PROCOAGULANT AND ANTI-FIBRINOLYTIC PROPERTIES OF CELL-FREE DNA AND HISTONES IN SEPSIS

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ABSTRACT

Sepsis is a devastating clinical condition characterized by a systemic inflammatory response to infection, with concomitant dysregulated pathological thrombus formation. Although sepsis is triggered by the release of microorganisms and/or microbial toxins into the circulation, the presence of infection itself is rarely the cause of death in these patients. Rather, mortality in sepsis is attributed to irreversible organ damage resulting from prolonged, uncontrolled activation of inflammatory and coagulation pathways within the microcirculation. Despite recent advances in clinical management, treatment continues to be largely supportive in nature. As a result, sepsis remains the leading cause of morbidity and mortality in non-coronary intensive care units with mortality rates ranging from 18-30%. Sepsis-induced mortality is further increased following the development of disseminated intravascular coagulation, a thrombohemorrhagic state defined by a primary thrombotic and secondary hemorrhagic diathesis that may culminate in multi-organ failure.

Clinical management of patients with sepsis is challenging and largely limited to supportive therapies, which is in part related to a limited understanding of the underlying pathophysiology. Recently, cell-free DNA (CFDNA) has emerged as an important link between innate immunity, coagulation, and inflammation. Furthermore, we have previously demonstrated that plasma levels of CFDNA have high discriminative power to predict ICU

mortality in patients with severe sepsis. Patients with higher plasma concentrations of CFDNA are more likely to face severe complications such as organ dysfunction/failure, and death. The evidence presented in this thesis suggests that CFDNA may not simply be an innocuous marker of disease severity, but may itself exert pathological effects and contribute to the fatal coagulation abnormalities observed in sepsis patients.

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List of Abbreviations

α2AP	Alpha-2-antiplasmin
ADAMTS13	A disintegrin and metalloproteinase with a thrombospondin type 1 motif, member13
ADP	Adenosine diphosphate
ANOVA	Analysis of variance
AP	Alkaline phosphatase
APACHE	Acute physiology and chronic health evaluation
APC	Activated protein C
APTT	Partial thromboplasmin time
AT	Antithrombin III
ATP	Adenosine triphosphate
AUC	Area under the cuve
BSA	Bovine serum albumin
Ca^{2+}	Calcium
$CaCl_2$	Calcium chloride
CAT	Calibrated automated thrombin generation
CFDNA	Cell-free DNA
CG	Cathespin G
CGD	Chronic granulomatous disease
CLFA	Clumping factor A
CLP	Cecal ligation and puncture
CLT	Clot lysis time
CRP	C-reactive protein
CTI	Corn trypsin inhibitor
DAMP	Damage-associated molecular pattern
DAPI	4',6-diamidino-2-phenylindole
DIC	Disseminated intravascular coagulation
DNA	Deoxyribonucleic acid
DNase	Deoxyribonuclease
DVT	Deep vein thrombosis
ECM	Extracellular matrix
EDTA	Ethylenediaminetetraacetic acid
ELISA	Enzyme-linked immunosorbant assay
EPCR	Endothelial protein C receptor
ETP	Endogenous thrombin potential
F	Factor
FDP	Fibrin degradation product
FITC	Fluorescein isothiocyanate
fMLP	N-Formylmethionyl-leucyl-phenylalanine
Fp	Fibrinopeptide

Gla	γ-carboxyglutamic
GP	Glycoprotein
H	Histone subunit
HBSS	Hank's buffered saline solution
HK	High molecular weight kininogen
HMGB1	High mobility group box 1
HUVEC	Human umbelical vein endothelial cell
ICU	Intensive care unit
IFN	Interferon
Ig	Immunoglobulin
II	Prothrombin
IIa	Thrombin
IL	Interleukin
K	Kunitz domain
LPS	Lipopolysaccharide
LTA	Lipoteichoic acid
mAb	Monoclonal antibody
Mal	MyD88-adaptor like
MODS	Multiple organ dysfunction syndrome
MP	Microparticle
mRNA	Messenger RNA
MyD88	Myeloid differentiation factor
NADPH	Nicotinamide adenine dinucleotide phosphate
NE	Neutrophil elastase
NET	Neutrophil extracellular trap
NF-κB	Nuclear factor kappa B
PAD4	Peptidyl arginine deiminase 4
PAI-1	Plasminogen activator inhibitor 1
PAMP	Pathogen-associated molecular pattern
PAR	Protease-activated receptor
PBS	Phosphate buffered saline
PC	Protein C
PG	Peptidoglycan
PK	Prekallikrein
PMA	Phorbol myristate acetate
PolyP	Polyphosphate
PPP	Platelet-poor plasma
PRP	Platelet-rich plasma
PRR	Pattern recognition receptor
PS	Phosphatidylserine
PT	Prothrombin time
RAGE	Receptor for advanced glycation end-products

RBC	Red blood cell
RNA	Ribonucleic acid
RNase	Ribonuclease
ROS	Reactive oxygen species
SARM	Sterile alpha and heat-armadillo motifs
SDS-PAGE	Sodium dodecyl sulfate polyacrylamide gel electrophoresis
SE	Standard error
SEM	Scanning electron microscopy
SPR	Surface plasmon resonance
TAFI	Thrombin-activatable fibrinolysis inhibitor
TEG	Thromboelastography
TF	Tissue factor
TFAM	Transcription factor A, mitochondrial
TFPI	Tissue factor pathway inhibitor
TIR	Toll/Interleukin-1 receptor
TLR	Toll-like receptor
TM	Thrombomodulin
TNF	Tissue necrosis factor
tPA	Tissue plasminogen activator
TRAM	TRIF-relaated adaptor molecule
TRIF	TIR domain-containing adaptor inducing interferon
UFH	Unfractionate heparin
uPA	Urokinase-type plasminogen activator
vWF	von Willebrand factor

1.0 General Introduction

1.1 Introduction to Hemostasis & Thrombosis

1.1.1 Overview of Hemostasis

Maintenance of blood fluidity within the vasculature is an essential physiological process. Hemostasis is the process that maintains the integrity of the host's circulatory system following injury. Hemostasis serves three primary purposes: i) to cordon off damaged blood vessels at the site of injury, ii) to maintain circulating blood in a fluid state, and; iii) to remove blood clots after restoration of vascular integrity. The hemostatic system is one that is highly evolutionarily conserved and can be found in a variety of organisms ranging from zebrafish to humans (Versteeg et al. 2013).

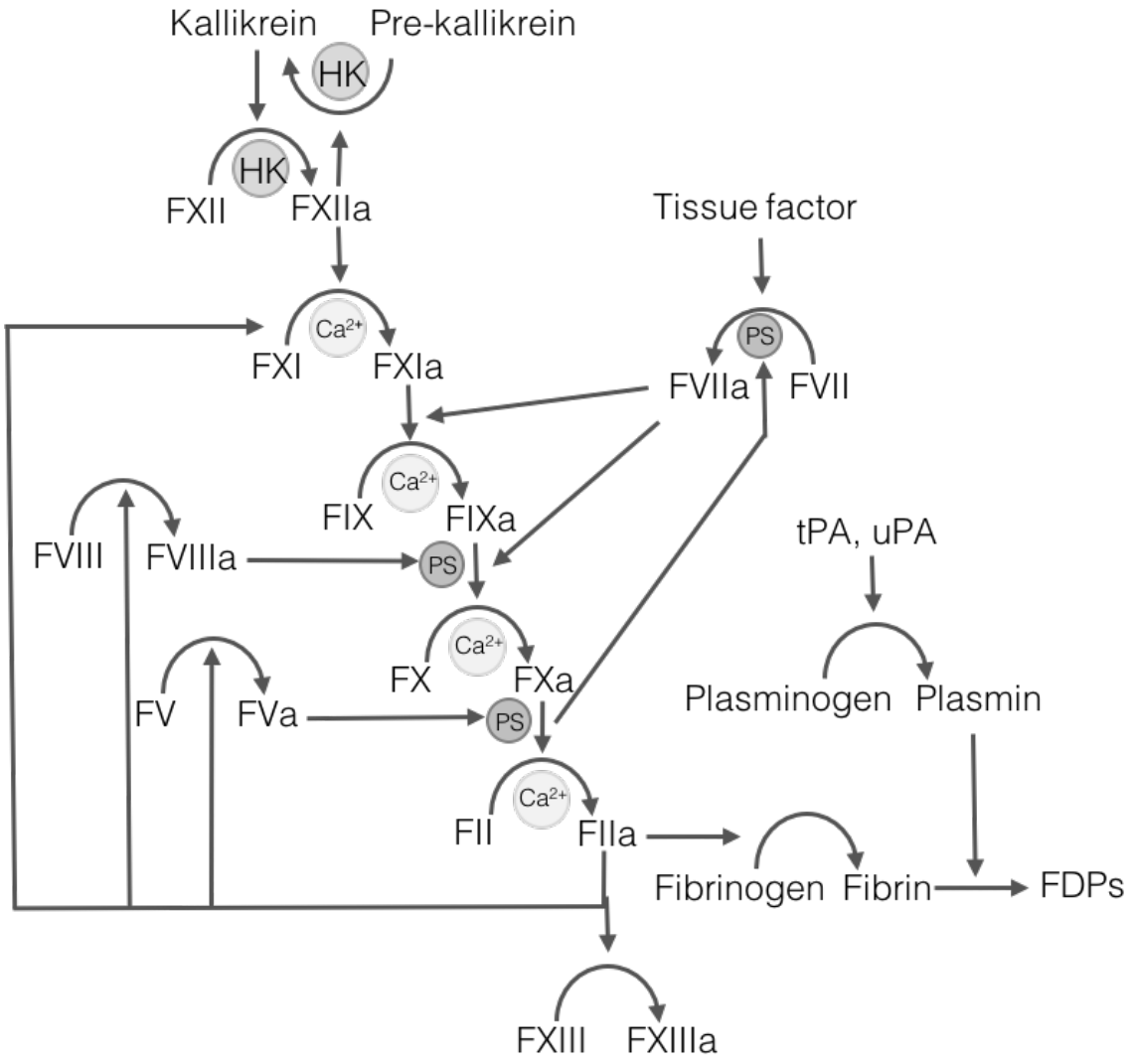
Primary hemostasis refers to the immediate formation of a 'platelet plug' at the site of vessel injury. Following insult, exposure of subendothelial collagen and von Willebrand factor (vWF) tethers platelets to the vessel wall and facilitates their activation. Activated platelets will then degranulate, releasing platelet agonists and ultimately propagating the formation of a platelet thrombus (reviewed in Furie and Furie 2008).

Simultaneous to platelet activation, secondary hemostasis occurs which involves the activation of a complex cascade of coagulation proteins to generate a fibrin network. Secondary hemostasis is initiated via two distinct pathways: the extrinsic (tissue factor) and the intrinsic (contact) pathways of coagulation. In the

extrinsic pathway, vascular damage exposes subendothelial tissue factor (TF) to the blood. Exposed TF complexes with circulating activated (a) factor (F) VIIa and zymogen FX to form the extrinsic tenase complex, which functions to catalytically activate FX to FXa and FIX to FIXa. Conversely, the intrinsic pathway becomes activated once blood comes into contact with negatively charged, hydrophilic surfaces such as glass, kaolin, or endogenous activators such as nucleic acids or polyphosphates. Contact activation is triggered by auto-activated FXII cleaving prekallikrein into kallikrein, which leads to a subsequent activation pathway of FXI, FIX, and ultimately FX (Versteeg et al. 2013).

Both the extrinsic and intrinsic pathways converge upon the generation of FXa (referred to as the common pathway of coagulation), which converts a small amount of prothrombin into thrombin (FIIa) in the presence of cofactors FVa, calcium (Ca^{2+}), and phosphatidylserine (PS). The trace amounts of thrombin generated at this stage activate FV, VIII, and FXI, initiating multiple positive feedback loops that potentiate additional thrombin generation referred to as a "thrombin burst". Thrombin, in turn, converts soluble fibrinogen into insoluble strands of fibrin, which reinforce the initial platelet plug. In addition, thrombin also activates FXIII to FXIIIa which crosslinks individual fibrin polymers to stabilize the fibrin network and confers fibrinolytic resistance to the newly formed clot (Versteeg et al. 2013). Both arms of the coagulation cascade are depicted in **Figure 1.1**.

Figure 1.1 – The coagulation cascade. Vascular injury initiates the extrinsic pathway by exposing TF to the blood. TF complexes with low levels of circulating factor VIIa, to convert factor IX to IXa, or X to Xa. The contact (or intrinsic) pathway is initated by the activation of prekallikrein to kallikrein and factor XII to XIIa. Factor XIIa activates factor XI, which in turn activates factor IX. Factor Xa converts small amounts of prothrombin to thrombin (IIa), the enzymatic endpoint of the coagulation cascade. Thrombin converts soluble fibrinogen into an insoluble fibrin clot, which further stabilized by cross-linking mediated by factor XIIIa. Dissolution of a fibrin clot is regulated by the activation of liver-produced plasminogen into plasmin by tissue-type plasminogen activator (tPA) and urokinase-type plasminogen activator (uPA).


Kallikrein
Pre-kallikrein
HK
HK
FXII
FXIIa
Tissue factor
Ca2+
FXI
FXIa
PS
FVIIa
FVII
Ca2+
FIX
FIXa
FVIII
FVIIIa
PS
tPA, uPA
Ca2+
FX
FXa
FV
FVa
PS
Plasminogen
Plasmin
Ca2+
FII
FIIa
Fibrinogen
Fibrin
FDPs
FXIII
FXIIIa

Dissolution and clearance of a fibrin clot is mediated via a process termed fibrinolysis, whereby tissue-type plasminogen activator (tPA) and urokinase-type plasminogen activator (uPA) are released from damaged endothelial cells and convert clot-bound plasminogen into plasmin. Plasmin degrades insoluble fibrin into soluble fibrin degradation products (FDPs) that are readily cleared from the circulation (reviewed in Chapin and Hajjar 2015).

1.1.2 Thrombosis & Disseminated Intravascular Coagulation

Under pathological conditions, hemostatic regulation becomes overwhelmed resulting in the excessive formation of thrombin and fibrin. This hypercoagulable state is termed 'thrombosis'. Thrombotic events can occur in both large and small vessels. Of the large vessels, there are two primary types of thrombus formation: arterial and venous thrombosis. Arterial thrombosis refers to the formation of a thrombus within an artery and, in most cases, is the result of a ruptured atherosclerotic plaque. Arterial thrombi may embolize and cause infarction of almost any organ in the body. Conversely, venous thrombosis occurs commonly in the deep veins of the leg, such as the femoral vein. This form of thrombosis is the typically the result of impaired circulation (stasis), alterations in clotting factor levels, and/or endothelial dysfunction. Embolization of a venous clot commonly results in blockage of the pulmonary arteries and subsequent hypoxia (Furie and Furie 2008).

However, the most severe form of pathologic coagulation is known as disseminated intravascular coagulation (DIC), which primarily affects the small vessels of the microcirculation. DIC is characterized by the simultaneous development of systemic thrombosis within the microvasculature and does not occur by itself: rather, DIC is always associated with an underlying pathological condition, such as cancer, trauma, or sepsis (Levi and Cate 1999).

The most common consequence of DIC is the development of terminal organ failure as a result of widespread microvascular thrombi formation, which contributes to insufficient vascular perfusion and inadequate oxygen delivery to vital organ tissues (Wheeler and Bernard 1999a). Indeed, autopsy results in patients with DIC reveal the presence of microthrombi contained within vessels of various organ tissue beds. Fibrin deposition in small and midsize vessels in these patients was also associated with ischemia, tissue necrosis, and ultimately the deterioration of organ function (Robboy et al. 1972; Wada 2004; Levi et al. 2013). Simultaneously, the unregulated activation of coagulation in DIC leads to the consumption of circulating coagulation factors and platelets, and can potentially result in severe thrombocytopenia and major bleeding (Levi and Cate 1999). Though bleeding only occurs in a minority of patients who develop DIC, these events can often be fatal and include intracranial, intrathoracic, or intra-abdominal bleeding requiring transfusion (Bernard et al. 2001; Dhainaut et al. 2005).

Clinically, DIC has been demonstrated to be a strong predictor of organ dysfunction in both trauma and septic patients. In patients with sepsis, mortality is almost twice as high in patients who develop DIC when compared with those who do not (Dhainaut et al. 2004; Bakhtiari et al. 2004). Thus, early identification and treatment of DIC is vital to ensuring survival in these patients.

1.2 Primary Hemostasis

1.2.1 Platelets in Hemostasis

Platelets are small, anucleated cells that are derived from megakaryocytes in the bone marrow (Italiano and Shivdasani 2003) but contain megakaryocyte messenger (m)RNA and the translational machinery needed for protein synthesis. Platelets are also characterized by the presence of cytoplasmic granules that harbor a variety of contents, including growth factors, pro- and anti-inflammatory cytokines, as well as adhesion molecules. Platelets play a central role during primary hemostasis and the formation of the initial platelet plug at the site of vascular injury to minimize blood loss. The recruitment of platelets to a site of injury has occurs in three distinct phases: 1) platelet adhesion; 2) platelet activation and degranulation; and 3) platelet aggregation.

1.2.2 Platelet Adhesion

Platelet adhesion to the vascular wall can occur under conditions of high shear (as found within arterial vessels) and low shear (such as the venous

systems). Upon vascular injury, subendothelial collagen is exposed to the circulating blood which immobilizes circulating vWF (Dopheide et al. 2002; Goto et al. 1995). Platelets interact with immobilized vWF via the surface glycoprotein (GP)Ibα, resulting in platelet tethering and arrest at the site of injury (Ruggeri 2007). Stable adhesion is subsequently mediated by several integrin interactions between the platelet and the vessel wall, namely integrin αIIbβ3 and fibrin/fibronectin, αVβ1 to fibronectin and collagen, and α2β1 to collagen (Ni and Freedman 2003).

1.2.3 Platelet Activation & Granule secretion

Interactions between platelet surface receptors and their ligands during adhesion induces platelet activation (Berndt et al. 2014; Furie and Furie 2008). In addition, the coagulation cascade is activated following vascular injury, generating a small amount of thrombin. Thrombin is considered to be the most potent platelet activator and exerts its effects by cleaving protease-activated receptors (PARs) on the platelet surface (Sambrano et al. 2001; Celikel et al. 2003). The process of platelet activation is characterized by the reorganization of the platelet cytoskeleton, transforming the platelet from discoid in shape to a sphere with protruding pseudopods extending onto the subendothelial matrix (Hartwig 1992). Platelet activation is negatively regulated by prostacyclin, which inhibits G-protein activity (Furie and Furie 2008).

Upon activation, platelets shuttle the contents of their intraceullar granules into the extracellular space. Contents of α-granules include platelet adhesion molecules, P-selectin (Yang et al. 2009), integrins, fibrinogen, fibronectin, vWF (Blair and Flaumenhaft 2009), platelet factor 4, and approximately 300 other proteins of various functions (Rao 2013). In contrast, dense granules contain inorganic polyphosphates (Ruiz et al. 2004), adenosine diphosphate (ADP), adenosine triphosphate (ATP), and ionized calcium which promote subsequent platelet activation (Jin et al. 2002).

1.2.4 Platelet Aggregation

Activated platelets recruit additional platelets to the growing hemostatic plug by multiple feedback loops. The release of platelet agonists from granules (namely ADP and serotonin), as well as the *de novo* synthesis of proaggregatory thromboxane A_2, promotes the activation of local quiescent platelets and their subsequent involvement in the growing thrombus (Jin et al. 2002; Arthur et al. 2008; Ni and Freedman 2003). In addition, platelets are also involved in secondary hemostasis (described in greater detail below) by serving as a negatively charged catalytic surface for amplification of the coagulation cascade. These signaling events serve to grow the initial platelet plug and, in conjunction with fibrin formation, consolidate thrombus formation at the site of vascular injury.

1.3 Secondary Hemostasis

1.3.1 Tissue Factor & Extrinsic Pathway Activation

Concurrent to primary hemostasis, secondary hemostasis is initiated by two distinct yet convergent pathways: the extrinsic (tissue factor) and intrinsic (contact) pathways of coagulation. Tissue factor (TF) is an integral transmembrane glycoprotein and is the primary initiator of coagulation in response to vascular injury (Mackman 2004). Though cells that come in contact with blood do not typically express TF, cells surrounding the vascular wall (such as smooth muscle cells, pericytes, and adventitial fibroblasts) constitutively express it (Fleck et al. 1990). Following injury, the sequestered TF is exposed to circulating blood, which initiates clotting (Mackman 2004). Elsewhere, TF expression is largely tissue-specific, with high levels found in the brain, lungs, heart, and placenta. This distribution of TF is consistent with its essential role in embryonic development and hemostasis. Low levels of TF also circulate within the blood during pathologic conditions, existing primarily on the surface of monocytes or as part of circulating microparticles (MPs)(Butenas 2005).

The extrinsic pathway of coagulation is initiated by the formation of the TF-VIIa complex. Under normal physiological conditions, FVII circulates in the plasma predominantly in its zymogen form, though a small amount (<1% of total) exists in the active state, FVIIa (Morrissey et al. 1993). TF serves as the allosteric cofactor for FVIIa, and upon vessel damage, FVIIa complexes to the extracellular domain of TF with high affinity. The γ-carboxyglutamic (Gla) domain

of FVIIa facilitates binding to phospholipids on the cell surface, while the serine protease domain binds to TF and catalyzes the activation of downstream coagulation factors, namely FX to FXa (Bach et al. 1986). In addition to activation of FX, TF-VIIa also activates FIX to FIXa, which complex together with FVIIIa to form what is referred to as the intrinsic tenase and generate additional FXa (Osterud and Rapaport 1977).

1.3.2 Regulation of Tissue Factor Procoagulant Activity

Not all TF is biologically active. Maynard et al. (1975) first reported that cell surface TF may exist in a latent or "cryptic" form, exhibiting limited ability to activate coagulation unless first exposed to trypsin or other proteases. Though both cryptic and non-cryptic TF are capable of binding FVIIa with similar affinities, only TF that has been decrypted exerts procoagulant activity (Bach 2005). Encrypted cell surface TF can be decrypted in several ways in vitro, including freeze-thaw cycles, exposure to non-ionic detergents, activation by calcium ionophore treatment, as well as pathophysiological stimuli such as lipopolysaccharide (LPS), thrombin, cytokines, or several apoptotic stimuli (Rao et al. 2012).

The primary method by which latent TF is decrypted is mediated by membrane phospholipid asymmetry (Shaw et al. 2007). The outer leaflet of the plasma membrane is abundant with phospholipids including

phosphatidylcholine and sphingomyelin while only containing traces of phosphatidylserine (PS). In contrast, the inner leaflet is enriched with PS, phosphatidylethanolamine, phosphatidylinositols, and phosphatidic acid. This asymmetry is maintained by several ATP-dependent transmembrane lipid transporter proteins (flippases, floppases) and scramblases that catalyze the bidirectional movements of lipids across the plasma membrane (Daleke 2002). The inhibition or activation of these enzymes during pathological conditions can result in the loss of lipid asymmetry, resulting in the increased exposure of negatively charged PS on the outer membrane leaflet. It has been well established *in vitro* that TF procoagulant activity requires the presence of phospholipids. TF reconstitution studies demonstrate that the presence of anionic phospholipids (including PS) greatly accelerate clotting times (Krishnaswamy et al. 1992; Neuenschwander et al. 1995; Shaw et al. 2007). However, when phosphatidylcholine- or sphingomyelin-containing liposomes were incorporated into the reaction mixture, TF procoagulant activity was diminished (Neuenschwander et al. 1995). These studies suggest that the limited availability of PS on the outer leaflet, coupled with the high concentrations of phosphatidylcholine and sphingomyelin present on the outer leaflet, contribute to the restrictions on procoagulant activity of cell surface TF.

The mechanisms by which PS regulates TF-VIIa coagulant activity are incompletely understood. It is postulated that externalized PS binds circulating

vitamin-K dependent coagulation factors via their Gla domains. In doing so, PS effectively increases the local concentration of these proteins on the lipid surface (Ruf et al. 1991). Therefore, one potential mechanism by which PS increases TF-VIIa activity is by facilitating FX binding to the cell surface, allowing it to come in contact with membrane anchored TF-VIIa. Alternate lines of evidence suggest a more direct role of PS in modulating TF-VIIa activity. Possible electrostatic interactions between the polar head of PS and Lys^{165}/Lys^{166} in TF induces a conformational change in TF that alters the orientation of the extracellular domain relative to the membrane surface. This shift may facilitate the precise alignment of the TF-VIIa active site to the activation peptides of both FIX and FX (Bach and Moldow 1997; Bach 2005).

1.3.3 The Intrinsic Pathway

The intrinsic (contact) pathway of coagulation consists of four serine proteinases: coagulation factors FXII, XI, IX, plasma prekallikrein (PK) and the nonenzymatic cofactor high molecular weight kininogen (HK) (Colman and Schmaier 1997; Kaplan and Silverberg 1987). In plasma, HK circulates bound noncovalently to PK, with the remaining HK forming a heterodimer with circulating FXI. The nonenzymatic cofactor HK is responsible for binding to negatively charged surfaces and facilitating the assembly of contact pathway proteins. Once FXII is bound to a negatively charged surface, it undergoes a

conformational change, resulting in its auto-activation. Activated FXII, in turn, catalyzes the activation of PK to PKa that subsequently converts additional FXII to FXIIa. This established feedback loop potentiates the sustained generation of FXIIa, which activates FXI to FXIa. FXIa subsequently activates FIX, which in turn activates FX. It is at this point that the intrinsic and extrinsic pathways converge (Colman and Schmaier 1997; Maas et al. 2011).

For decades, FXII and the contact system were thought to be of no biological significance for *in vivo* coagulation. Indeed, humans deficient in FXII and murine knockout models of FXII retain normal hemostatic functionality with no notable bleeding phenotype (Renné et al. 2012). Interest in the contact pathway of coagulation was renewed, however, when it was discovered that FXII-deficient mice exhibit a thrombo-protective phenotype when challenged with chemical (ferric chloride) and mechanical models of vascular injury (Renne 2005; Cheng et al. 2010). Likewise, FXII-deficient mice are protected from cerebral ischemia in an experimental stroke model (Kleinschnitz 2006), suggesting that the physiological events that drive pathologic thrombus formation (perhaps via the contact pathway) are distinct from hemostatic coagulation that is required to produce a clot at a site of vascular injury.

1.3.4 Polyanionic Activators of the Contact System

Historically, *in vitro* activation of contact pathway-driven coagulation was achieved by the addition of exogenous negatively charged molecular surfaces such as kaolin, glass, and silica (Maas et al. 2011). The requirement of these non-physiological stimuli to activate the contact system is largely responsible for the dismissal of the contact pathway as a biologically relevant component of coagulation. However, recent studies have identified numerous endogenous activators such as collagen, misfolded protein aggregates, polyphosphates, and nucleic acids that can serve as the negatively charged surface required to initiate coagulation (Long et al. 2015).

Nucleic acids (including DNA and RNA) are potent activators of the contact pathway. The negatively charged phosphate backbone of nucleic acids serves as template for contact activation (Gansler et al. 2012). In the presence of negatively charged surfaces, the heavy chain of FXII undergoes a conformational change, resulting in proteolytic cleavage at amino acid residues Arg^{353}-Pro^{354} and subsequent auto-activation of the light chain serine protease domain (Tankersley and Finlayson 1984). In purified systems, both types of nucleic acids decrease clotting time by promoting auto-activation of FXII, and to a lesser degree FXI (Gansler et al. 2012). In addition, administration of RNase or a FXIIa inhibitor has been shown to attenuate thrombus formation in a ferric chloride-induced model of arterial thrombosis, implicating nucleic acids as

disease mediators (Kannemeier et al. 2007). It is not surprising, then, that increased concentrations of circulating DNA are commonly found in pathological conditions associated with increased thrombotic risk, such as sepsis (Dwivedi et al. 2012; Saukkonen et al. 2008; Ziegler et al. 2002).

Long-chain polyphosphates (polyP) have also been shown to potentiate FXIIa generation in a similar manner. PolyP is an inorganic polymer comprised of tens to many hundreds of negatively charged phosphate residues linked by high-energy phophoanhydride bonds (Müller et al. 2009). PolyP is released from granules of activated platelets, as well as from certain bacteria such as *Escherichia coli*, *Vibrio cholerae*, and *Haemophilus influenza*, which all contain large quantities of long-chain polyP (Ruiz et al. 2004). Platelet polyP binds directly to FXII and HK with high affinity (Müller et al. 2009; Choi et al. 2011), and has been shown to trigger coagulation in both human and murine plasma in a FXII-dependent manner. Moreover, treatment with phosphatase, an enzyme that cleaves phosphoester bonds and degrades polyP, attenuates the formation of occlusive thrombi *in vivo* (Müller et al. 2009).

1.3.5 The Common Pathway & Thrombin Generation

Thrombin (FIIa) is the enzymatic endpoint of the coagulation cascade and is derived from its zymogen prothrombin (FII) upon proteolytic cleavage. Thrombin is a serine protease that converts soluble fibrinogen into insoluble

strands of fibrin, as well as potentiating many other coagulation-based reactions (Crawley et al. 2007; Wolberg and Campbell 2008). The generation of thrombin is typically described in three sequential phases: the initiation phase, the amplification phase, and the propagation phase (Hoffman and Monroe 2001).

The initiation phase is characterized by the generation of low amounts of active coagulation factors through the intrinsic or extrinsic pathways (described in detail above). If the procoagulant stimulus is sufficiently strong, a small amount of FXa, FIXa, and thrombin are generated to successfully initiate coagulation (Hoffman and Monroe 2001).

Amplification occurs as the procoagulant activity begins to shift from the initial stimulus to the platelet surface. The small amount of thrombin generated during initiation begins to amplify the procoagulant signal predominantly by activating platelets via PAR-1 and PAR-4 cleavage (Hung et al. 1992). Once activated, platelets release FV from granules that becomes localized on the platelet cell surface. The initial thrombin generated also serves to activate additional circulating FV, FVIII, and FXI, thereby locally amplifying coagulation (Monković and Tracy 2001).

Unlike the initiation phase which takes place on TF-expressing surfaces, the propagation phase occurs on surfaces containing procoagulant phospholipids, such as activated platelets. FIXa generated during the initiation phase binds to thrombin-activated FVIIIa on the platelet surface, forming the

intrinsic tenase complex. Along with Ca^{2+}, the intrinsic tenase rapidly catalyzes the conversion of FX to FXa on the platelet surface. Newly formed FXa then associates with FVa released to the platelet surface during amplification, resulting in the formation of the prothrombinase complex that catalyzes the conversion of large quantities of prothombin into thrombin (Hoffman and Monroe 2001; Versteeg et al. 2013; Hoffman 2003). The mass amounts of thrombin generated during the propagation phase are sufficient to catalyze the conversion of fibrinogen to fibrin, resulting in fortification of the platelet thrombus by the newly formed fibrin clot.

1.3.6 Regulation of Coagulation

Thrombin generation is regulated predominantly by three endogenous anticoagulants: antithrombin, tissue factor pathway inhibitor, and activated protein C. Antithrombin III (AT) is a plasma serpin that is produced by the liver and has a plasma half life of 2-3 days (Roemisch et al. 2002). In addition to a potent inhibitory effect on thrombin, AT has also been shown to inhibit many contact pathway factors including FIXa, FXa, FXIa, and FXIIa (Roemisch et al. 2002). In the presence of exogenous heparin or endogenous heparin-like glycosaminoglycans, FVIIa is also inhibited (Rao et al. 1995). Though heparin is non-essential to AT function, AT activity is increased more than 1000-fold in its presence (Opal et al. 2002). Heparin functions by providing a template on which

both AT and thrombin can interact. Heparin is also able to induce a conformational change in AT which is required for AT-mediated inactivation of FXa (Opal et al. 2002).

Conversely, tissue factor pathway inhibitor (TFPI) is the primary regulator of the extrinsic pathway *in vivo*. TFPI is produced constitutively by microvascular endothelial cells and pooled in the endothelium (50-80%), plasma (10-50%), and platelets (less than 2.5%)(Kasthuri et al. 2010). Low levels of TFPI have been found on monocytes/macrophages. Structurally, TFPI consists of three Kunitz-type domains which are integral for its inhibitor functions. In a two-stage process, the second Kunitz domain (K2) binds to and directly inhibits FXa. K1 then binds to a TF-VIIa complex to abolish subsequent Xa generation (Broze et al. 1988). Though the binding of TFPI to Xa is reversible, the formation of the inhibitory quaternary complex between TFPI, Xa, and TF-VIIa is permanent (Broze and Girard 2012). TFPI has also been shown to stimulate monocytes to internalize and degrade cell surface TF-VIIa complexes, further restricting additional thrombin generation (Lupu et al. 1999).

The protein C (PC) anticoagulant pathway is the third regulator of coagulation, and involves thrombomodulin (TM), the endothelial cell protein C receptor (EPCR), PC, and protein S (Esmon 2003). As thrombin is generated it binds to to TM on the endothelial cell surface, losing its procoagulant activity while simultaneously driving the activation of PC to activated protein C (APC).

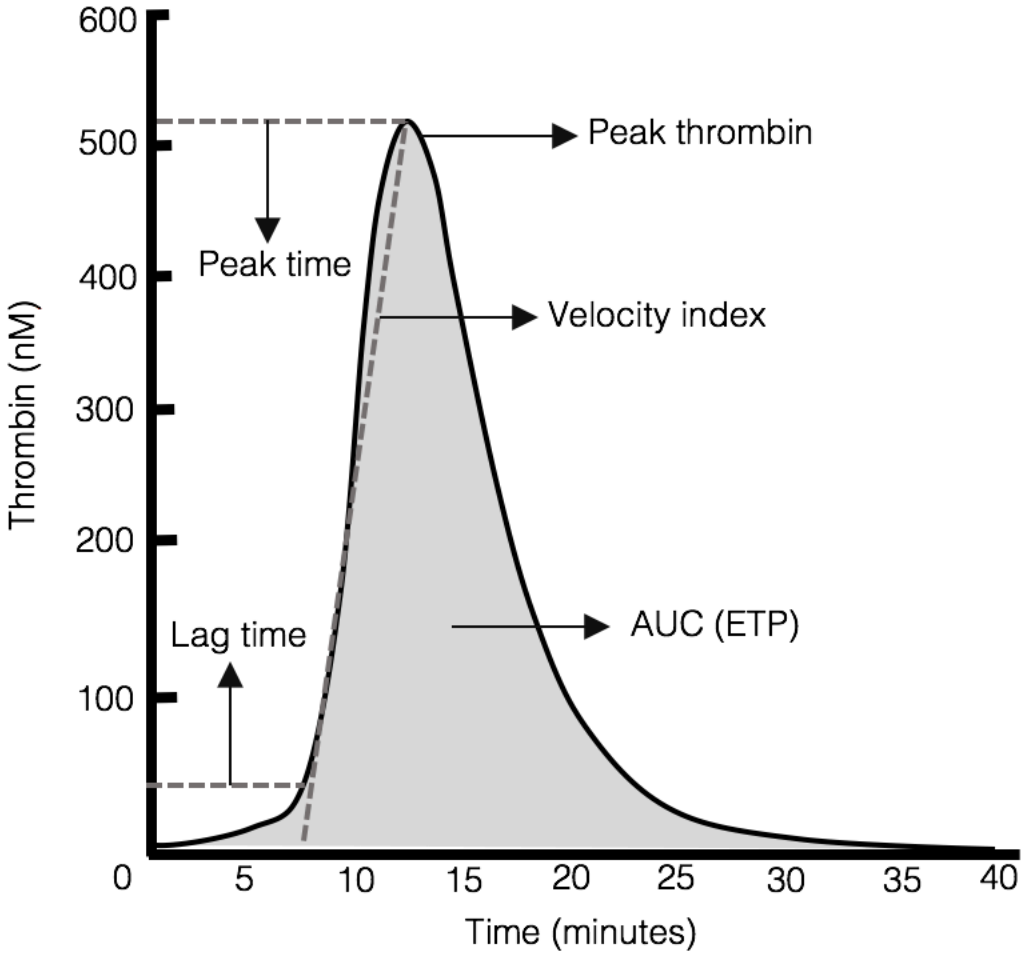
EPCR augments the activation of PC by 20-fold *in vivo* by binding to PC and presenting it to the thrombin-TM activation complex (Taylor et al. 2001). Newly formed APC, together with its cofactor protein S, inactivates FVa and VIIIa, limiting thrombin generation. Several clinical studies have demonstrated that deficiencies in the PC pathway can result in the development of microvascular thrombosis (Esmon 2001; Esmon 2002; Bereczky et al. 2010).

1.3.7 The Thrombogram

The estimation of how well a particular individual can generate thrombin may correlate with either a risk of bleeding or thrombosis. Over time, several laboratory methods have been developed to to quantify thrombin generation. Historically, these tests included the prothrombin time (PT) or the partial thromboplastin time (APTT) assays which examine clotting times initiated via the extrinsic or intrinsic pathways, respectively. Early versions of these assays employed chromogenic reagents used to monitor thrombin generation, as well as a technically difficult method requiring the subsampling of reaction mixtures at regular intervals into secondary tubes containing fibrinogen allowing clotting to proceed. The clotting times of the fibrinogen solutions were used to estimate thrombin concentration against a thrombin standard (Baglin 2005).

Calibrated automated thrombography (CAT) is a more recently developed

Figure 1.2 – **The thrombogram**. The thrombin generation curve can be described in terms of lag time (period of undetectable thrombin generation before the propagation phase), peak time and peak thrombin (period when thrombin formation and inhibition reach an equilibrium), velocity index (rate of thrombin generation during propagation phase), and AUC/ETP (total amount of free thrombin generated during the course of the assay).


600
500
400
300
200
100
0
Thrombin (nM)
Peak thrombin
Peak time
Velocity index
Lag time
AUC (ETP)
5
10
15
20
25
30
35
40
Time (minutes)

thrombin generation assay that serves as a general physiologic function test of the hemostatic system (Hemker et al. 2006). The assay is initiated by the recalcification of a given plasma sample, and the conversion of prothrombin to thrombin is measured by cleavage of a fluorogenic substrate to produce a thrombin generation curve, or 'thrombogram' (**Figure 1.2**). Like thrombin generation *in vivo*, the thrombogram is divided into three distinct phases (Hemker et al. 2006). The 'lag phase' captures the initiation/amplification phase, where trace amounts of thrombin are generated via the intrinsic or extrinsic pathways and are not detectable by the system. Following this, a large burst of thrombin generation occurs during the propagation phase, the rate of which is represented by the velocity index. As thrombin is generated, it is continuously inactivated by endogenous inhibitors (namely AT in platelet-poor plasma) until it reaches an equilibrium state that is indicated by the thrombin peak height or time to peak. Following the peak, thrombin generation decreases and eventually returns to baseline levels due to continued AT inhibition. The total amount of thrombin generated during the period of observation is described by the area under the curve (AUC) or endogenous thrombin potential (ETP)(Hemker et al. 2006). In addition to being a invaluable research tool, the thrombin generation assay is also useful clinically. Thrombin generation can not only be used to assess genetic or acquired thrombotic disorders (Vlieg et al. 2007), but also to monitor the treatment of hemophilic disorders (such as treatment with FVIII or

VIIa)(Brummel-Ziedins et al. 2009), as well as to monitor treatment with oral anticoagulants such as warfarin or antiplatelet agents (Gatt et al. 2008).

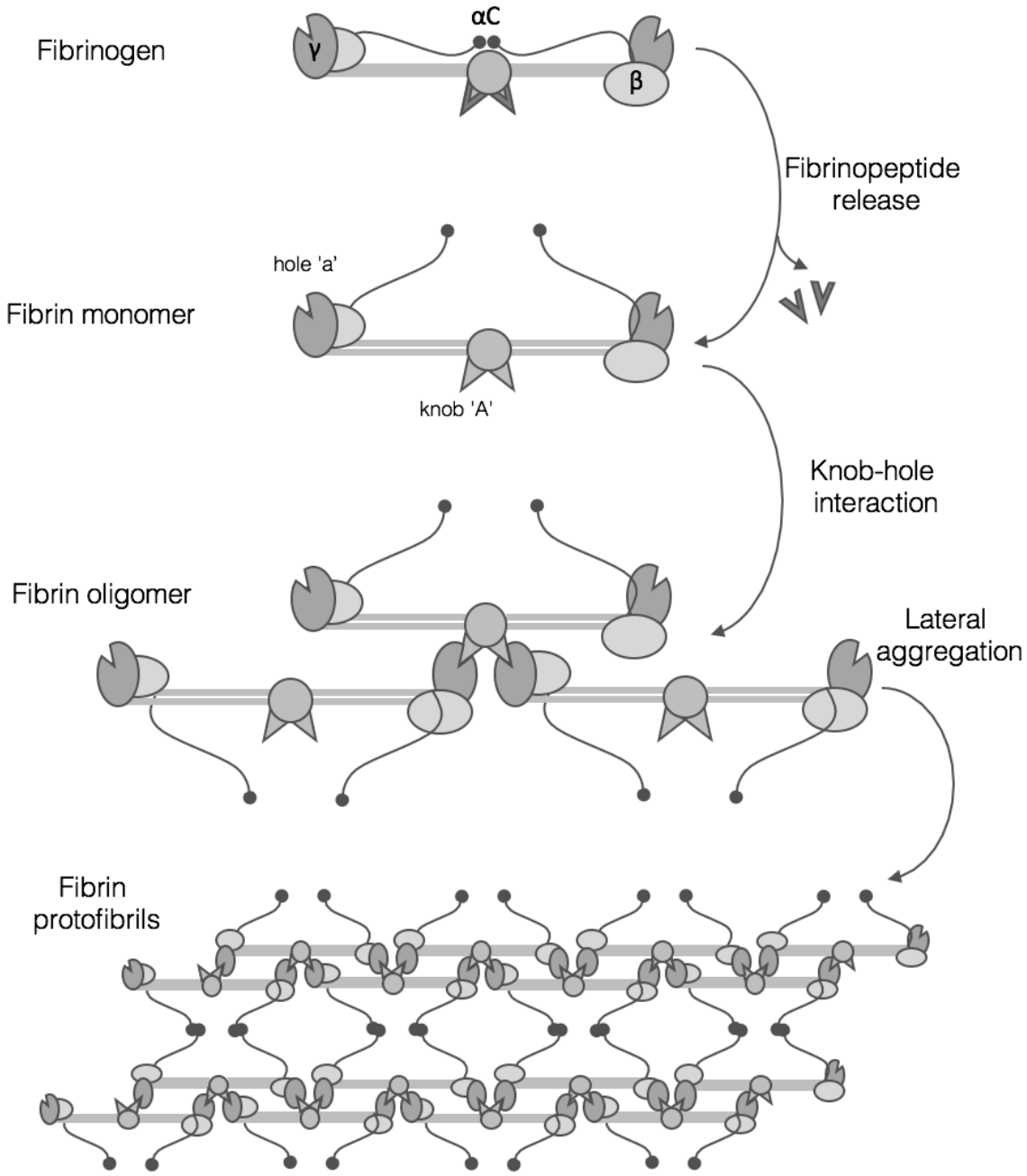
1.4 Fibrin Formation & Fibrinolysis

1.4.1 Regulation of Fibrin Formation

Fibrinogen is a soluble 340kDa trinodular protein that circulates at high concentrations (6-12μM) in plasma. Structurally, fibrinogen is comprised of three pairs of disulfide-bridged polypeptide chains, Aα, Bβ, and γ that are assembled with their N-termini converging onto the central (or "E") domain of the molecule. The C-termini of the Bβ, and γ chains project outward, constituting the distal (or "D") domains, while the C-termini region of the Aα chains are globular and positioned near the E domain (Medved et al. 1983; Weisel 2007; Lord 2007; Wolberg 2007). The structure of fibrinogen and fibrin are detailed in **Figure 1.3**.

Conversion of soluble fibrinogen to insoluble fibrin begins with the thrombin-mediated cleavage of fibrinopeptides A (FpA) and B (FpB) from the N-termini of the Aα- and Bβ-chains of fibrinogen to generate a single fibrin monomer (Medved et al. 2009; Litvinov 2005). Thrombin selectively cleaves the Arg-Gly bonds of fibrinogen, where the optimal cleavage sites for thrombin have been determined to be A-B-Pro-Arg-||-X-Y where A and B are hydrophobic amino acids and X and Y are nonacidic amino acids (Chang 1985). The subsequent release of FpA exposes an N-terminal α-chain Gly-Pro-Arg-Val motif, known as 'knob A', which is complimentary to pockets or holes 'a' located on the

Figure 1.3 – Fibrin polymerization and aggregation. Fibrinogen is composed three sets of polypeptide chains that form the central E-domain, which is flanked by two lateral D-domains. The D-regions contain the distal portions of the C-terminal Bβ and γ-nodules, while the E-region contains the central N-terminal portions. The E-region also has two pairs of binding sites termed A- and B-knobs that are exposed following the cleavage of FpA and FpB by thrombin. This process is also associated with the partial dissociation of the αC regions. The D-regions have constitutively open a- and b-holes located in the γ- and β-modules, respectively. The self-assembly of monomeric fibrin is mediated via knob-hole interactions, resulting in the formation of a half-staggered two-stranded fibrin protofibril. Lateral aggregation is promoted by αC-αC-interactions within and between protofibrils, which is further reinforced by cross-linking mediated by thrombin-activated factor XIIIa (adapted from Weisel and Litvinov 2013).


Fibrinogen
αC
γ
β
Fibrinopeptide release
hole 'a'
Fibrin monomer
knob 'A'
Knob-hole interaction
Fibrin oligomer
Lateral aggregation
Fibrin protofibrils

γ-nodules of other fibrin molecules. Conversely, FpB release exposes the new N-terminal β-chain Gly-His-Arg-Pro motif, termed 'knob B' which is complementary to hole 'b' located in the globular β-nodule (Litvinov 2005). Though FpB releaseis considered non-essential for fibrin formation, B:b interactions appear to be involved in lateral aggregation of fibrin protofibrils, as incomplete FpB cleavage produces thinner fibrin strands (Litvinov et al. 2007).

Two fibrin monomers produced following FpA release interact with each other in a staggered, overlapping end-to-middle manner, so that knob 'A' fits into hole 'a' with two A:a knob-hole interactions holding the individual monomers together (Weisel and Medved 2001; Yermolenko et al. 2011). The addition of subsequent fibrin monomers to a half-staggered dimer produces an end-to-end junction where the ends of two molecules bridge each other resulting in a double-stranded, twisted fibrin protofibril.

Once the protofibril oligomers reach a sufficient length (typically 0.5-0.6 μm), they begin to undergo lateral aggregation to form individual fibrin fibers (Chernysh et al. 2011; Medved et al. 1990). Though the exact mechanisms underlying the process of lateral aggregation remain largely unknown, the C-terminal portions of the Aα-chains play an important role. During fibrin polymerization, the αC regions between protofibrils interact with each other, enhancing lateral aggregation (Veklich et al. 1993; Gorkun et al. 1994). These interactions are further consolidated by thrombin-activated FXIIIa, a plasma

transglutaminase that crosslinks lysine residues between adjacent αC regions (Mosesson et al. 1995).

A number of endogenous and exogenous factors are known to modulate fibrin clot structure, including temperature, pH, ionic strength of the reaction solution (Nair et al. 1986), concentrations of metals such as calcium and zinc (Carr et al. 1986; Henderson et al. 2016), as well as the concentration of coagulation factors such as fibrinogen and FXIIa (Blombäck et al. 1994; Konings et al. 2011). However, the most potent influence on fibrin structure is thrombin concentration, which has been shown to alter not only the properties of individual fibers, but the fibrin network as a whole. Low thrombin concentrations produce turbid, loosely woven clots that are composed of thick fibrin strands. In contrast, high thrombin concentrations produce clots composed of thin individual fibrin fibers that are tightly packed (Blombäck et al. 1994; Shah et al. 1985; Blombäck et al. 1989; Carr and Hermans 1978). Differences in fibrin morphology have been shown directly affect multiple clot properties, including clot stability, viscoelastic properties, and susceptibility to fibrinolysis whereby tightly compacted clots are more resistant to lysis and exhibit less viscoelastic malleability (Wolberg 2007).

1.4.2 Fibrinolysis

Much like the coagulation cascade, the process of fibrinolysis involves a series of serine proteases, their respective serpins, and cofactors that serve to precisely regulate fibrin deposition and to remove existing thrombi (Chapin and Hajjar 2015).

Plasmin is an 86 kDa serine protease and is the primary protease involved in fibrinolysis (Cesarman-Maus and Hajjar 2005). Active plasmin is generated from its plasma zymogen plasminogen upon a single cleavage at Arg^{560}-Val^{561} by either tissue- or urokinase-type plasminogen activators (tPA and uPA, respectively) as well as by bacteria-derived streptokinase (Hoylaerts et al. 1982; Holvoet et al. 1985; Bergmann and Hammerschmidt 2007). While tPA is both synthesized and secreted by endothelial cells, uPA is derived primarily from monocytes and macrophages. Both of these plasminogen activators have short half-lives in circulation (approximately 5-15 minutes) due to rapid inhibition by endogenous plasminogen activator inhibitor 1 (PAI-1), which is released into the circulation by endothelial cells, hepatocytes, and platelets (Chapin and Hajjar 2015). Levels of PAI-1 have been shown to be upregulated in a number of prothrombotic disease states, and transgenic mice overexpressing PAI-1 exhibit thrombotic occlusion of tail veins and swelling of hind limbs shortly after birth (Erickson et al. 1990).

Once generated, plasmin acts upon its primary substrate, fibrin, by cleaving the polymer at Lys-Arg sites to subsequently expose carboxy-terminal lysine residues (Cesarman-Maus and Hajjar 2005). The binding domains of both tPA and plasminogen (referred to as the 'Kringle' domains) contain lysine-binding sites, which mediate the binding of tPA/plasminogen to fibrin, ultimately resulting in enhanced plasmin generation and fibrin degradation (Miles et al. 1988). Continuous plasmin generation is reinforced by an additional feedback mechanism, whereby plasmin cleaves both tPA and uPA, transforming them from single chain to two-chain polypeptides that possess enhanced catalytic activity (Cesarman-Maus and Hajjar 2005; Hoylaerts et al. 1982; Tate et al. 1987). Plasminogen activation is regulated largely by thrombin-activatable fibrinolysis inhibitor (TAFI), a carboxypeptidase that removes exposed C-terminal lysine residues on fibrin, preventing the binding of plasminogen and its activators to fibrin and subsequently attenuating plasmin generation (Foley et al. 2013). Plasmin activity itself is also regulated by its endogenous inhibitor, the serpin α2-antiplasmin (α2AP). Plasmin is immediately neutralized by α2AP when circulating freely in the blood, or in the vicinity of a platelet-rich clot. However, plasmin is immune from α2AP-mediated inhibition while it is bound to fibrin (Schneider 2004), allowing fibrinolysis to proceed and localizing fibrinolytic activities to the area of the clot.

As fibrin is degraded by plasmin, a distinct set of soluble FDPs are released into the circulation. When fibrin polymers are cleaved by plasmin at the D fragment site, the resulting D-dimer fragments reflect the degree of thrombosis and plasmin activity. The quantification of D-dimer levels have been shown to have modest predictive and prognostic value in a number of disease states, including DIC and DVT (Voss et al. 1990; Eichinger et al. 2003).

Clinically, measuring fibrinolysis directly has proven difficult, and assays that measure individual fibrinolytic components (such as tPA, PAI-1, or plasmin-antiplasmin complexes) are of limited prognostic utility. To address this limitation, the clot lysis time (CLT) assay has been developed as a global test of total fibrinolytic activity in plasma. This test is performed on platelet-poor plasma samples whereby coagulation is initiated by the addition of Ca^{2+}, and the resulting fibrin clot is lysed by the addition of exogenous tPA. The reaction is then observed spectrophometrically over time, where increases and decreases in solution turbidity correspond with the formation and subsequent lysis of the fibrin clot, respectively (Carr and Hermans 1978). In patients with recurrent venous thrombosis, insufficient fibrinolytic activity as determined by CLT predicted a two-fold risk of thrombosis (Lisman et al. 2005). This assay has also been validated in larger cohorts of patients and CLT has maintained its predictive power for venous thrombosis in the MEGA study, which evaluated over 1000 patients (Meltzer et al. 2008).

1.5 Coagulation, immunity, and immunothrombosis

In addition to preventing blood loss, the initiation of coagulation and subsequent clot formation is a critical event in the host's innate immune response to infection. Indeed, it has been suggested that the coagulation system itself is an evolutionary divergence of the innate immune system, with complement factors giving rise to coagulation factors themselves (Krem and Di Cera 2002).

Immediately following infection, the rapid activation of coagulation leads to entrapment of pathogens within the fibrin clot and limiting their ability to disseminate within the host. Engelmann and Massberg recently coined the term 'immunothrombosis' to describe the process whereby the coagulation system is used by the innate immune system as a first line defense against invading microorganisms (Engelmann and Massberg 2012).

Several lines of evidence highlight the importance of an intact coagulation system for an efficient immune response. Sun et al. (2009) demonstrate that FV deficiency in mice infected with *S. pyogenes* resulted in increased mortality, suggesting a role of thrombin generation in host defense. The authors hypothesized that insufficient fibrin deposition mediated by both plasma- and platelet-derived FV enhance the survival and hematogenous spread of *S. pyogenes* in these mice. In accordance, fibrinogen-deficient mice not only exhibit increased instance of hemorrhage, but also exacerbated organ

pathology and markedly increased mortality rates following parasitic infection (Johnson et al. 2003). Levels of FXIII were also shown to be an important factor for the entrapment of bacteria within a fibrin clot, as FXIII-deficient individuals demonstrate limited capacity to immobilize bacteria (Wang et al. 2010). It has also been suggested that FXIII mediates cross-linking of bacterial surface structures, such as the M1 protein of *S. pyogenes*, directly to fibrin fibers, effectively anchoring the pathogen to the clot (Loof et al. 2011).

Immunothrombosis is initiated and maintained by the accumulation of innate immune cells – particularly monocytes and neutrophils – following exposure to pathogens. While these leukocytes are traditionally considered for their phagocytic and pathogen-killing abilities, there is mounting evidence to suggest that these cells are also involving in mediating procoagulant activity during infection. However, dysregulation of immunothrombosis may be a major contributor to the development of thrombotic disorders. Interestingly, many of the molecular mediators that potentiate immunothrombosis are distinct from those involved in the formation of hemostatic clots, making these mechanisms alluring targets for therapeutic intervention.

1.6 PAMPs, DAMPs, & Toll-like Receptors

1.6.1 Pathogen-associated molecular patterns (PAMPs)

A fundamental property of the innate immune system is its ability to distinguish innocuous foreign antigens from infectious pathogens. Pathogen-associated molecular patterns (PAMPs) are an assortment of exogenous, pathogen-derived molecules that share a number of distinct biochemical properties or motifs that alert the host organism to the presence of invading organisms (Janeway and Medzhitov 2002). A non-exhaustive list of common PAMPs include microbial nucleic acids, such as unmethylated CpG DNA, double- and single-stranded RNA, as well as microbe-derived lipoproteins, surface glycoproteins, and membrane components such as peptidoglycans, lipoteichoic acid (LTA), and LPS (Janeway and Medzhitov 2002; Creagh and O'Neill 2006; Kumar et al. 2011).

PAMPs are recognized by various cells of both the innate and adaptive immune system (including monocytes, macrophages, neutrophils, and dendritic cells) via pattern recognition receptors (PPRs)(Janeway and Medzhitov 2002). Of these PRRs, the Toll-like receptor (TLR) family is the most prominent, which activate several signaling pathways resulting in the generation of proinflammatory and antimicrobial responses to destroy the pathogen or pathogen-infected cells. Recognition of infectious signals is the first and most critical event in the initiation of immunothrombosis.

1.6.2 Damage-associated molecular patterns (DAMPs)

In contrast, damage-associated molecular patterns (DAMPs) are endogenously released molecules that originate from inside various compartments of host cells or from the extracellular space itself. Similar to PAMPs, DAMPs possess the ability to initiate and perpetuate an immune response during instances of tissue damage and sustained inflammation, including trauma, ischemia, and cancer (Medzhitov and Janeway 2002; Beutler 2007; Bianchi 2006; Ito 2014).

Intracellular DAMPs may be derived from the cytosol (uric acid (Shi et al. 2003), heat shock proteins (Lehnardt et al. 2008)), the plasma membrane (syndecans (Karamanos 2014), glypicans (Mani 2006)), the endoplasmic reticulum (calreticulin (Obeid et al. 2006)), and the mitochondria (ATP, formyl peptides (Zhang et al. 2010; Manfredi and Rovere-Querini 2010)). Of particular interest are DAMPs derived from the nuclei of damaged and/or necrotic cells, as the translocation of these molecules signifies the most complete and irreversible form of cellular destruction (Pisetsky 2014). There are three major constituents of nuclear DAMPs: DNA, histones, and high mobility group box-1 protein.

1.6.2.1 Cell-free DNA

Deoxyribonucleic acid (DNA) is the most abundant intra-nuclear molecule. Extracellular or cell-free DNA (CFDNA) may be released into the circulation by various host cells including neutrophils (Brinkmann et al. 2004;

Clark et al. 2007; Urban et al. 2006), macrophages (Choi et al. 2004), eosinophils (Yousefi et al. 2008), or tumor cells (Diehl et al. 2008). Accumulation of CFDNA in the circulation may be a result of increased cell death and/or activation, and impaired clearance (Tamkovich et al. 2006). Indeed, elevated levels of CFDNA have been shown to have prognostic utility in many disease states, and increases in CFDNA are considered to be indicative of the degree of tissue damage and/or cellular activation (Jahr et al. 2001a; Stroun et al. 2001; Chang et al. 2003; Lo et al. 2000). In many autoimmune disorders such as systemic lupus erythematosus and rheumatoid arthritis, CFDNA itself has been implicated in disease pathogenesis. Antibodies directed against CFDNA are generated by the adaptive immune system, resulting in the accumulation of CFDNA-antibody complexes that trigger a proinflammatory response following immune recognition (Zhong et al. 2007; Christensen and Shlomchik 2007).

1.6.2.2 Histones

Histones are nuclear proteins that form hetero-octamers consisting of two copies each of histone subunits H2A, H2B, H3, and H4. Under normal physiological conditions, histones are essential for organizing double-stranded DNA to form chromatin and chromosomes (Richmond and Davey 2003). During instances of infection, histones are released (along with CFDNA) from dying tubular epithelial cells, or from neutrophils during the formation of neutrophil

extracellular traps (NETs) where they exert antimicrobial activity towards invading pathogens (Kawasaki and Iwamuro 2008; Brinkmann et al. 2004). Interestingly, histones are known potent antimicrobials, and are among the very first antimicrobial proteins described. However, the bactericidal effects of extracellular histones also damage self-tissues; the release of histones directly contributes to fatal outcomes in murine endotoxemia by activating and killing vascular endothelial cells (Abrams et al. 2013; Xu et al. 2009; Xu et al. 2011).

1.6.2.3 High Mobility Group Box-1

Finally, high mobility group box-1 (HMGB1) is a non-histone intra-nuclear protein that possesses multiple functions. Under normal conditions, HMGB1 binds nuclear DNA to facilitate gene transcription (Pisetsky 2014). In pathological settings, HMGB1 is released from damaged or dying cells and is a key mediator of the inflammatory response. HMGB1 has little to no proinflammatory properties of its own, but through its interactions with other mediators of inflammation (such as LPS, DNA, or IL-1β) it induces signaling pathways that culminate in an exacerbated inflammatory response (Park et al. 2004; Park 2005). The signaling pathways driven by HMGB1 are not as of yet fully understood; however, several lines of evidence points to receptor interactions with the multiligand receptor for advanced glycation end products (RAGE) and TLRs (Klune et al. 2008).

1.6.3 Toll-like receptors

TLRs are type I transmembrane proteins that consist of a leucine-rich extracellular domain and a cytoplasmic tail containing a conserved region called the Toll/IL-1 receptor (TIR) domain (Medzhitov et al. 1997; Choe et al. 2005). TLRs are expressed primarily in tissues involved in immune functions, such as the spleen, peripheral blood leukocytes, as well as cells exposed to the external environment such as lung epithelial and gastrointestinal tract cells (Nishiya and DeFranco 2004). With respect to function, TLRs are pivotal in initiating an early immune response to invading organisms. TLRs function by recognizing both endogenous and exogenous structural motifs (PAMPs, DAMPs) and, in response, stimulate various intracellular signaling cascades that culminate in the production of interferons (IFN), proinflammatory cytokines, and effector cytokines that direct an immune response (Medzhitov et al. 1997).

Ten human and twelve murine TLRs have been characterized to date: TLR1 to TLR10 exist in humans, with additional TLR11-13 existing exclusively in mice. In addition to TLRs being differentially expressed on various tissue types, they also vary in their location within an individual cell. While most TLRs are located on the plasma membrane, TLR3, TLR7, and TLR9 are localized intracellularly to endosomal compartments (reviewed in Piccinini and Midwood 2010).

Various TLRs are responsible for recognizing various PAMPs and DAMPs. TLR2 is well established in its recognition of Gram-positive bacteria components, including bacterial lipoproteins (Piccinini and Midwood 2010). TLR3 has been implicated in the detection of double-stranded RNA derived from viral pathogens (Piccinini and Midwood 2010). TLR4 recognizes LPS, and recent evidence has demonstrated that TLR4 is also involved in the recognition of endogenous DAMPs such as histone protein and HMGB1 (Tadie et al. 2013; Xu et al. 2011). TLR9 is required for response to bacteria-derived umethylated CpG DNA (Fischer and Preissner 2013). This repertoire of specificities of individual TLRs may be further broadened by the ability of TLRs to form heterodimers with each other. For example, dimers of TLR2+6 have recently been shown to initiate diacylated lipoprotein signaling, while TLR1+2 interact to recognize triacylated lipoproteins (Ozinsky et al. 2000). A comprehensive list of TLRs and their respective ligands are summarized in **Table 1.1**.

In addition to ligand recognition, individual TLRs also initiate a diverse array of intracellular signaling pathways. Stimulation of TLRs leads to the dimerization of the cytoplasmic TIR domain with specific adaptor molecules, of which five subtypes have been described to date: myeloid differentiation factor 88 (MyD88), MyD88-adaptor like (Mal), TIR domain-containing adaptor inducing IFN-beta (TRIF), TRIF-related adaptor molecule (TRAM), and sterile alpha and HEAT-Armadillo motifs (SARM)(Kenny and O'Neill 2008).

Table 1. Expression and activation of Toll-like receptors by various PAMPs and DAMPs. Mo, monocytes; M_{Φ}, macrophages; DC, dendritic cells; MC, mast cell; B, B-cells; T, T-cells; IE, intestinal epithelium; IC, inflammatory cytokines; IFN, interferon; HSP, heat shock proteins; HMGB1, high mobility group box protein 1.

Receptor	Localization	PAMPs	DAMPs	Response
TLR1+2	Cell surface (Mo, M_Φ, DC, B)	Triacylated lipoproteins (Pam3CSK4, peptidoglycans, lipopolysaccharides)	(TLR2 DAMPs listed below)	IC
TLR2+6	Cell surface, Mo, M_Φ, DC, B	Diacylated lipoproteins (FSL-1)	HSPs (60, 70, Gp96), HMGB1, Histone, Proteoglycans (versican, Hyaluronic acid)	IC
TLR3	Endosomes (B, T, NK, DC)	dsRNA , tRNA, siRNA	mRNA, tRNA	IC, type1 IFN
TLR4	Cell surface/ endosomes (Mo, MC, IE, M_Φ, DC)	Lipopolysaccharide (LPS)	HSPs (22, 60, 70,72, Gp96), HMGB1, Histone, Proteoglycans (versican, herparin sulfate, hyaluronic acid) Fibronectin, Tenascin-C	IC, type1 IFN
TLR5	Cell surface (Mo, IE, M_Φ, DC, B)	Flagellin	N/A	IC
TLR7	Endosomes (Mo, M_Φ, DC, B)	ssRNA, guanosine analogs	ssRNA	IC, type1 IFN
TLR8	Endosomes (Mo, M_Φ, DC, MC)	ssRNA	ssRNA	IC, type1 IFN
TLR9	Endosomes (Mo, M_Φ, DC, B, T)	CpG DNA motifs	Chromatin IgG complex	IC, type1 IFN
TLR10	Endosomes (Mo, M_Φ, DC, B, T)	Profilin-like proteins	N/A	IC

At present it is well recognized that individual TLRs are able to induce different signaling responses by usage of different adaptors, though it is unclear what factors contribute to the recruitment of individual adaptor molecules. Once ligand recognition occurs, TLR signaling may proceed via two distinct pathways. The first is a MyD88-dependent pathway (which may be activated by all TLRs except for TLR3) that leads to the production of NF-κB-regulated inflammatory cytokines (Kenny and O'Neill 2008). The second pathway is known as the TRIF or MyD88-independent pathway that occurs upon activation of TLR3 and 4. It leads to the activation of IFN-regulated factors (IRF) family of transcription factors that results in IFN-β synthesis and subsequent maturation of dendritic cells (Kenny and O'Neill 2008). This combination of multiple ligand recognition, receptor dimerization, along with multiple signaling pathway options contributes to the ability of a small number of individual TLRs to recognize such a wide array of PAMPs and DAMPs.

1.7 The Monocyte

1.7.1 Monocyte structure & function

Mononuclear leukocytes (herein referred to as monocytes) are a subset of circulating leukocyte that originate from long-term hematopoietic stem cells in the bone marrow (Rees 2010). Matured monocytes are released into the circulation, where they circulate for 1-3 days and have the ability to migrate to

various tissues and subsequently differentiate into a wide range of tissue resident macrophages and dendritic cells (Rees 2010). In addition, monocytes are also mediators of host antimicrobial defense, and have been shown to participate in pathogen phagocytosis, antigen presentation, and cytokine production upon activation by microbial stimuli. Several studies have revealed the diverse functionality of monocytes within varying inflammatory environments. The ability of monocytes to mobilize and traffic to an infectious focus is essential for their functions in promoting immune defense during infection.

1.7.2 Role of the monocyte in (immuno)thrombosis

Several lines of evidence suggest that monocytes possess a variety of mechanisms to initiate and amplify thrombin generation during instances of infection. Among the leukocytes, monocytes have been shown to be unique in that their cell surface membrane serves as a template for the assembly and function of TF-mediated coagulation. While surface TF typically exists in its cryptic form on quiescent monocytes, treatment with calcium ionophore or cell lysis agents substantially increases the procoagulant potential of monocytes, suggestive of TF decryption to its active form (Wolberg et al. 1999). In addition, monocytes have the ability to synthesize TF *de novo* and subsequently express it on the cell surface following exposure to bacterial components (such as LPS) and other various proinflammatory cytokines and ligands (Gregory et al. 1989; Cermak et al. 1993; Celi et al. 1994). Monocyte-derived TF-bearing MPs have

also been observed in peripheral blood. Upon activation, monocytes may release small membrane vesicles that disseminate within the circulation. These MPs have been shown to express various P-selectin ligands, which facilitate platelet-MP interactions and subsequent platelet activation (Rauch et al. 2000; Kleinjan et al. 2012).

Monocyte-bound TF is able to bind FVIIa in circulation, forming functional extrinsic tenase complexes. The coagulant response is further propagated by the formation of prothrombinase complexes, again on the monocyte surface. Monocytes express approximately 16 000 prothrombinase binding sites per cell (Tracy et al. 1985). Prothrombinase assembly and function on the monocyte can be augmented by exposure to LPS, resulting from enhanced binding of FVa and Xa (Robinson et al. 1992). Thrombin generated at this stage is not only able to convert FV to FVa, but also activates FVIII to FVIIIa to facilitate FVIIIa:FIXa functional interaction with the monocyte surface to assemble intrinsic tenase and potentiate additional FXa generation (McGee and Li 1991; McGee et al. 1992). Interestingly, FX activation mediated by monocyte intrinsic tenase is approximately 25 times more efficient than FX activation via TF:VIIa complex (McGee and Li 1991). The assembly of the tenase complex is essential for sustained thrombin generation by the monocyte, as the co-expression of TFPI on the monocyte surface inhibits extrinsic pathway activity once FXa is formed. Taken together, these studies demonstrate that monocyte-associated TF, either

expressed on the cell surface or released into the blood in the form of MPs, is likely involved in thrombus development.

1.8 The Neutrophil

1.8.1 Neutrophil structure & function

Neutrophil granulocytes, commonly referred to as simply neutrophils, are a subset of polymorphonuclear leukocyte and are well recognized as being one of the key mediators involved in the effector functions of the innate immune system. Neutrophils are the most abundant leukocyte in circulation, comprising 70-90% of total leukocyte count, and are characterized by their multi-lobed nucleus and the presence of enzyme-containing granules (Amulic et al. 2012). Neutrophils mature in the bone marrow, and are subsequently released into the circulation where they circulate for up to 24 hours before undergoing apoptosis and clearance by macrophages or dendritic cells (Weissman et al. 2001; Amulic et al. 2012).

Neutrophil granules contain more than 800 variations of antimicrobial peptides and enzymes (Brogden 2005). Many of these peptides are charged, which promotes their interaction with microbial membrane surfaces and disrupts membrane integrity. Of particular interest are α-defensins, neutrophil elastase (NE), and cathespin G (CG) which cleave bacterial virulence factors as well as permeabilize bacterial membrane bilayers. Because the antimicrobial properties

of the granule contents are generally non-specific in nature, they remain sequestered within the neutrophil to avoid inadvertently damaging host cells and tissues (Brogden 2005). As a result, the turnover for circulating neutrophils is typically a highly regulated process, as it is critical to remove spent neutrophils from circulation to prevent the accidental release of cytotoxic effector molecules (Bratton and Henson 2011).

During instances of infection, neutrophils are rapidly recruited to the infectious focus in response to chemotactic stimuli released by the host or invading microorganism. Once arrived, neutrophils rapidly become activated through TLR or formyl peptide receptor (fMLP) receptor engagement and mount an antimicrobial response. Phagocytosis by neutrophils is the major mechanism by which pathogens and cell debris are removed (Rigby and DeLeo 2011). As with other phagocytes, the mechanisms involved in internalization depend on the type of interaction between the neutrophil and the target. Interactions can be direct, through recognition of PAMPs by PRRs, or opsonin-mediated (Underhill and Ozinsky 2002). Once the pathogen has been engulfed, neutrophil granules fuse with the newly internalized phagosome, delivering their antimicrobial contents into the phagosomal lumen (Rigby and DeLeo 2011; Amulic et al. 2012). Simultaneously, the NADPH oxidase complex assembles on the phagosomal membranes and initiates the reactive oxygen cascade by reducing molecular oxygen to superoxide. Downstream, superoxide is dismutated into

several different species, including hydrogen peroxide, hypochlorous acid, and chloramines which are considered to be the most potent antimicrobial reactive oxygen species (ROS)(Hampton et al. 1998; Winterbourn 2008). Together, the high concentration of antimicrobial enzymes and ROS production creates an environment that is lethal to most pathogens. However, several microbial species have evolved ways to circumvent phagosome-mediated killing by the neutrophil. For example, *Staphylococcus aureus* expresses a polysaccharide capusule that confers anti-phagocytic properties (Luong and Lee 2002), while *Salmonella typhimurium* (Joiner et al. 1989) and *S. pyogenes* (Staali et al. 2006) block granule fusion with the phagosome, permitting these species to survive within the neutrophil.

1.8.2 Neutrophil extracellular traps

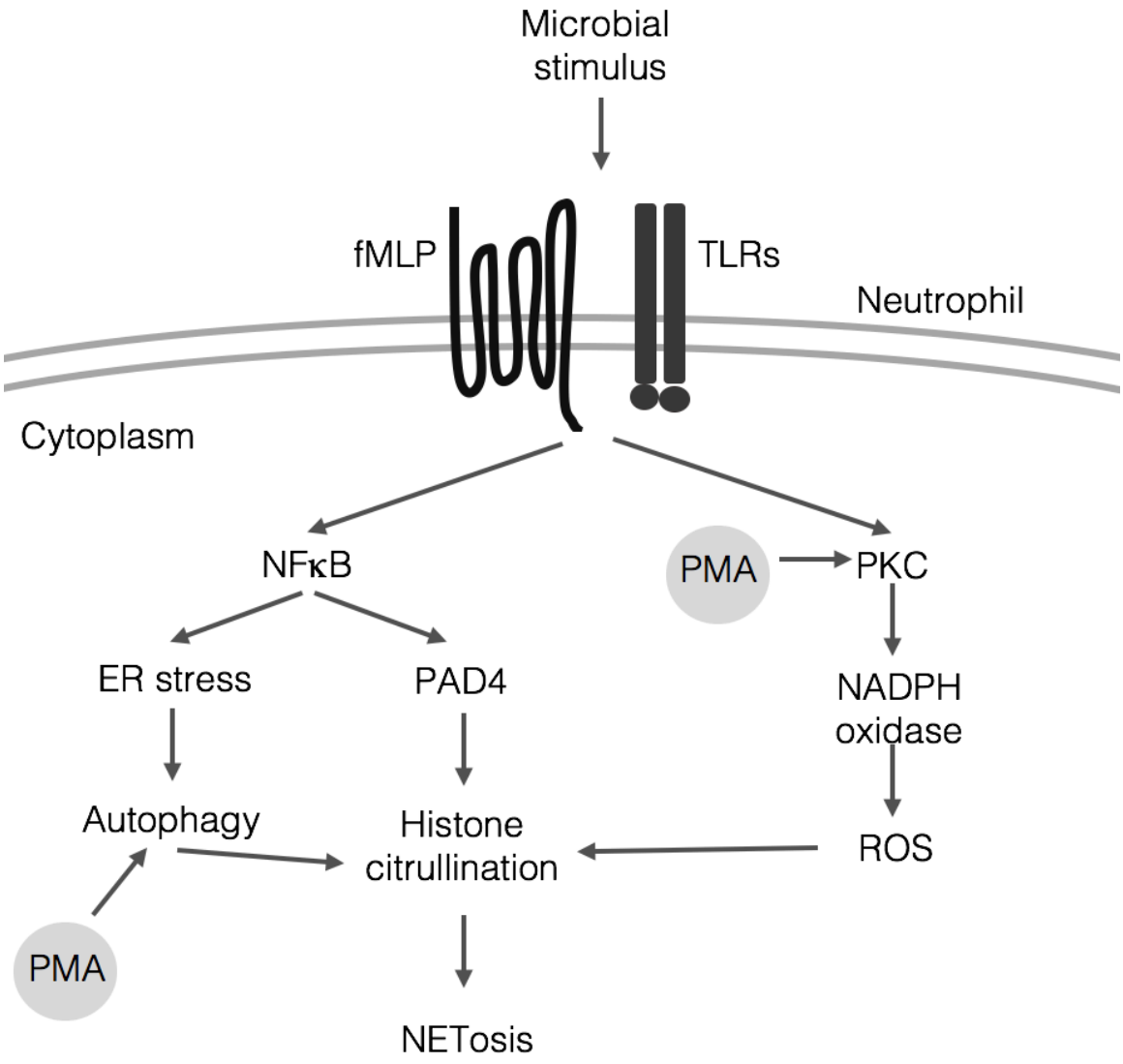
In 2004, Brinkmann and colleagues described an additional neutrophil function: when stimulated with phorbol myristate acetate (PMA), LPS, or IL-8, neutrophils would release web-like structures comprised of nuclear chromatin that was decorated with histone protein, various granule enzymes, and antimicrobial molecules (Brinkmann et al. 2004). These formations, since termed neutrophil extracellular traps (NETs), have been shown to ensnare various types of microbes and kill them by exposing them to high concentrations of granule-derived antimicrobial enzymes (Brinkmann et al. 2004).

Though significant progress has been made towards delineating the cellular events of NET formation, many of the underlying mechanisms remain incompletely understood. NET formation is generally initiated by the stimulation of neutrophils with microbial or chemical components, followed by the loss of cytosolic membranes organization and chromatin decondensation. As the nuclear envelope degrades, decondensed chromatin mixes with neutrophil granular enzymes within the cytoplasm. Finally, eruption of the cell membrane follows, and the DNA/granular enzyme mixture is released into the extracellular space (Fuchs et al. 2007). This process has been termed 'NETosis', and is considered a cell death pathway that is distinct from apoptosis or necrosis (Steinberg and Grinstein 2007). Unlike apoptosis or necrosis, NETosis is not associated with DNA fragmentation or PS exposure on the external cellular membrane. The lack of PS limits the clearance of NETosing cells by macrophages, allowing the neutrophil to remain in circulation to exert its immunoprotective functions (Huynh et al. 2002).

Several lines of evidence suggest that NETosis relies on the formation of ROS generated by NADPH oxidases. The generation of ROS has been reported to inactivate the function of several caspases, thereby leading to inhibition of apoptotic cell death pathways and prolonged neutrophil survival (Fadeel et al. 1998). Pharmacologic inhibition of NADPH oxidase attenuates NET formation *in vitro* (Metzler et al. 2011), and patients with chronic granulomatous disease

(CGD) who lack NADPH oxidase function are unable to form NETs and are susceptible to infection (Fuchs et al. 2007). Interestingly, CGD patients can effectively clear catalase-negative bacteria which produce their own hydrogen peroxide, providing a substrate for downstream NADPH oxidase reactions (Segal et al. 2000). However, certain microorganisms such as *S. aureus* (Pilsczek et al. 2010) or *Leishmania. donovani* (Gabriel et al. 2010) are able to induce NETosis through ROS-independent mechanisms, suggesting that there may be multiple cellular pathways that culminate in NET formation depending on the initial stimulus. ROS generation in neutrophils also induces endoplasmic reticulum (ER) stress within the neutrophil and initiates autophagy pathways which have been show to be necessary for NETosis (Remijsen et al. 2010). The cellular mechanisms associated with neutrophil autophagy include an influx in intracellular Ca^{2+} levels which trigger Ca^{2+}-dependent activation of peptidyl arginine deiminase 4 (PAD4), an intranuclear enzyme that citrulinates histones, ultimately reducing their net positive charge and facilitating chromatin decondensation (Rohrbach et al. 2012). Indeed, neutrophils isolated from mice with PAD4 deficiency are unable to generate NETs when stimulated with bacterial components (Li et al. 2010; Martinod et al. 2013). In addition, neutrophil autophagy contributes to the degradation of intracellular membrane components, permitting the mixing of nuclear granular contents in the cytoplasm

Figure 1.4 – **Cellular mechanisms of NETosis**. Stimulation of formyl peptide receptor (fMLP) or Toll-like receptors on the neutrophil cell surface with PAMPs and/or DAMPs results in the activation of specific G protein-coupled intracellular signaling events. These events potently activate NADPH oxidase, leading to reactive species production. In parallel, induction of NFκB drives autophagy and consequently induces PAD4 activation and subsequent chromatin decondensation. Similarly, stimulation of neutrophils with phorbol 12-myristate 13-acetate (PMA) also results in protein kinase C (PKC) activation and induction of autophagy to initiate NETosis (adapted from Medina 2013).


Microbial stimulus
fMLP
TLRs
Neutrophil
Cytoplasm
NFκB
PMA
PKC
ER stress
PAD4
NADPH oxidase
Autophagy
Histone citrullination
ROS
PMA
NETosis

prior to NET release (Remijsen et al. 2010). **Figure 1.4** summarizes the key signaling events known to potentiate NETosis.

Despite their protective and bactericidal functions, the release of NETs can also be detrimental to the host and as a result, NETosis is considered by many to be a 'last resort' measure for neutrophils. The externalization of NETs and granular contents has been associated with a wide range of cytotoxic effects and collateral damage to host tissue (Smith 1994; Saffarzadeh et al. 2012; Cui et al. 2012). Furthermore, the release of NETs has been implicated in autoimmune disorders such as systemic lupus erythematosus and rheumatoid arthritis, both disorders characterized by the formation of autoantibodies against chromatin (Villanueva et al. 2011).

1.8.3 Role of the neutrophil in (immuno)thrombosis

Many neutrophil effector functions are known to modulate the procoagulant activities associated with immunothrombosis. Unlike with monocytes, the role of TF in neutrophil-driven thrombosis remains a highly contested area of research. Expression of TF on neutrophils was first described more than 40 years ago, and more recently it was demonstrated that neutrophils may synthesize and express functional TF following stimulation with P-selectin (Maugeri et al. 2006). Similarly, exposure to immunoglobulin (Ig) G obtained from patients with antiphospholipid syndrome induces functional TF expression

by neutrophils (Ritis 2012). In contrast, several reports have demonstrated that isolated neutrophils fail to produce TF when stimulated with LPS or PMA (Osterud et al. 2000). Though trace amounts of TF activity was detected in these experiments, the authors attributed this to the presence of contaminating monocytes. Unlike monocytes, neutrophils do not support the assembly of tenase on their cell surface (McGee and Li 1991). Furthermore, an *in vivo* study utilizing a murine sepsis model demonstrated clusters of TF-positive granulocytes infiltrating the spleen. However, even though these neutrophils expressed TF on their cell surface, they did not possess TF mRNA (de Waard et al. 2006). Here the authors hypothesized that neutrophils uptake TF from other cells, possibly in the form of MPs. This was later confirmed by Egorina and colleagues who demonstrated that neutrophils acquire preformed monocyte-derived TF rather than derive it through *de novo* synthesis themselves (Egorina et al. 2007). Together this evidence suggests that neutrophils, though potentially a source of trace amounts of functional TF, are unlikely to be a major contributor of extrinsic pathway-mediated thrombosis.

NET formation represents an entirely new mechanism by which neutrophils modulate coagulation and several NET components have been examined in isolation for their ability to induce coagulation reactions. In a baboon model of DVT, the DNA component of newly formed NETs provided a template for fibrin deposition and stabilized the developing thrombus (Fuchs et

al. 2010). Similarly, in a murine DVT model, NETs were demonstrated to promote thrombus formation by binding and activating FXII (Bruhl et al. 2012). Neutrophil depletion or administration of DNase to degrade potential NET formations abrogated thrombus development.

The histone component of NETs is also known to modulate coagulation through several mechanisms. In murine models of endotoxemia, NET formation within the vasculature was associated with the entrapment and subsequent activation of platelets, which culminated in endothelial injury and impaired blood flow (Clark et al. 2007). It was later revealed that the histone component of NETs (specifically histone subunits H3 and H4) were responsible for platelet activation through engagement of platelet TLR2 and 4 (Xu et al. 2011). Histones also promote a procoagulant phenotype through auto-activation of prothrombin to thrombin (Barranco-Medina et al. 2013), as well as inducing PS exposure on red blood cells (Semeraro et al. 2014). In addition, histones have also been implicated in the impairment of endogenous anticoagulant pathways by impairing TM-mediated binding and subsequent activation of PC (Ammollo et al. 2011) and interfering with AT-mediated thrombin inhibition (Varju et al. 2015).

1.9 Introduction to Sepsis

1.9.1 Sepsis as a Clinical Challenge

Sepsis is a devastating condition that is best characterized as a systemic infection with concomitant pathologic thrombus formation, often described as "blood poisoning," and is the result of invasion by pathogenic organisms that subsequently disseminate within the host's circulation. Despite advances in management and treatment, sepsis remains the leading cause of morbidity and mortality in non-coronary intensive care units (ICUs) in North America (Zeerleder et al. 2003; Angus et al. 2001; Margraf et al. 2008; Dwivedi et al. 2012). In the United States during the late 1970s, it was estimated that 164 000 cases of sepsis occurred annually (Martin et al. 2003). Recent estimates are higher with more than 650 000 cases of sepsis reported each year, and this trend continues to be observed in both Canada and European countries as well (Angus et al. 2001; Harrison et al. 2006; Husak et al. 2010).

Risk factors for sepsis include a combination of the patient's predisposition to infection as well as likelihood of developing organ dysfunction. There are many risk factors of infections that are known to precipitate severe sepsis, including chronic conditions such as cancer, HIV/AIDS, chronic obstructive pulmonary disease, as well as the use of immunosuppressive agents (Angus et al. 2001). The growing proportion of multidrug-resistant infections makes antibiotic policy more difficult and may also increase the incidence of sepsis (Esper and Martin 2009). The risk factors for organ dysfunction among

patients that do develop infections are less well studied but include the causative organism, the genetic composition of the patient, the underlying health status of the patient and preexisting organ function, as well as the timeliness of therapeutic intervention (Angus and Wax 2001). Age, sex, and ethnicity all influence the incidence of severe sepsis, which is higher in infants and the elderly, higher in males than females, and higher in African Americans compared to Caucasians (Angus et al. 2001; Mayr et al. 2010).

The clinical features associated with sepsis are extremely heterogeneous, and often vary depending on the origin of infection, the causative pathogen, degree and location of organ dysfunction, the medical case history of the patient, as well as the delay between the onset of sepsis and the initiation of treatment (Remick 2007; Levy et al. 2003). Symptomatic manifestations in sepsis are often nuanced and non-specific, making a diagnosis of sepsis difficult to discern. The most recent international consensus guidelines provide a list of clinical features characteristic of incipient sepsis (**Table 1.2**).

One similarity among patients with sepsis is the increased risk of developing multiple organ dysfunction syndrome (MODS), which results in cumulative alterations in organ function and ultimately terminal organ failure. Sepsis-associated mortality increases exponentially in accordance with the degree of organ dysfunction. In one early study, case fatality figures for sepsis, severe sepsis, and septic shock were 16, 20, and 46 per cent, respectively

Table 1.2. Diagnostic criteria for sepsis, severe sepsis, and septic shock. Data adapted from Levy et al. (2003)

Sepsis (suspected or confirmed infection with ≥2 of the following)

General variables

- Fever (>38.3°C)
- Hypothermia (<36°C)
- Elevated heart rate (>90 beats per min.)
- Tachypnea
- Altered mental status
- Substantial edema or positive fluid balance (>20ml/kg of body weight)
- Hyperglycemia (>120mg/dl plasma glucose)

Inflammatory variables

- Leukocytosis (>12 000/mm^3 leukocytes)
- Leukopenia (<4000/mm^3 leukocytes)
- Normal white-cell count with >10% immature forms.
- Elevated plasma C-reactive protein (>2 SD above normal range)
- Elevated plasma procalcitonin (>2 SD above normal range)

Hemodynamic variables

- Arterial hypotension (systolic <90mmHg; arterial <70mmHg)
- Elevated venous oxygen saturation (>70%)
- Elevated cardiac index (>3.5 L/min/m^2 of body-surface area)

Organ dysfunction variables

- Arterial hypoxemia
- Acute oliguria (urine output, <0.5ml/kg/hr or 45ml/hr for 2h)
- Increased creatinine (>0.5mg/dl)
- Coagulation abnormalities (INR >1.5; APPT >60s)
- Paralytic ileus
- Thrombocytopenia (<100 000/mm^3 platelet count)
- Hyperbilirubinemia (>4mg/dl)

Tissue perfusion variables

- Hyperlactatemia (>1mmol/l)
- Decreased capillary refill or mottling

Severe sepsis (sepsis with organ dysfunction)

Septic shock (sepsis with hypotension)

(Rangel-Frausto et al. 1995). Similar trends have also been reported within several retrospective analyses examining septic patient mortality, where sepsis-associated mortality ranges between 27 to 59 per cent (Martin et al. 2003; Padkin et al. 2003).

Treatment strategies for sepsis can be as heterogeneous as the disease itself. The fundamental principles of sepsis management are largely supportive in nature, and consist of cardiorespiratory resuscitation, and to mitigate the immediate threat of uncontrolled infection (Dellinger et al. 2007). Resuscitation often mandates intravenous fluids and vasopressor administration, along with oxygen therapy and mechanical ventilation if required. Specific guidelines for resuscitation strategies remain the subject of ongoing debate, with the type and volume of fluid, intensity of hemodynamic monitoring, and selection of vasoactive agents currently left up to the discretion of the attending healthcare team (Dellinger et al. 2007). Similarly, the selection of antibiotic therapy varies depending on the site of infection, the setting in which the infection developed, and the patient's medical history (Dellinger 2003). Drotrecogin Alfa (Xigris/recombinant APC), the only approved drug for sepsis indication, was withdrawn from market in 2012 following a failure to demonstrate a survival benefit during the landmark PROWESS-SHOCK trial (Ranieri et al. 2012). As a result, identification of new therapeutic targets in sepsis remains the subject of intense investigation.

1.9.1 Sepsis Pathophysiology

Though sepsis is initiated by microbial invasion, the observed clinical phenomena cannot be fully explained by a diagnosis of infection. Despite the development of modern antibiotics, patients with sepsis continue to die despite successful elimination of the offending pathogen. As a result, current research identifies that it may be the host response to the pathogen – not the pathogen itself – that drives the pathophysiology of sepsis (van der Poll and Opal 2008; Medzhitov 2008).

The onset of sepsis is invariably associated with coagulation abnormalities that promote a hypercoagulable state (Remick 2007; Levi et al. 2003; Stief et al. 2007). During sepsis, coagulation activation is ubiquitous and induced by PAMPs such as LPS and exotoxins released by offending pathogens. Activation of innate immune defense mechanisms, triggered by microbial PAMPs, results in a generalized proinflammatory, procoagulant state. Sepsis-related coagulopathies range from mild laboratory alterations to severe DIC, resulting in the formation of microvascular thrombosis, hypoperfusion, and ultimately MODS and death (Levi et al. 2013). Moreover, these damaged tissues continue to release additional DAMPs into the extracellular environment which activate circulating leukocytes to perpetuate a cycle of continuous tissue injury (Rittirsch et al. 2008). The activation of coagulation, reinforced by the

downregulation of anticoagulant and fibrinolytic pathways, plays a crucial role in the progression of sepsis pathophysiology.

Conventionally, the initiation of coagulation in sepsis is thought to be TF-dependent and this has been demonstrated in numerous sepsis models. Generation of thrombin in humans injected with a low dose of LPS was preceded by an increase in TF mRNA levels and TF expression on circulating monocytes, and the release of TF-containing MPs (Aras 2004; Franco et al. 2000). Similarly, baboons infused with a lethal dose of *E. coli* and patients with severe bacterial infection express sustained TF activity on the surface of peripheral blood monocytes. Furthermore, mice with an almost complete absence of TF had reduced coagulation, inflammation, and mortality relative to control mice following infusion of LPS (Pawlinski 2003). However, several groups have reported that sepsis patients display no signs of systemic hypercoagulability when evaluated with the thrombin generation assay, even in the early stages of sepsis. Importantly, thrombin generation in these studies was analyzed in platelet-poor plasma triggered with relipidated TF (Picoli-Quaino et al. 2013; Petros et al. 2012; Collins et al. 2006; Seo et al. 2009). Thus, previous studies may have overlooked the importance of the intrinsic pathway of coagulation in the hypercoagulable state observed in sepsis.

In addition to enhanced coagulation, several anticoagulant pathways are impaired during sepsis progression. Markedly lower plasma levels of AT are

found in sepsis due to diminished protein synthesis and enhanced consumption due to sustained thrombin generation (van der Poll and Opal 2008). Following exposure to proinflammatory cytokines, the synthesis of glycosaminoglycans by endothelial cells is reduced (Kobayashi et al. 1990), impairing the inhibitory potential of AT. TFPI, PC, and its cofactor protein S are also found in lower circulating concentrations in septic patients, and their rapid depletion is associated with poor prognosis (Asakura et al. 2001; Tang et al. 2007; Liaw 2004; Shorr et al. 2006). In addition, elevated levels of soluble plasma TM and EPCR has been consistently observed in sepsis, suggesting that endothelial damage resulting from exposure to inflammatory mediators occurs, resulting in the shedding of both anticoagulant participants *in vivo* (Semeraro et al. 2010; Levi 2010).

There is also evidence of impaired fibrinolytic activity in sepsis as a result of increased PAI-1 synthesis and secretion from cytokine-activated vascular endothelial cells (Yipp and Kubes 2013; Levi et al. 2003; Brinkmann et al. 2004; Semeraro et al. 2012; Fuchs et al. 2012). Increased circulating PAI-1 levels have been shown to correlate with disease severity and poor outcome in patients with sepsis (Lorente et al. 2014; Madoiwa et al. 2006; Dofferhoff et al. 1992). Previous studies in which LPS was administered to human (van Deventer et al. 1990; Cadena et al. 1996) or nonhuman primates (Biemond et al. 1995) demonstrated that endotoxemia results in a transient increase in tPA activity and activation of

fibrinolysis that is followed by a robust elevation in PAI-1 levels, ultimately driving a hypofibrinolytic state. Interestingly, activation of plasminogen by tPA and subsequent fibrinolytic activity in sepsis patients has been shown to remain ongoing despite increases in PAI-1 as determined by plasma levels of plasmin-antiplasmin complexes and D-dimer (Hesselvik et al. 1989; Raaphorst et al. 2001; Madoiwa et al. 2006). Therefore, while the inhibitory effects of elevated PAI-1 may contribute to impaired fibrinolysis in sepsis, there are likely other critical anti-fibrinolytic mechanisms involved.

1.8.3 Diagnostic and Prognostic Biomarkers in Sepsis

The most significant disappointment in sepsis management has been the failure to translate our understanding of sepsis pathophysiology into successful clinical therapies. This is due, in part, to an inability of clinical studies to incorporate a vastly diverse sepsis patient population into the study design (Marshall 2014). For example, most sepsis clinical studies utilize blanket enrollment criteria (outlined in **Table 1.2**), and pharmacologic interventions are usually given on the basis of a standard dosing formula for a small duration of time. There is seldom any information collected on how an intervention impacts the host response or host-pathogen interactions, and the primary endpoint of these studies is typically death from any cause. This over-simplistic approach to clinical study design in sepsis has been criticized for its inability to distinguish

patients most likely to benefit from a particular therapy, a refusal to adjust a given therapy based on changes in patient response, as well as disguising potential important effects on non-fatal outcomes (Marshall 2014). As a result, there is an obvious need to assess patient-to-patient variability and employ strategies that consider and incorporate individual patient information.

Biological markers (biomarkers) are critical to this process. Biomarkers are defined as alterations to biochemical or molecular constituents found normally in the biological environment, including tissues and fluids and provide us with an approach to observe and understand the dynamic state of a disease (Biomarkers Definitions Working Group. 2001). In sepsis, various biomarkers have been evaluated in their potential to guide antibiotic therapy, differentiate between causative pathogens (i.e: Gram-negative versus Gram-positive), gauge patient response to a given therapy, or predict sepsis-related complications (such as organ dysfunction) (Marshall and Reinhart 2009; Venteluoto 2008; The Surviving Sepsis Campaign Guidelines Committee including The Pediatric Subgroup et al. 2013). Several biomarkers have demonstrated modest prognostic utility to stratify septic patients into high- or low-risk groups and predict overall mortality, including TNF-α (Calandra et al. 1990), IL-6 (Miguel-Bayarri et al. 2012), procalcitonin (Meng et al. 2009), IL-8 (Harbarth et al. 2001), APC (Fourrier et al. 1992), and C-reactive protein (CRP) (Meng et al. 2009).

Recently, we have identified plasma levels of CFDNA as a useful prognostic biomarker in sepsis. Levels of CFDNA in plasma at the onset of admission to the ICU had better prognostic utility than did MODS or the acute physiology and chronic health evaluation (APACHE) II scores, or other biomarkers evaluated in this study. The area under the receiver operating characteristic (ROC) curves for CFDNA to predict ICU mortality is 0.97 and to predict hospital mortality is 0.84. Furthermore, CFDNA had a sensitivity of 88% and specificity of 94% for predicting ICU mortality compared to APACHEII or MODS scores alone (Dwivedi et al. 2012). This pilot study suggested that incorporating plasma levels of CFDNA into sepsis risk-stratification systems may be clinically valuable for assessing disease severity in sepsis patients.

2.0 Hypothesis and Objectives

2.1 Rationale and Hypothesis

Mortality in sepsis is not attributed to the presence of infection itself, but rather the result of an exaggerated innate immune response directed towards the invading pathogen. The recent discovery of NETs has established an important link between innate immunity, coagulation, and inflammation. Elevated levels of NET-derived components, namely CFDNA and histones, have been found in various prothrombotic, pathologic disease states and are a hallmark of the immune response to proinflammatory processes. Furthermore, we have recently identified levels of CFDNA as a valuable prognostic marker in sepsis patients, suggesting that elevated levels of CFDNA may contribute to the progression of sepsis pathophysiology. Therefore, the overall aim of the research performed for this thesis is to identify the mechanisms by which CFDNA/histones modulate coagulation in sepsis.

We hypothesize that elevations in levels of CFDNA and histones drive a procoagulant state in sepsis by (A) promoting contact pathway-mediated coagulation in a platelet-dependent and platelet-independent manner; (B) modulating fibrin clot structure and impairing fibrinolysis via interactions with fibrinogen and plasmin; and (C) by functioning as endogenous circulating DAMPs, resulting in the induction of TF expression in circulating peripheral blood monocytes.

To test our hypotheses, we measured the effects of CFDNA and histones, both purified and in the form of NETs, on thrombin generation and clot lysis times in a plasma environment. We also quantified TF activity, expression, and global procoagulant potential of monocytic cells. Finally, we confirmed our *in vitro* findings in plasmas obtained from patients with severe sepsis, and examined the ability of various DNA/histone inhibitors to modulate their procoagulant effects.

2.2 Objectives

Our specific objectives were as follows:

1. To determine the procoagulant potential of intact NETs released from activated neutrophils, and to assess the relative contributions of CFDNA and histones to thrombin generation.
2. To investigate the relationship between CFDNA levels and fibrinolytic activity in sepsis, and determine the mechanisms by which CFDNA may modulate fibrinolysis.
3. To investigate the ability of CFDNA and histones to modulate the procoagulant potential of monocytic cells, and examine the effects of septic plasmas to induce a procoagulant phenotype on peripheral blood monocytes.

3.0 Neutrophil Extracellular Traps promote thrombin generation through platelet-dependent and –independent mechanisms

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Foreword: In Chapter 3, we investigate the procoagulant activities of cell-free DNA (CFDNA) and histones in the context of intact neutrophil extracellular traps (NETs). We demonstrate that intact NETs promote thrombin generation in platelet-poor plasma and that (1) thrombin generation is triggered via the intrinsic pathway, and (2) thrombin generation in platelet-poor plasma is attenuated with DNase but not RNase. Thrombin generation in PRP containing intact NETs was further enhanced with DNase addition, an effect that was abolished with anti–Toll-like receptor antibodies. This suggests that degradation of CFDNA in the NET scaffold increases histone-mediated, platelet-dependent thrombin generation. Our studies also implicate a role for CFDNA–mediated activation of the intrinsic pathway of coagulation in the pathogenesis of sepsis. We observed a direct correlation between plasma CFDNA levels and indices of thrombin generation in patients with sepsis, implicating that CFDNA may contribute to coagulation abnormalities typically observed in sepsis.

Running title: NETs enhance thrombin generation

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References for this manuscript have been incorporated into the Bibliography (Chapter 8) at the end of this thesis.

3.1 Abstract

Objective - Activation of neutrophils by microbial or inflammatory stimuli results in the release of neutrophil extracellular traps (NETs) which are comprised of DNA, histones, and antimicrobial proteins. In purified systems, cell-free DNA (CFDNA) activates the intrinsic pathway of coagulation whereas histones promote thrombin generation through platelet-dependent mechanisms. In this study, we examined the procoagulant potential of intact NETs released from activated neutrophils.

Approach and Results - NETs released from phorbyl myristate (PMA)-activated neutrophils enhance thrombin generation in platelet-poor plasma (PPP). This effect was DNA-dependent (confirmed by DNase treatment) and occurred via the intrinsic pathway of coagulation (confirmed with FXII- and FXI-depleted plasma). In platelet-rich plasma treated with corn-trypsin inhibitor, addition of PMA-activated neutrophils increased thrombin generation and shortened the lag time in a TLR-2- and TLR-4-dependent mechanism. Addition of DNase further augmented thrombin generation, suggesting that dismantling of the NET scaffold increases histone-mediated, platelet-dependent thrombin generation. In PPP plasma samples from septic patients, we found a positive correlation between endogenous CFDNA and thrombin generation, and addition of DNase attenuated thrombin generation.

Conclusions - These studies are the first to examine the procoagulant activities of CFDNA and histones in the context of NETs. Our studies also implicate a role for the intrinsic pathway of coagulation in sepsis pathogenesis.

3.2 Introduction

Sepsis is the leading cause of morbidity and mortality in non-coronary intensive care units in the Western world (Angus et al. 2001). Severe sepsis, defined as sepsis associated with at least one dysfunctional organ, afflicts approximately 750 000 individuals in the United States annually, with an estimated mortality rate of 30 to 50% (Angus et al. 2001). Sepsis is often initiated by release of microorganisms and/or microbial toxins into the circulation (Wheeler and Bernard 1999a; Hotchkiss and Karl 2003). Under these conditions, morbidity and mortality are the result of uncontrolled activation of inflammatory and coagulation pathways, which leads to microvascular thrombosis and subsequent multiple organ dysfunction syndrome (MODS).

Although many clinical trials have explored the utility of agents designed to attenuate inflammatory and/or coagulation pathways, all have failed and the outcome of patients with severe sepsis remains poor (Ulloa et al. 2009; The Surviving Sepsis Campaign Guidelines Committee including The Pediatric Subgroup* et al. 2013). Thus, a better understanding of the pathogenesis of sepsis is needed. Recently, cell-free DNA (CFDNA) has emerged as an

important link between innate immunity, coagulation, and inflammation (Yipp and Kubes 2013; Brinkmann et al. 2004; Fuchs et al. 2012). When activated by microbial or inflammatory stimuli, neutrophils release web-like structures known as neutrophil extracellular traps (NETs), which are comprised of CFDNA, histones, and antimicrobial proteins (Brinkmann et al. 2004). These structures bind to microorganisms, prevent them from spreading, and ensure a high local concentration of neutrophil granule enzymes to kill bacteria.

CFDNA is the major structural component of NETs, as shown by the ability of DNA-intercalating dyes to stain NETs and by the ability of deoxyribonuclease (DNase) to dismantle NETs (Brinkmann et al. 2004). However, CFDNA might also have deleterious effects on the host. CFDNA triggers the intrinsic pathway of blood coagulation (Swystun et al. 2011), and elevated levels of CFDNA are found in patients with deep vein thrombosis (DVT) (Fuchs et al. 2012). It has been proposed that the presence of CFDNA and platelet-neutrophil interactions in the microcirculation results in microvascular thrombosis, leading to tissue hypoxia and endothelial damage (Clark et al. 2007; Gupta et al. 2010).

Histones, the other principal component of extracellular traps, are important contributors to the bacteriocidal and cytotoxic properties of NETs (Kawasaki and Iwamuro 2008). Histones are cationic nuclear proteins that associate with DNA to form nucleosomes, the repeating units of chromatin.

When injected into mice, histones result in death due to an extreme prothrombotic response including diffuse microvascular thrombosis, fibrin deposition, platelet aggregation, and thrombocytopenia (Xu et al. 2009). Histone H4 is cytotoxic towards endothelial cells, and blocking histone-mediated cytotoxicity protects mice from endotoxemia (Xu et al. 2009). In purified systems, histones H3 and H4 directly induce platelet aggregation through interactions with Toll-like receptors 2 and 4 (Xu et al. 2011).

Many of the studies on the procoagulant/proinflammatory properties of CFDNA and histone proteins have examined these components in isolation. However, the majority of CFDNA in plasma is likely histone-bound. Thus, the overall procoagulant effects of CFDNA/histone complexes as part of intact NETs released from activated neutrophils are unknown. Importantly, the interaction between CFDNA and histones may shield many of the pathophysiological effects observed when components are examined in isolation. To address this possibility, we (a) identified the cells responsible for release of CFDNA in blood, (b) compared the capacity of NETs released from activated neutrophils to promote thrombin generation in platelet-poor and platelet-rich plasma, and (c) determined the relative contribution of CFDNA and/or histones to thrombin generation in plasma from septic patients and non-septic controls

3.3 Materials and Methods

3.3.1 Materials

Factor VII-, XI-, FXII-deficient plasmas, human FVII, FXI, FXII, and corn trypsin inhibitor (CTI) were from Haematologic Technologies (Essex Junction, VT). Bovine DNase and RNase were from Promega (Madison, WI). Heparin was purchased from Leo Pharma (Thornhill, ON). Lipopolysaccharide (LPS), lipoteichoic acid (LTA), peptidoglycan, phorbol 12-myristate 13-acetate (PMA), bovine alkaline phosphatase (AP), and protamine sulfate (PS) were from Sigma-Aldrich (St Louis, MO). Sytox Green and DAPI were purchased from Invitrogen (Burlington, ON). Blocking mAbs against human TLR2 (clone T2.5), TLR4 (HTA125), and isotype control (IgG2a) were all purchased from eBiosciences.

3.3.2 DNA isolation and quantification

To isolate DNA from plasma or cell supernatants, samples were subjected to centrifugation at 10 000 x g for 10 min to sediment cellular debris and DNA in the supernatant was then isolated using the QIAMP DNA blood mini kit (QIAGEN, Mississauga, ON) according to the manufacturer's instructions. CFDNA was isolated from 200 µL plasma or supernatant and eluted into 200 µL elution buffer (10mM Tris-Cl, 0.5mM EDTA, pH 9.0). The concentrations of isolated DNA was determined by spectrophotometry by measuring absorbance at 260 nm using a BioPhotometer Plus spectrophotometer (Eppendorf,

Mississauga, ON) and purity was confirmed by calculating the ratio of absorbance determined at 260 and 280 nm.

3.3.3 Neutrophil isolation

Blood was collected from the antecubital veins of drug- and caffeine-free healthy volunteers into heparin (10 UmL^{-1}). Neutrophils were isolated as previously described (Oh et al. 2008). Briefly, whole blood layered onto lympholyte poly cell separation media (Cedarlane, Burlington, ON) was subjected to centrifugation for 40 min at 500 x g at 22°C. The neutrophil layer was harvested and residual red blood cells were lysed using RBC lysis buffer (Roche Applied Science, Basel, Switzerland). Neutrophils were then washed and re-suspended in HBSS containing $CaCl_2$ $MgCl_2$ (Gibco Invitrogen, Carlsbad, CA) and 2% human serum albumin (Canadian Blood Services, Ottawa, ON).

3.3.4 Stimulation of whole blood and neutrophils by bacterial components

Increasing concentrations of LPS (0.1µg/mL or 1µg/mL) or LTA (10µg/mL or 30µg/mL) co-incubated with peptidoglycan (30µg/mL) were added to whole blood or purified neutrophils obtained from healthy volunteers at a concentration of 1 x 10^6 cells/mL. Whole blood or purified neutrophils were incubated for varying lengths of time at 37°C after which the cells were pelleted, the plasma was collected (by centrifugation at 1500 x g), and the CFDNA was quantified.

3.3.5 Visualization of NETs

Isolated neutrophils seeded onto glass coverslips at 1 x 10^7 cells/mL were incubated with 100 nM PMA for 30min at 37°C. PMA was chosen as our NET-stimulator for this and subsequent experiments due to its consistent induction of NET formation and limited capacity to activate platelets (compared to LPS). After washing five times with 1x PBS, total DNA was stained with 5μM DAPI (which passes through intact cell membranes), whereas extracellular DNA was stained with 1μM Sytox Green (which does not cross cell membranes) as previously described (Brinkmann et al. 2010). Fluorescent images were acquired using an Olympus BX41 fluorescent microscope fitted with an Olympus DP72 camera and analyzed using Slidebook software v.5.0.

3.3.6 Thrombin generation assays

To prepare platelet-poor plasmas (PPP), peripheral venous blood was collected from healthy volunteers into 3.8% trisodium citrate. PPP was prepared by centrifugation at 1500 x g for 10 minutes at room temperature. Simultaneously, platelet-rich plasma (PRP) was prepared by collecting venous blood into citrate supplemented with 50μg/mL CTI. PRP was prepared immediately by centrifugation at 180 x g for 10 minutes at room temperature. To 40μL aliquots of PPP or PRP in wells of a 96-well black Costar plate were added 1 x 10^5 neutrophils. Where indicated, PPP or PRP was pretreated for 30 minutes with the following enzymes or inhibitors before the addition of neutrophils: bovine

DNase (20µg/mL), RNase (20µg/mL), protamine sulfate (PS; 50µg/mL), anti-TLR2, anti-TLR4, or IgG2a mAbs (50 µg/mL), or bovine AP (10 U/mL). After incubation with 100nM PMA for 30 min at 37°C undisturbed, 15 mM $CaCl_2$ and 1mM Z-Gly-Gly-Arg-AMC (Bachem, Bubendorf, Switzerland) were added and thrombin generation was monitored using the Technothrombin TGA thrombin generation assay (Technoclone, Vienna, Austria) as previously described (Bunce et al. 2011). Thrombin generation profiles were analyzed using Technothrombin TGA software (Technoclone).

3.3.7 Quantification of circulating DNA/histone complexes

DNA-histone complex levels were quantified using the Cell Death Detection ELISA Plus kit from Roche Applied Science according to the manufacturer's instructions.

3.3.8 Gel Electrophoresis

Extracted DNA was prepared in DNA loading buffer (Thermo Scientific, ON) and 20µL were added to 1.5% agarose gels for electrophoresis. After staining with ethidium bromide, gels were photographed using UV transillumination.

3.3.9 Patient plasma samples

Frozen plasma samples from patients with severe sepsis were collected as part of our ongoing DYNAMICS Study (DNA as a Prognostic Marker in ICU

Patients Study; ClinicalTrials.gov Identifier: NCT01355042), an investigator-initiated multi-center prospective observational study that was undertaken to validate CFDNA as a prognostic biomarker in patients with severe sepsis. Patients with severe sepsis, as defined as previously described (Dwivedi et al. 2012), were recruited between September 2010 and January 2013 from tertiary care ICUs in Hamilton, Ontario. Blood samples were collected within 24 h of enrollment and processed within 2 h. Briefly; 9 mL blood was collected from indwelling venous catheters and transferred into 15-mL polypropylene tubes containing 0.5mL of 0.105M buffered trisodium citrate (pH 5.4). After centrifugation at 1500 x g for 10 min at 20°C, plasma was harvested and stored in aliquots at -80°C. The study was approved by the Research Ethics Board of McMaster University and Hamilton Health Sciences, Hamilton,(REB approval 10-532). Signed informed consent was obtained from all patients or substitute decision-makers) or from the healthy controls before blood collection.

3.3.10 Plasma samples from healthy volunteers

Using the same methods, plasma samples were obtained from 10 healthy adult volunteers who had been free of medication for at least 48 h. No attempt was made to age or sex match controls with cases.

3.3.11 Statistical analyses

Statistical analysis was performed on experiments with an n = 3 or greater. Values are expressed as means ± standard error. Significance of differences was determined by one-way ANOVA and Tukey's pair-wise comparisons or by t-tests using SIGMAPLOT Software (San Jose, CA, USA).

3.4 Results

3.4.1 Neutrophils are the major source of plasma CFDNA released from activated whole blood

Previously, we reported that high levels of CFDNA in plasma predicts poor clinical outcome in patients with severe sepsis (Dwivedi et al. 2012). DNA-sequence analyses and studies with Toll-like receptor 9 (TLR9) reporter cells suggest that the circulating CFDNA from sepsis patients is host-derived (Dwivedi et al. 2012). In this study, we determined whether neutrophils are the major source of circulating CFDNA released from activated whole blood. Incubation of whole blood with LPS (**Fig. 3.1A**) or LTA+PG (**Fig. 3.1B**) produced a rapid increase in plasma CFDNA. Likewise, incubation of purified neutrophils with LPS (**Fig. 3.1C**) or LTA+PG (**Fig. 3.1D**) also resulted in a rapid increase in plasma CFDNA. The amount of CFDNA released by activated neutrophils was similar to that released by activated whole blood, suggesting that neutrophils are the primary source of CFDNA when whole blood is incubated with LPS or LTA+PG.

To visualize the released CFDNA, neutrophils mounted on coverslips were imaged after stimulation with increasing concentrations of PMA. As shown in **Fig 3.2A**, extracellular chromatin was observed in neutrophils stimulated with PMA, but not in resting neutrophils. Similar results were observed when neutrophils were stimulated with LPS or LTA+PG (data not shown).

3.4.2 NETs enhance thrombin generation through the intrinsic pathway.

To date, there have been no studies that have examined plasma thrombin generation in the presence of intact NETs released from activated neutrophils. To study the effects of NETs on thrombin generation, neutrophils isolated from healthy volunteers were added to PPP and the plasma was then incubated for 30 min in the absence or presence of PMA prior to recalcification and quantification of thrombin generation. In the presence of PMA, the lag time and time to peak thrombin were shorter, and peak thrombin and total thrombin (AUC) were higher compared with those measured in the absence of PMA, findings consistent with a procoagulant effect (**Fig. 3.2B; Table 3.1**). This activity was diminished with DNase I, but not with RNase (data not shown), findings that suggest that the procoagulant activity is mediated by CFDNA.

Figure 3.1: **CFDNA release from activated neutrophils**. Human whole blood was stimulated for varying lengths of time with increasing concentrations of lipopolysaccharide (A) or lipoteichoic acid/peptidoglycan (B), and levels of CFDNA were measured. Isolated human neutrophils were treated with increasing doses of lipopolysaccharide (C) or lipoteichoic acid/peptidoglycan, and levels of CFDNA were measured. (n = 4 independent experiments). * ($P < 0.05$) and ** ($P < 0.01$) indicates significance relative to untreated conditions.

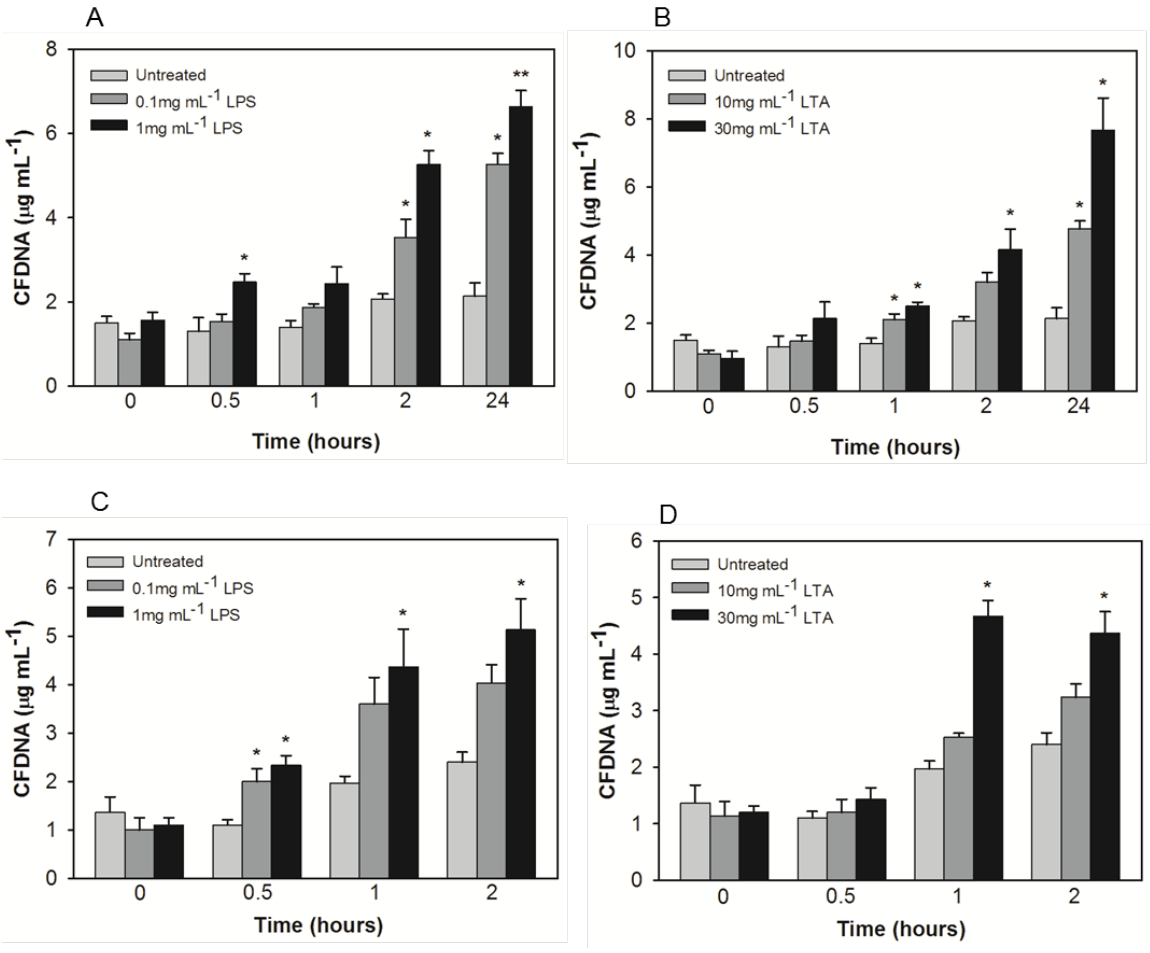
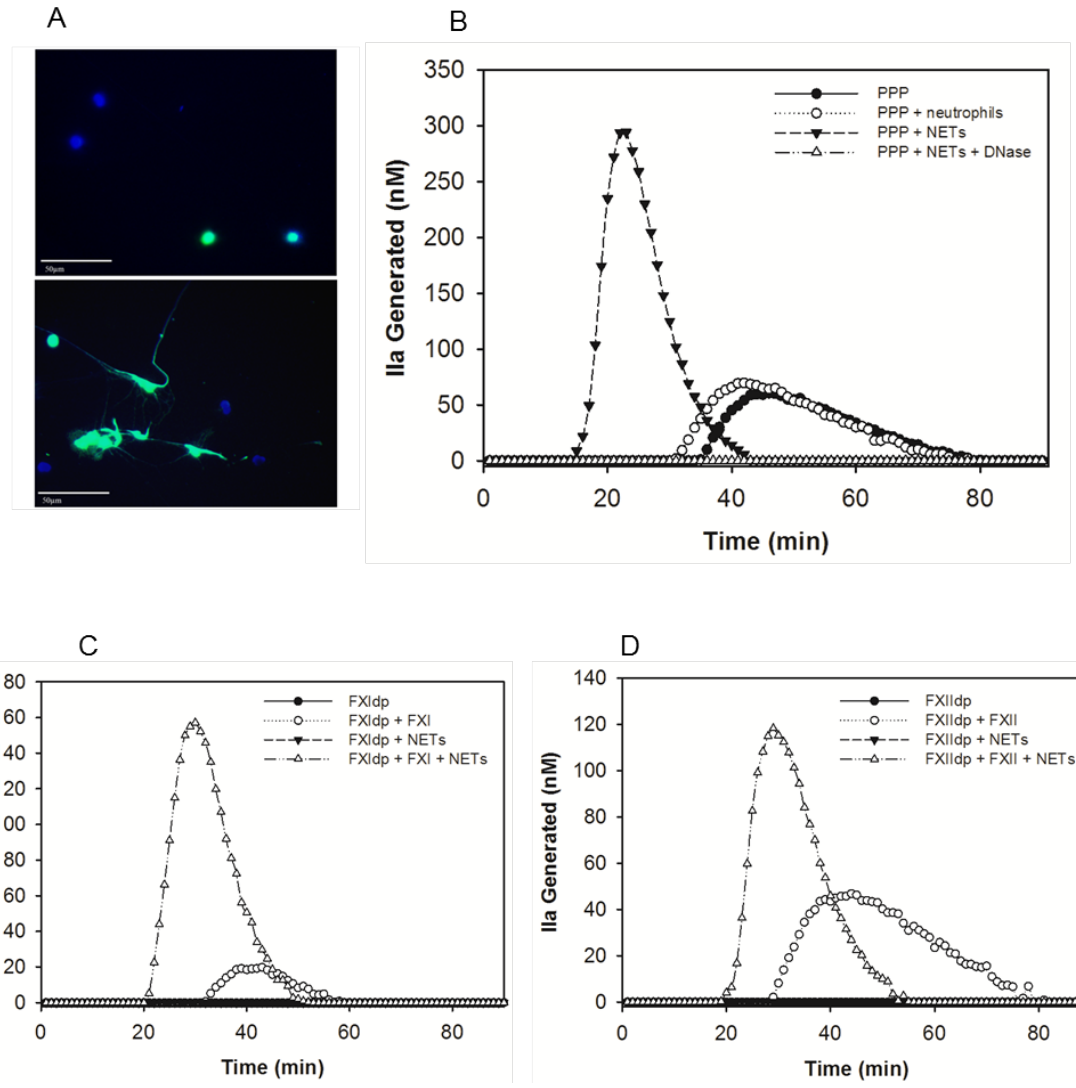

A
Untreated
0.1mg mL-1 LPS
1mg mL-1 LPS
CFDNA (µg mL-1)
Time (hours)
B
Untreated
10mg mL-1 LTA
30mg mL-1 LTA
CFDNA (µg mL-1)
Time (hours)
C
Untreated
0.1mg mL-1 LPS
1mg mL-1 LPS
CFDNA (µg mL-1)
Time (hours)
D
Untreated
10mg mL-1 LTA
30mg mL-1 LTA
CFDNA (µg mL-1)
Time (hours)

Figure 3.2. **PMA-stimulated neutrophils released NETs which enhance thrombin generation via the intrinsic pathway**. Human neutrophils were incubated with 100nM PMA for 30min and stained with DAPI (blue) and Sytox Green (green) (A). The effects of NETs on thrombin generation was examined as described in Materials and methods (B). The effects of NETs on thrombin generation were also measured in FXI- (C) and FXII-deficient (D) plasma. A summary of coagulation parameters for NET release in normal plasma is described in Table 1. Thrombograms shown are representative of 4-6 independent experiments.


A
B
PPP
PPP + neutrophils
PPP + NETs
PPP + NETs + DNase
IIa Generated (nM)
Time (min)
C
FXIdp
FXIdp + FXI
FXIdp + NETs
FXIdp + FXI + NETs
IIa Generated (nM)
Time (min)
D
FXIIdp
FXIIdp + FXII
FXIIdp + NETs
FXIIdp + FXII + NETs
IIa Generated (nM)
Time (min)

Although similar results were obtained in FVII-deficient plasma, when thrombin generation was quantified in FXII- or FXI-deficient plasma, incubation with PMA-activated neutrophils had little effect. The results suggest that the procoagulant activity of CFDNA is mediated via the intrinsic pathway. This concept is supported by the observations that (a) supplementation of FXII- or FXI-deficient plasma with FXII and FXI, respectively, restores procoagulant activity (**Fig. 3.2C, 3.2D**), and (b) addition of CTI, a potent and specific inhibitor of FXIIa, to control plasma abolishes procoagulant activity (**Table 3.2**).

3.4.3 NETs enhance thrombin generation in a platelet-dependent manner

Purified histones have been reported to enhance thrombin generation in platelet-rich plasma (PRP) through a polyphosphate-dependent mechanism (Semeraro et al. 2011). However, it remains unclear whether the platelet-activating effects of histones are shielded when in complex with CFDNA and other NET components. To determine whether platelets enhance thrombin generation when neutrophil-containing plasma is incubated with PMA, results in PRP were compared with those in PPP. In the presence of neutrophils, incubation of PRP with PMA shortened the lag time by half and increased peak thrombin compared with the lag time and peak thrombin determined in PPP. We induced thrombin generation in corn trypsin inhibitor (CTI)-inhibited PRP in the absence or presence of PMA-activated neutrophils. In this system, CTI prevents

Table 3.1. **The effect of NET release on thrombin generation in platelet-poor plasma.** Summary of coagulation parameters for NET release in CTI-supplemented PPP. Results reflect the mean ± SE of at least 3 determinations. Asterisks denote $p < 0.05$ compared with platelet-poor plasma.

	Lag time (min)	Peak IIa (nM)	Peak time (min)	AUC
PPP only	28 ± 4	58 ± 17	41 ± 5	1188 ± 375
Neutrophils	24 ± 2	62 ± 6	42 ± 4	1375 ± 140
NETs	13 ± 1 *	225 ± 23 *	20 ± 2 *	2898 ± 383 *

Table 3.2. **The effect of NET release on thrombin generation in platelet-rich plasma**. Summary of coagulation parameters for NET release in CTI-supplemented PRP. Results reflect the mean ± SE of at least 3 determinations. Asterisks denote $p < 0.05$ compared with platelet-rich plasma.

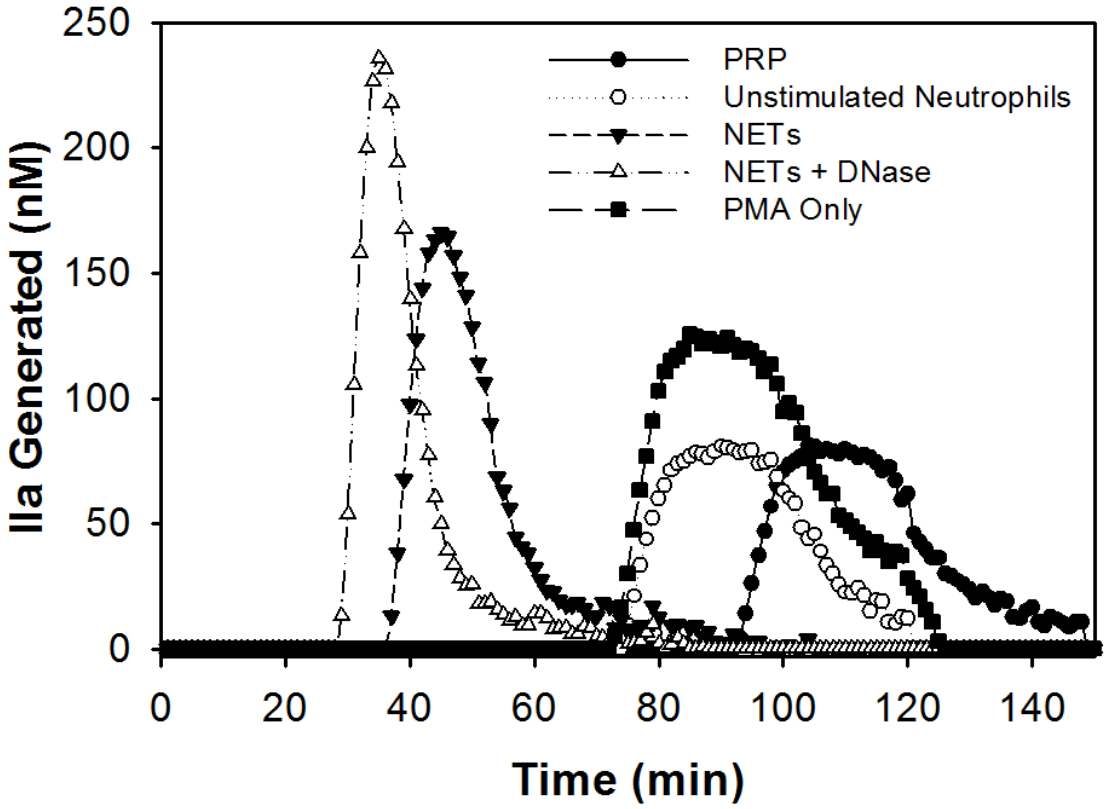
	Lag time (min)	Peak IIa (nM)	Peak time (min)	AUC
PRP	81 ± 3	109 ± 11	101 ± 4	2648 ± 378
Neutrophils	73 ± 1	85 ± 3	94 ± 2	2695 ± 243
NETs	37 ± 2 *	203 ± 13 *	46 ± 2	3608 ± 324 *
DNase	32 ± 2 *	234 ± 10 *	40 ± 2 *	3631 ± 329 *

CFDNA-mediated FXII activation and subsequent thrombin generation but is unable to inhibit platelet-mediated (polyphosphate-dependent) FXII activation (Semeraro et al. 2011). The addition of PMA to PRP caused a modest increase in thrombin generation (consistent with the known platelet-activating effects of PMA)(Elzagallaai et al. 2000), whereas the addition of PMA-activated neutrophils significantly exacerbated this effect. Compared to PMA-treated PRP, the addition of PMA-activated neutrophils to PRP resulted in a 50% decrease in lag time, accompanied by a significant increase in peak and total thrombin (**Fig. 3.3; Table 3.2**). The enhanced procoagulant effect was attenuated with bovine alkaline phosphatase, platelets. Thrombin generation in PRP was further enhanced with DNase addition, but not with RNase addition. These findings suggest that dismantling of the DNA network of NETs with DNase releases more procoagulant material (presumably histones) (**Table 3.2**). Addition of TLR2- and/or TLR4-directed inhibitory antibodies attenuated this enhancement (**Fig. 3.4**), whereas a control IgG had no effect. Taken together, these findings suggest that digestion of the DNA network of NETs with DNase exposes the platelet-activating functions of histones.

3.4.4 DNA-histone complexes in plasma from patients with severe sepsis enhance thrombin generation.

To determine the physiological relevance of our in vitro studies, we measured plasma levels of DNA-histone complexes in septic patients.

Figure 3.3. **Effect of intact NETs on platelet-mediated thrombin generation**. Thrombin generation induced by NETting neutrophils in CTI-inhibited PRP with or without DNase I (20ug/mL) treatment to degrade NET structures. Thrombograms shown are representative of three independent experiments. A summary of coagulation parameters for NET release in normal plasma is described in Table 2. Thrombograms shown are representative of 4 independent experiments.


250
200
150
100
50
0
IIa Generated (nM)
0
20
40
60
80
100
120
140
Time (min)
PRP
Unstimulated Neutrophils
NETs
NETs + DNase
PMA Only

Compared with plasma from healthy controls, plasma from patients with severe sepsis contained increased levels of DNA-histone complexes (**Fig. 3.5**), suggesting that CFDNA circulates in complex with histones. Next, we investigated if there is a correlation between plasma CFDNA levels and plasma thrombin generation. Plasma samples from severe sepsis patients were divided into those that contained low, intermediate or high levels of CFDNA as arbitrarily defined as CFDNA levels less than 5μg mL^{-1}, 5.0-14.9 μg mL^{-1}, and over 15μg mL^{-1}, respectively. Thrombin generation in these samples was then determined in the absence or presence of DNase. In the absence of DNase, there was a direct correlation between CFDNA levels and total thrombin as determined by AUC (r = 0.6; **Fig. 3.6F**) and an inverse correlation between CFDNA levels and lag times (r = 0.56; **Fig. 3.6G**). Likewise, higher CFDNA levels were associated with shorter lag times (**Fig. 3.6B**) and times to peak (**Fig. 3.6C**), higher peak thrombin values (**Fig. 3.6D**), as well as greater AUC (**Fig. 3.6E**) relative to control. Incubation with DNase (confirmed by gel electrophoresis; data not shown) attenuated thrombin generation in septic plasma and DNase addition to control plasma reduced thrombin generation to undetectable levels (**Fig. 3.6**). The addition of protamine sulfate, which is a small cationic protein that binds and precipitates DNA, also reduced thrombin generation to undetectable levels (**Figure 6A**). In addition, although CTI inclusion resulted in an abrogation of thrombin generation, no effect was seen when tissue factor–inhibitory antibody

Figure 3.4. **NET-induced platelet activation is partially mediated through TLR2 and TLR4**. Thrombin generation induced by NETting neutrophils in CTI-inhibited PRP with inhibitory mAbs against TLR2, TLR4, control IgG, or bovine phosphatase (Psp). Thrombograms shown are representative of three independent experiments. A summary of coagulation parameters for NET release in CTI-supplemented PRP is described in Table 3. Thrombograms shown are representative of 4 independent experiments. * indicates $p < 0.05$ relative to NETting neutrophil only conditions.

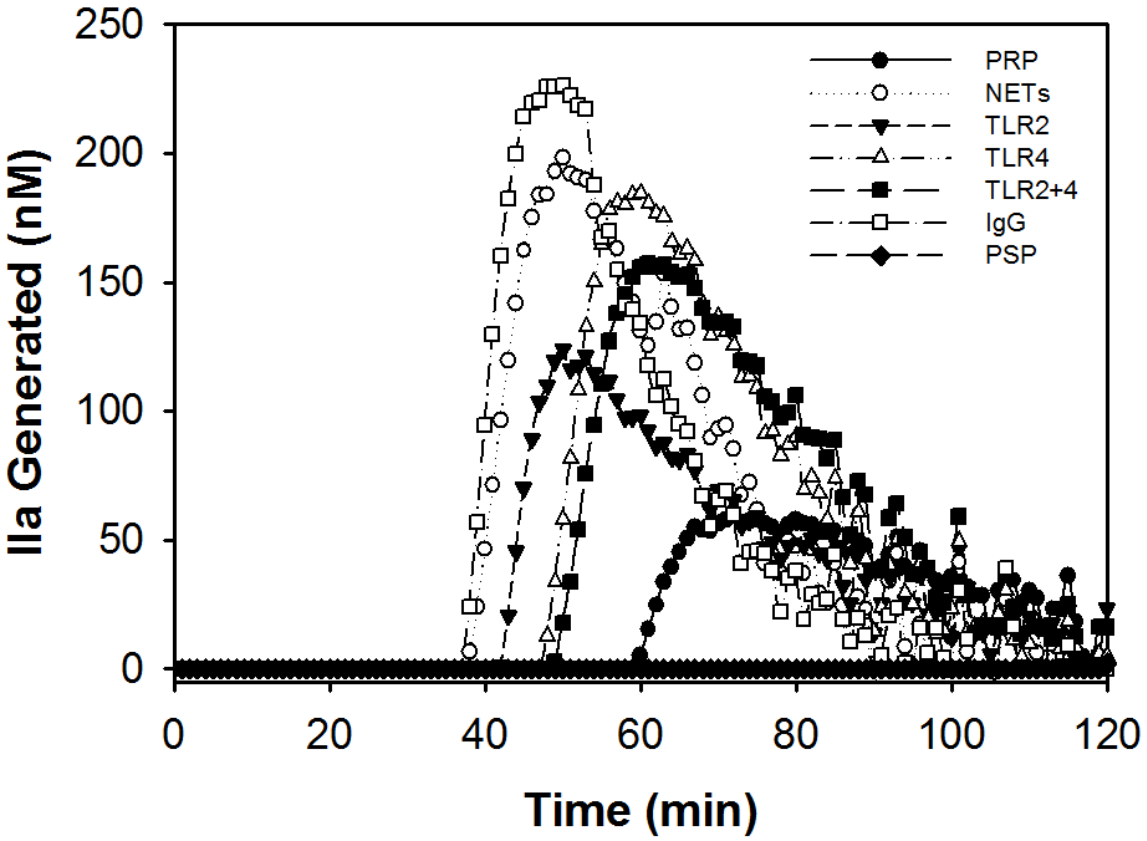
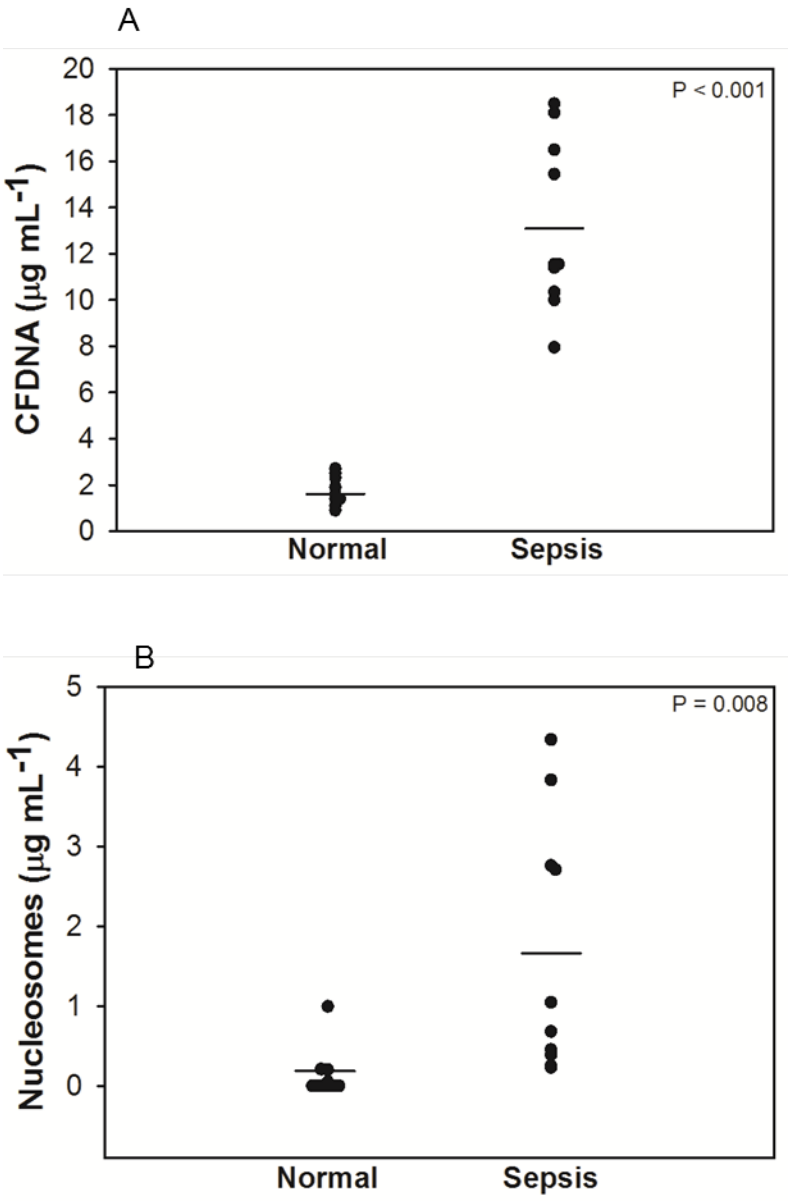

250
200
150
100
50
0
IIa Generated (nM)
0
20
40
60
80
100
120
Time (min)
PRP
NETs
TLR2
TLR4
TLR2+4
IgG
PSP

Table 3.3. **The effect of TLR inhibitors on NET-induced thrombin generation**. Summary of coagulation parameters for NET release in CTI-supplemented PRP. Results reflect the mean ± SE of at least 3 determinations. Asterisks denote $p < 0.05$ compared with stimulated neutrophils.

	Lag time (min)	Peak IIa (nM)	Peak time (min)	AUC
PRP	56 ± 2	63 ± 1	69 ± 2	2730 ± 224
NETs	36 ± 1	192 ± 5	46 ± 3	4939 ± 451
TLR2	41 ± 1	133 ± 6 *	48 ± 1	3219 ± 594
TLR4	46 ± 2 *	174 ± 8	54 ± 4	3831 ± 1076
TLR2&4	51 ± 2 *	140 ± 14 *	59 ± 1 *	3644 ± 1117
IgG	35 ± 2	202 ± 18	44 ± 4	5226 ± 278
AP	89 ± 2 *	3 ± 1 *	111 ± 6 *	22 ± 14 *

Figure 3.5: **Increased levels of nucleosomes correspond with increases in CFDNA in sepsis**. DNA was isolated from the plasma of healthy volunteers and septic patients (A). Nucleosome analysis was performed using the Cell Death Detection ELISA PLUS from Roche Diagnostics (B); (n = 10 for all conditions).


A
20
18
16
14
12
10
8
6
4
2
0
CFDNA (μg mL-1)
P < 0.001
Normal
Sepsis
B
5
4
3
2
1
0
Nucleosomes (μg mL-1)
P = 0.008
Normal
Sepsis

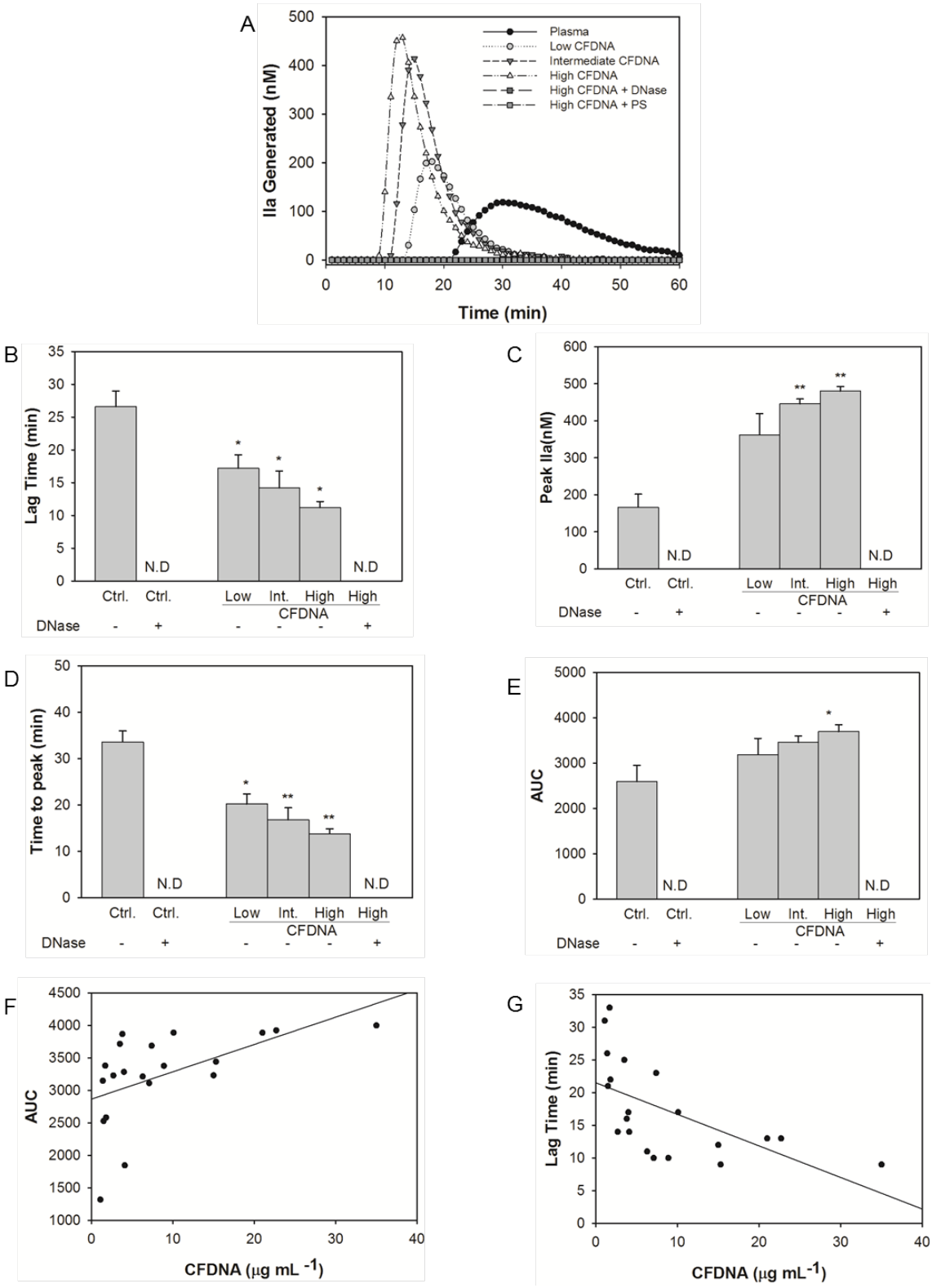
HTF-1 was added to septic plasmas, suggesting negligible contributions by tissue factor to this system (**Table 3**). Taken together, these data suggest that elevations in plasma CFDNA levels result in a hypercoagulable state.

3.5 Discussion

Cell-free DNA (CFDNA) is acellular deoxyribose nucleic acid fragments that circulate within peripheral blood. CFDNA circulates at low levels in healthy individuals, with elevated levels observed in an array of clinical conditions including trauma (Lo et al. 2000), cancer (Swystun et al. 2011), stroke (Rainer et al. 2003), and myocardial infarction (Chang et al. 2003). In a previous study, we demonstrated that CFDNA appears to have high discriminative power to predict ICU mortality in patients with severe sepsis (Dwivedi et al. 2012). Patients with higher plasma concentrations of CFDNA are more likely to face severe complications, such as organ dysfunction/failure, and death. As a prognostic indicator of mortality in septic ICU patients, CFDNA alone appears to possess the greatest predictive power, even when combined with existing clinical scoring systems such as MODS and APACHEII scores (Dwivedi et al. 2012).

In this study, we identified neutrophils stimulated with LPS or LTA+PG as a likely source of CFDNA in whole blood. We demonstrated that intact NETs promote thrombin generation in PPP and that (a) thrombin generation is triggered via the intrinsic pathway, and (b) thrombin generation in PPP is attenuated with DNase, but not RNase. Conversely, thrombin generation in PRP

Figure 3.6. **Effects of elevated levels of CFDNA in septic plasmas on thrombin generation.** Plasmas obtained from patients with severe sepsis was recalcified to initiate coagulation and thrombin generation was measured as described in Materials and methods (A). Patients were categorized based on CFDNA concentrations: low CFDNA levels (o) <5ug/mL; intermediate CFDNA levels (▼) 5.1-14.9ug/mL, and; high CFDNA levels (△) >15ug/mL. DNase (+/-) indicates the presence or absence of a 4h DNase pre-treatment prior to initiating thrombin generation. Lag time (B), time to peak (C), peak thrombin (D), and AUC (E) analysis was performed with Technothrombin TGA software. Correlation curves for AUC and CFDNA levels (F) as well as lag time and CFDNA levels (G). * ($P < 0.05$) and ** ($P < 0.01$) indicates significance relative to plasma only. ND = no thrombin generation detected. Thrombograms shown are representative of 5 independent experiments (n = 5 for all subgroups).


A
IIa Generated (nM)
Time (min)
Plasma
Low CFDNA
Intermediate CFDNA
High CFDNA
High CFDNA + DNase
High CFDNA + PS
B
Lag Time (min)
N.D
Ctrl. Ctrl. Low Int. High High
CFDNA
DNase - + - - - +
C
Peak IIa(nM)
D
Time to peak (min)
E
AUC
F
AUC
CFDNA (μg mL -1)
G
Lag Time (min)
CFDNA (μg mL-1)

was further enhanced with DNase addition, an effect that was abolished with anti-TLR antibodies, suggestive of histone-induced platelet activation. Finally, we showed a direct correlation between CFDNA levels and indices of thrombin generation in plasma from sepsis patients.

For many years, the hemostatic abnormalities in sepsis have been described as an initial hypercoagulable phase driven by aberrant expression of tissue factor, downregulation of endogenous anticoagulant pathways, and impairment of fibrinolysis due to elevations in plasminogen activator inhibitor-1 (PAI-1) (Wheeler and Bernard 1999a; Hotchkiss and Karl 2003; Ulloa et al. 2009). Currently, the thrombin generation assay is one of the most extensively used global hemostatic assays used in hemostasis research (Hemker et al. 2006). However, several groups have reported that patients with sepsis present no signs of systemic hypercoagulability when evaluated with the thrombin generation assay, even in the early stages of sepsis (Picoli-Quaino et al. 2013; Petros et al. 2012; Collins et al. 2006; Massion et al. 2012; Seo et al. 2009). It should be noted that thrombin generation in previous studies was analyzed in PPP triggered with relipidated tissue factor (ie. via the extrinsic pathway) rather than with $CaCl_2$ as was done in the current study. Our study is the first to demonstrate elevations in indices of thrombin generation in plasma samples from septic patients, and implicates an important role of the intrinsic pathway of coagulation in the pathogenesis of sepsis.

Given that CFDNA is a potent activator of coagulation, lowering levels of CFDNA may be beneficial to the host in disease states. In humans and in mouse models, there is precedence for the therapeutic efficacy of DNase. For example, in patients with cystic fibrosis, a condition often associated with *P. aeruginosa* infection of the lung epithelium, inhalation of recombinant human DNase I reduces the viscosity of purulent sputum and inhibits bacterial biofilm formation (Hodson and Shah 1995). In a mouse model of systemic lupus erythmatosus (SLE), an autoimmune disease characterized by high circulating DNA levels, intraperitoneal injection of recombinant mouse DNase interferes with the disease process (Macanovic et al. 1996). In the current study, we observed that addition of DNase to NETs actually results in increases in thrombin generation in PRP, suggesting that removal of the CFDNA component of NETs may be detrimental to the host. Consistent with this finding, Meng et al. showed that digestion of NETs by DNase in a mouse model of sepsis results in advanced sepsis progression accompanied by an increase in mortality (Meng et al. 2012). Thus, it is possible that digestion of NETs with DNase exposes histones that are potent activators of platelets and are cytotoxic to vascular endothelial cells. In addition, dismantling of NETs may also impair their bacteriocidal capabilities (Brinkmann et al. 2004).

Since histones are presumably exposed when the CFDNA scaffold is degraded by DNase, the absence of thrombin generation in PPP incubated with

PMA/DNase-treated neutrophils suggests that histones themselves do not trigger the intrinsic pathway of coagulation. It also suggests that neutrophil granular enzymes do not trigger the intrinsic pathway. However, it is possible that neutrophil enzymes promote coagulation by inactivating endogenous anticoagulants. Neutrophil elastase (NE) has been shown to degrade antithrombin, and both elastase and cathespin G (both released during neutrophil degranulation) proteolyze tissue factor pathway inhibitor (TFPI) (Higuchi et al. 2003; Massberg et al. 2010).

Semeraro et al. have recently demonstrated that purified histones activate platelets through TLR2 and TLR4, inducing the secretion of inorganic polymer polyp (Semeraro et al. 2011). When thrombin generation was performed in the presence of NETs in CTI-treated PRP (which inhibits CFDNA-mediated but not polyphosphate-mediated contact activation) there was a robust reduction in lag time coupled with an increase in peak thrombin, suggesting that, similar to purified histone proteins, intact NETs are able to activate platelets. This effect was platelet-dependent as no thrombin generation was detected when CTI-inhibited PPP was used. However, platelet activation in the presence of NETs was not completely abrogated with TLR2 and TLR4-blocking antibodies, suggesting that alternative mechanisms may also regulate platelet activation in the presence of NETs. Histones have been shown to increase membrane

permeability of cells, which contribute to their cytotoxic effects (Kleine et al. 1995; Xu et al. 2009).

In addition to histones, platelet polyP may be another therapeutic target in sepsis. Recent studies have suggested a therapeutic use for alkaline phosphatase (AP) in the treatment of sepsis-associated organ dysfunction (Peters et al. 2012). Two phase II studies demonstrated that parenteral administration of the dephosphorylating enzyme AP to ICU patients with sepsis and associated acute kidney injury improved kidney function and reduced markers of inflammation and kidney injury (Heemskerk et al. 2009; Pickkers et al. 2012). The therapeutic efficacy of AP has been attributed to AP-mediated dephosphorylation/detoxification of LPS, and dephosphorylation of ATP, a proinflammatory energy molecule released by inflamed renal tissue (Peters et al. 2012). In the current study, we have shown that the addition of AP abolished thrombin generation in PRP, suggesting that AP may also exert beneficial effects by impairing platelet polyP-dependent activation of coagulation.

In summary, these studies are the first to examine the procoagulant activities of CFDNA and histones in the context of intact NETs. Our studies also implicate a role for CFDNA-mediated activation of the intrinsic pathway of coagulation in the pathogenesis of sepsis. Our findings support the concept that NET components may be important therapeutic targets for the treatment of sepsis.

4.0 Cell-free DNA modulates clot structure and impairs fibrinolysis in sepsis

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Foreword: In Chapter 4, we examine the interplay between CFDNA and sepsis pathophysiology by (i) investigating the relationship between CFDNA levels and fibrinolytic activity in sepsis, and (ii) determining the mechanisms by which CFDNA modulates fibrinolysis. We demonstrate that elevated levels of CFDNA correlate with impaired fibrinolytic activity in the plasma of severe sepsis patients, and that this effect can be reversed upon the addition of DNase. The changes in fibrinolytic activity in septic plasmas correlated with changes in clot morphology, a phenomenon that appears to be mediated by increases in thrombin generated by CFDNA itself. In a purified system, CFDNA delays fibrinolysis and does not impact tPA-mediated plasmin generation. Our studies also show that CFDNA is able to bind both plasmin and fibrin to form a non-productive ternary complex that inhibits plasmin-mediated fibrinolysis.

Running title: DNA impairs fibrinolysis in sepsis

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References for this manuscript have been incorporated into the Bibliography (Chapter 9) at the end of this thesis.

4.1 Abstract

Objectives: Sepsis is characterized by systemic activation of inflammation and coagulation in response to infection. In sepsis, activated neutrophils extrude neutrophil extracellular traps comprised of cell-free DNA (CFDNA) that not only trap pathogens, but also provide a stimulus for clot formation. Although the effect of CFDNA on coagulation has been extensively studied, much less is known about the impact of CFDNA on fibrinolysis. To address this, we (a) investigated the relationship between CFDNA levels and fibrinolytic activity in sepsis; and (b) determined the mechanisms by which CFDNA modulates fibrinolysis.

Approach and Results: Plasma was collected from healthy and septic individuals and CFDNA was quantified. Clot lysis assays were performed in plasma and purified systems and lysis times were determined by monitoring absorbance. Clot morphology was assessed using scanning electron microscopy. Clots formed in plasma from septic patients containing >5μg mL^{-1} CFDNA were dense in structure and resistant to fibrinolysis, a phenomenon overcome by DNase addition. These effects were recapitulated in control plasma supplemented with CFDNA. In a purified system, CFDNA delayed fibrinolysis, but did not alter tPA-induced plasmin generation. Using surface plasmon resonance, CFDNA bound plasmin with a K_d value of 4.2 ± 0.3 μM and increasing concentrations of CFDNA impaired plasmin-mediated degradation of

fibrin clots via the formation of a non-productive ternary complex between plasmin, CFDNA, and fibrin.

Conclusions: Our studies suggest that increased levels of CFDNA in sepsis impair fibrinolysis by inhibiting plasmin-mediated fibrin degradation, thereby identifying CFDNA as a potential therapeutic target for sepsis treatment.

4.2 Introduction

Sepsis is initiated by the release of microorganisms and/or microbial toxins into the circulation, though infection itself is rarely the cause of death in these patients (Rainer et al. 2003; Wheeler and Bernard 1999a; Hotchkiss and Karl 2003). Rather, mortality in septic patients is attributed to irreversible organ failure from prolonged, uncontrolled activation of inflammatory and coagulation pathways within the microcirculation. Despite many recent advances in management and treatment, sepsis remains the leading cause of morbidity and mortality in non-coronary intensive care units (ICUs) in North America (Zeerleder et al. 2003; Angus et al. 2001; Margraf et al. 2008; Dwivedi et al. 2012). Severe sepsis, defined as sepsis associated with at least one dysfunctional organ, affects approximately 750,000 individuals in the United States annually (Levi et al. 2003; Angus et al. 2001; Gando 2010). Sepsis-induced mortality remains high, ranging from 18-30%, and is further increased if disseminated intravascular coagulation (DIC) occurs (Levi et al. 2003; Angus et al. 2001; Semeraro et al. 2012; Kaukonen et al. 2014).

Clinical management of patients with sepsis is challenging and largely limited to supportive therapies, which is in part related to a limited understanding of the underlying pathophysiology (Ulloa et al. 2009; The Surviving Sepsis Campaign Guidelines Committee including The Pediatric Subgroup* et al. 2013). Recently, cell-free DNA (CFDNA) has emerged as an important link between innate immunity, coagulation, and inflammation (Yipp and Kubes 2013; Brinkmann et al. 2004; Fuchs et al. 2012). Furthermore, we have previously demonstrated that plasma levels of CFDNA have high discriminative power to predict ICU mortality in patients with severe sepsis (Dwivedi et al. 2012). Patients with higher plasma concentrations of CFDNA are more likely to face severe complications such as organ dysfunction/failure, and death. This evidence suggests that CFDNA may not simply be an innocuous biomarker of disease severity, but may itself exert pathological effects in sepsis.

CFDNA is released through various cellular processes including apoptosis, necrosis, or by neutrophils as a component of neutrophil extracellular traps (NETs) (Stroun et al. 2001; Atamaniuk et al. 2012; Jiang 2003; Margraf et al. 2008; Fuchs et al. 2010; Choi et al. 2004). NETs are comprised of extracellular DNA, histones, and neutrophil granular proteins. CFDNA, either in the presence or absence of histones, has been shown to modulate several procoagulant pathways. CFDNA circulates at low levels in health individuals (0.02 – 1.7μg mL^{-1}) (Arnalich et al. 2013; Diaz et al. 2013; Dwivedi et al. 2012),

but elevated circulating CFDNA levels (0.1 – 5μg mL^{-1}) (Saukkonen et al. 2008; Dwivedi et al. 2012; Moreira et al. 2010) have been detected in a variety of disease states, including sepsis. Elevated levels of CFDNA in septic patients increase thrombin generation by activating the intrinsic pathway of blood coagulation (Gould et al. 2014), while DNA-histone complexes trigger platelet activation and aggregation by signaling through Toll-like receptors (TLR)-2 and TLR-4 (Gould et al. 2014; Xu et al. 2009; Xu et al. 2011). In addition, recent evidence suggests that fibrin, along with von Willebrand factor (vWF) and chromatin, form a co-localized network within the thrombus that provides a scaffold for localized coagulation activation coupled with platelet and red blood cell adhesion, thereby promoting thrombus formation (Fuchs et al. 2012; Brill et al. 2012; Fuchs et al. 2010).

While the contributions of CFDNA to coagulation activation and thrombus formation have been well characterized, studies on the influence of CFDNA on the fibrinolytic system are limited. NETs have previously been shown to intercalate with fibrin to form a structural network that is resistant to lysis by tissue plasminogen activator (tPA) or degradation by DNase (Fuchs et al. 2010). Conversely, CFDNA has been shown to facilitate the recruitment of pro-fibrinolytic enzymes such as tPA, urokinase plasminogen activator (uPA), plasminogen, and plasmin, as well as their endogenous inhibitors, plasminogen activator inhibitor-1 (PAI-1) and alpha 2-antiplasmin (α_2AP)(Komissarov et al.

2011). Thus, while CFDNA may augment fibrinolytic activity by enhancing fibrin-independent plasminogen activation, it may also suppress fibrinolysis by increasing the susceptibility of fibrinolytic enzymes to inhibition (Komissarov et al. 2011).

Levels of CFDNA are elevated in patients with severe sepsis. Consequently, samples from septic patients provide an opportunity to examine the influence of CFDNA on fibrinolysis in the plasma milieu. Therefore, in this study, we (a) measured fibrinolysis in plasma samples from septic patients and examined the influence of endogenous CFDNA on this process; (b) investigated the impact of CFDNA on the structure of plasma clots, and (c) defined the mechanisms by which CFDNA modulates fibrinolytic activity.

4.3 Materials and Methods

4.3.1 Materials

Human alpha-thrombin (IIa), unfractionated, FXIII-free fibrinogen, and Glu-Plasminogen were from Enzyme Research Laboratories (South Bend, IN, USA). Plasmin and fluorescein-labeled FPR-chloromethylketone (PPACK) were from Haematologic Technologies Inc. (Vermont, USA). Recombinant human DNase I (Pulmozyme® dornase alpha) and alteplase (recombinant single-chain tPA) were from Genentech (San Francisco, CA, USA). RNase was from Promega (Madison, WI, USA). The PAI-1 inhibitory antibody MA-55F4C12 was purchased from Hycult Biotech (Plymouth, PA, USA). Sequencing grade trypsin was

purchased from Roche Diagnostics GmbH (Mannheim, Germany). Heparin was purchased from Leo Pharma (Thornhill, ON). H-D-Val-Leu-Lys-p-nitroaniline-dihydrochloride, the plasmin-directed chromogenic substrate S-2251 was from Chromogenix (Bedford, MA, USA). Batroxobin and streptavadin were purchased from Sigma Aldrich (St. Louis, MO, USA). PHOTOPROBE kit (long arm) biotin for nucleic acid labeling was purchased from Vector Laboratories (Burlingame, CA, USA). HOOK-sulfo-NHS-LC-biotin was purchased from G-Biosciences (St. Louis, MO, USA). Plasma levels of D-dimer, PAI-1, and fibrinogen were quantified by immunoassay using kits from Ray Biotech, Inc. (Norcross, GA, USA), Abcam (Cambridge, MA, USA), and Affinity Biologicals (Ancaster, ON), respectively.

4.3.2 Human Sample Collection

Frozen plasma samples were obtained from an available biobank that contains samples from 400 patients with severe sepsis (DYNAMICS Study, ClinicalTrials.gov Identifier: NCT01355042). The patients were recruited between September 2010 and January 2013 from tertiary care ICUs from 9 centers across Canada. Patients with severe sepsis were identified using the inclusion and exclusion criteria previously described by (Dwivedi et al. 2012). The study was approved by the Research Ethics Boards of all participating centers.

Patient blood samples, which were collected within 24 hours of meeting the inclusion criteria for severe sepsis, were processed within 2 hours. Briefly,

9mL of arterial blood was withdrawn from an indwelling catheter and transferred into 15mL polypropylene tube containing 0.5mL of 0.105M buffered trisodium citrate (pH 5.4). After centrifugation at 1500 x g for 10 min at 20°C, platelet poor plasma was harvested and stored in 200µL aliquots at -80°C until used.

Control plasma samples were obtained via venipuncture from 10 healthy adult volunteers who were not receiving any medication at the time of blood collection. There was no attempt to match controls with cases. The blood was processed as described above, and plasma was pooled and stored in aliquots at -80°C until used.

4.3.3 DNA isolation and quantification

To isolate DNA from plasma or buffy coat, samples were subjected to centrifugation at 1500 x g for 10 min to sediment cellular debris and DNA in the supernatant was then isolated using the QIAMP DNA blood mini kit (QIAGEN, Mississauga, ON) according to the manufacturer's instructions. CFDNA was isolated from 200µL plasma or supernatant and eluted into 200µL elution buffer (10mM Tris-Cl, 0.5mM EDTA, pH 9.0). Concentrations of isolated DNA were determined by measuring measuring absorbance at 260 nm using a BioPhotometer Plus spectrophotometer (Eppendorf, Mississauga, ON) and purity was confirmed by calculating the ratio of absorbance determined at 260 and 280 nm. Integrity of isolated DNA was confirmed by gel electrophoresis on

2% agarose gels. To generate DNA fragments, genomic DNA was subjected to sonication using an M220 Focused-ultrasonicator™ (Covaris, Woburn, MA) according to instrument instructions.

4.3.4 Plasma Clot Lysis Assays

Clot formation and lysis times were determined in plasma from septic patients or healthy controls in the absence or presence of 20μg mL^{-1} DNase/RNase or 20nM of inhibitory PAI-1 antibody MA-55F, which were pre-incubated at 37°C for 4 or 1 h, respectively. Where indicated, 'ctrl' refers to control plasma obtained from pooling platelet-poor plasma from 10 healthy volunteers. After adding 50μL of plasma to wells of a flat-bottomed 96-well plate maintained at 37°C, 50μL of a solution containing 1nM tPA and 30mM $CaCl_2$ in 20mM Tris, pH 7.0 was added. Absorbance was monitored at 405nm for up to 5 h in a SpectraMax M5e plate reader (Molecular Devices, Sunnyvale CA, USA) and clot formation and lysis times were determined as the time to half maximal increase and decrease in absorbance, respectively, as calculated using the instrument software. Where indicated, complete clot lysis was defined as a given sample reaching the half maximal decrease in absorbance during the 5-hour period of observation.

4.3.5 Clot Lysis Assays in Buffer Systems

The effect of CFDNA on clot formation and lysis was also examined in a purified system. Aliquots of 10nM thrombin and 1nM tPA were placed separately in wells of a 96-well plate prior to addition of a solution containing 5μM fibrinogen, 1μM Glu-plasminogen, and 2mM $CaCl_2$ in 20mM Tris-pH 7.0 in the absence or presence of CFDNA up to 50μg mL^{-1}. In some experiments, 1nM plasmin or 5μM trypsin was added in place of tPA and Glu-plasminogen. In all cases, absorbance was monitored at 405nm for up to 2 h and the clotting and lysis times were determined as described above.

4.3.6 Evaluation of Plasma Clot Structure using Scanning Electron Microscopy

After adding 15mM $CaCl_2$ to 50μL aliquots of plasma from either healthy or septic individuals, 30μL aliquots were removed, deposited on etched glass slides, and incubated for 2 hours at 37°C. Clots were then gently washed 5 times with PBS and submerged and fixed overnight at 4°C in 2% glutaraldehyde. Clots were imaged digitally in 3 different areas using a Tescan Vega II scanning electron microscope (Tescan, USA, PA) at a magnification of 20 000X. Fibrin fiber diameter was determined using ImageJ software (v.1.6). Individual fibers from 10 random areas were measured per image. Fibrin clot porosity was quantified by calculating the number of black pixels relative to total pixels in a given image using Adobe Photoshop CS5 software. For these determinations, a

'black pixel' was defined as 75% pixel grayscale pixel intensity, with 0% being equal to white and 100% being equal to completely black.

4.3.7 SDS-PAGE Analysis of Fibrin Degradation

Clots were formed and fibrinolysis initiated by incubating 5μM fibrinogen with 5nM thrombin and 1nM plasmin in HEPES-buffered saline (HBS), pH 7.0, containing 0.005% Tween-20 in the absence or presence of DNA up to 50μg mL^{-1}. At various intervals, reactions were stopped and clots were solubilized by the addition 0.1M acetic acid. Aliquots containing 5μg of protein were added to the sample preparation buffer [final concentrations of 1% DodSO4, 0.05M Tris–HCl (pH 8.0), 0.025M EDTA (pH 8.0), 0.05mg mL^{-1} 1 bromophenol blue,10% β-mercaptoethanol and 5% glycerol, 20μL total], and degradation products were resolved by SDS–PAGE using 4–15% gradient gels (Bio-Rad, Mississauga, ON, USA). Gels were stained with Bio-safe Coomassie G-250 stain (Bio-Rad), digitally scanned, and band density was quantified using Image Lab™ Software v.4.1 (Bio-Rad). Band identity was determined by apparent molecular weight (Kim et al. 2007).

4.3.8 Plasminogen Activation Assay

The effect of CFDNA on plasminogen activation by tPA was assessed by incubating 1μM Glu-plasminogen with 400nM S-2251 at 37°C in wells of a 96-well flat-bottom plate in the absence or presence of CFDNA up to 50μg mL^{-1}.

After adding 50nM tPA, S-2251 hydrolysis was monitored at 405nm for 1h. Turbidity was corrected for substrate hydrolysis by subtracting the absorbance measured at 450nm. Corrected absorbance values were then plotted against time-squared and plasminogen activation rates were determined from the linear portions of the plots using the specific activity of plasmin for S-2251, which was determined in a separate experiment to be 1.64 mOD/min/nM. Values in the presence of varying concentrations of CFDNA were then normalized relative to that measured in its absence.

4.3.9 Surface Plasmon Resonance

Binding interactions were studied by surface plasmon resonance (SPR) using a Biacore T200 (GE Healthcare, Piscataway, NJ). FXIII-free fibrinogen in 10mM sodium acetate, pH 5.5, was covalently linked to carboxyl groups on a CM4 sensor chip (GE Healthcare) using an amine coupling kit (GE Healthcare) at a flow rate of 5µL/min until 5000 response units (RU) were attained. Where indicated, immobilized fibrinogen was converted to fibrin by injecting 500nM thrombin in HBS containing 10mM $CaCl_2$ and 0.005% Tween-20 at a flow rate of 5µL/min for 60 min. This step was repeated until no further reduction in RU was detected, indicating complete release of fibrinopeptide A (Fp) A and/or FpB. Genomic DNA (isolated from buffy coat of healthy volunteers) was diluted in HBS-Tw and injected at a flow rate of 20 µL/min for 1 min. Flow cells were then

washed with HBS-Tw to monitor dissociation. Between runs, flow cells were regenerated with HBS containing 1M NaCl, 10mM EDTA and 0.005% Tween-20.

Alternatively, biotinylated genomic DNA was immobilized to streptavidin-coated flow cells. Flow cells were then washed with 0.02M HEPES, 1M NaCl, 2mM $CaCl_2$, pH 8.0 (HBS) containing 0.005% Tween-20 (Tw). Glu-plasminogen or plasmin diluted in HBS-Tw (pH 8.0) were injected at a flow rate of 20 µL/min for 1 min and flow cells were then washed with HBS-Tw to monitor dissociation. Between runs, flow cells were regenerated with HBS containing 1M NaCl, 10mM EDTA and 0.005% Tween-20, pH 8.0. For each condition, RU values at equilibrium were determined by subtracting the RU values obtained in the control flow cell containing unmodified carboxymethyl-dextran (for fibrinogen/fibrin binding studies) or streptavidin (for DNA binding studies). Equilibrium values were then plotted against the starting concentrations of fibrinogen/fibrin/DNA and data were fit to a rectangular hyperbola using SigmaPlot v.11.0 software (San Jose, CA, USA) to determine the affinity (K_d) values.

4.3.10 Determination of plasmin binding to DNA-containing clots

To study the effects of CFDNA on the plasmin-fibrin interaction, a fluorometric displacement assay was used. Plasmin was active site labeled by incubation with a 10-fold molar excess of fluorescein-labeled FPR-

chloromethylketone (f-PPACK) for 2h at 25°C. After dialysis against phosphate buffered saline (PBS) pH 7.4 four times for 2h at 25°C to remove unincorporated f-PPACK, the material was incubated with S-2251 to confirm complete absence of activity.

The affinity of FITC-labeled plasmin for DNA/fibrin was determined by measuring unbound plasmin in supernatants of clots. Briefly, 3μM fibrinogen, 9μM f-PPACK-inhibited plasmin, and increasing concentrations of genomic DNA (0-500nM) were added to 1.5mL Eppendorf tubes, mixed carefully, and incubated at room temperature for 10min. After adding 10nM thrombin to initiate clotting, clots were incubated for 1 h at 25°C. Fibrin clots were then dislodged from the tubes by vortexing, and collapsed by centrifugation at 16 000 x g. The supernatant from each tube was collected and 100μL of each sample was added to wells of a 96-well black Costar plate. The fluorescent intensity of the samples was determined using a SpectraMax M5e plate reader (Molecular Devices, Sunnyvale, CA, USA) with excitation and emission wavelengths of 495nm and 520nm, respectively.

4.3.11 Statistical analyses

Values are expressed as mean ± standard error. Significance of differences was determined by one-way ANOVA and Tukey's pair-wise

comparisons or by t-tests using SigmaPlot software. For all analyses, p values less than 0.05 were considered to be statistically significant.

4.4 Results

4.4.1 Elevated levels of Endogenous CFDNA impairs fibrinolysis in septic patient plasma.

To study the effects of elevated endogenous CFDNA levels on fibrinolysis, plasma samples from 60 patients with severe sepsis and 10 healthy donors were examined. The baseline (day 1) characteristics of the 60 severe sepsis patients are shown in **Table 4.1**. CFDNA was quantified, and based on levels, plasma samples were arbitrarily categorized into 'low' (0-4.9μg mL^{-1}), 'intermediate' (5-9.9μg mL^{-1}), and 'high' (>10μg mL^{-1}) CFDNA subgroups.

Plasma samples were supplemented with 1nM tPA and clotting was initiated by addition of $CaCl_2$. Clots formed in plasma samples from healthy controls (n = 10) and patients with low levels of endogenous CFDNA (n = 20) underwent complete lysis. In contrast, of the clots formed in plasma samples containing intermediate or high levels of CFDNA, only 35% and 10%, respectively, underwent complete lysis during the 5-hour period of observation (**Fig. 4.1A**). The delay in clot lysis correlated with decreased plasma levels of D-dimer, consistent with reduced fibrin degradation (**Table 4.2**). No significant differences were observed between other potential mediators of fibrinolysis,

Table 4.1. Baseline characteristics of 60 patients with severe sepsis.

Characteristic		Value
Age, years Mean ± SEM (min, max)		61.33 ± 2.2 (22, 93)
Gender, % female (no./total)		45 (27/60)
APACHE II score Mean ± SE (min, max).		26.47 ±1.11 (7, 52)
MODS score Mean ± SE (min, max).		7.25 ± 0.47 (1, 17)
Positive cultures, % positive (no./total)		70 (42/60)
Infection type, % (no./total)	Gram-positive	21.4 (9/42)
	Gram-negative	31 (13/42)
	Fungal	16.7 (7/42)
	Viral	28.6 (12/42)
	Mixed	2.4 (1/42)

including thrombin activatable fibrinolysis inhibitor (TAFI) or α2-antiplasmin (**Table 4.2**).

To determine the contribution of CFDNA to the reduced fibrinolysis, plasma samples were pre-incubated with 20μg mL^{-1} DNase I. RNase and the PAI-1 inhibitory antibody MA-55F (which was first confirmed to be effective in inhibiting PAI-1 activity in a plasma environment; data not shown) were used as controls. Neither pre-incubation with RNase nor MA-55F affected clot lysis. In contrast, pre-treatment with DNase partially restored fibrinolytic activity in samples containing intermediate and high levels of CFDNA (**Fig. 4.1A**). In the absence of tPA, DNase alone exerted no fibrinolytic activity (data not shown). Collectively, these results suggest that the presence of elevated levels of CFDNA in septic patient plasmas impairs fibrinolytic activity.

4.4.2 Evidence of altered structure of clots formed in plasmas from septic patients with elevated levels of CFDNA.

Turbidity, as determined by spectrophometric absorbance, is indicative of fibrin clot structure – thick fibers and dense clot morphology results in an increase in final turbidity (Wolberg and Campbell 2008). Although maximum absorbance values in samples with low levels of CFDNA were similar to those in control samples, maximum absorbance values were 3-and 2-fold higher in

Figure 4.1. **CFDNA in septic plasmas impairs fibrinolysis and modulates clot structure**. Plasmas from patients with severe sepsis were classified as having 'low' (0 – 4.9μg mL^{-1}), 'intermediate' (5 – 9.9 μg mL^{-1}), or 'high' (10μg mL^{-1} or above) concentrations of CFDNA. Where indicated, plasmas were treated with 20μg mL^{-1} of DNase or RNase for 4 hours, or 20nM MA-55F4C12 (PAI-1 inhibitory antibody) for 1h at RT. Clotting was initiated by the addition of 15mM $CaCl_2$ supplemented with 1nM of tPA and clot formation and clot lysis was observed for up to 5 hours. Clot lysis times were assessed by observing turbidity at A_{450}. Lysis time was taken as the time required for absorbance to reach the midpoint between clot formation and clot lysis (A). Changes in clot structure in septic patient plasmas as indicated by plasma turbidity were also quantified (B). Changes in clot structure were confirmed by scanning electron microscopy in control plasma (C) and in septic patient plasmas containing low (D), intermediate (E), and high (F) levels of CFDNA.

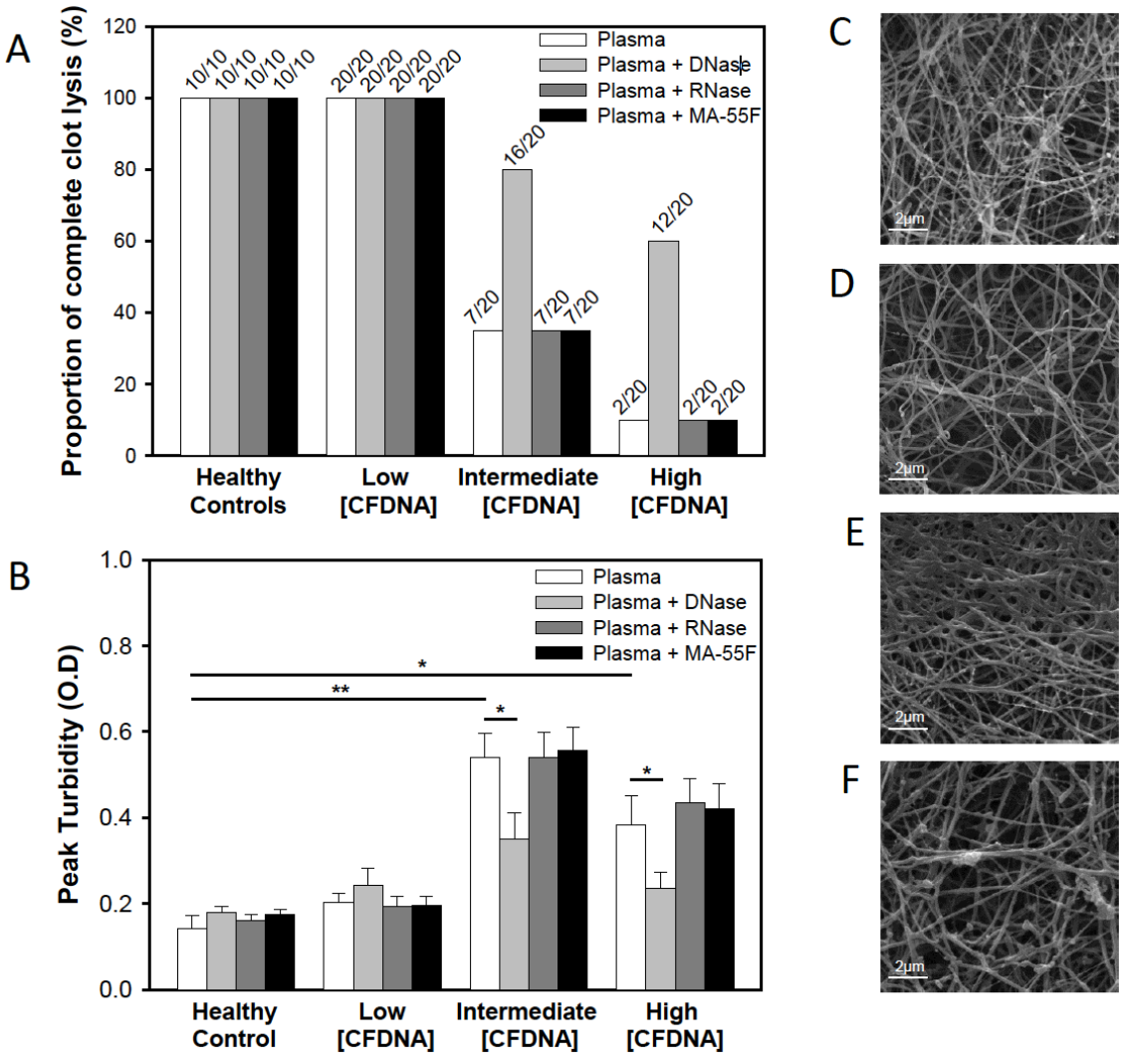

A
Proportion of complete clot lysis (%)
120
100
80
60
40
20
0
10/10
10/10
10/10
10/10
20/20
20/20
20/20
20/20
7/20
16/20
7/20
7/20
2/20
12/20
2/20
2/20
Plasma
Plasma + DNase
Plasma + RNase
Plasma + MA-55F
Healthy Controls
Low [CFDNA]
Intermediate [CFDNA]
High [CFDNA]
B
Peak Turbidity (O.D)
1.0
0.8
0.6
0.4
0.2
0.0
*
**
*
*
Plasma
Plasma + DNase
Plasma + RNase
Plasma + MA-55F
Healthy Control
Low [CFDNA]
Intermediate [CFDNA]
High [CFDNA]
C
D
E
F
2µm
2µm
2µm
2µm

Table 4.2. Levels of CFDNA, D-dimer, fibrinogen, and PAI-1 in healthy control and septic patient plasmas. (n = 10 for healthy controls; n = 20 for septic subgroups). Asterisks (*) denote $p < 0.05$ compared with healthy control plasma.

Cohort	CFDNA (μg mL^{-1})	D-dimer (μg mL^{-1})	Fibrinogen (μg mL^{-1})	PAI-1 (ng mL^{-1})	α2-AP (% of control)	TAFI (ng mL^{-1})
Control	2.45 ± 0.233	61.2 ± 12.6	4874 ± 78	4.68 ± 2.13	100	65 ± 2.6
Low	1.72 ± 0.086	44.9 ± 28.7	4712 ± 136	27.23 ± 3.16 *	84 ± 7.2	54 ± 1.8
Intermediate	8.78 ± 0.37 *	49.9 ± 15.3	4939 ± 415	32.32 ± 12.05 *	97 ± 7.7	54 ± 3.2
High	17.6 ± 2.6 *	20.4 ± 4.7 *	5000 ± 63	76.86 ± 3.24 *	75 ± 6.5	52 ± 3.8

samples with intermediate or high CFDNA levels, respectively ($p < 0.001$; $p = 0.004$) (**Fig. 4.1B**). Addition of DNase but not RNase or MA-55F reduced the peak turbidity of the plasmas containing intermediate or high CFDNA levels (**Fig. 4.1B**). This suggests that the presence of elevated CFDNA levels correspond with altered clot structure comprised of thicker fibers in a densely-packed fibrin network.

To investigate the effect of CFDNA on clot morphology, we used scanning electron microscopy (SEM) to compare the clot structure of clots formed in plasma from septic patients with those generated in control plasma. Whereas clots formed in control plasmas were loosely packed and composed of thin fibrin fibers (**Fig. 4.1C; Table 4.3**), those formed in plasma from septic patients were more tightly packed and composed of thicker individual fibrin fibers (**Fig. 4.1D, E; Table 4.3**). Interestingly, clots formed in plasmas containing the highest levels of CFDNA appeared to be less dense, though the individual fibers remained thicker (**Fig. 4.1F; Table 4.3**).

To identify potential factors that modulate clot formation or fibrinolytic activity, we measured levels of fibrinogen and PAI-1. Although the levels of fibrinogen were similar in plasma from septic patients and controls, levels of PAI-1 were increased in plasma from septic patients containing low or intermediate amounts of CFDNA, and markedly elevated in samples with high levels of CFDNA (**Table 4.2**).

Table 4.3. Parameters of clot morphology in septic patient plasmas observed by SEM. Results reflect the mean ± SE of at least 3 independent determinations. Asterisks (*) denote $p < 0.05$ compared with healthy control plasma.

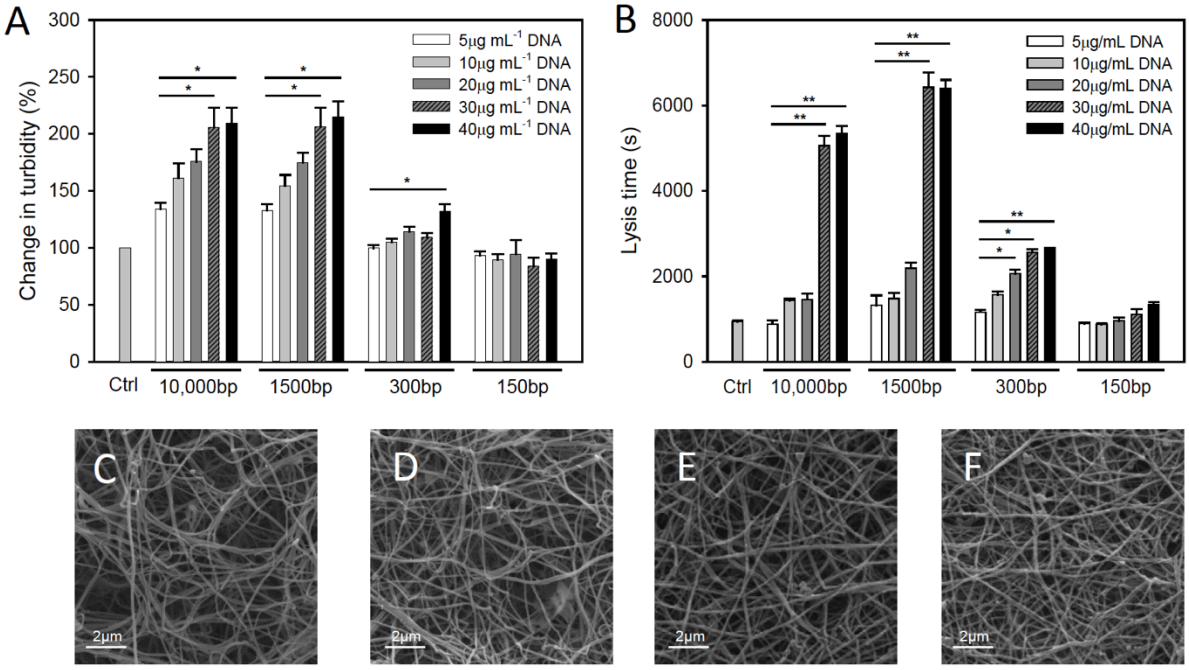
Cohort	Fiber Diameter (μm)	Density (% pores)
Control	0.132 ± 0.004	67.41 ± 1.78
Low	0.190 ± 0.006 *	46.39 ± 2.14 *
Intermediate	0.235 ± 0.005 *	35.04 ± 1.21 *
High	0.277 ± 0.008 *	64.93 ± 1.80

4.4.3 Addition of CFDNA to normal plasma delays fibrinolysis.

In order to determine the effects of DNA on clot turbidity and lysis, we performed clot lysis assays on control plasma (pooled from 10 healthy volunteers) supplemented with increasing concentrations of exogenous genomic DNA (isolated from buffy coats of healthy volunteers). Addition of 30 or 40μg mL^{-1} CFDNA to control plasma increased maximum absorbance by ~2-fold ($p = 0.017$; $p = 0.007$, respectively) (**Fig. 4.2A**), consistent with formation of thicker fibers, and produced a 5-fold prolongation of the clot lysis time ($p < 0.001$) (**Fig. 4.2B**). These results are in agreement with our SEM data, demonstrating that the presence of DNA increases clot turbidity. With CFDNA addition, clot structure transitioned from a loose collection of fibrin fibers to a densely-packed clot structure (**Fig. 4.2C-F**). These results suggest that DNA is responsible for the changes in clot structure and lysis observed in plasma from septic plasmas.

We next sought to investigate the effects of DNA size on fibrinolysis. CFDNA fragments of varying sizes were generated by sonication. The size of CFDNA is known to be dependent on the cellular process by which it was liberated; apoptotic cells release a 'ladder' patter of DNA at ~150bp intervals (Nagata et al. 2003), while necrotic and NETting neutrophils release high molecular weight fragments greater than 10,000bp (Jahr et al. 2001a; Fuchs et al. 2007). Whereas CFDNA fragments comprised of 300, 1500, or 10 000bp significantly increased maximum absorbance and prolonged clot lysis times,

Figure 4.2. **CFDNA impairs fibrinolysis in normal plasma**. Clot lysis assays were performed in normal plasma supplemented with increasing concentrations of CFDNA in the presence of 1nM tPA (A). Changes in clot structure were measured by observing clot turbidity at A_{450} (B). Changes in clot morphology were subsequently confirmed by SEM in normal control plasma (C), and control plasma supplemented with 5μg mL^{-1} (D), 15μg mL^{-1} (E), and 40μg mL^{-1} (F) of exogenous genomic DNA. * indicates $P < 0.05$ and ** indicates $P < 0.001$.


A
Change in turbidity (%)
300
250
200
150
100
50
0
5μg mL-1 DNA
10μg mL-1 DNA
20μg mL-1 DNA
30μg mL-1 DNA
40μg mL-1 DNA
Ctrl
10,000bp
1500bp
300bp
150bp
B
Lysis time (s)
8000
6000
4000
2000
0
5μg/mL DNA
10μg/mL DNA
20μg/mL DNA
30μg/mL DNA
40μg/mL DNA
Ctrl
10,000bp
1500bp
300bp
150bp
C
D
E
F
2μm

CFDNA fragments of 150bp did not (**Fig. 4.2A,B**). This suggests that only DNA fragments larger than 150bp impair clot lysis.

4.4.4 CFDNA alters clot structure by modulating thrombin generation.

Thrombin concentrations are known to impact fibrin assembly and clot morphology (Wolberg 2007). High concentrations of thrombin promote the formation of fibrin clots consisting of thin, densely-packed fibrin strands. Previously, we demonstrated that the elevated levels of CFDNA in plasma from septic patients promoted thrombin generation (Gould et al. 2014). To determine the contribution of DNA-dependent thrombin generation to the observed changes in fibrinolytic activity and clot morphology, thrombin and tPA were added to control plasma samples and samples from septic patients and maximum absorbance values and lysis times were measured. The addition of exogenous thrombin normalized the differences in maximum absorbance values observed between subgroups (**Fig. 4.3A**), but clot lysis remained incomplete in samples with intermediate and high levels of CFDNA (**Fig. 4.3B**). These results were confirmed by clot lysis assays in plasmas where coagulation was triggered by the addition of batroxobin, a thrombin-like snake venom that only releases FPA (data not shown). This evidence suggests that promotion of thrombin generation may contribute to the more tightly-packed clot morphology, but

thrombin levels alone do not appear to be the sole determinant of the impaired fibrinolysis.

4.4.5 CFDNA delays plasmin-mediated lysis of fibrin clots.

To delineate the mechanism by which elevated concentrations of CFDNA impair fibrinolytic activity, studies were performed in a purified system. After clotting 5μM fibrinogen with 5nM thrombin in the absence or presence of increasing concentrations of CFDNA, fibrinolysis was initiated by the addition of 1μM Glu-Plasminogen and 1nM tPA. CFDNA significantly delayed tPA-mediated clot lysis times by up to 2-fold when CFDNA concentrations exceeded 20μg mL^{-1} ($p = 0.002$) (**Fig. 4.4A**). Despite this delay, however, CFDNA appeared to have no impact on tPA-mediated conversion of plasminogen to plasmin (**Fig. 4.4B**).

To explore the possibility that CFDNA impairs the catalytic activity of plasmin directly, experiments were repeated using 1nM plasmin or trypsin in place of tPA/plasminogen. Whereas CFDNA had no effect on lysis times with trypsin (data not shown), at concentrations of 20μg mL^{-1}, CFDNA prolonged plasmin-mediated lysis times by 1.7-fold ($p < 0.001$) (**Fig. 4.4C**). To confirm that CFDNA impaired clot degradation by plasmin, clots were incubated with plasmin in the absence or presence of 40μg mL^{-1} CFDNA and at intervals, reactions were stopped and clots were solubilized by the addition of acetic acid

Figure 4.3. **Effects of thrombin on clot formation and lysis in septic plasmas**. Turbidity was monitored in septic plasma samples where coagulation was initiated by the addition of 15mM $CaCl_2$ and 50nM thrombin (A). Clot lysis times in the same septic plasma samples were assessed by observing turbidity at A_{450} and the proportion of plasma clots undergoing complete lysis were determined (B).

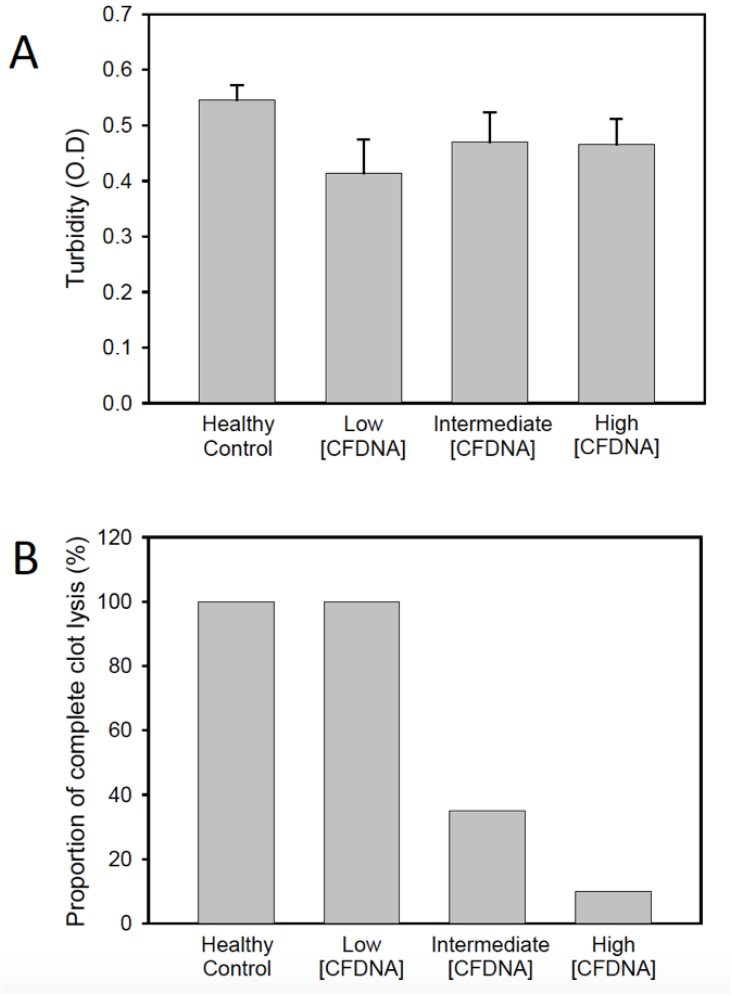
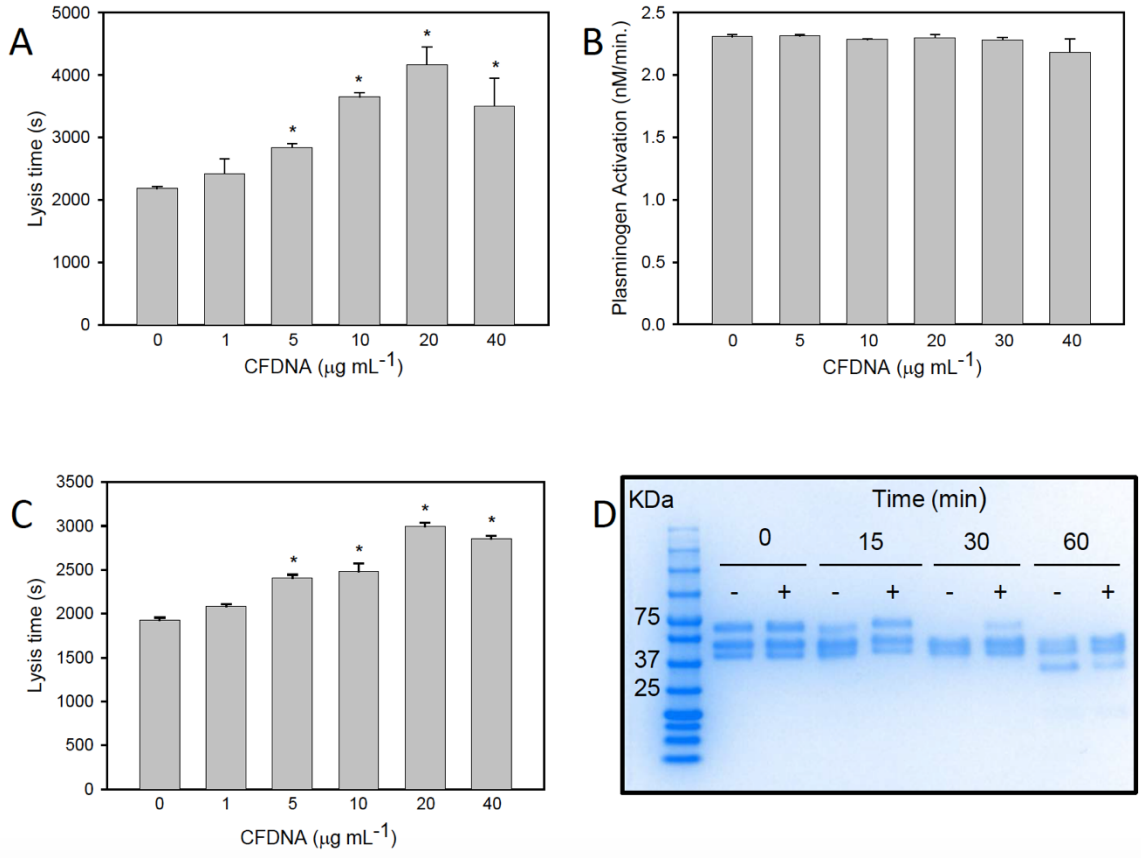

A
Turbidity (O.D)
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0.0
Healthy Control
Low [CFDNA]
Intermediate [CFDNA]
High [CFDNA]
B
Proportion of complete clot lysis (%)
120
100
80
60
40
20
0
Healthy Control
Low [CFDNA]
Intermediate [CFDNA]
High [CFDNA]

Figure 4.4. **Effects of CFDNA on clot formation and lysis in a purified system**. (A) Fibrin clots were formed with 10μM fibrinogen and 5nM thrombin in the presence of 1nM tPA and 1μM Glu-plasminogen. Clot lysis times were assessed by observing turbidity at A_{450}. Lysis time was taken as the time required for absorbance to reach the midpoint between clot formation and clot lysis. (B) Plasminogen activation by 1nM tPA and 1μM Glu-plasminogen in the presence of increasing concentrations of CFDNA as determined by plasmin-specific chromogenic substrate (S-2251) cleavage. (C) Clot lysis times of clots where fibrin degradation is mediated by 6μM plasmin. Fibrin clots were formed with 10μM fibrinogen and 5nM thrombin. (D) Analysis of plasmin-mediated fibrin degradation by SDS-PAGE. Identical clots were formed with or without the addition of 50μg mL^{-1} CFDNA. Clot breakdown was halted at various time points by the addition of acetic acid. * indicates $P < 0.05$.


A
Lysis time (s)
5000
4000
3000
2000
1000
0
0 1 5 10 20 40
CFDNA (μg mL-1)
B
Plasminogen Activation (nM/min.)
2.5
2.0
1.5
1.0
0.5
0.0
0 5 10 20 30 40
CFDNA (μg mL-1)
C
Lysis time (s)
3500
3000
2500
2000
1500
1000
500
0
0 1 5 10 20 40
CFDNA (μg mL-1)
D
KDa
Time (min)
0 15 30 60
- + - + - + - +
75
37
25

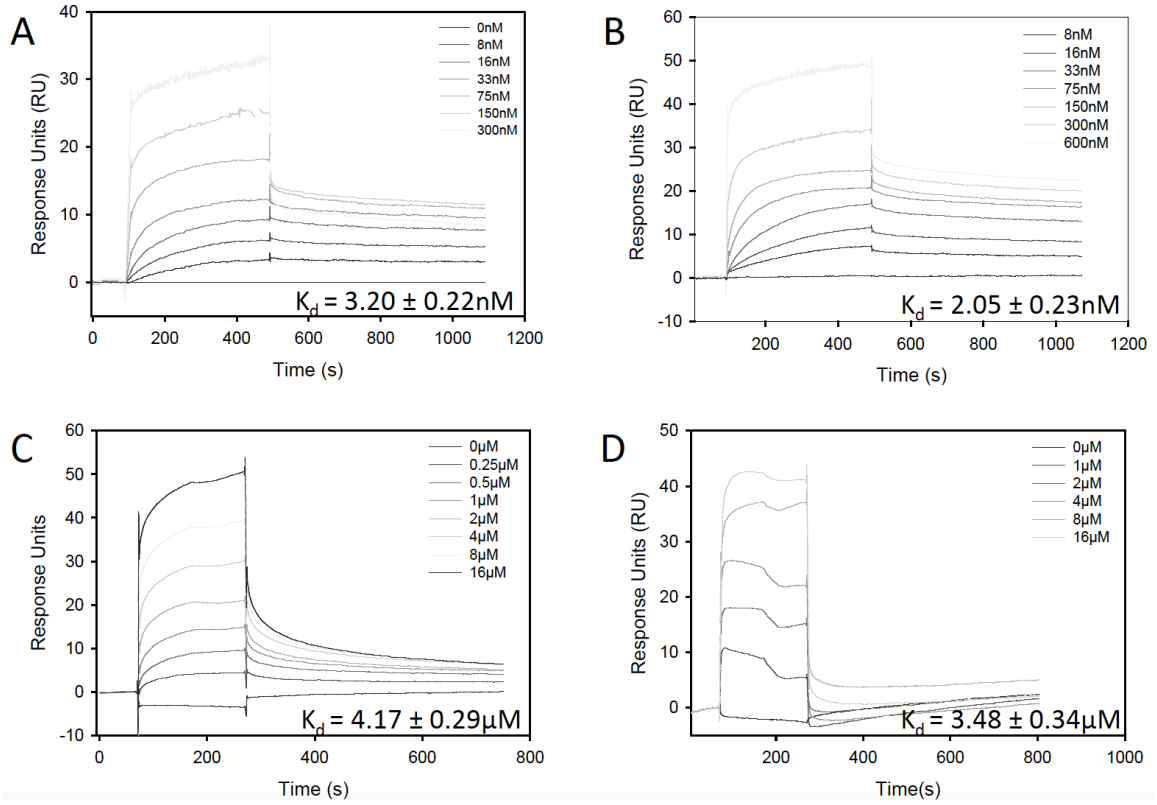
and subjected to SDS-PAGE analysis (**Fig. 4.4D**). Compared with plasmin-mediated fibrin lysis in the absence of CFDNA, the addition of CFDNA reduced fibrin α-chain degradation ~3-fold after 60min of lysis, as determined by densitometry ($p < 0.001$). Together, this evidence suggests that CFDNA does not impact the ability of tPA to generate plasmin, but rather inhibits fibrinolytic activity by attenuating plasmin activity.

4.4.6 Interaction of DNA with fibrinogen, fibrin, plasminogen, and plasmin.

SPR was used to quantify the interaction of DNA with fibrinogen (**Fig. 4.5A**) and fibrin (**Fig. 5B**). As shown in the sensorgrams, genomic DNA exhibits rapid association and slow dissociation with both fibrinogen and fibrin. Kinetic analysis of the on- and off-rates demonstrates that genomic CFDNA binds fibrinogen and fibrin with similar affinities (K_d values of 3.2 ± 0.2nM and 2.1 ± 0.2nM, respectively). In contrast, binding of smaller CFDNA fragments (~150bp in size) to fibrinogen was undetectable (data not shown).

To investigate if genomic CFDNA is able to compete with fibrinogen/fibrin for plasmin binding, the interaction of Glu-plasminogen or plasmin with immobilized biotinylated-DNA was investigated using SPR. Kinetic analyses of the sensorgrams show that Glu-plasminogen binds DNA with a K_d value of 4.2 ± 0.3μM (**Fig. 4.5C**), whereas plasmin binds DNA with a K_d value of 3.5 ± 0.3μM

Figure 4.5. **Binding of fibrinogen, fibrin, plasminogen, and plasmin to DNA as determined by SPR**. (A) Fibrinogen was covalently linked to carboxyl groups on a CM4 sensor chip. An unmodified carboxymethyl-dextran-containing cell served as the reference control. Genomic CFDNA was injected into the flow cells for 400 seconds to assess binding, followed by buffer injection to assess dissociation. (B) Biotinylated fibrinogen was subsequently converted to fibrin after multiple injections of 500nM thrombin. Genomic CFDNA was injected to assess binding to Fn. (C) Biotinylated DNA was adsorbed on flow cells containing streptavadin, and 0-16μM of Glu-plasminogen was passed through the flow cells to assess binding. Following regeneration, injections were repeated using 0-16uM plasmin (D). Data represent the mean ± S.D. of three determinations.


A
Response Units (RU)
Time (s)
0nM
8nM
16nM
33nM
75nM
150nM
300nM
K_d = 3.20 ± 0.22nM
B
Response Units (RU)
Time (s)
8nM
16nM
33nM
75nM
150nM
300nM
600nM
K_d = 2.05 ± 0.23nM
C
Response Units
Time (s)
0μM
0.25μM
0.5μM
1μM
2μM
4μM
8μM
16μM
K_d = 4.17 ± 0.29μM
D
Response Units (RU)
Time(s)
0μM
1μM
2μM
4μM
8μM
16μM
K_d = 3.48 ± 0.34μM

(**Fig. 4.5D**). Therefore, DNA binds fibrin(ogen) and plasmin(ogen), and may interfere with plasmin-mediated degradation of fibrin clots.

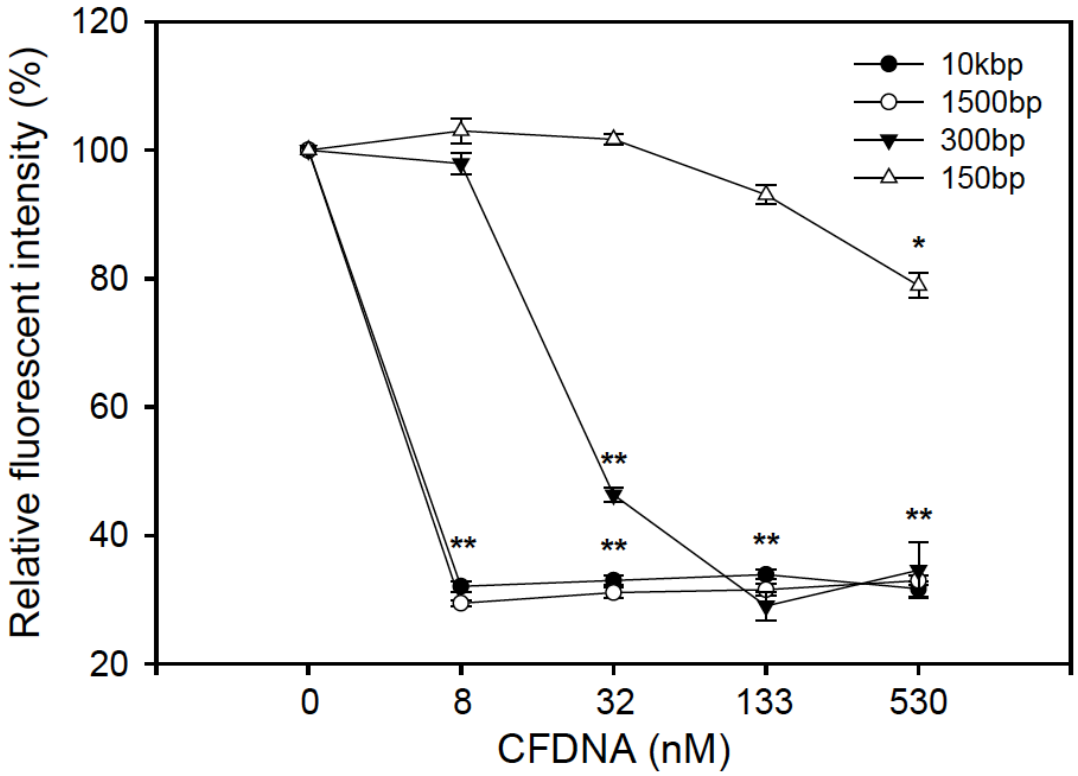
4.4.7 CFDNA inhibits fibrinolysis via formation of DNA/plasmin/fibrin ternary complex.

Though DNA binds both fibrin and plasmin, it was unclear whether CFDNA impairs plasmin-mediated fibrinolysis by competing with plasmin for fibrin binding or if DNA is able to bind both simultaneously. To examine these possibilities, we performed a fluorometric microplate assay to quantify plasmin binding to clots formed in the presence or absence of CFDNA. The addition of ~10kbp CFDNA fragments to fibrin clots resulted in an increase in f-PPACK-plasmin binding, as indicated by a decrease in fluorescent intensity of the supernatant (**Fig. 4.6**). Subsequent increases in DNA concentration did not have any additional effect on fluorescent intensity. However, in the presence of DNA fragments of ~150bp in size, there was little effect on plasmin binding. Collectively, these data suggest that larger CFDNA fragments are able to bind both plasmin and fibrin simultaneously, thereby forming a ternary complex that hinders the enzymatic activity of fibrin.

4.5 Discussion

Levels of CFDNA have been reported to be increased in various coagulopathy-associated disease states, including trauma (Lo et al. 2000),

Figure 4.6. **Effect of CFDNA on the displacement of plasmin from fibrin**. Fluorescently labeled, active site-inhibited plasmin was incubated with fibrinogen and increasing concentrations of genomic DNA of various lengths. F-PPACK-plasmin displacement was determined by quantifying fluorescent intensity of clot supernatant. * $p < 0.05$, ** $p < 0.01$ relative to clots formed with no DNA present.


Relative fluorescent intensity (%)
120
100
80
60
40
20
0
8
32
133
530
CFDNA (nM)
10kbp
1500bp
300bp
150bp
*
**
**
**
**
**

cancer (Angus et al. 2001; Swystun et al. 2011), stroke (Angus et al. 2001; Rainer et al. 2003), and sepsis (Angus et al. 2001; Zeerleder et al. 2003; Kaukonen et al. 2014; Margraf et al. 2008; Dwivedi et al. 2012). Of particular interest is the ability of CFDNA to potentiate disease pathology through activation of coagulation via the contact pathway. In a previous study, we demonstrated that increased concentrations of endogenous CFDNA correlated with increased endogenous thrombin potential in plasmas obtained from patients with severe sepsis. Yet, in addition to coagulation activation and the down-regulation of endogenous anticoagulant pathways, impairment of the fibrinolytic system plays a central role in the pathogenesis of microvascular thrombosis and organ dysfunction in sepsis (Ulloa et al. 2009; Levi et al. 2003; The Surviving Sepsis Campaign Guidelines Committee including The Pediatric Subgroup* et al. 2013; Gando 2010). To date, the mechanisms that modulate fibrinolysis in sepsis remain incompletely understood.

The present study has revealed three major findings. First, we demonstrated that elevations in endogenous CFDNA levels in the plasma of patients with severe sepsis correlate with the production of clots that are resistant to lysis. Only when plasma from septic patients was treated with DNase was fibrinolytic activity restored, thus highlighting the involvement of CFDNA in fibrinolysis. Second, we showed that increased CFDNA concentrations in plasma result in altered clot morphology, and that this effect is likely due to

potentiation of thrombin generation by CFDNA. Finally, we demonstrated that CFDNA is able to bind both fibrin and plasmin to form a non-productive ternary complex that results in delayed clot lysis in both plasma and purified systems.

Impaired fibrinolysis in sepsis is well documented, and has previously been attributed to the increased levels of PAI-1 because of its release from vascular endothelial cells (Yipp and Kubes 2013; Levi et al. 2003; Brinkmann et al. 2004; Semeraro et al. 2012; Fuchs et al. 2012). Elevations in PAI-1 levels have been shown to correlate with disease severity and poor outcome in patients with sepsis (Lorente et al. 2014; Madoiwa et al. 2006; Dofferhoff et al. 1992). Previous studies in which lipopolysaccharide (LPS) was administered to human (van Deventer et al. 1990; Cadena et al. 1996) or nonhuman (Biemond et al. 1995) primates demonstrated that endotoxemia produces a temporary increase in tPA activity and activation of fibrinolysis that is followed by a robust increase in PAI-1 levels that results in an anti-fibrinolytic state. Interestingly, activation of plasminogen by tPA and subsequent fibrinolytic activity in sepsis patients has been shown to remain ongoing despite increases in PAI-1 as determined by plasma levels of plasmin-α_2-antiplasmin complexes and D-dimer (Hesselvik et al. 1989; Raaphorst et al. 2001; Madoiwa et al. 2006). Thus, while the inhibitory effects of PAI-1 may contribute to impaired fibrinolysis in sepsis, there are likely other critical anti-fibrinolytic mechanisms involved. In the current study, although levels of PAI-1 were determined to be elevated in septic

plasmas, inhibition of PAI-1 activity alone was not sufficient to restore fibrinolysis. This evidence suggests that CFDNA and PAI-1 may both contribute to the suppression of fibrinolytic activity observed in septic patients.

In addition to a delay in clot lysis, we also observed significant alterations in clot structure when CFDNA levels were elevated. There are many factors that contribute to modified clot structure, including pH, ionic strength (Nair et al. 1986), and concentrations of calcium (Carr et al. 1986), fibrinogen (Undas and Ariens 2011), or dextran (Sasaki and Noguchi 1959). One of the most important physiologic regulators of clot structure is the concentration of thrombin at the time of clot formation (Wolberg 2007). Sub-nanomolar concentrations of thrombin are capable of cleaving fibrinopeptides and catalyzing fibrin polymerization. Low thrombin concentrations produce clots that are composed of loosely-packed fibrin fibers whereas high concentrations of thrombin produce tightly-packed fibrin clots that are more resistant to lysis (Wolberg 2007). In our studies, increased CFDNA in plasmas from septic patients correlated with denser fibrin networks. We were able to normalize clot structure by supplementing the plasmas with exogenous thrombin, but this had no effect on clot lysis. Therefore, while CFDNA appears to modulate clot structure by enhancing thrombin generation, changes in clot morphology do not appear to contribute to fibrinolytic resistance.

Recent work by Varjú et al. reported on the effect of histones on clot structure (Varju et al. 2015). The addition of at least 250µg mL^{-1} of histone protein to a forming clot resulted in more opaque clots that correlated with thicker fibrin fibers. Histones are often released into the circulation along with CFDNA, particularly during NETosis (Esmon 2013). We previously quantified the levels of circulating DNA-histone complexes in our septic patient plasmas; although the levels were higher than those in healthy controls, they were well below 250µg mL^{-1} (Gould et al. 2014). Therefore, the low levels of histones in our septic patient plasma samples are unlikely to directly influence clot morphology.

Previous studies suggested that DNA may potentiate plasminogen activation by tPA and uPA via a template mechanism that is dependent on ionic strength (Komissarov et al. 2011). As a result, it was suggested that DNA may compete with fibrin for binding plasminogen and tPA to ultimately promote fibrinolysis. The results from our present study support the findings that DNA is able to bind both plasminogen and plasmin with physiologically relevant affinities; however, the cumulative effect of this interaction in plasma correlates with a decrease in clot dissolution. Similarly, Varju et al. recently demonstrated that DNA has a modest effect on tPA-mediated activation of plasminogen, despite a concurrent impairment in fibrinolytic activity (Varju et al. 2015). Our studies demonstrate that only large CFDNA fragments are able to bind plasmin and fibrin simultaneously, inhibiting clot lysis through the formation of a non-

productive ternary complex. This observation is supported by recent work by Longstaff et al. who showed that the addition of exogenous DNA extended lysis times in a purified system (Longstaff et al. 2013). The authors concluded that DNA serves to bind large fibrin degradation products and stabilize the dismantling clot, ultimately prolonging fibrinolysis. Here, we demonstrated that CFDNA binds with high affinity to fibrinogen and fibrin. Therefore, it is likely that larger CFDNA molecules are able to associate with a forming clot and confer additional stability. However, as suggested by visualization of fibrin degradation products in our system (**Fig. 4**), the presence of CFDNA does not simply serve as an adhesive molecule for FDPs, but rather prevents fibrin from being degraded by plasmin. Thus, the ability of CFDNA to impair plasmin-mediated clot lysis coupled with its potential to stabilize the degrading fibrin scaffold may work in concert to delay fibrinolysis.

Particular emphasis has been placed on the harmful effects of elevated levels of CFDNA in various disease states (Kessenbrock et al. 2009; Ritis 2012; Lu et al. 2012; Fuchs et al. 2010). Yet, it is important to consider that coagulation activation and subsequent deposition of fibrin at sites of infection represents a critical mechanism of host defense against invading pathogens (Degen et al. 2007; Bergmann and Hammerschmidt 2007). In fact, several species of bacterial pathogens have evolved ways to circumvent entrapment by exploiting host plasmin-mediated proteolysis to facilitate their own dissemination. For

example, *Yersinia pestis* directly activates plasminogen via its surface Pla protease (Sodeinde et al. 1988), while several other species of bacteria potentiate plasmin-mediated fibrinolysis by recruiting plasminogen to their surface where it is subsequently activated by host tPA (Bergmann and Hammerschmidt 2007).

Similarly, the release of CFDNA in the form of NETs also represents an evolutionarily conserved mechanism of host defense that is essential for infection control. Using a cecal ligation and puncture (CLP) model of polymicrobial sepsis, Meng et al. showed that the dismantling of NETs in vivo by DNase in the early stage of sepsis progression is deleterious, resulting in increased bacterial dissemination and enhanced inflammation (Meng et al. 2012). In contrast, we have shown that delayed administration of DNase in mice subjected to CLP is beneficial, resulting in a reduction of CFDNA-mediated coagulation and a reduction in bacterial dissemination presumably because the bulk of the microbes are already disarmed by NETs (Mai et al. 2015). The findings of the present study suggest that the CFDNA component of NETs not only traps circulating pathogens, but may also work in tandem with fibrin to prevent systemic dissemination of microbes by limiting plasmin-mediated fibrin degradation. Thus, understanding the balance between the beneficial and deleterious effects of a hypofibrinolytic state in sepsis is critical to the development of therapeutic strategies.

In summary, our studies are the first to examine the effect of CFDNA on fibrinolysis in the context of sepsis. Our findings suggest that CFDNA inhibits fibrinolysis in septic patients by impairing plasmin-mediated fibrin degradation, thereby identifying CFDNA as a potential therapeutic target for sepsis.

5.0 Extracellular histones increased tissue factor activity and enhance thrombin generation by human blood monocytes

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Foreword: In Chapter 5, we identify a novel mechanism by which extracellular histones modulate hemostasis. We demonstrate that exposure of monocytic cells to histones results in increased levels of cell surface TF antigen and phosphatidylserine, which contributes to increased TF procoagulant activity. We identify that histones modulate monocyte procoagulant activity through interactions with Toll-like receptors 2/4, and this effect can be attenuated by the addition of C-reactive protein or heparin, which bind and neutralize histones. In addition, we demonstrate that plasmas from septic patients receiving an infusion of unfractionated heparin were less procoagulant towards monocytes compared with septic plasmas without UFH. These findings demonstrate a novel role for histones in potentiating a procoagulant environment in sepsis.

Running title: Histones enhance tissue factor activity

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Authorship Contributions: T.J. Gould and Z. Lysov performed the experiments. T. J. Gould and P.C. Liaw wrote the paper. Z. Lysov, L.L. Swystun, D.J. Dwivedi, A.E. Fox-Robichaud, R. Zarychanski, and P.C. Liaw edited the paper. T.J.

Gould, Z. Lysov, L.L. Swystun, and P.C. Liaw designed the research study.

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References for this manuscript have been incorporated into the Bibliography (Chapter 9) at the end of this thesis.

5.1 Abstract

Objectives: Sepsis is characterized by the systemic activation of inflammatory and coagulation pathways in response to infection. Recently, it was demonstrated that extracellular histones released into the circulation by dying/activated cells may contribute to sepsis pathology. Although the ability of extracellular histones to modulate the procoagulant activities of several cell types has been investigated, the influence of histones on the hemostatic functions of circulating monocytes is unknown. To address this, we (a) investigated the ability of histones to modulate the procoagulant potential of THP-1 cells and peripheral blood monocytes, and (b) examined the effects of plasmas obtained from septic patients to induce a procoagulant phenotype on monocytic cells.

Approach and Results: Tissue factor (TF) activity assays were performed on histone-treated THP-1 cells and blood monocytes. Exposure of monocytic cells to histones resulted in increases in TF activity, TF antigen, and phosphatidylserine exposure. Histones modulate the procoagulant activity via engagement of Toll-like receptors 2 and 4, and this effect was abrogated with inhibitory antibodies. Increased TF activity of histone-treated cells corresponded with enhanced thrombin generation in plasma determined by calibrated automated thrombography. Finally, TF activity was increased on monocytes

exposed to plasma from septic patients, an effect that was attenuated in plasma from patients receiving unfractionated heparin (UFH).

Conclusions: Our studies suggest that increased levels of extracellular histones found in sepsis contribute to dysregulated coagulation by increasing TF activity of monocytes. These procoagulant effects can be partially ameliorated in sepsis patients receiving UFH, thereby identifying extracellular histones as a potential therapeutic target for sepsis treatment.

5.2 Introduction

Sepsis is a devastating clinical condition characterized by a systemic inflammatory response to infection, with concomitant dysregulated pathological thrombus formation (Hotchkiss and Karl 2003). Although sepsis is triggered by the release of microorganisms and/or microbial toxins into the circulation, the presence of infection itself is rarely the cause of death in these patients. Rather, mortality in sepsis is attributed to irreversible organ damage resulting from prolonged, uncontrolled activation of inflammatory and coagulation pathways within the microcirculation. Despite recent advances in clinical management, treatment continues to be largely supportive in nature. As a result, sepsis remains the leading cause of morbidity and mortality in non-coronary intensive care units with mortality rates ranging from 18-30%(Angus et al. 2001). Sepsis-induced mortality is further increased following the development of disseminated

intravascular coagulation, a thrombohemorrhagic state defined by a primary thrombotic and secondary hemorrhagic diathesis that may culminate in multi-organ failure(Levi et al. 2003).

Although the pathogenesis of sepsis is not precisely understood, several studies have suggested that the aberrant overexpression of tissue factor (TF) by circulating cells is involved in the pathological derangement of coagulation in septic patients (Pawlinski and Mackman 2004; Semeraro et al. 2010; Levi et al. 2013). TF is the primary initiator of the extrinsic pathway of blood coagulation. Plasma levels of soluble TF have been demonstrated to be elevated in septic patients and are associated with increased concentrations of procoagulant biomarkers (Gando et al. 2015; Xue et al. 2015). Furthermore, impairment of the TF pathway of coagulation has been shown to diminish coagulation activation and lethality in animal models of sepsis or endotoxemia (Semeraro et al. 2010; Pawlinski and Mackman 2004). Circulating monocytes are considered to be the major source of intravascular TF (Pawlinski and Mackman 2010), and it has been well-established that monocytes possess the ability to modulate their levels of functional surface TF in response to proinflammatory stimuli such as lipopolysaccharide (LPS), interleukin (IL)-1β, and tumor necrosis factor (TNF-α) (Gregory et al. 1989; Cermak et al. 1993; Ito et al. 2007).

Recently, intensive research has focused on investigating novel biological targets that are expressed or exposed in sepsis patients in an attempt to better

understand the pathogenic mechanisms underlying this disorder. Histones are highly cationic nuclear proteins that form hetero-octamers consisting of two copies each of histone subunits H2A, H2B, H3, and H4. Under normal physiological conditions, histones are localized to the nucleus and are essential for organizing double-stranded DNA into chromatin and chromosomes (Richmond and Davey 2003). However, in several proinflammatory disease states, histones can released into the extracellular space, and high plasma concentrations of histones have been detected in sepsis patients that correlate with disease severity (Zeerleder et al. 2003; Xu et al. 2009). Histones can be released passively into the circulation by necrotic or apoptotic cells (Jahr et al. 2001b), or actively secreted during the formation of neutrophil extracellular traps (NETs) that are comprised of chromatin, histones, and antimicrobial molecules (Brinkmann et al. 2004).

Extracellular histones have been shown to contribute to microvascular thrombosis and subsequent organ failure in experimental sepsis models. Extracellular histones have been demonstrated to induce vascular necrosis and are highly cytotoxic to endothelial cells (Abrams et al. 2013; Kumar et al. 2015). Histones activate platelets through engagement of Toll-like receptors (TLRs) to induce platelet aggregation and thrombocytopenia *in vivo* (Xu et al. 2011). Histones also induce a procoagulant phenotype in red blood cells through the externalization of phosphatidylserine (PS) (Semeraro et al. 2014). Administration

of neutralizing antibodies against histones resulted in diminished fibrin deposition and vascular occlusion during systemic infection, further highlighting the importance of histones in infection-associated thrombosis (Massberg et al. 2010). In addition, the activity of circulating histones can also be modified by several anticoagulant mechanisms such as activated protein C which proteolytically cleaves histones (Xu et al. 2009), as well as thrombomodulin (TM) (Ammollo et al. 2011; Nakahara et al. 2013) and heparin (Wildhagen et al. 2013) which both neutralize their procoagulant activities.

It is well characterized that circulating leukocytes including neutrophils and monocytes can separately modify the risk for thrombosis in sepsis via the release of procoagulant NETs and/or the expression of TF. Although the ability of extracellular histones to modulate the procoagulant activities of several circulating cell types has been investigated, the influence of histones on the hemostatic functions of circulating monocytes is currently unknown. Therefore, in this study, we examined the effects of histones on TF expression and activity on peripheral blood monocytes and a human monocytic cell line (THP-1). We also determined the total procoagulant potential of histone-exposed monocytes and THP-1 cells in a plasma system using calibrated automated thrombin generation, and evaluated the ability of extracellular histones in plasmas obtained from septic patients to enhance the procoagulant activity of monocytes.

5.3 Materials & Methods

5.3.1 Materials

Human FVIIa and FX were from Haematologic Technologies (Essex Junction, VT). Recombinant human histones H1, H2A, H2B, H3, and H4 were purchased from New England Biolabs (Toronto, ON). Blocking mAbs against human TLR2 (clone T2.5), TLR4 (HTA125), and isotype control (IgG2a) were all purchased from eBiosciences. Inhibitory TF mAb (HTF-1) was purchased from BD Biosciences (Mississauga, ON). Monoclonal inhibitory antibodies against histone H3 (MHIS1947) and histone H4 (MHIS1952) were generously provided by Dr. Charles Esmon (Oklahoma Medical Research Foundation, OK). Heparin was purchased from Leo Pharma (Thornhill, ON). RPMI 1640 growth medium and penicillin-streptomycin were from Invitrogen (Carlsbad, CA, USA). C-reactive protein from human plasma was purchased from Sigma-Alrich (Oakville, ON). Factor Xa chromogenic substrate S-2765 was purchased from DiaPharma (West Chester, OH). Endotoxin-free PBS was purchased from Cedarlane (Burlington, ON).

5.3.2 Human Sample Collection

Frozen plasma samples were obtained from two available biobanks containing samples from patients with severe sepsis. The first biobank was obtained as part of a prospective cohort study (DYNAMICS Study, ClinicalTrials.gov identifier: NCT01355042). The patients were recruited between

September 2010 and January 2013 from tertiary care ICUs from 9 centers across Canada. Patients with severe sepsis were identified using the inclusion and exclusion criteria previously described by Dwivedi et al. (2012). All samples selected for the present experiments were obtained from patients with refractory hypotension requiring the institution of ongoing use of vasopressor agents and did not receive heparin for thromboprophylaxis. The study was approved by the Research Ethics Boards of all participating centers.

The second biobank utilized was obtained as part of a randomized double-blind clinical trial (HALO study, ClinicaTrials.gov identifier: HALO NCT01648036). The patients were recruited between August 2012 and December 2013 from tertiary care ICUs from 9 centers across Canada. Patients with severe sepsis and septic shock were identified using the inclusion and exclusion criteria previously employed by Zarychanski et al. (2008) as part of a retrospective, propensity matched cohort study. Patients were randomized to receive a continuous IV infusion of UFH 18 IU/kg/hr or to usual care consisting of subcutaneous dalteparin at 5000 IU daily. Patient samples utilized in the present study were exclusively from the UFH intervention arm. The study was approved by the Research Ethics Boards of all participating centers.

For both biobanks, patient blood samples, which were collected within 24 hours of meeting the inclusion criteria for severe sepsis, were processed within 2 hours. Briefly, 9 mL of blood was withdrawn from an indwelling catheter and

transferred into 15 mL polypropylene tube containing 0.5 mL of 0.105 M buffered trisodium citrate and 100 mM benzamidine (pH 5.4). After centrifugation at 1500 x g for 10 min at 20°C, platelet poor plasma was harvested and stored in 200 µL aliquots at -80°C until used. All plasmas selected for study were collected on the second day following ICU admission, allowing for 24 hours of UFH infusion prior to blood collection.

5.3.3 Plasma samples from healthy controls

Plasma samples were obtained via venipuncture from 5 healthy adult volunteers who were not receiving any medication at the time of blood collection. There was no attempt to match controls with cases. The blood was processed as described above, and plasma was stored in aliquots at -80°C until used.

5.3.4 Cell culture

Human acute monocytic leukemia suspension cell line (THP-1) was purchased from American Type Culture Collection (Manassas, VA). Cells were maintained in RMPI 1640 medium supplemented with 10% FBS and 100 U/mL penicillin-streptomycin. Cultures were maintained at a concentration between 2 x 10^5 and 1 x 10^6 cells/mL, with medium being added every 3 days. Cells were subcultured by total medium replacement using centrifugation at 4000xg every 5-6 days and incubated at 37°C in a 5% CO_2 humidified incubator.

5.3.5 Isolation of peripheral human monocytes from whole blood

Peripheral human monocytes were isolated from the whole blood of healthy volunteers by magnetic cell sorting (MACS) as previously described (Stephenson et al. 2006). Isolated monocytes were resuspended in RPMI 1640 (10% FBS and 100 U/mL penicillin-streptomycin). Cell density of $1x10^6$ cells/mL as determined by hemocytometer counting was used for monocyte culture experiments.

5.3.6 Viability assays

Cell viability was assessed using propidium iodide exclusion. Following incubation with histones, cells were washed twice with endotoxin-free PBS and incubated with propidium iodide (4μM) for 1 min. Dead or dying cells were identified by flow cytometry based on propidium iodide uptake.

5.3.7 Flow cytometric analysis of tissue factor expression and phosphatidylserine exposure

Following exposure to histones, cultured blood monocytes or THP-1 cells were washed twice and resuspended with endotoxin-free PBS. Cells were incubated at room temperature in the absence of light with 2 μg/mL of FITC-conjugated anti-human TF antibody for 30 minutes. Incubation with annexin V-FITC was performed in annexin V-binding buffer according to the manufacturer's instruction. IgG isotype antibodies were used as controls. Cell-bound

fluorescence was determined using a FACSCalibur flow cytometer (Becton Dickinson, San Jose, California) with 3500 events counted per sample in duplicates. Data acquisition was performed using CellQuest Pro software.

5.3.8 Tissue factor activity assays

Tissue factor activity of histone-treated cell types was measured by FVIIa-dependent activation of FX using a modification of the method described by (Hoffman et al. 1995). Briefly, 1 x 10^6 blood monocytes or THP-1 cells were seeded into a 24-well dish and were exposed to purified histone protein for up to 24 hours. Cells were pelleted by centrifugation at 4000 x g for 3 min, washed twice with endotoxin-free PBS, and were then resuspended in TBS containing 5 nM FVIIa, 150 nM FX, and 0.005 mM $CaCl_2$. Cells were incubated at 37°C for 30 min, at which point cells were once again pelleted and the reaction mixture was removed and placed in a 96-well plate. To determine FXa generation, 0.2 mM (final concentration) of chromogenic substrate S-2765 was added and allowed to incubate at room temperature for 5 min. The reaction was terminated by the addition of acetic acid and absorbance was determined at 405 nm.

5.3.9 Thrombin generation assays

To prepare platelet-poor plasmas (PPP), peripheral venous blood was collected from healthy volunteers into 3.8% trisodium citrate. PPP was prepared by centrifugation at 1500 x g for 10 minutes at room temperature. Blood

monocytes or THP-1 cells, either untreated or pre-treated for 24 hours with 100 µg/mL unfractionated histones, carefully washed 3 times with endotoxin-free PBS, and were added at a final concentration of 5 x 10^4 cells to 40 µL aliquots of PPP in wells of a 96-well black Costar plate. Where indicated, cells were pretreated for 30 min with the following inhibitors before being added to plasma: anti-TLR2 (50 µg/mL), anti-TLR4 (50 µg/mL), IgG2a (50 µg/mL), or HTF-1 (10 µg/mL) monoclonal antibodies. Also where indicated, 200 µg/mL each of C-reactive protein (CRP), unfractionated heparin (UFH), or anti-histone H3 and H4 inhibitory antibodies were added at the same time as histone or plasma application. Coagulation was initiated by the addition of 15 mM $CaCl_2$ and 1mM Z-Gly-Gly-Arg-AMC (Bachem, Bubendorf, Switzerland) and thrombin generation was monitored using the Technothrombin TGA thrombin generation assay (Technoclone, Vienna, Austria) as previously described (Bunce et al. 2011). Thrombin generation profiles were analyzed using Technothrombin TGA software (Technoclone).

5.3.10 Quantification of circulating DNA/histone complexes

DNA-histone complex levels in patient plasma samples were quantified using the Cell Death Detection ELISA Plus kit from Roche Applied Science according to the manufacturer's instructions.

5.3.11 Statistical analyses

Statistical analysis was performed on experiments with an n = 3 or greater. Values are expressed as means ± standard error. Significance of differences was determined by one-way ANOVA and Tukey's pair-wise comparisons or by t-tests using SIGMAPLOT Software (San Jose, CA, USA).

5.4 Results

5.4.1 Histones enhance TF activity, antigen, and phosphatidylserine exposure on THP-1 and human blood monocytes.

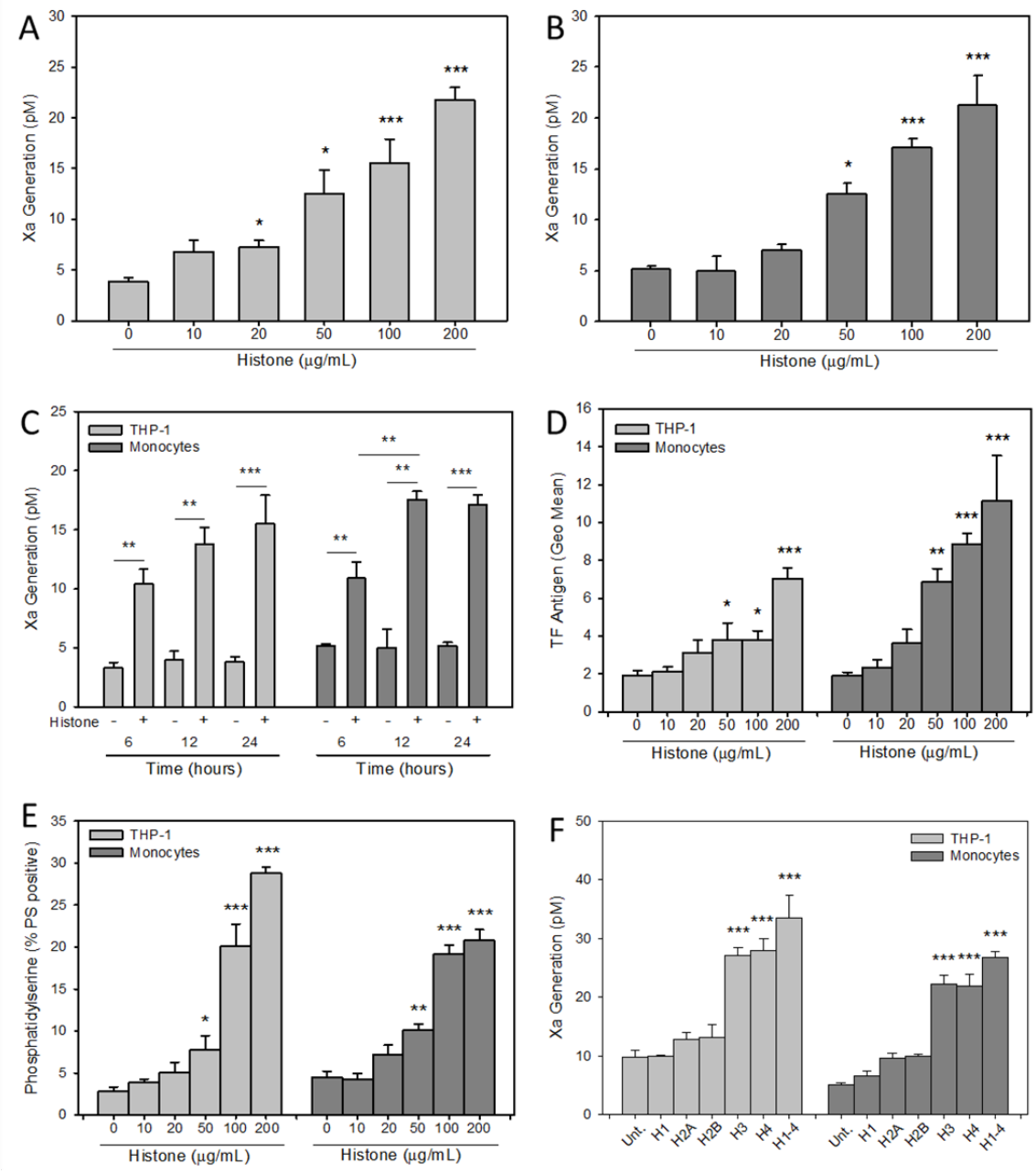
To study the procoagulant effects of histones on monocytic cells, increasing concentrations (0-200 μg/mL) of unfractionated bovine histones were incubated for 24 hours with human acute monocytic leukemia suspension cell line (THP-1) or peripheral blood monocytes immediately after isolation from healthy volunteers. TF activity was determined by the conversion of factor X to factor Xa in the presence of factor VIIa and Ca^{2+}. As shown in **Figure 1A**, exposure of THP-1 cells to histones upregulated TF activity in a dose-dependent fashion. Similarly, treatment of blood monocytes with increasing concentrations of histones resulted in increased TF activity (**Figure 1B**). Both THP-1 cells and monocytes exhibited increased surface TF activity in as little as 6 hours following exposure to 100μg/mL of histones (**Figure 1C**). In contrast, no changes in TF activity were observed when THP-1 cells or monocytes were exposed to histones subjected to heat denaturation (data not shown). The cytotoxicity of

histones towards monocytes was also determined by propidium iodide exclusion. Monocytes incubated with up to 100μg/mL of histones for 24 hours demonstrated >75% viability, which was not significantly different ($p = 0.51$) from untreated cells.

It is well established that increases in tissue factor activity may be a result of increased expression of surface TF, or the result of decryption of latent TF to a more biologically active form in the presence of negatively-charged cell surface phospholipid phosphatidylserine (PS) (Bach 2005). Both THP-1 cells and blood monocytes exposed to histones exhibited a ~3- and ~5-fold increase in surface TF antigen levels, respectively (**Figure 1D**). In addition, incubation with histones resulted in a dose-dependent increase in surface PS for both THP-1 cells and blood monocytes alike (**Figure 1E**).

It was previously demonstrated that histone subunits H3 and H4 are the main contributors to histone cytotoxicity toward endothelial cells (Xu et al. 2009). To determine which histone subunits are responsible for the observed increase in TF activity, we incubated THP-1 and blood monocytes with 100 μg/mL of purified, recombinant histone subunits. While histone subunits H1, H2A, and H2B demonstrated only a modest ability to modulate TF activity, exposure to histone subunits H3 and H4 significantly increased TF activity on both THP-1 cells and monocytes (**Figure 1F**). Collectively, these results suggest that the exposure of THP-1 cells and peripheral blood monocytes to histones,

Figure 5.1. **Effects of histones on tissue factor activity, antigen, and phosphatidylserine exposure on THP-1 cells and blood monocytes.** TF activity (as determined by FXa generation) was quantified on THP-1 cells (A) and peripheral blood monocytes (B) following exposure to increasing concentrations of unfractionated bovine histone protein for 24 hours. (C) Tissue factor activity quantified on THP-1 and monocytes exposed to 100 μg/mL of bovine histones for 6, 12, and 24 hours. Cell surface TF antigen (D) and phosphatidylserine (E) were determined by flow cytometry. (F) TF activity of THP-1 cells and blood monocytes following exposure to 100 μg/mL each of recombinant, purified histone subunits H1-H4. * indicates $P < 0.05$, ** indicates $P < 0.01$, and *** indicates $P < 0.001$ ($n = 3$-4 for all conditions).


A
Xa Generation (pM)
Histone (µg/mL)
B
Xa Generation (pM)
Histone (µg/mL)
C
THP-1
Monocytes
Xa Generation (pM)
Histone
Time (hours)
D
THP-1
Monocytes
TF Antigen (Geo Mean)
Histone (µg/mL)
E
THP-1
Monocytes
Phosphatidylserine (% PS positive)
Histone (µg/mL)
F
THP-1
Monocytes
Xa Generation (pM)
Unt.
H1
H2A
H2B
H3
H4
H1-4

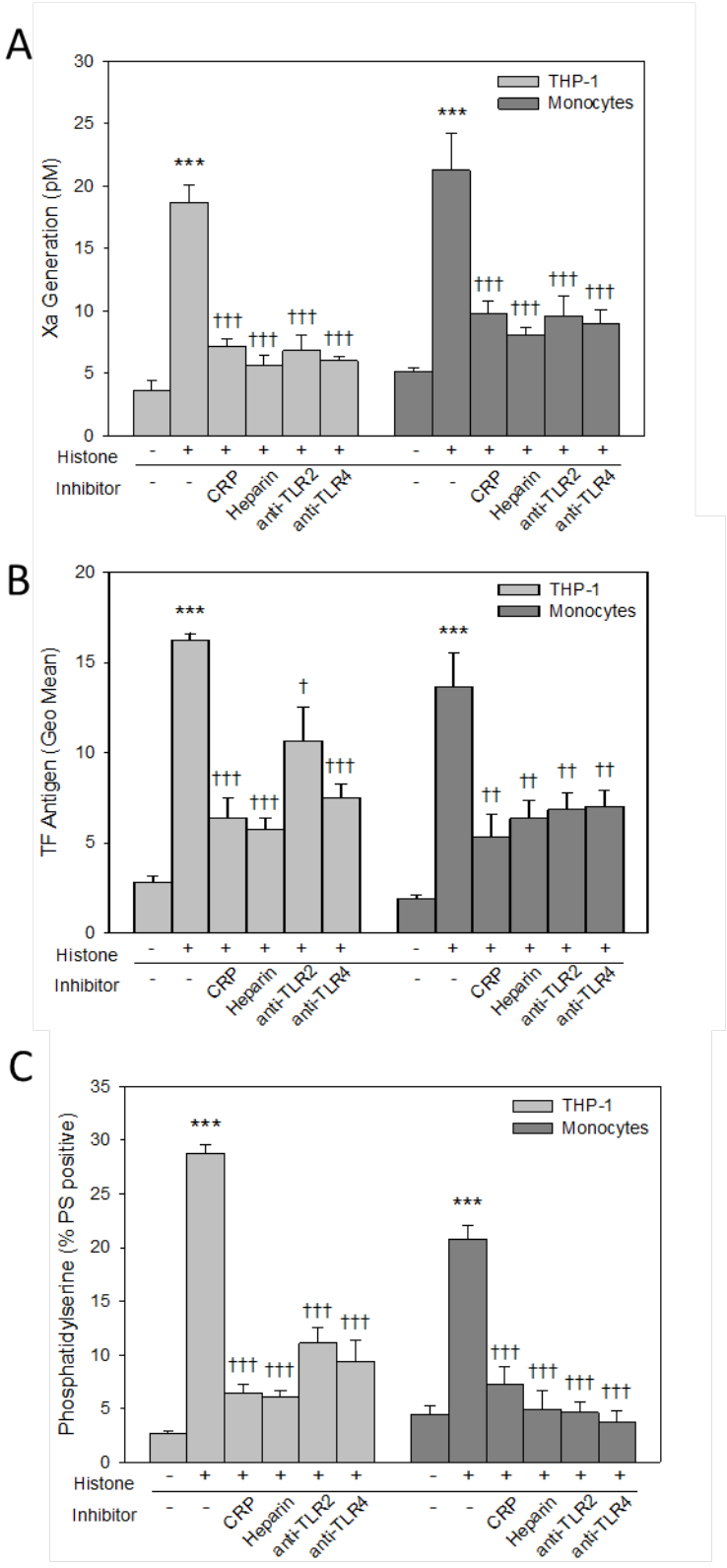
particularly subunits H3/H4, increases surface TF activity via increased surface TF antigen and PS exposure.

5.4.2 The procoagulant effects of histones on THP-1 and blood monocytes are abrogated by C-reactive protein, heparin, and Toll-like receptor blockade.

Recently it was demonstrated that co-administration of C-reactive protein (CRP) or unfractionated heparin (UFH) along with LPS dampens coagulation and reduces mortality in sepsis models (Abrams et al. 2013; Wildhagen et al. 2013). To investigate whether CRP and/or heparin could diminish the procoagulant effects of histones, TF activity assays were performed on THP-1 cells and blood monocytes exposed to 100 μg/mL unfractionated histones with or without 200 μg/mL each of CRP or heparin for 24 hours. As shown in **Figure 2A**, the presence of either CRP or heparin attenuated TF activity on both cell types. In accordance, we observed reduced levels of both surface TF antigen and PS exposure (**Figures 2B and 2C**, respectively).

Histones have been shown to engage with surface TLRs on numerous cell types to induce procoagulant effects (Xu et al. 2011; Semeraro et al. 2014). To determine if histone-TLR interactions were involved in the ability of histones to upregulate TF activity, we repeated the previous experiments using THP-1 and blood monocytes pre-incubated with inhibitory antibodies directed toward TLR-2 and TLR-4. Following exposure to histone, TF activity of these cells was partially attenuated (**Figure 2A**). Similarly, blockade of TLR-2 and 4 resulted in a

Figure 5.2. **Mechanisms of histone-induced procoagulant activity on THP-1 cells and blood monocytes**. (A) TF activity, (B) surface TF antigen, and (C) phosphatidylserine exposure of THP-1 cells and blood monocytes treated with 100 µg/mL of unfractionated histones for 24 hours. Where indicated, 200 µg/mL of C-reactive protein (CRP), 200 µg/mL unfractionated heparin, and 50 µg/mL of each TLR-2 and TLR-4 inhibitory antibodies were included for the duration of the incubation. *** indicates $P < 0.001$ compared to untreated conditions, † indicates $P < 0.05$, †† indicates $P < 0.01$, and ††† indicates $P < 0.001$ relative to histone-only conditions (n = 3-4 for all groups.).


A
Xa Generation (pM)
THP-1
Monocytes
Histone
Inhibitor
CRP
Heparin
anti-TLR2
anti-TLR4
B
TF Antigen (Geo Mean)
C
Phosphatidylserine (% PS positive)

decrease in both surface TF antigen (**Figure 2B**) and PS (**Figure 2C**). Taken together, these results demonstrate that histones modulate monocyte procoagulant activity through TLR-2 and TLR-4 interactions, and these effects can be attenuated by CRP- or heparin-induced inhibition.

5.4.3 Histone exposure enhances thrombin generation mediated by THP-1 cells and blood monocytes.

Next, we assessed the ability of histone-treated THP-1 cells and blood monocytes to generate thrombin using a calibrated automated thrombin generation assay. THP-1 cells and blood monocytes were exposed to 100 µg/mL of unfractionated bovine histones for 24 hours. The histones were then removed following several washes with PBS and the cells were resuspended in platelet-poor plasma which was subsequently recalcified to initiate coagulation. Thrombin generation was measured by cleavage of a fluorogenic substrate at one minute intervals.

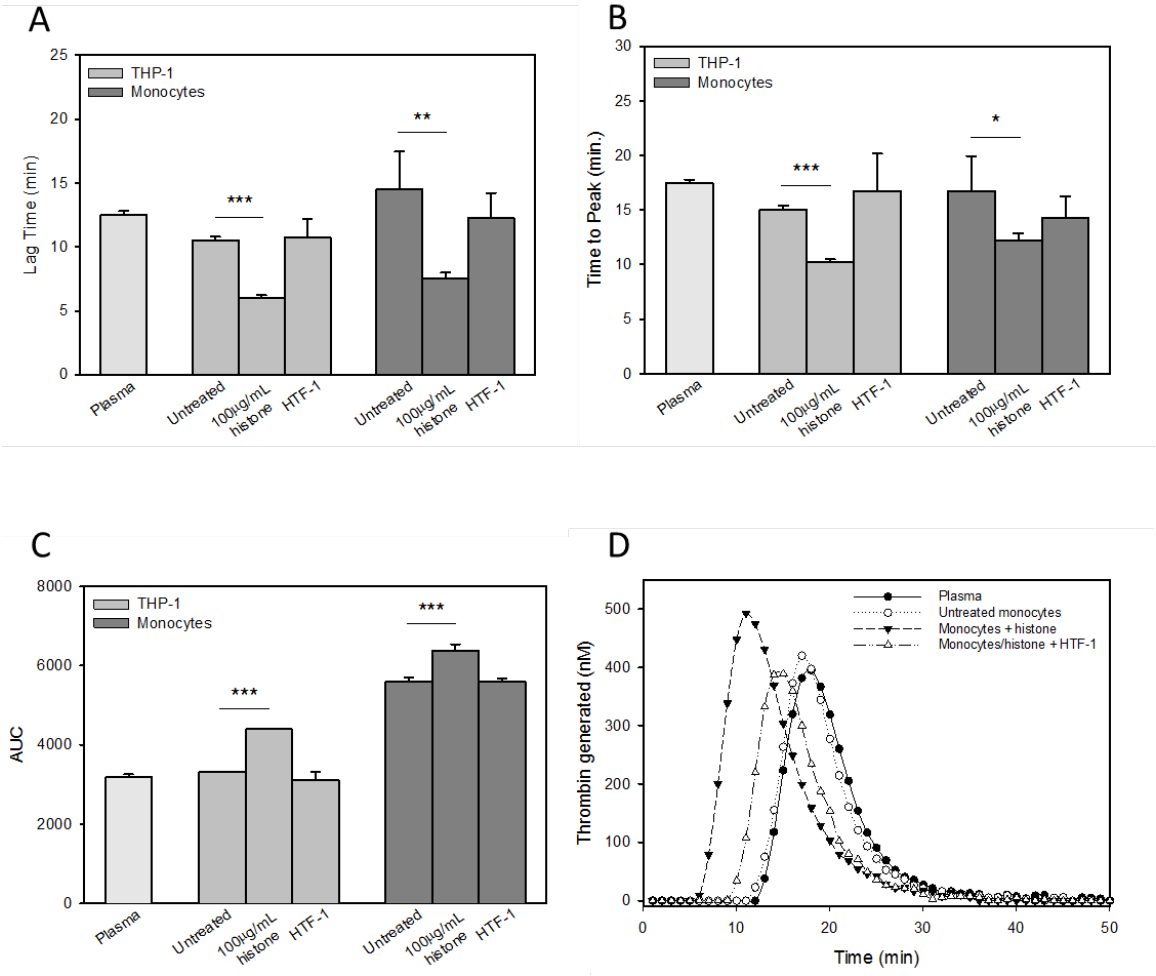
As shown in **Figure 5.3**, exposure of platelet-poor plasma to untreated THP-1 cells or monocytes resulted in only modest changes in thrombin generation parameters. However, THP-1 and blood monocytes exposed to histones significantly decreased the lag time (**Figure 5.3A**) and the time to peak thrombin generated (**Figure 5.3B**). The area under the curve (AUC) which indicates the total amount of thrombin generated confirmed the ability of histone-treated cells to enhance thrombin generation (**Figure 5.3C**).

To confirm the contributions of TF to the augmented thrombin generation parameters observed, the thrombin generation assays were repeated using histone-treated cells which had been blocked with an inhibitory TF antibody (HTF-1). Inhibition of TF abrogated the procoagulant effects of both histone-treated THP-1 and monocytes and restored lag time, time to peak, and AUC to basal levels (**Figures 5.3A-C**). Representative thrombin generation curves of histone-exposed monocytes are depicted in **Figure 5.3D**. This data suggests that increases in TF activity as a result of histone exposure translates to enhanced thrombin generation in plasma.

5.4.4 Extracellular DNA-histone complexes are elevated in septic patient plasma and enhance TF activity of blood monocytes.

To explore the potential clinical relevance of our in vitro studies, we utilized plasma samples from two severe sepsis biobanks. Plasmas were obtained from patients with severe sepsis (as outlined in Materials & Methods), with one patient subgroup receiving prophylactic UFH while the other patient subgroup received usual care without UFH intervention. Patient characteristics from both groups are summarized in **Table 5.1**. Plasma levels of DNA-histone complexes were quantified in both UFH and non-UFH patient plasmas. Plasmas from patients receiving UFH contained significantly lower ($P = 0.005$) levels of DNA-histone complexes compared to non-UFH patients (**Figure 5.4A**). Levels of

Figure 5.3. **Effects of histone exposure on thrombin generation in platelet-poor plasma by THP-1 cells and blood monocytes**. THP-1 cells and blood monocytes isolated from healthy volunteers were treated for 24 hours with 100 μg/mL of unfractionated bovine histone protein and thrombin generation in platelet-poor plasma was assessed using calibrated automated thrombin generation. Where indicated, histone-exposed cells were treated with 10 μg/mL of a TF-inhibiting antibody (HTF-1) for 30 minutes prior to the addition of cells to plasma. (A) Lag times, (B) time to peak, and (C) area under the curve (AUC) were determined for both cell lines. (D) representative thrombin generation profile for monocytes treated with 100 μg/mL histone protein with and without HTF-1. * indicates $P < 0.05$, ** indicates $P < 0.01$, and *** indicates $P < 0.001$ ($n = 3$-4 for all conditions).


A
Lag Time (min)
THP-1
Monocytes
***
**
Plasma
Untreated
100µg/mL histone
HTF-1
Untreated
100µg/mL histone
HTF-1
B
Time to Peak (min.)
THP-1
Monocytes
***
*
Plasma
Untreated
100µg/mL histone
HTF-1
Untreated
100µg/mL histone
HTF-1
C
AUC
THP-1
Monocytes
***
***
Plasma
Untreated
100µg/mL histone
HTF-1
Untreated
100µg/mL histone
HTF-1
D
Thrombin generated (nM)
Plasma
Untreated monocytes
Monocytes + histone
Monocytes/histone + HTF-1
Time (min)

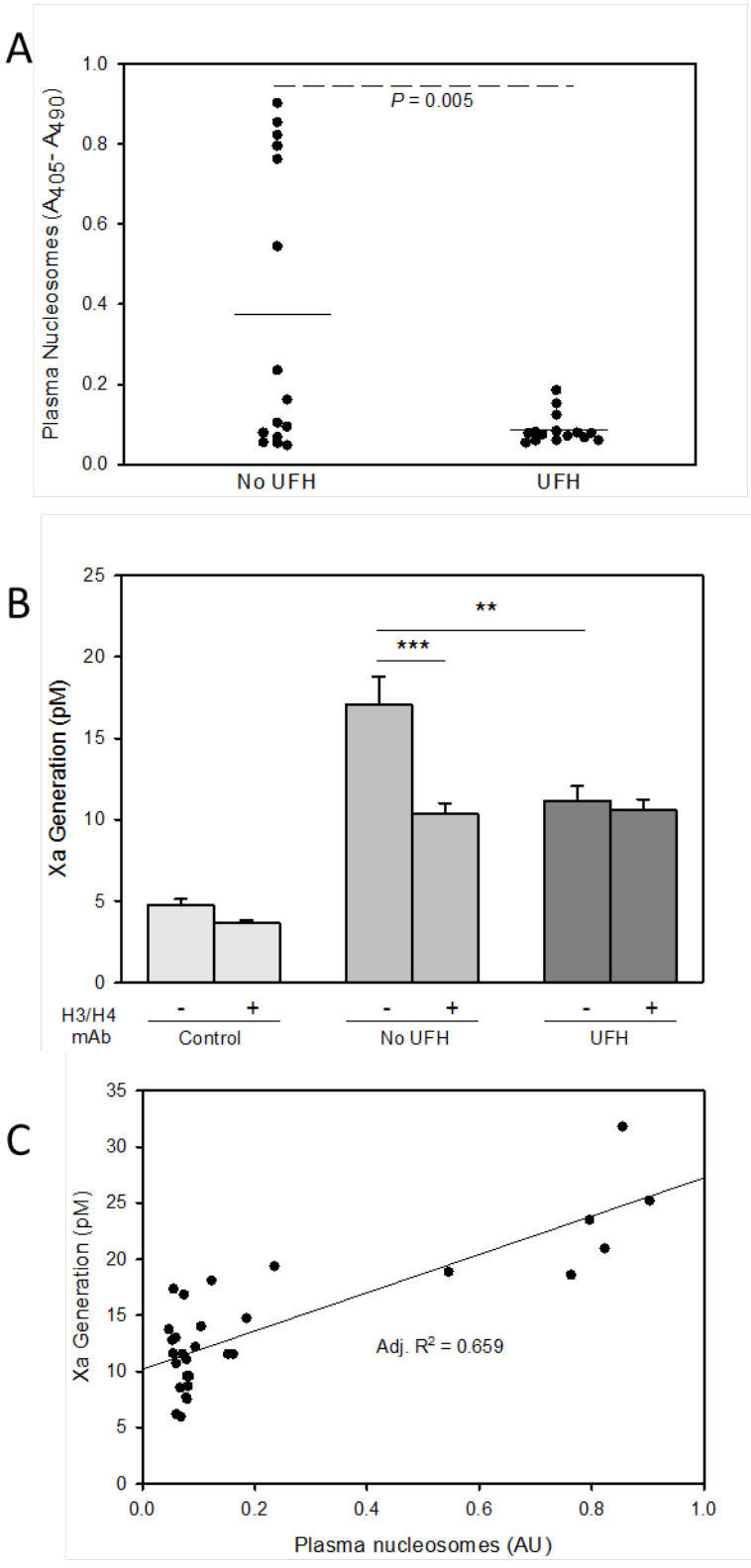
DNA-histone complexes from healthy control plasmas were below the lower limit of detection for the assay (data not shown).

To determine if elevated DNA-histone complexes could modulate TF activity of monocytes, we incubated peripheral blood monocytes with 75% septic plasma containing citrate and benzamidine diluted with media for 24 hours. Compared to control plasma, all septic plasmas enhanced TF activity of monocytes. However, plasmas from patients receiving UFH induced significantly lower TF activity compared to non-UFH patients (**Figure 5.4B**). In a separate set of experiments, monocytes were incubated with both septic plasma and a mixture of 100μg/mL each of histone H3 and H4 inhibitory antibodies. The addition of inhibitory histone antibodies significantly reduced monocyte TF activity in non-UFH sepsis patients, decreasing TF activity levels to those observed in UFH patients. In contrast, the inclusion of inhibitory histone antibodies had no effect in UFH patient plasma (**Figure 5.4B**). In addition, levels of plasma DNA-histone complexes positively correlated with the degree of TF activity induced by these plasmas. Linear regression analysis demonstrated an $r^2 = 0.67$, with a slope of 17.06 that was significantly different from zero ($P < 0.0001$; **Figure 5.4C**). Taken together, this data suggests that extracellular histones found within the circulation of septic patients may induce a procoagulant phenotype of circulating monocytes, and this effect may be attenuated by inhibiting histone activity.

Table 5.1. Baseline characteristics of patients with severe sepsis with or without UFH administration.

Septic Patient Subgroup		**Non-UFH Cohort (n = 15)**	**UFH Cohort (n = 15)**
Characteristic		**Value**	**Value**
Age, years Mean ± SEM (min, max)		70.1 ± 3.5 (45, 93)	62.8 ± 4.1 (28, 82)
Gender, % female (no./total)		40 (6/15)	60 (9/15)
APACHE II score Mean ± SE (min, max).		27.2 ±1.9 (7, 36)	27.1 ± 1.2 (21, 37)
MODS score Mean ± SE (min, max).		6.9 ± 1.1 (1, 16)	6.3 ± 0.6 (4, 14)
Positive cultures, % positive (no./total)		73 (11/15)	80 (12/15)
Infection type, % (no./total)	Gram-positive	18.2 (2/11)	41.7 (5/12)
	Gram-negative	9.1 (1/11)	16.7 (2/12)
	Fungal	27.2 (3/11)	8.3 (1/12)
	Viral	0 (0/11)	8.3 (1/12)
	Mixed	45.5 (5/11)	25 (3/12)

Figure 5.4. **Effects of extracellular histones in septic plasma on TF activity of blood monocytes**. Concentrations of circulating DNA-histone complexes were determined using the Cell Death Detection ELISA PLUS (A; n = 15 for each group). (B) TF activity of monocytes was assessed after exposure to 75% septic plasmas obtained from healthy volunteers, septic patients who received prophylactic unfractionated heparin in the ICU, and septic patients not treated with heparin. (C) Correlation curves for TF activity and plasma levels of DNA-histone complexes in septic patients. * indicates $P < 0.05$, ** indicates $P < 0.01$, and *** indicates $P < 0.001$ (n = 15 patients for each group).


A
Plasma Nucleosomes (A405- A490)
1.0
0.8
0.6
0.4
0.2
0.0
P = 0.005
No UFH
UFH
B
Xa Generation (pM)
25
20
15
10
5
0
**
***
H3/H4 mAb
-
+
Control
No UFH
UFH
C
Xa Generation (pM)
35
30
25
20
15
10
5
0
Adj. R2 = 0.659
0.0
0.2
0.4
0.6
0.8
1.0
Plasma nucleosomes (AU)

5.5 Discussion

The underlying host response to sepsis involves the interplay of complex biological mechanisms and cell types that culminate in severe dysregulation of inflammation and coagulation. To date, over 100 randomized clinical trials have attempted to identify therapies that modulate the septic response and improve patient outcomes; however, none of these potential therapies have been successfully translated into the clinic (Marshall 2014). Therefore, an improved understanding of the molecular basis of sepsis pathophysiology, including interactions between inflammatory, coagulation, and fibrinolytic systems is needed. Recently, there has been renewed enthusiasm for the identification of novel therapeutic targets in sepsis, and circulating histones have emerged not only as a promising biomarker for assessing disease severity, but also as a potential target for pharmacological intervention.

Circulating monocytes are responsible for modulating hemostasis through multiple mechanisms. Among the leukocytes, monocytes have been shown to be unique in that their cell surface membrane serves as a template for the assembly and function of TF-mediated coagulation (McGee et al. 1992). While quiescent monocytes display limited procoagulant activity, they possess the ability to synthesize TF *de novo* and subsequently express it on the cell surface following exposure to bacterial components, proinflammatory cytokines, as well as host-derived ligands (Gregory et al. 1989; Cermak et al. 1993; Celi et al.

1994; Ito et al. 2007). The present study demonstrates that histones also serve as a potent procoagulant stimulus for monocytes, resulting in the upregulation of TF expression on the cell surface as well as inducing the exposure of the anionic phospholipid PS.

In addition to TF, monocytes also express several anticoagulant factors including endothelial protein C receptor (Galligan et al. 2001), TM (McCachren et al. 1991), and tissue factor pathway inhibitor (TFPI)(McGee et al. 1994). Notably, TF and TFPI are the primary regulators of coagulation initiation on the monocyte surface, and TF/TFPI ratios are essential for modulating the procoagulant activity of monocytes (Basavaraj et al. 2010). In resting monocytes, levels of TFPI are approximately 2-fold higher than TF, which serve to rapidly neutralize surface TF. However, common proinflammatory agents such as LPS (Basavaraj et al. 2010) result in a concurrent upregulation of TF and, to a lesser degree, TFPI, potentiating a hemostatic imbalance that favors TF activity. Furthermore, histones have been shown to associate with TM, diminishing its ability to bind thrombin and facilitate protein C activation (Ammollo et al. 2011). Similarly, our studies suggest that there is a cumulative shift to a procoagulant phenotype for histone-stimulated monocytes, as evidenced by a shortened thrombin lag phase and a net increase in the total amount of thrombin generated.

Many of the pathobiological effects exerted by histones are regulated by

TLR2/4 signaling including the induction of cell death in an animal model of kidney inflammation (Allam et al. 2012), inducing cytotoxicity during liver injury (Xu et al. 2011; Huang et al. 2011), and stimulating platelet activation and subsequent aggregation (Semeraro et al. 2011). More recently, it was demonstrated that histones may induce TF expression on endothelial cells and in a murine macrophage-like cell line, again through TLR2/4 involvement (Yang et al. 2016), though these studies did not explore the functional significance of elevated TF to modulate coagulation. Furthermore, in murine models of endotoxemia, reciprocal bone marrow transfer studies between normal mice and mice expressing ~1% human TF demonstrate that the activation of coagulation can be attributed to hematopoietic TF rather than vessel wall-derived TF (Pawlinski et al. 2010). Therefore, the contribution of histones to activating monocyte TF may be of greater pathophysiological relevance. The present results demonstrate mechanistically that TLR2 and TLR4 are the primary receptors for extracellular histones on human monocytes, and demonstrate that antagonizing the histone-TLR interactions may be an attractive target for modulating the procoagulant effects of histones *in vivo*.

Anti-histone treatments in various animal models of sepsis have been shown to confer protective effects. In particular, the administration of heparin has been shown to reduce the cellular cytotoxicity of histones in vitro and decrease mortality in animal models of LPS-induced endotoxemia and cecal

ligation and puncture (Saffarzadeh et al. 2012; Wildhagen et al. 2013; Iba et al. 2015). It is hypothesized that since histones are polycationic peptides, negatively-charged heparins bind and inactivate circulating histones through high-affinity electrostatic interactions, ultimately attenuating their cytotoxicity (Alcantara et al. 1999; Saffarzadeh et al. 2012). In the presence of heparin, we observed diminished procoagulant activity of monocytes exposed to histones. We were able to recapitulate this effect in plasmas obtained from septic patients, whereby plasmas from patients receiving IV administration of UFH induced less procoagulant activity on monocytes compared with plasmas without UFH. In accordance with these results, a recent meta-analysis has shown that heparin therapy may be beneficial to severe sepsis patients without an increased risk for adverse bleeding events (Wang et al. 2014). Although the presence of heparin in plasma was unable to attenuate TF activity to basal levels, the complex proinflammatory milieu of the septic plasmas utilized for this study likely contain a number of mediators, including LPS and lipoteichoic acid, or proinflammatory cytokines such as IL-6 and TNF-α that may also contribute to monocyte procoagulant activity. The attenuation of TF activity by anti-histone antibodies in our plasma experiments demonstrates that pathophysiological levels of circulating histones are sufficient to induce a procoagulant phenotype on monocytes and that our *in vitro* observations were not simply a phenomenon initiated by supraphysiological histone concentrations.

For years, hemostasis has been thought to be regulated by a coagulation factor cascade coupled with platelet activation. However, this conventional view is being challenged by mounting new evidence which suggests that leukocytes play an indispensable role in mediating coagulation under pathological conditions. During infection, the sequence of leukocyte recruitment comprises an initial recruitment of neutrophils to an infectious focus. Once localized to the site of infection, neutrophils are activated and begin to exert effector functions, including phagocytosis, degranulation, and NET release. In addition to bacterial trapping, the DNA component of NETs also serves as an initial stimulus for coagulation and a scaffold for clot formation (Fuchs et al. 2010). The release of NETs is also coupled with the externalization of granular proteins including azurocidin, LL37, and cathespin G which promote the secondary recruitment of monocytes to the site of infection (Soehnlein et al. 2008; Soehnlein and Lindbom 2010). Indeed, not only do neutropenic septic patients have lower numbers of monocytes present within bronchoalveolar fluids (Mokart et al. 2008), but low numbers of circulating neutrophil in sepsis patients are associated with increased 28-day hospital mortality (Bermejo-Martín et al. 2014), highlighting the importance of neutrophil-monocyte cooperation to the host immune response.

A critical event in NET formation is the decondensation of nuclear chromatin which is mediated by peptidyl arginine deiminase 4 (PAD4). PAD4 converts histone arginine residues to citrulline via a deamination process

(Rohrbach et al. 2012), ultimately reducing the positive charge of histones and leading to the uncoiling of DNA and release of free histones (Martinod et al. 2013). The results of our present study suggest that recruited monocytes, upon encountering activated neutrophils and their liberated intracellular contents, are activated by externalized histones and adopt a procoagulant phenotype. Thus, the initial neutrophil response to infection may work synergistically with monocytic procoagulant activity to initiate and sustain coagulation following an infectious insult. It is also important to consider that, while thrombus formation at sites of infection represents a critical host defense mechanism that limits dissemination of dangerous pathogens, sustained activation of coagulation can result in pathological microthrombus formation that contributes to organ dysfunction and failure (Degen et al. 2007; Bergmann and Hammerschmidt 2007). Therefore, understanding the balance between the beneficial and deleterious effects of a hypercoagulable state in sepsis is critical to the development of therapeutic strategies.

In summary, these studies are the first to examine the effects of histones on monocytic cells, specifically within the context of sepsis. Our findings suggest that histones potentiate a procoagulant phenotype on circulating monocytes, thereby establishing a novel mechanism by which extracellular histones modulate hemostasis and identify histones as a potential therapeutic target in sepsis treatment.

6.0 General Discussion & Future Directions

6.1 General Discussion

Sepsis is a devastating condition that is characterized by a systemic inflammatory response to infection, coupled with concomitant dysregulated pathological thrombus formation and a hypofibrinolytic state. Mounting evidence suggests that coagulation abnormalities in sepsis are mediated by leukocytes, namely circulating monocytes and neutrophils. Of particular interest are the formation of NETs, which are comprised of CFDNA, histones, and neutrophils granular proteins, and represent a newly identified link between innate immunity and coagulation. The main objective of this thesis was to evaluate the mechanisms by which CFDNA and/or histones modulate coagulation in sepsis. The findings presented in this thesis demonstrate that (i) neutrophil extracellular traps released from live, activated neutrophils promote coagulation via platelet-dependent and -independent pathways (Chapter 3); (ii) CFDNA, the primary component of NETs, attenuates fibrinolysis by impairing plasmin-mediated fibrin degradation (Chapter 4), and; (iii) histones, the other principle component of NETs, induces TF activity and expression in monocytic cells (Chapter 5). Taken together, these studies identify novel mechanisms by which leukocytes and their procoagulant effector molecules potentiate a hypercoagulable and anti-fibrinolytic state in septic patients. This work also has important implications for leukocyte-mediated coagulation abnormalities found in other disease states.

6.2 Leukocytes support antimicrobial defense via microthrombi formation

Coagulation serves not only to prevent hemorrhage following injury, but it also plays a critical role in host defense during infection by limiting the spread of organisms. The coordinated activation of intravascular coagulation in response to circulating pathogens has recently been termed 'immunothrombosis' (Engelmann and Massberg 2012). During immunothrombosis, cells of the innate immune system employ multiple procoagulant mechanisms that include the local delivery of active TF, the degradation of endogenous anticoagulants, and the formation of a procoagulant matrix consisting of extracellular DNA and histones at the site of infection. Importantly, the procoagulant mechanisms that underlie immunothrombosis are distinct from those involved in hemostasis.

The TF pathway is an important initiator of coagulation during instances of infection. Indeed, for many years, the hemostatic abnormalities in sepsis have been described as an initial hypercoagulable phase driven by aberrant expression of TF (Wheeler and Bernard 1999b; Hotchkiss and Karl 2003). During immunothrombosis, the primary source of intravascular TF is expressed and delivered by monocytes, rather than being derived from sequestered subendothelial stores. The initial importance of TF has been shown in experimental models of Gram-negative sepsis, where low myeloid TF-expressing mice exhibit an impaired ability to limit pathogen dissemination that is accompanied by an increased bacterial load and increased mortality (Luo et al.

2011). This evidence suggests that leukocyte-derived TF is a critical component in the events that precipitate immunothrombosis during infection. Moreover, the presence of infectious pathogens and the induction of TF are intimately linked. The detection of PAMPs, such as LPS, by monocytic TLRs results in the *de novo* synthesis of and expression of functional TF (Robinson et al. 1992). Similarly, platelets also express PRRs that are able to recognize and promote coagulation in response to PAMPs (Ma and Kubes 2008). Monocytes and platelets require only a trace amount of LPS to become fully activated, and this sensitivity suggests that these cells play a key role in the detection of blood-borne organisms. However, several studies have reported that patients with sepsis present no signs of systemic TF-mediated hypercoagulability, even in the early stages of sepsis (Petros et al. 2012; Collins et al. 2006). Our studies in Chapter 3 support these findings, as thrombin generation in septic plasmas was not attenuated by the addition of TF inhibitory antibody, HTF-1. Taken together this evidence suggests that, while the extrinsic pathway may be involved in the initiation of sepsis-associated coagulation, the role of the intrinsic pathway may have been largely overlooked.

Another major contributor to immunothrombosis in sepsis is the neutrophil via the formation of NETs. The adhesive properties of DNA have been shown to trap and ensnare bacteria, limiting their ability to spread within the host (Brinkmann et al. 2004). Dismantling of these DNA matrices are detrimental

during infection, and often results in increased mortality in animal models (Meng et al. 2012; Mai et al. 2015). In addition to bacterial trapping, NETs also initiate localized coagulation to ensure the successful entrapment of pathogens. As demonstrated in Chapter 3, the procoagulant activity of NETs is mediated by its ability to activate factors XI and XII to trigger the contact pathway of coagulation. This is largely owing to the negatively-charged backbone of DNA, as dismantling of NETs with DNase abolished thrombin generation. The histone component of NETs further amplifies the procoagulant functions of NETs. In addition to robust antimicrobial activities (Brinkmann et al. 2004), histones also exert a wide array of procoagulant functions including platelet activation (Esmon 2011), inhibition of endogenous anticoagulants (Ammollo et al. 2011), and, as demonstrated in Chapter 5, an ability to potentiate a procoagulant phenotype in monocytes. Furthermore, our studies also implicate NETs in the suppression of fibrinolysis that is frequently observed in sepsis. Although anti-fibrinolytic properties of NETs are currently less well-known, we have identified that the DNA component of NETs impairs plasmin-mediated degradation of fibrin clots, ultimately prolonging clot lysis (Chapter 4). Collectively, NETs serve to localize thrombus development following neutrophil activation by microorganisms to prevent systemic spread within the host. The procoagulant mechanisms of NET components are summarized in **Table 6.1**.

While the innate immune and coagulation systems co-exist to contain

Table 6.1. Procoagulant effector functions of neutrophil extracellular trap (NET) components. CFDNA, cell-free DNA; HMGB1, high-mobility group box 1; PS, phosphatidylserine; TF, tissue factor; RBC, red blood cell; PC, protein C; AT, antithrombin; TFPI, tissue factor pathway inhibitor.

Effector Molecule	Procoagulant Activity	Reference
CFDNA	• **Contact pathway activation (FXI, FXII)** • **Modulate clot structure** • **Impair fibrinolysis**	(Gould et al. 2014) (Gould et al. 2015)
Histone	• Platelet activation • Induce PS exposure on RBCs • Induce TF expression on endothelial cells • **Induce TF expression on monocytes** • Auto-activation of prothrombin • Impair PC pathway • Impair AT activity • Cytotoxic to endothelial cells	(Esmon 2011) (SEMERARO et al. 2014) (X. Yang et al. 2016) (Barranco-Medina et al. 2013) (F. Semeraro et al. 2011) (Varju et al. 2015) (Kleine et al. 1995)
Neutrophil Elastase	• Activation of coagulation proteases (FV, FVIII, FX) • Degradation of anticoagulant TFPI	(Higuchi et al. 2003; Liou and Campbell 1996)
HMGB1	• Induce TF expression on monocytes • Induce NETosis	(Ito et al. 2007) (Tadie et al. 2013)

potentially lethal pathogens, several organisms have evolved ways to circumvent entrapment by exploiting mechanisms of immunothrombosis to facilitate their own dissemination. For example, group A, C, and G streptococci express streptokinase which is able to convert plasminogen to plasmin, as well as inhibit circulating α_2-antiplasmin (Bergmann and Hammerschmidt 2007). This allows the bacteria to degrade a confining fibrin network and disseminate freely within the host. *Yersinia pestis* directly activates plasminogen via its surface Pla protease (Sodeinde et al. 1988), while several other species of bacteria potentiate plasmin-mediated fibrinolysis by recruiting plasminogen to their surface where it is subsequently activated by host tPA (Bergmann and Hammerschmidt 2007). In addition, several species have evolved mechanisms to evade entrapment by NETs. Examples include *S. pyogenes* (Beiter et al. 2006) and *Streptococcus pneumonia* (Buchanan et al. 2006), which secrete bacterial DNases that dismantle the DNA scaffold of NETs and limits their procoagulant and antimicrobial capabilities (**Table 6.2**).

The findings of this thesis suggest that the CFDNA component of NETs not only traps circulating pathogens and sequesters them by inducing coagulation, but CFDNA may also work in tandem with fibrin and elevated PAI-1 to prevent systemic dissemination of microbes by limiting plasmin-mediated fibrin degradation. Furthermore, the procoagulant phenotype of platelets and monocytes induced by NET-derived histones facilitates sustained thrombin

Table 6.2. Mechanisms by which invasive pathogens exploit coagulation and fibrinolysis. CLFA, clumping factor A; NET, neutrophil extracellular trap; DNase, deoxyribonuclease; tPA, tissue plasminogen activator; ECM, extracellular matrix.

Pathogen	Molecular Mediator	Mechanism	Reference
Group A, C, and G streptococci	• Streptokinase	• Plasmin generation • Inhibition of α_2-antiplasmin	(Bergmann and Hammerschmidt 2007)
Yersinia pestis	• Pla protease	• Plasmin activation on cell surface • Inhibition of α_2-antiplasmin • Degradation of complement factors	(Sodeinde et al. 1988)
Escherichia coli	• OmpT	• ECM adhesion • Plasminogen activation • Degradation of cationic bactericidal peptides	(Stumpe et al. 1998)
Staphylococcus aureus	• Staphylocoagulase • CLFA	• Activation of prothrombin, preventing pathogen clearance • Platelet aggregation, reducing pathogen clearance	(A. G. Cheng et al. 2010; Clemetson 2011)
Streptococcus pneumoniae	• DNase	• Degradation of NETs	(Buchanan et al. 2006)
Streptococcus pyogenes	• DNase	• Degradation of NETs	(Beiter et al. 2006)

generation and fibrin deposition at the infectious focus. These redundant procoagulant and anti-fibrinolytic mechanisms help to ensure the successful detainment and subsequent clearance of invading pathogens despite their acquired mechanisms of host exploitation.

6.2.1 Investigating the effect of NETs on thrombolysis

Our studies presented in Chapter 4 are the first to characterize the anti-fibrinolytic properties of NET components in the context of sepsis. To date, the effects of NET components on thrombolysis in vivo remain uncharacterized. To examine this, mice could be subjected to the cecal ligation and puncture model of sepsis followed by blood collection and plasma isolation as previously described (Mai et al. 2015). Levels of CFDNA should be quantified and clot lysis assays could be performed on these samples to determine if CFDNA levels correspond with a delay in clot lysis times as was observed in patient plasmas in Chapter 4. In addition, DNase could be administered to these septic mice at incremental time points during the period of observation (Mai et al. 2015). Clot lysis time in DNase-treated mice would presumably be restored to basal levels and further support a role for CFDNA in impaired fibrinolysis in sepsis.

Furthermore, *in vitro* and *in vivo* observations indicate that chromatin, fibrin, and VWF form a colocalized network within the thrombus that is similar to extracellular matrix (Fuchs et al. 2010; Brill et al. 2012). It is likely that each of the components will need to be cleaved by their own appropriate enzyme and also

that the presence of one component may in influence the degradation or stability of the other. Combinations of tPA, DNase, and/or ADAMTS13 could be administered to CLP-subjected mice to evaluate their effects on thrombolysis (Zhao et al. 2009).

The specific interactions between CFDNA and fibrin are also incompletely understood. In Chapter 4, we demonstrate that DNA is able to interact directly with both fibrinogen and fibrin. As described above, a final step during fibrin polymerization is the cross-linking and stabilization by FXIII transglutaminase. Whether NET components provide a substrate to Factor XIII and could be cross-linked to fibrin is unknown. In addition, PAD4, which is eventually released from neutrophils during NET formation, has been shown to citrullinate fibrin in rheumatoid arthritis (Masson-Bessiere et al. 2001). Whether this occurs in sepsis and how it may affect plasmin-mediated fibrin degradation is unclear. These experiments would yield important insights into the interactions between CFDNA and fibrin and reveal potential additional mechanisms by which CFDNA and/or additional NET components attenuate fibrinolysis.

6.3 Dysregulated immunothrombosis potentiates DIC in sepsis

As described in Chapter 1, severe sepsis is associated with the development of DIC, a condition that manifests as coagulation activation and microvascular thrombosis formation in organ tissues (Levi et al. 2003). Similar to

immunothrombosis, DIC occurs in the microvasculature, is initiated in response to infectious stimuli, and is sustained by effector molecules such as intravascular TF (Pawlinski and Mackman 2010) and NET components (Ma and Kubes 2008). DIC is triggered when immunothrombosis becomes overwhelmed and is no longer able to restrict the spread of pathogens. The failure of immunothrombosis to contain microorganisms results in the unrestricted formation of microvascular thrombi and the excessive activation of inflammation, which is aggravated by the ability of both pathways to potentiate each other (Schouten et al. 2007). Thus, immunothrombosis may represent an early event in the development of DIC.

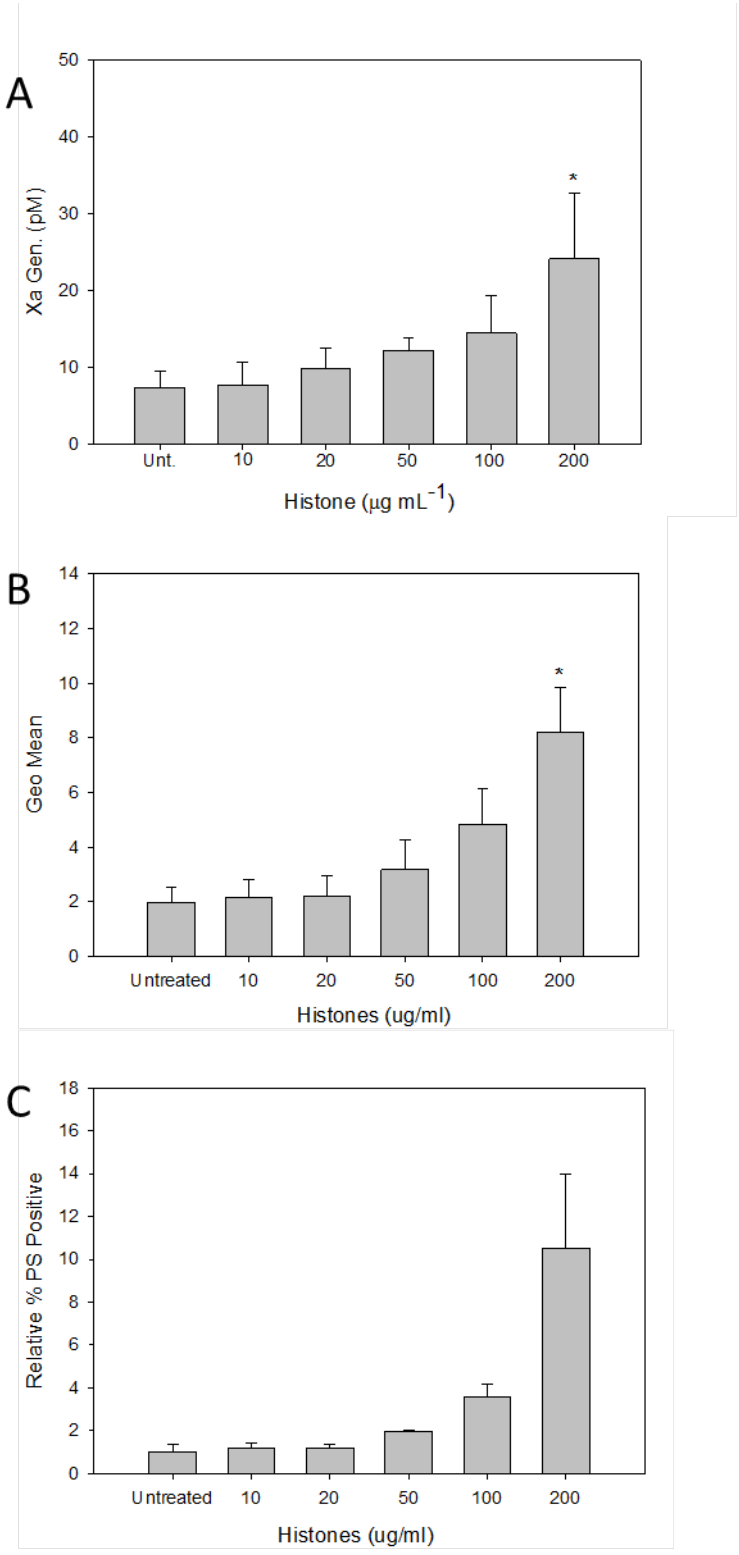
Neutrophils and the formation of NETs represents a critical juncture between regulated immunothrombosis and uncontrolled, systemic coagulation. As discussed previously, monocytes are likely responsible for the initiation of coagulation reactions in response to infection via the TF pathway (Luo et al. 2011). However, should monocyte-mediated coagulation be insufficient to contain the infection, neutrophils may become activated and form NETs as a second line of defense to control dissemination.

NETs aggressively modulate coagulation by inducing a procoagulant phenotype on many different cell types, including monocytes, platelets, vascular endothelial cells, and even additional neutrophils. Recent evidence suggests that NET components (namely histones and thrombin generated via CFDNA) are able to potently activate platelets. These activated platelets, in turn, are able to

form platelet-neutrophil conjugates which stimulate additional NET release (Clark et al. 2007). In addition, we and others have also found that histones are able to induce TF expression and PS exposure on vascular endothelial cells, suggesting that NETs contribute to the activation of the endothelium during sepsis and septic shock. As depicted in **Figure 6.1**, co-incubation of increasing concentrations of histones resulted in an upregulation of TF expression, TF activity, and PS exposure. Similarly, histones have been found to induce a procoagulant phenotype via PS exposure on red blood cells (Semeraro et al. 2014).

Neutrophil activation during infection also results in the recruitment of additional leukocytes to the infectious focus. Granular contents, specifically LL-37 and heparin-binding protein, have been shown to promote the recruitment of proinflammatory monocytes (Soehnlein and Lindbom 2010). In our current studies, we identified that the histone component of NETs is able to induce TF expression and activity on monocytes, resulting in enhanced thrombin generation in plasma. This effect may be further potentiated by neutrophil elastase, which has been shown to exacerbate monocyte TF activity through the degradation of monocyte surface TFPI (Higuchi et al. 2003). Furthermore, HMGB1, a nuclear DAMP that is released during NETosis, has also been shown to induce NET formation in resting neutrophils (Tadie et al. 2013), ultimately potentiating a cyclical series of immune cell activation. These effects, coupled

Figure 6.1. **Effects of histones on tissue factor activity, antigen, and phosphatidylserine exposure on human umbilical vein endothelial cells**. TF activity (as determined by FXa generation) was quantified on HUVEC (A) following exposure to increasing concentrations of unfractionated bovine histone protein for 24 hours. Cell surface TF antigen (B) and phosphatidylserine (C) were determined by flow cytometry. * indicates $P < 0.05$, ** indicates $P < 0.01$, and *** indicates $P < 0.001$ ($n = 3$-4 for all conditions).


A
Xa Gen. (pM)
0
10
20
30
40
50
*
Unt.
10
20
50
100
200
Histone (µg mL-1)
B
Geo Mean
0
2
4
6
8
10
12
14
*
Untreated
10
20
50
100
200
Histones (ug/ml)
C
Relative % PS Positive
0
2
4
6
8
10
12
14
16
18
Untreated
10
20
50
100
200
Histones (ug/ml)

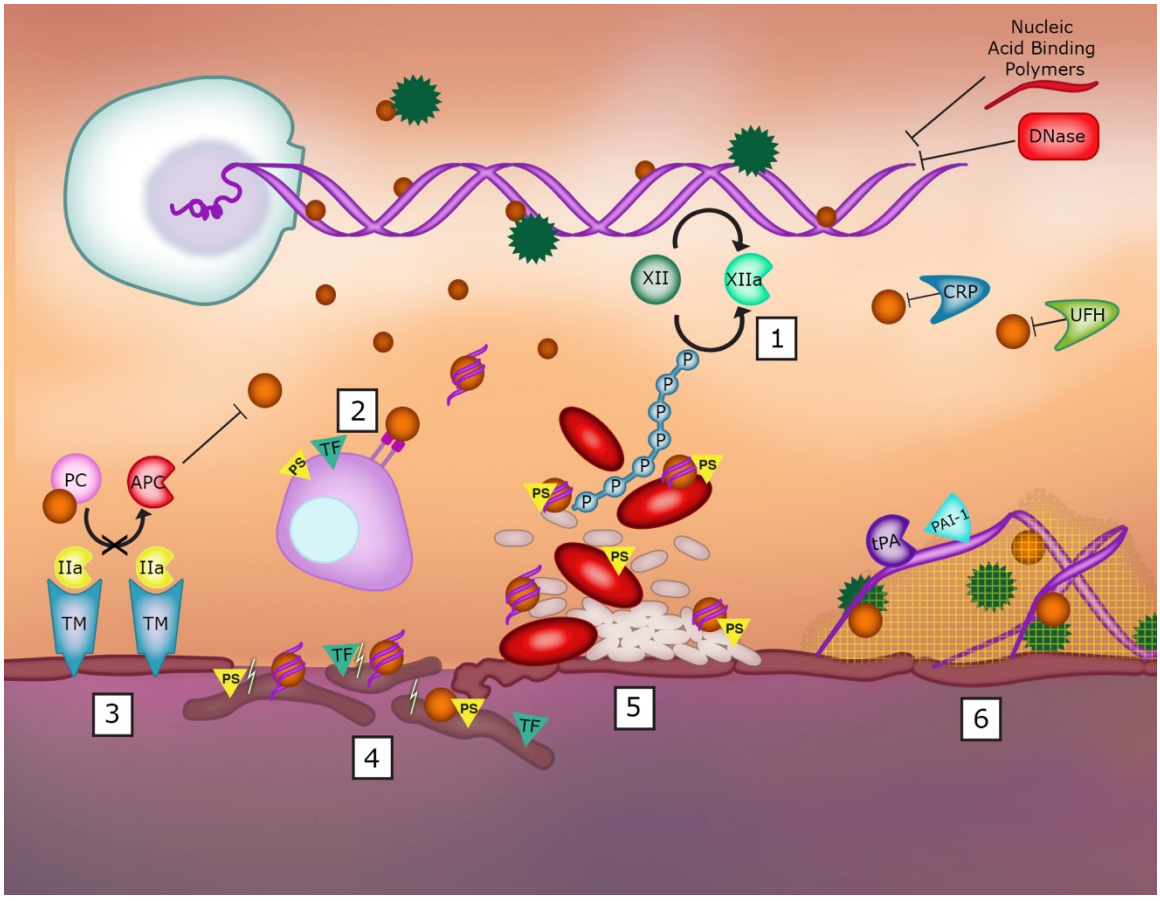
with the newly-established PS-rich environment created by histone release, suggests that neutrophils are able to orchestrate a coordinated, procoagulant response to severe infection that involves numerous cell types. Thus, the formation of NETs during infection may 'release the brakes' of immunothrombosis, inducing a procoagulant phenotype in the key cellular mediators of coagulation in an attempt to contain circulating pathogens. While this last-ditch effort on the part of the neutrophil may result in the successful elimination of infectious organisms, such severely dysregulated coagulation is often accompanied by several deleterious (and sometimes fatal) consequences for the host. These events are collectively summarized in **Figure 6.2**.

6.3.1 Investigating the mechanisms of NET clearance in sepsis

Currently, it is unclear how NETs and NET degradation products are removed from circulation. Studies in healthy human volunteers demonstrate a rapid, transient increase in CFDNA levels following exercise that are restored to basal levels within minutes (Beiter et al. 2014). However, in pathological conditions like sepsis, CFDNA levels remain elevated over time. It is unclear whether these persistently elevated levels of CFDNA are the result of impaired clearance mechanisms or the sustained production of NETs.

To determine clearance rates of DNA in sepsis, wild-type mice and mice subjected to cecal ligation and puncture could be infused with radio-labelled or biotinylated DNA fragments. At regular intervals, retro-orbital plasma samples

Figure 6.2. **Modulation of coagulation and fibrinolysis by cell-free DNA and histones**. Cell-free DNA and histones are released into the circulation through NETosis. DNA triggers the contact pathway of coagulation by catalyzing the autoactivation of FXII to FXIIa [Box 1]. Histones induce a procoagulant phenotype in circulating monocytes via TLR2/4 interactions which drive tissue factor (TF) and phosphatidylserine (PS) expression [Box 2]. Histones also bind to protein C and impair TM-dependent protein C activation [Box 3]. Histones activate inflammation and cell death pathways by direct interaction with the cell membrane or via TLR-2, TLR-4, and TLR-9 signaling pathways [Box 4]. Histones, alone or in complex with DNA, activate platelets resulting in aggregation and increased cell surface expression of PS, P-selectin, and FV/FVa. Activated platelets secrete polyphosphates which further potentiate coagulation by activating FXII, accelerating FXI activation by thrombin, and enhancing FV activation [Box 5]. NETs suppress fibrinolysis by intercalating into fibrin clots, which results in thicker fibers that are more stable and resistant to shear forces. DNA also delays fibrinolysis by accelerating the inactivation of tPA by PAI-1 and by competing with fibrin for plasmin [Box 6]. DNA can be degraded by DNase 1 or neutralized by synthetic nucleic acid-binding polymers. Inhibitors of histones include APC, CRP, and heparin. Adapted from Gould et al. (2015).


Nucleic
Acid Binding
Polymers
DNase
XII
XIIa
1
CRP
UFH
2
TF
PS
PC
APC
IIa
IIa
TM
TM
3
4
5
6
tPA
PAI-1
P

could be collected and examined for labelled DNA content to determine DNA clearance kinetics.

Previous animal studies suggest that it is therapeutically beneficial to clear NETs from the circulation. Indeed, the studies presented here demonstrate that removal of CFDNA from septic plasmas attenuates the procoagulant and anti-fibrinolytic effects. DNase is currently the most commonly utilized method of removing NETs within experimental sepsis models. Early in sepsis, levels of DNaseI is elevated, perhaps to reduce the risk of thrombosis (Meng et al. 2012). Future investigations should examine DNaseI knockout mice (Apostolov et al. 2009) subjected to cecal ligation and puncture. Compared to wild-type mice, DNaseI knockout mice would presumably exhibit a prolonged procoagulant phenotype, with elevated procoagulant markers (i.e. thrombin-antithrombin complexes). Furthermore, monocytes and macrophages have been implicated in the clearance of NETs, largely owing to their ability to synthesize and release DNase (Farrera and Fadeel 2013). To assess the specific contributions of monocyte-derived DNase to NET clearance, mice may also be subjected to clodronate liposome injections prior to the start of experiments to deplete circulating monocytes. These studies would yield novel insights into the role of DNase in the clearance of NETs, as well as its potential as a natural thrombolytic.

6.4 Contributions of leukocytes to large vessel thrombosis

Though the studies conducted in this thesis maintained a sepsis focus, the novel procoagulant mechanisms of NET components may have important implications for other pathologies associated with leukocyte-mediated coagulation abnormalities. In particular, recent investigation has begun to explore the role of innate immune cells in the pathogenesis of both arterial and venous thrombosis.

6.4.1 Contributions of leukocytes to atherothrombosis

Atherothrombosis is characterized by the superimposition of an arterial thrombus over the site of a ruptured atherosclerotic plaque comprised of an accumulation of inflammatory cells and lipid deposits. Plaque rupture within a high-shear arterial environment triggers platelet adhesion, activation, and aggregation, and involves many of the same platelet receptors and subendothelial ligands involved in hemostatic clot formation (Furie and Furie 2008; Jackson 2011). Although arterial thrombosis is initiated by platelets, the subsequent activation of coagulation and fibrin deposition is critical for thrombus stabilization and growth. While activated platelets can directly enhance coagulation via the release of inorganic polyphosphates (Müller et al. 2009), fibrin formation in arterial thrombi is primarily regulated by leukocyte participation. Early studies have shown that the inhibition of neutrophil and monocyte recruitment to the site of arterial thrombus formation results in the

cessation of fibrin deposition and destabilization of the thrombus (Palabrica et al. 1992).

Recent evidence suggests that the procoagulant mechanisms employed by immune cells during immunothrombosis are similar to those involved in arterial thrombi formation. In particular, the contributions of monocyte-derived TF are central to arterial thrombus development. During atherosclerotic plaque formation, monocytes are recruited into the intima and subintima where they differentiate into macrophages and foam cells. The proinflammatory environment of the plaque induces TF expression and activity on these resident cells (Wilcox et al. 1989), as well as facilitates the shedding of PS- and TF-positive microparticles (Mallat et al. 1999). Following plaque rupture, the established TF-rich microenvironment is exposed to the circulating blood, which initiates arterial thrombus formation (Day et al. 2005). Although this localized exposure precipitates atherothrombosis, the additional recruitment of leukocyte-derived procoagulant material is important for regulating thrombus growth and stability (Chou et al. 2004). Activated platelets regulate the recruitment of peripheral blood monocytes and monocyte-derived MPs to the thrombotic site via the release of cytokines and P-selectin (Falati et al. 2003; Gross et al. 2005). Correspondingly, plasma levels of TF-expressing monocytes have been demonstrated to be elevated in conditions associated with increased arterial thrombogenesis in animal models (Owens et al. 2012).

In addition to monocytes, circulating neutrophils have recently been implicated in the development of atherosclerosis. Neutrophils have been shown to migrate into atherosclerotic plaques and, upon activation by cholesterol crystals, release NETs within the lesion that prime resident macrophages for proinflammatory cytokine release (Warnatsch et al. 2015). NETs have been visualized in association with the lumen overlying the atherosclerotic plaque in both animal models and in human plaques, suggesting that NETs may contribute to a localized procoagulant environment (Megens et al. 2012). A recent study demonstrated that NETs formed at the site of an atherosclerotic plaque are decorated with TF, suggesting that NETs may serve to capture and localize monocyte-derived TF-bearing microparticles to the arterial thrombus (Stakos et al. 2015). Moreover, elevated levels of extracellular nucleosomes and myeloperoxidase-DNA complexes, the major hallmarks of NET release, are independently associated with coronary artery disease, a prothrombotic state, and occurrence of adverse cardiac events in patients (Borissoff et al. 2013).

The evidence presented in this thesis suggest novel mechanisms by which monocyte-neutrophil cooperation may contribute to arterial thrombus development. Following plaque rupture, the proinflammatory microenvironment of the atherosclerotic lesion is exposed to the circulation. The induction of NETosis and subsequent NET release, mediated by proinflammatory cytokines or localized cholesterol crystals, contributes to the procoagulant environment by

supporting the activation of the contact pathway of coagulation. In addition, exposed histones serve to reinforce monocyte-mediated TF coagulation of newly recruited monocytes by upregulating TF surface expression and activity. Histones may also contribute to a PS-rich environment by inducing PS on monocytes and endothelial cells. Furthermore, free or NET-associated histones may also serve to facilitate the growth of the platelet-rich thrombus by reinforcing additional localized platelet activation. Thus, targeting triggers of leukocyte-mediated coagulation may be beneficial to therapies that seek to prevent arterial thrombosis.

6.4.2 Venous thrombosis

The leukocyte-mediated procoagulant mechanisms described in this thesis may also contribute to the development of deep vein thrombosis (DVT). With an incidence of 2-3 cases per 1000 individuals, DVT and its major complication, pulmonary embolism, remain the third leading cause of cardiovascular death in the world (Goldhaber and Bounameaux 2012). Thrombi that form within the deep veins are typically rich in fibrin containing layers of platelets, red blood cells, and leukocytes. Previously, the cellular events contributing to DVT development have remained incompletely understood due to an absence of a relevant experimental model (Goldhaber and Bounameaux 2012). However, recent findings in mice suggest that aberrant activation of

leukocyte-mediated coagulation is a critical event in the initiation and propagation of DVT.

In particular, the formation of NETs have been demonstrated to be critical in DVT formation. NET components (namely DNA, histones, and neutrophil elastase) have been identified within venous thrombi in both murine and baboon models of DVT (Fuchs et al. 2010; Brill et al. 2012). In a murine stenosis model of DVT, PAD4 knockout mice exhibit reduced venous thrombi formation, and administration of DNase in wild-type mice significantly suppressed DVT growth, further implicating the participation of neutrophils and NETs in DVT (Martinod et al. 2013). Our findings suggest that the CFDNA component of NETs may not only promote thrombus formation by enhancing contact pathway activation, but CFDNA may also contribute to thrombus stability in DVT by impairing fibrinolysis via interactions with fibrin and plasmin. This is supported by observations that thrombi containing NETs are resistant to tPA-mediated fibrinolysis, an effect which is resolved by the co-incubation of DNase (Fuchs et al. 2012). Furthermore, histones, the other central component of NETs, has been reported to promote DVT formation following infusion in mice (Brill et al. 2012). Although platelets are less frequent in venous thrombi than in arterial thrombi, they support leukocyte procoagulant activity via neutrophil interaction and subsequent induction of NETosis (Clark et al. 2007).

The role of TF-mediated coagulation, though perhaps less vital in DVT than in arterial thrombosis, has recently been implicated in DVT propagation. Imaging studies have revealed that DVT is initiated as sterile inflammation with a massive influx of neutrophils and monocytes. In addition, this study demonstrated that intravascular activation of the extrinsic pathway via TF derived from myeloid cells is indispensable for DVT formation (Bruhl et al. 2012). Correspondingly, mice that were deficient in myeloid TF were protected from DVT formation. Our findings suggest that thrombus-resident NETs may facilitate the upregulation of myeloid TF via histone-myeloid interactions and contribute to TF-driven coagulation in DVT. Thus, the cross talk between monocytes and neutrophils may be responsible for the initiation and amplification of DVT and for inducing its unique clinical features.

6.4.3 Identification of signaling events in venous & arterial thrombosis

While NET release is conventionally triggered by pathogens or microbial components, it remains unclear what triggers NET formation in venous and arterial thrombosis. Likely stimuli include the interaction of neutrophils with activated cells, such as platelets or the endothelium (Clark et al. 2007; Massberg et al. 2010; Gupta et al. 2010). This could be confirmed if the neutrophil receptors involved in these interactions (i.e P-selectin) were inhibited and tested in animal models of DVT and atherothrombosis. Alternatively, environmental factors including hypoxia, ROS, cytokines, or coagulation factors

generated in thrombosis could induce NETosis. *In vitro*, generation of ROS typically precipitates NET formation by neutrophils and eosinophils (Fuchs et al. 2007; Yousefi et al. 2008). In addition, platelets may potentiate ROS production in platelet-neutrophil conjugates (Haselmayer et al. 2007), and endothelial ROS were shown to trigger NET formation *in vitro* (Gupta et al. 2010). Studies should move from purified neutrophils to more complex *in vitro* or *in vivo* systems which more accurately model DVT/atherothrombosis and include endothelium, platelets, and neutrophils under hypoxic and inflammatory experimental conditions.

6.5 Leukocyte targets for anti-thrombotic therapies

As discussed earlier, the procoagulant mechanisms that underlie pathological immunothrombosis are distinct from those that are responsible for hemostasis. Current strategies for the clinical management of thrombosis involve the use of anticoagulant therapies that target hemostatic coagulation pathways and are often associated with an increased bleeding risk. Recently, the role of leukocytes in the initiation of pathologic thrombosis has become more well-recognized, and this has prompted the development of therapeutic strategies that specifically target leukocyte-mediated coagulation reactions. Throughout this thesis, the use of various inhibitors of NETosis, CFDNA, and histones have revealed several important potential therapeutic avenues for thrombosis management.

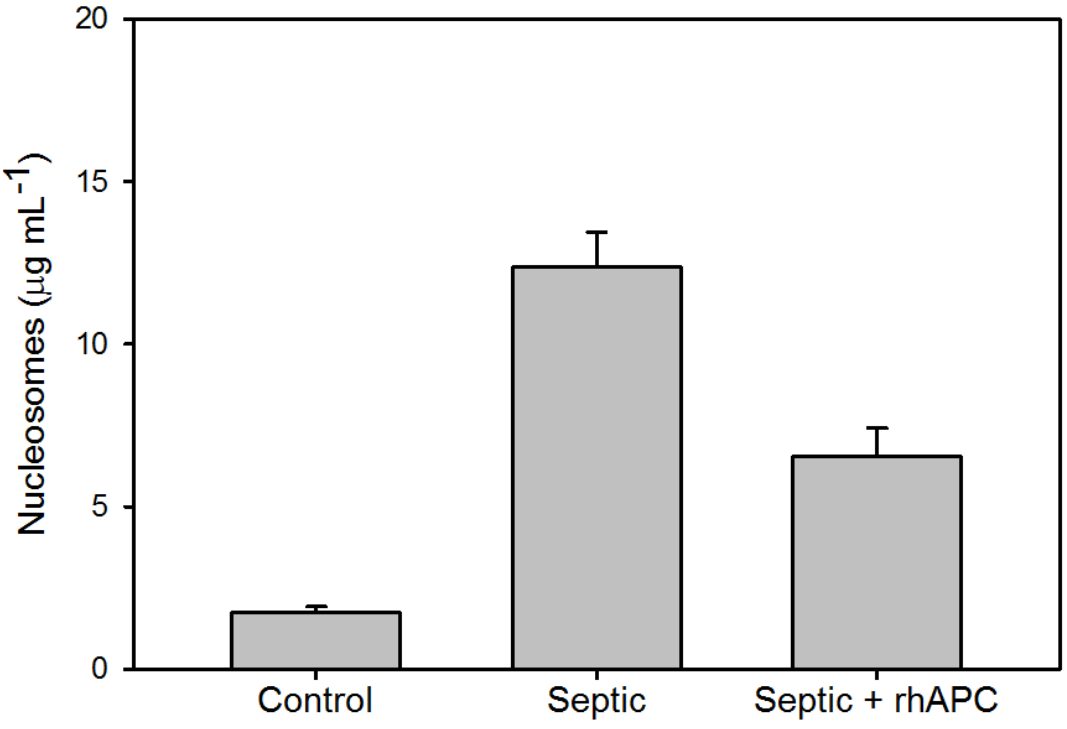
In Chapter 3, we explored the use of Cl-amidine, a non-specific inhibitor of peptidylarginine deiminases capable of blocking NET release from PMA-stimulated neutrophils. We demonstrated that Cl-amidine potently reduces thrombin generation induced by NETting neutrophils in both platelet-rich and platelet-poor plasmas. In other studies, the use of Cl-amidine has been shown to attenuate venous thrombus development and reduces atherosclerotic lesion areas in murine models (Knight et al. 2013; Knight et al. 2014). However, the complete inhibition of NET release may have deleterious consequences during infection. Using a PAD4 knockout model, (Li et al. 2010) show that mice with an impaired ability to generated NETs are more susceptible to bacterial infection. In contrast, a recent report by (Martinod et al. 2015) demonstrated that PAD4 deficiency and impaired NET formation is not associated with increased bacteremia in a polymicrobial sepsis model, and is in fact partially protective. Thus, therapeutic strategies that inhibit NET formation directly may be best suited for use in non-infectious pathologies.

Our studies also explored several therapies that decrease the procoagulant activity of histones by themselves, including APC, heparin, and neutralizing antibodies. Notably, recombinant APC was previously considered the first-line therapy for sepsis management prior to its withdrawal from the market following the negative results of the PROWESS clinical trial (Ranieri et al. 2012). In our studies, APC significantly diminished the activity of histone H3

towards platelets, resulting in diminished thrombin generation and polyphosphate release (Chapter 3). In addition, levels of circulating nucleosomes were markedly lower in severe sepsis patients receiving recombinant APC compared to those who did not (**Figure 6.3**). This suggests that, in addition to regulating thrombin generation via FVa and FVIIa inactivation, APC may also exert cytoprotective effects by interfering with extracellular histone activity.

Next, we investigated the effects of UFH as a potential histone inhibitor in our experiments. In Chapter 5, we have shown that UFH largely inhibits histone-mediated increases in TF activity on THP-1 cells and blood monocytes *in vitro*. Similarly, the plasmas of heparinated sepsis patients induced significantly less procoagulant activity on monocytes compared to septic plasmas without UFH. Heparins, namely low molecular weight heparins such as Dalteparin, are routinely administered to patients as part of standard thromboprophylaxis in sepsis. The studies performed in Chapter 5 have identified a new mechanism by which they exert protective effects. Though our studies utilized unfractionated heparin, it has recently been demonstrated that non-anticoagulant heparins retain their ability to bind and neutralize histones without altering coagulation parameters (Wildhagen et al. 2013), suggesting that heparins of various molecular weights are able to ameliorate histone activity.

Figure 6.3. **Effects of APC on plasma nucleosome concentrations**. Concentrations of circulating nucleosomes were determined in the plasma of healthy volunteers, sepsis patients receiving rhAPC, and sepsis patients receiving only standard care. Nucleosomes were determined using the Cell Death Detection ELISA PLUS kit from Roche Diagnostics.


20
15
10
5
0
Nucleosomes (μg mL-1)
Control
Septic
Septic + rhAPC

We also impaired histone-receptor interactions through the use of TLR2 and TLR4 inhibitory antibodies. In Chapter 3, we demonstrated that TLR2/4 blockade impaired histone-induced platelet activation and attenuated thrombin generation. Similarly, inhibition of monocyte TLR2/4 (as performed in Chapter 5) abrogated TF activity and diminished monocyte-mediated thrombin generation. These results are consistent with previous findings that identify TLR2 and TLR4 as the primary receptors for extracellular histones. However, despite promising preclinical findings, the use of anti-TLR strategies in patients has not been translated successfully to clinical practice (Rice et al. 2010; Opal et al. 2013). Since TLRs are known to be expressed on a multitude of cell types, and since histones have been documented to exert numerous differential effects, it may prove more efficient to target histones directly rather than attempting to modulate the growing number of cellular targets.

Therapeutic use of DNase may also be a viable strategy for targeting NET-mediated coagulation. In humans and mouse models, there is precedence for the therapeutic efficacy of DNase in several disease states. For example, in patients with cystic fibrosis, a condition often associated with *Pseudomonas aeruginosa* infection of the lung epithelium, inhalation of recombinant human DNase I reduces the viscosity of purulent sputum and inhibits bacterial biofilm formation (Hodson and Shah 1995). In a mouse model of systemic lupus erythematosus, intraperitoneal injection of recombinant mouse DNase interferes

with disease progression (Macanovic et al. 1996). Dismantling of NETs with DNase is also protective in models of flow-restricted venous thrombosis, as well as arterial thrombosis induced by photochemical injury in a murine model of chronic inflammation. However, in Chapter 3 we demonstrate that dismantling of the DNA scaffold with DNase results in the liberation of histones, resulting in enhanced platelet-dependent thrombin generation. This is consistent with the findings of (Meng et al. 2012) and (Mai et al. 2015), who both demonstrate that the early digestion of NETs with DNase in a mouse model of sepsis results in advanced sepsis progression, altered organ pathology, accompanied by an increase in mortality. Moreover, a recent report demonstrated that while DNase is effective at eliminating the DNA component of NETs in circulation, it fails to remove the majority of histones from the vessel wall and only partly reduces injury (Kolaczkowska et al. 2015). Thus, effective therapeutic strategies should attempt to target both the DNA and histone components of NETs simultaneously.

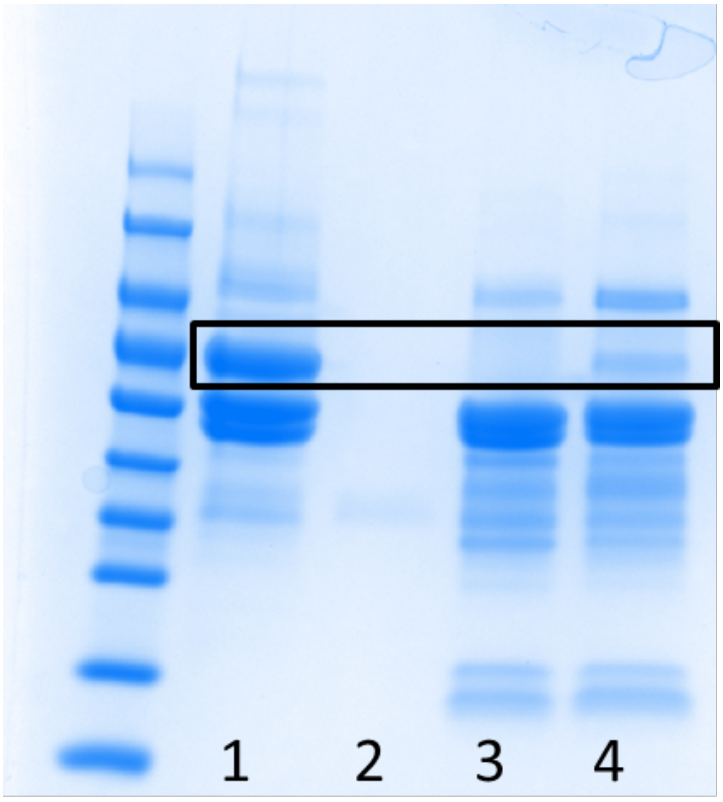
6.5.1 Investigating the anticoagulant effects of DNase

The administration of DNase is currently the most commonly employed method of degrading NETs *in vivo*. Many of these studies report the resolution of procoagulant activity and improved mortality following DNase therapy (Fuchs et al. 2010; Mai et al. 2015). It is presumed that the degradation of NETs facilitates the clearance of procoagulant CFDNA and histones and thus confers protective effects. However, our preliminary findings suggest that DNase may exert non-

specific activity towards circulating coagulation factors, namely fibrinogen. We incubated 20μg mL^{-1} of recombinant DNaseI with fibrinogen. As shown by **Figure 6.4**, co-incubation of fibrinogen with DNase results in the degradation of the Aα chain of fibrinogen which is integral to lateral fibrin polymerization. To confirm that Aα degradation is occurring, we can analyze excised protein bands using mass spectrometry. The identify of these fragments can be confirmed using peptide sequencing and, if necessary, Western blots. These studies can be repeated by incubating DNase with purified coagulation factors (such as FV, FVII, FVXI, and FXII) to determine if DNase exerts proteolytic effects towards these components of the coagulation cascade.

To explore the *in vivo* consequences of DNase administration, plasmas from mice infused with DNaseI can be analyzed. Using the thromboelastography (TEG) functional fibrinogen assay, clot strengths of plasmas obtained from mice with or without DNase can be determined. In addition, tail bleeding assays may be performed on the same cohorts of mice to determine if DNase administration contributes to an increased bleeding tendency. These studies could have important implications for the safety and efficacy of DNase administration as a thrombolytic therapy. It is possible that, instead of diminishing the procoagulant activity of NET components directly, studies that employ DNaseI as a therapeutic are in fact impairing hemostatic mechanisms.

Figure 6.4. **Effects of DNase on fibrinogen**. 20μg mL^{-1} of DNaseI was incubated for up to 24h with 5μg of fibrinogen. Lane 1: fibrinogen only; 2) DNase only; 3) fibrinogen after 12 hours with DNase; 4) fibrinogen after 24 hours with DNase. Analysis of DNase-mediated fibrinogen degradation was assessed by SDS-PAGE.


1
2
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4

7.0 Concluding remarks

Though the activation of coagulation cascades are essential for hemostasis and controlling infection, dysregulation of these pathways can result in severe, often fatal, consequences for the host. The findings presented in this thesis reveal novel biological mechanisms through which CFDNA and histones contribute to the development of immunothrombosis. First, CFDNA augments contact pathway-mediated coagulation in a FXII-dependent manner. Simultaneously, histones augment contact-pathway mediated coagulation via the activation of platelets and subsequent release of polyphosphate. Second, CFDNA but not histones modulate clot structure and delay fibrinolysis by forming a non-productive ternary complex comprised of fibrin, DNA, and plasmin. Finally, histones enhance monocyte TF activity by increasing surface TF antigen and phosphatidylserine exposure. These studies provide insights into the mechanisms that underlie coagulation abnormalities in sepsis, and identify CFDNA and/or histones as potential therapeutic targets in sepsis treatment. Furthermore, the procoagulant mechanisms that contribute to immunothrombosis in sepsis may also have important implications for pathological thrombus formation in large vessels, where growing evidence suggests that leukocyte-mediated coagulation activation may also play a role.

8.0 Bibliography

Abrams, S T, N Zhang, C Dart, S S Wang, J Thachil, Y Guan, G Wang, and C H Toh. 2013. “Human CRP Defends Against the Toxicity of Circulating Histones.” *The Journal of Immunology* 191 (5): 2495–2502. doi:10.4049/jimmunol.1203181.

Abrams, Simon T, Nan Zhang, Joanna Manson, Tingting Liu, Caroline Dart, Florence Baluwa, Susan Siyu Wang, et al. 2013. “Circulating Histones Are Mediators of Trauma-Associated Lung Injury.” *American Journal of Respiratory and Critical Care Medicine* 187 (2): 160–69. doi:10.1164/rccm.201206-1037OC.

Alcantara, F F, D J Iglehart, and R L Ochs. 1999. “Heparin in Plasma Samples Causes Nonspecific Binding to Histones on Western Blots..” *Journal of Immunological Methods* 226 (1-2): 11–18.

Allam, R, C R Scherbaum, M N Darisipudi, S R Mulay, H Hagele, J Lichtnekert, J H Hagemann, et al. 2012. “Histones From Dying Renal Cells Aggravate Kidney Injury via TLR2 and TLR4.” *Journal of the American Society of Nephrology* 23 (8): 1375–88. doi:10.1681/ASN.2011111077.

Ammollo, Concetta T, Fabrizio Semeraro, J Xu, N L Esmon, and C T Esmon. 2011. “Extracellular Histones Increase Plasma Thrombin Generation by Impairing Thrombomodulin-Dependent Protein C Activation.” *Journal of Thrombosis and Haemostasis* 9 (9): 1795–1803. doi:10.1111/j.1538-7836.2011.04422.x.

Amulic, Borko, Christel Cazalet, Garret L Hayes, Kathleen D Metzler, and Arturo Zychlinsky. 2012. “Neutrophil Function: From Mechanisms to Disease.” *Annual Review of Immunology* 30 (1): 459–89. doi:10.1146/annurev-immunol-020711-074942.

Angus, D C, and R S Wax. 2001. “Epidemiology of Sepsis: an Update..” *Critical Care Medicine* 29 (7 Suppl): S109–16.

Angus, D C, W T Linde-Zwirble, J Lidicker, G Clermont, J Carcillo, and M R Pinsky. 2001. “Epidemiology of Severe Sepsis in the United States: Analysis of Incidence, Outcome, and Associated Costs of Care..” *Critical Care Medicine* 29 (7): 1303–10.

Apostolov, Eugene O, Izoumroud Soultanova, Alena Savenka, Osman O Bagandov, Xiaoyan Yin, Anna G Stewart, Richard B Walker, and Alexei G Basnakian. 2009. “Deoxyribonuclease I Is Essential for DNA Fragmentation Induced by Gamma Radiation in Mice.” *Radiation Research* 172 (4): 481–92. doi:10.1667/RR1647.1.

Aras, O. 2004. “Induction of Microparticle- and Cell-Associated Intravascular Tissue Factor in Human Endotoxemia.” *Blood* 103 (12): 4545–53. doi:10.1182/blood-2003-03-0713.

Arnalich, Francisco, Maria Constanza Maldifassi, Enrique Ciria, Rosa Codoceo, Jaime Renart, Carmen Fernández-Capitán, Rafael Herruzo, Francisco Garcia-Rio, Eduardo López-Collazo, and Carmen Montiel. 2013. “Plasma Levels of Mitochondrial and Nuclear DNA in Patients with Massive Pulmonary Embolism in the Emergency Department: a Prospective Cohort Study.” *Critical Care* 17 (3). BioMed Central Ltd: R90. doi:10.1186/cc12735.

Arthur, Jane F, Elizabeth E Gardiner, Dermot Kenny, Robert K Andrews, and Michael C Berndt. 2008. “Platelet Receptor Redox Regulation.” *Platelets* 19 (1): 1–8. doi:10.1080/09537100701817224.

Asakura, H, Y Ontachi, T Mizutani, M Kato, T Ito, M Saito, E Morishita, et al. 2001. “Decreased Plasma Activity of Antithrombin or Protein C Is Not Due to Consumption Coagulopathy in Septic Patients with Disseminated Intravascular Coagulation..” *European Journal of Haematology* 67 (3): 170–75.

Atamaniuk, J, C Kopecky, S Skoupy, M D Saemann, and T Weichhart. 2012. “Apoptotic Cell-Free DNA Promotes Inflammation in Haemodialysis Patients.” *Nephrology Dialysis Transplantation* 27 (3): 902–5. doi:10.1093/ndt/gfr695.

Bach, R R. 2005. “Tissue Factor Encryption.” *Arteriosclerosis, Thrombosis, and Vascular Biology* 26 (3): 456–61. doi:10.1161/01.ATV.0000202656.53964.04.

Bach, R R, and C F Moldow. 1997. “Mechanism of Tissue Factor Activation on HL-60 Cells..” *Blood* 89 (9): 3270–76.

Bach, R, R Gentry, and Y Nemerson. 1986. “Factor VII Binding to Tissue Factor in Reconstituted Phospholipid Vesicles: Induction of Cooperativity by Phosphatidylserine..” *Biochemistry* 25 (14): 4007–20.

Baglin, Trevor. 2005. “The Measurement and Application of Thrombin Generation.” *British Journal of Haematology* 130 (5): 653–61. doi:10.1111/j.1365-2141.2005.05612.x.

Bakhtiari, Kamran, Joost C M Meijers, Evert de Jonge, and Marcel Levi. 2004. “Prospective Validation of the International Society of Thrombosis and Haemostasis Scoring System for Disseminated Intravascular Coagulation*.” *Critical Care Medicine* 32 (12): 2416–21. doi:10.1097/01.CCM.0000147769.07699.E3.

Barranco-Medina, S, N Pozzi, A D Vogt, and E Di Cera. 2013. “Histone H4 Promotes Prothrombin Autoactivation.” *Journal of Biological Chemistry* 288 (50): 35749–57. doi:10.1074/jbc.M113.509786.

Basavaraj, Manjunath Goolyam, Franz X Gruber, Mikhail Sovershaev, Hege I Appelbom, Bjarne Østerud, Lars C Petersen, and John-Bjarne Hansen. 2010. “The Role of TFPI in Regulation of TF-Induced Thrombogenicity on the Surface of Human Monocytes..” *Thrombosis Research* 126 (5): 418–25. doi:10.1016/j.thromres.2010.07.014.

Beiter, Katharina, Florian Wartha, Barbara Albiger, Staffan Normark, Arturo Zychlinsky, and Birgitta Henriques-Normark. 2006. “An Endonuclease Allows

Streptococcus Pneumoniae to Escape From Neutrophil Extracellular Traps." *Current Biology* 16 (4): 401–7. doi:10.1016/j.cub.2006.01.056.

Beiter, T, A Fragasso, J Hudemann, M Schild, J Steinacker, F C Mooren, and A M Niess. 2014. "Neutrophils Release Extracellular DNA Traps in Response to Exercise." *Journal of Applied Physiology* 117 (3): 325–33. doi:10.1152/japplphysiol.00173.2014.

Bereczky, Zsuzsanna, Kitti B Kovács, and László Muszbek. n.d. "Protein C and Protein S Deficiencies: Similarities and Differences Between Two Brothers Playing in the Same Game." *Clinical Chemistry and Laboratory Medicine* 48. doi:10.1515/CCLM.2010.369.

Bergmann, Simone, and Sven Hammerschmidt. 2007. "Fibrinolysis and Host Response in Bacterial Infections." *Thrombosis and Haemostasis*, October. doi:10.1160/TH07-02-0117.

Bermejo-Martín, Jesús F, Eduardo Tamayo, Gema Ruiz, David Andaluz-Ojeda, Rubén Herrán-Monge, Arturo Muriel-Bombín, Maria Fe Muñoz, et al. 2014. "Circulating Neutrophil Counts and Mortality in Septic Shock.." *Critical Care (London, England)* 18 (1): 407. doi:10.1186/cc13728.

Bernard, G R, J L Vincent, P F Laterre, S P LaRosa, J F Dhainaut, A Lopez-Rodriguez, J S Steingrub, et al. 2001. "Efficacy and Safety of Recombinant Human Activated Protein C for Severe Sepsis.." *The New England Journal of Medicine* 344 (10): 699–709. doi:10.1056/NEJM200103083441001.

Berndt, M C, P Metharom, and R K Andrews. 2014. "Primary Haemostasis: Newer Insights." *Haemophilia* 20 (April): 15–22. doi:10.1111/hae.12427.

Beutler, Bruce. 2007. "Neo-Ligands for Innate Immune Receptors and the Etiology of Sterile Inflammatory Disease.." *Immunological Reviews* 220 (December): 113–28. doi:10.1111/j.1600-065X.2007.00577.x.

Bianchi, M E. 2006. "DAMPs, PAMPs and Alarmins: All We Need to Know About Danger." *Journal of Leukocyte Biology* 81 (1): 1–5. doi:10.1189/jlb.0306164.

Biemond, B J, M Levi, H ten Cate, T van der Poll, H R Buller, C E Hack, and J W Ten Cate. 1995. "Plasminogen Activator and Plasminogen Activator Inhibitor I Release During Experimental Endotoxaemia in Chimpanzees: Effect of Interventions in the Cytokine and Coagulation Cascades.." *Clinical Science (London, England : 1979)* 88 (5): 587–94.

Biomarkers Definitions Working Group. 2001. "Biomarkers and Surrogate Endpoints: Preferred Definitions and Conceptual Framework.." *Clinical Pharmacology and Therapeutics* 69 (3): 89–95. doi:10.1067/mcp.2001.113989.

Blair, Price, and Robert Flaumenhaft. 2009. "Platelet A-Granules: Basic Biology and Clinical Correlates." *Blood Reviews* 23 (4): 177–89. doi:10.1016/j.blre.2009.04.001.

Blombäck, B, K Carlsson, B Hessel, A Liljeborg, R Procyk, and N Aslund. 1989. "Native Fibrin Gel Networks Observed by 3D Microscopy, Permeation and

Turbidity.." *Biochimica Et Biophysica Acta* 997 (1-2): 96–110.
Blombäck, B, K Carlsson, K Fatah, B Hessel, and R Procyk. 1994. "Fibrin in Human Plasma: Gel Architectures Governed by Rate and Nature of Fibrinogen Activation.." *Thrombosis Research* 75 (5): 521–38.
Borissoff, Julian I, Ivo A Joosen, Mathijs O Versteylen, Alexander Brill, Tobias A Fuchs, Alexander S Savchenko, Maureen Gallant, et al. 2013. "Elevated Levels of Circulating DNA and Chromatin Are Independently Associated with Severe Coronary Atherosclerosis and a Prothrombotic State.." *Arteriosclerosis, Thrombosis, and Vascular Biology* 33 (8): 2032–40. doi:10.1161/ATVBAHA.113.301627.
Bratton, Donna L, and Peter M Henson. 2011. "Neutrophil Clearance: When the Party Is Over, Clean-Up Begins." *Trends in Immunology* 32 (8): 350–57. doi:10.1016/j.it.2011.04.009.
Brill, A, T A Fuchs, A S Savchenko, G M Thomas, K Martinod, S F De Meyer, A A Bhandari, and D D Wagner. 2012. "Neutrophil Extracellular Traps Promote Deep Vein Thrombosis in Mice." *Journal of Thrombosis and Haemostasis* 10 (1): 136–44. doi:10.1111/j.1538-7836.2011.04544.x.
Brinkmann, Volker, Britta Laube, Ulrike Abu Abed, Christian Goosmann, and Arturo Zychlinsky. 2010. "Neutrophil Extracellular Traps: How to Generate and Visualize Them." *Journal of Visualized Experiments*, no. 36. doi:10.3791/1724.
Brinkmann, Volker, Ulrike Reichard, Christian Goosmann, Beatrix Fauler, Yvonne Uhlemann, David S Weiss, Yvette Weinrauch, and Arturo Zychlinsky. 2004. "Neutrophil Extracellular Traps Kill Bacteria.." *Science (New York, N.Y.)* 303 (5663): 1532–35. doi:10.1126/science.1092385.
Brogden, Kim A. 2005. "Antimicrobial Peptides: Pore Formers or Metabolic Inhibitors in Bacteria?." *Nature Reviews Microbiology* 3 (3): 238–50. doi:10.1038/nrmicro1098.
Broze, G J, L A Warren, W F Novotny, D A Higuchi, J J Girard, and J P Miletich. 1988. "The Lipoprotein-Associated Coagulation Inhibitor That Inhibits the Factor VII-Tissue Factor Complex Also Inhibits Factor Xa: Insight Into Its Possible Mechanism of Action.." *Blood* 71 (2): 335–43.
Broze, George J, and Thomas J Girard. 2012. "Tissue Factor Pathway Inhibitor: Structure-Function.." *Frontiers in Bioscience (Landmark Edition)* 17: 262–80.
Bruhl, von, M L, K Stark, A Steinhart, S Chandraratne, I Konrad, M Lorenz, A Khandoga, et al. 2012. "Monocytes, Neutrophils, and Platelets Cooperate to Initiate and Propagate Venous Thrombosis in Mice in Vivo." *Journal of Experimental Medicine* 209 (4): 819–35. doi:10.1161/ATVBAHA.109.185678.
Brummel-Ziedins, K E, M F Whelihan, M Gissel, K G Mann, and G E Rivard. 2009. "Thrombin Generation and Bleeding in Haemophilia A." *Haemophilia* 15 (5): 1118–25. doi:10.1111/j.1365-2516.2009.01994.x.
Buchanan, John T, Amelia J Simpson, Ramy K Aziz, George Y Liu, Sascha A

Kristian, Malak Kotb, James Feramisco, and Victor Nizet. 2006. “DNase Expression Allows the Pathogen Group a Streptococcus to Escape Killing in Neutrophil Extracellular Traps.” *Current Biology* 16 (4): 396–400. doi:10.1016/j.cub.2005.12.039.

Bunce, M W, R Toso, and R M Camire. 2011. “Zymogen-Like Factor Xa Variants Restore Thrombin Generation and Effectively Bypass the Intrinsic Pathway in Vitro.” *Blood* 117 (1): 290–98. doi:10.1182/blood-2010-08-300756.

Butenas, S. 2005. “Tissue Factor Activity in Whole Blood.” *Blood* 105 (7): 2764–70. doi:10.1182/blood-2004-09-3567.

Cadena, DeLa, R A, A Majluf-Cruz, A Stadnicki, J M Agosti, R W Colman, and A F Suffredini. 1996. “Activation of the Contact and Fibrinolytic Systems After Intravenous Administration of Endotoxin to Normal Human Volunteers: Correlation with the Cytokine Profile..” *Immunopharmacology* 33 (1-3): 231–37.

Calandra, T, J D Baumgartner, G E Grau, M M Wu, P H Lambert, J Schellekens, J Verhoef, and M P Glauser. 1990. “Prognostic Values of Tumor Necrosis Factor/Cachectin, Interleukin-1, Interferon-Alpha, and Interferon-Gamma in the Serum of Patients with Septic Shock. Swiss-Dutch J5 Immunoglobulin Study Group..” *The Journal of Infectious Diseases* 161 (5): 982–87.

Carr, M E, and J Hermans. 1978. “Size and Density of Fibrin Fibers From Turbidity..” *Macromolecules* 11 (1): 46–50.

Carr, M E, D A Gabriel, and J McDonagh. 1986. “Influence of Ca2+ on the Structure of Reptilase-Derived and Thrombin-Derived Fibrin Gels..” *The Biochemical Journal* 239 (3): 513–16.

Celi, A, G Pellegrini, R Lorenzet, A De Blasi, N Ready, B C Furie, and B Furie. 1994. “P-Selectin Induces the Expression of Tissue Factor on Monocytes..” *Proceedings of the National Academy of Sciences of the United States of America* 91 (19): 8767–71.

Celikel, Reha, Richard A McClintock, James R Roberts, G Loredana Mendolicchio, Jerry Ware, Kottayil I Varughese, and Zaverio M Ruggeri. 2003. “Modulation of Alpha-Thrombin Function by Distinct Interactions with Platelet Glycoprotein Ibalpha..” *Science (New York, N.Y.)* 301 (5630): 218–21. doi:10.1126/science.1084183.

Cermak, J, N S Key, R R Bach, J Balla, H S Jacob, and G M Vercellotti. 1993. “C-Reactive Protein Induces Human Peripheral Blood Monocytes to Synthesize Tissue Factor..” *Blood* 82 (2): 513–20.

Cesarman-Maus, Gabriela, and Katherine A Hajjar. 2005. “Molecular Mechanisms of Fibrinolysis.” *British Journal of Haematology* 129 (3): 307–21. doi:10.1111/j.1365-2141.2005.05444.x.

Chang, Christine P-Y, Rhu-Hsin Chia, Tsu-Lan Wu, Kuo-Chien Tsao, Chien-Feng Sun, and James T Wu. 2003. “Elevated Cell-Free Serum DNA Detected in Patients with Myocardial Infarction..” *Clinica Chimica Acta* 327 (1-2): 95–101.

Chang, J Y. 1985. “Thrombin Specificity. Requirement for Apolar Amino Acids Adjacent to the Thrombin Cleavage Site of Polypeptide Substrate..” *European Journal of Biochemistry / FEBS* 151 (2): 217–24.

Chapin, John C, and Katherine A Hajjar. 2015. “Fibrinolysis and the Control of Blood Coagulation..” *Blood Reviews* 29 (1): 17–24. doi:10.1016/j.blre.2014.09.003.

Cheng, Alice G, Molly McAdow, Hwan K Kim, Taeok Bae, Dominique M Missiakas, and Olaf Schneewind. 2010. “Contribution of Coagulases Towards Staphylococcus Aureus Disease and Protective Immunity.” Edited by Ambrose Cheung. *PLoS Pathogens* 6 (8): e1001036. doi:10.1371/journal.ppat.1001036.s008.

Cheng, Q, E I Tucker, M S Pine, I Sisler, A Matafonov, M f Sun, T C White-Adams, et al. 2010. “A Role for Factor XIIa-Mediated Factor XI Activation in Thrombus Formation in Vivo.” *Blood* 116 (19): 3981–89. doi:10.1182/blood-2010-02-270918.

Chernysh, Irina N, Chandrasekaran Nagaswami, and John W Weisel. 2011. “Visualization and Identification of the Structures Formed During Early Stages of Fibrin Polymerization..” *Blood* 117 (17): 4609–14. doi:10.1182/blood-2010-07-297671.

Choe, Jungwoo, Matthew S Kelker, and Ian A Wilson. 2005. “Crystal Structure of Human Toll-Like Receptor 3 (TLR3) Ectodomain..” *Science (New York, N.Y.)* 309 (5734): 581–85. doi:10.1126/science.1115253.

Choi, J-J, C F Reich, and D S Pisetsky. 2004. “Release of DNA From Dead and Dying Lymphocyte and Monocyte Cell Lines in Vitro..” *Scandinavian Journal of Immunology* 60 (1-2): 159–66. doi:10.1111/j.0300-9475.2004.01470.x.

Choi, Sharon H, Stephanie A Smith, and James H Morrissey. 2011. “Polyphosphate Is a Cofactor for the Activation of Factor XI by Thrombin..” *Blood* 118 (26): 6963–70. doi:10.1182/blood-2011-07-368811.

Chou, Janet, Nigel Mackman, Glenn Merrill-Skoloff, Brian Pedersen, Barbara C Furie, and Bruce Furie. 2004. “Hematopoietic Cell-Derived Microparticle Tissue Factor Contributes to Fibrin Formation During Thrombus Propagation..” *Blood* 104 (10): 3190–97. doi:10.1182/blood-2004-03-0935.

Christensen, Sean R, and Mark J Shlomchik. 2007. “Regulation of Lupus-Related Autoantibody Production and Clinical Disease by Toll-Like Receptors.” *Seminars in Immunology* 19 (1): 11–23. doi:10.1016/j.smim.2006.12.005.

Clark, Stephen R, Adrienne C Ma, Samantha A Tavener, Braedon McDonald, Zahra Goodarzi, Margaret M Kelly, Kamala D Patel, et al. 2007. “Platelet TLR4 Activates Neutrophil Extracellular Traps to Ensnare Bacteria in Septic Blood.” *Nature Medicine* 13 (4): 463–69. doi:10.1038/nm1565.

Clemetson, K J. 2011. “The Role of Platelets in Defence Against Pathogens.” *Hämostaseologie* 31 (4): 264–68. doi:10.5482/ha-1152.

Coenraad Hemker, H, Raed Al Dieri, Erik De Smedt, and Suzette Béguin. 2006.

"Thrombin Generation, a Function Test of the Haemostaticthrombotic System." *Thrombosis and Haemostasis*, October. doi:10.1160/TH06-07-0408.

Collins, Peter W, Luis I Macchiavello, Sarah J Lewis, Nichola J Macartney, Anton G Saayman, Roger Luddington, Trevor Baglin, and George P Findlay. 2006. "Global Tests of Haemostasis in Critically Ill Patients with Severe Sepsis Syndrome Compared to Controls.." *British Journal of Haematology* 135 (2): 220–27. doi:10.1111/j.1365-2141.2006.06281.x.

Colman, R W, and A H Schmaier. 1997. "Contact System: a Vascular Biology Modulator with Anticoagulant, Profibrinolytic, Antiadhesive, and Proinflammatory Attributes.." *Blood* 90 (10): 3819–43.

Crawley, J T B, S Zanardelli, C K N K Chion, and D A Lane. 2007. "The Central Role of Thrombin in Hemostasis.." *Journal of Thrombosis and Haemostasis : JTH* 5 Suppl 1 (July): 95–101. doi:10.1111/j.1538-7836.2007.02500.x.

Creagh, Emma M, and Luke A J O'Neill. 2006. "TLRs, NLRs and RLRs: a Trinity of Pathogen Sensors That Co-Operate in Innate Immunity." *Trends in Immunology* 27 (8): 352–57. doi:10.1016/j.it.2006.06.003.

Cui, Bei-Bei, Chun-Yu Tan, Christine Schorn, Hong-Hu Tang, Yi Liu, and Yi Zhao. 2012. "Neutrophil Extracellular Traps in Sterile Inflammation: the Story After Dying?." *Autoimmunity* 45 (8): 593–96. doi:10.3109/08916934.2012.719952.

Daleke, D L. 2002. "Regulation of Transbilayer Plasma Membrane Phospholipid Asymmetry." *The Journal of Lipid Research* 44 (2): 233–42. doi:10.1194/jlr.R200019-JLR200.

Day, Sharlene M, Jennifer L Reeve, Brian Pedersen, Diana M Farris, Daniel D Myers, Michael Im, Thomas W Wakefield, Nigel Mackman, and William P Fay. 2005. "Macrovascular Thrombosis Is Driven by Tissue Factor Derived Primarily From the Blood Vessel Wall.." *Blood* 105 (1): 192–98. doi:10.1182/blood-2004-06-2225.

de Waard, Vivian, Hjalmar R Hansen, Henri H M Spronk, Janneke J Timmerman, Hans Pannekoek, Sandrine Florquin, Pieter H Reitsma, and Hugo ten Cate. 2006. "Differential Expression of Tissue Factor mRNA and Protein Expression in Murine Sepsis. the Role of the Granulocyte Revisited." *Thrombosis and Haemostasis*, January. doi:10.1160/TH05-07-0512.

Degen, J L, T H Bugge, and J D Goguen. 2007. "Fibrin and Fibrinolysis in Infection and Host Defense.." *Journal of Thrombosis and Haemostasis : JTH* 5 Suppl 1 (July): 24–31. doi:10.1111/j.1538-7836.2007.02519.x.

Dellinger, R Phillip. 2003. "Inflammation and Coagulation: Implications for the Septic Patient.." *Clinical Infectious Diseases : an Official Publication of the Infectious Diseases Society of America* 36 (10): 1259–65. doi:10.1086/374835.

Dellinger, R Phillip, Mitchell M Levy, Jean M Carlet, Julian Bion, Margaret M Parker, Roman Jaeschke, Konrad Reinhart, et al. 2007. "Surviving Sepsis

Campaign: International Guidelines for Management of Severe Sepsis and Septic Shock: 2008." *Intensive Care Medicine* 34 (1): 17–60. doi:10.1007/s00134-007-0934-2.

Dhainaut, J F, S B Yan, D E Joyce, V Pettila, B Basson, J T Brandt, D P Sundin, and M Levi. 2004. "Treatment Effects of Drotrecogin Alfa (Activated) in Patients with Severe Sepsis with or Without Overt Disseminated Intravascular Coagulation.." *Journal of Thrombosis and Haemostasis : JTH* 2 (11): 1924–33. doi:10.1111/j.1538-7836.2004.00955.x.

Dhainaut, Jean-Francois, Andrew F Shorr, William L Macias, Marin J Kollef, Marcel Levi, Konrad Reinhart, and David R Nelson. 2005. "Dynamic Evolution of Coagulopathy in the First Day of Severe Sepsis: Relationship with Mortality and Organ Failure*." *Critical Care Medicine* 33 (2): 341–48. doi:10.1097/01.CCM.0000153520.31562.48.

Diaz, Jose Antonio, Tobias A Fuchs, Tatum O Jackson, Johanna A Kremer Hovinga, Bernhard Lämmle, Peter K Henke, Daniel D Myers Jr., Denisa D Wagner, and Thomas W Wakefield. 2013. "Plasma DNA Is Elevated in Patients with Deep Vein Thrombosis." *Journal of Vascular Surgery: Venous and Lymphatic Disorders* 1 (4): 341–41. doi:10.1016/j.jvsv.2012.12.002.

Diehl, Frank, Kerstin Schmidt, Michael A Choti, Katharine Romans, Steven Goodman, Meng Li, Katherine Thornton, et al. 2008. "Circulating Mutant DNA to Assess Tumor Dynamics.." *Nature Medicine* 14 (9): 985–90. doi:10.1038/nm.1789.

Dofferhoff, A S, V J Bom, H G de Vries-Hospers, J van Ingen, J vander Meer, B P Hazenberg, P O Mulder, and J Weits. 1992. "Patterns of Cytokines, Plasma Endotoxin, Plasminogen Activator Inhibitor, and Acute-Phase Proteins During the Treatment of Severe Sepsis in Humans.." *Critical Care Medicine* 20 (2): 185–92.

Dopheide, Sacha M, Mhairi J Maxwell, and Shaun P Jackson. 2002. "Shear-Dependent Tether Formation During Platelet Translocation on Von Willebrand Factor.." *Blood* 99 (1): 159–67.

Dwivedi, Dhruva J, Lisa J Toltl, Laura L Swystun, Janice Pogue, Kao-Lee Liaw, Jeffrey I Weitz, Deborah J Cook, Alison E Fox-Robichaud, Patricia C Liaw, and the Canadian Critical Care Translational Biology Group. 2012. "Prognostic Utility and Characterization of Cell-Free DNA in Patients with Severe Sepsis." *Critical Care* 16 (4). BioMed Central Ltd: R151. doi:10.1186/cc11466.

Egorina, E M, M A Sovershaev, J O Olsen, and B Osterud. 2007. "Granulocytes Do Not Express but Acquire Monocyte-Derived Tissue Factor in Whole Blood: Evidence for a Direct Transfer." *Blood* 111 (3): 1208–16. doi:10.1182/blood-2007-08-107698.

Eichinger, Sabine, Erich Minar, Christine Bialonczyk, Mirko Hirschl, Peter Quehenberger, Barbara Schneider, Ansgar Weltermann, Oswald Wagner,

and Paul A Kyrle. 2003. “D-Dimer Levels and Risk of Recurrent Venous Thromboembolism..” *JAMA : the Journal of the American Medical Association* 290 (8): 1071–74. doi:10.1001/jama.290.8.1071.

Elzagallaai, A, S D Rosé, and J M Trifaró. 2000. “Platelet Secretion Induced by Phorbol Esters Stimulation Is Mediated Through Phosphorylation of MARCKS: a MARCKS-Derived Peptide Blocks MARCKS Phosphorylation and Serotonin Release Without Affecting Pleckstrin Phosphorylation..” *Blood* 95 (3): 894–902.

Engelmann, Bernd, and Steffen Massberg. 2012. “Thrombosis as an Intravascular Effector of Innate Immunity.” *Nature Publishing Group* 13 (1). Nature Publishing Group: 34–45. doi:10.1038/nri3345.

Erickson, L A, G J Fici, J E Lund, T P Boyle, H G Polites, and K R Marotti. 1990. “Development of Venous Occlusions in Mice Transgenic for the Plasminogen Activator Inhibitor-1 Gene..” *Nature* 346 (6279): 74–76. doi:10.1038/346074a0.

Esmon, C T. 2001. “Protein C Anticoagulant Pathway and Its Role in Controlling Microvascular Thrombosis and Inflammation..” *Critical Care Medicine* 29 (7 Suppl): S48–51–discussion51–2.

Esmon, C T. 2011. “Extracellular Histones Zap Platelets.” *Blood* 118 (13): 3456–57. doi:10.1182/blood-2011-07-364380.

Esmon, C T. 2013. “Molecular Circuits in Thrombosis and Inflammation.” *Thrombosis and Haemostasis* 109 (3): 416–20. doi:10.1160/TH12-08-0634.

Esmon, Charles T. 2002. “Protein C Pathway in Sepsis..” *Annals of Medicine* 34 (7-8): 598–605.

Esmon, Charles T. 2003. “The Protein C Pathway..” *Chest* 124 (3 Suppl): 26S–32S.

Esper, Annette M, and Greg S Martin. 2009. “Extending International Sepsis Epidemiology: the Impact of Organ Dysfunction.” *Critical Care* 13 (1): 120. doi:10.1186/cc7704.

Fadeel, B, A Ahlin, J I Henter, S Orrenius, and M B Hampton. 1998. “Involvement of Caspases in Neutrophil Apoptosis: Regulation by Reactive Oxygen Species..” *Blood* 92 (12): 4808–18.

Falati, S, Liu, Q., Gross, P., Merrill-Skoloff, G., Chou, J., Vandendries, E., Celi, A., Croce, K., Furie, BC., and B. Furie. 2003. “Accumulation of Tissue Factor Into Developing Thrombi in Vivo Is Dependent Upon Microparticle P-Selectin Glycoprotein Ligand 1 and Platelet P-Selectin.” *Journal of Experimental Medicine* 197 (11): 1585–98. doi:10.1016/0092-8674(93)80055-J.

Farrera, C, and B Fadeel. 2013. “Macrophage Clearance of Neutrophil Extracellular Traps Is a Silent Process.” *The Journal of Immunology* 191 (5): 2647–56. doi:10.4049/jimmunol.1300436.

Fischer, S, and K T Preissner. 2013. “Extracellular Nucleic Acids as Novel Alarm Signals in the Vascular System.” *Hämostaseologie* 33 (1): 37–42.

doi:10.5482/HAMO-13-01-0001.
Fleck, R A, L V Rao, S I Rapaport, and N Varki. 1990. “Localization of Human Tissue Factor Antigen by Immunostaining with Monospecific, Polyclonal Anti-Human Tissue Factor Antibody..” *Thrombosis Research* 59 (2): 421–37.
Foley, J H, P Y Kim, N J Mutch, and A Gils. 2013. “Insights Into Thrombin Activatable Fibrinolysis Inhibitor Function and Regulation.” *Journal of Thrombosis and Haemostasis* 11 (June): 306–15. doi:10.1111/jth.12216.
Fourrier, F, C Chopin, J Goudemand, S Hendrycx, C Caron, A Rime, A Marey, and P Lestavel. 1992. “Septic Shock, Multiple Organ Failure, and Disseminated Intravascular Coagulation. Compared Patterns of Antithrombin III, Protein C, and Protein S Deficiencies..” *Chest* 101 (3): 816–23.
Franco, R F, E de Jonge, P E Dekkers, J J Timmerman, C A Spek, S J van Deventer, P van Deursen, et al. 2000. “The in Vivo Kinetics of Tissue Factor Messenger RNA Expression During Human Endotoxemia: Relationship with Activation of Coagulation..” *Blood* 96 (2): 554–59.
Fuchs, Tobias A, Alexander Brill, and Denisa D Wagner. 2012. “Neutrophil Extracellular Trap (NET) Impact on Deep Vein Thrombosis.” *Arteriosclerosis, Thrombosis, and Vascular Biology* 32 (8): 1777–83. doi:10.1161/ATVBAHA.111.242859.
Fuchs, Tobias A, Alexander Brill, Daniel Duerschmied, Daphne Schatzberg, Marc Monestier, Daniel D Myers, Shirley K Wrobleski, Thomas W Wakefield, John H Hartwig, and Denisa D Wagner. 2010. “Extracellular DNA Traps Promote Thrombosis..” *Proceedings of the National Academy of Sciences of the United States of America* 107 (36): 15880–85. doi:10.1073/pnas.1005743107.
Fuchs, Tobias A, U Abed, C Goosmann, R Hurwitz, I Schulze, V Wahn, Y Weinrauch, V Brinkmann, and A Zychlinsky. 2007. “Novel Cell Death Program Leads to Neutrophil Extracellular Traps.” *The Journal of Cell Biology* 176 (2): 231–41. doi:10.1083/jcb.200606027.
Furie, Bruce, and Barbara C Furie. 2008. “Mechanisms of Thrombus Formation..” *The New England Journal of Medicine* 359 (9): 938–49. doi:10.1056/NEJMra0801082.
Gabriel, C, W R McMaster, D Girard, and A Descoteaux. 2010. “Leishmania Donovani Promastigotes Evade the Antimicrobial Activity of Neutrophil Extracellular Traps.” *The Journal of Immunology* 185 (7): 4319–27. doi:10.4049/jimmunol.1000893.
Galligan, L, W Livingstone, Y Volkov, K Hokamp, C Murphy, M Lawler, K Fukudome, and O Smith. 2001. “Characterization of Protein C Receptor Expression in Monocytes..” *British Journal of Haematology* 115 (2): 408–14.
Gando, S, S Nanzaki, S Sasaki, and O Kemmotsu. 2015. “Significant Correlations Between Tissue Factor and Thrombin Markers in Trauma and Septic Patients with Disseminated Intravascular Coagulation..” *Thrombosis and Haemostasis* 79 (6): 1111–15.

Gando, Satoshi. 2010. “Microvascular Thrombosis and Multiple Organ Dysfunction Syndrome.” *Critical Care Medicine* 38 (February): S35–S42. doi:10.1097/CCM.0b013e3181c9e31d.

Gansler, Julia, Miriam Jaax, Silke Leiting, Bettina Appel, Andreas Greinacher, Silvia Fischer, and Klaus T Preissner. 2012. “Structural Requirements for the Procoagulant Activity of Nucleic Acids.” Edited by Michael P Bachmann. *PLoS ONE* 7 (11): e50399. doi:10.1371/journal.pone.0050399.g007.

Gatt, Alex, Joost J van Veen, Anita M Woolley, Steve Kitchen, Peter Cooper, and Michael Makris. 2008. “Thrombin Generation Assays Are Superior to Traditional Tests in Assessing Anticoagulation Reversal in Vitro.” *Thrombosis and Haemostasis*, July. doi:10.1160/TH07-05-0357.

Goldhaber, Samuel Z, and Henri Bounameaux. 2012. “Pulmonary Embolism and Deep Vein Thrombosis..” *Lancet* 379 (9828): 1835–46. doi:10.1016/S0140-6736(11)61904-1.

Gorkun, O V, Y I Veklich, L V Medved, A H Henschen, and J W Weisel. 1994. “Role of the Alpha C Domains of Fibrin in Clot Formation..” *Biochemistry* 33 (22): 6986–97.

Goto, S, D R Salomon, Y Ikeda, and Z M Ruggeri. 1995. “Characterization of the Unique Mechanism Mediating the Shear-Dependent Binding of Soluble Von Willebrand Factor to Platelets..” *The Journal of Biological Chemistry* 270 (40): 23352–61.

Gould, T J, T T Vu, L L Swystun, D J Dwivedi, S H C Mai, J I Weitz, and P C Liaw. 2014. “Neutrophil Extracellular Traps Promote Thrombin Generation Through Platelet-Dependent and Platelet-Independent Mechanisms.” *Arteriosclerosis, Thrombosis, and Vascular Biology* 34 (9): 1977–84. doi:10.1161/ATVBAHA.114.304114.

Gould, T J, T T Vu, A R Stafford, D J Dwivedi, P Y Kim, A E Fox-Robichaud, J I Weitz, and PC Liaw. 2015. “Cell-Free DNA Modulates Clot Structure and Impairs Fibrinolysis in Sepsis..” *Arteriosclerosis, Thrombosis, and Vascular Biology* 35 (12): 2544–53. doi:10.1161/ATVBAHA.115.306035.

Gregory, S A, J H Morrissey, and T S Edgington. 1989. “Regulation of Tissue Factor Gene Expression in the Monocyte Procoagulant Response to Endotoxin..” *Molecular and Cellular Biology* 9 (6): 2752–55.

Gross, Peter L, Barbara C Furie, Glenn Merrill-Skoloff, Janet Chou, and Bruce Furie. 2005. “Leukocyte-Versus Microparticle-Mediated Tissue Factor Transfer During Arteriolar Thrombus Development..” *Journal of Leukocyte Biology* 78 (6): 1318–26. doi:10.1189/jlb.0405193.

Gupta, Anurag Kumar, Manjunath B Joshi, Maria Philippova, Paul Erne, Paul Hasler, Sinuhe Hahn, and Therese J Resink. 2010. “Activated Endothelial Cells Induce Neutrophil Extracellular Traps and Are Susceptible to NETosis-Mediated Cell Death.” *FEBS Letters* 584 (14). Federation of European Biochemical Societies: 3193–97. doi:10.1016/j.febslet.2010.06.006.

Hampton, M B, A J Kettle, and C C Winterbourn. 1998. “Inside the Neutrophil Phagosome: Oxidants, Myeloperoxidase, and Bacterial Killing..” *Blood* 92 (9): 3007–17.

Harbarth, S, K Holeckova, C Froidevaux, D Pittet, B Ricou, G E Grau, L Vadas, J Pugin, Geneva Sepsis Network. 2001. “Diagnostic Value of Procalcitonin, Interleukin-6, and Interleukin-8 in Critically Ill Patients Admitted with Suspected Sepsis..” *American Journal of Respiratory and Critical Care Medicine* 164 (3): 396–402. doi:10.1164/ajrccm.164.3.2009052.

Harrison, David A, Catherine A Welch, and Jane M Eddleston. 2006. “The Epidemiology of Severe Sepsis in England, Wales and Northern Ireland, 1996 to 2004: Secondary Analysis of a High Quality Clinical Database, the ICNARC Case Mix Programme Database..” *Critical Care (London, England)* 10 (2): R42. doi:10.1186/cc4854.

Hartwig, J H. 1992. “Mechanisms of Actin Rearrangements Mediating Platelet Activation..” *The Journal of Cell Biology* 118 (6): 1421–42.

Haselmayer, P, L Grosse-Hovest, P von Landenberg, H Schild, and M P Radsak. 2007. “TREM-1 Ligand Expression on Platelets Enhances Neutrophil Activation.” *Blood* 110 (3): 1029–35. doi:10.1182/blood-2007-01-069195.

Heemskerk, Suzanne, Rosalinde Masereeuw, Olof Moesker, Martijn P W J M Bouw, Johannes G van der Hoeven, Wilbert H M Peters, Frans G M Russel, and Peter Pickkers. 2009. “Alkaline Phosphatase Treatment Improves Renal Function in Severe Sepsis or Septic Shock Patients*.” *Critical Care Medicine* 37 (2): 417–23. doi:10.1097/CCM.0b013e31819598af.

Henderson, S J, J Xia, H Wu, A R Stafford, B A Leslie, J C Fredenburgh, D A Weitz, and J I Weitz. 2016. “Zinc Promotes Clot Stability by Accelerating Clot Formation and Modifying Fibrin Structure.” *Thrombosis and Haemostasis* 115 (3). doi:10.1160/TH15-06-0462.

Hesselvik, J F, M Blombäck, B Brodin, and R Maller. 1989. “Coagulation, Fibrinolysis, and Kallikrein Systems in Sepsis: Relation to Outcome..” *Critical Care Medicine* 17 (8): 724–33.

Higuchi, D A, T C Wun, K M Likert, and G J Broze. 2003. “The Effect of Leukocyte Elastase on Tissue Factor Pathway Inhibitor..” *Blood* 79 (7): 1712–19.

Hodson, M E, and P L Shah. 1995. “DNase Trials in Cystic Fibrosis.” *European Respiratory Journal* 8 (10): 1786–91. doi:10.1183/09031936.95.08101786.

Hoffman, M, and D M Monroe. 2001. “A Cell-Based Model of Hemostasis..” *Thrombosis and Haemostasis* 85 (6): 958–65.

Hoffman, M, D M Monroe, J A Oliver, and H R Roberts. 1995. “Factors IXa and Xa Play Distinct Roles in Tissue Factor-Dependent Initiation of Coagulation..” *Blood* 86 (5): 1794–1801.

Hoffman, Maureane. 2003. “Remodeling the Blood Coagulation Cascade..” *Journal of Thrombosis and Thrombolysis* 16 (1-2): 17–20.

doi:10.1023/B:THRO.0000014588.95061.28.
Holvoet, P, H R Lijnen, and D Collen. 1985. “A Monoclonal Antibody Specific for Lys-Plasminogen. Application to the Study of the Activation Pathways of Plasminogen in Vivo..” *The Journal of Biological Chemistry* 260 (22): 12106–11.
Hotchkiss, Richard S, and Irene E Karl. 2003. “The Pathophysiology and Treatment of Sepsis..” *The New England Journal of Medicine* 348 (2): 138–50. doi:10.1056/NEJMra021333.
Hoylaerts, M, D C Rijken, H R Lijnen, and D Collen. 1982. “Kinetics of the Activation of Plasminogen by Human Tissue Plasminogen Activator. Role of Fibrin..” *The Journal of Biological Chemistry* 257 (6): 2912–19.
Huang, Hai, John Evankovich, Wei Yan, Gary Nace, Lemeng Zhang, Mark Ross, Xinghua Liao, et al. 2011. “Endogenous Histones Function as Alarmins in Sterile Inflammatory Liver Injury Through Toll-Like Receptor 9 in Mice.” *Hepatology* 54 (3): 999–1008. doi:10.1002/hep.24501.
Hung, D T, T K Vu, V I Wheaton, K Ishii, and S R Coughlin. 1992. “Cloned Platelet Thrombin Receptor Is Necessary for Thrombin-Induced Platelet Activation..” *Journal of Clinical Investigation* 89 (4): 1350–53. doi:10.1172/JCI115721.
Husak, Liudmila, Annette Marcuzzi, Jeremy Herring, Eugene Wen, Ling Yin, Dragos Daniel Capan, and Geta Cernat. 2010. “National Analysis of Sepsis Hospitalizations and Factors Contributing to Sepsis in-Hospital Mortality in Canada..” *Healthcare Quarterly (Toronto, Ont.)* 13 Spec No: 35–41.
Huynh, Mai-Lan N, Valerie A Fadok, and Peter M Henson. 2002. “Phosphatidylserine-Dependent Ingestion of Apoptotic Cells Promotes TGF-B1 Secretion and the Resolution of Inflammation.” *Journal of Clinical Investigation* 109 (1): 41–50. doi:10.1172/JCI200211638.
Iba, Toshiaki, Naoyuki Hashiguchi, Isao Nagaoka, Yoko Tabe, Katsuhiko Kadota, and Koichi Sato. 2015. “Heparins Attenuated Histone-Mediated Cytotoxicity in Vitro and Improved the Survival in a Rat Model of Histone-Induced Organ Dysfunction.” *Intensive Care Medicine Experimental*, December. Intensive Care Medicine Experimental, 1–11. doi:10.1186/s40635-015-0072-z.
Italiano, J E, and R A Shivdasani. 2003. “Megakaryocytes and Beyond: the Birth of Platelets..” *Journal of Thrombosis and Haemostasis : JTH* 1 (6): 1174–82.
Ito, T, K Kawahara, T Nakamura, S Yamada, T Nakamura, K Abeyama, T Hashiguchi, and I Maruyama. 2007. “High-Mobility Group Box 1 Protein Promotes Development of Microvascular Thrombosis in Rats..” *Journal of Thrombosis and Haemostasis : JTH* 5 (1): 109–16. doi:10.1111/j.1538-7836.2006.02255.x.
Ito, Takashi. 2014. “PAMPs and DAMPs as Triggers for DIC..” *Journal of Intensive Care* 2 (1): 67. doi:10.1186/s40560-014-0065-0.
Jackson, Shaun P. 2011. “Arterial Thrombosis—Insidious,Unpredictable and

Deadly." *Nature Medicine* 17 (11). Nature Publishing Group: 1423–36. doi:10.1038/nm.2515.

Jahr, S, H Hentze, S Englisch, D Hardt, F O Fackelmayer, R D Hesch, and R Knippers. 2001a. "DNA Fragments in the Blood Plasma of Cancer Patients: Quantitations and Evidence for Their Origin From Apoptotic and Necrotic Cells.." *Cancer Research* 61 (4): 1659–65.

Jahr, S, H Hentze, S Englisch, D Hardt, F O Fackelmayer, R D Hesch, and R Knippers. 2001b. "DNA Fragments in the Blood Plasma of Cancer Patients: Quantitations and Evidence for Their Origin From Apoptotic and Necrotic Cells.." *Cancer Research* 61 (4): 1659–65.

Janeway, Charles A, Jr., and Ruslan Medzhitov. 2002. "Innate Immune Recognition." *Annual Review of Immunology* 20 (1): 197–216. doi:10.1146/annurev.immunol.20.083001.084359.

Jiang, N. 2003. "Role of Macrophages in the Generation of Circulating Blood Nucleosomes From Dead and Dying Cells." *Blood* 102 (6): 2243–50. doi:10.1182/blood-2002-10-3312.

Jin, Jianguo, Todd M Quinton, Jin Zhang, Susan E Rittenhouse, and Satya P Kunapuli. 2002. "Adenosine Diphosphate (ADP)-Induced Thromboxane a(2) Generation in Human Platelets Requires Coordinated Signaling Through Integrin Alpha(IIb)Beta(3) and ADP Receptors.." *Blood* 99 (1): 193–98.

Johnson, L L, K N Berggren, F M Szaba, W Chen, and S T Smiley. 2003. "Fibrin-Mediated Protection Against Infection-Stimulated Immunopathology." *Journal of Experimental Medicine* 197 (6): 801–6. doi:10.1084/jem.20021493.

Joiner, K A, T Ganz, J Albert, and D Rotrosen. 1989. "The Opsonizing Ligand on Salmonella Typhimurium Influences Incorporation of Specific, but Not Azurophil, Granule Constituents Into Neutrophil Phagosomes.." *The Journal of Cell Biology* 109 (6 Pt 1): 2771–82.

Kannemeier, Christian, Aya Shibamiya, Fumie Nakazawa, Heidi Trusheim, Clemens Ruppert, Philipp Markart, Yutong Song, et al. 2007. "Extracellular RNA Constitutes a Natural Procoagulant Cofactor in Blood Coagulation.." *Proceedings of the National Academy of Sciences of the United States of America* 104 (15): 6388–93. doi:10.1073/pnas.0608647104.

Kaplan, A P, and M Silverberg. 1987. "The Coagulation-Kinin Pathway of Human Plasma.." *Blood* 70 (1): 1–15.

Karamanos, Nikos K. 2014. "Syndecans as Modulators and Potential Pharmacological Targets in Cancer Progression," January, 1–11. doi:10.3389/fonc.2014.00004/abstract.

Kasthuri, Raj, Sam Glover, Jeremiah Boles, and Nigel Mackman. 2010. "Tissue Factor and Tissue Factor Pathway Inhibitor as Key Regulators of Global Hemostasis: Measurement of Their Levels in Coagulation Assays." *Seminars in Thrombosis and Hemostasis* 36 (07): 764–71. doi:10.1055/s-0030-1265293.

Kaukonen, Kirsi-Maija, Michael Bailey, Satoshi Suzuki, David Pilcher, and Rinaldo Bellomo. 2014. “Mortality Related to Severe Sepsis and Septic Shock Among Critically Ill Patients in Australia and New Zealand, 2000-2012.” *JAMA : the Journal of the American Medical Association* 311 (13): 1308. doi:10.1001/jama.2014.2637.

Kawasaki, H, and S Iwamuro. 2008. “Potential Roles of Histones in Host Defense as Antimicrobial Agents..” *Infectious Disorders Drug Targets* 8 (3): 195–205.

Kenny, Elaine F, and Luke A J O'Neill. 2008. “Signalling Adaptors Used by Toll-Like Receptors: an Update.” *Cytokine* 43 (3): 342–49. doi:10.1016/j.cyto.2008.07.010.

Kessenbrock, Kai, Markus Krumbholz, Ulf Schönermarck, Walter Back, Wolfgang L Gross, Zena Werb, Hermann-Josef Gröne, Volker Brinkmann, and Dieter E Jenne. 2009. “Netting Neutrophils in Autoimmune Small-Vessel Vasculitis.” *Nature Medicine* 15 (6): 623–25. doi:10.1038/nm.1959.

Kim, P Y, R J Stewart, S M Lipson, and M E Nesheim. 2007. “The Relative Kinetics of Clotting and Lysis Provide a Biochemical Rationale for the Correlation Between Elevated Fibrinogen and Cardiovascular Disease..” *Journal of Thrombosis and Haemostasis : JTH* 5 (6): 1250–56. doi:10.1111/j.1538-7836.2007.02426.x.

Kleine, T J, A Gladfelter, P N Lewis, and S A Lewis. 1995. “Histone-Induced Damage of a Mammalian Epithelium: the Conductive Effect..” *The American Journal of Physiology* 268 (5 Pt 1): C1114–25.

Kleinjan, Ankie, Anita N Böing, Auguste Sturk, and Rienk Nieuwland. 2012. “Thrombosis Research.” *Thrombosis Research* 130 (S1). Elsevier Ltd: S71–S73. doi:10.1016/j.thromres.2012.08.281.

Kleinschnitz, C. 2006. “Targeting Coagulation Factor XII Provides Protection From Pathological Thrombosis in Cerebral Ischemia Without Interfering with Hemostasis.” *Journal of Experimental Medicine* 203 (3): 513–18. doi:10.1084/jem.20052458.

Klune, John R, Rajeev Dhupar, Jon Cardinal, Timothy R Billiar, and Allan Tsung. 2008. “HMGB1: Endogenous Danger Signaling..” *Molecular Medicine (Cambridge, Mass.)* 14 (7-8): 476–84. doi:10.2119/2008-00034.Klune.

Knight, J S, W Luo, A A O'Dell, S Yalavarthi, W Zhao, V Subramanian, C Guo, et al. 2014. “Peptidylarginine Deiminase Inhibition Reduces Vascular Damage and Modulates Innate Immune Responses in Murine Models of Atherosclerosis.” *Circulation Research* 114 (6): 947–56. doi:10.1161/CIRCRESAHA.114.303312.

Knight, Jason S, Wenpu Zhao, Wei Luo, Venkataraman Subramanian, Alexander A O'Dell, Srilakshmi Yalavarthi, Jeffrey B Hodgin, Daniel T Eitzman, Paul R Thompson, and Mariana J Kaplan. 2013. “Peptidylarginine Deiminase Inhibition Is Immunomodulatory and Vasculoprotective in Murine Lupus..” *Journal of Clinical Investigation* 123 (7): 2981–93. doi:10.1172/JCI67390.

Kobayashi, M, K Shimada, and T Ozawa. 1990. “Human Recombinant Interleukin-1 Beta- and Tumor Necrosis Factor Alpha-Mediated Suppression of Heparin-Like Compounds on Cultured Porcine Aortic Endothelial Cells..” *Journal of Cellular Physiology* 144 (3): 383–90. doi:10.1002/jcp.1041440304.

Kolaczkowska, Elzbieta, Craig N Jenne, Bas G J Surewaard, Ajitha Thanabalasuriar, Woo-Yong Lee, Maria-Jesus Sanz, Kerri Mowen, Ghislain Opdenakker, and Paul Kubes. 2015. “Molecular Mechanisms of NET Formation Anddegradation Revealed by Intravital Imaging in Theliver Vasculature.” *Nature Communications* 6 (March). Nature Publishing Group: 1–13. doi:10.1038/ncomms7673.

Komissarov, Andrey A, Galina Florova, and Steven Idell. 2011. “Effects of Extracellular DNA on Plasminogen Activation and Fibrinolysis..” *The Journal of Biological Chemistry* 286 (49): 41949–62. doi:10.1074/jbc.M111.301218.

Konings, J, J W P Govers-Riemslag, H Philippou, N J Mutch, J I Borissoff, P Allan, S Mohan, G Tans, H ten Cate, and R A S Ariens. 2011. “Factor XIIa Regulates the Structure of the Fibrin Clot Independently of Thrombin Generation Through Direct Interaction with Fibrin.” *Blood* 118 (14): 3942–51. doi:10.1182/blood-2011-03-339572.

Krem, Maxwell M, and Enrico Di Cera. 2002. “Evolution of Enzyme Cascades From Embryonic Development to Blood Coagulation..” *Trends in Biochemical Sciences* 27 (2): 67–74.

Krishnaswamy, S, K A Field, T S Edgington, J H Morrissey, and K G Mann. 1992. “Role of the Membrane Surface in the Activation of Human Coagulation Factor X..” *The Journal of Biological Chemistry* 267 (36): 26110–20.

Kumar, Himanshu, Taro Kawai, and Shizuo Akira. 2011. “Pathogen Recognition by the Innate Immune System.” *International Reviews of Immunology* 30 (1): 16–34. doi:10.3109/08830185.2010.529976.

Kumar, S V R, O P Kulkarni, S R Mulay, M N Darisipudi, S Romoli, D Thomasova, C R Scherbaum, et al. 2015. “Neutrophil Extracellular Trap-Related Extracellular Histones Cause Vascular Necrosis in Severe GN.” *Journal of the American Society of Nephrology* 26 (10): 2399–2413. doi:10.1681/ASN.2014070673.

Lehnardt, S, E Schott, T Trimbuch, D Laubisch, C Krueger, G Wulczyn, R Nitsch, and J R Weber. 2008. “A Vicious Cycle Involving Release of Heat Shock Protein 60 From Injured Cells and Activation of Toll-Like Receptor 4 Mediates Neurodegeneration in the CNS.” *Journal of Neuroscience* 28 (10): 2320–31. doi:10.1523/JNEUROSCI.4760-07.2008.

Levi, M. 2010. “The Coagulant Response in Sepsis and Inflammation..” *Hämostaseologie* 30 (1): 10–2–14–6.

Levi, M, and H ten Cate. 1999. “Disseminated Intravascular Coagulation..” *New England Journal of Medicine* 341 (8): 586–92. doi:10.1056/NEJM199908193410807.

Levi, Marcel, Evert de Jonge, and Tom van der Poll. 2003. “Sepsis and Disseminated Intravascular Coagulation..” *Journal of Thrombosis and Thrombolysis* 16 (1-2): 43–47. doi:10.1023/B:THRO.0000014592.27892.11.

Levi, Marcel, Marcus Schultz, and Tom van der Poll. 2013. “Sepsis and Thrombosis.” *Seminars in Thrombosis and Hemostasis* 39 (05): 559–66. doi:10.1055/s-0033-1343894.

Levy, Mitchell M, Mitchell P Fink, John C Marshall, Edward Abraham, Derek Angus, Deborah Cook, Jonathan Cohen, Steven M Opal, Jean-Louis Vincent, and Graham Ramsay. 2003. “2001 SCCM/ESICM/ACCP/ATS/SIS International Sepsis Definitions Conference.” *Critical Care Medicine* 31 (4): 1250–56. doi:10.1097/01.CCM.0000050454.01978.3B.

Li, P, M Li, M R Lindberg, M J Kennett, N Xiong, and Y Wang. 2010. “PAD4 Is Essential for Antibacterial Innate Immunity Mediated by Neutrophil Extracellular Traps.” *Journal of Experimental Medicine* 207 (9): 1853–62. doi:10.1084/jem.20100239.

Liaw, P C Y. 2004. “Patients with Severe Sepsis Vary Markedly in Their Ability to Generate Activated Protein C.” *Blood* 104 (13): 3958–64. doi:10.1182/blood-2004-03-1203.

Liou, T G, and E J Campbell. 1996. “Quantum Proteolysis Resulting From Release of Single Granules by Human Neutrophils: a Novel, Nonoxidative Mechanism of Extracellular Proteolytic Activity..” *Journal of Immunology (Baltimore, Md. : 1950)* 157 (6): 2624–31.

Lisman, Ton, Philip G De Groot, Joost C M Meijers, and Frits R Rosendaal. 2005. “Reduced Plasma Fibrinolytic Potential Is a Risk Factor for Venous Thrombosis..” *Blood* 105 (3): 1102–5. doi:10.1182/blood-2004-08-3253.

Litvinov, R I. 2005. “Polymerization of Fibrin: Specificity, Strength, and Stability of Knob-Hole Interactions Studied at the Single-Molecule Level.” *Blood* 106 (9): 2944–51. doi:10.1182/blood-2005-05-2039.

Litvinov, R I, O V Gorkun, D K Galanakis, S Yakovlev, L MEDVED, H Shuman, and J W Weisel. 2007. “Polymerization of Fibrin: Direct Observation and Quantification of Individual B:B Knob-Hole Interactions.” *Blood* 109 (1): 130–38. doi:10.1182/blood-2006-07-033910.

Lo, Y M, T H Rainer, L Y Chan, N M Hjelm, and R A Cocks. 2000. “Plasma DNA as a Prognostic Marker in Trauma Patients..” *Clinical Chemistry* 46 (3): 319–23.

Long, Andrew T, Ellinor Kenne, Roman Jung, Tobias A Fuchs, and Thomas Renné. 2015. “Contact System Revisited: an Interface Between Inflammation, Coagulation, and Innate Immunity.” *Journal of Thrombosis and Haemostasis*, December, n/a–n/a. doi:10.1111/jth.13235.

Longstaff, C, I Varju, P Sotonyi, L Szabo, M Krumrey, A Hoell, A Bota, Z Varga, E Komorowicz, and K Kolev. 2013. “Mechanical Stability and Fibrinolytic Resistance of Clots Containing Fibrin, DNA, and Histones.” *Journal of*

Biological Chemistry 288 (10): 6946–56. doi:10.1074/jbc.M112.404301.
Loof, T G, M Morgelin, L Johansson, S Oehmcke, A I Olin, G Dickneite, A Norrby-Teglund, U Theopold, and H Herwald. 2011. "Coagulation, an Ancestral Serine Protease Cascade, Exerts a Novel Function in Early Immune Defense." *Blood* 118 (9): 2589–98. doi:10.1182/blood-2011-02-337568.
Lord, Susan T. 2007. "Fibrinogen and Fibrin: Scaffold Proteins in Hemostasis.." *Current Opinion in Hematology* 14 (3): 236–41. doi:10.1097/MOH.0b013e3280dce58c.
Lorente, Leonardo, María M Martín, Juan M Borreguero-León, Jordi Solé-Violán, José Ferreres, Lorenzo Labarta, César Díaz, Alejandro Jiménez, and José A Páramo. 2014. "Thrombosis Research." *Thrombosis Research* 134 (1). Elsevier Ltd: 182–86. doi:10.1016/j.thromres.2014.04.013.
Lu, Thea, Scott D Kobayashi, Mark T Quinn, and Frank R DeLeo. 2012. "A NET Outcome.." *Frontiers in Immunology* 3: 365. doi:10.3389/fimmu.2012.00365.
Luo, D, F M Szaba, L W Kummer, E F Plow, N Mackman, D Gailani, and S T Smiley. 2011. "Protective Roles for Fibrin, Tissue Factor, Plasminogen Activator Inhibitor-1, and Thrombin Activatable Fibrinolysis Inhibitor, but Not Factor XI, During Defense Against the Gram-Negative Bacterium Yersinia Enterocolitica." *The Journal of Immunology* 187 (4): 1866–76. doi:10.4049/jimmunol.1101094.
Luong, T T, and C Y Lee. 2002. "Overproduction of Type 8 Capsular Polysaccharide Augments Staphylococcus Aureus Virulence." *Infection and Immunity* 70 (7): 3389–95. doi:10.1128/IAI.70.7.3389-3395.2002.
Lupu, C, E Poulsen, S Roquefeuil, A D Westmuckett, V V Kakkar, and F LUPU. 1999. "Cellular Effects of Heparin on the Production and Release of Tissue Factor Pathway Inhibitor in Human Endothelial Cells in Culture.." *Arteriosclerosis, Thrombosis, and Vascular Biology* 19 (9): 2251–62.
Ma, A C, and P Kubes. 2008. "Platelets, Neutrophils, and Neutrophil Extracellular Traps (NETs) in Sepsis." *Journal of Thrombosis and Haemostasis* 6 (3): 415–20. doi:10.1111/j.1538-7836.2007.02865.x.
Maas, Coen, Chris Oschatz, and Thomas Renné. 2011. "The Plasma Contact System 2.0." *Seminars in Thrombosis and Hemostasis* 37 (04): 375–81. doi:10.1055/s-0031-1276586.
Macanovic, M, D Sinicropi, S Shak, S Baughman, S Thiru, and P J Lachmann. 1996. "The Treatment of Systemic Lupus Erythematosus (SLE) in NZB/W F1 Hybrid Mice; Studies with Recombinant Murine DNase and with Dexamethasone.." *Clinical and Experimental Immunology* 106 (2): 243–52.
Mackman, N. 2004. "Role of Tissue Factor in Hemostasis, Thrombosis, and Vascular Development." *Arteriosclerosis, Thrombosis, and Vascular Biology* 24 (6): 1015–22. doi:10.1161/01.ATV.0000130465.23430.74.
Madoiwa, Seiji, Shin Nunomiya, Tomoko Ono, Yuichi Shintani, Tsukasa Ohmori, Jun Mimuro, and Yoichi Sakata. 2006. "Plasminogen Activator Inhibitor 1

Promotes a Poor Prognosis in Sepsis-Induced Disseminated Intravascular Coagulation." *International Journal of Hematology* 84 (5): 398–405. doi:10.1532/IJH97.05190.

Mai, Safiah H C, Momina Khan, Dhruva J Dwivedi, Catherine A Ross, Ji Zhou, Travis J Gould, Peter L Gross, Jeffrey I Weitz, Alison E Fox-Robichaud, and Patricia C Liaw. 2015. "Delayed but Not Early Treatment with DNase Reduces Organ Damage and Improves Outcome in a Murine Model of Sepsis." *Shock*, May, 1. doi:10.1097/SHK.0000000000000396.

Mallat, Z, B Hugel, J Ohan, G Lesèche, J M Freyssinet, and A Tedgui. 1999. "Shed Membrane Microparticles with Procoagulant Potential in Human Atherosclerotic Plaques: a Role for Apoptosis in Plaque Thrombogenicity.." *Circulation* 99 (3): 348–53.

Manfredi, Angelo A, and Patrizia Rovere-Querini. 2010. "The Mitochondrion--a Trojan Horse That Kicks Off Inflammation?." *The New England Journal of Medicine* 362 (22): 2132–34. doi:10.1056/NEJMcibr1003521.

Mani, K. 2006. "Defective Nitric Oxide-Dependent, Deaminative Cleavage of Glypican-1 Heparan Sulfate in Niemann-Pick C1 Fibroblasts." *Glycobiology* 16 (8): 711–18. doi:10.1093/glycob/cwj121.

Margraf, Stefan, Tim Lögters, Jenny Reipen, Jens Altrichter, Martin Scholz, and Joachim Windolf. 2008. "Neutrophil-derived circulating free DNA (cfDNA/NETs)." *Shock* 30 (4): 352–58. doi:10.1097/SHK.0b013e31816a6bb1.

Marshall, John C. 2014. "Why Have Clinical Trials in Sepsis Failed?." *Trends in Molecular Medicine* 20 (4). Elsevier Ltd: 195–203. doi:10.1016/j.molmed.2014.01.007.

Marshall, John C, and Konrad Reinhart. 2009. "Biomarkers of Sepsis." *Critical Care Medicine* 37 (7): 2290–98. doi:10.1097/CCM.0b013e3181a02afc.

Martin, Greg S, David M Mannino, Stephanie Eaton, and Marc Moss. 2003. "The Epidemiology of Sepsis in the United States From 1979 Through 2000.." *The New England Journal of Medicine* 348 (16): 1546–54. doi:10.1056/NEJMoa022139.

Martinod, Kimberly, Mélanie Demers, Tobias A Fuchs, Siu Ling Wong, Alexander Brill, Maureen Gallant, Jing Hu, Yanming Wang, and Denisa D Wagner. 2013. "Neutrophil Histone Modification by Peptidylarginine Deiminase 4 Is Critical for Deep Vein Thrombosis in Mice.." *Proceedings of the National Academy of Sciences of the United States of America* 110 (21): 8674–79. doi:10.1073/pnas.1301059110.

Martinod, Kimberly, Tobias A Fuchs, Naamah L Zitomersky, Siu Ling Wong, Mélanie Demers, Maureen Gallant, Yanming Wang, and Denisa D Wagner. 2015. "PAD4-Deficiency Does Not Affect Bacteremia in Polymicrobial Sepsis and Ameliorates Endotoxemic Shock.." *Blood* 125 (12): 1948–56. doi:10.1182/blood-2014-07-587709.

Massberg, Steffen, Lenka Grahl, Marie-Luise von Bruehl, Davit Manukyan,

Susanne Pfeiler, Christian Goosmann, Volker Brinkmann, et al. 2010. "Reciprocal Coupling of Coagulation and Innate Immunity via Neutrophil Serine Proteases." *Nature Medicine* 16 (8). Nature Publishing Group: 887–96. doi:10.1038/nm.2184.

Massion, Paul B, Pierre Peters, Didier Ledoux, Valentine Zimermann, Jean-Luc Canivet, Pierre P Massion, Pierre Damas, and André Gothot. 2012. "Persistent Hypocoagulability in Patients with Septic Shock Predicts Greater Hospital Mortality: Impact of Impaired Thrombin Generation." *Intensive Care Medicine* 38 (8): 1326–35. doi:10.1007/s00134-012-2620-2.

Masson-Bessiere, C, M Sebbag, E Girbal-Neuhauser, L Nogueira, C Vincent, T Senshu, and G Serre. 2001. "The Major Synovial Targets of the Rheumatoid Arthritis-Specific Antifilaggrin Autoantibodies Are Deiminated Forms of the - and -Chains of Fibrin." *The Journal of Immunology* 166 (6): 4177–84. doi:10.4049/jimmunol.166.6.4177.

Maugeri, N, M Brambilla, M Camera, A Carbone, E Tremoli, M B Donati, G de Gaetano, and C Cerletti. 2006. "Human Polymorphonuclear Leukocytes Produce and Express Functional Tissue Factor Upon Stimulation.." *Journal of Thrombosis and Haemostasis : JTH* 4 (6): 1323–30. doi:10.1111/j.1538-7836.2006.01968.x.

Maynard, J R, C A Heckman, F A Pitlick, and Y Nemerson. 1975. "Association of Tissue Factor Activity with the Surface of Cultured Cells.." *Journal of Clinical Investigation* 55 (4): 814–24. doi:10.1172/JCI107992.

Mayr, Florian B, Sachin Yende, Walter T Linde-Zwirble, Octavia M Peck-Palmer, Amber E Barnato, Lisa A Weissfeld, and Derek C Angus. 2010. "Infection Rate and Acute Organ Dysfunction Risk as Explanations for Racial Differences in Severe Sepsis.." *JAMA : the Journal of the American Medical Association* 303 (24): 2495–2503. doi:10.1001/jama.2010.851.

McCachren, S S, J Diggs, J B Weinberg, and W A Dittman. 1991. "Thrombomodulin Expression by Human Blood Monocytes and by Human Synovial Tissue Lining Macrophages.." *Blood* 78 (12): 3128–32.

McGee, M P, and L C Li. 1991. "Functional Difference Between Intrinsic and Extrinsic Coagulation Pathways. Kinetics of Factor X Activation on Human Monocytes and Alveolar Macrophages.." *The Journal of Biological Chemistry* 266 (13): 8079–85.

McGee, M P, L C Li, and M Hensler. 1992. "Functional Assembly of Intrinsic Coagulation Proteases on Monocytes and Platelets. Comparison Between Cofactor Activities Induced by Thrombin and Factor Xa.." *The Journal of Experimental Medicine* 176 (1): 27–35.

McGee, M P, S Foster, and X Wang. 1994. "Simultaneous Expression of Tissue Factor and Tissue Factor Pathway Inhibitor by Human Monocytes. a Potential Mechanism for Localized Control of Blood Coagulation.." *The Journal of Experimental Medicine* 179 (6): 1847–54.

MD, Corey E Ventetuolo, and Mitchell M Levy MD. 2008. “Biomarkers: Diagnosis and Risk Assessment in Sepsis.” *Clinics in Chest Medicine* 29 (4). Elsevier Ltd: 591–603. doi:10.1016/j.ccm.2008.07.001.

Medina, Oliver Goldmann and Eva. 2013. “The Expanding World of Extracellular Traps: Not Only Neutrophils but Much More,” January, 1–10. doi:10.3389/fimmu.2012.00420/abstract.

Medved, L V, O V Gorkun, and P L Privalov. 1983. “Structural Organization of C-Terminal Parts of Fibrinogen a Alpha-Chains..” *FEBS Letters* 160 (1-2): 291–95.

Medved, L, J W Weisel, on behalf of fibrinogen and factor XIII subcommittee of the scientific standardization committee of the International Society on Thrombosis and Haemostasis. 2009. “Recommendations for Nomenclature on Fibrinogen and Fibrin.” *Journal of Thrombosis and Haemostasis* 7 (2): 355–59. doi:10.1111/j.1538-7836.2008.03242.x.

Medved, L, T Ugarova, Y Veklich, N Lukinova, and J Weisel. 1990. “Electron Microscope Investigation of the Early Stages of Fibrin Assembly. Twisted Protofibrils and Fibers..” *Journal of Molecular Biology* 216 (3): 503–9. doi:10.1016/0022-2836(90)90376-W.

Medzhitov, R, P Preston-Hurlburt, and C A Janeway. 1997. “A Human Homologue of the Drosophila Toll Protein Signals Activation of Adaptive Immunity..” *Nature* 388 (6640): 394–97. doi:10.1038/41131.

Medzhitov, Ruslan. 2008. “Origin and Physiological Roles of Inflammation.” *Nature* 454 (7203): 428–35. doi:10.1038/nature07201.

Medzhitov, Ruslan, and Charles A Janeway. 2002. “Decoding the Patterns of Self and Nonself by the Innate Immune System..” *Science (New York, N.Y.)* 296 (5566): 298–300. doi:10.1126/science.1068883.

Megens, R T A, S Vijayan, D Lievens, Y Döring, M A M J van Zandvoort, J Grommes, C Weber, and O Soehnlein. 2012. “Presence of Luminal Neutrophil Extracellular Traps in Atherosclerosis.” *Thrombosis and Haemostasis* 107 (3): 597–98. doi:10.1160/TH11-09-0650.

Meltzer, Mirjam E, Ton Lisman, Carine J M Doggen, Philip G De Groot, and Frits R Rosendaal. 2008. “Synergistic Effects of Hypofibrinolysis and Genetic and Acquired Risk Factors on the Risk of a First Venous Thrombosis..” *PLoS Medicine* 5 (5): e97. doi:10.1371/journal.pmed.0050097.

Meng, Fan-Su, Lei Su, You-Qing Tang, Qiang Wen, Yun-Song Liu, and Zhi-Feng Liu. 2009. “Serum Procalcitonin at the Time of Admission to the ICU as a Predictor of Short-Term Mortality.” *Clinical Biochemistry* 42 (10-11). The Canadian Society of Clinical Chemists: 1025–31. doi:10.1016/j.clinbiochem.2009.03.012.

Meng, Wei, Adnana Paunel-Görgülü, Sascha Flohé, Almuth Hoffmann, Ingo Witte, Colin MacKenzie, Stephan E Baldus, Joachim Windolf, and Tim T Lögters. 2012. “Depletion of Neutrophil Extracellular Traps in Vivo Results in

Hypersusceptibility to Polymicrobial Sepsis in Mice." *Critical Care* 16 (4). BioMed Central Ltd: R137. doi:10.1186/cc11442.

Meng, Wei, Adnana Paunel-Görgülü, Sascha Flohé, Ingo Witte, Michael Schädel-Höpfner, Joachim Windolf, and Tim Tobias Lögters. 2012. "Deoxyribonuclease Is a Potential Counter Regulator of Aberrant Neutrophil Extracellular Traps Formation After Major Trauma." *Mediators of Inflammation* 2012 (4): 1–8. doi:10.1002/jor.20911.

Metzler, K D, T A Fuchs, W M Nauseef, D Reumaux, J Roesler, I Schulze, V Wahn, V Papayannopoulos, and A Zychlinsky. 2011. "Myeloperoxidase Is Required for Neutrophil Extracellular Trap Formation: Implications for Innate Immunity." *Blood* 117 (3): 953–59. doi:10.1182/blood-2010-06-290171.

Miguel-Bayarri, V, E B Casanoves-Laparra, L Pallás-Beneyto, S Sancho-Chinesta, L F Martín-Osorio, C Tormo-Calandín, and D Bautista-Rentero. 2012. "Prognostic Value of the Biomarkers Procalcitonin, Interleukin-6 and C-Reactive Protein in Severe Sepsis.." *Medicina Intensiva / Sociedad Española De Medicina Intensiva Y Unidades Coronarias* 36 (8): 556–62. doi:10.1016/j.medin.2012.01.014.

Miles, L A, C M Dahlberg, and E F Plow. 1988. "The Cell-Binding Domains of Plasminogen and Their Function in Plasma.." *The Journal of Biological Chemistry* 263 (24): 11928–34.

Mokart, Djamel, Eric Kipnis, Pierre Guerre-Berthelot, Norbert Vey, Christian Capo, Antoine Sannini, Jean-Paul Brun, et al. 2008. "Monocyte Deactivation in Neutropenic Acute Respiratory Distress Syndrome Patients Treated with Granulocyte Colony-Stimulating Factor." *Critical Care* 12 (1): R17. doi:10.1186/cc6791.

Monković, D D, and P B Tracy. 2001. "Functional Characterization of Human Platelet-Released Factor v and Its Activation by Factor Xa and Thrombin.." *The Journal of Biological Chemistry* 265 (28): 17132–40.

Moreira, V G, B Prieto, J S M Rodriguez, and F V Alvarez. 2010. "Usefulness of Cell-Free Plasma DNA, Procalcitonin and C-Reactive Protein as Markers of Infection in Febrile Patients." *Annals of Clinical Biochemistry* 47 (3): 253–58. doi:10.1258/acb.2010.009173.

Morrissey, J H, B G Macik, P F Neuenschwander, and P C Comp. 1993. "Quantitation of Activated Factor VII Levels in Plasma Using a Tissue Factor Mutant Selectively Deficient in Promoting Factor VII Activation.." *Blood* 81 (3): 734–44.

Mosesson, M W, K R Siebenlist, J F Hainfeld, and J S Wall. 1995. "The Covalent Structure of Factor XIIIa Crosslinked Fibrinogen Fibrils.." *Journal of Structural Biology* 115 (1): 88–101. doi:10.1006/jsbi.1995.1033.

Müller, Felicitas, Nicola J Mutch, Wolfdieter A Schenk, Stephanie A Smith, Lucie Esterl, Henri M Spronk, Stefan Schmidbauer, William A Gahl, James H Morrissey, and Thomas Renné. 2009. "Platelet Polyphosphates Are

Proinflammatory and Procoagulant Mediators in Vivo." *Cell* 139 (6): 1143–56. doi:10.1016/j.cell.2009.11.001.

Nagata, S, H Nagase, K Kawane, N Mukae, and H Fukuyama. 2003. "Degradation of Chromosomal DNA During Apoptosis.." *Cell Death and Differentiation* 10 (1): 108–16. doi:10.1038/sj.cdd.4401161.

Nair, C H, G A Shah, and D P Dhall. 1986. "Effect of Temperature, pH and Ionic Strength and Composition on Fibrin Network Structure and Its Development.." *Thrombosis Research* 42 (6): 809–16.

Nakahara, Mayumi, Takashi Ito, Ko-ichi Kawahara, Mika Yamamoto, Tomoka Nagasato, Binita Shrestha, Shingo Yamada, et al. 2013. "Recombinant Thrombomodulin Protects Mice Against Histone-Induced Lethal Thromboembolism." Edited by Toshiyuki Miyata. *PLoS ONE* 8 (9): e75961. doi:10.1371/journal.pone.0075961.s004.

Neuenschwander, P F, E Bianco-Fisher, A R Rezaie, and J H Morrissey. 1995. "Phosphatidylethanolamine Augments Factor VIIa-Tissue Factor Activity: Enhancement of Sensitivity to Phosphatidylserine.." *Biochemistry* 34 (43): 13988–93.

Ni, Heyu, and John Freedman. 2003. "Platelets in Hemostasis and Thrombosis: Role of Integrins and Their Ligands." *Transfusion and Apheresis Science* 28 (3): 257–64. doi:10.1016/S1473-0502(03)00044-2.

Nishiya, T, and A L DeFranco. 2004. "Ligand-Regulated Chimeric Receptor Approach Reveals Distinctive Subcellular Localization and Signaling Properties of the Toll-Like Receptors." *Journal of Biological Chemistry* 279 (18): 19008–17. doi:10.1074/jbc.M311618200.

Obeid, Michel, Antoine Tesniere, François Ghiringhelli, Gian Maria Fimia, Lionel Apetoh, Jean-Luc Perfettini, Maria Castedo, et al. 2006. "Calreticulin Exposure Dictates the Immunogenicity of Cancer Cell Death." *Nature Medicine* 13 (1): 54–61. doi:10.1038/nm1523.

Oh, Hana, Brian Siano, and Scott Diamond. 2008. "Neutrophil Isolation Protocol.." *Journal of Visualized Experiments*, no. 17: 745. doi:10.3791/745.

Opal, Steven M, Craig M Kessler, Juergen Roemisch, and Sigurd Knaub. 2002. "Antithrombin, Heparin, and Heparan Sulfate.." *Critical Care Medicine* 30 (5 Suppl): S325–31.

Opal, Steven M, Pierre Francois Laterre, Bruno Francois, Steven P LaRosa, Derek C Angus, Jean-Paul Mira, Xavier Wittebole, et al. 2013. "Effect of Eritoran, an Antagonist of MD2-TLR4, on Mortality in Patients with Severe Sepsis: the ACCESS Randomized Trial.." *JAMA : the Journal of the American Medical Association* 309 (11): 1154–62. doi:10.1001/jama.2013.2194.

Osterud, B, and S I Rapaport. 1977. "Activation of Factor IX by the Reaction Product of Tissue Factor and Factor VII: Additional Pathway for Initiating Blood Coagulation.." *Proceedings of the National Academy of Sciences of the United States of America* 74 (12): 5260–64.

Osterud, B, L V Rao, and J O Olsen. 2000. “Induction of Tissue Factor Expression in Whole Blood: Lack of Evidence for the Presence of Tissue Factor Expression in Granulocytes..” *Thrombosis and Haemostasis* 83 (6): 861–67.

Owens, A Phillip, III, Freda H Passam, Silvio Antoniak, Stephanie M Marshall, Allison L McDaniel, Lawrence Rudel, Julie C Williams, et al. 2012. “Monocyte Tissue Factor–Dependent Activation of Coagulation in Hypercholesterolemic Mice and Monkeys Is Inhibited by Simvastatin.” *Journal of Clinical Investigation* 122 (2): 558–68. doi:10.1172/JCI58969DS1.

Ozinsky, A, D M Underhill, J D Fontenot, A M Hajjar, K D Smith, C B Wilson, L Schroeder, and A Aderem. 2000. “The Repertoire for Pattern Recognition of Pathogens by the Innate Immune System Is Defined by Cooperation Between Toll-Like Receptors..” *Proceedings of the National Academy of Sciences of the United States of America* 97 (25): 13766–71. doi:10.1073/pnas.250476497.

Padkin, Andrew, Caroline Goldfrad, Anthony R Brady, Duncan Young, Nick Black, and Kathy Rowan. 2003. “Epidemiology of Severe Sepsis Occurring in the First 24 Hrs in Intensive Care Units in England, Wales, and Northern Ireland.” *Critical Care Medicine* 31 (9): 2332–38. doi:10.1097/01.CCM.0000085141.75513.2B.

Palabrica, T, R Lobb, B C Furie, M Aronovitz, C Benjamin, Y M Hsu, S A Sajer, and B Furie. 1992. “Leukocyte Accumulation Promoting Fibrin Deposition Is Mediated in Vivo by P-Selectin on Adherent Platelets..” *Nature* 359 (6398): 848–51. doi:10.1038/359848a0.

Park, J S. 2005. “High Mobility Group Box 1 Protein Interacts with Multiple Toll-Like Receptors.” *AJP: Cell Physiology* 290 (3): C917–24. doi:10.1152/ajpcell.00401.2005.

Park, J S, D Svetkauskaite, Q He, J Y Kim, D Strassheim, A Ishizaka, and E Abraham. 2004. “Involvement of Toll-Like Receptors 2 and 4 in Cellular Activation by High Mobility Group Box 1 Protein.” *Journal of Biological Chemistry* 279 (9): 7370–77. doi:10.1074/jbc.M306793200.

Pawlinski, R. 2003. “Role of Tissue Factor and Protease-Activated Receptors in a Mouse Model of Endotoxemia.” *Blood* 103 (4): 1342–47. doi:10.1182/blood-2003-09-3051.

Pawlinski, R, J G Wang, A P Owens, J Williams, S Antoniak, M Tencati, T Luther, et al. 2010. “Hematopoietic and Nonhematopoietic Cell Tissue Factor Activates the Coagulation Cascade in Endotoxemic Mice.” *Blood* 116 (5): 806–14. doi:10.1182/blood-2009-12-259267.

Pawlinski, Rafal, and Nigel Mackman. 2004. “Tissue Factor, Coagulation Proteases, and Protease-Activated Receptors in Endotoxemia and Sepsis.” *Critical Care Medicine* 32 (Supplement): S293–97. doi:10.1097/01.CCM.0000128445.95144.B8.

Pawlinski, Rafal, and Nigel Mackman. 2010. “Cellular Sources of Tissue Factor in Endotoxemia and Sepsis.” *Thrombosis Research* 125 (April): S70–S73. doi:10.1016/j.thromres.2010.01.042.

Peters, E, A van Elsas, S Heemskerk, L Jonk, J van der Hoeven, J Arend, R Masereeuw, and P Pickkers. 2012. “Alkaline Phosphatase as a Treatment of Sepsis-Associated Acute Kidney Injury.” *Journal of Pharmacology and Experimental Therapeutics* 344 (1): 2–7. doi:10.1124/jpet.112.198226.

Petros, S, P Kliem, T Siegemund, and R Siegemund. 2012. “Thrombosis Research.” *Thrombosis Research* 129 (6). Elsevier Ltd: 797–800. doi:10.1016/j.thromres.2011.08.004.

Piccinini, A M, and K S Midwood. 2010. “DAMPening Inflammation by Modulating TLR Signalling.” *Mediators of Inflammation* 2010 (2): 1–21. doi:10.1007/s10555-009-9182-y.

Pickkers, Peter, Suzanne Heemskerk, Jeroen Schouten, Pierre Francois Laterre, Jean-Louis Vincent, Albertus Beishuizen, Philippe G Jorens, et al. 2012. “Alkaline Phosphatase for Treatment of Sepsis-Induced Acute Kidney Injury: a Prospectiverandomized Double-Blind Placebo-Controlled Trial.” *Critical Care* 16 (1). BioMed Central Ltd: R14. doi:10.1186/cc11159.

Picoli-Quaino, Susan K, Brunna E Alves, Vanessa B Faiotto, Silmara A L Montalvao, Carmino A De Souza, Joyce M Annichino-Bizzacchi, and Erich V De Paula. 2013. “Impairment of Thrombin Generation in the Early Phases of the Host Response of Sepsis.” *Journal of Critical Care*, September. Elsevier Inc., 1–6. doi:10.1016/j.jcrc.2013.08.015.

Pilsczek, F H, D Salina, K K H Poon, C Fahey, B G Yipp, C D Sibley, S M Robbins, et al. 2010. “A Novel Mechanism of Rapid Nuclear Neutrophil Extracellular Trap Formation in Response to Staphylococcus Aureus.” *The Journal of Immunology* 185 (12): 7413–25. doi:10.4049/jimmunol.1000675.

Pisetsky, David S. 2014. “The Translocation of Nuclear Molecules During Inflammation and Cell Death.” *Antioxidants & Redox Signaling* 20 (7): 1117–25. doi:10.1089/ars.2012.5143.

Raaphorst, J, A B Johan Groeneveld, A W Bossink, and C Erik Hack. 2001. “Early Inhibition of Activated Fibrinolysis Predicts Microbial Infection, Shock and Mortality in Febrile Medical Patients..” *Thrombosis and Haemostasis* 86 (2): 543–49.

Rainer, Timothy H, Lawrence K S Wong, Wynnie Lam, Eddie Yuen, Nicole Y L Lam, Constantine Metreweli, and Y M Dennis Lo. 2003. “Prognostic Use of Circulating Plasma Nucleic Acid Concentrations in Patients with Acute Stroke..” *Clinical Chemistry* 49 (4): 562–69.

Rangel-Frausto, M S, D Pittet, M Costigan, T Hwang, C S Davis, and R P Wenzel. 1995. “The Natural History of the Systemic Inflammatory Response Syndrome (SIRS). a Prospective Study..” *JAMA : the Journal of the American Medical Association* 273 (2): 117–23.

Ranieri, V Marco, B Taylor Thompson, Philip S Barie, Jean-Francois Dhainaut, Ivor S Douglas, Simon Finfer, Bengt Gårdlund, et al. 2012. “Drotrecogin Alfa (Activated) in Adults with Septic Shock.” *The New England Journal of Medicine* 366 (22): 2055–64. doi:10.1056/NEJMoa1202290.

Rao, A Koneti. 2013. “Inherited Platelet Function Disorders: Overview and Disorders of Granules, Secretion, and Signal Transduction..” *Hematology/Oncology Clinics of North America* 27 (3): 585–611. doi:10.1016/j.hoc.2013.02.005.

Rao, L V, O Nordfang, A D Hoang, and U R Pendurthi. 1995. “Mechanism of Antithrombin III Inhibition of Factor VIIa/Tissue Factor Activity on Cell Surfaces. Comparison with Tissue Factor Pathway Inhibitor/Factor Xa-Induced Inhibition of Factor VIIa/Tissue Factor Activity..” *Blood* 85 (1): 121–29.

Rao, L Vijaya Mohan, Hema Kothari, and Usha R Pendurthi. 2012. “Tissue Factor: Mechanisms of Decryption..” *Frontiers in Bioscience (Elite Edition)* 4: 1513–27.

Rauch, U, D Bonderman, B Bohrmann, J J Badimon, J Himber, M A Riederer, and Y Nemerson. 2000. “Transfer of Tissue Factor From Leukocytes to Platelets Is Mediated by CD15 and Tissue Factor..” *Blood* 96 (1): 170–75.

Rees, A J. 2010. “Monocyte and Macrophage Biology: an Overview.” *Ysnep* 30 (3). Elsevier Inc.: 216–33. doi:10.1016/j.semnephrol.2010.03.002.

Remick, Daniel G. 2007. “Pathophysiology of Sepsis.” *The American Journal of Pathology* 170 (5): 1435–44. doi:10.2353/ajpath.2007.060872.

Remijsen, Quinten, Tom Vanden Berghe, Ellen Wirawan, Bob Asselbergh, Eef Parthoens, Riet De Rycke, Sam Noppen, Michel Delforge, Jean Willems, and Peter Vandenabeele. 2010. “Neutrophil Extracellular Trap Cell Death Requires Both Autophagy and Superoxide Generation.” *Nature Publishing Group* 21 (2). Nature Publishing Group: 290–304. doi:10.1038/cr.2010.150.

Renne, T. 2005. “Defective Thrombus Formation in Mice Lacking Coagulation Factor XII.” *Journal of Experimental Medicine* 202 (2): 271–81. doi:10.1084/jem.20050664.

Renné, Thomas, Alvin H Schmaier, Katrin F Nickel, Margareta Blombäck, and Coen Maas. 2012. “In Vivo Roles of Factor XII..” *Blood* 120 (22): 4296–4303. doi:10.1182/blood-2012-07-292094.

Rice, Todd W, Arthur P Wheeler, Gordon R Bernard, Jean-Louis Vincent, Derek C Angus, Naoki Aikawa, Ignace Demeyer, et al. 2010. “A Randomized, Double-Blind, Placebo-Controlled Trial of TAK-242 for the Treatment of Severe Sepsis*.” *Critical Care Medicine* 38 (8): 1685–94. doi:10.1097/CCM.0b013e3181e7c5c9.

Richmond, Timothy J, and Curt A Davey. 2003. “The Structure of DNA in the Nucleosome Core..” *Nature* 423 (6936): 145–50. doi:10.1038/nature01595.

Rigby, Kevin M, and Frank R DeLeo. 2011. “Neutrophils in Innate Host Defense

Against Staphylococcus Aureus Infections." *Seminars in Immunopathology* 34 (2): 237–59. doi:10.1007/s00281-011-0295-3.

Ritis, Konstantinos. 2012. "The Emerging Role of Neutrophils in Thrombosis—the Journey of TF Through NETs," December, 1–8. doi:10.3389/fimmu.2012.00385/abstract.

Rittirsch, Daniel, Michael A Flierl, and Peter A Ward. 2008. "Harmful Molecular Mechanisms in Sepsis." *Nature Publishing Group* 8 (10): 776–87. doi:10.1038/nri2402.

Robboy, S J, M C Major, R W Colman, and J D Minna. 1972. "Pathology of Disseminated Intravascular Coagulation (DIC). Analysis of 26 Cases.." *Human Pathology* 3 (3): 327–43.

Robinson, R A, L Worfolk, and P B Tracy. 1992. "Endotoxin Enhances the Expression of Monocyte Prothrombinase Activity.." *Blood* 79 (2): 406–16.

Roemisch, J, E Gray, J N Hoffmann, and C J Wiedermann. 2002. "Antithrombin: a New Look at the Actions of a Serine Protease Inhibitor.." *Blood Coagulation & Fibrinolysis : an International Journal in Haemostasis and Thrombosis* 13 (8): 657–70.

Rohrbach, Amanda S, Daniel J Slade, Paul R Thompson, and Kerri A Mowen. 2012. "Activation of PAD4 in NET Formation.." *Frontiers in Immunology* 3: 360. doi:10.3389/fimmu.2012.00360.

Ruf, W, A Rehemtulla, J H Morrissey, and T S Edgington. 1991. "Phospholipid-Independent and -Dependent Interactions Required for Tissue Factor Receptor and Cofactor Function.." *The Journal of Biological Chemistry* 266 (4): 2158–66.

Ruggeri, Zaverio M. 2007. "The Role of Von Willebrand Factor in Thrombus Formation." *Thrombosis Research* 120 (January): S5–S9. doi:10.1016/j.thromres.2007.03.011.

Ruiz, Felix A, Christopher R Lea, Eric Oldfield, and Roberto Docampo. 2004. "Human Platelet Dense Granules Contain Polyphosphate and Are Similar to Acidocalcisomes of Bacteria and Unicellular Eukaryotes." *Journal of Biological Chemistry* 279 (43): 44250–57. doi:10.1074/jbc.M406261200.

Saffarzadeh, Mona, Christiane Juenemann, Markus A Queisser, Guenter Lochnit, Guillermo Barreto, Sebastian P Galuska, Juergen Lohmeyer, and Klaus T Preissner. 2012. "Neutrophil Extracellular Traps Directly Induce Epithelial and Endothelial Cell Death: a Predominant Role of Histones." Edited by Dominik Hartl. *PLoS ONE* 7 (2): e32366. doi:10.1371/journal.pone.0032366.g009.

Sambrano, G R, E J Weiss, Y W Zheng, W Huang, and S R Coughlin. 2001. "Role of Thrombin Signalling in Platelets in Haemostasis and Thrombosis.." *Nature* 413 (6851): 74–78. doi:10.1038/35092573.

Sasaki, S, and H Noguchi. 1959. "Interaction of Fibrinogen with Dextran Sulfate.." *The Journal of General Physiology* 43 (September): 1–12.

Saukkonen, K, P Lakkisto, V Pettila, M Varpula, S Karlsson, E Ruokonen, K

Pulkki, for the Finnsepsis Study Group. 2008. “Cell-Free Plasma DNA as a Predictor of Outcome in Severe Sepsis and Septic Shock.” *Clinical Chemistry* 54 (6): 1000–1007. doi:10.1373/clinchem.2007.101030.

Schneider, M. 2004. “A Study of the Protection of Plasmin From Antiplasmin Inhibition Within an Intact Fibrin Clot During the Course of Clot Lysis.” *Journal of Biological Chemistry* 279 (14): 13333–39. doi:10.1074/jbc.M313164200.

Schouten, M, W J Wiersinga, M Levi, and T van der Poll. 2007. “Inflammation, Endothelium, and Coagulation in Sepsis.” *Journal of Leukocyte Biology* 83 (3): 536–45. doi:10.1189/jlb.0607373.

Segal, B H, T L Leto, J I Gallin, H L Malech, and S M Holland. 2000. “Genetic, Biochemical, and Clinical Features of Chronic Granulomatous Disease..” *Medicine* 79 (3): 170–200.

Semeraro, F, C T Ammollo, N L Esmon, and C T Esmon. 2014. “Histones Induce Phosphatidylserine Exposure and a Procoagulant Phenotype in Human Red Blood Cells.” *Journal of Thrombosis and Haemostasis* 12 (10): 1697–1702. doi:10.1111/jth.12677.

Semeraro, Fabrizio, Concetta T Ammollo, J H Morrissey, G L Dale, P Friese, N L Esmon, and C T Esmon. 2011. “Extracellular Histones Promote Thrombin Generation Through Platelet-Dependent Mechanisms: Involvement of Platelet TLR2 and TLR4.” *Blood* 118 (7): 1952–61. doi:10.1182/blood-2011-03-343061.

Semeraro, Nicola, Concetta T Ammollo, Fabrizio Semeraro, and Mario Colucci. 2010. “Sepsis-Associated Disseminated Intravascular Coagulation and Thromboembolic Disease.” *Mediterranean Journal of Hematology and Infectious Diseases* 2 (3): 2010024. doi:10.4084/mjhid.2010.024.

Semeraro, Nicola, Concetta T Ammollo, Fabrizio Semeraro, and Mario Colucci. 2012. “Thrombosis Research.” *Thrombosis Research* 129 (3). Elsevier Ltd: 290–95. doi:10.1016/j.thromres.2011.10.013.

Seo, Ji-Weon, Hyun Kyung Kim, Ji-Eun Kim, Seonyang Park, and Han-Ik Cho. 2009. “Prognostic Values of the Factor Xa-Activated Clotting Time and Endogenous Thrombin Potential in Patients Suspected of Having Disseminated Intravascular Coagulation.” *Thrombosis Research* 123 (4). Elsevier Ltd: 565–72. doi:10.1016/j.thromres.2008.03.017.

Shah, G A, C H Nair, and D P Dhall. 1985. “Physiological Studies on Fibrin Network Structure..” *Thrombosis Research* 40 (2): 181–88.

Shaw, Andrew W, Vincent S Pureza, Stephen G Sligar, and James H Morrissey. 2007. “The Local Phospholipid Environment Modulates the Activation of Blood Clotting..” *The Journal of Biological Chemistry* 282 (9): 6556–63. doi:10.1074/jbc.M607973200.

Shi, Yan, James E Evans, and Kenneth L Rock. 2003. “Molecular Identification of a Danger Signal That Alerts the Immune System to Dying Cells..” *Nature* 425 (6957): 516–21. doi:10.1038/nature01991.

Shorr, Andrew F, Gordon R Bernard, Jean-Francois Dhainaut, James R Russell, William L Macias, David R Nelson, and David P Sundin. 2006. “Protein C Concentrations in Severe Sepsis: an Early Directional Change in Plasma Levels Predicts Outcome..” *Critical Care (London, England)* 10 (3): R92. doi:10.1186/cc4946.

Smith, J A. 1994. “Neutrophils, Host Defense, and Inflammation: a Double-Edged Sword..” *Journal of Leukocyte Biology* 56 (6): 672–86.

Sodeinde, O A, A K Sample, R R Brubaker, and J D Goguen. 1988. “Plasminogen Activator/Coagulase Gene of Yersinia Pestis Is Responsible for Degradation of Plasmid-Encoded Outer Membrane Proteins..” *Infection and Immunity* 56 (10): 2749–52.

Soehnlein, O, A Zernecke, E E Eriksson, A G Rothfuchs, C T Pham, H Herwald, K Bidzhekov, M E Rottenberg, C Weber, and L Lindbom. 2008. “Neutrophil Secretion Products Pave the Way for Inflammatory Monocytes.” *Blood* 112 (4): 1461–71. doi:10.1182/blood-2008-02-139634.

Soehnlein, Oliver, and Lennart Lindbom. 2010. “Phagocyte Partnership During the Onset and Resolution of Inflammation.” *Nature Publishing Group* 10 (6). Nature Publishing Group: 427–39. doi:10.1038/nri2779.

Staali, Leila, Susanne Bauer, Matthias Mörgelin, Lars Bjorck, and Hans Tapper. 2006. “Streptococcus Pyogenes Bacteria Modulate Membrane Traffic in Human Neutrophils and Selectively Inhibit Azurophilic Granule Fusion with Phagosomes.” *Cellular Microbiology* 8 (4): 690–703. doi:10.1111/j.1462-5822.2005.00662.x.

Stakos, D A, K Kambas, T Konstantinidis, I Mitroulis, E Apostolidou, S Arelaki, V Tsironidou, et al. 2015. “Expression of Functional Tissue Factor by Neutrophil Extracellular Traps in Culprit Artery of Acute Myocardial Infarction.” *European Heart Journal* 36 (22): 1405–14. doi:10.1093/eurheartj/ehv007.

Steinberg, Benjamin E, and Sergio Grinstein. 2007. “Unconventional Roles of the NADPH Oxidase: Signaling, Ion Homeostasis, and Cell Death..” *Science's STKE : Signal Transduction Knowledge Environment* 2007 (379): pe11. doi:10.1126/stke.3792007pe11.

Stephenson, Daniel A, Lisa J Toltl, Suzanne Beaudin, and Patricia C Liaw. 2006. “Modulation of Monocyte Function by Activated Protein C, a Natural Anticoagulant..” *Journal of Immunology (Baltimore, Md. : 1950)* 177 (4): 2115–22.

Stief, Thomas W, Ola Ijagha, Bettina Weiste, Ileana Herzum, Harald Renz, and Martin Max. 2007. “Analysis of Hemostasis Alterations in Sepsis..” *Blood Coagulation & Fibrinolysis : an International Journal in Haemostasis and Thrombosis* 18 (2): 179–86. doi:10.1097/MBC.0b013e328040bf9a.

Stroun, M, J Lyautey, C Lederrey, A Olson-Sand, and P Anker. 2001. “About the Possible Origin and Mechanism of Circulating DNA Apoptosis and Active DNA Release..” *Clinica Chimica Acta* 313 (1-2): 139–42.

Stumpe, S, R Schmid, D L Stephens, G Georgiou, and E P Bakker. 1998. "Identification of OmpT as the Protease That Hydrolyzes the Antimicrobial Peptide Protamine Before It Enters Growing Cells of Escherichia Coli.." *Journal of Bacteriology* 180 (15): 4002–6.

Sun, Hongmin, Xixi Wang, Jay L Degen, and David Ginsburg. 2009. "Reduced Thrombin Generation Increases Host Susceptibility to Group a Streptococcal Infection.." *Blood* 113 (6): 1358–64. doi:10.1182/blood-2008-07-170506.

Swystun, Laura L, S Mukherjee, and Patricia C Liaw. 2011. "Breast Cancer Chemotherapy Induces the Release of Cell-Free DNA, a Novel Procoagulant Stimulus." *Journal of Thrombosis and Haemostasis* 9 (11): 2313–21. doi:10.1111/j.1538-7836.2011.04465.x.

Tadie, J M, H B Bae, S Jiang, D W Park, C P Bell, H Yang, J F Pittet, et al. 2013. "HMGB1 Promotes Neutrophil Extracellular Trap Formation Through Interactions with Toll-Like Receptor 4." *AJP: Lung Cellular and Molecular Physiology* 304 (5): L342–49. doi:10.1152/ajplung.00151.2012.

Tamkovich, S N, A V Cherepanova, E V Kolesnikova, E Y Rykova, D V Pyshnyi, V V Vlassov, and P P Laktionov. 2006. "Circulating DNA and DNase Activity in Human Blood." *Annals of the New York Academy of Sciences* 1075 (1): 191–96. doi:10.1196/annals.1368.026.

Tang, Haiwang, Lacramioara Ivanciu, Narcis Popescu, Glenn Peer, Erik Hack, Cristina Lupu, Fletcher B Taylor Jr, and Florea Lupu. 2007. "Sepsis-Induced Coagulation in the Baboon Lung Is Associated with Decreased Tissue Factor Pathway Inhibitor.." *The American Journal of Pathology* 171 (3): 1066–77. doi:10.2353/ajpath.2007.070104.

Tankersley, D L, and J S Finlayson. 1984. "Kinetics of Activation and Autoactivation of Human Factor XII.." *Biochemistry* 23 (2): 273–79.

Tate, K M, D L Higgins, W E Holmes, M E Winkler, H L Heyneker, and G A Vehar. 1987. "Functional Role of Proteolytic Cleavage at Arginine-275 of Human Tissue Plasminogen Activator as Assessed by Site-Directed Mutagenesis.." *Biochemistry* 26 (2): 338–43.

Taylor, F B, G T Peer, M S Lockhart, G Ferrell, and C T Esmon. 2001. "Endothelial Cell Protein C Receptor Plays an Important Role in Protein C Activation in Vivo.." *Blood* 97 (6): 1685–88.

The Surviving Sepsis Campaign Guidelines Committee including The Pediatric Subgroup*, R P Dellinger, Mitchell M Levy, Andrew Rhodes, Djillali Annane, Herwig Gerlach, Steven M Opal, et al. 2013. "Surviving Sepsis Campaign: International Guidelines for Management of Severe Sepsis and Septic Shock, 2012." *Intensive Care Medicine* 39 (2): 165–228. doi:10.1007/s00134-012-2769-8.

Tracy, P B, L L Eide, and K G Mann. 1985. "Human Prothrombinase Complex Assembly and Function on Isolated Peripheral Blood Cell Populations.." *The Journal of Biological Chemistry* 260 (4): 2119–24.

Ulloa, Luis, Michael Brunner, Laura Ramos, and Edwin A Deitch. 2009. “Scientific and Clinical Challenges in Sepsis..” *Current Pharmaceutical Design* 15 (16): 1918–35.

Undas, A, and R A S Ariens. 2011. “Fibrin Clot Structure and Function: a Role in the Pathophysiology of Arterial and Venous Thromboembolic Diseases.” *Arteriosclerosis, Thrombosis, and Vascular Biology* 31 (12): e88–e99. doi:10.1161/ATVBAHA.111.230631.

Underhill, David M, and Adrian Ozinsky. 2002. “Phagocytosis of Microbes: Complexity in Action.” *Annual Review of Immunology* 20 (1): 825–52. doi:10.1146/annurev.immunol.20.103001.114744.

Urban, Constantin F, Ulrike Reichard, Volker Brinkmann, and Arturo Zychlinsky. 2006. “Neutrophil Extracellular Traps Capture and Kill Candida Albicans Yeast and Hyphal Forms.” *Cellular Microbiology* 8 (4): 668–76. doi:10.1111/j.1462-5822.2005.00659.x.

van der Poll, Tom, and Steven M Opal. 2008. “Host-Pathogen Interactions in Sepsis..” *The Lancet. Infectious Diseases* 8 (1): 32–43. doi:10.1016/S1473-3099(07)70265-7.

van Deventer, S J, H R Buller, J W Ten Cate, L A Aarden, C E Hack, and A Sturk. 1990. “Experimental Endotoxemia in Humans: Analysis of Cytokine Release and Coagulation, Fibrinolytic, and Complement Pathways..” *Blood* 76 (12): 2520–26.

van Hylckama Vlieg, A, S C Christiansen, R Luddington, S C Cannegieter, F R Rosendaal, and T P Baglin. 2007. “Elevated Endogenous Thrombin Potential Is Associated with an Increased Risk of a First Deep Venous Thrombosis but Not with the Risk of Recurrence.” *British Journal of Haematology* 138 (6): 769–74. doi:10.1111/j.1365-2141.2007.06738.x.

Varju, I, C Longstaff, L Szabo, Á Z Farkas, V J Varga-Szabó, A Tanka-Salamon, R Machovich, and K Kolev. 2015. “DNA, Histones and Neutrophil Extracellular Traps Exert Anti-Fibrinolytic Effects in a Plasma Environment.” *Thrombosis and Haemostasis* 113 (6). doi:10.1160/TH14-08-0669.

Veklich, Y I, O V Gorkun, L V Medved, W Nieuwenhuizen, and J W Weisel. 1993. “Carboxyl-Terminal Portions of the Alpha Chains of Fibrinogen and Fibrin. Localization by Electron Microscopy and the Effects of Isolated Alpha C Fragments on Polymerization..” *The Journal of Biological Chemistry* 268 (18): 13577–85.

Versteeg, H H, J W M Heemskerk, M Levi, and P H Reitsma. 2013. “New Fundamentals in Hemostasis.” *Physiological Reviews* 93 (1): 327–58. doi:10.1152/physrev.00016.2011.

Villanueva, E, S Yalavarthi, C C Berthier, J B Hodgin, R Khandpur, A M Lin, C J Rubin, et al. 2011. “Netting Neutrophils Induce Endothelial Damage, Infiltrate Tissues, and Expose Immunostimulatory Molecules in Systemic Lupus Erythematosus.” *The Journal of Immunology* 187 (1): 538–52.

doi:10.4049/jimmunol.1100450.
Voss, R, F R Matthias, G Borkowski, and D Reitz. 1990. “Activation and Inhibition of Fibrinolysis in Septic Patients in an Internal Intensive Care Unit..” *British Journal of Haematology* 75 (1): 99–105.
Wada, Hideo. 2004. “Disseminated Intravascular Coagulation.” *Clinica Chimica Acta* 344 (1-2): 13–21. doi:10.1016/j.cccn.2004.02.015.
Wang, Changsong, Chunjie Chi, Lei Guo, Xiaoyang Wang, Libo Guo, Jiaxiao Sun, Bo Sun, Shanshan Liu, Xuenan Chang, and Enyou Li. 2014. “Heparin Therapy Reduces 28-Day Mortality in Adult Severe Sepsis Patients: a Systematic Review and Meta-Analysis..” *Critical Care (London, England)* 18 (5): 563. doi:10.1186/s13054-014-0563-4.
Wang, Zhi, Christine Wilhelmsson, Pavel Hyrsl, Torsten G Loof, Pavel Dobes, Martina Klupp, Olga Loseva, et al. 2010. “Pathogen Entrapment by Transglutaminase—a Conserved Early Innate Immune Mechanism.” Edited by David S Schneider. *PLoS Pathogens* 6 (2): e1000763. doi:10.1371/journal.ppat.1000763.s006.
Warnatsch, Annika, Marianna Ioannou, Qian Wang, and Venizelos Papayannopoulos. 2015. “Inflammation. Neutrophil Extracellular Traps License Macrophages for Cytokine Production in Atherosclerosis..” *Science (New York, N.Y.)* 349 (6245): 316–20. doi:10.1126/science.aaa8064.
Weisel, J W. 2007. “Structure of Fibrin: Impact on Clot Stability..” *Journal of Thrombosis and Haemostasis : JTH* 5 Suppl 1 (July): 116–24. doi:10.1111/j.1538-7836.2007.02504.x.
Weisel, J W, and L MEDVED. 2001. “The Structure and Function of the Alpha C Domains of Fibrinogen..” *Annals of the New York Academy of Sciences* 936: 312–27.
Weisel, John W, and Rustem I Litvinov. 2013. “Mechanisms of Fibrin Polymerization and Clinical Implications..” *Blood* 121 (10): 1712–19. doi:10.1182/blood-2012-09-306639.
Weissman, I L, D J Anderson, and F Gage. 2001. “Stem and Progenitor Cells: Origins, Phenotypes, Lineage Commitments, and Transdifferentiations..” *Annual Review of Cell and Developmental Biology* 17: 387–403. doi:10.1146/annurev.cellbio.17.1.387.
Wheeler, A P, and G R Bernard. 1999a. “Treating Patients with Severe Sepsis..” *The New England Journal of Medicine* 340 (3): 207–14. doi:10.1056/NEJM199901213400307.
Wheeler, A P, and G R Bernard. 1999b. “Treating Patients with Severe Sepsis..” *New England Journal of Medicine* 340 (3): 207–14. doi:10.1056/NEJM199901213400307.
Wilcox, J N, K M Smith, S M Schwartz, and D Gordon. 1989. “Localization of Tissue Factor in the Normal Vessel Wall and in the Atherosclerotic Plaque..” *Proceedings of the National Academy of Sciences of the United States of*

America 86 (8): 2839–43.
Wildhagen, K C A A, P Garcia de Frutos, C P Reutelingsperger, R Schrijver, C Areste, A Ortega-Gomez, N M Deckers, H C HEMKER, O Soehnlein, and G A F Nicolaes. 2013. "Non-Anticoagulant Heparin Prevents Histone-Mediated Cytotoxicity in Vitro and Improves Survival in Sepsis." *Blood* 123 (7): 1098–1101. doi:10.1182/blood-2013-07-514984.
Winterbourn, Christine C. 2008. "Reconciling the Chemistry and Biology of Reactive Oxygen Species." *Nature Chemical Biology* 4 (5): 278–86. doi:10.1038/nchembio.85.
Wolberg, A S, D M Monroe, H R Roberts, and M R Hoffman. 1999. "Tissue Factor De-Encryption: Ionophore Treatment Induces Changes in Tissue Factor Activity by Phosphatidylserine-Dependent and -Independent Mechanisms.." *Blood Coagulation & Fibrinolysis : an International Journal in Haemostasis and Thrombosis* 10 (4): 201–10.
Wolberg, Alisa S. 2007. "Thrombin Generation and Fibrin Clot Structure." *Blood Reviews* 21 (3): 131–42. doi:10.1016/j.blre.2006.11.001.
Wolberg, Alisa S, and Robert A Campbell. 2008. "Thrombin Generation, Fibrin Clot Formation and Hemostasis." *Transfusion and Apheresis Science* 38 (1): 15–23. doi:10.1016/j.transci.2007.12.005.
Xu, J, X Zhang, M Monestier, N L Esmon, and C T Esmon. 2011. "Extracellular Histones Are Mediators of Death Through TLR2 and TLR4 in Mouse Fatal Liver Injury." *The Journal of Immunology* 187 (5): 2626–31. doi:10.4049/jimmunol.1003930.
Xu, Jun, Xiaomei Zhang, Rosana Pelayo, Marc Monestier, Concetta T Ammollo, Fabrizio Semeraro, Fletcher B Taylor, Naomi L Esmon, Florea Lupu, and Charles T Esmon. 2009. "Extracellular Histones Are Major Mediators of Death in Sepsis." *Nature Medicine* 15 (11): 1318–21. doi:10.1038/nm.2053.
Xue, Mingming, Zhan Sun, Mian Shao, Jun Yin, Zhi Deng, Jin Zhang, Lingyu Xing, et al. 2015. "Diagnostic and Prognostic Utility of Tissue Factor for Severe Sepsis and Sepsis-Induced Acute Lung Injury." *Journal of Translational Medicine*, June. Journal of Translational Medicine, 1–8. doi:10.1186/s12967-015-0518-9.
Yang, H, S Lang, Z Zhai, L Li, W H A Kahr, P Chen, J Brkic, et al. 2009. "Fibrinogen Is Required for Maintenance of Platelet Intracellular and Cell-Surface P-Selectin Expression." *Blood* 114 (2): 425–36. doi:10.1182/blood-2008-03-145821.
Yang, Xinyu, Lin Li, Jin Liu, Ben Lv, and Fangping Chen. 2016. "Extracellular Histones Induce Tissue Factor Expression in Vascular Endothelial Cells via TLR and Activation of NF-κB and AP-1.." *Thrombosis Research* 137 (January): 211–18. doi:10.1016/j.thromres.2015.10.012.
Yermolenko, Ivan S, Valeryi K Lishko, Tatiana P Ugarova, and Sergei N Magonov. 2011. "High-Resolution Visualization of Fibrinogen Molecules and

Fibrin Fibers with Atomic Force Microscopy." *Biomacromolecules* 12 (2): 370–79. doi:10.1021/bm101122g.

Yipp, B G, and P Kubes. 2013. "NETosis: How Vital Is It?." *Blood* 122 (16): 2784–94. doi:10.1182/blood-2013-04-457671.

Yousefi, Shida, Jeffrey A Gold, Nicola Andina, James J Lee, Ann M Kelly, Evelyne Kozlowski, Inès Schmid, et al. 2008. "Catapult-Like Release of Mitochondrial DNA by Eosinophils Contributes to Antibacterial Defense." *Nature Medicine* 14 (9): 949–53. doi:10.1038/nm.1855.

Zarychanski, Ryan, Steven Doucette, Dean Fergusson, Daniel Roberts, Donald S Houston, Satendra Sharma, Harlena Gulati, and Anand Kumar. 2008. "Early Intravenous Unfractionated Heparin and Mortality in Septic Shock*." *Critical Care Medicine* 36 (11): 2973–79. doi:10.1097/CCM.0b013e31818b8c6b.

Zeerleder, Sacha, Bas Zwart, Walter A Wuillemin, Lucien A Aarden, A B Johan Groeneveld, Christoph Caliezi, Annemarie E M van Nieuwenhuijze, et al. 2003. "Elevated Nucleosome Levels in Systemic Inflammation and Sepsis*." *Critical Care Medicine* 31 (7): 1947–51. doi:10.1097/01.CCM.0000074719.40109.95.

Zhang, Qin, Mustafa Raoof, Yu Chen, Yuka Sumi, Tolga Sursal, Wolfgang Junger, Karim Brohi, Kiyoshi Itagaki, and Carl J Hauser. 2010. "Circulating Mitochondrial DAMPs Cause Inflammatory Responses to Injury." *Nature* 464 (7285). Nature Publishing Group: 104–7. doi:10.1038/nature08780.

Zhao, B Q, A K Chauhan, M Canault, I S Patten, J J Yang, M Dockal, F Scheiflinger, and D D WAGNER. 2009. "Von Willebrand Factor-Cleaving Protease ADAMTS13 Reduces Ischemic Brain Injury in Experimental Stroke." *Blood* 114 (15): 3329–34. doi:10.1182/blood-2009-03-213264.

Zhong, X Y, I von Muhlenen, Y Li, A Kang, A K Gupta, A Tyndall, W Holzgreve, S Hahn, and P Hasler. 2007. "Increased Concentrations of Antibody-Bound Circulatory Cell-Free DNA in Rheumatoid Arthritis." *Clinical Chemistry* 53 (9): 1609–14. doi:10.1373/clinchem.2006.084509.

Ziegler, Annemarie, Uwe Zangemeister-Wittke, and Rolf A Stahel. 2002. "Circulating DNA: a New Diagnostic Gold Mine?." *Cancer Treatment Reviews* 28 (5): 255–71. doi:10.1016/S0305-7372(02)00077-4.