CELLULAR AND PLASMA PROTEIN CROSSTALK IN ARTERIAL AND VENOUS THROMBOSIS

Maria M. Aleman

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Approved by:

Alisa S. Wolberg

Frank C. Church

William B. Coleman

Maureane Hoffman

Nigel S. Key

ABSTRACT

Maria M. Aleman: Cellular and Plasma Protein Crosstalk in Arterial and Venous Thrombosis
(Under the direction of Dr. Alisa Wolberg)

Coagulation is an enzymatic cascade culminating in the formation of a clot. Intravascular coagulation is termed thrombosis. Studies using platelet-rich plasma, whole blood, and animal models reveal the complex crosstalk between clotting factors and the cellular environment that promote thrombosis. Three distinct, yet related, studies included in this dissertation exemplify this crosstalk.

First, the vascular bed-dependent prothrombotic effects of elevated prothrombin were examined. We found that elevated prothrombin increased venous, but not arterial, thrombosis in mice. The prothrombotic effects of elevated prothrombin were manifested by increased fibrin deposition but no significant increase in platelet accumulation. These data show that elevated prothrombin would not be expected to contribute to platelet-dominated arterial thrombosis.

Second, the procoagulant properties of microparticles from different cell types were investigated. We found that monocyte-derived microparticles contributed to initiation and propagation of clotting via the extrinsic coagulation pathway, while platelet-derived microparticles contributed to primarily to propagation of clotting via the intrinsic coagulation pathway. These data suggest monocyte-derived microparticles may contribute to the initiation of a thrombotic event, while platelet-derived microparticles may propagate an existing thrombotic event.

Third, the role of fibrinogen and factor XIII (FXIII) in venous thrombosis was studied. We discovered the binding site of FXIII on fibrinogen, the necessity for FXIII activity for red blood cell retention in clots, and that reduction or deficiency of FXIII activity reduces venous thrombus size. These data indicate a novel role for FXIII in the pathogenesis of thrombosis and implicate FXIII as a novel therapeutic target.

Together, these studies emphasize the importance of complementary *in vitro* and *in vivo* studies of coagulation and thrombosis to properly elucidate the pathophysiological relevance of clotting factor and cell function.

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PREFACE

Chapter 2 represents work that was published previous to writing this dissertation with the following citation:

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α	Alpha
A23187	Calcium ionophore
ANOVA	Analysis of variance
APC	Allophycocyanin
AU	Arbitrary unit
β	Beta
CTI	Corn trypsin inhibitior
CVD	Cardiovascular disease
DSS	Dextran sodium sulfate
DTT	Dithiothreitol
DVT	Deep vein thrombosis
EDTA	Ethylenediamine tetraacetic acid
ELISA	Enzyme-linked immunosorption assay
EPCR	Endothelial protein C receptor
Fgn	Fibrinogen
FVIII	Factor VIII
FXIII	Factor XIII
FXIIIa	Activated factor XIII
g	Gram
γ	Gamma
GPIX	Glycoprotein IX
HBS	HEPES-buffered saline

HBSS	Hank's balanced salt solution
HMW	Higher molecular weight
IgG	Immunoglobulin G
II	Prothrombin
IIa	Thrombin
IVC	Inferior vena cava
L	Liter
LPS	Lipopolysaccharide
M	Molar
M-MP	Monocyte-derived microparticle
MDP	Microparticle-depleted plasma
Min	Minute
mg	Milligram
mL	Milliliter
mM	Millimolar
MP	Microparticle
ng	Nanogram
nM	Nanomolar
NTA	Nanoparticle tracking analysis
OD	Optical density
PBMC	Peripheral blood mononuclear cell
PBS	Phosphate-buffered saline
PE	Pulmonary embolism

PFP	Platelet-free plasma
pM	Picomolar
PMP	Platelet-derived microparticle
PPP	Platelet-poor plasma
PS	Phosphatidylserine
RBC	Red blood cell
SCD	Sickle cell disease
SD	Standard deviation
SDS	Sodium dodecyl sulfate
SEM	Standard error of the mean
T_1	Magnetic resonance longitudinal relaxation time
TAT	Thrombin-antithrombin complex
TEM	Transmission electron microscopy
TF	Tissue factor
THP-1	Human monocyte cell line
THP-MP	THP-1 cell microparticles
TM	Thrombomodulin
tPA	Tissue plasminogen activator
TRAP	Thrombin receptor agonist peptide
TTO	Time to occlusion
U	Unit
μL	Microliter
μM	Micromolar

Va	Activated factor V
VTE	Venous thromboembolism
WBC	White blood cell
Xa	Activated factor X

CHAPTER 1: INTRODUCTION

1.1 Coagulation & Blood Cells

Blood coagulation is a complex enzymatic cascade culminating in the formation of a fibrin protein meshwork that turns liquid blood into a gel-like solid. Two initiating coagulation pathways—intrinsic and extrinsic—converge on a common pathway that produces the serine protease thrombin (Figure 1.1). Thrombin activates platelets and cleaves fibrinogen into fibrin monomers that polymerize into fibers forming a net-like structure that is stabilized by the transglutaminase factor XIII (FXIII). Misbalance of this system leads to a failure of hemostasis (bleeding) or excessive clotting (thrombosis).

Clotting can be reduced down to two essential components—thrombin and fibrinogen. However, physiologic coagulation involves the entire cascade and, importantly, anionic phospholipid surfaces such as on an activated platelet. Thus, the simple coagulation cascade has evolved into a cell-based model[1] that emphasizes the role of blood cells in initiation or propagation of coagulation (Table 1.1). Platelets, when activated, support coagulation by expression of anionic phosphatidylserine (PS) on their plasma membrane, release of granules rich in clotting factors such as factor V and fibrinogen, and by their ability to aggregate and occlude vessels at sites of trauma or atherosclerotic plaque rupture.[2] Leukocytes, such as monocytes and neutrophils, contribute to coagulation by various means: a) expression of tissue factor (TF)—the key initiator of extrinsic coagulation—can be induced following activation of monocytes[3,4], and (mouse) neutrophils[5], b) formation of

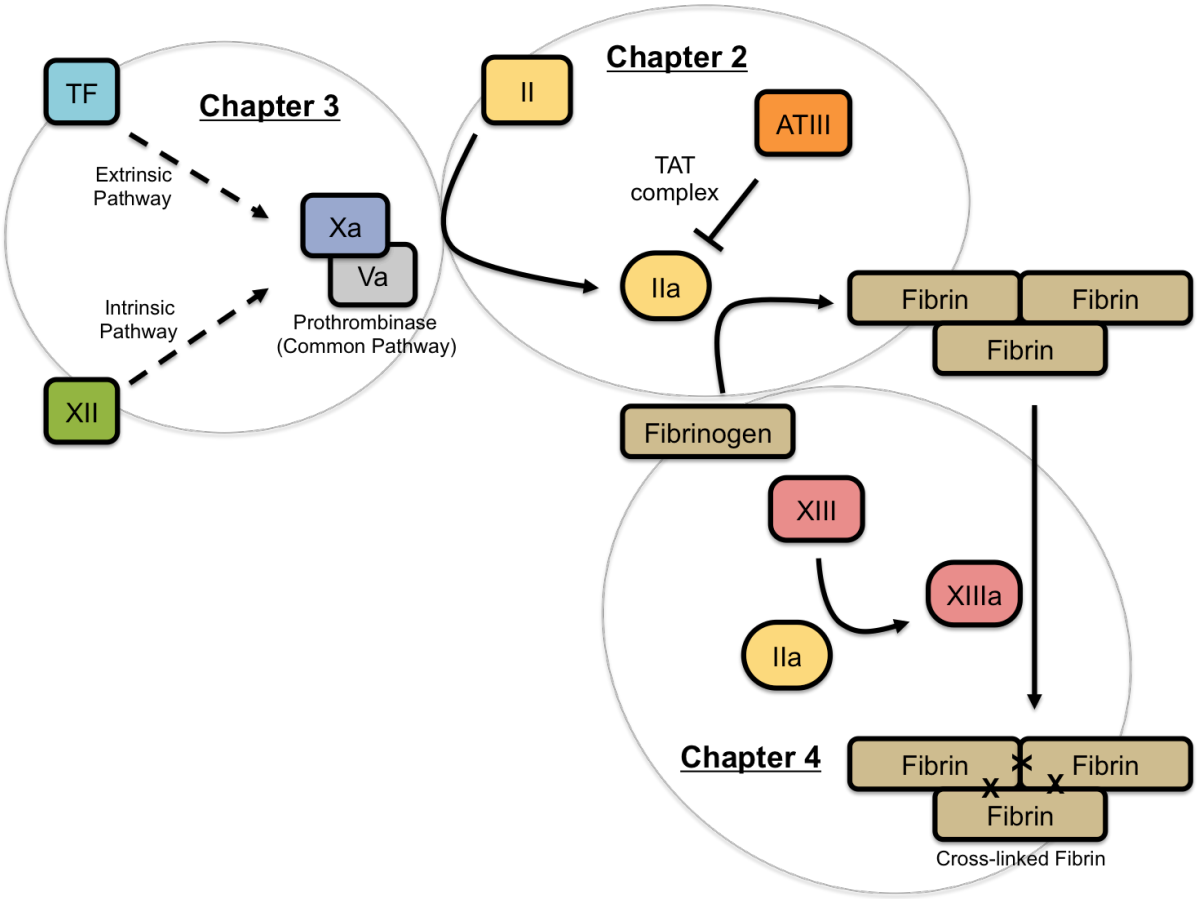


Figure 1.1. Simplified coagulation cascade. Extrinsic and intrinsic coagulation converge on the common pathway that activates prothrombin (II) to thrombin (IIa). Thrombin then converts fibrinogen into fibrin and activates FXIII. Chapter 2 examines the effects of elevated prothrombin levels in mouse models of arterial and venous thrombosis. Chapter 3 examines the contribution of TF-bearing microparticles and non-TF-bearing microparticles to thrombin generation. Chapter 4 investigates the relationship between fibrin(ogen), FXIII, and thrombus formation. Abbreviations: TF, tissue factor; XII, factor XII; Xa, activated factor X; Va, activated factor V; II, prothrombin; IIa, thrombin; ATIII, antithrombin III; TAT, thrombin-antithrombin complex; XIII, factor XIII.

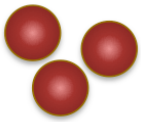
microparticles, especially TF-bearing microparticles[6-8], and c) by neutrophil extracellular trap-mediated activation of the intrinsic coagulation pathway[9,10]. Endothelial cells contribute to clotting by release of von Willebrand Factor which binds platelets[11,12], their ability to bind fibrin and modulate its network structure[13], and by induced expression of TF[14], though their ability to express it *in vivo* is controversial[15]. Finally, red blood cells (RBCs) contribute to coagulation by exposure of PS that supports thrombin generation[16,17], and RBCs increase blood viscosity[18] and marginate platelets toward the endothelium[19], placing them in close proximity to sites of vascular trauma. Lastly, recent studies from our laboratory indicate RBCs directly dictate the size of clots (Chapter 4).

1.2 Arterial & Venous Thrombosis

Thrombosis is a pathologic process leading to inappropriate intraluminal blood coagulation that is a key component of myocardial infarction, ischemic stroke, and venous thromboembolism (VTE; deep vein thrombosis (DVT)/pulmonary embolism (PE)). Cardiovascular disease (CVD), which includes myocardial infarction and ischemic stroke, currently afflicts 83.6 million Americans, caused 32.3% of all deaths in the United States in 2009, and costs an estimated $312.6 billion each year.[20] One million Americans suffer from VTE each year[21], and incidence rates increase with age in both men and women[20]. The annual health care costs for VTE, including hospital-acquired and recurrent events, are estimated to range from $15.4 to $34.4 billion.[22] These pathologies are significant health and economic burdens and the need for improved understanding and treatment is essential.

Thrombosis can occur in different vascular beds—arterial or venous. Arterial thrombosis is associated with atherosclerotic plaque rupture and exposure of highly

Table 1.1. Procoagulant Properties of Blood Cells & Microparticles

Procoagulant Property	 **Platelets**	 **Leukocytes**	 **RBCs**	 **Endothelial Cells**	 **Microparticles**
Clotting Factor Expression	Granules contain FV, fibrinogen	Inducible TF		Inducible TF, vWF	TF
Anionic Phospholipids	PS	PS	PS	PS	PS
Aggregation or Adhesion	Aggregation & plug formation	Adhesion to EC	Aggregation, adhesion to EC	Supports adhesion	Adhesion to other cells
Other	Binds fibrin(ogen)	Neutrophil Extracellular Traps	Viscocity, blood margination	Binds fibrin(ogen)	

Abbreviations: EC, endothelial cells; FV, factor V; PS, phosphatidylserine; TF, tissue factor; vWF, von Willebrand factor.

procoagulant sub-endothelial material and cells (Figure 1.2).[23] This process occurs under high shear leading to the formation of platelet-rich thrombi—so called "white clots"—and detrimental vascular occlusion. Treatment of arterial thrombosis primarily involves anti-platelet drugs, sometimes with anticoagulation, and/or thrombolytic therapy. Risk factors for arterial thrombosis include CVD, hypertension, diabetes mellitus, obesity, and smoking.[24]

Venous thrombosis, in contrast, is thought to originate in the hypoxic environment of venous valve pockets (Figure 1.3).[25,26] In these areas, zones of blood flow recirculation lead to endothelial activation, leukocyte accumulation and activation, and the exposure of tissue factor and release of neutrophil extracellular traps thereby initiating coagulation and the formation of a thrombus rich in RBCs and fibrin—so called "red clots." Treatment for acute DVT is heparin, followed by warfarin for 3 to 6 months. Novel anticoagulants to replace warfarin include anti-Xa and anti-thrombin drugs. Like arterial thrombosis, risk factors for venous thrombosis include CVD, hypertension, diabetes mellitus, obesity, and smoking, but additional risk factors include trauma, infection, immobility, and cancer.[27] The differences between arterial and venous thrombosis triggering events, cellular compositions, treatments, and risk factors highlight the context-dependent nature of coagulation *in vivo*.

1.3 Hyperprothrombinemia

What happens to coagulation when a single clotting factor is elevated? The answer depends on which factor is elevated and its functionality in the clotting cascade. Previous studies by our laboratory painstakingly determined the effect of elevated clotting factors on *in vitro* thrombin generation and fibrin formation[28-31], as well as thrombus formation *in vivo*[30,31]. For instance, while elevated fibrinogen increased the rate of fibrin formation

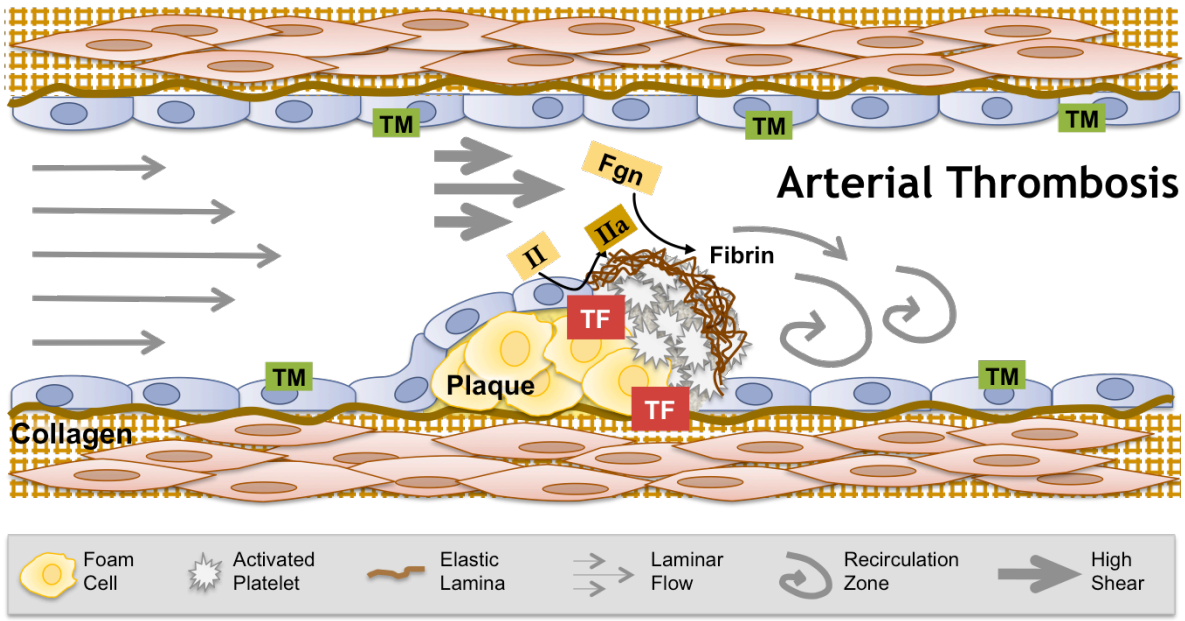


Figure 1.2. Arterial thrombosis. Interplay between abnormalities in blood components, the vasculature, and blood flow contribute to the development of arterial thrombosis. Arterial thrombosis involves the formation of platelet-rich "white clots" that form after rupture of atherosclerotic plaques and exposure of procoagulant material such as lipid-rich macrophages (foam cells), collagen, tissue factor, and/or endothelial breach, in a high shear environment. Abbreviations: Fgn, fibrinogen; II, prothrombin; IIa, thrombin; TF, tissue factor; TM, thrombomodulin. Figure originally appeared in Wolberg, et al.[65] Reprinted with permission from Lippincott, Williams, & Wilkins.

regardless of TF level used to initiate clotting[30], elevated factor VIII (FVIII, a cofactor in the intrinsic pathway) only increased fibrin formation with low levels of TF[31]. Similarly, while elevated fibrinogen shortened the time to occlusion (TTO) in a $FeCl_3$ injury model[30], shortening of the TTO with elevated FVIII was dependent on the extent of injury[31]. These data show how the effects of elevated factors on coagulation kinetics are context-dependent.

Prothrombin is the inactive zymogen form of thrombin. Previous studies by our laboratory described the *in vitro* procoagulant effects of elevated prothrombin levels (hyperprothrombinemia). In both purified and plasma-based systems, elevated prothrombin increases thrombin generation[28,29] and significantly increases fibrin formation and network density[29]. Interestingly, elevated prothrombin has no effect on platelet activation[29], suggesting prothrombotic effects of hyperprothrombinemia are due to effects on fibrin formation.

Risk of thrombosis for hyperprothrombinemia patients is vascular bed-specific. While there is a clear association between venous thrombosis and elevated prothrombin levels[32,33], the association is less clear between elevated prothrombin and arterial thrombosis risk.[34,35] This suggests that vascular bed-specific mechanisms dictate the prothrombotic effects of elevated prothrombin. To investigate the role of elevated prothrombin in vascular bed-specific thrombosis, we utilized multiple mouse models of venous and arterial thrombosis in a study described in Chapter 2. Briefly, we determined that acute hyperprothrombinemia increases venous thrombosis, but has no detectable effect on arterial thrombosis.[36] As we found with elevated FVIII, the effects of elevated prothrombin on thrombogenesis are context-dependent.

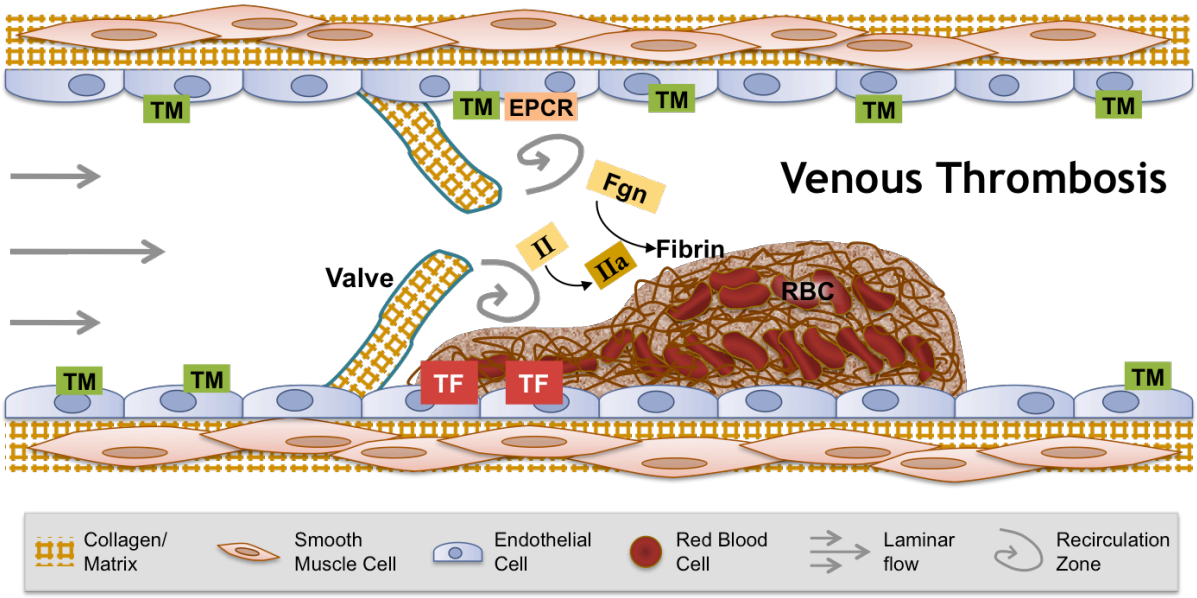


Figure 1.3. Venous thrombosis. Interplay among abnormalities in blood components, the vasculature, and blood flow contribute to the development of venous thrombosis. Venous thrombosis involves the formation of fibrin-rich "red clots" that result from exposure of procoagulant activity on intact endothelium plus plasma hypercoagulability, in reduced or static blood flow. Venous thrombi are thought to initiate behind valve pockets, in which reduced or static flow decreases wall shear stress that normally regulates endothelial cell phenotype. Abbreviations: EPCR, endothelial protein C receptor; Fgn, fibrinogen; II, prothrombin; IIa, thrombin; RBC, red blood cells; TF, tissue factor; TM, thrombomodulin. Figure originally appeared in Wolberg, et al.[65] Reprinted with permission from Lippincott, Williams, & Wilkins.

1.4 Microparticles & Thrombosis

Cellular microparticles, also known as microvesicles, are sub-micron plasma membrane blebs released from various cell types. In particular, platelets, monocytes, red blood cells, endothelial cells, and tumor cells, have been shown to release microparticles.[37-39] These microparticles circulate in the blood and are primarily of platelet origin in healthy individuals.[40] However, microparticles of various cellular origins are elevated in diseases including CVD[41-43], VTE[44], and cancer[45,46].

Like their parent cells, microparticles have procoagulant properties that can include the presence of TF and/or PS on the outer leaflet of their plasma membrane (Table 1.1). While PS exposure appears to be a hallmark of microparticles from all cell types, TF expression is restricted to microparticles stemming from TF-expressing parent cells such as monocytes and tumor cells. The presence or absence of TF confers differential procoagulant activity on microparticles. In work detailed in Chapter 3, we show that TF-bearing microparticles from monocytes or a monocytic cell line support *extrinsic* thrombin generation and fibrin formation, whereas non-TF-bearing microparticles from platelets support *intrinsic* thrombin generation and fibrin formation.[6] This study suggests TF-bearing microparticles may be causative for VTE, whereas non-TF-bearing microparticles may only contribute to thrombosis after an initiating event.

Cancer patients, particularly pancreatic cancer patients, with VTE have increased microparticle-associated tissue factor activity[47], suggesting these cellular blebs may be causative for the development of VTE. To test this concept, we performed a study to determine the role of tumor-derived TF in the activation of coagulation and thrombus formation in xenograft models of human pancreatic cancer in mice. We found that activation

of coagulation only occurred in mice with TF-positive tumors.[48] Interestingly, of two TF-positive pancreatic tumor cell lines that activated coagulation, only one provided detectable levels of circulating TF-positive MPs. These data suggested activation of coagulation was due to TF expression by the tumor itself rather than by the MPs. Tumor-bearing mice with elevated levels of TF-positive MPs exhibited increased thrombosis in a saphenous vein model, while mice injected with TF-bearing microparticles showed increased thrombus formation in a concentration-dependent manner in an inferior vena cava stenosis model.[48] These data suggest tumors contribute to activation of coagulation and circulating TF that might contribute to thrombosis.

1.5 FXIII & RBCs

FXIII is a 320 kDa pro-transglutaminase that circulates in plasma at ~70 nM and consists of two catalytic subunits (FXIII-A) and two non-catalytic subunits (FXIII-B) in a noncovalent, heterotetramer (FXIII-A_2B_2). Essentially all FXIII in plasma circulates in complex with fibrinogen[49,50], whereas less than 1% of fibrinogen has FXIII bound. FXIII is activated by thrombin[51,52] and calcium[53-55]. Of note, FXIII activation is accelerated when it is bound to fibrinogen[56,57] suggesting localization of FXIII on circulating fibrinogen is an important factor in its activation. However, the residues in fibrinogen that mediate interaction with FXIII have not been defined. A study by our group, described in Chapter 4, indicates FXIII binds to fibrinogen at the c-terminus of the γ chain and that localization of FXIII on fibrinogen is important for normal FXIII activation and fibrin cross-linking. Intriguingly, disruption of FXIII binding to fibrinogen causes a defect in retention of RBCs within clots (Figure 1.4). However, the role of RBCs in clots is not fully understood.

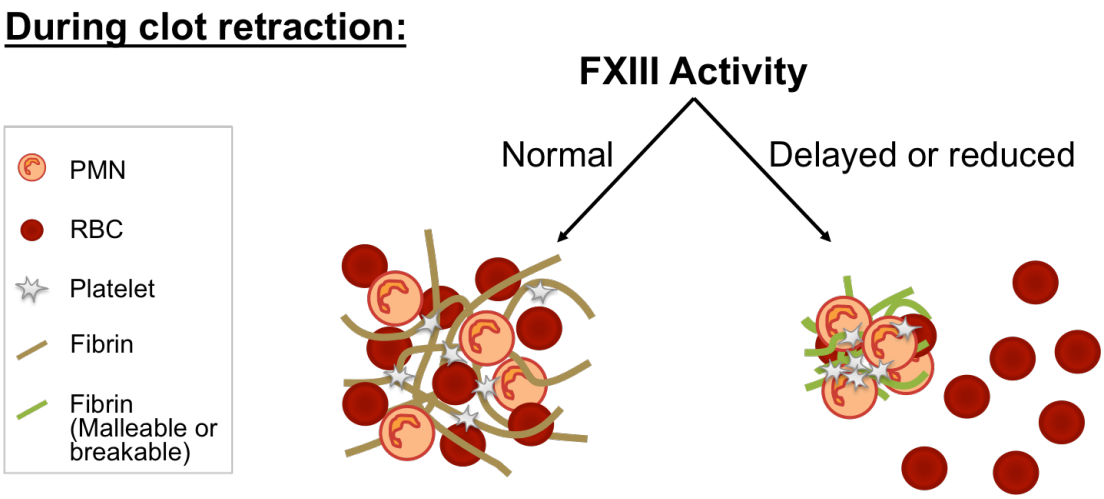


Figure 1.4. Model of FXIII-dependent RBC retention during clot retraction. Studies described in Chapter 4 examine how delayed or deficient FXIII activity results in reduced RBC retention in mouse and human clots.

The most abundant cell in blood, RBCs are flexible, biconcave, anucleate cells derived from bone marrow that circulate at 4-6.x10^9/mL in humans. Primary RBC function is oxygen transport via its hemoglobin-rich cytoplasm. RBCs are readily identifiable by most macro and microscopic techniques, but are often discarded during blood processing for hematological tests. However, this centrifugal waste may be more important than realized as recent studies suggest RBCs are not just passive bystanders, but play an active role in coagulation (Table 1.1).

A growing body of evidence suggests that abnormal RBC quantity and quality contribute to clot formation *in vivo*. Bleeding times shorten as hematocrit rises in anemic, normal, and polycythemic individuals[58], and elevated RBC levels are associated with increased risk of venous thrombosis in patients with polycythemia vera or patients on erythropoietin[59,60]. Patients with sickle cell disease (SCD) have abnormal hemoglobin polymerization resulting in dysfunctional RBCs with a characteristic "sickled" appearance; notably, SCD patients have an increased incidence of large-vessel thrombosis, including pulmonary embolism.[61] However, an etiologic role for RBCs in VTE remains unclear.

Once in the clot, RBCs appear to exert direct, complex effects on clot structure and stability. For example, RBCs have been shown to increase the size of pores in the fibrin network[62], but decrease fibrin network permeability[63]. Finally, the presence of RBCs in clots suppresses plasmin generation and reduces clot dissolution.[64] These observations suggest that decreasing thrombus RBC content would accelerate thrombus resolution. Our recent discovery that FXIII activity is required for RBC retention in clots and that FXIII inhibition reduces venous thrombus size in mice suggests FXIII may be an attractive target for the development of novel anti-thrombotic agents.

1.6 Focus of this Dissertation

The pathophysiologic interplay between plasma clotting factors and blood cells is an important research topic to improve our understanding of mechanisms that tip the hemostatic balance towards thrombosis in both arterial and venous vascular beds. The studies comprised within this dissertation emphasize the importance of complementary *in vitro* and *in vivo* studies of coagulation and thrombosis. We investigated 1) the role of elevated prothrombin in arterial and venous thrombosis; 2) the differential procoagulant activities of microparticles from different cells; and 3) the role of fibrin(ogen) and FXIII in venous thrombosis.

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CHAPTER 2: ELEVATED PROTHROMBIN PROMOTES VENOUS, BUT NOT ARTERIAL, THROMBOSIS IN MICE[1]

2.1 Introduction

Arterial thrombosis and venous thrombosis/thromboembolism are traditionally regarded as distinct diseases with respect to their epidemiology and treatment strategies (reviewed in[2,3]). The presence of certain non-overlapping risk factors suggests that distinct features in the arterial and venous environments confer differential pathophysiology. Venous thrombosis is often associated with acquired or inherited plasma hypercoagulability and is thought to be triggered by expression of cell adhesion molecules and procoagulant activity on intact endothelium in low shear. In contrast, arterial thrombosis is typically associated with atherosclerotic plaque rupture and exposure of subendothelial cells and highly procoagulant material (tissue factor [TF] and collagen) to blood in high shear. Consequently, venous thrombi are high in erythrocyte and fibrin content; whereas, arterial thrombi are platelet-rich. Treatment strategies to minimize venous thrombosis/thromboembolism and arterial thrombosis (anticoagulants and platelet antagonists, respectively) have reduced efficacy *vice versa*[2-4], supporting the premise that unique pathophysiological mechanisms promote thrombosis in veins and arteries.

Individuals with elevated prothrombin (hyperprothrombinemia), including those with the G20210A mutation in the prothrombin 3'-untranslated region[5,6], have ~3-fold increased risk for venous thrombosis. In particular, the G20210A mutation is associated with ~115-170% of normal prothrombin activity levels.[5-7] This mutation is present in 1-4% of the

general European population[8], making it the 2nd most common genetic risk factor for venous thrombosis in Caucasians. In contrast, association of either elevated prothrombin or the G20210A mutation with arterial thrombosis is unclear.[9,10] Although the G20210A mutation has been weakly-associated with arterial disease[11] including coronary heart disease[12], ischemic stroke[13], and risk of myocardial infarction in young women[14] and men[15], other investigations have failed to support these findings in patients with cerebral ischemia[16,17], myocardial infarction[18], or peripheral arterial events[19]. Patient heterogeneity may reconcile differences between these studies since arterial risk is increased in patients with additional risk factors (e.g., smoking, hypertension, diabetes and/or obesity)[14,15]; however, the independent association between elevated prothrombin and arterial thrombosis is difficult to discern in a human cohort and remains unresolved.

The operant pathological mechanisms of hyperprothrombinemia in either venous or arterial vascular beds are also unknown. Individuals with elevated prothrombin do not have increased circulating prothrombin fragment 1.2 levels[7], suggesting thrombosis does not result from constitutive activation of coagulation. However, *in vitro* studies show that following coagulation activation, high prothrombin levels increase thrombin generation[20-23], induce activated protein C resistance[24], and promote formation of abnormal fibrin networks[20] in clots that resist fibrinolysis[24,25]. These findings suggest elevated prothrombin levels promote thrombosis; however, this effect has never been directly demonstrated *in vivo*.

The objectives of the present study were to define the prothrombotic role(s) of elevated prothrombin *in vivo* and distinguish pathologic mechanisms differentiating these effects in venous and arterial thrombosis. We found that elevated prothrombin increased plasma thrombin generation *ex vivo* following TF-dependent initiation of coagulation, but did

not activate coagulation or increase baseline thrombin generation *in vivo* in the absence of overt vascular injury. Following vascular injury, elevated prothrombin increased *in vivo* thrombin generation, but did not increase the rate of platelet accumulation in either arterial or venous thrombi. Elevated prothrombin increased the rate of fibrin deposition and produced larger thrombi in models of venous thrombosis. In contrast, elevated prothrombin did not increase the rate of fibrin deposition or shorten the time to occlusion (TTO) in models of arterial thrombosis. These data are the first to show that elevated prothrombin levels directly promote venous thrombosis *in vivo*, and show elevated prothrombin has little to no independent contribution to arterial thrombosis in the absence of additional risk factors.

2.2 Materials & Methods

Proteins and materials. Human and murine prothrombin and human thrombin were from Haematologic Technologies, Inc (Essex Junction, VT). Murine thrombin was from Enzyme Research Laboratories (South Bend, IN). Human prothrombin was treated with inhibitors[21,26], concentrated, and dialyzed into sterile 20 mM HEPES (pH 7.4)/150 mM NaCl (HBS). Prothrombin concentrate contained less than 0.004% thrombin (the lower level of detection). Murine thrombomodulin and protein C were from R&D Systems (Minneapolis, MN), Pefachrome PCa from Pentapharm (Switzerland), and hirudin from Calbiochem (La Jolla, CA). Rhodamine 6G was from Sigma-Aldrich. Rabbit polyclonal antibodies against human fibrinogen (A0080) and human prothrombin (A0325) were from DAKO (Carpinteria, CA). Fluorescent secondary antibodies and protein labeling kits were from Invitrogen (Carlsbad, CA). Anti-fibrin antibody (59D8) was from a clone generously provided by Drs.

Marschall Runge [University of North Carolina (UNC)] and Charles Esmon (Oklahoma College of Medicine).

Murine platelet-poor plasma (PPP) was prepared from blood drawn from the inferior vena cava (IVC) of 49 female C57BL/6 mice into 3.2% sodium citrate and processed by centrifugation (4,000*xg*, 20 minutes).

Thrombosis models. Procedures were approved by the UNC and Medical College of Wisconsin Institutional Animal Care and Use Committees. Mice (6-8 week old male C57BL/6) were purchased from Charles River Laboratories (Raleigh, NC) or Harlan Laboratories (Indianapolis, IN). The left saphenous vein or jugular vein was exposed and catheterized as described. [27-29] Prothrombin or vehicle (HBS) was administered through the catheter on a per weight basis [blood volume (mL) is 7% of body weight (g), plasma is 50% of blood volume] 5 minutes before injury.

The electrolytic injury models were performed as described.[30,31] Briefly, mice were anesthetized with intraperitoneal pentobarbital, and rhodamine 6G (platelet label), Alexa Fluor 647-labeled anti-fibrin (59D8), and prothrombin (to 200%, final) or saline were infused via a jugular vein branch 3-5 minutes before thrombus induction. Antibody 59D8 does not inhibit fibrin polymerization.[30] Injury was induced with 30-second, 3-V direct current application via 140-µm diameter steel blunt-end needle on the carotid artery surface, or with a 30-second, 1.5-V direct current application via 70-µm blunt-end needle to the femoral vein surface. Vessels were illuminated with beam-expanded green (532 nm) and red (650 nm) laser light. Fluorescent time-lapse video images were captured over 60 minutes with a low-light camera attached to a microscope at 100X magnification. Video images at 2-minute

intervals were analyzed for relative fluorophore intensity (ImageJ 1.45s) within the thrombus zone and normalized for inter-animal comparisons. Rates of platelet and fibrin accumulation were determined by linear fit to 3 or more points exhibiting the maximal rate of increase.

The $FeCl_3$/carotid artery thrombosis model was performed as described.[28,29] Briefly, mice were anesthetized with 1.5-2% isoflurane in oxygen and infused with prothrombin (to 200%, final) or vehicle (HBS) via left saphenous vein catheter 5 minutes before thrombus induction. The right common carotid artery was dissected and placed on parafilm. $FeCl_3$ (7.5%) was applied to the artery for 2 minutes. The artery was washed with warm saline and blood flow was monitored via Doppler transonic flow probe (Indus Instruments, Webster, TX). The TTO was defined as the time between $FeCl_3$ administration and lack of flow for 60 consecutive seconds.

The IVC stasis model was performed as described.[32,33] Briefly, mice were anesthetized with 1.5-2% isoflurane in 2% oxygen and prothrombin (to 300%, final) or vehicle was infused via tail vein injection. Following sterile laparotomy, the intestines were exteriorized, the IVC was dissected bluntly, and side branches were ligated with 8-0 Prolene suture and lumbar branches closed by cautery. The IVC was separated from the aorta by blunt dissection and completely ligated with 8-0 Prolene suture. After replacing the intestines, the muscle layer was closed with 5-0 vicryl suture and skin closed with 8-0 Prolene suture and skin glue. Mice recovered with analgesia (buprenorphine, 0.05 mg/kg subcutaneous). After 12 hours, blood was drawn from the IVC above the ligation site into 3.2% sodium citrate and processed to PPP by centrifugation at 5,000*xg* for 10 minutes. Thrombi were collected and weighed.

Detection of circulating human prothrombin antigen. PPP (1:10 dilution) was separated on Novex 10% Tris-Glycine gels and transferred to polyvinylidene difluoride membranes (Invitrogen). Membranes were blocked for 1 hour at room temperature with Odyssey blocking buffer (LI-COR Biosciences, Lincoln, NE). Primary antibodies were incubated overnight at 4°C. After washing three times with 10 mM phosphate (pH 7.4), 150 mM NaCl containing 0.1% Tween-20, membranes were incubated with fluorescently-labeled secondary antibodies (1:15,000 dilution) for 1 hour at room temperature. Membranes were then washed 3 times and scanned using an Odyssey® Infrared Imaging System (LI-COR Biosciences). Band intensity was quantified by densitometry.

Detection of circulating human prothrombin activity. The final level of circulating prothrombin was confirmed by clotting assay in separate, uninjured mice as described.[29] Briefly, blood was drawn from the IVC 5 minutes after infusion into 3.2% sodium citrate and processed to PPP by centrifugation (5,000*xg*, 10 minutes). Murine PPP was mixed with human prothrombin-deficient PPP (5% murine PPP, 95% prothrombin-deficient PPP), and clotting was initiated with Kontact aPTT reagent (40%, final) and calcium chloride (10 mM, final). Clot formation was followed in a SpectraMax Plus 340 plate reader (Molecular Devices, Sunnyvale, CA)[34] and compared to a standard curve. The prothrombin level of mice infused to 300% prothrombin (final, endogenous murine plus infused human) was as expected (323±12% of normal activity, n=2).

Calibrated automated thrombography. Blood was drawn from the IVC into 3.2% sodium citrate and processed to PPP by centrifugation (5,000*xg*, 10 minutes). Thrombin generation

was measured as described.[35] Briefly, murine PPP (10 µL) was added to 30 µL HBS, and 10 µL of PPP Reagent Low or Calibrator Reagent. After 10 minutes at 37 °C, reactions were initiated by addition of 10 µL fluorogenic substrate/$CaCl_2$. Thrombin generation was detected on a Fluoroskan Ascent fluorometer (Thermo Labsystems) and evaluated using Thrombinoscope software v3.0.0.29.

Measurement of circulating thrombin-antithrombin (TAT) complexes. TAT levels were measured by ELISA (Enzygnost TAT microELISA, Siemens Healthcare Diagnostics Inc., Deerfield, IL) using citrated PPP prepared from IVC blood draws. This kit detects mouse antithrombin in complex with either mouse or human thrombin, but is slightly (1.3-fold) more sensitive to human/mouse chimeric TAT complexes than mouse/mouse TAT complexes (data not shown).

Statistical Methods. Descriptive statistics (mean and standard deviation [SD] or standard error of the mean [SEM], as indicated) were calculated for each experiment. Analysis of variance (ANOVA) was used to identify significance in experiments with more than two dosing groups, with posthoc Student-Newman-Keuls tests for between-groups comparisons. Datasets with only two comparison groups (control and elevated prothrombin) were compared using a Student t test (normally-distributed data) or Wilcoxon-Mann-Whitney Rank Sum Test (non-normally distributed data) in Kaleidagraph version 4.1.3. $P<0.05$ was considered statistically significant.

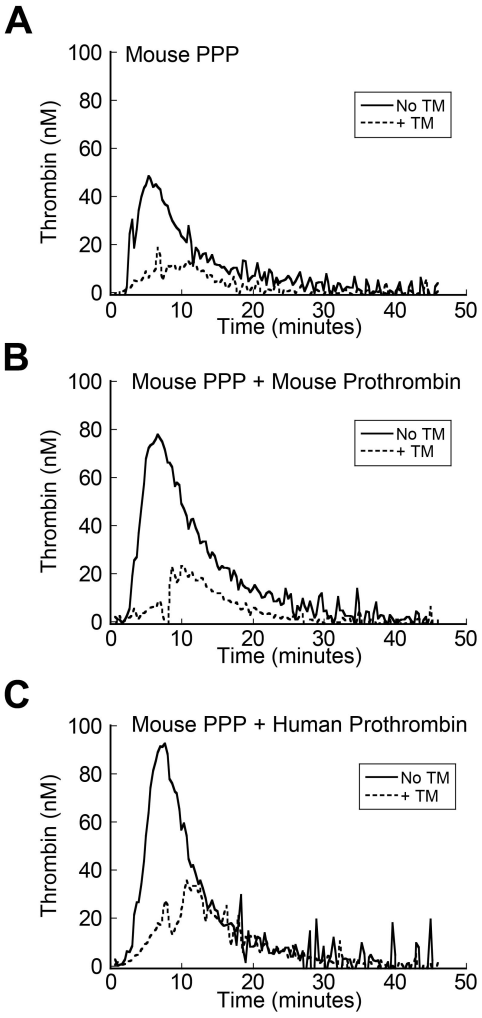

Figure 2.1. Human prothrombin supports thrombin generation in murine plasma and mouse thrombomodulin reduces thrombin generation in mouse plasma spiked with human prothrombin. Thrombin generation was measured in the absence and presence of 200 nM thrombomodulin in **A)** murine PPP spiked with vehicle, **B)** murine PPP spiked to 200% with murine prothrombin, and **C)** murine PPP spiked to 200% with human prothrombin.

2.3 Results

Human prothrombin is active in murine plasma. The *in vivo* hyperprothrombinemia model was developed by infusing mice with human prothrombin. Human prothrombin and the thrombin B chain have 81.4% and 88.8% amino acid identity with murine prothrombin and thrombin, respectively, and highly-conserved substitutions in non-identical residues.[36] Human and mouse thrombin bind and cleave human and mouse fibrinogen[37], activate platelets to form aggregates with pseudopodia[38], bind murine thrombomodulin, and support activated protein C generation[39,40]. To assess the ability of human (pro)thrombin to support thrombin generation in murine plasma, murine plasma was spiked with vehicle or murine or human prothrombin to 200% (final, endogenous plus spiked human prothrombin) and thrombin generation was measured by calibrated automated thrombography in the absence and presence of 200 nM murine thrombomodulin. Thrombin generation peaks were 49.4±7.2, 82.4±13.8 and 90.4±5.2 nM (mean±SD, n=2-3) for plasma plus vehicle, plasma plus murine prothrombin, or plasma plus human prothrombin, respectively, and addition of murine thrombomodulin reduced the thrombin peaks by 73, 74, and 64%, respectively (Figure 2.1). These data demonstrate that human (pro)thrombin is compatible with the murine procoagulant and anticoagulant systems.

In the absence of vessel injury, elevated prothrombin does not activate coagulation. Platelet-poor plasma (PPP) from patients with elevated prothrombin demonstrates increased thrombin generation *ex vivo*[7,23]; however, these patients do not have higher circulating prothrombin fragment 1.2 compared to age-matched controls.[7] We hypothesized that elevated prothrombin does not independently cause thrombin generation in the absence of vascular

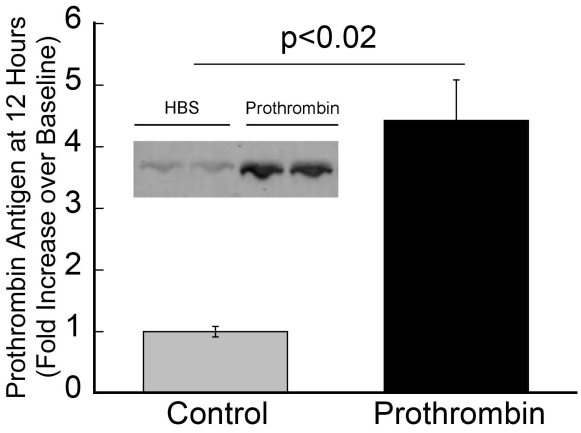

Figure 2.2. Human prothrombin circulates in mice 12 hours post-infusion. Twelve hours after infusion, blood was drawn from the IVC into 3.2% sodium citrate and processed to PPP. PPP samples were separated by 4-12% SDS-PAGE, transferred to PVDF membranes, and probed with rabbit polyclonal anti-human prothrombin antibody. Band intensity was quantified by densitometry. Since human prothrombin is better detected by the rabbit polyclonal antibody, the blot readily indicates the presence of human prothrombin in these samples.

injury, but increases thrombin generation following TF exposure. To test this hypothesis, we infused prothrombin (to 300% of normal levels; total prothrombin equals endogenous murine prothrombin plus infused human prothrombin) or vehicle [20 mM HEPES (pH 7.4)/150 mM NaCl (HBS), Control] into uninjured mice and measured prothrombin antigen by western blotting, thrombin generation *ex vivo* by calibrated automated thrombography, and thrombin-antithrombin complex (TAT) levels by ELISA 12 hours after infusion. Human prothrombin still circulated in mice 12 hours post-infusion and was strongly detected by the rabbit anti-human prothrombin antibody (Figure 2.2). Consistent with humans, thrombin generation was significantly elevated in PPP from prothrombin-infused mice compared to controls (49.5±2.1 *versus* 39.3±2.5 peak thrombin, respectively, mean±SEM, $P<0.04$) (Figure 2.3A) following initiation of coagulation *ex vivo*. However, also consistent with that seen in humans, circulating TAT levels in mice with elevated prothrombin, even at this high level, were not elevated compared to controls (2.4±1.4 *versus* 3.8±4.0 ng/mL, respectively, mean±SEM, $P=0.26$) (Figure 2.3B). These data reconcile outwardly discordant findings regarding thrombin generation in hyperprothrombinemic individuals measured *ex vivo* and *in vitr*o by showing that elevated prothrombin does not increase baseline hemostatic "idling" in the absence of vascular injury, but augments thrombin generation following a procoagulant trigger.

Elevated prothrombin accelerates fibrin deposition and produces larger thrombi in venous thrombosis models. To characterize the effect of elevated prothrombin on venous thrombosis *in vivo*, we first triggered thrombosis in the murine femoral vein via electrolytic injury and used intravital fluorescence detection to characterize the temporal and spatial contributions of

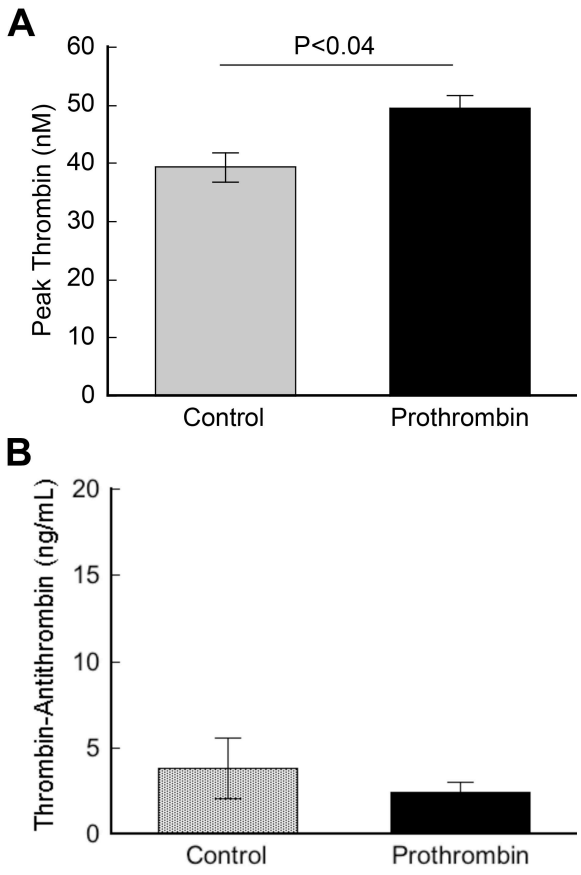

Figure 2.3. In the absence of vessel injury, elevated prothrombin does not activate coagulation. HBS (Control) or human prothrombin was infused into mice via tail vein injection to 300%, final (mouse plus human). Twelve hours after infusion, blood was drawn from the IVC into 3.2% sodium citrate and processed to PPP. **A)** PPP from mice infused with prothrombin (to 300%) or HBS (Control) was diluted 1:3 and coagulation was triggered by addition of TF/lipid. Thrombin generation was measured by calibrated automated thrombography (n=3/group). **B)** TAT levels were measured by ELISA (n=5/group). Bars show mean±SEM.

elevated prothrombin to thrombus formation. The electrolytic injury model induces mural thrombus formation via iron-mediated injury that causes early platelet accumulation followed by fibrin accumulation[30] (Figure 2.4A, Supplemental Videos I and II, available online[1]). Thrombus formation in this model is reduced by heparin, consistent with the sensitivity of venous thrombosis to thrombin generation.[41] We tested 2 levels of prothrombin: 130% and 200% (final); these levels were chosen to approximate the mean and upper end of the pathophysiologic range. Neither prothrombin concentration significantly increased the rate or total amount of platelet accumulation in femoral vein thrombi (Figures 2.4B, D). However, the plasma prothrombin level showed a dose-dependent effect on fibrin accumulation in the vein. At 60 minutes, fibrin accumulation in mice infused to 130% and 200% prothrombin was 1.7- (P<0.06) and 3.5- (P<0.002) fold higher, respectively, than control mice (Figure 2.4C), and mice infused with 200% prothrombin exhibited a significantly (2.3-fold, P=0.006) increased fibrin accumulation rate than control mice (Figure 2.4D). Furthermore, in contrast to control thrombi that remained relatively localized to the thrombus induction site, thrombi in prothrombin-infused mice showed considerable downstream elongation of a mass containing both fibrin and platelets (Supplemental Videos I, II).

We also characterized the effect of elevated prothrombin on venous thrombosis in an inferior vena cava (IVC) ligation (stasis) model that triggers venous thrombosis via vessel distention, blood stasis, and dysfunction (exposure of vessel wall TF) of intact endothelium.[42] For both control and prothrombin-infused mice, circulating TATs were significantly higher in mice that underwent IVC ligation than in mice that did not (Figure 2.5A), demonstrating activation of coagulation following vessel ligation. Circulating prothrombin was spiked to

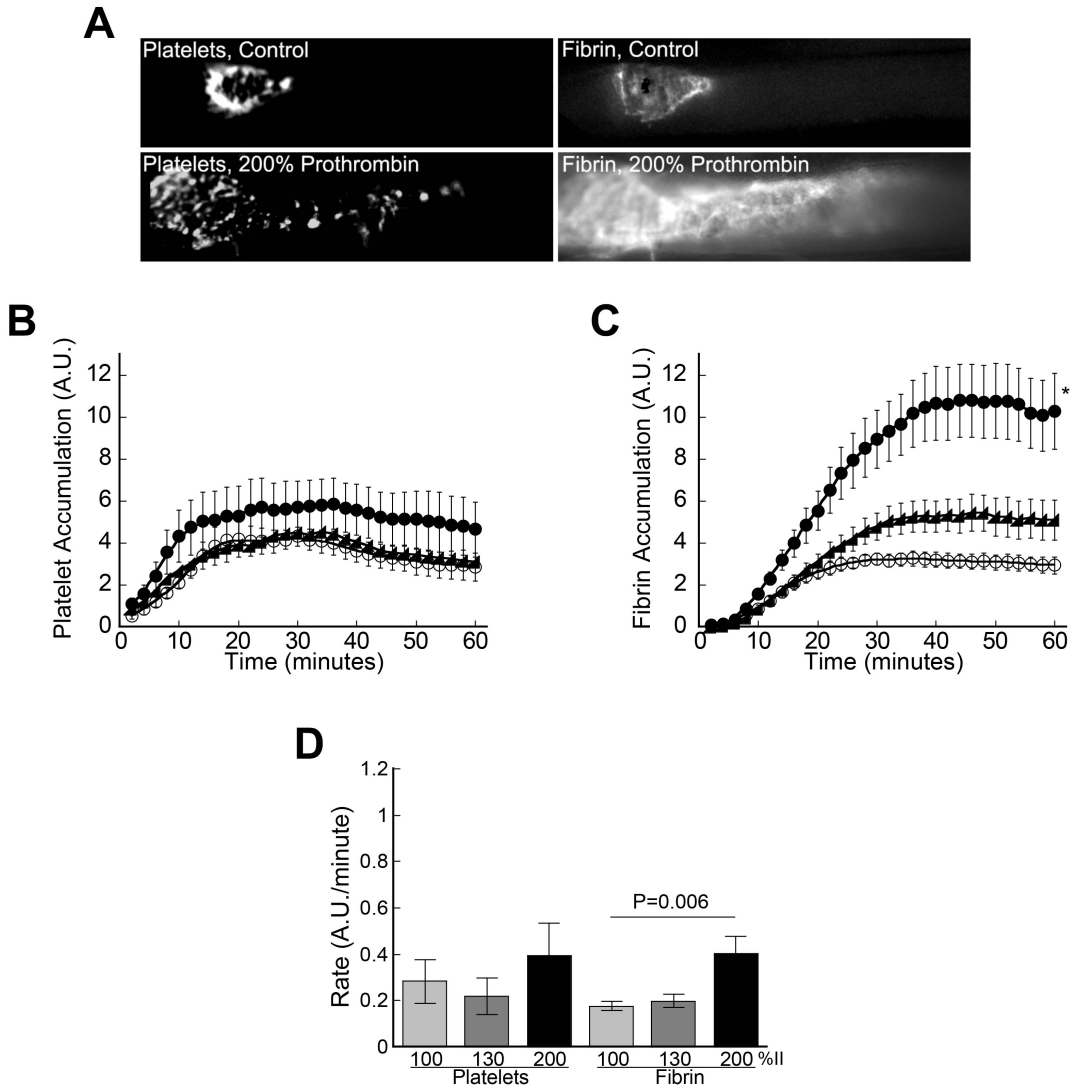

Figure 2.4. Elevated prothrombin increases the rate and extent of fibrin deposition following electrolytic injury to the femoral vein. Mice were infused with vehicle (control) or prothrombin to 200% of normal. Thrombosis was induced by electrolytic injury as described in Methods. **A)** Representative images of platelet and fibrin accumulation 60 minutes after induction of femoral vein thrombosis via application of electric current are shown. Flow is from left to right; field dimensions are 3.20 x 0.85 mm (width x height). **B-C**) Relative fluorescence intensity of platelet (**B**) and fibrin (**C)** accumulation following electrolytic injury to the femoral vein (mean±SEM, n=8/group, **P*<0.002). Symbols are: control (open circles) and prothrombin-infused (closed triangles, 130%; closed circles, 200%). **D)** Maximum rate of platelet and fibrin accumulation during femoral vein thrombus formation following electrolytic injury (mean±SEM, n=8/group).

300% so that levels remained elevated for the duration of thrombus formation (12 hours). Following IVC ligation, TATs were more than 2-fold higher in prothrombin-infused mice compared to control mice (47.9±6.5 *versus* 21.2±4.5 ng/mL, respectively, mean±SEM, $P<0.009$, Figure 2.5A), suggesting elevated prothrombin augmented thrombin generation during venous thrombogenesis. Following IVC ligation, all prothrombin-infused and control mice developed thrombi within 12 hours. Similar to that seen in the electrolytic injury model, thrombi in prothrombin-infused mice were significantly larger than thrombi in control mice [27.6 (26.1-43.6) *versus* 22.6 (9.5-27.7) mg, respectively, median (range), $P=0.01$, Figure 2.5B], and in some mice extended into the iliac branches. Together, these data show that elevated prothrombin augments venous thrombus formation *in vivo* by increasing thrombin generation and intravascular fibrin deposition.

Elevated prothrombin has little to no effect on the TTO or rate of platelet accumulation in arterial thrombosis models. We then used the electrolytic injury and real-time fluorescence detection method to measure the kinetics of platelet and fibrin accumulation in control and prothrombin-infused mice during arterial thrombosis (Figures 2.6A-D, Supplemental Videos III and IV, available online[1]). Platelet accumulation in the artery was 3.2-fold faster ($P<0.003$) than that in the vein (Figure 2.6B, D *versus* 2.4B, D) and was complete within 10 minutes, consistent with the strongly platelet-dependent mechanism associated with arterial thrombosis. At 60 minutes, arterial thrombi also had 50% less fibrin deposition than venous thrombi (Figure 2.6C *versus* 2.4C), further illustrating mechanistic differences in thrombus formation in these two vessels. Arterial clots exhibited some embolization, indicated by the loss of mean fluorescence. As in the venous model, prothrombin levels were raised to 130

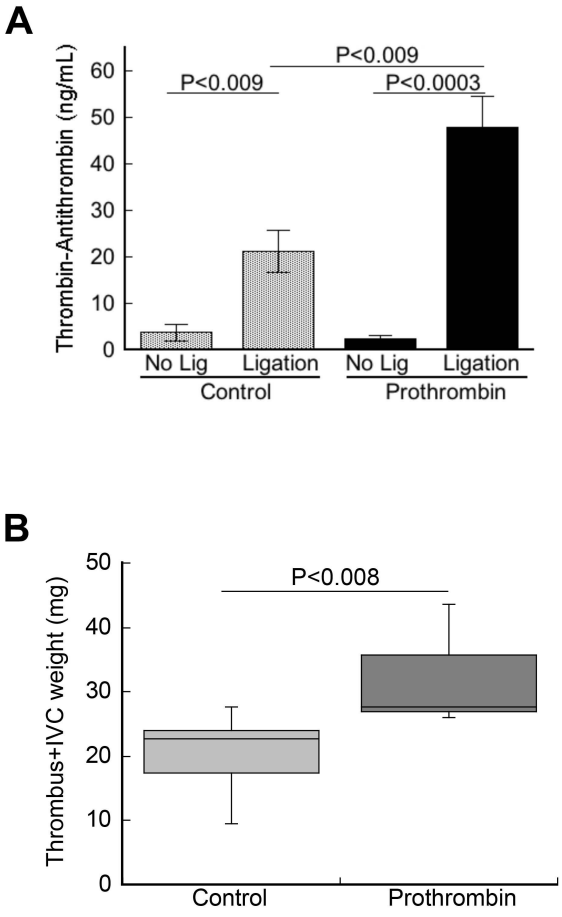

Figure 2.5. Elevated prothrombin produces larger venous thrombi by increasing thrombin generation following IVC ligation. A) IVC stasis was induced in mice infused with prothrombin (to 300%) or vehicle control. Twelve hours after ligation, blood was collected into citrate from just above the ligation site. TAT levels were measured from ligated (n=6-8/group) and uninjured mice (n=5/group) in parallel. Bars show mean±SEM. **B)** Twelve hours after ligation, the IVCs plus thrombi were excised and weighed. The box plot indicates medians and upper and lower quartiles (n=6-8/group).

and 200% of normal (final). Neither prothrombin concentration significantly increased either the rate or total amount of platelet accumulation, or the rate of fibrin accumulation in carotid artery thrombi (Figures 2.6B-D). Elevated prothrombin increased fibrin deposition at 60 minutes (~2-fold, Figure 2.6C), but this difference did not reach statistical significance (P<0.085).

We then tested the effect of elevated prothrombin in a second model of arterial thrombosis. The $FeCl_3$ application/carotid artery model triggers arterial thrombosis via generation of reactive oxygen species and exposure of collagen, resulting in a platelet-rich thrombus.[43-45] Since neither prothrombin concentration significantly altered arterial thrombus formation in the electrolytic model, we only tested the higher concentration of prothrombin (infusing to 200%, final) in the $FeCl_3$/carotid artery model. Prothrombin infusion transiently elevated TAT levels (12.6±3.2 ng/mL), likely reflecting trace (<0.004%) thrombin contamination in the prothrombin concentrate that was immediately inhibited by endogenous antithrombin (present in 1000-fold excess, 2.5 μM plasma level) with no physiologic effects (data not shown and [46,47]). For both control and prothrombin-infused mice, circulating TATs were significantly higher in mice that underwent $FeCl_3$ injury than in mice that did not (Figure 2.6E), demonstrating activation of coagulation following $FeCl_3$ injury. Following $FeCl_3$ injury, TAT levels were significantly different between control and prothrombin-infused mice (13.9±1.7 *versus* 23.5±2.6, for control and prothrombin-infused mice, respectively, P<0.007, Figure 2.6E). Interestingly, the absolute increase in TAT levels following arterial injury was similar in both control and prothrombin-infused mice, suggesting arterial injury activated coagulation to a similar degree in both groups. Consistent with this observation, both control and prothrombin-infused mice developed stable, occlusive

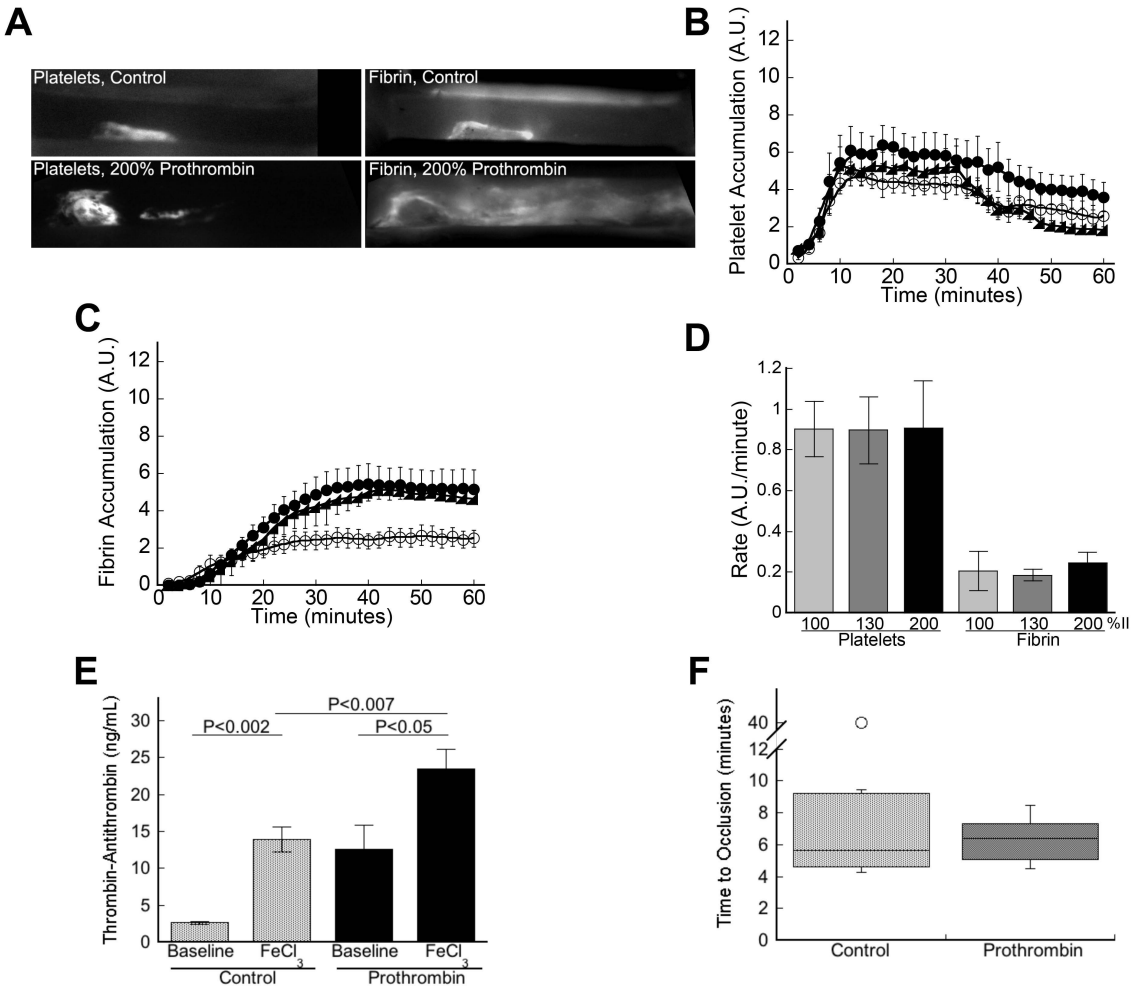

Figure 2.6. Elevated prothrombin does not increase thrombin generation or the rate of platelet or fibrin accumulation, and does not shorten the TTO in arterial injury models. **A)** Mice were infused with vehicle (Control) or prothrombin to 130 or 200% of normal. Thrombosis was induced by electrolytic injury as described in Methods. Representative images of platelet and fibrin accumulation 60 minutes after induction of carotid artery thrombosis via electrolytic injury are shown. Flow is from left to right; field dimensions are 3.20 x 0.85 mm (width x height). **B-D**) Relative fluorescence intensity of platelet **(B)** and fibrin **(C)** accumulation following electrolytic injury to the carotid artery (mean±SEM, n=7-8/group). Symbols are: control (open circles) and prothrombin-infused (closed triangles, 130%; closed circles, 200%). **D)** Maximum rate of platelet and fibrin accumulation during carotid artery thrombosis following electrolytic injury (mean±SEM, n=7-8/group). **E)** Mice were infused with vehicle (Control) or prothrombin to 200% of normal. Thrombosis was induced by 7.5% $FeCl_3$ application to the carotid artery for 2 minutes. Following thrombus formation, blood was collected from the IVC into citrate and processed to PPP. TAT levels were measured in $FeCl_3$-treated (n=7-10/group) and uninjured mice (n=4/group) in parallel. Bars show mean±SEM. **F)** Following $FeCl_3$ injury, the TTO was determined by flow probe. The box plot indicates medians and upper and lower quartiles (n=10/group); the open circle indicates one mouse that did not occlude.

thrombi, and the time to vessel occlusion in prothrombin-infused mice was not different from controls (9.5 [4.3-40.0] *versus* 6.4 [4.5-8.5] minutes, respectively, median [range], Figure 2.6F). Thus, elevated prothrombin did not significantly increase platelet or fibrin accumulation during arterial thrombus formation or shorten the time to artery occlusion. Together, these data show that although elevated prothrombin promotes venous thrombus formation, it does not significantly augment arterial (platelet-dependent) thrombosis.

2.4 Discussion

Elevated prothrombin is a well-established risk factor for venous thrombosis, but its relationship to arterial thrombosis is unclear. Using state-of-the-art *in vivo* models of venous thrombosis and arterial thrombosis, we show that elevated prothrombin did not increase baseline prothrombotic markers in unchallenged mice, but did increase thrombin generation following venous injury. The presence of elevated prothrombin did not accelerate intravascular platelet accumulation following either venous or arterial injury. In venous thrombosis models, mice with elevated prothrombin exhibited an increased rate and amount of fibrin accumulation, thrombus extension and formation of thrombi with increased mass. However, in arterial thrombosis models, elevated prothrombin slightly (non-significantly) increased the total amount of fibrin deposited, but did not increase the rate of fibrin accumulation or shorten the TTO. These findings suggest elevated prothrombin has little to no independent contribution to arterial thrombosis, and are the first to show that elevated prothrombin levels directly promote venous thrombosis *in vivo.*

The choice of murine thrombosis model for investigating human thrombosis has been the subject of considerable debate because many models fail to recapitulate key aspects of the

arterial and venous thrombogenic processes.[48] An important strength of our study was the use of complementary arterial and venous models to delineate both kinetic processes and their consequences for thrombus composition. Complementary, integrated information from the two arterial models and two venous models reveals both common and vascular bed-specific processes operant in these vessels that are consistent with the histological appearance of arterial and venous thrombi isolated from humans. Arterial injury produced rapid platelet accumulation; whereas, venous injury resulted in slower thrombus formation with fibrin accumulation. We used models that produced both occlusive ($FeCl_3$/carotid and IVC) and non-occlusive (electrolytic injury) thrombi. Findings were consistent within vessels, but differed between arteries and veins, suggesting these models are sensitive to the unique physical and biochemical environments within the different vessels.

We detected both common and vascular bed-specific effects of elevated prothrombin on arterial and venous thrombosis. Elevated prothrombin significantly augmented endogenous thrombin generation in the venous model, but only slightly (non-significantly) in the arterial model (Figures 2.5A, 2.6E). Consistent with our findings, a recent study showed that *Apo*E$^{-/-}$ mice expressing half of the prothrombin level of wild type mice (*FII*$^{/+}$) are not protected from arterial thrombosis, supporting the conclusion that variation in the prothrombin level does not mediate arterial occlusion times.[49] Notably, although thrombin is a potent agonist for platelet activation, elevated prothrombin did not accelerate the rate of platelet accumulation in either arteries or veins (Figures 2.4D, 2.6D). These findings are consistent with our prior observation that 5% prothrombin is both necessary and sufficient to maximize platelet activation.[20] Consequently, elevated prothrombin did not accelerate arterial occlusion, a platelet-dominated process. In contrast, the increased thrombin

generation significantly increased fibrin deposition and therefore, venous thrombosis, a fibrin-dominated process. We previously showed that elevated prothrombin also promotes the thrombin concentration-dependent formation of plasma clots with an abnormally dense fibrin network.[20] Although fibrin network structure is difficult to assess in thrombi in the presence of cells, combined, these results suggest that in veins, elevated prothrombin promotes thrombi with fibrin networks that have *both* increased mass and increased network density. Both properties are associated with increased clot stability *in vitro* and *in vivo,* and have been correlated with increased thrombosis risk.[50]

It is interesting that while elevated prothrombin did not accelerate arterial occlusion after $FeCl_3$ injury, elevated plasma factor VIII does[51-53]. Both elevated prothrombin and elevated factor VIII increase thrombin generation *in vitro*[20-22,53,54] and *in vivo* (Figure A and [51]). However, elevated factor VIII significantly shortens the lag time to platelet aggregation *in vitro*[51] and trends towards increased platelet accumulation *in vivo*[53]; whereas, elevated prothrombin did not significantly change the rate of platelet aggregation *in vitro*[20] or *in vivo* (Figures 2.4D, 2.6D). These data suggest elevated factor VIII modulates an early (amplification) phase of coagulation when thrombin levels are relatively low and platelet activation is taking place. Consequently, platelet-dependent arterial thrombosis models are sensitive to the effects of elevated factor VIII, but not to elevated prothrombin. This observation is consistent with the premise that procoagulant factors have complementary, but distinct, roles in different phases of coagulation.[55]

Previous studies on the association between elevated prothrombin and arterial thrombosis (myocardial infarction or ischemic stroke) have been inconsistent, showing either no or a modest relationship.[9-19] One explanation for these differences is that risk is present

only in specific groups. Of interest are observations that relative risk increases when another identifiable cardiovascular risk factor is also present and appears higher than from either risk factor alone, suggesting an additive or synergistic interaction.[14,15] A strength of our murine model in which prothrombin levels were acutely elevated in healthy wild type mice is the clear absence of other risk factors. Nonetheless, the co-existence of additional known or unidentified risk factors may augment the positive associations detected in prior studies with human cohorts. For example, on an atherosclerosis-prone background (*Apo*E$^{-/-}$), chronic plasma hypercoagulability (TM$^{Pro/Pro}$) increases atherogenesis and plaque formation, both of which are associated with atherothrombosis, and reduced prothrombin levels attenuate atherosclerotic lesion formation.[49]

This study has potential limitations. First, we used human prothrombin to increase circulating levels in the mouse. However, published studies[36-40] as well as our data show human prothrombin is stable in murine circulation and participates in murine pro- and anti-coagulant pathways. Moreover, the infusion strategy enabled us to precisely control the level of circulating prothrombin. Second, the infusion model used in these experiments does not reflect pathologic effects that chronic exposure to elevated prothrombin could have on the vasculature. Atherosclerotic disease reflects chronic vascular injury with occurrences of acute injury (plaque disruption and TF exposure). However, a major strength of the infusion model is that it enabled us to isolate and investigate the immediate, direct effects of elevated prothrombin on thrombus formation. These data on acute effects will be critical for interpreting findings from mice with genetically-induced chronic plasma hypercoagulability (*e.g.*, factor V Leiden mice); comparison of short-term and long-term exposure to hypercoagulability is likely to reveal interesting mechanisms that predispose these

individuals to thrombosis. Third, the thrombosis models we used differed in methodologic aspects, including anesthesia and analgesia protocols. However, the observation that elevated prothrombin exhibited consistent effects in each of the venous and each of the arterial models suggests the observed effects were not due to the methodologies, but to the prothrombin level, itself. Finally, the arterial and venous models used in this study were sensitive to thrombus formation, but did not reflect additional effects elevated prothrombin may have on thrombus stability. For example, although groups have demonstrated increased activation of the thrombin activatable fibrinolysis inhibitor in plasma with increased prothrombin, we did not evaluate the long-term resistance of thrombi to fibrinolysis.

In summary, our findings demonstrate that elevated prothrombin does not trigger endogenous thrombin generation in the absence of vascular injury, suggesting that in lieu of a signal that *initiates* coagulation, plasma hypercoagulability is not independently prothrombotic. These data suggest that increased coagulation biomarkers (*e.g.*, fragment 1.2 or TATs) indicate vascular dysfunction that, when coupled to additional plasma prothrombotic potential, promote thrombosis. Our findings further show that elevated prothrombin increases thrombin generation following vascular injury. Elevated prothrombin does not accelerate platelet activation in either the artery or the vein, but significantly increases the rate and amount of fibrin deposition following venous injury. These findings are consistent with findings that elevated prothrombin is associated with venous thrombosis in humans[5-7,56], but is only weakly associated with arterial thrombosis in the absence of other risk factors.[14-19,56] These results support the relevance of murine thrombosis models to

studies of hypercoagulability-related thrombosis in humans. Integrating complementary *in vivo* models is a powerful approach to investigate the underlying mechanisms of hemostatic and thrombotic processes.

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CHAPTER 3: DIFFERENTIAL CONTRIBUTIONS OF MONOCYTE- AND PLATELET-DERIVED MICROPARTICLES TOWARDS THROMBIN GENERATION AND FIBRIN FORMATION AND STABILITY[1]

3.1 Introduction

Once considered simply cell debris, microparticles (MPs) are bioactive sub-micron (0.1-1 μm) membrane vesicles shed from activated and apoptotic cells in culture and *in vivo*. While detectable in healthy controls[2], MP levels are greatly elevated in patients with diseases including venous thromboembolism[3], hypertension[4], diabetes mellitus[5], and cancer[6,7]. Platelet-derived MPs (PMPs) comprise the largest fraction of MPs in healthy controls[2] and patients[6,8]. PMP concentrations in patients range from ~3000-11,000/μL.[3,5,9] Monocyte-derived MPs (M-MPs) are low or undetectable in healthy controls, but circulate at levels from ~300-1300/μL in sickle cell disease, cancer, and other diseases.[3,5,7,10]

Despite studies demonstrating circulating MPs in healthy individuals and patients, MP-mediated mechanisms contributing to hemostasis or thrombosis are not understood. MPs bear surface antigens from their parent cells and anionic phospholipids such as phosphatidylserine (PS).[11] These properties are thought to define MP function. Observations that MPs support thrombin generation[2,12] suggest MPs contribute to hemostasis via "idling" of the coagulation system, evidenced by the presence of activation peptides and prothrombin fragment 1.2 in healthy individuals[13]. However, it is difficult to confirm the role of MP procoagulant activity in hemostasis *in vivo* because MPs cannot be depleted from circulation. Increased levels of MPs in thrombotic disease suggest MP procoagulant activity tips

coagulation "idling" towards full-fledged activation and promotes thrombosis. TF-bearing MPs, in particular, may independently initiate or propagate coagulation via recruitment to developing thrombi.[14-16] Human M-MPs promote fibrin accumulation in a murine carotid artery ligation model[17], and injection of MPs isolated from mice after inferior vena cava thrombosis into new mice prior to inferior vena cava ligation increases thrombus weight at early time points.[18] The mechanism(s) by which MP procoagulant activity increases fibrin deposition, thrombus growth, and/or weight are not known.

Thrombin generation promotes clotting and clot stability by modulating fibrin properties, including its network structure and resistance to fibrinolysis.[19,20] These studies suggest MP procoagulant activity directly increases fibrin formation and stability. However, although procoagulant activity of heterogeneous MP pools isolated from plasma has been examined, to our knowledge, no study has examined how MPs from different parent cells promote procoagulant activity or fibrin production or quality. This information gap limits clinical interpretation of MP function in healthy individuals or pathologic contributions of elevated levels of certain MP subtypes in patients.

The goal of this study was to determine the specific contributions of monocyte- and platelet-derived MPs to thrombin generation and fibrin formation, structure, and stability. We prepared MPs from isolated human platelets, monocytes, and a monocytic cell line (THP-1) and compared their physical and biochemical properties. Our data show monocyte-derived MPs initiate thrombin generation and fibrin formation via their TF activity, whereas platelet-derived MPs propagate thrombin and fibrin production in TF- or contact-triggered plasma. These findings suggest MPs from different parent cells uniquely contribute to

coagulation, and that the relative concentration of circulating MPs from different cell types influences thrombosis risk.

3.2 Materials & Methods

Materials. Monocyte Negative and Positive Selection Kits were from Miltenyi BioTec (Auburn, CA). Accu-Prep lymphocyte gradient medium was from Accurate Chemical Co. (Westbury, NY). Prostacyclin I_2 was from Cayman Chemical Co. (Ann Arbor, MI). Thrombin receptor agonist peptide (TRAP) was from Bachem Inc. (Torrence, CA). Convulxin was from Centerchem Inc. (Norwalk, CT). Calcium Ionophore (A23187), lipopolysaccharide (LPS), control IgG (MOPC-1), and bovine serum albumin (BSA) were from Sigma Aldrich Corp. (St. Louis, MO). Fluorescein isothiocyanate (FITC)-Annexin V antibody and Annexin V Binding Buffer were from Beckman Coulter (Brea, CA). Phycoerythrin-anti-CD41 antibody and Megamix beads were from BioCytex (Marseille, France). Allophycocyanin (APC)-anti-CD14 antibody was from R&D Systems (Minneapolis, MN). Innovin was from Siemens Healthcare Diagnostics (Deerfield, IL). Mouse anti-human TF antibody (HTF-1, "anti-TF")[21] was provided by Dr. Ronald Bach (Minneapolis VA Medical Center, MN), and recombinant factor VIIa by Novo Nordisk (Denmark). Human factor X was from Enzyme Research Laboratories (South Bend, IN). Human factors Xa, Va, prothrombin, and corn trypsin inhibitor (CTI) were from Haematologic Technologies, Inc. (Essex Junction, VT). Thrombin fluorogenic substrate (Z-Gly-Gly-Arg-AMC) and calibrator (α2-macroglobulin/thrombin) were from Diagnostica Stago (Parsippany, NJ). Tissue plasminogen activator (tPA) was from Calbiochem (La Jolla, CA). Citrated, contact-inhibited (18.6 μg/mL CTI[22]), normal-pooled, platelet-free plasma[19]

was prepared from 27 individuals, with final centrifugation (20,000x*g*, 20 minutes) to prepare MP-depleted plasma (MDP).

Primary cell isolation. Peripheral blood mononuclear cells (PBMCs), monocytes, and platelets were freshly-isolated from whole blood from healthy donors in a protocol approved by the University of North Carolina (UNC) Institutional Review Board. Blood was collected into 3.2% sodium citrate and centrifuged (150x*g*, 20 minutes). The platelet-rich plasma was treated with 50 ng/mL prostacyclin I_2 to prevent nonspecific activation of platelets during preparation and centrifuged (400x*g*, 20 minutes) to pellet platelets. Platelets were re-suspended in warm (37 °C) Tyrode's buffer [1.5 mM N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid, 0.33 mM NaH_2PO_4 (pH 7.4), 13.8 mM NaCl, 0.27 mM KCl, 0.1 mM $MgCl_2$, 0.55 mM dextrose, 1 mg/mL BSA] with 50 ng/mL prostacyclin I_2 and incubated for 30 minutes at 37 °C before stimulation. To isolate PBMCs and monocytes, the buffy coat and erythrocytes were diluted with Hank's balanced salt solution containing 5 mM ethylenediamine tetraacetic acid (HBSS/EDTA), layered over Accu-prep Lymphocyte gradient medium, and centrifuged (400x*g*, 30 minutes). The PBMC buffy coat fraction was removed, washed twice by centrifugation at 250x*g*, and re-suspended in HBSS/EDTA. For negative and positive monocyte selection, the PBMC buffy coat was washed twice and re-suspended in ice-cold MACS sorting buffer (phosphate-buffered saline/0.5% BSA/2 mM EDTA). Negative and positive monocyte selection kits were used according to the manufacturer's instructions. Final enriched monocyte populations were suspended in Macrophage-Serum-Free Media (Gibco, Grand Island, NY).

Cell culture. Human monocytic leukemia cell line (THP-1) was obtained from Dr. Nigel Mackman (UNC), and cultured in RPMI 1640 media with 10% fetal bovine serum.

Microparticle isolation. Platelets were stimulated for 30 minutes at room temperature with 6 or 50 μg/mL TRAP, 6 or 50 μg/mL TRAP plus 500 ng/mL convulxin (TRAP/convulxin), or 10 μM Ca^{2+} ionophore (A23187). There were no differences in peak thrombin generation between PMPs generated with 6 or 50 μg/mL TRAP. Samples were centrifuged (1,500x*g*, 15 minutes) and MP-containing supernatants were collected. PBMCs, monocytes, and THP-1 cells were stimulated with 100 ng/mL LPS (6 hours, 37 °C) in Macrophage Serum-Free Media; this condition supports cell stimulation in the absence of LPS-binding protein.[23] Trypan blue (HyClone, Logan, UT) exclusion experiments indicated less than 10% of monocytes and less than 15% of THP-1 cells were dead following the 6-hour incubation. Cells were removed by sequential centrifugation (500x*g* for 10 minutes, 1,500x*g* for 15 minutes, 13,000x*g* for 2 minutes). MPs were pelleted (20,000x*g*, 15 minutes), re-suspended in HEPES-buffered saline with 0.1% BSA (HBS/BSA), and re-pelleted (20,000x*g*, 15 minutes). MP pellets were re-suspended in HBS/BSA and stored at 4 °C up to one week. Control experiments showed no difference in thrombin generation in fresh and 1 week-old MPs (9.6±2.7 *versus* 12.0±0.9 nM peak thrombin, respectively).

Microparticle enumeration. Flow cytometry of washed MPs was performed on an LSR-II flow cytometer (BD Biosciences, San Jose, CA), using Megamix polystyrene beads (0.5, 0.9, and 3 μm) to gate the MP region by forward- and side-scatter.[24] MPs were incubated with antibodies in Annexin V Binding Buffer. FITC-Annexin V was used to detect PS.

Phycoerythrin-anti-CD41 was used as a cell marker for PMPs and APC-anti-CD14 as a marker for PBMC-MPs and M-MPs. Fluorospheres (10 μm) were added and samples were analyzed for PS-positive/cell marker-positive (dual positive) events. MP concentration was determined per: [(Dual positive Events/Fluorosphere Bead Events)* Fluorosphere Bead Concentration]*Dilution Factor.

Microparticle physical characterization. Transmission electron microscopy (TEM) was performed by depositing MPs onto carbon-coated Formvar copper grids treated with 0.01% poly-L-lysine. MPs were negatively stained with 3% ammonium molybdate (pH 7.0)/0.1% trehalose and imaged on a LEO EM910 transmission electron microscope (Carl Zeiss SMT, Peabody, MA) operating at 80 kV. Digital images were recorded using a Gatan Orius CCD Digital Camera and Digital Micrograph 3.11.0 (Gatan INC, Pleasanton, CA). MP diameters were measured using Adobe Photoshop 8.0.

Nanoparticle Tracking Analysis (NTA) was performed using the Nanosight NS500 system (NanoSight, Amesbury, UK), which focuses a laser beam through a suspension of the particles of interest, visualizes particles resident within the beam using a conventional optical microscope aligned normally to the beam axis, and collects light scattered from all particles in the field of view. Videos (60 seconds) were recorded using an electron multiplying charge-coupled device, and particle movement was analyzed by NTA software. Each particle was identified and its Brownian movement tracked and measured frame-to-frame. The particle movement velocity was used to calculate particle size by applying the Stokes-Einstein equation.

TF and prothrombinase activity. MPs were pre-incubated with anti-TF antibody (10 μg/mL, final) or isotype-matched control IgG (10 minutes, 37 °C) and then with factor VIIa (100 pM, final, 5 minutes) and factor X (135 nM, final, 30 minutes, 37 °C), in calcium (5 mM, final). Factor Xa generation per MP (per number of cell-marker- and Annexin V-positive events) was measured by chromogenic substrate cleavage[25] and referenced to a standard curve of lipidated recombinant human TF (Innovin). Prothrombinase activity (per Annexin V-positive events) was measured as described.[19]

Thrombin generation by calibrated automated thrombography. MPs (10 μL) and 20 μL HBS/BSA were spiked into 70 μL contact-inhibited MDP with anti-TF or control IgG (10 μg/mL, final) (58% MDP, final). Thrombin generation was initiated by automatically dispensing fluorogenic substrate (Z-Gly-Gly-Arg-AMC) in $CaCl_2$ (416 μM and 16 mM, final, respectively), calibrated against wells containing α2-macroglobulin/thrombin complex and plasma, and analyzed with Thrombinoscope software v3.0.0.29 (Thrombinoscope BV, Maastricht, Netherlands).

Fibrin formation, network structure and clot stability. MPs were added to re-calcified (10 mM, final) MDP (88% MDP, final) in the absence or presence of 0.5 μg/mL tPA. Fibrin formation and lysis were measured by turbidity as described.[20] Fibrin networks were imaged in 10 μm z-stacks (4 stacks/clot) using laser scanning confocal microscopy in the presence of Alexa488-conjugated fibrinogen, as described.[19,20] Fibrin density was measured by counting fibers intersecting a randomly-applied grid, as described.[19,20]

Statistical analysis. Data are described using descriptive statistics [mean (±standard error of the mean) or mean (range), as indicated]. TF-dependent activity (anti-TF *versus* control IgG) and PMP-dependent activity (±PMPs) were analyzed by paired student's t test. For all other data, Analysis of Variance (ANOVA) with Dunnett's post hoc test using buffer as the index group was used to control type I error. $P<0.05$ was considered significant.

3.3 Results

MPs from platelets and monocytes have a similar size distribution. We first characterized MPs from TRAP, TRAP/convulxin, and A23187-treated platelets, and LPS-treated PBMCs, monocytes, and THP-1 cells using flow cytometry with Annexin V- and cell-specific marker positivity, the gold standard for MP characterization and enumeration.[24] Figure 3.1A shows the MP gate (determined by 500 nm and 900 nm Megamix fluorescent beads) from representative PMPs from TRAP-stimulated platelets; MP from TRAP/convulxin- and A23187-treated platelets appeared similar (data not shown). M-MP contamination in PMP preparations was <5%. PMP contamination in MP preparations from LPS-treated PBMCs, negatively-selected monocytes, and positively-selected monocytes was 45.4±7.4%, 33.4±8.7%, and 16.6±2.9% of Annexin V-positive events, respectively, likely from platelets contaminating monocyte preparations. M-MPs derived from positively-selected monocytes (Figure 3.1B) were used for subsequent experiments. We also used THP-1-derived MPs (THP-MPs) as a platelet-free source of monocytic MPs, as indicated.

Although flow cytometry detects particles as small as 300 nm, studies indicate MPs may be smaller (100-300 nm).[26] To explicitly determine MP size we utilized two independent techniques: TEM and NTA. TEM enables imaging and measurement of

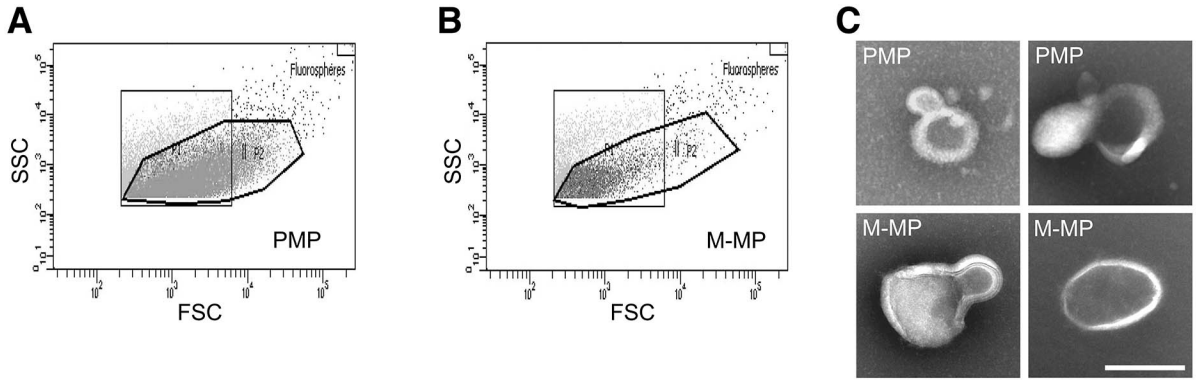

Figure 3.1. M-MPs and PMPs have similar size distributions. Representative bivariate flow cytometry plots (forward and side scatter) of **(A)** TRAP-derived PMPs and **(B)** M-MPs. **(C)** TEM of representative TRAP-derived PMPs and M-MPs. Scale bar is 200 nm.

Table 3.1. Comparison of MP sizes by TEM and NTA.

	TEM (nm, n=3-6)		**NTA (nm, n=2)**	
	Mean	Range	Mean	Range
M-MP	270	32	227	46
PMP (TRAP)	220	199	246	69
PMP (TRAP/convulxin)	290	182	184	4.3
PMP (A23187)	267	480	233	61

individual MP (Figure 3.1C), while NTA enables enumeration and size analysis of fully hydrated MPs (from 20-1000 nm) in suspension[27]. Both methodologies indicated MPs have similar diameters (150-300 nm), regardless of the parent cell (monocytes or platelets), or stimulation method (LPS, TRAP, TRAP/convulxin, or A23187) (Table 3.1). NTA indicated LPS-stimulated monocytes generated approximately 60 M-MP/monocyte, and platelets stimulated with TRAP, TRAP/convulxin, or A23187 produced approximately 1.0 PMP/platelet; both were approximately 85-fold higher than that suggested by flow cytometry, consistent with reports that flow cytometry detects only a modest proportion of MPs[27]. However, although NTA can detect fluorescently-labeled placental MPs[27], it has not been validated for fluorescently detecting M-MPs or PMPs. Since MP enumeration in prior studies of human plasmas utilized flow cytometry, we used flow cytometry-determined enumeration in all subsequent assays.

M-MPs, but not PMPs, promote thrombin generation in a TF-dependent manner. To compare inherent functional properties of MPs from platelets and monocytes, we measured both TF- and PS-dependent procoagulant activity. Figure 2A shows TF activity on M-MPs (16.7±2.4 pM TF/10^6 MP) and THP-MPs (2.88±0.8 pM TF/10^6 MP). However, PMPs, regardless of the agonist used for their derivation, did not support factor Xa generation (Figure 3.2A), suggesting PMPs do not express TF.

We next compared the ability of these MPs to support prothrombinase activity. Regardless of the agonist used for their derivation, PMPs had similar prothrombinase activity (Figure 3.2B). PMPs did not support thrombin generation in the absence of exogenous factor Va (data not shown), indicating functional, platelet-derived factor Va was not present on

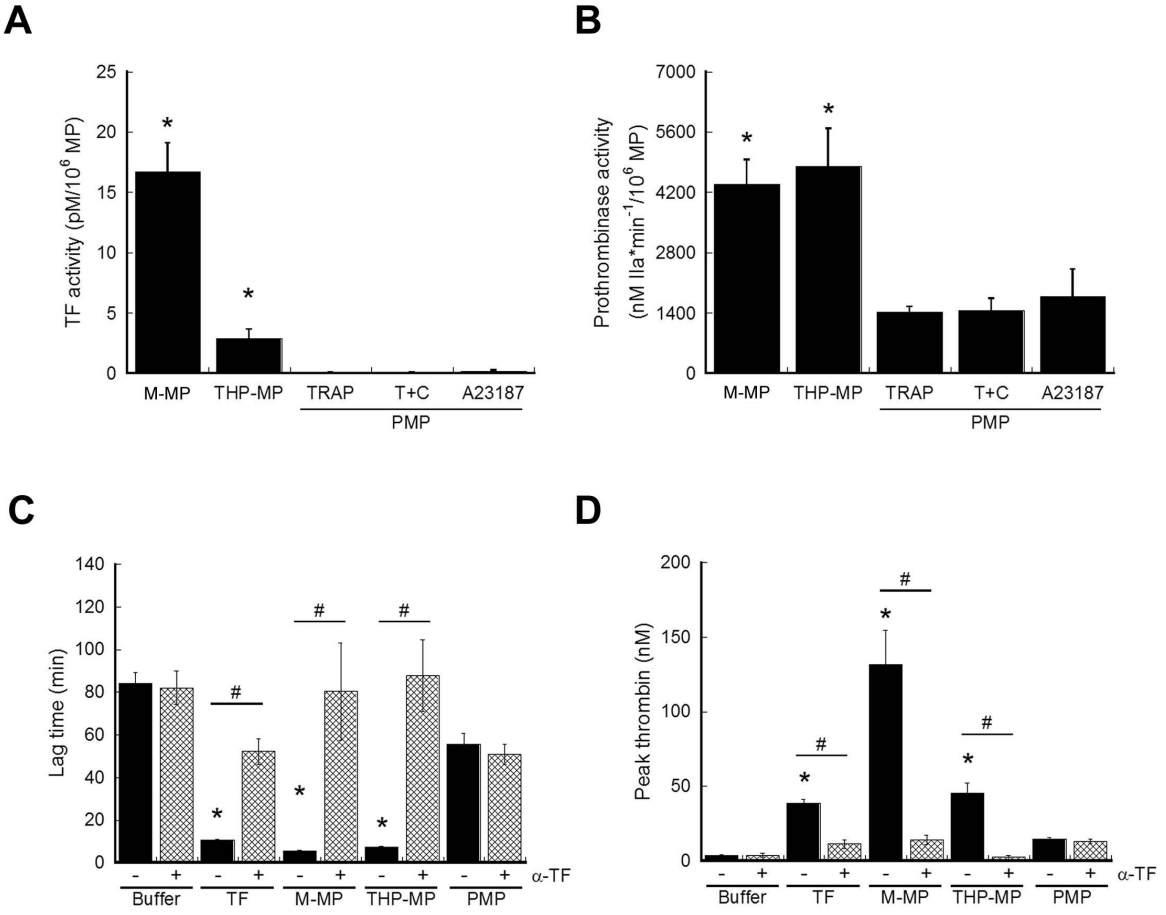

Figure 3.2. M-MPs, but not PMPs, promote thrombin generation in a TF-dependent manner. (A) TF activity (±SEM) was determined by factor Xa chromogenic substrate cleavage (n=5). **P*<0.02 by ANOVA *versus* PMPs. **(B)** Prothrombinase activity (±SEM) was determined by thrombin chromogenic substrate cleavage (n=3-4). MP counts were determined by Annexin V-positive events in flow cytometry. **P*<0.01 by ANOVA *versus* all PMPs. **(C-D)** Thrombin generation supported by 5000 PMP/μL or 1000 M-MP or THP-MP/μL was measured in the presence of anti-TF or control IgG. **(C)** Thrombin lag time and **(D)** peak (±SEM, n=3-6). Data from PMP from TRAP-stimulated platelets are shown; PMP from TRAP/convulxin- or A23187-stimulated platelets were similar. "Buffer" is HBS/BSA. TF is 1 pM (Innovin). **P*<0.05 by ANOVA *versus* buffer controls. $^{\#}P$<0.05 by paired Student's t test between anti-TF and IgG controls.

PMPs. Similarly, M-MPs and THP-MPs did not support thrombin generation in the absence of exogenous factor Va (data not shown). Flow cytometry indicated M-MPs had higher Annexin V mean fluorescence intensity than PMPs (711.0±71.0 *versus* 418.7±43.8 arbitrary units, $P<0.002$). Accordingly, M-MPs showed 2.8-fold higher prothrombinase activity than PMPs (4382±584 nM thrombin*min^{-1}/10^6 M-MP *versus* 1556±189 nM thrombin*min^{-1}/10^6 PMP, Figure 3.2B]. Although THP-MPs had lower Annexin V mean fluorescence intensity than PMPs (111.8±2.7 arbitrary units, $P<0.001$), they demonstrated higher prothrombinase activity (4807±879 nM thrombin*min^{-1}/10^6 THP-MP, Figure 3.2B) than PMPs. These data suggest functional prothrombinase activity is not necessarily predicted by Annexin V fluorescence intensity, and confirm that M-MP prothrombinase activity was not due to PMP contamination in M-MP preparations.

Given these inherent differences in the nature of procoagulant activity of monocyte (TF and PS)- and platelet (only PS)-derived MPs, we compared their ability to support thrombin generation in plasma. We spiked MPs at levels associated with thrombosis in humans (1000 M-MP/μL and 5000 PMP/μL)[3,5,7,10] into re-calcified MDP and measured thrombin generation by calibrated automated thrombography. PMPs from TRAP-, TRAP/convulxin-, or A23187-stimulated platelets slightly but non-significantly shortened the lag time and increased the peak over buffer controls (Figures 3.2C-D, data not shown). These activities were not blocked by anti-TF antibody, indicating this activity was TF-independent. Although MDP was treated with CTI to inhibit contact activation, studies have reported time-dependent loss of CTI-mediated contact inhibition.[28] Accordingly, PMPs did not trigger clotting in factor XI- or IX-deficient plasma (data not shown). These data are consistent with observations that PMP-enriched plasma from healthy donors supports contact

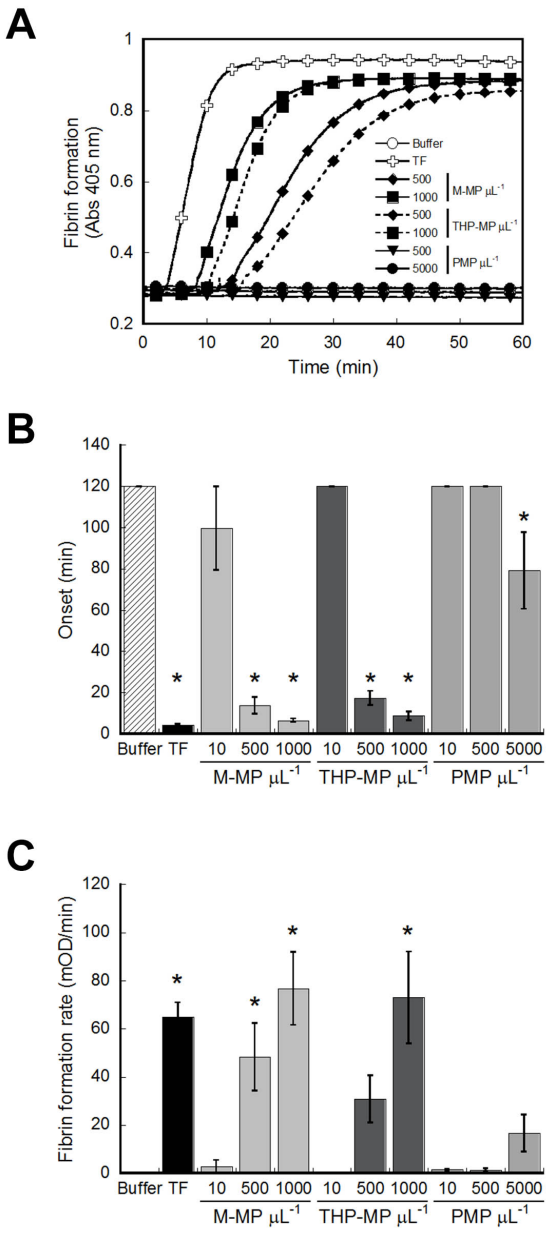

Figure 3.3. M-MPs initiate fibrin formation. MPs were spiked into re-calcified MDP and fibrin formation was followed by turbidity. **(A)** Representative fibrin formation curves. **(B)** Onset and **(C)** rate of fibrin formation. For conditions in which clots did not form, onsets were censored at 120 minutes and rates at 0 mOD/min. Data show mean (±SEM, n=4-6). TF is 1 pM (Innovin). **P*<0.0001 by ANOVA *versus* buffer controls.

pathway-driven thrombin generation[2], and attribute this finding specifically to the PMP fraction.

In contrast to PMPs, compared to buffer controls, both M-MPs and THP-MPs significantly (P<0.001) shortened the thrombin lag time (5.4±0.5 min and 7.5±0.4 min, respectively, *versus* 84.2±4.8 min, Figure 3.2C) and increased the peak (131.5±23.4 and 45.6±6.9 nM, respectively, *versus* 3.6±0.8 nM, Figure 3.2D) in a TF-dependent manner. These data indicate monocyte-derived MPs initiate thrombin generation in a TF-dependent manner, while PMPs support thrombin generation only in the presence of an intact contact pathway.

M-MPs promote fibrin formation. Given differences in PMP and M-MP procoagulant activity, we compared the ability of MPs to support fibrin formation by spiking MPs into re-calcified MDP and following fibrin formation by turbidity. PMPs from TRAP- (Figures 3.3A, B), TRAP/convulxin- (data not shown) or A23187- (data not shown) stimulated platelets inconsistently triggered fibrin formation after 60 minutes only at the highest concentrations tested, and in a TF-independent mechanism. As in thrombin generation assays (Figure 3.2), PMPs did not trigger clotting in contact (factor XII)-deficient plasma for up to 120 minutes (data not shown), indicating PMP activity in MDP reflected time-dependent loss of contact inhibition[28]. In contrast, M-MPs and THP-MPs significantly (P<0.0001) shortened the onset (Figures 3.3A, B) and increased the rate (Figures 3.3A, C) of fibrin formation in a concentration-dependent manner that was completely blocked by anti-TF antibody (data not shown). These data indicate M-MPs and THP-MPs trigger TF-

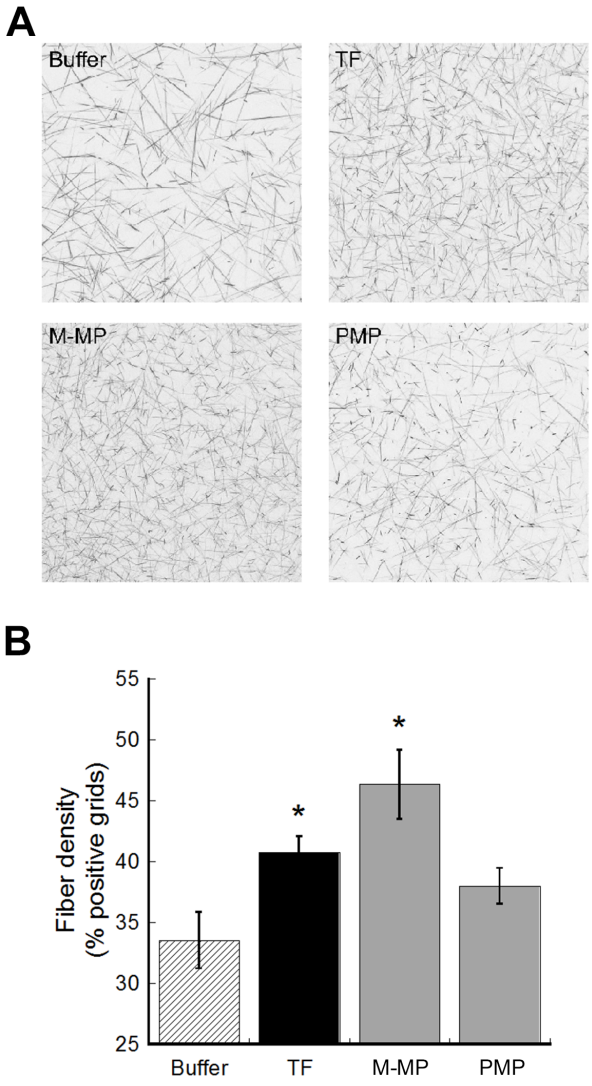

Figure 3.4. M-MPs, but not PMPs, increase fibrin network density. Clots were formed by incubating MPs (5000 PMP/μL and 1000 M-MP/μL, final) in re-calcified MDP containing Alexa488-conjugated fibrinogen, and imaged by confocal microscopy. **(A)** Micrographs of fibrin networks after clots were fully-formed (180 minutes). **(B)** Fibrin fiber density shown as mean percent positive grids (±SEM, n=3-5). TF is 1 pM (Innovin). **P*<0.05 by ANOVA *versus* buffer controls.

dependent fibrin production, while PMPs support fibrin formation only in the presence of an intact contact pathway.

M-MPs, but not PMPs, increase fibrin network density. The fibrin formation rate is correlated with fibrin network density.[19,20] We used laser scanning confocal microscopy to compare effects of PMP and M-MPs on network density *versus* networks produced by the background level of contact activation in MDP. Addition of TRAP-derived PMPs to clotting reactions did not significantly alter network density compared to buffer controls (Figure 3.4), although fibers appeared slightly more branched in samples clotted in the presence of 5000 PMP/μL. TRAP/convulxin- and A23187-derived PMPs similarly had no significant effect on network density (data not shown). Since platelets increase fibrin density via interactions between their integrins and the fibrin network[20,29], this observation suggests integrin density on PMP is lower than on intact platelets and does not sufficiently interact with the fibrin network to alter structure. In contrast to PMPs, M-MPs significantly increased fiber density (P=0.001) over controls (Figure 3.4).

M-MPs increase clot resistance to fibrinolysis. Fibrin network density is positively associated with network stability.[19,20] To determine the ability of MPs to promote clot stability, we spiked MPs into re-calcified MDP in the presence of tPA, and followed fibrin formation and lysis by turbidity (Figure 3.5A). The time to peak and peak turbidity reflect the time to maximum fibrin formation and peak incorporation of fibrin into the clot, respectively.[20] Both M-MPs and THP-MPs shortened the time to peak (P<0.0001, Figure 3.5B) and increased the peak (P<0.03, Figure 3.5C) turbidity *versus* control (buffer), whereas

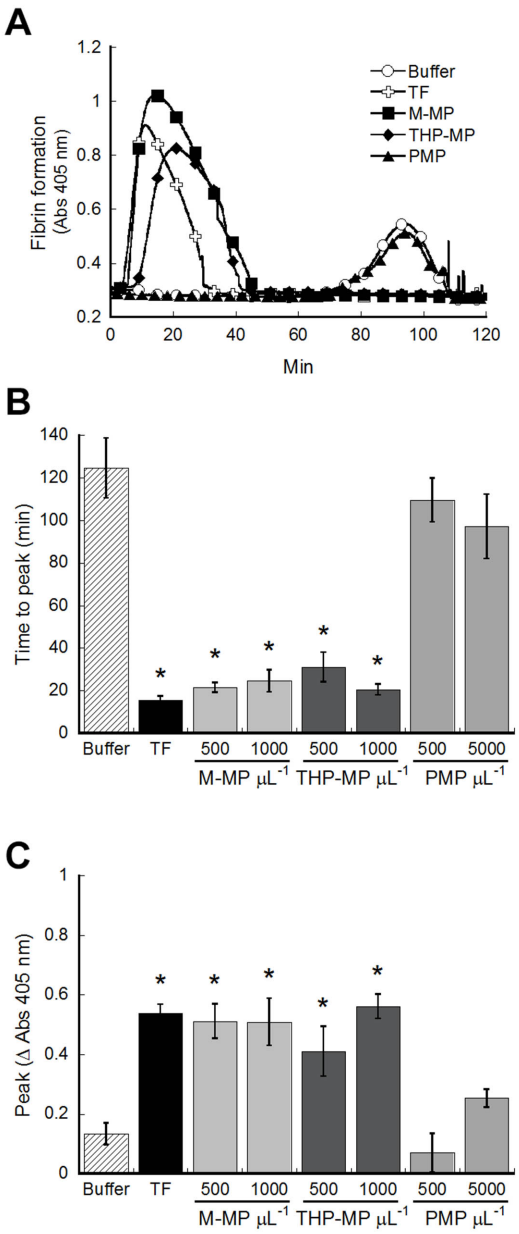

Figure 3.5. M-MPs increase clot resistance to fibrinolysis. MPs (1000 M-MP or THP-MP/μL and 5000 PMP/μL) were spiked into re-calcified MDP in the presence of tPA and fibrin formation and lysis were followed by turbidity. **(A)** Representative turbidity curves. **(B)** Time to peak and **(C)** peak turbidity change. Data show mean (±SEM, n=4-7). TF is 1 pM (Innovin). **P*<0.05 by ANOVA *versus* buffer controls.

PMPs did not enhance fibrin formation over buffer (contact activation), alone. These data suggest monocyte-derived MPs trigger rapid accumulation of fibrin, even in the presence of fibrinolytic activity.

PMPs increase thrombin generation and the fibrin formation rate during TF-initiated clotting. Our data indicate PMPs support little to no fibrin generation in the absence of a procoagulant stimulus (contact activation), consistent with their apparently non-pathogenic presence in healthy individuals.[2] However, since PMP supported procoagulant activity (Figure 3.2) and fibrin formation following contact activation (Figures 3.2-5), and MPs are recruited to sites of vascular injury (TF exposure)[14-16], we tested the hypothesis that PMPs augment coagulation in reactions triggered by TF. We spiked PMPs into re-calcified MDP, triggered clotting with TF (1 pM Innovin or THP-MPs as a model monocyte TF-bearing MP to avoid any influence of contaminating PMPs), and followed thrombin generation and fibrin formation. Addition of PMPs from TRAP-stimulated platelets to Innovin-triggered reactions shortened the thrombin lag time from 10.4±0.8 to 8.7±0.5 minutes (P<0.01). PMPs also shortened the lag time in 500/μL and 1000/μL THP-MP-triggered reactions from 4.6±0.2 to 4.0±0.1 minutes, and 3.7±0.6 to 3.2±0.5 minutes, respectively (mean ± SEM, P<0.04). PMPs increased the thrombin peak (P<0.05, Figure 3.6A). These data reflect the ability of PMPs to support thrombin propagation, as well positively feedback on the initiation phase. Consequently, addition of PMPs to Innovin- or THP-MP-triggered clotting assays consistently increased the fibrin formation rate over Innovin or THP-MP, alone (P<0.01, Figure 3.6B), and slightly, but non-significantly, shortened the time to peak (Figure 3.6C) and increased the peak (Figure 3.6D) turbidity in fibrinolysis assays. These findings suggest

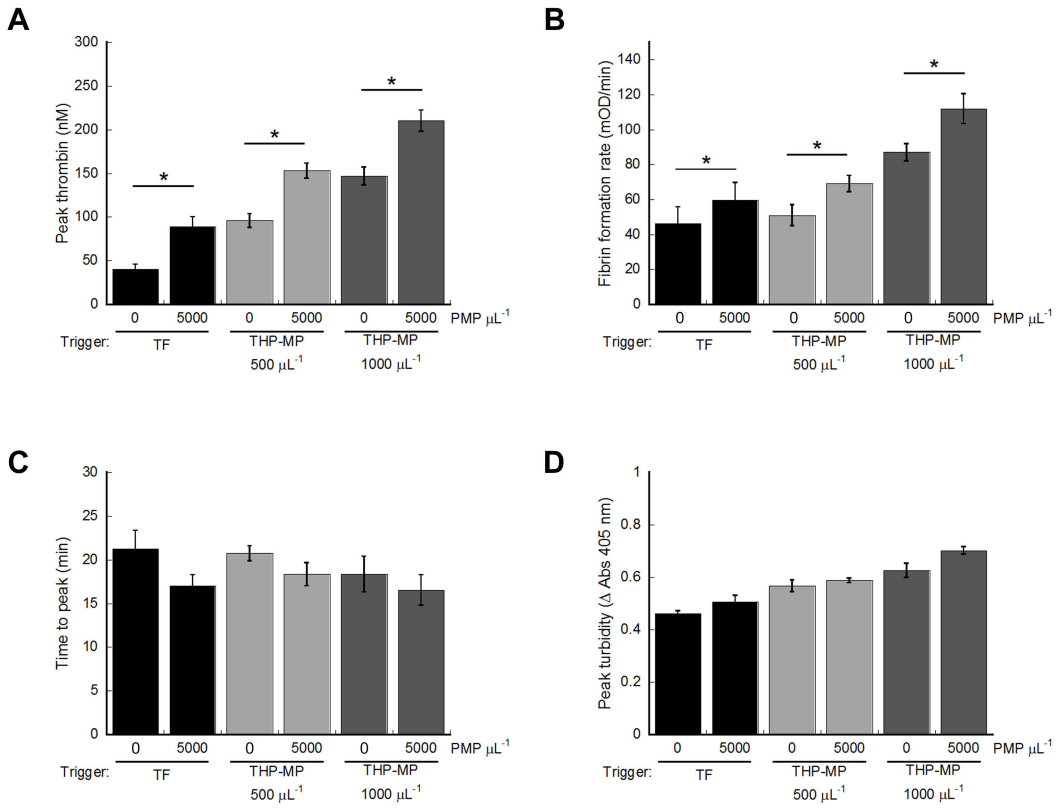

Figure 3.6. PMPs increase thrombin generation and the rate of fibrin formation during TF-initiated clotting. PMPs were mixed with THP-MPs or Innovin and spiked into re-calcified MDP, and thrombin generation and fibrin formation were measured. **(A)** Peak thrombin and **(B)** fibrin formation rate in the absence of tPA. **(C)** Time to peak and **(D)** peak turbidity in the presence of tPA. Data show mean (±SEM, n=3-4). TF is 1 pM (Innovin). **P*<0.02 by Student's t test *versus* 0 PMP/μL.

that following initiation, PMPs enhance clotting propagation and promote faster fibrin growth.

3.4 Discussion

The presence of circulating, cell-derived MPs in healthy individuals and their increased numbers in disease is well-documented; however, their potential mechanistic role(s) in hemostasis and/or thrombosis are poorly-defined. This information gap results, in part, from the lack of studies investigating independent procoagulant contributions of pure MP populations. We specifically characterized properties of MPs from isolated platelets and monocytes—MP subtypes most frequently implicated in procoagulant/prothrombotic disorders. Our data show TF-bearing MPs (M-MPs, THP-MPs) initiated plasma thrombin generation, promoted fibrin formation, and increased fibrin network density and resistance to lysis. In contrast, PMPs did not support plasma thrombin generation or clotting in the absence of a procoagulant trigger (contact or TF). However, following initiation, PMPs significantly increased thrombin generation and the fibrin formation rate. These data support findings demonstrating MP-enriched plasma supports thrombin generation[2,12], but extend these observations to show consequential, functional effects of MPs from specific parent cells on fibrin formation and stability.

Fueled by reports that MPs are smaller (<0.5 μm) than initially reported (≤1 μm)[26], use of flow cytometry to characterize and enumerate MPs is controversial; the refractive index of cellular vesicles appears lower than that of polystyrene beads used to gate MP populations by flow cytometry, leading to underestimation of size.[30] Our data are consistent with reports that NTA detects more MPs than flow cytometry[27] due to increased detection of

small (<0.5 μm) particles. However, using NTA and TEM in addition to flow cytometry to assess physical characteristics of platelet and monocyte-derived MPs, our data show MPs from both cell types, regardless of the agonist used for their derivation, are ~150-300 nm in diameter. These findings support observations that MPs from LPS- and P-selectin-activated monocytes are similar in size[26], and extend the observations to include similarity with PMPs, suggesting cellular mechanisms producing MPs are common between cell types.[11] Nonetheless, MP characterization may be enhanced by continued refinement of methods that detect smaller vesicles.[30]

Whereas PMPs circulate in healthy individuals, M-MPs are present only in prothrombotic disease. Our findings provide a rationale for different roles of these MPs in hemostasis and thrombosis. Consistent with the role of activated platelets in coagulation propagation[31], PMPs augmented thrombin generation and fibrin formation only following a procoagulant trigger. Although it is well-accepted that such a trigger may arise *in vivo* from vascular injury that exposes subendothelial collagen or TF, the contact pathway contributes to thrombosis in murine models.[32,33] Thus, PMPs may promote thrombus propagation following TF- or contact-initiated clotting in humans. In contrast to PMPs, M-MPs independently initiated thrombin generation and fibrin formation, and increased fibrin network density and stability, stemming from their ability to support both TF and prothrombinase activities. These findings are consistent with previous findings that leukocyte-derived MPs promote thrombogenesis, whereas PMPs are a marker of on-going thrombosis.[18] Together, these data suggest MPs derived from different parent cells uniquely contribute TF-dependent and-independent activities that promote thrombus formation and growth.

Given reports of platelet TF expression, it is notable that we did not detect TF activity in PMPs. Discrepancies between findings of TF mRNA processing and de novo protein synthesis in PAR- or A23187-stimulated platelets in some[34,35] but not other[36,37] studies have been potentially explained by the transient nature of TF expression following platelet activation[38]. Given the time required for MP production and isolation following platelet stimulation (~30 minutes in our study), it is unclear whether the lack of PMP TF reflects a lack of platelet TF expression, or that TF is not packaged into PMPs. Further studies are required to resolve this controversy. Similarly interesting was our finding that M-MPs and THP-MPs had greater prothrombinase activity than PMPs. Since NTA indicated not all MPs were detected by flow cytometry, the higher prothrombinase activity of M-MPs and THP-MPs could indicate higher numbers of small MPs were generated by monocytic cells and were therefore present in the prothrombinase assay. However, NTA and TEM indicated MPs generated by monocytes and platelets had similar physical characteristics. Although high lipid concentrations cause an inhibitory effect in prothrombinase assays, testing additional MP dilutions confirmed that MP concentrations used in these assays were below concentrations that caused this effect (data not shown). Tracy et al. previously observed 15-fold higher prothrombinase activity on monocytes than platelets.[39] Our findings support and extend this observation by demonstrating these differences are not due to different cellular surface area.

Our study has potential limitations. First, MP function was analyzed from isolated parent cells; MP produced *in vivo* may possess unique properties. However, our findings, as well as those of others[26] indicate similar physical and procoagulant properties of MPs isolated from given cell types, suggesting MPs have defined characteristics whether

generated *in vivo* or *in vitro*. Second, while we focused on MPs from monocytes and platelets, MPs from other parent cells may possess other procoagulant or anticoagulant/profibrinolytic properties.[40] Since reports suggest circulating CD41-positive MPs derive from megakaryocytes[41], caution is advised when extrapolating our findings with PMPs to CD41-positive MPs *in vivo*. Third, we examined M-MP and PMP procoagulant activity; however, their impact depends on their ability to be recruited to and retained in, clots. Accumulation of TF-bearing MPs in thrombi but not hemostatic clots[42] suggests not all circulating MPs contribute procoagulant activity to all clots. Fourth, even by positive selection, we were unable to reduce the level of platelet contamination in M-MP preparations to less than 17%. Therefore, at least some procoagulant activity exhibited by M-MPs may derive from contaminating PMPs. However, we were able to recapitulate activities observed in M-MPs with THP-MPs, supporting our conclusion that monocyte-derived MPs exhibited both TF and prothrombinase activity in the assays. Finally, given 10-15% cell death that occurred during monocyte/THP-MPs generation, it is possible that a minor population of MPs were generated from dying cells. However, the recent study by Boles et al. (2011)[43] demonstrates apoptosis-derived THP-MPs have procoagulant properties (TF and PS activity) similar to those produced by LPS-stimulated cells, and therefore would be expected to exhibit similar behavior as LPS-derived MP in our thrombin generation and fibrin formation assays.

In conclusion, MPs from monocytes and platelets exhibit unique procoagulant activities; M-MPs initiated the extrinsic pathway, PMPs supported intrinsic pathway-dependent clotting. Both MP types contributed to propagation of clotting. These data imply

a pathogenic role for M-MPs, and suggest PMPs exhibit prothrombotic activity once initiation has occurred. It will be important to compare the contributions of MP from different parent cells in *in vivo* thrombosis models.

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CHAPTER 4: FACTOR XIII ACTIVITY IS REQUIRED FOR RETENTION OF RED BLOOD CELLS IN VENOUS THROMBI

4.1 Introduction

Venous thrombosis is thought to initiate in the hypoxic environment of venous valve pockets[1,2], where intravascular activation of coagulation triggers thrombin-mediated intraluminal fibrin deposition. The prevailing paradigm asserts that red blood cells (RBCs) are incorporated into venous thrombi via passive trapping during fibrin deposition, culminating in the production of a fibrin- and RBC-rich venous "red" thrombus. However, little is known about the mechanisms regulating this process, or how the presence of RBCs in venous thrombi contributes to thrombus size.

Fibrinogen is a 340 kDa plasma glycoprotein consisting of 2 Aα chains, 2 Bβ chains, and 2 γ chains that circulates at high (2-5 mg/mL) concentrations. Following activation of coagulation, fibrinogen is cleaved by thrombin and polymerized into an insoluble network (reviewed in [3,4]). The transglutaminase factor XIII(a) (FXIIIa) produces ε-N-(γ-glutamyl)-lysine cross-links between residues Q398/399 and K406 in the γ-chain and subsequently between glutamine and lysine residues in the α-chain that stabilize the fibrin network. This stabilization is required for critical functions of cross-linked fibrin which: 1) promotes clot mechanical stability via its extraordinary extensibility and elasticity, and 2) protects the clot against premature dissolution during wound healing.

Factor XIII (FXIII) is a protransglutaminase that circulates in plasma at ~70 nM and consists of two catalytic subunits (FXIII-A) and two non-catalytic subunits (FXIII-B) in a

noncovalent, heterotetramer (FXIII-A_2B_2, M_r 325 kDa). Essentially all FXIII-A_2B_2 in plasma circulates in complex with fibrinogen.[5,6] FXIII-A_2B_2 is activated by thrombin-catalyzed release of a 37-amino acid activation peptide from the N-terminus of the FXIII-A_2 subunits[7,8] and calcium-mediated dissociation of the FXIII B subunits from FXIII-A_2, yielding activated FXIII-A_2 (FXIII-A_2*).[9-11] FXIII activation is accelerated when it is bound to fibrinogen, which facilitates dissociation of the regulatory FXIII-B subunits.[12-14] However, the residues in fibrinogen that mediate these interactions have not been defined.

The C-terminus of the fibrinogen γ chain is a site for interactions between fibrinogen and a myriad of soluble and cell-associated proteins (reviewed in [15]). In particular, fibrinogen residues γ390-395 are immediately upstream of the FXIII cross-linking sites on the fibrin(ogen) γ-chain, and mediate interactions with the CD11b subunit of CD11b/CD18 (Mac-1). Mice with a mutation of this region (Fibγ$^{390\text{-}396A}$) have normal levels of fibrinogen and were reported to exhibit normal fibrin polymerization, platelet aggregation and hemostasis, do not develop spontaneous hemorrhage, and undergo normal thrombus formation following $FeCl_3$-mediated carotid artery injury[16]. However, fibrinogen isolated from Fibγ$^{390\text{-}396A}$ mice does not support CD11b-mediated adhesion of neutrophils or macrophages.[16] Fibγ$^{390\text{-}396A}$ mice have defective clearance of *S. aureus*[16], but are protected against auto-inflammatory disorders including autoimmune central nervous system disease[17,18], inflammatory joint disease[19], colitis-associated cancer[20], and Duchenne muscular dystrophy[21], and this protection is thought to stem from the lack of fibrin-driven leukocyte responses during inflammation. In this study, we analyzed the role of fibrinogen residues γ390-396 in venous thrombosis. Our findings indicate fibrinogen residues γ390-396 mediate

FXIII-A_2B_2 binding and activation, and reveal a critical role for FXIIIa activity in venous thrombosis.

4.2 Materials & Methods

Mice. Fib$\gamma^{390\text{-}396A}$ mice[16] and *FXIII-A*$^{-/-}$ mice[22] were independently backcrossed six generations to C57BL/6J background. *CD11b*$^{-/-}$ mice[23], also on a C57Bl/6 background, were backcrossed more than seven generations. All animal procedures were approved by the UNC and Cincinnati Children's Hospital Institutional Animal Care and Use Committees.

Murine venous thrombosis models. The IVC stasis model was performed on 8-10-week-old male Fib$\gamma^{390\text{-}396A}$, *FXIII-A*$^{-/-}$ and WT mice as described.[24,25] Briefly, anesthetized mice were subject to sterile laparotomy and exposure of the IVC. Side branches were ligated and lumbar branches were closed by cautery. The IVC was separated from the aorta by blunt dissection and completely ligated. The IVC stenosis model was performed as described.[26] Briefly, side branches were ligated but no lumbar branches were cauterized. Stenosis was achieved by partial ligation of the IVC using a 30G needle as a temporary spacer during ligation, after which it was removed. Mice recovered with analgesia (buprenorphine, 0.05 mg/kg, subcutaneous) and were maintained on acetaminophen (6 mg/mL) in their drinking water post-surgery. After 24 hours, mice were anesthetized and blood drawn from the suprarenal IVC into 3.2% sodium citrate (10% v/v, final). Blood samples were centrifuged at 5,000x*g* for 10 minutes to prepare platelet-poor plasma (PPP). Thrombi were separated from the vein wall, weighed, and frozen.

Thrombus analysis by western blot. Frozen thrombus samples from WT and Fib$\gamma^{390\text{-}396A}$ mice were homogenized in lysis buffer (20 mM Tris-HCl pH 7.5, 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1% Triton, 2.5 mM sodium pyrophosphate, 1 mM β-glycerophosphate, 1 mM Na_3VO_4 and 1 mg/mL leupeptin [Cell Signaling, Danvers, MA]) supplemented with 1 mM PMSF using a Dounce Tissue Homogenizer (Bellco Glass, Vineland, NJ). Thrombus lysates were kept on ice for 1 hour before centrifugation (7,000x*g*, 15 minutes) at 4 °C. Supernatants were collected and mixed with 6X non-reducing SDS-sample buffer (Boston BioProducts, Boston, MA). Non-reduced samples were used for blotting leukocyte (Ly6G) and platelet (CD41) antigens. For a subset of samples, the cross-linked fibrin enriched pellet was dissolved in 8 M Urea containing 40 mM DTT and 12.5 mM EDTA at 55 °C overnight, and then mixed with 6X reducing SDS-sample buffer.

All samples were separated on 4-12% or 10% Tris-Glycine gels (Novex) and transferred to PVDF membranes (Invitrogen, Carlsbad, CA). Membranes were blocked for 1 hour at room temperature with Odyssey blocking buffer (LI-COR Biosciences, Lincoln, NE). Primary antibodies against Ly6G (rat anti-mouse clone RB6-8C5 from eBiosciences, San Diego, CA, 1:1500), CD41 (goat polyclonal from Santa Cruz Biotechnology, Dallas, TX, 1:500), or fibrin (clone 59D8 generously provided by Dr. Charles Esmon [Oklahoma Medical Research Foundation], 1:500) were incubated overnight at 4°C. After washing, membranes were incubated with fluorescence-labeled secondary antibodies for 1 hour at room temperature. Membranes were then washed and scanned using an Odyssey® Infrared Imaging System (LI-COR Biosciences) or GE Typhoon FLA-9000 Imager (GE Healthcare, Pittsburgh, PA).

Thrombus RBC content. Thrombus lysates were diluted 10-fold in 20 mM sodium phosphate (pH 7.4), 150 mM NaCl (PBS) and absorbance was measured at 575 nm.

Measurement of circulating thrombin-antithrombin (TAT) complexes. Post-ligation TAT levels of PPP prepared from IVC blood draws were measured by ELISA (Enzygnost TAT microELISA, Siemens Healthcare Diagnostics, Deerfield, IL).

Measurement of plasma thrombin generation. Plasma thrombin generation was measured in 6-fold diluted plasma by calibrated automated thrombography, as described.[27]

Clot retraction assays. Blood was drawn from mice into 3.2% sodium citrate (10% v/v, final) and corn trypsin inhibitor (CTI, 18.3 μg/mL final). For whole blood clot retraction, blood was used undiluted or was diluted 3-fold in HEPES-buffered saline (HBS; 20 mM HEPES [pH 7.4], 150 mM NaCl), in the absence or presence of the FXIII inhibitor T101 (Zedira, 0-200 μM, final), as indicated. For platelet-rich plasma (PRP) clot retraction, blood was centrifuged twice (150x*g*, 5 minutes) and PRP was collected and used undiluted. Clotting was triggered in re-calcified (10 mM, final) whole blood or PRP by addition of TF (Innovin diluted to 1:12,000 [1 pM] final) or thrombin (2 U/mL bovine thrombin, final), as indicated. In all experiments, clotting and subsequent retraction proceeded at 37 °C for 90 minutes in siliconized glass tubes. Retracted clots were imaged to determine final percent retraction. Serum was collected, and RBCs were measured by directly counting cells on a HV950FS Hemavet cell counter (Drew Scientific, Dallas, TX), or by diluting serum 10 to 100-fold in

HBS, measuring absorbance at 575 nm, and comparing to a standard curve of diluted RBCs enumerated with a Hemavet cell counter. Retracted clots were also collected and weighed.

RBC washout assay in the absence of platelets. Blood was drawn from mice into 3.2% sodium citrate (10% v/v, final) and centrifuged (250x*g*, 10 minutes). PRP fractions were separated and processed to platelet-free plasma (PFP, <20 platelets/μL) by sequential centrifugation (2000x*g* for 15 min, followed by 7000x*g* for 10 min). The buffy coat was removed from the RBC fraction and washed three times in 1.29 mM sodium citrate, 3.33 mM glucose, 124 mM NaCl (pH 7.2) with centrifugation (250x*g*, 5 minutes). RBCs were resuspended in PBS with 0.1% BSA and centrifuged (500x*g*, 10 minutes) to pack cells. Packed RBCs (4 μL) were added for every 100 μL PFP. Flow cytometry of PFP and washed RBCs revealed less than 0.01% and 10% platelet contamination (% of initial platelet count by GPIX-positive events) in PFP and RBCs, respectively. The initial absorbance of RBC-PFP mix was determined by 50-fold dilution in PBS. RBC-PFP mix was clotted with TF (1 pM, final) and $CaCl_2$ (10 mM, final) at room temperature. After 1 hour, 700 μL HBS was added and absorbance at 575 nm was measured and used to calculate percent change from initial absorbance.

Isolation of fibrinogen from plasma with glycine precipitation. PPP was supplemented with 10 mM PMSF and 5 mM benzamidine (final). Glycine (165 mg per mL plasma) was added and samples were rotated at room temperature for 1 hour. The precipitate was collected via centrifugation and resuspended in HBS before re-precipitation with glycine. The precipitate was resuspended in HBS and fibrinogen concentration measured by absorbance at 280 nm

with a Nanodrop 2000 (Thermo Scientific, Waltham, MA), using an extinction coefficient of 1.506 mL/(mg/cm). Fibrinogen was assessed by SDS-PAGE. Samples were stored at -80 °C.

RBC adhesion assay. Microfluidic channels (43.5 x 500 μm in cross-section) were coated with 10 μg/mL BSA, purified WT fibrinogen, or Fib$\gamma^{390\text{-}396A}$ fibrinogen for 2 hours at room temperature and then rinsed with PBS. Washed, packed RBCs from WT mice were diluted 100-fold in PBS (~20,000/μL), loaded into channels, and allowed to adhere for 10 minutes under static conditions. PBS was then flowed through the channels at 1000 s^{-1} for 5 minutes. Bright field images of the channels before and after flow were captured on an Olympus IX81 inverted light microscope with a 40X objective. Percent adhesion was measured by counting cells using ImageJ software.

Mass spectrometry-based identification of FXIII in WT fibrinogen. Purified fibrinogen was reduced and separated on a 10% Tris-Glycine gel. Bands were visualized by colloidal "Blue Silver" stain[28], excised, and analyzed by the Michael Hooker UNC Proteomics Center using a MALDI TOF/TOF 4800 Mass Analyzer (ABSciex, Framingham, MA).

Detection of FXIII in murine plasma. PPP was diluted 1:120 in water and added 5:1 to reducing SDS-Sample Buffer. Samples were separated on a 10% Tris-Glycine gel and transferred to PVDF membranes. Western blotting was performed as above, using sheep anti-FXIII-A domain primary antibody (Enzyme Research Laboratories, South Bend, IN, 1:1000).

ELISA for FXIII binding to fibrinogen. Purified mouse fibrinogen (0.029 μM diluted in HBS containing 1 mM $CaCl_2$, [HBS/Ca^{2+}]) was coated onto a Corning 96-well high binding microplate. Control wells were coated with HBS/Ca^{2+} alone. Wells were blocked with HBS/Ca^{2+} containing 0.1% BSA and incubated with a 1:5 dilution series of Fibrogammin®-P (1.95 μM to 0.2 pM). Wells were probed with sheep anti-human FXIII-A subunit antibody (1:1000) and HRP-conjugated donkey anti-sheep secondary (Cappel, 1:10,000). All incubation steps were performed for 1 hour at room temperature and followed by washing with HBS/Ca^{2+} containing 0.5% Tween 20. ELISAs were developed with SureBlue peroxidase substrate (KPL, Gaithersburg, MD) and generation of blue color was monitored at 600 nm with a SpectraMax 340PC microplate reader. The maximum rate of substrate cleavage was determined using SoftMax software.

Plasma clot formation and detection of FXIIIa and cross-linked fibrin. PPP from WT and Fib$\gamma^{390\text{-}396A}$ mice was re-calcified (10 mM, final) and clotted with TF (1 pM, final). At indicated time points, quencher solution (50 mM DTT, 12.5 mM EDTA, 8 M Urea) was added. Samples were then incubated at 60 °C for 1 hour with occasional agitation. Samples were reduced, boiled, separated on 10% Tris-glycine gels, and transferred to PVDF membranes. Membranes were probed with rabbit anti-human fibrinogen polyclonal antibodies (rabbit anti-fibrin(ogen)). Membranes were then stripped with 10 mM DTT in 7 M guanidine for 20 minutes at 60 °C and re-probed with sheep anti-human FXIII-A polyclonal antibody. Band intensity (arbitrary units, AU) was measured by densitometry using Photoshop CS6 version 13.0. Bands were normalized to initial intensity at time 0 with

the following exceptions: FXIII-A* was normalized to FXIII-A at time 0, γ-γ dimers were normalized to γ chain at time 0, and HMW cross-linked species were normalized to α chain at time 0.

Clot retraction of reconstituted human FXIII-deficient blood. All procedures were approved by the UNC and University of Colorado Institutional Review Boards. Normal and FXIII-deficient plasmas were prepared from blood drawn from consenting healthy individuals and a patient with FXIII-deficiency. The patient is a 7 year old female with congenital severe deficiency of FXIII (<5%) who receives prophylactic FXIII concentrate infusions every 4 weeks. The blood sample was obtained before an infusion. The trough level at that point was 14%. RBCs and platelets were isolated from blood drawn from consenting healthy donors into 3.2% citrate (10% v/v, final) and CTI (18.3 μg/mL, final) and centrifuged (150x*g*, 20 minutes). The PRP fraction was treated with prostaglandin-I_2 (50 ng/mL, final) and centrifuged (400x*g*, 20 minutes). Pelleted platelets were resuspended in Tyrode's buffer (15 mM HEPES, 3.3 mM NaH_2PO_4 (pH 7.4), 138 mM NaCl, 2.7 mM KCl, 1 mM $MgCl_2$, 5.5 mM dextrose), allowed to rest for 30 minutes at 37 °C, counted on a Hemavet, and diluted to 1 million/μL. After removing the buffy coat, the RBC fraction was washed 3 times with CGS buffer (1.29 mM sodium citrate, 3.33 mM glucose, 124 mM NaCl; pH 7.2), centrifuged (400x*g*, 5 minutes), resuspended in HBS, and packed by centrifugation (400x*g*, 10 minutes). Packed RBCs were diluted 2-fold in HBS and counted on a Hemavet. Plasma-derived FXIII-A_2B_2 (Fibrogammin®-P) or HBS was incubated with citrated normal and FXIII-deficient plasmas (no CTI) for 10 minutes at room temperature. Washed platelets (100,000/μL, final) and RBCs (4-5 million/μL, final) were combined with plasmas in siliconized wells of a 96-

well plate containing TF (1 pM, final) and $CaCl_2$ (10 mM, final). Clot formation and retraction was allowed to proceed for 90 minutes at 37 °C. RBC content of serum was measured by absorbance as described above.

Statistical methods. Descriptive statistics (mean, median, SD, SEM) were calculated for each condition, and Lilliefors test was used to assess normality. Data were compared by student's t test with equal or unequal variance, as appropriate, for experiments with two groups. ANOVA was used for experiments with more than two conditions, with Bonferroni or Dunnet post hoc tests for between-group comparisons.

4.3 Results

Thrombi from Fib$\gamma^{390\text{-}396A}$ mice are smaller and have reduced RBC content compared with thrombi from WT mice. Previous studies have shown that Fib$\gamma^{390\text{-}396A}$ mice have normal complete blood counts (WBC, RBC, Hg, HCT, platelets), tail bleeding times, thrombin time, wound healing, normal levels of circulating fibrinogen, and exhibit normal platelet aggregation and fibrin polymerization[16]. However, Fib$\gamma^{390\text{-}396A}$ mice have defective leukocyte function due to a failure of fibrin(ogen) to engage leukocyte integrin CD11b/CD18.[16] Therefore, to first identify leukocyte-independent functions of fibrinogen residues γ390-396 on venous thrombosis, we used the inferior vena cava (IVC) ligation (stasis) venous thrombosis model that produces thrombi independent of leukocyte TF activity ([29,30] and data not shown). Surprisingly, thrombi from Fib$\gamma^{390\text{-}396A}$ mice were 50% smaller than thrombi from WT mice 1 day after ligation (Figure 4.1A). Reduced thrombus weight was not due to reduced procoagulant activity; plasma from WT and Fib$\gamma^{390\text{-}396A}$ mice had

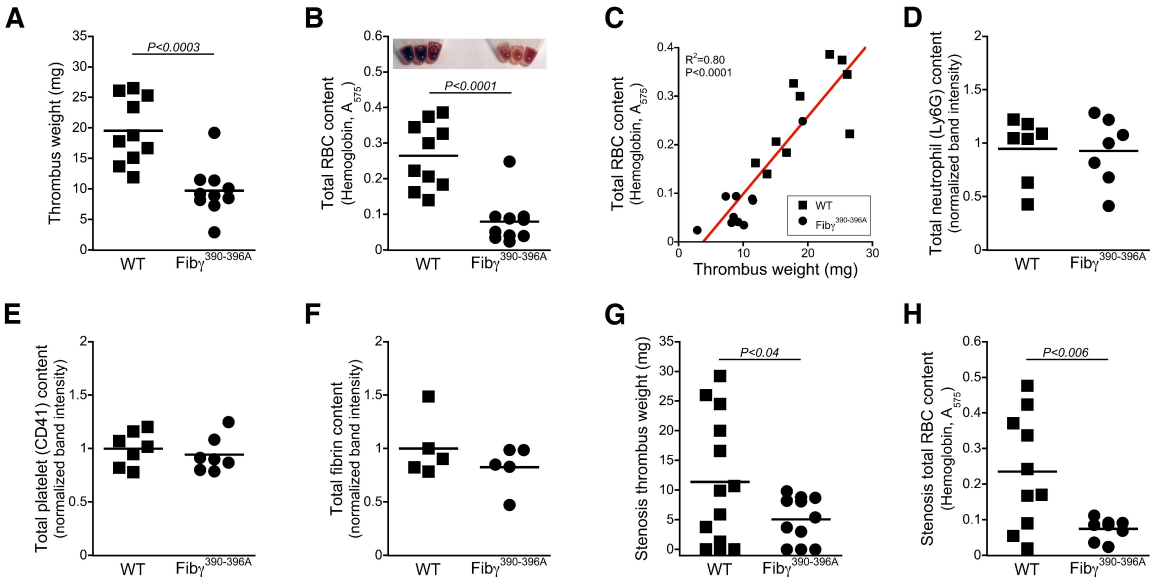

Figure 4.1. Compared to thrombi from WT mice, thrombi from Fibγ$^{390-396A}$ mice are smaller and have reduced RBC content. **(A)** Thrombus weights 1-day post-ligation (stasis model). **(B)** Total RBC content of stasis thrombus lysates, measured as hemoglobin absorbance at 575 nm. Inset: lysate samples. **(C)** Correlation of in vivo thrombus weight with total RBC content. Linear regression for all data points shown in red. **(D)** Total neutrophil content of thrombus lysates, measured by Ly6G antigen. **(E)** Total platelet content of thrombus lysates, measured by CD41 antigen. **(F)** Total fibrin (β chain) antigen of thrombus lysates. **(G)** Thrombus weights 1-day post-ligation (stenosis model). **(H)** Total RBC content of stenosis thrombus lysates, measured as hemoglobin absorbance at 575 nm. Each dot represents an individual mouse. Lines are means.

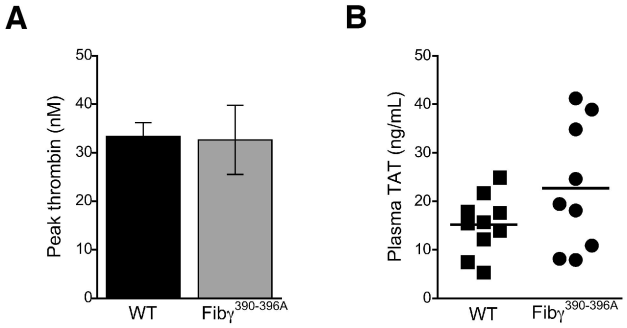

Figure 4.2. **Procoagulant activity is normal in Fibγ$^{390-396A}$ mice.** **(A)** Ex vivo plasma thrombin generation in WT and Fibγ$^{390-396A}$ mice (n=4-5). Data are means ± SEM. **(B)** Circulating TAT levels in WT and Fibγ$^{390-396A\ mice}$ following thrombus formation (stasis model). Lines are means.

similar thrombin generation *in vitro* (Figure 4.2A), and circulating TAT levels were similar in WT and Fibγ$^{390\text{-}396A}$ mice following thrombus formation *in vivo* (Figure 4.2B). To characterize the venous thrombi produced in these mice, we dissolved thrombi and analyzed thrombus lysates. Strikingly, thrombus lysates from Fibγ$^{390\text{-}396A}$ mice had significantly reduced absorbance at 575 nm (Figure 4.1B), indicating that they contained fewer RBCs than WT thrombi. Furthermore, the RBC content of the thrombus correlated positively and significantly with thrombus weight (Figure 4.1C). Western blots of thrombus lysates showed WT and Fibγ$^{390\text{-}396A}$ mice did not differ in Ly6G content, a neutrophil marker (Figure 4.1D), indicating the differences in thrombus weights were not due to differences in leukocyte recruitment or infiltration. WT and Fibγ$^{390\text{-}396A}$ thrombi also did not differ in CD41 content (a platelet marker, Figure 4.1E) or total fibrin (Figure 4.1F). Results in a second venous thrombosis model that involves IVC stenosis rather than complete ligation[26,31] were similar; despite the typical high degree of variability in clot sizes from WT mice, Fibγ$^{390\text{-}396A}$ mice still displayed significantly reduced thrombus weights and reduced RBC presence in thrombi compared to WT mice (Figures 4.1G-H). Together, these data suggest Fibγ$^{390\text{-}396A}$ mice produce smaller venous thrombi because of reduced RBC incorporation and/or retention during thrombogenesis.

RBCs are extruded from Fibγ$^{390\text{-}396A}$ clots during clot retraction, resulting in decreased clot weight. Our *in vivo* studies suggested that Fibγ$^{390\text{-}396A}$ mice had smaller thrombi due to reduced RBC content. We determined if we could recapitulate this phenotype ex vivo with a whole blood coagulation assay in which blood was clotted with tissue factor (TF) and $CaCl_2$. In this assay, clot formation is immediately followed by platelet-mediated clot retraction,

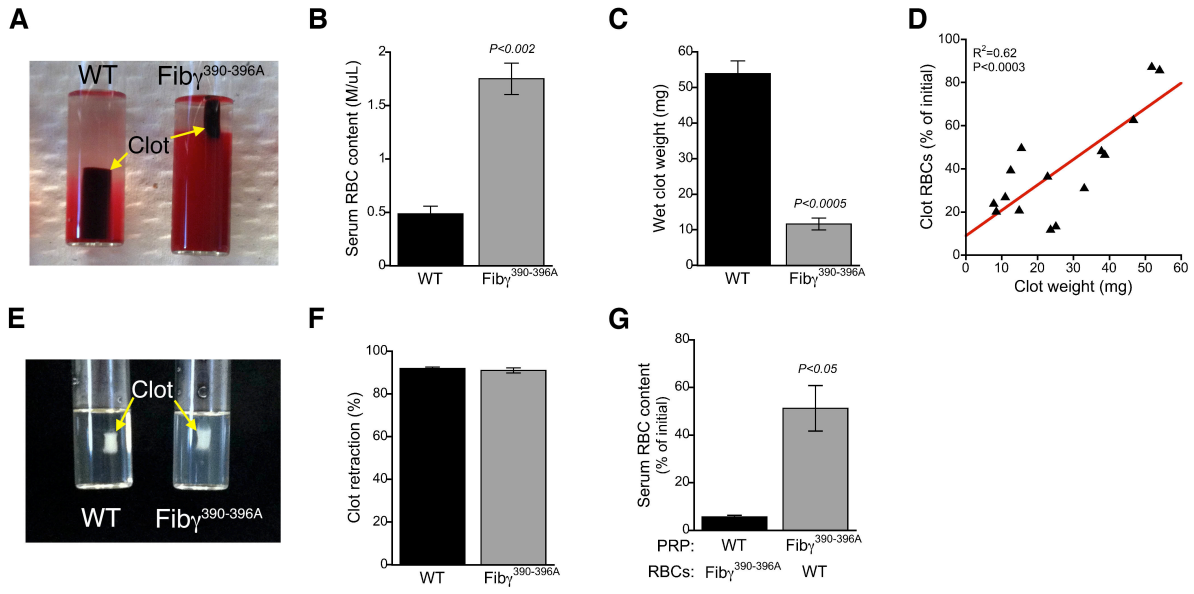

Figure 4.3. RBCs are extruded from Fibγ$^{390-396A}$ clots during clot retraction, resulting in decreased clot weight. After clot formation, platelet contractile force retracts clots away from siliconized tube walls leaving the clots surrounded by serum. **(A)** Image of fully retracted WT and Fibγ$^{390-396A}$ clots 90 minutes after initiation of clot formation. **(B)** Number of RBCs present in the serum following clot retraction (n=3). **(C)** Wet clot weights following clot retraction (n=3). **(D)** Correlation of clot weight with clot RBC content. Linear curve fit shown in red. **(E)** Image of fully retracted PRP clots 90 minutes after initiation of clot formation. **(F)** Percent retraction of PRP clots (n=3). **(G)** Serum RBC content of retracted clots containing WT PRP with Fibγ$^{390-396A}$ RBCs and Fibγ$^{390-396A}$ PRP with WT RBCs (n=3). Data are mean ± SEM.

which is complete within 60-90 minutes. Initially, clotting proceeded similarly for both WT and Fib$\gamma^{390\text{-}396A}$ blood. However, compared to WT clots, Fib$\gamma^{390\text{-}396A}$ clots exhibited strikingly reduced RBC retention (Figures 4.3A-B), significantly increased clot retraction (Figure 4.3A and data not shown), and significantly reduced weight of fully-retracted clots (Figure 4.3C). Consistent with observations *in vivo* (Figure 4.1C), clot RBC content correlated positively and significantly with clot weight (Figure 4.3D). Loss of RBCs during retraction of Fib$\gamma^{390\text{-}396A}$ clots was not due to alterations in platelet-mediated contraction because in the absence of RBCs, clot retraction was identical in WT and Fib$\gamma^{390\text{-}396A}$ platelet-rich plasma (PRP) (Figures 4.3E-F). RBC extrusion was also not due to a defect in RBC function; when we separately prepared PRP and washed RBCs from WT and Fib$\gamma^{390\text{-}396A}$ and mixed WT RBCs with Fib$\gamma^{390\text{-}396A}$ PRP and vice versa, RBC extrusion was specifically associated with the PRP component from Fib$\gamma^{390\text{-}396A}$ mice (Figure 4.3G). When WT and Fib$\gamma^{390\text{-}396A}$ whole blood were mixed in equal parts, retracted clot weights were intermediate between WT and Fib$\gamma^{390\text{-}396A}$ clots (data not shown).

Since RBCs bind to platelet $\alpha_{IIb}\beta_3$[32,33], we tested the hypothesis that RBC-platelet interactions mediated RBC retention in clots. We prepared platelet-free plasma (PFP) from WT and Fib$\gamma^{390\text{-}396A}$ mice, combined these with washed RBCs from WT mice, and initiated clotting with TF and $CaCl_2$. We then manually dislodged fully-formed clots from tube walls, added buffer, and measured RBCs released from the clot. Under these conditions Fib$\gamma^{390\text{-}396A}$ clots released more RBCs than WT clots (3.6±0.6 versus 1.3±0.1 % of initial, $P<0.02$, n=5). However, compared to clots formed in the presence of platelets (Figure 4.3G), both WT and Fib$\gamma^{390\text{-}396A}$ platelet-free clots released significantly (>10-fold) *fewer* RBCs. These data show platelet-mediated clot retraction promotes extrusion of RBCs from Fib$\gamma^{390\text{-}396A}$ clots and that

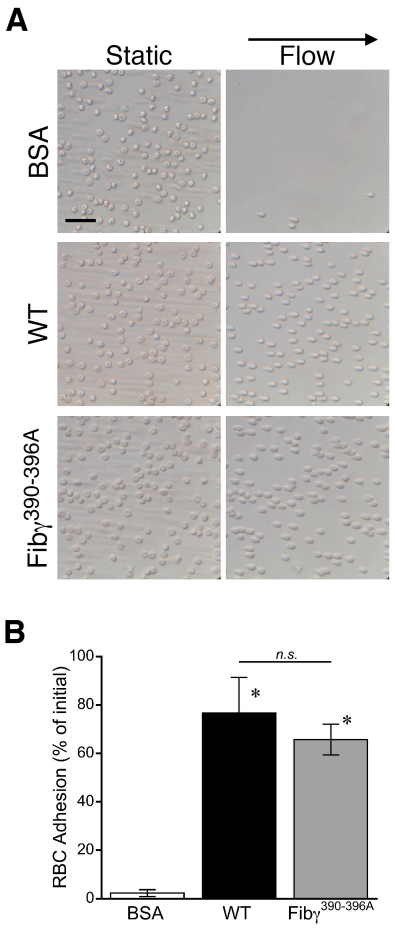

Figure 4.4. RBCs adhere to fibrinogen from WT and Fibγ$^{390\text{-}396A}$ mice. (A) Adherence of RBCs to BSA, WT fibrinogen, or Fibγ$^{390\text{-}396A}$ fibrinogen before and after application of flow with a shear rate of 1000 s^{-1}. Scale bar is 3 μm. **(B)** Quantitation of RBC adhesion after application of flow. Data are mean ± SEM (n=3). **P*<0.009 versus BSA by ANOVA.

platelets are not required to retain RBCs in either WT or Fib$\gamma^{390\text{-}396A}$ plasma clots. Together, these findings show defective retention of RBCs in clots made from blood containing the mutant (Fib$\gamma^{390\text{-}396A}$) fibrinogen.

Fibrinogen residues γ390-396 do not bind to RBCs. Previous studies have shown that RBCs bind to fibrin(ogen) via a specific ligand-mediated interaction involving either CD47[34] or a β_3-like integrin[35] on the RBC surface, and an unknown motif on fibrinogen.[36,37] To test the hypothesis that residues γ390-396 comprise the specific motif on fibrinogen that mediates RBC binding, we used a microfluidic-based adhesion assay to measure binding of RBCs to purified, adherent WT and Fib$\gamma^{390\text{-}396A}$ fibrin(ogen). Whereas RBCs did not adhere to BSA-coated microchannels, RBCs adhered to both WT and Fib$\gamma^{390\text{-}396A}$ purified fibrin(ogen) and remained bound even when subject to high (1000 s^{-1}) shear wash rates (Figures 4.4A-B). Importantly, there were no differences in the percent of RBCs that remained bound to WT and Fib$\gamma^{390\text{-}396A}$ fibrin(ogen) (P=1.0). These data indicate residues γ390-396 do not mediate a direct interaction between RBCs and fibrin(ogen).

Fibrinogen isolated from Fib$\gamma^{390\text{-}396A}$ mice has reduced binding to FXIII. Our findings strongly associated the mechanism mediating RBC retention in thrombi with the mutated fibrin(ogen); however, prior findings indicated purified fibrinogen from Fib$\gamma^{390\text{-}396A}$ mice was expressed at normal levels, contained Aα, Bβ, and γ chains of fibrinogen, expressed both normal (γ) and alternatively-spliced (γ') fibrinogen (data not shown), and polymerized normally.[16] We speculated that the deficiency in Fib$\gamma^{390\text{-}396A}$ mice stemmed from a disrupted interaction between residues γ390-396 and another protein present in plasma. Therefore, we

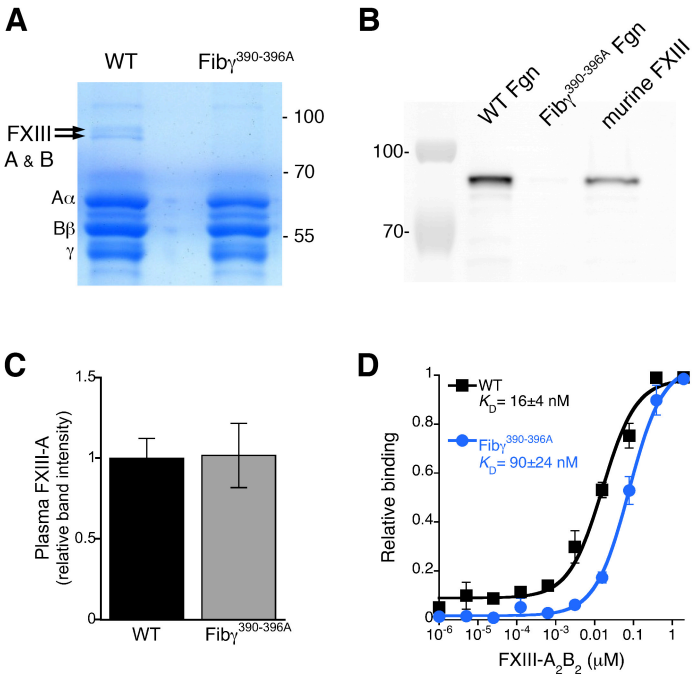

Figure 4.5. FXIII-A_2B_2 does not co-precipitate with Fibγ$^{390\text{-}396A}$ fibrinogen. (A) Blue Silver-stained 10% Tris-Glycine gel containing purified fibrinogens from WT and Fibγ$^{390\text{-}396A}$ mice. Bands indicated with arrows were analyzed by mass spectrometry. **(B)** Western blot for FXIII-A subunit in purified fibrinogen (Fgn) samples shown in (A) and commercial murine FXIII as a positive control. **(C)** Relative level of FXIII-A in WT and Fibγ$^{390\text{-}396A}$ plasmas. Data are means ± SEM (n=4). **(D)** Relative binding curves of plasma purified FXIII-A_2B_2 (Fibrogammin®-P) to WT or Fibγ$^{390\text{-}396A}$ purified fibrinogen. Data are normalized means ± SEM (n=4).

isolated fibrinogen from WT and Fib$\gamma^{390\text{-}396A}$ plasma by glycine precipitation and used SDS-PAGE and silver staining to identify unique plasma proteins that co-precipitated with WT, but not Fib$\gamma^{390\text{-}396A}$ fibrinogen. Two protein bands (M_r ~83 and ~76 kDa) were present with equal staining intensity in WT but not Fib$\gamma^{390\text{-}396A}$ fibrinogen preparations (Figure 4.5A). Mass spectrometry identified these bands as the A and B subunits of FXIII-A_2B_2, and western blotting with an anti-FXIII-A domain antibody confirmed the presence of FXIII in purified WT but not purified Fib$\gamma^{390\text{-}396A}$ fibrinogen (Figure 4.5B). Since Fib$\gamma^{390\text{-}396A}$ mice have normal levels of circulating FXIII in plasma (Figure 4.5C), and since FXIII binding to WT fibrinogen is well-established, these data suggest plasma FXIII-A_2B_2 binds fibrinogen from Fib$\gamma^{390\text{-}396A}$ mice with reduced affinity. Binding assays support this hypothesis; compared to WT fibrin(ogen), plasma-derived FXIII-A_2B_2 bound Fib$\gamma^{390\text{-}396A}$ fibrin(ogen) with 6-fold weaker affinity (16±4 versus 90±24 nM, respectively, Figure 4.5D). These data suggest FXIII-A_2B_2 binds to fibrinogen γ chain residues 390-396.

Fib$\gamma^{390\text{-}396A}$ mice exhibit delayed FXIII activation and delayed fibrin cross-linking in plasma. The initial characterization of Fib$\gamma^{390\text{-}396A}$ mice showed that purified fibrinogen from these mice can be cross-linked by activated FXIII.[16] However, these prior experiments were performed with low fibrinogen (0.25 mg/mL, 10-fold lower than is found in plasma) and high human FXIII-A_2B_2 (470 nM, 6.7-fold higher than is found in plasma). These conditions would have masked reduced binding affinity. Therefore, we used TF to trigger clotting in re-calcified plasma and measured the kinetics of FXIII activation and fibrin cross-linking by SDS-PAGE and western blotting. Compared to WT, plasma from Fib$\gamma^{390\text{-}396A}$ mice exhibited 5-fold slower conversion of FXIII-A_2B_2 to FXIII-A_2* (58.3±19.2 versus 12.2±2.5 AU/min

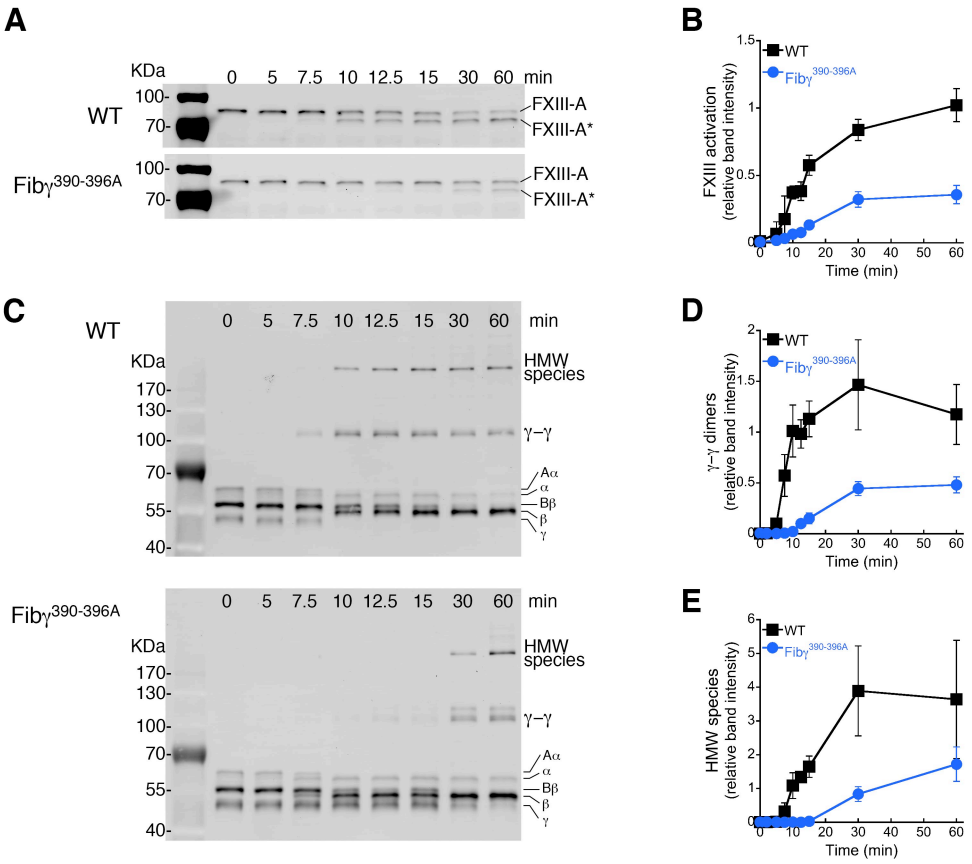

Figure 4.6. Compared to WT, plasma from Fibγ$^{390-396A}$ mice exhibits delayed FXIII activation and consequently, delayed fibrin cross-linking during TF-initiated coagulation. **(A)** Representative western blots for FXIII-A during time course of TF-initiated clotting in WT and Fibγ$^{390-396A}$ plasma. Activated FXIII (FXIII-A*) appears over time as a lower molecular weight band. **(B)** Quantitation of FXIII-A* appearance over time in blots from (A). **(C)** Representative western blots for fibrin(ogen) during time course of TF-initiated clotting in WT and Fibγ$^{390-396A}$ plasma showing γ-γ dimer and higher molecular weight (HMW) cross-linked species (α-α and α-γ polymers). **(D)** Quantitation of γ-γ dimer formation over time in western blots. **(E)** Quantitation of HMW cross-linked species over time in western blots. Data are means ± SEM (n=5-6).

[x10^{-3}], P<0.04, Figures 4.6A-B). Since association of FXIII-A_2B_2 with fibrin(ogen) accelerates its activation by enhancing the release of the regulatory B domain[38], these data are consistent with a loss of binding between FXIII-A_2B_2 and Fib$\gamma^{390\text{-}396A}$ fibrinogen. Accordingly, compared to WT plasma clots, plasma clots from Fib$\gamma^{390\text{-}396A}$ mice exhibited 6-fold reduced rate of formation of both γ-γ dimers (P<0.0005, Figures 4.6C-D) and higher molecular weight species (P<0.004, Figures 4.6C, 4.6E), consistent with the observed delay in FXIII-A_2* generation. Notably, the time course of fibrin cross-linking in these experiments mirrors the time frame during which RBCs were extruded during *in vitro* clot retraction. Together, these data demonstrate a novel defect in FXIII-A_2B_2 activation and activity in Fib$\gamma^{390\text{-}396A}$ mice and suggest decreased FXIII activity permits RBC extrusion during clot retraction, resulting in smaller thrombi.

FXIII-deficient mice have reduced RBC retention following clot retraction ex vivo and smaller thrombus weights following IVC ligation. To determine whether FXIIIa activity mediates RBC retention during clot retraction, we measured RBC retention during clot retraction in whole blood from mice deficient in the FXIII catalytic A subunit (*FXIII-A*$^{-/-}$)[22]. For these experiments, we triggered clotting in re-calcified blood with the addition of bovine thrombin, and measured clot retraction and RBC extrusion. Strikingly, whole blood from *FXIII-A*$^{-/-}$ mice exhibited reduced RBC retention during clot retraction, phenocopying that seen in Fib$\gamma^{390\text{-}396A}$ clot retraction (Figures 4.7A-B). In contrast, whole blood from *CD11b*$^{-/-}$ mice[23] – the molecular interaction previously shown to be deficient in Fib$\gamma^{390\text{-}396A}$ mice[16] – had normal RBC retention during clot retraction (Figure 4.7C). These results strongly suggest an essential role for FXIII activity in mediating RBC retention within murine clots.

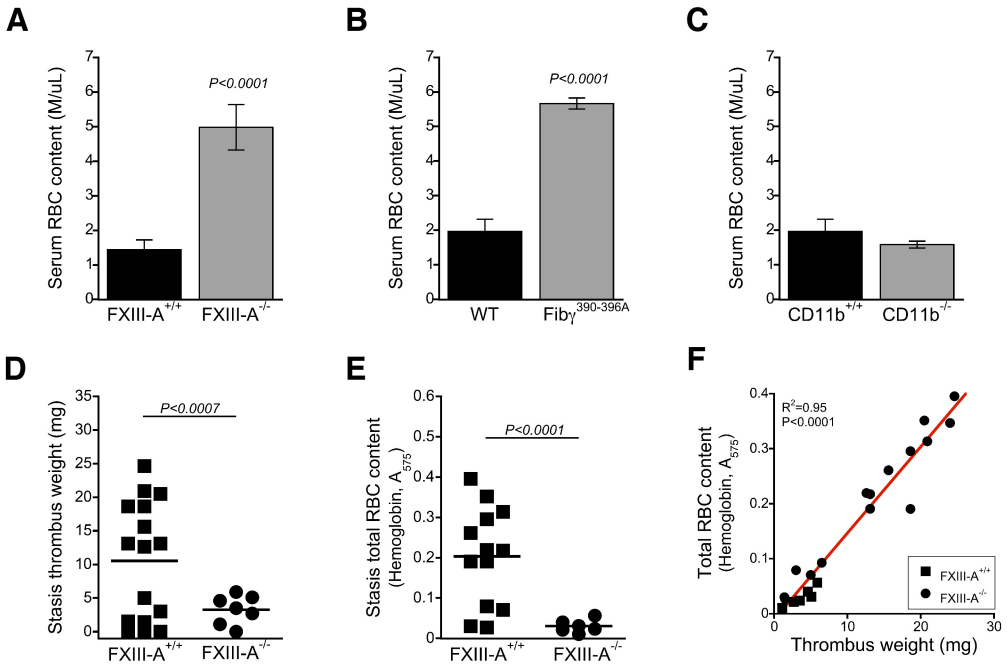

Figure 4.7. FXIII-deficient mice retain fewer RBCs after clot retraction, and RBC extrusion from clots can be induced in normal blood with FXIII inhibition. Serum RBC content following thrombin-initiated clot retraction of re-calcified blood from: **(A)** *FXIII-A*$^{-/-}$, **(B)** Fibγ$^{390\text{-}396A}$, and **(C)** *CD11b*$^{-/-}$ mice (n=4-7). Data are mean ± SEM. **(D)** Thrombus weights 1-day post-ligation (stasis model). Lines are medians. **(E)** Total RBC content of stasis thrombus lysates, measured as hemoglobin absorbance at 575 nm. Each dot represents an individual mouse. Lines are means. **(F)** Correlation of thrombus weight with RBC content. Linear regression shown in red.

To examine the effect of FXIII on thrombus formation *in vivo* we performed the IVC ligation stasis model on *FXIII-A*$^{-/-}$ mice and WT controls. Similar to that seen in Fib$\gamma^{390\text{-}396A}$ mice (Figure 4.1A), *FXIII-A*$^{-/-}$ mice had significantly reduced thrombus weights compared to WT controls (Figure 4.7D). Moreover, thrombi from *FXIII-A*$^{-/-}$ mice had significantly fewer RBCs than WT controls (Figure 4.7E). Although some WT controls also produced small thrombi, the RBC density of these smaller thrombi was similar to larger thrombi from WT controls (data not shown). Importantly, the RBC content of thrombi from both *FXIII-A*$^{-/-}$ and WT mice correlated positively and significantly with thrombus weight (Figure 4.7F). Together these data indicate FXIII activity dictates RBC retention in venous thrombi.

Inhibition of FXIII reduces RBC content in clots formed in human whole blood. To determine whether plasma FXIII activity is also required for RBC retention in *human* clots, we reconstituted plasma from a FXIII-deficient human patient with washed platelets and RBCs from healthy individuals. Reactions were performed in the absence and presence of exogenous plasma-derived FXIII-A_2B_2 (Fibrogammin®-P) added at doses recommended for prophylaxis (10 IU/kg, 0.15 IU/mL) and surgery (33 IU/kg, 0.5 IU/mL), and a dose to return FXIII-A_2B_2 levels to normal (67 IU/kg, 1 IU/mL). FXIII-deficient clots exhibited reduced RBC retention following clot retraction, to a similar degree as *FXIII-A*$^{-/-}$ and Fib$\gamma^{390\text{-}396A}$ mice. Importantly, RBC retention in FXIII-deficient human plasma was corrected by addition of FXIII-A_2B_2 in a dose-dependent manner (Figure 4.8A). These results demonstrate an essential role for FXIII activity in mediating RBC retention in both mouse and human clots.

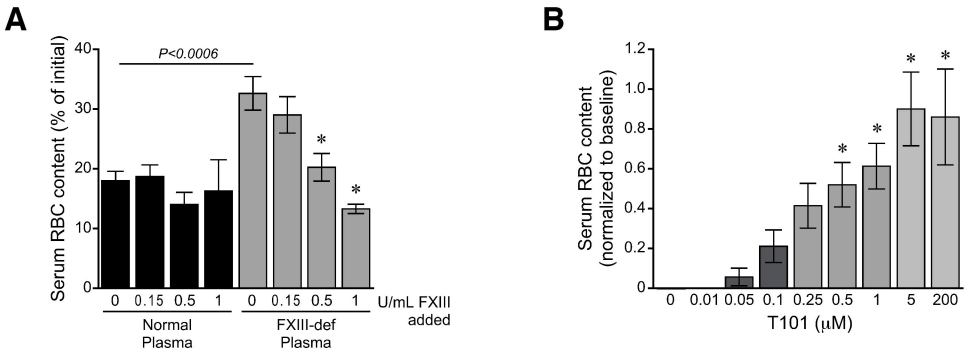

Figure 4.8. FXIII-deficient humans retain fewer RBCs after clot retraction, and RBC extrusion from clots can be induced in normal human blood with FXIII inhibition. (A) Serum RBC content following TF-initiated clot retraction of human FXIII-deficient patient plasma reconstituted with washed platelets and washed RBCs from normal donors and treated with increasing doses of FXIII (0-1 U/mL, n=3-6). Data are means ± SEM. **P*<0.02 versus FXIII-deficient plasma with no additional FXIII by ANOVA. **(B)** Serum RBC content following TF-initiated clot retraction of normal human whole blood in the presence of the irreversible FXIIIa inhibitor, T101 (0.1-5 μM, n=5). Data are normalized means ± SEM. **P*<0.02 versus absence of T101 by ANOVA.

Finally, we tested the hypothesis that blocking FXIII activity reduces RBC retention and clot size. We treated normal human whole blood with the irreversible FXIII active site inhibitor, T101, and induced clot formation and retraction. Following clot retraction, the baseline levels of RBCs present in serum exhibited inter-individual variability (44.7% CV); however, for all donors T101 produced a dose-dependent increase in serum RBC content (Figure 4.8B), resulting in a 46±8% reduction in clot size at 5 μM T101. Moreover, the IC_{50} for T101 was 0.43±0.2 μM, consistent with the reported IC_{50} for FXIII inhibition.[39] Together, these data show FXIII activity is a major determinant of clot RBC content and clot size in mice and humans.

4.4 Discussion

Although the defining characteristic of venous thrombi is their prominent RBC content, little is known about mechanisms that mediate the incorporation or retention of RBCs in venous clots. The prevailing view has been that RBCs are sterically trapped by the fibrin network during thrombogenesis; however, this premise neglects the dynamic nature of a growing thrombus which reflects both biochemical (fibrin deposition) and biophysical (clot retraction) processes. In this study, we identified critical residues in the γ chain of fibrinogen that mediate fibrinogen's interaction with FXIII, and show this interaction is required for appropriately-timed FXIII activation and fibrin cross-linking. We further demonstrate that early FXIII activation is required to retain RBCs in clots during platelet-mediated clot retraction. Importantly, we show that loss of FXIII activity results in the formation of smaller thrombi via decreased RBC retention in clots. These novel findings show that RBCs

are retained in venous thrombi by a specific, active mechanism that contributes to both venous thrombus size and composition.

Essentially all FXIII-A_2B_2 circulates bound to fibrinogen[5], and following activation, FXIII-A_2* cross-links intermolecular residues between both the γ and α chains of fibrinogen. These observations suggest multiple points of interaction between FXIII and fibrinogen; however, the identity of the fibrin(ogen) residues mediating these interactions has been elusive. Older studies suggested FXIII-A_2B_2 binds preferentially to a minor species of circulating fibrinogen produced by alternative-splicing of the fibrinogen γ chain (termed γ')[40,41]; however, studies with recombinant fibrinogens demonstrated FXIII-A_2B_2 binds to γ- and γ'-containing fibrinogen molecules with similar affinity (K_D ~40 nM).[42] Smith et al. showed high affinity binding of FXIII-A_2B_2 to a peptide containing amino acid residues 371-425 of the fibrinogen αC domain (K_D 5-30 nM).[43] However, because those authors were unable to verify binding of FXIII-A_2B_2 to the αC region on full length fibrinogen, they concluded that the interaction between FXIII-A_2B_2 and the αC region arises during FXIII activation, and that other fibrinogen residues fulfill the carrier function for FXIII-A_2B_2. Our data show that mutations within fibrinogen residues γ390-396 disrupt the interaction between fibrinogen and FXIII-A_2B_2, suggesting these residues mediate this carrier function. Given that the γ chain residues cross-linked by FXIII (Q398, Q399, and K406) are immediately C-terminal to residues γ390-396, localization of FXIII zymogen at this site would conveniently position FXIIIa for rapid cross-linking following its activation. Subsequent contributions from the αC domain would facilitate cross-linking of α chain residues Q221, Q237, Q328, and Q366 immediately following formation of γ-γ dimers. Thus, one hypothesis consistent with available data is that the FXIII-A_2B_2 binding site is a composite between residues in the

Aα and γ chains and that disrupting either of these sites reduces binding. Alternately, FXIII may be "handed off" between the fibrinogen γ chain and the αC domain during its activation and activity, such that disrupting this process delays cross-linking and reduces fibrin stability.

Our data show that mutation of residues γ390-396 delayed, but did not abolish, FXIII activation and fibrin cross-linking. The observed delay in FXIII activation and fibrin cross-linking (Figure 4.6) was temporally-associated with the timing of clot retraction (60-90 minutes), suggesting these processes also occur in concert *in vivo*. These findings imply the timing of fibrin formation, FXIII activation, cross-linking, and platelet-mediated clot retraction is precisely orchestrated to promote hemostasis, and that alteration in the relative timing of these events leads to the formation of abnormal clots. It is therefore interesting that Fib$\gamma^{390\text{-}396A}$ mice have normal thrombus formation following $FeCl_3$-induced arterial injury[16], but have significantly reduced thrombus size in a venous thrombosis model. Arterial thrombosis is a rapid event triggered by high TF in high shear. Consequently, arterial thrombi are predominantly composed of platelets, and platelet degranulation may release sufficient FXIII-A_2 transglutaminase activity to stabilize thrombi even in the absence of fibrinogen-bound FXIII-A_2B_2. In addition, artery occlusion – which typically occurs within 8-10 minutes – may precede FXIII activation and/or clot retraction, minimizing differences between the vessel occlusion time in WT and Fib$\gamma^{390\text{-}396A}$ mice. Since both FXIII activation and clot retraction likely occur after arterial occlusion, Fib$\gamma^{390\text{-}396A}$ mice may exhibit decreased thrombus stability and/or decreased resistance to lysis. Studies are ongoing to probe the consequences of delayed FXIII activation on arterial thrombus stability.

Fib$\gamma^{390\text{-}396A}$ mice have previously been used to probe the contribution of fibrinogen-leukocyte integrin $\alpha_M\beta_2$ interactions to physiological and pathological inflammatory

responses. The current data raise the possibility that the diminished inflammatory phenotypes observed in Fib$\gamma^{390\text{-}396A}$ mice are a function of delayed FXIII activation and fibrin cross-linking rather than, or in addition to, loss of fibrin(ogen) $\alpha_M\beta_2$-binding activity. In this scenario, *FXIII-A*$^{-/-}$ mice would be expected to phenocopy Fib$\gamma^{390\text{-}396A}$ mice following inflammatory challenges. In the context of bacterial infection, Fib$\gamma^{390\text{-}396A}$ mice have a compromised antimicrobial host response to *S. aureus* peritonitis challenge[16], and FXIII-deficient mice have compromised antimicrobial activity following *S. pyogenes* skin infection due to a loss FXIII-mediated cross-linking of bacteria to fibrin clots[44]. However, in contrast to Fib$\gamma^{390\text{-}396A}$ mice, the infection phenotype in FXIII-deficiency is accompanied by increased, not decreased, local leukocyte activity and inflammation[44], and in contrast to reduced bacterial clearance in Fib$\gamma^{390\text{-}396A}$ mice, *FXIII-A*$^{-/-}$ mice have peritoneal *S. aureus* clearance kinetics identical to WT mice (unpublished data). Thus, fibrinogen and FXIII likely support host defense through multiple, non-mutually exclusive functions that include mechanisms linked to leukocyte activation through $\alpha_M\beta_2$, as well as transglutaminase-dependent bacterial entrapment. Fib$\gamma^{390\text{-}396A}$ mice are significantly protected in inflammatory disease settings, including central nervous system autoimmune disease[17,18], inflammatory joint disease[19], and Duchenne muscular dystrophy[21]. Fib$\gamma^{390\text{-}396A}$ mice are also protected against inflammatory colitis following dextran sodium sulfate (DSS) challenge and from colitis-associated cancer (i.e., inflammation-driven colonic adenoma formation).[20] At present, similar comparable analyses of inflammatory disease progression in *FXIII-A*$^{-/-}$ mice remain to be reported. However, in patients with either active or inactive inflammatory bowel disease, circulating levels of FXIII-A_2B_2 are significantly lower than that of controls.[45] Reduced FXIII levels have been thought to be a consequence of the disease process and

attributed to consumption of FXIII from microthrombi formation or accelerated turnover of FXIII, but decreased FXIII activity may also contribute to the disease pathogenesis. Consistent with this hypothesis, preliminary studies indicate that unlike the Fib$\gamma^{390\text{-}396A}$ mice, *FXIII-A*$^{-/-}$ mice are not protected from colonic injury, but rather display prolonged colonic injury following DSS challenge (J. Palumbo, personal communication). Additional studies are warranted to elucidate the contribution of FXIII and FXIII-fibrinogen interactions to these inflammatory disease pathologies. The present data support the hypothesis that fibrin(ogen) can contribute to local inflammation through multiple mechanisms including $\alpha_M\beta_2$ engagement and/or FXIII interactions.

The finding that FXIIIa activity is required to mediate RBC retention in clots reveals a novel role for this enzyme in clot formation and composition. The established targets of FXIII(a) activity are the fibrin γ- and α-chains, and FXIII(a)-mediated cross-linking of these fibrin chains imparts critical properties to clots. Compared to uncross-linked fibers, cross-linked fibers are thinner and more elastic[46,47] and require greater force to rupture.[48] Compared to uncross-linked clots, cross-linked clots have smaller pores, increased network density, and a higher viscoelastic storage modulus[47] (and reviewed in [6,49]). It is currently unclear whether these effects directly mediate RBC retention during clot retraction. Since increasing the TF activity increases fibrin network density[50] and RBC retention in WT clots (data not shown), fibrin structure partly mediates RBC retention in thrombi. Since RBCs largely remained in clots in the absence of clot retraction, deficiencies in fibrin integrity needed during clot retraction may permit RBC loss. For example, uncross-linked fibers may extend or even break in response to the contractile forces exerted during clot retraction[51], enabling RBCs to escape. It is also possible that RBCs, themselves, become cross-linked to

the clot, either to fibrin or other clot-bound proteins or cells. RBCs have been shown to directly bind fibrin(ogen)[36,37] (Figure 4.4), though this interaction does not require transglutaminase activity and is insufficient to maintain RBCs in retracting clots. Studies are ongoing to determine if RBCs are cross-linked by FXIIIa during clot formation.

Our findings raise the possibility that current clinical guidelines for identifying FXIII deficiency fail to recognize the role of FXIII in determining clot composition, and therefore, the relationship between low FXIII levels and bleeding. While congenital FXIII deficiency is rare, acquired FXIII deficiency is considered an under-diagnosed disorder due to the relative insensitivity of the standard tests, and the supposition that 5% of FXIII levels are sufficient for hemostasis. However, mounting evidence suggests patients with "mild" FXIII deficiency due to heterozygosity or surgery have increased risk of bleeding.[52] Accordingly, there is considerable controversy regarding the level of FXIII antigen required to maintain hemostasis in these patients. Our finding that FXIII mediates clot composition in a dose-dependent manner across the FXIII reference range (Figure 4.8) suggests that 1 U/mL FXIII levels are required to achieve "normal" FXIII function, and that even mildly reduced levels of FXIII can result in the formation of abnormal clots. In addition, FXIII administration is being tested as an adjunct hemostatic approach in patients with hemophilia.[53,54] Although hemophilic patients are not known to have low FXIII levels at baseline, FXIII infusion may accelerate the rates of FXIII activation and fibrin cross-linking, and increase both fibrin stability and clot RBC content. Accordingly, observations that certain hemophilic patients experience less-than-expected bleeding[55,56] suggest a subset of patients may have a co-existing polymorphism that alters clot stability, such as the FXIII V34L mutation that accelerates FXIII activation.[57,58] Studies are ongoing to test this hypothesis.

Importantly, our findings have significant implications for treating venous thrombosis and accelerating thrombus resolution. Thrombus burden (size) is associated with increased morbidity and mortality after venous thromboembolism. Large, saddle pulmonary embolus is associated with 15% mortality at presentation, and treatments for massive PE involve fibrinolytic therapy or surgical embolectomy, both of which are associated with complications and high mortality. Following venous thrombosis, larger reductions in thrombus mass (clot-burden change) are associated with decreased risk of recurrent venous thromboembolism.[59] Consequently, a FXIII inhibitor that can reduce thrombus burden may be an attractive thromboprophylactic agent. Since FXIII activity both increases RBC retention in clots and increases clot mechanical stability and resistance to fibrinolysis, this approach would be expected to both reduce thrombus burden via decreased thrombus mass, and accelerate clot dissolution. Although total FXIII deficiency is associated with bleeding[60], Fib$\gamma^{390\text{-}396A}$ mice do not exhibit any bleeding tendency. Thus, it may be possible to reduce venous thrombosis without promoting bleeding by specifically blocking FXIII binding to fibrin(ogen). Given that residues γ390-396 are surface exposed[61], this region may be readily targeted by antibodies or small molecule inhibitors.

Finally, although the prevailing paradigm suggests that RBCs are non-interacting and only incidentally present in thrombi, several lines of evidence suggest RBCs actively promote clot formation and stability. Elevated levels of RBCs are associated with increased risk of thrombosis in patients with polycythemia vera or patients on erythropoietin[62-64], and increasing the hematocrit decreases the bleeding time in anemic patients.[65-69] While this effect has been attributed to RBC-mediated margination of platelets within the vessels, studies show RBCs support thrombin generation[70-72] and suppress plasmin generation.[73]

Consequently, RBCs may directly promote thrombus stability, and therapeutic approaches that limit thrombus RBC content may reduce thrombus size and/or stability. Recently, the magnetic resonance longitudinal relaxation time (T_1) of experimental venous thrombi was used to determine effective timing of thrombolytic therapy. Notably, the shortest T_1 times correlated with highest Fe^{3+} in methemoglobin from lysed RBCs, highest percent of fibrin, and greatest response to lysis[74], suggesting RBC and fibrin content within thrombi are central determinants of thrombolytic success. Our finding that FXIII activity mediates clot RBC presence provides a novel tool for investigating the specific contributions of RBCs to venous thrombus formation and stability *in vivo*.

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CHAPTER 5: SUMMARY AND FUTURE DIRECTIONS

5.1 Summary & Future Directions

Cellular and plasma protein crosstalk is an important feature that can vary between arterial and venous vascular beds. The studies comprised within this dissertation emphasize the importance of complementary *in vitro* and *in vivo* studies to explore this crosstalk.

We investigated the prothrombotic potential and vascular bed-specific effects of elevated prothrombin *in vivo*. These studies followed up on *in vitro* work demonstrating that elevated prothrombin significantly increases thrombin generation and fibrin formation without increasing platelet activation.[1,2] In our *in vivo* study, we found that elevated prothrombin significantly increased venous thrombosis, but not arterial thrombosis (Chapter 2).[3] The prothrombotic effects of elevated prothrombin were manifested by increased fibrin deposition but no significant increase in platelet accumulation. These data show that, at least in an acute setting, elevated prothrombin would not be expected to contribute to arterial thrombosis. Given that the highest risk of arterial thrombosis seen in humans with elevated prothrombin occurred in patients who had other underlying risk factors such as hypertension, diabetes mellitus, obesity, and smoking[4,5], it would be worthwhile to repeat our *in vivo* studies in mice with these additional risk factors.

As sub-cellular particles, microparticle procoagulant activities mirror that of their parent cells. Monocytes produce TF/PS-bearing microparticles (M-MP), whereas platelets produce PS-bearing microparticles (PMP). As a result, while M-MPs affect the initiation and

propagation of thrombin generation and clotting via the extrinsic pathway, PMPs affect the propagation of thrombin generation and clotting via the intrinsic pathway (Chapter 3).[6] These data imply a pathogenic role for M-MPs, and suggest PMPs exhibit prothrombotic activity once initiation has occurred. Follow up on this work was done in collaboration with Jian-Guo Wang and Nigel Mackman. We found that TF-bearing microparticles from pancreatic tumor cell lines increased venous thrombosis in a concentration-dependent manner, while pancreatic tumor-bearing mice increased venous thrombosis in only 1 out of 2 venous thrombosis models.[7] The impact of TF/PS-bearing microparticles and PS-bearing microparticles on *in vivo* thrombus development in other settings, such as CVD, would be interesting for future studies. Interestingly, some microparticles have also been shown to have fibrinoytic activity. Microparticles from monocytes, endothelial cells, and tumor cells express urokinase- and tissue-type-plasminogen activators.[8] The significance of the fibrinolytic activity is worthy of further investigation.

In Chapter 4, we investigated the role of fibrinogen and factor XIII (FXIII) in venous thrombosis. From studies using mice expressing a mutant form of fibrinogen ($Fib\gamma^{390\text{-}396A}$) and FXIII-deficient mice and humans, we made four important discoveries: 1) we determined fibrinogen residues involved in FXIII binding; 2) we discovered that FXIII activity is required for retention of red blood cells in venous thrombosis in mice; 3) we demonstrated that inhibition or deficiency of FXIII reduces RBC retention and reduces clot size; and 4) we have redefined the venous thrombosis paradigm to show that RBC retention in clots is an active process and not merely passive trapping. Together, these observations imply that the fibrin(ogen)-FXIII axis are determinants of VTE risk and actively contribute to the pathophysiology of VTE. Clearly, continued research into the prothrombotic nature of

RBCs, fibrin(ogen), and FXIII is warranted, with a particular emphasis on the physical and biochemical interactions between RBCs, fibrin(ogen), and vascular cells. Disrupting the incorporation of FXIII, fibrin(ogen) and/or RBCs into venous thrombi has high potential therapeutic value for reducing VTE and VTE-related sequelae.

Given our finding that delayed FXIII activation led to delayed fibrin cross-linking and reduced RBC retention in Fib$\gamma^{390\text{-}396A}$ clots, it would be interesting to explore the effects of accelerated FXIII activation. One might expect accelerated FXIII activation to increase RBC retention and form bigger clots. The Val34Leu mutation in the FXIII A subunit allows for faster release of the activation peptide, resulting in accelerated activation.[9] Surprisingly, this polymorphism is associated with moderate protection from myocardial infarction in some studies[10,11] but not others[12,13], and meta-analysis of the literature suggests the Val34Leu mutation also provides protection from VTE.[14] These studies in humans suggest we do not yet fully understand how the kinetics of FXIII activation play a role in the pathophysiology of thrombosis and suggest studies of the Val34Leu polymorphism in RBC retention are warranted.

In conclusion, the studies in this dissertation offer insight into the pathophysiology of both arterial and venous thrombosis. In particular, these studies highlight the context-dependent mechanisms that drive thrombogenesis, and the importance of understanding cellular and plasma protein crosstalk.

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