THE ROLE OF FACTOR XI DURING MURINE POLYMICROBIAL SEPSIS

By

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PUBLICATIONS

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LIST OF ABBREVIATIONS

Factor XI – FXI

Cecal Ligation and Puncture – CLP

FXI-deficient (mice) – $FXI^{-/-}$

Factor XII – FXII

Factor IX – FIX

Factor VII – FVII

Factor X – FX

Factor V – FV

Factor VIII - FVIII

Prekallikrein – PK

α-kallikrein – α-kal

High molecular weight kininogen – HK

Prothrombin time – PT

Activated partial thromboplastin time – aPTT

Tissue factor – TF

Disseminated intravascular coagulation – DIC

Nitric oxide - NO

Hereditary angioedema – HAE

C1-inhibitor – C1INH

Polyphosphate – polyP

Ferric chloride – $FeCl_3$

Systemic inflammatory response syndrome – SIRS

Pathogen-associated molecular pattern – PAMP

Damage-associated molecular pattern – DAMP

Pattern recognition receptor – PRR

Lipopolysaccharide – LPS

Toll-like receptor – TLR

Tumor necrosis factor α – TNFα

Interleukin – IL

Macrophage inflammatory protein-2 – MIP-2

Keratinocyte chemoattractant – KC

Monocyte chemotactic protein-1 – MCP-1

Thrombin-antithrombin – TAT

Serum amyloid P – SAP

CHAPTER I

PURPOSE OF STUDIES DESCRIBED IN THIS DISSERTATION

The work described in this dissertation focuses on the plasma protein factor XI (FXI), which is the zymogen of a protease, called factor XIa (FXIa), that contributes to blood coagulation. For this project, we investigated the role of FXI in the mortality and pathophysiology of murine sepsis. We hypothesized that FXI contributes to mortality during sepsis, and that it influences both the coagulopathy, as well as the inflammatory response, that occurs during sepsis. To test our hypotheses, we induced polymicrobial sepsis in mice using the cecal ligation and puncture (CLP) model to fulfill three Specific Aims. For Aim 1, we evaluated the contribution of FXI to survival during sepsis by comparing FXI deficient ($FXI^{-/-}$) mice to their wild-type ($FXI^{+/+}$) and $FXI^{+/-}$ littermates. We determined survival for each genotype over a period of 7 days after CLP. To fulfill Aim 2, we explored the role of FXI on the activation of coagulation in mice after CLP. We collected blood and tissue from $FXI^{-/-}$ and $FXI^{+/+}$ littermates sacrificed at several time points after CLP to evaluate systemic and histologic markers of coagulopathy over time. For Aim 3, we assessed the role of FXI on the inflammatory response to CLP by looking at markers of inflammation in blood and tissue from $FXI^{-/-}$ and $FXI^{+/+}$ mice. Chapter V describes our findings for these experiments, as well as the results of additional *in vitro* and *in vivo* experiments that further investigated the influence of FXI on the pro-inflammatory contact activation system. Our data provide novel insight into the influence of FXI on coagulation and inflammation, and suggest there are therapeutic advantages to targeting FXI for the treatment of patients who suffer from pathologies arising from each system.

CHAPTER II

REVIEW OF COAGULATION

Introduction to the Thesis Project

Formation of the plasma enzyme thrombin is critical for normal formation of a blood clot (hemostasis). Thrombin generation is beneficial when generated locally to seal damaged blood vessels during hemostasis, but may contribute to pathology when generated inappropriately during thrombosis. Thrombin is the product of a series of reactions involving several plasma serine proteases and their cofactors. One of these proteases, factor FXIa, is generated from its precursor FXI by the protease factor XIIa (FXIIa), or by thrombin itself. FXIa contributes to thrombin generation by cleaving factor IX (FIX) to form the protease factor IXa (FIXa). Epidemiologic data indicate that FXI makes a disproportionately greater contribution to thrombosis than to hemostasis in humans. These data, bolstered by studies with animal models that utilize FXI deficient mice, have led to the development of FXI inhibitors, which may be safer alternatives for anticoagulant therapy. In addition to being a valuable tool for determining the role of FXI in hemostasis and thrombosis, studies with $FXI^{-/-}$ mice are also providing clues about the role of FXI during the host response to invading micro-organisms. These studies, combined with *in vitro* findings, highlight several unique features of FXI that place it at a junction between coagulation and inflammation. In this project, I evaluated the effect of FXI on the coagulopathy and inflammatory response during the crucial early stages of murine sepsis.

Introduction to Plasma Coagulation

The plasma coagulation system is a tightly regulated host response mechanism that minimizes blood loss after vascular injury through formation of a fibrin and platelet-rich clot (Dahlback 2000). Blood clots result from an interaction between subendothelial collagen, platelets, and numerous proteins within blood plasma. Clot formation begins when circulating platelets near a vascular lesion attach to exposed subendothelial collagen. This attachment is mediated by von Willebrand factor, a multimeric glycoprotein that tethers platelets to collagen by binding to glycoprotein (GP) Ib of the platelet GPIb/IX/V receptor complex (Ruggeri and Mendolicchio 2015). Platelets are then activated through several mechanisms, and bind to each other through a process called aggregation. Platelet adherence and subsequent aggregation on transected vessels occurs within 30 seconds after injury (Weiss and Lages 1988). In addition to forming a vital platelet plug to staunch bleeding, this initial stage of hemostasis sets the conditions for effective fibrin formation, which is essential for clot integrity and stability. Phospholipids, which are expressed by activated platelets and damaged endothelial cells, provide a scaffold for the cell surface reactions that result in fibrin formation (Davie, Fujikawa et al. 1991), while activated platelets secrete factors that support the process (Monroe and Hoffman 2014). Vascular damage also exposes molecules that trigger the fibrin-forming plasma coagulation system, a set of enzymatic reactions that result in generation of thrombin. Thrombin converts soluble fibrinogen into insoluble fibrin and contributes to platelet activation. One of the plasma enzymes that contribute to thrombin generation is the protease FXIa, the subject of my thesis work.

Models of Plasma Coagulation

The plasma reactions that result in thrombin generation and subsequent fibrin formation at a wound site involve a group of proteins that are known as coagulation factors. With the exception of fibrinogen (the precursor of fibrin), these proteins are either zymogens of serine proteases, or inactive cofactors that facilitate coagulation reactions on surfaces (Monroe and Hoffman 2014). Most coagulation factors are designated by a Roman numeral, and are numbered according to their order of discovery. The active form of each factor is indicated by a lowercase "a" to the right of the Roman numeral, or is referred to by name. The zymogens are prothrombin (factor II, precursor of thrombin), prekallikrein [PK, precursor of α-kallikrein (α-kal)], and factors VII, IX, X, XI, and XII. The cofactors are high molecular weight kininogen (HK), tissue factor (TF), and factors V and VIII. A process for fibrin generation was first introduced in two separate publications in 1964. Both papers proposed the same mechanism, but Macfarlane called it a "cascade" (Macfarlane 1964), while Davie and Ratnoff called it a "waterfall" (Davie and Ratnoff 1964). **Figure 1** shows a version of the original cascade model, which is the basis for the *in vitro* prothrombin

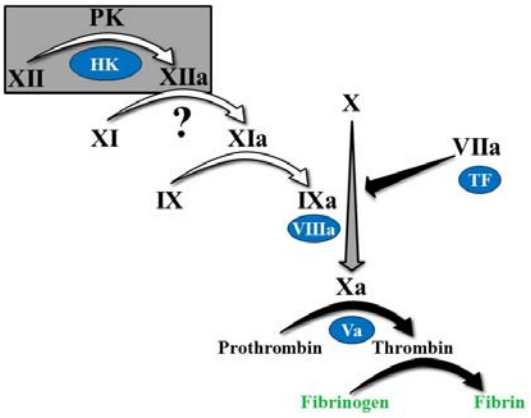

Figure 1. Cascade model of plasma coagulation. Fibrin generation is the result of a sequence of enzymatic reactions in plasma. The process is initiated through intrinsic (white arrows), or extrinsic (black arrow) pathways. Both pathways result in activation of FX to FXa, triggering a common pathway (grey arrows) for fibrin formation. In the cascade model, FXI is activated by FXIIa. However, FXII, PK, and HK (enclosed in gray box) deficient patients do not have a bleeding disorder. This raises questions about the role of these proteins in coagulation *in vivo*, and suggests there are other mechanisms (indicated by the "?" symbol) for FXI activation.

time (PT) and activated partial thromboplastin time (aPTT) clotting assays. The model illustrates a series of reactions wherein a zymogen is converted to an active protease, which then acts upon the next zymogen in the cascade until prothrombin is converted to thrombin by the protease FXa. According to the cascade model, there are two distinct biochemical pathways for thrombin generation. In the intrinsic pathway, the basis for the aPTT assay, FXII is activated on a surface in a process that also involves PK and HK. The resulting FXIIa then activates FXI to complete a set of reactions that are collectively referred to as contact activation (**Figure 2)**. Contact activation triggers the sequential activation of FIX, FX, and prothrombin. In the extrinsic pathway, the basis for the PT assay, tissue factor (TF) binds to factor VII (FVII), forming a TF/FVII(a) complex that subsequently activates factor X (FX). The aPTT and PT clotting assays are useful for detecting deficiencies of clotting factors in plasma because factor-deficient plasmas clot more slowly than normal plasma *in vitro*. In many cases, this *in vitro* phenomenon is translated to the clinical setting, wherein

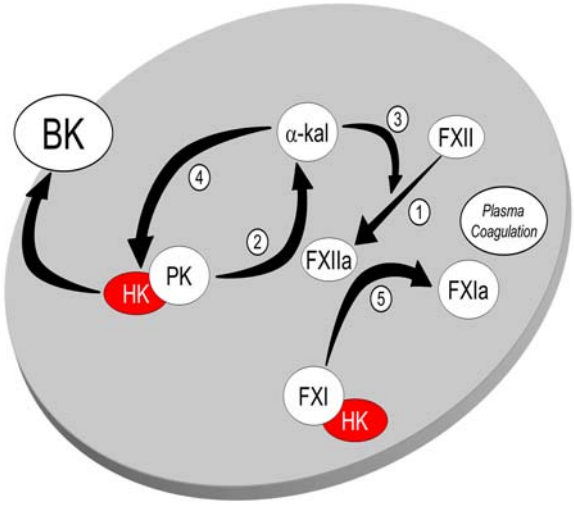

Figure 2. Contact Activation. On a surface (gray disk) FXII is auto-activated to form FXIIa (1). FXIIa activates PK to generate α-kal (2), which reciprocally activates additional FXII (3). α-kal also cleaves HK to liberate BK (4). FXIIa activates FXI (5) to propagate coagulation. The cofactor HK (shown in red) facilitates FXI and PK binding to the surface.

patients with a deficiency of a clotting factor will exhibit a clotting defect and therefore tend to bleed. However, this is not always the case. For example, patients with a hereditary deficiency of FXII, PK, or HK have a markedly prolonged aPTT, but no bleeding disorder, while patients with a deficiency of FXI, the substrate for FXIIa in the cascade model, have a prolonged aPTT and may bleed excessively, but only in certain instances. This places the role of contact activation in hemostasis in doubt, and suggests there are other mechanisms for FXI activation *in vivo*.

Advances in coagulation research have resulted in a revised model of thrombin generation **(Figure 3)** that excludes proteins that are not required for hemostasis (FXII, PK, and HK). This model proposes that tissue factor is the initiator of thrombin generation *in vivo*, based upon clinical observations and *in vitro* work showing that (a) in addition to activating FX, the TF/FVIIa complex also activates FIX (Osterud and Rapaport 1977), and

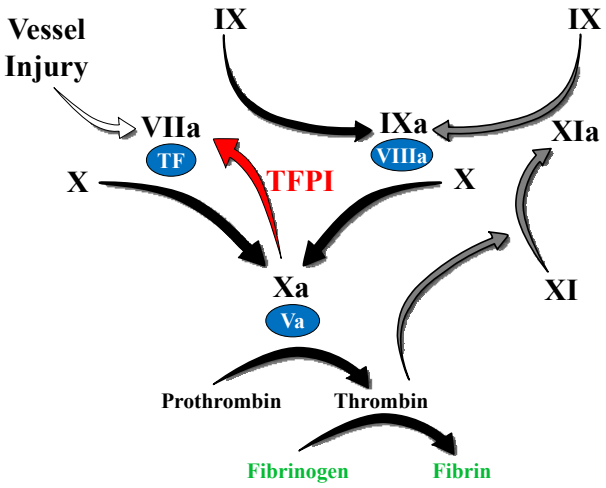

Figure 3. Revised model of plasma coagulation. Vascular injury exposes TF to FVII, and the resulting TF/FVIIa complex initiates thrombin generation by activating FX to FXa. The activation of FX by TF/FVIIa is limited by Tissue Factor Pathway Inhibitor (TFPI) (red arrow), so that sustained thrombin generation depends upon activation of FIX by TF/FVIIa. FXIa activates FIX (grey arrows) to provide additional thrombin to offset TF/FVIIa inhibition. In the revised model, FXI is activated by thrombin. For both figures, zymogens and proteases are shown in black. When Roman numerals are used, the active protease is designated by the letter "a." Cofactors are shown in blue.

(b) thrombin activates FXI (Gailani and Broze 1991, Naito and Fujikawa 1991). According to the revised model, thrombin generation is initiated after vascular injury exposes tissue

factor (TF), an integral membrane protein on the surface of a variety of non-vascular cells, to the circulation (Dahlback 2000, Morrissey 2004, Gailani and Renne 2007). TF quickly binds to zymogen FVII within the circulation forming a 1:1 complex. This interaction on the surface of cells allows a variety of plasma proteases to convert zymogen FVII to its active form, FVIIa (Davie, Fujikawa et al. 1991, Furie and Furie 1992, Monroe and Key 2007). The TF/FVIIa enzyme complex then binds and activates FX to FXa, which, in a reaction that is accelerated by the cofactor FVa, converts the plasma zymogen prothrombin to active thrombin. FX is also activated by FIXa, which is primarily produced by cleavage of FIX by the TF/FVIIa complex. Efficient activation of FX by FIXa requires the cofactor factor VIIIa (FVIIIa). The activation of FIX by TF/FVIIa is an important mechanism for supporting thrombin generation because the initiating pathway (activation of FXI by TF/FVIIa) is inhibited by the Kunitz-type protease inhibitor tissue factor pathway inhibitor (TFPI) (Broze and Girard 2012, Monroe and Hoffman 2014). In the revised model, the protease FXIa, generated by the cleavage of zymogen FXI by thrombin (and FXIa), completes a positive feedback loop for thrombin generation by activating FIX (Gailani and Broze 1991, Borissoff, Spronk et al. 2009). Thus, FXIa functions in clot maintenance, after thrombin generation is first initiated via the "tissue factor pathway". This current understanding of the role of FXI, which is illustrated by the revised model (**Figure 3**), helps explain why FXI deficiency contributes to abnormal bleeding in some patients (described in Chapter III), and why FXII and HK-deficient individuals appear clinically normal. It also illustrates why the revised model currently is the more clinically relevant representation of hemostasis.

Coagulant mechanisms have been described as operating along a clinical spectrum, with hemostasis at the center of a delicate balance **(Figure 4)**. Inadequate coagulation results in hemorrhage (bleeding), while excessive or inappropriate coagulation results in thrombosis (blood vessel occlusion). Several therapeutic strategies are employed to treat

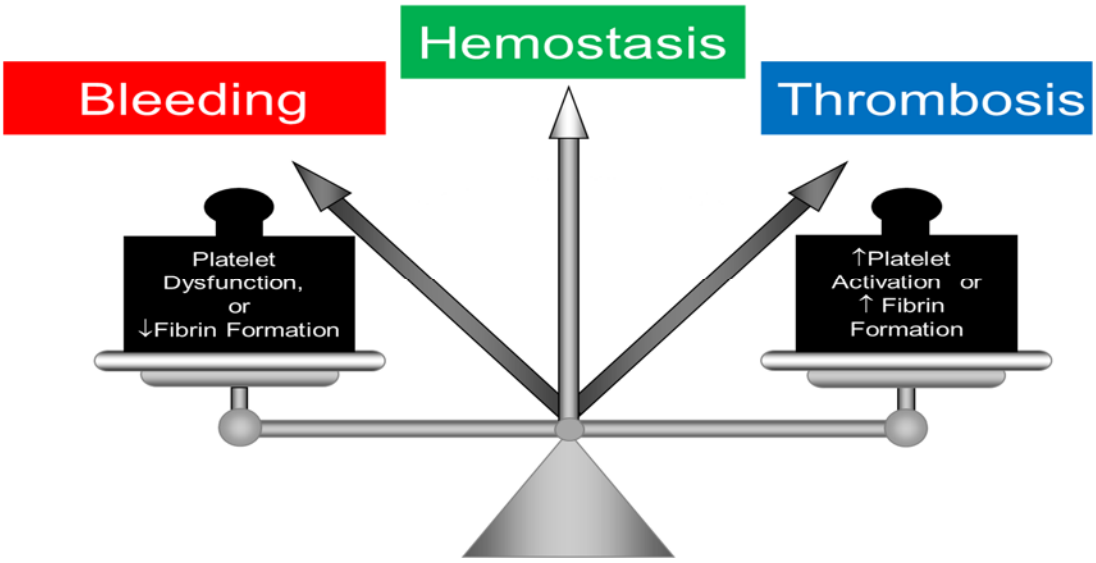

Figure 4. Current theory of coagulant mechanisms. Current treatment strategies place hemostasis at the center of a physiologic balance between hemorrhage and thrombosis.

and/or prevent disorders that represent each extreme of the coagulation spectrum, based upon the premise that thrombosis and hemorrhage result from a dysregulation of normal hemostasis. Consequently, in most cases, a therapy that is intended to treat a disorder at one end of the spectrum carries the risk of causing a disorder at the opposite extreme of the spectrum. In the case of anticoagulant therapy for prevention of thrombosis, treatment per the current paradigm carries the unfortunate risk of severe bleeding because traditional anticoagulants target proteins such as thrombin and FXa that are vital for hemostasis. For this reason, much of the effort in coagulation research is focused on the development of

novel anticoagulants that have reduced bleeding side effects. As will be discussed in Chapter 2, FXI has emerged as a target for such therapies because of its relatively minor role in hemostasis.

Cross-Talk Between Coagulation and Inflammation

The coagulation system functions alongside other host defense systems, including inflammation. Inflammation is a host response to injury that, when directed against microorganisms, is a key component of innate immunity (Tizard 2008). Many (perhaps most) clinical situations involve "cross-talk" between coagulation and inflammation (Levi, Keller et al. 2003, Levi and van der Poll 2010, van der Poll, de Boer et al. 2011, van der Poll and Levi 2012). The two systems are often activated by a common stimulus (Markiewski, Nilsson et al. 2007), and each pathway modulates the other through a variety of mechanisms to restore normal physiologic function after an insult. Like coagulation, inflammation operates along a clinical spectrum. Localized inflammation of an appropriate intensity is at the center of the balance, and results in microbial elimination and tissue repair. At the extremes are inadequate inflammation, which may contribute to microbial dissemination, and overwhelming inflammation, which manifests as an infection-induced systemic inflammatory response syndrome, or sepsis. Thus, the interaction between coagulation and inflammation, which is normally beneficial, contributes to increased morbidity and mortality during sepsis. This interaction presents many challenges for sepsis therapy. For example, systemic inflammation may trigger a coagulopathy [disseminated intravascular coagulation (DIC)] that is characterized by enhanced systemic thrombin generation (Levi and Ten Cate 1999). Thrombin can then propagate a vicious cycle by amplifying inflammation through various mechanisms (Borissoff, Spronk et al. 2009). Although anticoagulant therapy is a

tempting strategy for limiting thrombin generation and breaking this vicious cycle, the bleeding risk of currently available anticoagulants outweighs any potential benefit during sepsis. Individuals with sepsis therefore represent an additional patient population that may benefit from treatment with anticoagulant therapies that do not compromise normal hemostasis.

The Plasma Contact System

FXI is traditionally considered to be a component of the contact activation pathway, or contact system **(Figure 2)**. The contact system consists of FXII, PK, FXI, and the cofactor HK. Contact activation is initiated when FXII binds to negatively-charged surfaces. This binding causes a conformational change in zymogen FXII, resulting in the generation of a small amount of FXIIa. FXIIa then cleaves PK to generate α-kal, which then reciprocally cleaves FXII to generate more FXIIa (Bjorkqvist, Nickel et al. 2014). In addition to its role in FXII activation, α-kal also cleaves HK to liberate BK, a pro-inflammatory peptide that stimulates prostaglandin and nitric oxide (NO) production, resulting in fever, pain, and tissue swelling (Hall 1992).

Contact activation was initially assumed to be important for coagulation because of the observation that blood clots rapidly when exposed to negatively charged (anionic) artificial surfaces such as glass or kaolin. However, current discussions exclude all but one contact factor, FXI, when describing hemostasis. Although the clinical data do not support a role for contact activation in hemostasis, there is evidence that contact activation contributes to clinically relevant inflammation. For example, hereditary angioedema (HAE), is a rare, life-threatening disorder that is characterized by acute episodes of tissue swelling, particularly in the skin, gastrointestinal mucosa, and oropharynx (Bjorkqvist, Nickel et al.

2014). HAE results from a functional or quantitative deficiency of C1-inhibitor (C1INH), a serine protease inhibitor. A consistent clinical finding in HAE patients is the presence of high levels of BK. Because C1INH regulates the contact factors FXIIa and α-kal (Gailani 2010), BK release during HAE episodes is consistent with unchecked contact activation. Patients with HAE do not appear to have an increased risk of thrombosis (Bjorkqvist, Nickel et al. 2014), suggesting, consistent with the current model described in **Figure 3**, that contact activation does not contribute to coagulation in this setting.

Contact activation is likely to be an important host defense mechanism against microorganisms. Similar to components of innate immunity, such as the complement system, the contact factors can assemble and become activated on the surfaces of bacteria, including the human pathogens *Streptococcus pyogenes*, *Staphylococcus aureus*, *Escherichia coli*, and *Salmonell*a (Mattsson, Herwald et al. 2001, Herwald, Morgelin et al. 2003, Frick, Akesson et al. 2006). Contact activation is triggered through recognition of molecular patterns on bacterial surfaces in a manner that is strikingly similar to that observed with the complement system (Frick, Bjorck et al. 2007). Furthermore, consistent with *in vitro* observations of contact activation on negatively-charged surfaces, bacteria and damaged cells provide a ready supply of polyanions in the form of phosphate polymers [polyphosphate (polyP)], DNA, and RNA (Gailani, Bane et al. 2015). In addition to enhancing the innate immune response to microorganisms through BK release, contact activation-induced HK cleavage also generates antimicrobial peptides that directly target bacterial pathogens (Frick, Akesson et al. 2006). The importance of contact activation during infection was displayed in a mouse model of *S. pyogenes* infection using a synthetic peptide (H-D-Pro-Phe-Arg-CMK) that specifically inhibits FXIIa and α-kal. Inhibitor-treated mice experienced increased bacterial dissemination, suggesting that a functional

contact system is instrumental in the host response to bacterial infection (Frick, Akesson et al. 2006). The contact system, like the coagulation system, can also be harmful if activated inappropriately. Because bacteria and damaged cells can trigger contact activation, it stands to reason that bacterial infection and cellular injury above a certain threshold may activate the contact system to a level that is detrimental to the host. This is especially true in cases where bacteria disseminate and reach the bloodstream (bacteremia), allowing products of contact activation to reach the systemic circulation. BK is a potent vasodilator that may cause severe hypotension during sepsis. Septic patients with low blood pressure display a higher level of contact activation than septic patients with normal blood pressure (Mason, Kleeberg et al. 1970), while contact system inhibition corrects hypotension in animal models of lethal bacteremia (Pixley, De La Cadena et al. 1993).

While *in vitro* studies suggest an important role for contact activation in host defense, the *in vivo* significance of this process remains uncertain (Gailani 2010). Many studies have investigated possible mechanisms for FXII activation on a surface. A notable finding is that FXIa was shown to activate FXII in plasma and purified systems (Griffin 1978, Matafonov, Sarilla et al. 2011). This is perhaps not surprising, given that (a) FXI is structurally similar to PK, the zymogen for a protease (α-kal) that activates FXII, and (b) FXI and PK arose from a common gene during the course of vertebrate evolution (Ponczek, Gailani et al. 2008) and may therefore display similar features. This raises the possibility that FXIa is an effector, rather than simply a product, of contact activation, and that FXI links and influences two complex systems: one for thrombin generation, and the other for inflammation.

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CHAPTER III

INTRODUCTION TO FACTOR XI

Factor XI Structure and Function

FXI, the zymogen of the trypsin-like protease FXIa, is a 160kd protein that is made primarily in the liver. The protein circulates in a complex with the glycoprotein HK at a plasma concentration of approximately 30 nM (range of 15-45 nM) (Smith and Gailani 2008). FXI is the most recent member of the plasma coagulation system, having arisen from a duplication of the gene for the protease zymogen PK (Ponczek, Gailani et al. 2008, Bane and Gailani D. 2014). In humans, FXI has a plasma half-life of approximately 48 hours (range of 45-52 hours). Unlike the vitamin K ["Koagulations-Vitamin"(Kresage N 2005)] dependent coagulation proteins (prothrombin, FVII, FIX, and FX), FXI lacks a calcium-binding γ-carboxyglutamic acid (Gla) domain (Gailani and Smith 2009). Consequently, FXI synthesis does not require vitamin K, and treatment with vitamin K antagonists such as warfarin does not affect plasma FXI levels. Each FXI molecule circulates as a dimer of two identical 80kd subunits, which is a unique feature among the coagulation proteins. The functional significance of the dimeric structure is unclear, but may be related to activation of zymogen FXI by FXIIa (Geng, Verhamme et al. 2013), or to an interaction between platelets and FXI during hemostasis (Gailani and Smith 2009). Each 607-amino acid FXI subunit contains an N-terminal heavy chain consisting of four 90 to 91 amino acid repeats termed "apple" domains (numbered A1-A4), and a C-terminal trypsin-like catalytic domain (**Figure 5A**) (Emsley, McEwan et al. 2010). An inter-chain disulfide bond at Cys^{321} in the A4 domain (**Figures 5A & 5B**) links each subunit within the dimer. The apple domains form a

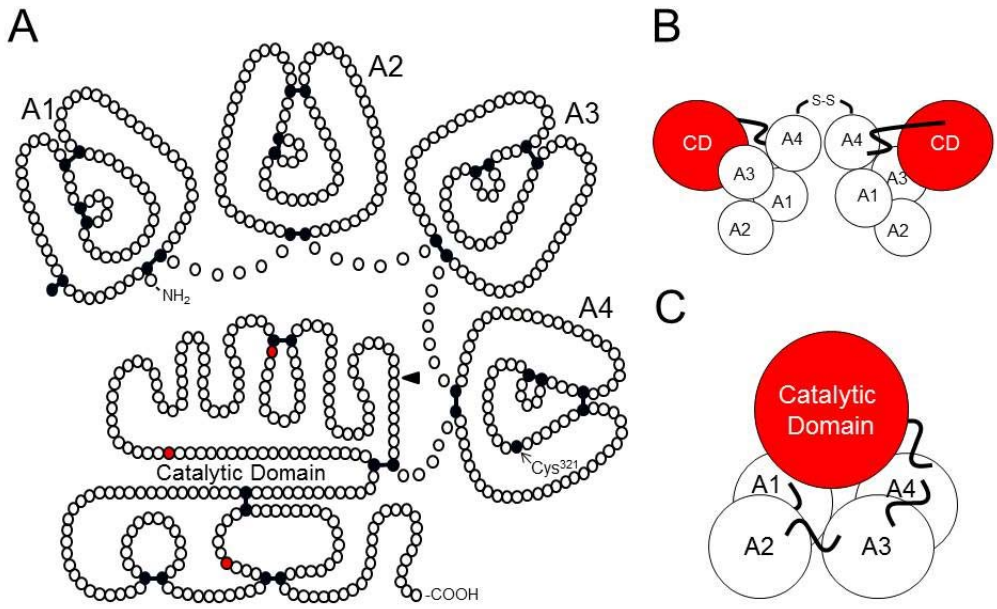

Figure 5. Factor XI structure. (A) Diagram indicating the domain organization of a factor XI (FXI) subunit. Each circle represents one amino acid. Cysteine residues that are associated with disulfide bonds are shown in black. FXI is activated through the cleavage of the peptide bond (indicated by the black arrowhead) between Arg^{369} and Ile^{370}. The amino acids of the catalytic triad (described in text) are shown in red. A disulfide bond between Cys^{321} residues on the A4 domains link FXI subunits to form (B) a FXI dimer. (C) The four "apple" domains form a platform upon which the catalytic domain rests. Panels B and C are models based on the crystal structure of FXI.

disc-like structure that serves as a platform for binding of FXI to other molecules (**Figure 5C**) (Bane and Gailani D. 2014). For example, HK binds to the FXI monomer within a groove formed by A1, A2, and A4 on the side of the platform opposite the catalytic domain, while the FXIa substrate FIX binds to the A3 domain (Emsley, McEwan et al. 2010, Bane and Gailani 2014). FXI activation occurs through cleavage of the peptide bond between the Arg^{369} and Ile^{370} residues, which are located between the A4 and catalytic domains (indicated by the black arrowhead on **Figure 5A**) of each FXI subunit (Gailani and Smith 2009). The proteases thrombin, FXIIa, and FXIa (autoactivation) convert FXI to FXIa *in vitro*. The

process involves an intermediate, ½-FXIa (only one of the two monomers is activated) that may be a major form of activated FXI (Emsley, McEwan et al. 2010). FXI activation induces a conformational change that facilitates FXIa activation of substrate FIX by exposing a binding site for FIX on the A3 domain of FXIa (Gailani and Smith 2009). Proteolysis of FXIa substrates is mediated by a "catalytic triad" (indicated by the solid red circles in **Figure 5A**) of serine (Ser^{557}), aspartic acid (Asp^{462}), and histidine (His^{413}). As discussed in chapter I, FXIa does not appear to be instrumental in initiating thrombin generation. Rather, it supports the formation of additional thrombin to bolster existing fibrin clots that are constantly subjected to numerous endogenous anticoagulant and fibrinolytic processes. The bleeding pattern of FXI deficient patients (described below) supports this interpretation of FXI's role in coagulation.

Factor XI Deficiency in Humans and Domestic Animals

This dissertation describes studies that compared mice with normal levels of FXI ($FXI^{+/+}$ mice) to those that possessed approximately 50% and 0% of normal levels ($FXI^{+/-}$ and $FXI^{-/-}$ mice, respectively) of this protein. As will be described later in this chapter, FXI deficiency is not associated with abnormal hemostasis in laboratory mice. This section is included to briefly summarize the clinical presentation of FXI deficiency in humans (and domestic animals) so that the reader is more equipped to relate FXI deficiency in humans to FXI deficiency in laboratory mice.

Although rare in the general population [approximately 1 in 1 million (Asakai, Chung et al. 1991)], severe (FXI activity <20% of normal) FXI deficiency is common (1 in 450) in people of Ashkenazi Jewish descent (Seligsohn 2009). The true incidence of FXI deficiency may be higher, as some patients may not experience bleeding that is of sufficient

intensity to require medical attention. The disorder may remain undiscovered until a prolonged aPTT is revealed during routine pre-surgical screening. In contrast to the X-linked hemophilias, people with FXI deficiency do not, with the exception of menorrhagia (Seligsohn 2009), bleed spontaneously. For example, hemarthrosis, a hallmark of the classical hemophilias, rarely occurs in FXI-deficient patients (Bolton-Maggs 2009). Significant hemorrhage, if observed, is usually related to trauma or surgery, particularly in tissues where fibrinolysis is most active, such as the oropharynx or genitourinary tract. For instance, approximately 20% of women with FXI deficiency experience excessive bleeding related to childbirth (Seligsohn 2009, Santoro, Prejano et al. 2011). It is postulated that FXIa is more important for hemostasis after trauma to these tissues, both for sustaining thrombin generation and for providing resistance to fibrinolysis by promoting activation of thrombin activatable fibrinolysis inhibitor (Von dem Borne, Bajzar et al. 1997, Bolton-Maggs 2009, Bane and Gailani D. 2014). Bleeding in FXI-deficient patients may occur immediately after injury, or may be delayed for several hours (Seligsohn 2009). Interestingly, there is a poor correlation between FXI levels and bleeding tendency. Some individuals with severe FXI deficiency do not bleed at all after trauma (Rapaport, Proctor et al. 1961), while others may experience bleeding that varies over time, even after re-exposure to a similar type of injury (Asakai, Chung et al. 1991, Bolton-Maggs, Patterson et al. 1995). The complex clinical presentation of FXI deficiency, combined with the potentially serious side effects of FXI replacement therapy, provide a difficult challenge to clinicians who manage these patients (Bolton-Maggs 2009).

Congenital FXI deficiency has also been reported in several species of domestic animals, where multiple modes of inheritance are described (Marron, Robinson et al. 2004, Ohba, Takasu et al. 2008). The disorder is most widely reported in cattle (Marron, Robinson

et al. 2004, Ohba, Takasu et al. 2008), but has also been described in dogs (Knowler, Giger et al. 1994) and in a domestic cat (Troxel, Brooks et al. 2002). Some reports describe injury-related bleeding in FXI-deficient animals (Knowler, Giger et al. 1994, Troxel, Brooks et al. 2002, Marron, Robinson et al. 2004), while others indicate no apparent bleeding phenotype (Ohba, Takasu et al. 2008).

Factor XI and Thrombosis

In healthy individuals, the mechanisms that promote coagulation are balanced by numerous anticoagulant mechanisms that localize clots to the area of tissue injury, where they are eventually broken down through fibrinolysis. A disruption to the procoagulant-anticoagulant balance may result in excessive bleeding (hemorrhage) or pathologic clot formation within blood vessels (thrombosis). The pathophysiology of thrombosis is complex, but has been attributed to increased thrombin generation and/or decreases in intrinsic fibrinolytic mechanisms. In keeping with this hypothesis, most antithrombotic therapies are directed at minimizing thrombin generation through inhibition of proteins that are vital for normal coagulation. Such therapies are very effective at preventing thrombosis, but, predictably, carry a significant bleeding risk. This fact is perhaps most vividly illustrated by the anticoagulant drug warfarin, a vitamin K-antagonist that is the drug of choice for treatment of venous thrombosis and prevention of stroke in patients with non-valvular atrial fibrillation. This anticoagulant has been in use since the early 1950's, but was first registered as a rodenticide in 1948 (van Montfoort and Meijers 2013). Interestingly, warfarin is a synthetic analogue of dicoumarol, a compound within spoiled sweet clover that was found to be the causative agent of an outbreak of hemorrhagic cattle disease in the 1920's (Kresage N 2005).

Consistent with traditional reasoning, proteins that are not vital for normal hemostasis would be predicted to play a proportionally smaller role in thrombosis. In the case of FXI, studies in humans have challenged this reasoning. In the Leiden Thrombophilia study, patients with plasma FXI levels in the top 10% of the normal distribution were nearly twice as likely to develop venous thromboembolism compared to the rest of the study population (Meijers, Tekelenburg et al. 2000). In the same study, men with higher levels of FXI were at a 2-fold greater risk for myocardial infarction. Another study showed a correlation between elevated FXI levels and stroke (Yang, Flanders et al. 2006). Conversely, population studies of individuals with severe FXI deficiency reveal a lower risk for stroke and deep-vein thrombosis (Salomon, Steinberg et al. 2008, Salomon, Steinberg et al. 2011). Severe FXI deficiency has not been shown to be protective against myocardial infarction (Salomon, Steinberg et al. 2003), indicating that FXI deficiency may not uniformly influence thrombus formation in all tissues.

Factor XI in Animal Models of Thrombosis

The observation that humans with FXI deficiency appear to be protected against certain thrombotic events has important therapeutic implications, and has supported an ongoing interest in research that investigates the role of FXI in experimental thrombosis. A mouse model of FXI deficiency was developed on the C57Bl/6 background by Gailani, *et al*., using homologous recombination in embryonic stem cells. The mice are fertile, and offspring from heterozygous matings follow the expected Mendelian ratio of approximately 25% wild type (WT), 50% heterozygous null, 25% homozygous null, indicating that partial or complete FXI deficiency is not embryonic lethal (Gailani, Lasky et al. 1997). The aPTT in plasma from FXI-deficient ($FXI^{-/-}$) mice is prolonged compared to WT mice, while

clotting times in plasma from mice that are homozygous for the FXI null allele ($FXI^{+/-}$) are intermediate between $FXI^{-/-}$ and WT ($FXI^{+/+}$) mice. Under normal conditions, these mice have no discernable phenotype and do not bleed excessively when subjected to amputation of the tail tip or other invasive procedures (Gailani, Lasky et al. 1997, Renne, Oschatz et al. 2009). This is in contrast, for example, to FIX-deficient mice, which experience injury-related (tail transection) hemorrhage that is comparable to animals given high doses of the anticoagulant heparin (Wang, Cheng et al. 2005).

Despite the lack of a bleeding disorder, $FXI^{-/-}$ mice are remarkably resistant to experimentally-induced arterial and venous thrombosis (Bane and Gailani 2014). In an important proof of concept study, Rosen, *et al*. discovered that, in contrast to WT mice, $FXI^{-/-}$ mice failed to form occlusive thrombi after application of a 20% solution of ferric chloride ($FeCl_3$) to the external surface of the carotid artery (Rosen, Gailani et al. 2002). The ability of $FXI^{-/-}$ mice to generate thrombi in response to $FeCl_3$ was restored after infusion of human FXI. Wang and colleagues (Wang, Cheng et al. 2005) used a modified version of this model to show that $FXI^{-/-}$ mice were protected from arterial thrombosis to a degree that was comparable to FIX deficiency. Importantly, the Wang study also reported that FXI deficiency was superior to aspirin therapy, and conferred a level of thrombosis resistance that was similar to treatment with doses of heparin that was well above the therapeutic range. Further work has confirmed that FXI is also instrumental in formation of venous thrombi (Wang, Smith et al. 2006), and that it contributes to experimental thrombus formation in numerous species, including rabbits and baboons (Minnema, Friederich et al. 1998, Gruber and Hanson 2003, Yamashita, Nishihira et al. 2006, Tucker, Marzec et al. 2009). Interestingly, the animal data also implicate FXII in thrombosis. For instance, WT mice that received a monoclonal antibody that blocks activation of FXI by FXIIa (but not thrombin or

FXIa) displayed a resistance to $FeCl_3$ thrombosis that was similar to FXI-deficient mice (Cheng, Tucker et al. 2010). These findings raise the profile of the contact system in thrombosis, and support the proposition that thrombus-generating mechanisms may be different from hemostatic mechanisms in some instances. Additional animal models, described in the following chapter, indicate that FXI and the contact system also participate in inflammation-induced pathology.

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CHAPTER IV

FACTOR XI AND THE CONTACT SYSTEM IN SEPSIS

Introduction

As discussed in Chapter II, numerous lines of evidence point to cross-talk between coagulation and inflammation. This chapter describes how both systems are triggered after infection, and provides examples of how coagulation and inflammation are intricately linked during sepsis. It is intended to provide a framework to help explain how FXI may contribute to morbidity and mortality during sepsis, thus setting the stage for a more complete understanding of the data reported in the next chapter.

An inflammatory syndrome known as sepsis is almost always accompanied by some degree of activation of coagulation (Levi 2010, Stearns-Kurosawa, Osuchowski et al. 2011). An extreme example of this relationship is DIC, a complication of sepsis that is associated with higher mortality (Hook and Abrams 2012). Contact activation is another complication of sepsis. Septic patients undergo systemic contact activation, as evidenced by consumption of contact factors and release of BK (Saugstad, Buo et al. 1992, Mattsson, Herwald et al. 2001). Notably, low levels of contact factors are associated with death among patients with sepsis (Oehmcke and Herwald 2010), suggesting that contact activation contributes to mortality. The contact activation and coagulation systems contribute to the pathology of sepsis through distinct mechanisms. Although it is extremely difficult to determine the precise mechanism of mortality during sepsis, inflammation and contact activation appear to figure more prominently than a coagulopathy in the mortality of sepsis in some animal models (Pixley, De La Cadena et al. 1993, Jansen, Pixley et al. 1996, Ganopolsky and

Castellino 2004, Corral, Yelamos et al. 2005, Iwaki, Cruz-Topete et al. 2008). FXI, which may participate in both coagulation and inflammation, may primarily influence mortality in some animal sepsis models through a pro-inflammatory effect.

Overview of Sepsis

Sepsis is defined as a systemic inflammatory response to infection (Bone, Balk et al. 1992). Clinically, the disorder is the result of a bacterial (gram-positive, gram-negative, or mixed) or fungal infection that originates primarily within the lungs, peritoneum, or urinary tract (Angus and van der Poll 2013). Sepsis is diagnosed when there is evidence of a systemic inflammatory response syndrome (SIRS) in the face of a confirmed or suspected infection (Bone, Balk et al. 1992). Criteria for the diagnosis of SIRS include an elevated heart rate, increased respiratory rate, and body temperature and white blood cell counts that are either above or below the normal range (Stearns-Kurosawa, Osuchowski et al. 2011). Sepsis may progress to *severe* sepsis, which is defined as SIRS complicated by organ dysfunction, and finally to *septic shock*, which is noted by the development of persistent hypotension in addition to the features described for less severe sepsis (Levy, Fink et al. 2003). Mortality from sepsis is thought to be a result of either an exaggerated inflammatory response, which typically occurs early in the course of the disease, or to prolonged immunosuppression, where patients succumb to secondary bacterial infections (Rittirsch, Flierl et al. 2008, Stearns-Kurosawa, Osuchowski et al. 2011). Additional complications, such as systemic activation of the coagulation and complement systems, may also contribute to mortality, particularly in the later stages of sepsis (Russell 2006, Rittirsch, Flierl et al. 2008, Amara, Flierl et al. 2010). Following is a discussion of the acute inflammatory response during the initial stages of sepsis, which is of relevance to this research project.

The Inflammatory Response to Infection

Inflammation is a feature of innate immunity, which is a nonspecific host response to infection and tissue injury. At its core, acute inflammation is a process of transporting cellular and soluble inflammatory factors from the bloodstream to damaged tissues. This occurs through a standard sequence of events: A local increase in blood flow is followed by increased vascular permeability, which facilitates entry of protein-rich edema fluid and leukocytes into extravascular sites of injury (Slauson and Cooper 1990). Edema fluid contains nutrients and beneficial proteins, such as coagulation factors and complement components, while leukocytes, predominantly neutrophils, augment the local response of resident macrophages to engulf, kill, and remove invading pathogens. The process is orchestrated by several types of inflammatory mediators, including cytokines, chemokines, and numerous vasoactive molecules. These mediators are protective when resolving infections that are contained locally. However, they contribute to sepsis morbidity and mortality when infections become more widespread.

Inflammation is triggered during infections when pathogens and damaged host cells express molecules called pathogen-associated molecular patterns (PAMPs) and damage- associated molecular patterns (DAMPs), respectively. These molecules are recognized by pattern recognition receptors (PRRs) on resident "sentinel" cells, such as macrophages and dendritic cells (Rittirsch, Flierl et al. 2008, Tizard 2008, Angus and van der Poll 2013). The classical PAMPs are lipopolysaccharide (LPS) and peptidoglycan within the cell walls of gram-negative and gram-positive bacteria, respectively, but PAMPs are also expressed by yeasts and viruses (Tizard 2008). DAMPs, also called alarmins, include high mobility group box protein-1 (Tizard 2008), and extracellular DNA, RNA, and histones (Chan, Roth et al. 2012). Toll-like receptors (TLRs) are the most prominent class of PRRs

(Tizard 2008). PAMP/DAMP recognition by PRRs results in the activation of receptor complexes on sentinel cells. This triggers intracellular signaling pathways, resulting in the activation of transcription factors, such as NF-κB. NF-κB and related proteins then regulate transcription of the cytokines, chemokines, and vasoactive molecules that contribute to acute inflammation.

Cytokines are polypeptides that mediate cellular interactions (Tizard 2008). They exert a variety of effects, including activation of immune cells, induction of fever, and initiation of the acute phase response. The major cytokines of acute inflammation are tumor necrosis factor α (TNFα), interleukin-1 (IL-1) and interleukin-6 (IL-6). TNFα and IL-1 have nearly identical effects (Sherwood and Toliver-Kinsky 2004) and play a central role in the vascular changes that exemplify the acute inflammatory response (Tizard 2008). IL-6 is a major mediator of the acute phase response of the liver (Sherwood and Toliver-Kinsky 2004, Tizard 2008), and also stimulates production of additional cytokines, including the anti-inflammatory cytokine IL-10 (Tizard 2008). Chemokines are a subset of cytokines that regulate the emigration of leukocytes from the bloodstream to the tissues (Tizard 2008). Among these are macrophage inflammatory protein-2 (MIP-2) and keratinocyte chemoattractant (KC), which attract neutrophils, and monocyte chemotactic protein-1 (MCP-1), which exerts chemotactic activity on monocytes.

Vasodilation is a critical step in acute inflammation. It facilitates the opening of gaps between adjacent vascular endothelial cells to allow passage of edema fluid and leukocytes from the intravascular to the extravascular space (Slauson and Cooper 1990, Sherwood and Toliver-Kinsky 2004). Vasoactive molecules, NO, BK, histamine, and vasoactive lipids, such as phospholipase, mediate this process (Sherwood and Toliver-Kinsky 2004, Tizard 2008). NO is the most important contributor to vasodilation during

sepsis (Vallance and Chan 2001, Sherwood and Toliver-Kinsky 2004). It is derived from the amino acid L-arginine by the enzyme nitric oxide synthase, and acts through cyclic guanosine monophosphate-dependent mechanisms to cause vascular smooth muscle relaxation. There are three forms of NOS: endothelial, neuronal, and inducible (Vallance and Chan 2001). The inducible form is released by numerous cell types, primarily endothelial cells and macrophages (Moncada, Palmer et al. 1991) in response to inflammatory cytokines and BK (Vallance and Chan 2001, Zhao, Qiu et al. 2001). In addition to its role in vasodilation, NO is also thought to enhance the microbial killing efficiency of macrophages (Moncada, Palmer et al. 1991).

Severe bacterial infection and/or massive trauma lead to excessive, systemic release of the inflammatory mediators described above. If left unchecked, this systemic inflammatory response ultimately results in septic shock, which is characterized by irreversible hypotension, organ injury, and death. The coagulation and contact systems (described below) are also excessively activated during sepsis. Dysregulation of each pathway presents unique challenges that may compromise the host's ability to survive.

Disseminated Intravascular Coagulation

DIC is a dysregulated coagulation response that accompanies a number of primary conditions, including sepsis, neoplasia, and trauma (Levi, de Jonge et al. 1999). The syndrome is characterized by intravascular activation of the coagulation system, with simultaneous down-regulation of intrinsic anticoagulant and fibrinolytic mechanisms (Russell 2006, Hook and Abrams 2012). This condition tips the coagulant-anticoagulant balance in favor of coagulation, resulting in ischemic organ damage secondary to widespread microvascular thrombosis. Eventually, DIC causes depletion of platelets and plasma

coagulation proteins, resulting in diffuse hemorrhage. The impact of DIC during sepsis depends on several factors, including the intensity of the coagulopathy and any existing disease process that may complicate the management of septic patients. Although individuals that develop DIC subsequent to sepsis or severe trauma are twice as likely to die, it is unclear if DIC is a definitive mechanism of mortality, or simply an indicator of disease severity, in such patients (Levi, de Jonge et al. 1999).

The coagulation system is activated during sepsis in a number of ways. Gram-negative infections trigger coagulation when the LPS component of these organisms associates with extracellular LPS-binding protein. This initiates toll-like receptor 4 (TLR4)-mediated intracellular signaling cascades, resulting in TF expression by circulating mononuclear cells (Guha, O'Connell et al. 2001). Sepsis-induced TF expression is also mediated by pro-inflammatory cytokines, particularly IL-6 (Levi 2010). The consequence of inflammation-induced TF expression is the intravascular formation of thrombin, the central enzyme in coagulation. In addition to driving DIC, thrombin also perpetuates the inflammation-coagulation cycle by stimulating cytokine production in mononuclear cells through protease activated receptor (PAR) cleavage (Borissoff, Spronk et al. 2009).

Gram-negative sepsis is the best understood cause of DIC (Hook and Abrams 2012), and most animal models of DIC employ LPS as the inciting agent (Berthelsen, Kristensen et al. 2011). The role of gram-positive organisms in coagulation is less clear, but is probably also related to toll receptor signaling upon recognition of gram-positive associated peptidoglycans by TLR2 (Russell 2006, Hook and Abrams 2012). Patel and co-workers used a “two hit” model to explore the influence of gram-negative (LPS) and polymicrobial (CLP) sepsis on TLR-mediated thrombosis. In this study, groups of mice were given IP injections of LPS or saline, or were subjected to CLP or sham surgery. Four to six hours

later, a light-dye laser injury was applied to a section of the cremaster vasculature of each (living) animal. Time to thrombosis at this discrete site was then evaluated using intravital microscopy. Thrombosis was enhanced (compared to controls) in laser-injured WT animals after CLP and LPS infusion. LPS infusion enhanced thrombosis in TLR2 deficient mice, but did not enhance thrombosis in TLR4 deficient mice. Conversely, CLP enhanced thrombosis in TLR4, but not TLR2, deficient mice. The authors concluded that CLP promoted laser-induced thrombosis through a mechanism that required TLR2 signaling, while the pro-thrombotic manifestations of *E. coli* endotoxemia required TLR4. This is consistent with our understanding of TLR4 and TLR2 signaling in gram-negative and gram-positive sepsis, respectively, since LPS is a component of gram-negative bacteria, while bacteria that enter the bloodstream of mice during the initial stages of CLP-induced sepsis are reported to be predominantly gram-positive (Hyde, Stith et al. 1990). Apart from the TLR data, the Patel study highlighted additional key differences in the coagulation response elicited by LPS and CLP. Notably, the pro-thrombotic phenotype of the CLP-operated mice disappeared when the mice were given intravenous fluid replacement with isotonic saline post-operatively, while fluid replacement had no effect on thrombosis in LPS-treated mice. This is telling, considering that they also showed that, in contrast to LPS-treated mice, thrombin-antithrombin (TAT) complex levels did not rise in mice after CLP (Patel, Soubra et al. 2010). Antithrombin is the major plasma inhibitor of thrombin (Egberg and Blomback 2014), and TAT complex levels are a surrogate marker of thrombin levels (Teitel, Bauer et al. 1982). The authors provided several hypotheses that could explain their observations, speculating, for example, that fluid replacement improves microvascular hemodynamics, leading to enhanced shear-dependent release of the antiplatelet molecules NO and prostacyclin (Saba and Saba 2014). While these data indicate sepsis promotes a pro-

coagulant state *in vivo*, the influence of the coagulopathy of sepsis on mortality is controversial (Stearns-Kurosawa, Osuchowski et al. 2011). Corral *et al* (Corral, Yelamos et al. 2005) inhibited the nuclear enzyme PARP-1 in mice and observed reduced inflammation and improved survival after LPS infusion or CLP. Notably, they saw comparable evidence of DIC in both PARP-1 deficient and WT mice, leading them to conclude that inflammation, not DIC, influenced mortality in their studies. Conversely, Opal and colleagues (Opal, Palardy et al. 2001), observed improved survival in recombinant human tissue factor pathway inhibitor (rhTFPI)-treated mice after CLP. Unfortunately, the authors did not evaluate DIC in this study, but TFPI inhibits the TF (extrinsic) pathway of coagulation (Lwaleed and Bass 2006), which is the central mechanism for intravascular coagulation during sepsis (Opal and Esmon 2003). Accordingly, it is reasonable to assume that rhTFPI treatment would reduce DIC if it were present. Interestingly, rhTFPI anticoagulant treatment improved survival when the cecum was punctured with a small-bore needle (defined as 23 gauge), but not when injury was inflicted with a large bore needle (defined as 21 gauge). This suggests that higher levels of injury may trigger or enhance additional pathways, such as contact activation, that exacerbate sepsis pathology.

In theory, anticoagulant therapy is a rational approach for treating sepsis because it addresses both the coagulopathy and the inflammatory response of the disorder by inhibiting thrombin. In practice, such therapies have met with disappointing results, probably because anticoagulants increase the bleeding risk in septic patients. This is illustrated by studies that describe hemorrhage in fibrinogen-deficient mice infected with the protozoal parasite *Toxoplasma gondii* and the gram-positive bacteria *Listeria monocytogene*s (Johnson, Berggren et al. 2003, Mullarky, Szaba et al. 2005). Another hypothesis for treatment failures is that antithrombotic therapy actually contributes to sepsis pathology by dampening the

protective effects of coagulation during infections. Many authors speculate the coagulation system limits infection through various mechanisms, including the formation of a protective fibrin barrier around invading microbes (Ahrenholz and Simmons 1980, Echtenacher, Weigl et al. 2001) and up-regulation of inflammation (Szaba and Smiley 2002, Flick, Du et al. 2004, Adams, Schachtrup et al. 2007). These effects undoubtedly depend on the degree of infection, type of organism, and numerous host factors.

The Contact System and Sepsis

In contrast to DIC, which some consider to be a "bystander" in the pathology of sepsis (Stearns-Kurosawa, Osuchowski et al. 2011), vasodilation secondary to excessive NO production is the primary feature of septic shock (Vallance and Chan 2001). The contact activation product BK contributes to vasodilation through the canonical pathway by stimulating endothelial NO production (Vallance and Chan 2001, Zhao, Qiu et al. 2001). BK also activates phospholipase (Sriskandan and Cohen 2000), which stimulates the release of prostaglandins and other vasoactive lipids from cell membranes (Tizard 2008). Despite the traditional association between contact activation and coagulation, animal models have demonstrated that hypotension, not coagulopathy, is the primary consequence of infection-induced contact activation. Furthermore, there is strong evidence that hypotension is the mechanism through which the contact system contributes to sepsis mortality. Using a baboon model of lethal *Escherichia coli* sepsis, Pixley and colleagues (Pixley, De La Cadena et al. 1993) found that an anti-FXII monoclonal antibody reduced contact activation and hypotension, and prolonged survival. Importantly, the antibody did not influence the development of DIC. Consistent with the baboon work, Iwaki, et al(Iwaki, Cruz-Topete et al. 2008) found that FXII deficiency improved hypotension but not coagulopathy in

endotoxemic mice. A later study showed that anti-FXII antibody treatment lowered IL-6 levels in septic baboons (Jansen, Pixley et al. 1996), indicating that contact activation also influences cytokine release. The mechanism of contact activation-induced cytokine release during sepsis is not known, but may be related to monocyte stimulation by FXIIa and HK fragments (Toossi, Sedor et al. 1992, Khan, Bradford et al. 2006). These animal studies provide evidence that the contact system operates independently of the coagulation system in some cases of sepsis. This proposition is supported by a retrospective analysis of streptococcal toxic shock syndrome (STSS) in humans, which noted the absence of DIC in patients with an "isolated prolongation of aPTT" (prolonged aPTT with normal PT) (Sriskandan and Cohen 2000). Recall that the aPTT evaluates the intrinsic (contact activation) pathway, while the PT evaluates the extrinsic (TF) pathway. If activation of one or both pathways *in vivo* results in consumption (depletion) of the corresponding coagulation factors, the *ex vivo* observation of isolated prolongation of aPTT suggests that contact activation predominates in this syndrome. Apart from reinforcing the role of the contact system in sepsis, the animal and STSS studies suggest contact activation-induced hypotension has a relatively greater role than coagulopathy in the morbidity and mortality that accompanies some infections.

Factor XI in Animal Models of Sepsis

The *in vitro* data, discussed in Chapter II, that indicate FXI influences both contact activation/inflammation and coagulation are bolstered by recent findings that point to a role for this protein in the host response to infection. In a murine study of peritonitis induced by intraperitoneal injection of the gram-positive intracellular bacteria *Listeria monocytogenes* (Luo, Szaba et al. 2012), $FXI^{-/-}$ mice had improved survival (67% $FXI^{-/-}$ alive at 10 days

compared to 30% for WT, p= 0.04) when challenged with a high dose of *L. monocytogenes*, and displayed reduced levels of plasma IL-6 (p=0.03), and hepatic fibrin (p=0.02). Markers of DIC indicated a less intense coagulopathy in $FXI^{-/-}$ mice compared to WT mice (higher fibrinogen [p=0.0005], lower TAT levels [p=0.008], and increased platelet numbers [p=0.009]). Dissemination of the organism to liver and spleen was markedly reduced in $FXI^{-/-}$ mice (p=0.007 and 0.0006, respectively). An interesting finding from this study was the discovery that hepatic FXI mRNA levels were elevated in WT mice infected with lethal doses of *L. monocytogenes*. This suggests FXI synthesis is increased in response to infection with this organism. It should be noted that FXI does not appear to contribute to the pathophysiology of all types of infection. For example, FXI deficiency was not protective in mice infected with the gram-negative bacteria *Yersinia enterocolitica* (Luo, Szaba et al. 2011). Furthermore, $FXI^{-/-}$ mice were not resistant to the detrimental effects of injections of *Escherichia coli* endotoxin (Tucker, Gailani et al. 2008).

In a study of sepsis accompanying bacterial peritonitis induced by CLP, Tucker and co-workers showed that $FXI^{-/-}$ mice had a survival advantage over WT mice (Tucker, Gailani et al. 2008). One week after CLP, 46% of $FXI^{-/-}$ mice were alive, while only 13% of WT mice were alive (P=0.0001). Markers of DIC [platelet counts, fibrinogen levels, TAT complex levels] in blood drawn 24 hours after CLP indicated a less severe coagulopathy in $FXI^{-/-}$ mice compared to WT mice. $FXI^{-/-}$ mice also had lower peritoneal leukocyte counts 24 hours post CLP compared to WT littermates, suggesting that FXI influenced leukocyte trafficking. In contrast to the *Listeria* study, the authors found no differences in peritoneal or blood levels of bacteria between $FXI^{-/-}$ and WT mice, suggesting there are fundamental differences in the disease processes that follow *Listeria* infection and CLP, respectively. More recently, Tucker *et al* tested an anti-FXI antibody that blocks FXI activation by FXIIa

(14E11) *in vitro* in the CLP model (Tucker, Verbout et al. 2012). Treatment with 14E11 improved survival by 30% (p=0.001) in WT mice, when administered up to 12 hours after CLP. 14E11-treated mice also had significant attenuation of the inflammatory markers TNFα and IL-6 in plasma at 12 hours after CLP. Surprisingly, WT mice given 14E11 after CLP had peritoneal leukocyte counts that were comparable to vehicle (PBS) treated animals at 24 hours. This contrasts with the study using $FXI^{-/-}$ mice, where peritoneal leukocyte counts were noted to be lower in the knockout animals, and suggests that FXI-associated leukocyte migration is not dependent on the contact system.

The earlier CLP data indicate FXI is associated with enhanced thrombin generation and consumption of platelets and fibrinogen during CLP-induced sepsis. However, this is insufficient evidence to conclude that the mice were in fulminant DIC after CLP. Hemorrhage and microvascular thrombosis are hallmarks of DIC in laboratory animals (Berthelsen, Kristensen et al. 2011), yet histology of tissues taken 24 hours after CLP was unremarkable in the study that compared $FXI^{-/-}$ to WT mice. These histologic findings were similar to those of a prior study by Ganopolsky and colleagues (Ganopolsky and Castellino 2004) that failed to show severe pathology (including evidence for DIC) in mice 24 hours after CLP. Ganopolsky, *et al.* concluded that severe DIC is not a feature of CLP-induced sepsis, and attributed the unremarkable histology to the acute nature of septic shock after CLP. Considering that plasma inflammatory cytokines correlate with death after CLP in mice (Remick, Bolgos et al. 2002, Osuchowski, Welch et al. 2006), the finding that plasma TNFα and IL-6 was lower in 14E11-treated mice after CLP is perhaps a better explanation for the difference in mortality observed between treatment groups in both Tucker studies.

Taken together, the earlier studies suggest a role for FXI in the host response to CLP injury (summarized in **Figure 6**). However, our knowledge of the course of this response in

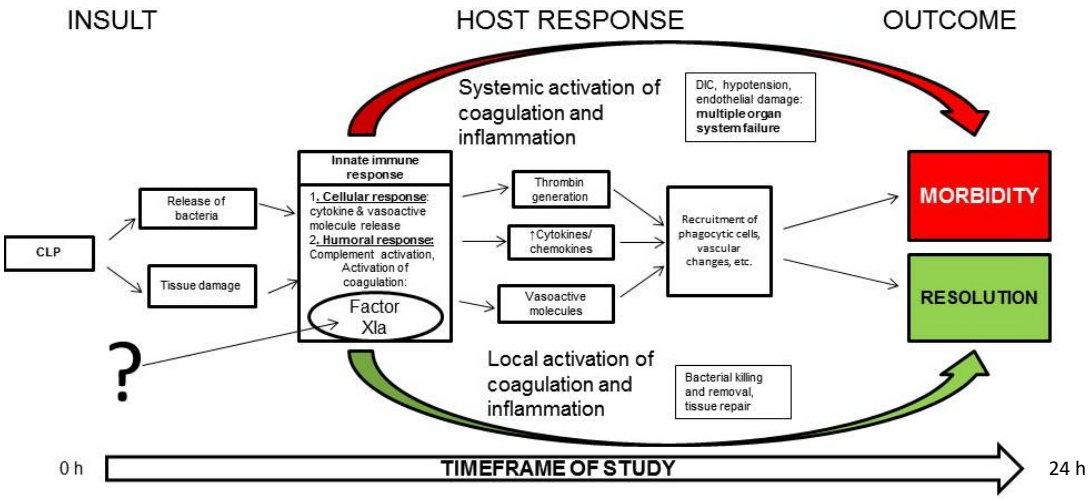

Figure 6. Role of FXI in polymicrobial sepsis. Coagulation and inflammation are both triggered because of the tissue damage and bacterial release that occurs after CLP. Both systems work synergistically and lead to resolution of the infection when contained at the locus of infection (green arrow and box). However, systemic activation of coagulation and inflammation may contribute to morbidity through DIC, endothelial damage, and multiple system organ failure (red arrow and box). Using FXI deficient mice, we attempted to define the role of FXI in polymicrobial sepsis by evaluating the effects of this protein on survival, and on the coagulopathy and inflammatory response induced by CLP.

WT and $FXI^{-/-}$ mice during polymicrobial sepsis over time was limited to a single time point (24 hours). Studies with the 14E11 antibody were informative, but administration of this antibody did not result in FXI deficiency, and thus to blockage of all FXI-related activities. The goal of my research project was to address important gaps in our current understanding of the mechanisms by which FXI contributes to the pathophysiology of sepsis. Chapter V describes the findings of my project.

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CHAPTER V

FACTOR XI DEFICIENCY ALTERS THE CYTOKINE RESPONSE AND ACTIVATION OF CONTACT PROTEASES DURING MURINE POLYMICROBIAL SEPSIS

Introduction

Sepsis, a systemic inflammatory response that may develop with severe bacterial, fungal, or viral infection (Levy, Fink et al. 2003), is a major cause of mortality (Dellinger, Levy et al. 2013). It is estimated that nearly twenty million cases of sepsis occur each year worldwide, with death occurring in more than 25% of those with severe sepsis (Angus, Linde-Zwirble et al. 2001). Inflammation and coagulation are key players in the initial host response to infection, and while both are beneficial when localized to an injury site, they can be destructive when activated systemically. The hyper-inflammatory response during sepsis causes tissue damage and organ dysfunction, resulting in morbidity and mortality, while perturbations in coagulation (both thrombotic and hemorrhagic) signal a worsening prognosis, further complicating the management of septic patients.

FXIa contributes to thrombin generation through proteolytic activation of several plasma coagulation factors, most prominently FIX (Emsley, McEwan et al. 2010, Matafonov, Cheng et al. 2013). While FXIa serves a relatively modest role hemostasis, it makes major contributions to pathologic thrombus formation in a variety of animal models (Tucker, Marzec et al. 2009, Cheng, Tucker et al. 2010, Yau, Liao et al. 2014), and appears to contribute to thromboembolic disease in humans (Meijers, Tekelenburg et al. 2000, Doggen, Rosendaal et al. 2006, Suri, Yamagishi et al. 2010). Previously, we observed that complete deficiency of FXI, the zymogen of FXIa, improved survival in a mouse polymicrobial sepsis model induced by ligation and puncture of the cecum (CLP) (Tucker,

Gailani et al. 2008). There was evidence of a consumptive coagulopathy in WT C57Bl/6 mice 24 hours post-CLP that was not evident in FXI-deficient mice. Subsequent work showed that a monoclonal anti-mouse FXI IgG (14E11) improved survival in WT mice subjected to CLP, with blunting of the coagulopathy (Tucker, Verbout et al. 2012). In that study, mice treated with 14E11 immediately after CLP had ~50% reductions in the levels of the cytokines IL-6 and TNF-α 12 hours after CLP, suggesting FXI inhibition produced an anti-inflammatory effect in addition to an anti-thrombotic effect. As inflammatory and hemodynamic changes characteristic of septic shock become evident within the first few hours after CLP in mice (Walley, Lukacs et al. 1996, Ganopolsky and Castellino 2004, Murphy, Morgelin et al. 2011), we investigated the importance of FXI to inflammation and coagulation early in the course of CLP-induced polymicrobial sepsis. We observed improved survival in $FXI^{-/-}$ mice, and found significant differences in cytokine responses between WT and $FXI^{-/-}$ mice as early as four hours post-CLP. In contrast to our earlier work, which employed a less severe level of injury, we did not find histologic or hematologic evidence of consumptive coagulopathy. Interestingly, while there was evidence of contact activation in the plasma of WT mice after CLP, the process was blunted in FXI-deficient mice. A mechanism that might explain this observation was investigated.

Materials and Methods

Mice

For CLP survival studies, C57Bl/6 mice heterozygous for a null FXI allele ($FXI^{+/-}$) (Gailani, Lasky et al. 1997) were crossed to generate WT ($FXI^{+/+}$), heterozygous null ($FXI^{+/-}$), and homozygous null ($FXI^{-/-}$) mice. FXI genotypes for study mice were determined by

PCR. Age-matched 3 to 6-month-old littermates were subjected to CLP. For time course studies, $FXI^{+/+}$ and $FXI^{-/-}$ littermates were used. Animals were housed under a 12-hour day/night cycle with unrestricted access to food and water. Experiments were approved by the Institutional Animal Care and Use Committee at Vanderbilt University.

Cecal Ligation and Puncture (CLP) model of polymicrobial sepsis

CLP was carried out as described (Tucker, Gailani et al. 2008, Rittirsch, Huber-Lang et al. 2009, Tucker, Verbout et al. 2012). Briefly, mice were anesthetized with isoflurane and aseptically prepared for surgery. A 1 cm midline celiotomy was performed, and the cecum was exteriorized. The cecum was ligated using 4-0 coated Vicryl™ (Ethicon, Inc., Somerville, NJ) either 1 cm from the distal end (low-grade injury) or at the base just distal to the ileocecal valve (high-grade injury). Care was taken to avoid mechanical obstruction of the bowel. The cecum was punctured twice using a sterile 21-gauge hypodermic needle, and a drop of cecal contents was expressed. The cecum was returned to the abdominal cavity, the abdominal wall was closed with 4-0 Vicryl™, and the skin was closed with wound clips (Roboz Surgical, Gaithersburg, MD). Mice received a 1 mL subcutaneous (SC) injection of warmed 0.9% NaCl immediately after surgery, and were monitored on a warming pad until recovery. After consultation with a veterinarian, it was decided not to use analgesics because they alter the process being studied. After CLP, mice were evaluated every 6 hours, and received 1 mL warm 0.9% NaCl SC daily. At each inspection, animals that (1) were moribund (no response to stimulation), (2) in respiratory distress, (3) were cool to touch, (4) had dusky discoloration of the tail or ears, or (5) had lost >30% body weight were euthanized (CO2 inhalation, followed by cervical dislocation). For pilot studies, WT mice underwent low-grade CLP. At 2, 4, 8, 12, and 24 hours after surgery, mice were

anesthetized with isoflurane, blood was collected by cardiac puncture, and the animals were sacrificed by cervical dislocation while under isoflurane anesthesia. For later time course studies that compared $FXI^{+/+}$ and $FXI^{-/-}$ littermates, mice underwent high-grade CLP or sham (cecum manipulated without ligation or puncture) surgery, and samples were collected at 4, 8, and 24 hours post-CLP. No animals met criteria for euthanasia prior to reaching designated time points.

Sample collection and cell counts

Blood was drawn from control and post-surgical animals by cardiac puncture into a 1/10th volume of 100 mM EDTA. Complete blood counts were measured using a Hemavet® 950 FS analyzer (Drew Scientific, Waterbury, CT). Blood samples were then immediately centrifuged to obtain platelet poor plasma. Plasma was stored at -80°C.

Measurements of plasma proteins

Plasma cytokine (IL-1β, IL-6, IL-10, TNFα) levels were measured with a multi-plex assay on a Luminex 100 system (Luminex Corporation, Austin, TX). Mouse specific ELISA kits were used to measure plasma levels of TAT complex, fibrinogen, and serum amyloid P (SAP) (Innovative Research, Novi, MI). Changes in plasma FXII, PK and FXI concentration were determined by densitometry of western immunoblots. One microliter samples of mouse plasma were size fractionated under non-reducing conditions on 10% polyacrylamide-SDS gels, and transferred to nitrocellulose paper. FXII and PK were detected by HRP-conjugated goat anti-human FXII and sheep anti-human PK (Affinity Biologicals, Ancaster, Ontario), respectively, while FXI was detected with a biotinylated monoclonal antibody to murine FXI (14E11) (Cheng, Tucker et al. 2010), followed by streptavidin-HRP. Detection was by

chemiluminescence. Films were evaluated with BioRad Quantity 1 software (BioRad, Richmond, CA). Mean signals for samples obtained from mice that did not undergo surgery were arbitrarily assigned a value of 100%, and served as a baseline reference for signals at various time points

Histology

Liver, spleen, thymus, kidney, and brain samples were collected from controls and from mice sacrificed at 4, 8, and 24 hours post-CLP or sham surgery, fixed in 10% buffered neutral formalin for 24 hours, then processed routinely, embedded in paraffin, longitudinally sectioned at 4 microns, and stained with hematoxylin-eosin. Light microscopic evaluation was performed by an experienced veterinary pathologist. For each sample, at least 20 high-power fields of view were evaluated for evidence of fibrin formation, platelet clumping, inflammation, lymphoid apoptosis, hemorrhage, and necrosis. Samples were submitted to the pathologist with a numeric code to facilitate blinding.

Chromogenic assay for FXII activation

Human FXII, FXIa, α-kal and HK were purchased from Enzyme Research Laboratory (South Bend, IN). Genomic DNA was isolated from human leukocytes, and total RNA from mouse liver. Polyphosphate (60-100 phosphate units) was a gift from Thomas Renné (Karolinska Institute). Activation of FXII (200 nM) was studied in 20 mM HEPES, pH 7.4, 100 mM NaCl, 0.1% PEG-8000, ZnCl2 (10 μM) at 37 ºC. Reactions included DNA (5.0 μg/ml), RNA (5.0 μg/ml), polyphosphate (20 μg/ml), HK (20 nM), α-kal (2.0 nM), or FXIa (1.0 nM). At various times, 20 μl of reactions were mixed with 10 μl Polybrene (0.4 mg/ml) and 40 nM of an anti-kallikrein IgG H03 (Kenniston, Faucette et al. 2014) (for

reactions with α-kal) or 40 μM aprotinin (for reactions with FXIa). Ten microliters S-2302 (2 mM in H2O) was added and ΔOD 405 nm was monitored on a microplate reader. Results were compared to a standard curve prepared with pure FXIIa.

Polyphosphate-induced FXII activation in mice

WT C57Bl/6 mice, and C57Bl/6 lacking FXII (Pauer, Renne et al. 2004), PK (Stavrou, Fang et al. 2015), or FXI (Gailani, Lasky et al. 1997) underwent general anesthesia with sodium pentobarbital. The abdomen was opened, and polyphosphate (60 mg/kg) in 100 μL of PBS was infused into the inferior vena cava over 30 seconds. Five minutes later, blood was collected from the inferior vena cava into 1/10th volumes of 3.2% sodium citrate, and plasma was prepared by centrifugation. Mice were sacrificed by cervical dislocation after phlebotomy while still under anesthesia. Plasma samples (1 μL) were size fractionated under non-reducing conditions on 12% polyacrylamide-SDS-gels, transferred to nitrocellulose paper, and developed with HRP-conjugated goat anti-human FXII (Affinity Biologicals). Detection was by chemiluminescence.

Statistical analysis

Data were analyzed using GraphPad Prism® 5.0 software (San Diego, CA). Survival differences between groups were evaluated by log-rank test. Differences in continuous variables (cytokines, plasma proteins, cell counts) were assessed with a non-parametric Mann-Whitney test. For densitometric measurements of Western blots, the one-sample t-test was used to differentiate between mean intensity for each group from signal intensity at the pre-surgical baseline (assigned a value of 100%). Data are presented as mean

± standard error of the mean (SEM). A p value of < 0.05 was considered significant for all analyses.

Results

FXI deficiency confers a survival advantage after high-grade CLP injury

$FXI^{-/-}$ and $FXI^{+/-}$ mice had significantly better survival than WT mice (p=0.002 and 0.003, respectively) after CLP using a high-grade injury level (**Figure 7A**). Here, in contrast to our previous work (Tucker, Gailani et al. 2008), study animals of all genotypes were littermates, to address a concern that our earlier observations of a survival difference between unrelated $FXI^{-/-}$ and WT mice may have been due to differences in gut flora or background genetics. Overall survival at 7 days post-CLP was 3% in WT mice (n=36), 17% in $FXI^{+/-}$ mice (n=41), and 30% in $FXI^{-/-}$ mice (n=33). Hazard ratios were 2.4 (95% confidence interval 1.4-4.1) for WT compared to $FXI^{-/-}$ mice, and 2.1 (95% confidence interval 1.3-3.6) for WT compared to $FXI^{+/-}$ mice. Median survival times for WT, $FXI^{+/-}$, and $FXI^{-/-}$ mice were 42, 61, and 54 hours, respectively, while median time to death for mice dying after CLP was 42, 52, and 44 hours, respectively. Survival curves between $FXI^{-/-}$ and $FXI^{+/-}$ mice were not significantly different (p=0.3). FXI deficiency conferred a survival advantage in male (**Figure 7B**) and female (**Figure 7C**) mice, with no differences between genders. Due to concerns regarding effects of the female estrus cycle on the CLP model (Zellweger, Wichmann et al. 1997), the remainder of the study was performed using male mice.

Survival was similar between genotypes subjected to CLP with a low-grade injury (**Figure 7D**, p=0.3 for WT vs $FXI^{-/-}$, p=1.0 for WT vs $FXI^{+/-}$). In fact, survival was unrelated

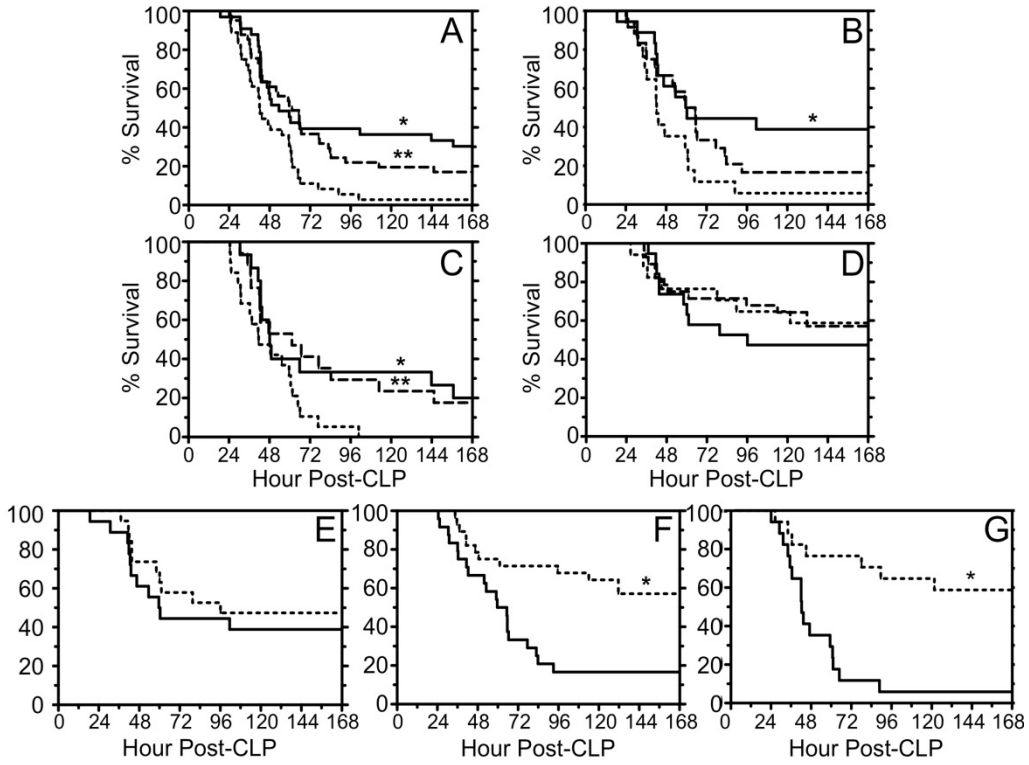

Figure 7. Survival after CLP. (A) Survival of WT (----, n=36), $FXI^{+/-}$ (---, n=41), or $FXI^{-/-}$ (—, n=33) mice after high-grade CLP injury. *p= 0.002 for WT vs $FXI^{-/-}$ and **p= 0.003 for WT vs $FXI^{+/-}$ mice. Survival in $FXI^{-/-}$ mice was higher than $FXI^{+/-}$ mice (30% vs. 17%), but the difference was not significantly different (p= 0.3). **(B)** Survival for male mice in panel A (n=17, 24, and 18 for WT, $FXI^{+/-}$, and $FXI^{-/-}$ mice, respectively). Survival was significantly higher (*p= 0.03) in $FXI^{-/-}$, but not $FXI^{+/-}$ (p= 0.09) compared to WT littermates. **(C)** Survival for female mice in panel A (n=19, 17, and 15 for WT, $FXI^{+/-}$, and $FXI^{-/-}$ mice, respectively). Survival was significantly higher (*p= 0.04) in $FXI^{-/-}$ and $FXI^{+/-}$ (**p= 0.01) compared to WT littermates. **(D)** Survival of WT (n=17), $FXI^{+/-}$ (n=28), or $FXI^{-/-}$ (n=19) male mice after low-grade CLP injury. No difference in survival was noted between genotypes (p=0.4 for WT vs $FXI^{-/-}$, p=1.0 for WT vs $FXI^{+/-}$, p=0.5 for $FXI^{-/-}$ vs $FXI^{+/-}$). **(E-G)** Survival curves for male mice from panels B and D, comparing high-grade injury (—) to low-grade injury (----) for **(E)** $FXI^{-/-}$, **(F)** $FXI^{+/-}$, and **(G)** WT mice. For $FXI^{-/-}$ mice, there was no difference in survival after low- or high- grade injury (p= 0.5), while survival was significantly different in $FXI^{+/-}$ (p=0.001) and WT (p= 0.0002) mice between the two levels of injury. Curves were compared using the log-rank test.

to injury grade in $FXI^{-/-}$ mice (**Figure 7E**), while it was influenced by injury grade for $FXI^{+/-}$ (**Figure 7F**) and WT (**Figure 7G**) mice. Survival rates for WT and $FXI^{+/-}$ mice, which have ~100% and ~50% of the normal plasma levels of FXI, respectively, were significantly different at low- and high-grade injuries (p=0.0002 for WT, p=0.001 for $FXI^{+/-}$), while there

was no difference in survival in $FXI^{-/-}$ mice at the two levels of injury. Taken together, these data suggest that FXI contributes to mortality during more severe sepsis, that reduction of plasma FXI levels to as much as ~50% of normal may be protective, and that differences in gut flora do not explain differences in survival. Because the high-grade injury model revealed differences in survival between genotypes, we used this model to assess the effects of FXI deficiency on inflammation and coagulation in subsequent studies.

FXI contributes to inflammation during the early stages of sepsis

We measured plasma levels of cytokines in response to CLP or sham injury, concentrating on responses within the first 24 hours to avoid losing study subjects, as deaths started occurring within 24 hours of high-grade CLP. Studies focused on cytokines that increase early in sepsis, as determined in pilot studies using WT C57Bl/6 mice. In our pilot studies, CLP resulted in marked elevations of the pro-inflammatory cytokines TNF-α, IL-1β, and IL-6, and the anti-inflammatory cytokine IL-10 (**Figures 8A and 8B**). For TNF-α, IL-1β, and IL-6 there was a peak of activity four hours post-CLP, and for IL-10, 2 hrs. post-CLP. A biphasic pattern was observed for some cytokine responses, which is consistent with reports from previous investigators (Walley, Lukacs et al. 1996, Torres and De Maio 2005). By 8 hours, TNF-α and IL-10 levels were decreased from the initial peak, but were again increased (compared to 8 hour levels) at 24 hours (**Figures 8B and 8A**, respectively). Notably, sham surgery did not elicit the profound cytokine response that was observed after CLP. **Figures 8C and 8D** compare plasma levels of TNF-α, IL-1β, IL-6, and IL-10 four hours after CLP in pilot studies to levels measured at four hours after sham surgery. The plasma level of serum SAP, a major acute phase protein in rodents that is considered comparable to C-reactive protein as an indicator of inflammation in humans (Tizard 2008),

was significantly elevated by 24 hours in our pilot studies of WT mice undergoing CLP (**Figure 8E**), consistent with an intense inflammatory response.

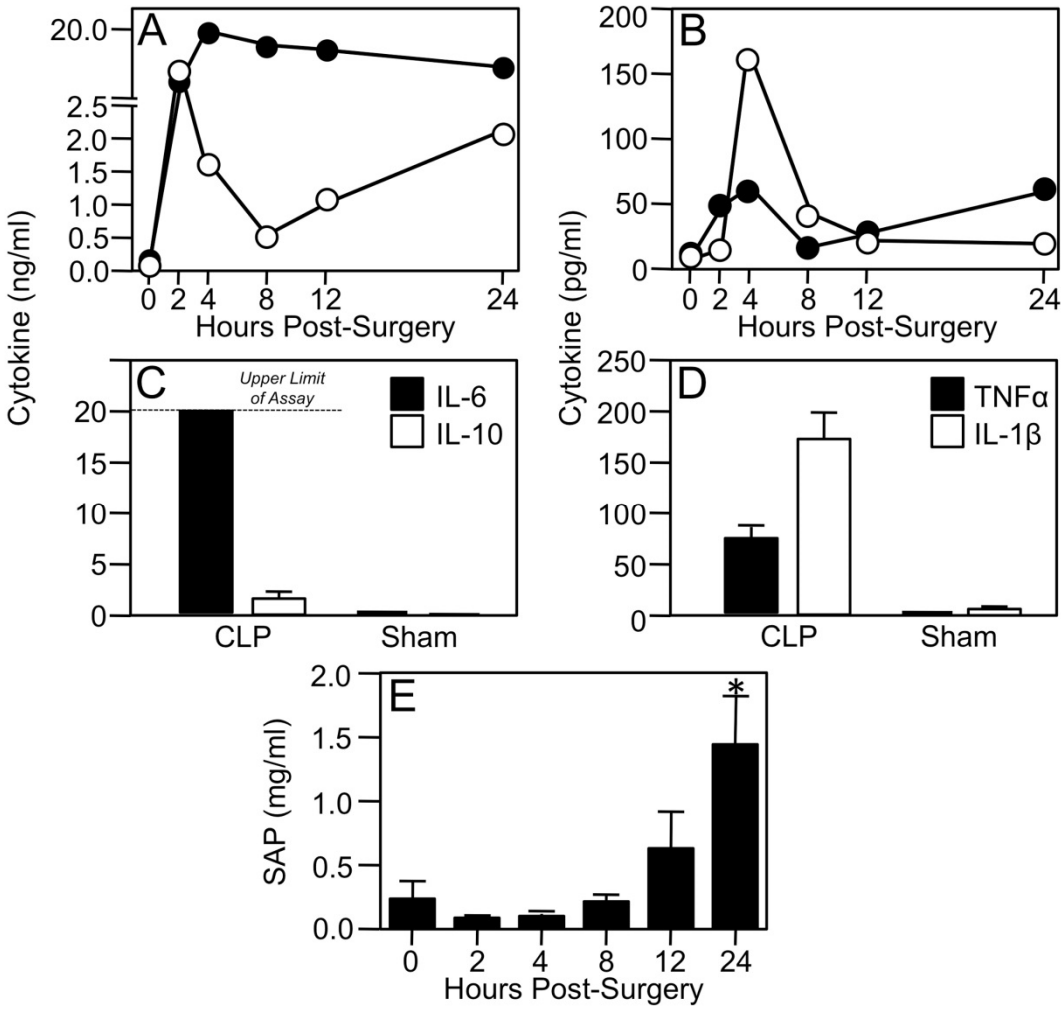

Figure 8. Pilot studies-plasma cytokine and SAP expression after CLP. Plasma levels of **(A)** IL-6 (●) and IL-10 (○) and **(B)** IL-1β (○) and TNFα (●) at various time points after CLP in WT mice. Plasma levels of **(C)** IL-6 (○) and IL-10 (●) and **(D)** IL-1β (○) and TNFα (●) in WT mice 4 hours after CLP or sham surgery (sham levels are from a separate study). Note that the IL-6 levels at 4 hr. in CLP mice exceeded the upper limit of the assay (20 ng/ml). **(E)** Plasma levels of serum amyloid P (SAP) at various time points after CLP in WT mice. Error bars represent SEM. N=3-4 mice per group.

Cytokine data for time course studies comparing $FXI^{+/+}$ mice with $FXI^{-/-}$ littermates are displayed in **Figure 9**, and are expressed as fold-increase compared to sham surgery to illustrate the impact of high-grade CLP over and above anesthesia and celiotomy alone. Increases in plasma cytokine levels were significantly greater in $FXI^{+/+}$ mice than in $FXI^{-/-}$ mice 4 hours post-CLP for TNF-α (**Figure 9A**, p=0.009), IL-1β (**Figure 9B**, p=0.0009), IL-6 (**Figure 9C**, p=0.003), and IL-10 (**Figure 9D**, p=0.0003). For TNF-α (**Figure 9E)** and IL-6 (**Figure 9G**) the second increase in cytokine levels was present at 24 hours in both genotypes. Indeed, for IL-6, the increases at 8 and 24 hours were significantly greater in $FXI^{-/-}$ mice than WT mice (p=0.04). The levels of IL-1β increased more in $FXI^{-/-}$ mice than WT mice 8 hours post-CLP (**Figure 9F**, p=0.08), suggesting a delay in the early increase at 4 hours observed in WT mice. For IL-10, both early (4 hr.) and late (24 hr.) changes were reduced in $FXI^{-/-}$ mice (**Figure 9H**), but only the early reduction reached significance (4 hrs. p=0.0003, 24 hrs. p=0.9) compared to WT mice. Analysis of absolute cytokine levels (**Figure 10**) revealed that Plasma TNF-α (**Figure 10A**) and IL-10 (**Figure 10D**) levels were significantly greater in WT mice than in $FXI^{-/-}$ mice 4 hours post-CLP (p= 0.006 and 0.0003, respectively), while there was a trend toward higher IL-1β and IL-6 levels four hours post-CLP in WT animals (p= 0.16 and 0.06, respectively). Later increases out to 24 hours for TNF-α, IL-6, and IL-10 were comparable between genotypes. Peak IL-1β levels were similar between WT and $FXI^{-/-}$ mice, however, they occurred later (8 hrs.) in $FXI^{-/-}$ mice than in WT mice (4 hrs.). The data demonstrate that absence of FXI alters the normal (WT) cytokine response pattern induced by CLP, with prominent blunting of the early response within the first few hours after injury.

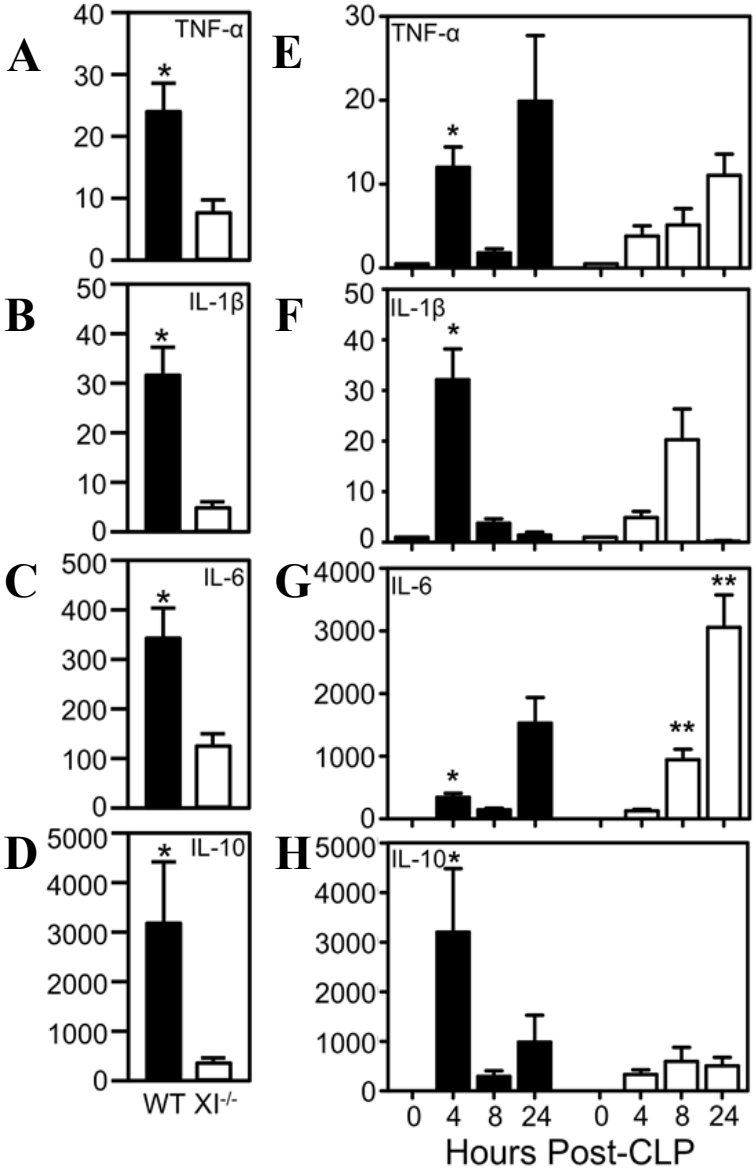

Figure 9. Fold increases in plasma cytokine levels after CLP. Plasma cytokine concentrations were measured by multiplex (Luminex 100) assay, and are displayed as fold-increases for TNFα, IL-1β, IL-6 and IL-10 in WT (black bars) and FXI$^{-/-}$ (white bars) littermates after high-grade CLP, compared to sham treated animals. Panels A to D are comparisons 4 hr. post-CLP, and panels E to H are comparisons 0, 4, 8, and 24 hr. post-CLP. Eight or nine mice were tested at each time point for each genotype for CLP, and three mice were used at each time point for each genotype for sham surgery. Increases in plasma levels of TNFα, (*p*=0.009), IL-1β, (*p*=0.009), IL-6 (*p*=0.003) and IL-10 (*p*=0.0003) were significantly greater in WT mice than in FXI$^{-/-}$ mice 4 hr. post-CLP. For IL-6, FXI$^{-/-}$ mice had significantly greater increases in plasma levels 8 (***p*=0.005) and 24 hr. (***p*=0.04) post-CLP than did WT mice. Error bars represent SEM.

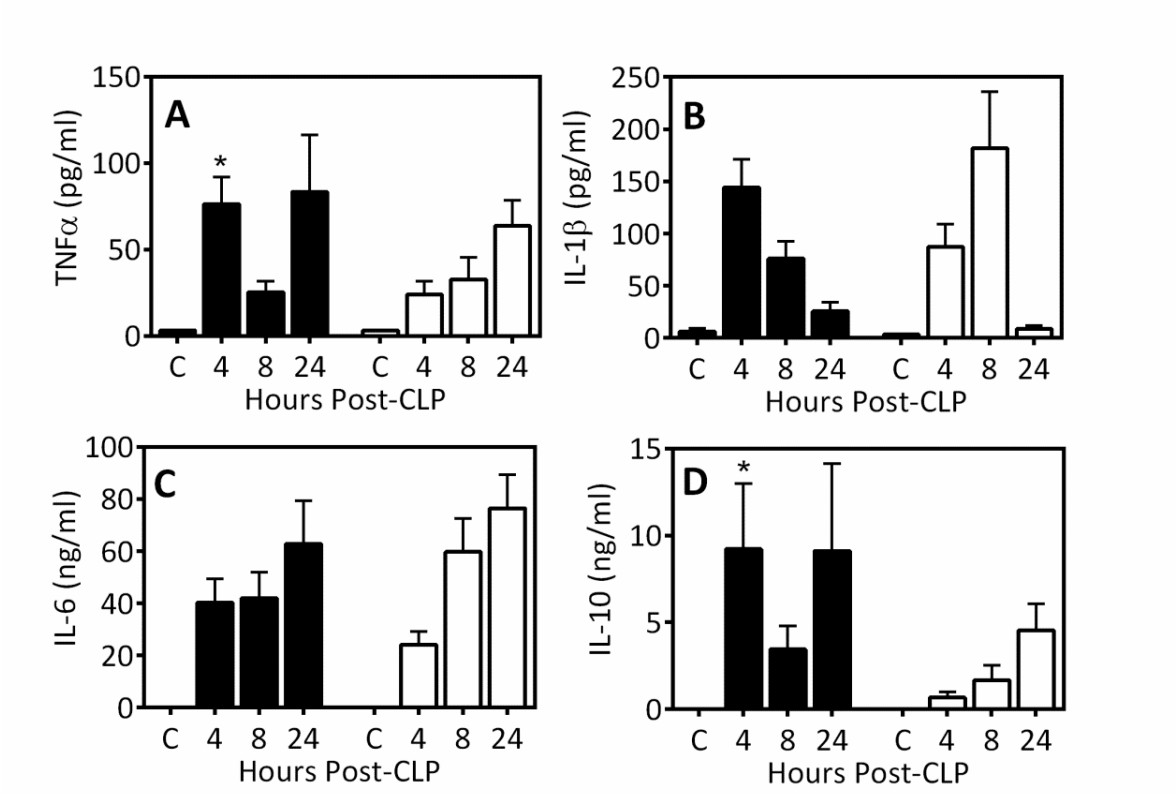

Figure 10. Absolute increases in plasma cytokine levels after CLP. Shown are plasma concentrations of (**A**) TNFα, **(B)** IL-1β, (**C**) IL-6 and (**D**) IL-10 in WT (black bars) and FXI$^{-/-}$ (white bars) littermates at various times after high-grade CLP. Eight or nine mice were tested at each time point for each genotype for CLP; n=8-9 for non-operated controls. Plasma TNFα **(A,** p=0.006) and IL-10 **(C,** p=0.0003) levels were significantly greater in WT mice than in FXI$^{-/-}$ mice 4 hr. post-CLP. Error bars represent SEM.

Plasma concentrations of SAP were ~5-fold lower in FXI$^{-/-}$ mice compared to WT mice 24 hours post-CLP (**Figure 11**). In fact, SAP levels in FXI$^{-/-}$ mice undergoing CLP were comparable to those observed in mice undergoing sham surgery. This finding suggests that the overall effect of FXI deficiency in this model is anti-inflammatory.

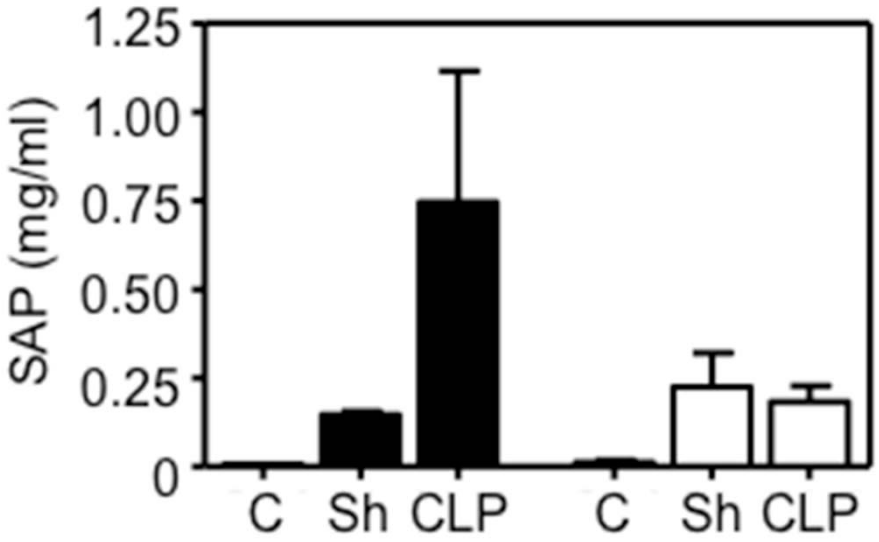

Figure 11. SAP levels post-CLP. Plasma levels of SAP measured by ELISA in control mice (C, n=4) not undergoing surgery, and 24 hr. post-CLP (n=8-9) or sham (Sh n=3) surgery. Black bars are results for WT mice and white bars for FXI$^{-/-}$ mice. Error bars represent SEM.

Evaluation of evidence for CLP-induced coagulopathy

Previously, we observed decreased fibrinogen and platelets, and elevated TAT complex in blood from WT mice 24 hours after low-grade CLP, consistent with disseminated intravascular coagulation (DIC). In the present study, which utilized high-grade CLP, we saw little evidence for severe DIC in WT or FXI$^{-/-}$ mice over the 24-hr. period post-CLP. Reductions in platelet counts were present for both genotypes (**Figure 12A**, WT baseline 559 ± 37 X 10^3/mm^3, 24 hours post-CLP 339 ± 47 X 10^3/mm^3, p=0.003;

$FXI^{-/-}$ baseline 664 ± 34 X $10^3/mm^3$, 24 hours post-CLP 362 ± 37 X $10^3/mm^3$, p=0.001), although the mean platelet counts remained above 300,000 cells/μL in both groups (**Figure 12A**). Platelet count reductions were significantly greater with CLP than with sham surgery (**Figure 12B**). For both genotypes, plasma TAT levels did not increase significantly above baseline (**Figure 12C**), and were not different from animals undergoing sham operations (**Figure 12D**). Levels of fibrinogen decreased modestly over the first 4 to 8 hours post-CLP, then increased in both genotypes (**Figure 12E**, WT: baseline 1.7 ± 0.2 mg/mL, 24 hrs. 3.3 ± 1.0 mg/mL; $FXI^{-/-}$: baseline 1.7 ± 0.2, 24 hrs. 2.1 ± 0.4). The results at 24 hours for CLP-treated mice and sham-treatment did not reach statistical significance for either genotype (**Figure 12F)**. These results are consistent with work from Patel *et al*. that showed large increases in TAT in C57Bl6 mice after LPS administration, but not after CLP (Patel, Soubra et al. 2010). While changes in fibrinogen were not significant for either genotype, there is an impression that the level at 24 hrs. was somewhat greater in WT mice than in $FXI^{-/-}$ mice. Fibrinogen levels rise with inflammation, and the modest difference could reflect the greater inflammatory response induced by CLP in WT mice.

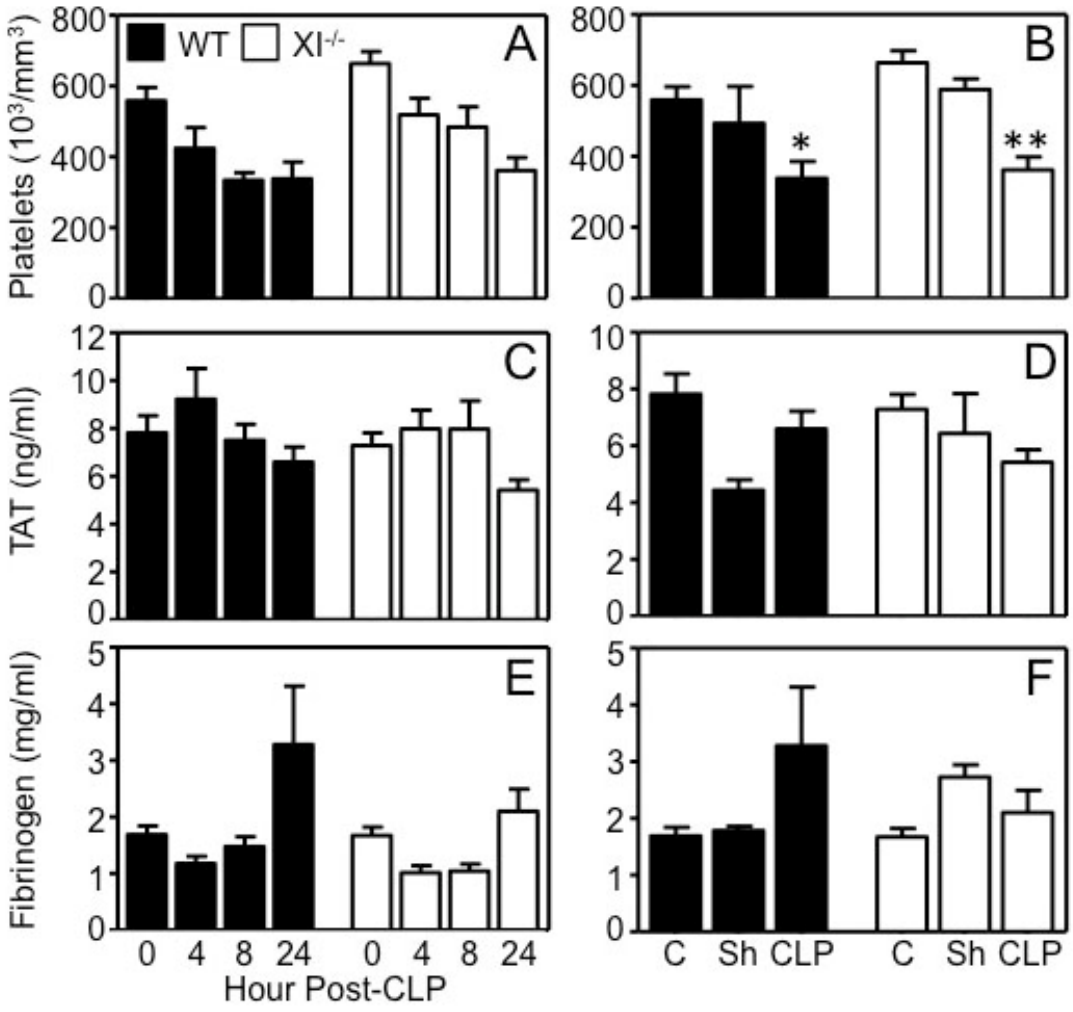

Figure 12. Effects of CLP on markers of coagulation. (A, B) Whole blood platelet counts, **(C, D)** plasma thrombin-antithrombin complex (TAT) levels, and **(E, F)** plasma fibrinogen levels in WT (black bars) and FXI$^{-/-}$ (white bars) mice after CLP or sham surgery. Panels A, C and E show values various times post-CLP. Panels B, D and F compare values 24 hour post-CLP or sham (Sh) surgery to 0 hr. controls. CLP induced significant platelet reductions in WT (*p=0.003) and FXI$^{-/-}$ (**p=0.001) mice. For panels **A** to **F**, error bars represent SEM.

At necropsy 24 hours post-CLP, lymphocyte apoptosis, a common finding in murine sepsis (Lang and Matute-Bello 2009), was noted in spleen and thymus for both genotypes (**Figure 13**). There was no evidence of hemorrhage. Histologic analysis of multiple organs including liver, kidney, brain, spleen, and thymus (**Figures 13 and 14)** did not reveal

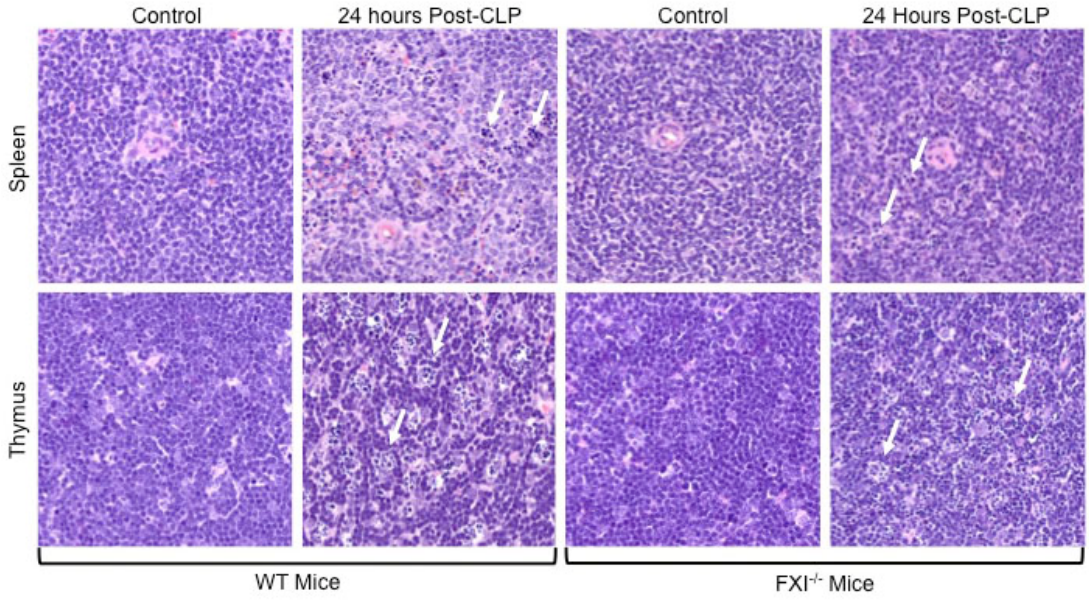

Figure 13. Histology of thymus and spleen. Photomicrographs (400X magnification) of paraffin embedded sections of spleen and thymus stained with hematoxylin and eosin before, and 24 hours after, CLP in WT and $FXI^{-/-}$ mice. Evidence of lymphocyte apoptosis (indicated by white arrows) was present in both organs for mice of both genotypes 24 hrs. post-CLP.

microvascular thrombus accumulation, which is considered essential for diagnosing DIC in laboratory animals (Berthelsen, Kristensen et al. 2011). Cumulatively, these data are consistent with published studies indicating that DIC is not a prominent feature of sepsis in mice during the first 24 hours after high-grade CLP (Ganopolsky and Castellino 2004, Patel, Soubra et al. 2010).

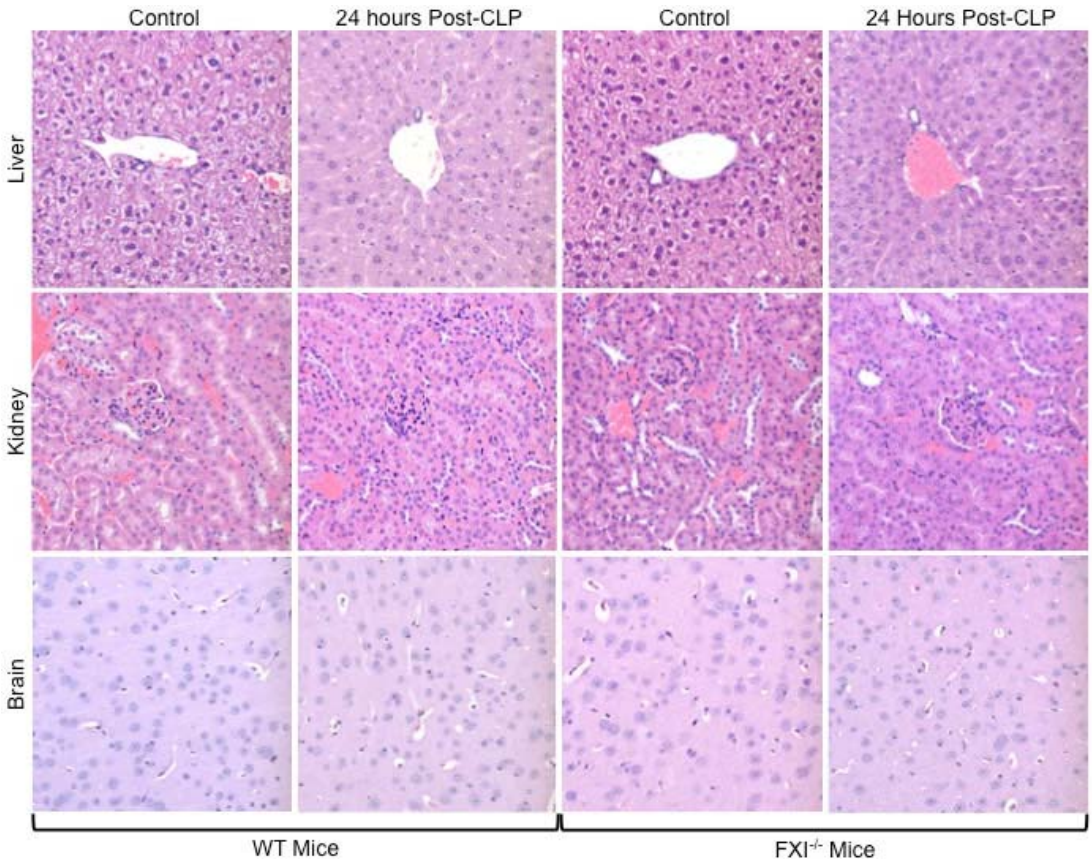

Figure 14. Histology of liver, brain, and kidney. Photomicrographs (400X magnification) of paraffin embedded sections of mouse liver, kidney and brain stained with hematoxylin and eosin before, and 24 hours after, CLP in WT and $FXI^{-/-}$ mice. There was no evidence of disseminated intravascular coagulation in animals of either genotype following cecal ligation and puncture.

Effects of CLP on plasma levels of contact protease zymogens

During contact activation-initiated coagulation *in vitro*, the plasma zymogen FXII is activated on a surface to generate the protease FXIIa, which cleaves PK and FXI to release α-kal and FXIa, respectively (Bjorkqvist, Nickel et al. 2014, Schmaier 2014, Wu 2015). According to current models of contact activation, α-kal amplifies the process by reciprocally activating FXII and also contributes to inflammation by cleaving HK to release the pro-inflammatory peptide BK. FXIa augments thrombin generation by activating FIX, but is not considered important for FXII activation.

During sepsis, blood may be exposed to a variety of substances that induce contact activation, including polyphosphate (polyP) released from platelets (Muller, Mutch et al. 2009) or microorganisms (Herwald, Morgelin et al. 2003, Frick, Bjorck et al. 2007, Yun and Morrissey 2009), and extracellular nucleic acids including DNA and RNA from damaged tissue and chromatin extruded from activated neutrophils (neutrophil extracellular traps)(Esmon 2013, Gould, Lysov et al. 2015). Noting that *in vivo* activation of contact system proteins has been assessed by measuring reductions in plasma levels of their respective zymogens (Oschatz, Maas et al. 2011), we measured changes in plasma FXII, PK, and FXI levels to assess contact system activation. In WT mice, FXI decreased by ~50% by 8 hours after CLP (**Figures 15A and 15B**), and PK was comparably reduced by 24 hours (**Figures 15B and 15C**). While FXII levels remained similar to baseline in WT mice post-CLP, we observed a modest increase in plasma FXII levels in $FXI^{-/-}$ mice (**Figure 15D)**. FXII levels were also moderately increased in sham-treated animals (not shown), suggesting a reaction to surgery. The data, therefore, indicate that there is activation and consumption of FXII in WT mice after CLP that was not as prominent in $FXI^{-/-}$ mice. Interestingly, PK levels were not reduced 24 hours post-CLP in $FXI^{-/-}$ mice (**Figure 15D)**. Taken as a whole,

the data are consistent with activation of the contact proteases during CLP-induced sepsis in WT mice, but suggest that FXI deficiency blunts this process.

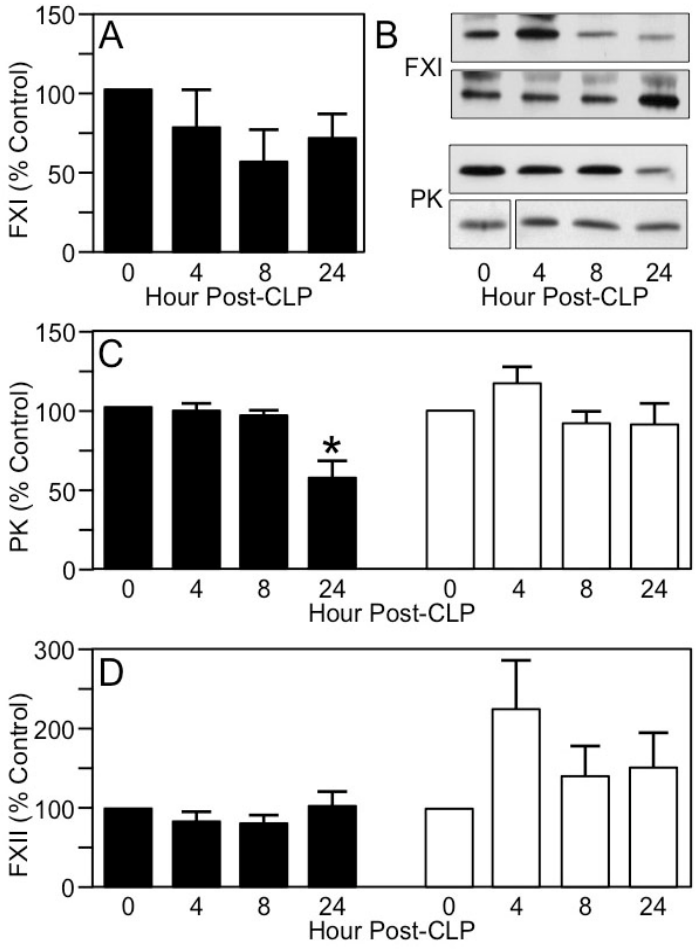

Figure 15. The effect of CLP on plasma contact proteases. Plasma FXI, PK and FXII levels at various times after high-grade CLP were determined using densitometry of western blots and are reported as percent of baseline (0 hr.) control. **(A)** Plasma FXI levels after CLP in WT mice **(B)** Examples of western blots for FXI and PK for WT mouse plasma at various times after CLP (upper panels of each pair) or sham surgery (lower panels of each pair). **(C)** Plasma PK levels after high-grade CLP in WT (black bars) or $FXI^{-/-}$ (white bars) mice. The asterisk above the 24-hr. bar for PK in WT animals indicates the value is significantly different than 0 hr. control ($p<0.05$). **(D)** Plasma FXII levels after high-grade CLP in WT (black bars) or $FXI^{-/-}$ (white bars) mice. In panels A, C, and D, each bar represents data for eight mice. For all panels, error bars represent SEM.

FXII activation by α-kal and FXIa

To explore the possibility that FXI may have contributed to contact activation in our study, we first evaluated FXII activation by α-kal or FXIa *in vitro* in the presence of the polyanions DNA, RNA, and polyphosphate. FXII was converted to FXIIa at comparable relatively low rates by α-kal and FXIa in the absence of a polyanion (**Figure 16A**). The FXIa-mediated reaction was enhanced 56-fold by DNA (**Figure 16B**) and 90-fold by RNA (**Figure 16C**). With α-kal, DNA and RNA enhanced FXII activation modestly (2.8 and 5.6-fold, respectively). In reactions with polyP, contributions of α-kal and FXIa to FXII activation appeared comparable, but were difficult to assess because FXII autoactivation was prominent (**Figure 16D**). FXI and PK are homologs (Emsley, McEwan et al. 2010), and can be difficult to separate chromatographically when purified from plasma. To address the concern that FXIa was contaminated with α-kal, we ran reactions in the presence of an anti-kallikrein IgG (H03) that binds to the protease active site (Kenniston, Faucette et al. 2014). H03 blocked cleavage of chromogenic substrate S-2302 by α-kal, but not FXIa or FXIIa (**Figure 17A**). H03 blocked FXII activation by α-kal (**Figure 17B**) but did not affect FXII activation by FXIa (**Figure 17C**), thus illustrating that FXIa can cleave FXII under conditions that block the enzymatic activity of α-kal.

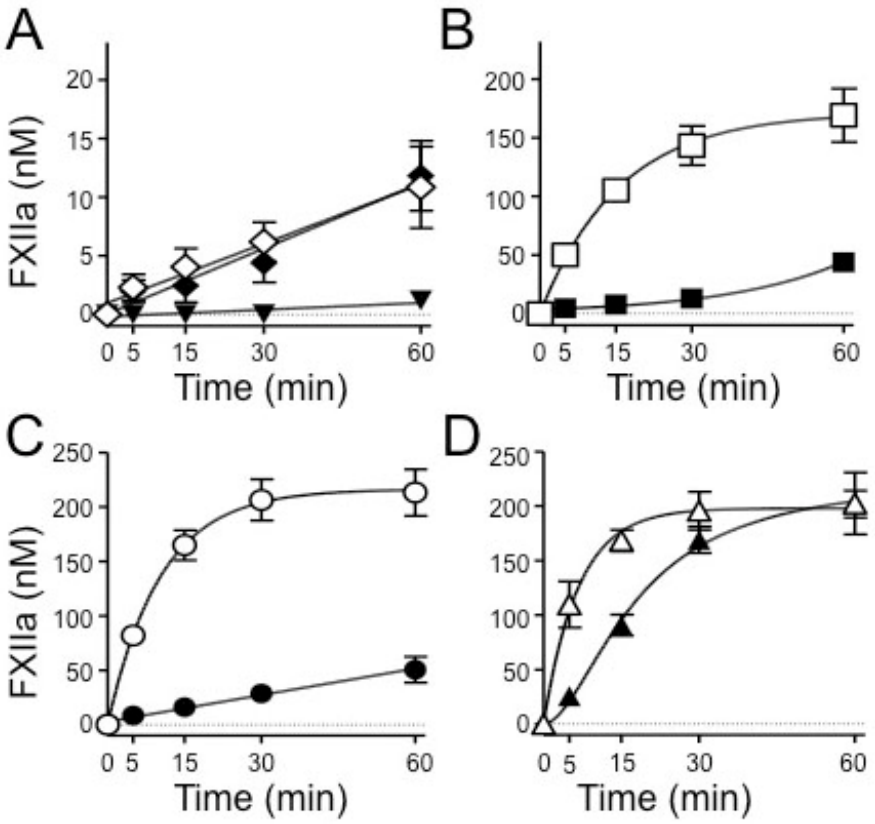

Figure 16. FXII activation by α-kal or FXIa in the presence of polyanions. (A) FXII (200 nM) was incubated with vehicle (▼), 1nM FXIa (◇), or 2 nM α-kal (◆). **(B-D)** FXII (200 nM) incubated with 5 ug/ml **(B)** DNA (□,■), **(C)** 5 ug/ml RNA (○,●) or **(D)** 20 μg/ml Poly-P (△,▲), in the presence of 1 nM FXIa (□,○,△) or 2 nM α-kal (■,●,▲). At the indicated times, samples were tested for FXIIa activity by chromogenic assay. Error bars are +/– one standard deviation.

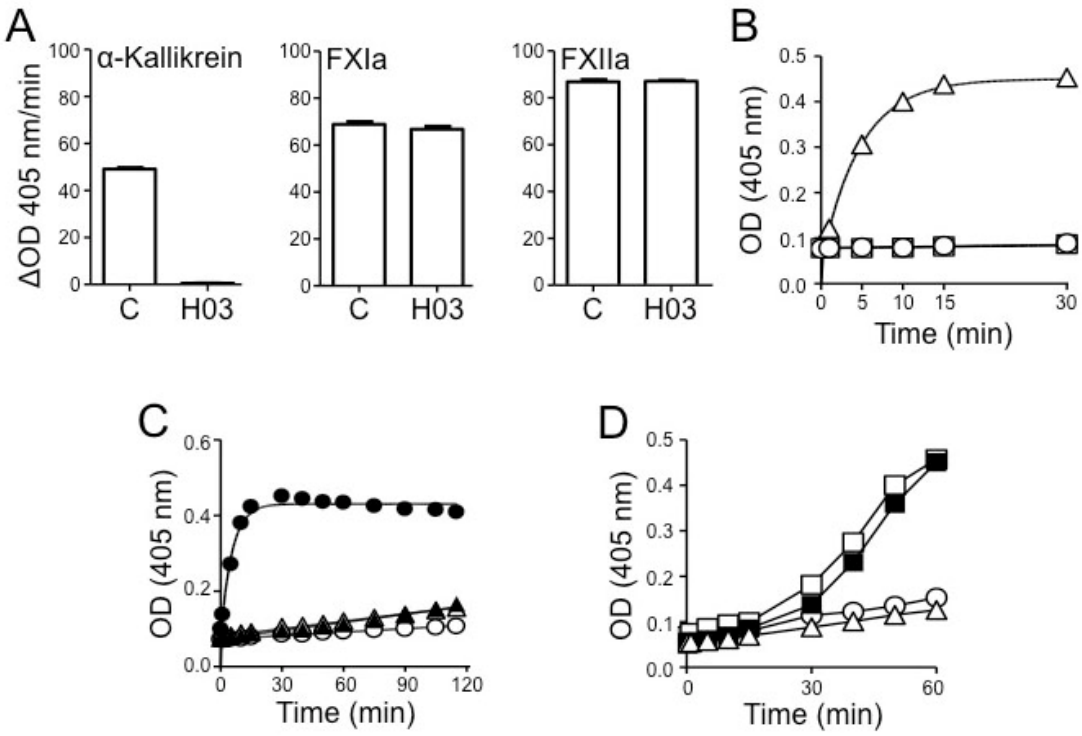

Figure 17. Effect of anti-kallikrein antibody H03. (A) FXIIa (100 nM), α-kal (5 nM) or FXIa (5 nM) were incubated at room temperature for 3 min with 10-fold molar excess of anti-kal antibody (H03) respectively or control vehicle (C) in a buffer with 10 μM $ZnCl_2$, and residual activity was determined by chromogenic substrate. Error bars are +/– one standard deviation. **(B)** α-al (5 nM) and 10 μM $ZnCl_2$ were incubated with the chromogenic substrate S-2302 (200 uM) in the presence of vehicle (△), 100 nM H03 (○) or 100 nM H03 and 10 ug/ml DNA (□) and changes in OD 405 nm were monitored. **(C)** FXII (200 nM) and 10 μM $ZnCl_2$ were incubated with vehicle (○), 5 nM α-kal (●), 5 nM α-kal and 100 nM H03 (△), or 5 nM α-kal with 100 nM H03 and 10 ug/ml DNA (▲) in the presence of S-2302 (200 uM). Changes in OD 405 nm were monitored. **(D)** FXII (200 nM) and 10 μM $ZnCl_2$ were incubated with vehicle (△), 5 nM FXI (○), 5 nM FXI and 10 ug/ml DNA (□), or 5 nM FXI and 10 ug/ml DNA with 100 nM H03 (ν) in the presence of S-2302 (200 uM). Changes in OD 405 nm were monitored

Three-quarters of PK and nearly all FXI in plasma circulates as a complex with the glycoprotein HK (Mandle, Colman et al. 1976, Thompson, Mandle et al. 1977, Thompson, Mandle et al. 1979). HK is thought to be required for proper binding of PK and FXI to the contact surface during contact activation. Consistent with the importance of HK during contact activation on non-biologic surfaces such as kaolin (Meier, Pierce et al. 1977, Wiggins, Bouma et al. 1977), HK enhanced FXII activation by α-kal with polyP (**Figure 18A**), DNA (**Figure 18B**) or RNA (**Figure 18C**). In contrast, HK had no appreciable effect on FXII activation by FXIa in the presence of polyP (**Figure 18D**), and a modest inhibitory

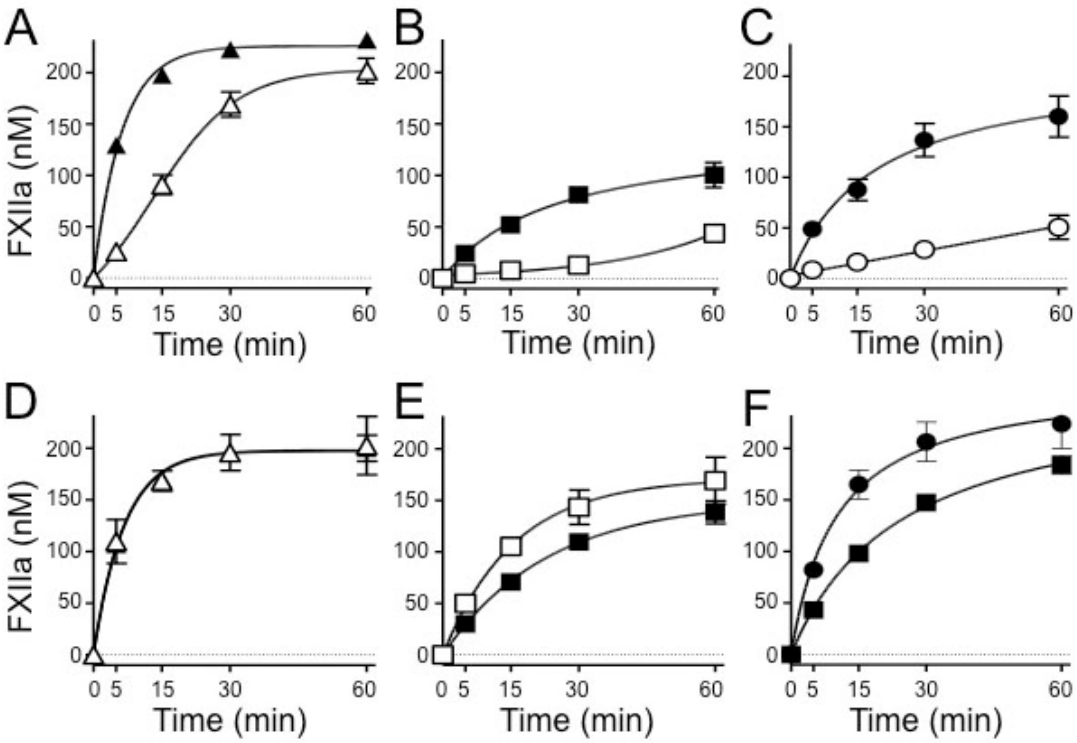

Figure 18. Effect of HK on FXII activation in the presence of polyanions. Plasma FXII (200 nM) was incubated with (**A-C**) 2 nM α-kal or (**D-F**) 1 nM FXIa and **(A, D)** 20 μg/ml poly-P, **(B, E)** 5 μg/ml DNA or **(C, F)** 5 μg/ml RNA in the absence (○,□,△) or presence (●,■,▲) of 20 nM HK. At the indicated times, samples were tested for FXIIa activity by chromogenic assay. Error bars are +/– one standard deviation.

effect with DNA (**Figure 18E**) or RNA (**Figure 18F**). These data suggest that α-kal (in an HK-dependent manner) and FXIa (in an HK-independent manner) can both activate FXII *in vitro*, and support the notion that FXIa may be an activator FXII *in vivo*.

Effects of polyphosphate infusion on FXII in mice.

To determine if polyanions induce FXIa-mediated FXII activation *in vivo*, we injected polyphosphate into WT mice and mice lacking PK, FXI, or FXII, and the effect on plasma FXII was analyzed by Western blot (**Figure 19**). Intravenous infusion of polyphosphate induces rapid FXII-dependent thrombosis in mice (Muller, Mutch et al. 2009), indicating that polyphosphate induces contact activation in this model. In WT mice,

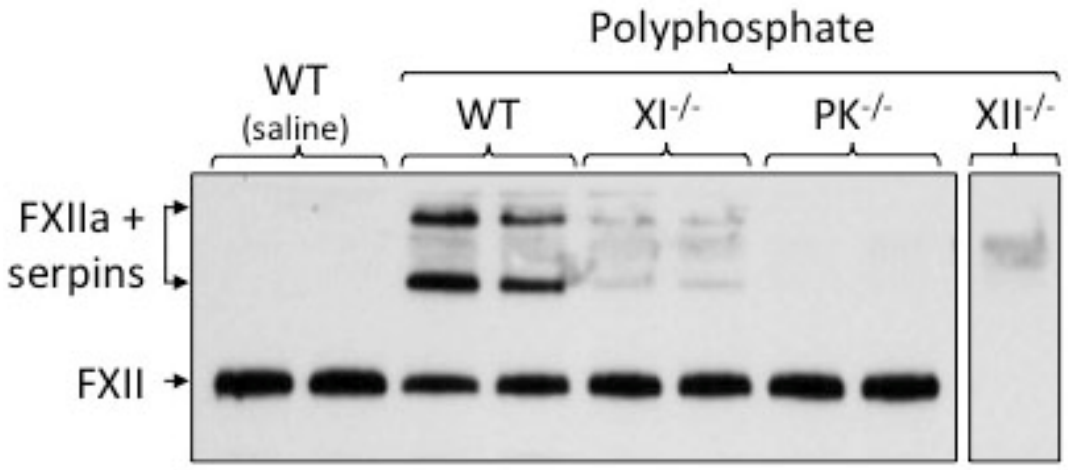

Figure 19. Polyphosphate-induced changes in FXII in mice. WT C57Bl/6 mice or C57Bl/6 mice lacking FXI ($XI^{-/-}$), PK ($PK^{-/-}$) or FXII ($XII^{-/-}$) received a bolus infusion of phosphate buffered saline (saline) or PBS containing 60 μg of polyphosphate into the inferior vena cava. Five minutes later blood was drawn from the inferior vena cava into sodium citrate anticoagulant. Plasma samples were analyzed by western blot for FXII under non-reducing conditions. The blot contains samples for two mice of each genotype. The position of the FXII zymogen band is indicated on the left (FXII). Free FXIIa would also run in this position. The higher molecular mass FXII specific-species likely represent FXIIa in SDS-stable complexes with plasma serine protease inhibitors (serpins).

infusion of polyP, but not saline, caused a modest decrease in intensity of the band representing FXII, and produced higher molecular weight species that likely represent FXIIa in SDS-stable complexes with serpins such as C1-inhibitor and antithrombin. There were no bands on blots with plasma from polyP treated FXII-deficient mice, indicating the high molecular mass bands on blots for WT mice are specific FXII/XIIa signals. No changes were apparent in the FXII band in PK-deficient mice after polyP infusion, and no higher molecular mass species appeared. This is consistent with the impression that PK is required for normal FXII turnover in healthy mice (Revenko, Gao et al. 2011). Surprisingly, FXI deficiency also affected polyphosphate-induced changes in FXII, although perhaps not quite as profoundly as did PK deficiency. Bands for the higher molecular weight species were markedly less intense in blots for $FXI^{-/-}$ mice than for WT mice. These data support the hypothesis that α-kallikrein and FXIa are required for optimal FXII activation after polyphosphate infusion in mice, and are consistent with the notion that FXI deficiency may interfere with activation of contact proteases in the CLP model.

Discussion

The protease FXIa, which plays a relatively modest role in hemostasis, has been identified as contributing to pathologic coagulation in humans (Meijers, Tekelenburg et al. 2000, Doggen, Rosendaal et al. 2006, Suri, Yamagishi et al. 2010, Bane and Gailani 2014, Key 2014). Consistent with this, we previously reported evidence for DIC in WT mice after CLP but not in similarly treated $FXI^{-/-}$ mice, or WT mice receiving the anti-FXI IgG 14E11 (Tucker, Gailani et al. 2008, Tucker, Verbout et al. 2012). We concluded that the survival advantage after CLP conferred by absence of FXI was primarily due to blunting of sepsis-induced coagulation. However, WT mice treated with 14E11 also had lower plasma levels of

TNFα and IL-6 twelve hours post-CLP than vehicle-treated mice (Tucker, Verbout et al. 2012), suggesting FXI inhibition affected cytokine responses. The study presented here explored the importance of FXI in early cytokine and coagulation responses after CLP.

The survival data from WT mice show that mortality increased as injury severity increased. This is in keeping with the literature, which indicates that mortality after CLP correlates with the proportion of the cecum that is ligated (Rittirsch, Huber-Lang et al. 2009, Toscano, Ganea et al. 2011). In contrast to this paradigm, the data show that FXI deficiency confers a survival advantage after high-grade, but not low grade, CLP. This suggests the contribution of FXI is more prominent in severe sepsis. The findings are consistent with a previous observation that $FXI^{-/-}$ mice had better survival when exposed to a dose of *Listeria monocytogenes* that was uniformly lethal in WT mice, but not after inoculation with a lower concentration of organisms (Luo, Szaba et al. 2012). It is unclear why FXI deficiency did not reduce mortality with low-grade injury in the current study. In contrast to the earlier study, WT and $FXI^{-/-}$ mice were littermates in the work presented here. It is plausible that differences in gut flora between separate inbred lines of WT and $FXI^{-/-}$ affected earlier results. In septic patients, death is likely due to a constellation of factors, including hypotension, the systemic inflammatory response, DIC, and the specific microorganism involved (Russell 2006). We did not determine causes of death post-CLP, but previous work provides some insight. Death in our study occurred during the "early" or "acute" phase (within the first 4 or 5 days) of sepsis (Osuchowski, Welch et al. 2006, Xiao, Siddiqui et al. 2006). Deaths during this phase correlate with elevations in pro- and anti-inflammatory cytokines (Osuchowski, Welch et al. 2006). The altered cytokine pattern after high-grade CLP in $FXI^{-/-}$ mice suggests that a FXI contribution to systemic inflammation figures prominently in mortality in WT animals.

The difference in levels of the acute phase protein SAP in WT and $FXI^{-/-}$ mice 24 hours post-CLP suggests that the altered cytokine response in $FXI^{-/-}$ mice was associated with a less robust inflammatory response compared to WT mice. Previous work with mouse models indirectly suggested that FXI contributions to inflammation. Mice lacking the key coagulation regulatory protease protein C typically die *in utero* from extensive thrombosis. Chan and coworkers (Chan, Ganopolsky et al. 2001) reported that superimposing total FXI deficiency on protein C deficiency partially overcomes this phenotype, with some animals living to adulthood. Interestingly, despite the well described anti-inflammatory effect of protein C, mice lacking both protein C and FXI had similar plasma and tissue TNFα and IL-6 levels to littermates with normal protein C levels. Kleinschnitz and co-workers (Kleinschnitz, Stoll et al. 2006) showed that mice lacking either FXII or FXI display a marked reduction in cerebral ischemia-reperfusion injury compared to WT mice after temporary occlusion of the middle cerebral artery. The results were attributed to reduced fibrin deposition in the null animals, but injury in this model is known to have a significant inflammatory component (Carmichael 2005). The mechanisms by which FXI contributes to inflammation in the CLP-model are not clear. In earlier work with a low-grade injury CLP model, we noted evidence of DIC in WT mice that was not present in FXI-deficient animals, raising the possibility that the pro-inflammatory effects of FXI are related to its well-characterized role in generating thrombin. Surprisingly, we did not observe evidence for consumptive coagulation during the 24 hours post-CLP in WT or $FXI^{-/-}$ mice; findings that are discrepant with our prior experience. Several factors may have contributed to the different results. First, CLP, unlike LPS, does not consistently induce DIC in mice (Ganopolsky and Castellino 2004, Patel, Soubra et al. 2010). It is also possible that differences in gut flora between inbred lines were a factor in our original studies.

Alternatively, our earlier studies used a lower grade injury than was used in the present work, and differences in injury level may create models with different features (Rittirsch, Huber-Lang et al. 2009). These issues aside, an important point that can be taken from the cumulative experience is that FXI deficiency confers a survival advantage after CLP, regardless of the presence or absence of DIC. This in turn suggests that the detrimental effects of FXI in this model are not primarily tied to consumptive coagulation.

Our findings indicate that it is likely that FXI makes thrombin-independent contributions to inflammation. For example, while FXIa is classically considered a product of contact activation, and not a contributor to the process itself, our results raise the possibility that FXIa may contribute to the turnover of the two main zymogens (FXII and PK) involved in contact activation. During contact activation, FXIIa converts PK to α-kal, which then cleaves HK, liberating the nonapeptide BK, and forming cleaved kininogen (HKa) and antimicrobial peptides. BK is a potent vasodilator and inducer of vascular permeability (Couture, Blaes et al. 2014, Sharma 2014), and contact activation-induced hypotension may be an important contributor to mortality in sepsis. Contact activation is probably triggered by traces of FXIIa and/or α-kal present in plasma, and are enhanced by substances such as the polyanions used in our experiments. HK is an essential co-factor for the process, facilitating PK binding to the surface (Meier, Pierce et al. 1977, Wiggins, Bouma et al. 1977). Our data indicate FXIa activates FXII in the presence of certain types of surfaces in an HK-independent manner. In 1978 Griffin reported that FXIa activates FXII on celite (diatomaceous earth), although significantly more slowly than the α-kal-mediated reaction (Griffin 1978). In our experiments with polyP or nucleic acids, FXIa was a significantly better activator of FXII than α-kal in the absence of HK, with the difference largely disappearing when HK was included in reactions. The reason for this is not certain,

but FXI does contain anion-binding sites not found on PK that may allow it to interact properly with polyanions in the absence of HK (Geng, Verhamme et al. 2013). In addition to the effects mentioned above, products of contact activation may also contribute to increased cytokine levels. FXIIa (Toossi, Sedor et al. 1992) and peptides derived from HK cleavage (Khan, Bradford et al. 2006) can elicit cytokine release in human mononuclear cells. FXIa may indirectly contribute to a cytokine response by affecting cleavage of these contact proteins.

Targeting thrombin generation with anticoagulants such as heparin appears to be of some benefit in patients with severe sepsis (Wang, Chi et al. 2014), probably by limiting tissue damage from thrombosis, DIC, and perhaps inflammation. However, such therapy increases bleeding in patients who are already coagulopathic, limiting its applicability. The anti-inflammatory effects of activated protein C (rhAPC, a.k.a., Xigris®) in sepsis were probably partially offset by increased bleeding due to proteolysis of factors Va and VIIIa (Warren, Suffredini et al. 2002). Our work suggests that FXI inhibition may provide a benefit early in sepsis by altering the inflammatory response. While the CLP-model used in the current study did not include a major coagulopathic component, earlier work suggests that FXI inhibition may also blunt the DIC that frequently accompanies severe sepsis in humans. The importance of FXI to hemostasis in the setting of sepsis in humans remains to be established, but based on clinical observations of patients with congenital FXI deficiency, FXI inhibition would likely be safer than anticoagulation with heparin.

Additional Experiments

Effect of CLP on plasma C5a and chemokine levels.

The blunted early cytokine response in $FXI^{-/-}$ mice undergoing CLP raises the possibility of a suboptimal initial immune response to infection. With this in mind, we measured factors known to affect leukocyte migration in response to CLP. C5a is a proteolytic fragment of the complement protein C5 that enhances neutrophil migration and adherence. It is also an anaphylatoxin that has been linked to deleterious outcomes in experimental sepsis (Ward 2010). As FXIa cleaves C5 to form C5a *in vitro* (Amara, Flierl et al. 2010), we measured plasma C5a levels by ELISA (R & D Systems, Minneapolis, MN) 24 hours after CLP or sham surgery in WT and $FXI^{-/-}$ mice. Both types of surgery induced comparable elevations of plasma C5a 24 hours post-procedure in WT and $FXI^{-/-}$ mice (**Figure 20A**). Levels of the murine neutrophil chemoattractants KC (**Figure 20B**) and MIP-2 (**Figure 20C**) were elevated in WT and $FXI^{-/-}$ mice within four hours of CLP (**Figures 20E & 20G**), with levels reaching comparable levels by 24 hours. We also measured levels of the monocyte chemoattractant protein MCP-1, which is reported to affect leukocyte recruitment through a mechanism that is at least partly independent of MIP-2 and KC (Matsukawa, Hogaboam et al. 1999). While MCP-1 levels were somewhat lower in $FXI^{-/-}$ mice compared to WT animals at 4 and 24 hours after CLP **(Figure 20D**) the difference was not statistically significant. Cumulatively, the data indicate that processes that stimulate leukocyte migration are largely intact in $FXI^{-/-}$ mice, despite the blunted initial cytokine response.

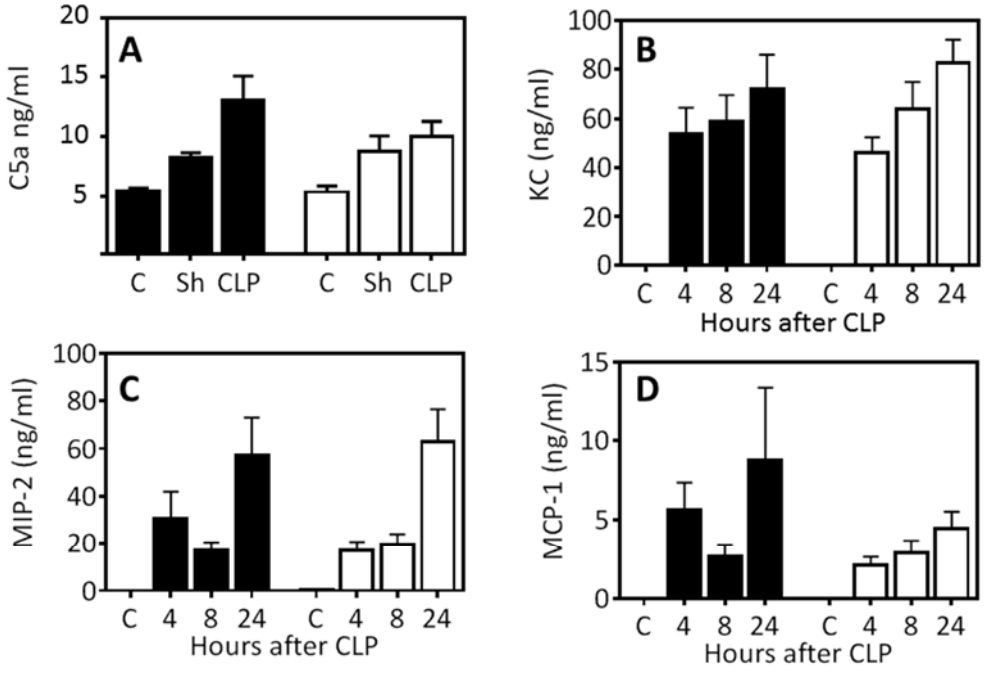

Figure 20. Complement component C5a and chemokine responses to CLP. For all panels, results for WT mice are represented by black bars and for $FXI^{-/-}$ mice by white bars. C5a levels were measured by ELISA, while chemokine levels were measured by Luminex technology. **(A)** Plasma levels of C5a in control mice (C) not undergoing surgery, and 24 hr. post-CLP or sham (Sh) surgery. For both genotypes, sham surgery and CLP both caused significant increases in C5a compared to untreated control (C) mice ($p<0.05$); however, differences in C5a after CLP or sham surgery were not significant for either genotype, nor between genotypes. Plasma levels of KC **(B)**, MCP-1 **(C)**, and MIP-2 **(D)** at various time points after CLP.

Leukocyte migration into the peritoneal cavity

Since the chemokine response appeared to be intact, we assessed leukocyte infiltration into the peritoneum after high-grade CLP (**Figure 21**). We noted significant increases in total leukocytes in the peritoneal cavity in both WT (baseline 9 ± 0.7 X 10^3 cells, 24 hours post-CLP 50 ± 12 X 10^3, $p=0.0007$) and $FXI^{-/-}$ (baseline 9 ± 1.1 X 10^3 cells, 24 hours post-CLP 29 ± 4 X 10^3 $p=0.0002$) mice 24 hours post-CLP, (**Figure 21A**). While the increase appeared to be somewhat greater in WT mice (50 ± 12 X 10^3 for WT vs. 29 ± 4 X 10^3 for $FXI^{-/-}$), the difference was not significant ($p=0.4$), a trend that was repeated in the

various leukocyte subsets [neutrophils (**Figure 21B**), lymphocytes (**Figure 21C**), and monocytes (**Figure 21D**]. Notably, there was a significant increase in lymphocyte infiltration above baseline in WT mice (baseline 6 ± 0.5 X 10^3 cells, 24 hrs. post-CLP 14 ± 4 X 10^3 cells, p=0.008) that was not observed in FXI$^{-/-}$ mice (baseline 6 ± 0.7 X 10^3 cells, 24 hrs. post-CLP 8 ± 1 X 10^3 cells, p=0.2) (**Figure 21C**). Taken as a whole, the data do not support a clear influence of FXI on leukocyte migration after CLP, but do introduce the possibility that lymphocyte response may be more robust in WT mice.

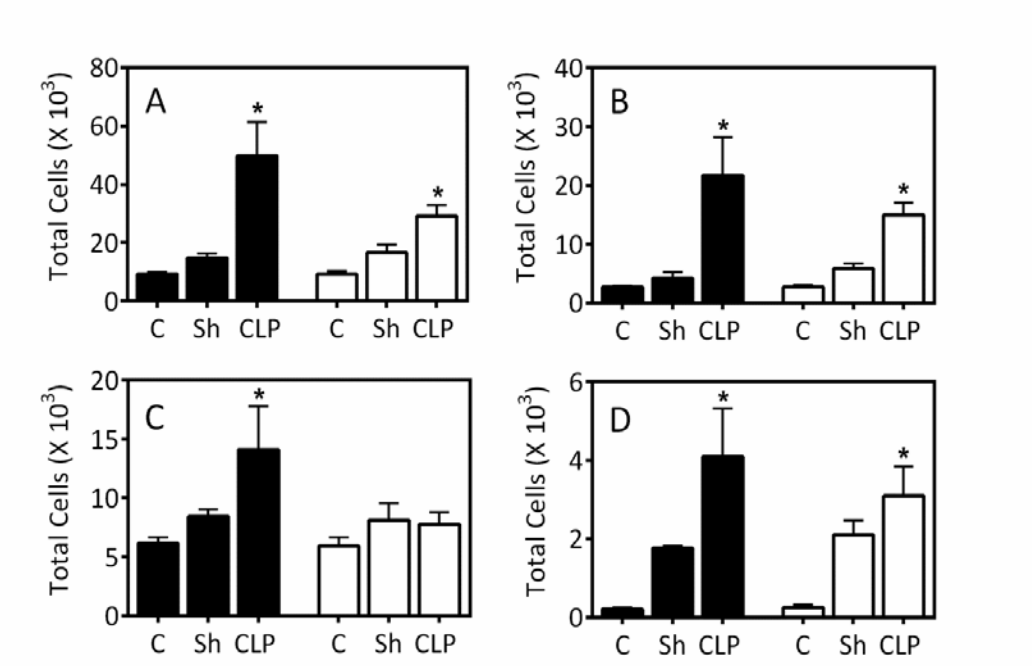

Figure 21. Peritoneal leukocyte infiltration after CLP. Leukocyte counts in fluid aspirated after peritoneal wash with 5ml NaCl, 2mM EDTA, as measured by an automated hematology analyzer (Hemavet 950FS). Data are from 24 hours after CLP in WT (black bars) and FXI$^{-/-}$ (white bars) mice compared to untreated controls (noted by the letter "C") and to samples taken 24 hours after sham (Sh) surgery. **(A)** Total leukocyte counts were significantly greater than control for both WT (p=0.0007) and FXI$^{-/-}$ (p=0.0002) mice. This was also true for **(B)** neutrophils and **(D)** monocytes. However, only WT mice had a significant elevation in lymphocyte counts (*p=0.0008) compared to controls. For all panels, error bars represent SEM.

Evaluation of microorganisms during CLP-induced sepsis.

As mortality after CLP has been associated with bacteremia (Cuenca, Delano et al. 2010), we performed aerobic and anaerobic CFU counts on blood collected 24 hours after CLP or sham surgery in groups of 6 mice to see if differences in the level of bacteremia might explain the survival difference between WT and $FXI^{-/-}$ mice. No bacteria were cultured from blood obtained from either group after sham surgery, while 4 of 6 WT and 6 of 6 $FXI^{-/-}$ mice had positive cultures for either aerobic or anaerobic bacteria 24 hours after CLP. There was no difference in CFU counts between WT and $FXI^{-/-}$ mice in this study (**Figure 22**).

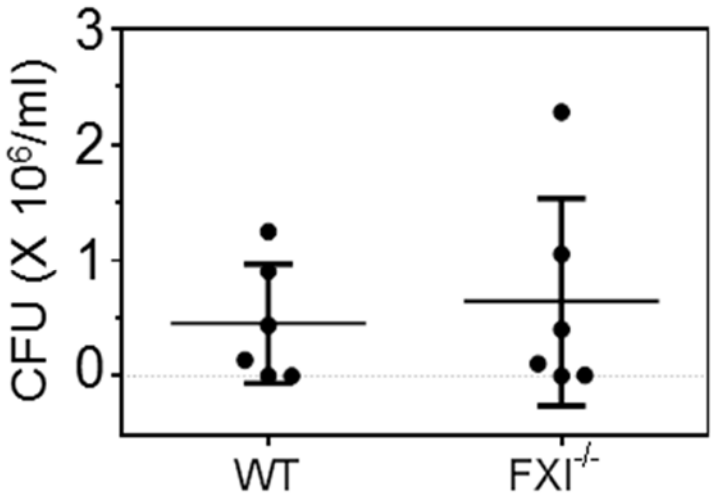

Figure 22. Evaluation of microorganisms during CLP-induced sepsis. Whole blood obtained 24 hours post-CLP or sham surgery was diluted 1:10 in pre-reduced anaerobically sterilized (PRAS) media (Anaerobe Systems, Morgan Hill, CA) (Hyde, Stith et al. 1990) for aerobic and anaerobic bacteria colony forming unit (CFU) counts. Samples were processed by 10-fold serial dilution in PBS, pH 7.00. Aliquots (100 µl) of each dilution were plated on BBL™ Columbia Sheep Blood Agar (SBA), BBL™ CDC Anaerobic SBA, and BBL™ CDC Anaerobic SBA with kanamycin and vancomycin. Columbia SBA was incubated aerobically at 35.0 °C, while other plates were incubated anaerobically at 35.0 °C for 48 hours. CFU counts were recorded for plates containing 30-300 CFUs. Data are presented as combined aerobic and anaerobic CFU counts in WT and $FXI^{-/-}$ mice. There were

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CHAPTER VI

CONCLUSIONS AND FUTURE DIRECTIONS

Factor XI is at a Junction Between Coagulation and Inflammation

The primary finding of this work is that FXI influences systemic inflammation during murine polymicrobial sepsis through mechanisms that are not exclusively related to thrombin generation, a finding that has not been reported to our knowledge. We present compelling evidence that suggests this influence is at least partly due to an effect on contact activation. Taken together with our earlier findings that assigned a more "traditional" role for FXI in sepsis-induced DIC, the accumulated data place FXI at a junction between pro-inflammatory and pro-coagulant mechanisms, where it is positioned to contribute to either or both, depending on the nature of the insult. **Figure 23** summarizes the various mechanisms by which FXI may influence the two pathways. First, FXI is a component of the thrombin generating mechanism that is triggered during hemostasis (**Figure 23, panel 1**). Here, FXI is activated by thrombin, and the resulting FXIa cleaves FIX to sustain thrombin production by participating in a feedback loop. Thrombin generation may ultimately amplify the inflammatory response by, for example, enhancing cytokine production through PAR cleavage on vascular endothelial cells and leukocytes (**Figure 23, panel 3**). FXIa could also serve as a bridge between contact activation and thrombin generation (**Figure 23, panel 2**). In this case, FXIa is generated during contact activation on polyanions (such as platelet or bacterial polyphosphates, and nucleic acids) and bacterial surfaces, where it then exerts a procoagulant effect by cleaving FIX. Our results indicate that FXIa also indirectly contributes to inflammation by promoting the activation of the contact proteins FXII and PK,

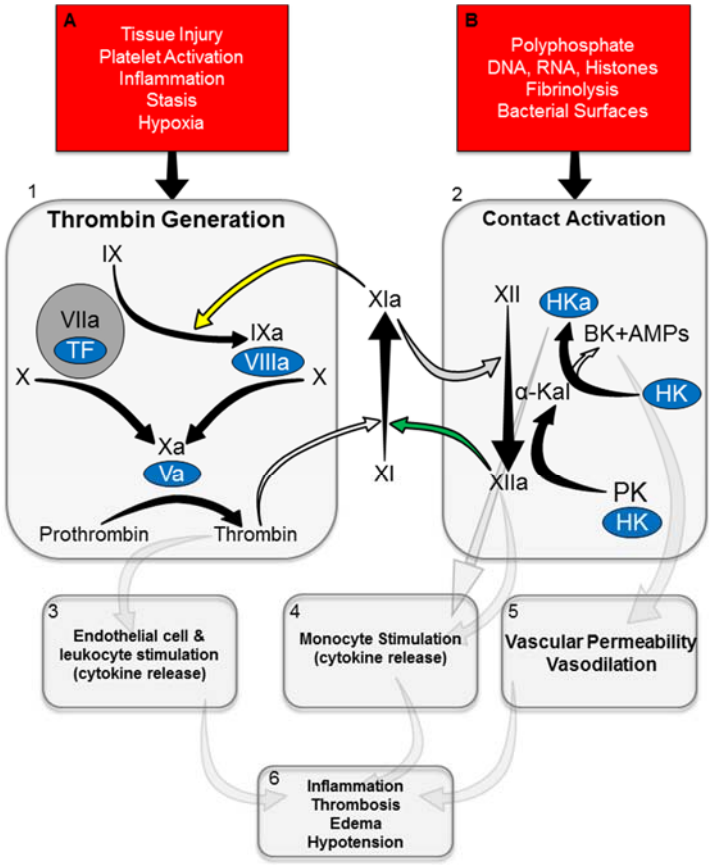

Figure 23. FXI in thrombin generation and contact activation during sepsis. Thrombin Generation (**Panel 1**). Depicted are proteolytic reactions that generate thrombin at a site of vascular injury. The process is initiated by activation of factors X and IX by the factor VIIa/tissue factor (TF) complex. Vitamin-K dependent protease zymogens are shown in black type with the active protease forms indicated by a lower case "a". The blue ovals represent cofactors. During hemostasis, FXI is converted to FXIa by thrombin (white arrow). FXIa then activates FIX (yellow arrow). Contact activation (**Panel 2**). On a surface, FXII and PK undergo reciprocal activation to FXIIa and α-kal. HK serves as a cofactor for this reaction. FXIIa can promote thrombin generation by activating FXI (green arrow). α-kal cleaves HK liberating bradykinin (BK) and antimicrobial peptides (AMPs). FXIa, can also contribute to contact activation through activation of FXII (gray arrow). **Panels A and B** list factors that could trigger or enhance thrombin generation (**A**) or contact activation (**B**). **Panels 3, 4, and 5** list processes mediated by thrombin (**3**), FXIIa and HKa (**4**) or α-kal (**5**) that could contribute to sepsis. **Panel 6** lists some of the consequences of those processes.

perhaps resulting in BK-mediated vasodilation and tissue edema, as well as cytokine release (**Figure 23, panels 4 and 5**).

Future Studies

More work is needed to dissect the precise role of FXIa in cytokine release and inflammation, particularly considering earlier data, which suggest there are FXIIa-independent mechanisms through which FXI/FXIa operates. Host responses in WT mice treated with the anti-FXI monoclonal antibody 14E11, which blocks activation of FXI by FXIIa (Kravtsov, Matafonov et al. 2009), were not identical to those of FXI deficient mice after CLP or infection with lethal doses of *L. monocytogenes* (Tucker, Gailani et al. 2008, Luo, Szaba et al. 2012, Tucker, Verbout et al. 2012). FXI deficient mice had a survival advantage compared to WT mice after inoculation with high concentrations of *L. monocytogenes*, but pre-treatment of WT mice with 14E11 was only protective in similarly-infected mice when sub-therapeutic doses of ampicillin were given (Luo, Szaba et al. 2012). One explanation for this is that FXI participates in host defense through mechanisms that do not involve contact activation. One mechanism may be through a direct effect on leukocyte trafficking, especially considering that peritoneal leukocyte counts were significantly lower in $FXI^{-/-}$ mice compared to WT mice 24 hours after low-grade CLP (Tucker, Gailani et al. 2008), but not in CLP-operated WT mice after pre-treatment with 14E11 (Tucker, Verbout et al. 2012). This introduces the possibility that FXI alters neutrophil migration in a FXIIa-independent manner. FXI binds to neutrophils, and may affect neutrophil chemotaxis (Itakura, Verbout et al. 2011) and activation (Coomber, Galligan et al. 1997). Although our current study does not indicate a clear influence of FXI on leukocyte migrate into the peritoneum, they do show a non-significant increase in the various leukocyte subsets in WT

mice compared to their $FXI^{-/-}$ littermates. We used an automated cell counter to determine peritoneal leukocyte counts and obtained results that, although inconclusive, do not rule out a role for FXI in leukocyte migration. With this in mind, future studies should employ flow cytometry to more accurately determine peritoneal levels of both the myeloid (monocytes, macrophages, and neutrophils) and lymphoid (CD4+ T cells, CD8+ T cells, NK cells, etc.) leukocyte populations.

Our work does not exclude the possibility that FXI activation through the extrinsic (TF) pathway contributes to pathology during sepsis. The CLP model does not cause overt DIC in mice in our hands, but it is still possible that pathology from thrombin-mediated FXI activation occurs. To further investigate this possibility, WT and $FXI^{-/-}$ mice should be compared with $FXII^{-/-}$ and $PK^{-/-}$ mice in the CLP model to determine the influence of these proteins on survival, thrombin generation, inflammation (measurement of plasma cytokines and SAP, and peritoneal leukocyte counts), and bacterial dissemination. If survival rates after CLP are comparable among $FXI^{-/-}$, $FXII^{-/-}$, and $PK^{-/-}$ animals, we may conclude that contact activation-induced FXI activation is largely responsible for the mortality observed in WT mice. An observation of improved survival in $FXI^{-/-}$ mice relative to $FXII^{-/-}$ and $PK^{-/-}$ mice would suggest a more complex pathophysiology. It could indicate that FXI activation is mediated through additional mechanisms, such as the TF pathway, and/or, that FXII and PK are more important, relative to FXI, for the mounting of an effective host defense against infection. Time course studies should follow the survival experiments so that the complex host responses that contribute to morbidity and mortality during CLP-induced sepsis may be further understood. For example, if mortality in $FXII^{-/-}$ or $PK^{-/-}$ results from increased susceptibility to infection, we may expect to see elevated peritoneal and blood levels of bacteria in these animals.

Clinical relevance

The clinical relevance of this work remains to be determined. Most sepsis therapies that have shown promise in animal models do not advance to clinical trials, and those that do often fail to show a benefit in humans (Deitch 1998). Anticoagulants are certainly no exception to this trend. In the most recent update to the International Guidelines for the Management of Severe Sepsis and Septic Shock (Dellinger, Levy et al. 2013), heparin therapy for thrombus prophylaxis is the only recommended anticoagulant strategy, but this therapy can only be used in septic patients that are not experiencing a coagulopathy. The present study did not reproduce the apparent coagulopathy that was observed in previous work with the CLP model (Tucker, Gailani et al. 2008, Tucker, Verbout et al. 2012). However, the earlier studies, along with studies with mouse thrombosis models, do suggest that targeting FXI may be safer than using heparin when treating patients that are in DIC. Considering this, the major advantage of FXI as a target for sepsis therapy over other anticoagulant strategies may be its ability to "first, do no harm."

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APPENDIX

VALIDATION OF THE CECAL LIGATION AND PUNCTURE MODEL FOR EVALUATING THE CONTRIBUTION OF FXI DURING MURINE SEPSIS

Introduction

A multitude of animal sepsis models are described in the literature. These models have been divided into three categories: exogenous administration of a viable pathogen (e.g., a bacteria), exogenous administration of a toxin (e.g. LPS), or techniques that alter the permeability of the bowel (e.g., CLP) (Buras, Holzmann et al. 2005). Of these, CLP most closely mimics the hemodynamic and cytokine response observed in humans with sepsis, (Wichterman, Baue et al. 1980, Deitch 1998, Buras, Holzmann et al. 2005, Hubbard, Choudhry et al. 2005, Dyson and Singer 2009, Dejager, Pinheiro et al. 2011), probably because it provides a continuous source of endogenous gut flora instead of a massive bolus of laboratory strains of bacteria or toxin (Deitch 1998). Additionally, because sepsis in humans typically arises from a local infection that subsequently becomes systemic (Freise, Bruckner et al. 2001), the infection that is induced by CLP is also considered to be more clinically relevant. Considering this, we chose the CLP model to evaluate the role of FXI during sepsis. This model was new to our laboratory, requiring us to perform several preliminary studies. These studies in WT C57Bl/6 mice allowed us to refine our technique (and thus improve reproducibility), evaluate survival after CLP, and identify which inflammatory mediators change reliably during sepsis. The pilot studies also helped us to gain insight into any histologic changes that are apparent during sepsis.

Our pilot studies of WT C57Bl/6 mice featured an in-depth examination of the cytokine response, along with a cursory examination of the impact of CLP on the coagulation system. Of note is that we examined a greater range of time points (2, 4, 8, 12,

24, and 48 hours after CLP) in the pilot study, while we limited our investigations to 4, 8, and 24 hours after CLP in the full time course study. Pilot studies were conducted using low-grade CLP as, at this stage in our investigation, we had yet to discover that $FXI^{-/-}$ mice only exhibited a survival advantage after high-grade CLP in our hands. Indeed, this observation is what led us to use high-grade CLP in follow-on time course studies, and contributed to our decision to limit the latter studies to the first 24 hours of CLP-induced sepsis. Our survival studies predicted that a large proportion of animals would succumb to sepsis within 48 hours after high-grade CLP, so that too few animals would be available for investigating the inflammatory and coagulation system response at this stage of sepsis. The lower grade of injury in the pilot study may have resulted in a disease process that was different than what we observed with high grade injury (Rittirsch, Huber-Lang et al. 2009), but it did allow us to look at changes at a later time point than in the full study. Histology findings at 24 hours after high-grade CLP in the full study were largely unremarkable, but we were able to see significant changes in tissue histology at 48 hours after low-grade CLP in the pilot study. Of note is that we saw no clear evidence of DIC, even at 48 hours, in this model. This appendix includes a brief review of CLP, followed by some interesting findings from our preliminary work.

Cecal Ligation and Puncture

CLP was introduced by Wichterman and colleagues, who first used the technique to induce polymicrobial sepsis in rats (Wichterman, Baue et al. 1980). The procedure, which is described in **Figure 24** and in the Materials and Methods section of Chapter IV, involves ligation of the cecum below the ileocecal valve after midline celiotomy, followed by puncture of the organ with a hypodermic needle (Rittirsch, Huber-Lang et al. 2009). It is a

modification of a procedure, cecal ligation, that was originally used to study sepsis in dogs (Clowes, Zuschneid et al. 1968) and pigs (Imamura and Clowes 1975). Although Ryan *et al*., who adapted the model for use in laboratory rodents, observed peritonitis in rats after cecal ligation (Ryan, Blackburn et al. 1974), Wichterman added the "P" (puncture) to "CL" (cecal ligation) after failing to induce peritonitis and death (no deaths among 71 rats) with cecal ligation alone. The procedure is now in common use in mice as well as rats, and has also been described in neonatal piglets (Kato, Hussein et al. 2004) and sheep (Richmond, Walker et al. 1985).

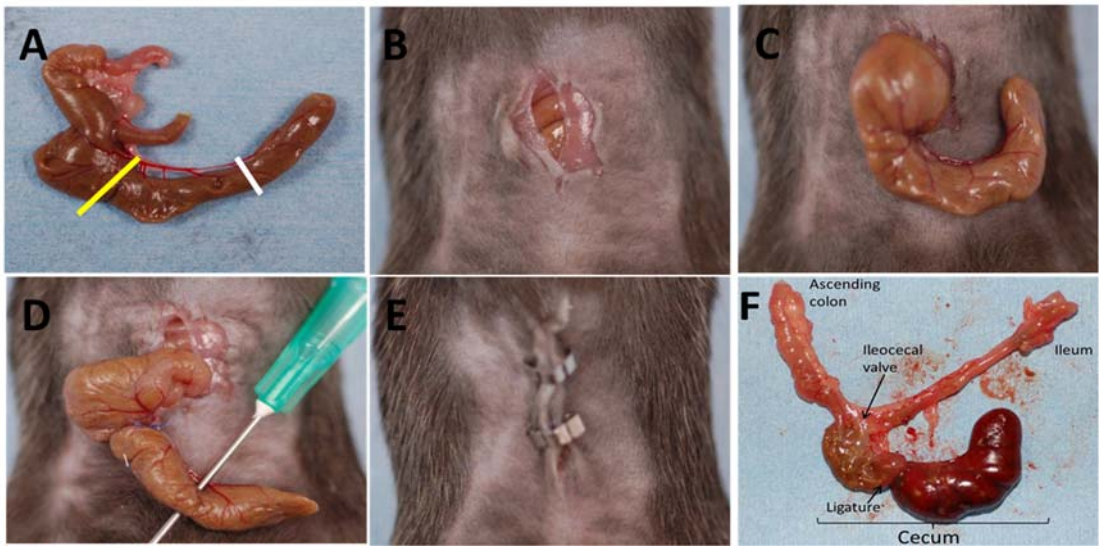

Figure 24. Cecal ligation and puncture procedure. (**A**). Photograph of the murine distal ileum, cecum, and proximal ascending colon. The yellow line indicates a ligature location that induces a high grade of sepsis; the white line indicates a ligature location that induces a low-grade sepsis. Surgery is initiated through a midline celiotomy (**B**). The cecum is located and exteriorized (**C**), then ligated according to the desired level of severity and punctured (**D**). The abdomen is closed with a simple continuous suture pattern, and the skin is closed with wound clips (**E**). The surgery takes approximately 10-15 minutes. The image shown in panel **F** is a necropsy photograph showing the cecum after high-grade CLP.

Microfil Experiments

There are many factors that affect morbidity and mortality in the CLP model of experimental sepsis, such as amount of cecum ligated, needle gauge used for cecal perforations, and number of perforations (Singleton and Wischmeyer 2003, Rittirsch, Huber-Lang et al. 2009). It is important to conduct the CLP surgeries in a systematic manner to reduce variability. To accomplish this, we used the same surgeon (the author) for all CLP procedures, and refined our surgical technique, specifically the cecal ligation portion of CLP, using experiments with Microfil (MV-122 Flow Tech Inc., Carver, MA). This allowed us to evaluate the cecal microcirculation of mice subjected to cecal ligation (no puncture) to assess the effectiveness of our ligation. Microfil is a compound that fills and opacifies blood vessels, allowing the user to visualize the microcirculation (http://www.flowtech-inc.com/microfil.htm). Because the goal of the procedure is to cut off circulation to the affected portion of the organ, we reasoned that an effective ligation would result in no Microfil infiltration distal to the ligation. We conducted a small study (**Figure 25**) where we ligated two mice with the "traditional" method (suture), and two mice with metallic vascular clips. One control mouse, in which no ligation was performed, was used for comparison. After euthanasia, mice were perfused with Microfil as previously described (Yuasa, Mignemi et al. 2014) (methods summarized in the figure legend). Microvascular infiltration of Microfil was evident in the control mouse (**Figure 25A).** The vascular clips (**Figure 25B**) did not prevent infiltration of Microfil, indicating that this method is not an effective means of achieving tissue ischemia. Interestingly, we noted a difference in the degree of blood vessel occlusion between the two suture-ligated mice. One mouse (**Figure 25C**) displayed complete tissue ischemia, with no Microfil infiltration noted distal to the ligature. This indicated that our ligature successfully occluded both arteries and veins. In the other mouse,

however, it was evident that the suture had been place inappropriately. In this mouse (**Figure 25D**), the cecum was engorged with blood distal to the ligation and a small amount of Microfil infiltration was observed, indicating that we had left the arterial circulation intact while rendering the veins unable to drain blood out of the organ. We determined that this difference was likely due to variability in the method we used to tighten our ligatures. We had previously experienced premature breakage of the suture if we grasped one end of the knot with a needle holder (surgical instrument) while tightening the ligature. This likely led to differences in the tightening pressure applied to avoid breakage. The results of the Microfil experiments led us to tighten our ligatures by hand, which did not result in ligature breakage, even when sutures were applied very firmly. We feel that this modification to our technique further reduced variability in our model.

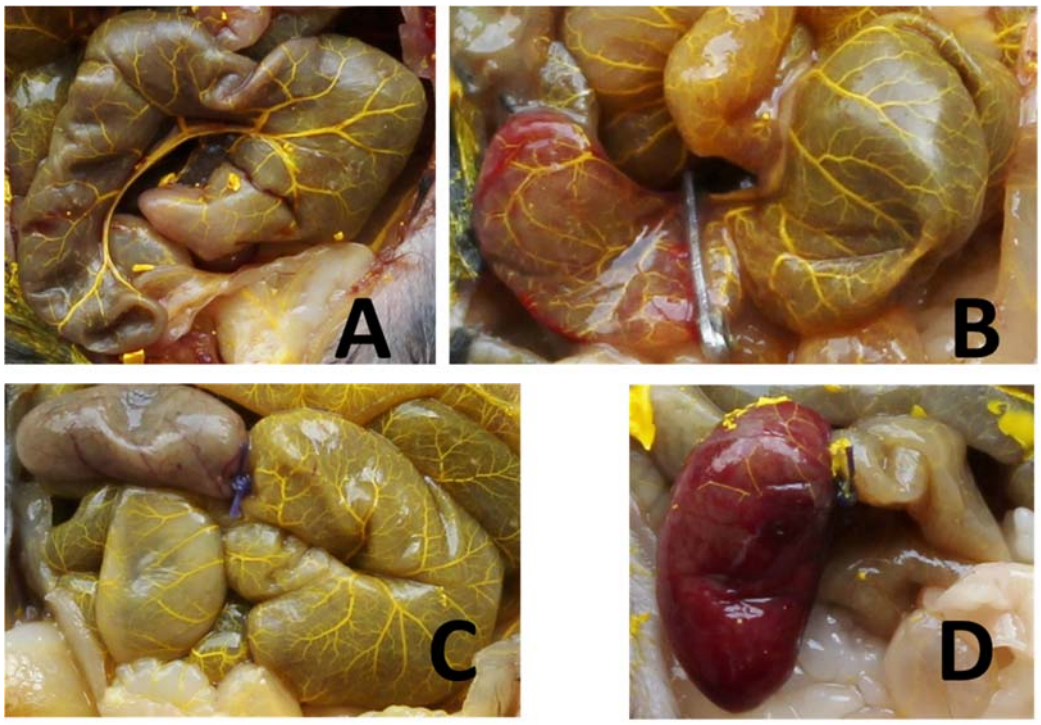

Figure 25. Microfil experiments. Two hours after cecal ligation, mice were euthanized and a ventral midline incision extending from the thoracic inlet to the caudal abdomen was made. The thorax and abdomen were opened and a 25G butterfly catheter was placed in the left ventricle of the heart and glued in place. The caudal vena cava was transected proximal to the liver and approximately 9ml of warm heparinized saline was flushed through the butterfly catheter and vasculature. The vasculature was then perfused with a 10% solution of buffered neutral formalin, then injected with 3ml of Microfil contrast polymer. The control mouse exhibited complete perfusion of contrast polymer throughout the cecal microvasculature (A), while the metallic vascular clips failed to prevent Microfil infiltration (B). The suture ligation in one mouse (C) effectively prevented all Microfil infiltration. In contrast, some Microfil infiltration was noted in the other suture ligated mouse (D). Additionally, the cecum of this mouse was engorged with blood, indicating that the cecal ligation had left the arterial circulation intact while blocking venous drainage, thereby allowing blood to pool within the organ before euthanasia. Microfil experiments were performed by Dr. Nicholas Mignemi, who also provided the images shown.

Pilot studies

We conducted a pilot time course study of WT mice to evaluate the feasibility of our sample collection strategy and experimental technique for follow-on work, and to determine the optimum time points for collection. We collected samples from groups of mice at 2, 4, 8,

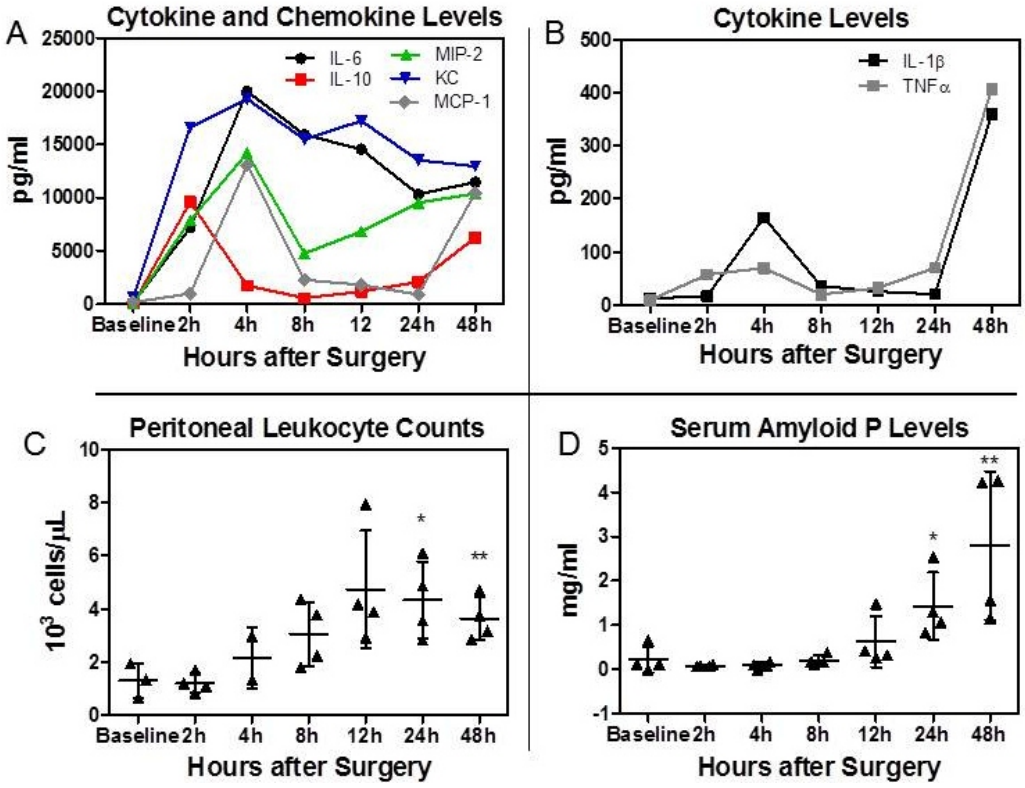

Figure 26. Markers of inflammation after CLP-pilot studies. Blood was collected from WT C57BL/6 mice at the indicated times after CLP. Plasma cytokine and chemokine (A & B) and SAP (D) levels were measured with a Luminex assay. Leukocyte counts in peritoneal wash fluid were measured with an automated hematology analyzer (C)**.** There were significant increases in peritoneal leukocyte (p= 0.02) and SAP (p=0.03) levels starting at 24 hours post-CLP.

12, 24, and 48 hours after CLP to evaluate markers of inflammation and coagulopathy. For the cytokine and chemokine markers, we constructed simple XY plots with each point representing the mean levels from 3 or 4 mice (no error bars shown), and lines drawn

between points to more easily identify the time points that would yield the most information in further studies. **Figure 26A** shows mean levels of IL-6, IL-10, MIP-2, KC, and MCP-1. (Note: levels of these markers exceeded the maximum limit of detection (20,000 pg/ml) for the assay). **Figure 26B** shows mean levels of IL-1 beta and TNFα. We also measured peritoneal leukocyte counts (**Figure 26C**) and plasma SAP (**Figure 26D**) in these mice, and found significant increases beginning at 24 hours after CLP. These data confirmed that the markers we chose were useful for gauging the inflammatory response after CLP.

We then evaluated FXI levels by ELISA because of an earlier finding that showed elevated FXI mRNA after infection (Luo, Szaba et al. 2011). In contrast to the earlier study, FXI levels decreased modestly, though not significantly, in the 48 hours after CLP (**Figure 27A**). Mean platelet counts were moderately decreased from baseline (not significantly) between 4 and 24 hours, and were significantly lower than baseline at 48 hours (p=0.03) (**Figure 27B**).

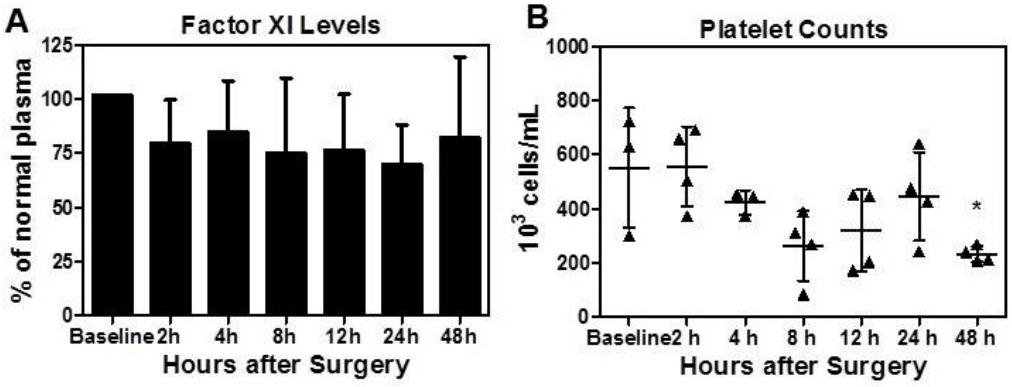

Figure 27. FXI levels and platelet counts after CLP-pilot studies. CLP was conducted on groups of 4 male, 4-month-old C57BL/6 mice at the indicated time points, and blood was collected by cardiac puncture. (A) Plasma factor XI levels. (B) Platelet counts, measured with an automated hematology analyzer. * p=0.03.

We evaluated hematoxylin and eosin stained sections of several organs (liver, kidney, spleen, heart, and brain) taken from WT mice at 48 hours after CLP surgery to look for evidence of inflammation, bacterial dissemination, or DIC. We observed a number of histologic lesions, but, notably, we did not see obvious evidence of thrombosis. Microhemorrhage with perivascular inflammation and edema was commonly observed within brain tissue collected from WT mice (**Figures 28A and 28B**). A photograph of brain section from a control (no surgery) mouse is provided in figure **28C** for comparison.

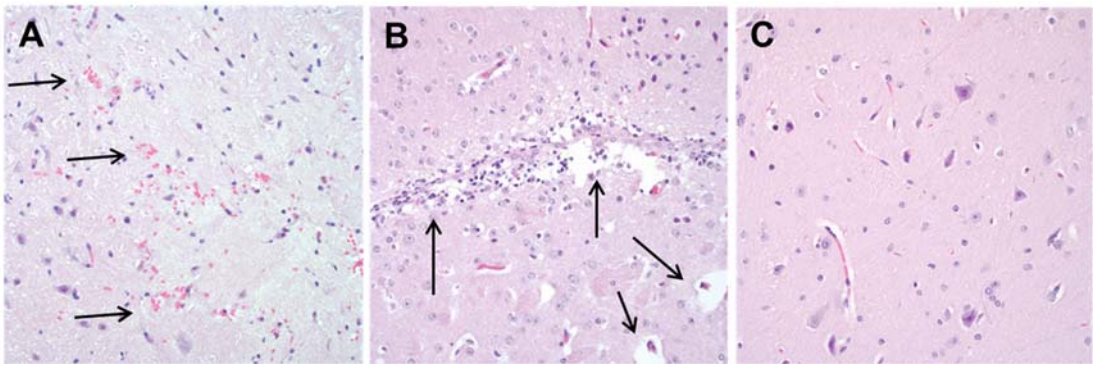

Figure 28. Histology of brain tissue-pilot studies. Photomicrographs (400X magnification) of paraffin embedded sections of brain stained with hematoxylin and eosin. (A&B) mice 48 hours after CLP and control mouse that did not undergo surgery (C). (A) Microhemorrhages are commonly observed throughout the brain(arrows). Perivascular inflammation and edema are also present (B, arrows) This is not observed in the control brain (C).

Histologic evaluation of spleen provided additional information on the inflammatory course of disease. In samples taken from WT mice 48 hours post-CLP, we observed severe congestion and white pulp depletion of the spleen with lymphoid apoptosis (**Figures 29A and 29B**), suggestive of acute bacteremia (Carlton, McGavin et al. 1995). This contrasts with the marked hyperplasia of splenic white pulp that was observed in a mouse that survived the 7-day study period of an earlier survival pilot experiment (**Figure 29E**).

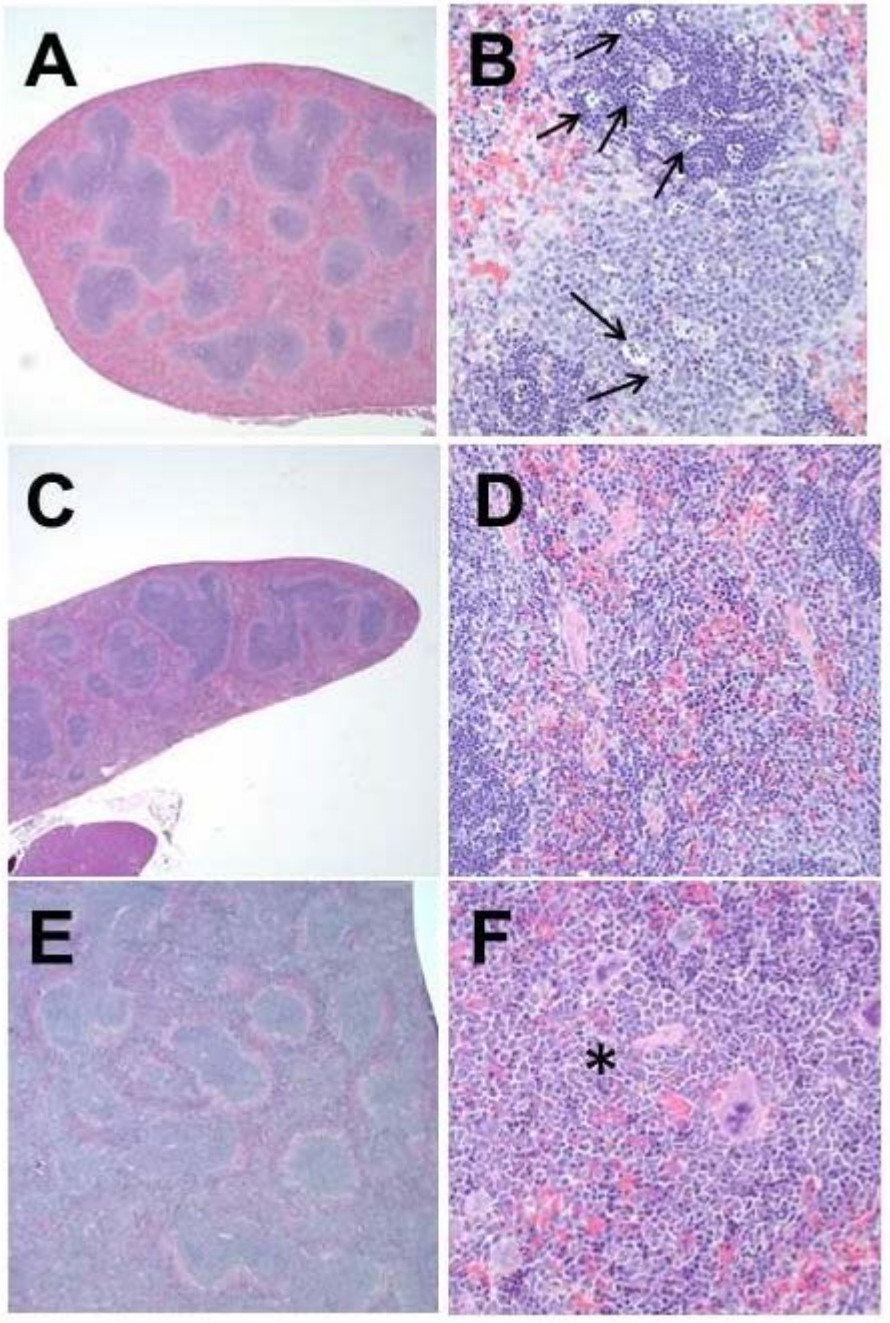

Figure 29. Lymphoid response to CLP in pilot studies. Photomicrographs of paraffin embedded sections of spleen stained with hematoxylin and eosin. Images A, C, & E are 40X; images B, D, & F are 400X. 48 hours after CLP, splenic sections from a WT mouse show depletion of the white pulp, while the red pulp is expanded by congestion (A). Apoptosis of lymphocytes is prominent in the white pulp (arrows) (B). Images C and E are of a non-operated WT control mouse. Images E and F are from a $FXI^{-/-}$ mouse that was still alive at the end of a 7-day pilot survival study. There is hyperplasia of the white pulp (E), and the red pulp is expanded by extramedullary hematopoiesis.

Perhaps the most striking finding from the histological evaluation of mouse organs 48 hours after CLP was the clear evidence of bacterial dissemination. **Figures 30A** and **30B** show heavy bacterial colonization of the surface of the liver, along with areas of hepatic necrosis, while the photograph in **Figure 30F** shows bacterial dissemination to a thoracic lymph node with concurrent apoptosis of lymphocytes. Figures **30C** and **30D** are photographs of a liver section from a mouse that survived to 7 days, and are similar to images from a control mouse (not shown).

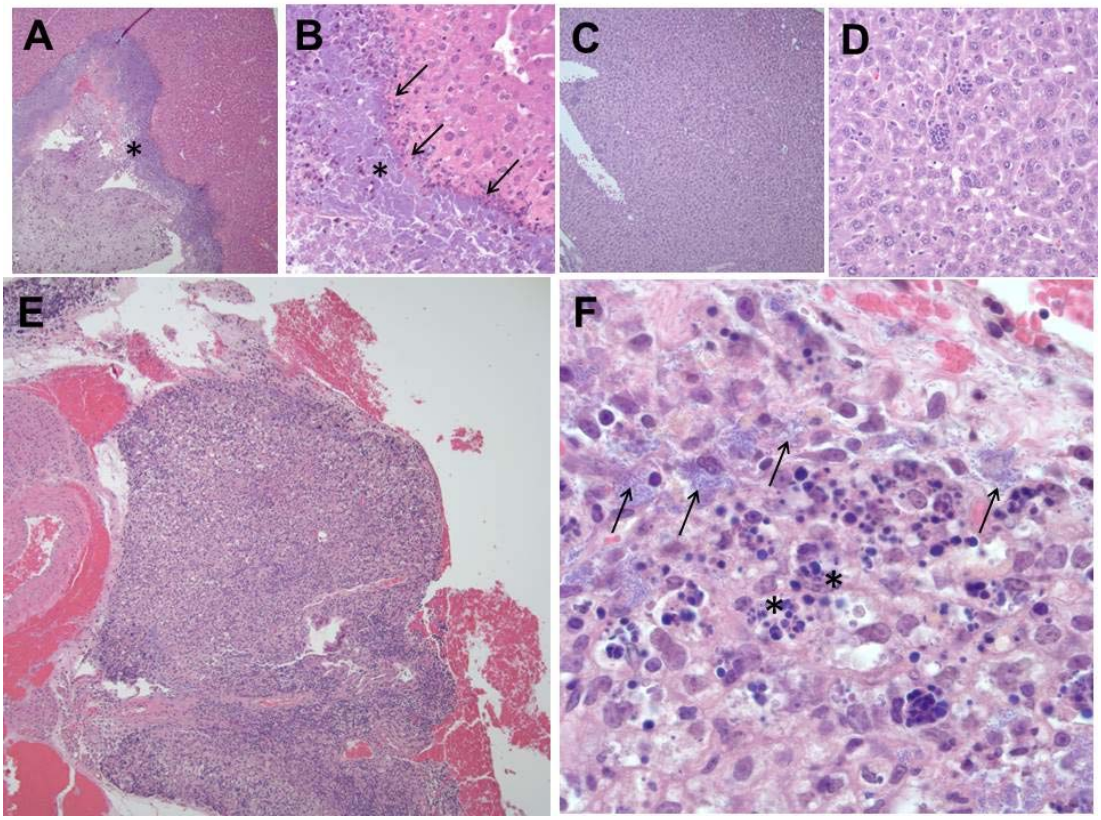

Figure 30. Evidence of bacterial dissemination after CLP-pilot studies. H&E stained sections of paraffin-embedded liver (A-D) and thoracic lymph node (E & F). Images A, C, and E are 40X, while images B, D, and F are 400X. 48 hours after CLP, the surface of the liver is heavily colonized with bacteria (arrows) and there are areas of hepatic necrosis (asterisks) (A & B). This is not observed in a mouse that survived to 7 days after CLP (C & D). Images E and F show bacterial dissemination to thoracic lymph node (arrows in image F) and apoptotic bodies (asterisks in image F) that are indicative of apoptosis of lymphocytes.

Discussion

Given the results from earlier work with the CLP model (Tucker, Gailani et al. 2008), as well as our understanding of the role of FXI in thrombosis, we were somewhat surprised to find no evidence of thrombosis in our pilot studies of WT mice. We initially postulated that the microhemorrhage observed in some brain sections of WT mice 48 hours after CLP could indicate that mice were entering the latter stages of DIC, wherein hemorrhage predominates. In retrospect, this now seems unlikely. First, we did not see evidence of hemorrhage at necropsy or during the histologic evaluation of other organs. Second, mean platelet counts remained above 200,000 cells/µl, with a count of 172,000 cells/µl being the lowest observed. Using a tail bleeding time assay, Morowski and colleagues recently demonstrated that platelet counts as low as 25,000 cells/µl are adequate for supporting normal hemostasis in C56BL/6 mice (Morowski, Vogtle et al. 2013). The levels we observed were at least 8-fold higher than this, suggesting that the observed hemorrhage in mouse brains was not a result of platelet consumption secondary to DIC. This notion is reinforced by the findings from our published study of WT and $FXI^{-/-}$ mice (Bane, Ivanov et al. 2016), which utilized a broader array of markers of DIC (TAT and fibrinogen levels, platelet counts, and histology) and showed no evidence of DIC in mice of either genotype at 24 hours after CLP. Considering this, we are inclined to conclude that CLP, at least in our hands, is an inappropriate model for assessing DIC in mice.

In contrast to markers of coagulopathy, we saw ample evidence of inflammation after CLP in our pilot studies, with elevations in numerous plasma cytokines and chemokines. SAP levels rose appreciably after CLP, as did peritoneal leukocyte counts. The histologic findings provide compelling evidence that CLP triggered a profound lymphoid response, resulting in marked lymphocyte apoptosis in spleen and lymph node. In

the tissue sections that we examined, the lymphoid depletion was accompanied by widespread bacterial dissemination, with areas of hepatocyte necrosis at 48 hours after CLP, suggesting that immunosuppression was the predominant feature of the disease process at this point. We have no way of knowing if the animals sacrificed at 48 hours would have ultimately survived this insult. We did note splenic white pulp hyperplasia and no evidence of bacterial dissemination or hepatocyte necrosis in a mouse that survived to 7 days after CLP. Two possible scenarios for mice that survived to 7 days in pilot studies are that some mice (1) mounted a robust immune response that prevented bacterial dissemination altogether, or (2) mounted an insufficient response early on, but then recovered to clear the bacterial infections.

In summary, the pilot experiments were instructive and guided many of our decisions for follow-on work. Both the pilot and follow-on studies appeared to implicate the inflammatory response (whether it be overwhelming or insufficient) in the morbidity and mortality of sepsis. However, neither study ruled out a role for FXI in the coagulopathy of sepsis because CLP did not result in DIC in our hands. A different model is required to make this determination.

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