REGULATION OF THE INNATE IMMUNE RESPONSE BY THE BLOOD COAGULATION CASCADE

By

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List of Abbreviations

ADAMTS-13	a disintegrin and metalloproteinase with a thrombospondin type 1 motif, member 13
aPTT	activated partial thromboplastin time
APC	Activated protein C
ATP	adenosine triphosphate
ApoER2	apolipoprotein E receptor 2
RGD	Arg-Gly-Asp motif
βAR-2	β-arrestin 2
Bcl-2	B-cell lymphoma 2
BBB	blood brain barrier
CNS	central nervous system
CGD	chronic granulomatous disease
H3Cit	citrullinated histone 3
cAMP	cyclic adenosine monophosphate
DAMPs	damage-associated molecular patterns
DNA	deoxyribonucleic acid
Dab1	Disabled-1
PPACK	D-phenylalanyl-prolyl-arginyl chloromethyl ketone
EPCR	endothelial protein C receptor
EGF	epidermal-like growth factor domain
EAE	experimental autoimmune encephalomyelitis
ECM	extracellular matrix
ERK1/2	extracellular signal-regulated kinases 1/2
FVIII	Factor VIII
FAK	focal adhesion kinase

GPIb	glycoprotein Ib
GPIbR	glycoprotein Ib receptor
GPCR	G-protein coupled receptor
G-CSF	granulocyte colony-stimulating factor
GM-CSF	granulocyte macrophage colony-stimulating factor
IP3	inositol triphosphate
ICAM	intercellular adhesion molecule
IL-8	interleukin 8
LDV	Leu-Asp-Val
LAD I	leukocyte adhesion deficiency I
LFA-1	lymphocyte function-associated antigen 1 (CD11a/CD18)
Mac-1	macrophage-1 antigen (CD11b/CD18)
MIDAS	metal ion-dependent adhesion site motif
MAPK	mitogen-activated protein kinases
MS	multiple sclerosis
MPO	myeloperoxidase
NE	neutrophil elastase
NETs	neutrophil extracellular traps
NADPH	nicotinamide adenine dinucleotide phosphate
NFκB	nuclear factor kappaB
PAMPs	pathogen-associated molecular patterns
PRRs	pattern recognition receptors
PAD4	peptidyl arginine deiminase type IV
PMA	phorbol 12-myristate 13-acetate
PLC	phospholipase C

PI3K	phospoinositide 3-kinase
PECAM	platelet endothelial cell adhesion molecule (CD31)
PDGF	platelet-derived growth factor
PSI	plexin-semaphorin-integrin domain
PMNs	polymorphonuclear leukocytes
PolyP	polyphosphate
PCNA	proliferating cell nuclear antigen
PGI_2	prostaglandin
PARs	protease activated receptors
PKC	protein kinase C
PSGL-1	P-selectin glycoprotein ligand-1
CD62P	P-selectin; cluster of differentiation
ROS	reactive oxygen species
rhoGEFs	Rho guanine nucleotide exchange factors
RNA	ribonucleic acid
SFKs	Src family kinases
TM	thrombomodulin
TF	tissue factor
TEM	transendothelial migration
TNFα	tumor necrosis factor α
VCAM	vascular cell adhesion molecule
vWF	von Willebrand factor
WE-thrombin	W215A/E217A thrombin

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Abstract

Regulation of the innate immune response by the blood coagulation cascade

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Polymorphonuclear leukocytes, specifically neutrophils, are highly dynamic, multi-functional innate immune cells. Neutrophils are among the first cellular responders to bacterial host tissue infection. At sites of inflammation, neutrophils can undergo activation, releasing their nuclear content to form microbicidal neutrophil extracellular traps (NETs). NETs provide a structural framework for pathogen clearance; however, recent studies have demonstrated that NETs may additionally contribute to thrombotic complications, including deep vein thrombosis. The crosstalk and underlying mechanisms in the blood microenvironment that regulate NETs formation and potentiate thrombus formation remain ill-defined. This gap in knowledge has guided my research thus far, driving investigations into how the blood microenvironment influences neutrophil activation, specifically NETosis, and how the thrombogenic potential of NETs is dynamically regulated.

Our initial experiments sought to determine the binding and spatial localization of select coagulation factors to neutrophils and NETs. The coagulation factor fibrinogen was found to bind both the neutrophil cell body and NETs, while prothrombin, coagulation factor (F)X and FVIIa binding was restricted to the neutrophil cell body. The Gla domain of FX was required for FX binding to neutrophils. Activated protein C (APC) but not Gla-less APC bound to neutrophil cell bodies and NETs. Moreover, FX and APC required neutrophils to express phosphatidylserine, a marker of activation, to bind to the neutrophil membrane. Lastly, fibrinogen binding was dependent on neutrophil extracellular DNA. Overall, these results suggest activated neutrophils and NETs may serve as a platform for assembly and possible activation of coagulation.

Studies were subsequently focused on the role of activated protein C (APC) and how this protease affects neutrophil function. APC is a multifunctional enzyme capable of exerting antithrombotic, anti-inflammatory, and cytoprotective effects. APC is known to inhibit the migration of neutrophils, but the role of APC in NETosis is ill-defined. This thesis presents results demonstrating that APC negatively regulates NETs formation, which is reversed in the presence of the protease inhibitor PPACK. Equimolar concentrations of thrombin or the zymogen protein C did not affect NETs formation by PMA, a PKC agonist that induces NETs. Similarly, use of autologous platelet secretome was found to induce NETs formation, which was inhibited when neutrophils were pretreated with APC. Similar to pretreatment with APC, the use of the APC-derived P3R peptide, which is generated after the cleavage of PAR3 at Arg41 by APC, significantly reduced PMA-induced NETosis either in the presence or absence of APC. Treatment of APC with a specific physiologic inhibitor of protease activity also reversed the inhibition of APC on NETs formation, suggesting that under physiologic conditions, there may be a role for APC regulation of neutrophil activation. APC is known to regulate cytoprotective signaling in endothelial cells via binding endothelial protein C receptor (EPCR). Different inhibitors were used to determine whether

APC plays a similar role in neutrophils, and results confirm that APC regulation of signaling to promote cell survival may play a role in reducing NETs formation.

Finally, experiments were performed to screen APC proteins containing specific mutations to determine the structure-function relationship between APC and inhibition of NETs formation. Results suggest that mutations near the active site of APC result in loss of its inhibitory activity, and mutations near the Gla domain that facilitate binding EPCR also partially lose its inhibitory effect on NETs formation. A pilot proof-of-concept *in vivo* experiment utilizing a non-human primate model of bacterial sepsis determined that pharmacological doses of APC reduced neutrophil activation via a decrease in myeloperoxidase levels. Overall, our data suggest that soluble APC can inhibit platelet secretome- and PMA-induced NETosis in a EPCR/Mac-1/PAR3 dependent manner. This work has defined an additional and novel anti-inflammatory function of APC to modulate neutrophil survival. Fundamental questions remain about how these three receptors ligate APC and initiate the specific intracellular signaling pathways that are activated to result in inhibition. Future work will aim to identify the precise signaling network that results in this alteration of NETosis.

Chapter 1. Introduction to neutrophil activation and thrombus formation

1.1 Roles for the innate immune response and blood coagulation in infectious disease

The annual incidence of severe sepsis cases in the United States is estimated to be 300 per 100,000 in the population [1]. Hospitalized patients that are diagnosed with severe sepsis are defined as presenting with evidence of systemic infection and acute organ dysfunction. The approximate survival in the most severe sepsis cases approaches 30%, making this disease an important health problem [1,2]. Moreover, the causative invading pathogen is often difficult to diagnose and treat, making proper supportive care essential and difficult for many patients with severe sepsis. In the development of sepsis, inflammation is initiated in response to infection, resulting in activation of the immune response and activation of the blood coagulation cascade, which further potentiates inflammation. The crosstalk between the immune response and coagulation amplifies both responses, resulting in consumption of immune cells and coagulation factors, exhausting the immune and coagulation responses and increasing the risk for adverse bleeding events and/or death. Historically, use of traditional anticoagulants in the treatment of sepsis has shown short-term benefit but ultimately proved unsuccessful; thus, the treatment of sepsis remains an unmet clinical need [3–5].

Lung infections (pneumonia) are the most common cause of sepsis. In positive blood cultures of septic patients, the most common species are Gram-negative bacteria, including *Psuedomonas aeruginosa*, *Escherichia coli* and the Klebsiella species, whilst the Gram-positive species include *Staphylococcus aureus* and *Streptococcus pneumoniae* [3]. Antibiotic administration as part of the supportive care guidelines to treat the underlying infection is often insufficient to halt the acute organ dysfunction stemming from dysregulation of the immune and coagulation systems [4]. Over the course of sepsis, the aberrant activation and subsequent consumption of coagulation factors and blood cells results in consumptive coagulopathy and an increased risk for bleeding. Disseminated intravascular coagulation

(DIC) can cause blood vessel occlusion that will result in microcirculatory damage, ischemia, organ dysfunction, or even necrosis. In particular, an inverse correlation between mortality and levels of the essential antithrombotic and cytoprotective blood serine protease protein C in sepsis patients demonstrates a link between coagulation pathways and prognosis of sepsis and suggests causality between poor outcomes and an acquired protein C-deficient state [6].

After the characterization and identification of plasma protein C and its active form, activated protein C (APC), researchers developed a non-human primate model of *E. coli*-induced sepsis to evaluate the pharmacological activity and therapeutic potential of exogenous APC. In this model, termed the LD_{100} baboon model of sepsis, *E. coli* infused at 1×10^{10} colony forming units (CFU)/mL has been found to mimic the phenotype of severe sepsis with acute organ dysfunction and induction of cardiovascular shock and DIC [7]. The initially chosen *E. coli* strain, Type B, was isolated from a stool specimen at Childrens Memorial Hospital, Oklahoma City; however, currently the lab strain used *in vivo* is the nonvirulent *E. coli* strain, ATCC 12701 serotype O86:K61(B7) [7–9]. By use of the LD_{100} baboon model of sepsis, Taylor et al. demonstrated that administration of APC reduced early markers of sepsis and increased survival, which suggested that pharmacologic APC could be beneficial in sepsis [7]. It was also shown that in canines, generation of the procoagulant serine protease thrombin led to APC generation, which was protective [10]. Canine studies also suggested that APC's combined anticoagulant and anti-inflammatory activity was responsible for protection from bacterial lipopolysaccharide toxicity [11]. The early observations on the antithrombotic and *E. coli* neutralizing activities of APC in baboons prompted the commercial development of recombinant human (rh)APC Xigris© (Lilly) [7,12]. The PROWESS trial, which infused rhAPC in severe sepsis patients, initially showed improved outcomes [13], but the follow-on PROWESS-SHOCK trial failed to demonstrate any outcome benefit of rhAPC administration, and Xigris was removed from the world market [14]. Since reasons for the failure to demonstrate patient outcome benefit in the PROWESS-SHOCK study have not been identified, research continues to define

the mechanisms by which endogenous APC may play a role in the anti-inflammatory and antithrombotic responses to pathological conditions. APC has been rationally mutated to select for these specific functions, and one anti-inflammatory APC analog progressed to phase 1 and is currently in phase 2 studies in human stroke subjects [15]. Furthermore, defining the molecular mechanisms by which the cytoprotective and the antithrombotic activities of endogenous or exogenous APC occur will generate knowledge that may help the development of novel sepsis treatment strategies that improve patient outcome.

My thesis research has centered on defining coagulation factors that bind to the innate immune cells, neutrophil leukocytes and their various components, and how these interactions may modulate downstream activation-associated processes and cell death. Specifically, my studies identified a novel mechanism for APC to bind and activate neutrophil-expressed receptors to result in a reduction in one form of cell death, thus demonstrating that APC, in pharmacological settings, has significant effects on neutrophil function and the immune response. This discovery may help in elucidating why APC could have different effects in various types of sepsis, depending on the causal pathogen.

1.2 Innate immunity

1.2.1 *Overview of innate immunity and neutrophils*

The immune system has evolved to protect the host from invading pathogens and tissue damage. In vertebrates, the immune system is split into two broad systems, the innate immune system and the adaptive immune system. The main function of the immune system is to recognize and halt the spread of infectious or inflammatory agents and promote resolution of infection and inflammation [16,17]. The innate immune response is comprised of leukocytes, which are produced in the bone marrow, and from the time of birth are capable of recognizing foreign pathogens and mounting a response. Innate immune

cells derive from the myeloid progenitor cell and mature into granulocytes, macrophages, dendritic cells, and mast cells. Leukocytes circulate in the blood and extravasate into tissue only in response to infection or injury where they nonspecifically recognize pathogen- or damage-associated molecular patterns (PAMPs/DAMPs) via pattern recognition receptors (PRRs), initiating intracellular signaling pathways that result in cell activation. In response to activation, leukocytes undergo several cellular responses. First, leukocytes release their granular contents containing antimicrobial proteases to promote degradation of bacterial cell walls. Second, they secrete granules containing cytokines to recruit additional innate immune cells and adaptive immune cells to promote pathogen clearance. Lastly, leukocytes including dendritic cells are capable of engulfing and phagocytosing the pathogen, promoting degradation and intracellular processing of the foreign body. In the case of dendritic cells, they then migrate into lymphoid organs and act as antigen presenting cells to the lymphoid-derived adaptive immune system, training lymphocytes to selectively recognize that antigen or produce antigen-specific antibodies.

Granulocytic leukocytes derive their name from the dark staining seen of their granular contents after hematoxylin and eosin preparations; they are also called polymorphonuclear leukocytes (PMNs) for their lobulated nuclei and are considered an essential first line of defense [18]. The work of Elie Metchnikoff in starfish larvae in the 1800s is credited as the first description of phagocytosis in the immune response [19]. Of the PMNs, neutrophils are the most populous innate immune cell found in circulation. Neutrophils are released from the hematopoietic bone marrow in their mature form at a steady state at a rate of 5-10 $\times$ 10^{10} cells/day in normal human adults. Neutrophils survey the circulation and select organs including the liver and spleen for signs of PAMPs/DAMPs. They are short-lived cells, surviving 6-8h in circulation before undergoing senescence if they remain unactivated, followed by phagocytosis and clearance by macrophages residing within the bone marrow and the reticuloendothelial system [20]. The primary functions of neutrophils are to migrate to sites of infection or inflammation and begin clearing the

infectious agent through phagocytosis and releasing of antimicrobial molecules that amplify inflammation. Neutrophil amplification of inflammation recruits additional innate immune cells. Lastly, neutrophils can undergo several different cell death mechanisms to facilitate the eventual resolution of inflammation. Whilst neutrophils have historically been considered inelegant phagocytic "suicide cells," a resurgence in neutrophil research in the last three decades has uncovered novel functions that demonstrate neutrophils participate in exquisite signaling and dynamic interactions in order to drive the reactions comprising the inflammatory response.

1.2.2 *Neutrophil adhesive proteins and neutrophil function*

Integrin Structure and function. Integrins are type I integral membrane glycoproteins that are heterodimers comprising an α and β subunit. Integrins are responsible for mediating cell adhesion to proteins of the extracellular matrix (ECM) as well as cell-cell interactions, and they are considered critical for organism development and growth, hemostasis and the immune response. Integrins serve as mechanochemical sensors and transducers of signals into and out of the cells to result in directed cytoskeletal rearrangements. Integrins are able to undergo conformational changes upon ligand and divalent metal ion binding from a low-binding, bent state to extend out into a high-affinity state via inside-out signaling. Whether intermediate states exist is thought to depend on the specific integrin. In mammals, 24 integrins have been identified thus far with 18 α- and 8 β- subunits, and different cell types express specific integrins [21]. The crystal structure of αvβ3 with and without its ligand demonstrates the flexibility of integrins to adopt either bent or extended conformations, leading to the conclusion that both conformations could play physiologically relevant roles in directing cellular functions [22,23].

The basic structure of the integrin α-subunit is typically 1000 amino acids, containing four or five extracellular domains, including a seven-bladed β-propeller responsible for binding Ca^{2+}, a thigh domain

and two calf domains. The linker between the thigh and β-propeller as well as the linker between the calf-1domain and thigh both promote interdomain flexibility. The N-terminal extracellular domains are followed by membrane spanning regions and cytoplasmic tails. In the β-subunit, typically 750 amino acids long, there are seven domains that provide the flexibility associated with integrin activation. First, there is a β-I domain, a hybrid domain, and a plexin-semaphorin-integrin (PSI) domain. After these domains, there are four epidermal growth factor (EGF) domains and lastly a β-tail domain [24]. The β-I domain contains the metal-ion-dependent adhesion site (MIDAS), facilitating binding of Mn^{2+}, Ca^{2+} and Mg^{2+}, which drives open and closed conformations of β-I domain and subsequent activation of the integrin [25]. Specific to leukocytes are the β_1, β_2, β_3 and β_7 integrins. The αV integrins, β_1 and $\alpha_{IIb}\beta_3$ integrins recognize the Arg-Gly-Asp (RGD) motif at the interface between the α and β subunit but can also recognize the acidic motif Leu-Asp-Val (LDV). The β_2 integrin (CD18) is the principal integrin expressed on neutrophils that when bound to α_L (CD11a) forms lymphocyte-function associated antigen 1 (LFA-1) and when bound to α_M (CD11b) forms macrophage-1 antigen (Mac-1). LFA-1 and Mac-1 are both critical to neutrophil adhesion and extravasation out of the vasculature, important functions of neutrophils that will be further described shortly. Mutations of the CD18 subunit causes a reduction in or loss of association with either CD11a or CD11b, resulting in leukocyte adhesion deficiency type I (LAD I) [26]. CD11a primarily recognizes intercellular adhesion molecules (ICAMs), whereas CD11b recognizes a host of ligands, including fibrin(ogen), fibronectin, factor X, platelet Ibα, polysaccharides, and heparin. CD11c and CD11d also recognize vascular cell adhesion proteins (VCAMs) and several matrix proteins [27]. The intracellular cytoplasmic tails are responsible for the assembly of signaling complexes in part due to the NPXY motifs in the β integrin tails, and promote assembly of talin and kindlin proteins that recruit Src-family kinases (SFKs) and promote cytoskeletal rearrangement [28].

As neutrophils are the first line of host defense, they constantly survey their surroundings for sites of infection or inflammation, at which they are recruited to the vessel wall in order to extravasate into the

surrounding tissue in four main steps. To recruit neutrophils to a specific tissue, inflamed cells release cytokines like tumor necrosis factor α (TNF-α), or in cases of infection, the pathogen associated lipopolysaccharide (LPS) is capable of activating endothelial cells to release Weibel-Palade bodies containing P-selectin, which drives synthesis and expression of E-selectin on the endothelium. E-selectin recognizes and binds sulfated sialyl-Lewis^X, the carbohydrate ligand expressed on neutrophils. This is a transient interaction, but it slows the neutrophils enough to promote rolling along the endothelium. Endothelial cell expression of ICAM-1, together with the chemokine interleukin-8 (IL-8) bound to endothelial proteoglycans will induce conformational changes in LFA-1 and Mac-1 integrins on the neutrophil, promoting stronger adhesion and crawling. Third, the neutrophil extravasates via diapedesis, moving through the vessel wall at intercellular junctions of endothelial cells, in a process called transendothelial migration (TEM). Neutrophils utilize LFA-1 and Mac-1 binding to endothelial platelet-endothelial cell adhesion molecule (PECAM; CD31) in order to squeeze between the endothelial cells. Finally, neutrophils undergo chemotaxis to the primary site of infection or inflammation using the concentration gradient of chemokines generated by the resident macrophages that first encounter the pathogen, and they release their granules to amplify the immune response [17,18,29]. Once the neutrophils arrive to the tissue, they can phagocytose the invading pathogen, releasing granular contents like myeloperoxidase (MPO), aiding in generation of reactive oxygen species (ROS) by nicotinamide adenine dinucleotide phosphate (NADPH) oxidase to promote degradation of the bacterial cell wall. Neutrophils may also release cytokines to amplify the immune response or undergo programmed cell death pathways including apoptosis or formation of neutrophil extracellular traps to promote and amplify resolution of inflammation (Figure 1.1).

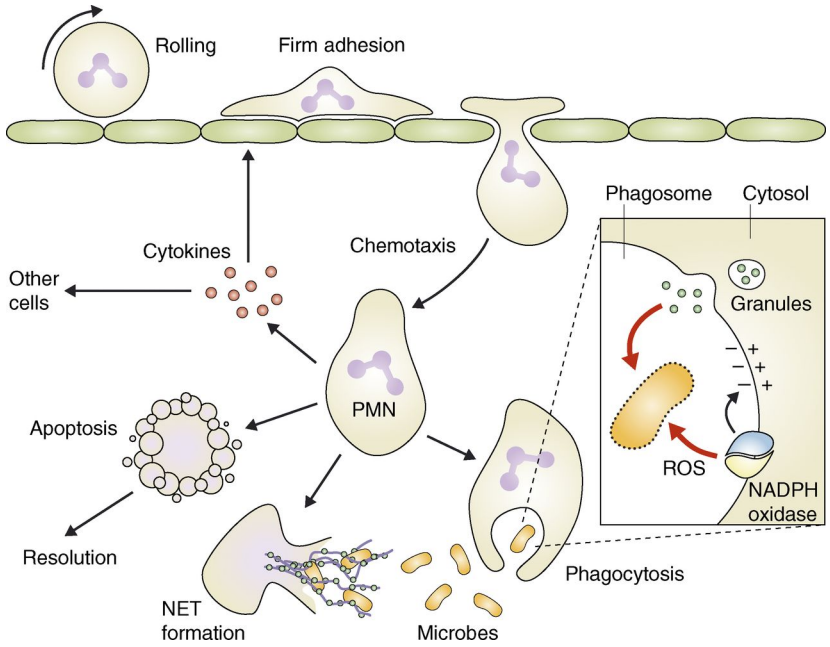

Figure 1.1. Neutrophil functions.

In response to chemokines and exposure of E-selectins on the endothelium, neutrophils begin rolling on the vessel. Neutrophils then stably adhere and extravasate, undergoing chemotaxis in response to the chemokine gradient. Once neutrophils reach the target tissue site, they are capable of phagocytosis and pathogen clearance via ROS generation, releasing cytokines to amplify the immune response or undergoing programmed cell death pathways, namely via formation of NETs or apoptosis, ultimately promoting resolution of inflammation. Figure was adapted from ©Mocsai et al., 2013, originally published in J Exp Med 210(7): 1283-99. Reprinted with permission from Rockefeller University Press.

1.2.3 *Neutrophil extracellular traps*

First described in 2004 as a novel mechanism to promote pathogen clearance, neutrophils were found to undergo an alternative form of cell death. Neutrophil elastase (NE) from the azurophil granules moves into the nucleus, the chromatin decondenses, and peptidyl arginine deiminase 4 (PAD4) converts arginine residues on histone 3 to citrulline, facilitating chromatin decondensation [30–32]. Finally, the chromatin is extruded out of the cell complexed with granule proteins into the extracellular space, termed neutrophil extracellular traps (NETs). These NETs were initially described as capable of capturing gram-positive and

-negative bacteria including *Staphlococcus aureus* and *Shigella flexneri*, as well as the yeast and hyphal forms of *Candida albicans*, demonstrating that neutrophils were capable of recognizing different microbes of varying sizes and undergoing NETosis to clear the invading pathogen [30,33].

The signaling pathways driving NETosis include protein kinase C (PKC) -dependent and -independent pathways and are considered to be specific to the agonist used to initiate NETosis [34]. Moreover, mitogen activated protein kinase (MAPK) kinases and generation of ROS generated by NADPH oxidase are also considered required for the formation of NETs [35–37]. This supports the discovery by Bianchi et al. that chronic granulomatous disease (CGD) patients, who have impaired NADPH oxidase activity and experience prolonged infections due to the decrease in antimicrobial activity from neutrophils were unable to undergo NETosis, suggesting that NETosis may serve a functional role in response to aspergillosis infection. Gene therapy to increase NADPH oxidase activity in these patients restored NETosis and promoted neutrophilic antifungal activity [38]. The observation that NETs formed in the pulmonary airways and liver microvasculature in sepsis highlighted that in select infectious disease states, NETosis might be beneficial to survival [39]. On the opposite side of the spectrum, aberrant induction of NETosis is associated with rheumatoid arthritis, thrombosis, fibrosis and acute respiratory distress syndrome [40–43]. Associations between these inflammatory disease states and incidence of NETosis led researchers to conclude that NETs play a dual role in modulating inflammation and infection, either acting to the benefit or detriment of the patient in a disease-dependent manner.

1.3 Vascular cells in hemostasis and thrombosis

1.3.1 *Overview of vascular cells*

Vascular cells, including endothelial cells and platelets, all play an essential and critical role in maintaining the normal flow of blood and ensuring proper exchange of nutrients, oxygen and removal of

waste products within the body. The endothelium is comprised of a single cell layer, separating the blood from the ECM and surrounding tissue. Under homeostatic conditions, the endothelium expresses and produces a glycocalyx, comprised of proteoglycans and glycoproteins that are membrane-bound. While it is recognized that the glycocalyx plays an important role in (patho)physiology of the vasculature, the precise role for the function and composition of the glycocalyx remains ill-defined. Thus, research is ongoing to define glycocalyx expression, components and functional relevance under homeostatic and pathologic states [44].

Endothelial cells play critical roles as sensors of infection and inflammation, releasing Weibel-Palade bodies containing P-selectins that promote neutrophil rolling and adhesion, in addition to releasing von Willebrand Factor (vWF), a blood glycoprotein capable of promoting coagulation and platelet adhesion after endothelial cell activation or injury (Figure 1.2.) [45,46]. Chains of the vWF polymer unravel in a shear-dependent manner, exposing twelve domains, which were recently re-annotated and the proposed structure revised [47]. First, the D'D3 domain binds and prevents degradation of the coagulation factor VIII (FVIII). The A1 domain is responsible for binding the platelet glycoprotein Ib (GPIb), heparin and collagen. The A1 domain was also reported to bind extracellular histones, though the biological significance of this observed result was questioned until the discovery of NETs [48]. The A2 domain contains the recognition motif for degradation by a disintegrin and metalloproteinase with a thrombospondin type 1 motif, member 13 (ADAMTS-13), but it also binds heparin as well as purified DNA, likely due to the high negative charge of these molecules [49]. Lastly, the C4 domain contains the RGD motif, which is recognized and bound by platelet integrin $\alpha_{IIb}\beta_3$.

Platelets are small, anucleate cells that circulate in the blood at a steady-state of 1×10^{12} cells, typically circulating with little adhesion and activation along the vessel wall, as endothelial cells secrete nitric oxide and prostaglandin (PGI_2) to keep platelets quiescent [50,51]. Platelets originate from

megakaryocytes, which form proplatelet extensions in the bloodstream and release platelets into the blood. While the best characterized niche for platelet production is in the bone marrow, the lungs of mice have recently been described to harbor hematopoietic progenitor cells and megakaryocytes, which may lead to exciting new mechanisms by which megakaryocytes and other hematopoietic progenitors may circulate and extravasate out of the bloodstream, possibly via utilizing mechanisms similar to immune cells [52,53].

Activation of platelets occurs in response to endothelial cell dysregulation or injury, whereby platelets undergo shape change from a discoid to a round shape with pseudopods, express integrins and are considered procoagulant, facilitating formation of a platelet plug. Under hemostatic conditions, the endothelium is injured, exposing ECM proteins and releasing vWF, promoting platelet adhesion, activation and aggregation. Platelet receptor integrin $\alpha_2\beta_1$ promotes platelet adhesion and anchoring to the ECM proteins collagen type I and III. Glycoprotein (GP)VI mediates platelet intracellular signaling and activation and is the main collagen receptor. Platelets contain different granules that are secreted after activation that act in an autocrine and paracrine manner to amplify the platelet response. Release of granular contents potentiates platelet-platelet binding via secretion of ADP, thromboxane A_2, epinephrine and fibrinogen. This allows for conformational activation of GPIIb/IIIa, enabling it to bind vWF and fibrinogen, bridging platelets together (Figure 1.2). Platelet granules also contain coagulation factors including fibrinogen and coagulation factors V, VII, XI and XIII to amplify coagulation. Granule secretion of short-chain polyphosphates (PolyP) is also capable of activating coagulation via the intrinsic cascade; this will be described in more detail in section 1.4. The neutrophil receptor Mac-1 tethers with platelet GPIb, and firm adhesion between platelet P-selectin (CD62P) promotes binding via neutrophil P-selectin glycoprotein ligand-1 (PSGL-1), resulting in platelets and neutrophils “rosettes” or clusters. Platelet-neutrophil adhesion is also enhanced when fibrinogen is bound to platelet GPIIb/IIIa, making platelets an important player in mediating innate immune cell responses. Platelet-neutrophil interactions

may facilitate neutrophil activation, as proteomics studies have demonstrated that platelet release of TNFα, IL-6, IL-8, thrombin, granulocyte colony stimulating factor (G-CSF), and platelet-derived growth factor (PDGF) are capable of activating neutrophils and possibly even potentiating NETs formation [39,40,54–57].

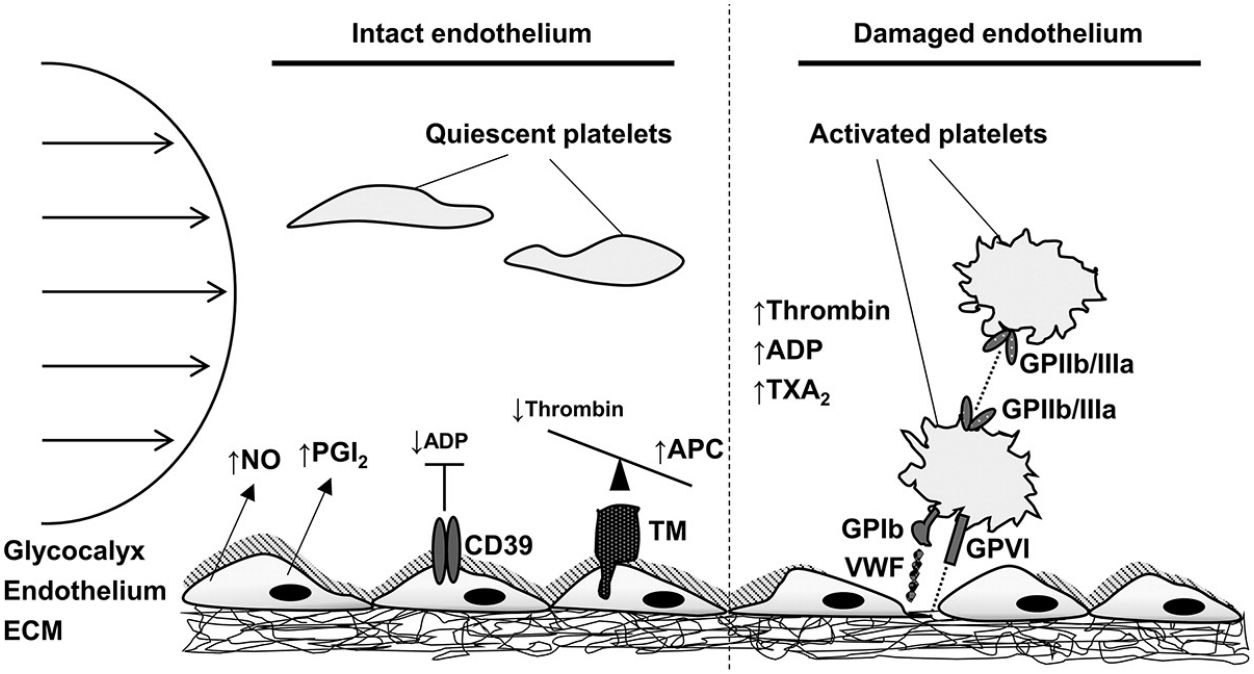

Figure 1.2. Vascular cells in homeostasis and damage.

Vascular cells including endothelial cells and platelets play essential roles in hemostasis and thrombosis. Under normal conditions, the intact endothelium secretes molecules to keep circulating platelets quiescent and expresses a glycocalyx. In response to endothelial damage, platelets roll and adhere to the extracellular matrix (ECM) protein collagen via GPVI, as well as bind vWF released from endothelial cells via GPIb. Endothelium and platelets upon activation release granules containing thrombin, ADP and thromboxane A_2 (TXA_2). Platelets then aggregate and bind together via the fibrinogen receptor GPIIb/IIIa. Figure was adapted from ©Zilberman-Rudenko et al., 2017, originally published in Platelets 2017 Mar 30:1-8. Reprinted with permission from Taylor & Francis Group.

1.3.2 *Protease activated receptors*

Protease activated receptors are rhodopsin-like G-protein coupled receptors (GPCRs) containing seven transmembrane domains. PARs are best studied to understand the cell signaling effects of thrombin, a coagulation factor essential to fibrin formation in coagulation, but also capable of initiating cellular responses. PARs are expressed primarily in vascular and immune cells, but are also seen in epithelial cells, neurons and astrocytes. To date, there are four PARs that have been identified (PAR1-4). Human platelets express PAR1 and PAR4, however mouse platelets express PAR3 and PAR4, an important consideration when using a mouse model to study platelet biology. In the GPCR family, PARs are unique as activation occurs after cleavage of the N-terminus by a protease, resulting in the uncovering of the ligand or new N-terminus which binds to the body of the receptor to initiate intracellular signaling. Whilst PARs are classified as GPCRs and typically use G proteins to initiate intracellular signaling, they can signal through different proteins. Interestingly, the different proteases that cleave PARs generate unique N-termini or tethered ligands that result in biased, or opposing intracellular signaling. Thrombin cleaves PAR1, -3 and -4, whereas coagulation factor (F)X recognizes and cleaves PAR3 [58–63]. Trypsin, tryptase and FX recognize PAR2 [64]. Lastly, activated protein C recognizes and catalyzes cleavage of PAR1 and PAR3 [58,65].

PAR1 signaling is the best studied receptor of the PAR family to date. In response to PAR1 activation by thrombin, PAR1 can couple to the $G_{12/13}$, G_q, and G_i families. The α subunit of G_i inhibits adenylyl cyclase, which is responsible for catalyzing ATP into the second messenger, cAMP. The α subunits of G_{12} and G_{13} bind RhoGEFs (guanine exchange factors associated with the small G protein Rho) that promote activation of Rho which is involved in cytoskeletal rearrangements within platelets. The α subunit of G_q binds and activates phospholipase C (PLC), which hydrolyzes phosphoinositide, generating

diacylglycerol and inositol triphosphate (IP_3) that drives intracellular Ca^{2+} flux and activation of protein kinase C (PKC). This initiates broad downstream signaling to result in integrin activation and granule secretion. The β and γ subunits are capable of activating phosphoinositide 3-kinase (PI(3)K), PLC and ion channels. The activation of PI(3)K results in a myriad of signaling events, including recruitment of serine/threonine kinases, non-receptor tyrosine kinases, GEFs, and focal adhesion kinases (FAK) to the cell membrane, facilitating actin reorganization. Activation of PAR1 using *in vitro* cell systems has shown that the distribution of receptors like integrin $\alpha_5\beta_3$ changes around the cell surface [64,66].

1.4 Blood coagulation proteins

1.4.1 *Extrinsic coagulation*

Within the blood, plasma proteins that participate in coagulation are called coagulation factors and circulate in their inactive, or zymogen form. Activation of the coagulation cascade occurs concomitant to platelet activation and aggregation, resulting in the formation of fibrin from fibrinogen, a peptide that polymerizes and crosslinks to provide stability to the platelet plug. The coagulation process is highly conserved in eukaryotes, and the observation that blood was capable of solidifying and clotting was first described by the Greek philosopher Plato, who coined the term fibrin. The 20th century saw the identification of coagulation factors as serine proteases, whose mechanisms of action result in fibrin formation, and the subsequent development of the waterfall cascade model to explain activation of the coagulation factors [53]. Coagulation factors are primarily made in the liver. Coagulation factors II, VII, IX, and X as well as proteins C, S and Z are considered vitamin K-dependent, as their NH_2-termini undergo post-translational modifications in the liver to gain a γ-carboxyglutamic acid (Gla)-rich domain. Upon secretion, this Gla domain facilitates binding to phospholipid cell membranes, increasing the efficiency of coagulation.

In response to vessel injury or inflammation, endothelial cells expose tissue factor (TF) that binds and activates coagulation factor (F)VII into activated FVII (FVIIa), thus initiating the extrinsic coagulation cascade. This TF-FVIIa complex proteolytically cleaves FIX and FX into FIXa and FXa, respectively. The tenase complex of FIXa with its cofactor FVIIIa cleaves additional FX into FXa. From there, FXa and its cofactor FVa together form the prothrombinase complex which binds and cleaves prothrombin (FII) into thrombin (FIIa). Thrombin is a serine protease that recognizes and cleaves many substrates; however one of its most important roles is the cleavage of fibrinogen into fibrin. As platelets undergo activation and aggregation, they release FV, and coagulation is amplified by thrombin cleavage of FV into FVa and conversion of FVIII into FVIIIa, the cofactor for FIXa. Thrombin also positively feeds back by activating FXI into FXIa, which cleaves FIX into FIXa, amplifying thrombin generation and fibrin formation (Figure 1.3). Lastly, thrombin activates FXIIIa, a transglutaminase that catalyzes covalent crosslinks between fibrin chains. Deficiencies in FVIII result in hemophilia A, and deficiencies in FIX result in hemophilia B, highlighting the importance of the extrinsic coagulation cascade in humans.

A hemostatic plug is formed with the generation of the platelet plug and fibrin crosslinking, resulting in the cessation of blood flow from the vessel. In contrast, aberrant activation of coagulation, recruitment of leukocytes to the endothelium and platelet activation result in a clot or thrombus in the vasculature that impedes the normal flow of the blood, in a process termed thrombosis [54,67]. Thrombosis can result in ischemia to organs or if the thrombus breaks away or embolizes from the endothelium, it can be caught in the pulmonary system, causing a dangerous pathological disease known as pulmonary embolism. In order to treat thrombosis, anticoagulant drugs or antiplatelet therapies are administered. However, a major limitation of current therapies is an increased risk for bleeding, as current therapies target cellular pathways and proteases that are essential to hemostasis. Thus, it is important to better understand the mechanisms underlying thrombosis and hemostasis as well as where these mechanisms diverge, in order

to identify safer antithrombotic targets and ultimately reduce patient complications. In infectious disease states like sepsis, the occurrence of microvascular thrombosis drives tissue hypoxia and ischemia before coagulation factors and platelets are consumed, exacerbating severe bleeding and DIC, complicating treatment strategies that can only inhibit thrombosis but also increase bleeding.

1.4.2 *Intrinsic coagulation*

In contrast to the extrinsic coagulation cascade, partial or full deficiencies of the intrinsic coagulation cascade result in mild bleeding, such as deficiency of FXI (hemophilia C), or no discernible effects, as when patients are deficient in FXII [68,69]. The intrinsic or contact activation pathway of coagulation is initiated when FXII associates with a negatively charged surface and undergoes a conformational change to autoactivate into FXIIa. FXIIa then activates FXI into FXIa, which then cleaves and activates FIXa, feeding into the common cascade (Figure 1.3). The negatively charged surfaces capable of activating FXII include platelet- or bacterial-derived PolyP, glass, or kaolin, like that used in the activated partial thromboplastin time (aPTT) test, a clinical assay used to asses contact activation. FXII was originally discovered as Hageman's factor in 1955 by the hematologist Dr. Oscar Ratnoff, after Hageman, a train conductor, fell and was administered routine blood tests. Hageman presented with normal test results, except for a prolonged aPTT time that could be rescued with the addition of normal plasma, suggesting a factor existed that initiated coagulation on foreign surfaces [70]. The biological significance of the intrinsic cascade is proposed to be important in thrombosis, as FXII inhibition reduces incidence of thrombosis and stroke in mouse models and non-human primate models [71–73]. Furthermore, patients with severe FXI deficiency are considered protected from deep vein thrombosis [69]. Extracellular histones, DNA and RNA have been shown to also contribute to FXII activation and fibrin formation, suggesting that NETs formation may potentiate thrombus formation in an FXII-dependent manner [54,74–76]. A subsequent mechanistic study found that histones and DNA in an *in vitro* purified system

promoted activation of coagulation; however, intact NETs failed to activate coagulation, likely due to the complex histone-DNA interactions that are the main components of NETs [77].

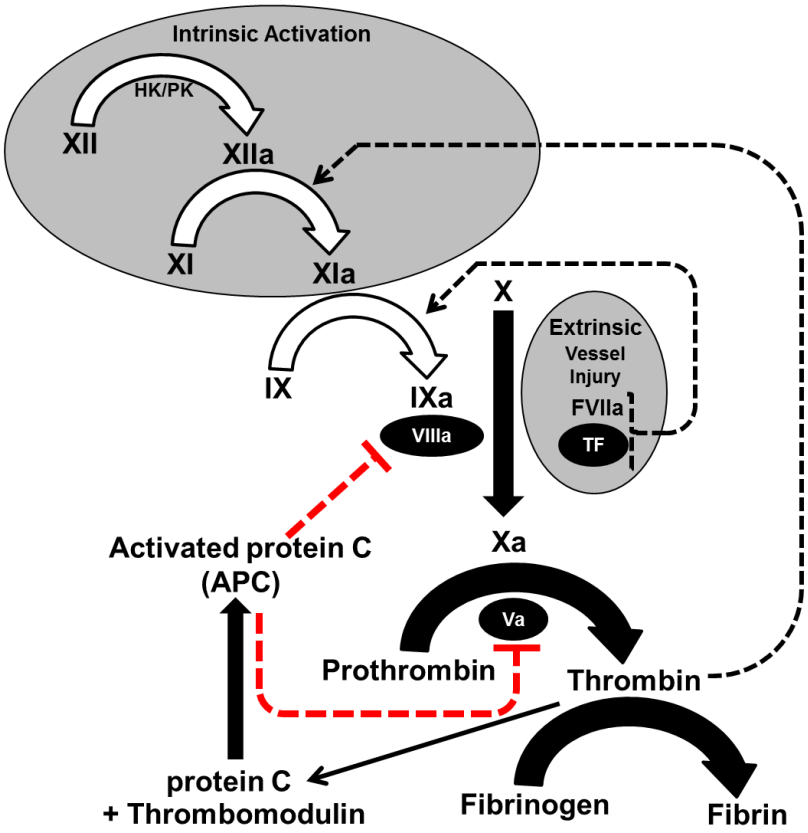

Figure 1.3. Coagulation Cascade.

Coagulation factors circulate in the bloodstream in their zymogen forms. In response to activation of the intrinsic or extrinsic pathways, coagulation factors cleave the coagulation factors immediately downstream, ultimately generating thrombin and potentiating fibrin formation as well as initiating negative feedback of thrombin generation via APC activation.

1.5 Crosstalk between the anticoagulant protein C pathway and inflammation

1.5.1 *Overview of protein C structure and activation*

Protein C is a serine protease that circulates in the bloodstream in its inactive zymogen form, similar to other vitamin K-dependent coagulation factors. The systemic concentration of protein C is ~65 nM, and

protein C is synthesized and secreted from the liver [78–80]. Protein C is 56.2 kDa and contains a Gla domain, two epidermal growth factor (EGF) domains, and a disulfide bridge linking the light chain and heavy chain of the catalytic domain. Following post-translational modification and processing, protein C is 419 amino acids long. After activation of coagulation, thrombin generation activates protein C by cleavage at Arg169, generating activated protein C (APC). APC generation increases 1000-fold in an Ca^{2+}- and thrombomodulin- (TM) dependent manner [81–84]. The presence of endothelial protein C receptor (EPCR) increases the rate of APC generation further by 10-fold [85]. Similar to other chymotrypsin-like serine proteases, the active site contains the triad His-Asp-Ser. The Gla domain is required for binding cell membrane phospholipids, EPCR, and Ca^{2+}. TM binds the basic residues in the anion–binding exosite 1, which includes the EGF-1 and Gla domains of protein C, to facilitate activation and cleavage of protein C by thrombin [82,85–88]. In whole blood, APC has a half-life of 18 minutes before being inactivated by serine protease inhibitors (serpins), heparin-dependent inhibitors or divalent metal ion-dependent inhibitors [89,90]. Congenital heterozygous autosomal deficiency of protein C results in an increased risk for venous thrombosis and is considered rare, as only 2-5% of patients presenting with venous thromboembolism (VTE) are determined to be deficient [91,92]. Homozygous deficiency of protein C results in neonatal *purpura fulminans*, which is life-threatening without protein C replacement therapy. There are two types of congenital protein C deficiency; type I results in reduced levels of protein C, and type II results in protein C that is unable to be properly activated. In severe sepsis, progression to DIC can result in acquired protein C deficiency, which is associated with worse outcomes [13]. APC has well characterized anticoagulant functions and a steadily emerging role in promoting cytoprotective signaling on cells, and will be described in more detail below.

1.5.2 *Anticoagulant functions*

The primary function of APC following disassociation from EPCR is to act as a key negative feedback regulator to dampen thrombin generation by inactivating coagulation factors immediately upstream of thrombin. APC binds Protein S in a 1:1 stoichiometry, and protein S acts as a cofactor to increase the rate of APC activity in a Ca^{2+}- and phospholipid- dependent manner. The APC anion-binding exosite is required for recognition of FVa and FVIIIa to promote cleavage. FVa is enzymatically inactivated by APC in a sequential manner, first via cleavage at Arg506 and then at Arg306. Cleavage at Arg306 is cell membrane- dependent, as Protein S increases the rate of FVa inactivation by APC by 20-fold [93–95]. In the patient population, Arg506 can be mutated reducing FVa inactivation by APC; Arg506Gln results in the disorder FV-Leiden, Arg506Thr results in FV-Cambridge, and Arg506Gly results in FV-Hong Kong. FV-Leiden patients tend to be at higher risk for thrombotic events throughout their life, and interestingly, especially in women taking birth control [96–98]. Inactivation of FVIIIa by APC can occur at two residues; proteolysis at Arg336 or Arg562 results in the loss of FVIII activity [99,100]. This dampens thrombin generation as FVIII and FV are important upstream cofactors of the extrinsic cascade (Figure 1.3).

1.5.3 *Anti-inflammatory functions*

The other primary function of APC appears to be initiating cell signaling pathways that culminate in cytoprotection and a reduction in pro-apoptotic signaling pathways. A role for APC-mediated cytoprotective signaling has been described for endothelial cells, neurons, monocytes, podocytes, neutrophils and platelets. Initially, it was demonstrated that APC remains bound to EPCR on the endothelium, overall reducing APC's anticoagulant activity. Gene expression profiling of endothelial cells in response to APC treatment, combined with stimulation of tumor necrosis factor (TNF) to activate

the endothelium, demonstrated that APC could suppress pro-apoptotic gene expression and promote expression of genes associated with cell survival. Specifically, it was observed that there were increases in gene expression for the endothelial analog B-cell lymphoma 2 (Bcl-2), an apoptosis regulator and the proliferating cell nuclear antigen (PCNA) that promotes cell survival. Moreover, APC was found to suppress NFκB expression and cytokine signaling initiated by TNF treatment, including induction of cell surface adhesion molecules like VCAM, ICAM and E-selectin, which promote platelet and neutrophil rolling and subsequent activation. Lastly, induction of apoptosis was inhibited in the presence of APC and the activation of these pathways [101].

Following this discovery, the receptors driving these signaling pathways were identified. APC was shown to require binding to EPCR, followed by the activation of PAR1-associated signaling, preventing p53-mediated apoptosis [102,103]. Moreover, the APC/EPCR/PAR1 complex was found to colocalize in lipid rafts and clathrin-associated caveolae to drive Rac1 activation, desensitization and endocytosis [104,105]. Whilst these results were generating much interest in the field, PAR1 was considered a thrombin-cleaved receptor that activated pro-apoptotic signaling; thus, resolving how one receptor initiated unique signaling and cell fate pathways in a ligand-dependent manner remained unresolved. In 2012, it was discovered that APC cleaved PAR1 at Arg46 whilst thrombin cleaved PAR1 at Arg41, resulting in unique N-termini generated from each serine protease that could then promote distinct signaling, a concept proposed as "biased agonism" [65]. Similar to PAR1, PAR3 was subsequently demonstrated to substantiate the biased agonism hypothesis, in which thrombin cleaved the N-terminus of PAR3 at Lys38 whereas APC cleaved PAR3 at Arg41 [58]. Moreover, there is some evidence suggesting PAR1 and PAR3 are capable of heterodimerizing and undergoing transactivation [106]. Whereas activation of PARs by thrombin results in G protein-coupled signaling, Rho activation and PLC activation, PAR cleavage and activation by APC results in signaling initiated by β-arrestin-2, PI(3)K/Akt and Rac1 (Figure 1.4) [64,107,108] .

In addition to the PAR-related signaling mechanisms, APC was found to signal via apolipoprotein E receptor 2 (ApoER2) in an adaptor protein disable-1 (Dab1) -dependent manner in the monocytic U937 cell line [109]. In platelets, APC bound ApoER2 in a GPIb-dependent manner [110]. APC signaling via transactivation between EPCR and the lipid sphingosine 1-phosphate receptor-1 resulted in Akt activation to decrease endothelial cell permeability [111]. Whilst the cell protective effects of APC are increasingly appreciated, there is still a need to define the signaling pathways initiated in response to APC treatment by cells expressing receptors that recognize APC.

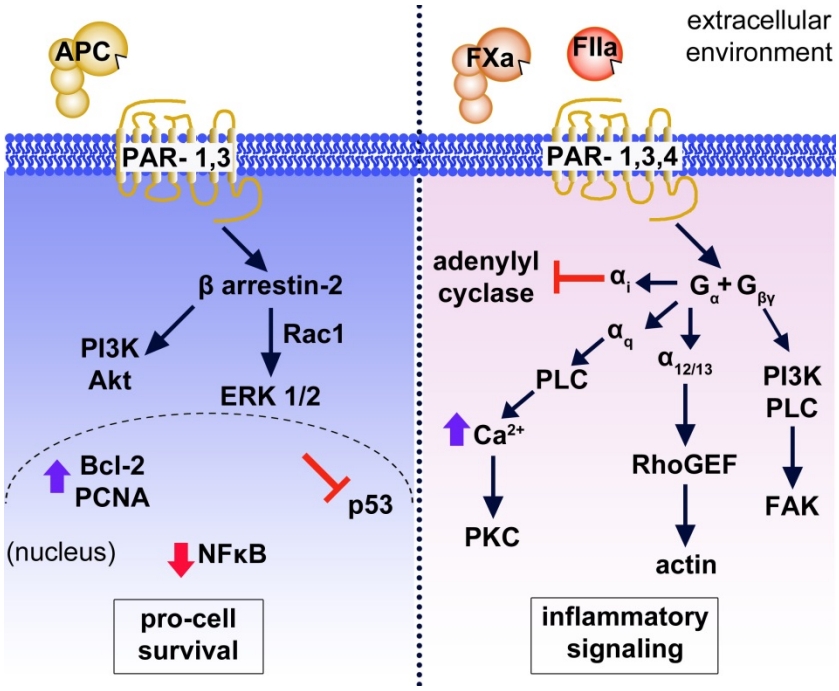

Figure 1.4. PAR biased agonism.

Model of PAR activation in response to different proteases. APC cleavage of the N-terminus of PARs results in regulation of gene expression pathways that promotes cell survival. In contrast, the response to thrombin (FIIa) or FXa cleavage of the N-terminus of a PAR results in intracellular signaling to promote cell activation, inflammation and apoptosis.

1.6 Immunothrombosis in relation to disease states

In the past decade, there has been an increase in the acceptance within the field that components of the innate immune system, specifically neutrophils, are not merely bystanders in diseases associated with thrombosis but are capable of potentiating dysregulation of hemostatic events. In 2013 the phrase ”immunothrombosis” was coined to emphasize that innate immunity in select diseases may drive aberrant activation of platelets and coagulation factors, resulting in thrombosis [112]. The discovery of NETosis and subsequent studies suggesting NETs can form in the vasculature and amplify thrombus formation raise the hypothesis that disease states associated with increased thrombotic risk may be driven in part through NETosis. However, a homeostatic role for NETosis independent of grossly pathologic states, remains in large part undefined. One study demonstrating a correlation between exercise and elevated markers of NETosis suggests that NETosis may be tightly regulated under homeostatic conditions in response to select stressors [113]. However, due to limitations of current experimental systems and quantitative methodologies, most studies have initially focused on the contribution of NETosis to dysregulated pathologic disease states rather than homeostasis.

The current state of the research at the intersection between innate immunity and thrombosis has focused on two main approaches to test the immunothrombosis hypothesis. First, by determining the specific infectious and noninfectious disease states where NETosis might occur, the pathophysiologic relevance can be interrogated [114,115]. Currently, a positive correlation between markers for NETosis and known risk for thrombosis is observed in severe sepsis, stroke, cancer and venous thrombosis, where *in vitro* systems, animal models and clinic data have been used [39,40,54,114,116–118]. The second approach primarily focuses on defining the components of coagulation and platelet activation that drive NETosis and the immediate downstream activation of platelet or proteases in the presence of NETs [39,54,74,119,120]. As the interactions that drive immunothrombosis are uncovered and the

(patho)physiologic relevance tested, the logical next questions will address the mechanisms that negatively regulate or even inhibit immunothrombotic processes. Namely, these future studies may work to determine whether pathways exist whereby the immune response via NETosis is blunted, the crosstalk between coagulation and immunity is depressed, or if there exist positive and negative feedback reactions between coagulation to the immune system.

1.7 Thesis Overview

In this thesis, the interactions between the innate immune system, specifically neutrophils, and the components of coagulation and thrombus formation are characterized. As neutrophils and NETs are thought to play dual roles in promoting pathogen clearance but also potentiating thrombosis, there is a need to identify the complex interactions between neutrophil function and coagulation. Although studies have described the interactions between NETs and coagulation demonstrating NETs can serve as a platform for platelet adhesion and coagulation factor localization, there remained a need to systematically define the coagulation factors that bind to neutrophils, NETs, and the specific components required for this association. This investigation is described in Chapter 3. The data demonstrates that select coagulation factors bind NETs in both a DNA- and phosphatidylserine-dependent manner. These results demonstrate that NETs may serve as a platform for the assembly and eventual polymerization of fibrinogen, suggesting a mechanism by which NETs can potentiate thrombosis.

While the results in Chapter 3 demonstrate a possible mechanism by which NETs drive thrombus formation, Chapter 4 explores whether mechanisms exist to reduce or inhibit formation of NETs. APC is a multi-functional serine protease that serves as an anticoagulant, dampening thrombin generation as well as binding cell receptors to induce intracellular signaling that ultimately promotes cell survival and induction of anti-inflammatory pathways. First, our results indicate that APC can inhibit NETosis in a

concentration-dependent manner. Moreover, APC's inhibition of NETosis appears to be receptor-mediated, dependent specifically on EPCR, PAR3, and Mac-1. Additionally, the key domains and residues of APC required to inhibit NETosis were investigated through rational mutation. APC's inhibition of NETosis was retained in the presence of two alternative agonists. Lastly, as a proof-of-concept experiment, it was found that using a lethal *in vivo* baboon model of bacterial sepsis, APC infusion reduced plasma levels of MPO, a marker of neutrophil activation. This demonstrates a novel mechanism by which APC exerts anti-inflammatory activity and indirect anti-thrombotic activity. Finally, in Chapter 5, conclusions from these studies and future directions are considered.

The studies presented provide new mechanistic insight into the dynamic interactions between neutrophil cell function, platelets and coagulation factors and provide additional rationale to develop new treatments for sepsis. Specifically, these studies show that NETs only bind select coagulation factors, providing insight into how NETs can modulate thrombosis in infectious disease settings. The findings presented describe for the first time a new anti-inflammatory function of APC in inhibiting one form of neutrophil cell death, demonstrating a significant effect of APC on the immune response. Lastly, as sepsis is an acquired protein C-deficient state, this thesis adds to the body of knowledge by suggesting that induction of NETosis in the absence of APC regulation may play a critical role in contributing to poor patient outcomes that is dependent on the causal pathogen.

Chapter 2. Materials & Methods

2.1 Ethical Considerations

Studies in this thesis were conducted using human blood. All human donors were healthy and gave full informed consent in accordance with the Declaration of Helsinki. Experiments using human donors were performed with approval of the Oregon Health & Science University Institutional Review Board.

2.2 Common Reagents

Polymorphprep was purchased from Axis-Shield (Oslo, Norway). Hank's Balanced Salt Solution (HBSS) was from Corning (Manassas, VA, USA). The cell-permeable DNA dye Hoechst 33342 was from Invitrogen (Grand Island, NY). Alexa Fluor conjugated anti-mouse and anti-rabbit antibodies were from Abcam (Cambridge, MA). All other reagents were from Sigma Aldrich (St. Louis, MO, USA) unless otherwise specified.

2.3 Blood cell collection and neutrophil isolation

2.3.1 *Blood collection*

Human venous blood was collected by venipuncture from healthy adult male and female volunteers directly into syringes containing the anticoagulant, citratephosphate-dextrose (1:7 vol/vol).

2.3.2 *Neutrophil purification*

Blood was layered over an equal volume of Polymorphprep and centrifuged at 500 *g* for 45 min at 18°C. The lower layer containing neutrophils was collected and washed with HBSS by centrifugation at 400 *g* for 10 min. To remove contaminating red blood cells, the pellet was resuspended in sterile H_2O for 30 s, followed by the immediate addition of 10× PIPES buffer (250 mM PIPES, 1.1 mM NaCl, 50 mM KCl, pH 7.4). After centrifugation at 400 *g* for 10 min, the pellet was resuspended in HBSS containing 2 mM $CaCl_2$, 2 mM $MgCl_2$ and 1% wt/vol Bovine Serum Albumin (BSA).

2.4 Immunofluorescence microscopy

2.4.1 *General protocol*

Acid-washed glass coverslips were coated with 20 µg/mL fibronectin and then blocked with denatured BSA (5 mg/mL). Initially, purified human neutrophils (2×10^6/mL) were plated on the coverslips for 30 min at 37°C. Cells were subsequently stimulated with HBSS or phorbol 12-myristate 13-acetate (PMA; 10 nM) for 3 hr at 37°C. Subsequently, samples were fixed with 4% paraformaldehyde (PFA) followed by incubation with blocking buffer. Cells and proteins of interest were incubated with appropriate primary antibodies in blocking buffer at 4°C overnight. Following washing coverslips with PBS, samples were incubated with secondary goat anti-rabbit or –mouse IgG antibody conjugated to either Alexa Fluor 488/546 (1:500) and Hoechst 33342 (1:1000) in blocking buffer were added and incubated for 2 hr in the dark followed by washing again with PBS. Coverslips were mounted onto glass slides using Fluoromount G and visualized with a Zeiss Axiovert fluorescence microscope (Axio Imager; Carl Zeiss, Gittingen, Germany) equipped with a ×40/1.3 numerical aperture (NA) oil immersion objective and an air-coupled lens providing Köhler illumination at an NA of 0.17. All images were recorded with a charge-coupled

device camera (AxioCam MRc5 12-bit camera; Carl Zeiss) under software control by Slidebook 5.5 (Intelligent Imaging Innovations, Denver, CO).

2.4.2 *Image analysis*

The fluorescent intensities of each image were adjusted based on signals detected in leukocyte or platelet samples in the absence of primary antibodies. Quantification of fluorescent images was performed using a custom algorithm in MATLAB (The Mathworks, Inc., Natick, MA). This algorithm first establishes global thresholding parameters based on a training set of fluorescence image data to then systematically quantify intensity profiles across treatment conditions. The area of the total field of view is calculated to be 12510 μm^2. Quantitative comparison of treatment conditions was achieved by the normalization of fluorescence intensities per image to determine the area of DNA or respective fluorescent channels. The code can be accessed on Github (https://github.com/lauradhealy/NETAnalysis).

Chapter 3. Regulation of thrombus formation through coagulation factor localization to NETs

Laura D. Healy, Cristina Puy, Asako Itakura, Tiffany Chu, David K. Robinson, Alan Bylund, Kevin G. Phillips, Elizabeth E. Gardiner, Owen J.T. McCarty

3.1 Abstract

Neutrophils, the most populous innate immune cell type, are the first responders to sites of infection and inflammation. Neutrophils can release their DNA to form extracellular traps (NETs), webs of DNA and granular proteases that contribute to pathogen clearance and promote thrombus formation. At present, the study of NETs is in part limited to the qualitative analysis of fluorescence microscopy-based images, thus quantification of the interactions between NETs and coagulation factors remains ill-defined. The aim was to develop a quantitative method to measure the spatial distribution of DNA and colocalization of coagulation factor binding to neutrophils and NETs utilizing fluorescence-based microscopy. Human neutrophils were purified from peripheral blood, bound to fibronectin and treated with the PKC-activator phorbol myristate acetate (PMA) to induce neutrophil activation and NETs formation. Samples were incubated with purified coagulation factors or plasma before staining with a DNA-binding dye and coagulation factor-specific antibodies. The spatial distribution of DNA and coagulation factors was imaged via fluorescence microscopy and quantified via a custom-built MATLAB-based image analysis algorithm. The algorithm first established global thresholding parameters on a training set of fluorescence image data and then systematically quantified intensity profiles across treatment conditions. Quantitative comparison of treatment conditions was enabled through the normalization of fluorescent intensities using the number of cells per image to determine the percent and area of DNA and coagulation factor binding per cell. Upon stimulation with PMA, NETs formation resulted in an increase in the area of DNA per cell. The coagulation factor fibrinogen bound to both the neutrophil cell body as well as NETs, while

prothrombin, FX and FVIIa binding was restricted to the neutrophil cell body. The Gla domain of FX was required to mediate FX-neutrophil binding. Activated protein C (APC), but not Gla-less APC, bound to neutrophil cell bodies and NETs in a punctate manner. Neither FXIIa nor FXIa were found to bind either neutrophil cell bodies or NETs. Fibrinogen binding was dependent on extracellular DNA, while FX and APC required phosphatidylserine exposure for binding to activated neutrophils. We have developed a quantitative measurement platform to define the spatial localization of fluorescently-labeled coagulation factor binding to neutrophils and extracellular DNA during NETosis.

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3.2 Introduction

Studies in this chapter were designed to develop a quantitative method to measure the spatial distribution of select coagulation factors binding to neutrophils and NETs. Our immunofluorescence experiments demonstrate select coagulation factors bound to NETs in a Gla-domain dependent manner. Moreover, the binding of coagulation factors occurred in a DNA- and PS- dependent manner, suggesting both the chromatin of the NETs and the remaining cell membrane may act as a platform for assembly of coagulation factors.

3.3 Background

Polymorphonuclear leukocytes, or neutrophils, are the most abundant circulating white blood cell type in humans, and play an indispensable role in innate immune host defense. Conversely, in the setting of

inflammatory disease, excessive neutrophil activation has been shown to contribute to thrombotic complications. At sites of inflammation, activated neutrophils release intracellular granule proteins and chromatin that together form neutrophil extracellular traps (NETs) in an alternative form of cell death [30,121]. These decondensed chromatin fibers are decorated with antimicrobial proteins and proteases including elastase, cathepsin G and myeloperoxidase (MPO), forming a physical trap to sequester and clear pathogens, considered an additional host defense mechanism [18,122]. In addition to these microbial clearance functions, NETs have been shown to promote thrombin generation, fibrin formation and platelet activation in both *in vitro* and *in vivo* models to promote thrombus formation [57,75,123]. Previous studies have indicated that fibrinogen and coagulation factors of the intrinsic pathway associate with the DNA, histones or proteases comprising NETs [54,120,124]. However, it is unclear whether NETs are involved in an active or passive manner in promoting thrombus formation. Specifically, it is unclear whether NETs directly promote activation of coagulation factors, such as factor XII, or act as a scaffold for the assembly and activation of coagulation factors. In contrast, it is possible that NETs serve to bind and sequester active serine proteases, analogous to the binding and inactivation of thrombin on fibrin. These questions are difficult to address experimentally as they require systems that permit quantitative assessment of the binding, assembly and activation of coagulation factors on NETs. Here, we report on the development of a custom-built MATLAB-based image analysis algorithm that coupled with fluorescence microscopy-based images can provide quantitative analysis and spatial localization of coagulation factors binding to neutrophils as they undergo NETosis.

3.4 Materials and Methods

3.4.1 *Reagents*

Activated factor XII(a), factor XIa, factor X, FX-GD, factor VIIa, protein C, APC-GD, prothrombin, fibrinogen, and anti-human mouse antibodies to protein C/APC (AHPC-5071), FVII (AHFVII-5031), FX

(AHX-5050), and prothrombin (AHP-5013) were purchased from Hematologic Technologies Inc. (Essex Junction, VT, USA). Activated protein C (APC) was a gift from Dr. András Gruber (Oregon Health & Science University, Portland, OR, USA). Polymorphprep was from Axis-Shield PoC AS (Oslo, Norway). Rabbit polyclonal antibody to fibrinogen was from MP Biomedicals (Santa Ana, CA). Mouse monoclonal antibody to factor XII heavy chain (sc-59517) was from Santa Cruz Biotech (Dallas, TX). The cell-permeable DNA dye Hoechst 33342 was from Invitrogen (Grand Island, NY). Alexa Fluor conjugated anti-mouse antibodies and rabbit polyclonal anti-histone H3 (ab5103) were from Abcam (Cambridge, MA). The antibody 1A6, against the A3 domain of human FXI was generated as described.[125] All other reagents were purchased from Sigma-Aldrich (St. Louis, MO).

3.4.2 *Preparation of human neutrophils*

Human neutrophils were purified as previously described [126]. Briefly, human blood was drawn in accordance with an Oregon Health & Science University Institutional Review Board-approved protocol from healthy donors by venipuncture into citrate-phosphate-dextrose (1:7 vol/vol). Blood was layered over an equal volume of Polymorphprep and centrifuged at 500 *g* for 45 min at 18°C. The lower layer containing neutrophils was subsequently collected and washed with HBSS by centrifugation at 400 *g* for 10 min. To remove red blood cells from the sample, the pellet was resuspended in sterile H_2O for 30 s, followed by immediate addition of 10× PIPES buffer (250 mM PIPES, 1.1 mM $CaCl_2$, and 50 mM KCl, pH 7.4). After centrifugation at 400 *g* for 10 min, the pellet was resuspended in buffer (HBSS containing 2 mM $CaCl_2$, 2 mM $MgCl_2$ and 1% wt/vol BSA).

3.4.3 *Immunofluorescence microscopy*

Purified human neutrophils (2×10^6/mL) were stimulated with HBSS or PMA (10 nM) for 3 h at 37°C on fibronectin-coated glass coverslips. For initial colocalization experiments, cell samples were washed and treated with vehicle (HBSS buffer), fibrinogen (2.6 mg/mL), prothrombin (FII, 100 µg/mL), FX (10 µg/mL), FX-GD (10 µg/mL), protein C (300 nM), APC (300 nM), APC-GD (300 nM), FVIIa (300nM), FXIa (20 µg/mL), FXIIa (20 µg/mL) in the presence of HK (20 µg/mL) and $ZnCl_2$ (25 µM) was then incubated for 15 min with the cell samples at 37°C. For select experiments, cell samples were washed and treated with vehicle (HBSS buffer), DNase I (10,000U/mL), RGDS (20 µM), Annexin V (10 µg/mL) for 10 min at 37°C. In these experiments, cell samples were then washed and treated with vehicle (HBSS buffer containing BSA), fibrinogen (2.6 mg/mL), FX (10 µg/mL), and APC (300 nM) for 15 min with the cell samples at 37°C. In select experiments cells were otherwise incubated with platelet-poor plasma or vehicle (HEPES containing 2mM $CaCl_2$, 2mM $MgCl_2$ and 0.1% BSA) (1:1) for 15 min at 37°C.

Subsequently, samples were washed with PBS and fixed with 4% PFA followed by incubation with blocking buffer (PBS containing 10% FBS and 5 mg/mL Fraction V BSA). Cells and coagulation factors were stained with anti-FXII (50 µg/mL), 1A6 (anti-FXI, 1:50), anti-PC/APC (100 µg/mL), anti-FVII (100 µg/mL), anti-fibrinogen (1:100), anti-FX (50 µg/mL) or anti-prothrombin (50 µg/mL) in blocking buffer at 4°C overnight. Secondary goat anti-rabbit IgG antibody conjugated with AlexaFluor 488 (1:500) and goat anti-mouse IgG antibody conjugated with AlexaFluor 546 (1:500) and Hoescht 33342 (10 µg/mL) in blocking buffer were added and incubated for 2 h in the dark. Coverslips were mounted onto glass slides and visualized with a Zeiss Axiovert fluorescence microscope.

3.4.4 *Preparation of endothelial cells*

Human umbilical endothelial cells (HUVECs) were grown to confluency on glass coverslips in 24-well plates, cells were then washed once with buffer and treated with vehicle (HBSS buffer), fibrinogen (2.6 mg/mL), FX (10 μg/mL), APC (300 nM), FXIa (20 μg/mL), FXIIa (20 μg/mL) in the presence of $ZnCl_2$ (25 μM) for 15 min at 37°C. Cells were then washed again with HBSS buffer and fixed with 4% paraformaldehyde (PFA), then followed previously described antibody labeling as described in Section 3.2.3.

3.4.5 *Image Analysis*

For presentation of data, the fluorescent intensities of each image were adjusted based on signals detected in neutrophil samples in the absence of primary antibodies. Quantification of fluorescent images was performed using a custom algorithm in MATLAB (The Mathworks, Inc., Natick, MA). This algorithm first establishes global thresholding parameters on training set fluorescence image data to then systematically quantify intensity profiles across treatment conditions. Quantitative comparison of treatment conditions was achieved by the normalization of fluorescence intensities to the number of cells per image to determine the percent and area of DNA and coagulation factor binding per cell. Two-way analysis of variance with Tukey's *post hoc* correction or one-way analysis of variance with Welch's *post hoc* correction was used to assess statistical significance among parameters across multiple normally distributed cell parameters. P values < 0.05 were considered significant.

3.5 Results

3.5.1 *Quantification of coagulation factor binding to neutrophils during NETosis in a purified system*

Activation of coagulation factors involved in thrombus formation, including fibrinogen and members of the intrinsic pathway of coagulation, have been reported to be associated with NETs formation in the context of immunothrombosis [120,124]. The overall goal was to design a platform to quantify the binding and spatial localization of coagulation factor-NETs interactions. Neutrophils were treated with PMA to induce NETs, followed by incubation with vehicle (HBSS buffer) or the following coagulation factors: fibrinogen (2.6 mg/mL), prothrombin (FII, 100 µg/mL), FX (10 µg/mL), Gla-less FX (FX-GD; 10 µg/mL), protein C (300 nM), APC (300 nM), Gla-less APC (APC-GD; 300 nM), FVIIa (300nM), and FXIa (20 µg/mL) or FXIIa (20 µg/mL) in the presence of HK (20 µg/mL) and $ZnCl_2$ (25 µM) or $ZnCl_2$ alone. Samples were fixed and stained for DNA, while coagulation factor binding was detected following staining with monoclonal anti-human mouse antibodies followed by secondary labeling with fluorescent anti-mouse antibodies. Coverslips were then mounted onto microscope slides, and at least 4 images were taken per condition. After exportation of images, a MATLAB code was utilized containing an algorithm that uploaded each image, converted each image into greyscale and created a jet scale image of each signal undergoing analysis. The images in greyscale and jet were then adjusted for threshold levels of signal and the signal was quantified for both the sum area and the area per cell, where the area of the total field of view is calculated to be 12510 μm^2. The pixel overlap between the signal resulting from fluorescently labeled coagulation factors and DNA was subsequently computed (Pearson's correlation coefficient, R_r) where the values from -1 to +1 described the overlap between two colored patterns and was independent of the pixel intensity values.[127]

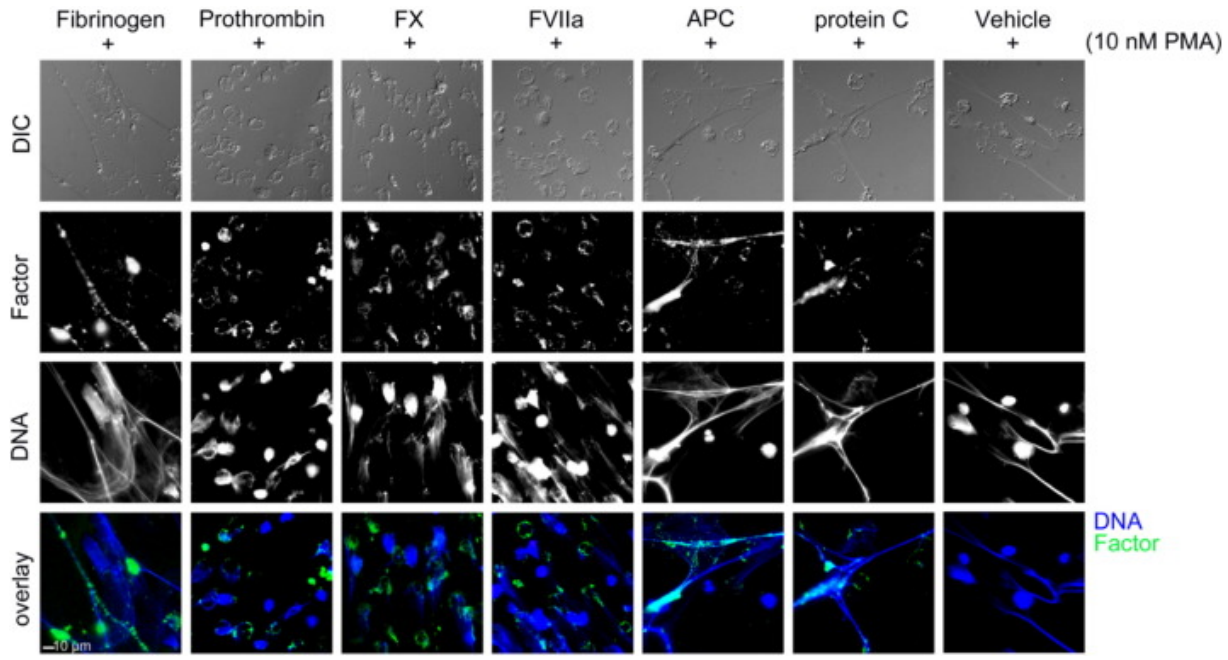

Figure 3.1. NETs promote binding of select purified coagulation factors.

Acid-washed glass coverslips were coated with 20 µg/mL fibronectin and then blocked with denatured BSA (5 mg/mL). Purified human neutrophils (2×10^6/mL) were plated on the coverslips, and were treated with HBSS or PMA (10 nM) for 3 hours at 37°C. Cell samples were washed and treated with vehicle (HBSS buffer), fibrinogen (2.6 mg/mL), prothrombin (FII, 100 µg/mL), FX (10 µg/mL), FVIIa (300nM), APC (300 nM), protein C (300 nM) was then incubated for 15 minutes with the cell samples at 37°C. Samples were then fixed with 4% PFA. (A) Samples were incubated overnight with primary antibodies. Samples were then incubated with Hoechst 33342 (1:1000) and secondary antibodies Alexa Fluor 488 goat anti-rabbit and Alexa Fluor 546 goat anti-mouse (Invitrogen, 1:500). Images were normalized to secondary antibody alone images and quantified in a custom MATLAB program to quantify each pixel positive signal, shown above are representative images of coagulation factors with positive cell staining.

We first validated that this platform was able to detect and quantify NETs formation following stimulation of neutrophils with the PKC activator PMA. As shown in Figure 3.1, PMA induced robust NETs formation, as observed by the presence of extracellular DNA. Quantification of the surface area coverage of DNA per field of view demonstrated that PMA stimulation promoted an increase in DNA surface area coverage from 297.5 ± 47.1 μm^2 for resting neutrophils under basal conditions to 1767.4 ± 201.3 μm^2 for neutrophils that had undergone NETosis, as shown in Figure 3.2A. When quantified on a

per cell basis, the results show that PMA stimulation promoted an increase in DNA staining from 13.4 ± 0.9 μm^2 per cell to 87.1 ± 10.1 μm^2 per cell (Figure 3.2B). These data are in accord with previous qualitative observations that neutrophils expel their DNA to form extracellular NETs following PMA stimulation, in contrast to DNA fragmentation and condensation observed during apoptosis [121].

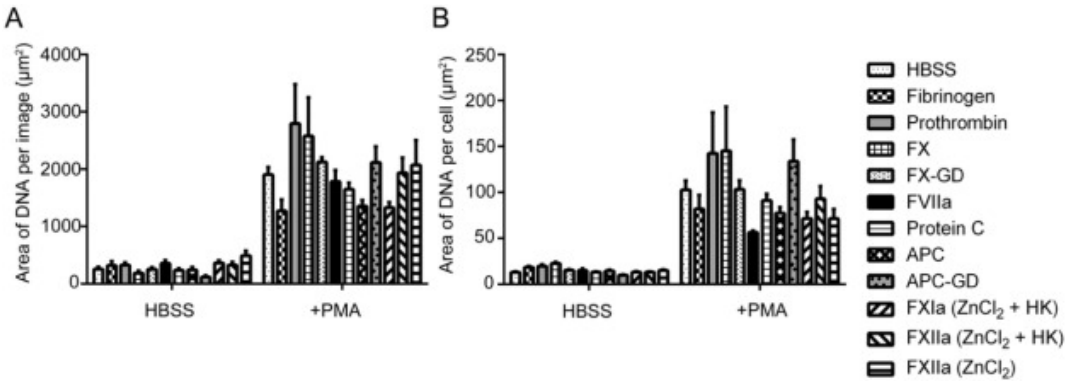

Figure 3.2. DNA quantified as area per image and per cell.

Fluorescent images were normalized to secondary antibody alone images and quantified in a custom MATLAB program to quantify each pixel positive signal as (A) the area of DNA per image and (B) area DNA per cell. Data are mean±SEM n=3.

We next utilized the platform to quantify the binding and spatial localization of coagulation factor-NETs interactions. Resting or PMA-stimulated neutrophils were incubated with the coagulation factor fibrinogen in a purified system. The results show that purified fibrinogen bound to resting adherent neutrophils, as evidenced by an area of 182.57 ± 41.73 μm^2 for fibrinogen labeling per field of view. A dramatic increase in fibrinogen binding was observed following PMA-induced NETosis, as evidenced by a greater than 3-fold increase in fibrinogen staining, when calculated as area coverage (Figure 3.3A). A similar trend was observed when the signal for fibrinogen binding was normalized per cell, as shown in Figure 3.3B. Moreover, the correlation coefficient between fibrinogen binding and DNA staining after

NETs formation increased to 0.82, reflecting the observation that fibrinogen colocalized with NETs as seen in Figure 3.1.

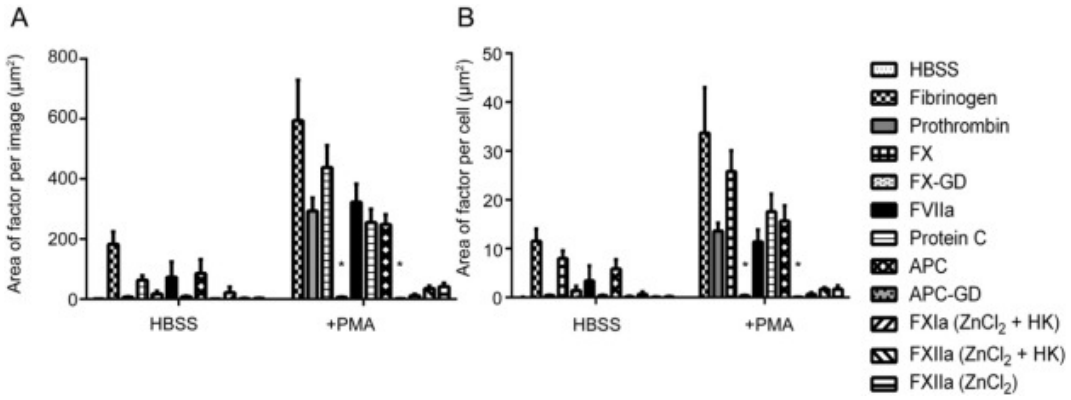

Figure 3.3. Purified coagulation factor proteins quantified as area per image and per cell.

Acid-washed glass coverslips were coated with 20 μg/mL fibronectin and then blocked with denatured BSA (5 mg/mL). Purified human neutrophils (2×10^6/mL) were plated on the coverslips, and were treated with HBSS or PMA (10 nM) for 3 hours at 37°C. Cell samples were washed and treated with vehicle (HBSS buffer), fibrinogen (2.6 mg/mL), prothrombin (FII, 100 μg/mL), FX (10 μg/mL), FX-GD (10 μg/mL), protein C (300 nM), APC (300 nM), APC-GD (300 nM), FVIIa (300nM), FXIa (20 μg/mL), FXIIa (20 μg/mL) in the presence of HK (20 μg/mL) and $ZnCl_2$ (25 μM) was then incubated for 15 minutes with the cell samples at 37°C. Samples were then fixed with 4% PFA. (A) Samples were incubated overnight with primary antibodies. Samples were then incubated with Hoechst 33342 (1:1000) and secondary antibodies Alexa Fluor 488 goat anti-rabbit and Alexa Fluor 546 goat anti-mouse (Invitrogen, 1:500). Images were normalized to secondary antibody alone images and quantified in a custom MATLAB program to quantify each pixel positive signal as (A) the area of factor per image and (B) area of signal per cell. *, $p < 0.05$ vs. FX or APC + PMA. Data are mean±SEM n=3.

We next evaluated whether FX and prothrombin bound to neutrophils following NETosis, as FX and prothrombin have been shown to assemble on surfaces of blood cells, such as platelets and endothelial cells as part of the prothrombinase complex [128–130]. The results show that while FX binding to neutrophils was minimal under resting conditions, FX bound to neutrophil cell bodies following PMA stimulation (Figure 3.1). Quantification of FX labeling demonstrated that the mean surface area of FX

binding to neutrophils increased from 62.9 ± 15.5 μm^2 for resting cells to 438.4 ± 72.9 μm^2 after PMA stimulation (Figure 3.3A). On a per cell basis, FX labeling was shown to increase from 7.96 ± 1.57 μm^2 on resting cells to 25.8 ± 4.3 μm^2 after PMA stimulation as seen in Figure 3.3B. The correlation coefficient between FX and DNA from NETs formation was 0.13, indicative of the fact that FX bound in a punctate manner to the neutrophil cell body, rather than to the DNA-rich NETs. Based on the colocalization pattern, we hypothesized the γ-carboxyglutamic acid (Gla)-rich domain of FX could promote the binding to neutrophils underwent NETosis. To test this, a Gla domain-less derivative of FX was utilized to determine whether the Gla-domain was required for FX-NETs binding. After quantification, the results demonstrated that FX-GD labeling accounted for less than <20 μm^2 for either resting or PMA-activated neutrophils, which is approximately a 14-fold reduction in binding as compared to wild-type FX (Figure 3.3A &B). Overall these data suggesting that the Gla domain of FX plays a critical role in mediating the binding of FX to neutrophils during NETosis. Next, prothrombin was incubated with neutrophils under resting conditions and following NETs formation with PMA. The results show that binding of prothrombin to the cell body increased after PMA stimulation to form NETs (Figure 3.1). Upon quantification, the mean area of prothrombin on resting cells increased from 6.8 ± 1.5 μm^2 to 292.9 ± 43.8 μm^2 following PMA stimulation (Figure 3.3A). Prothrombin labeling per cell was determined on resting cells to be 0.4 ± 0.06 μm^2 and increased after PMA stimulation to 13.62 ± 1.6 μm^2 (Figure 3.3B). After NETs formation, the correlation coefficient between prothrombin and DNA was found to be 0.25, again indicative of the fact that prothrombin bound in a punctate manner to the neutrophil cell body rather than to NETs.

Neutrophils are known to contain and, upon activation, express tissue factor, the initiator of the extrinsic coagulation pathway [131]. However, it is unknown whether neutrophil tissue factor can complex with FVII to activate FX. The results show that after incubation with FVIIa, the mean area coverage of FVIIa

labeling was 73.3 ± 50.2 μm^2 for resting cells and increased to 323.3 ± 59.8 μm^2 after PMA stimulation (Figure 3.3A). After normalization per cell, the mean area of FVIIa labeling was 3.4 ± 3.1 μm^2 for resting cells and again increased to 11.4 ± 2.4 μm^2 after PMA stimulation (Figure 3.3B).

Following the initiation and propagation of the coagulation cascade to generate thrombin, thrombin itself feeds back to downregulate thrombin generation through the generation of APC. Thrombin in complex with thrombomodulin cleaves protein C to generate APC, an endogenous anticoagulant which inactivates FVIIIa and FVa to dampen thrombin activation. Moreover, APC has been demonstrated to cleave histones, the most abundant protein associated with DNA [9]. Our group has previously shown that APC binding to leukocytes inhibits endotoxin-induced tissue factor procoagulant activity [109]. Experiments were designed to determine whether APC bound to neutrophils during NETosis. Thus, resting neutrophils and neutrophils that had undergone NETosis were incubated with APC as well as the zymogen, protein C in a purified system. These data show that the mean area of APC labeling was 85.5 ± 46.3 μm^2 for resting neutrophils, while induction of NETs resulted in a 3-fold increase in APC binding, to 248.5 ± 32.1 μm^2 after PMA stimulation (Figure 3.3A). After normalization, the mean area of APC binding per cell was 5.9 ± 1.9 μm^2 for resting neutrophils as compared to 15.63 ± 3.16 μm^2 per cell after NETs formation (Figure 3.3B). The correlation coefficient between labeling for APC and DNA after NETs induction was 0.54, reflective of the binding of APC to both the cell body and DNA-rich NETs as observed in Figure 3.1. In the presence of protein C, a mean area of 8.1 ± 2.2 μm^2 was found for protein C-labeling of neutrophils. Following PMA stimulation to form NETs, the area increased to 255.3 ± 43.5 μm^2 for protein C. Following normalization per cell, the pattern of increased area after NETs formation in response to PMA was again observed. The correlation coefficient between PC and DNA after NETs induction was calculated to be 0.45, again reflective of the binding of PC to NETs as observed in Figure 3.1.

We have previously shown that APC binding to leukocytes is mediated in part by the endothelial protein C receptor (EPCR) [109]. Based on the fact that the N-terminus of APC also contains a Gla-rich domain which mediates binding to EPCR [132], a Gla domain-less derivative of APC was utilized to determine whether the Gla-domain was required for APC-NETs binding. The results show that incubation of neutrophils with Gla-less APC only resulted in a mean area of <2 μm^2 for either resting or PMA-activated (Figure 3.3A). After normalization per cell, Gla-less APC labeling was quantified as less than 0.2 μm^2 for resting or PMA-stimulated neutrophils stimulation (Figure 3.3B), a nearly 40-fold reduction in labeling as compared to wild-type APC. This data suggests that the Gla domain of APC plays a critical role in mediated APC binding to activated neutrophils and NETs.

To generate thrombin, the serine protease essential to formation of fibrin, coagulation can be activated and propagated through two main pathways, namely the extrinsic or the intrinsic pathways of coagulation. Activation of the intrinsic pathway of coagulation is initiated when the coagulation factor XII comes into contact with negatively charged surfaces, causing FXII to undergo autoactivation resulting in the generation of FXIIa, which subsequently proteolytically activates FXI to form the serine protease, FXIa. As DNA is a highly charged negative molecule, it has been hypothesized that NETs, by being comprised primarily of DNA, can promote activation of the intrinsic pathway of coagulation, specifically through activation of FXII [54,74]. Thus, experiments were designed to test detection of labeled NETs with either FXIa or FXIIa. Our quantitative analysis failed to show labeling of either neutrophils or NETs with FXIIa or FXIa, either alone or in the presence of the cofactors HK and/or $ZnCl_2$ (Figure 3.3A & B), suggesting that under these experimental conditions, NETs may not be able to support the binding of the coagulation factors of the intrinsic pathway.

Conversely, as a positive control we tested if we could detect FXIIa and FXIa binding to cultured endothelial cells (Figure 3.4A & B) [133,134]. Quantification of FXIIa labeling of endothelial cells in the presence of $ZnCl_2$ demonstrated a mean surface area of 1001.2 ± 68.5 μm^2 that was normalized to 37.4 ± 3.3 μm^2 per cell. The correlation coefficient between FXIIa and DNA of endothelial cells was 0.55, reflecting the colocalization and strong signal of FXIIa observed on the endothelium. Similarly, incubation of endothelial cells with FXIa in the presence of 25μM $ZnCl_2$ resulted in a surface area of 977.3 ± 44.5 μm^2 that after normalization per cell was found to be 35.8 ± 1.7 μm^2. The correlation coefficient was calculated to be 0.36 for FXIa and DNA found in endothelial cells, reflective of the more diffuse staining pattern of FXIa to the endothelium in these studies. Our quantitative platform was also able to detect the binding of fibrinogen, FX and APC to endothelial cells (Figure 3.4A & B). These results demonstrate that our quantitative analysis platform is able to detect purified coagulation factor binding to different cell types, such as endothelial cells.

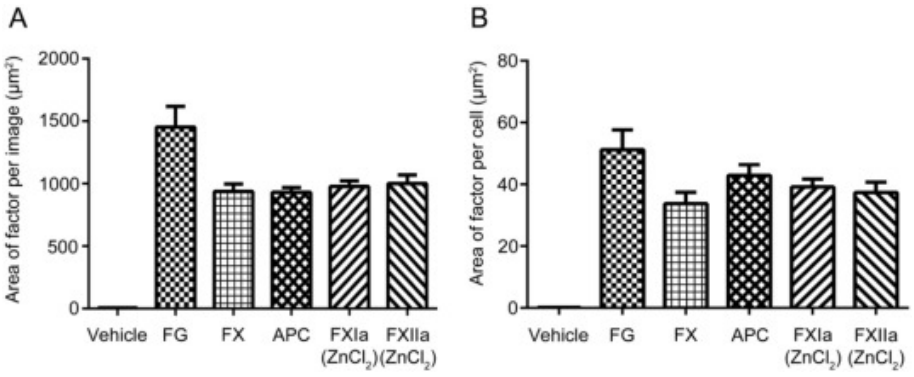

Figure 3.4. Coagulation factors bind cultured endothelial cells quantified as area per image and per cell.

Glass coverslips were seeded with endothelial cells and allowed to grow to confluency. Cell samples were washed once with buffer and treated with vehicle (HBSS buffer), fibrinogen (2.6 mg/mL), FX (10 μg/mL), APC (300 nM), FXIa (20 μg/mL), FXIIa (20 μg/mL) in the presence of $ZnCl_2$ (25 μM) was then incubated for 15 minutes with the cell samples at 37°C. Samples were then fixed with 4% PFA followed by incubation with blocking buffer. (A) Samples were incubated overnight with primary antibodies. Samples were then incubated with Hoechst 33342 (1:1000) and secondary antibodies Alexa Fluor 488 goat anti-rabbit and Alexa Fluor 488 goat anti-mouse (Invitrogen, 1:500). Images were normalized to secondary antibody alone images and quantified in a custom MATLAB program to quantify each pixel positive signal as (A) the area of factor per image and (B) area of signal per cell. Data are mean±SEM n=3.

3.5.2 *Quantification of coagulation factor binding to neutrophils during NETosis in plasma*

We next designed experiments to utilize platelet-poor plasma in this assay in order to quantify endogenous coagulation factor binding to neutrophils during NETosis. As shown in Figure 3.5, neutrophils were observed to form DNA-rich NETs following stimulation with PMA. First, results show that resting neutrophil DNA area was 316.8 ± 78.0 μm^2, which after normalization per cell corresponded to a mean area of DNA/cell of 19.8 ± 1.8 μm^2. After NETs formation, the total DNA area increased to 2234.6 ± 129.3 μm^2, which after normalization per cell corresponded to a mean area of DNA/cell of 116.5 ± 14.1 μm^2. These results in the presence of plasma were similar to the extent of NETosis observed in the presence of purified coagulation factors.

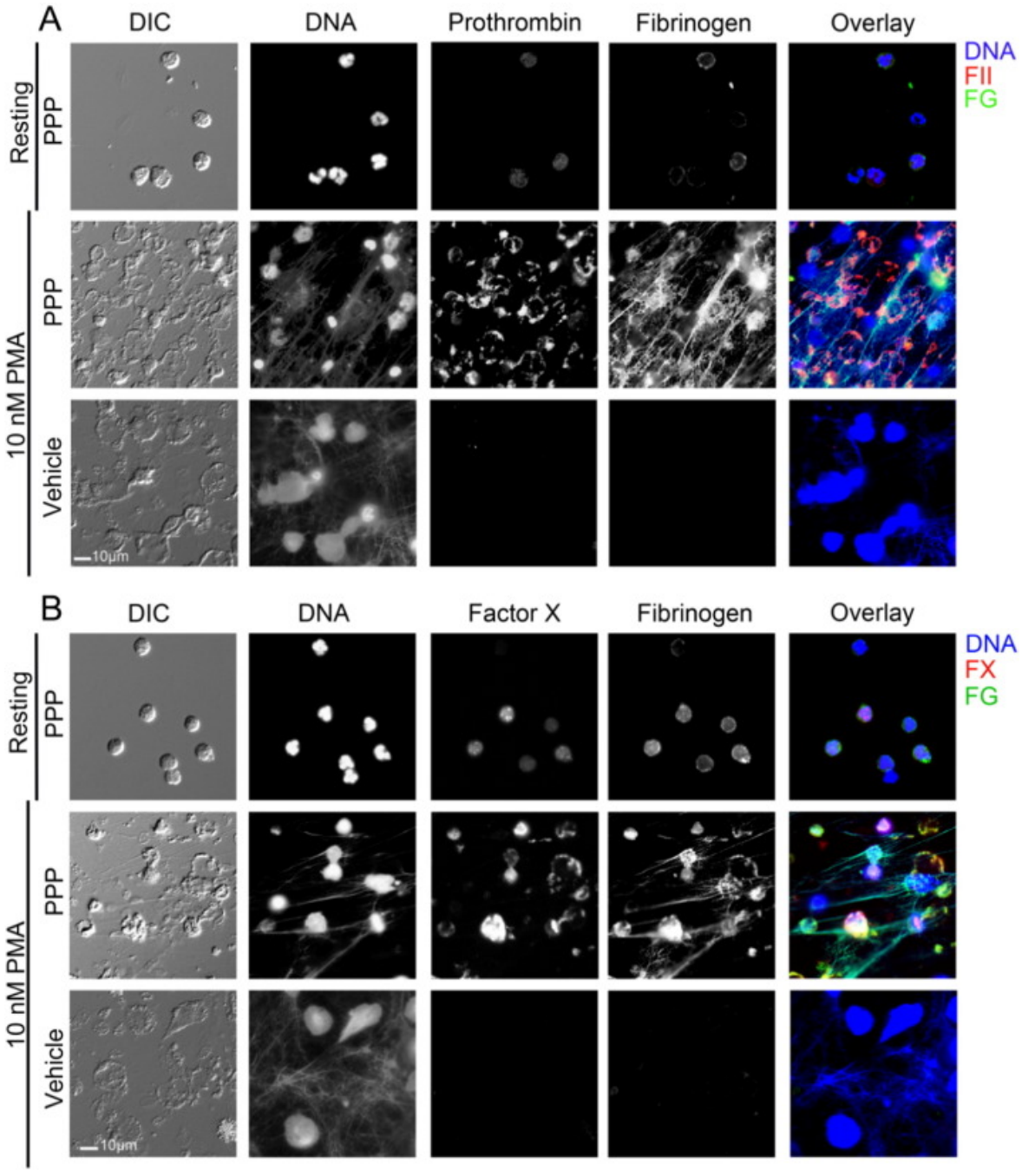

Figure 3.5. NETs promote localization of plasma prothrombin, FX and fibrinogen.

Representative images of neutrophils and NETs treated with buffer or plasma and stained for DNA, fibrinogen and either (A) prothrombin or (B) Factor X.

The binding of plasma coagulation factors to neutrophils during NETosis was then quantified. In the presence of plasma, no prothrombin, FX nor fibrinogen labeling of resting neutrophils was observed. However, as shown in Figure 3.5, the coagulation factors prothrombin, FX and fibrinogen bound to PMA-stimulated neutrophils. The mean area of prothrombin binding to PMA-stimulated neutrophils was 1434.1 ± 107.9 μm^2, which upon normalization per cell was quantified to be 67.9 ± 5.0 μm^2 (Figure 3.6A & B). The mean area of FX binding to PMA-stimulated neutrophils was 1417.5 ± 188.4 μm^2, which upon normalization per cell was quantified to be 40.0 ± 7.4 μm^2 (Figure 3.6A & B). The mean area of fibrinogen binding to PMA-stimulated neutrophils was 1483.9 ± 174.5 μm^2, which upon normalization per cell was quantified to be 96.4 ± 11.8 μm^2. The correlation coefficient between fibrinogen, prothrombin and FX binding and DNA staining after NETs formation was quantified to be 0.75, 0.65 and 0.49, respectively, reflective of the observation that fibrinogen colocalized with NETs, whereas both prothrombin and FX were observed to bind to the neutrophil cell body (Figure 3.5).

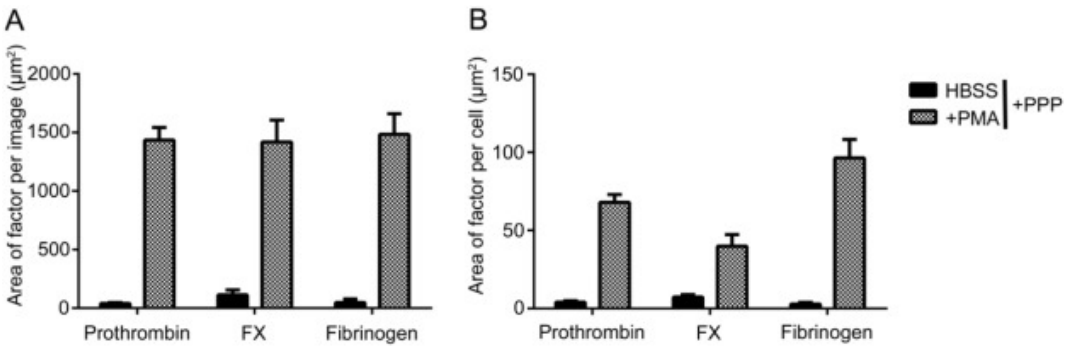

Figure 3.6. Coagulation factors in platelet poor plasma colocalize to NETs, quantified as area per image and per cell.

Acid-washed glass coverslips were coated with 20 μg/mL fibronectin and then blocked with denatured BSA (5 mg/mL). Purified human neutrophils (2×10^6/mL) were plated on the coverslips, and were treated with HBSS or PMA (10 nM) for 3 hours at 37°C. Cell samples were washed and incubated with platelet-poor plasma or vehicle (HEPES containing 2mM $CaCl_2$, 2mM $MgCl_2$ and 0.1% BSA) (1:1) for 15 min at 37°C. Samples were incubated overnight with primary antibodies. Samples were then incubated with Hoechst 33342 (1:1000) and secondary antibodies Alexa Fluor 488 goat anti-rabbit and Alexa Fluor 546 goat anti-mouse (Invitrogen, 1:500). Images were normalized to secondary antibody alone images and quantified in a custom MATLAB program to quantify each pixel positive signal as (A) the area per image and (B) area per cell. Data are mean±SEM n=3.

3.5.3 *Quantification of coagulation factor binding to DNA and phospholipids comprising NETs*

We next designed experiments to determine the role of DNA and phospholipids that respectively comprise NETs, may play in promoting the binding of coagulation factors [124,127,135]. To first validate our platform and determine the role of DNA, we quantified the DNA extruded after NETs were pretreated with 10,000U/mL DNase. After imaging and analysis the surface area of DNA was significantly reduced to 329.2 ± 57.9 μm^2 after pretreatment with DNase, and the DNA area was normalized to 16.8 ± 2.4 μm^2 per cell.

After validating that DNase treatment could significantly reduce the degree of extracellular DNA in our system, we incubated DNase-treated NETs with fibrinogen, FX and APC. First, fibrinogen binding was found to be significantly reduced in absence of extracellular DNA, where the total surface area was 298.65 ± 24.9 μm^2 and normalized to 14.0 ± 1.5 μm^2 per cell, similar to the result for resting neutrophil-fibrinogen binding (Figure 3.7A & B). Similarly, the surface area of FX was reduced to 200.7 ± 19.9 μm^2 which normalized to 8.6 ± 1.0 μm^2 per cell. Lastly, APC labeling was significantly reduced in the presence of DNase-treatment, with an area of 149.6 ± 23.5 μm^2, which normalized to 5.9 ± 0.9 μm^2 per cell (Figure 3.7A & B). Overall, these results confirm the role of extracellular DNA in supporting the binding of select coagulation factors during NETosis.

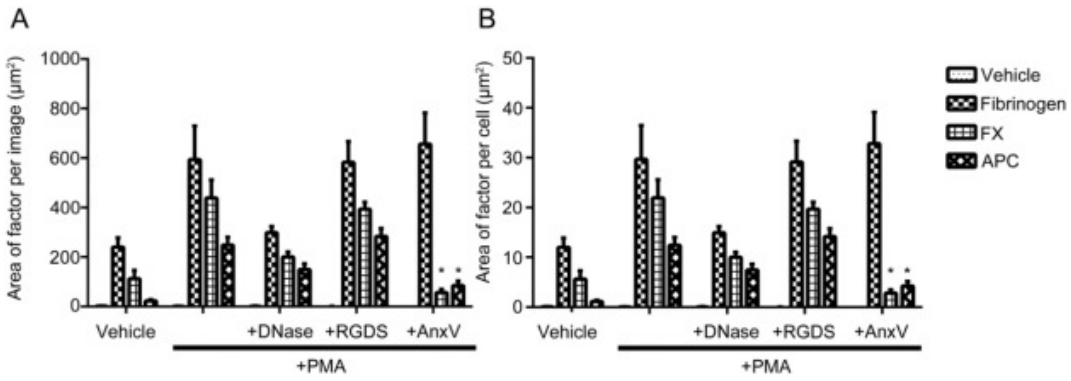

Figure 3.7. Coagulation factors bind neutrophils and neutrophil extracellular traps in a DNA- and phospholipid-dependent manner quantified as area per image and per cell.

Fibronectin-coated coverslips were blocked with denatured BSA (5 mg/mL). Purified human neutrophils (2×10^6/mL) were allowed to adhere, then treated with HBSS or PMA (10 nM) for 3 h at 37°C. Cells were washed and treated with vehicle (HBSS), DNase I (10,000U/mL), RGDS (20 μM), Annexin V (10 μg/mL) for 10 min at 37°C. Samples were then washed and treated with vehicle (HBSS), fibrinogen (2.6 mg/mL), FX (10 μg/mL), and APC (300 nM) for 15 min at 37°C. Samples were then fixed with 4% PFA. (A) Samples were incubated overnight with primary antibodies. Samples were then incubated with Hoechst 33342 (1:1000) and secondary antibodies Alexa Fluor 488 goat anti-rabbit and Alexa Fluor 546 goat anti-mouse (Invitrogen, 1:500). Images were normalized to secondary antibody alone images and quantified in a custom MATLAB program to quantify each pixel positive signal as (A) the area of factor per image and (B) area of signal per cell. *, $p < 0.05$ vs. vehicle + PMA. Data are mean±SEM n=3.

Following activation, the cell membrane of blood cells 'flip' to expose phosphatidylserine to the extracellular environment. Moreover, phospholipid exposure is considered a marker of cell activation and enhancer of coagulation factor activation, in part through binding of calcium ions and the Gla-domain of vitamin K-dependent coagulation factors [136]. As our results demonstrated a critical role for the Gla domain of FX and APC in supporting the binding of these coagulation factors to activated neutrophils, we designed experiments to determine the role of the exposure of phosphatidylserine by activated neutrophils in mediating FX and APC binding. In order to test this hypothesis, activated neutrophils were pretreated with purified Annexin V (AnxV) in order to competitively inhibit binding of coagulation factors to phosphatidylserine. Our results demonstrate that fibrinogen binding to NETs was relatively unchanged in the presence of AnxV, where the total surface area was 655.9 ± 126.3 μm^2 and normalized to 37.7 ± 6.8 μm^2 per cell (Figure 3.7A & B). Next, after inhibition of activated neutrophils with Annexin V, the surface area of FX labeling of activated neutrophils was significantly reduced to 56.5 ± 13.4 μm^2 which normalized to 3.4 ± 0.9 μm^2 per cell. Lastly, APC labeling of activated neutrophils was significantly reduced in the presence of AnxV, with an area of 84.9 ± 18.4 μm^2, which normalized to 3.7 ± 1.0 μm^2 per cell (Figure 3.7A & B). Overall, these results demonstrate additional evidence that the Gla domains in FX and APC may be essential to mediate binding to the cell body during NETosis in a phosphatidylserine-dependent manner.

3.6 Discussion

Fluorescence- and brightfield-based microscopy imaging modalities have been extensively utilized to visualize and study the mechanisms of NETs formation. Herein, we present a quantitative platform to study neutrophil-coagulation factor binding to neutrophils during NETosis.

Using this custom-built MATLAB-based image analysis algorithm, it was first validated that NETs support the binding of fibrinogen in both purified and plasma-based systems, in line with previous observations [124]. The fibrinogen-NETs interaction was found to occur in an extracellular DNA-dependent manner. These findings show that FVIIa, FX and prothrombin bound to the neutrophil cell body during NETosis, suggesting that the surface of activated neutrophils may serve as a site to assemble the prothrombinase complex and promote thrombin generation at sites of inflammation. FX binding could be abrogated with the engineered loss of its Gla domain, as well as after inhibition of phosphatidylserine exposed on the neutrophil cell membrane during NETosis. Using cultured endothelial cells, we observed binding of fibrinogen, FX and APC, as well as the intrinsic factors FXIIa and FXIa in a $ZnCl_2$-dependent manner. Conversely, we failed to observe a measurable direct interaction of either FXII or FXI with neutrophils during NETosis. As several reports have suggested that NETs activate the intrinsic pathway of coagulation [54,120], perhaps indirect pathways may be involved in the activation of FXII by NETs rather than direct binding and autoactivation of FXII by DNA.

As coagulation is activated and propagated, protein C is cleaved to become APC, whereupon the active serine protease exerts its anticoagulant function to dampen the amplification of thrombin generation. The proteolytic activity of APC as an essential endogenous mechanism to downregulate coagulation, and together with its activity as an anti-inflammatory protease APC plays a critical role in dampening thromboinflammation associated with disease states such as sepsis. Neutrophil activation leading to NETs formation has been implicated to promote pathologic thrombus formation at sites of inflammation. Thus, uncovering the molecular mechanisms by which APC binds to neutrophils during NETosis, and determining whether this interaction downregulates NETs formation may provide clues for the development of therapies targeted to safely reduce thromboinflammation. These results demonstrated that

APC and protein C binds to DNA-rich NETs and the cell membrane, which support previous studies suggesting that histone degradation is associated with the proteolytic activity of APC, and supports the hypothesis that APC may regulate neutrophil cell death [9,137].

In conclusion, we have developed and utilized a quantitative fluorescence-based imaging platform to study the spatial distribution of DNA and coagulation factors binding to neutrophils during NETosis. We demonstrated an increase in fibrinogen binding to NETs that is dependent upon extracellular DNA, while the coagulation factors prothrombin, FX and FVIIa bind to the neutrophil cell bodies following NETosis in both purified systems and in plasma. We found that the Gla domain of FX played an essential role in mediating binding of FX to activated neutrophils during NETosis, and that this is dependent upon the exposure of anionic phospholipids on the activated neutrophil cell membrane. Conversely, we were not able to measure the binding of FXIIa or FXIa to neutrophils during NETosis, but were able to measure the binding of FXIIa and FXIa to endothelial cells. We also found that the zymogen protein C, as well as APC, both bound DNA-rich NETs, and identified a critical role for the Gla domain of APC in mediating binding to the neutrophil cell body during NETosis, mediated by phosphatidylserine exposure. Overall, this study suggests that NETs may serve as a scaffold for the assembly of select coagulation factors, providing rationale for the targeted inhibition of NETosis to reduce thromboinflammation.

Chapter 4. Regulation of neutrophil function by coagulation enzymes

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4.1 Abstract

Activated protein C (APC) is a multi-functional serine protease with anticoagulant, cytoprotective, and anti-inflammatory activities. In addition to the cytoprotective effects of APC on endothelial cells, podocytes, and neurons, APC cleaves and detoxifies extracellular histones, a major component of neutrophil extracellular traps (NETs). NETs promote pathogen clearance but also can lead to thrombosis; the pathways that negatively regulate NETosis are largely unknown. Thus, we studied whether APC is capable of directly inhibiting NETosis via receptor-mediated cell signaling mechanisms. Here, by quantifying extracellular DNA or myeloperoxidase, we demonstrate that APC binds human leukocytes and prevents activated platelet supernatant or phorbol 12-myristate 13-acetate (PMA) from inducing NETosis. Of note, APC proteolytic activity was required for inhibiting NETosis. Moreover, antibodies against the neutrophil receptors endothelial protein C receptor (EPCR), protease activated receptor 3 (PAR3), and macrophage-1 antigen (Mac-1) blocked APC inhibition of NETosis. Select mutations in the Gla and protease domains of recombinant APC caused a loss of NETosis. Interestingly, pretreatment of neutrophils with APC prior to induction of NETosis inhibited platelet adhesion to NETs. Lastly, in a non-human primate model of *E. coli*-induced sepsis, pre-treatment of animals with APC abrogated release of myeloperoxidase from neutrophils, a marker of neutrophil activation. These findings suggest that the anti-inflammatory function of APC at therapeutic concentrations may include the inhibition of NETosis in an EPCR-, PAR3-, and Mac-1-dependent manner, providing additional mechanistic insight into the diverse functions of neutrophils and APC in disease states including sepsis.

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4.2 Introduction

The studies conducted in this chapter were designed to determine a role for the anti-inflammatory functions of APC in regulating neutrophil cell survival. APC functions as both a key endogenous anticoagulant and anti-inflammatory molecule in the blood. Administration of APC as a therapeutic in select diseases appears to be an attractive target, however defining the multifunctional actions of APC in detail remain ill-defined. In Chapter 4, studies were designed to test the effect of APC administration on purified neutrophils undergoing NETosis. The aim of the subsequent experiments presented herein were to determine the receptors on the neutrophil surface that bound APC, and define the structure-function relationship of APC on neutrophil cell survival. Lastly, a pilot proof-of-concept experiment sought to determine if *in vivo* relationship between APC administration and neutrophil activation existed. These data overall describe additional mechanistic roles into the varied functions of neutrophils and APC.

4.3 Background

Activated protein C (APC) is a 56 kDa serine protease that acts as an anticoagulant and anti-inflammatory molecule. In complex with protein S, APC inactivates coagulation factors (F)Va and VIIIa, dampening the generation of the thrombin [93,78,138,139]. APC is generated from the zymogen protein C via enzymatic cleavage by thrombin. On endothelial cells this reaction is enhanced in a calcium-dependent manner in the presence of thrombomodulin and endothelial protein C receptor (EPCR) [81,85,87,140]. Due to the combined antithrombotic and anti-inflammatory properties of APC, recombinant APC was

used clinically as a therapeutic for adult severe sepsis before its removal in 2011, as no overall benefit to patient outcome was observed in a second phase 3 trial performed 10 years after the first successful phase 3 trial [13,14]. In severe sepsis therapy, APC increased risk for serious bleeding [13]. Thus, a comprehensive understanding of the structure-activity relationships for the various functional activities of APC may provide the rationale for developing different engineered APC mutants that maintain the anti-inflammatory or cytoprotective functions of APC without adversely affecting thrombin generation or compromising hemostasis [100,107].

Neutrophils are the most populous innate immune cells found in circulation, playing an indispensable role in surveying the body for signs of infection and inflammation [141]. Neutrophils express several of the key receptors responsible for binding and mediating cell signaling of APC, and APC can inhibit neutrophil migration [132,142–145].

Classically, the cytoprotective mechanisms of APC have been best characterized on the endothelium, where APC canonically binds EPCR and cleaves protease activated receptor (PAR) 1 at Arg46, a unique site different from thrombin's cleavage site at Arg41, activating an intracellular signaling cascade to promote barrier function and inhibit apoptosis, a concept known as biased agonism [65,103,146,147]. Moreover, subsequent studies have made a similar observation that PAR3 on the endothelium is cleaved by APC at Arg41, a noncanonical position, which may in part explain the ability of APC to produce a PAR3-dependent cytoprotective effect observed in podocytes [58,148]. We aimed to determine whether APC was able to ligate EPCR and employ PAR3 to regulate neutrophil cell survival.

One form of neutrophil cell death that has garnered much interest since its initial report in 2004 is the ability of neutrophils to form extracellular traps [30]. Neutrophil extracellular traps (NETs) are comprised

of extruded nuclear DNA and associated nuclear proteins including histones and granular proteins. NETs are hypothesized to act as an additional mechanism to promote pathogen clearance. Recent studies have focused on the characterization of the mechanisms that result in NETosis, as well as the (patho)physiological relevance of this process [34,36,38,39,121,126,149,150]. Since their initial discovery, NETs have been shown to promote activation of select coagulation factors and platelets, thereby exacerbating several disease states including thrombosis [40,54,124]. Based on studies linking the activation of blood cells and coagulation factors with inflammatory processes leading to thrombosis, the concept of immunothrombosis was proposed to encompass the complex reactions and crosstalk occurring in disease states such as sepsis [112]. APC has been proposed to play a role in inhibiting immunothrombosis, based in part on the observation that APC was found to cleave extracellular histones in animal models of sepsis, suggesting a possible link between NETs and APC function [9,137,151]. While it is known that neutrophils express cognate receptors that bind APC, it is unknown if APC can inhibit signaling mechanisms that potentiate NETosis. We have recently shown that APC binds to leukocytes and NETs in a Gla domain-dependent manner. Herein, we present evidence that APC binds neutrophils to elicit a receptor-mediated intracellular signaling cascade to inhibit neutrophil activation and NETosis. Moreover, infusion of pharmacological levels of APC abrogated neutrophil activation and MPO release in a non-human primate model of bacterial sepsis. Together these results suggest that the anti-inflammatory properties of APC may extend to the inhibition of neutrophil cell death, including NETosis.

4.4 Material and Methods

4.4.1 *Reagents*

Human plasma-derived APC was a gift from the American Red Cross.[152] Recombinant human (rh)APC and mutant APC proteins were cloned, expressed and purified as previously described.(64–66) The protein kinase C (PKC) inhibitors GF109203X and Ro31-8220; as well as the $G_{\beta\gamma}$ inhibitor gallein

were purchased from Tocris Bio-Techne (Minneapolis, MN, USA). Polymorphprep was from Axis-Shield (Oslo, Norway). The cell-permeable DNA dye Hoechst 33342 was from Invitrogen (Grand Island, NY). Alexa Fluor conjugated anti-mouse antibodies, rabbit polyclonal anti-CD41 (ab63983), rabbit polyclonal anti-histone H3 (ab5103), polyclonal rabbit anti-neutrophil elastase antibody (ab21595), rabbit monoclonal anti-CD11a (ab52895), rat monoclonal anti-C11b (ab8878) and rat monoclonal anti-EPCR (RCR-252, ab81712) were from Abcam (Cambridge, MA). Mouse monoclonal antibody to CD-18 (CBL 158) was from EMD Millipore (Darmstadt, Germany). Blocking antibody to protease activated receptor, PAR-3 (H-103, sc-5598) was purchased from Santa Cruz Biotechnology (Dallas, TX, USA). Anti-human mouse antibody to protein C/APC (AHPC-5071) was purchased from Hematologic Technologies Inc. (Essex Junction, VT, USA). PAR3-derived Peptides GAPPNSFEEFPFS (P3R) and TFRGAPPNSFEEF (P3K) were synthesized and purified to >95% purity from Synthetic Biomolecules (San Diego, CA). Pierce™ LDH Cytotoxicity Assay Kit (88953) was purchased from Thermo Fisher Scientific (Rockford, IL). NE Activity Assay Kit (600610) was purchase from Cayman Chemical (Ann Arbor, MI). *Escherichia coli* Serotype O86:K61(B7), ATCC© 12701, was purchased from ATCC (Manassas, VA) All other reagents were purchased from Sigma-Aldrich (St. Louis, MO) or previously described sources.[156]

4.4.2 *Preparation of leukocytes*

Human leukocytes were purified as previously described [157]. Briefly, human blood was drawn in accordance with an Oregon Health & Science University Institutional Review Board-approved protocol from healthy donors by venipuncture into citrate-phosphate-dextrose (1:7 vol/vol). Blood was layered over an equal volume of Polymorphprep™ and centrifuged at 500 *g* for 45 min at 18°C. The lower layer containing neutrophils was subsequently collected and washed with Hank's Balanced Salt Solution (HBSS) by centrifugation at 400 *g* for 10 min. To remove red blood cells from the sample, the pellet was resuspended in sterile H_2O for 30 sec, followed by immediate addition of 10× PIPES buffer (250 mM

PIPES, 1.1 mM $CaCl_2$, and 50 mM KCl, pH 7.4), to dilute the leukocyte suspension. After centrifugation at 400 *g* for 10 min, the pellet was resuspended in HBSS containing 2 mM $CaCl_2$, 2 mM $MgCl_2$ and 1% wt/vol Bovine Serum Albumin (BSA).

4.4.3 *Platelet isolation and secretome preparation*

Washed platelet isolation was carried out as previously described.[158] Thrombin (1U/mL)-induced platelet aggregation was allowed to proceed for 15 min at 37ºC after its onset and the platelet secretome, containing platelet-derived soluble molecules, was isolated as previously described [55]. Briefly, activated platelets were pelleted by sequentially centrifuging twice at 1000 *g* for 10 min in the presence of a protease inhibitor cocktail to prevent protein degradation. Platelet pellet was discarded and the autologous supernatant (secretome) was collected and recentrifuged at 13,000g for 10 min to remove any microparticles. Hirudin (40 μg/mL) was added to the supernatant to neutralize thrombin activity. The platelet supernatant was used to stimulate NETosis as described below.

4.4.4 *Immunofluorescence microscopy*

Acid-washed glass coverslips were coated with 20 μg/mL fibronectin and then blocked with denatured BSA (5 mg/mL). Initially, purified human neutrophils (2×10^6/mL) were plated on the coverslips for 30 min at 37°C. In select experiments, APC or thrombin was pretreated with PPACK dihydrochloride (40 μM) for 15 min prior to incubation with neutrophils. Samples were subsequently treated with HBSS or phorbol 12-myristate 13-acetate (PMA; 10 nM) for 3 hr at 37°C prior to fixation with 4% paraformaldehyde (PFA) followed by incubation with blocking buffer (PBS containing 10% Fetal Bovine Serum (FBS) and 5 mg/mL Fraction V BSA). Cells were stained with primary antibody (1:100 or 1:250) in blocking buffer at 4°C overnight. Secondary goat anti-mouse IgG antibodies conjugated with

AlexaFluor 546 (1:500) and Hoechst 33342 (10 μg/mL) in blocking buffer were added and incubated for 2 hr in the dark. Coverslips were mounted onto glass slides using Fluoromount G and visualized with a Zeiss Axiovert fluorescence microscope (Axio Imager; Carl Zeiss, Gittingen, Germany) equipped with a ×40/1.3 numerical aperture (NA) oil immersion objective and an air-coupled lens providing Köhler illumination at an NA of 0.17.

To examine platelet binding, following fixation with 4% paraformaldehyde, platelets were permeabilized with blocking solution (1% BSA and 0.1% SDS in PBS). Platelets were then stained with indicated antibodies (1:100) overnight at 4°C in blocking buffer. Secondary Alexa Fluor antibodies (1:500) with Hoechst (1:1000) were added in blocking buffer for 2 hr in the dark. After mounting with Fluoromount G, platelets and neutrophils were imaged on a Zeiss Axiovert fluorescence microscope (Axio Imager; Carl Zeiss, Gottingen, Germany) equipped with a ×63 numerical aperture (NA) oil immersion objective and an air-coupled lens providing Köhler illumination at an NA of 1.40. All images were recorded with a charge-coupled device camera (AxioCam MRc5 12-bit camera; Carl Zeiss) under software control by Slidebook 5.5 (Intelligent Imaging Innovations, Denver, CO) as previously described [158].

4.4.5 *Neutrophil static binding assay*

Acid-washed glass coverslips were coated with BSA (denatured and filtered 5 mg/mL), IgG (20 μg/mL), fibronectin (20 μg/mL) or APC (100 μg/mL) for one hr, followed by washing and then blocked with denatured BSA (5 mg/mL) for one hr. Initially, purified human leukocytes (2×10^6/mL) were plated on the coverslips for one hr at 37°C. Cells were then washed again with PBS and fixed with 4% PFA followed by mounting and imaging.

4.4.6 *APC binding assay*

Purified human leukocytes (2×10^6/mL) were stimulated with HBSS or PMA (10 nM) for 3 hr at 37°C on fibronectin-coated glass coverslips. For colocalization experiments, adherent cells were washed and incubated with vehicle (HBSS buffer) or APC (300 nM) for 15 min at 37°C. Subsequently, samples were washed with PBS and fixed with 4% PFA followed by incubation with blocking buffer (PBS containing 10% FBS and 5 mg/mL Fraction V BSA). Cells and coagulation factors were stained anti-PC/APC Abs (100 µg/mL) in blocking buffer at 4°C overnight. Secondary goat anti-mouse IgG antibodies conjugated with AlexaFluor 488 (1:500) and Hoechst 33342 (10 µg/mL) in blocking buffer were added and incubated for 2 hr in the dark. Coverslips were mounted onto glass slides and visualized.

4.4.7 *NETs formation assays*

In select experiments, purified human leukocytes (2×10^6/mL) were plated on fibronectin-coated coverslips for 30 min at 37°C. APC (300 nM) and thrombin (300 nM) were incubated for 15 min in the presence or absence of PPACK (40 µM) or α1-antitrypsin (α1-AT, 20 µM) prior to incubation with leukocytes for 30 min at 37°C. For select experiments, neutrophils were pretreated with platelet activating factor (PAF, 100 nM) for 30 min at 37°C prior to incubation with 100 nM APC for an additional 30 min at 37°C.

In separate experiments, cells were incubated with PKC inhibitors GF109203X (5 µM) or Ro31-8220 (380 µM); the PI(3)K inhibitor wortmannin (100 nM); the Phospholipase C (PLC) inhibitor U73122 (5 nM) or the inactive analog U73343 (5 nM); the JAK/STAT inhibitor Curcurbitacin I (500 nM); the blocking CD-11b antibody (ab8878; 10 µg/mL), the blocking CD-18 antibody (CBL158; 1 µg/mL); the blocking EPCR antibody (RCR-252; 10 µg/mL); the blocking PAR3 antibody (H-103; 25 µg/mL); or the

PAR3 peptides derived from cleavage at Arg41 (P3R; 50 μM) or Lys38(P3K; 50 μM) for 10–30 min at 37°C before use. In select experiments, leukocytes were pretreated with 30-300 nM recombinant wild-type or mutant APC for 30 min at 37°C. Samples were then washed once with PBS and subsequently treated with HBSS, PMA (10 nM), platelet secretome treated with PPACK or platelet secretome for 3 hr at 37°C. Cell samples were then fixed and imaged.

4.4.8 *Platelet adhesion assay to leukocytes and NETs*

In select experiments, purified human leukocytes (2×10^6/mL) were plated on fibronectin-coated coverslips for 30 min at 37°C. Leukocytes were pretreated with APC (300 nM) for 30 min at 37°C prior to washing and stimulation with HBSS or PMA (10 nM) for 3 hr at 37°C. Samples were then washed once with PBS and incubated with autologous purified platelets (2×10^7/mL) and allowed to adhere for 45 min at 37°C. In select wells, platelets were allowed to adhere to a fibronectin-coated coverslip in the absence of neutrophils. Samples were then washed once with PBS and fixed with 4% PFA followed by staining, mounting and imaging.

4.4.9 *Image analysis*

The fluorescent intensities of each image were adjusted based on signals detected in leukocyte or platelet samples in the absence of primary antibodies. Quantification of fluorescent images was performed using a custom algorithm in MATLAB (The Mathworks, Inc., Natick, MA). This algorithm first establishes global thresholding parameters based on a training set of fluorescence image data to then systematically quantify intensity profiles across treatment conditions. The area of the total field of view is calculated to be 12510 μm^2. Quantitative comparison of treatment conditions was achieved by the normalization of fluorescence intensities per image to determine the area of DNA or respective fluorescent channels.

4.4.10 *LDH activity assay*

Primary human leukocytes (4×10^5/mL) were purified and incubated in solution with 300 nM APC for 30 min at 37°C in a 96 well plate format. In brief, samples were then incubated with HBSS or PMA (20 nM, final) and after mixing were incubated for 3 hr at 37°C. 10× Lysis Buffer was added to duplicate columns and incubated for 45 min at 37°C. Supernatant from all triplicate samples, including lysed samples and an LDH positive control were transferred to new 96-well plates prior to incubation with reaction mixture for 30 min at 37°C. The reaction was stopped and absorbance was measured at 490nm and 680nm. Percent cytotoxicity was quantified as per the manufacturer's instructions (Thermo Fisher, Rockford, IL).

4.4.11 *Non-human primate model of bacterial sepsis*

Studies were reviewed and approved by the Institutional Animal Care and Use Committee at Oklahoma Medical Research Foundation. In this established baboon (*Papio anubis*) model of sepsis, animals (n=4) were initially dosed with 3 mg/kg bolus of APC T-10 min. At T-0 min to T+320 min, APC was infused at 16 µg/kg/min i.v. A lethal dose ($1\text{-}2\times10^{10}$ CFU/kg) of *E.coli*, ATCC 12701 Serotype O86:K61(B7) was infused intravenously over two hours (from T0 to T-120 min). The control group (n=8) consisted in historical experiments where animals were challenged with the same dose of bacteria under similar experimental conditions but not treated with APC. .Blood samples were obtained at 0, 2, 4, 8, and 24 hr and plasma samples were isolated. MPO in plasma samples was determined using Fluoro MPO myeloperoxidase detection kit (Cell Technology, Fremont, CA) as per the manufacturer's instructions.

4.4.12 *Statistical analysis*

Two-way analysis of variance with Tukey's *post hoc* correction or one-way analysis of variance with Welch's *post hoc* correction was used to assess statistical significance among parameters across multiple normally distributed cell parameters. P-values of 0.05 or less were considered statistically significant. At least four images per treatment conditions were obtained and experiments were performed in triplicate. All values are reported at mean ± standard error of the mean unless otherwise stated.

4.5 Results

4.5.1 *APC binds leukocytes and inhibits NETosis*

It was first validated that APC bound neutrophils in a static adhesion assay. Neutrophils were plated on coverslips coated with immobilized APC and assessed for adhesion using DIC microscopy. Neutrophils bound to APC to a similar degree as to fibronectin, whilst significantly fewer neutrophils bound to BSA- or IgG-coated coverslips (Figure 4.1A). It was next studied whether APC in solution bound to neutrophils undergoing NETosis. The data show that soluble APC bound to both the activated neutrophil cell body and to extracellular DNA during NETosis (Figure 4.1B).

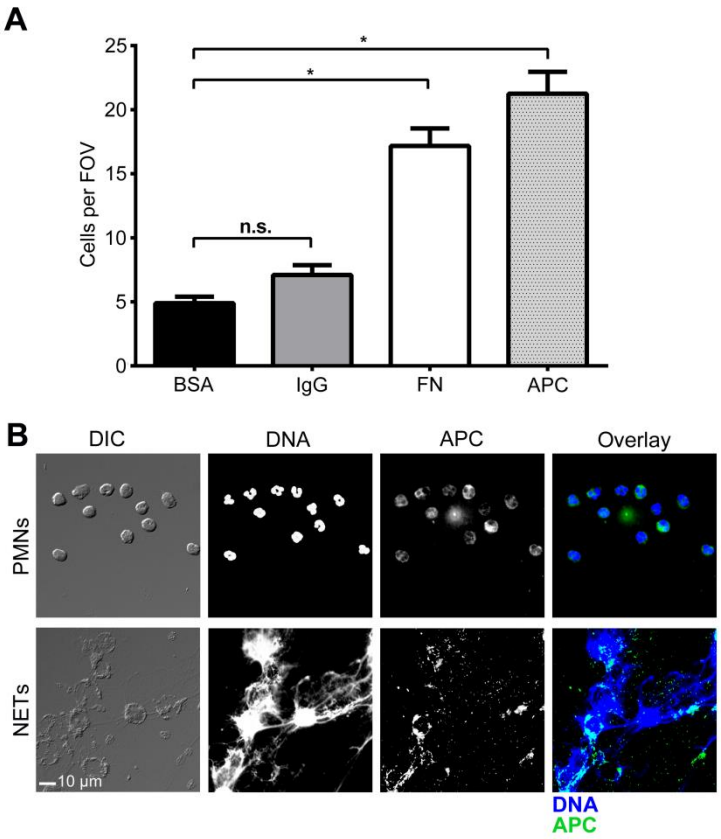


Figure 4.1. Neutrophils bind APC.

Acid-washed glass coverslips were coated with denatured BSA (5mg/mL), 20 µg/mL IgG, 20 µg/mL fibronectin (FN) or 100 µg/mL immobilized APC for one hr, followed by PBS wash and then blocked with denatured BSA (5 mg/mL). (A) Purified human neutrophils (2×10^6/mL) were plated on the coverslips for one hr at 37°C, followed by washing and fixation. After imaging, cell adhesion was counted in Image J and quantified as cell per field of view (FOV). (B) Neutrophils (2×10^6/mL) were plated on fibronectin-coated coverslips and were treated with HBSS or PMA (10 nM) for 3 hr at 37°C. Cell samples were washed and vehicle (HBSS buffer) or APC (300 nM) was then incubated for 15 min with the cell samples at 37°C. Samples were then fixed with 4% PFA. Samples were incubated overnight with primary antibodies. Samples were then incubated with Hoechst 33342 (1:1000) and secondary antibody Alexa Fluor 488 goat anti-mouse (Invitrogen, 1:500). Images were normalized to secondary antibody alone images. Shown above are representative images of APC-positive staining in the presence of NETs and neutrophils. *, p <0.05 vs. vehicle. Data are mean±SEM n=3.

Subsequently, whether APC binding to neutrophils had a functional effect on the process of NETosis was investigated. NETs were formed upon incubation of neutrophils with either autologous platelet secretome or the PKC activator, PMA. NETs formation was characterized by an increase in surface area of DNA, detection of citrullinated histone 3 (H3) and extracellular appearance of myeloperoxidase (MPO) (Figure 4.2A). Prior incubation of neutrophils with APC caused a significant reduction in the area of DNA and MPO extruded by neutrophils during NETosis induced by either platelet secretome or PMA (Figure 4.2B & Figure 4.3).

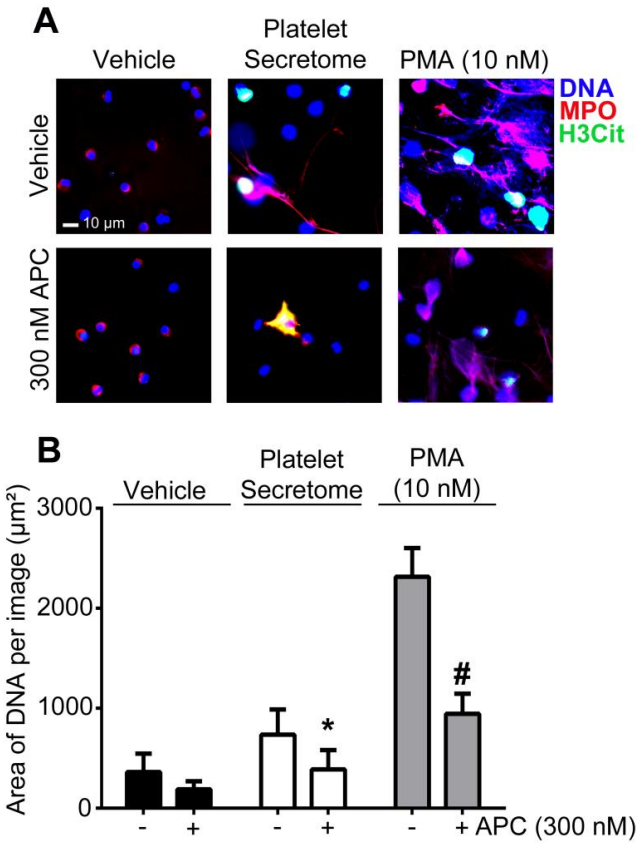


Figure 4.2. APC treatment inhibits NETosis.

Acid-washed glass coverslips were coated with 20 µg/mL fibronectin and then blocked with denatured BSA (5 mg/mL). Purified human neutrophils (2×10^6/mL) were plated on the coverslips for 30 min at 37°C, then incubated with APC (300 nM) for 30 min at 37°C. Samples were then washed once with PBS and treated with HBSS, platelet secretome or PMA (10 nM) for 3 hr at 37°C. All samples were then fixed with 4% PFA. Samples were incubated overnight with polyclonal mouse anti-myeloperoxidase antibody (MPO, 1:100) and rabbit anti-citrullinated histone 3 antibody (H3Cit, 1:250). Samples were then incubated with Hoechst 33342 (1:1000) and secondary antibodies Alexa Fluor 488 goat anti-rabbit and 546 goat anti-mouse IgG (Invitrogen, 1:500). Images were normalized to secondary antibody alone (vehicle) images. (A) Shown are representative images of neutrophils and NETs with DNA, MPO and H3Cit staining. Images were then analyzed in a custom MATLAB program to quantify each pixel positive signal as area DNA per image in (B). *, p <0.05 vs. DMSO + platelet secretome. #, p <0.001 vs. DMSO + PMA. Data are mean±SEM n=4.

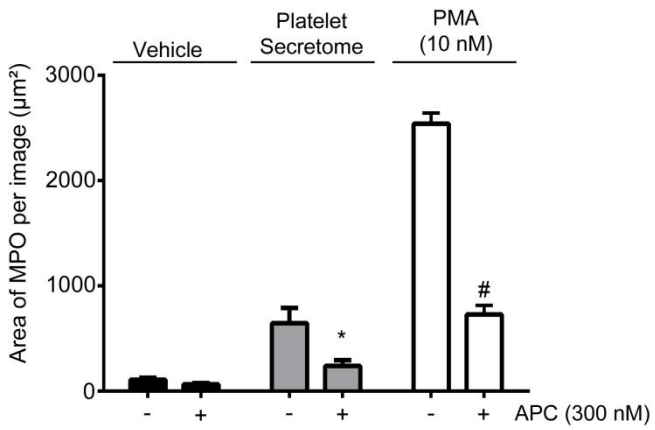


Figure 4.3. MPO release is inhibited by APC treatment prior to NETs induction.

Acid-washed glass coverslips were coated with 20 µg/mL fibronectin and then blocked with denatured BSA (5 mg/mL). Purified human neutrophils (2×10^6/mL) were plated on the coverslips for 30 min at 37°C, then incubated with APC (300 nM) for 30 min at 37°C. Samples were then subsequently treated with HBSS, platelet secretome or PMA (10 nM) for 3 hr at 37°C. All samples were then fixed with 4% PFA. Samples were incubated overnight with polyclonal mouse anti-myeloperoxidase antibody (MPO, 1:100) and rabbit anti-citrullinated histone 3 antibody (H3Cit, 1:250). Samples were then incubated with Hoechst 33342 (1:1000) and secondary antibodies Alexa Fluor 488 goat anti-rabbit and 546 goat anti-mouse (Invitrogen, 1:500). Images were normalized to secondary antibody alone images. Images were then analyzed in a custom MATLAB program to quantify each pixel positive signal as area MPO per image. *, p <0.05 vs. DMSO + platelet secretome. #, p <0.001 vs. DMSO + PMA. Data are mean±SEM n=4.

PMA was used to induce NETosis in the subsequent mechanistic studies due to the enhanced signal-to-noise ratio for surface area of DNA observed when NETosis was induced by PMA as compared to platelet secretome. Results show that a minimal concentration of 75 nM APC was sufficient to reduce the extent of DNA surface area, whereas 300 nM APC potently reduced PMA-induced NETs formation (Figure 4.4A-C). Pretreatment of APC with the serine protease inhibitor, PPACK, reversed the inhibitory effect of APC on NETosis. Equimolar concentrations of the zymogen protein C or the serine protease thrombin failed to inhibit the extent of DNA extruded by neutrophils during NETosis (Figure 4.4A-B).

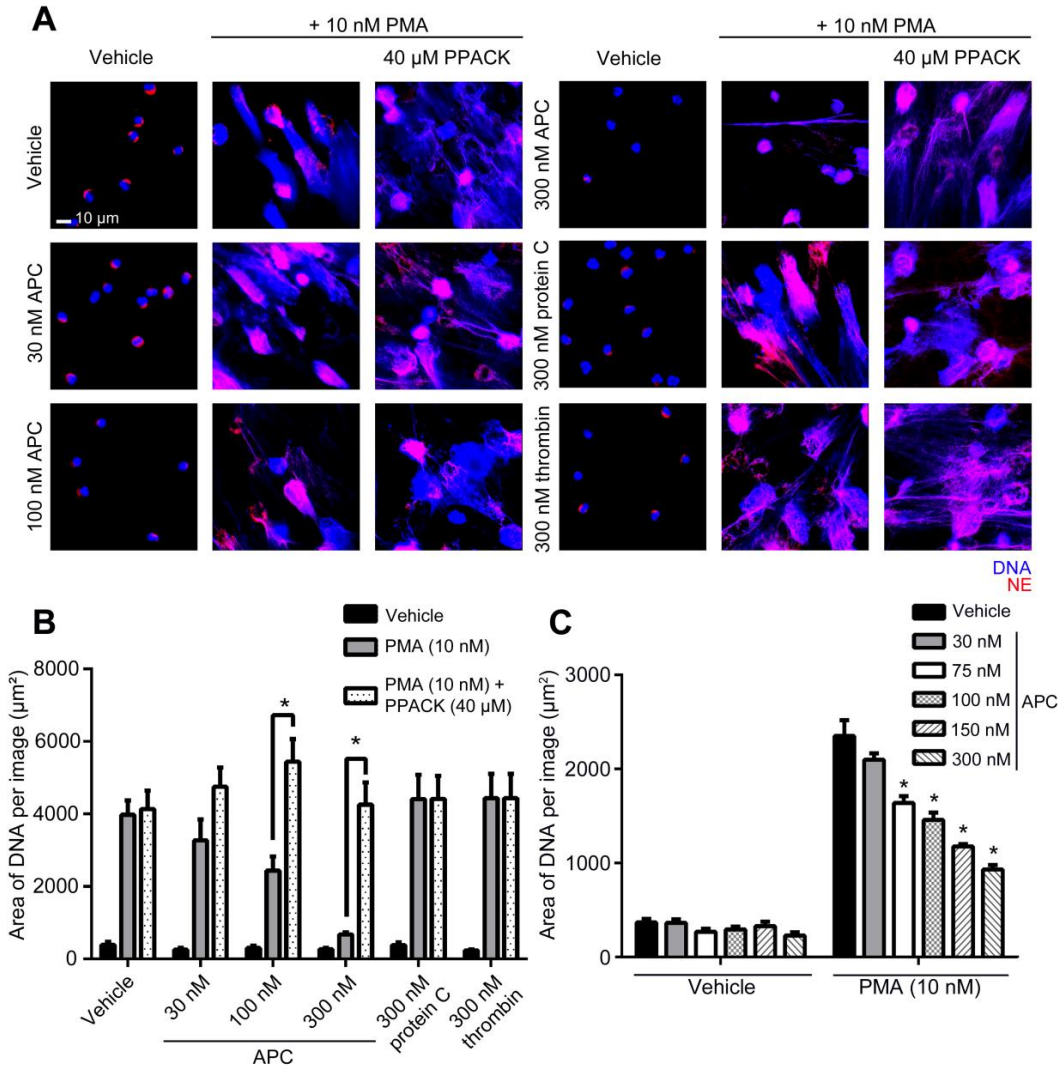


Figure 4.4. APC treatment inhibits PMA-induced NETosis.

Purified human neutrophils (2×10^6/mL) were plated on fibronectin-coated coverslips for 30 min at 37°C, then in (A) and (B) incubated with increasing concentrations of APC (30 nM to 300 nM), protein C (300 nM) and thrombin (300 nM) ± PPACK (40 μM) for 30 min at 37°C. Samples were washed and treated with HBSS or PMA (10 nM) for 3 hr at 37°C. In (C) neutrophils (2×10^6/mL) adhered to coverslips for 30 min at 37°C, then incubated with increasing concentrations of APC (30 nM to 300 nM) for 30 min at 37°C. Samples were washed then treated with HBSS or PMA (10 nM) for 3 hr at 37°C. All samples were fixed with 4% PFA. Samples were incubated with polyclonal rabbit anti-NE antibody (1:100). Samples were then incubated with Hoechst 33342 (1:1000) and Alexa Fluor 546 goat anti-rabbit IgG (1:500). In (A) shown are representative images of NETs in the presence of increasing concentrations of coagulation factors. Images were analyzed in a custom MATLAB program to quantify pixel positive signal as area DNA per image, in (B) and (C), respectively. *, p <0.001 vs. vehicle + PMA. Data are mean±SEM n=3.

In order to determine whether the effect of APC on NETosis was due to a receptor-ligand interaction or due to the direct enzymatic cleavage of extracellular DNA, a wash step was introduced after incubation of neutrophils with APC and prior to PMA stimulation. As shown in Figure 4.4C, an equivalent level of inhibition of NETosis was observed for experiments in which APC was washed away prior to induction of NETosis, with a minimal concentration of 75 nM APC causing a reduction in PMA-induced NET formation. Moreover, inclusion of PPACK in this experiment eliminated the inhibitory effect of APC without having an effect on vehicle-treated, PMA-induced NETosis (Figure 4.5A), whilst pretreatment of neutrophils with an equimolar concentration of thrombin had no significant effect on the ability of neutrophils to undergo PMA-induced NETosis (Figure 4.5B). Since PPACK irreversibly inhibits APC enzyme activity, this implies APC's proteolytic activity is required.

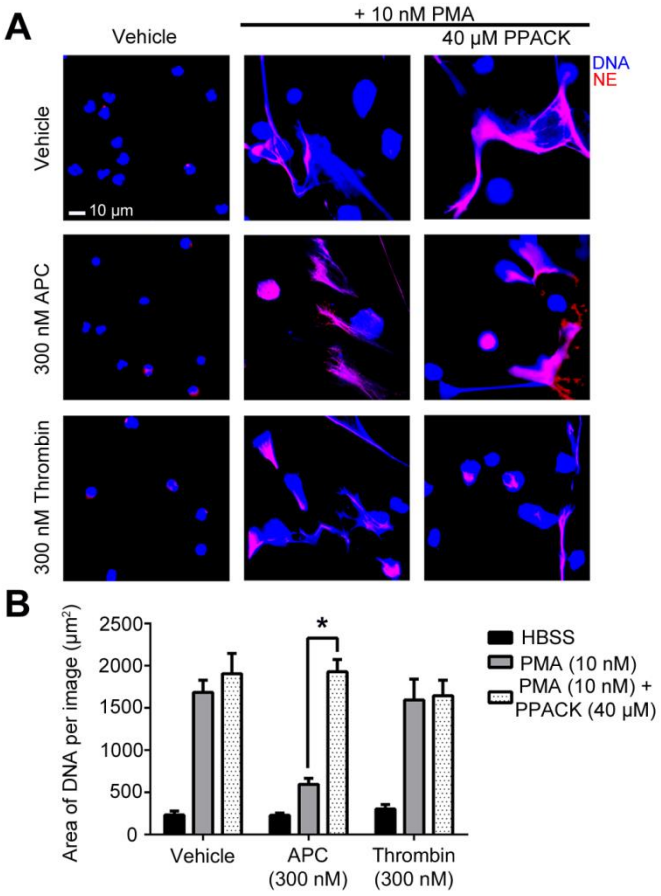


Figure 4.5. PPACK blocks the ability of APC to inhibit NETosis.

Acid-washed glass coverslips were coated with 20 μg/mL fibronectin and then blocked with denatured BSA (5 mg/mL). Purified human neutrophils (2×10^6/mL) were plated on the coverslips for 30 min at 37°C, then incubated with APC (300 nM) or thrombin (300 nM) in the presence or absence of PPACK (40 μM) for 30 min at 37°C. Samples were then washed once with PBS and subsequently treated with HBSS or PMA (10 nM) for 3 hr at 37°C. Samples were then fixed with 4% PFA. Samples were incubated overnight with polyclonal rabbit anti-neutrophil elastase antibody (1:100). Samples were then incubated with Hoechst 33342 (1:1000) and secondary antibody Alexa Fluor 546 goat anti-rabbit (Invitrogen, 1:500). Images were normalized to secondary antibody alone images (A) shown above are representative images of coagulation factors with positive cell staining. Images were then analyzed in a custom MATLAB program to quantify each pixel positive signal as (B) area DNA per image. *, p <0.05 Vehicle + PMA. Data are mean±SEM n=3.

4.5.2 *Proteins PAF and A1-AT eliminate APC inhibition of NETosis*

Other inhibitors of APC activity were studied. Platelet activating factor (PAF) is a phospholipid that binds EPCR, thereby inhibiting APC binding to EPCR and signaling [159]. NETs formation was detected by staining for DNA and citrullinated H3 (Figure 4.6A), and PAF eliminated the inhibitory effect of APC on PMA-induced NETosis, as evidenced by an overall DNA area increase from $1380 \pm 47.8\ \mu m^2$ in the presence of APC alone to $2140 \pm 40.9\ \mu m^2$ in the presence of APC with PAF (Figure 4.6B).

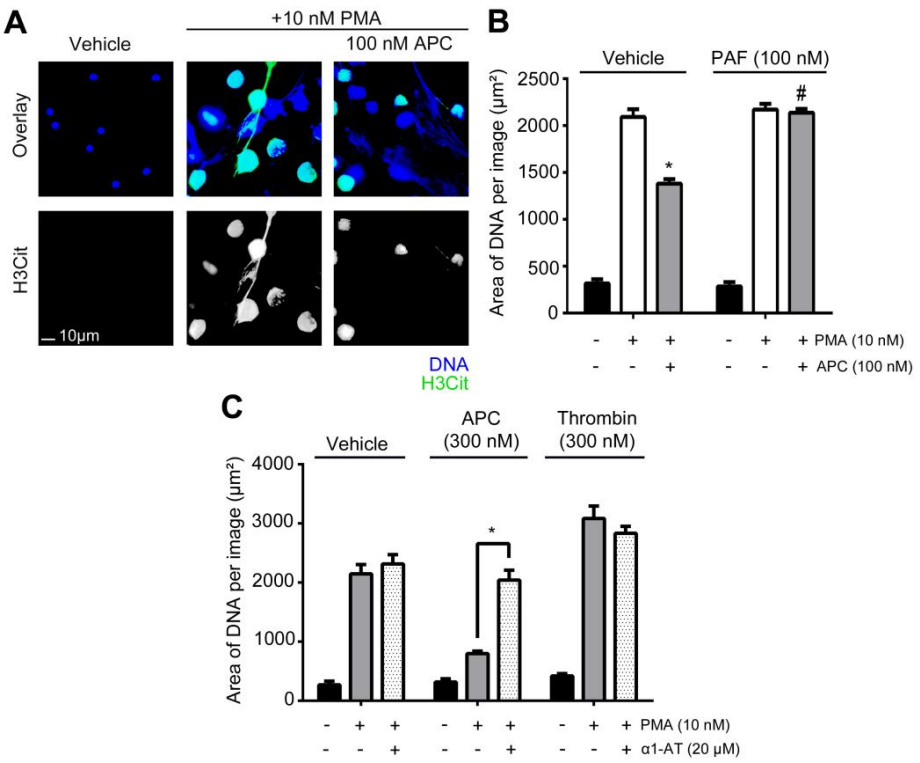


Figure 4.6. APC inhibition of NETosis is abrogated by proteins platelet activating factor and α1-antitrypsin.

Acid-washed glass coverslips were coated with 20 μg/mL fibronectin and then blocked with denatured BSA (5 mg/mL). Purified human neutrophils (2×10^6/mL) were plated on the coverslips for 30 min at 37°C and allowed to adhere before APC treatments. (A) Cells were washed once with PBS and incubated with APC (100 nM) for 30 min at 37°C. To test for inhibition of APC as seen in (B), cells were washed once with PBS and incubated with buffer (vehicle) or Platelet Activating Factor (PAF; 100 nM) for 10 min at 37°C prior to incubation with APC (100 nM) for 30 min at 37°C. As seen in (C), surface-bound cells were incubated with APC (300 nM) and thrombin (300 nM) in the presence or absence of α1-antitrypsin (α1-AT; 20 μM) for 30 min at 37°C. Samples were then washed once with PBS and subsequently treated with HBSS or PMA (10 nM) for 3 hr at 37°C. Samples were then fixed with 4% PFA. Samples were incubated overnight with polyclonal rabbit anti-citrullinated histone H3 antibody (1:250). Samples were then incubated with Hoechst 33342 (1:1000) and secondary antibody Alexa Fluor 488 goat anti-rabbit IgG (Invitrogen, 1:500). Images were normalized to secondary antibody alone images and analyzed in a custom MATLAB program to quantify each pixel positive signal as the area of DNA per image. *, p <0.05 vs. vehicle + PMA. #, p <0.05 vs. APC + PMA. Data are mean±SEM n=3.

Under physiologic conditions, APC is regulated in part by the heparin-independent plasma inhibitor, α1-antitrypsin (A1-AT), which blocks the proteolytic activity of APC [89]. Experiments were designed to test if A1-AT would block the ability of APC to inhibit NETosis. When APC was pretreated with A1-AT, the DNA area increased from 1380 ± 47.8 μm^2 for the presence of APC alone to 2040.5 ± 168.2 μm^2 when APC was pretreated with A1-AT (Figure 4.6C). As a control, thrombin was used in lieu of APC, and either in the presence or absence of A1-AT, there was no significant change in the ability of PMA to induce NETosis, suggesting specificity for APC with regards to inhibiting NETosis.

4.5.3 *Neutrophil receptors Mac-1, EPCR and PAR3 mediate APC inhibition of NETosis*

As our results indicated that both the proteolytic activity and binding of APC to EPCR are required for APC-mediated inhibition of NETosis, we sought to identify the receptors that mediate this potential cytoprotective function of APC. Purified human neutrophils were treated with function blocking antibodies prior to incubation with APC and subsequent induction of NETosis with PMA. APC pretreatment resulted in a significant decrease in DNA area from 2380 ± 45.7 μm^2 to 1010 ± 39.9 μm^2. After pretreatment of neutrophils with blocking antibodies to either CD11b or CD18, it was found that only in combination was there blockade of APC-mediated inhibition of NETosis, as evidenced by the increase to 2230 ± 93.4 μm^2 of DNA area in the presence of the combination of these antibodies (Figure 4.7A). The ability of APC to inhibit PMA-induced NETosis was eliminated in the presence of a blocking antibody to EPCR (RCR-252), as evidenced by a corresponding increase in DNA area to 2260 ± 34.4 μm^2. Notably, neutrophil pretreatment with a blocking antibody to PAR3 blocked the ability of APC to inhibit NETosis, as evidenced by the increase of DNA area to 2080 ± 104 μm^2. In contrast, pretreatment with a specific small molecule PAR1 antagonist, SCH79797, was found to have no significant effect on the ability of APC to inhibit NETosis (Figure 4.8).

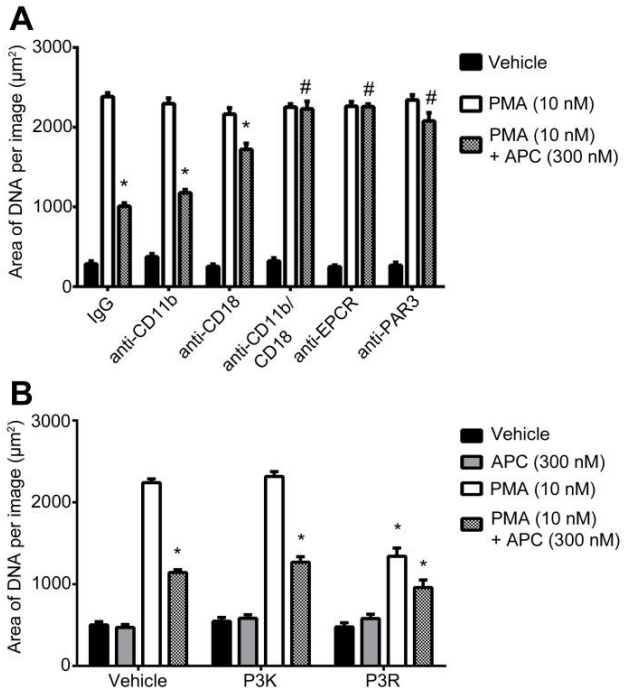


Figure 4.7. EPCR, PAR-3, and Mac-1 are required for APC-mediated inhibition of NETosis.

Acid-washed glass coverslips were coated with 20 μg/mL fibronectin and then blocked with denatured BSA (5 mg/mL). Purified human neutrophils (2×10^6/mL) were plated on the coverslips and allowed to adhere for 30 min at 37°C. Cells were washed once with PBS and in (A) incubated with CD-11b blocking antibody (ab8878; 10 μg/mL), CD-18 blocking antibody (CBL158; 1 μg/mL), EPCR blocking antibody (RCR-252; 10 μg/mL), or PAR3 blocking antibody (ab-5598; 25 μg/mL) for 10 min at 37°C prior to incubation with APC (300 nM) for 30 min at 37°C. In (B), cells were incubated with PAR3 peptides P3K or P3R (50 μM) representative of cleavage at Lys38 (P3K) or Arg41 (P3R) either in the presence or absence of APC (300 nM) for 30 min at 37°C. Samples were then washed once with PBS and subsequently treated with HBSS or PMA (10 nM) for 3 hr at 37°C. Samples were then fixed with 4% PFA. Samples were incubated overnight with polyclonal rabbit anti-neutrophil elastase antibody (1:100) or polyclonal rabbit anti-citrullinated histone H3 antibody (1:250). Samples were then incubated with Hoechst 33342 (1:1000) and secondary antibody Alexa Fluor 546 goat anti-rabbit IgG (Invitrogen, 1:500). Images were normalized to secondary antibody alone images and analyzed in a custom MATLAB program to quantify each pixel positive signal as the area of DNA per image. *, p <0.001 vs. vehicle + PMA. #, p <0.05 vs. APC + PMA. Data are mean±SEM n=3.

As the use of an antibody that blocked the cleavage of PAR3 resulted in the loss of APC-mediated inhibition of NETosis, we sought to test if the PAR3 tethered-ligand peptides generated by APC or thrombin cleavages could inhibit NETs formation [58]. The thrombin-derived P3K peptide, which is generated after the cleavage of PAR3 at Lys38 by thrombin, had no effect on PMA-induced NETs

formation. In contrast to the P3K peptide, the use of the APC-derived P3R peptide, which is generated after the cleavage of PAR3 at Arg41 by APC, significantly reduced PMA-induced NETosis either in the presence or absence of APC (Figure 4.7B), implying that PAR3 agonism was sufficient to inhibit NETosis.

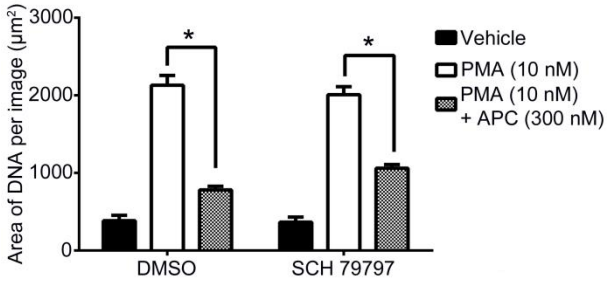


Figure 4.8. PAR1 inhibition does not reduce APC-mediated inhibition of NETosis.

Acid-washed glass coverslips were coated with 20 μg/mL fibronectin and then blocked with denatured BSA (5 mg/mL). Purified human neutrophils (2×10^6/mL) were plated on the coverslips, and allowed to adhere for 30 min at 37°C. Cells were washed once with PBS and incubated with the small molecule inhibitor to PAR1, SCH 79797 (70 nM) 30 min at 37°C prior to incubation with APC (300nM) for 30 min at 37°C. Samples were then washed once with PBS and subsequently treated with HBSS or PMA (10 nM) for 3 hr at 37°C. Samples were then fixed with 4% PFA. Samples were incubated overnight with polyclonal rabbit anti-neutrophil elastase antibody (1:100). Samples were then incubated with Hoechst 33342 (1:1000) and secondary antibody Alexa Fluor 546 goat anti-rabbit (Invitrogen, 1:500). Images were normalized to secondary antibody alone images and analyzed in a custom MATLAB program to quantify each pixel positive signal as the area of DNA per image. *, p <0.05 DMSO + PMA. Data are mean±SEM n=3.

4.5.4 *APC signals via Gβγ to inhibit PKC and PI3K-signaling dependent NETs formation*

The signaling pathways that regulate NETosis were studied, first by validation of the pathways by which platelet secretome and PMA potentiate NETosis. Pretreatment of neutrophils with the PI3K inhibitor, wortmannin, eliminated NETs formation induced by either platelet secretome or the PKC activator, PMA (Figure 4.9A). Moreover, pretreatment of neutrophils with U73122, an inhibitor to PLC which acts

upstream of PKC, abrogated NETs formation stimulated by platelet secretome but not PMA, whilst the inactive analog of the PLC inhibitor, U73343, had no effect on NETosis potentiated by either agonist. Whether a role for cytokine receptor-mediated signaling in platelet secretome-induced NETosis was tested next. Pretreatment of neutrophils with the JAK/STAT inhibitor, cucurbitacin I, significantly reduced the degree of DNA extruded from neutrophils following exposure to platelet secretome, whilst having no effect on PMA-induced NETosis (Figure 4.9A).

Next, the signaling pathways by which APC inhibits PMA-induced NETosis were characterized. As PMA is a diacylglycerol analog, it activates PKC to drive NETosis *in vitro* in a PI3K-dependent manner [34,36,37]. First, the role of PKC signaling pathway in PMA-induced NETosis was confirmed. As seen in Figure 4.9B, pharmacological inhibition of PKC with either GF109203X or Ro31-8220 ablated the ability of PMA to induce NETosis. In contrast, inhibition of PLC or Gβγ signaling with U73122 or gallein, respectively, had no effect on PMA-induced NETosis, yet eliminated the ability of APC to inhibit NETosis, consistent with the findings suggesting that APC induces signaling downstream of the GPCR PAR3 to inhibit NETosis. Of note, a partial inhibition of NETosis was observed for gallein in the absence of APC, likely due to the fact that gallein is also known to exhibit a partial off-target inhibitory effect on the PI3K pathway (Figure 4.9B).

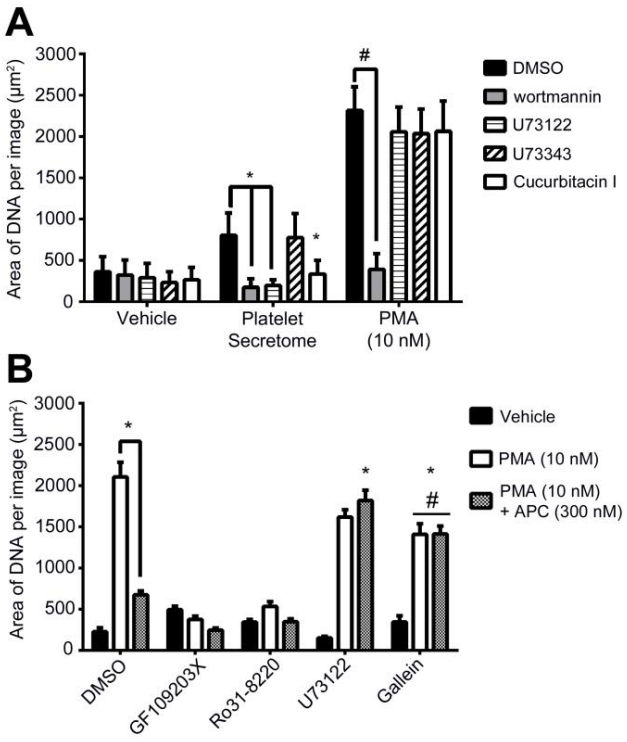


Figure 4.9. APC-mediated inhibition of NETosis requires Gβγ signaling to inhibit PKC and PI3K.

Acid-washed glass coverslips were coated with 20 μg/mL fibronectin and then blocked with denatured BSA (5 mg/mL). Purified human neutrophils (2×10^6/mL) were plated on the coverslips, and allowed to adhere for 30 min at 37°C. Cells were washed once with PBS and incubated in (A) with PI3K inhibitor wortmannin (100 nM), PLC inhibitor U73122 (5 nM) the inactive analog U73343 (5 nM), or JAK/STAT inhibitor cucurbitacin I (500 nM) for 30 min at 37°C. In (B) cells were incubated with PKC inhibitors GF109203X (5 μM) and Ro31-8220 (380 μM), PLC inhibitor U73122 (5 nM), or $G_{\beta\gamma}$ inhibitor Gallein (10 μM) for 30 min at 37°C prior to incubation with APC (300 nM) for 30 min at 37°C. Samples were then washed once with PBS and subsequently treated with HBSS, autologous platelet secretome or PMA (10 nM) for 3 hr at 37°C. Samples were then fixed with 4% PFA. Samples were incubated overnight with polyclonal rabbit anti-citrullinated histone H3 antibody (1:250). Samples were then incubated with Hoechst 33342 (1:1000) and secondary antibody Alexa Fluor 488 goat anti-rabbit IgG (Invitrogen, 1:500). Images were normalized to secondary antibody alone images and analyzed in a custom MATLAB program to quantify each pixel positive signal as the area of DNA per image. *, p <0.001 vs. vehicle + PMA. #, p <0.001 vs. APC + PMA. Data are mean±SEM n=3.

4.5.5 *The protease and Gla domain of APC mediate the inhibition of NETs formation*

Subsequent experiments were designed to determine the regions of APC responsible for inhibiting NETosis. A panel of recombinant APC mutants were rationally created with mutations within the active site and charged exosite of the protease domain, as well as within the Gla domain, as depicted in Figure 4.10A. Equimolar concentrations of recombinant human APC (rhAPC) inhibited PMA-induced NETosis to the same degree as plasma-derived APC (Figure 4.10B). The proteolytic activity of APC was required to inhibit NETosis. Consistent with the findings that demonstrated the action of APC was blocked by the serine protease inhibitor, PPACK (Figure 4.4), the inhibitory function of APC was lost when the active site residue Ser360 was mutated to Ala, rendering APC catalytically inactive (S360A-APC) (Figure 4.10C).

Recombinant APC mutants with protease domain mutations were used to determine structure-activity relationships for inhibition of NETosis. APC with the mutation, Asn329 to Gln (N329Q-APC), exhibited a loss-of-function and was unable to inhibit NETosis. The N329Q mutation results in the loss of an attached carbohydrate that possibly participates in APC-receptor interactions. In the same sequence as N329, substitution of two glutamic acid residues for alanine (EE330/333AA-APC) resulted in only partial inhibition of PMA-induced NETosis, suggesting that residues 329-333 are required for normal inhibition of NETosis by APC. Mutation of Ser252 located along the opening of the catalytic pocket to the acidic residue Glu (S252E-APC) also eliminated the ability of APC to inhibit NETosis. However, mutation at the same residue to alanine (S252A-APC) did not impair the ability of APC to inhibit NETosis (Figure 4.10C).

As mutations in and around the active site seem to play a critical role for APC inhibition of NETosis, we next sought to determine if charged exosites on the face opposite from the face containing the active site

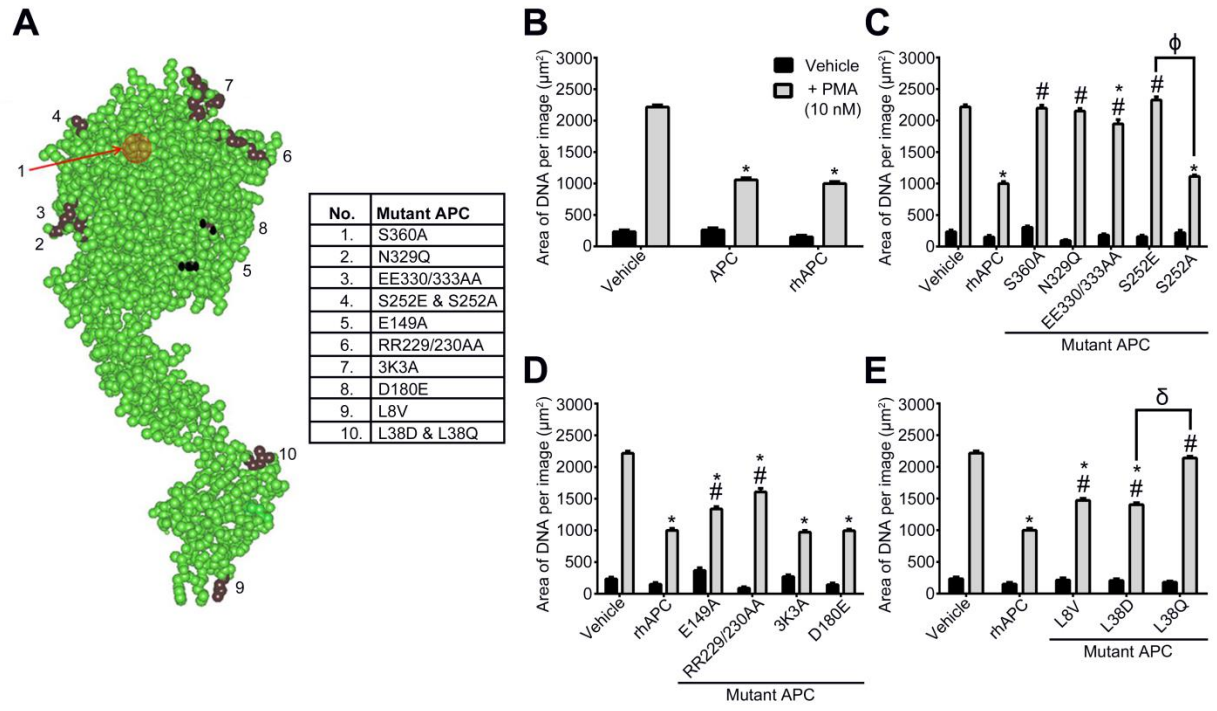
were necessary to inhibit NETosis. A mutation that enhances Protein S cofactor activity by mutating the negatively charged Glu149 to Ala caused a partial reduction in the ability of E149A-APC to inhibit PMA-induced NETosis. Next, it was determined if the positively charged exosites in the protease domain played a role in the functional inhibition of NETs formation by APC. There was a partial loss of function for APC when the residues in the calcium binding-binding loop were mutated to alanine (RR229/230AA-APC). In contrast, mutation of several positively charged lysine residues 191-193 to alanine in the 37-loop had no effect on the inhibitory function of APC, as evidenced by an equivalent degree of inhibition of NETosis for the 3K3A-APC mutant and rhAPC (Figure 4.10D).

The protease domain of APC contains an Arg-Gly-Asp (RGD) triad that is canonically recognized by integrins; thus, Asp180 in the RGD motif was mutated in order to determine if the RGD motif of APC played a role in the regulation of NETosis. D180E-APC had normal ability for inhibition of NETosis (Figure 4.10D).

The γ-carboxyglutamic acid-rich (Gla) domain of APC plays an essential role mediating binding to both EPCR and phosphatidylserine exposed on the surface of activated blood and endothelial cells [160]. Consistent with data in Figure 4.7A implicating a role for EPCR in mediating the inhibitory function of APC, the Gla domain mutation of Leu8 to Val (L8V-APC) caused a partial loss of activity (Figure 4.10E).

The anticoagulant function of APC is enhanced when bound to the cofactor protein S, increasing the rate of inactivation of FVa and FVIIIa. Recently, there was demonstrated a critical role for protein S and cleaved FVa acting as cofactors for APC's anti-inflammatory function in a mouse model of septic peritonitis.[161] Moreover, studies have demonstrated that binding of protein S to APC requires the APC residue Leu38 in the Gla domain, as mutation to Asp (L38D-APC) impairs APC and protein S

interactions [162]. The L38D mutation caused a partial loss of the NETosis inhibitory activity of APC. The substitution of the bulky polar uncharged amino acid, Gln for hydrophobic L38 in APC produced a complete loss-of-function (Figure 4.10E) indicating that residue L38 located on the top of the Gla domain is critical for inhibition of NETosis.



No.	Mutant APC
1.	S360A
2.	N329Q
3.	EE330/333AA
4.	S252E & S252A
5.	E149A
6.	RR229/230AA
7.	3K3A
8.	D180E
9.	L8V
10.	L38D & L38Q

Figure 4.10. Identification of APC residues required for APC-mediated inhibition of NETosis.

(A) Structural scheme for APC with residue mutations numbered beginning with the active site Ser 360 in the protease domain. (B-E) Acid-washed glass coverslips were coated with 20 μg/mL fibronectin and then blocked with denatured BSA (5 mg/mL). Purified human neutrophils (2×10^6/mL) were plated on the coverslips for 30 min at 37°C, then incubated with 300 nM APC or APC mutants for 30 min at 37°C. Samples were then washed once with PBS subsequently treated with HBSS or PMA (10 nM) for 3 hr at 37°C. Samples were then fixed with 4% PFA. Samples were incubated overnight with polyclonal rabbit anti-neutrophil elastase antibody (1:100). Samples were then incubated with Hoechst 33342 (1:1000) and secondary antibody Alexa Fluor 488 goat anti-rabbit IgG (Invitrogen, 1:500). Images were normalized to secondary antibody alone images and analyzed in a custom MATLAB program to quantify each pixel positive signal as the area of DNA per image. (B) Compares wtAPC versus rhAPC used to make all mutants, where all mutations within the active site are presented in (C), the charged exosite mutations are presented in (D) and the Gla domain mutations in APC are presented in (E). *, p <0.001 vs. vehicle + PMA. #, p <0.05 vs. rhAPC + PMA. δ, p <0.05 vs. L38D + PMA. ϕ , p <0.05 vs. S252A + PMA. Data are mean±SEM n=3.

4.5.6 *Platelet adhesion to NETs is inhibited by APC*

Experiments were designed to determine if APC could inhibit platelet-NETs binding, which has been implicated to play a pathological role in promoting immunothrombosis [40,54,124]. First, it was validated that platelets bound immobilized fibronectin, as measured by staining for the platelet integrin CD41 (Figure 4.11A). Next, platelet adhesion to fibronectin-bound neutrophils was quantified. The results show that induction of NETosis with PMA potentiated platelet adhesion. Pre-treatment of neutrophils with APC reduced the degree of platelet adhesion by over 50%, as measured by either the total degree of CD41 staining (Figure 4.11A) or the overlap of CD41 with DNA (Figure 4.11B).

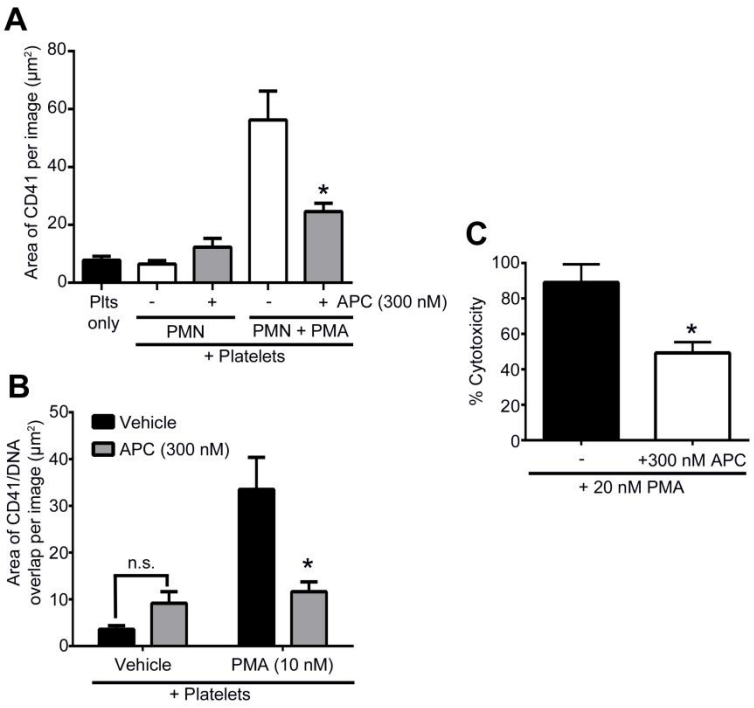


Figure 4.11. APC inhibits platelet adhesion to NETs and LDH release.

(A & B) Acid-washed glass coverslips were coated with 20 μg/mL fibronectin and then blocked with denatured BSA (5 mg/mL). Purified human neutrophils (2×10^6/mL) were plated on the coverslips, and allowed to adhere for 30 min at 37°C. Cells were washed once with PBS and incubated in (A & B) with HBSS or 300 nM APC 30 min at 37°C. Samples were then washed once with PBS and subsequently treated with HBSS or PMA (10 nM) for 3 hr at 37°C. Samples again were washed once with PBS and incubated with autologous platelets (2×10^7/mL) and allowed to adhere for 45 min at 37°C. Samples were then washed once with PBS and fixed with 4% PFA. Samples were incubated overnight with polyclonal rabbit anti-CD41 antibody (1:100). Samples were then incubated with Hoechst 33342 (1:1000) and secondary antibody Alexa Fluor 488 goat anti-rabbit (Invitrogen, 1:500). Images were normalized to secondary antibody alone images and analyzed in a custom MATLAB program to quantify each pixel positive signal as (A) the area of FITC (CD41) per image. In (B) each pixel positive for both CD41 & DNA signals per image was quantified. *, p <0.001 vs. vehicle + PMA. In (C) purified human neutrophils (4×10^5/mL) were incubated in solution with 300 nM APC for 30 min at 37°C. Samples were then incubated with HBSS or PMA (20 nM, final) and incubated for 3 hr at 37°C. 10× Lysis buffer was added to duplicate columns and incubated for 45 min at 37°C. Supernatants from samples and the LDH positive controls were transferred to new 96-well plates prior to incubation with reaction mixture for 30 min at 37°C. The reaction was stopped and absorbance was measured at 490 nm and 680 nm. Percent cytotoxicity was quantified per the manufacturer's instructions (Thermo Fisher, Rockford, IL). *, p <0.05 vs. vehicle + PMA. Data are mean±SEM n=3.

4.5.7 *LDH release is reduced by APC pretreatment*

Experiments were next designed to test if neutrophil cell death was inhibited by APC. Lactate dehydrogenase (LDH) is an enzyme found in almost all cells including neutrophils, playing an integral role in changing lactate to pyruvate, and LDH release is a marker of cell death [33]. Neutrophil cell death was induced with PMA and quantified as percent cytotoxicity, where under vehicle conditions resulted in nearly 90% cytotoxicity (Figure 4.11C). When neutrophils were pretreated with APC, the percentage of cytotoxic cells was significantly reduced by nearly 40%.

4.5.8 *MPO plasma levels are inhibited by APC infusion in a non-human primate model of sepsis*

As an *in vivo* proof-of-concept experiment, we studied whether the administration of pharmacological doses of APC under pathophysiologic conditions could reduce neutrophil activation using an established baboon model of bacterial sepsis [7,9,163]. In this model, either pharmacological doses of APC (3 mg/kg initial bolus followed by infusion of APC at 16 μg/kg/min for 5.4 hrs) or of vehicle were infused prior to challenge with a lethal dose (LD_{100}) of *E.coli* (ATCC© 12701) that resulted in a total concentration of 1-2×10^{10} colony forming units (CFU)/kg. Using this model, the effect of APC on neutrophil myeloperoxidase (MPO) release was assessed. MPO is a critical antimicrobial enzyme released by activated neutrophils as well as a biomarker for NETs formation [32,33]. As seen in Figure 4.12, *E.coli* challenge induced a nearly 7-fold increase in MPO release from a baseline of 31.1 ± 4.3 mU/mL to 196.4 ± 45.8 mU/mL in non-treated animals; in striking contrast, pretreatment of baboons with APC nearly abrogated MPO release, as evidenced by the level of MPO release only increasing to 38.6 ± 2.7 mU/mL 24 hrs post-challenge. All animals treated with APC survived to the end of the study, whereas vehicle-treated animals challenged to *E.coli* did not survive past 48 h post-challenge.

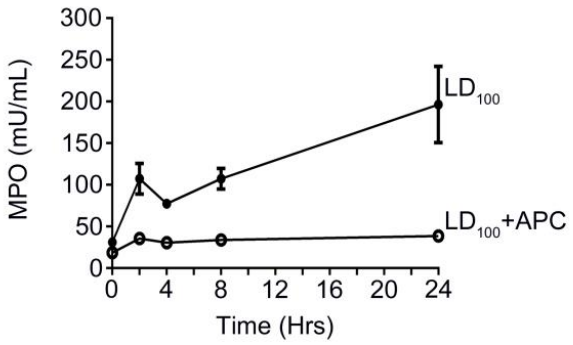


Figure 4.12. Infusion of APC reduces plasma levels of MPO in an LD100 *E. coli* baboon model of sepsis.

In this established baboon model of sepsis, *Papio c. anubis* treated animals were initially dosed with 3 mg/kg bolus of APC at time (T)-10 min. From T-0 min to T+320 min, APC was infused intravenously at 16 µg/kg/min. A lethal dose ($1\text{-}2\times10^{10}$ CFU/kg) of *E.coli*, ATCC 12701 Serotype O86:K61(B7) was infused intravenously over two hours (from T-0 to T-120 min). Blood samples were obtained at 0, 2, 4, 8, and 24 hr and plasma samples were isolated. MPO in plasma samples was determined using Fluoro MPO myeloperoxidase detection kit (Cell Technology, Fremont, CA) per the manufacturer's instructions. LD_{100} animals n = 8; LD_{100} + APC animals n = 4. Data are mean±SEM. *This data was generated by Dr. Ravi Keshari (Oklahoma Medical Research Foundation).

4.6 Discussion

Here, we report that in a purified neutrophil system and in an initial proof-of-concept experiment using an established *in vivo* non-human primate model of sepsis, using therapeutic concentrations of APC was found to inhibit neutrophil activation and induction of cell death. Utilizing a mechanistic *in vitro* approach, APC inhibition of PKC- and PI3K-dependent NETosis was found to be in part dependent on the neutrophil receptors Mac-1, EPCR and PAR3 (Figure 4.13). EPCR and β integrins expressed on neutrophils are known to bind APC resulting in a reduction in migration [132,142,143], and our results now suggest a role for the receptors EPCR and Mac-1 in APC-mediated inhibition of NETosis. Based on molecular studies with endothelial cells and podocytes, APC ligates EPCR and cleaves PAR1 and PAR3 at noncanonical residues differing from the thrombin cleavage sites [58,65]. Together with our results,

these studies substantiate the biased agonism hypothesis where thrombin and APC differentially cleave the same receptors, thus initiating different signaling pathways to culminate in disparate effects on cell survival [65,104,107,148]. PAR3 was identified in 1997 as the second thrombin receptor in humans, following PAR1 [59]. Initially, PAR3 was thought to be a nonsignaling receptor. PAR3 was shown to replace PAR1 and facilitate PAR4 activation by thrombin [164]. PAR1 and PAR3 heterodimerize on endothelial cells to help explain thrombin-mediated signaling [165]. Thus far, it has been shown that thrombin, APC and FXa can cleave PAR3 [58,59,166,167]. APC-mediated cytoprotective signaling on neuronal cells, podocytes and endothelial cells occurs in part via the activation of PAR3 and requires EPCR [58,102,168,169]. It remains to be determined whether APC generated in plasma from endogenous protein C inhibits neutrophil activation or extends cell survival.

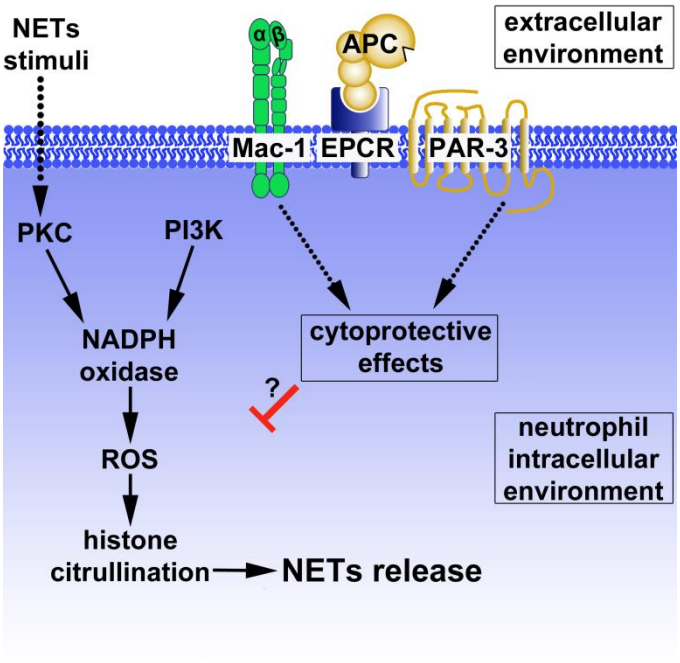


Figure 4.13. Schematic overview for APC-mediated inhibition of NETosis.

In vitro, select stimuli including cytokines, bacteria, the PKC-agonist PMA and the platelet secretome induce PKC-dependent NETosis. Our observations show a mechanism whereby proteolytically active APC requires Mac-1, EPCR and PAR3 to induce intracellular, cytoprotective signaling that results in the downstream inhibition of NETs formation.

In neutrophils, basal mRNA and cell surface expression of PAR3 remains relatively constant in response to various inflammatory agonists [144,170]. APC-mediated PAR3 signaling on neutrophils inhibited NETosis, whilst equimolar concentrations of thrombin-mediated PAR3 signaling had no effect on NETosis. The striking finding that the PAR3 P3R peptide inhibits NETosis implies that neutrophil-expressed PAR3 biased agonism is sufficient to regulate neutrophil survival.

With the novel observation that PAR3 but not PAR1 had an essential role in APC-mediated inhibition of NETosis, it is an open question whether there are two signaling pathways initiated that feed into one common path under pathophysiological conditions. It is possible that Mac-1 and a PAR3-EPCR complex

each initiate distinct pathways to activate inhibitory signaling; alternatively, Mac-1/PAR3/EPCR may form a larger complex to initiate signaling, similar to the clustering of EPCR/PAR1 in caveolae found on endothelial cells [105]. Our NETosis inhibition data add to the repertoire of mechanisms by which administration of APC exerts anti-inflammatory effects.

Prior to its removal from clinical practice, recombinant APC was used in adult patients with severe sepsis based on the rationale that the anticoagulant and anti-inflammatory effects of APC would provide a clinical benefit in this patient population [14]. Additional benefits may derive from the ability of APC to cleave cytotoxic extracellular histones in animal models of sepsis [9], supporting the concept that APC exerts its anti-inflammatory effects through both cell-dependent and cell-independent pathways. To date, it has not been clear whether APC has a direct cytoprotective action on neutrophil cell survival. As our results demonstrated APC inhibited neutrophil cell death, this raises the possibility that APC may exert anti-inflammatory activity by inhibiting cellular functions that potentially drive or amplify pathophysiological disease, whilst APC may preserve functions required for homeostatic surveillance. Under select inflammatory conditions, the interactions between platelets and NETs are hypothesized to potentiate immunothrombosis, thus, we tested if APC reduced platelet-NETs binding [40,54,171]. Our results suggest that through inhibition of neutrophil cell death, APC reduces platelet-NET binding and thus may reduce the prothrombotic phenotype of NETs-promoted immunothrombosis.

As APC has well characterized anticoagulant and multiple cell signaling actions, it should become possible through rational design to select for specific functions of APC [107]. Thus, one of our goals is to develop APC mutants that can provide improved treatments for a variety of disease states, as in sepsis where these proteins may allow for the better control of inflammation and immune system homeostasis without compromising hemostatic mechanisms [107]. Introduction of single or double amino acid substitutions within APC can significantly modulate its anticoagulant or anti-inflammatory functions.

Proof-of-concept studies using APC mutants have demonstrated beneficial selection for specific functions of APC on a variety of cell types and through use of *in vivo* mouse models, where select mutants have now advanced to clinical trial [82,153–155,162,168,172,173]. Using the protein C database ProCMD [91], five of the sites where mutations were introduced into APC for our study were found to occur in humans; however the amino acids introduced in our study did not reflect the mutated amino acid replacements seen in affected humans. Multiple APC mutants inhibited PMA-induced NETosis. Select mutations in the protease domain, as in 3K3A-APC, yielded a normal degree of inhibition of NETosis, similar to rhAPC. This APC signaling selective mutant is in clinical trials for ischemic stroke [15]. In contrast, multiple mutations in the protease domain have a partial or total loss-of-function, demonstrating that certain residues in the protease domain are essential for the ability of APC's signaling to inhibit NETosis. Moreover, mutations within the Gla domain of APC result in the partial or entire loss-of-function of APC, highlighting the additional importance of the Gla domain to mediate both the binding and orientation of APC on neutrophils to inhibit NETs formation. These findings support the rationale development of APC mutants that retain cytoprotective and anti-inflammatory functions but shed anticoagulant activity for potential clinical use in selected inflammatory disease states.

Since the *in vitro* studies show that APC modulates neutrophil cell death, we determined whether infusion of APC at a pharmacologically relevant dose could reduce neutrophil activation using an established non-human primate model of bacterial sepsis as an initial proof-of-concept experiment. 3K3A-APC is currently being studied at high doses, up to 0.5 mg/kg, in healthy adults and is well tolerated [15]. Here we showed that baboons infused with lethal doses of *E. coli*, when using recombinant wildtype APC at 3mg/kg followed by infusion from >5 hrs at 0.96 mg/kg/hr, inhibited MPO release. This suggests a link in vivo between APC and neutrophil function and point towards future *in vivo* studies. Of potential relevance, a previous study observed that in heart surgery patients, a negative correlation between neutrophil MPO activity in the myocardium and generation of APC was observed post-surgery [174].

In conclusion, our discoveries uncover a novel mechanism by which pharmacologic concentrations of APC utilizes neutrophil expressed Mac-1, EPCR and PAR3 to activate intracellular signaling that culminates in the inhibition of PMA-induced NETosis. Furthermore, a pilot proof-of-concept experiment using a non-human primate model of bacterial sepsis demonstrates that infusion of APC reduced neutrophil activation *in vivo*. Fundamental questions remain about how these three receptors ligate APC and initiate the specific intracellular signaling pathways that are activated to result in inhibition, and the role of endogenous APC in reducing NETosis *in vivo*. Future work will aim to identify the precise signaling networks that result in this inhibition of NETosis by APC.

Chapter 5. Conclusions and Future Directions

5.1 Conclusions

Initially, the experiments performed in this thesis developed an imaging platform that demonstrated only select coagulation factors can bind to NETs components or the remaining neutrophil cell bodies. In contrast to previous studies, our systematic testing determined that not all coagulation factors tested bound to either NETs or remaining cell membranes at detectable levels, leading to the conclusion that the interactions modulating immunothrombosis in select diseases are likely more limited than previously hypothesized. This will impact the development of future anticoagulants, as it will be necessary to determine whether they are capable of reducing immunothrombosis by inhibiting coagulation factor binding to various components of NETs.

My studies next elucidated an additional anti-inflammatory function of APC, which includes a novel mechanism by which APC inhibits induction of NETosis, in part due to neutrophil-expressed Mac-1, EPCR and PAR3 receptors. These findings lead to the major conclusion that inhibition of neutrophil death by APC has an impact on the pathophysiological immune response in sepsis. This suggests that in the original PROWESS trial, the beneficial effects of rhAPC might have been initiated by preventing patients from entering an acquired protein C deficient state, which overall limited unchecked neutrophil activation and death, resulting in the initial improved patient outcomes. The discovery of this interplay of APC and neutrophils substantiates future testing of the hypothesis that signaling-specific APC mutants in sepsis models will blunt induction of neutrophil cell death to reduce acute organ dysfunction. Thus, these data are the first description of a direct regulation of neutrophil cell survival by APC, expanding the study of the innate immune response and its regulation. Overall, we can conclude that there exists dynamic crosstalk between coagulation and neutrophils in the progression of sepsis, and applying this new

mechanistic knowledge will translate into safer and more specific therapeutics for sepsis patients in the future.

5.2 Summary

5.2.1 *Coagulation factor localization to NETs*

Induction of NETosis is hypothesized to contribute to immunothrombosis via activation of coagulation and recruitment of platelets. Our initial experiments presented in Chapter 3 sought to systematically test select coagulation factors in their binding to neutrophils and NETs as well as identify the components of NETs required for binding. To approach this, we developed a quantitative imaging platform to quantify the spatial distribution of coagulation factors in immunofluorescent images. First, we validated a previous study to demonstrate that fibrinogen, either purified or in plasma, colocalized to neutrophils and NETs [54]. We then demonstrated that fibrinogen binding was dependent on extracellular DNA. Furthermore, we observed increased binding of prothrombin, FX, FVIIa, protein C and APC in the presence of PMA-induced NETosis. The use of Gla domain-less FX and APC eliminated their binding to NETs, suggesting FX and APC may bind to the remaining cell membrane after NETs induction. Future functional work will need to utilize purified and plasma-based enzymatic assay systems in order to define whether the rate of coagulation factor activation is amplified after adhering to NETs. Additionally, it will be necessary to determine if NETs formed in solution promote similar adhesion of coagulation factors and what differences, if any, for solution- or surface-induced NETs to potentiate fibrin formation. These experiments would biochemically address the functional significance of NETs-mediated coagulation.

In contrast to previous studies demonstrating colocalization between NETs and the coagulation factors FXIIa and FXIa of the intrinsic cascade [54,120], our system was unable detect these coagulation factors on NETs. As a control, both FXIIa and FXIa were detected and quantified when incubated with

endothelial cells under similar conditions, demonstrating our system was capable of detecting FXIIa and FXIa binding to cells. Interestingly, our findings support a recent mechanistic study demonstrating that the individual components comprising NETs, namely histones and DNA, were able to drive activation of FXII and FXI, whereas intact NETs were unable to potentiate their activation [77]. Thus, it is tempting to speculate that when NETs are in their intact chromatin form, the cationic charge of the histones and the anionic charge of the DNA collectively neutralize the overall charge of the complex, rendering it unable to initiate intrinsic coagulation. To resolve these conflicting results, it is possible NETs may associate with additional, as of yet unidentified cofactors to allow FXII and FXI binding and activation.

5.2.2 *APC inhibition of NETosis*

APC is a multifactorial serine protease that plays important roles in coagulation and inflammation. Therefore, elucidating the effector cells and subsequent pathways regulated by APC at a mechanistic level is an important therapeutic challenge. In our studies described in Chapter 4, we demonstrate a role for APC inhibition of neutrophil death through APC pretreatment inhibiting the induction of NETosis. Our mechanistic studies demonstrate that the enzymatic activity of APC was required for this inhibition and that APC could inhibit induction of NETosis by either platelet secretome or the conventional agonist PMA. Platelet adhesion to NETs was significantly reduced with APC pretreatment, suggesting that APC may indirectly reduce the prothrombotic potential of NETosis. Moreover, we determined that APC-mediated inhibition of NETosis was, in part, dependent on Mac-1, EPCR and PAR3. These data add to the current literature demonstrating that APC initiates intracellular effects on cells, culminating in extending cell survival. In particular, the novel finding that PAR3 may play a functional role in neutrophil activation and survival warrants further exploration. Lastly, the immediate downstream signaling proteins in neutrophils that associate with these receptors in response to APC treatment remain unknown. Future directions to address these gaps in knowledge will be described shortly.

5.2.3 *Rational mutation screen of APC*

Given the dual role of APC in coagulation and inflammation, it is hypothesized that specific functions of APC can be selected for using rational mutation. One APC mutant, 3K3A-APC, has been shown to be well-tolerated in healthy volunteers at pharmacologically relevant doses, substantiating this hypothesis [15,107]. In order to elucidate specific functions of APC and identify the critical domains and residues required for these functions, the construction and use of mutant APC proteins are critical. Selecting for specific functions of APC also provides rationale to test these mutants in select disease states where a role for inflammation or coagulation is described; thus, targeting one of these global pathways by APC administration could eventually improve patient outcome. Our studies demonstrate that select mutations in the Gla domain and protease domain of APC were required to inhibit NETosis, showing that APC can differentially regulate neutrophil function. Specifically, our results corroborate our previous finding that the proteolytic activity of APC was required for inhibition of NETosis, as the catalytically inactive S360A-APC was unable to inhibit PMA-induced NETosis. To date, this is the largest panel of mutant APC proteins systematically tested using a purified *in vitro* system on neutrophils. Whether select mutant APC proteins are capable of regulating other neutrophil functions *in vitro* and whether similar effects between mutant APC and neutrophils occur *in vivo* remain to be defined.

5.2.4 *Non-human primate models of sepsis*

As our purified studies demonstrated a significant role for APC at pharmacologic doses to inhibit NETosis, we sought to determine if any relationship existed between neutrophil activation and response to APC treatment. Previously, studies using various animal models of sepsis found that APC

administration resulted in cleavage of extracellular histones, a major protein component of NETs [9,137]. However, whether these histones only derive from neutrophils or are a result of global inflammation and induction of general cell death in severe sepsis is unclear. Thus, using an LD_{100} non-human primate model of *E. coli*-induced sepsis, we measured plasma levels of the neutrophil marker MPO in response to APC treatment. As an initial proof-of-concept study, this demonstrates that APC reduces blood plasma MPO levels in comparison to untreated conditions, suggesting that pharmacologic doses of APC can regulate neutrophil activation and cell death *in vivo* which may be of benefit or detriment to the host. Our results add to the body of evidence elucidating the mechanisms underlying the effects observed when recombinant APC (Xigris©) was used for patients with severe sepsis. It remains an intriguing question, however, why APC failed to improve patient outcome in the PROWESS-SHOCK trial, whether APC's failure was due to its anticoagulant effects, cellular functions, or some combination thereof. Future work to resolve this complex mechanistic question may require use of rationally mutated APC proteins in an *in vivo* model of sepsis.

5.3 Future directions

5.3.1 *Signaling*

Future work will define the proximal intracellular signaling downstream of EPCR, PAR3 and Mac-1 in response to APC that culminates in inhibition of PMA-induced NETosis. One hypothesis is that PAR3 cleavage by APC results in a bias for G-protein independent signaling mediated by β-arrestin 2 (β-AR2) to induce intracellular signaling, similar to PAR1 signaling by APC on endothelial cells [108]. First, whether APC treatment induces G-protein-dependent or -independent signaling will be addressed by use of the cAMP accumulation assay. Performing the β-galactosidase fragment compartmentalization assay will indicate whether there is an increase in β-AR2 biased signaling after APC treatment [175], which would suggest that APC initiates G protein-independent signaling in neutrophils. Next, to determine

whether β-AR2 is activated and recruited to the cell membrane, neutrophils will be pretreated with APC, lysed and fractionated followed by western blotting for the relative levels of β-AR2, Akt and ERK1/2 in the cell membrane, cytoplasmic and nuclear fractions. Immunoprecipitation experiments for PAR3 and EPCR will be performed, followed by western blotting for β-AR2 to determine whether stable binding occurs between PAR3 and β-AR2. In order to determine whether PAR3 is internalized and degraded in response to APC binding, ubiquitination of the cytoplasmic tail of PAR3 will be measured. Furthermore, these experiments will be repeated in the presence of the PAR3 peptides, P3K and P3R, in order to determine whether internalization differs in response to thrombin or APC, respectively.

Alternatively, immunofluorescence microscopy will be utilized to determine the spatial localization of Mac-1, EPCR and PAR3 on neutrophils in the presence or absence of APC. This approach will define whether the receptors rearrange and cluster to form a larger complex in response to APC treatment or form separate complexes. To determine whether the receptors are internalized, a time course experiment will be performed. Colocalization of lysosomal-associated membrane protein 1 and EPCR or PAR3 will be tested, demonstrating whether the receptors are targeted to lysosomes for degradation.

As the integrin Mac-1 is known to activate Src-family tyrosine kinases (SFKs) via phosphorylation, it remains an open question how Mac-1 is regulated by APC. In order to determine whether Mac-1 is activated in response to APC, neutrophils will be pretreated with the phosphorylation inhibitor PP2 prior to APC treatment and induction of NETosis. Global phosphorylation in neutrophils in response to APC treatment will be measured by western blot. Next, Mac-1 and the SFK member Hck will be immunoprecipitated and blotted for phosphorylation, to determine whether APC inhibits Mac-1 signaling. Altogether, these proposed studies would begin to provide mechanistic insight into the downstream signaling events induced by APC to drive inhibition of NETosis.

5.3.2 *Models of immunothrombosis & clinical implications*

Multiple sclerosis (MS) is a debilitating, chronic autoimmune disease that affects nearly 2.5 million people worldwide, in which the immune system attacks the central nervous system (CNS), causing lesions within the brain and spinal cord as the neurons are demyelinated [176,177]. In the pathogenesis of MS, the blood-brain barrier (BBB) can be compromised and is associated with the inflammatory response; concomitantly, there is an increase in the influx of procoagulant molecules into the CNS. Analysis of MS lesions has shown positive staining for fibrinogen and fibrin, which are critical to coagulation and thought to contribute to activation of microglial cells, the resident macrophages in the CNS [178]. Thus, it is hypothesized that anticoagulant strategies may be beneficial in the treatment of MS.

In order to understand the pathogenesis of MS, a mouse model of experimental autoimmune/allergic encephalomyelitis (EAE) is used [179]. The EAE model partially mimics induction of an inflammatory immune response, with lesion formation in the brain and the spinal cord, thus allowing researchers to test whether anticoagulation reduces the inflammatory response of EAE. It has been shown that administration of APC in EAE reduced neuroinflammation and decreased neurological deficits [178]. To extend these studies, we hypothesized that activation of the endogenous protein C system in EAE would decrease activation of immune cells without increasing the potential risk for bleeding with pharmacologic doses of APC administration. Our initial studies sought to determine whether pharmacologic administration of the enzyme WE thrombin reduced inflammation in the EAE model by activation of endogenous protein C. The mutant WE (W215A/E217A) thrombin contains two mutations that significantly reduce the procoagulant activity of thrombin and has been found to improve neurological

outcomes in mouse models of stroke [180–182]. First, EAE was induced using mouse myelin oligodendrocyte glycoprotein, peptides 35-55 (MOG35-55), combined with Freund's adjuvant containing heat-inactivated *M. tuberculosis*, as previously described [183]. All mice were then injected with 75 ng and 200 ng pertussis toxin intraperitoneally on days 0 and 2. Mice were then assessed for signs of EAE until moderate to severe hind limb weakness or more severe symptoms were observed. Mice were then divided into two groups and treated intravenously every other day with 50 ng of WE thrombin in 100 µL volumes (25 µg/kg) or saline vehicle. Mice were evaluated and sacrificed on day 30. To evaluate the anti-inflammatory function of WE thrombin, it was determined whether WE thrombin blunted the activation of the macrophage subpopulation of splenocytes during EAE [184]. In response to WE thrombin treatment, a reduction in the overall frequency of TNFα and ICAM-1 expression on CD11b+ macrophages was observed (Figure 5.1). This demonstrates that WE thrombin treatment decreases frequency of activated immune cells in EAE, possibly through activation of endogenous protein C.

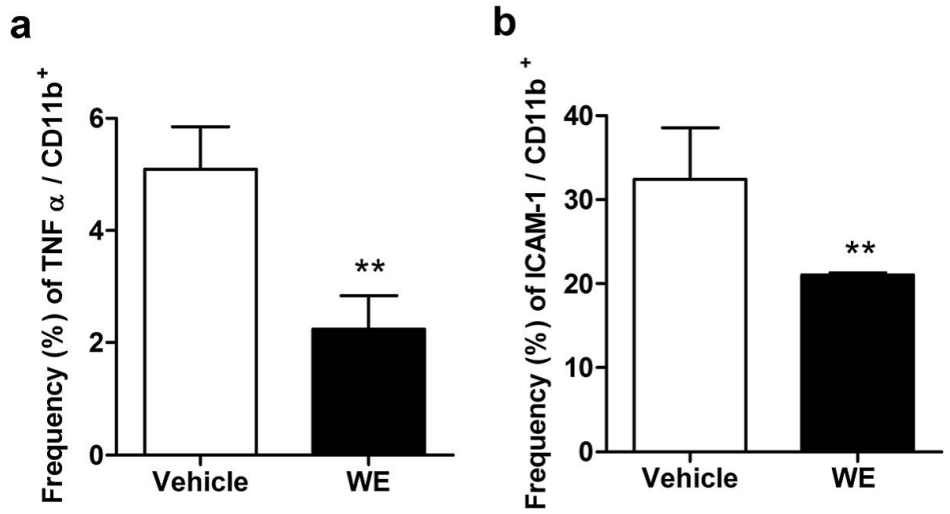


Figure 5.1. Inflammatory response in the spleen is attenuated by WE thrombin treatment of EAE.

In a mouse model of EAE, mice were treated with WE thrombin and the influx of macrophages was measured, gated as the frequency in the spleen of CD11b+ cells that were also positive for (A) TNFα- or (B) ICAM-1-positive. Data are the mean ±SEM, n=3. Figure was adapted from ©Verbout et al., 2015, originally published in Metabolic Brain Disease 2015 Feb 30(1): 57-65. Reprinted with permission from Springer.

A previous study demonstrated fibrinogen and other procoagulant molecules were present in EAE, showing that in MS the autoimmune response increases the permeability of the BBB, allowing blood components into the CNS and potentiating inflammation. Thus, we hypothesized that WE thrombin administration in EAE would reduce the influx of fibrin(ogen) in the CNS. We assessed fibrin(ogen) accumulation in spinal cord sections from vehicle- and WE thrombin-treated EAE mice by immunofluorescence microscopy. As seen in Figure 5.2, a reduction in positive staining for fibrin(ogen) was observed in WE thrombin-treated mice, suggesting that activation of endogenous protein C reduces the influx of coagulation proteins into the CNS, potentially by preserving the integrity of the endothelium.

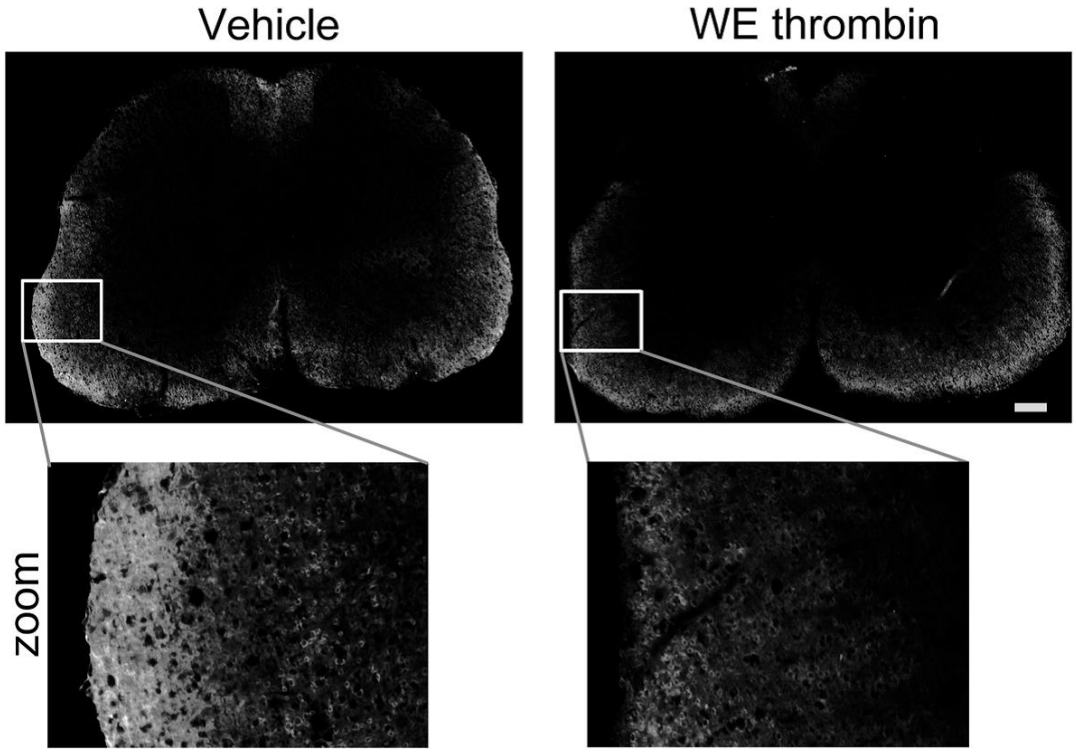


Figure 5.2. WE thrombin treatment reduces fibrin accumulation in the spinal cord.

Spinal cords sampled from the lumbar region of one representative animal per treatment group were fixed, paraffin embedded, sectioned and rehydrated. Fibrin(ogen) deposition was detected using a rabbit polyclonal antibody against fibrin(ogen). Sections were photographed under a fluorescent microscope under 20× and images processed under identical conditions. Images were compiled in Photoshop to create complete construction of the entire spinal cord section. Shown are representative photographs of lumbar spinal cords labeled with an antibody to fibrin(ogen) treated with either vehicle (left) or WE thrombin (right). Scale bar is 100 μm. Figure was adapted from ©Verbout et al., 2015, originally published in Metabolic Brain Disease 2015 Feb 30(1): 57-65. Reprinted with permission from Springer.

As the role of innate immune cell activation in MS remains ill-defined, future experiments will determine whether neutrophil activation and death are reduced in response to WE thrombin administration in EAE. First, plasma levels of MPO will be measured, and neutrophil counts in the spleen and brain will be determined by flow cytometry. Although a correlation has been observed between MS severity in patients and serum levels of NETs measured by the MPO-DNA complex [185], it remains unknown whether WE thrombin administration in EAE would reduce neutrophil activation. In light of our results presented in Chapter 4 demonstrating that APC can inhibit NETosis, it is hypothesized that WE thrombin administration will reduce NETosis indirectly via activation of endogenous protein C to APC to cause a

decrease in immunothrombotic markers in the CNS of the EAE model. We will measure the serum levels of MPO-DNA complexes in order to determine if there is any change in neutrophil cell death. Brain and spinal cord sections will be stained for the NETs marker H3Cit and DNA, in addition to fibrinogen. This will determine whether NETs form in the CNS and if there exists colocalization between fibrinogen and NETs in EAE. Lastly, neutrophils will be purified from the blood of vehicle- and WE thrombin-treated EAE mice, plated on coverslips and stimulated with PMA to induce NETosis. This will test whether neutrophils from WE thrombin-treated mice can undergo similar NETosis as vehicle-treated animals. Overall, these *in vivo* experiments would determine whether WE thrombin has any functional effect on neutrophil function via activation of the endogenous protein C system.

The proposed studies would extend the results presented in Chapter 4 that examined the relationship between neutrophils and pharmacologic doses of APC and finally begin to address whether activation of endogenous protein C to APC can modulate neutrophil activation and survival. The results described herein and the future experiments will provide additional mechanistic insight into the dynamic relationship between innate immunity and thrombotic-related disease.

Clinical Implications. As basic research continues to identify possible antithrombotic targets to translate into clinical medicine, there are several criteria to be met. Biologic therapies must first demonstrate scalability. As formation of antibodies to biologic therapies is of major concern in patient populations, minimizing their occurrence must be addressed in therapy design by considering frequency, method and length of administration, as well as identifying the qualified personnel needed to treat and monitor efficacy in patients. In high risk thrombosis patients, treatment can be prolonged indefinitely. Given the emerging link between innate immunity and thrombosis, whether it is possible to retain both normal innate immune functions and hemostatic processes during treatment remains to be investigated; meeting this unmet need will help accomplish the larger goal of improving patient outcomes.

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Biographical Sketch

Laura Day Healy was born on September 2, 1990 in Tucson, Arizona to Kathy Day and Dennis Healy.

Laura attended Catalina Foothills High School and, upon graduation, enrolled at University of Portland (UP) in 2008. Throughout her undergraduate studies she was on the UP President's scholarship and on the UP Dean's List from 2010-2012. During her undergraduate studies, she became involved in research through being a teaching assistant at UP in the general and organic chemistry labs, as well as the genetics lab. She was a Murdock undergraduate research scholar in her final year of college, working in the lab of Dr. Cheryl Maslen at Oregon Health & Science University (OHSU). In May of 2012, she earned her Bachelor of Science degree in Biology.

Laura continued her education at OHSU, joining the Program in Molecular and Cellular Biology. She then joined the lab of Dr. Owen McCarty in April of 2013, joining the department of Cell and Developmental Biology. Laura's research has centered on determining the roles and crosstalk between coagulation and neutrophil function.

During her graduate studies at OHSU, she was awarded a Keystone Symposia Future of Science Fund scholarship, a Young Investigator award from the International Society for the Thrombosis and Haemostasis Standardization and Scientific Committee, and was a Student Presidential Award Finalist for the Society of Leukocyte Biology. In July 2014, Laura received research support from the Oregon Clinical & Translational Research Institute (OCTRI) TL1 fellowship. In September 2015, she received research support from the Interactions at the Microbe-Host Interface Training Grant (T32) for the remainder of her studies on neutrophils. Laura has presented her research in peer-reviewed journals and at conferences throughout the U.S. and Europe. Current publications and presentations are listed below:

Publications:

Peer-reviewed

1. Aslan JE, Phillips KG, **Healy LD**, Itakura A, Pang J, McCarty OJ. Histone deacetylase 6 (HDAC6)-mediated deacetylation of α-tubulin coordinates cytoskeletal and signaling events during platelet activation. *American Journal of Physiology: Cell Physiology* 2013 Dec;305(12):C1230-39. (Featured article with Editorial) PMCID: PMC3882361
2. Verbout NG, Yu X, **Healy LD**, Phillips KG, Tucker EI, Gruber A, McCarty OJ, Offner H. Thrombin mutant W215A/E217A treatment improves neurological outcome and attenuates central nervous system damage in experimental autoimmune encephalomyelitis. *Metab Brain Dis* 2015 Feb; 30(1):57-65. NIHMSID: 594013
3. Loren CP, Aslan JE, Rigg RA, Nowak MS, **Healy LD**, Gruber A, Druker BJ, McCarty OJ. The BCR-ABL inhibitor ponatinib inhibits platelet immunoreceptor tyrosine-based activation motif (ITAM) signaling, platelet activation and aggregate formation under shear. *Thrombosis Research* 2015 Jan;135(1):155-60. PMCID: PMC4272760
4. Baker-Groberg SM, Phillips KG, **Healy LD**, Itakura A, Porter JE, Newton PK, Nan X, McCarty OJT. Critical behavior of subcellular density organization during neutrophil activation and migration. *Cellular and Molecular Bioengineering* 2015 Dec;8(4):543-552. PMID: 26640599
5. Rigg RA, Aslan JE, **Healy LD**, Wallisch M, Thierhiemer ML, Loren CP, Pang J, Hinds MT, Gruber A, McCarty OJT. Oral administration of Bruton's Tyrosine Kinase (Btk) inhibitors impairs GPVI-mediated platelet function. *American Journal of Physiology: Cell Physiology* 2016 Mar 1;310(5):C373-80. PMID: 26659727
6. Rigg RA, **Healy LD**, Nowak MS, Mallet J, Thierhiemer ML, Pang J, McCarty OJ, Aslan JE. Heat shock protein 70 (Hsp70) regulates platelet integrin activation, granule secretion and aggregation.

American Journal of Physiology: Cell Physiology 2016 Apr; 310(7): C568-C575. PMID: 26764050 PMCID: PMC4824157 (Editor's Pick as Featured Article)

7. **Healy LD**, Puy C, Itakura A, Chu T, Robinson DK, Bylund A, Phillips KG, Gardiner EE, McCarty OJT. Colocalization of neutrophils, extracellular DNA and coagulation factors during NETosis: development and utility of an immunofluorescence-based microscopy platform. *Journal of Immunological Methods*. 2016 Aug;435:77-84.PMID: 27286714 PMCID: PMC4935600
8. **Healy LD**, Puy C, Fernández JA, Mitrugno A, Keshari RS, Taku N, Chu TT, Xu X, Gruber A, Lupu F, Griffin JH, McCarty OJT. Activated protein C inhibits neutrophil extracellular trap formation *in vitro* and activation *in vivo*. *Journal of Biological Chemistry*. Accepted April 13, 2017. *In Press*. PMID:28408624

Reviews, Editorials and Book Chapters

Jones CM, Baker-Groberg SM, Cianchetti FA, Glynn JJ, **Healy LD**, Lam WY, Nelson JW, Parrish DC, Phillips KG, Scott-Drechsel DE, Tagge IJ, Zelaya J, Hinds MT, McCarty OJ. Measurement science in the circulatory system. *Cellular and Molecular Bioengineering* 2014 Mar;7(1):1-14. NIHMSID: 551340

Conference Abstracts:

1. **Healy LD**, Itakura A, Wong ZC, Gailani D, Tucker EI, Gruber A, McCarty OJ, "Contact activation supports prothrombotic effect of neutrophil extracellular traps (NETs)", *New Frontiers in the Discovery and Treatment of Thrombosis*, Keystone Conference, Keystone, CO (Jan, 2014). [Poster & Oral Presentation]
2. Verbout NG, Yu X, **Healy LD**, Phillips KG, Tucker EI, Gruber A, McCarty OJ, Offner H, "Thrombin mutant W215A/E217A treatment improves neurological outcome and attenuates central nervous system damage in experimental autoimmune encephalomyelitis", *New Frontiers in the Discovery and Treatment of Thrombosis*, Keystone Conference, Keystone, CO (Jan, 2014). [Poster]
3. Loren CP, Rigg RA, Aslan JE, **Healy LD**, Gruber A, McCarty OJ, "The prothrombotic profile of BCR-ABL inhibitors ponatinib, nolotinib and imatinib", *Arteriosclerosis, Thrombosis and Vascular Biology Scientific Sessions*, Toronto, Canada (May, 2014). [Poster]
4. **Healy LD**, Robinson DK, Chu TT, Oldenkamp H, Puy C, Gruber A, McCarty OJ, "Rapamycin increases the thrombogenic potential of neutrophil extracellular traps (NETS): potential mechanism of prothrombotic drug-eluting stents", *British Society for Haemostasis & Thrombosis*, Edinburgh, Scotland, UK (Oct, 2014). [Poster]
5. Baker-Groberg SM, Phillips KG, **Healy LD**, Itakura A, Porter JE, Newton PK, Nan X, McCarty OJT. "Critical behavior of subcellular density organization during neutrophil activation and migration." *International Society for Thrombosis and Hemostasis*, ISTH 2015 Congress, Toronto, CA (June, 2015). [Poster]
6. **Healy LD**, Puy C, Fernández JA, Chu T, Gruber A, Griffin JH, McCarty OJT. "Activated protein C (APC) down regulates neutrophil extracellular traps (NETs): potential contributing mechanism for anti-inflammatory and antithrombotic effects of APC." *Scientific and Standardization Committee (SSC)*, ISTH SSC 2016, Montpellier, France (May, 2016). [Featured Abstract; Poster & Oral Presentation]
7. Chu T, **Healy LD**, Itakura A, Robinson DK, Bylund A, Phillips KG, Gardiner EE, McCarty OJT. "Development of a platform to quantify the colocalization of neutrophil extracellular DNA and coagulation factors using immunofluorescence-based microscopy." *Scientific and Standardization Committee (SSC)*, ISTH SSC 2016, Montpellier, France (May, 2016). [Poster]
8. **Healy LD**, Puy C, Fernández JA, Taku N, Chu TT, Gruber A, Griffin JH, McCarty OJT. "Activated protein C (APC) binds leukocytes and signals to inhibit neutrophil extracellular trap formation; possible additional mechanism for anti-inflammatory and cytoprotective effects of APC." Joint

Meeting: *Society for Leukocyte Biology (SLB) and Neutrophil Conference*, SLB & Neutrophil 2016, Verona, Italy (September, 2016). [Presidential Award Finalist; Poster & Oral Presentation]

9. Rigg RA, Mitrugno A, **Healy LD**, Ngo ATP, Duvernay MT, Hamm HE, Gruber A, McCarty OJT. "Role of Protease-Activated Receptor 4 in Regulating Platelet-Leukocyte Interactions in Whole Blood." *International Society on Thrombosis and Haemostasis*, Berlin, Germany (July, 2017). [Poster]

Undergraduate Research Mentor

1. Tiffany T Chu (2014-2015): Currently enrolled in her first year at Johns Hopkins University, Baltimore, MD in Biomedical Engineering.
2. D. Kyle Robinson (2014): Currently an OHSU Med4 Student, matched in anesthesiology fellowship program at OHSU.
3. Alan Bylund (2015): Currently graduated with Master's in Biomedical Engineering from University of Portland.
4. Nyiawung Taku (2016): Currently graduated with Master's in Biomedical Engineering from University of Portland.

Teaching

Lecturer, ME461/561, Biomaterials (University of Portland), Spring 2014