INVESTIGATION OF THE PATHOLOGICAL EFFECTS OF EXTRACELLULAR DNA AND HISTONES IN SEPSIS

INVESTIGATION OF THE PATHOLOGICAL EFFECTS OF EXTRACELLULAR DNA AND HISTONES IN SEPSIS

By

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TITLE: Investigation of the Pathological Effects of Extracellular DNA and Histones in Sepsis

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Lay abstract

Sepsis is a type of blood poisoning that occurs when the body has an over reactive response to an infection. This can lead to tissue damage, organ failure, and death. Sepsis is recognized as a global health priority. The death rate from sepsis is high between 15% to 30%, suggesting that an improved understanding of how sepsis leads to death may develop into new therapies. Recently, it was discovered that high levels of free-floating DNA and histones in the blood can predict death in sepsis. The DNA and histones are likely released by white blood cells in response to trying to fight off the infection. In test tubes, free-floating DNA can trigger clotting of blood. DNA often exists in blood together with histones. In test tubes, histones can kill blood vessels and make blood thicker. However, no one has confirmed that DNA and/or histones are harmful to mammals and contributes to death in sepsis. Some new studies show that getting rid of DNA with injections of DNase I minimally increases survival in mice. Other studies show that removing histones with a treatment called heparin shows a small increase in survival in mice. Heparin is also a blood thinner and decreases inflammation. No one knows if these drugs used together can improve sepsis survival. Because both drugs on their own show some survival improvement in sepsis, perhaps using them together will cure sepsis.

This thesis has three objectives: (1) to confirm the clotting properties of free-floating DNA, (2) to find out if DNA and/or histones contributes to death in

sepsis, and (3) if using a combination of DNase I and heparin can cure sepsis in a mouse model. Finding new therapies for sepsis can save millions of people's lives and decrease the financial burden on society and healthcare systems.

Abstract

Sepsis is defined as a life-threatening organ dysfunction that results in systemic activation of coagulation and inflammation in response to microbial infection. Neutrophil extracellular traps (NETs) have shown to be an important interface between innate immunity and coagulation in sepsis. The major structural components of NETs are nucleosomes (DNA-histone complexes). Although nucleosomes do not modulate coagulation, there are conditions where DNA and histones dissociate from each other in the circulation (e.g. in the presence of heparan sulfate or therapeutic heparin binding histones, or DNase digestion of DNA). *In vitro*, purified DNA was reported to activate coagulation, but this procoagulant activity has been questioned due to isolation methods that yield DNA that is contaminated with other procoagulant molecules. On the other hand, histones have been shown to not only activate coagulation but are cytotoxic to endothelial cells. However, their contribution to the pathogenesis of sepsis has yet to be determined in an *in vivo* model. Understanding the contribution of DNA, histones, and nucleosomes to the pathogenesis of sepsis may allow us to develop novel therapies that may prove targeting multiple components of NETs (i.e. DNA and histones) may be beneficial.

Consequently, in this thesis, we (1) identified methods of DNA purification that produce DNA that is free of contamination and confirmed the procoagulant properties of the isolated DNA, (2) determined the harmful effects of DNA,

histones, and nucleosomes cytotoxicity, coagulation, and inflammation *in vitro and in vivo,* (3) and then we explored the possibility of targeting both DNA and histones using a combination approach of DNase I and heparin in a mouse model of sepsis. Since heparin is administered to patients as a thromboprophylaxis and DNase I is a potential therapy in sepsis, it is important to understand any potential drug-drug interactions.

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List of Abbreviations

ADAM	A disintegrin and metalloproteinase
ADP	Adenosine diphosphate
α2-AP	α2-antiplasmin
ALT	Alanine transaminase
ANOVA	Analysis of variance
APC	Activate protein C
ARRIVE	Animal Research: Reporting of In Vivo Experiments
AT	Antithrombin
ATP	Adenosine triphosphate
AUC	Area under the curve
AUP	Animal utilization protocol
bp	Base pairs
BSA	Bovine serum albumin
Ca^{2+}	Calcium
$CaCl_2$	Calcium chloride
CFDNA	Cell-free deoxyribonucleic acid
CIHR	Canadian Institute for Health Research
CLP	Cecal-ligation and puncture
COVID	Coronavirus disease
CRP	C-reactive protein
CT-DNA	Calf-thymus DNA
CTI	Corn trypsin inhibitor
DAMP	Damage-associate molecular pattern
DAPI	4', 6'-diamidino-2-phenylindole
DBL-DNA	QuickGene DNA from whole blood
dsDNA	Double-stranded deoxyribonucleic acid
DIC	Disseminated intravascular coagulation
DMEM	Dulbecco's Modified Eagle Medium
DNA	Deoxyribonucleic acid
DNase I	Deoxyribonuclease I
DRFD	DNase/RNase-free distilled water
DVT	Deep vein thrombosis
EDTA	Ethylenediaminetetraacetic acid
ELISA	Enzyme-linked immunosorbent assay

EPCR	Endothelial protein C receptor
ERK	Extracellular-signal regulated kinase
ET-1	Endothelin-1
ETA	Endothelin receptor A
ETB	Endothelin receptor B
ETP	Endogenous thrombin potential
F	Factor
FBS	Fetal bovine serum
FDP	Fibrin degradation product
FIP	Fecal-induced peritonitis
H	Histone subunit
HCL	Hydrochloric acid
HEK 293	Human embryonic kidney cells 293
HIT	Heparin-induced thrombocytopenia
HiREB	Hamilton Integrated Research Ethics Board
HMWK	High-molecular-weight kininogen
HPSE-1	Heparanase-1
ICU	Intensive care unit
Ig	Immunoglobulin
IL	Interleukin
IP	Intraperitoneal
ICU	Intensive care unit
IU	International unit
KO	Knock out
LDH	Lactate dehydrogenase
LMWH	Low-molecular-weight heparin
LPS	Lipopolysaccharide
LTA	Lipoteichoic acid
MyD88	Myeloid differentiation primary response 88
Mg^{2+}	Magnesium
MGS	Mouse grimace score
MQTiPSS	Minimum Quality Threshold in Preclinical Sepsis Studies
MMP	Matrix metalloproteinase
MODS	Multiple organ dysfunction syndrome
MPO	Myeloperoxidase
MRSA	Methicillin-resistant Staphylococcus aureus
MSS	Murine sepsis score

NaCl	Sodium chloride
NADPH	Nicotinamide adenine dinucleotide phosphate
NCT	National clinical trial
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
PAX-DNA	PAXgene Blood DNA
PBS	Phosphate buffered saline
PC	Protein C
PCF	Peritoneal cavity fluid
PCI	Peritoneal contamination and infection
PCR	Polymerase chain reaction
PKC	Protein kinase C
Plt	Platelet
PolyP	Polyphosphate
PPP	Platelet pooled plasma
PRR	Pattern recognition receptor
PS	Phosphatidylserine
RBC	Red blood cell
RCT	Randomized controlled trial
RNA	Ribonucleic acid
RNase	Ribonuclease
ROS	Reactive oxygen species
SARS	Severe acute respiratory syndrome
SD	Standard deviation
SDS-PAGE	Sodium dodecyl sulfate polyacrylamide gel electrophoresis
SEM	Standard error
Serpin	Serine protease inhibitor
SLE	Systemic lupus erythematosus
SUBQ	Subcutaneous
TAFI	Thrombin-activatable fibrinolysis inhibitor
TAT	Thrombin-antithrombin
TBE	Tris-borate buffer

TF	Tissue factor
TFPI	Tissue factor pathway inhibitor
TGA	Thrombin generation assay
TLR	Toll-like receptor
TM	Thrombomodulin
TNFα	Tumor necrosis factor α
TPA	Tissue plasminogen activator
UFH	Unfractionated heparin
uPA	Urokinase-type plasminogen activator
UV	Ultraviolet
VCAM-1	Vascular cell adhesion protein
VSMC	Vascular smooth muscle cell
VTE	Venous thromboembolism
vWF	von Willebrand factor
WBC	White blood cell
WHO	World Health Organization

Declaration of Academic Achievement

I, Sarah K. Medeiros, declare that this thesis titled "**INVESTIGATION OF THE PATHOLOGICAL EFFECTS OF EXTRACELLULAR DNA AND HISTONES IN SEPSIS"** is a record of my independent research work under the supervision of Dr. Patricia Liaw. I am the sole author of this work. No part of this thesis has been submitted or published for the award of any other degree.

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1.0. General Introduction

1.1. Introduction to hemostasis

1.1.1. Overview of hemostasis

Hemostasis is a well-controlled process of blood clot formation at a site of vessel injury. Since maintenance of vessel integrity is essential for the conservation of the circulatory system, hemostasis is highly conserved in evolution from zebrafish to humans (Mariz & Nery, 2020). In hemostasis, the vascular system, cellular components, and non-cellular components work together to form a stable clot, which prevents bleeding and allows the body to repair the damage. Normally, the vascular endothelium provides an antithrombotic surface, but can promote coagulation under pathophysiological conditions such as injury, infection, or stress. Dissolution of the clot is then required to restore vascular integrity. There are three major branches to hemostasis: primary, secondary, and tertiary (fibrinolysis) hemostasis (Scridon, 2022; Stassen & Deckmyn, 2004; Versteeg et al., 2013).

Primary hemostasis is defined by platelet plug formation that initiates at a site of injury. Platelet plug formation begins with endothelial damage. When damaged, endothelial cells expose subendothelial collagen and release von Willebrand factor (vWF) and inflammatory mediators allowing platelets to adhere to the vessel wall through surface glycoproteins and then activate. Activation of platelets leads to the release of granular content, which results in aggregation and initiation of secondary hemostasis. Ultimately, the formation of a weak platelet plug is essential to temporarily halt bleeding until further stabilization occurs by secondary hemostasis (Scridon, 2022).

Secondary hemostasis involves clotting factors in the coagulation cascade, which inevitably converts fibrinogen to fibrin to stabilize the platelet plug. Extrinsic and intrinsic coagulation pathways are required to activate the coagulation factors, which convert prothrombin into thrombin. Thrombin can then cleave soluble fibrinogen to insoluble fibrin and convert factor (F) XIII to its active form FXIIIa. FXIIIa then crosslinks fibrin, forming a stabilized clot (Garmo et al., 2022).

Tertiary hemostasis is the final phase of blood clotting, which involves the breakdown of fibrin and restoration of normal blood flow. It is also known as fibrinolysis, which is triggered by the activation of plasminogen to plasmin. Plasmin cleaves the fibrin meshwork to dissolve the blood clot and release the trapped cells and nutrients back into circulation. Tertiary hemostasis is a critical step in preventing excessive clotting and ensuring proper wound healing (Chapin & Hajjar, 2015; Stassen & Deckmyn, 2004).

1.1.2. Thrombosis pathophysiology

Normally, the body has highly regulated mechanisms that prevent clot formation. However, hemostatic abnormalities may lead to either excessive bleeding or unwanted clot formation. Unwanted clot formation can block blood vessels and cut off blood supply to organs causing organ damage. The underlying causes are typically due to widespread inflammation and/or infection (Ashorobi et al., 2022; Garmo et al., 2022).

1.2. The Vascular Endothelium

The vascular endothelium is essential for maintaining hemostasis as it is a natural barrier to the prothrombotic collagen underneath. Under normal conditions, it also prevents unwanted clotting by providing an antithrombotic surface to prevent platelet adhesion, inhibit coagulation, and promote fibrinolysis (Figure 1.1) (Bombeli et al., 1997). In pathophysiological conditions such as injury or stress (i.e. inflammation, hypoxia, or infection), subendothelial collagen and tissue factor (TF) is exposed or expressed to activate platelets and initiate thrombosis. Endothelial cells will also secrete vWF, antifibrinolytic proteins, and express adhesion molecules to promote platelet adhesion and activation (Golebiewska & Poole, 2015). The localization of platelets to the surface is essential for the initiation of primary hemostasis.

1.2.1. The glycocalyx

The glycocalyx is a gel-like negatively charged molecular layer covering the surface of the vascular endothelium. It consists of proteoglycans, glycoproteins, glycosaminoglycans (GAGs), and adherent plasma proteins (Weinbaum et al., 2007). Together, these work together to (a) regulate vascular permeability, (b) regulate leukocyte adhesion, and (c) inhibit coagulation (Alphonsus & Rodseth, 2014; Ince et al., 2016; Woodcock & Woodcock, 2012).

The apical surface of endothelial cells is covered by proteoglycans, to which GAG chains are covalently bound (Li et al., 2012). There are numerous proteoglycans, but

Figure 1.1. The dynamic vascular endothelium. The endothelium is responsible for regulating coagulation by providing an antithrombotic surface to prevent unwanted clot formation in healthy conditions. Usually, endothelial cells have a gel-like negatively charged molecular layer covering the surface called the glycocalyx. During pathophysiological conditions, proteoglycans in the glycocalyx are cleaved by MMPs (-7, -9, -13) and ADAMs (-15, -17). GAGs can be degraded by sheddases such as Heparanase 1 (HPSE1) and hyaluronidases. Endothelial cells can also facilitate anticoagulation by expressing thrombomodulin (TM), endothelial protein C receptor (EPCR), and tissue factor pathway inhibitor (TFPI). Tissue plasminogen activator (tPA) is secreted by endothelial cells in healthy environments to directly promote fibrinolysis. During injury or stress (i.e. inflammation, hypoxia, or infection), endothelial cells promote coagulation by upregulating tissue factor (TF) and adhesion molecules (i.e. P-selectin, E-selectin and VCAM-1), which facilitates adhesion and activation of leukocytes and platelets. Endothelial cells also secrete plasminogen activator inhibitor-1 (PAI-1), which inhibits fibrinolysis to promote a procoagulant environment. CD39 is an integral membrane protein expressed on the cell surface that hydrolyzes ADP in a cation-dependent manner to generate AMP, which dissipates adenosine diphosphate (ADP) to regulate platelet activation. Vascular tone is also regulated by endothelial cells. In healthy conditions, nitric oxide (NO) and prostaglandins are released to promote vasodilation. Under injury or stress, endothelin-1 (ET) is released to enable vasoconstriction. Figure created with BioRender.

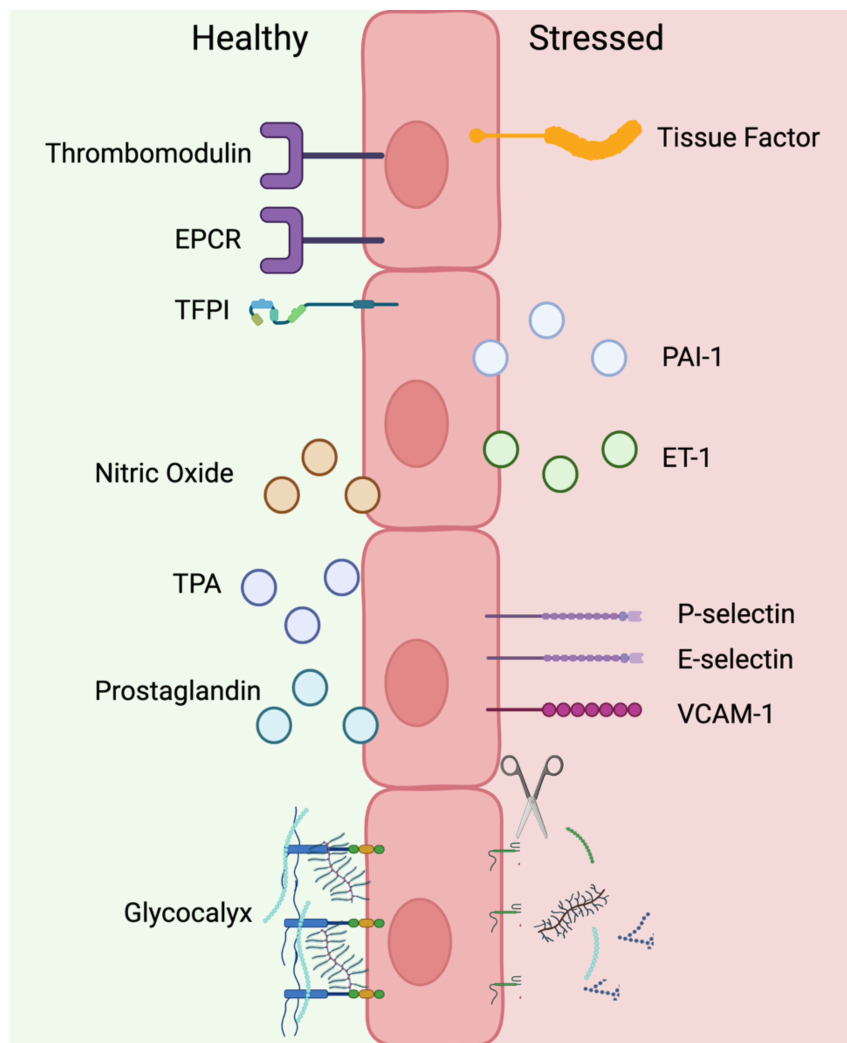

Figure 1.1. The dynamic vascular endothelium.

syndecan-1 is the focus of the majority of glycocalyx explorations (Becker et al., 2015). GAGs such as chondroitin sulfate, dermatan sulfates, keratin sulfates, and—most importantly—heparan sulfates (comprises of 50% of GAGs) bind proteoglycans forming a negative surface (Broekhuizen et al., 2009). A neutral-charged linear GAG, hyaluronan, interacts with other GAGs resulting in the ability to sequester water, thereby forming the gel-like structure of the glycocalyx (Chappell et al., 2009). This surface binds many circulating plasma proteins such as albumin, fibrinogen, fibronectin, anti-thrombin (AT), histones, and cell adhesion molecules (Uchimido et al., 2019).

During pathophysiological inflammatory conditions, the glycocalyx is degraded by matrix metalloproteinases (MMPs) and sheddases leading to increased vascular permeability, tissue inflammation, enhanced leukocyte adhesion, platelet aggregation, initiation of coagulation, inhibition of fibrinolysis, and dysregulated vasodilation (Becker et al., 2015). Together, this further contributes to a proinflammatory and procoagulant environment.

1.3. Primary hemostasis

Primary hemostasis occurs in four phases: (1) vasoconstriction, (2) platelet adhesion, (3) platelet activation, and (4) platelet aggregation. Together, they work to seal off the damaged vessel wall through the aggregation of platelets at the site of injury by forming a “platelet plug” (Scridon, 2022; Versteeg et al., 2013).

1.3.1. Vasoconstriction

When damage to a vessel occurs, vascular factors initially reduce blood loss through vasoconstriction. Damaged endothelial cells release endothelin-1 (ET-1), a potent vasoconstrictor, which primarily binds to G-protein coupled receptors ETA/ETB found on vascular smooth muscle cells (VSMCs), enabling contraction (Kowalczyk et al., 2015).

ET-1 can also be released when endothelial cells are activated by infection, inflammation, hypoxia, and thrombin. The released ET-1 plays a role in vascular wall inflammation, reduced cardiac output, and impaired circulation contributing to organ damage in vascular diseases (Kowalczyk et al., 2015).

1.3.2. Platelet adhesion

Platelets, derived from megakaryocytes, are small, discoid-shaped, anucleated cells known for their vasoprotective responses to injury and inflammation (Scridon, 2022; Yun et al., 2016). Platelet adhesion occurs when platelets attach to the exposed subendothelial collagen and vWF from damaged endothelium. Specifically, the platelet receptor glycoprotein (GP) Ibα is primarily responsible for binding vWF and tethering to the site of injury (Ruggeri, 1997). GPIb-IX-V complex and GPVI receptors can also directly bind to the subendothelial collagen contributing to adhesion (Ruggeri, 1997; Scridon, 2022; Yun et al., 2016). Platelet integrin GPIIb/IIIa is express to further facilitate adhesion and aggregation through binding fibrinogen and fibronectin (Hou et al., 2015).

1.3.3. Platelet activation

When vascular injury occurs, a small amount of thrombin is generated from the extrinsic pathway, which can bind protease-activated receptors (PARs) on platelet surfaces to potently activate platelets (Sambrano et al., 2001). Once platelet receptors are bound to their activating ligands, they (a) undergo an irreversible shape change, from smooth discoid shapes to multi-pseudopodial plugs, and (b) secrete cytoplasmic granules. Granular content is used to characterize platelets and is essential to their function. ADP, ATP, thromboxane A_2, integrins, fibrinogen, fibronectin, vWF, polyphosphates (polyP), and calcium are among the molecules that are contained in granules. The secreted granular content promotes subsequent platelet adhesion, activation, and aggregation (Scridon, 2022; Yun et al., 2016).

1.3.4. Platelet aggregation

Activated platelets recruit additional platelets creating a positive feedback loop to contribute to the growing hemostatic plug. Once activated, platelet integrin adhesion receptors, most importantly GPIIb/IIIa, crosslinks fibrinogen between platelet receptors resulting in platelet aggregation and a weak platelet plug (Scridon, 2022; Yun et al., 2016).

1.4. Secondary hemostasis

Once a platelet plug is formed, the secondary hemostasis process begins on the surface of platelets and endothelial cells through the activation of the coagulation cascade via the extrinsic and intrinsic pathways. This results in the production of thrombin, which converts fibrinogen to fibrin to form a stabilized fibrin clot (Figure 1.2). It is important to

note that calcium ions and negatively-charged phospholipid surfaces are essential for secondary hemostasis (Versteeg et al., 2013).

1.4.1. The extrinsic pathway of coagulation

The extrinsic coagulation pathway is initiated by tissue factor (TF), a transmembrane glycoprotein. Normally, blood cells and plasma proteins are not exposed to TF. Upon vascular injury, TF is exposed on subendothelial cells to initiate coagulation and maintain vascular integrity (Mackman, 2004). TF can also be expressed on endothelial cells and monocytes that have been activated in response to inflammatory mediators or infection (Butenas et al., 2005; Grover & Mackman, 2020). Exposed TF then binds circulating FVIIa and FX, which activates FXa in the presence of calcium ions (Garmo et al., 2022; Versteeg et al., 2013).

Under physiological conditions, most of the FVII circulates in plasma in its zymogen form. However, a small percentage (less than 1%) also exists in the active state, FVIIa (Morrissey et al., 1993). When a vessel is damaged, TF acts as a cofactor for FVIIa forming a high-affinity complex that produces FXa to generate thrombin.

The extrinsic pathway is essential for hemostasis *in vivo.* TF deficiencies have not been discovered in humans and mice genetically deficient in TF die during embryonic development (Toomey et al., 1997). Mice deficient in FVII die during perinatal development due to hemorrhage (Rosen et al., 1997).

Figure 1.2. The mammalian coagulation cascade. The coagulation cascade is dependent on the activation of clotting factors. Clotting factors are predominantly zymogens, precursors of proteolytic enzymes, that circulate in an inactive form. The activation of a zymogen is depicted by "a" as a suffix. During the initiation phase, there are two pathways that are activated, the extrinsic and intrinsic pathway, which merge into a common pathway to create a fibrin clot. The extrinsic pathway is initiated in response to tissue damage or activated monocytes where tissue factor (TF) is expressed on the surface. TF then forms a complex with circulating factor (F) VIIa to convert FX to FXa. In the intrinsic pathway, negatively charged surfaces and molecules such as DNA, RNA, and polyP can promote the autocatalytic activation of FXII (Gould, Lysov, et al., 2015; Smith et al., 2015). FXIIa in complex with cofactor high-molecular-weight kininogen (HMWK) can activate kallikrein from prekallikrein further promoting FXII activation. FXIIa can then activate FXI, which then cleaves and activates FIX. FIXa with cofactor FVIIIa can bind and activate FX. FXa generated from the intrinsic and extrinsic pathway along with cofactor FVa can then convert prothrombin into thrombin. Small amounts of thrombin can activate platelets to further propagate thrombosis. Thrombin then cleaves soluble fibrinogen into insoluble fibrin to initiate the propagation of the clot. Thrombin also plays a crucial role in the amplification of coagulation by activating FV, FVIII, FXI, and FXIII. FXIIIa is required to stabilize fibrin clots (created with BioRender).

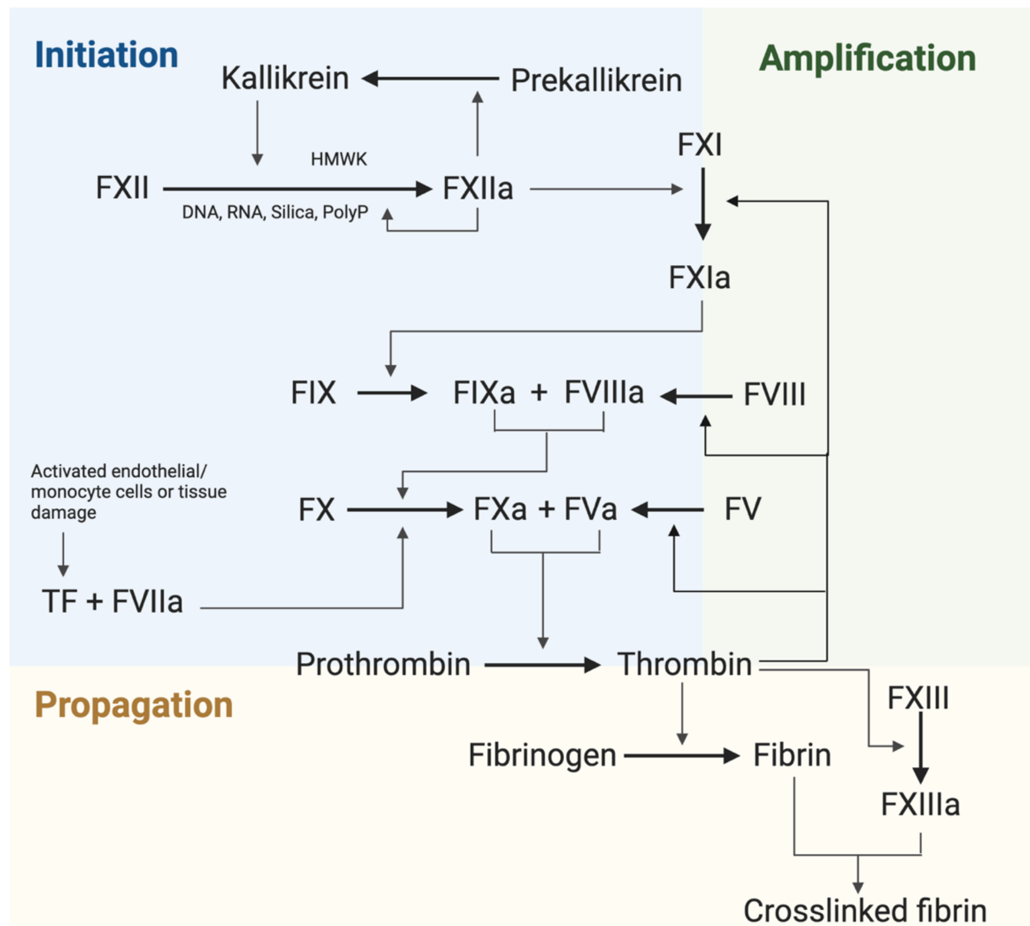

Figure 1.2. The mammalian coagulation cascade

1.4.2. The intrinsic pathways of coagulation

The intrinsic pathway of coagulation, or contact pathway, is initiated by four plasma proteins: FXII, FXI, prekallikrein, and cofactor, high-molecular-weight kininogen (HMWK) (Grover & Mackman, 2019; Kaplan & Silverberg, 1987). HMWK is a cofactor responsible for binding to negatively charged surfaces and assembling the plasma proteins into a complex (Colman & Schmaier, 1997). Initiation occurs by the autoactivation of FXII upon exposure to negatively charged surfaces or molecules from injured cells or pathogens. Activated FXII (FXIIa) can then activate prekallikrein to kallikrein, which can then activate more FXII to create a positive feedback loop. FXIIa can then convert FXI into FXIa. FXIa then activates FIX, which forms a complex with cofactor VIIIa on the surface of cells to activate FX. At this point, the intrinsic and extrinsic pathways converge into the common pathway to generate thrombin (Colman & Schmaier, 1997; Garmo et al., 2022; Versteeg et al., 2013). Thrombin can activate more FXI creating a positive feedback reaction to amplify thrombosis (Gailani & Broze, 1991).

The contact pathway is not required for hemostasis as patients with hereditary deficiencies in FXII, HMWK, or prekallikrein do not suffer from bleeding tendencies (Broze, 1995; Davidson et al., 2001; Girolami et al., 2010; Key, 2014). A deficiency in FXI results in a mild to moderate bleeding phenotype in patients (Preis et al., 2017; Seligsohn, 2009) since it is important for maximizing thrombin generation (Gailani & Broze, 1991). Clinical deficiency of FXI, but not FXII, is associated with a reduced risk for thrombotic events, such as venous thromboembolism (VTE) and ischemic stroke (Gailani et al., 2015; Key, 2014; Preis et al., 2017; Wheeler & Gailani, 2016). In mouse

models, FXII or FXI deficiency attenuated arterial and venous thrombosis. Furthermore, the thrombi formed were unstable and more susceptible to degradation compared to wild-type controls (Renné et al., 2005, 2005, 2009; Renné & Stavrou, 2019). Altogether, this suggests that the contact pathway is important in thrombus growth, but not hemostasis.

Since the contact pathway is not essential to hemostasis, it is considered a safe target for anticoagulation. The holy grail of anticoagulant therapies is to reduce thrombosis, without altering hemostasis or bleeding risks. Data suggests that therapies targeting FXII and FXI safely reduces thrombosis without any significant bleeding risk (Weitz & Fredenburgh, 2017). Targeting FXI would likely be associated with mild bleeding, which occurs in patients with FXI deficiency. However, strategies that target FXII would not cause bleeding as it plays no role in hemostasis and deficiency is not associated with bleeding. Since thrombin has the potential to activate FXI, targeting FXII may be of little value when thrombosis is started by TF (Gailani & Broze, 1991). Although targeting FXI may reduce thrombosis more than FXII, targeting FXII activation may reduce thrombosis without increasing bleeding risks.

There are 3 different strategies to target the intrinsic pathway: (1) specific antisense oligonucleotides (ASO) that reduce FXII and FXI hepatic production, (2) antibodies, aptamers, or molecules that inhibit activation or activity of FXII or FXI, or (3) neutralizing circulating polyanionic intrinsic pathway activators (Weitz & Fredenburgh, 2017).

1.4.3. Polyanionic activators of the intrinsic pathway

In vitro activation of the intrinsic pathway was historically achieved by adding a negatively charged surface such as kaolin, glass, or silica (Davie, 2003). However, *in vivo*, negatively charged molecules such as platelet polyP and nucleic acids (e.x. RNA and DNA) have also been shown to activate the intrinsic pathway (Choi et al., 2011; Gould et al., 2014; Kannemeier et al., 2007; Smith et al., 2018). The activation of FXII by polyanions causes a procoagulant response that enhances the production of thrombin and fibrin, thereby promoting thrombosis (Nickel et al., 2017; Stavrou & Schmaier, 2010).

PolyP, which is found in all living organisms, is an inorganic polymer containing linear, highly anionic, orthophosphate residues connected by phosphoanhydride bonds (Smith et al., 2010). In mammals, 60 to 100 phosphate unit polyP can be released from dense platelet granules during activation to modulate coagulation in numerous ways (Ruiz et al., 2004). PolyP can (1) accelerate the autoactivation of FXII, (2) enhance FV activation by thrombin and FXIa, (3) accelerate FXI activation by thrombin, and (4) bind fibrin(ogen) to improve fibrin clot stability and attenuate fibrinolysis (Choi et al., 2011; Engel et al., 2014; Malik et al., 2021; Smith et al., 2010; Smith & Morrissey, 2008).

Nucleic acids are also procoagulant and enhance activation of the intrinsic pathway to promote thrombosis. Upon vascular injury, intracellular material from damaged cells such as RNA and DNA activates coagulation in a FXI- and FXII-dependent manner (Bhagirath et al., 2015; Gansler et al., 2012; Gould et al., 2014; Kannemeier et al., 2007; Swystun et al., 2011). Investigations into the roles that extracellular nucleic acids play in coagulation and inflammation are ongoing, and various studies have concluded that DNA

has procoagulant properties that are essential for pathothrombosis. Contrary to this, Smith et al. (2017) reported in a Letter to Blood that isolating nucleic acids using common silica-based kits results in silica and polyP contamination that may confound our interpretation of the procoagulant effects of nucleic acids *in vitro* (Smith et al., 2017 & 2018). Therefore, further investigation on alternative sources of DNA that is free of silica and polyP contamination is required to assess the procoagulant properties of DNA *in vitro*.

1.4.4. Common pathway and thrombin generation

The extrinsic and intrinsic pathways converge upon the production of FXa. FXa and its cofactor FVa form a complex, prothrombinase, that converts prothrombin into thrombin. Thrombin then cleaves soluble fibrinogen to insoluble fibrin and activates FXIII to FXIIIa, which crosslinks fibrin polymers. Together, this creates a fibrin network and forms a strong hemostatic plug (Grover & Mackman, 2019; Versteeg et al., 2013).

Thrombin is generated in 3 phases: (1) initiation, (2) amplification, and (3) propagation (Hoffman & Monroe, 2001). Initially, activation of the extrinsic and intrinsic pathways generate small amounts of thrombin that is insufficient for clot formation. Consequently, numerous positive feedback loops are present to amplify thrombin production. Thrombin generated in the initiation phase can further activate platelets, FV, FVIII, and FXI to accelerate the activation of FX to FXa, thereby amplifying thrombin generation. Propagation occurs on the platelet surface to support sufficient thrombin generation, platelet activation, and fibrin formation to form a stable crosslinked fibrin clot (Hoffman & Monroe, 2001; Versteeg et al., 2013).

1.5. Cell-based model of coagulation

The cell-based model of coagulation describes how the initial activation of platelets plays a crucial role in the generation of thrombin (Hoffman & Monroe, 2001). When platelets are activated, they expose negatively charged phospholipids on their surface, which provide a negatively charged phospholipid surface where the amplification of coagulation occurs in secondary hemostasis to form a stable fibrin clot. The reason is that phospholipids provide a surface for the assembly and activation of the prothrombinase complex, composed of FV and FX. The prothrombinase complex then converts prothrombin to thrombin, which in turn cleaves fibrinogen to form fibrin, the main protein component of a blood clot. Phospholipids also play a role in the activation of other proteins involved in coagulation, such as FVII, FIX, and FXII. These proteins all contain regions that are able to bind to phospholipids, which helps to localize and activate them at the site of injury. Without platelet phospholipids, coagulation would be much slower and less efficient, and bleeding could continue for longer periods of time, leading to potentially life-threatening consequences (Hoffman & Monroe, 2001; Scridon, 2022; Yun et al., 2016). Overall, the cell-based model of coagulation provides a comprehensive framework for understanding the complex process of thrombin generation and its role in the formation of a stable clot.

1.6. Naturally occurring anticoagulants

The anticoagulant system provides regulation over coagulation to inevitably control clot formation (Palta et al., 2014). AT, TF pathway inhibitor (TFPI), and the protein C (PC) pathway are common physiological anticoagulants.

1.6.1. Antithrombin (AT)

AT is an important serine protease inhibitor (serpin) of thrombin and FXa (Buchanan et al., 1985; Rosenberg & Damus, 1973). AT can also inhibit FIXa, XIa, and XIIa (Kurachi et al., 1976; Scott & Colman, 1989; Stead et al., 1976). AT activity can be accelerated 1000-fold by binding endogenous heparins such as heparan sulphate on endothelial cells to localize coagulation (de Agostini et al., 1990; Palta et al., 2014; Rao et al., 1995). When in circulation, thrombin quickly binds AT and, thus, thrombin-AT (TAT) complexes can be detected to quantify thrombin generation *in vivo* (Rimpo et al., 2018) .

1.6.2. Tissue factor pathway inhibitor (TFPI)

TFPI is a polypeptide serpin, which naturally inhibits the extrinsic pathway. TFPI is primarily found on the surface of endothelial cells, but is also found in the plasma, platelets, and monocytes at low levels (Kasthuri et al., 2010; J. Wood & Ellery, 2014). Its structure allows for the reversible binding of FXa, which can then irreversibly bind TF-FVIIa to prevent thrombin generation (Broze & Girard, 2012; J. P. Wood et al., 2017). TFPI can also stimulate monocytes to internalize and degrade cell surface TF-VIIa complexes to further restrict thrombin production (Lupu et al., 1999; J. Wood & Ellery, 2014).

1.6.3. Protein C (PC)

The PC pathway is also responsible for regulating anticoagulation. Thrombin is responsible for its activation into activated PC (APC). This activation is enhanced when thrombin is bound to thrombomodulin (TM) and PC is bound to endothelial PC receptor (EPCR), a transmembrane receptor on the surface of healthy endothelial cells. APC can inhibit FVa and FVIIIa when in complex with its cofactor, protein S, thereby reducing thrombin generation. APC can also cleave extracellular histones, which can be cytotoxic and prothrombotic (Esmon, 2003).

APC is a serine protease that is not only anticoagulant but is anti-inflammatory and profibrinolytic. Through the binding of PARs, APC can reduce the activation of inflammatory pathways and is cytoprotective, thus reducing exposure of procoagulant cellular components (Esmon, 2012). Its profibrinolytic effects can be attributed to APC's ability to limit thrombin generation, which reduces activation of antifibrinolytic proteins such as thrombin-activatable fibrinolysis inhibitor (TAFIa) (Bajzar et al., 1996).

1.7. Fibrinolysis

Similar to the coagulation cascade, fibrinolysis is a highly regulated process that controls fibrin deposition to prevent unnecessary clotting and degrade existing thrombi to restore blood flow (Chapin & Hajjar, 2015). Plasmin is the primary fibrinolysin responsible for cleaving fibrin generating fibrin degradation products (FDPs) (Cesarman-Maus & Hajjar, 2005). FDPs can be measured to reflect the degree of thrombosis and plasmin activity (Khalafallah et al., 2014).

The initiation of fibrinolysis begins with the conversation of plasminogen to plasmin by tissue plasminogen activator (tPA; released by endothelial cells) or urokinase plasminogen activator (uPA; released by monocytes and macrophages) (Cesarman-Maus & Hajjar, 2005). Activated plasmin is then able to cleave single-chain tPA and uPA into their more active two-chain structures creating a positive feedback loop (Cesarman-Maus & Hajjar, 2005; Higgins et al., 1990). The presence of fibrin increases the catalytic efficiency of tPA activation of plasmin by approximately 500 times (Hoylaerts et al., 1982). Despite this, tPA and uPA have short half-lives due to the high circulating levels of plasminogen activator inhibitor-1 (PAI-1) secreted from endothelial cells and platelets. When plasmin is generated, it can be rapidly inactivated by α2-antiplasmin (α2-AT). If plasmin is bound to fibrin, it is protected from inhibition (Schneider & Nesheim, 2004). Plasmin generation can also be reduced by TAFIa. Upon activation by thrombin, TAFIa removes C-terminal lysines from fibrin, which prevents plasminogen binding to fibrin and subsequent activation (W. Wang et al., 1998).

1.8. Immunothrombosis: The relationship between coagulation and infection

Not only does the coagulation cascade maintain vascular integrity, but it is also important in the innate immune response to infection. Infection and inflammation can activate coagulation, which allows the trapping of pathogens as a first line of defence in a process coined "immunothrombosis" (Engelmann & Massberg, 2013). The significance of coagulation in the context of innate immunity was emphasized when mice deficient in FV and infected with S. pyogenes exhibited lower survival rates as compared to their wild-type

controls. The lack of FV results in insufficient fibrin deposition resulting in the dissemination of *S. pyogenes* and increased mortality (Sun et al., 2009). Furthermore, fibrinogen-deficient mice demonstrated increased organ damage and mortality in response to parasitic infections (Johnson et al., 2003).

Innate immune cells such as neutrophils and monocytes bridge infection and coagulation through pathogen recognition receptors (PRRs) that recognize conserved molecular motifs called pathogen-associated molecular patterns (PAMPs). Toll-like receptors (TLRs) are the most common PRR that sense PAMPs such as microbial-derived nucleic acids (e.x. DNA, dsRNA, ssRNA), endotoxins, and microbial membrane components such as flagellin, lipopolysaccharides (LPS), lipoteichoic acid (LTA), and peptidoglycans (Vijay, 2018) (Table 1.1). Platelets are also considered an innate immune cell as they also possess PRRs that can recognize microbial pathogens (Dib et al., 2020; Semple et al., 2011). Activation of these innate immune cells results in the generation of proinflammatory mediators, which can further activate platelets and the vascular endothelium to promote thrombosis and pathogen clearance.

1.8.1. The role of TLRs in immunothrombosis

TLRs are a family of PRRs that play a critical role in the recognition of microbial pathogens by the immune system (Fitzgerald et al., 2020). Recent studies have suggested that TLRs may also play a role in immunothrombosis (Antoniak et al., 2014; Engelmann et al., 2013). TLRs are expressed on various immune cells, including platelets, neutrophils, and monocytes, and their activation by microbial pathogens leads to the release of

Table 1-1. The major TLRs and their PAMPs in humans

TLR	Recognized PAMPs	
TLR1/TLR2	Bacterial lipoproteins	Triacylated lipopeptides from gram-positive bacteria, such as Staphylococcus aureus and Streptococcus pneumoniae
TLR2/TLR6	Bacterial lipoproteins	Diacylated lipopeptides from gram-positive bacteria, such as Mycobacterium tuberculosis and Borrelia burgdorferi
TLR3	dsRNA	dsRNA from viruses, such as picornaviruses and flaviviruses
TLR4	LPS	LPS from gram-negative bacteria, such as Escherichia coli and Salmonella typhimurium
TLR5	Bacterial flagellin	Flagellin from motile bacteria, such as Salmonella typhimurium and Pseudomonas aeruginosa
TLR7	ssRNA	ssRNA from viruses, such as influenza virus and HIV
TLR8	ssRNA	ssRNA from viruses, such as influenza virus and HIV
TLR9	DNA	Unmethylated CpG motifs found in bacterial and viral DNA (also can detect host DNA)
TLR10	Function not well understood	Hypothesized similar ligand specificity to TLR2
TLR11-TLR13	Functions not well understood	Various PAMPs

TLR: Toll-like receptor; PAMP: pathogen-associated molecular patterns; LPS: lipopolysaccharide; dsRNA: double-stranded; ssRNA: single-stranded RNA; HIV: human immunodeficiency virus.

Table adapted from previously published data (Fitzgerald et al., 2020; Kawai et al., 2008, & O'Neill et al., 2007).

proinflammatory cytokines and chemokines, which contribute to the recruitment of immune cells to the site of infection. Activation of monocytes can lead to TF expression, thereby directly activating coagulation through the extrinsic pathway (Bode & Mackmanm 2014). TLRs can also directly activate platelets, which releases prothrombotic materials and induces platelet aggregation, a critical step in the formation of thrombi (Antoniak et al., 2014; Engelmann et al., 2013). Recent studies have shown that mice deficient in platelet TLR-4 have been shown to exhibit reduced thrombus formation and improved survival in a model of sepsis (Vallance et al., 2017) suggesting a critical relationship between inflammation and thrombosis.

While the exact mechanisms underlying the role of TLRs in immunothrombosis are not yet fully understood (Vallance et al., 2017), it is clear that TLRs are an important component of the immune response to microbial pathogens and may play a significant role in the regulation of thrombosis in both physiological and pathological settings.

1.9. Introduction to sepsis

1.9.1. Clinical sepsis

Sepsis is defined as a life-threatening organ dysfunction that results in systemic activation of coagulation and inflammation in response to microbial infection (Singer et al., 2016). This excessive response can lead to tissue damage, organ failure, and death. In 2017, sepsis was reported to be the most common cause of in-hospital deaths world-wide and accounted for almost 20% of all global deaths highlighting its burden on society (Rudd et al., 2020). In Canada, the cost of sepsis is estimated to be $1.7 billion (CAD) per year

(Farrah et al., 2021). With an aging population, the cost of sepsis is only expected to increase (Iwashyna et al., 2012). Current management strategies include early administration of antibiotics, fluid resuscitation, and mechanical ventilation. Despite these strategies, the mortality rate from sepsis remains high (22.5%) (Rhee et al., 2019; Rudd et al., 2020), suggesting that an improved understanding of sepsis pathophysiology is required to develop novel therapeutic strategies.

1.9.2. COVID-19 related sepsis

In December 2019, a new zoonotic coronavirus called severe acute respiratory syndrome coronavirus-2 (SARS-CoV-2) was responsible for the global coronavirus disease 2019 (COVID-19) pandemic. Studies indicated that patients with COVID-19 suffered from dysregulated inflammation, coagulation, and fibrinolytic systems (An et al., 2021; Cani et al., 2021; Juneja et al., 2021). As SARS-CoV-2 continued to spread, COVID-related sepsis increased the clinical burden of sepsis with intensive care unit (ICU) 31-day mortality rates reported as high as 20-65% (Arentz et al., 2020; Oliveira et al., 2021). Therefore, further investigation into the development of sepsis may be essential to create innovative treatment approaches to reduce its burden.

1.9.3. Relationship between infection, inflammation, and coagulation

There are several important themes in our current understanding of sepsis pathophysiology. Infection, inflammation, and coagulation are intimately linked in sepsis (Aird, 2003; Hotchkiss & Karl, 2003; van der Poll et al., 2017). It is rare for the initial

infection to cause mortality; rather, mortality is due to the body's maladaptive response to infection. PAMPs activate PRRs on host immune cells such as monocytes, neutrophils, platelets, and endothelial cells. As a result, host immune cells synthesize and secrete large amounts of cytokines and chemokines. These inflammatory mediators damage tissue, which then secretes even more inflammatory mediators creating a positive cycle of tissue injury. Damaged tissues can release damage-associated molecular patterns (DAMPs). Similar to PAMPs, DAMPs bind PRRs on immune cells contributing to sustained inflammation and coagulation. Consequently, this activates platelets and upregulates TF expression on endothelial cells and monocytes, thereby triggering coagulation (Semeraro et al., 2015).

1.9.4. Coagulopathy in sepsis

Sepsis is almost always associated with hemostasis abnormalities. The procoagulant and antifibrinolytic state observed in sepsis is due to (a) anticoagulant pathway impairment (i.e. reduced AT, APC, and TFPI), (b) suppression of fibrinolysis via elevated PAI-1, and (c) up-regulation of procoagulant molecules such as TF (Semeraro et al., 2010).

Coagulopathy is common in sepsis and can result in disseminated intravascular coagulation (DIC). DIC is a condition associated with microvascular and macrovascular thrombosis that leads to poor blood flow and contributes to multiple organ dysfunction (van der Poll et al., 2017). Subsequently, this leads to hemorrhage due to the consumption of clotting factors and platelets (van der Poll et al., 2017). DIC occurs in approximately 25% of the sepsis population and has a significantly higher mortality rate of 45% to 78% (Singh

et al., 2013). In a retrospective observational study including 357 patients, hemostatic markers such as clotting time, platelets, AT, PC, D-Dimer, and fibrinogen levels could be used to identify patients at risk for DIC (Jackson-Chornenki et al., 2020).

1.9.5. Historical sepsis clinical trials

Numerous phase III clinical trials have focused on attenuating inflammation, neutralizing microbial toxins, or dampening coagulation without any success (N. Semeraro et al., 2015). These failures highlight a gap in our understanding of sepsis pathophysiology that is required to develop beneficial therapeutic strategies. A targeted personalized approach may also be necessary to direct treatments to patients who would benefit most. Our lab has recently published a novel longitudinal and multivariable prognostic approach to predict the probability of death and to generate personalized mortality risk profiles in patients with sepsis. These personalized profiles communicate which biological indicator contributes most to the patient's mortality risk, allowing the development of custom-made therapies (Liaw et al., 2019). For example, patients at a high risk of death due to elevated levels of circulating DNA may benefit from deoxyribonuclease I (DNase I) therapy. In contrast, patients with whom deficiencies in PC contribute the most to their mortality risk, may benefit from anticoagulant therapies. We believe that this personalized approach can improve survival and success of future clinical trials.

1.10. Targeting cell-free DNA and histones in sepsis

1.10.1. Using cell-free DNA as a prognostic indicator in sepsis

We previously reported that cell-free DNA (CFDNA) is a useful prognostic indicator in patients with sepsis (Dwivedi et al., 2012). DNA sequence analysis revealed that the circulating DNA in sepsis originates from the host and is not pathogen-derived (Bhagirath et al., 2015). Using DNA methylation analyses, the most significant contributor to CFDNA levels in most septic patients (20-fold greater than normal levels) was granulocytes, with neutrophils being the most abundant (Dor et al., 2018). Interestingly, a correlation was observed between hepatocyte CFDNA and serum levels of alanine aminotransferase (ALT), a standard biomarker for hepatocyte damage, suggesting that the CFDNA in sepsis originates from a combination of neutrophil and tissue injury (Dor et al., 2018).

1.10.2. Neutrophil-derived cell-free DNA

Neutrophils are the most abundant innate immune cells in the human immune system (Mócsai, 2013; Papayannopoulos, 2017). The mechanisms used by neutrophils to eliminate pathogens include phagocytosis, degranulation, and the release of neutrophil extracellular traps (NETs) containing CFDNA. The formation of NETs has been extensively studied in infection and sepsis. NETs are comprised of chromatin (DNA and histones), cathepsin G, neutrophil elastase (NE), myeloperoxidase (MPO), catalase, and bactericidal-permeability-increasing protein, which work together to have antimicrobial activity (Brinkmann et al., 2004; Ramos-Kichik et al., 2009; Shapiro et al., 2006). *In vitro*,

incubation of neutrophils with septic patient plasma triggered NET formation highlighting their importance in human sepsis (Clark et al., 2007). In animal models of sepsis, NET production can be visualized in organs in real-time after infection (Kolaczkowska et al., 2015; McDonald et al., 2012, 2017).

The formation of NETs occurs via a regulated process termed NETosis. NETosis is a unique form of cell death that is distinct from apoptosis and necrosis (Steinberg & Grinstein, 2007). Unlike apoptosis and necrosis, NETosis does not involve DNA fragmentation or phosphatidylserine exposure on the extracellular membrane (Steinberg & Grinstein, 2007).

Bacteria, fungi, viruses, or immune complexes can activate neutrophils through various receptors to induce NETosis (Figure 1.3). Activation of neutrophil surface receptors causes the intracellular calcium concentration to increase, thereby activating protein kinase C (PKC) (Papayannopoulos, 2017). Through NADPH oxidase, reactive oxygen species (ROS) are produced, which results in the degradation of the nuclear envelope and granule rupture (Papayannopoulos, 2017). Mixing of cytosolic, nuclear, and granule components allows peptidylarginine deiminase-4 (PAD4) to convert arginine to citrulline on histone H1, H3, and H4 (P. Li et al., 2010; Y. Wang et al., 2009). Citrullination reduces histones' positive charge and prevents chromatin condensation (Martinod et al., 2013; Y. Wang et al., 2004, 2009). PAD4-deficient mice cannot produce NETs, but retain their neutrophil ability to phagocytose (Hemmers et al., 2011; P. Li et al., 2010; Martinod et al., 2013). MPO released from granules oxidizes and activates NE resulting in the degradation of the actin cytoskeleton to block phagocytosis. NE can also aid chromatin decondensation

through histone processing (Papayannopoulos, 2017). Lastly, the DNA/granular enzyme mixture is released into the extracellular space through membrane rupture (Fuchs et al., 2007). These pose as DAMPs which can perpetuate the proinflammatory and prothrombotic environment.

1.10.3. The role of NETs in inflammation and hemostasis

NETs serve as a scaffold for platelet aggregation, leukocyte interactions, and red blood cell (RBC) accumulation resulting in microvascular thrombosis and tissue ischemia (Brill et al., 2011, 2012; Clark et al., 2007). At the site of infection and acute inflammation, NETs are widely distributed (Clark et al., 2007). Mice that are unable to produce NETs (i.e. PAD4 deficient mice) have an increased susceptibility to bacterial infections highlighting their importance in sepsis (P. Li et al., 2010; Martinod et al., 2013). PAD4-deficiency also protects from venous and carotid artery thrombosis and vascular injury (Knight et al., 2014; Martinod et al., 2013). In thrombosis studies, through interactions with vWF, fibronectin, and fibrinogen, NETs have been shown to stabilize platelet-rich clots and promote platelet aggregation, thereby contributing to thrombosis (Fuchs et al., 2010; Martínez Valle et al., 2008; Napirei et al., 2004).

NETs can also stimulate inflammation by creating areas of ischemia, endothelial damage, and promoting more neutrophil recruitment and NETosis forming a positive feedback loop. Proinflammatory cytokines, such as IL-1β, tumor necrosis factor α (TNFα), and IL-8, can stimulate neutrophils to produce NETs by activating NADPH oxidase and MPO (Keshari et al., 2012). The stimulation of neutrophils, in turn, leads to the further release of inflammatory mediators, creating a positive feedback loop. MPO released during

Figure 1.3. Cellular mechanisms of NETosis. NETosis can be triggered in neutrophils by various microorganisms and endogenous stimuli. Activation of cell surface receptors leads to reactive oxygen species (ROS) production by induction of MEK-extracellular-signal regulated kinase (ERK) signaling triggering the myeloperoxidase (MPO) pathway. MPO oxidation releases neutrophil elastase (NE), which degrades the actin cytoskeleton inhibiting phagocytosis. NE then translocates to the nucleus to drive histone processing and chromatin decondensation. Further chromatin decondensation occurs through histone deamination by peptidylarginine deiminase-4 (PAD4) resulting in the release of neutrophil extracellular traps (NETs) by membrane rupture. Figure adapted from (Jorch & Kubes, 2017; Papayannopoulos, 2017) and created with BioRender.

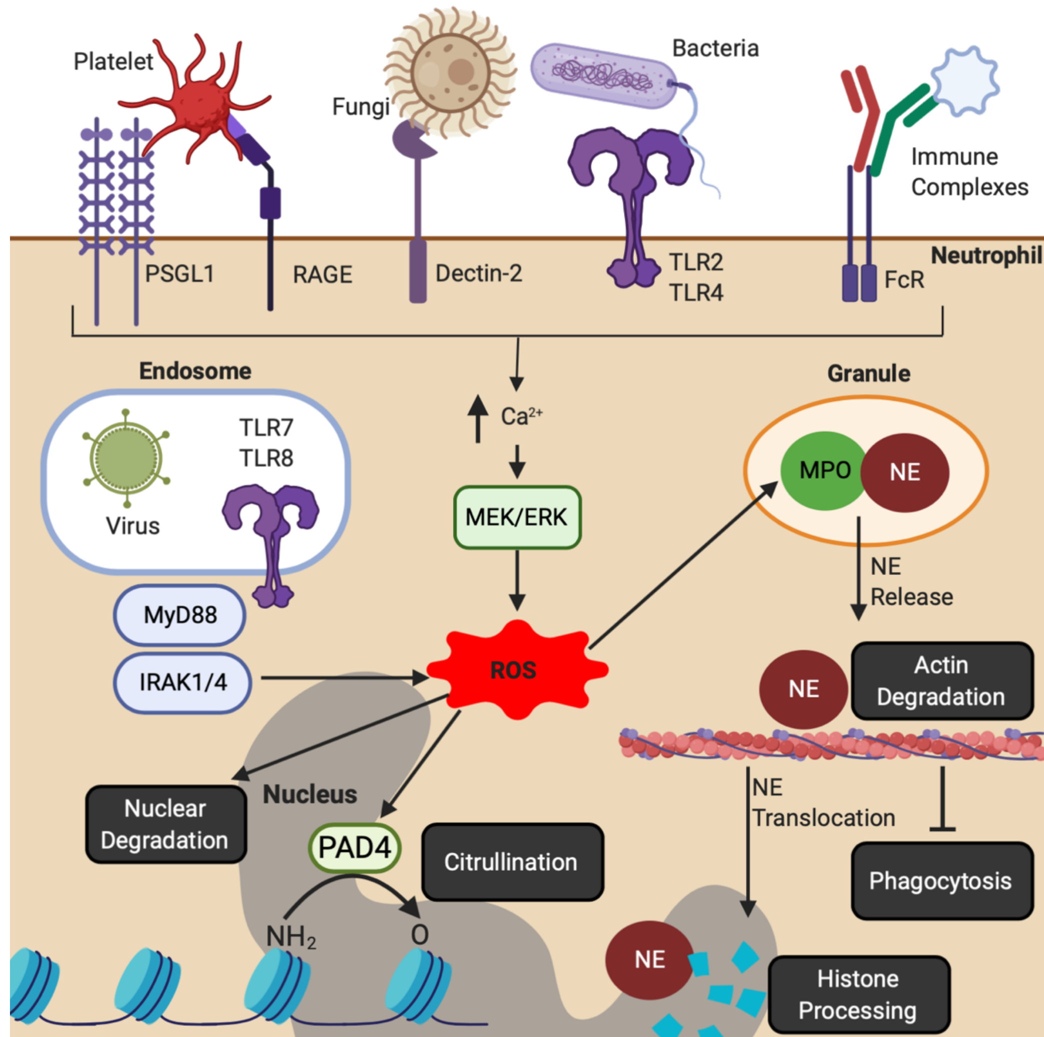

Figure 1.3. Cellular mechanisms of NETosis.

NETosis induces endothelial cell damage through binding negatively charged GAGs in the glycocalyx (Baldus et al., 2001). Endothelial damage, as discussed in section 1.2, can contribute to a proinflammatory and procoagulant environment. Endothelial damage can also occur when stimulated neutrophils are perfused along an activated endothelial cell monolayer via platelet-neutrophil interactions (Clark et al., 2007). Depletion of neutrophils or platelets reduces NET formation and liver damage in LPS and E. coli-induced sepsis (Clark et al., 2007).

1.10.4. The role of cell-free DNA in NETs

NETs can be degraded by DNase I, but not by proteases which demonstrates the importance of DNA in the structural integrity of NETs (Brinkmann et al., 2004). When NETs are dismantled with DNase I, the killing of bacteria is reduced, suggesting that the fibrous structure of DNA is necessary for sequestering and killing bound microbes (Brinkmann et al., 2004).

CFDNA might exert damage to the host as *in vitro* studies suggest CFDNA modulates inflammation, coagulation, and fibrinolysis (Figure 1.4). DNA can pose as a DAMP binding through TLR-9 (Bamboat et al., 2010), potentially contributing to the proinflammatory environment seen in sepsis. Extracellular DNA can also trigger blood coagulation via the intrinsic pathway in a FXII- and FXI-dependent manner (Gould et al., 2014). This is supported by the observation that the procoagulant activity of DNA is inhibited when corn-trypsin inhibitor (CTI) is added to block FXIIa (Gould et al., 2014). CFDNA also activates platelet integrin $\alpha_{IIb}\beta_3$ (GPIIb/IIIa) in a TLR-dependent manner (Bhagirath et al., 2015). In addition to activating coagulation, CFDNA inhibits fibrinolysis

by impairing plasmin-mediated fibrin degradation and enhancing PAI-1-mediated inhibition of tPA (Gould et al., 2015; Komissarov et al., 2011).

In a retrospective observational study of septic patients, our research group reported that incorporating CFDNA into the multi-organ dysfunction score (MODS) provided higher predictive power than MODS alone (Dwivedi et al., 2012). In plasma samples from septic patients, there was a positive correlation between plasma levels of CFDNA and thrombin generation (Dwivedi et al., 2012; Gould et al., 2014). Addition of DNase I to septic plasma samples attenuates the procoagulant potential of the samples (Dwivedi et al., 2012; Gould et al., 2014). In addition to activating coagulation, CFDNA can also inhibit fibrinolysis by impairing plasmin-mediated fibrin degradation. Clots formed from septic patients' plasma are resistant to fibrinolysis, a phenomenon overcome by DNase I addition (Gould et al., 2015).

Together, these *in vitro* studies suggest that CFDNA may contribute to the procoagulant and antifibrinolytic state observed in sepsis. Although elevated levels of CFDNA have been reported to accelerate thrombus growth in a mouse model of thrombosis, (Gaitzsch et al., 2017) it is still unknown if CFDNA is solely a prognostic biomarker or contributes to the pathogenesis of sepsis *in vivo*.

1.10.5. Targeting cell-free DNA in sepsis with DNase I

CFDNA can be degraded by endogenous DNases, enzymes responsible for the hydrolytic cleavage of phosphodiester bonds in the DNA backbone (Napirei et al., 2009). DNase I has been extensively studied and is an ubiquitously expressed endonuclease that

Figure 1.4. Harmful effects of cell-free DNA on inflammation, coagulation, and fibrinolysis. *In vitro* studies have shown that CFDNA can activate inflammation through toll-receptor (TLR) -9, found on immune cells and endothelial cells. CFDNA can activate coagulation by activating platelets in a TLR-dependent manner, accelerating FXII activation in the presence of prekallikrein and high-molecular-weight kininogen (HMWK), and accelerating FXI activation by thrombin (FIIa). CFDNA can also inhibit fibrinolysis by binding fibrin, binding plasmin (pln) to inhibit plasmin mediated degradation of fibrin, and enhancing plasminogen-activator inhibitor-1 (PAI-1) inhibition of tissue plasminogen activator (tPA). DNases are enzymes responsible for the degradation of DNA. Figure created in BioRender.

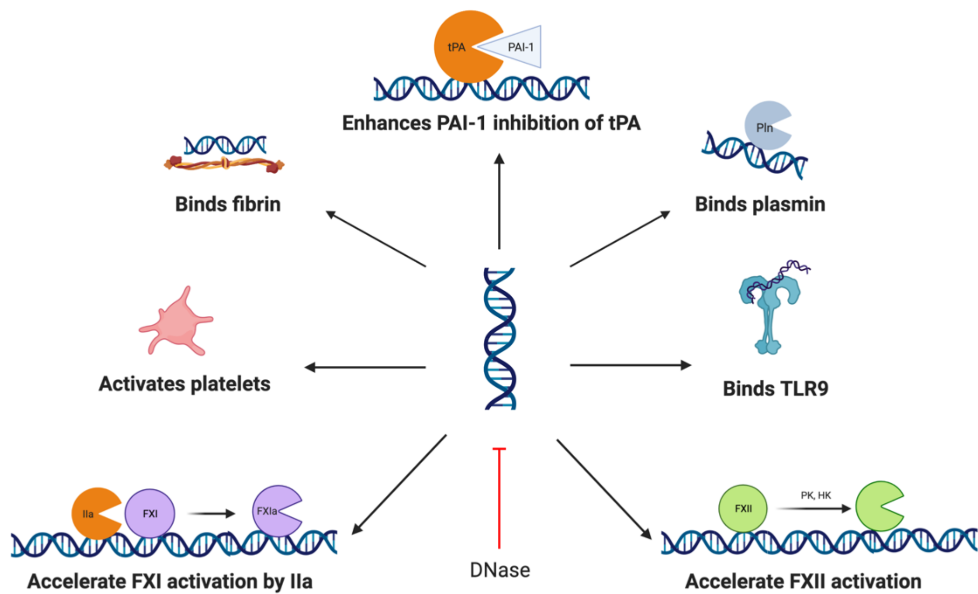

Figure 1.4. Harmful effects of cell-free DNA on inflammation, coagulation, and fibrinolysis.

cleaves the DNA backbone in the presence of divalent cations (Mg^{2+} and Ca^{2+}) under neutral pH conditions (Guéroult et al., 2010). It is a member of a family including four nucleases (DNase I, DNase1L1, DNase1L2, and DNase1L3) that have a high degree of sequence homology but differ from one another in terms of their preferred substrates and modes of production (Ludwig et al., 2009; Shiokawa & Tanuma, 2001). Specifically, DNase I creates single-stranded nicks through nonspecific hydrolysis of phosphodiester bonds yielding oligonucleotide fragments with 5'-phosphorylated and 3'-hydroxylated ends (Guéroult et al., 2010).

DNase I is essential for the depolymerization of actin in cytoskeleton regulation, DNA clearance during apoptosis, and DNA degradation in the alimentary tract (Counis & Torriglia, 2000; dos Remedios et al., 2003; Grimble, 1994). The serum endonuclease, found at plasma concentrations of 3 ng/mL (W. S. Prince et al., 1998), is produced by various places (e.x. the liver, pituitary gland, organs of the gastrointestinal tract, thyroid, pancreas, salivary glands, etc.) and then cleared by the liver (Ludwig et al., 2009). DNase activity deficiency is associated with elevated levels of CFDNA in diseases such as sepsis, systemic lupus erythematosus (SLE), psoriasis, and cancer (Gould et al., 2011; Keyel, 2017; Sohrabipour et al., 2021).

Currently, therapeutic DNase I (Pulmozyme by Roche) is only approved as a pulmonary inhalant in patients with cystic fibrosis. By reducing DNA buildup in the sputum and lungs, DNase I treatment lowers neutrophil recruitment, lung damage, and sputum viscosity to improve pulmonary function in cystic fibrosis (Rahman et al., 2011; Robinson, 2002). Therapeutic DNase I (either as an inhalant or intravenous administration) is also

being considered for use in other diseases that have elevated levels of circulating CFDNA such as sepsis, inflammatory lung diseases, SLE, and cancer (Alcazar-Leyva et al., 2009; Benmerzoug et al., 2018; Davis et al., 1999, Juneja et al., 2021).

In support of using DNase I as a treatment in sepsis, administration of delayed DNase I treatment (>6 h post-sepsis) in a CLP mouse model of sepsis reduced organ damage and partially improved survival outcomes (Mai et al., 2015). A decrease in CFDNA, IL-6, organ damage, and an increase in IL-10 was observed in mice administered DNase I (Mai et al., 2015). If DNase I was administered too early, mice succumbed to bacterial burden suggesting CFDNA is important to the progression of sepsis (Czaikoski et al., 2016; Mai et al., 2015; Meng et al., 2012). Taken together, these studies suggest that high levels of CFDNA may contribute to sepsis pathophysiology and that delayed targeting of CFDNA is a promising therapeutic strategy.

As a result, this has led to the use of DNase I in sepsis clinical trials. Clinical studies have begun to recruit patients to investigate aerosolized DNase I (NCT04541979 and NCT04445285) in COVID-19 patients with respiratory failure and intravenous DNase I (NCT05453695) as an intervention in septic ICU patients.

1.10.6. Targeting histones in sepsis

Another potential strategy in sepsis is to neutralize the harmful effects of histones (Figure 1.5). CFDNA likely circulates in complex with histones in the form of nucleosomes (Ekaney et al., 2014). Nucleosomes consist of ~150 bp of DNA wrapped around a core

histone octamer, which contains two copies of histone H2A, H2B, H3, and H4 (Figure 1.6). Histones are cationic, nuclear proteins essential for the structure and function of chromatin.

Previous studies have shown that histones are a prognostic biomarker in patients with sepsis. In a retrospective observational study including 126 patients with sepsis, mortality was associated with elevated levels of histone H4 (Lu et al., 2020). H3 and citrullinated-H3 derived from NETosis were also shown to be associated with coagulopathy, multiple organ failure, and mortality suggesting histones may contribute to the pathogenesis of sepsis (Nakahara et al., 2013; Yokoyama et al., 2019). Levels of histones in humans have been estimated to reach up to 50 mg/kg (Nakahara et al., 2013; Y. Zhang et al., 2022).

In mice, intravenous injections of purified histones resulted in thrombocytopenia, neutrophil migration, and organ failure mimicking the pathophysiological nature of sepsis (Xu et al., 2009). Administration of 50 mg/kg of histones into mice led to DIC, and administration at 75 mg/kg was lethal (Wu et al., 2020; Xu et al., 2009). C-reactive protein (CRP) can counteract histone toxicity and can save mice from histone-induced death (Abrams et al., 2013). Targeting histones with an anti-H4 antibody significantly improved survival in various animal models of sepsis highlighting their importance in the pathogenesis of sepsis (Xu et al., 2009). In a LPS mouse model and a baboon *E. coli* model of sepsis, APC can cleave histones, and co-injection of histones with APC lowers histone cytotoxicity and mortality (Xu et al., 2009). Altogether, this suggests histones may contribute to the pathogenesis of sepsis.

Figure 1.5. The harmful effects of histones on cytotoxicity and hemostasis. Histones are cytotoxic to endothelial cells via TLR-2 and TLR-4. Histones can also stimulate the production of fibrin by promoting the expression of phosphatidylserine (PS) on erythrocytes, activating platelets in a TLR-2 and TLR-4 dependent manner, binding FXa to create an alternative prothrombinase, and promoting autoactivation of prothrombin to generate thrombin independent of the coagulation cascade. Histones can also impair the anticoagulant pathway through the inhibition of protein C (PC) and thrombomodulin (TM). Histones can also bind fibrin leading to clots that are thicker and more resistant to fibrinolysis. Activated PC (APC), C-reactive protein (CRP), heparan sulfates on the surface on the endothelium, and therapeutic heparins can bind and neutralize the harmful properties of histones. Figure created with BioRender.

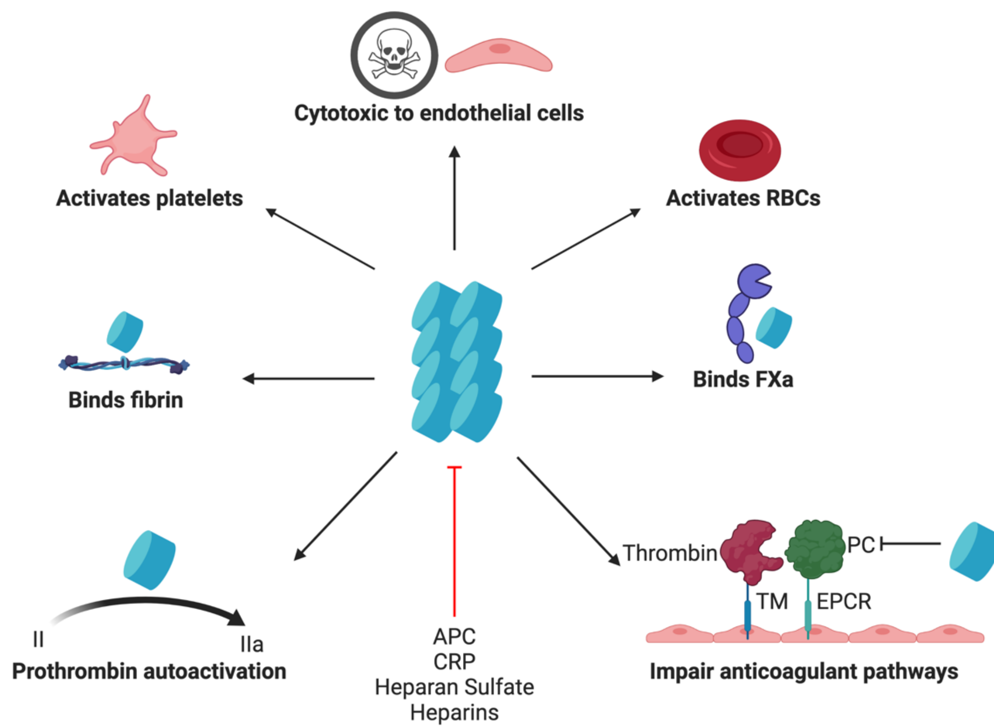

Figure 1.5. Harmful effects of histones on cytotoxicity and hemostasis.

Figure 1.6. Nucleosome structure. Nucleosomes are made up of a histone octamer core (2 copies of histone H2A, H2B, H3, and H4) which DNA (147 bp) wraps around in 1.65 turns (Luger et al., 2012). Figure created in BioRender.

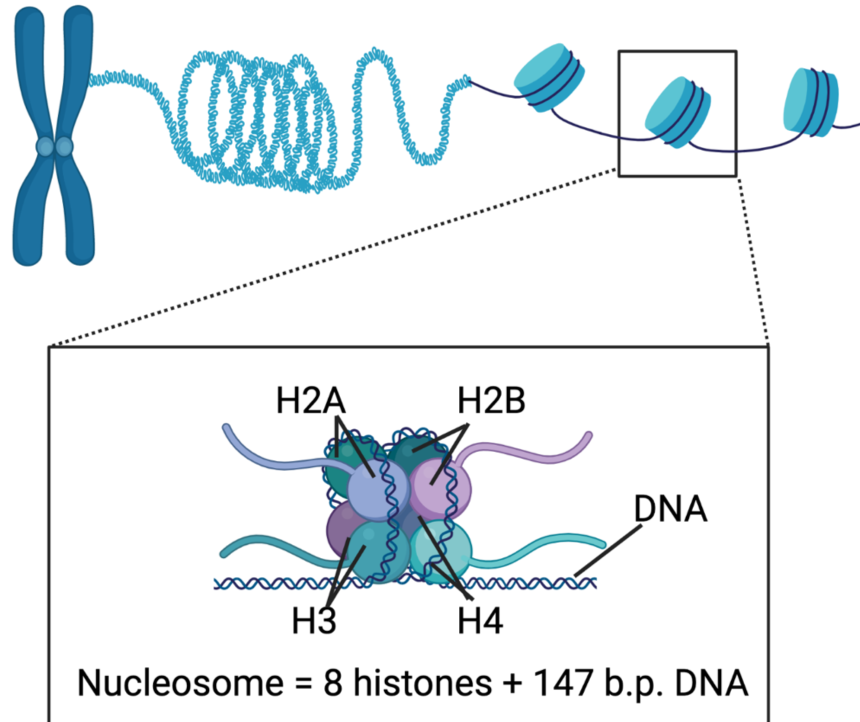

Figure 1.6. Nucleosome structure.

In vitro, histones can activate endothelial cells in a TLR-2 and -4 manner which increases endothelial cell dysfunction markers such as vWF, P-selection expression, and inflammatory mediators (Lam et al., 2016; F. Semeraro et al., 2011). At high concentrations, histones induce Ca^{2+} influx leading to cytotoxicity (Abrams et al., 2013). Small polyanions (SPAs) that interact with histones can inhibit endothelial-mediated cytotoxicity and improve sepsis outcomes in a murine model of sepsis. However, the SPA's ability to bind histones was altered when interacting with free versus NET-bound histones (Meara et al., 2020). Histones can also affect clot formation and structure. In the presence of histones, clots become resistant to fibrinolysis as histones bind and increase fibrin fiber thickness (Longstaff et al., 2013). Histones can also bind FXa and form an alternative prothrombinase that can activate thrombin without FVa and phospholipids (Abrams et al., 2021). They can also bind prothrombin allowing it to autoactivate and generate thrombin independent of the coagulation cascade (Barranco-Medina et al., 2013). Histones also promote the expression of phosphatidylserine on erythrocytes and activate platelets in a TLR-2 and TLR-4 dependent manner. Activated platelets release their granule contents, which promotes coagulation and fibrin formation (F. Semeraro et al., 2008, 2011). Not only can histones promote coagulation, but they can impair the anticoagulation pathway as they can reduce TM-dependent PC activation (Ammollo et al., 2011). The harmful effects of histones can be neutralized by physiological heparan sulphate on the surface of the endothelium or through the administration of prophylactic/therapeutic heparins or antibodies.

1.11. Heparin

Heparins are GAGs that possess histone neutralization, anti-inflammatory, anticoagulant, and profibrinolytic effects (Bazzoni et al., 1993; Wildhagen et al., 2014; Xu et al., 2009). Heparins inhibit inflammation in several ways: they (a) inhibit neutrophil activation (Bazzoni et al., 1993), (b) inhibit LPS-induced inflammatory mediators' secretions (Li et al., 2015), and (c) inhibit NF-κB activation (Li et al., 2013). Heparins are also anticoagulant and profibrinolytic, which is why they are common thromboprophylactic agents used in ICU septic patients to prevent VTE. There are two main categories of heparins that inhibit coagulation in slightly different ways: unfractionated heparin (UFH) and low-molecular-weight heparin (LMWH).

1.11.1. Unfractionated heparin (UFH)

UFH inhibits coagulation by (a) binding AT via its pentasaccharide sequence forming a ternary complex with FXa or thrombin, which inhibits thrombin generation by 1000-fold; (b) inhibiting thrombin-induced activation of FV and FVIII; and (c) promoting the release of TFPI (Alban, 2005; X. Li & Ma, 2017).

In patients diagnosed with sepsis, UFH reduced thrombin generation and clot lysis times, thereby reducing a procoagulant state (Houston et al., 2015). A large meta-analysis reported that UFH reduced 28-day mortality, organ dysfunction, and ICU length of stay (Fu et al., 2022). However, UFH is not commonly used in sepsis thromboprophylaxis as it can induce heparin-induced thrombocytopenia (HIT) and therapeutic dosing requires close monitoring. Consequently, LMWHs are preferred in clinical practice.

1.11.2. Low-molecular weight heparin (LMWH)

LMWH is an anticoagulant that exerts its effect by also binding AT, however, it has a reduced ability to inhibit thrombin and greater activity against FXa due to its smaller size (Nutescu et al., 2016). For VTE occurrence, there was no difference between UFH and LMWH administration (The PROTECT Investigators for the Canadian Critical Care Trials Group and the Australian and New Zealand Intensive Care Society Trials Group, 2011). Despite this, LMWH is more commonly used in thrombosis prophylaxis due to a lower risk of HIT, once daily dosing, and a more predictable pharmacodynamic and pharmacokinetic profiles (Nutescu et al., 2016).

Enoxaparin and dalteparin are the two most widely used LMWHs for thromboprophylaxis in North America (Miano et al., 2018). In the past, American hospitals have used these drugs interchangeably and in a trauma population, they have been shown to be equally as effective at preventing VTE (Miano et al., 2018). In Canada, where this thesis has taken place, dalteparin is more commonly used in ICU critically ill patients (Cook et al., 2011; Przybysz & Huang, 2011).

1.11.3. Nonanticoagulant heparin

Nonanticoagulant heparins were created to reduce any risk of bleeding that UFH and LMWH had. They are created by removing or inactivating the AT binding sequence, which can be achieved by complexation with AT applying affinity chromatography or precipitation experiments (Casu et al., 2009). These nonanticoagulant heparins retain the

ability to neutralize histone-mediated cytotoxicity and can effectively be used without increased bleeding risks (Wildhagen et al., 2014).

1.11.4. Heparins in sepsis

Heparins target the interplay between immunity and thrombosis, which improves survival (Ding et al., 2011). In noncritically COVID-19 patients, a therapeutic dose of heparin improved probability of hospital discharge and reduced use of organ support compared to prophylactic doses (REMAP-CAP Investigators et al., 2021). However, in moderately and critically ill patients, therapeutic heparins do not significantly improve survival and probability of hospital discharge compared to prophylactic doses (ATTACC Investigators et al., 2021; Scholzberg et al., 2021).

In pre-clinical animal studies, prophylactic and therapeutic heparin doses have been shown to increase survival compared to controls (Li et al., 2011). This is likely, in part, due to its histone-neutralizing abilities since co-administration of UFH with histones can improve histone-mediated cytotoxicity in a CLP murine model of sepsis (Wang et al., 2020). In the same study, UFH was concluded to alleviate apoptosis and inflammation by neutralizing histones, thereby improving survival (Wang et al., 2020). In a histone-induced sepsis model (50 mg/kg of H3), administration of prophylactic doses of both UFH and LMWH improved sepsis outcomes such as reducing WBCs counts, endothelial cell death, and leukocyte death which improved renal function (Iba et al., 2015). Even nonanticoagulant heparins were shown to be beneficial in murine models of sepsis, suggesting that the mechanism by which heparins are beneficial may be independent of

their anticoagulant properties. Nonanticoagulant heparins can still bind histones and inhibit histone-mediated cytotoxicity, which reduces sepsis and sterile inflammation-related mortality in mice models without altering hemostasis and bleeding risks (Wildhagen et al., 2014). The ability of nonanticoagulant heparins to attenuate histone induced inflammation has also been confirmed *ex vivo* in whole blood (Hogwood et al., 2020). As a result, heparins are hypothesized to be beneficial in sepsis partly due to their histone-neutralizing ability and not just their anticoagulant properties (Buijsers et al., 2020).

1.12. Introduction to preclinical animal models

1.12.1. Types of animal models in sepsis

Since it is not ethical to test new therapies in critically ill patients with sepsis, animal models are used as a platform for testing treatments. The goal of preclinical animal studies is to reproduce a clinically relevant pathogenesis of sepsis that allows treatments to be translatable to human sepsis. There are many animal models to utilize, but murine models of sepsis are the most common due to their small size, short-life, relatively low cost, and genetic similarity to humans. The link between inflammation, coagulation, and innate immunity could not have been established if it were not for mouse models (Henke et al., 2011; Wakefield et al., 2008). However, despite these models, most of the proposed therapies for sepsis have failed.

There are 3 major categories for murine models of sepsis: (1) administration of a toxin such as LPS, (2) administration of a pathogen such as bacteria (ex. fecal induced

peritonitis; FIP), or (3) alteration of the mouse's protective barrier such as colonic puncture (ex. cecal-ligation and puncture; CLP) (Buras et al., 2005).

The endotoxin LPS is the most common microbial mediator in sepsis (Opal, 2010). LPS is a component of gram-negative bacteria membranes and is recognized by TLR-4. Binding initiates a signalling pathway through MyD88 enabling the release of proinflammatory stimuli such as interleukins (Yamamoto & Akira, 2010). This upregulation of proinflammatory mediators leads to the development of sepsis. Since sepsis can also be caused by gram-positive bacteria, fungi, viruses, and parasites, LPS would not play a role in their pathogenesis, limiting its clinical relevance. Additionally, the LPS model does not reproduce the characteristics of human sepsis as LPS administration leads to a large early wide-spread cytokine activation (Deitch, 1998; Seemann et al., 2017). Therefore, this model has been recommended as a way to study the pathophysiological processes of endotoxemia and endotoxic shock, but not sepsis in general (Seemann et al., 2017).

FIP, also referred to as peritoneal contamination and infection (PCI) in literature, is a polymicrobial sepsis model that was developed to compensate for the weaknesses in the LPS model (Seemann et al., 2017). FIP injections lead to a slower development of sepsis compared to LPS making it more clinically relevant to human sepsis. However, human patients rarely have massive bacteremia, which limits its clinical translation (Seemann et al., 2017).

The most common sepsis model is the CLP polymicrobial murine model of sepsis, which entails a ligation and then puncture of the cecum resulting in bacterial translocation

from the colon to the intraperitoneal cavity. This mimics the pathophysiology of human sepsis as it is similar to a polymicrobial peritonitis or human abdominal perforation model of sepsis (Wichterman et al., 1980). Therefore, this model has been considered a gold-standard for preclinical murine studies (Seemann et al., 2017).

1.12.2. Improving translatability

Since numerous clinical trials have failed in human sepsis, translatability from animal models is considered poor. To combat this, the Animal Research: Reporting of In Vivo Experiments (ARRIVE) guidelines are used to improve reporting of animal research to maximize the quality/reliability of research (Kilkenny et al., 2010; Percie du Sert et al., 2020). Specifically in sepsis, the Minimum Quality Threshold in Preclinical Sepsis Studies (MQTiPSS) was created to improve translatability and humane animal sepsis (Hellman et al., 2019; Libert et al., 2019; Zingarelli et al., 2019). The three reviews a part of the MQTiPSS provide guidelines on study design, humane modelling, endpoints, fluid resuscitation, and antimicrobial therapies. Since patients with sepsis always get fluids and antibiotics, it is important to include these in sepsis animal models. However, we noted that almost all critically ill patients with sepsis in the ICU, will also receive some sort of thromboprophylaxis. While some researchers will add fluids or antibiotics, it is also important to understand how treatments are affected by thromboprophylaxis. By implementing these guidelines, animal models become more clinically relevant, and thus, may have better translatability.

2.0. Hypothesis and Objectives

2.1. Rationale and Hypothesis

CFDNA is an important prognostic biomarker in sepsis and has been hypothesized to contribute to sepsis pathophysiology due to its procoagulant phenotype (Dwivedi et al., 2012; Gould et al., 2015). However, the role of DNA in coagulation has mainly been studied using DNA that has been contaminated with silica and polyP that may confound our interpretation of its procoagulant effects (Smith et al., 2017 & 2018). As a result, it is important to characterize alternative isolation methods that yield silica- and polyp-free DNA (Smith et al., 2017).

Despite this, reporting the effects of individual components *in vitro* may not be representative of their effects *in vivo.* Since DNA typically exists bound to histones in a complex (i.e. a nucleosome), it is also important to understand the differences between the purified components and their complexes *in vivo*.

Understanding the effect of nucleosomes and their individual components *in vivo* will allow us to further understand how to potentially develop new strategies for targeting nucleosomes. Both DNase I (target DNA) and heparins (target histones) have been shown to improve outcomes in experimental models of sepsis, however, no one has compared them to each other in a single study or used a combination approach. The biological rationale for adjunctive treatment of DNase I and LMWH is 3-fold. Firstly, circulating DNA likely exists in complex with histones in the form of a nucleosome. We show in this thesis that digestion of the DNA component in nucleosomes releases histones, which have potent cytotoxic and platelet-activating abilities. Secondly, DNase I and heparins have been

shown to function synergistically *in vitro* as heparins increase in the ability of DNase I to cleave DNA by 3 to 5-fold (Brotherton et al., 1989; Napirei et al., 2009). Lastly, heparin is administered to all ICU patients as standard thromboprophylaxis. If we want to explore the therapeutic efficacy of DNase I in septic patients, it would be important to consider the therapy in the context of heparins.

We hypothesize that (i) individual components of nucleosomes, but not nucleosomes, will be harmful *in vitro* and/or *in vivo* and (ii) targeting both DNA and histones by using a combination of DNase I and heparin would be beneficial in a mouse model of sepsis compared to monotherapy.

2.2. Objectives

The specific objectives for this thesis are as follows:

Objective 1: To identify and analyze various methods of DNA purification that result in DNA that is free of contamination by procoagulant silica and polyP molecules.

Objective 2: To investigate the pathological effects of purified DNA, histones, and nucleosomes (DNA-histone) complexes in a murine model of sepsis.

Objective 3: To determine the therapeutic efficacy of DNase I alone or in combination with LMWH in a murine model of sepsis.

3.0. Isolation of silica- and polyphosphate-free DNA and characterization of its role in coagulation

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Foreword: In chapter 3, we investigate:

1. Current methods of DNA isolation result in silica and polyphosphate contamination.
2. Two alternate methods of isolating contaminant-free DNA are reported.
3. Contaminant-free DNA is procoagulant whereby this activity is neutralized by DNase I.
4. The two new methods are appropriate for isolating DNA to study its procoagulant properties.

Running title: Properties of silica- and polyphosphate-free DNA

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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

Background: Although extracellular DNA has been reported to activate coagulation, its direct effects and consequent interpretations have recently been questioned due to silica and DNA polyphosphate (polyP) contaminations when DNA is isolated using common silica-based kits.

Objectives: To identify and characterize alternative methods of isolating DNA that is free of silica and polyP.

Methods: DNA was isolated from the whole blood or buffy coat using 3 different DNA isolation kits: (i) the silica-based QIAGEN QIAMP DNA Blood mini kit (silica-DNA) (ii) the non-silica- based QIAGEN PAXgene Blood DNA kit (PAX-DNA), and (iii) the non-silica-based QuickGene DNA whole blood kit large (DBL-DNA). The procoagulant properties of DNA were assessed by thrombin generation and plasma clotting assays. A polyP detection assay was used to detect polyP contamination.

Results and Conclusions: Unlike the isolated DNA, commercially available calf thymus contains thrombin-like amidolytic activity. PAX-DNA and DBL-DNA contained no silica- or polyP-contamination. Both PAX- and DBL-DNA were

procoagulant in a dose-dependent manner, which is neutralized with DNase I. Thus, we recommend the use of PAX-DNA or DBL-DNA for functional studies to investigate the role of extracellular DNA.

3.2. Introduction

The intrinsic pathway of coagulation has emerged as an important interface between innate immunity, inflammation, and thrombosis. Negatively charged molecules such as DNA, RNA, and polyphosphates (polyP) have been shown to activate the intrinsic pathway (Choi et al., 2011; Gould et al., 2014; Kannemeier et al., 2007; Smith et al., 2018). Extracellular DNA, a major component of neutrophil extracellular traps, has been intensely examined and reported to activate coagulation in a factor (F) XII- and FXI- dependent manner (Bhagirath et al., 2015; Gansler et al., 2012; Gould et al., 2015; Swystun et al., 2011). Recently, Smith *et al.* reported in a Letter to Blood that isolating nucleic acids using common silica-based kits results in silica contamination that may confound our interpretation of the procoagulant effects of DNA (Smith et al., 2017). Since DNA is primarily isolated by silica-based columns, it is important to clarify the role and consequence of silica contamination and explore alternative silica-free isolation methods (Smith et al., 2017). The negative charge of silica promotes coagulation through the intrinsic pathway by autocatalytic activation of FXII and FXI (Salloum-Asfar et al., 2018). In addition, a recent study reported that polyP co-purifies with DNA or RNA that is isolated using several common nucleic acid isolation methods (Smith et al., 2018), which casts uncertainty in assessing the apparent role of DNA in coagulation. These studies highlight the importance of investigating alternative sources of DNA that is free of both silica and polyP contamination. In this study,

we identify potential alternative sources of isolating silica-free and/or polyP-free DNA by commercially kits that do not require silica.

3.3. Materials and Methods

3.3.1. Materials

RedSafe was purchased from FroggaBio (North York, ON, Canada). QIAMP DNA blood mini kit, PAXgene Blood DNA tubes, and PAXgene Blood DNA kits were purchased from QIAGEN (Mississauga, ON, Canada). QuickGene DNA whole blood kit large was purchased from Kurabo Industries (Osaka, Osaka, Japan). Calf-thymus DNA (CT-DNA) was purchased from Calbiochem (San Diego, CA, USA). UltraPure™ DNase/RNase-Free distilled (DRFD) water and calf intestinal alkaline phosphate (20,000 U/µL) were purchased from Thermo Fisher Scientific (Mississauga, ON, Canada). A thrombin-specific chromogenic substrate (S-2238) was purchased from DiaPharma (West Chester, OH, USA). Technothrombin thrombin generation assay (TGA) and Technothormbin TGA software were purchased from Technoclone (Vienna, Austria). Recombinant human deoxyribonuclease I (DNase I; Pulmozyme® dornase alpha) was from Genentech (San Francisco, CA, USA). Polyacrylamide (10%) TBE-urea (7 M) Ready Gels (8.6 x 8.6 cm^2) were purchased from Bio-Rad (Hercules, CA, USA). Corn trypsin inhibitor (CTI), a contact pathway inhibitor, was purchased from Molecular Innovations (Novi, MI, USA). $PolyP_{70}$ was purchased from BK Giulini

Gmbh (Werk Ladenburg, Germany).

3.3.2. Preparation of platelet-poor plasma (PPP)

Plasma samples were obtained by collecting blood via venipuncture into 3.2% trisodium citrate (0.109 M) from healthy adult volunteers who were 18-56 years old and not receiving medication. Whole blood was centrifuged at 1500 × g for 10 minutes at room temperature and the plasma layer was collected. PPP from 10 to 15 volunteers were pooled, aliquoted, and stored at -80°C. Approval was obtained by the Hamilton Integrated Research Ethics Board (HiREB #12-712-T).

3.3.3. Isolation of DNA using commercial kits

To isolate DNA using a silica-based column (silica-DNA), DNA was extracted from buffy coat using the QIAMP DNA blood mini kit according to the manufacturer's instructions with minor modifications. Buffy coat was isolated from citrated whole blood from healthy human volunteers as previously described (Repnik et al., 2003). DNA was then eluted using the DRFD water. The procedure was then repeated using water as control (silica-blank).

DNA was also extracted using the PAXgene Blood DNA kit (PAX-DNA) or the QuickGene DNA whole blood kit (DBL-DNA) with minor modifications. For PAX-DNA, blood was collected via venipuncture from healthy volunteers into PAXgene Blood DNA tubes, which was then transferred to processing tubes that

contained lysis buffer. Cell nuclei and mitochondria were pelleted by centrifugation, washed, and suspended in digestion buffer. Protein contaminants were removed by incubation with a protease mixture. DNA was precipitated with isopropanol, washed in 70% ethanol, dried, and suspended in DRFD water instead of the supplied buffer.

For DBL-DNA, blood was again collected into trisodium citrate. Cells were then lysed, and DNA was precipitated using 99% ethanol. To increase the DNA yield per column while increasing the concentration of each isolation, each column was loaded with 4-times the recommended volume of whole blood lysate (from 2 mL to 8 mL). The lysate was then loaded onto the column, washed with the buffer provided, and eluted with DRFD water instead of the elution buffer. All DNA samples were then visualized for purity assessment by 1.5% agarose gel and stained with RedSafe, aliquoted and stored at -20°C.

3.3.4. Thrombin activity assay

Thrombin-like activity in DNA was assessed by monitoring hydrolysis of the thrombin-specific substrate S-2238 in the presence or absence of DNA. Wells containing 400 µM S-2238 and 5 mM $CaCl_2$ were incubated at 37°C. The reactions were initiated by the addition of silica-blank, silica-DNA, PAX-DNA, DBL-DNA, CT-DNA (40 µg/mL), or thrombin (5 nM). The reactions were monitored at 405 nm at 37°C, with readings at 1-minute intervals.

3.3.5. Thrombin generation assay

TGAs were performed using a black 96-well microtitre plate, with the well containing PPP (40 μL) in the presence of PAX-DNA at varying concentrations (0, 5, 10, 20 μg/mL). Thrombin generation was initiated by the addition of 50 μL of the Technothrombin TGA substrate mixture (containing 15 mM $CaCl_2$ and a fluorogenic thrombin substrate). Where specified, 20 μg/mL of DNase I was added to the DNA samples followed by a 1-hour incubation at 37°C. Fluorescence was set at 360 nm / 460 nm (excitation/emission) with a cutoff at 455 nm, and the signal was monitored every minute at 37°C in SpectraMax M2 plate reader (Molecular Devices, Sunnyvale, CA, USA). Thrombin production was calculated from a calibration curve and parameters of thrombin generation (*e.g.* lag time, endogenous thrombin potential) were analyzed using the Technothrombin TGA software.

3.3.6. Plasma clotting assay

Mixtures containing degassed PPP (1:3 final dilution) were incubated at 37°C with either silica-blank, silica-DNA, PAX-DNA, or DBL-DNA at varying concentrations (0, 2, 5, 10, 20, 40 μg/mL). Mixtures were added to wells containing 25 μL of 40 mM $CaCl_2$ (10 mM final, 100 μL final volume). Clot formation was monitored by turbidity at 405 nm at 37°C, with readings at 1-minute intervals. DNase I (20 μg/mL) was added to samples followed by a 1-hour incubation at 37°C where stated. To determine the involvement of the contact pathway (Gould et al.,

2014), the plasma clotting experiments were repeated in the presence of CTI (100 μg/mL). Clot time was determined as the time to reach half-maximal increase in absorbance.

3.3.7. Polyphosphate detection assay

PolyP was detected as described previously (Smith & Morrissey, 2007). Briefly, polyP was resolved by electrophoresis using 10% polyacrylamide TBE-urea (7 M) at 100 V for 1 hour. As a positive control, 500 ng of $polyP_{70}$ was used, while 3 μg of DNA (silica-DNA, PAX-DNA, or DBL-DNA) were used per lane. The polyP/DNA samples were then treated with either DNase I (100 μg/mL) or calf intestinal alkaline phosphatase (200 U/μL) as described previously (Smith & Morrissey, 2007). Gels were then stained by agitation with a DAPI solution (25% methanol. 5% glycerol, 1 mg/mL p-phenylenediamine, and 2 μg/mL DAPI, pH 8.0) for 30 minutes at room temperature in a foil-covered container. Gels were then destained in two 1-hour changes of fixative (25% methanol, 5% glyercol, 1 mg/mL p-phenylenediamine, 10 mM EDTA). Gels were then exposed to ultraviolet light and imaged.

3.3.8. Statistical analysis

Comparison between groups were performed using one-way analysis of variance test (ANOVA) followed by Tukey's multiple comparisons test. *P* values

<0.05 were considered statistically significant. GraphPad Prism was used to analyze data (La Jolla, CA, USA).

3.4. Results and Discussion

DNA was isolated from whole blood or buffy coat using 3 different DNA isolation kits. When visualized by electrophoresis, CT-DNA, silica-DNA, PAX-DNA, and DBL-DNA were all comparable in size (Figure 3.1A).

When the various DNA preparations were incubated with S-2238, only CT-DNA demonstrated inherent thrombin-like activity and thus was excluded in our subsequent studies (Figure 3.1B). We next investigated the procoagulant activities of the PAX-DNA and DBL-DNA using the TGA (Fotiou et al., 2018; Gould et al., 2015; Park et al., 2018; R. Prince et al., 2018). When PAX-DNA (0 to 20 µg/mL) was added to PPP, the lag time decreased with increasing DNA concentrations in a dose-dependent manner (Table 1). These effects were verified to be dependent on DNA as the addition of DNase I ameliorated any shortened lag times observed. Neither peak thrombin concentration nor endogenous thrombin potential (ETP) values were affected by increasing PAX-DNA concentration. The TGA could not be performed with DBL-DNA as the addition of DBL-DNA to any mixture containing the fluorogenic substrate, either in buffer or plasma, lead to immediate and irreversible precipitation of the fluorogenic substrate and/or DNA (data not shown).

To further verify the apparent procoagulant activity of DNA, a plasma clotting test was used. Briefly, clotting of PPP was initiated by the addition of $CaCl_2$ in the presence or absence of exogenous DNA and clot formation was monitored in real-time by turbidity at 405 nm. Consistent with previous findings (Smith et al., 2017), silica-DNA enhanced coagulation as shown by the shortened clot time (Figure 3.1C). Both PAX-DNA and DBL-DNA also demonstrated dose-dependent procoagulant activity, albeit DBL-DNA was less procoagulant than PAX-DNA at all concentrations investigated. It has been shown previously that DNase I cannot neutralize the procoagulant effects of silica-isolated DNA (Smith et al., 2017). Similarly, we also observed that the procoagulant activity of silica-DNA could not be removed by the addition of DNase I (Figure 3.1D), as well as high-speed centrifugation, or size exclusion chromatography, suggesting that the potential contaminants may be tightly bound to the DNA (data not shown). The presence of PAX-DNA or DBL-DNA significantly reduced the clot times, and subsequent addition of DNase I normalized the clot times to be comparable to control (Figure 3.1D). These data suggest that the apparent procoagulant activity of either PAX- or DBL-DNA is highly DNA-specific. We previously reported that there is a positive correlation between plasma levels of cell-free DNA and thrombin generation in plasma samples obtained from patients with sepsis, whereby the procoagulant activity is ameliorated by the addition of DNase I (Dwivedi et al., 2012; Gould et

al., 2014). Taken together, these results suggest that DNA is procoagulant and digestion of DNA neutralizes this effect.

To study the mechanism of the observed procoagulant effect of DNA, CTI was included during plasma clot formation (Figure 3.1E). The inclusion of CTI neutralized the procoagulant effect exerted by all exogenous DNA, further demonstrating that the procoagulant effect of DNA is mediated through contact pathway activation.

Smith *et. al.* reported the presence of polyP as a contaminant in DNA isolated using silica columns (Smith et al., 2018). PolyP is a chain of highly negatively charged orthophosphate residues connected by a high energy phosphoanhydride bonds (Travers et al., 2015). The dense granules of platelets contain short-chain polyP (<200 monomers) that can be secreted in response to platelet activation (Ruiz et al., 2004). This polyP can activate FXII, enhance FXIa generation by thrombin, and accelerate FVa generation (Travers et al., 2015). Thus, the contamination of DNA by polyP would confound our interpretations of the impact that DNA may impose on promoting coagulation. Therefore, we tested for the presence of polyP in silica-DNA, PAX-DNA, and DBL-DNA (Figure 3.2). Although polyP alone as a positive control was detectable (Smith & Morrissey, 2007), there was variability in the levels of polyP contamination in the silica-DNA, with the large majority of the preparations having undetectable levels of polyP. Large majority of PAX-DNA also appeared to contain no detectable amounts of

polyP, while it was completely undetectable with DBL-DNA. The potential polyP levels, if present, are substantially lower as the procoagulant effects can be completely neutralized by the inclusion of DNase I alone (Figure 3.1D). Unfortunately, similar functional analyses could not be performed with calf-intestinal alkaline phosphatase as it alone prolonged clotting, with or without its inactivation as described previously (Smith et al., 2017).

In summary, we have identified two methods of DNA isolation that are free of silica while having undetectable functional levels of polyP, PAX-DNA and DBL-DNA. DNA isolated using these methods demonstrate procoagulant activity that can be neutralized with DNase I digestion. In addition, PAX-DNA or DBL-DNA appears to be appropriate for use in investigating the role of DNA in various biochemical processes. While we recommend the use of PAX-DNA or DBL-DNA for functional studies involving extracellular DNA, it is worth noting that DNA isolated with silica columns can still be useful for DNA quantification purposes.

Figure 3.1. Characterization of DNA from varying sources. (A) Agarose gel electrophoresis of 4 μg each of calf-thymus DNA (CT-DNA), PAXgene isolated DNA (PAX-DNA), QuickGene DNA whole blood kit large isolated DNA (DBL-DNA), silica isolated DNA (silica-DNA), or silica control (silica-blank). (B) Effects of thrombin (5 nM) and DNA on the cleavage of chromogenic substrate (S-2238). Plasma clot times observed by the addition of (C) DNA at varying concentrations, or DNA (30 μg/mL) (D) without or with DNase I (20 μg/mL), or (E) without or with corn trypsin inhibitor (CTI; 200 μg/mL). Results are presented as mean ± SEM and analyzed by one-way ANOVA followed by Tukey's post-hoc analysis (n=3-6). A p-value of 0.05 was considered statistically significant (*: $p<0.05$).

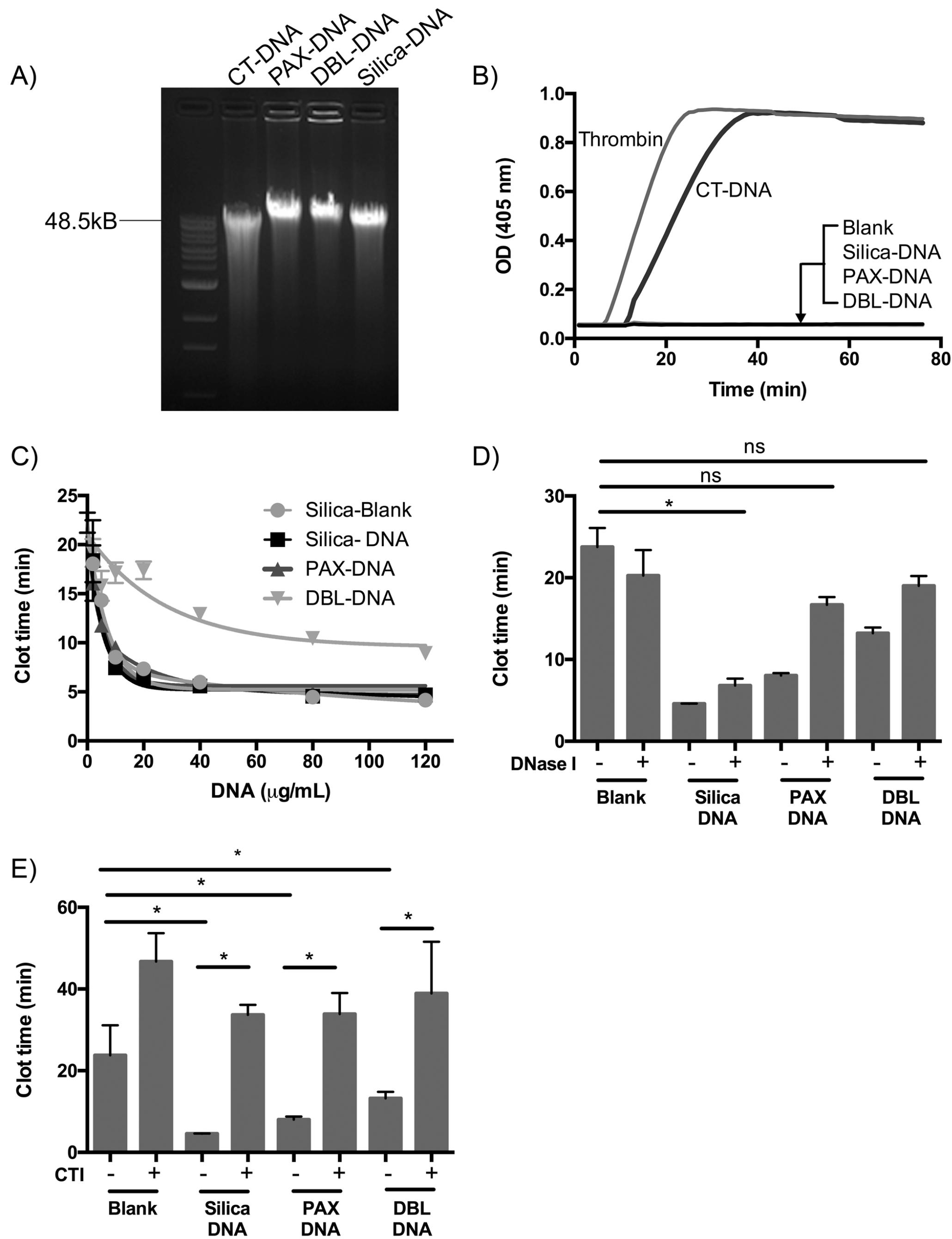

Figure 3.1. Characterization of DNA from varying sources.

Figure 3.2. Detection of polyphosphates (polyP) in DNA from varying sources. DNA (3 μg) isolated using the silica column (silica-DNA), the PAXgene kit (PAX-DNA), or the QuickGene DNA kit (DBL-DNA) was digested with DNase I (100 μg/mL) or calf intestinal alkaline phosphatase (200 U/μL) and then resolved by electrophoresis on a 10% polyacrylamide TBE-urea (7 M) gel. The gel was then stained with DAPI and exposed to UV transillumination to detect for polyP. Gel is a representative image of 5 independent experiments.

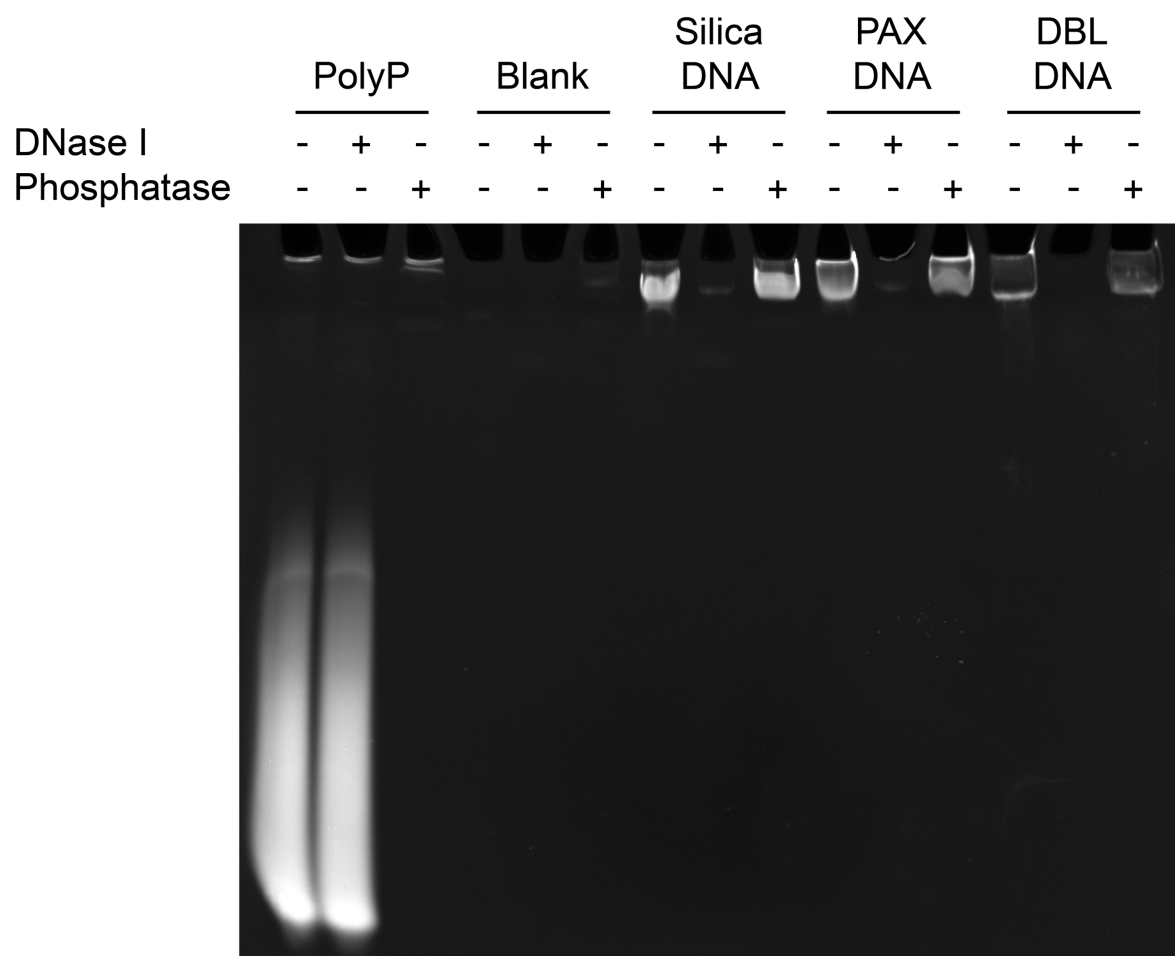

Figure 3.2. Detection of polyphosphates (polyP) in DNA from varying sources.

Table 3-1: Dose-dependence of PAX-DNA on clotting as measured by thrombin generation assay. The procoagulant property of PAX-DNA is neutralized by DNase I digestion.

	[DNA] (µg/mL)	Lag Time (min)	Peak Thrombin (nM)	ETP (nM*min)
Blank	0	21.7 ± 2.5	344.6 ± 60.7	4327.9 ± 439.9
PAX-DNA	5	16.5 ± 1.9	338.6 ± 44.2	4317.4 ± 314.6
	10	*13.5 ± 0.8	323.3 ± 39.2	4291.7 ± 287.4
	20	*11.7 ± 0.4	291.0 ± 32.6	4236.6 ± 263.6
DNase I	0	17.3 ± 1.9	244.8 ± 54.3	4193.0 ± 373.1
PAX-DNA + DNase I	5	20.8 ± 2.0	178.0 ± 22.4	4092.7 ± 294.7
	10	19.3 ± 2.0	218.0 ± 11.0	4098.0 ± 265.2
	20	20.7 ± 1.3	229.0 ± 10.5	4141.0 ± 336.4

ETP: endogenous thrombin potential; PAX-DNA: PAXgene DNA.

Results were analyzed by a one-way ANOVA (*: $p<0.05$) and compared to the blank.

4.0. The investigation of the pathological effects of histones, DNA, and nucleosomes in a murine model of sepsis

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Foreword: In chapter 4, we investigate:

1. The cytotoxic effects of histones, DNA, and nucleosomes treated with DNase I and heparin.
2. The pathological effects of histones, DNA, and nucleosomes in a murine model of sepsis.

Running title: Histones, DNA, and nucleosomes in sepsis

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All authors were involved in mouse surgeries, monitoring, and harvesting. Patricia C. Liaw and Sarah K. Medeiros conceptualized the experiments. Sarah K. Medeiros performed the experiments. Sarah K. Medeiros wrote the manuscript and Patricia C. Liaw edited the manuscript.

This manuscript was accepted to *Shock* and is currently in press.

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

4.1. Abstract

Background: In sepsis, neutrophil extracellular traps (NETs) are an important interface between innate immunity and coagulation. The major structural component of NETs is nucleosomes (DNA-histone complexes). *In vitro,* DNA and histones exert procoagulant/cytotoxic effects whereas nucleosomes are not harmful. However, whether DNA, histones, and/or nucleosomes exert harmful effects *in vivo* remain unclear.

Objectives: (1) To investigate the cytotoxic effects of nucleosomes +/- DNase I and heparin *in vitro* and (2) to investigate whether DNA, histones, and/or nucleosomes are harmful when injected into healthy and septic mice.

Methods: The cytotoxic effects of DNA, histones, and nucleosomes (+/- DNase I or +/- heparin) were assessed in HEK293 cells. Mice underwent cecal ligation and puncture (CLP) or sham-surgery, then received injections of DNA (8 mg/kg), histones (8.5 mg/kg), or nucleosomes at 4h and 6h. Organs and blood were harvested at 8h. Cell-free DNA (CFDNA), IL-6, thrombin-anti-thrombin (TAT), and protein C were quantified from plasma.

Results: *In vitro*, incubation of HEK293 cells with DNase I-treated nucleosomes reduced cell survival compared with nucleosome-treated cells, suggesting that DNase I releases cytotoxic histones from nucleosomes. Addition of heparin to DNase I-treated nucleosomes rescued cell death. *In vivo,* administration of histones to septic mice increased markers of inflammation (IL-6) and coagulation (TAT), which was not observed in sham or septic mice administered DNA or nucleosomes.

Conclusions: Our studies suggest that DNA masks the harmful effects of histones *in vitro* and *in vivo*. Although administration of histones contributed to the pathogenesis of sepsis, administration of nucleosomes or DNA were not harmful in healthy or septic mice.

4.2. Introduction

Sepsis is defined as a life-threatening organ dysfunction resulting in inflammation and coagulation caused by a dysregulated response to infection (Singer et al., 2016). Sepsis accounts for approximately 20% of deaths world-wide and is recognized as a global health priority by the World Health Organization (Reinhart et al., 2017). The release of neutrophil extracellular traps (NETs) by activated neutrophils has emerged as an important interface between infection, inflammation, and coagulation (Levi et al., 2003). NETs are web-like networks of cell-free DNA (CFDNA) and histones, decorated with lysosomal enzymes and antimicrobial peptides (Brinkmann et al., 2004). These networks are released from neutrophils during a regulated form of cell death called NETosis. NETosis can be triggered by microorganisms as well as mediators of sterile inflammation such as cytokines, immune complexes, and autoantibodies (Thiam et al., 2020). NETs are considered a double-edged sword as they aid in host defense by trapping and killing microbial pathogens (Brinkmann et al., 2004). However, they can also cause tissue damage and organ dysfunction through hindered blood flow by activating blood coagulation, impairing fibrinolysis, and injuring endothelial cells (Brinkmann et al., 2004). Consequently, targeting components of NETs have become a promising potential therapy in sepsis.

The major structural component of NETs is chromatin or nucleosomes, a complex of extracellular DNA, also known as cell free DNA (CFDNA), and

histones (Brinkmann et al., 2004). Elevated levels of CFDNA and histones in disease states such as sepsis may exert collateral damage to the host. *In vitro* studies have shown that CFDNA can trigger blood coagulation via the intrinsic pathway in a factor (F) XII- and XI-dependent manner (Medeiros et al., 2019, Gould et. al. 2015). In addition to activating coagulation, CFDNA can also inhibit fibrinolysis by impairing plasmin-mediated fibrin degradation in a purified system (Gould et al., 2015; Komissarov et al., 2011). CFDNA has also been reported to be elevated in patients with sepsis and is a useful prognostic indicator (Liaw et. al., 2019). From these *in vitro* studies, CFDNA was hypothesized to contribute to the procoagulant and antifibrinolytic state observed in sepsis. Although there is a positive correlation between plasma levels of circulating CFDNA and thrombin generation in septic patient plasma (Dwivedi et al., 2012; Gould et al., 2014), it is still unknown if CFDNA is solely a prognostic biomarker or if elevated levels of CFDNA directly contributes to the pathogenesis of sepsis *in vivo*.

While CFDNA may contribute to the widespread activation of coagulation, histones are cytotoxic to endothelial cells *in vitro* and can also promote thrombosis in a variety of ways. For example, histones can activate platelets in a TLR-2 and TLR-4 manner and bind prothrombin allowing it to autoactivate, thereby generating thrombin independent of the coagulation cascade (Barranco-Medina et al., 2013; F. Semeraro et al., 2011). Histones also impair the anticoagulant pathway as they can reduce thrombomodulin (TM)-dependent protein C (PC) activation which enhances

thrombosis generation (Ammollo et al., 2011). Intravenous injection of purified histones (75 mg/kg) in mice resulted in thrombocytopenia, neutrophil migration, and organ failure mimicking the pathophysiological nature of sepsis (Xu et al., 2009). Targeting histones with an anti-H4 antibody significantly improved survival in various animal models of sepsis highlighting their importance in the pathogenesis of sepsis (Xu et al., 2009). Therapeutic heparins have also been shown to tightly bind and neutralize the cytotoxic effects of histones, thereby improving survival in a mouse model of sepsis (Wildhagen et al., 2014). For these reasons, histones have been considered a promising potential therapeutic target in sepsis.

Although histones may be harmful to the host, *in vitro* studies have shown that DNA may mask the harmful effects of histones while circulating in a complex. For instance, Noubouossie et. al. (2017) reported that intact nucleosomes are not procoagulant, despite individually purified components of DNA and histones being procoagulant (Noubouossie et al., 2017). Furthermore, the cytotoxic properties of histones are neutralized by the addition of polyanions such as DNA (Meara et al., 2020). Despite this, it is unknown if DNA can mask the effects of histones *in vivo*. Since there are instances where DNA or histones can be naturally displaced when nucleosomes are circulating (e.g. heparan sulfates or DNases), it is important to understand the different effects of circulating DNA, histones, and nucleosomes *in vivo*. The importance of this is highlighted by emerging therapies, such as DNase I or heparin, which may also displace DNA or histones from nucleosomes thereby

altering their cytotoxic properties. Therefore, the purpose of this paper is (1) to explore the potential cytotoxic effects of nucleosomes with heparin or DNase I therapy, and (2) to investigate the pathological effects of purified DNA, histones, and nucleosomes in a mouse model of sepsis. We hypothesize that (1) DNase I, a potential therapy, may degrade DNA and release histones which can induce cytotoxic effects. And (2) *in vivo*, intravenous administration of DNA or histones in a mouse model of sepsis will contribute to the pathogenesis of sepsis by increasing markers of coagulation and inflammation. We also hypothesize that the harmful properties of these molecules will be masked when they are in complex with each other in the form of nucleosomes.

4.3. Materials and Methods

4.3.1. Preparation of DNA, histone-octamers, and nucleosomes

Nucleosomes are the basic structural unit of DNA packaging in eukaryotes and are composed of a string of nucleosome monomers (mononucleosomes). Mononucleosomes created from an adapted protocol described by Rogge et. al. (Rogge et al., 2013). Mononucleosomes consist of an octameric core of histones around which 147 bp of DNA are wrapped in 1.65 turns. They can be reconstructed *in vitro* using purchased recombinant unfractionated bovine histones (Worthington Biochemical Corporation, Lakewood, New Jersey, US) and tandemly repeated positioning DNA (Rogge et al., 2013). The octamers were isolated using size

exclusion chromatography and a specialized DNA "601" (147 bp) sequence optimal for mononucleosome reconstitution was PCR amplified from pGEM-3z/601 plasmid grown in *E. coli.* The pGEM-3z/601 plasmid was a gift from Jonathan Widom (Addgene plasmid #26656; http://n2t.net/addgene:26656; RRID:Addgene_26656) (Lowary & Widom, 1998). The PCR primers used were 5' - CTG GAG AAT CCC GGT GCC G – 3' and 5' – ACA GGA TGT ATA TAT CTG ACA CG – 3' from Integrated DNA Technologies (Coralville, Iowa, US).

We also reconstituted nucleosomes using a simple modified salt-gradient dialysis protocol as described by Stein in 1989 (Stein, 1989). These nucleosomes would be larger than 10,000 bp of DNA. We have previously reported that the PAXgene Blood DNA Kit (Qiagen) is an appropriate kit to use that produces a high yield of purified DNA that is free of procoagulant contaminants such as silica (Medeiros et al., 2019). Therefore, DNA was extracted from the PAXgene Blood DNA Kit as per the manufacturer's instruction. Unfractionated bovine histones were then added in a 1:1.2 molar ratio and then nucleosomes were constituted using a modified step wise dialysis protocol. Buffer 1 consisted of 0.8 M NaCl, 20 mM Tris-HCL, 0.2 mM EDTA, 1mM 2-mercaptoethanol (pH = 7.2), buffer 2 consisted of 0.8 M NaCl, 20 mM Tris-HCl (pH = 7.2), and buffer 3 consisted of 0.6 M NaCl and 20 mM Tris-HCl (pH = 7.2). We refer to these nucleosomes as "simplified" as they form random DNA-histone complexes which do not contain histone octamer cores. Therefore, simple reconstituted nucleosomes were only used for *in vitro*

experiments and not used in animal experiments. Similar to simple reconstituted nucleosomes, mononucleosomes were not cytotoxic to HEK 293 cells (refer to Figure 4.1 and 4.5). Mononucleosomes were chosen for *in vivo* studies because their 3-dimensional structure is physiologically relevant (Rogge et al., 2013).

4.3.2. Assessing histone-mediated cytotoxicity using trypan blue and lactate dehydrogenase (LDH) release assays

Histone-mediated cytotoxicity was assessed on human embryonic kidney 293 (HEK 293) cells cultured in DMEM supplemented with 5% FBS and 1% penicillin-streptomycin (100 IU/mL). The cells were seeded at 100,000 cells/well in a 24-well plate or 10,000 cells/well in a 96-well plate and then grown to 60% confluency. The cells were then treated for 24 h with either unfractionated bovine histones (0 to 250 μg/mL), octamers (0 to 250 μg/mL), DNA (isolated from the Qiagen PAXgene Blood DNA kit or "601" amplified DNA (100 μg)), DNase I (Pulmozyme, Roche; 100 μg/mL), and/or dalteparin (Fragmin, Pfizer; 0 to 5 IU/mL). Trypan blue exclusion assays were performed by adding a 1:1 volume ratio of cell suspension to trypan blue and then manually counted under a microscope using a hemocytometer. A CyQuant LDH release Assay (ThermoFisher Scientific; Waltham, Massachusetts, US) was also used to further quantify cell survival according to the manufacturer's instruction.

4.3.3. Gel Electrophoresis

Enzymatic digestion of DNA by DNase I was confirmed using gel electrophoresis. Samples were added to a 1.5% agarose gel for electrophoresis at 110 V for 1 h. After staining with RedSafe (FroggaBio, North York, ON), the gels were photographed using UV transillumination.

4.3.4. Animals

10 to 12-week-old C57Bl6/J mice (Charles River) were used according to an animal utilization protocol (AUP) approved by the McMaster University's Animal Research Ethics Boards (AUP# 19-01-06). Each group contained equal numbers of female and male mice.

4.3.5. Cecal ligation, and puncture (CLP) procedure

Mice were placed under gaseous anesthesia (2.5% isoflurane, oxygen flow 1.5 L/min). Mice had their abdomen shaved and cleaned with iodine and 70% ethanol for sterility. CLP or sham-surgery was then performed beginning with a longitudinal skin midline dissection. Following blunt dissection of skin, a 1 cm midline dissection of linea alba was made to open abdominal musculature to gain access to the peritoneum and cecum. The cecum was then located and exteriorized. At this point, sham mice had their cecum returned to the abdominal cavity. For mice undergoing CLP, 1 cm of the cecum was ligated then punctured through-and-

through with an 18-G needle. Fecal matter was then extruded (0.5 cm, half from each of the mesenteric and antimesenteric puncture sites). The cecum and fecal matter were then returned to the abdominal cavity. The musculature was closed with 6-0 vicryl continuous sutures and skin was closed using surgical staples.

Mice were continuously monitored every 4 h for signs of distress or until study endpoint at 8 h. Every 4 h, the following parameters were recorded for ethical purposes in accordance with the ARRIVE guidelines (Kilkenny et al., 2010) (Figure 4.7): weight, temperature, mouse grimace score (MGS; a pain score that is the average of orbital tightening, ear positioning, nose/cheek bulge, and whiskers scored on a 3-point scale), and mouse surrogate markers of death (MSS; an average score of hunch, ambulation, responsiveness, and ruffled fur scored on a 3-point scale). All mice survived. Throughout the experiment, mice were given sub-cutaneous Ringer's Lactate (2.0 mL pre-operatively and 1.0 mL every 4 h post-operatively) and analgesia (buprenorphine, 0.1 mg/kg pre-operatively).

4.3.6. Administration of DNA, octamers, and mononucleosomes in septic mice

At 4- and 6-hours post-surgery, the mice were put under anesthesia and received two intravenous injections of either saline (0.9% NaCl), histone octamers (non-lethal dose of 8.5 mg/kg or 50 mg/kg), DNA (147 bp, 8 mg/kg), or mononucleosomes (containing an equivalent of 8.5 mg/kg octamers and 8 mg/kg DNA) via retroorbital injections at 4 h and 6 h post-CLP. The histone dose

administered was based on the maximum volume of fluid that can be injected into each eye (200 µL) as well as the solubility of the mononucleosomes. Since the mice started showing signs of sickness at 4h (Figure 4.7.), we decided to inject 8.5 mg/kg of histones (or nucleosomes containing 8.5 mg/kg of histones) at 4 h and 6 h post-CLP.

At study endpoint (8 h), the mice were put under anesthesia, peritoneal cavity fluid (PCF) was collected, blood was collected from the inferior vena cava in 3.2% trisodium citrate, organs were collected in neutralized formalin buffer, and then culled via cervical dislocation. The PCF and blood were plated to quantify the bacterial loads in the fluids. White blood cell (WBC), red blood cell (RBC), and platelet counts were obtained from whole blood using a Hemavet 950 (Drew Scientific, USA). Platelet poor plasma was then obtained by subjecting the blood to 5,000 x g for 20 min, aliquoted, and frozen at -80ºC until needed.

4.3.7. Quantification of CFDNA, IL-6, Thrombin-anti-thrombin complexes (TAT), protein C (PC), and cystatin C in plasma

CFDNA levels in plasma was determined using PicoGreen dsDNA assay kit (ThermoFisher Scientific; Waltham, Massachusetts, US). IL-6 was quantified using a mouse specific ELISA kit from R&D Systems (Minneapolis, Minnesota, US). Thrombin-anti-thrombin (TAT) complexes were quantified using an ELISA from Affinity Biologicals (Ancaster, Ontario, Canada) as per manufacturer's

instructions. Protein C (PC) was quantified as previously described (W. Li et al., 2005).

4.3.8. Histology

Histology was performed on lung, liver, and kidneys. Organ sections (5 μm thick) were stained with H&E. The sections were scored in a blinded fashion, by two individuals, on a scale of 0 to 5 (0 = normal, 5 = severe) based on inflammation, thrombosis, and overall organ morphology. A composite score was calculated as a sum of the 3 categories per organ.

4.3.9. Statistical analysis

Data was presented as mean (SD). Comparison between groups were performed using a one-way analysis of variance test (ANOVA) followed by Tukey's multiple comparison test using Prism 9 (Graphpad, San Diego, California, US).

4.4. Results

4.4.1. DNase I can degrade DNA in mononucleosomes to release cytotoxic histones

Mononucleosomes were created and their cytotoxicity was assessed using trypan blue and LDH release assays on HEK 293 cells (Figure 4.1 and 4.2). Histone

octamers were cytotoxic in a dose-dependent manner (Figure 4.1A and 4.2A). Addition of increasing concentrations of LMWH attenuated this cytotoxic effect demonstrating the ability of LMWH to bind and neutralize their harmful effects (Figure 4.1B and 4.2B). Addition of mononucleosomes to cells did not alter cell survival or increase LDH release, suggesting that mononucleosomes were not cytotoxic to the cells. However, addition of DNase I to mononucleosomes resulted in reduced cell survival and increased LDH release (Figure 4.1D and 4.2D). The cell cytotoxicity and LDH quantified reflected DNase I treatment degrading the DNA portion of the mononucleosomes releasing cytotoxic histones (Figure 4.1E). Addition of LMWH to DNase I-treated mononucleosomes improved cell survival and reduced LDH release (Figure 4.1D and 4.2D) suggesting that DNA can shield the harmful effects of histones *in vitro*. Similar findings were observed using simplified reconstituted nucleosomes (Figure 4.5).

4.4.2. Administration of histone-octamers increase inflammatory and coagulation biomarkers in CLP mice

Next, we investigated the pathologic effects of DNA, histones, and nucleosomes *in vivo*. Mice were subjected to CLP or sham (control) surgeries and then received two repeated intravenous injections of either DNA (8 mg/kg), histone octamers (8.5 mg/kg), or nucleosomes to determine if these molecules further the pathogenesis of sepsis. At study endpoint (8 h post-CLP), CFDNA was elevated in

all CLP mice compared to their sham controls (Figure 4.3A). Specifically, septic mice administered histone octamers or DNA had significantly elevated circulating CFDNA concentrations compared to their sham controls. In contrast, CFDNA was not elevated in mice administered nucleosomes suggesting that nucleosomes can be cleared efficiently and/or may not pose harm to the host.

To assess inflammation, we quantified circulating levels of IL-6. We observed that IL-6 levels were significantly elevated in CLP mice administered histone octamers compared to other treatment groups suggesting the histone octamers were inducing inflammation. Consequently, this may contribute to the pathogenesis of sepsis (Figure 4.3B). However, healthy sham mice administered the same non-lethal dose of histone octamers had no increase in IL-6 suggesting they were only harmful in mice who were septic. Administration of either DNA or nucleosomes had no effects on IL-6 in both sham and septic mice suggesting that DNA may mask the proinflammatory effects of histones *in vivo*.

Since histones and DNA are procoagulant and prothrombotic *in vitro*, we also investigated their effects in septic mice by quantifying TAT complexes. TATs were elevated in all CLP mice compared to controls. We also observed significantly elevated TAT complexes in septic mice administered histone octamers compared to saline septic controls (Figure 4.3C). However, no differences in TATs were seen between saline, DNA, or nucleosome-treated sham and septic mice. We concluded that DNA may mask the procoagulant properties of histones *in vivo*. Despite this,

we saw no difference in PC levels between shams and septic mice suggesting our treatments may not affect the anti-coagulation pathway in our model at 8 hours (Figure 4.3D).

To confirm our findings, we blindly scored lungs, liver, and kidneys for inflammation, thrombosis, and organ morphology. All sham treatment groups had similar organ morphology (Figure 4.6) and were not significantly different from each other (Figure 4.4). Although mean lung, liver, kidney scores were elevated in all CLP groups compared to shams, there was no significant difference observed between treatment groups in this model (Figure 4.4). Unexpectedly, DNA and nucleosome treated septic mice had significantly lower liver scores compared to saline septic controls. Therefore, DNA and nucleosomes may potentially reduce liver thrombosis, inflammation, and overall morphology.

4.4.3. No differences in physical appearance, bacterial burden, and cell differentials between CLP mice

Despite seeing differences in CFDNA, inflammation, and coagulation markers, we observed no physiological differences between groups when we monitored temperature, weight, and signs of distress (Figure 4.7). We also reported similar PCF and blood bacterial loads (Figure 4.8). Furthermore, WBC, platelet, and RBC counts were also normal (i.e. similar to the sham and naïve mice) and not different between groups (Figure 4.9). Consequently, we can conclude that

differences in inflammation and coagulation between groups can be attributed to the treatments themselves and not differences in bacterial burden or blood cell counts.

4.5. Discussion

In this study, we report that DNA can mask the harmful effects of histones *in vitro* and *in vivo.* We showed that treating nucleosomes with DNase I (100 μg/mL) releases harmful cytotoxic histones that can be neutralized with LMWH (Figure 4.1 and 4.2). This confirms the *in vitro* studies by Abrams et al. which demonstrated that intact nucleosomes are not toxic whereas nucleosomes disrupted by sonication are cytotoxic towards endothelial cells (Abrams et al., 2013). Additionally, we confirmed the *in vivo* phenomenon observed by Kolackowska et. al., where they hypothesized that DNase I treatment in a MRSA model of sepsis may release histones from nucleosomes resulting in organ damage (Kolaczkowska et al., 2015). As DNase I emerges as a potential therapy in sepsis, it is important to understand potential side effects of releasing histones from nucleosomes. It is also important to note that lower DNase I concentrations (14.5 μg/mL) are unable to degrade nucleosomes without heparin (Sohrabipour et al., 2021). Therefore, DNase I and LMWH may work synergistically to neutralize nucleosomes. Since LMWH is already used as a standard thromboprophylaxis, a combination approach using

DNase I and LMWH may provide a theoretical advantage in sepsis to target nucleosomes.

The ability of a negatively charged molecule to shield the harmful effects of histones is not novel. Previous studies have shown histone-mediated cytotoxicity can be neutralized by numerous other heparins and small negatively charged polyanions (Sharma et. al., 2021; Meara et. al., 2020). Our study shows that this can also be achieved by the addition of negatively charged DNA and can be reversed by degrading DNA using DNase I, a clinically relevant therapy. Altogether, we hypothesize that other negatively charged molecules mechanistically act in the same manner by neutralizing histones' positive charge, thereby, reducing their ability to induce cell damage.

We also explored differences between purified components (DNA or histones) and their complexes (nucleosomes) on inflammation and coagulation *in vivo* that may allow us to further understand the contribution of NETs to the pathophysiology of sepsis. We report that two non-lethal doses of histone octamers (8.5 mg/kg) increased circulating CFDNA in CLP mice. Since elevated levels of CFDNA are associated with poor sepsis outcomes (Dwivedi et al., 2012), histone octamers may be causing harm to the host furthering the pathogenesis of sepsis. We hypothesize the histones may be furthering the pathogenesis of sepsis through inflammation and coagulation, as seen by elevation of circulating IL-6 and TAT complexes. In contrast, nucleosomes were not harmful as they did not elevate these

markers. Consequently, our data suggests that DNA can mask the harmful effects of histones *in vivo*, a finding that is consistent with our *in vitro* data.

Although we show that two doses of histones at 8.5 mg/kg can induce proinflammatory and procoagulant effects in our murine model of sepsis, this was not observed in healthy mice administered the same dose of histones. The glycocalyx on the surface of endothelial cells may provide an explanation for this observation. Negatively charged heparan sulfates, present in the glycocalyx, are able to shield cells from histone-induced cytotoxicity (Chaaban et al., 2015). However, during pathophysiological inflammatory conditions such as sepsis, the glycocalyx is degraded by matrix metalloproteinases (MMPs) and sheddases, which reduces its ability to sequester and neutralize histones (Chaaban et al., 2015; Uchimido et al., 2019). As a result, the risk of histone-induced cytotoxicity may be elevated in our septic mice. While the dose of histones (8.5 mg/kg) used is representative of patients with sepsis, patients with disseminated intravascular coagulation (DIC) have been reported to have up to 50 mg/kg of circulating histones (X. Zhang & Li, 2022). We observed that administration of doses equivalent to 50 mg/kg of histones accelerated sepsis further as seen by elevated MGS and MSS scores, which can be also seen in sham mice (data not shown). This is consistent with previous studies that show histones at 50 mg/kg can induce a DIC sepsis-like state in healthy mice and administration can further reduce survival in a CLP mouse model of sepsis (Wu et al., 2020).

Although DNA has been reported to be procoagulant (Medeiros et al., 2019), it was unknown if elevated circulating DNA contributes to the pathogenesis of sepsis. We report that septic mice who received bolus doses of DNA had significantly higher circulating CFDNA levels compared to DNA sham controls potentially suggesting septic mice were unable to clear the DNA challenge in the same manner as sham controls. One potential explanation lies in differences of DNase activity in sepsis. In support of this, Jimenez-Alazar et. al. (2017) reported that healthy mice have DNases which are important for degradation of CFDNA *in vivo* and thus, can rapidly clear the DNA challenge in our sham mice (Jiménez-Alcázar et al., 2017). In patients with sepsis, there is an acquired deficiency of endogenous DNase I (Sohrabipour et al., 2021), which may contribute to the elevated levels of CFDNA

Despite elevations of CFDNA, there was no effect on inflammation and coagulation in DNA treated mice (Figure 4.3B and 4.3C). In support of this, many studies commonly use genetically engineered DNA plasmid administration for gene therapy (Armengol et al., 2004). However, to our knowledge, no studies have reported that control DNA plasmids were harmful to mice. Therefore, we conclude that intravenous injections of 150 bp DNA does not induce inflammation or coagulation in healthy mice. It is also important to note that DNA fragments in sepsis range from 150 bp to > 10,000 bp and are dependent on the biological mechanism by which they are released. For instance, necrotic cells and neutrophils

undergoing NETosis release high molecular weight fragments of > 10,000 bp, while apoptotic cells release a "ladder" pattern of DNA at 150 bp intervals (Fuchs et al., 2007; Jahr et al., 2001). In DNA methylation studies, the origin of circulating CFDNA is from a combination of tissue injury and NETs (Dor et al., 2018). When CFDNA isolated from septic plasma was run on an agarose gel, approximately 11% of the septic patients had DNA predominantly 150 to 300 bp (Dwivedi et al., 2012). The importance of this phenomenon is highlighted by Gould et. al. (2015) who demonstrated the size of DNA altered its antifibrinolytic properties, with larger fragments increasing maximum absorbance and prolonging clot lysis compared to smaller fragments (Gould et al., 2015). Altogether, larger fragments would better reflect the effects of NET-derived DNA and may potentially have a harmful effect. In support of this, previous studies have reported that larger networks of extracellular DNA may promote thrombosis by providing a scaffold for platelets and RBCs (Brinkmann et al., 2004). Consequently, it would be beneficial to investigate how DNA size affects the pathogenesis of sepsis *in vivo*. Therefore, while injections of 150 bp DNA fragments were not harmful, larger networks of DNA are hypothesized to contribute to the pathogenesis of sepsis.

In this study, HEK 293 cells were used for ease of use and cost (cells are immortalized and can be passaged indefinitely). However, we acknowledge that using endothelial cells would be a more clinically relevant model to test our hypothesis. We have previously published histone-mediated cytotoxicity research

that includes both human umbilical vein endothelial cells (HUVECs) and HEK 293s and saw no differences in cytotoxicity between them (Sharma et al., 2022). Consequently, HEK 293s were a suitable cell line to test if our nucleosomes were appropriate for injection into mice. For *in vivo* studies, we used a CLP murine mouse model of abdominal sepsis. The CLP model is considered the gold-standard for sepsis survival studies in mice as it mimics the slow physiological response of human sepsis reflective of a perforated bowel. The CLP procedure can be modified to alter severity/mortality by changing surgical parameters such as ligation length, puncture width, and the volume of fecal matter extruded. In our study, we altered the amount of fecal matter extruded (0.5 cm total) to mimic a non-lethal, mild sepsis model to observe if histones, DNA, or nucleosomes can enhance the progression of sepsis. While clinically relevant, the CLP model is not representative of all sepsis (i.e. the most common cause of sepsis is respiratory). Unexpectedly, septic mice treated with DNA or nucleosomes had a significantly lower liver damage score compared to saline septic controls (Figure 4.4). This may suggest a potential protective mechanism, which can be explored and confirmed in future studies. Another limitation to our model is the fact that no histological differences between CLP groups in lungs, liver, and kidneys were observed. Although an 8 h study endpoint was chosen to investigate the early proinflammatory phase of sepsis, future studies could focus on monitoring the response to treatments over a longer

period of time. Alternatively, a more severe model of CLP could be used that may reveal differences in organ histology between treatment groups.

In summary, we showed that DNA can mask the harmful effects of histone octamers *in vitro* and *in vivo.* Therefore, treatments targeting nucleosomes such as DNase I or heparin (Sohrabipour et al., 2021) can separate DNA or histones from one another, thereby enhancing their potentially harmful properties. Although we observed that administration of nucleosomes had no detectable effect in mice, injection of histones to CLP mice contributed to the pathogenesis of sepsis. The histone-mediated effects were less pronounced in sham-control mice compared to mice with sepsis, suggesting a protective histone mechanism in healthy mice. Since treatments targeting nucleosomes are emerging, it is important to understand the potential effects of targeting individual components.

Figure 4.1. Assessing cell viability using trypan blue in HEK 293 cells. (A) Increasing concentrations of octamer were assessed in a cell toxicity assay. (B) Increasing concentrations of LMWH was added to 50 μg/mL of octamers. (C) DNA (100 μg/mL), DNase I (100 μg/mL), LMWH (2 IU/mL), and a combination of DNase I + LMWH were assessed as controls. (D) DNase I and/or LMWH were added to 50 μg/mL of octamers or mononucleosomes and viability was assessed using trypan blue ($n = 3$). (E) An agarose gel (1.5%) with was run to confirm the degradation of DNA in mononucleosome treatments. Results are presented as mean (SD) and analyzed using a one-way ANOVA followed by Tukey's post-hoc test ($\alpha = 0.05$; * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, and *** $p < 0.0001$).

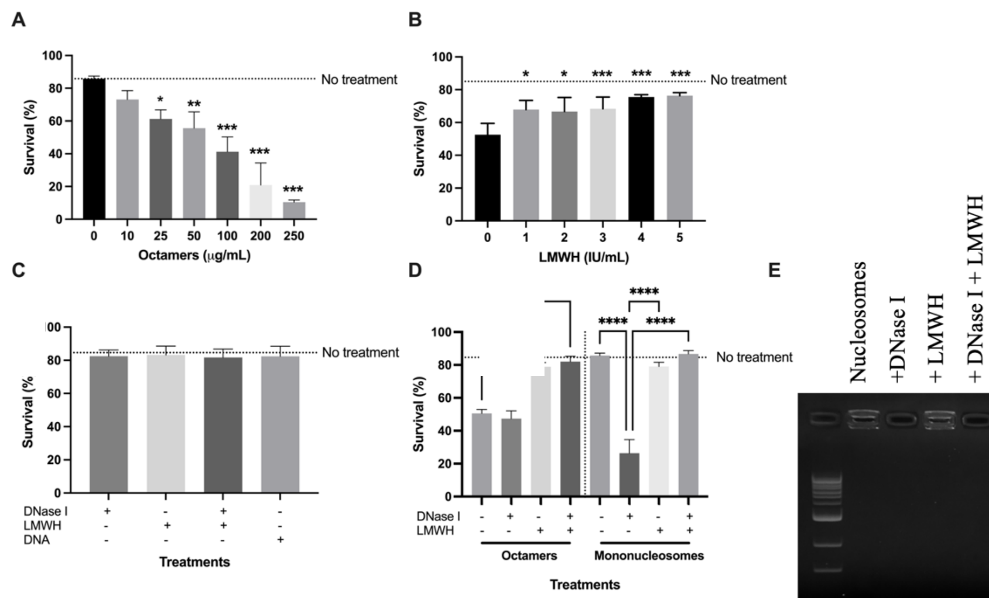

Figure 4.1. Assessing cell viability in nucleosomes treated with DNase I and/or LMWH.

Figure 4.2. Assessing LDH release from HEK 293 cells. (A) Increasing concentrations of octamer were assessed in a cell toxicity assay. (B) Increasing concentrations of LMWH was added to 200 μg/mL of octamers. (C) DNA (100 μg/mL), DNase I (100 μg/mL), LMWH (2 IU/mL), and a combination of DNase I + LMWH were assessed as controls. (D) DNase I and/or LMWH were added to 200 μg/mL of octamers or mononucleosomes and viability was assessed using trypan blue ($n = 6$). Results are presented as mean (SD) and analyzed using a one-way ANOVA followed by Tukey's post-hoc test ($\alpha = 0.05$; * $p < 0.05$ and ** $p < 0.01$).

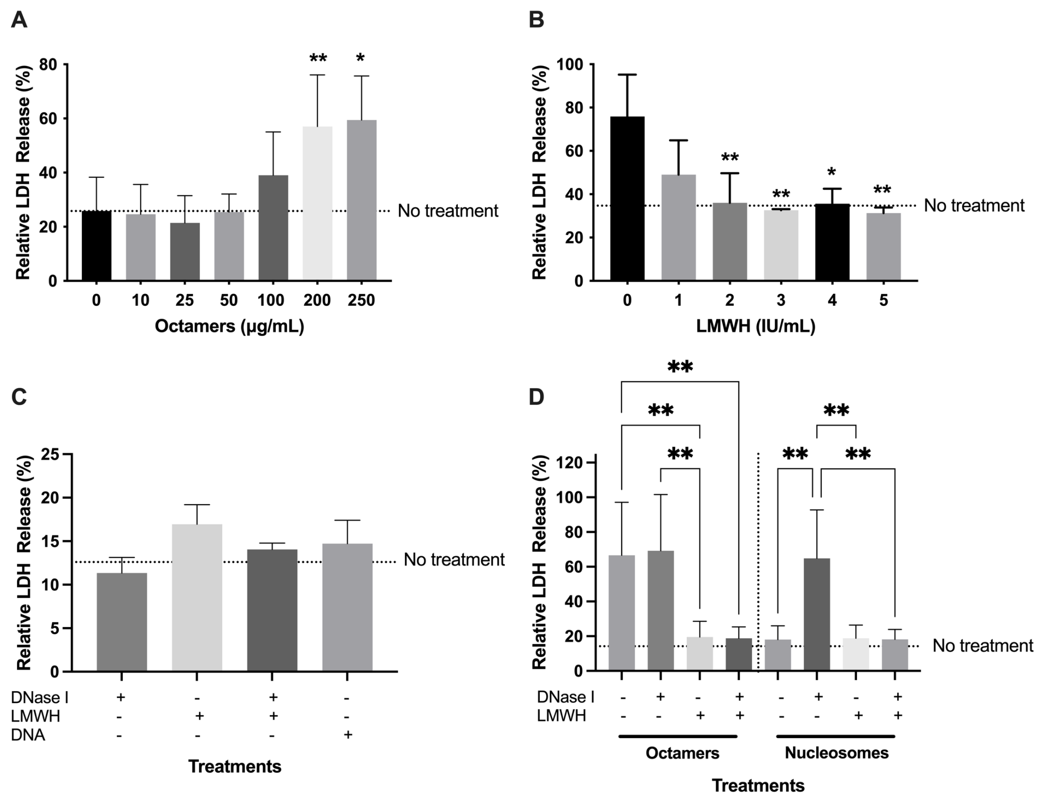

Figure 4.2. Assessing LDH release from HEK 293 cells.

Figure 4.3. Quantification of (A) CFDNA, (B) IL-6, (C) thrombin-anti-thrombin (TAT) complexes, and (D) protein C in sham (black) and CLP (teal) mice. Mice received two doses of either DNA (8 mg/kg), histones (8.5 mg/kg), and nucleosomes (composed of equivalent DNA and histones). Shams are represented by black circles and CLP are mice are represented by teal circles. Results are presented as mean (SD) and analyzed using a one-way ANOVA followed by Tukey's post-hoc test ($n = 6$-8). A p-value of 0.05 was considered statistically significant (*: $p<0.05$, **: $p<0.01$, ***: $p<0.001$, ****: $p<0.0001$).

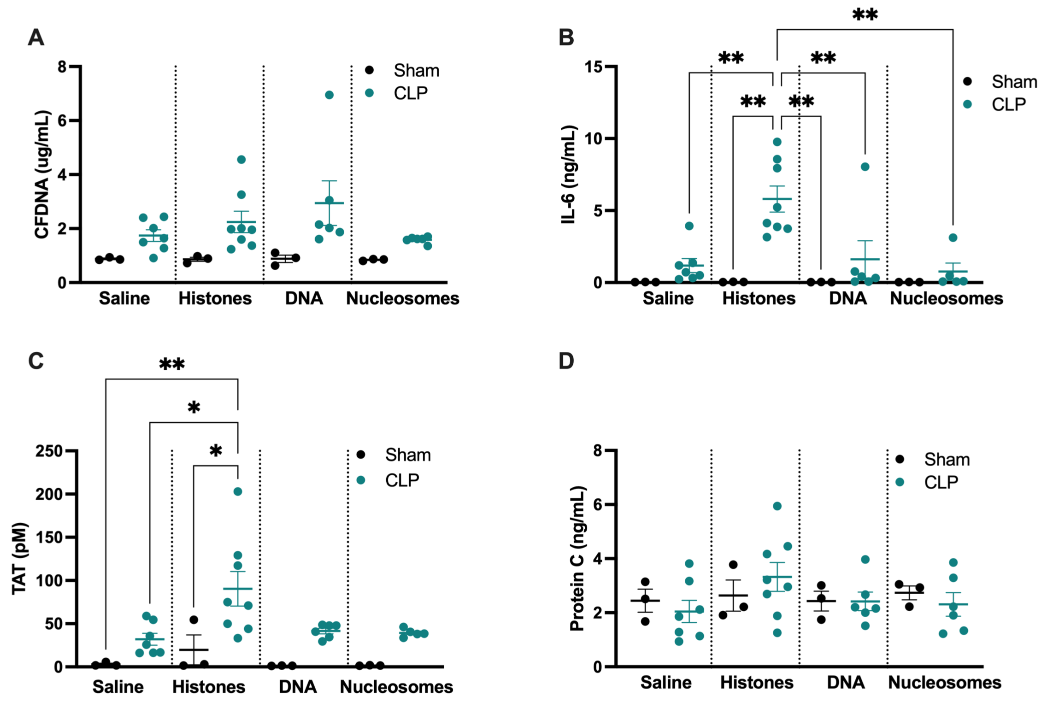

Figure 4.3. Quantification of CFDNA, IL-6, TAT, and PC.

Figure 4.4. Histology scoring. (A) Lung, liver, and kidney sections (5 μm) were stained for H&E. Sham (black) and CLP (teal) mice received two doses of either DNA (8 mg/kg), histones (8.5 mg/kg), and nucleosomes (composed of an equivalent of DNA and histones). Images are representative of 6 slides (n = 6). Saline sham controls are shown in panel A and is representative of all shams. Organs were then blindly scored for inflammation, thrombosis, and organ morphology on a 5-point scale. Composite scores per organ were calculated with results presented as mean (SD) and analyzed using a one-way ANOVA followed by Tukey's post-hoc test ($\alpha = 0.05$). Scale bars represent 100 μm.

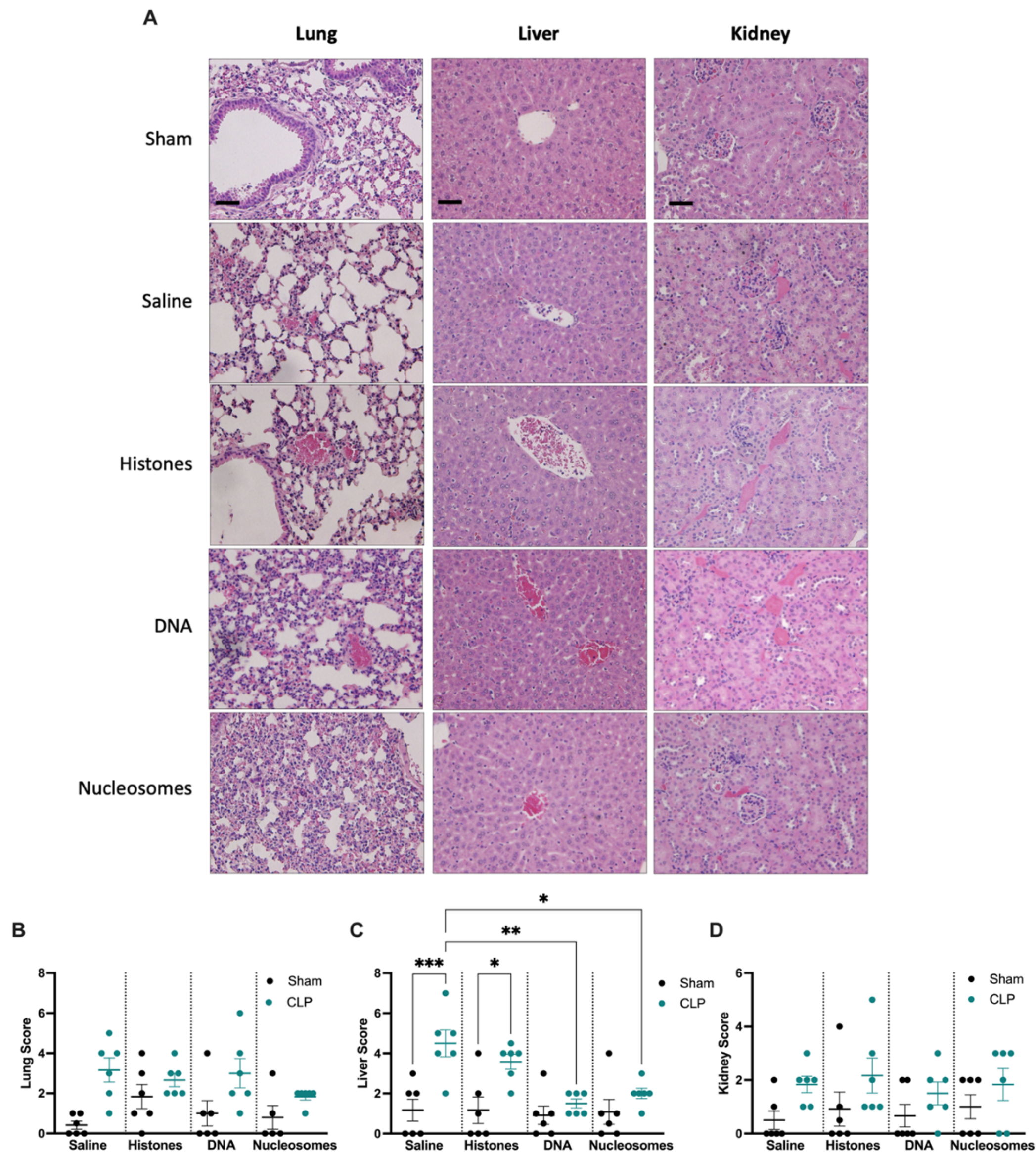

Figure 4.4. Histology scoring.

Figure 4.5. Assessing cell cytotoxicity using simplified reconstituted nucleosomes treated with DNase I and/or heparin (LMWH, dalteparin). (A) Increasing concentrations of histones were assessed in a cell toxicity assay. (B) Increasing concentrations of LMWH was added to 50 μg/mL of histones. (C) DNA (100 μg/mL), DNase I (100 μg/mL), LMWH (2 IU/mL), and a combination of DNase I + LMWH were assessed as controls. (D) DNase I and/or LMWH were added to 50 μg/mL of histones or nucleosomes and viability was assessed using trypan blue ($n = 3$). (E) An agarose gel (1.5%) was run to confirm the degradation of DNA in nucleosome treatments. Results are presented as mean (SD) and analyzed using a one-way ANOVA followed by Tukey's post-hoc test ($\alpha = 0.05$; * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, and *** $p < 0.0001$).

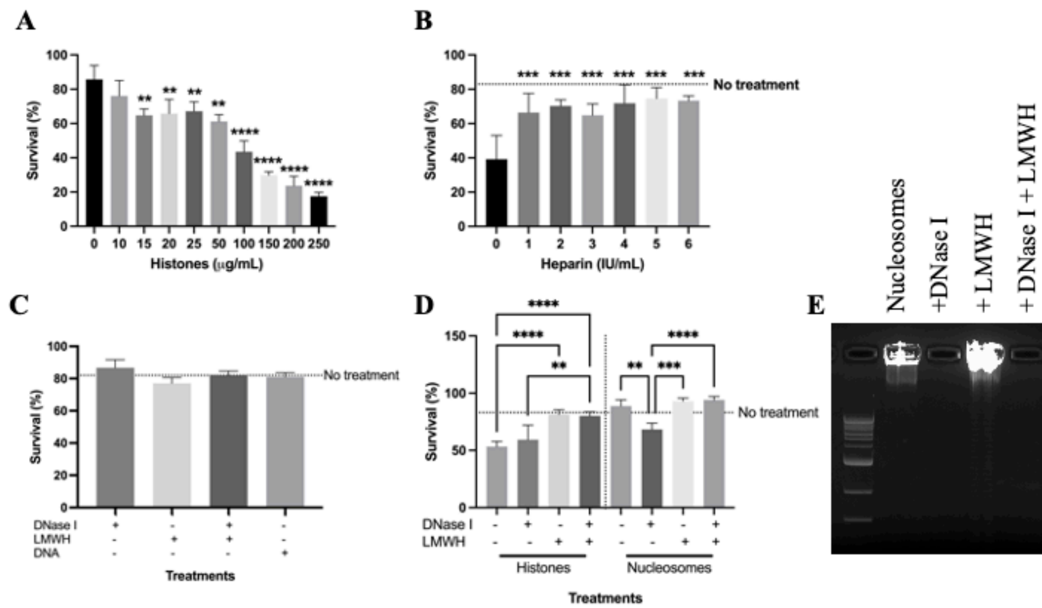

Figure 4.5. Confirming cell viability in reconstituted nucleosomes using a different protocol treated with DNase I and/or heparin

Figure 4.6. Sham histology scoring. (A) Lung, liver, and kidney sections (5 μm) were stained for H&E. Sham mice received two doses of either DNA (8 mg/kg), histones (8.5 mg/kg), and nucleosomes (composed of an equivalent of DNA and histones). Images are representative of 6 slides (n = 6). Scale bars represent 100 μm.

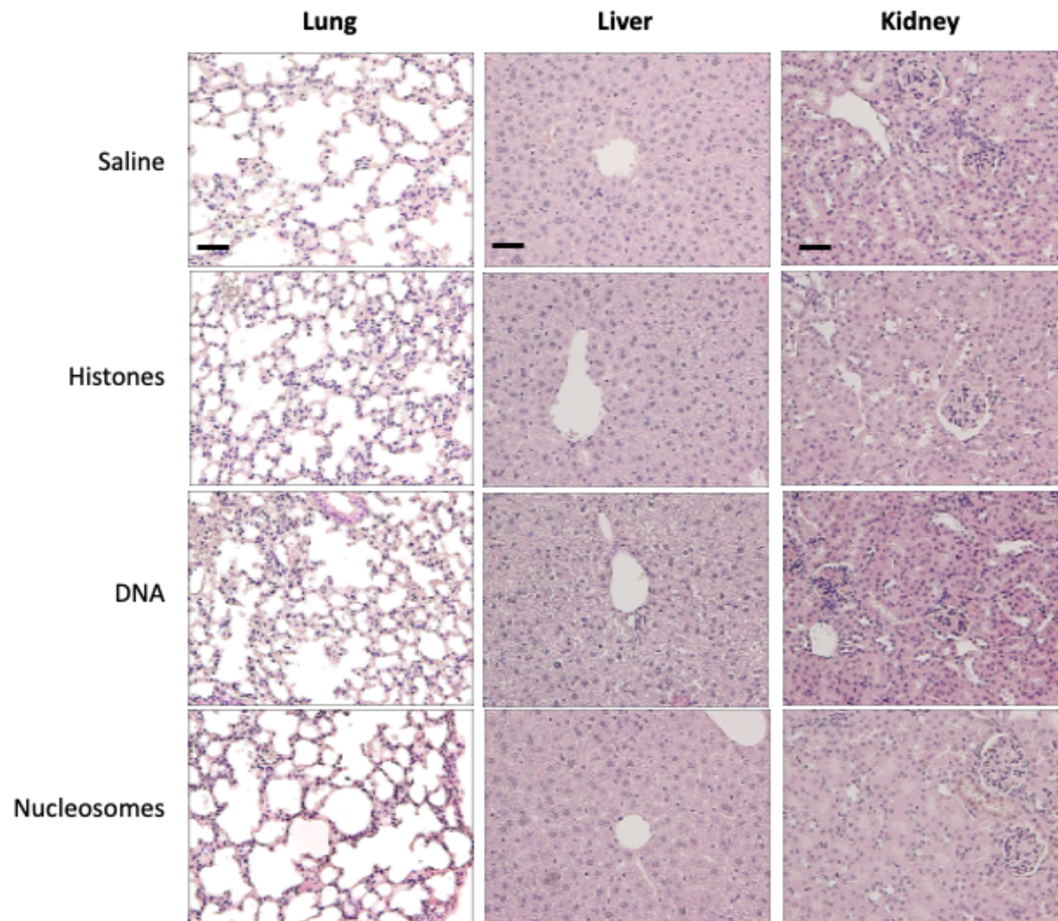

Figure 4.6. Sham histology scoring.

Figure 4.7. Mouse monitoring scores. Mice were subjected to a cecal-ligation and puncture (CLP) or sham surgery and then administered either saline, histone octamers (8.5 mg/kg) DNA (8 mg/kg), or nucleosomes. (A) Temperature, (B) weight, (C) mouse grimace score (MGS), and (D) mouse surrogate scores (MSS) were reported every 4 h until study endpoint was reached. Results are presented as mean (SD) (n = 6).

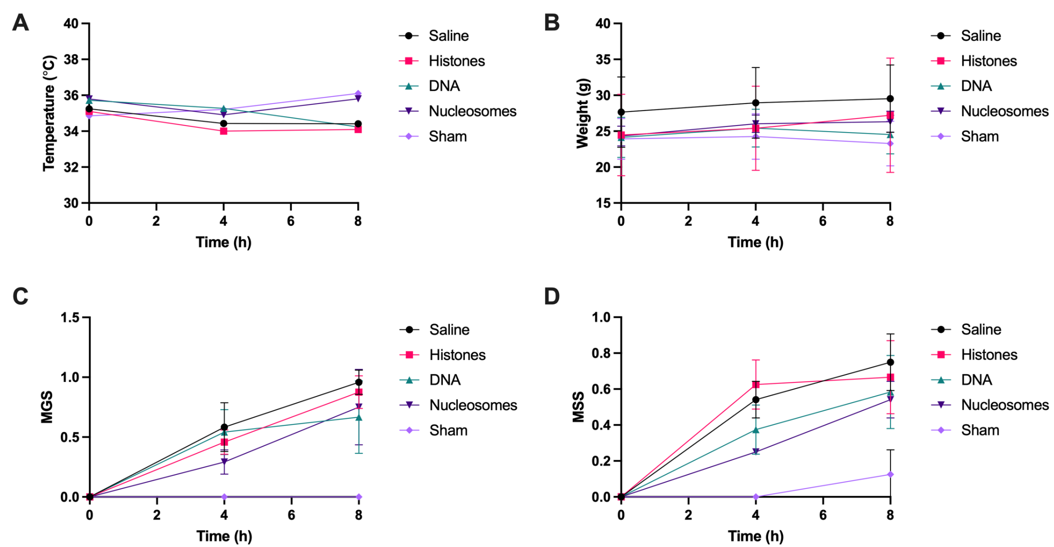

Figure 4.7. Mouse monitoring scores.

Figure 4.8. (A) Peritoneal cavity fluid (PCF) and (B) blood bacterial loads for sham and CLP mice administered either saline, histone octamers (8.5 mg/kg) DNA (8 mg/kg), or nucleosomes (n = 3-6). Results are presented as mean (SD).

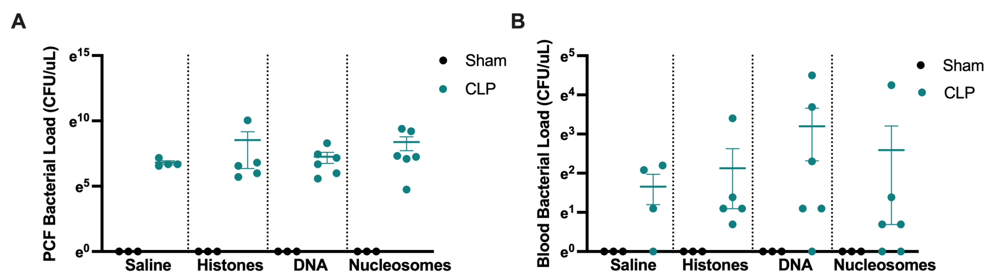

Figure 4.8. Bacterial burden.

Figure 4.9. (A) White blood cell (WBC), (B) red blood cell (RBC), and (C) platelet (Plt) counts from CLP mice administered either saline, histone octamers (8.5 mg/kg) DNA (8 mg/kg), or nucleosomes (n = 4-6). All groups were similar to naïve controls. Results are presented as mean (SD).

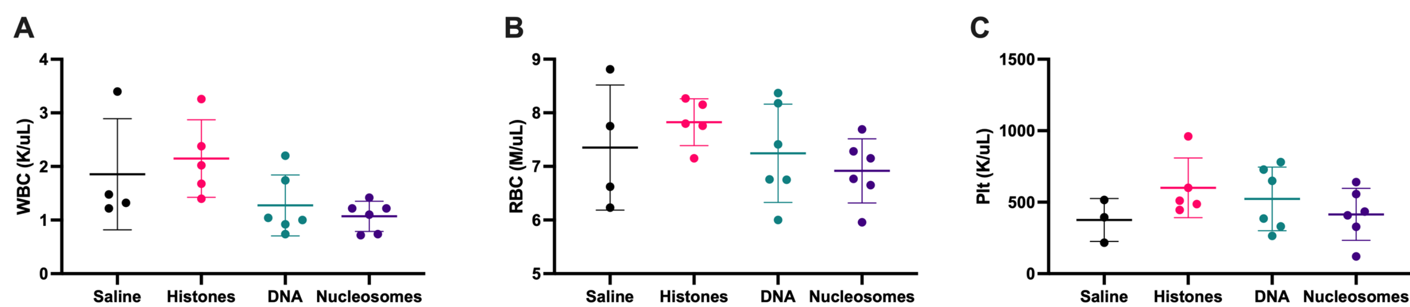

Figure 4.9. White blood cell, red blood cell, and platelet counts

5.0. The effects of DNase I and low-molecular-weight heparin in a murine model of polymicrobial abdominal sepsis

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Foreword: In chapter 5, we investigate whether using a combination approach of DNase I and low-molecular-weight heparin is beneficial in a murine model of sepsis.

Running title: DNase I and heparin in sepsis

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Author Contributions

All authors were involved in mouse monitoring and harvesting. Patricia C. Liaw, Sarah K. Medeiros, and Dhruva Dwivedi conceptualized the experiments. Sarah K. Medeiros performed the experiments. Sarah K. Medeiros wrote the manuscript and Patricia C. Liaw edited the manuscript.

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

5.1. Abstract

Introduction: Cell-free DNA (CFDNA) has emerged as a prognostic biomarker in patients with sepsis. Circulating CFDNA is hypothesized to be associated with histones in the form of nucleosomes. *In vitro,* DNA activates coagulation and inhibits fibrinolysis, whereas histones activate platelets and are cytotoxic to endothelial cells. Previous studies have targeted CFDNA or histones in animal models of sepsis using DNase I or heparins, respectively, which has reduced inflammatory and thrombosis markers, thereby improving survival. In this study, we explored the possibility that the combination of DNase I and a low-molecular weight heparin (LMWH) may be a better therapeutic approach than monotherapy in a murine model of abdominal sepsis.

Methods: C57Bl/6 mice (8–12 weeks-old, both sexes) were subjected to either cecal-ligation and puncture (CLP) or sham-surgery. Mice were given antibiotics, fluids, and either saline, DNase I (intraperitoneally; 20 mg/kg/8h), LMWH (dalteparin, subcutaneously 500 IU/kg/12h), or a combination of both (n = 12-31). Mice were monitored over 72h for survival. Organs and blood were harvested for analysis. Levels of LMWH, CFDNA, IL-6, citrullinated histone-H3 (H3-cit), thrombin-anti-thrombin complexes, protein C were measured in plasma.

Results: Administration of either DNase I (81.8%) or LMWH (83.3%, prophylactic range of 0.12 ± 0.07 IU/mL achieved) improved the survival of septic mice compared with saline- (38.7%) and combination-treated mice (48.8%; $p<0.05$). Combination-treated-mice also showed a small but insignificant improvement in survival compared to saline-treated CLP mice. Monotherapies may be improving survival by reducing blood bacterial loads, H3-cit, and thrombin-anti-thrombin complexes, and improving protein C levels.

Conclusions: Compared to saline- and combination-treated mice, administration of monotherapies to septic mice improved survival. These findings suggest that there may be a negative drug-drug interaction between DNase I and LMWH when DNase I is administered intraperitoneally in a murine model of polymicrobial abdominal sepsis.

5.2. *Introduction*

Sepsis is defined as life-threatening organ dysfunction induced by a dysregulated host response to infection (Singer et al., 2016). In the United States, the cost of sepsis is estimated to be $48 billion (USD) per year (Liang et al., 2006) and with an aging population, the cost of sepsis is only expected to increase (Iwashyna et al., 2012). As SARS-CoV-2 continues to spread, coronavirus disease (COVID)-related sepsis has increased the clinical burden of sepsis with intensive care unit 31-day mortality rates reported as high as 20-65% (Arentz et al., 2020; Oliveira et al., 2021). Therefore, an improved understanding of sepsis pathophysiology is required to develop novel therapeutic strategies to reduce the burden of sepsis.

We previously reported that circulating levels of extracellular DNA (also known as cell-free DNA; CFDNA) is a useful prognostic indicator in patients with sepsis (Dwivedi et al., 2012; Liaw et al., 2019). Most CFDNA in sepsis is derived from either organ damage (predominantly liver-derived) or neutrophils (Dor et al., 2018). CFDNA from neutrophils is likely produced from a unique form of cell death called NETosis, which releases neutrophil extracellular traps (NETs) (Dor et al., 2018). NETs are web-like networks of CFDNA and histones, decorated with lysosomal enzymes and antimicrobial peptides (Brinkmann et al., 2004). Although NETs aid in host defense by trapping microorganisms, excessive NET production may hinder blood flow by providing a scaffold for platelets and red blood cells,

thereby contributing to tissue hypoxia, organ damage, and ultimately mortality (Brinkmann et al., 2004; Clark et al., 2007).

The major structural component of NETs is chromatin, which consists of nucleosomes (also known as DNA and histones complexes) (Brinkmann et al., 2004). CFDNA itself can be harmful to the host as it can trigger coagulation via the intrinsic pathway and can also inhibit fibrinolysis (Dwivedi et al., 2012; Gould et al., 2014; Gould et al., 2015). In contrast, histones are cytotoxic to endothelial cells *in vitro* and can promote coagulation via platelet activation, impairing the anticoagulant pathway, and impairing fibrinolysis (Ammollo et al., 2011; Longstaff et al., 2013; F. Semeraro et al., 2011; Sharma et al., 2022). Intravenous injections of purified histones in mice result in thrombocytopenia, neutrophil migration, and organ failure mimicking the pathophysiological nature of sepsis (Xu et al., 2009). For these reasons, both DNA and histones have been considered potential targets in sepsis.

The harmful effects of histones can be neutralized via binding to heparins, which are negatively charged GAGs (Sharma et al., 2022). In murine and human sepsis, administration of various heparins improve survival (X. Li & Ma, 2017; Y. Li et al., 2011). Clinically, heparins are best known for their anti-coagulant properties via their ability to catalyze antithrombin-mediated inactivation of thrombin and factor (F) Xa (Nutescu et al., 2016). Heparins—either unfractionated heparin (UFH) or low molecular weight heparin (LMWH)—are also standard

thromboprophylaxis in all acutely hospitalized patients who are not at risk for bleeding, including patients with sepsis (Rhodes et al., 2017). LMWHs are replacing UFH for therapeutic anticoagulation due to improved predictable pharmacokinetics, less frequent dosing, and lower risk of heparin-induced thrombocytopenia (Hirsh et al., 2008).

While LMWH can bind and neutralize histones, the harmful effects of CFDNA can be reduced through degradation by DNase I. Targeting CFDNA with DNase I via intraperitoneal or intravenous administration has been shown to provide a survival benefit in preclinical murine studies as many studies report that DNase I treatment partially improves inflammation, thrombosis, organ damage, survival outcomes, and/or bacterial burden (Lauková et al., 2017; Mai et al., 2015). However, Kolackowska et. al. reported that although injection of DNase I into mice removed circulating DNA, the released histones from nucleosomes remain bound to vessel walls and contribute to liver injury (Kolaczkowska et al., 2015). Consequently, co-administration of heparins with DNase I may potentially neutralize the harmful effects of histones, thereby improving survival. Additionally, DNase I and heparins have been shown to function synergistically *in vitro* as heparins bind histones to release DNA resulting in a 3- to 5-fold increase in DNase I's enzymatic activity (Brotherton et al., 1989; Sohrabipour et al., 2021). Our lab has also shown that nucleosomes cannot be degraded by DNase I solely and requires a combination of a heparin and DNase I (Sohrabipour et al., 2021).

All together, these studies suggest that a combination approach may be a beneficial therapy in sepsis research. Therefore, the objective of this study was to investigate the therapeutic efficacy of targeting nucleosomes using a combination therapy of DNase I and LMWH in a murine model of sepsis. We investigated the protective effects of LMWH dalteparin (6 kDa) in our murine model as dalteparin is commonly used as thromboprophylaxis in critically ill patients (Cook et al., 2011; Przybysz & Huang, 2011).

5.3. Materials and Methods

5.3.1. Induction of sepsis: cecal ligation, and puncture (CLP) model

C57Bl6/J mice (8-12 weeks-old, both sexes) were placed under gaseous anesthesia (2.5% isoflurane, oxygen flow 1.5 L/min) for surgery. Ringer's Lactate (2 mL) and analgesia (buprenorphine 0.1 mg/kg) were administered pre-operatively. Mice then underwent either a sham or CLP surgery as previously described (Mai et al., 2015). For mice undergoing CLP, 1 cm of cecum was ligated, punctured through-and-through using an 18-G needle, and 1 cm of fecal matter was extruded.

Mice were continuously monitored for signs of distress until study endpoint at 72h. Every 4h, the following parameters were recorded (Figure 5.6): weight, temperature, mouse grimace score (MGS; a pain score that is the average of orbital tightening, ear positioning, nose/cheek bulge, and whiskers scored on a 3-point

scale), and mouse surrogate markers of death (MSS; an average score of hunch, ambulation, responsiveness, and ruffled fur scored on a 3-point scale). Mice were sacrificed at humane endpoint when they received average MSS or MGS scores greater than 1.75 or a score of 3 in any category.

Throughout the experiment, mice were given subcutaneous Ringer's Lactate (1 mL every 4h) and analgesia (buprenorphine, 0.05 mg/kg every 8h). An antibiotic (pipercillin/tazobactam) was administered (200 μL) intraperitoneally (IP) at the pediatric dose used in human sepsis (100 mg/kg) 8h post-surgery and every 8h thereafter. The ARRIVE guidelines were followed (Kilkenny et al., 2010) and all animal studies were approved by the McMaster University's Animal Research Ethics Boards (AUP# 19-01-06).

5.3.2. Administration of DNase I and/or LMWH in septic mice

DNase I (Pulmozyme, 20 mg/kg, 500 μL) or an equivalent volume of saline was administered IP every 8h. Dalteparin (Fragmin) was administered every 12h subcutaneously at 500 IU/kg (100 μL) to establish a prophylactic dose in our model. Refer to Table 5.1 for the full experimental schedule.

5.3.3. Organ harvest and blood isolation

At study or humane endpoint, mice were put under anesthesia, peritoneal cavity fluid (PCF) was collected, blood was collected from the inferior vena cava

in 3.2% trisodium citrate, organs were collected in neutralized formalin buffer or snap frozen in liquid nitrogen, and then mice were culled via cervical dislocation. PCF, blood, lungs, and livers were diluted in PBS at various concentrations then plated on blood agar to detect bacterial burden. Platelet poor plasma was obtained by centrifugation of whole blood at 5,000 x g for 20 min at room temperature. The plasma samples were then aliquoted and frozen at -80ºC until needed.

5.3.4. Detection of LMWH in plasma

Plasma levels of LMWH were measured using an anti-Xa assay. Briefly, anti-Xa levels were determined by the addition of diluted standards (dalteparin, 0-1.5 IU/mL) or samples (1:10 dilution, 40 µL) to 96-well plates containing anti-thrombin (5 IU/mL, 40 µL). FXa (10 µg/mL, 40 µL) and S-2765 substrate (Diapharma, Beckett Ridge, Ohio, US; 800 mM, 40 µL) was then added in a stepwise fashion. Wells were incubated for 2 min at 37˚C and then the reaction was stopped using 80 µL of 20% acetic acid. Plates were read at 405 nm and the amount of LMWH was determined based on a standard curve.

5.3.5. Quantification of CFDNA, citrullinated histone-H3, IL-6, TAT, PC, ALT, and cystatin C in plasma

CFDNA levels in plasma was determined using PicoGreen dsDNA assay kit (ThermoFisher Scientific; Waltham, Massachusetts, US). IL-6 was quantified

using a mouse specific ELISA kit from R&D Systems (Minneapolis, Minnesota, US). Citrullinated histone-H3 (H3-Cit) was measured according to a previously described protocol (Thålin et al., 2019). Thrombin-anti-thrombin (TAT) complexes were quantified using an ELISA from Affinity Biologicals (Ancaster, Ontario, Canada) as per manufacturer's instructions. Protein C (PC) was quantified as previously described (W. Li et al., 2005). Alanine aminotransferase (ALT) was quantified using an activity assay kit from Abcam (Cambridge, UK). Kidney damage was quantified using a mouse cystatin C Quantikine ELISA kit from R&D.

5.3.6. Histology and staining

Paraffin sections (5 µm) of liver, lungs, and kidneys were stained with H&E. The sections were scored in a blinded fashion, by two individuals, on a scale of 0–5 (0 = normal, 5 = severe) based on inflammation, thrombosis, and overall organ morphology (Table 5.2). Immunohistochemistry was also conducted on lung tissue sections to detect fibrin and confirm the presence of intravascular thrombi. Lungs sections were incubated with polyclonal rabbit anti-human fibrin antibody (1:200 dilution, Dako, Santa Clara, California, US). The anti-fibrin antibody was detected by incubation with a secondary antibody goat anti-rabbit IgG (H+L), Alexa Fluor 594 (1:200 dilution; Thermofisher Scientific). DAPI (1 µg/mL) was used to counter stain the sections. Confocal microscopy was performed to obtain images of entire sections using the Stellaris 5 microscope (Leica Microsystems, Wetzlar, Germany).

Area of fibrin coverage (%) was quantified using instrument software (Leica Application Suite X; LAS X).

5.3.7. Statistical analysis

Kaplan-Meier survival curves were created and analyzed using the Mantel-Cox Log-rank test. Data was presented as mean (SD). Comparison between groups were performed using a one-way analysis of variance test (ANOVA) followed by Tukey's multiple comparison test. *P* values less than 0.05 were considered statistically significant. Prism 9 (Graphpad, San Diego, California, US) was used to analyze data.

5.4. Results

5.4.1. Administration of DNase I or LMWH monotherapy improved survival compared to saline controls

Using an abdominal murine model of sepsis, administration of either DNase I (81.8%) or LMWH (83.3%) improved survival compared to treatments with either saline (38.7%) or combination therapy (48.0%, $p<0.05$; Figure 5.1A). In our study, LMWH levels were in prophylactic range at 0.12 ± 0.07IU/mL. Interestingly, the survival rate of the combination therapy was not statistically different from the saline-treated CLP mice ($p = 0.396$) suggesting that the combination therapy was

not synergistic in our model. These results suggest that the combination therapy reduced the protective effects of the monotherapies.

5.4.2. Blood bacterial burden is reduced in monotherapies, but not saline- or combination-treated mice

The mice subjected to CLP that survived to 72h (study-end point) were categorized as *"CLP-live"* and the mice that died within the study timeline (prior to 72h) were termed "*CLP-dead.*" We observed that PCF bacterial loads improved in all DNase I and combination treated mice compared to controls. This trend was not observed in LMWH treated mice (Figure 5.1B). However, mice treated with a monotherapy of DNase I or LMWH had reduced blood bacterial loads, which was not observed in mice treated with either saline or combination therapy. This difference could partially explain the survival benefit observed in mice treated with monotherapies (Figure 5.1C). Despite this, there were no significant differences in organ bacterial burden between treatment groups in the lungs and livers (Figure 5.1D and 5.1E). Interestingly, we observed all treatment groups had lower lung bacterial burden compared to the saline septic mice. However, both saline and combination treated septic mice that survived to 72h had higher bacterial burden than other treatments.

5.4.3. CFDNA is normal in DNase I groups and is elevated in early sepsis in saline and LMWH groups

CFDNA levels were elevated during the early phase of sepsis (between 8 and 32 hours) but returned to baseline levels by 72h in the saline and LMWH groups (Figure 5.2A and 5.3A). Although not significant, the saline-treated non-surviving septic mice (CLP-dead) had the highest CFDNA levels prior to 24h followed by those treated with LMWH suggesting that treatment with DNase I reduces levels of circulating CFDNA.

5.4.4. Plasma levels of IL-6 do not correlate with poor outcome

To investigate inflammation in our model, plasma levels of IL-6 were quantified. As shown in Figure 5.2B, IL-6 levels were elevated in all CLP mice—both in mice who died within 72h and mice who survived—similarly compared to sham surgery controls. However, mice that passed away had significantly higher plasma levels of IL-6 compared to mice that survived to 72h (Figure 5.3B). This trend was observed in all treatment groups suggesting that IL-6 may not explain the protective effects of DNase I or LMWH monotherapy in our sepsis model.

5.4.5. Monotherapies potentially reduce coagulation

PC is an essential anticoagulant protein that is reduced in severe sepsis (Koyama et al., 2014). In our model, plasma levels of PC were significantly

reduced in saline-treated CLP mice compared to sham-surgery controls (Figure 5.2C). PC in all treatment groups by 72h returned to normal levels (Figure 5.3C). To investigate coagulation, we quantified TAT complexes in plasma. We observed elevated TAT levels in mice who died (early sepsis) signifying a procoagulant state (Koyama et al., 2014). Mice who received monotherapies and survived to 72h had reduced TAT complexes compared to those that received saline or the combination (Figure 5.2E). Consequently, we hypothesize that the monotherapies may improve survival in part by reducing coagulation and reducing PC consumption.

5.4.6. Monotherapies may be improving survival by reducing NETosis

To explore the possibility that the combination treatment reduces NETosis, we quantified levels of H3-cit. We observed that H3-cit was elevated in saline septic mice compared to sham controls but reduced in DNase I or LMWH monotherapy treated mice who survived to 72h (Figure 5.2D). Combination therapies showed an intermediate but insignificant improvement at 72h, which suggests the monotherapies may be improving survival by reducing markers of NETosis.

5.4.7. Biomarkers of liver and kidney damage remained elevated in saline- and combination-treated mice

Liver and kidney damage was quantified by measuring ALT activity and cystatin C levels in plasma, respectively. ALT levels were elevated in all mice who died and in mice that received saline or combination therapy who survived to 72h, suggesting there was a trend to liver damage in these groups (Figure 5.4A and 5.4B). The data suggests that treatment with monotherapies may be improving sepsis-induced liver damage, although this was not significant. Cystatin C levels were normal in mice who survived to 72h, but levels in saline-treated mice were significantly elevated in early timepoints compared to mice who survived (Figure 5.4C and 5.4D). Altogether, this suggests our model may not be severe enough to see significant liver and kidney damage although trends are reported.

5.4.8. Inflammation, thrombosis, and organ morphology scores in organs

H&E-stained lung, liver, and kidney sections were blindly scored for inflammation, thrombosis, and organ morphology (Figure 5.5). Organ morphology scores were elevated in the CLP mice (Figure 5.5C), but there was no trend observed in necrosis, glomeruli congestion, or alveolar pocket morphology that could potentially explain the differences in survival between treatment groups. Inflammation scores (Figure 5.5D) were significantly elevated in all CLP mice compared to the sham surgery controls. Thrombosis scores for all the dead mice in

the CLP treatment groups were significantly higher than in the saline-treated controls (Figure 5.5E). However, both inflammation and thrombosis scores were not significantly different between CLP treatment groups. Despite this, fibrin meshes were observed in some organs that require further investigation and, thus, we quantified fibrin deposition through immunohistochemistry. We observed increases in fibrin deposition in septic mice compared to sham-surgery mice (data not shown). However, no difference in fibrin deposition was observed between the septic mice treated with either monotherapy or combination therapy.

5.5. *Discussion*

In this study, we explored the effects of DNase I and/or LMWH in a CLP murine model of sepsis. We observed that treatment with either DNase I or LMWH significantly improved survival compared to saline septic controls (Figure 5.1), which aligns with multiple *in vivo* and clinical studies that suggests these treatments are beneficial (Lauková et al., 2017; X. Li & Ma, 2017; Y. Li et al., 2011; Mai et al., 2015). Unexpectedly, there was no significant improvement of mice treated with the combination therapy compared to saline-treated controls suggesting that these agents do not function synergistically *in vivo* despite evidence that suggests synergy *in vitro* (Brotherton et al., 1989; Sohrabipour et al., 2021).

Bacterial burden in the PCF and blood may provide some insight to our results (Figure 5.1B and 5.1C). We observed that LMWH did not reduce PCF

bacterial burden compared to saline controls, however, blood bacterial burden was reduced, which could partly explain the survival improvement in this group. Therapies that included DNase I (both monotherapy and combination) reduced bacterial burden in PCF, but the combination did not reduce blood bacterial load suggesting a potential drug-drug interaction occurring that can increase blood bacterial burden. Unfortunately, our exploration into common markers of inflammation and coagulation does not provide significant mechanistic insights into our findings. We do observe our treatments elevate markers of thrombosis and inflammation early, as seen in elevation in IL-6, TAT, and H3-cit and a reduction in PC (Figure 5.2 and 5.3). By 72h, we observed a trend to recovery of these biomarkers, suggesting our mice were recovering from their abdominal sepsis challenge. In general, monotherapies improved biomarker levels at 72h, while the saline and combination therapy could not, however, this was not a significant trend.

The importance of this study is highlighted by the fact clinical studies have begun to recruit patients to investigate aerosolized DNase I (NCT04541979 and NCT04445285) in COVID-19 patients with respiratory failure and intravenous DNase I (NCT05453695) as an intervention in septic ICU patients. Since standard thromboprophylaxis in sepsis is the administration of an anticoagulant (such as LMWH), it is important to explore if there are any potential drug-drug interactions. Our study suggests that there may be potential drug-drug interactions between

LMWH (dalteparin) and DNase I when DNase I is administered intraperitoneally at 20 mg/kg/8h in our polymicrobial abdominal model of sepsis.

Dalteparin is not the only heparin used clinically. UFH and enoxaparin are also commonly used as thrombosis prophylaxis in septic patients. Previous *in vitro* data reported that UFH and other various LMWHs can similarly inhibit histone cytotoxicity (Sharma et al., 2022). However, it is unknown if our *in vivo* findings are unique to dalteparin or if they can be generalized to these other heparins. To answer this question, we explored a prophylactic treatment of UFH (18 IU/kg/h) (Houston et al., 2015) in our mouse model of sepsis. We observed a similar trend to improved survival in our model and that the combination of UFH and DNase I was not synergistic and no different to their saline controls (44.4% vs. 44.4%; $p>0.05$; data not shown). Therefore, we hypothesize that there would be a similar survival improvement when using different heparins with histone-neutralizing abilities.

There are several strengths to this study. We used both male and female mice in equal proportions per group, whereas previous mouse studies using DNase I focused on one sex (Lauková et al., 2017; Mai et al., 2015). We did not observe any significant sex differences in our study. Moreover, we administered antibiotics, fluids, and analgesia to our mice, which makes the study more clinically relevant. We also ensured blinding for subjective scores where possible, such as histology, which ensures reduced result bias. However, our study has several limitations.

Firstly, we used an abdominal polymicrobial sepsis model, which may not be representative of all sepsis causes (ie. the most common cause of sepsis is a respiratory tract infections) (Esper et al., 2006). However, the CLP model is considered the gold-standard for sepsis survival studies in mice as it mimics the physiological response of human sepsis. Secondly, only one dose and one timing of administration was investigated for DNase I and LMWH. Thus, it is unknown if these results can be generalized to other doses and other times of administration. Additionally, the route of administration of DNase I was intraperitoneal, which is a convenient method for mice, but is not translatable to humans. Finally, additional future studies are needed to investigate potential mechanisms for why the combination therapy was inferior to the monotherapies in our mouse model.

In summary, this is the first study to compare DNase I or LMWH monotherapy with combination therapy in a murine model of CLP. Our results suggest that there may be drug-drug interactions between DNase I and LMWH when DNase I is administered intraperitoneally at a specific dose and timing regimen in a murine model of polymicrobial abdominal sepsis. Future studies will focus on identifying the mechanisms by which monotherapy is more effective than combination therapy in the CLP model of sepsis.

Figure 5.1. Kaplan-Meier survival curve and bacterial loads. (A) Mice were subjected to a cecal-ligation and puncture (CLP) or sham surgery and then given various treatments. Mice received either saline, DNase I (20 mg/kg/8h), dalteparin (LMWH; 500 IU/kg/12h), or a combination of both (DNase I + LMWH). A Mantel-Cox Log-rank test was used to analyze results (n = 12-31). Bacterial loads for (B) peritoneal cavity fluid (PCF; n = 7-17), (C) blood (n = 3-15), (D) lung (n = 6), and (E) liver (n = 6) from CLP and sham mice. Bacterial loads were analyzed from shams at 72 h represented by black dots, mice that survived until 72 h are dark grey, and mice that succumbed within 72 h are represented in light grey. No bacterial growth was found in sham mice. Results are presented as mean (SD) and analyzed using a one-way ANOVA followed by Tukey's post-hoc test. A p-value of 0.05 was considered statistically significant (*: $p<0.05$).

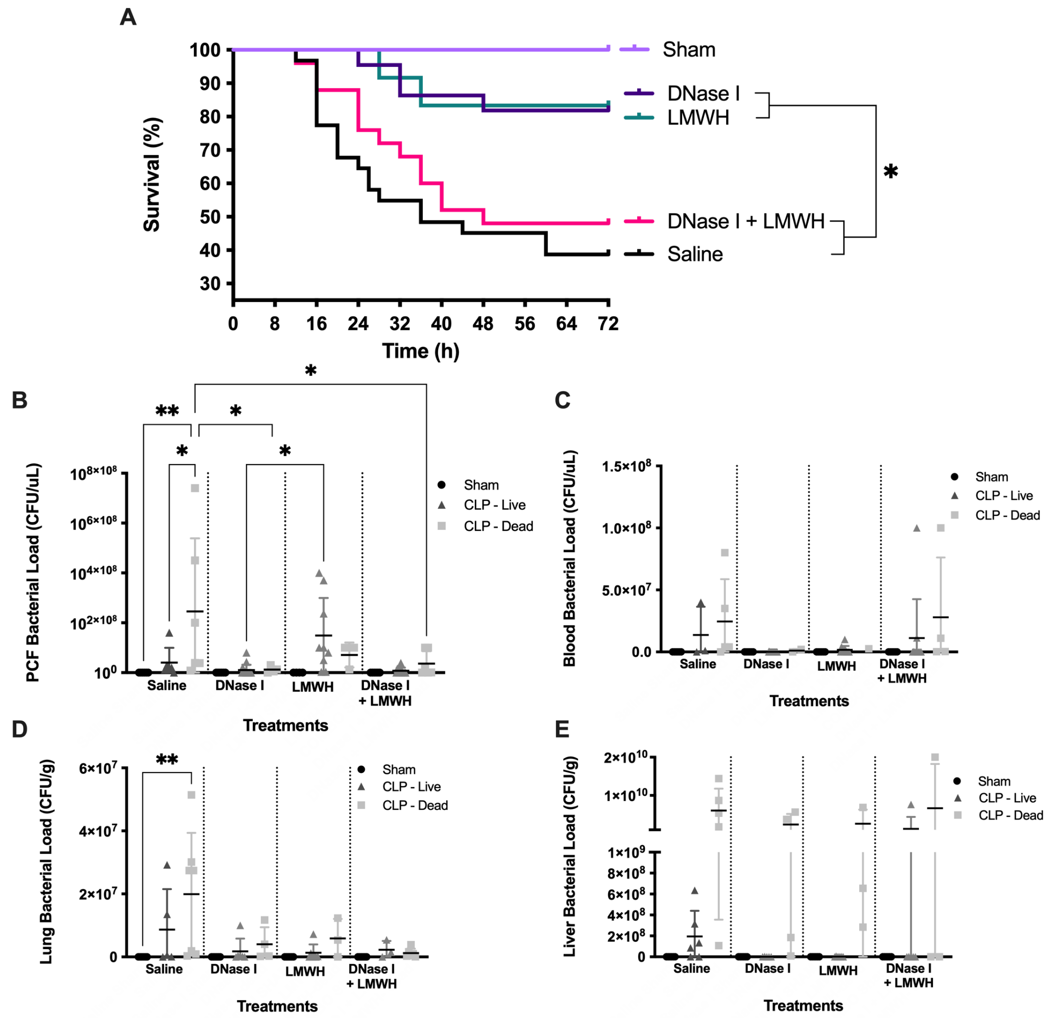

Figure 5.1. Kaplan-Meier survival curve and bacterial loads.

Figure 5.2. Quantification of (A) CFDNA, (B) IL-6, (C) protein C (PC), (D) citrullinated histone H3 (H3-cit), and (E) thrombin-anti-thrombin (TAT) complexes in sham and CLP mouse plasma (n = 3-6). Shams are represented by black bars, mice that survived until 72h are dark grey, and mice that succumbed within 72h are represented in light grey. Results are presented as mean (SD) and analyzed using a one-way ANOVA followed by Tukey's post-hoc test. The normal range of variables in C57Bl/6 mice are indicated by a shaded green area for reference. A p-value of 0.05 was considered statistically significant (*: $p<0.05$, **: $p<0.01$, ***: $p<0.001$, ****: $p<0.0001$).

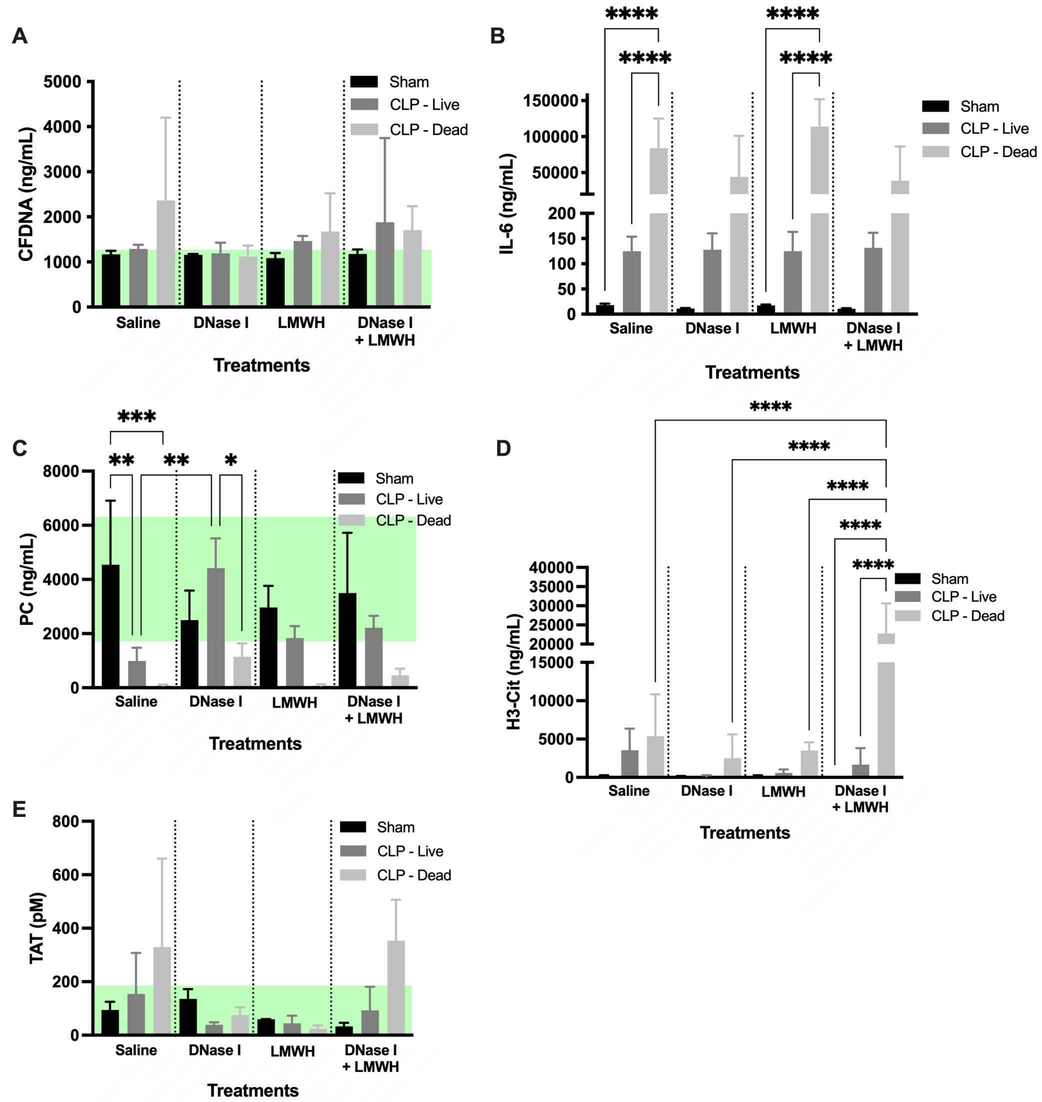

Figure 5.2. Quantification of plasma biomarkers.

Figure 5.3. Quantification over time of (A) CFDNA, (B) IL-6, (C) protein C (PC), (D) citrullinated histone H3 (H3-cit), and (E) thrombin-anti-thrombin (TAT) complex in sham and CLP mouse plasma (n = 6). The normal range of variables in C57Bl/6 mice are indicated by a shaded green area for reference.

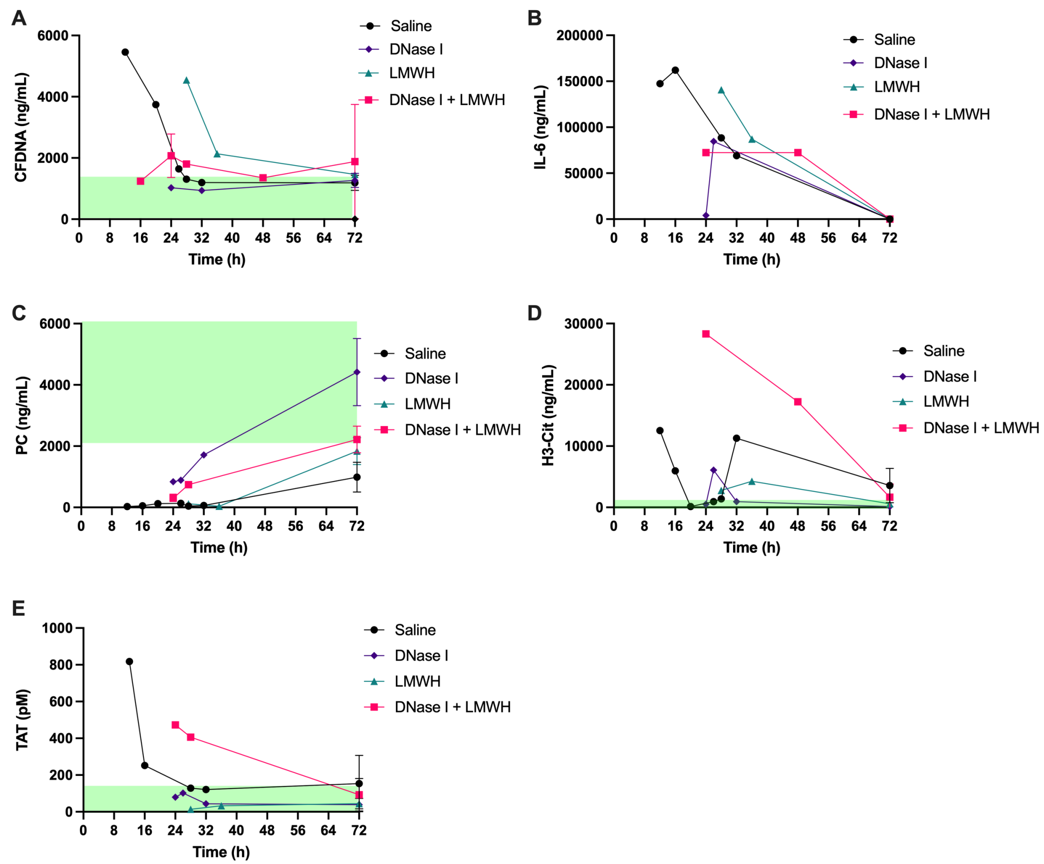

Figure 5.3. Quantification of plasma biomarkers overtime.

Figure 5.4. Markers of liver and kidney damage. (A-B) Alanine transferase activity and (C-D) cystatin C levels were used to assess organ damage. Shams are represented by black bars, mice that survived until 72h are dark grey, and mice that succumbed within 72h are represented in light grey. Results are presented as mean (SD) and analyzed using a one-way ANOVA followed by Tukey's post-hoc test. The normal range of variables in C57Bl/6 mice are indicated by a shaded green area for reference ($n = 3\text{-}6$). A p-value of 0.05 was considered statistically significant (*: $p<0.05$, **: $p<0.01$).

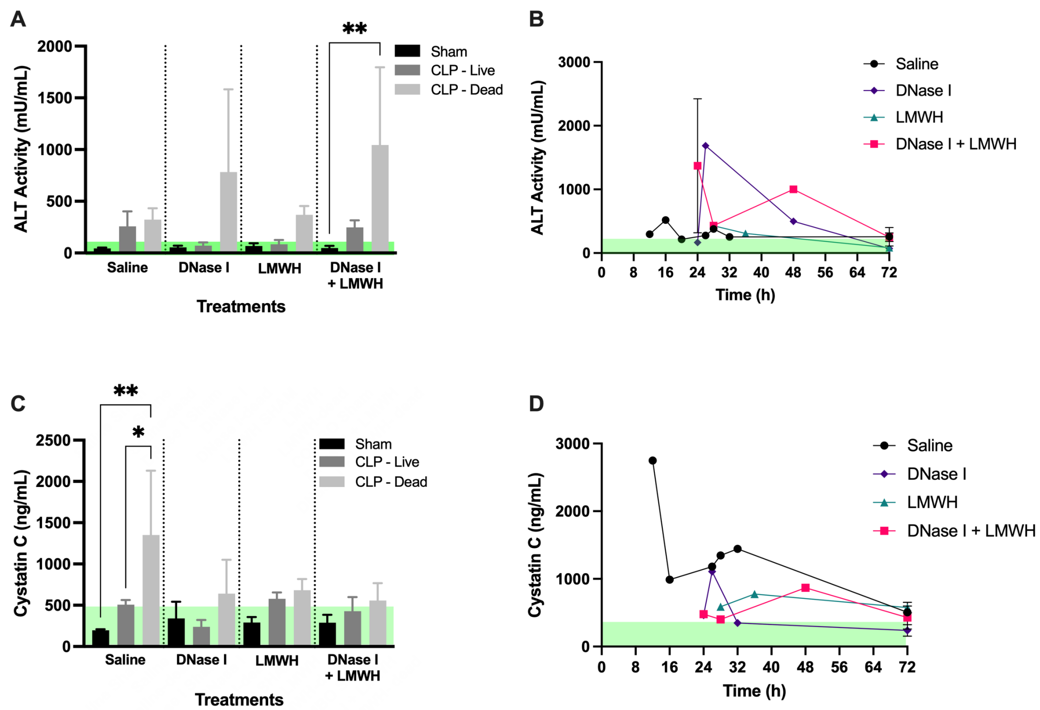

Figure 5.4. Markers of liver and kidney damage.

Figure 5.5. Histology scoring for mice who (A) survived and (B) died within 72 h. Lung, liver, and kidney sections (5 μm) were stained for H&E. Images are representative of 3-6 slides (n = 3-6). Slides were then blindly scored for (C) inflammation, (D) thrombosis, and (E) organ morphology. Results are presented as mean (SD) and analyzed using a one-way ANOVA followed by Tukey's post-hoc test ($\alpha = 0.05$). Scale bars represent 100 μm. A p-value of 0.05 was considered statistically significant (*: $p<0.05$, **: $p<0.01$, ***: $p<0.001$, ****: $p<0.0001$).

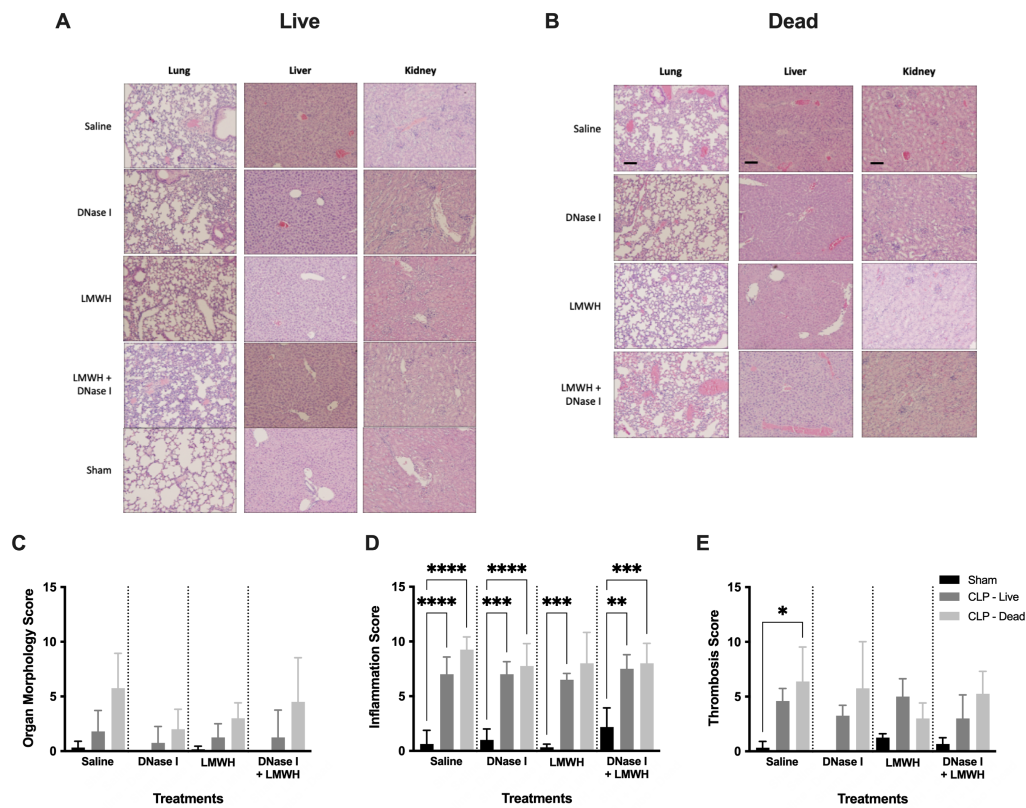

Figure 5.5. Histology scoring.

Figure 5.6. Mouse surrogate markers of death scores. Sham (blue) or CLP mice subjected with either saline (red), DNase I (green), LMWH (purple), and DNase I + LMWH (orange). (A) Temperature, (B) weight, (C) mouse grimace score, and (D) hunch scores were taken every 4 h until study endpoint (72 h) was reached. Results are presented as mean (SD) (n = 12-31).

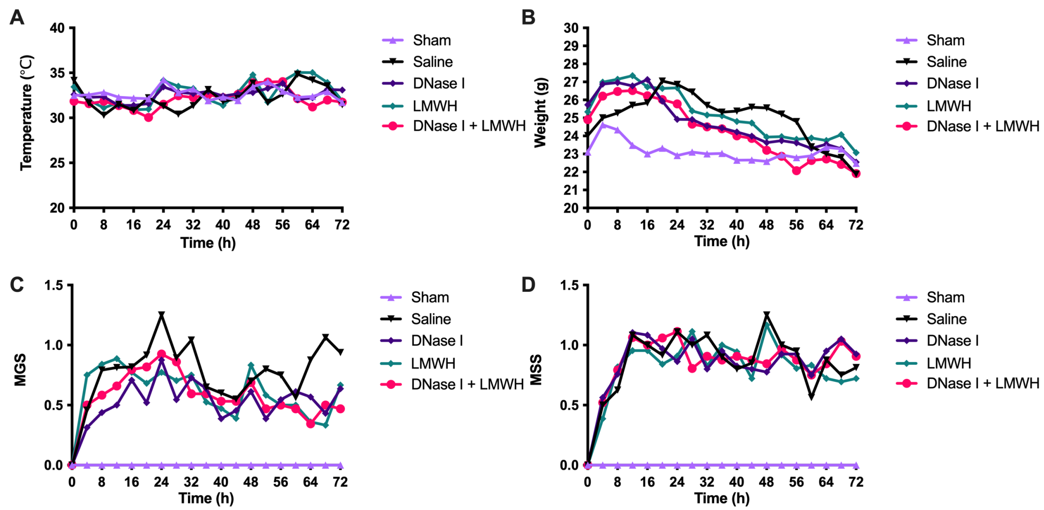

Figure 5.6. Mouse surrogate markers of death scores.

Table 5-1: Schedule of monitoring, fluid resuscitation, and treatments for 72h sepsis survival study

Time (h)	Ringers (mL) SUBQ	Buprenorphine SUBQ	Antibiotics IP	DNase I IP	LMWH SUBQ
0	2	0.1 mg/kg			
4	1				
8	1	0.05 mg/kg	100 mg/kg	20 mg/kg	500 IU/kg
12	1				
16	1	0.05 mg/kg	100 mg/kg	20 mg/kg	
20	1				500 IU/kg
24	1	0.05 mg/kg	100 mg/kg	20 mg/kg	
28	1				
32	1	0.05 mg/kg	100 mg/kg	20 mg/kg	500 IU/kg
36	1				
40	1	0.05 mg/kg	100 mg/kg	20 mg/kg	
44	1				500 IU/kg
48	1	0.05 mg/kg	100 mg/kg	20 mg/kg	
52	1				
56	1	0.05 mg/kg	100 mg/kg	20 mg/kg	500 IU/kg
60	1				
64	1	0.05 mg/kg	100 mg/kg	20 mg/kg	
68	1				500 IU/kg
72					

SUBQ: subcutaneous; IP: intraperitoneal; LMWH: low-molecular weight heparin.

Table 5-2: Histology scoring

Score	Thrombosis	Inflammation
0	No thrombi, no platelets, no fibrin mesh observed	No WBCs present
1	Transient state of thrombosis: Fibrin mesh or platelets	Few WBC's lining the vessel wall
2	Fibrin mesh and platelets seen in <20% of vessels	Some WBCs present in circulation
3	Fibrin mesh and platelets seen in 20% - 40% of vessels	A lot of WBCs present in circulation
4	Fibrin mesh and platelets seen in 40% - 60% of vessels	WBCs present in circulation and tissue
5	Fibrin mesh and platelet aggregates seen in >60% of vessels	Severe WBC infiltration into tissue

6. General Discussion and Future Directions

6.1. General Discussion

Sepsis is defined as the life-threatening response to infection from the overactivation of inflammation and coagulation (Singer et al., 2016). Accumulating evidence indicates that innate immune cells such as neutrophils mediate the interaction between infection, inflammation, and coagulation (Jing et al., 2021). Specifically, NETs, a product of neutrophil activation and death, have emerged as an important link for the progression of sepsis. The major structural component of NETs are nucleosomes. Previous studies have reported that the individual components of nucleosomes (i.e. DNA and histones) are harmful, but when bound together may have altered effects *in vitro*. For example, *in vitro* studies reported that DNA and histones individually can activate coagulation, which could be neutralized when bound to each other as a nucleosome (Noubouossie et al., 2017). However, this procoagulant activity was questioned due to isolation techniques that led to contaminated DNA.

While the procoagulant, antifibrinolytic, and cytotoxic effects of purified DNA, histones, and nucleosomes have been reported *in vitro*, their contribution *in vivo* had also yet to be explored. Understanding the role of DNA, histones, and nucleosomes in the pathogenesis of sepsis is essential for the development of novel therapies that may prove targeting multiple components of NETs (i.e. DNA and histones) may be beneficial.

Consequently, in this thesis we reported that: (1) when isolating contamination-free DNA using the DBL or PAXgene kit, DNA retains its procoagulant activity (Chapter 3.0); (2) elevated levels of histones, but not DNA fragments or mononucleosomes, contribute to a proinflammatory and procoagulant state in CLP mice, thereby contributing to the pathogenesis of sepsis. As a result, we concluded that DNA may mask the harmful effects of histones *in vivo* suggesting a combination approach to targeting DNA and histones may be a beneficial treatment (Chapter 4.0). However, (3) using a combination approach with DNase I and LMWH to target DNA and histones, respectively, was not beneficial in a CLP murine model of sepsis (Chapter 5.0).

6.2. The contribution of DNA to the pathogenesis of sepsis

Elevated levels of CFDNA have been reported to be associated with mortality in sepsis highlighting its importance (Dwivedi et al., 2012). This association was attributed to the harmful properties of DNA observed *in vitro*. *In vitro*, DNA was reported to be proinflammatory, procoagulant, and antifibrinolytic, which was hypothesized to contribute to the pathogenesis of sepsis. However, DNA's properties were questioned due to contamination. In literature, the role of DNA in coagulation was mainly studied using DNA isolated from common silica-based kits. These kits produced DNA contaminated with silica and polyP that confounded our interpretation of the procoagulant effects of DNA since DNA,

silica, and polyP influence coagulation in a similar manner (Smith et al., 2017 & 2018). DNA, silica, and polyP all carry negative charges that can influence the autocatalytic activation of FXII, thereby inducing coagulation through the intrinsic pathway (Salloum-Asfar et al., 2018). As a result, Chapter 3.0 focused on (a) identifying alternative methods of isolating DNA that are free of silica and polyP contamination, and (b) characterizing the potential procoagulant properties of the purified DNA. Firstly, we explored commercially available calf-thymus DNA (CT-DNA) but determined CT-DNA contained thrombin-like amidolytic activity rendering it inappropriate for use in functional studies (Figure 3.1). It is important to note that there are several other sources of commercially available DNA on the market. We show that simply purchasing DNA requires investigation of potential contaminants that may interfere with the performance of studies. We then explored silica column free isolation methods such as the PaxGene isolation kit (PAX-DNA) and DBL kit (DBL-DNA), which did not show any detectable polyP contamination (Figure 3.2). Consequently, these methods of isolation were recommended for any functional study involving purified DNA. We then confirmed that purified PAX- and DBL-DNA were procoagulant in a dose-dependent manner acting via the intrinsic pathway, as expected. However, it is important to note that this procoagulant activity was significantly lower than the commonly used silica-DNA. Therefore, we concluded that DNA is a weak activator of the intrinsic pathway.

While the properties of DNA have been extensively studied *in vitro*, its role *in vivo* was less known. In Chapter 4.0, we saw administration of high doses of DNA fragments (150 bp) to septic mice result in significantly elevated levels of CFDNA compared to DNA administered shams (Figure 4.3). To account for differences in CFDNA between sham and CLP mice, we hypothesize that the septic mice were unable to clear the DNA challenge in the same manner as sham controls. One potential explanation lies in differences of DNase activity in sepsis. Jimenez-Alazar et. al. (2017) reported that healthy mice have DNases which are important for degradation of CFDNA *in vivo* (Jiménez-Alcázar et al., 2017). Therefore, our sham mice could rapidly clear the DNA challenge due to a functioning DNase system. However, it has been reported that sepsis can reduce DNase I levels (Sohrabipour et al., 2021), which likely contributed to the elevated levels of CFDNA observed in our CLP mice. Consequently, repeating this study in DNase I-deficient mice may exacerbate the septic response and allow us to further understand the pathogenesis of DNA in sepsis. Another potential theory lies in the ability of DNA to be cleared by the liver. Purified injections of a plasmid can be rapidly cleared by scavenging receptors on liver cells with a half-life of 10 min (Kawabata et al., 1995). Therefore, liver damage from sepsis may also alter its ability to reduce CFDNA levels. Altogether, this highlights the importance of determining differences in the half-life of DNA between healthy and septic mice,

which can be explored by administering a bolus dose of DNA and sampling CFDNA levels over a period of time.

Despite elevations of CFDNA, there were no detectable effects on inflammation or coagulation (Figure 4.3). In support of this, administration of specifically engineered DNA plasmids are commonly used to induce targeted gene expression in mice (Armengol et al., 2004; Horton et al., 1999). However, the control plasmids (plasmid back-bone without the gene of interest) have never been reported to be harmful. To validate our observation that administration of DNA is not harmful *in vivo*, it is important to understand the rationale for the dose we used. Pilot studies demonstrated that the dose used was enough to initially induce levels of 160 µg/mL of circulating CFDNA in mice. However, the DNA was rapidly degraded in healthy mice within 60 min, with a half-life of approximately 10 to 11 minutes (Figure 6.1), which is consistent with previous literature (Kawabata et al., 1995). In relation to human sepsis, a retrospective observational study including 356 patients observed the range of CFDNA in sepsis was between 1.10 to 123.90 µg/mL, with plasma CFDNA levels in sepsis averaging 5.38 ± 6.53 µg/mL compared to healthy individuals at 2.19 ± 0.63 µg/mL (Liaw et al., 2019). Altogether, this suggests the dose of DNA we used was above what was clinically relevant. Any lack of coagulation and inflammation would not have been a result of limited DNA levels. Therefore, we concluded that elevated levels of 150 bp DNA does not induce inflammation or coagulation *in vivo*.

Figure 6.1. Injection of pathological levels of PAX-DNA (8 mg/kg) into healthy C56Bl/6J mice. (B) Cell-free DNA (CFDNA) was quantified from plasma at various time points (0, 1, 15, 30, 60 min) using the QIAGEN Blood mini kit. Results are presented as mean ± SEM (n = 3). A one-way ANOVA was used to analyze the results followed by a Bonferonni test. A p value of <0.05 was determined to be significant (*) compared to baseline values.

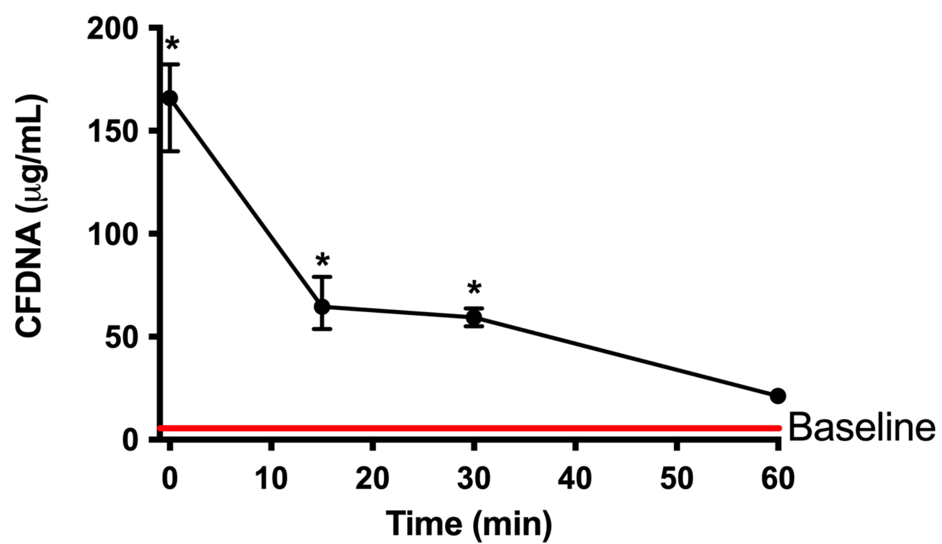

Figure 6.1. Injection of pathological levels of PAX-DNA (8 mg/kg) into healthy C56Bl/6J mice.

A potential limitation to these conclusions, is the use of 150 bp DNA fragments. It is important to note that DNA fragments in sepsis range from 150 bp to greater than 10,000 bp and are dependent on the biological mechanism by which it was released. For instance, necrotic cells and neutrophils undergoing NETosis release high-molecular-weight pieces of DNA more than 10,000 bp long, while apoptotic cells release a "ladder" pattern of DNA at 150 bp intervals (Fuchs et al., 2007; Jahr et al., 2001; Nagata et al., 2003). When CFDNA isolated from septic plasma was run on an agarose gel to visualize DNA size, approximately 11% of the septic patients had DNA predominantly 150 to 300 bp likely from tissue injury (Dwivedi et al., 2012). While 150 bp is clinically relevant, larger fragments would better reflect the majority of the sepsis population (89%) and effects of NET-derived DNA. The importance of this is highlighted by Gould et. al. (2015) who demonstrated that the size of DNA altered its antifibrinolytic properties, with larger fragments increasing maximum absorbance and prolonging clot lysis compared to smaller fragments (Gould et al., 2015). Large networks of DNA have also been reported to be structurally important and may promote thrombosis by providing a scaffold for platelets and RBCs (Brinkmann et al., 2004; Clark et al., 2007). Consequently, it would be beneficial to investigate how DNA size affects coagulation and the pathogenesis of sepsis *in vivo*.

In support of DNA being harmful and a useful target, we show in Chapter 5.0. that DNase I administration to septic mice improves survival and sepsis

outcomes. These findings are consistent with previous literature (Lauková et al., 2017; Mai et al., 2015). Therefore, while purified injections of 150 bp DNA fragments were not harmful, larger networks of DNA are hypothesized to be essential for the pathogenesis of sepsis.

Although we saw no effects on global inflammation and coagulation, looking at endothelial markers of damage may provide additional insight on how DNA may be potentially harmful. Previous studies showed that purchased commercially available DNA can induce endothelial dysfunction through upregulation of TF and PAI-1, resulting in accelerated clotting in mice. DNA also led to increased surface expression of vWF in endothelial cells resulting in increased platelet-endothelium interactions under flow conditions (Gaitzsch et al., 2017). However, results from Chapter 3.0 concluded that purchased DNA may come with contamination. Consequently, repeating these studies using our purified methods may also be beneficial to understand how DNA may contribute to the pathogenesis of sepsis through endothelial dysfunction and coagulation.

6.3. The contribution of histones to the pathogenesis of sepsis

Elevated levels of histones in sepsis have been also reported to be associated with mortality (Lu et al., 2020; Yokoyama et al., 2019). In Chapter 4.0, we explored how two doses of 8.5 mg/kg of histones can contribute to the pathogenesis of sepsis within an 8-hour time frame. We show that histones at this dose induce

proinflammatory and procoagulant effects in a murine model of sepsis, but this was not observed in healthy mice administered the same dose of histones (Figure 4.3). While the dose of histones administered was representative of the average levels in patients with sepsis, patients with DIC have been reported to have up to 50 mg/kg of circulating histones (X. Zhang & Li, 2022). We observed that administration of two 25 mg/kg of histones accelerated sepsis further as seen visually by elevated MGS and MSS scores revealing a dose-dependent effect. Meanwhile, visual symptoms of histone-induced sickness were now observed in sham controls at this higher dose (data not shown), suggesting that the ability of healthy mice to neutralize the harmful effects of histones is overcome at higher concentrations. Remarkably, preclinical studies have used a similar sub-lethal 50 mg/kg histone dose to induce a sepsis-like state to explore DIC, the cytotoxic effects of histones on leukocyte/renal cell death in sepsis, and the beneficial effects of histone-binding heparins (Iba et. al, 2015, Z. Wang et. al., 2020).

The mechanism of histone-induced coagulation and inflammation have been intensely studied. These observations may decipher how histones impact coagulation and inflammation in our mouse model of sepsis. Exogenous histones are considered DAMPs that can activate TLR-2 and -4 found on platelets, neutrophils, and endothelial cells. As a result, platelets and neutrophils are activated, and the endothelium can become stressed, thereby contributing to a proinflammatory and procoagulant environment (Figure 6.2). Purified free histones

can also induce cytotoxicity by Ca^{2+} influx, resulting in cell lysis (Abrams et al., 2013), which can contribute to this environment. However, the exact mechanism by which this occurs is controversial. Some *in vitro* studies suggest this is through TLR-2 or -4 mediated pathways, while others report TLR-2 or -4 neutralizing antibodies do not effect cytotoxicity (Chaaban et al., 2015; Ekaney et al., 2014). This was hypothesized to be due to differences in cell lines used. Consequently, studies turned their focus on the glycocalyx, which differs between cell lines. Chaaban et. al. (2015) reported that cells deficient in heparan-sulfate (a major component of the glycocalyx) were more sensitive to histone-induced cytotoxicity. This highlights the relationship between histones, the glycocalyx, and endothelial cell dysfunction. The glycocalyx plays an important role in sepsis. Normally, the glycocalyx regulates vascular permeability, leukocyte adhesion, and inhibits thrombosis. In sepsis, MMPs targeting proteoglycans and GAG sheddases are activated from proinflammatory mediators, (such as ROS, TNFα, and IL-1β) leading to a degradation of the glycocalyx (Becker et al., 2015; Chappell et al., 2009; Lipowsky & Lescanic, 2013; Manon-Jensen et al., 2013; Schmitt et al., 2001). In support of this, the addition of TNFα or LPS to human umbilical endothelial cells displayed a 50% decrease in glycocalyx thickness (Wiesinger et al., 2013). Furthermore, administration of a low-dose intravenous endotoxin to human volunteers resulted in a significant reduction in sublingual glycocalyx thickness. As a result, this was accompanied by an increase in plasma hyaluronan,

Figure 6.2. Proposed harmful effects of histones in sepsis. Based on previous *in vitro* literature, we propose histones are contributing to the development of sepsis in our murine model by binding and activating neutrophils, endothelial cells, and platelets likely through a TLR-dependent manner. (1) Neutrophils then produce proinflammatory mediators and neutrophils extracellular traps (NETs) to enhance the inflammatory environment and promote thrombosis. (2) Endothelial cells become dysfunctional and can promote coagulation and inflammation through reducing expression of tissue factor pathway inhibitor (TFPI) and tissue plasminogen activator (tPA), and degradation of the glycocalyx. Upregulation of tissue factor (TF), plasminogen activator inhibitor-1 (PAI-1), and adhesion molecules can activate coagulation, inhibit fibrinolysis, and recruit leukocytes/platelets. (3) Platelet activation leads to degranulation and aggregation leading to clot formation and a proinflammatory environment. Altogether these cells have multiple positive feedback mechanisms, which enhance a proinflammatory, procoagulant, and antifibrinolytic environment contributing to the progression of sepsis. Molecules such as DNA, heparan sulfates, and therapeutic heparins can neutralize histones and reduce their harmful effects. Figure created with BioRender.

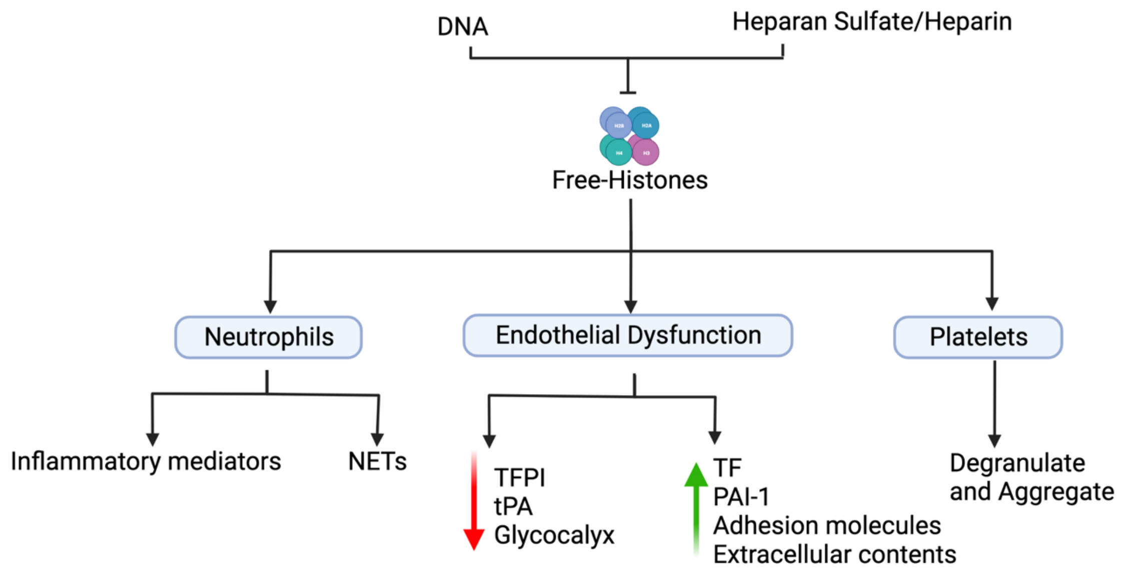

Figure 6.2. Proposed harmful effects of histones in sepsis.

also known as hyaluronic acid, suggesting glycocalyx shedding. In the same study, inhibition with an anti-TNFα antibody abolished this difference (Nieuwdorp et al., 2009). Since sepsis has been shown to increase glycocalyx shedding in mice (X. Huang et al., 2020), we propose that the CLP surgery induced a proinflammatory response as seen by an increase of IL-6, which led to the degradation of the glycocalyx. As a result, CLP mice would be more susceptible to histone-mediated activation of the endothelium compared to sham controls. Quantification of markers of endothelial dysfunction and glycocalyx degradation products would be beneficial to elucidate the mechanisms by which histones are harmful in septic mice and not sham mice.

Previous studies have shown that a single high bolus dose of purified histones at 75 mg/kg into mice resulted in death and organ failure similar to what is observed in sepsis. In the same study, anti-histone antibodies improved sepsis outcomes in murine models of sepsis (Xu et al., 2009). However, the mechanism by which histones contributed to the sepsis-like state was not investigated. In our study, we observed elevated IL-6 and TAT levels in histone-treated septic mice (Figure 4.3). Therefore, we concluded that clinically relevant low doses of purified histones contribute to the pathogenesis of sepsis likely through proinflammatory and procoagulant pathways. In previous studies, administration of sub-lethal doses of histones to healthy TLR-2 deficient mice did not produce any different immunostimulatory effects in a kidney injury model compared to wild-type controls

(Allam et al., 2012). Therefore, TLR-4 was hypothesized to play a more of an important role. Despite this, it is unknown if the same mechanism occurs in sepsis. Therefore, future studies can explore the exact mechanisms by which exogenous histones effect sepsis outcomes using TLR-2 or -4 deficient mice. Based on these studies, we hypothesize the effects of histones would be minimized in TLR-4, not TLR-2 genetically deficient septic mice.

It is important to note that most histone studies focus on their immunostimulatory and cytotoxic effects, but not their procoagulant effects. To our knowledge, our study is the first study to demonstrate how clinically relevant purified doses of histones contribute to the pathogenesis of sepsis through elevation of coagulation biomarkers. It is important to understand how histones contribute to immunothrombosis observed in sepsis, which may enhance the pathogenesis of sepsis. Quantifying other markers of inflammation (CRP, TNFα, NF-κB, IL-1β, IL-10), coagulation (clotting assays, fibrinogen), fibrinolysis (PAI-1, D-dimer), or NETosis (H3-cit, MPO) would also be beneficial to fully understand the role of histones in sepsis. These results would potentially aid in the development of novel therapeutic strategies.

6.4. The contribution of nucleosomes to the pathogenesis of sepsis

In addition to histone mediated TLR-2 and -4 signalling, histones act as a cofactor to DNA and signal through TLR-9 in immune cells when in complex with

each other in the form of a nucleosome (H. Huang et al., 2011). This highlights the notion that histones and DNA may have different functions when they are free or bound to other molecules. To our knowledge, our study is the first to compare the effects of free-histones, free-DNA, and DNA-bound histones (nucleosomes) in the pathogenesis of sepsis. As previously mentioned in section 6.2, purified DNA had no detectable harmful effects *in vivo*. However, histones may be enhancing the pathogenesis of sepsis through inflammation and coagulation. When histones were bound to DNA in a nucleosome complex, nucleosomes were not harmful as they did not elevate these markers. Consequently, we concluded that DNA could mask the harmful effects of histones *in vivo*.

While we show that free-histones and DNA-bound histones possess different properties, when measuring histones using ELISAs, it is unknown if free-histones and/or protein-bound histones are being measured. To combat this, a small proportion of ELISAs have been developed to capture DNA-histone complexes (M. Li et al., 2020). Despite this, no standardized histone measurement has been developed (i.e. free versus bound, one histone subunit, or overall concentration). Exploring methods of detecting free- versus bound-histones is essential to understanding the mechanisms by which histones contribute to the pathogenesis of sepsis.

Typically, histones are released in association with DNA as a nucleosome but can be displaced and bound to other proteins, altering their effects on

inflammation and coagulation. For example, histones can bind heparan sulfate on the endothelial vasculature or therapeutic heparin, which hypothetically displaces DNA. In Chapter 4.0, we reported that release of harmful histones can occur with therapies targeting nucleosomes/NETs such as DNase I. When nucleosomes were treated with DNase I (100 μg/mL), histones were released contributing to cytotoxicity (Figure 4.1). Since we know LMWH can neutralize this effect, we hypothesized a combination approach would be beneficial. A proposed *in vitro* mechanism is depicted in Figure 6.3. It is also important to note, DNase I is unable to degrade nucleosomes without heparin at lower physiologically relevant concentrations (<14.5 μg/mL) (Sohrabipour et al., 2021). Therefore, we hypothesized DNase I and LMWH can also work synergistically to neutralize nucleosomes in sepsis and potentially provide a theoretical advantage over monotherapy. Consequently, in Chapter 5.0 we explored the potential beneficial effects of targeting nucleosomes using a combination of DNase I and heparin. The importance of this study is highlighted by the fact that clinical studies have started recruiting patients to investigate aerosolized DNase I (NCT04541979 and NCT04445285) in COVID-19 patients with respiratory failure and intravenous DNase I (NCT05453695) as an intervention in septic ICU patients. Since standard thromboprophylaxis in sepsis is the administration of an anticoagulant (such as LMWH), it is also important to explore if there are any potential drug-drug interactions.

Figure 6.3. Proposed mechanism of action for cells treated with nucleosomes, DNase I, and/or LMWH. (A) Nucleosomes are not harmful to endothelial cells. (B) Addition of DNase I to nucleosomes releases histones, thereby inducing cell death. We hypothesize this is due to toll-like receptor (TLR) -2 or TLR-4 mediated signaling. (C) Treatment of DNase I and LMWH neutralizes this effect. Figure created with BioRender.

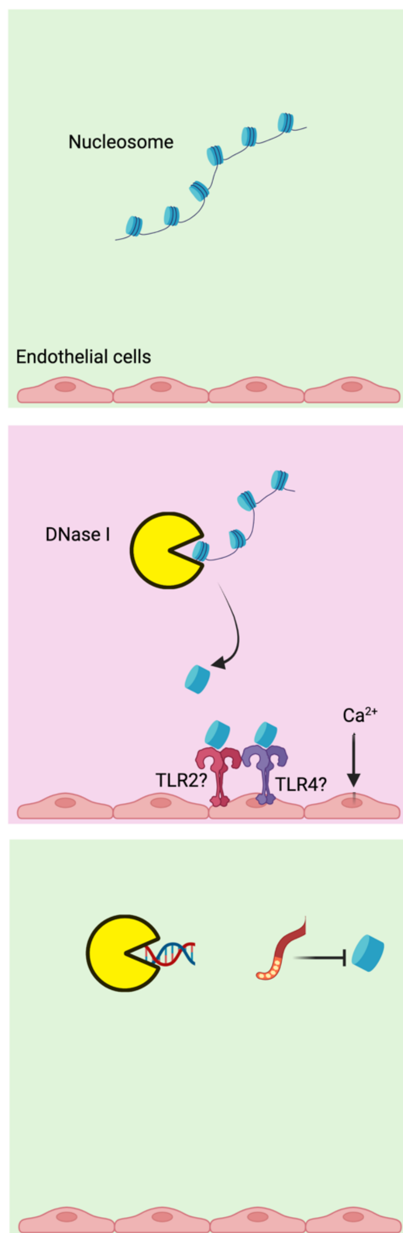

Figure 6.3. Proposed mechanism of action for cells treated with nucleosomes, DNase I, and/or LMWH.

We observed that monotreatment with either DNase I or LMWH significantly improved sepsis survival compared to saline septic controls (Figure 5.1). These results align with multiple *in vivo* and clinical studies that suggest that these treatments are beneficial (Lauková et al., 2017; X. Li & Ma, 2017; Y. Li et al., 2011; Mai et al., 2015). We hypothesize that the positive effect is in part due to the ability of the molecules to target NETs as both DNase I and heparins have been reported to dismantle NETs *ex vivo* (Fuchs et al., 2010). However, we unexpectedly observed no significant improvement in mice treated with the combination therapy compared to saline-treated controls. Therefore, we reported that these agents do not function synergistically *in vivo* as a treatment for sepsis, despite evidence that suggests synergy *in vitro* (Brotherton et al., 1989; Sohrabipour et al., 2021).

While common markers of coagulation and inflammation did not offer much explanation, bacterial burden in the PCF and blood potentially sheds light on what was occurring *in vivo*. LMWH and DNase I monotherapies reduced blood bacterial burden, likely contributing to improved survival. Therapies that included DNase I (both monotherapy and combination) reduced bacterial burden in PCF, but the combination did not reduce blood bacterial loads. Together, this suggests a potential drug-drug interaction occurring that can increase blood bacterial burden which likely reduced survival. One potential explanation lies in the fact that NETs are a double-edged sword (Gould et. al., 2015). While NETs may be harmful at later time-points, they are essential for the sequestering of bacteria in the early

stages of sepsis. For example, mice unable to produce NETS, PAD4-deficient mice, are more susceptible to bacterial infections compared to wild-type controls (Li et al., 2010). Moreover, in a CLP model of sepsis, our lab showed early administration of DNase I treatment resulted in poorer sepsis outcomes and survival compared to delayed treatment (Mai et al., 2015). Since DNase I and LMWH have been shown to be synergistic at degrading nucleosomes (ie. LMWH increases the catalytic efficiency of DNase) (Brotherton et al., 1989; Napirei et al., 2009; Sohrabipour et al., 2021), bacterial sequestering may be reduced, thereby neutralizing any potential beneficial effects the monotherapies had.

To fully understand this perplexing mechanism, future studies could vary treatment concentrations or timing. For example, DNase I administration at 20 mg/kg every 8h will theoretically result in plasma levels greater than 15,000-fold what is physiologically relevant. Potentially the use of physiologically relevant doses of DNase I may be more beneficial (i.e. 3 to 40 ng/mL) (W. S. Prince et al., 1998; Sohrabipour et al., 2021). Furthermore, altering the route of administration of therapies may also be beneficial. Altered routes of administration may alter bioavailability and confound potential drug-drug interactions. In our study, we administer DNase I via the intraperitoneal cavity, which is a convenient method for mice, but is not translatable to humans. Current human clinical trials administer DNase I through an IV (NCT05453695) or as an inhalant (NCT04541979 and

NCT04445285). It is possible that peritoneal cells or other mediators may modulate intraperitoneal DNase activity in the presence of heparin.

While this thesis focused on using dalteparin, UFH and enoxaparin are also commonly used as thromboprophylaxis in septic patients. Previous *in vitro* data reported that UFH and other various LMWHs can similarly inhibit histone cytotoxicity (Sharma et al., 2022) However, it was unknown if our *in vivo* findings were unique to dalteparin or if they can be generalized to these other heparins. To answer this question, we explored a prophylactic treatment of UFH (18 IU/kg/h) in our CLP mouse model of sepsis. We observed a similar trend towards improved survival in our model and that the combination of UFH and DNase I was not synergistic and no different to their saline controls (Figure 6.4). Therefore, we hypothesize that there would be a similar survival improvement when using different heparins with histone-neutralizing abilities.

Unexpectedly, results from Chapter 4.0 and 5.0 contradict each other. Chapter 4.0 suggests targeting nucleosomes with a monotherapy of DNase I can release cytotoxic histones which can be neutralized by LMWH, suggesting synergy. However, Chapter 5.0 shows a combination approach in a mouse model of sepsis neutralizes any positive effect the monotherapies had. There are two potential differences that can account for this contradiction: (1) differences in nucleosomes and NETs, and (2) differences between mononucleosomes and nucleosomes. (1) This thesis focuses on the effects of purified DNA, histones, and nucleosomes,

Figure 6.4. Kaplan-Meier survival curves of DNase I and/or UFH in a CLP model of sepsis. Mice received either saline, DNase I (30 mg/kg/day), unfractionated heparin (UFH; 18 IU/kg/hr), or DNase I + UFH. A Mantel-Cox Log-rank test was used to analyze results (n = 15-25).

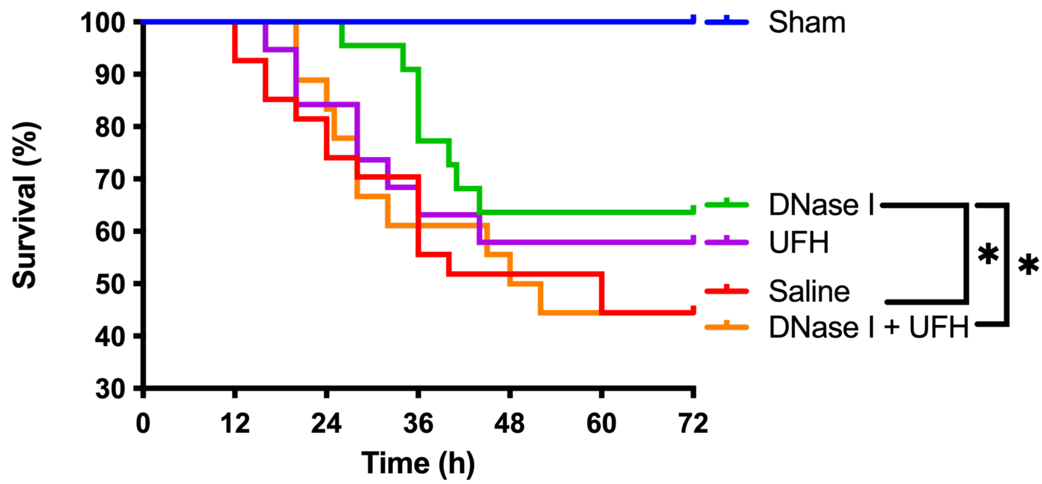

Figure 6.4. Kaplan-Meier survival curves of DNase I and/or UFH in a CLP model of sepsis.

which are the major structural components in NETs. While DNase I may release histones from nucleosomes, the combination of DNase I and heparin to NETs may also release other NET-associated proteins into circulation that may be more harmful than histones. For instance, nucleosomes do not contain NET-associated proteins such as cathepsin G, NE, MPO, catalase, and bactericidal-permeability-increasing protein, which work together to have antimicrobial activity. These proteins can be procoagulant, proinflammatory, and antifibrinolytic. Cathepsin G and NE are the two major serine proteases that can be harmful (Iba et al., 2014). Cathepsin G is released to activate receptors, enzymes, cytokines, and peptides to stimulate recruitment of inflammatory cells (chemotaxis) and apoptosis (Zamolodchikova et al., 2020). NE can cleave TFPI and activate platelets to increase procoagulant activity (Wohner et al., 2010). As a result, NE-deficient mice have significant issues with fibrin formation (Massberg et al., 2010). Consequently, our treatments may be doing more than just targeting DNA and histones. For example, we know LMWH has other properties such as its anticoagulant and anti-inflammatory effects that may also influence survival. While DNase I and LMWH may be targeting NETs, we acknowledge that there may be more specific ways to target NETosis and NETs such as PAD4 inhibition. (2) Furthermore, the use of mononucleosomes in Chapter 4.0 may be appropriate for the studies of purified DNA and histones but is structurally less clinically relevant. Mononucleosomes can be found in circulation as a by-product of apoptosis. As stated in section 6.2., 89%

of patients have DNA that is larger than 10,000 bp which is more indicative of NETs. We also know that DNA is structurally important and larger networks of extracellular DNA may promote thrombosis by providing a scaffold for platelets and RBCs (Brinkmann et al., 2004; Clark et al., 2007). Therefore, these differences may account for the unexpected contradictory results in Chapter 4.0 and 5.0. We propose that repeating studies in Chapter 4.0 using isolated nucleosomes and/or NETs may provide a better platform to understand the contribution of the nucleosomes and NETs to the pathogenesis of sepsis.

6.5. Issues with translation of murine sepsis to human sepsis

For the last 30 years, we have used wild-type and genetically-engineered mice treated with numerous drugs to further our understanding of infection-induced molecular pathways and organ dysfunction (Cavaillon et al., 2020). Despite the many animal studies and clinical trials, there has not been a single new, efficient treatment that has significantly improved patient outcomes. Consequently, one might ask themselves, what could explain 30 years of failed translational research? One potential explanation may be related to differences in murine and human sepsis due to physiology or immune dissimilarities (Table 6-1).

For example, septic mice develop bradypnea not tachypnea, bradycardia not tachycardia, hypothermia not fever, and hypoglycemia not hyperglycemia to name a few (Hoover et al., 2015; Iskander et al., 2013; Zolfaghari et al., 2013). The

Table 6-1: Human versus murine sepsis

	Human Sepsis	Murine Sepsis
Symptoms		
Respiratory rhythm	Tachypnea	Bradypnea
Cardiac rhythm	Tachycardia	Bradycardia
Temperature	Fever	Hypothermia
Glucose Levels	Elevate	Decrease
Main acute phase protein	CRP	SAP
Metabolic rate	Increases	Decreases
Physiology		
Circadian Rhythm	Diurnal	Nocturnal
Platelet levels	150-400 x 10^9/L	1000-1500 x 10^9/L
Main WBC	Neutrophils	Lymphocytes
Immune system		
Neutrophil enzyme content	High	low
Complement activity	High	Low
NETosis	Low	High
Missing IL's		IL-8, IL-32, IL-37
Endotoxin response	High sensitivity	High resistance

CRP: C-reactive protein; SAP: serum amyloid protein; WBC: white blood cell; IL: interleukin.

immune systems between mice and humans are also different (Mestas & Hughes, 2004) as mice have greater resistance to endotoxins such as LPS, infections, and sterile insults (Gentile et al., 2013; Warren et al., 2010). These differences can be attributed to variances in LPS sensing genes (McCarron et al., 2015), genetics, metabolism (Zolfaghari et al., 2013), circadian rhythms (Zhao et al., 2017), microflora (Klingensmith & Coopersmith, 2016) or complement activity (Banda & Takahashi, 2014). In regards to thrombosis, there are differences in platelet numbers, sizes, and mRNA content (Rowley et al., 2012; Schmitt et al., 2001). The major WBC in humans is neutrophils, but in mice is lymphocytes. Most importantly, neutrophil physiology differs between mice and humans. Mouse neutrophils possess low enzymatic content, but NETosis is enhanced compared to human neutrophils in sepsis (Hashiba et al., 2015; Meng et al., 2012). Mice and humans also produce different acute phase proteins which are used in the definition of sepsis; humans mainly produce CRP while mice produce serum amyloid protein (SAP) (Cray et al., 2010). While mice still produce a small amount of CRP in response to sepsis, this is minor compared to the levels found in humans. Since CRP is important for degrading histones and administration improves survival in mouse models of sepsis (Abrams et al., 2013), this highlights the translational burden of our study. Altogether, these differences in the host's response to infection and the development of inflammation and thrombosis may create limited translation. As a result, using mice as a model of sepsis may not be effective for testing treatments.

Further investigation of a standardized set of biomarkers for sepsis in human and animal sepsis is required to understand the limited translation between them.

When utilizing murine models of sepsis, there is no universal standard on sepsis procedures. There are numerous variables that create significant variations in murine research that limit its translatability to human sepsis. For example, time of day sepsis is inoculated, gender, mouse origin (Charles River or Jackson Laboratories), anesthesia, treatment time, antibiotics, and fluid resuscitation (Hubbard et al., 2005; Marshall et al., 2005; Poli-de-Figueiredo et al., 2008; van der Poll, 2012). The MQTiPSS have attempted to tackle this, but there is still no agreed upon magnitude for sepsis insult or sepsis definition for mice (i.e is it defined by systemic inflammation, organ injury, or mortality?) (Hellman et al., 2019; Libert et al., 2019; Zingarelli et al., 2019). Furthermore, most studies use healthy mice at 8-12 weeks old (adolescent aged), but human sepsis is observed mostly in the neonate or elderly population or patients with comorbidities such as obesity or diabetes (Angus et al., 2001; Chen et al., 2014; Rittirsch et al., 2007). Future studies should incorporate aged mice and include comorbidities to allow better translation to human sepsis.

In this study, we used the gold-standard CLP murine model of sepsis following the MQTiPSS guidelines to test our treatments. The CLP procedure mimics human polymicrobial intrabdominal sepsis and produces a response most similar to that seen in humans. The CLP model induces sepsis over a delayed

timeline and mice continue to exhibit signs of sepsis as determined by a persistently elevated inflammatory profile, consistent with human sepsis (Seemann et al., 2017). In contrast, LPS and FIP models induce sepsis quickly, leading to an acute inflammatory condition that can be rapidly resolved (Seemann et al., 2017).

The CLP procedure is also an inexpensive procedure and easy to modify severity/mortality (i.e. can alter ligation length, puncture width, and fecal matter extruded). Although this model is commonly used due to its ease and feasibility, there are several disadvantages. It can be difficult to standardize fecal matter extruded and there is no accountability of weight leading to large variations between animals (Seemann et al., 2017). Furthermore, the most common cause of community-acquired sepsis is respiratory pneumonia (Mayr et al., 2014), and thus, the CLP polymicrobial abdominal model may not be representative of all sepsis. Future studies should focus on repeating studies in other animal or mouse models such as acute lung injury (a response to sepsis) or intra-nasal/intra-tracheal pneumonia model.

To combat translational issues in murine and human sepsis, a targeted personalized approach may be beneficial to direct treatments to patients who would benefit most. Our lab has created a multivariable prognostic approach to develop personalized mortality profiles. The tool highlights a biological indicator contributing most to mortality, which can be used for personalized therapies (Liaw et. al., 2019). This tool can also be used to better define sepsis populations as the

failure of numerous clinical trials may be from unclear definitions of patient populations. Clear grouping of populations (or personalizing medicine to different groups) may be required to ensure the success of future trials.

7.0. Concluding Remarks

Since the procoagulant properties of DNA have been questioned due to contaminated isolation methods, we identified two methods of DNA isolation that are free of silica while having undetectable functional levels of polyP, PAX-DNA and DBL-DNA. DNA isolated using these methods demonstrate procoagulant activity that can be neutralized with DNase I digestion. While we recommend the use of PAX-DNA or DBL-DNA for functional studies involving extracellular DNA, it is worth noting that DNA isolated with silica columns can still be useful for DNA quantification purposes.

While DNA is procoagulant *in vitro*, injection of high doses of 150 bp DNA in mice did not cause detectable harm. However, DNA is typically circulating in complex with histones as a nucleosome. We show that low doses of free histones can be harmful in septic mice, but not healthy mice suggesting a protective mechanism. We hypothesize this is in part due to glycocalyx changes in sepsis. When nucleosomes were administered to mice, no detectable harmful effects were observed suggesting DNA can mask the harmful effects of histone *in vivo. In vitro* studies suggest that this may be due to DNA's ability to neutralize histones to reduce histone-induced cytotoxicity. Altogether, this shows DNA can mask the harmful effects of histones both *in vitro* and *in vivo*. Therefore, degradation of the DNA portion of nucleosomes using DNase I, an upcoming promising sepsis treatment, can potentially release histones that can be harmful and reduce survival.

We show *in vitro* that this can be neutralized by the combination of DNase I and LMWH, suggesting that targeting both DNA and histones at the same time may be potentially beneficial in sepsis.

When we compared monotherapy of DNase I or LMWH in a murine model of sepsis we saw similar significant improvements in sepsis outcomes. However, a combination approach to target nucleosomes was not beneficial. Our results suggest that there may be drug-drug interactions between DNase I and LMWH in our model resulting in blood bacterial dissemination. However, our mouse model has several limitations preventing us from concluding that these treatments do not work together in synergy. Future studies should focus on why targeting nucleosomes with DNase I and LMWH can be detrimental. Further understanding of the role of DNA, histones, and nucleosomes in the pathogenesis of sepsis may allow translation into beneficial therapies.

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