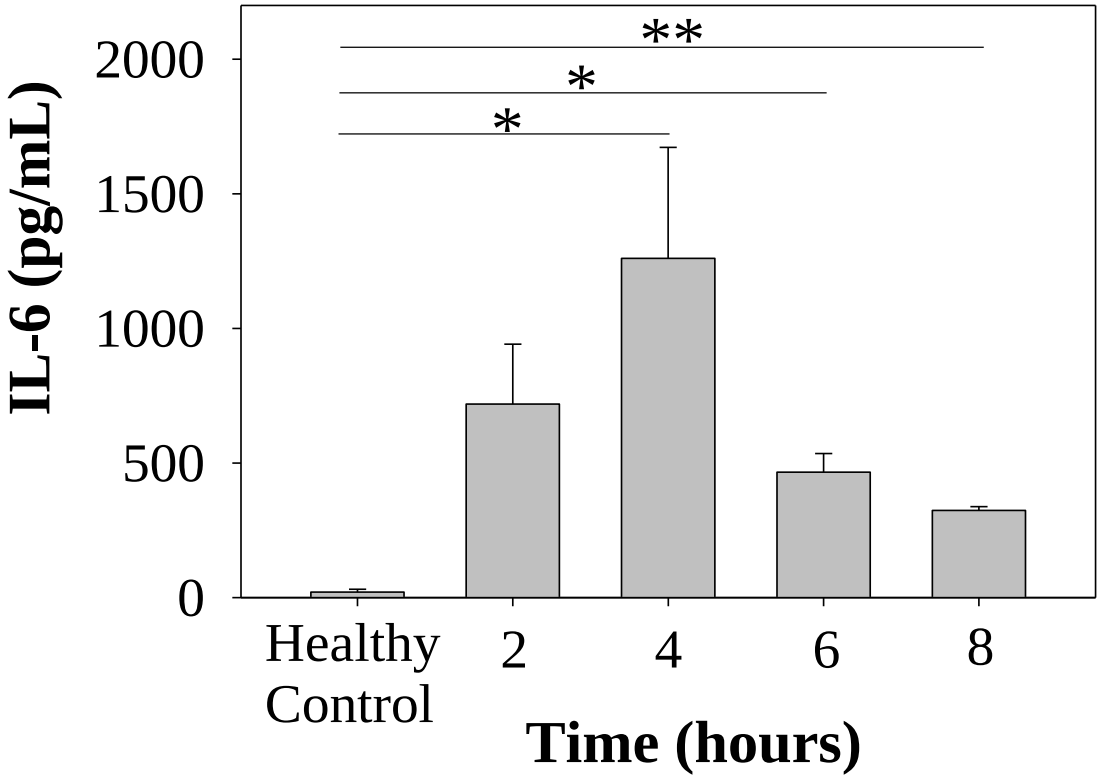

Cell-free DNA (µg/mL)
8
6
4
2
0
*
*
Healthy Control
2
4
6
8
Time (hours)

Figure 6.2. Time-course of IL-6 levels following LPS challenge. Mice were subjected to a model of endotoxemia via intraperitoneal injection of LPS. Mice were euthanized at 2, 4, 6, or 8 hours following LPS injection and blood was collected via the inferior vena cava. Levels of IL-6 were quantified and compared to those in healthy control mice (*$p < 0.050$, **$p < 0.010$).


**
*
*
2000
1500
1000
500
0
IL-6 (pg/mL)
Healthy Control
2
4
6
8
Time (hours)

by recruiting neutrophils which may cause lung damage by triggering the release of pro-inflammatory mediators such as CXCL2, TNFα, and HMGB1 (Hamaguchi et al., 2015; Luo et al., 2014). Our studies support this hypothesis as lung MPO, an indicator of neutrophil inflammation, increased significantly and an influx of neutrophils was observed in photomicrographs of lungs 6 and 8 hours post-CLP, when cfDNA levels were significantly elevated. In addition, it has been shown that isolated cfDNA induces the release of inflammatory IL-6 in monocytes (Atamaniuk et al., 2012) further supporting the link between cfDNA and inflammation. It is possible that cfDNA in the lungs contributes to increased inflammation and organ damage observed in mice subjected to CLP. Taken together, these data suggest that elevations in cfDNA contribute to increased inflammation, bacteria burden, and organ damage.

6.1.2 Cell-free DNA and coagulation in sepsis

Increases in cfDNA levels were also associated with the procoagulant response in sepsis. In mice subjected to CLP, increases in cfDNA levels accompanied RBC congestion and fibrin deposition in the lungs and kidneys as well as an early, sustained increase in TAT levels. Increased levels of TATs were also documented in other murine sepsis studies (Kaneider et al., 2007; Tucker et al., 2012) and are also supported by clinical studies which documented elevated TAT levels in early sepsis (Koyama et al., 2014) and severe sepsis patients (Kinasewitz et al., 2004; Zeerleder et al., 2003).

Increased cfDNA was associated with changes in thrombin generation. Plasma from mice subjected to CLP for 6 hours (with elevated cfDNA levels) generated more thrombin than plasma from sham-operated or healthy control mice in our studies. This is

consistent with the observation that thrombin generation in plasma from severe sepsis patients is increased compared to healthy controls (Gould et al., 2014). In our murine studies, thrombin generation in recalcified plasma from septic mice had increased peak thrombin levels (Figure 6.3E) and endogenous thrombin potential (Figure 6.3F) and decreased lag time (Figure 6.3C) and time to peak (Figure 6.3D) compared to plasma from sham-operated or healthy control mice, which did not generate thrombin. As with septic patient plasma (Gould et al., 2014), thrombin generation from septic mouse plasma is likely driven by the intrinsic pathway as co-incubation with corn trypsin inhibitor (CTI), an inhibitor of FXIIa, largely abolished thrombin generation (Figure 6.3B) indicated by reductions in peak thrombin (Figure 6.3G) and AUC (Figure 6.3H).

In murine sepsis, platelets may potentiate FXIIa-mediated thrombin generation as more thrombin (i.e. increased peak thrombin and AUC) was generated in platelet-rich plasma (PRP) than in platelet-poor plasma (PPP) from CLP-operated mice given saline (Figure 6.4) and CLP-operated mice given DNase (Figure 6.5). However, the differences in thrombin generation between PRP and PPP were modest. NETs containing histones and cfDNA have been shown to increase thrombin generation in a TLR2 and TLR4-dependent manner potentially through the release of platelet polyP (Semeraro et al., 2011; J. Xu et al., 2011). It is possible that histones and cfDNA activate platelets and increase thrombin generation in PRP (Gould et al., 2014; Semeraro et al., 2011), hence less thrombin generation is observed in the absence of platelets. In the absence and presence of platelets, thrombin generation from septic mouse plasma was driven by FXIIa as CTI largely suppressed thrombin generation in both PRP and PPP (Figures 6.4, 6.5). No

thrombin generation was observed in recalcified plasma from sham-operated mice given saline or DNase in PRP (Figure 6.6A) or PPP (Figure 6.6B). These findings suggest that both the intrinsic pathway and platelets contribute to thrombin generation which occurs through platelet-dependent and platelet-independent mechanisms in murine sepsis.

One other study investigated thrombin generation in CLP-operated mice and reported increases in thrombin generation marked by increased endogenous thrombin potential (Y. Wang et al., 2014). However, this increase was rapid and transient, appearing only at 1 hour post-CLP and lost in thrombin generation assays from mice subjected to CLP for 3, 6, and 24 hours (Y. Wang et al., 2014). This difference may be due to a decreased model severity however, interpreting this data has limitations as no data corresponding to levels of neutrophil accumulation, organ injury, or lung MPO to assess disease severity and progression were shown. Organ histology, plasma levels of TAT complexes, and thrombin generation data suggest that increases in cfDNA accompanies the early pro-coagulant response in murine sepsis which involves both intrinsic pathway and platelet activation, findings which were consistent with clinical sepsis.

6.2 *In vivo* effects of DNase administration in sepsis

Administration of exogenous DNase may reduce levels of cfDNA and modulate inflammation and outcome in sepsis. Extracellular DNA in plasma is digested predominantly by DNaseI. In our studies, administration of recombinant human DNaseI (DNase) in healthy mice exerted no pathological effects and levels of inflammatory

Figure 6.3. Thrombin generation in recalcified plasma from healthy control, sham-operated, and CLP-operated mice in the absence and presence of corn trypsin inhibitor. Mice were subjected to sham or CLP surgery. Blood was collected 6 hours post-operatively and platelet-poor plasma was isolated. Thrombin generation assays initiated in the presence of 15 mM $CaCl_2$ were run with plasma from healthy control, sham-operated, and CLP-operated mice. Recalcified plasma from CLP-operated mice generated thrombin (A) which was abolished when incubated with corn trypsin inhibitor (CTI), an inhibitor of FXIIa (B). Recalcified plasma from sham-operated and healthy control mice did not generate measurable amounts of thrombin (A). Lag time was reduced (C), time to peak was delayed (D), and peak thrombin (E) and AUC (F) were increased in thrombin generations with recalcified plasma from CLP-operated mice compared to sham-operated and healthy control mice. Blocking FXIIa with CTI suppressed peak thrombin (G) and AUC (H) in recalcified plasma from CLP-operated mice (n = 5-7 per group).

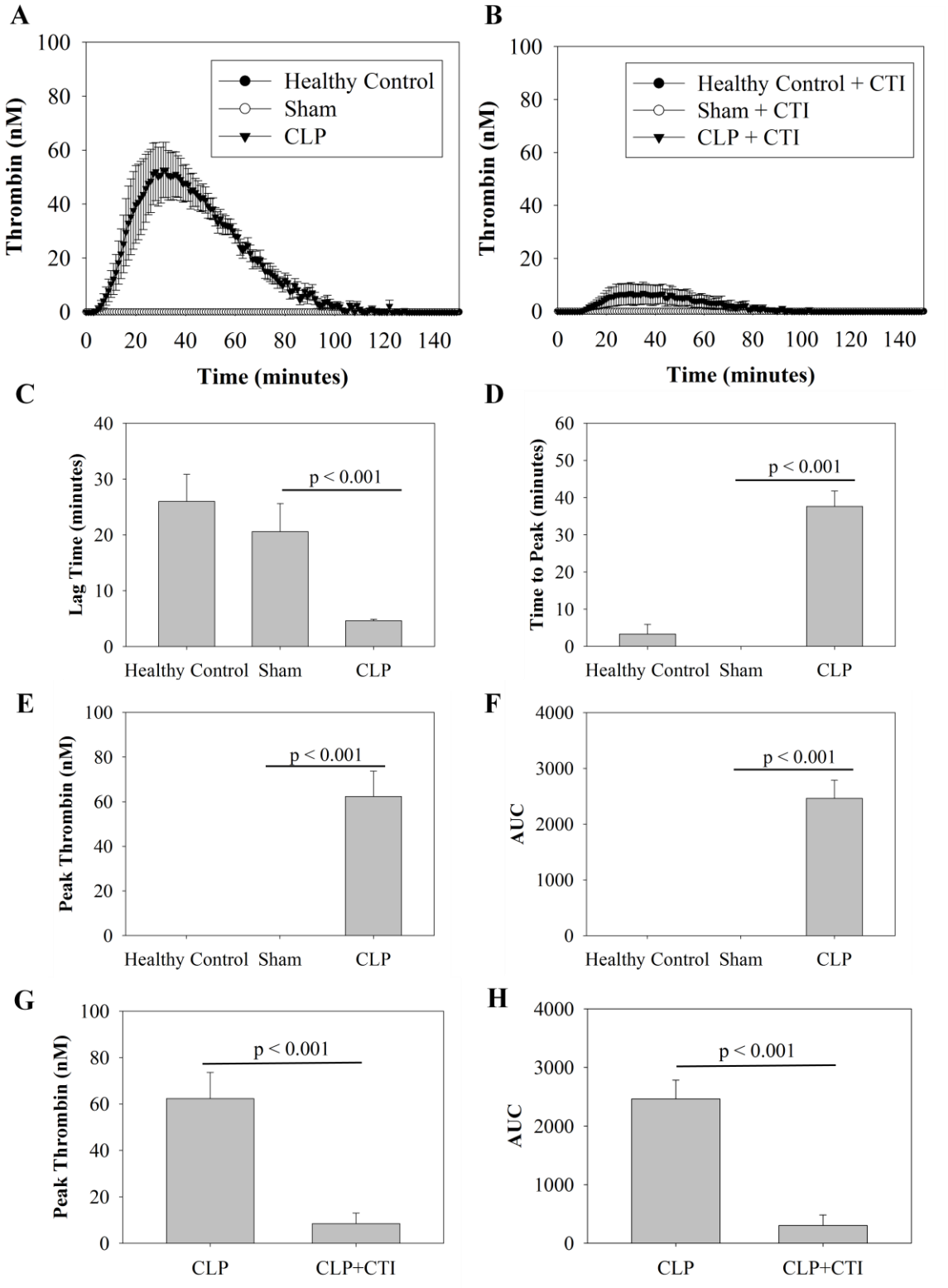

A
Thrombin (nM)
Time (minutes)
Healthy Control
Sham
CLP
B
Thrombin (nM)
Time (minutes)
Healthy Control + CTI
Sham + CTI
CLP + CTI
C
Lag Time (minutes)
p < 0.001
Healthy Control
Sham
CLP
D
Time to Peak (minutes)
p < 0.001
Healthy Control
Sham
CLP
E
Peak Thrombin (nM)
p < 0.001
Healthy Control
Sham
CLP
F
AUC
p < 0.001
Healthy Control
Sham
CLP
G
Peak Thrombin (nM)
p < 0.001
CLP
CLP+CTI
H
AUC
p < 0.001
CLP
CLP+CTI

Figure 6.4. Thrombin generation in recalcified platelet-rich versus platelet-poor plasma from CLP-operated mice given saline in the absence and presence of corn trypsin inhibitor. Mice were subjected to CLP surgery and given saline 6 hours post-operatively. Blood was collected 2 hours following saline administration, platelet-rich (PRP) and platelet-poor plasma (PPP) were prepared, and thrombin generation assays initiated in the presence of 15 mM $CaCl_2$ were run. Recalcified PRP from CLP-operated mice generated more thrombin than recalcified PPP (A). Peak thrombin (B) and AUC (C) were greater in PRP compared to PPP although these differences did not reach statistical significance. Incubation with CTI, an inhibitor of FXIIa, significantly reduced thrombin generation (A), peak thrombin (B), and AUC (C) in both PRP and PPP from CLP-operated mice (n = 8 per group).

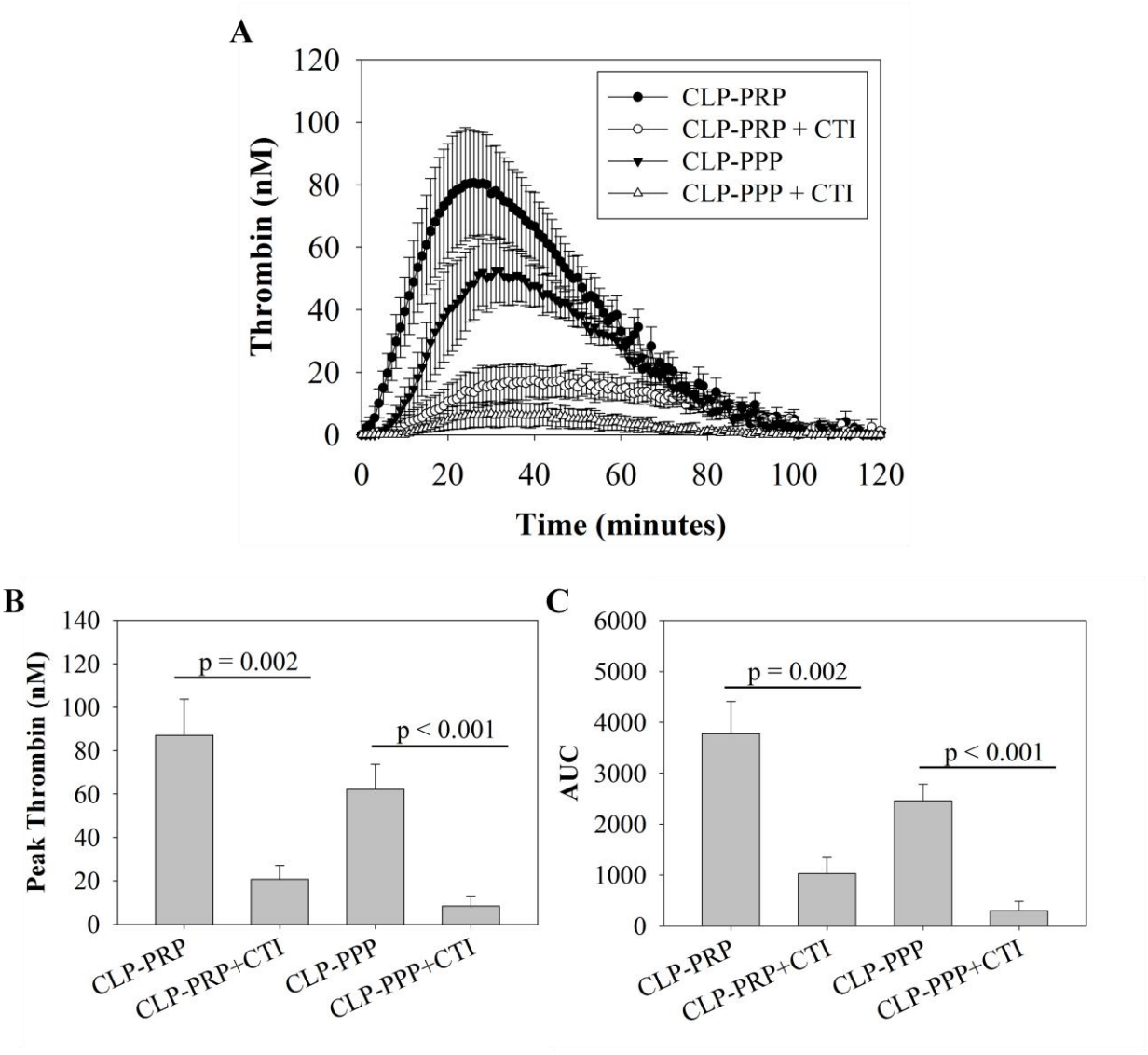

A
Thrombin (nM)
Time (minutes)
CLP-PRP
CLP-PRP + CTI
CLP-PPP
CLP-PPP + CTI
B
Peak Thrombin (nM)
p = 0.002
p < 0.001
CLP-PRP
CLP-PRP+CTI
CLP-PPP
CLP-PPP+CTI
C
AUC
p = 0.002
p < 0.001
CLP-PRP
CLP-PRP+CTI
CLP-PPP
CLP-PPP+CTI

Figure 6.5. Thrombin generation in recalcified platelet-rich versus platelet-poor plasma from CLP-operated mice given DNase in the absence and presence of corn trypsin inhibitor. Mice were subjected to CLP surgery and given DNase 6 hours post-operatively. Blood was collected 2 hours following DNase administration, platelet-rich (PRP) and platelet-poor plasma (PPP) were prepared, and thrombin generation assays initiated in the presence of 15 mM $CaCl_2$ were run. Recalcified PRP generated more thrombin than recalcified PPP (A). Incubation with CTI, an inhibitor of FXIIa, significantly reduced thrombin generation (A), peak thrombin (C) and AUC (E) in both PRP and PPP from CLP-operated mice given DNase (n = 9 per group).

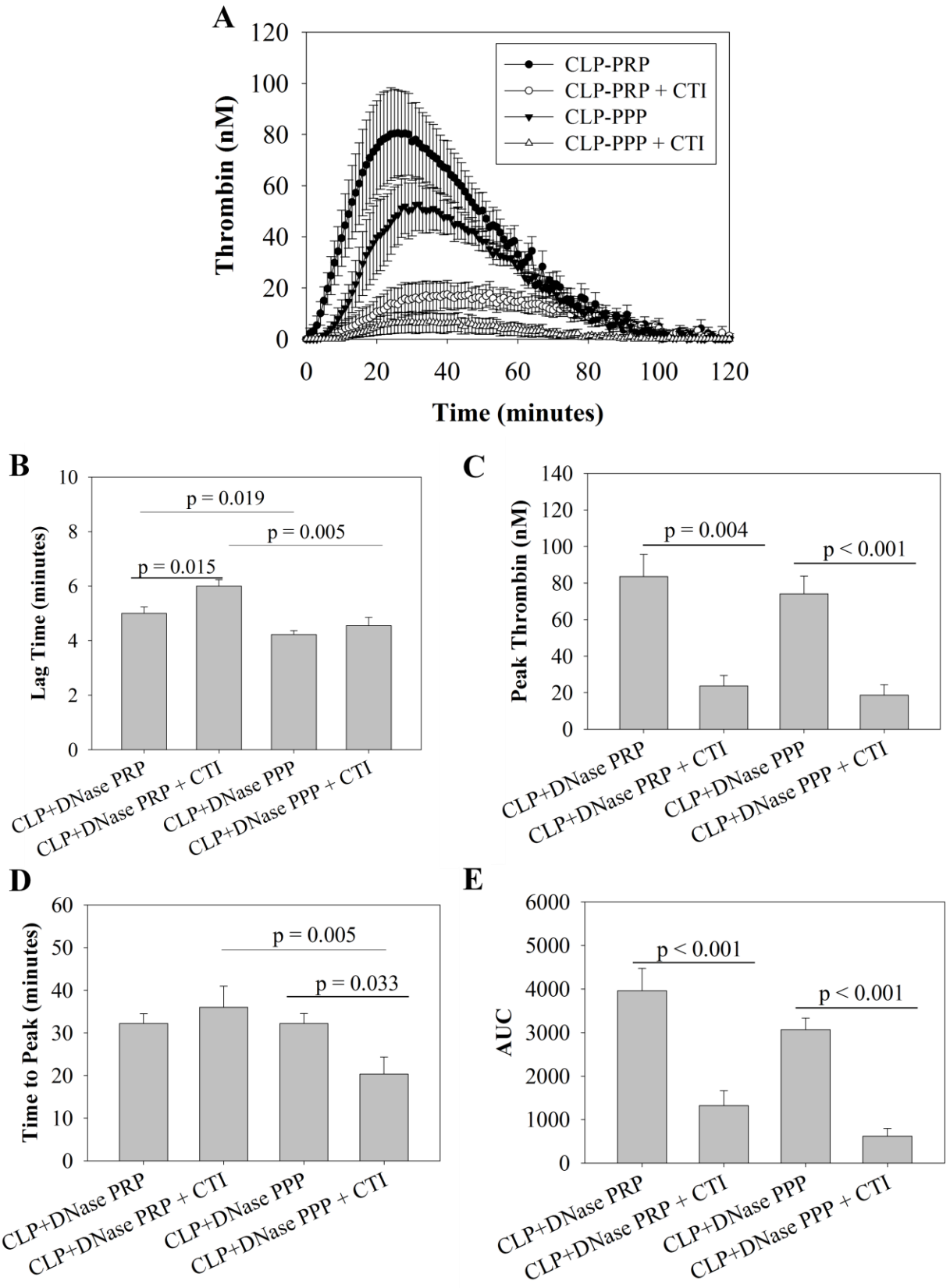
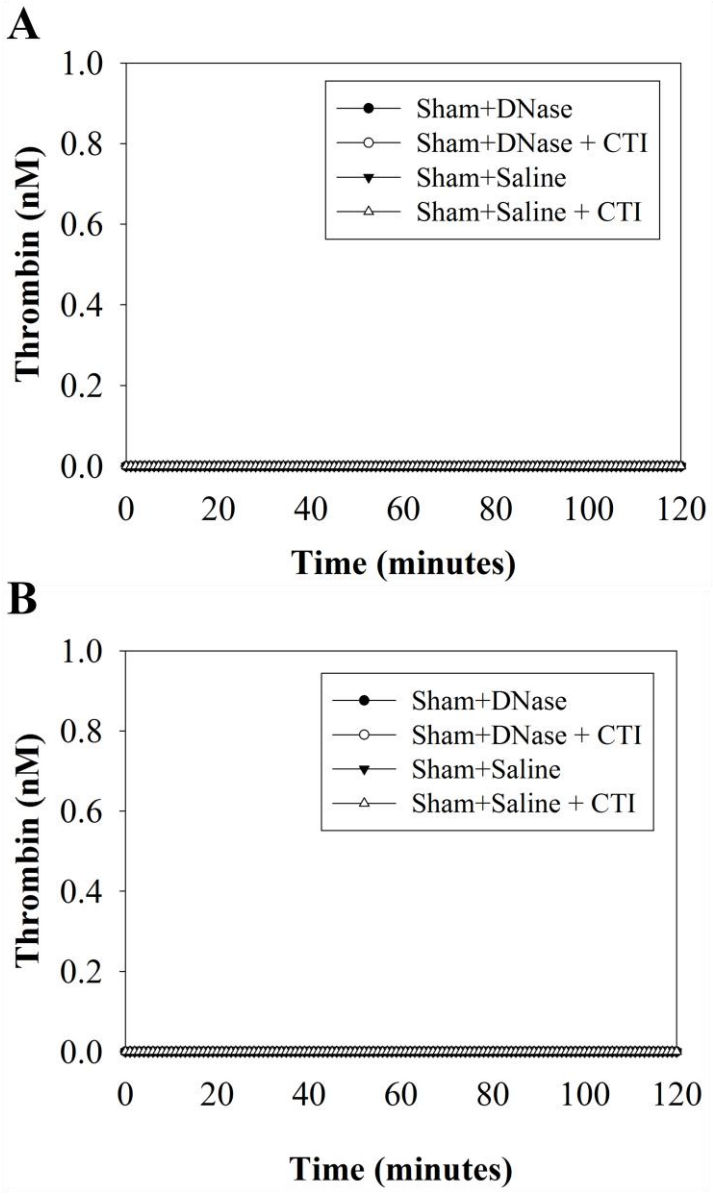

A
Thrombin (nM)
Time (minutes)
CLP-PRP
CLP-PRP + CTI
CLP-PPP
CLP-PPP + CTI
B
Lag Time (minutes)
p = 0.019
p = 0.005
p = 0.015
C
Peak Thrombin (nM)
p = 0.004
p < 0.001
D
Time to Peak (minutes)
p = 0.005
p = 0.033
E
AUC
p < 0.001
p < 0.001
CLP+DNase PRP
CLP+DNase PRP + CTI
CLP+DNase PPP
CLP+DNase PPP + CTI

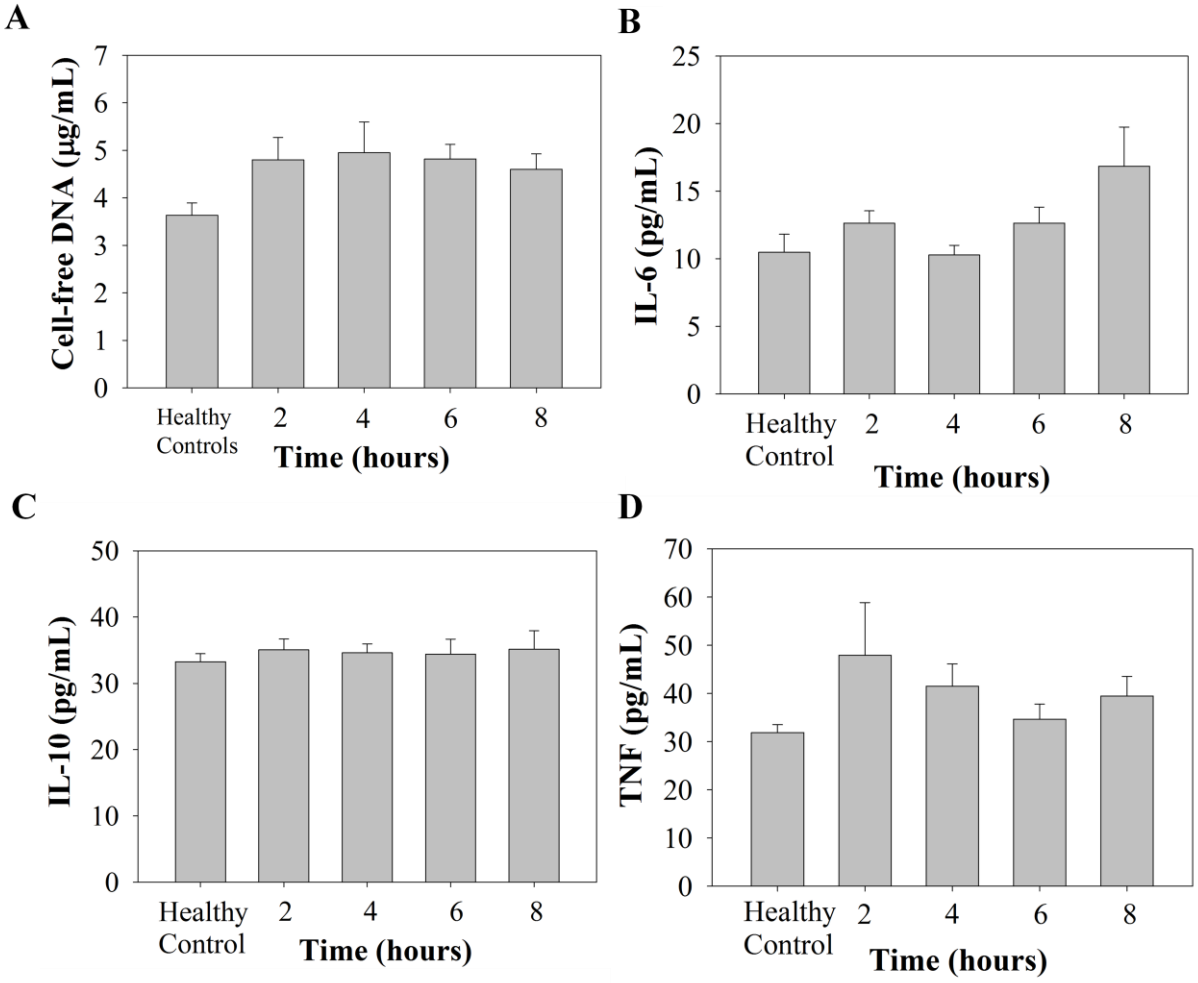
Figure 6.6. Thrombin generation in recalcified platelet-rich and platelet-poor plasma from sham-operated mice in the absence and presence of corn trypsin inhibitor. Mice were subjected to sham surgery and given saline or DNase 6 hours post-operatively. Blood was collected 2 hours following saline or DNase administration, platelet-rich (PRP) and platelet-poor plasma (PPP) were prepared, and thrombin generation assays initiated in the presence of 15 mM $CaCl_2$ were run. No thrombin generation was observed from PRP (A) or PPP (B) from sham-operated mice given saline or DNase (n = 7-9 per group).


A
1.0
0.8
0.6
0.4
0.2
0.0
Thrombin (nM)
Sham+DNase
Sham+DNase + CTI
Sham+Saline
Sham+Saline + CTI
0
20
40
60
80
100
120
Time (minutes)
B
1.0
0.8
0.6
0.4
0.2
0.0
Thrombin (nM)
Sham+DNase
Sham+DNase + CTI
Sham+Saline
Sham+Saline + CTI
0
20
40
60
80
100
120
Time (minutes)

mediators were not changed 2-8 hours following *in vivo* DNase administration (Figure 6.7). Similar observations were reported in studies involving healthy humans wherein intravenous rhDNaseI caused no ill effects (Dayan, 1994; Johnson, Goger, & Tillett, 1954; Prince et al., 1998). In the healthy host, DNaseI administered intravenously appears to be rapidly cleared from circulation by the liver, as evidenced by pharmaco-kinetic studies characterizing the short half-life of DNase (7-25 minutes) (Dayan, 1994) and the rapid appearance of DNase in the lungs, livers, and kidneys within 2-30 minutes (Johnson et al., 1954). The half-life of circulating cfDNA is also short, around 16.3 min (with a range of 4-30 min) in one study quantifying fetal DNA in the maternal circulation (Lo et al., 1999) and around 15 min in another study quantifying circulating cfDNA during exercise (Breitbach, Sterzing, Magallanes, Tug, & Simon, 2014).

The effects of DNase administration in septic mice on inflammation and outcome were dependent on the timing of DNase administration. Early DNase injection, just 2 hours following CLP surgery resulted in increased inflammation and increased lung damage. However, increased inflammation following early DNase administration may be independent of cfDNA as levels of cfDNA are not significantly increased in septic mice compared to non-septic mice 2 hours post-operatively. Another murine study also reported increased inflammation in mice given early DNase administration 1 hour post-CLP surgery (Meng, Paunel-Görgülü, Flohé, Hoffmann, et al., 2012). These mice had increased circulating IL-6, lung and liver damage, and polymorphonuclear cell infiltration in end organs. Mice given DNase early following CLP surgery also died earlier than mice given a PBS control (Meng, Paunel-Görgülü, Flohé, Hoffmann, et al., 2012).

Figure 6.7. DNase administration in healthy control mice. Healthy mice were given an intraperitoneal injection of recombinant human DNaseI. Blood was collected at 2, 4, 6, or 8 hours following DNase injection and levels of cfDNA (A), IL-6 (B), IL-10 (C), and TNF (D) were quantified. No significant changes were detected in levels of these biomarkers over the 8 hour study period (n = 3-6 per group).

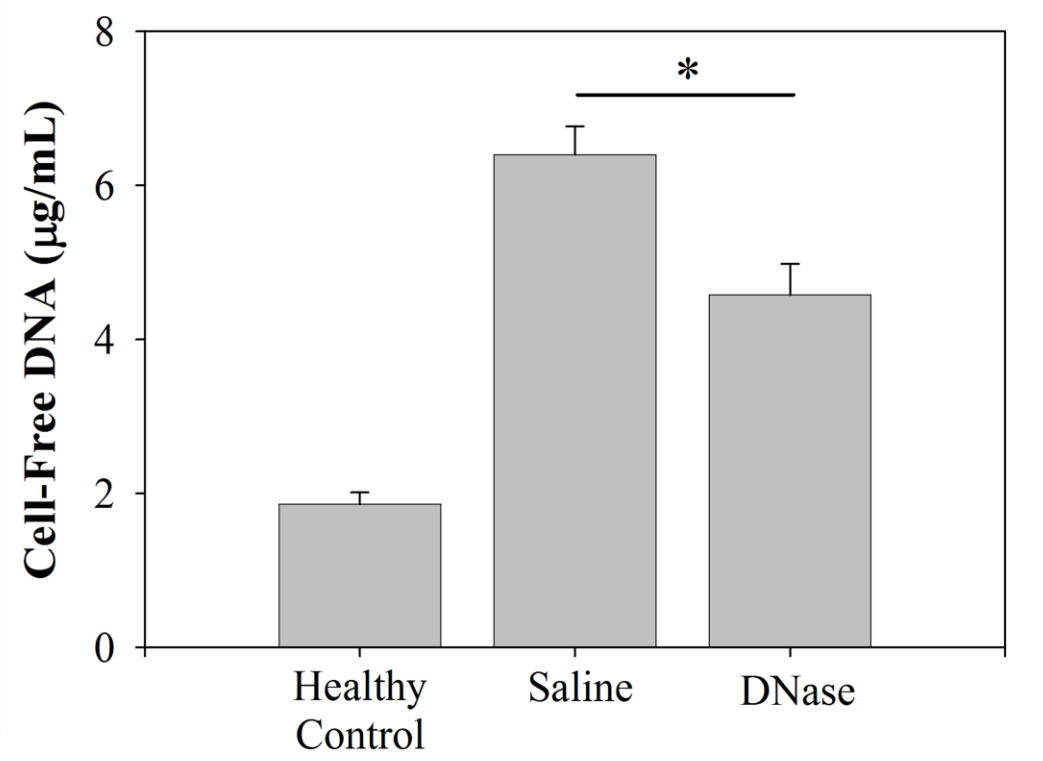

A
Cell-free DNA (µg/mL)
0
1
2
3
4
5
6
7
Healthy Controls
2
4
6
8
Time (hours)
B
IL-6 (pg/mL)
0
5
10
15
20
25
Healthy Control
2
4
6
8
Time (hours)
C
IL-10 (pg/mL)
0
10
20
30
40
50
Healthy Control
2
4
6
8
Time (hours)
D
TNF (pg/mL)
0
10
20
30
40
50
60
70
Healthy Control
2
4
6
8
Time (hours)

At this time-point, the release of extracellular DNA into the circulation in the form of NETs may support host defence and immunity by trapping and killing circulating microorganisms. The degradation of NETs in circulation with early DNase administration may be detrimental to the host by hindering the host's ability to contain circulating microorganisms, resulting in organ damage.

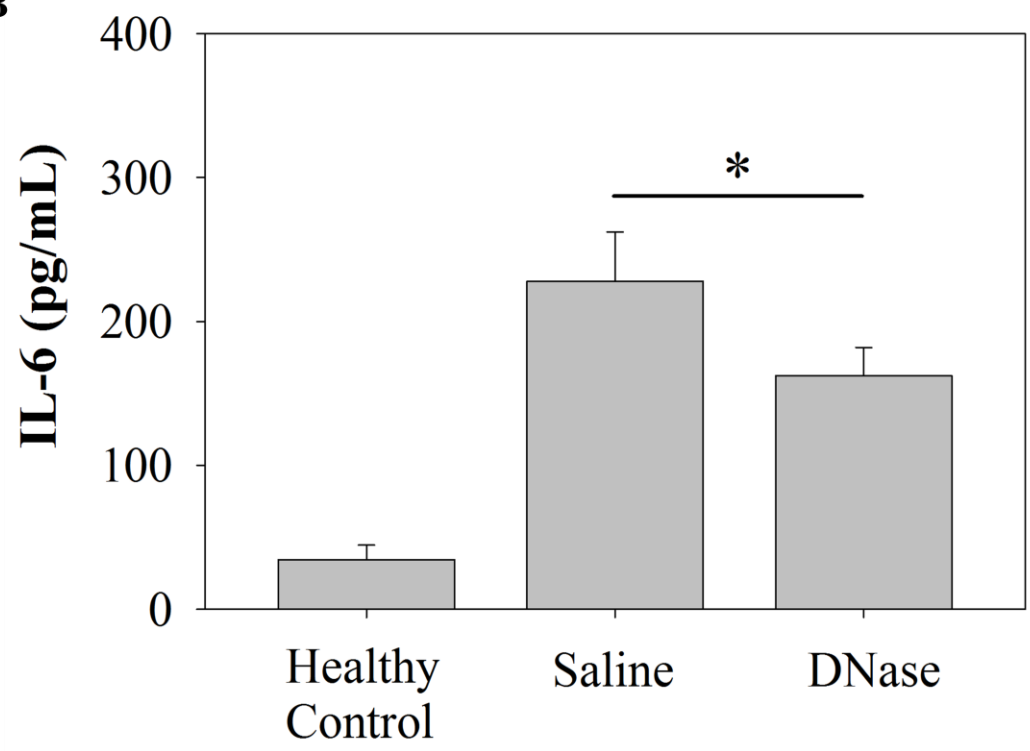
In contrast, administration of DNase when cfDNA levels are elevated may be protective to the septic host. Various studies have demonstrated that DNaseI can effectively degrade extracellular DNA. In a model of endotoxemia, LPS injection resulted in the release of extracellular DNA into the liver sinusoids stained by a cell-impermeable DNA stain (Sytox Green) (McDonald et al., 2012). Within seconds of intravenous DNase injection, staining of the extracellular chromatin was abolished. In *in vitro* studies, neutrophils isolated from the bone marrow released extracellular DNA following PMA stimulation and treatment with DNase also reduced the staining of extracellular DNA (Meng, Paunel-Görgülü, Flohé, Hoffmann, et al., 2012). In studies of thrombosis, anticoagulated blood passed over DNA caused platelet adherence and aggregation on the DNA web and treatment with DNase dismantled the extracellular DNA and removed platelets (Fuchs et al., 2010). In our endotoxemia studies, DNase injection following LPS injection produced similar results. Levels of cfDNA were elevated in LPS-challenged mice compared to mice given a saline control, a finding consistent with other murine endotoxemia studies (Clark et al., 2007). DNase administration resulted in decreases in levels of circulating cfDNA and IL-6, suggesting that DNase may degrade cfDNA and suppress inflammation in a model of endotoxemia (Figure 6.8).

Figure 6.8. DNase administration in LPS-challenged mice. Healthy mice were given an intraperitoneal injection of LPS endotoxin and an intraperitoneal injection of rhDNaseI or a saline control 2 hours following LPS injection. Blood was collected via the inferior vena cava 2 hours following DNase or saline injection. Levels of cfDNA (A) and IL-6 (B) were reduced in LPS-challenged mice given DNase compared to mice given saline or levels observed in healthy control mice.

A


Cell-Free DNA (µg/mL)
8
6
4
2
0
*
Healthy Control
Saline
DNase

B

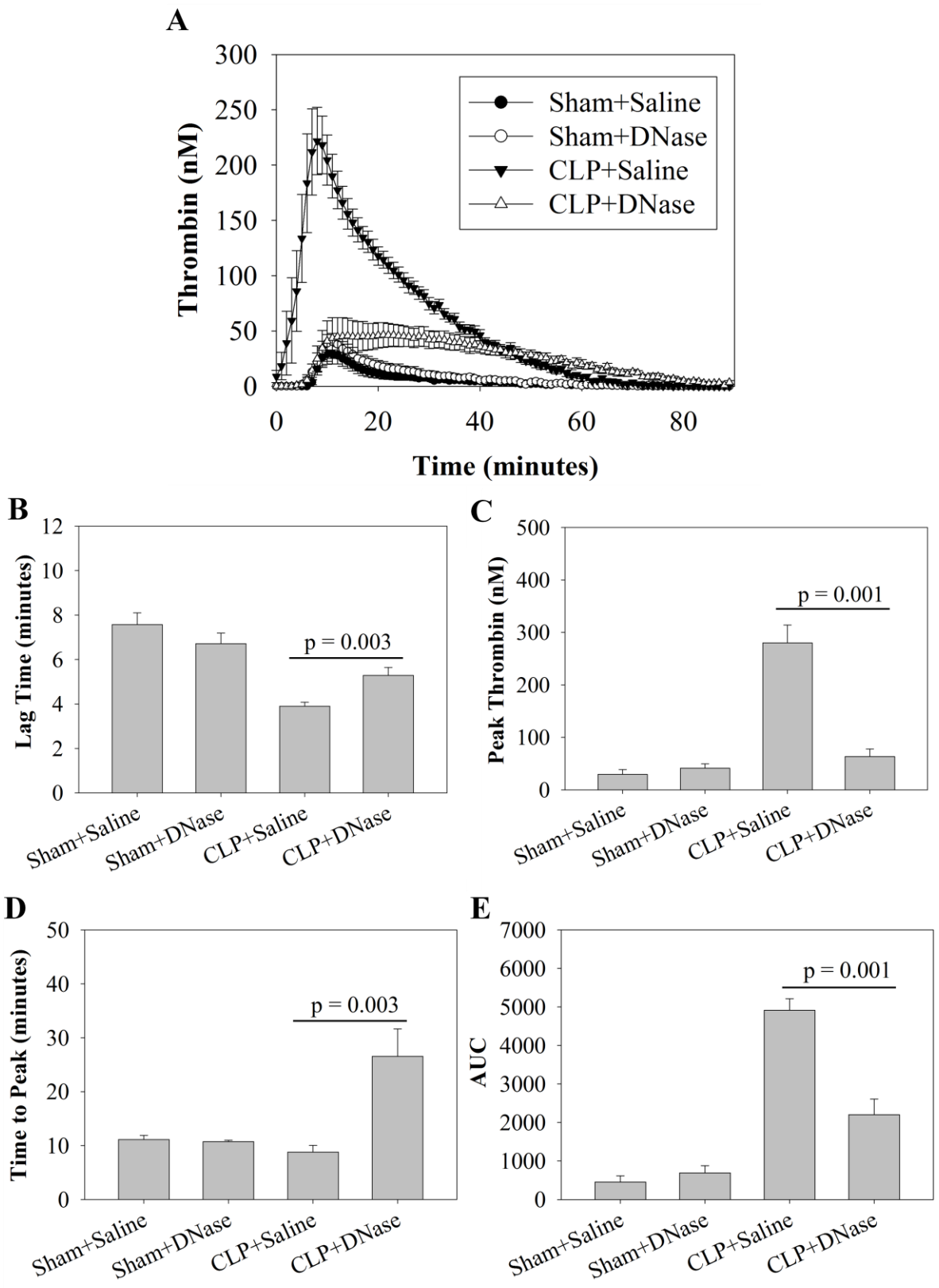

IL-6 (pg/mL)
400
300
200
100
0
*
Healthy Control
Saline
DNase

In vivo administration of DNase also resulted in decreased thrombin generation (Figure 6.9). While plasma from healthy control mice and sham-operated mice given either saline or DNase generated minimal amounts of thrombin, thrombin generation was significantly elevated in CLP-operated mice given saline *in vivo*. In plasma from CLP-operated mice given DNase *in vivo,* thrombin generation was suppressed compared to CLP-operated mice given a saline control (Figure 6.9). Previous studies have demonstrated that DNase treatment dismantles DNA traps and decreases platelet aggregation and thrombus formation (Brill et al., 2012; Fuchs et al., 2010) Taken together, these findings suggest that administering DNase *in vivo* when cfDNA levels are high may reduce cfDNA levels and suppress the procoagulant response in experimental sepsis.

In our CLP studies, injection of DNase following a period of sepsis progression (4 or 6 hours) resulted in decreased cfDNA levels, although we have not completed studies to investigate the binding of DNase to cfDNA and the ability of DNase to degrade cfDNA released in septic mice. Delayed DNase administration, when cfDNA levels are elevated in septic mice, resulted in reduced levels of circulating cfDNA, suppressed inflammatory and coagulant responses, decreased bacterial load, suppressed organ damage.

The observations that the therapeutic effect of DNase are dependent on time have several implications. DNase administration when cfDNA levels are elevated is therapeutic but pathological when cfDNA levels are low (such as in the sham condition). This should be considered when investigating DNase as a potential therapy. DNase should only be considered for patients in whom cfDNA levels are elevated as there is a

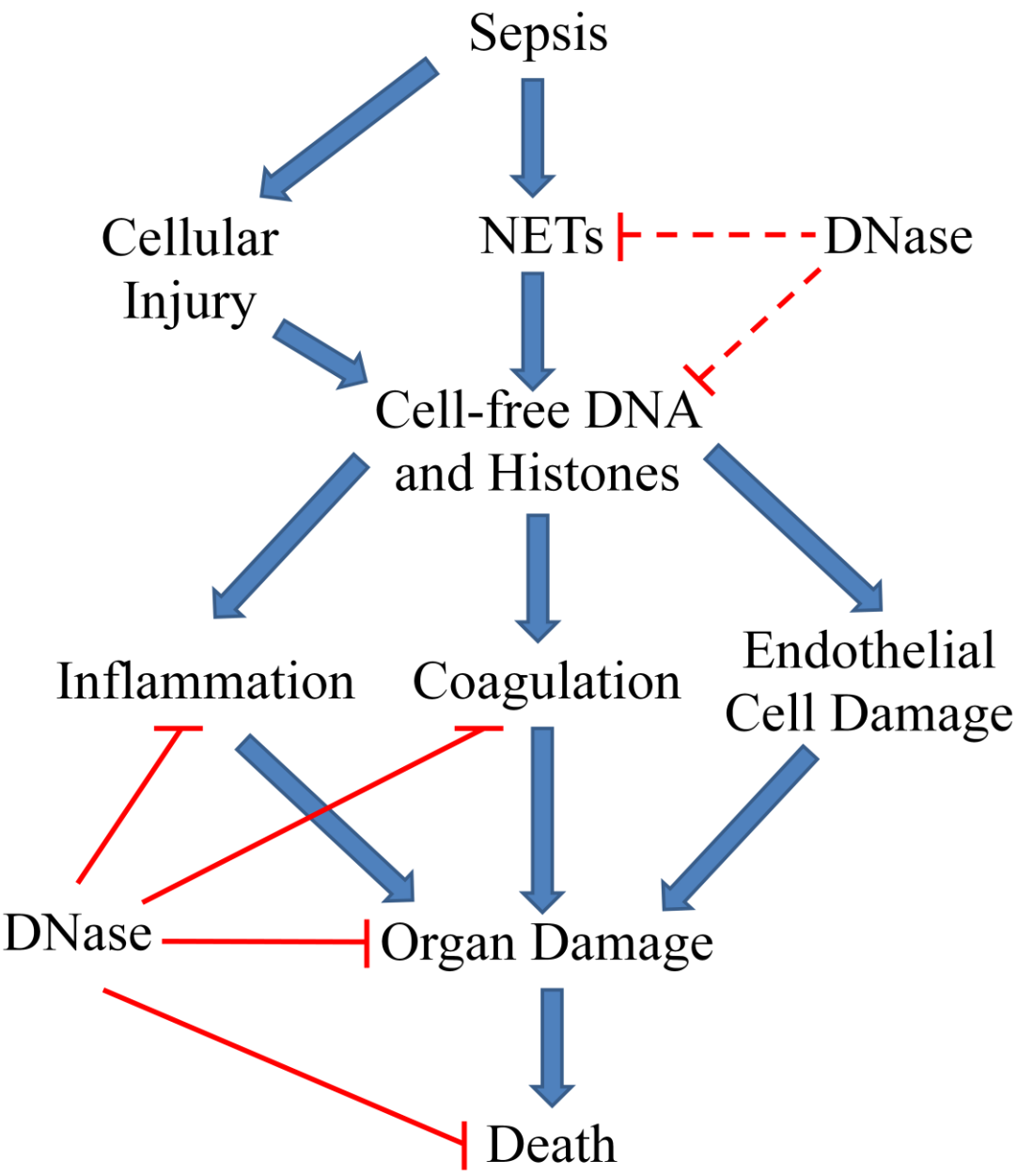
Figure 6.9. Thrombin generation in plasma from sham- and CLP-operated mice given saline or DNase initiated in the presence of calcium, dilute Recombiplastin, and phospholipids. Mice were subjected to sham or CLP surgery and given either DNase or saline 6 hours post-operatively. Blood was collected 2 hours following DNase or saline injection and platelet-poor plasma was isolated. Thrombin generation assays were initiated in the presence of 15 mM $CaCl_2$, 1/500 dilution of Recombiplastin (17 pM), and 30 μM PCPS in PBS. Plasma from CLP-operated mice given saline generated much more thrombin than plasma from sham-operated mice given saline or DNase (A). Thrombin generation was largely suppressed in CLP-operated mice given DNase treatment compared to CLP-operated mice given a saline control (A). In CLP-operated mice given DNase treatment, lag time (B) and time to peak (D) were increased and peak thrombin (C) and AUC (E) were reduced compared to CLP-operated mice given saline (n = 7 per group).


A
Thrombin (nM)
Time (minutes)
Sham+Saline
Sham+DNase
CLP+Saline
CLP+DNase
B
Lag Time (minutes)
p = 0.003
C
Peak Thrombin (nM)
p = 0.001
D
Time to Peak (minutes)
p = 0.003
E
AUC
p = 0.001

biological plausibility for the therapeutic effect of DNase. Given that our preclinical studies demonstrated that administration of DNase in mice with low cfDNA levels exacerbates inflammation and organ damage, levels of cfDNA should be quantified in patients in whom DNase may be tested. The failure of many therapeutic agents that showed early promise in preclinical studies highlight the importance of patient stratification (Iskander et al., 2013). For instance, clinical anti-TNF therapy was tested in patients with plasma TNF levels ranging from 8-1 550 000 pg/mL, which likely diluted the therapeutic efficacy of anti-TNF therapy (J. C. Marshall, 2014; Reinhart & Karzai, 2001). With over 1000 sepsis clinical trials and no approved therapeutic agent currently available for the treatment of sepsis and our findings of the differential effects of DNase, the negative effects of having an all-inclusive, unstratified patient cohort should be considered (Iskander et al., 2013; J. C. Marshall, 2014).

A limitation in assessing the effects of DNase injection in sepsis is that it is unknown if the potential therapeutic effects of DNase are via the degradation of cfDNA or if they are independent of DNase's activity on cfDNA (Figure 6.10). NETs released during sepsis contribute to increases in circulating cfDNA and histones which exacerbate inflammation and coagulation. Aberrant inflammatory and procoagulant responses drive organ damage leading to death. DNase suppresses inflammation, coagulation, and organ damage and delayed DNase administration rescues mice from death. It is possible that degradation of cfDNA by DNase dismantles DNA traps and attenuates inflammation and coagulation in the microvasculature, thus reducing organ damage in sepsis (Figure 6.10). However, we have not elucidated whether these effects occur via the degradation of

Figure 6.10. Cell-free DNA and DNase treatment in sepsis. NETs released during sepsis increase circulating cell-free DNA (cfDNA) and histones which potentiate inflammation, coagulation, and endothelial toxicity. Aberrant inflammation, coagulation, and endothelial activation drives organ damage and death. *In vivo* administration of DNase in sepsis results in suppressed inflammation, coagulation, and organ damage and rescues mice from death, potentially via the degradation of NETs and cfDNA in sepsis.


Sepsis
Cellular Injury
NETs
DNase
Cell-free DNA and Histones
Inflammation
Coagulation
Endothelial Cell Damage
DNase
Organ Damage
Death

cfDNA and/or NETs. The causality between DNase treatment and changes in cfDNA, inflammation, coagulation, and outcome remains to be characterized (Figure 6.10).

6.3 Cell-free DNA and NETs

While various mechanisms such as extracellular trap formation and necrosis contribute to the release of cfDNA, it is unclear which mechanisms and to what extent these mechanisms contribute to the cfDNA measured in our and other studies. Some clinical studies in trauma, sepsis, burns, and arthritis refer to circulating cfDNA and NETs interchangeably and extracellular DNA which is detected by Pico Green is considered to be NET-derived (Altrichter et al., 2010; Lögters et al., 2009; Margraf et al., 2008; Meng, Paunel-Görgülü, Flohé, Hoffmann, et al., 2012; Meng, Paunel-Görgülü, Flohé, Witte, et al., 2012).

In our studies, we observed that levels of cfDNA in PAD4-deficient mice did not increase following CLP surgery, suggesting that NETs contribute to cfDNA elevations in our model of CLP sepsis. Other murine sepsis studies demonstrated that depletion of neutrophils and administration of DNase resulted in significantly reduced levels of cfDNA deposited in the lungs and in circulation (Luo et al., 2014) suggesting that cfDNA is neutrophil-derived. Studies using endotoxemia and CLP models have also demonstrated that mice deficient in PAD4 protein have lower circulating levels of cfDNA than wildtype mice following disease induction (Martinod et al., 2015), suggesting that differences in cfDNA levels are due to the release of NETs in only wildtype mice. Our observations along with other CLP studies suggest that NETosis contributes to increases in cfDNA in our sepsis model.

However, a recent study aimed at characterizing the source of cfDNA released during CLP-induced sepsis suggests otherwise. Hamaguchi *et al*. observed that neutrophil depletion via administration of anti-Ly6G antibody resulted in no change in circulating cfDNA levels between neutrophil-depleted and wildtype mice subjected to CLP surgery (Hamaguchi et al., 2015). Moreover, neutrophils isolated from whole blood in mice following CLP or sham surgery showed no differences in the levels of citrullinated histone H3 (citH3), a marker of chromatin decondensation and NETosis. This study measured citH3 levels via Western blot analyses which showed similarly sized citH3 antibody bands from naive, sham-, and CLP-operated mice and confirmed these findings with FACS analyses which showed no changes or shifts in the fluorescent peaks of citH3 between naive, sham-, and CLP-operated mice (Hamaguchi et al., 2015). These findings suggest that circulating cfDNA released following CLP-induced sepsis may not be from NETosis and that neutrophils may not be the main source of cfDNA, although the group did not investigate other potential sources of cfDNA in circulation.

One potential reason for this discrepancy in the source of cfDNA is that not all neutrophils form NETs. The percentage of neutrophils that undergo NETosis following LPS challenge is quite low, around 3% of neutrophils (Tanaka et al., 2014). Other *in vitro* studies reported that around 35% of activated neutrophils form NETs (Brinkmann & Zychlinsky, 2007; Clark et al., 2007; Fuchs et al., 2007) and NETosis is inhibited by the antioxidant activity of serum (Fuchs et al., 2007; Yu, 1994). It is possible that different models of sepsis, and different models of CLP induce different amounts of neutrophils to undergo NETosis. In studies where neutrophils undergo degranulation and phagocytosis,

NETosis may occur less frequently. Alternatively, the differences between our findings versus those reported by Hamaguchi *et al*. which suggested that NETs are not a major source of circulating cfDNA may potentially be attributable to the methods of preventing NET formation. PAD4-/- mice were used in our study while Hamaguchi *et al*. used a neutrophil-depleting antibody to investigate the source of cfDNA. Given the potentially conflicting results of the source of cfDNA released following CLP-induced sepsis, further studies with PAD4-/- and other methods of NET-inhibition should be conducted.

6.4 Absence of NETs in PAD4-deficient mice

NETs have been shown to act as scaffolds for platelet recruitment, platelet aggregation, and leukocyte-platelet interactions in LPS sepsis models (Clark et al., 2007; Tanaka et al., 2014). TAT levels were decreased in PAD-/- mice following LPS challenge, but not in a CLP sepsis model (Martinod et al., 2015). In our studies, we also observed no changes in TAT levels following administration of DNase versus saline. It is possible that aberrant coagulant responses are part of the sequelae which occur later in sepsis progression (Bone, 1992) and that changes in mediators of coagulation may be observed at later time-points in our studies. However, in our PAD4-/- studies, thrombin generation in PAD4-/- mouse plasma was significantly reduced compared to C57Bl/6 mouse plasma in CLP-operated mice, suggesting that NETs contribute to thrombin generation in sepsis.

One study using PAD4-deficient mice as a model of removing NETs suggest that the absence of NETs results in no changes in inflammation, bacterial dissemination, or survival (Martinod et al., 2015). In our studies, we observed that inflammation and organ

damage are blunted in PAD4-deficient mice. The discrepancy may be due to a much less severe CLP model used by Martinod *et al.* which more closely resembles that of abscess formation as evidenced by the low bacterial burden and low levels of circulating inflammatory biomarkers (Martinod et al., 2015). Others have also observed that inflammation and liver injury are significantly reduced in PAD4-deficient mice subjected to MRSA-challenge (Kolaczkowska et al., 2015). However, no differences in survival were detected between PAD4-deficient and wildtype mice subjected to CLP.

6.5 Design of animal studies in sepsis

While surrogate markers of death have been validated in animal models of other disease states, endpoint markers have not been established for sepsis, despite recommendations from various animal ethics boards. Several scoring systems, the Mouse Clinical Assessment Score for Sepsis (M-CASS) (Huet et al., 2013), Murine Sepsis Score (MSS) (Shrum et al., 2014), and Mouse Grimace Scale (MGS) (Langford et al., 2010; Matsumiya et al., 2012) have been proposed as tools to monitor endpoints and assess disease progression. These scoring systems involve the monitoring of predetermined criteria to determine the time at which animals should be humanely euthanized.

Using mortality studies, we determined that the M-CASS, MSS, and MGS are effective scoring systems for predicting death and assessing disease severity in the CLP sepsis model and identified various components which can be removed to improve the utility of the scoring systems. Moreover, rectal temperature was an effective, objective surrogate marker which can be used to complement scoring systems to increase their utility. Symptomatic evaluation using these scoring systems and temperature measured by

a rectal probe offers significant advantages. Using these scoring systems may close the gap in the translation of data from animal sepsis studies to clinical sepsis as physiologically relevant surrogate markers similar to those used in the ICU (e.g. respiratory rate, temperature, level of consciousness, etc.) (Stenhouse et al., 2000; Subbe et al., 2001) are assessed in these scoring systems. Assessment of disease progression via scoring systems does not artificially exacerbate sepsis symptoms or sepsis pathology. In studies investigating changes induced by additional insults or therapeutic interventions, changes in the animal's health status can be detected immediately. Scoring systems provide reliable monitoring techniques to meet the increasingly stringent standards of ethical, humane animal research. Lastly, scoring systems using phenotypic and physiological endpoint markers offer a more feasible and cost-efficient means to obtain vital signs in small rodents. We have proposed scoring systems which can be used for assessing disease severity, endpoint monitoring, and predicting death to obviate inhumane methods of using death as an endpoint in sepsis studies.

In our murine sepsis studies, we have documented that time-dependent increases in cfDNA accompany pro-inflammatory and procoagulant responses. Administration of DNase following a period of sepsis progression during which levels of cfDNA increase offers a protective effect in a CLP sepsis model. The source of cfDNA detected in circulation in murine sepsis may be NET-derived and NETs may contribute to sepsis pathophysiology by driving inflammation and coagulation. Future sepsis studies using animal models of sepsis should consider the use of surrogate markers and scoring systems rather than death as an endpoint.

Chapter 7: Future directions and conclusions

7.1 Source of cell-free DNA

While some studies suggest that cfDNA released upon inflammatory challenge in *in vitro* conditions are NET-derived (Gould et al., 2014), the source of cfDNA following inflammatory challenge *in vivo* is unclear. There are currently no *in vivo* studies which clearly elucidate the degree to which NETs in the microcirculation contribute to circulating cfDNA during sepsis. Some groups classify all circulating DNA which stain for PicoGreen, a cell-impermeable DNA dye, as NET-derived (Meng, Paunel-Görgülü, Flohé, Hoffmann, et al., 2012) while others establish that cfDNA which stain for the cell-permeable Hoecht 33342 stain (and a neutrophil elastase antibody (Knight et al., 2013; Knight, Luo, et al., 2014; Knight, Subramanian, et al., 2014) are NET-derived. Other *in vitro* studies suggest that cfDNA released following inflammatory challenge may not be from NETosis. In one study, only 3% of neutrophils underwent NETosis following LPS challenge (Tanaka et al., 2014) and in other studies, around 35% of activated neutrophils formed NETs (Brinkmann & Zychlinsky, 2007; Clark et al., 2007; Fuchs et al., 2007). Moreover, a recent study reported no changes in levels of cfDNA released in neutrophil-depleted mice following CLP surgery (Hamaguchi et al., 2015). No increase in citrullinated histone H3, a marker of PAD4-mediated NET formation was observed via Western blotting or FACS analysis (Hamaguchi et al., 2015). In contrast, our studies and others where NET formation is abolished in PAD4 deficient mice show that NETs at least partially contribute to increases in cfDNA during sepsis (Martinod et al., 2015). Future *in vivo* sepsis studies which characterize NET formation via different methods such as

inhibiting NETs with Cl-amidine or treatment with DNase and histone inhibitors (CRP, APC, or heparin) should be conducted to determine the degree to which NETosis contributes to cfDNA increases observed in sepsis.

7.2 Pathogenic effects of cfDNA

To investigate whether cfDNA is pathogenic, healthy mice can be given intrajugular injections of cfDNA (isolated from healthy or septic plasma). A separate group of mice can be injected with cfDNA pretreated with DNase and any increases in inflammation, coagulation, and organ damage in the absence of DNase may indicate pathogenicity induced by cfDNA. In addition, LPS studies can be conducted to determine whether co-injection of cfDNA and LPS causes increased pathogenic effects compared to cfDNA injection alone.

7.3 DNase treatment and outcome

We have observed that following DNase treatment in sepsis, levels of cfDNA decrease, aberrant inflammation and coagulant responses are blunted, organ damage is reduced, and survival is improved (Mai et al., 2015). However, it is unclear whether the potential therapeutic effects of DNase occur through degradation of cfDNA or NETs thus abolishing the pathological effects of DNA and/or NETs in circulation. Future studies may be conducted to investigate the relationship between plasma levels of DNase and cfDNA in septic mice. It is also unclear whether there is continuous release of cfDNA after DNase is cleared. Further dosage studies can be conducted with either continuous injections via a pump or multiple bolus injections of DNase.

7.4 DNase in sham-operated mice

While DNase treatment in healthy mice appears to have no pathological effects, DNase treatment in sham-operated mice resulted in increased inflammation and lung damage (Mai et al., 2015). In cystic fibrosis, DNase treatment also exerts an initial pro-inflammatory response (Shah, Scott, Knight, & Hodson, 1996; Shah, Scott, Knight, Marriott, et al., 1996) however, the mechanism by which DNase may exacerbate inflammation has not been elucidated. It is possible that in the healthy condition, DNase is rapidly cleared but following injury (through sham surgery), DNase clearance is delayed. In the absence of high levels of cfDNA, DNase may cleave other targets. One known target of DNase is actin (dos Remedios et al., 2003), however it has not been shown that the cleavage of actin by DNase produces any pathological effects. β-actin knock-out mice may be used to determine whether addition of DNase following sham surgery results in increased inflammation (Bunnell, Burbach, Shimizu, & Ervasti, 2011). Future studies may focus on identifying the mechanism by which DNase potentiates inflammation.

7.5 Thrombin generation

Plasma from healthy control and sham-operated mice did not generate thrombin when initiated in the presence of 15 mM $CaCl_2$ (Figure 7.1) and generated minimal amounts of thrombin when initiated in the presence of 15 mM $CaCl_2$, 1/500 dilution of Recombiplastin (17 pM), and 30 μM PCPS in PBS (Figure 7.2). Plasma from CLP-operated mice generated more thrombin than plasma from sham-operated and healthy mice in the absence or presence of Recombiplastin. More thrombin was generated in

Figure 7.1. Thrombin generation in plasma from healthy control, sham-operated, and CLP-operated mice initiated in the presence of calcium, dilute Recombiplastin, and phospholipids. Mice were subjected to sham or CLP surgery. Blood was collected 6 hours post-operatively and platelet-poor plasma was isolated. Thrombin generation assays were initiated in the presence of 15 mM $CaCl_2$, 1/500 dilution of Recombiplastin (17 pM), and 30 μM PCPS in PBS. Plasma from sham-operated and healthy control mice generated thrombin, however, much greater levels of thrombin was generated with plasma from CLP-operated mice (A). Lag time was reduced (B) and peak thrombin (C) and AUC (E) were significantly increased in plasma from CLP-operated mice compared to plasma from sham-operated and healthy control mice (n = 8 per group).

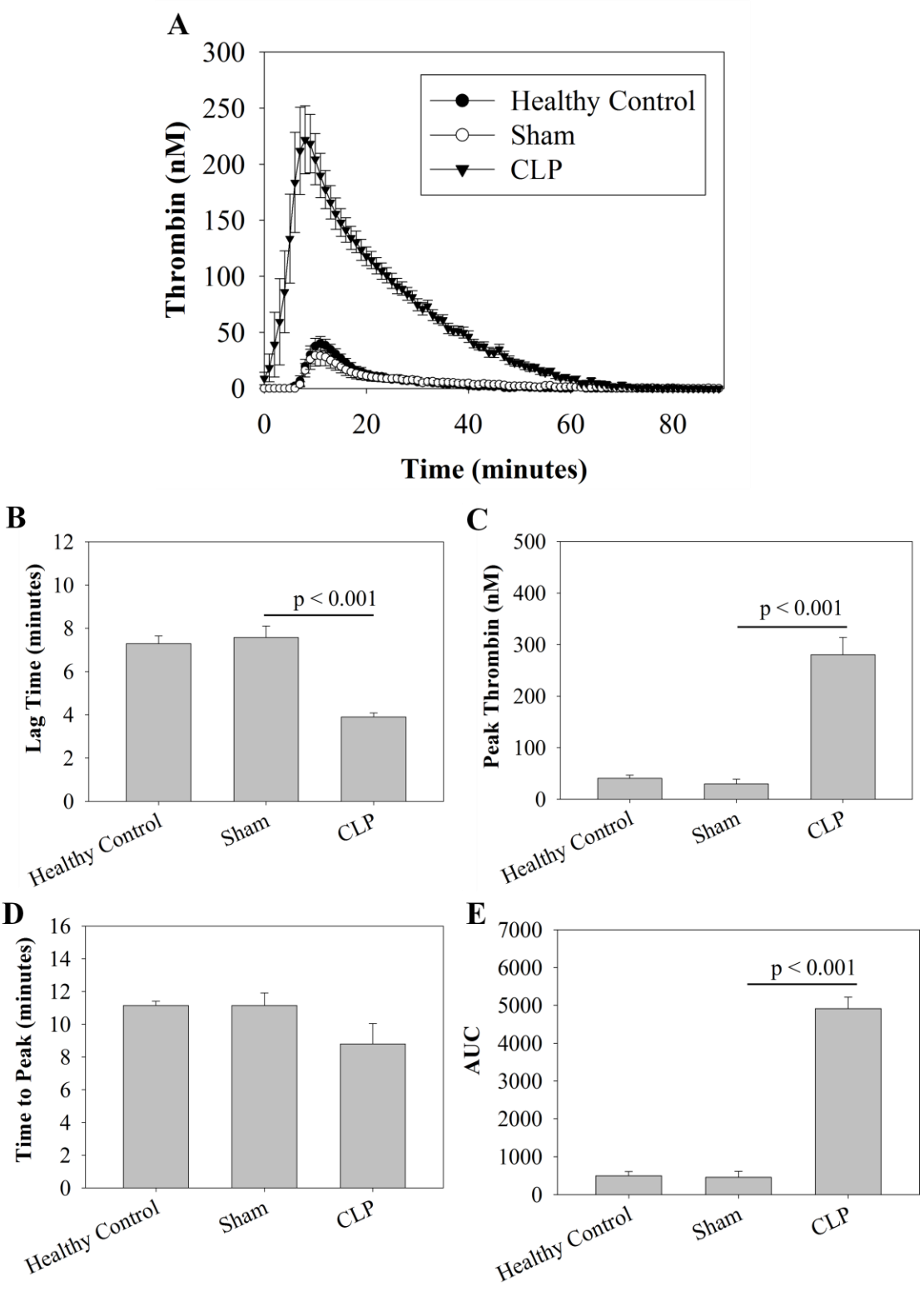

A
Thrombin (nM)
Time (minutes)
Healthy Control
Sham
CLP
B
Lag Time (minutes)
p < 0.001
C
Peak Thrombin (nM)
p < 0.001
D
Time to Peak (minutes)
E
AUC
p < 0.001
Healthy Control
Sham
CLP

Figure 7.2. Thrombin generation in plasma from healthy control, sham-operated, and CLP-operated mice in the presence of calcium. Mice were subjected to sham or CLP surgery. Blood was collected 6 hours post-operatively and platelet-poor plasma was isolated. Thrombin generation assays were initiated with 15 mM $CaCl_2$. Plasma from sham-operated and healthy control did not generate thrombin while plasma from CLP-operated mice generated thrombin (A). Lag time was reduced (B) and time to peak (C), peak thrombin (D), and AUC (E) were significantly increased in CLP-operated mice compared to sham-operated and healthy control mice (n = 7 per group).

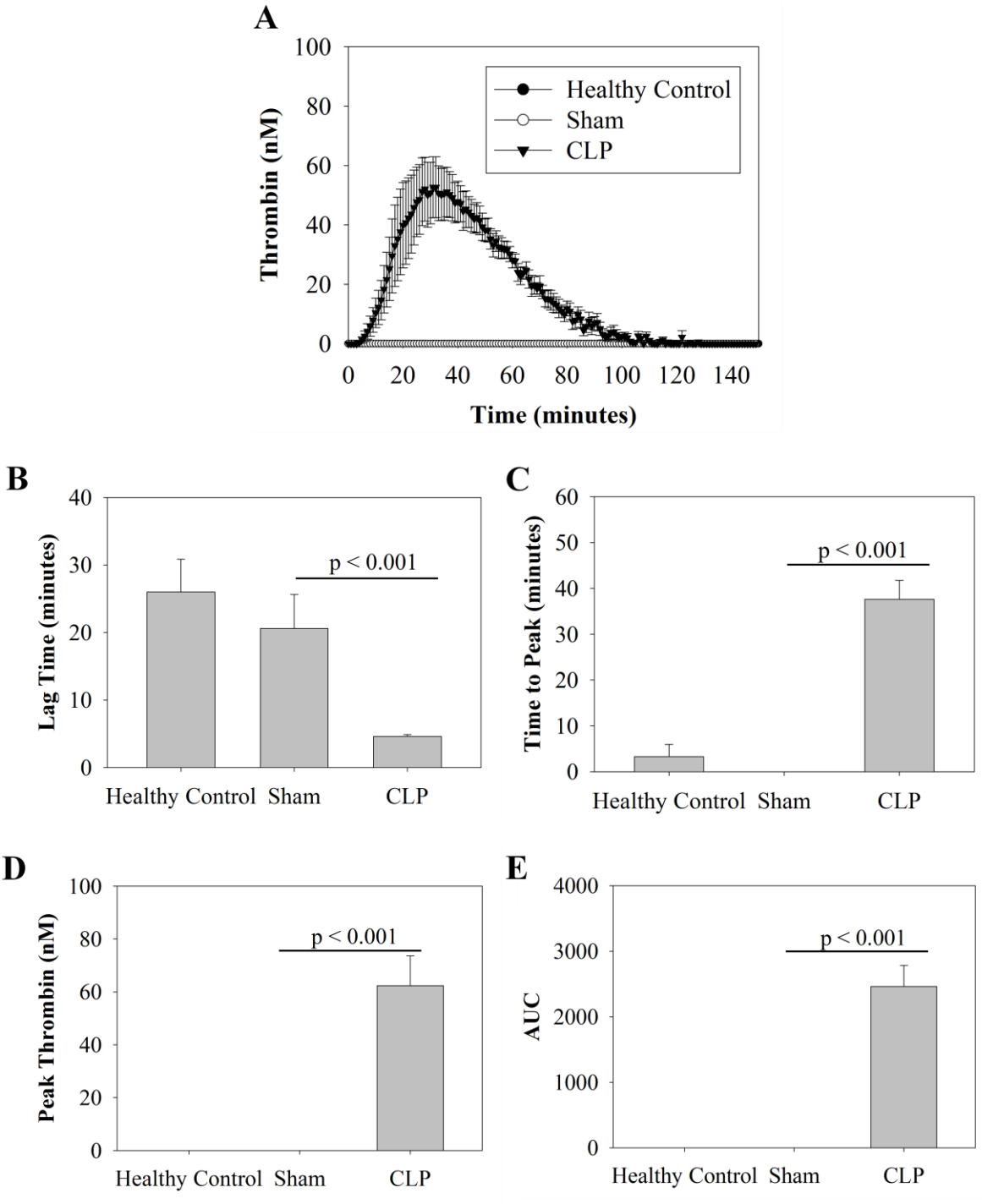
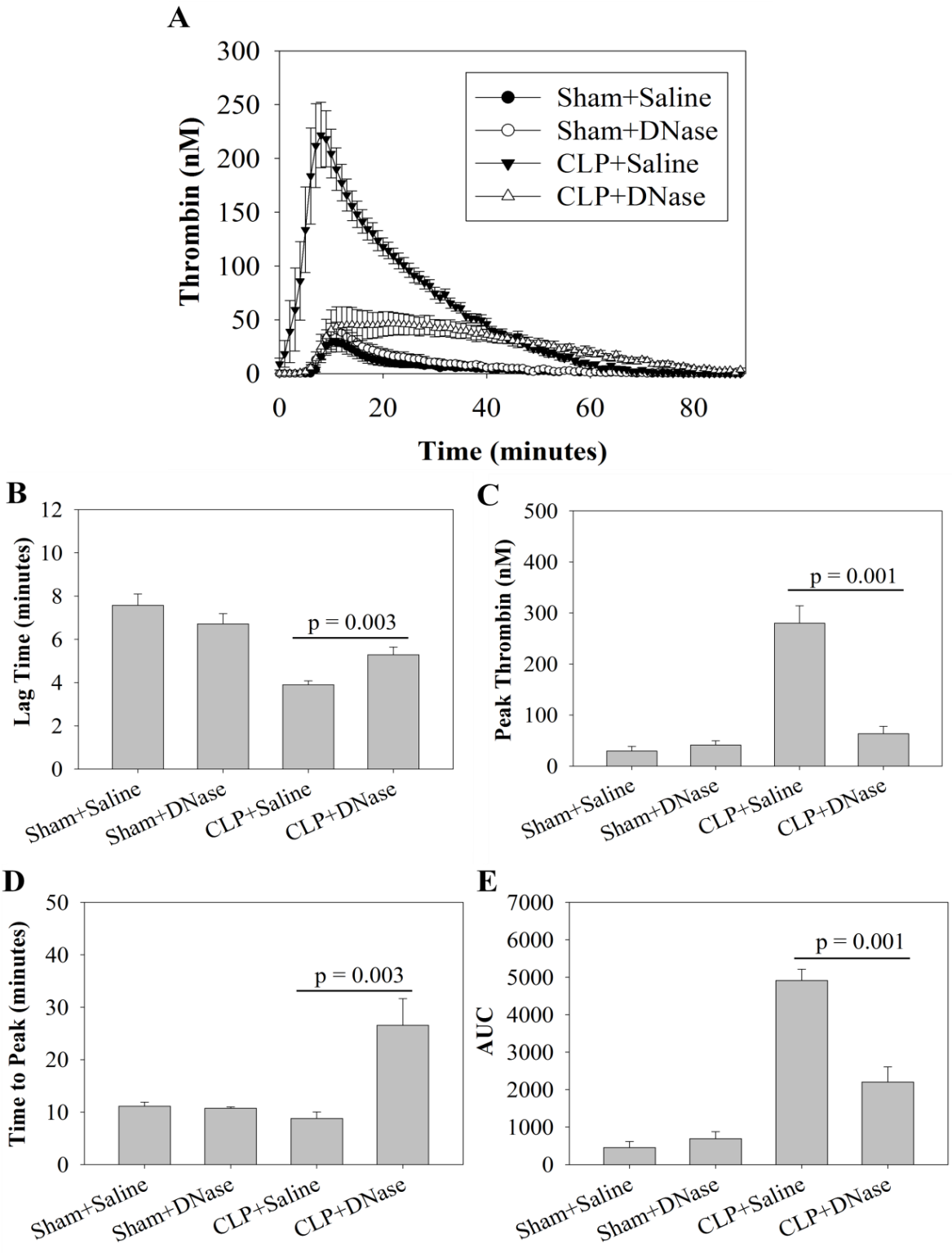

A
Thrombin (nM)
Time (minutes)
Healthy Control
Sham
CLP
B
Lag Time (minutes)
p < 0.001
Healthy Control
Sham
CLP
C
Time to Peak (minutes)
p < 0.001
Healthy Control
Sham
CLP
D
Peak Thrombin (nM)
p < 0.001
Healthy Control
Sham
CLP
E
AUC
p < 0.001
Healthy Control
Sham
CLP

Figure 7.3. Thrombin generation in plasma from sham- and CLP-operated mice given saline or DNase initiated in the presence of calcium, dilute Recombiplastin, and phospholipids. Mice were subjected to sham or CLP surgery and given either DNase or saline 6 hours post-operatively. Blood was collected 2 hours following DNase or saline injection and platelet-poor plasma was isolated. Thrombin generation assays were initiated in the presence of 15 mM $CaCl_2$, 1/500 dilution of Recombiplastin (17 pM), and 30 μM PCPS in PBS. Plasma from CLP-operated mice given saline *in vivo* generated much more thrombin than plasma from sham-operated mice given saline or DNase (A). Thrombin generation was largely suppressed in CLP-operated mice given DNase treatment compared to CLP-operated mice given a saline control (A). In CLP-operated mice given DNase treatment, lag time (B) and time to peak (D) were increased and peak thrombin (C) and AUC (E) were reduced compared to CLP-operated mice given saline (n = 7 per group).


A
Thrombin (nM)
Time (minutes)
Sham+Saline
Sham+DNase
CLP+Saline
CLP+DNase
B
Lag Time (minutes)
p = 0.003
C
Peak Thrombin (nM)
p = 0.001
D
Time to Peak (minutes)
p = 0.003
E
AUC
p = 0.001

plasma from CLP-operated mice when thrombin generation assays were initiated in the presence of dilute Recombiplastin, phospholipids, and $CaCl_2$ (Figure 7.1) compared to initiating in the presence of only $CaCl_2$ (Figure 7.2).

In thrombin generation assays initiated in the presence of 15 mM $CaCl_2$, 17 pM Recombiplastin, and 30 μM PCPS, a reduction in thrombin generation was observed in plasma from CLP-operated mice given DNase *in vivo* versus plasma from CLP-operated mice given saline (Figure 7.3). However, when initiating only in the presence of 15 mM $CaCl_2$, no differences were observed in thrombin generation with plasma from CLP-operated mice given saline versus DNase *in vivo* (Figure 7.4).

One potential mechanism by which thrombin generation is inhibited in septic mice administered DNase is if DNase cleaves FVIIa, however, we did not observe any changes in the cleavage of Biophen CS-11(32) by FVIIa in the presence or absence of DNase (Figure 7.5). It is possible that in the presence of TF, cfDNA drives thrombin generation via contact activation as well as via through the extrinsic pathway. Incubating plasma from CLP-operated mice with an anti-FVIIa antibody before triggering thrombin generation with dilute TF, phospholipids, and calcium is one method which can be used to investigate the possible role of TF pathway activation by cfDNA.

Figure 7.4. Thrombin generation in plasma from CLP-operated mice given saline or DNase initiated in the presence of calcium. Mice were subjected to CLP surgery and administered saline or DNase 6 hours post-operatively. Blood was collected 2 hours following saline or DNase injection and platelet-poor plasma was isolated. Thrombin generations were initiated in the presence of 15 mM $CaCl_2$. No differences in thrombin generation (A), lag time (B), peak thrombin (C), time to peak (D), or AUC (E) were observed between plasma from CLP-operated mice given saline versus CLP-operated mice given DNase *in vivo* (n = 9 per group).

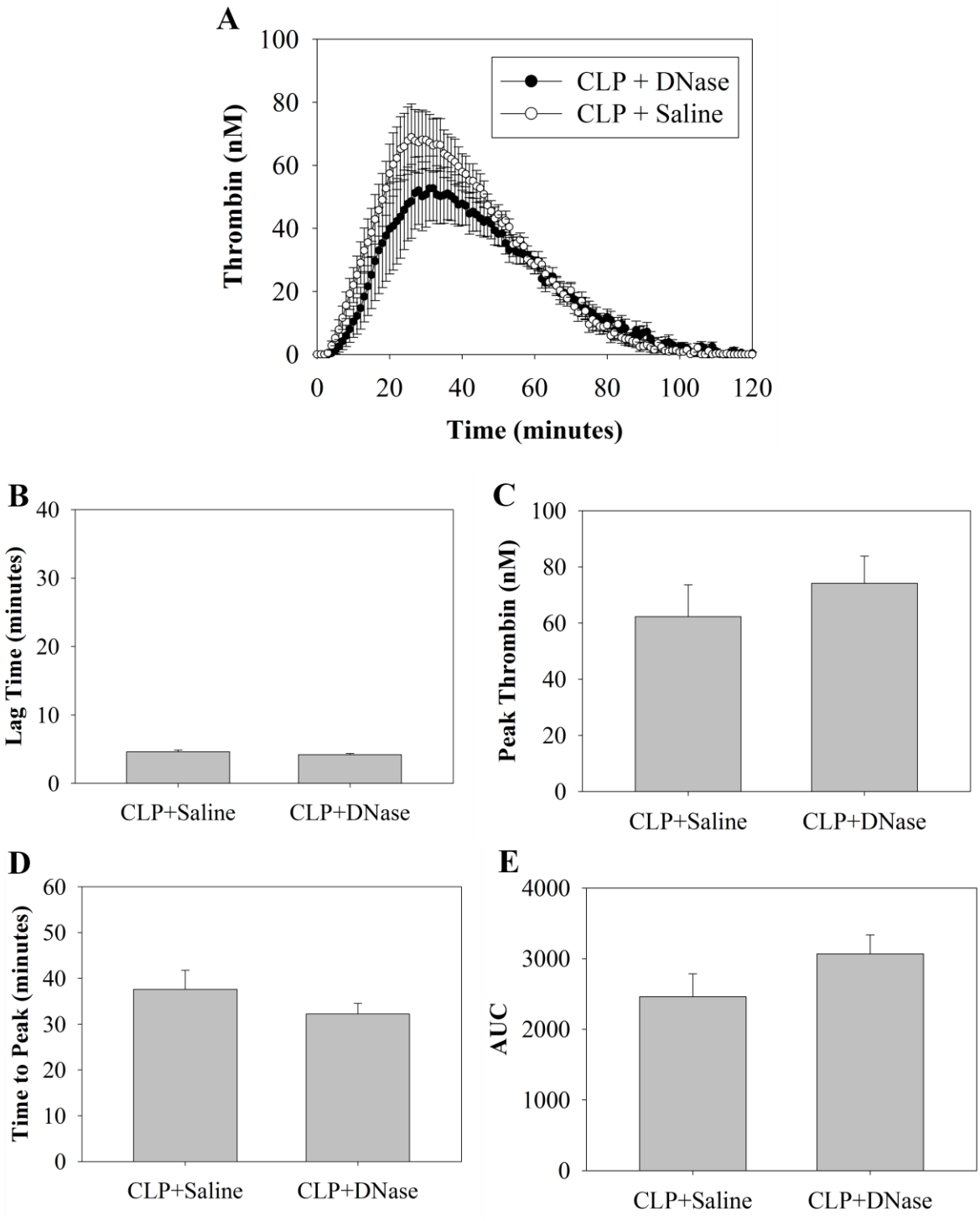
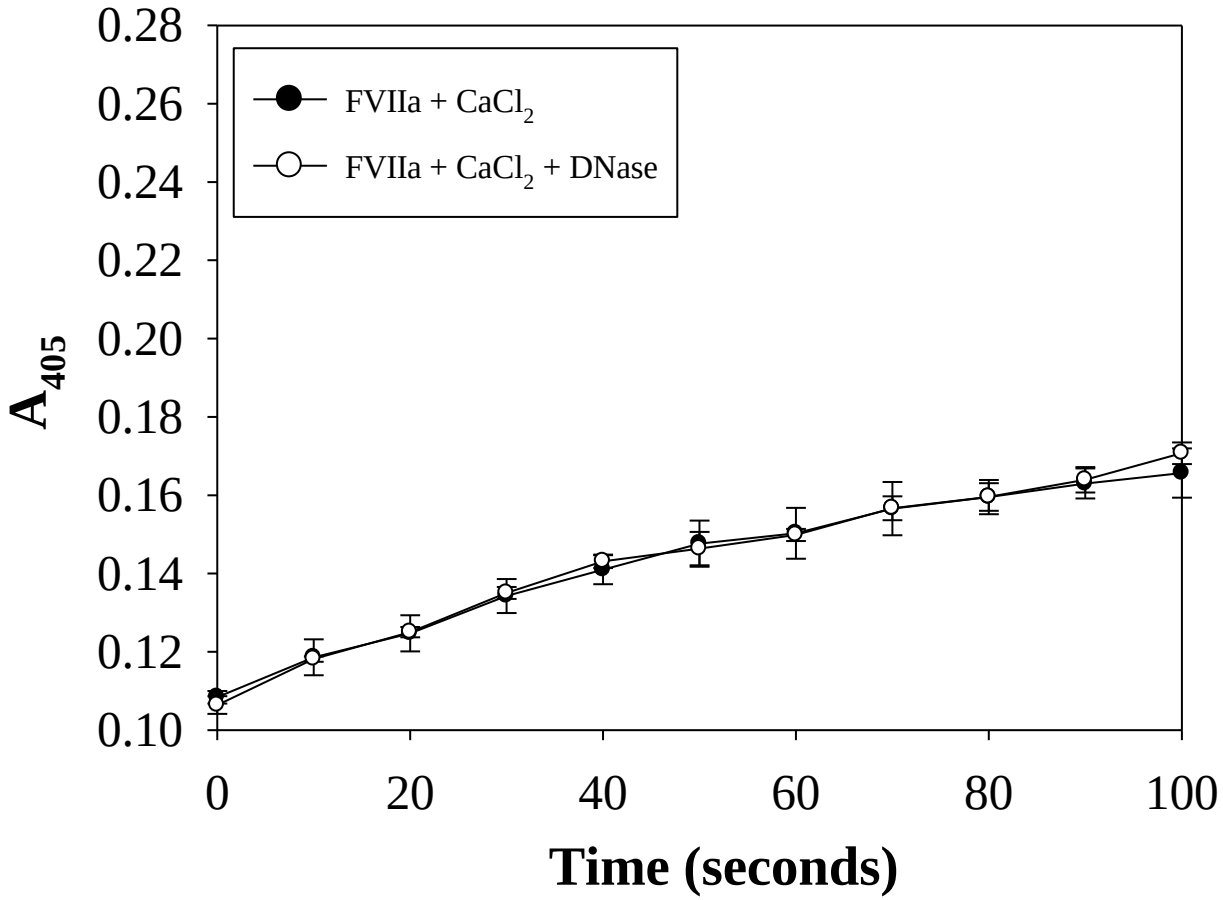

A
Thrombin (nM)
Time (minutes)
CLP + DNase
CLP + Saline
B
Lag Time (minutes)
CLP+Saline
CLP+DNase
C
Peak Thrombin (nM)
CLP+Saline
CLP+DNase
D
Time to Peak (minutes)
CLP+Saline
CLP+DNase
E
AUC
CLP+Saline
CLP+DNase

Figure 7.5. DNase does not inhibit FVIIa activity against Biophen CS-11(32). 50 nM FVIIa was incubated in the absence and presence of 10 nM DNase in PBS containing 2 mM $CaCl_2$ for 3 hours at room temperature. 200 μM of chromogenic substrate Biophen CS-11(32) was added and absorbance was monitored at 405 nm. A_{405} values are plotted versus time. No differences in absorbance in the presence or absence of DNase were observed (n = 4 per group).


0.28
0.26
0.24
0.22
0.20
0.18
0.16
0.14
0.12
0.10
A405
FVIIa + CaCl2
FVIIa + CaCl2 + DNase
0
20
40
60
80
100
Time (seconds)

7.6 Conclusions

In our murine sepsis studies, time-dependent increases in cfDNA accompany inflammation, coagulation, organ damage, and bacterial dissemination. Administration of DNase following a period of sepsis progression during which levels of cfDNA are elevated offers a protective effect in a CLP sepsis model.

Using PAD4-deficient mice, we demonstrated that NETs contribute to increased circulating cfDNA levels in our murine sepsis model. NETs exacerbate sepsis pathophysiology by driving inflammation, coagulation, and organ damage and the absence of NETs results in a blunted inflammatory response, but no change in survival. NETs may be a potential therapeutic target in the treatment of sepsis.

Surrogate scoring systems of sepsis which include clinically relevant physiological markers can be used to assess sepsis severity and disease progression in murine sepsis. Scoring systems provide immediate, non-invasive, feasible, and cost-efficient means to assess the health status of the septic mouse. Future studies using animal models of sepsis should consider the use of humane surrogate markers and scoring systems rather than use of death as an endpoint.

8.0 References

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