THE ROLE OF THE PLATELET-ENDOTHELIUM INTERFACE AND REGULATION OF PLATELET FUNCTION

By

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CERTIFICATE OF APPROVAL

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List of Abbreviations

ADAMTS-13	a disintegrin and metalloproteinase with a thrombospondin type 1 motif, member 13
APC	activated protein C
ATP	adenosine triphosphate
ADP	adenosine diphosphate
BM	basement membrane
cAMP	cyclic adenosine monophosphate
DIC	differential interference contrast
DNA	deoxyribonucleic acid
EC	endothelial cell
ECM	extracellular matrix
FV	coagulation factor V
FVII	coagulation factor VII
FVIII	coagulation factor VIII
FIX	coagulation factor IX
FX	coagulation factor X
FXI	coagulation factor XI
FXII	coagulation factor XII
GPCR	G-protein coupled receptors
GPV	glycoprotein V
GPVI	glycoprotein VI
GPIX	glycoprotein IX
GPIb	glycoprotein Ib
HUVEC	human umbilical vein endothelial cell

IKK	IκB kinase
ITAM	immunoreceptor tyrosine activation motif
NFκB	nuclear factor κB
NO	nitric oxide
eNOS	endothelial nitric oxide synthase
PAR	protease activated receptor
PDMS	polydimethyl siloxane
PGI_2	prostacyclin
PI3K	phosphoinositide 3-kinase
PLC	phospholipase C
PS	phosphatidylserine
RGD	Arg-Gly-Asp motif
SFK	Src family kinase
TF	tissue factor
TNF-α	tumor necrosis factor α
TM	thrombomodulin
TxA_2	thromboxane A_2
ULvWF	ultra-large von Willebrand Factor
VE-cadherin	vascular endothelial cadherin
vWF	von Willebrand Factor

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Dedication

I dedicate this Thesis to my parents Doug and Claudia. Without your sacrifice and love this would never have been possible. To my brother and sisters, you have always been there for me both as a friend and a role model. May this serve as our accomplishment, the first of many.

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Most of all, I dedicate this to my daughter Luna, may you always know the work that your parents and family have accomplished. Let this be an inspiration to you, so that you may achieve any dreams you pursue. We will always love you beyond all else.

Abstract

Platelets are the first responders to vascular injury, playing a critical role in the formation of a hemostatic plug to stop and prevent blood loss. This process requires a complex sequence of mechanisms that is initiated by exposed extracellular matrix and damaged endothelial cells and results in platelet adhesion, activation, and aggregation at the site of vascular injury. Platelet binding of extracellular matrix proteins is required for formation of platelet aggregates, and their interaction may have significant impact on platelet function in inflammation and thrombosis. The following studies identify the role of the extracellular matrix protein nidogen-1 in adhesion and activation of platelets and provide rationale for the development of microfluidic platforms for investigation of the platelet endothelium interface.

The focus of this research was to characterize the interaction between nidogen-1 and platelets, demonstrating that nidogen-1 surfaces support platelet adhesion and spreading *in vitro*. Platelet inhibition studies revealed that platelet adhesion on and activation by nidogen-1 surfaces induces glycoprotein VI (GPVI) mediated signaling and platelet spreading and suggests that platelets may adhere to nidogen-1 via an additional platelet receptor. Furthermore, nidogen-1 induced platelet activation of platelets adhered to von Willebrand Factor (vWF) during whole blood perfusion, leading to platelet aggregation under shear flow. These results confirm that GPVI is a redundant receptor for extracellular matrix proteins that contributes to platelet activation and aggregation and implicates GPVI may play a role in thrombus formation.

In conclusion, these studies identify nidogen-1 as a new ligand of platelet GPVI and provide evidence for utilization of more physiologic *in vitro* models for the investigation of platelet function in pathological settings. Future work will elucidate the signaling pathway and

mechanism driving platelet activation and spreading on nidogen-1 surfaces, as well as further the development of an endothelialized microfluidic chamber for investigation of the platelet-endothelium interface under physiologic conditions to better understand the contribution of blood cells, endothelial cells, and extracellular matrix proteins in hemostasis and thrombosis.

Chapter 1. Introduction to the Platelet-Endothelium Interface and its Role in Hemostasis and Thrombosis

1.1 Overview

The human vasculature is a closed network of blood vessels that extends throughout the body, pumping roughly 5 liters of blood to tissues and organs and supplying oxygen and nutrients to cells. Vital to this function is the regulation of blood flow and vascular tone by a layer of endothelial cells that respond to biological, chemical, and mechanical stimuli.[1, 2] Due to these stimuli, blood vessels regularly experience damage that causes gaps, tears, or more traumatic injuries to the endothelial cell layer and results in bleeding. Blood cells and proteins quickly respond to signs of damage to stop bleeding and to allow the blood vessel to regain vascular integrity. This process is known as hemostasis and is tightly regulated by endothelial cells, blood cells, and plasma proteins. An integral component of hemostasis are platelets, small blood cells that respond to vascular injury by creating a seal to prevent blood loss. Platelets are anucleate cells that have a lifespan of 7-10 days, wherein they are constantly surveying the blood vessel wall for damage. Platelets are responsible for forming a hemostatic plug at the site of vascular injury by adhering to the extracellular matrix (ECM) or stimulated endothelium and activating intracellular signaling pathways.[3] Activation of these pathways promotes activation of additional platelet receptors and release of secondary mediators for recruitment and activation of circulating platelets. After an initial layer of platelets forms at the site of injury, recruited platelets adhere on top of this layer and begin to aggregate together via integrin $\alpha_{IIb}\beta_3$. This step is crucial in the formation of a stable clot or thrombus, as it must be able to resist the shear stress generated by the flowing blood. During hemostasis, platelets will employ multiple receptors for adhesion and activation of intracellular processes, to work in tandem with the coagulation cascade to effectively limit blood loss and mediate further vascular damage.

The platelet-endothelium interface plays an important role in regulating the interaction between the blood and the surrounding tissue. Endothelial cells and the underlying extracellular matrix maintain barrier integrity of blood vessels while platelets circulate throughout the body ready to respond to vascular injury.[4] Circulating platelets survey the surface of the blood vessel wall in order to quickly respond to signs of vascular damage to seal the blood vessel and limit further blood loss. Due to a phenomenon called platelet margination, platelets are forced to the outer edges of flowing blood, resulting in a concentrated layer of platelets near the surface of endothelial cells.[5] This allows platelets to quickly react to vascular damage, but also requires active prevention of platelet adhesion and aggregation under healthy conditions. In the event of vascular injury, platelets quickly adhere to the endothelium/ECM, inducing activation and aggregation to form a platelet plug. In the following sections, the process of hemostasis and thrombosis will be examined with a focus on the contribution of the platelet-endothelium interface, in particular the role of ECM proteins in platelet function. Specifically, we will consider the ECM protein nidogen-1 and its interactions in the basement membrane and conclude with a thesis overview.

1.2 Hemostasis

Hemostasis is the physiological response to blood vessel injury that stops and prevents blood loss into the surrounding tissue. This tightly controlled process is dependent on platelet adhesion and aggregation and activation of the coagulation cascade for the formation of fibrin.[6] Platelets and proteins of the coagulation cascade work in concert for the formation and stabilization of a platelet plug, eventually forming a fibrin rich clot which allows for vascular repair. The initial response to vascular injury is dependent on platelet adhesion and aggregation to a vessel wall for formation of a platelet plug. This requires platelet receptor binding to exposed ECM proteins and

activation by soluble agonists generated in response to the injury.[7] The exposure of the ECM and the cells within the ECM initiates the coagulation cascade, a series of enzymatic reactions that leads to the formation of fibrin. Coagulation factors are proteins involved in the coagulation cascade and are found in two forms, (1) an inactive proenzyme form and (2) an active enzyme generated by an upstream activated factor. Initial activation of the coagulation cascade involves recognition of either tissue factor (TF) or negatively charged surfaces and results in rapid activation of downstream factors.

Upon vascular injury, endothelial cells are damaged or removed and the subendothelium is exposed. This introduces coagulation factors to the potent coagulation cascade activator TF.[8] The initial activation of coagulation factors is amplified through a positive feedback mechanism that converges on thrombin generation and culminates in fibrin formation for stabilization of the growing blood clot. Under normal hemostatic settings, while the endothelium is healing, coagulation factors are inhibited to help dissolve the clot and prevent excessive clot formation. It is critical to regulate clot formation by limiting excessive thrombin generation which can lead to aberrant blood coagulation and fibrin formation.

1.2.1 Coagulation Cascade

Hemostasis depends on the enzymatic activity of the coagulation cascade to rapidly generate thrombin for both platelet activation and fibrin formation. This is achieved through two different pathways, the extrinsic or TF pathway and the intrinsic or contact pathway, that are triggered by different initiators and converge on factor (F) X activation. Together with activated FX (FXa), thrombin and fibrin represent the common pathway of coagulation. Fibrin, or FIa, is the main product of the coagulation cascade and is polymerized with other fibrin strands by FXIIIa,

forming a fibrin mesh to reinforce platelet aggregates. Inactive FI, or fibrinogen, is one of the most concentrated blood proteins and is activated via thrombin-mediated cleavage.[6]

The TF pathway is initiated by the binding of TF with FVII. TF is an integral membrane protein that is expressed by cells in the vascular adventitia and other layers of the ECM that do not make contact with blood.[9] Vital to the coagulant activity of TF is its anchoring to the cell membrane, where it is able to sequester FVII or FVIIa.[10, 11] The formation of the TF-FVIIa complex leads to local activation of FIX and FX, both of which require interaction with their own cofactors, FVIIIa and FVa which are activated by thrombin.[8] FVIIIa binds FIXa to form the tenase complex which converts additional FX into FXa. FXa is then able to bind FVa, forming the prothrombinase complex which converts prothrombin into thrombin. Thrombin enhances this series of reactions by inducing activation of FXI and the feedback loop which increases FIXa and FXa activity, further propagating thrombin generation.

In contrast to the TF pathway, the contact pathway is dependent on the activation of FXII by interaction with negatively charged surfaces, such as deoxyribonucleic acid (DNA) or inorganic polyphosphate molecules, or foreign material like glass or kaolin.[12] This generates a limited amount of FXIIa that activates prekallikrein (PK) to form kallikrein and induces a reciprocal activation feedback loop by further activating FXII.[13] This leads FXIIa to convert FXI into FXIa, leading to the activation of FIX and formation of the tenase complex (FVIIIa-FIXa). Deficiency of FXI is associated with only a slight bleeding tendency, while FXII deficiency does not exhibit a bleeding phenotype, demonstrating that the contact pathway is not required for normal hemostatic function. However, FXII deficiency is associated with a reduced risk for thrombotic complications.[14, 15] Because of these observations, it has been suggested that inhibition of the contact pathway may safely protect against thrombosis without affecting proper hemostatic response via the TF pathway. Despite their role in the coagulation cascade, the

proteins of the contact pathway are implicated to have a larger role in inflammation and immunity.

A critical component for hemostasis and fibrin clot formation is the serine protease prothrombin (FII) that is cleaved into its active form thrombin (FIIa), which functions as a vital intermediate in both the coagulation cascade and platelet activation. Thrombin is responsible for the generation of insoluble fibrin from soluble fibrinogen and catalyzes the activation of coagulation factors FXI, VIII, V, and XIII as part of the feedback mechanism mentioned earlier. Furthermore, thrombin is a potent platelet agonist that binds to protease-activated receptors (PAR) 1 and PAR4 found on the platelet membrane.[16, 17] This leads to enhanced platelet activation and aggregation due to the release of soluble agonists. Regarded as the most potent platelet agonist, thrombin is a mediator of platelet shape change, secretion, release of thromboxane A_2 (TxA_2), localization of P-selectin and CD40 to the membrane, and most importantly, activation of $\alpha_{IIb}\beta_3$.[18] The conversion of prothrombin to thrombin is a key mechanism of the physiology of blood and the occurrence of abnormal levels of prothrombin are associated with pathological coagulation. Hypoprothrombinemia, or a deficiency of prothrombin limits the ability of blood to clot properly, while hyperprothrombinemia, or an abundance of prothrombin, leads to excessive coagulation.[19, 20]

1.3 Endothelial Cell Biology and the Regulation of Hemostasis

Vascular endothelial cells form the innermost layer of blood vessels and separate the blood from the surrounding tissue. This monolayer of endothelial cells, or endothelium, exhibits many functions, most notably maintaining proper blood flow and nutrient delivery, controlling barrier permeability, and preventing platelet activation and aggregation. Vital to these functions is the ability of the endothelium to maintain cell-cell interactions sealed via intracellular binding

complexes, such as tight junctions and adheren junctions. These junctional proteins support cell-cell interactions both structurally and functionally, for example vascular endothelial cadherin (VE-cadherin) molecules are involved in both actin-cytoskeletal rearrangements and angiogenesis.[21, 22] Inflammatory cytokines such as tumor necrosis factor α (TNF-α), can stimulate endothelial cells and activate intracellular signaling that compromises the regulation of intercellular junctions, causing gap formations and increasing permeability. Although the permeability is often temporary, chronic or prolonged stimuli can have detrimental effects and cause diseases such as chronic inflammation, edema, or sepsis.

In healthy blood vessels, blood is pumped through arteries and veins, supplying nutrients to tissues and transporting blood cells. An important role of the endothelium in regulating blood flow is to prevent the adhesion of blood cells. This protective function is supported by the non-adhesive glycocalyx membrane found on the luminal side of the endothelium. The vascular glycocalyx is composed of membrane-bound glycoproteins and glycolipids that form a non-adhesive network on the surface of endothelial cells. The glycocalyx plays a major role in preventing the adhesion of circulating blood cells to the endothelium. The glycocalyx is also involved in mechanotransduction and nitric oxide (NO) expression of the endothelium, aiding in the response to shear stress changes due to flowing blood.[2] Additionally, the polysaccharide heparan sulfate, a main component of the glycocalyx, is able to activate antithrombin and inhibit the activity of coagulation proteases.[23, 24] As the initial layer in contact with flowing blood, the glycocalyx has a significant role in regulating barrier integrity and permeability, along with the endothelium. Degradation of the glycocalyx can cause endothelial cell stimulation and dysfunction, resulting in expression of adhesion molecules that induce binding of blood cells and increased permeability.[25] Loss of the glycocalyx and these protective properties has been associated with diseases such as diabetes and atherosclerosis.[26]

As another measure to prevent blood cell adhesion, healthy endothelial cells do not have ECM proteins present on the luminal membrane. Rather, endothelial cells store ECM proteins in intracellular bodies known as Weibel-Palade bodies or they secrete these proteins from their basal membrane. The endothelial layer is attached to and further supported by the basement membrane, a layer of extracellular matrix proteins that form a thin layer around the blood vessel. Consistent with other basement membranes, the vascular basement membrane contains laminins, type IV collagen, nidogens and similar ECM proteins. However, endothelial cells and vascular basement membranes also play a unique role in hemostasis by preventing blood loss through the presence and function of von Willebrand Factor (VWF).[27, 28]

1.3.1 Regulation of platelet activity and blood flow

Vascular endothelial cells actively prevent platelet adhesion through secretion of platelet inhibitors, degradation of platelet agonists by ectonuleotidases, and most importantly, regulation of vascular tone via nitric oxide (NO) release (Figure 1). Vascular endothelial cells release NO, also known as endothelium-derived relaxing factor (EDRF), as a signaling molecule to relax surrounding smooth muscle cells and to inhibit platelet aggregation.[29] Vascular production of NO is mainly regulated by the activity of endothelial nitric oxide synthase (eNOS) and the supply of precursor molecules such as L-arginine or L-citrulline. NO is also a highly reactive biomolecule involved in both paracrine and autocrine signaling of endothelial cells as it is able to diffuse across cell membranes. Once synthesized, NO is quickly consumed in an enzymatic reaction or degraded by free radicals. Low levels of NO can lead to constriction of blood vessels causing hypertension. Under shear flow conditions, NO is able to inhibit platelet adhesion to endothelial cells and the ECM.[4] Upon production and release from endothelial cells, NO enters the bloodstream and passively diffuses across platelet membranes where it binds to soluble guanylyl cyclase.[30] Binding of NO to soluble guanylyl cyclase leads to an upregulation of

cyclic guanosine monophosphate that activates protein kinase G. Platelet activity is diminished by protein kinase G via phosphorylation of key proteins that provide Ca^{2+} for platelet activation.[31] NO further inhibits platelet activation and aggregation by diminishing platelet response to adenosine diphosphate (ADP) and TxA_2 and which in turn limits dimerization of platelet integrins, particularly $\alpha_{IIb}\beta_3$ and $\alpha_2\beta_1$.[32]

The endothelium also secretes prostacyclin (PGI_2) which has been demonstrated to be an inhibitor of ADP-induced platelet activation.[33] It has been suggested that PGI_2 and TxA_2 work to establish vascular balance by employing competing functions. Binding of PGI_2 to its receptor induces adenylyl cyclase production of cyclic adenosine monophosphate (cAMP), inhibiting platelet activation. Additionally, cAMP can mediate the calcium release due to TxA_2 binding, further inhibiting platelet activation. Alternatively, PGI_2 is also able to bind to its receptor on endothelial cells leading to generation of cAMP. cAMP activates protein kinase A and leads to smooth muscle cell relaxation and vasodilation through phosphorylation and inhibition of myosin light chain kinase.

Ectonucleotidases are enzymes that metabolize nucleotides and are expressed on the membrane of endothelial cells. They prevent unnecessary platelet activation by metabolizing the platelet agonists adenosine triphosphate (ATP) and ADP into AMP, preventing activation of the ADP receptors P2Y1 and P2Y12 that lead to activation of phospholipase C and downregulation of cAMP formation.[34-36] In particular, endothelial cells express the membrane ectonucleotidase cluster of differentiation (CD) 39 that neutralizes the activity of ADP and is capable of inhibiting platelet activation in the absence of PGI_2 and NO.[37] Studies have demonstrated that CD39 also functions in a thromboprotective manner against stroke.[38]

Endothelial cells express protease activated receptor PAR1, PAR2, and PAR4 in significant quantities, wherein recognition of thrombin, or typsin in the case of PAR2, induces procoagulant activity of endothelial cells via the release of vWF and the expression of TF and P-selectin.[39-41] This activity leads to the platelet and leukocyte adhesion. In addition, endothelial cells undergo morphological changes leading to gap formation and increased permeability upon PAR1 activation by thrombin. [42-44] In addition to activating platelets, thrombin also interacts with endothelial cells, binding to the receptors PAR1 and PAR2.[40] Binding of thrombin to PAR1 increases phospholipase activity and intracellular Ca^{2+} levels. Endothelial cells will also express adhesion proteins which promote white blood cell adhesion. Endothelial cells additionally express thrombomodulin on their surface to inhibit the coagulation factor thrombin, which activates platelets through the PAR1 and PAR4 receptors. Thrombomodulin binds thrombin at a 1:1 ratio and acts as a cofactor for the activation of protein C.[45] Binding of the two proteins leads to the activation of protein C (APC), which further inhibits activity of the coagulation cascade by inactivating FVa and FVIIIa. Formation of thrombin-thrombomodulin complex significantly increases the rate of protein C activation and negates the procoagulant activity of thrombin.

The endothelium functions as a vital barrier in many physiological processes centered around blood flow. Of these, the role of the endothelium in hemostasis is of the utmost importance as dysregulation or loss of the functions discussed above can lead to cardiovascular disease and thrombotic complications (atherosclerosis, stroke, stent/graft implants). Endothelial dysfunction is generally characterized by a reduction in the bioavailability of NO and as a result the endothelium is unable to regulate blood flow properly. Endothelial dysfunction is a major risk factor for cardiovascular disease. Exposure of endothelial cells to thrombin, lipopolysaccharide, or cytokines induces the expression and localization of TF at the luminal surface and increases the risk of thrombotic complications. [1, 46]

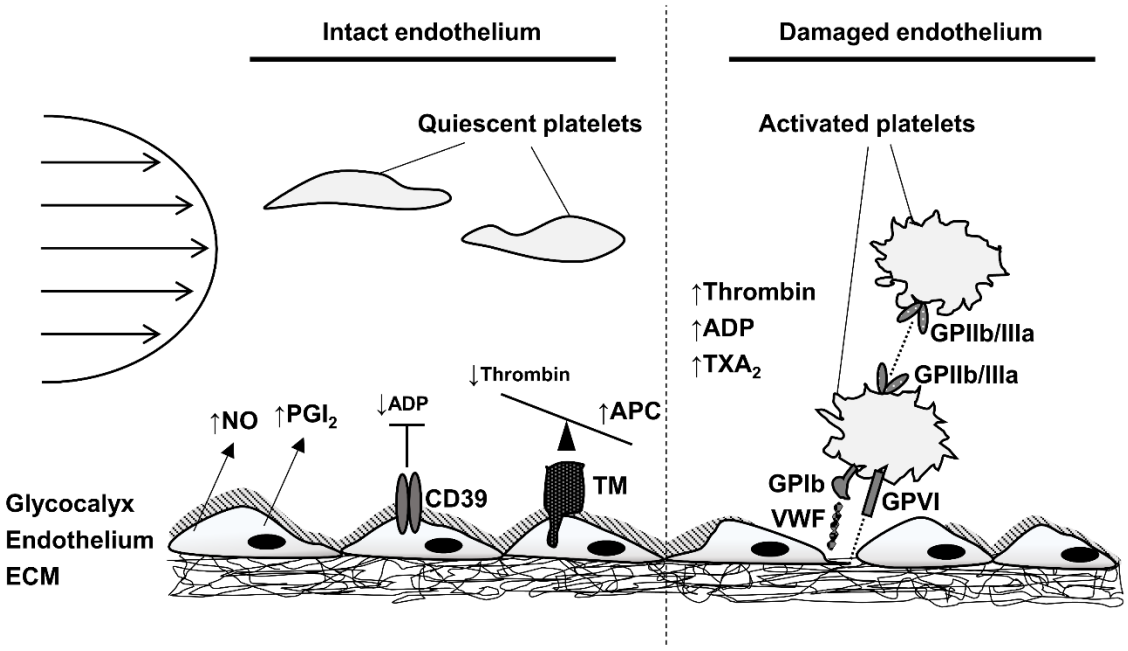


Figure 1. The Platelet-Endothelium interface

Under healthy conditions (left), platelet adhesion and activation are suppressed by an intact and functional endothelium that secretes platelet inhibitors nitric oxide (NO) and prostacyclin (PGI_2) and expresses membrane proteins CD39 and thrombomodulin (TM) to inactivate soluble platelet agonists. The glycocalyx is an anti-adhesive layer that covers the endothelium preventing platelet adhesion. Once damaged (right), platelets sense the exposed subendothelium and ECM proteins and begin to adhere to von Willebrand Factor (vWF) and collagen via glycoprotein Ib (GPIb) and GPVI. Platelets become activated and secrete soluble agonists thrombin, ADP and TxA_2 (TA_2) for secondary activation and recruitment of additional platelets that leads to GPIIb/IIIa mediated platelet aggregation at the site of injury. Figure was adapted from ©Zilberman-Rudenko et al., 2017, originally published in Platelets 2017 Mar 30:449-456. Reprinted with permission from Taylor & Francis Group.

1.4 Extracellular Matrix

1.4.1 ECM biology

In human tissue, the ECM is responsible for providing structural and functional support through the formation of a three-dimensional protein network. Within this network are multiple layers of varying stiffness and elasticity composed of proteins, mostly collagens, and glycoproteins.[47]

Although different ECMs have developed to fulfill specific roles dependent on their cellular microenvironment, the ECM is generally involved in cellular functions such as adhesion, differentiation, and migration. Most importantly, the ECM regulates intercellular communication and separates tissues. The formation of the ECM is a dynamic process in which proteins are secreted and self-assemble into insoluble cell scaffolding.

In blood vessels, the ECM lays beneath the endothelium supporting separation of blood from the surrounding tissue. The endothelium directly interacts with and is secured by a thin, sheet-like barrier of ECM proteins called the basement membrane (BM). Individual endothelial cells are bound through interactions with the basement membrane which contains laminins, type IV collagen, perlecan, and nidogens. Although the protein composition varies depending on the vasculature, nidogen-2 and laminins 8 and 10 are the main isoforms found in the vasculature.[48] Unique to vascular basement membranes is the presence of vWF, which plays a vital role in hemostasis.

Blood vessels are also secured by cells beyond the basement membrane (Figure 2). In smaller blood vessels, pericytes and endothelial cells share the same basement membrane, whereas larger vessels are supported by smooth muscle cells found within their own ECM.[49] As such, arteries are composed of more smooth muscle cells than veins to accommodate the force exerted by the flowing blood. In addition, beyond the smooth muscle cells, arteries have an additional adventitia layer containing fibroblasts, fibrillar collagens, and more ECM proteins. Veins and smaller vessels are also surrounded by an extra layer of ECM proteins similarly containing fibrillar collagens, fibronectin, and other ECM proteins. In the event of vascular injury, blood cells are exposed to a range of ECM proteins that is determined by the nature and depth of injury, as well as the type of blood vessel injured.

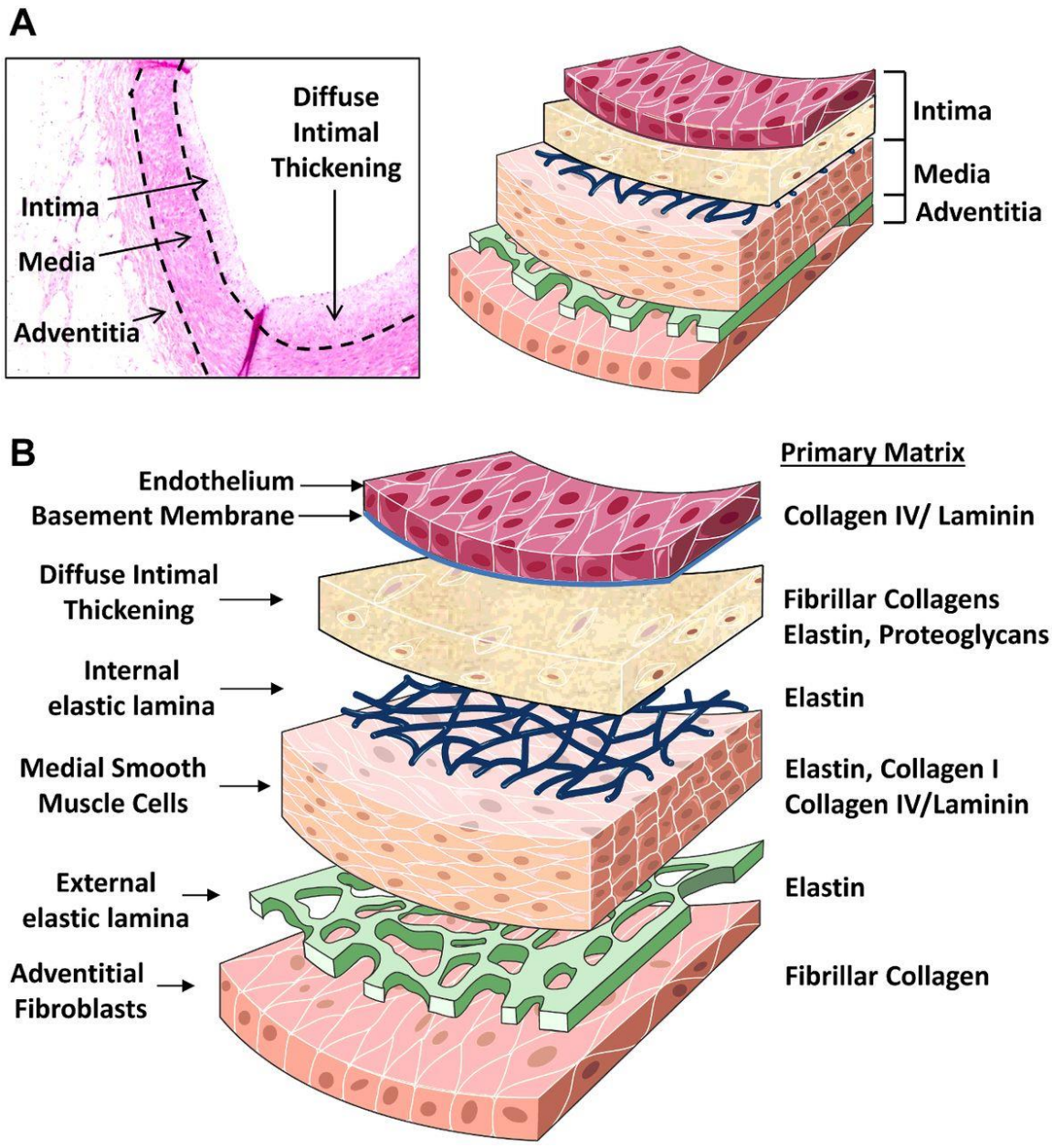


Figure 2. Vascular Extracellular Matrix

Representation of the human arterial ECM defining the intima, media, and adventitia with dashed lines indicating the internal and external elastic lamina (A). An expanded view of the vascular ECM and the main protein components supporting each layer (B). Figure was adapted from ©Yurdagul et al., 2016, originally published in Biochemical Journal 2016 May 11: 1281-1295. Reprinted with permission from Portland Press Limited.

1.4.2 Types of ECM proteins in the vasculature

Laminins are glycoproteins that are required for formation of BM, where they form heterotrimeric conformation composed of α-β-γ subunits which are joined by a long coiled-coil domain. In mammals there exist 16 laminin isoforms.[50, 51] The self-assembly process involves binding to the cell surface via integrins and dystroglycans, allowing laminins to adhere and polymerize into an initial scaffold.[52] Laminins offer the most binding of ECM proteins as their C-terminal laminin-type globular domain interacts with integrins $\alpha_3\beta_1$, $\alpha_6\beta_1$, $\alpha_7\beta_1$, and $\alpha_6\beta_4$. Subsequently, laminin scaffolds recruit other BM proteins for further polymerization, strengthening the growing matrix.

Laminin has been determined to interact with platelets by binding to the platelet receptors integrin $\alpha_6\beta_1$ and glycoprotein VI (GPVI).[53, 54] The adhesive interaction of laminin and platelets exhibits similar mechanisms to that of collagen-induced platelet adhesion. Initial binding of $\alpha_6\beta_1$ to laminin is required for GPVI binding, in contrast to collagen which interacts with integrin $\alpha_2\beta_1$ after GPVI binding. In addition, GPVI exhibits a 10-fold decrease in affinity for laminin as compared to collagen. This difference of binding affinity between collagen and laminin is also evident by the absence of platelet aggregation in suspension with laminin. In contrast, collagen induces significant aggregation and tyrosine phosphorylation of platelets in suspension, indicative of GPVI activation. Furthermore, the absence of GPVI reduced platelet spreading on laminin surfaces but did not affect platelet adhesion.

Similar to collagen, laminin has been shown to mediate platelet adhesion under flow and is strongly influenced by the interaction of vWF and platelet GPIb-IX-V.[55] This mechanism of platelet adhesion to laminin and collagen involves vWF binding to substrate, GPIb-IX-V

engagement, GPVI activation and integrin binding, which leads platelet aggregation and thrombus formation.[56]

Collagen is the most abundant protein in the body and serves as a key structural component of human tissue. There are many collagens present in the vascular ECM such as collagens type I, III, IV, V, VIII, and XVIII. These are divided into fibrillar collagens (type I, III, and V) or network-forming collagens (type IV, VIII, and XVIII). Fibrillar collagens are found in the outermost layers of the ECM, while the network-forming collagens provide structure to basement membrane.

Of interest to cardiovascular researchers is collagen type IV, which is located proximal to the endothelium and is easily accessible to blood cells in the event of a vascular injury. Collagen IV is responsible for providing stability to the basement membrane, but is not required for matrix assembly.[57] It has also been documented that collagen IV is able to support binding of both nidogen and the nidogen-laminin complex, however, laminin and degraded fragments of laminin were unable to bind to collagen IV.[58] Despite strong evidence demonstrating the potent activation of the platelet receptor GPVI by fibrillar collagens, these proteins require a more significant injury to be exposed to blood.[59] Type IV collagen does not form fibrils, instead it creates a large polymeric network that binds nidogens and other ECM proteins in the basement membrane. GPVI activation by type IV collagen has also been documented, however results are not consistent regarding the degree of GPVI activation induced.[60]

GPVI is a transmembrane platelet receptor for collagen, binding the Gly-Pro-Hyp peptide sequence, and is a member of the immunoglobin superfamily. It is a 60-65 kDa protein composed of two immunoglobulin domains, a mucin-rich stalk and a cytosolic sequence.[61] It is localized

in the membrane with a disulfide-linked Fc receptor (FcR) γ-chain homodimer that forms a salt-bridge between the transmembrane and cytosolic sequences of GPVI for signal transduction. GPVI contains motifs within its cytosolic tail for binding to calmodulin and the SH3 domain of Src family tyrosine kinases (SFK).[62] Additionally, the FcR γ-chain contains one copy of the immunoreceptor tyrosine-based activation motif (ITAM) that upon crosslinking of GPVI is phosphorylated at two tyrosine residues. Crosslinking of GPVI by binding to ECM proteins induces Src kinase-dependent tyrosine phosphorylation of the FcR γ-chain ITAM by two Src family kinases (SFK), Fyn and Lyn. Fyn and Lyn are bound to the GPVI cytosolic tail and upon crosslinking come into contact with the FcR γ-chain ITAM. This action leads to the recruitment and activation of the tyrosine kinase Syk. Syk undergoes autophosphorylation and phosphorylation by Src kinases upon binding to the phosphorylated ITAM, and subsequently initiates a downstream signaling cascade leading to platelet activation.[63] Vital to the signaling cascade is the formation of the LAT signalosome that is composed of a series of adapter and effector proteins necessary to regulate one of the major effector enzymes in the GPVI signaling cascade, phospholipase C (PLC) γ2, responsible for platelet secretion, aggregation and thrombus formation.[64] These functions are regulated by calcium and diacylglycerol produced by PLCγ2 activation and lead to platelet granule release. Intracellular calcium release from granules creates an influx of cytosolic calcium in platelets and is dependent on the receptor signaling pathway that initiates activation. GPVI signaling specifically promotes procoagulant activity of platelets by maintaining intracellular calcium levels, which distorts the asymmetry of the lipid bilayer and leads to the flipping of phosphatidylserine, exposing it on the extracellular surface of platelets. Phosphatidylserine is a negatively charged molecule and acts as a surface for coagulation factors, catalyzing the generation of thrombin and formation of fibrin. Interestingly, GPVI deficiency results in only a minor bleeding phenotype evidenced by a prolonged bleeding time and a lack of platelet activation in response to collagen. Murine platelets deficient in GPVI demonstrated the

requirement of GPVI for collagen-induced platelet aggregation without affecting the activity of other platelet receptors such as G-protein coupled receptors (GPCR).

Collagen is also a ligand for the platelet receptor integrin $\alpha_2\beta_1$, which enhances platelet adhesion to collagen.[65] Integrin $\alpha_2\beta_1$ is expressed on the platelet membrane and requires inside-out activation to enter a high-affinity state. Binding to collagen occurs at amino acid sequences of GFOGER. The contribution of $\alpha_2\beta_1$ helps to reinforce platelet adhesion to collagen and promotes secondary platelet activation through outside-in signaling. It has been demonstrated that lack of proper $\alpha_2\beta_1$ function limits the stability of thrombi, however, α2β1 is not required for collagen-induced platelet adhesion and activation.

The ECM proteins nidogen-1 and -2 are major constituents of the basement membrane, that were first isolated from electrophoresis of cell cultures or extraction from mouse Englbreth-Holm-Swarm tumor.[66, 67] Also known as entactins, nidogens are 120-150 kD sulfated glycoproteins that have three globular domains (G1, G2, G3) and a cysteine-rich E domain connectingG1 and G2 to G3.[68] Within these domains nidogen contains 7 epidermal growth factor regions and a Arg-Gly-Asp (RGD) peptide sequence.[69] Post translational modifications of nidogen include N-and O-linked glycosylation. The proteins are vital components of the basement membrane and are expressed ubiquitously in the human body. In vivo, nidogen is colocalized with laminins and collagens in the basement membrane. The function of nidogen in the basement membrane is to link individual polymeric networks of collagen and laminin together. The G3 domain of nidogen-1 binds to laminin γ1, while domains G2 is the main binding domain for collagen IV.[70] A single binding site on the γ1 chain of laminin is responsible for the high-affinity bond created with nidogen and is shared by nearly all laminins.[71]

Nidogen supports cell adhesion by binding to integrins $\alpha_3\beta_1$, via the G2 domain, and $\alpha_v\beta_3$, via the RGD tripeptide in the E domain.[72-74] Studies have also demonstrated that nidogen binds to perlecan, fibulins, fibronectin, and fibrinogen.[75-78]

Nidogen-2 is a larger protein, with more glycosylation, than nidogen-1 and is found in more specific tissues than the ubiquitous nidogen-1. Deficiency of nidogen-1 results in increased expression and incorporation of nidogen-2 into the basement membrane.[79] Likewise, the absence of nidogen-2 had no effect on mice or the formation of the basement membrane. However, mice deficient in both isoforms of nidogen died within 24 hours after birth.[80] Nidogen double-null mice experienced hemorrhaging within the walls of the heart, as well as having a smaller heart mass. In addition, mice showed histological signs of trabecular hypoplasia or thinning of the ventricular wall.

In addition, a recent proteomic analysis of the platelet releasate (platelet releasate from 1U/mL thrombin-induced, activated platelets) proteome of 32 healthy individuals revealed nidogen-1 and nidogen-2 as the 34^{th} and 87^{th} most identified proteins (out of 894 proteins) released from platelets activated by thrombin, respectively.[81] Furthermore, both isoforms were consistently found in all 32 platelet releasates suggesting an important role for nidogen secretion by activated platelets. With the recent discovery of laminin as a prothrombotic surface and the well characterized role of collagen in promoting arterial thrombosis, it is necessary to characterize the role of nidogen in hemostasis and thrombosis. Currently, an understanding of the role of nidogen in supporting thrombus formation is critically missing from a full comprehension of the platelet-endothelium interface in procoagulant settings.

Additional proteins associated with the vascular ECM are fibulin-1 and fibronectin. Fibulin-1 is an ECM protein found in the elastic lamina expressed by smooth muscle cells.[82, 83] Studies

have demonstrated that fibulin-1 binds with other ECM proteins such as fibronectin, laminin, and nidogen.[84] In addition, fibulin-1 was found to bind with fibrinogen leading to platelet adhesion as well as inclusion of fibulin-1 within fibrin clots.[85, 86] Fibronectin is unique ECM protein found primarily in the plasma and subendothelium with a diverse range of binding interactions.[7] Of these, the interaction between fibronectin and platelets has generated significant interest into the mechanism by which platelets adhere to fibronectin surfaces. Fibronectin contains a RGD sequence that is responsible for $\alpha_{IIb}\beta_3$ binding leading to platelet adhesion and spreading.[87] In addition, platelet adhesion was observed in the absence of $\alpha_{IIb}\beta_3$ function and was determined to be mediated by $\alpha_5\beta_1$ and $\alpha_v\beta_3$, however, platelet spreading was incomplete as platelets failed to form lamellipodia.[88] Under low shear rates (less than or equal to 500 s^{-1}), platelet adhesion to collagens type I and III was found to be dependent on fibronectin concentration.[89] At higher shear rates (greater than 500 s^{-1}), vWF was determined to be a vital component of the interaction, mediating platelet adhesion under shear flow through activity of GPIb.[90]

Integrin $\alpha_{IIb}\beta_3$ is a platelet receptor for both fibrinogen and vWF.[91] The activity of $\alpha_{IIb}\beta_3$is calcium dependent and requires prior platelet activation to induce binding.[92] Upon activation, platelet secretion of granules releases ADP and TxA2 which bind to their respective platelet surface receptors. This causes the release of calcium ions from the endoplasmic reticulum, which leads to activation of protein kinase C, as stated earlier. The presence of calcium induces $\alpha_{IIb}\beta_3$complex formation and binding of fibrinogen ensues, leading to platelet aggregation as platelets bind to the same fibrinogen strand. Inhibitors of $\alpha_{IIb}\beta_3$include abciximab, eptibatide, and tirofiban.

As mentioned earlier, vascular basement membranes are unique due to the incorporation of vWF secreted by endothelial cells. vWF is also stored in Weibel-Palade bodies of endothelial cells and a released into plasma. vWF is a glycoprotein that forms larger multimeric complexes to promote

platelet adhesion in the event of vascular injury.[93] Similar to endothelial cells, platelets also store vWF in intracellular bodies called α-granules, able to secrete vWF upon activation. When stored in granules, vWF exists as larger multimers called ultralarge vWF (ULvWF) that are more active, inducing enhanced platelet adhesion. vWF is regulated by enzymatic degradation by the protease adisintegrinlike and metalloprotease with thrombospondin type I repeats-13 (ADAMTS13), which cleaves vWF into smaller multimers.[94] This cleavage can only be achieved when vWF is extended due to shear stress. vWF also plays an interesting role in the protection of coagulation factor VIII (FVIII) by binding the protein in plasma. This function helps protect FVIII from proteolysis and colocalizes FVIII with vWF at the site of vascular injury.

Endothelial cells and platelets secrete vWF in response to agonist exposure, allowing the multimer to extend and expose numerous binding sites to circulating platelets. Platelets will bind to vWF through their GPIb-V-IX complex causing platelets to become tethered. This binding interaction is transient causing platelets to be released only to bind to another GPIb-V-IX complex. This creates a rolling effect as platelets slowly advance down the multimer, allowing for additional platelet receptors to interact with nearby ligands, specifically the collagen IV receptors GPVI and integrin $\alpha_2\beta_1$.[95] vWF localizes with collagen IV to increase platelet adhesion at the site of vascular injury. [96] The GPIb-V-IX complex plays a large role in the initial adhesion of circulating platelets. The complex is a combination of GPIbα, GPIbβ, GPV, and GPIX with 2, 4, 1, and 2 copies of each individual component, respectively. Most notably, GPIbα is a constitutively active receptor on the surface membrane of platelets that binds to vWF. This interaction is dependent on shear stress as vWF undergoes a conformational change and becomes extended due to the force of the flowing blood, which exposes the A1 domain of vWF to platelets. This complex has additional ligands such as thrombi, P-selectin, FXI, and FXII.

1.5 Platelet Biology

Platelets are small blood cells (2-4 μm in diameter) that are vital to the process of hemostasis that respond to vascular injury to stop and prevent blood loss. Platelets are anucleate cells that are produced during thrombopoiesis and have a lifespan of 7-10 days. Degradation of circulating platelets is regulated by the spleen and liver. Platelets are responsible for forming a hemostatic plug at the site of vascular injury by adhering to the ECM or stimulated endothelium and activating intracellular signaling pathways.[3] Activation of these pathways promotes activation of additional platelet receptors and release of secondary mediators for recruitment and activation of circulating platelets. After an initial layer of platelets forms at the site of injury, recruited platelets adhere to this layer and begin to aggregate together via integrin $\alpha_{IIb}\beta_3$. This step is crucial in the formation of a stable clot or thrombus, as it must be able to resist the shear stress generated by the flowing blood. During hemostasis, platelets will employ multiple receptors for adhesion and activation of intracellular processes to work in tandem with the coagulation cascade to effectively limit blood loss and mediate further vascular damage (Figure 3).

A crucial part of hemostasis is the formation of a hemostatic plug through the aggregation of platelets at the site of injury. This requires initial adhesion of platelets under shear stress, mediated by transient bonds formed between GPIb and vWF.[97] This allows platelets to further secure themselves to the site of injury by binding to other exposed ECM proteins such as collagen IV. Then, platelets become activated and secrete soluble agonists for recruitment of additional platelets. Aggregation is highly dependent on the platelet integrin $\alpha_{IIb}\beta_3$ binding to fibrinogen, which is its main ligand and has a plasma concentration of 2-4 mg/mL.[6] This reinforcement is crucial to the growth of the clot as it resists increased shear stress. As such, platelets contain between 60-80,000 copies of $\alpha_{IIb}\beta_3$, ready to link platelets together by binding to a single fibrinogen protein. Prior to activation, platelets circulate in a resting state with $\alpha_{IIb}\beta_3$ inactive and

unable to bind circulating fibrinogen. Activation of $\alpha_{IIb}\beta_3$ is achieved via inside-out signaling in response to platelet activation by ECM proteins or soluble agonists. In addition to fibrinogen, $\alpha_{IIb}\beta_3$ also interacts with vWF, fibronectin, fibulin-1 and vitronectin, which further contributes to the formation of stable platelet aggregates by binding RGD sequences within the proteins.[98]

1.5.1 Platelet receptors of soluble agonists

Platelets are subject to activation by the coagulation cascade due to their expression of PARs, for which thrombin is a potent ligand. PARs are GPCRs that become active upon protease cleavage. Of the four PARs identified, PAR1 and PAR4 are expressed in platelets and participate in thrombin-induced platelet activation.[16, 17]

PAR1 is well studied and known to bind thrombin. This receptor is found on many cell types of the vasculature including endothelial cells, leukocytes, neutrophils, and smooth muscle cells. Thrombin acts as a potent activator of platelets by inducing ATP and TxA2 release and activation of $\alpha_{IIb}\beta_3$.[16] PAR1 also aids in platelet adhesion by stimulating P-selectin and CD-40 mobilization to the platelet membrane, enabling platelet-endothelial cell binding. Furthermore, thrombin-induced activation of platelets plays a role in the regulation of kinase signaling pathways such as Rho kinases, JAK2, PI3-K, and Src family tyrosine kinases. PAR1 activation in endothelial cells also aids in platelet adhesion and coagulation, as thrombin-induced activation causes vWF release and expression of TF on the luminal surface. The other PAR receptor found on platelets is PAR4.[17] While PAR1 has significant affinity for thrombin, PAR4 has low affinity for thrombin itself and is inactivated much slower than PAR1. This may help PAR4 serve as a vital thrombin receptor during vascular injury, when thrombin concentrations increase dramatically and for an extended time. PAR4 activation contributes to platelet aggregation.

The platelet receptor P2Y12 is a GPCR on the surface of platelets that functions as a purinergic receptor for ADP. Platelet activation via ADP stimulation leads to activation of $\alpha_{IIb}\beta_3$, promoting platelet degranulation and aggregation. [99, 100] P2Y12 is a commonly targeted platelet receptor for reducing the risk of thrombotic complications. An essential part in the platelet activation process is the interaction of ADP with the platelet P2Y12 receptor. The P2Y12 receptor is the predominant receptor involved in the ADP-stimulated activation of the glycoprotein IIb/IIIa receptor. Activation of the glycoprotein IIb/IIIa receptor results in enhanced platelet degranulation and thromboxane production, and prolonged platelet aggregation. Platelets also express the purinergic receptor P2Y1 which binds ATP and ADP. ADP stimulation of P2Y1 is different than P2Y12 in that it activates phospholipase C to mobilize calcium and induce platelet morphological changes.

The thromboxane receptor (TP) belongs to the prostanoid receptor family and binds its ligand TxA_2 causing mobilization of intracellular calcium.[99] The production of TxA_2 and binding to the TP is an important near-end step of platelet activation that results in increased adhesion, aggregation and degranulation of secretory granules. Studies have demonstrated that mice lacking the receptor experience mild bleeding due to impaired platelet adhesion, aggregation, and degranulation.

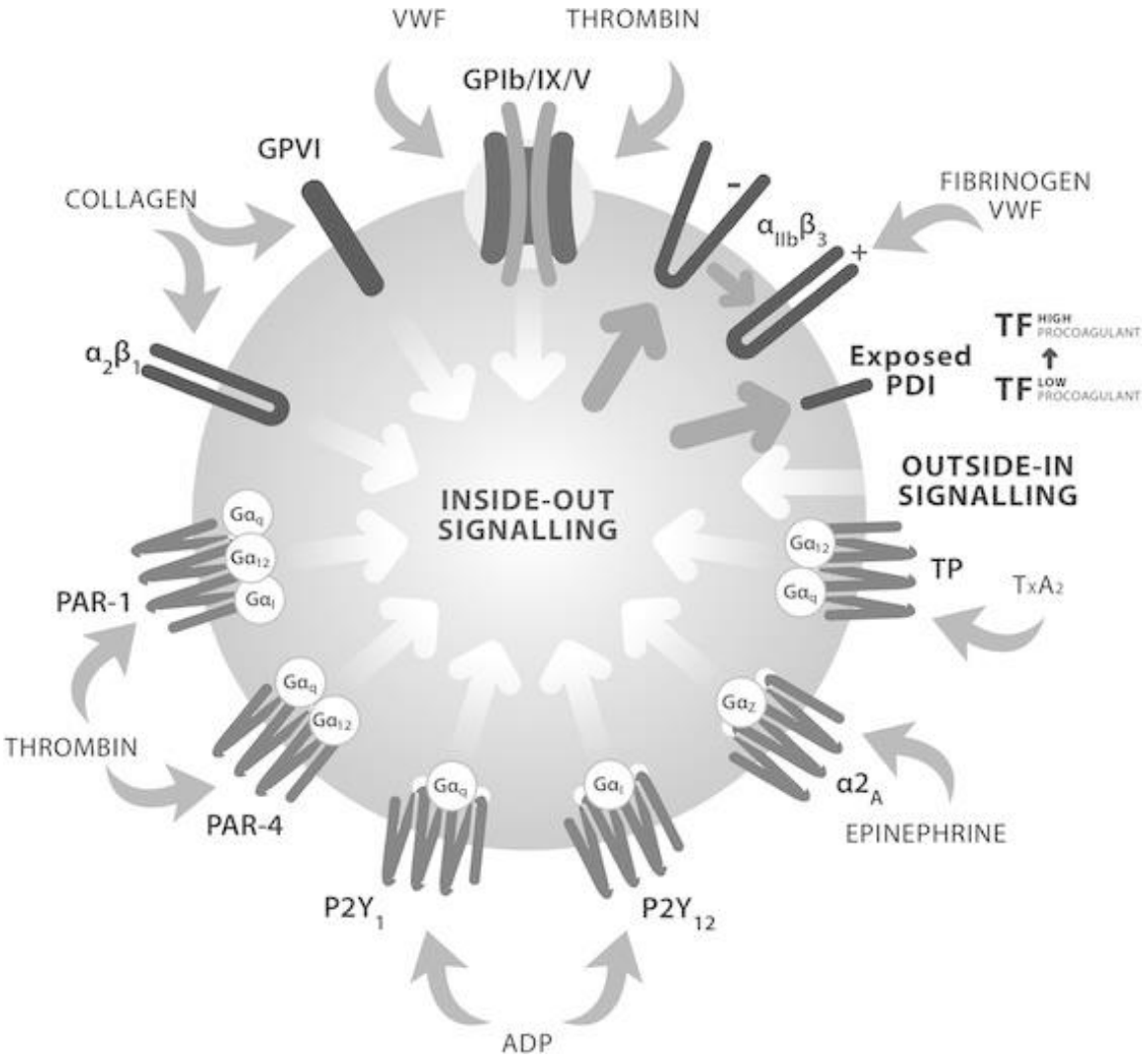


Figure 3. Platelet receptors and their agonists

Platelet activation occurs via multiple pathways and initiates inside-out signaling which results in platelet morphological changes and secretion of platelet granules. Activation of integrin $\alpha_{IIb}\beta_3$ enables binding of fibrinogen inducing platelet aggregation and is further propagated by soluble agonists that promote procoagulant activity. ADP activates platelets by binding to P2 purinergic receptors (P2Y) on the platelet membrane, strongly initiating a complex signaling cascade that induces platelet activation, aggregation and thrombus formation. Binding of thrombin to protease-activated receptors (PARs) leads to activation of PLC and increases platelet secretion. Platelets bind to collagen via GPVI and integrin $\alpha_2\beta_1$, promoting adhesion and triggering inside-out signalling. vWF serves as a vital ligand for platelet adhesion forming a transient bond through interaction with GPIb-IX-V complex. Figure was adapted from ©Kennedy et al., 2016, originally published in European Journal of Nutrition 2016 Jun 56: 461. Reprinted with permission from Springer Nature.

1.6 Thesis Overview

The studies described within this thesis characterize the contribution of the ECM protein nidogen-1 in platelet adhesion and activation. While it is well documented that exposure of the ECM proteins collagen and laminin to blood flow leads to platelet activation and aggregation, there is a need to fully characterize the interaction of platelets and additional ECM proteins of the BM. Despite evidence supporting the interaction between nidogen and ECM proteins involved in platelet activation and coagulation, the role of nidogen in these interactions remains to be determined. Chapter 3 characterizes the interaction between nidogen-1 and human platelets. The data obtained in this study demonstrate that nidogen-1 activates platelets through an interaction dependent on platelet GPVI binding, both in static and shear flow conditions. These results implicate nidogen-1 as a potential ligand of GPVI *in vivo,* suggesting platelet binding to BM proteins represents a redundant mechanism for platelet aggregation and thrombus formation.

The work described in Chapter 3 demonstrates platelet activation by nidogen-1*in vitro* and indicates a need to further define the mechanism of nidogen-induced platelet activation. As such, Chapter 4 describes future directions necessary to characterize nidogen-1 induced platelet activation and spreading. Furthermore Chapter 4 details the rationale and preliminary work for the development of an endothelialized flow chamber for characterization of the platelet-endothelium interface under physiological conditions. Collagen and laminin support platelet adhesion through different mechanisms involving integrins and GPVI, suggesting nidogen-1 may also induce platelet adhesion via GPVI and integrin activity. In addition, both collagen and laminin activate platelets via GPVI and induce platelet spreading and aggregation through the secretion of fibrinogen. Finally, whole blood perfusion over collagen and laminin demonstrated

that thrombus formation *in vitro* was FXII dependent. Altogether, this demonstrates a need to 1) identify additional receptors involved in platelet adhesion to nidogen-1, define nidogen-1 mediated spreading of platelets, and determine the procoagulant activity of nidogen-1; and 2) develop an endothelialized flow chamber for characterization of the platelet-endothelium interface under physiological conditions.

The studies presented identify nidogen-1 as a ligand of GPVI and demonstrate that nidogen-1 supports platelet activation under physiological shear flow. In particular, these studies demonstrated GPVI dependent nidogen-1 activation of vWF-adherent platelets under shear flow, leading to formation of platelet aggregates. This supports previous evidence that GPVI signaling is involved in platelet aggregation on ECM surfaces under shear flow. Furthermore, this work adds nidogen-1 to a number of potential ECM ligands capable of platelet activation *in vivo*. Finally, this thesis demonstrates the potential utility of endothelialized flow chambers for the investigation of the platelet-endothelium interface in pathological settings such as inflammation and thrombosis.

Chapter 2. Methods and Approach

2.1 Ethical Considerations

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

2.2 Reagents.

Reagents were purchased from Sigma-Aldrich (St Louis, MO, USA) unless specified otherwise. Recombinant human nidogen-1 was obtained from R&D Systems (Minneapolis, USA), soluble collagen from Corning (Corning, NY, USA), fibrillar collagen from Chrono-Log (Havertown, PA, USA) and human fibrinogen from Enzyme Research (South Bend, IN, USA). U73122 and U73343 were obtained from Tocris (Bristol, UK). Anti-GPVI ACT017 blocking antibody was donated by Acticor Biotech (Paris, France). Anti–β1 (clone: AIIB2) blocking antibody was purchased from Millipore (Burlington, MA, USA).

2.3 Isolation of human washed platelets.

Platelets were isolated from human venous blood drawn from healthy volunteers by venipuncture into sodium citrate (1:9; v/v), in accordance with an Institutional Review Board-approved protocol at Oregon Health & Science University as previously described.[101] Briefly, anticoagulated blood was centrifuged (200 × g, 20 min) to obtain platelet-rich plasma (PRP). PRP was centrifuged (1000 × g, 10 min) in the presence of prostacyclin (0.1 μg/mL) to obtain a

platelet pellet. The platelet pellet was resuspended in modified HEPES/Tyrode buffer (129 mM NaCl, 0.34 mM Na2HPO4, 2.9 mM KCl, 12 mM NaHCO3, 20 mM HEPES, 5 mM glucose, 1 mM MgCl2; pH 7.3) and washed once via centrifugation at 1000 × g for 10 min in modified HEPES/Tyrode buffer in the presence of prostacyclin (0.1 μg/mL). Purified platelets were resuspended in modified HEPES/Tyrode buffer at the indicated concentrations.

2.4 Static platelet adhesion and spreading assay.

Platelet adhesion and spreading assay was carried out as previously described.[102] Briefly, glass coverslips were coated with human fibrinogen (50 μg/mL), soluble collagen (50 μg/mL) or nidogen-1 (50 μg/mL) followed by surface blocking with bovine serum albumin (BSA) (5 mg/mL). Inhibitors or vehicle were added to platelets in solution (5×10^7/mL) for 15 minutes before exposure to indicated immobilized surfaces. After 45 minutes, nonadherent platelets were discarded and surface-bound platelets were washed 3 times with PBS. Platelets were imaged using Kohler illuminated Nomarski differential interference contrast (DIC) optics with a Zeiss 63x oil immersion 1.40 NA plan-apochromat lens on a Zeiss Axiovert 200M microscope.

2.5 Platelet adhesion and aggregate formation under flow.

Platelet adhesion and aggregation under flow was achieved using parallel plate flow systems prepared by coating capillary tubes (0.2 mm × 2 mm × 50 mm) with collagen (100 μg/mL), von Willebrand Factor (vWF, 50 μg/mL), or nidogen (100 μg/mL) alone or in combination with vWF (50 μg/mL) for 1 hour at RT. Channels were washed with PBS and then blocked with BSA (5 mg/mL) for 1 hour at RT. Capillaries were assembled on microscope slides prior to being mounted to an inverted microscope (Zeiss Axiovert 200M). Sodium citrate anticoagulated whole blood was recalcified immediately before perfusion through the channel at shear rates of 100 and

1000 s^{-1} for 10 minutes. In select experiments, blood was pretreated with inhibitors for 10 min prior to perfusion. Post-perfusion channels were washed 3 times with PBS and fixed using 4% parafomaldehyde. Images were captured using DIC on a Zeiss 63x oil immersion 1.40 NA plan-apochromat objective (Zeiss Axiovert 200).

2.6 Statistical analysis.

Data were analyzed using GraphPad PRISM 5.0 software (San Diego, CA, USA). To determine statistical significance, Student's paired t-test was used for comparison between treatment and control, while oneway-ANOVA was performed with Dunnet's multiple comparison test for experiments with multiple treatments. Results are expressed as the mean ± standard error of the mean (SEM). Differences were considered significant at $p < 0.05$.

Chapter 3. The basement membrane protein nidogen-1 stimulates platelet activation, adhesion and spreading in a GPVI-dependent manner

3.1 Abstract

Nidogen-1 is one of the main extracellular matrix (ECM) proteins of the basement membrane, ubiquitously found across human tissue along with collagens and laminins. While collagen and laminin are known to support platelet adhesion and activation via β1 integrins and glycoprotein (GP) VI, respectively, whether nidogen contributes to platelet activation and hemostasis is unknown. We sought to investigate the ability of nidogen-1 to induce platelet adhesion and spreading and to support platelet activation and aggregation, under static and shear flow conditions respectively. Recombinant human nidogen-1 supported platelet adhesion and induced complete spreading of platelets. Inhibitors of ADP and integrin $\alpha_{IIb}\beta_3$ reduced platelet adhesion to nidogen-1 surfaces, however, decreased platelet spreading was observed only in the presence of $\alpha_{IIb}\beta_3$ inhibitor. Treatment of human platelets with pharmacological inhibitors prevented platelet activation by significantly reducing platelet adhesion and spreading in a GPVI-dependent manner. Inhibition of the GPVI signaling effector protein PLCγ2, abolished platelet adhesion, while inhibition of IκB kinase prevented platelet spreading. Additionally, treatment with a GPVI blocking mAb, lowered platelet adhesion and eliminated platelet spreading on both nidogen-1 and soluble collagen. Under shear flow conditions, nidogen-1 was incapable of supporting platelet adhesion of whole blood. However, in combination with von Willebrand Factor (vWF), nidogen-1 induced activation of GPIb-adherent platelets, leading to platelet aggregate formation. In similar experiments under shear, platelet activation and aggregate formation was eliminated by inhibition of GPVI, while platelet adhesion to vWF was abolished by inhibition of GPIb. These results demonstrate that nidogen-1 supports platelet activation and aggregation *via* GPVI-dependent interaction.

3.2 Introduction

The major constituents of the subendothelial matrix include various isoforms of collagen and laminin, which assemble to form two independent networks. Collagen plays a major role in providing structural stability while laminin is required for the initial development of the basement membrane. As these networks have only a weak affinity for each other, the matrix protein nidogen acts as an integrating element for basement membrane assembly by binding and forming a complex with laminin which binds collagen. [66, 71, 104] Indeed, in mice, nidogen deficiency causes perinatal lethality due to impaired lung and heart development. [80] Studies including those in *C. elegans* revealed that nidogen may play other nonstructural roles including synaptic transmission and axonal pathfinding.{Ho, 2008 #147} As collagen and laminin are known to play nonstructural roles in hemostasis through activation of blood platelets and coagulation factors, we designed the current study to investigate whether nidogen likewise contributes to hemostasis by supporting platelet activation.

Platelet adhesion to and activation by subendothelial extracellular matrix (ECM) proteins at sites of vascular injury is a key primary event to the formation of a fibrin-rich hemostatic plug that prevents and stops bleeding.[3, 47] Firm adhesion, activation and spreading of platelets on the subendothelial ECM and basement membrane proteins is predominantly initiated by engagement of integrins, including $\alpha_2\beta_1$, $\alpha_6\beta_1$ and $\alpha_{IIb}\beta_3$, and activation of tyrosine-kinase mediated pathways downstream of the platelet receptor glycoprotein (GP) VI and GPIb.[3, 53, 55, 64, 65, 103] In particular, GPVI-mediated signaling is dependent on activation of PLCγ2 via phosphorylation of adaptor proteins like Src family kinases (SFK) and Syk.[92] These initial adhesive interactions by GPVI and GPIb are then potentiated by platelet release of secondary mediators of activation via PLCγ2, including ADP and TXA_2, which support aggregation of activated platelets and flipping of the platelet membrane to potentiate assembly of coagulation factors to promote thrombin

generation.[3] A complete understanding of the molecular signaling underlying platelet activation by ECM and basement membrane proteins is essential to develop novel therapies that prevent or limit undesired platelet activation in diseased vessels, yet maintain the hemostatic function of platelets at sites of vascular damage.

The discovery that the ECM protein laminin likewise binds and activates platelets in a GPVI-dependent manner to support thrombus formation under flow has brought to light GPVI as more than a faithful platelet receptor for collagen. Rather, GPVI is a promiscuous receptor for a growing number of ligands including adhesive proteins (fibrin)ogen, fibronectin and vitronectin acting in concert to support thrombus growth and stabilization. Herein this study adds nidogen-1 to the growing list of ligands that bind and activate GPVI contributing to thrombus formation under flow, providing further evidence that a number of redundant mechanisms have evolved to activate GPVI to maintain hemostasis.

3.3 Results

3.3.1 Adhesion and spreading of platelets onto recombinant human nidogen-1 surfaces.

We initially investigated the ability of platelets to adhere and spread on nidogen surfaces. As shown in Figure 4, platelets extensively adhered and spread on nidogen, indicated by formation of lamellipodia. Consistent with previous findings,[65, 105, 106] human platelets underwent complete spreading on collagen and fibrinogen (Figure 4). In contrast, very few platelets adhered on a BSA-coated surface which served as a negative control. The degree of platelet adhesion and spreading onto nidogen was significantly reduced by the presence of the αIIbβ3 inhibitor Integrilin, but not AIIB2, a β_1 blocking antibody (Table 1), demonstrating that $\alpha_{IIb}\beta_3$, but not β_1 integrin is crucial for the adhesion and spreading of platelets on nidogen. In addition, a drastic reduction in platelet adhesion and spreading on nidogen was also observed in the presence of

apyrase, which catalyzes the hydrolysis of ADP (Table 1). In line with previous reports,[65, 105, 106] we found that the integrin β_1 was shown to be crucial for platelet adhesion to collagen, but not fibrinogen, as evidenced by the loss of platelet binding in the presence of the β_1 blocking antibody, AIIB2 (Table 1). Furthermore, platelet adhesion to fibrinogen, but not collagen, was eliminated in the presence of integrillin (Table 1).

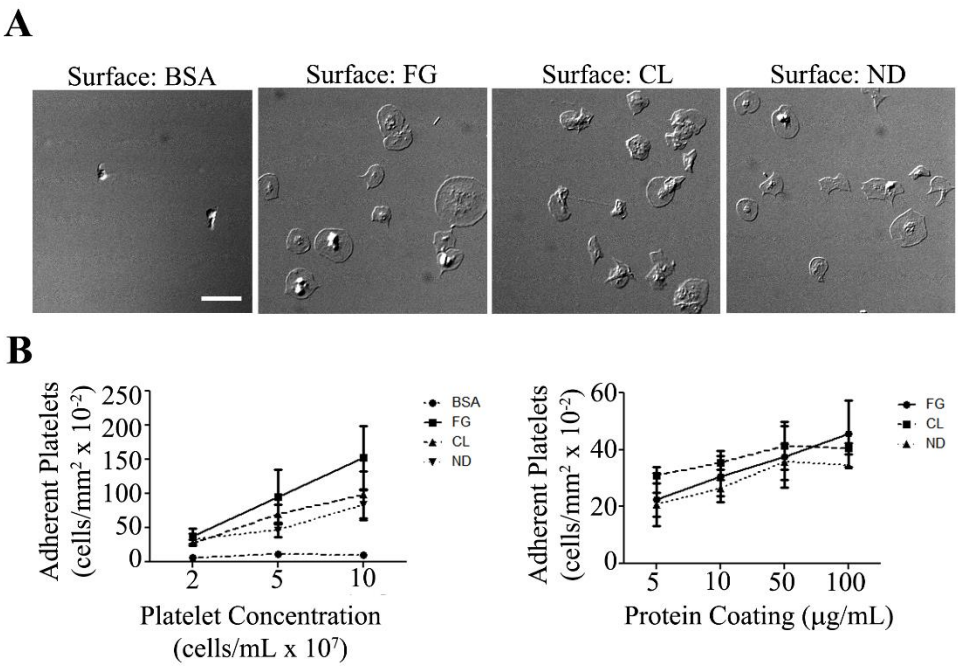


Figure 4. Adhesion and spreading of human platelets on nidogen-1.

Human platelets adhered and spread on collagen, fibrinogen, and nidogen-1. **A.** Human washed platelets (5×10^7/mL) were exposed to coverslips coated with fibrinogen (FG, 50 µg/mL), soluble collagen (CL, 50 µg/mL), recombinant nidogen (ND, 50 µg/mL) or BSA (5 mg/mL) for 45 min at 37º C and imaged using differential interference contrast (DIC) microscopy. Scale bar, 10 µm. **B.** In designated experiments, platelet concentration (2, 5, and 10×10^7/mL) was varied in the presence of protein surface coatings (50 µg/mL).(left) Separately, human washed platelets (2×10^7/mL) were exposed to varying concentrations of protein surface coating (5, 10, 50, and 100 µg/mL).(right) The number of spread adherent platelets were recorded for 3 fields of view and expressed as mean ± SEM from at least 3 different experiments.

Table 1. Adhesion and spreading of human platelets on nidogen-1: role of ADP and integrins αIIbβ3 and β1.

Human washed platelets (5×10^7/mL) were placed on coverslips coated with fibrinogen (FG, 50 µg/mL), soluble collagen (CL, 50 µg/mL), nidogen-1 (ND, 50 µg/mL) or BSA (5 mg/mL) for 45 min at 37º C and imaged using differential interference contrast (DIC) microscopy. In selected experiments, platelets were pre-treated with vehicle, apyrase (2 U/mL), integrilin (20 µg/mL) or anti-integrin β1 blocking antibody (20 µg/mL, clone AIIB2) for 15 min at RT prior to seeding. The number and surface area of spread adherent platelets were recorded for 3 fields of view and expressed as mean ± SEM from at least 3 different experiments. *, **, *** $P < 0.05, 0.01, 0.001$ with respect to platelet adhesion in the absence of inhibitors.

Surface	Treatment	Area/Plt (um^2)	# of Plts	% SA
BSA	−	11.8 ± 2.44	8.3 ± 2.88	0.6 ± 0.17
Fibrinogen	−	23.3 ± 0.98	162.7 ± 15.99	25.7 ± 2.49
Fibrinogen	Apyrase	14.6 ± 1.21**	134.3 ± 18.48	14.1 ± 2.77*
Fibrinogen	Integrilin	9.4 ± 0.77***	48.8 ± 7.72**	3.3 ± 0.75***
Fibrinogen	AIIB2	24.9 ± 3.00	167.4 ± 18.75	27.9 ± 3.00
Collagen	−	22.4 ± 1.43	87.7 ± 10.57	14.9 ± 0.76
Collagen	Apyrase	20.2 ± 1.49	80.6 ± 0.70	11.1 ± 0.72
Collagen	Integrilin	11.6 ± 0.31*	133.8 ± 11.14	10.6 ± 0.65
Collagen	AIIB2	12.7 ± 0.78*	15.1 ± 0.87***	1.6 ± 0.11***
Nidogen	−	26.5 ± 0.62	71.4 ± 5.36	15.1 ± 1.12
Nidogen	Apyrase	23.2 ± 1.68	55.7 ± 1.58	8.9 ± 0.85
Nidogen	Integrilin	8.8 ± 0.46***	35.1 ± 4.93**	2.1 ± 0.20***
Nidogen	AIIB2	27.1 ± 0.87	61.3 ± 6.81	14.4 ± 1.20

3.3.2 Elucidation of the signaling cascade underlying platelet spreading onto recombinant human nidogen-1 surfaces: role of GPVI receptor.

Platelet spreading downstream of integrins β3, β1 and GPVI is governed by the activation of tyrosine and lipid kinase signaling.[65, 91, 92, 107] To better define the signaling pathways that regulate the spreading of platelets onto nidogen, human platelets were pretreated with inhibitors of kinase signaling mediators, including the Src kinase inhibitor (PP2), the Syk-specific inhibitor (BAY 61-3606), or the lipid phosphoinositide 3-kinase (PI3K) inhibitor (wortmannin) before seeding, fixation and visualization by DIC microscopy. As seen in Figure 5A-B, PP2 inhibited filopodia and lamellipodia formation upon adhesion to nidogen, greatly reduced the number of adherent platelets, and significantly reduced mean platelet surface area. While to a lesser extent, inhibition of Syk with BAY 61-3606 also resulted in a significant reduction in mean platelet surface area, with a decrease in platelet adhesion. Furthermore, our data show that inhibition of PI3K with wortmannin drastically reduced platelet adhesion and spreading onto nidogen. In parallel experiments and in accordance with previous reports,[65, 92, 107] we show that PP2, BAY 61-3606 and wortmannin, significantly decreased platelet spreading, but not adhesion, on fibrinogen- and collagen-coated surfaces (Figure 6A-B).

Next, we extended our study to investigate the role of PLCγ2, an enzyme that plays an essential role in platelet activation, granule secretion, and aggregation. Platelet adhesion and spreading on nidogen was eliminated in the presence of the broad-spectrum PLC inhibitor U73122, but not the inactive analogue U73343 (Figure 5C-D). Similarly, PLC was confirmed to be crucial for platelet adhesion and spreading on fibrinogen and collagen (Figure 6C-D). In addition, we examined the role of the transcription factor NF-κB, which has recently been demonstrated to function in non-genomic roles in platelet activation, secretion and aggregation.[108-110] Inhibition of IκB kinase (IKK), an activator of NF-κB, with IKK-16 decreased the number of adherent platelets and

significantly reduced platelet spreading (Figure 5C-D). Additionally, inhibition of IKK decreased platelet adhesion and prevented platelet spreading on fibrinogen and collagen (Figure 6C-D).

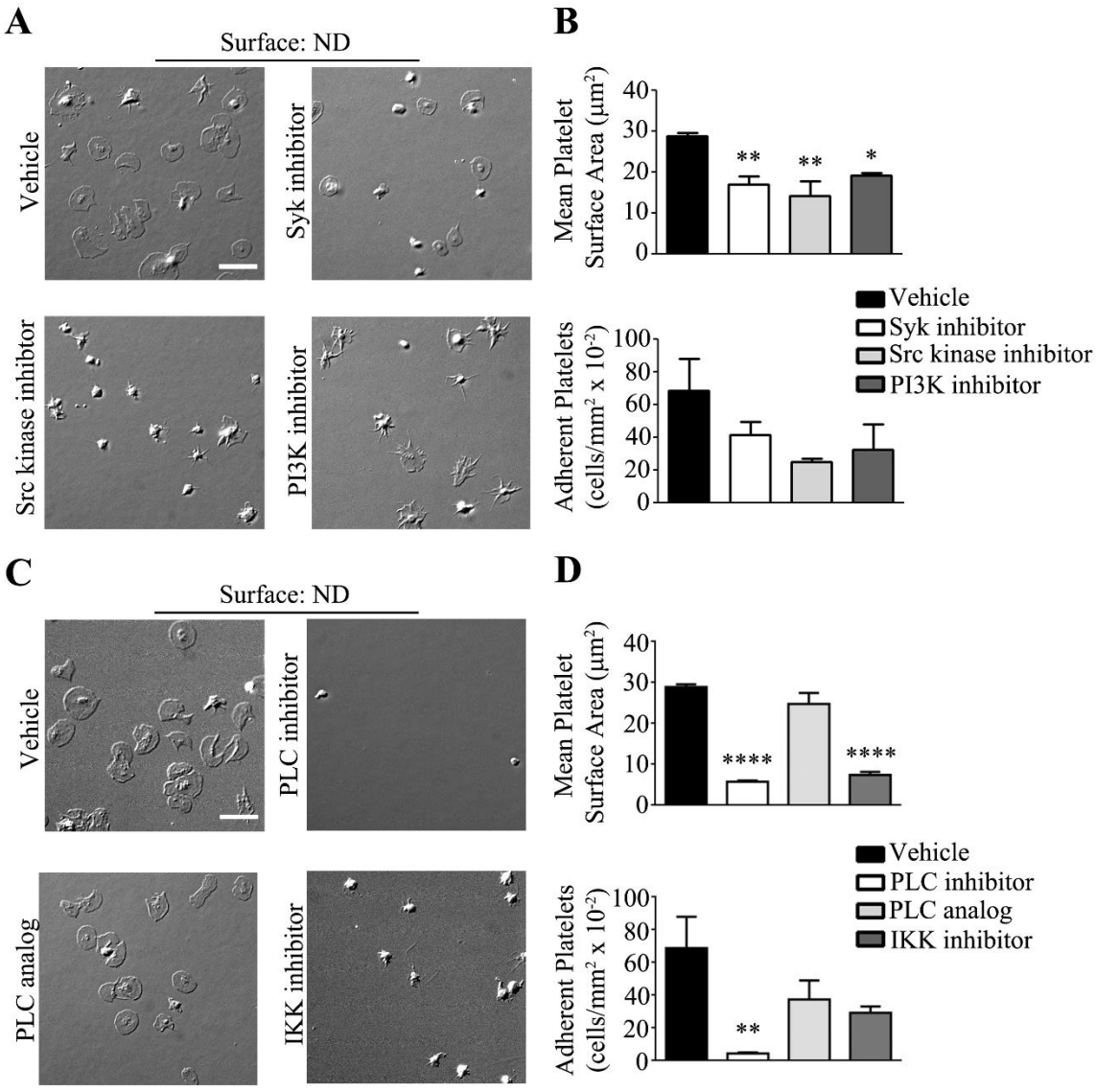
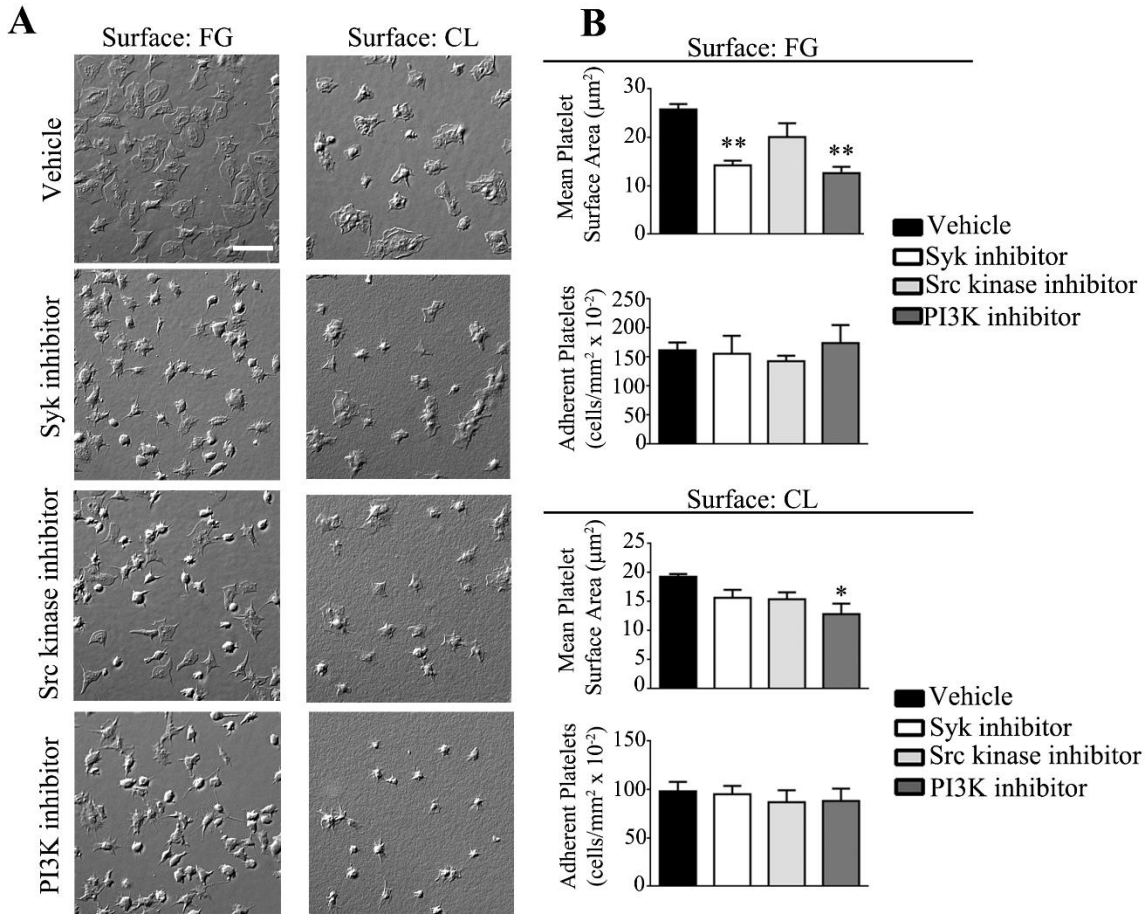


Figure 5. Spreading of human platelets on nidogen is dependent on Src-family kinase (SFK), Syk, PI3K and PLC.

Inhibition of tyrosine kinase signaling pathways led to decreased platelet adhesion and the incomplete platelet spreading on nidogen-1 surfaces. A,C. Human washed platelets (5 × 107/mL) were pre-treated with vehicle (DMSO), Bay-61-3606 (Syk inhibitor, 5 μM), PP2 (SFK inhibitor, 10 μM), wortmannin (PI3K inhibitor, 100 nM), IKK-16 (IκB kinase inhibitor, 10 μM), U73122 (PLC inhibitor, 10 μM) or U73343 (PLC inhibitor inactive analogue, 10 μM) for 15 min at RT

prior to seeding on coverslips coated with nidogen-1 (ND, 50 µg/mL) for 45 min at 37º C and imaged using differential interference contrast (DIC) microscopy. Images are representative of at least 3 independent experiments. Scale bar, 10 µm. B,D. The number and surface area of spread adherent platelets were recorded for 3 fields of view and expressed as mean ± SEM from at least 3 different experiments. *, **, **** P < 0.05, 0.01, 0.0001 with respect to platelet adhesion in the absence of inhibitors.



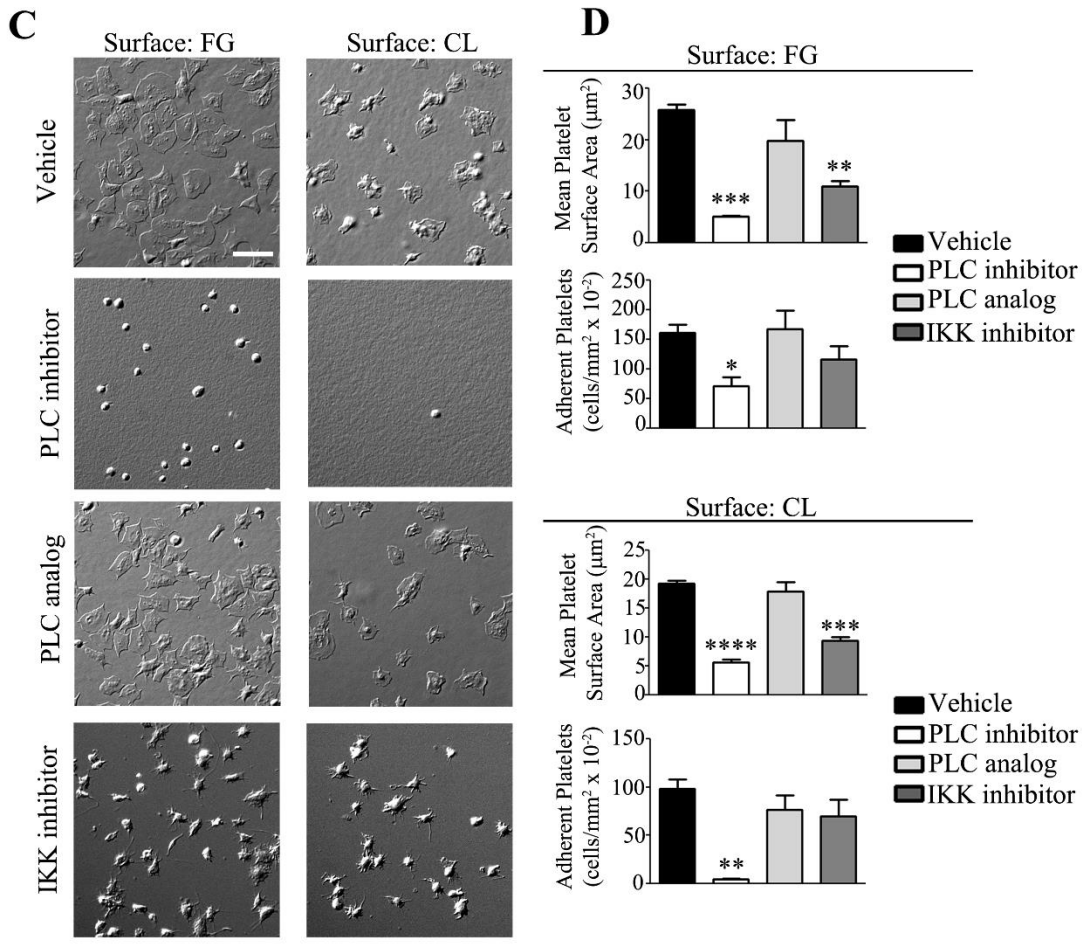


Figure 6. Spreading of human platelets on fibrinogen and soluble collagen.

Human washed platelets (5 × 107/mL) were pre-treated with vehicle (DMSO), Bay-61-3606 (Syk inhibitor, 5 μM), PP2 (SFK inhibitor, 10 μM), wortmannin (PI3K inhibitor, 100 nM), IKK-16 (IκB kinase inhibitor, 10 μM), U73122 (PLC inhibitor, 10 μM) or U73343 (PLC inhibitor inactive analogue, 10 μM) for 15 min at RT prior to seeding on coverslips coated with fibrinogen (FG, 50 μg/mL) or soluble collagen (CL, 50 μg/mL) for 45 min at 37º C and imaged using differential interference contrast (DIC) microscopy. Images are representative of at least 3 independent experiments. Scale bar, 10 μm. B,D. The number and surface area of spread adherent platelets were recorded for 3 fields of view and expressed as mean ± SEM from at least 3 different experiments. *, **, ***, **** $P < 0.05, 0.01, 0.001, 0.0001$ with respect to platelet adhesion in the absence of inhibitors.

The ECM proteins collagen and laminin are known to activate platelets downstream of the platelet receptor GPVI. A set of experiments was next designed to investigate the role of GPVI in supporting platelet adhesion and spreading onto nidogen-coated surfaces. As demonstrated in

Figure 7, treatment of human platelets with ACT017, a blocking antibody specific to GPVI, resulted in a significant decrease in the degree of adhesion and spreading of human platelets on nidogen. Consistent with previous studies,[111, 112] blockade of GPVI with ACT017 reduced the ability of platelets to spread on collagen-coated surfaces (Figure 7), although there was no detectable effect on the degree of adhesion. These observations reveal that platelets respond to nidogen by activating GPVI and downstream tyrosine kinase signaling pathways which require Src, Syk, PI3K and PLC activation.

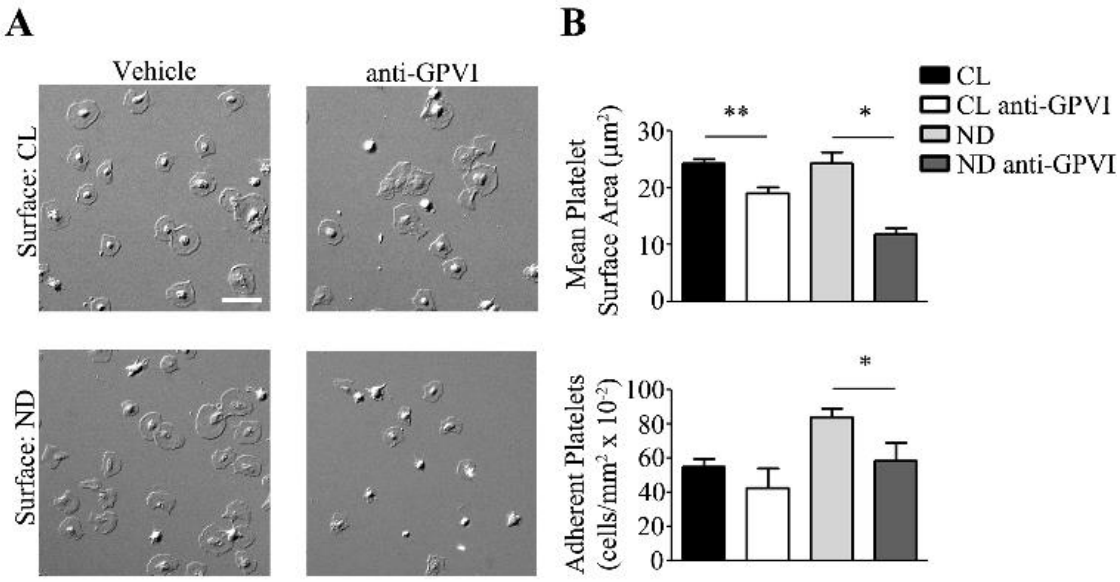


Figure 7. Adhesion and spreading of human platelets on nidogen-1 is dependent on GPVI.

ACT017 inhibited platelet adhesion and spreading on nidogen-1. A. Human washed platelets (5 × 107/mL) were pre-treated with vehicle or anti-GPVI blocking antibody (ACT017, 20 μg/mL) for 20 min at RT prior to seeding on coverslips coated with soluble collagen (CL, 50 μg/mL) or nidogen-1 (ND, 50 μg/mL) for 45 min at 37º C and imaged using differential interference contrast (DIC) microscopy. Images are representative of at least 3 independent experiments. Scale bar, 10 μm. B. The number and surface area of spread adherent platelets were recorded for 3 fields of view and expressed as mean ± SEM from at least 3 different experiments. *, ** $P < 0.05, 0.01$ with respect to platelet adhesion in the absence of inhibitors.

3.3.3 Investigation of platelet adhesion onto nidogen-1 surfaces under physiologic shear flow.

To assess the physiological significance of platelet activation by nidogen, we sought to explore whether nidogen was able to sustain platelet recruitment and activation during perfusion of whole blood under physiological flow conditions. As expected, collagen was able to support platelet adhesion and aggregation during whole blood perfusion, while vWF was only able to support platelet binding yet was unable to support robust platelet aggregate formation (Figure 8A-B).

Previous studies have shown that the ability of ECM proteins including laminin and collagen to support thrombus formation is dependent upon their individual ability to bind VWF, which recruits platelets to the surface facilitating subsequent activation by GPVI. Interestingly, under shear flow conditions nidogen was unable to support platelet adhesion or aggregation. Thus, we next tested the hypothesis that immobilizing VWF with nidogen-1 would induce robust thrombus formation as compared to either surface alone. As seen in Figure 8C-D, surfaces consisting of both vWF and nidogen supported platelet adhesion and activation when exposed to whole blood under shear flow, resulting in rampant platelet aggregate formation. The inhibition of GPVI with a blocking antibody (ACT017) significantly reduced the size of platelet aggregates formed on the combined surfaces of nidogen and VWF. Furthermore, blocking the function of platelet GPIb with 6D1 eliminated platelet recruitment and adhesion on the combination of nidogen and VWF. These results further implicate nidogen-1 as a ligand for platelet GPVI and demonstrate that nidogen-1 plays a redundant role in supporting hemostatic thrombus formation under flow in a VWF-dependent manner.

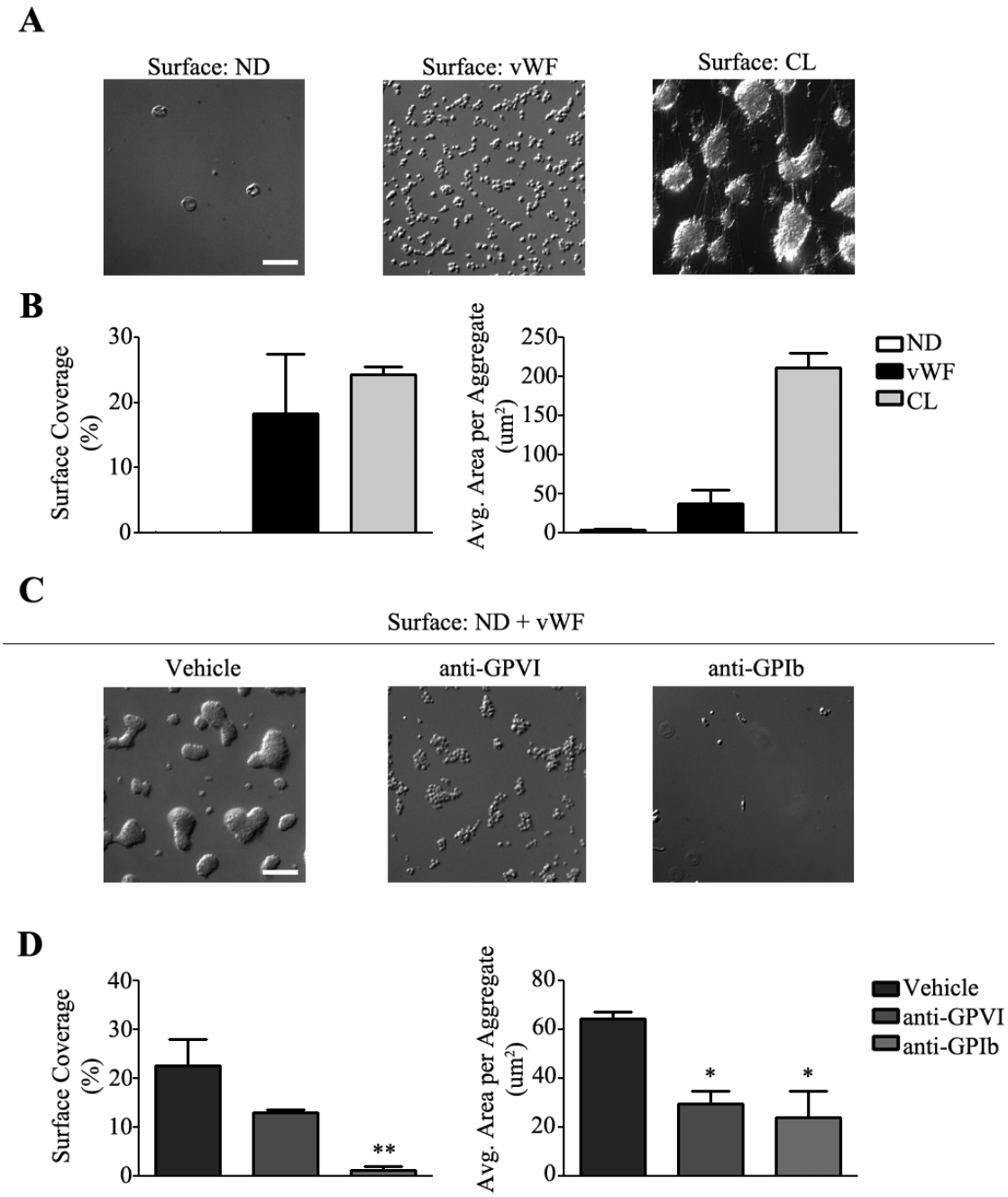


Figure 8. Platelet adhesion and aggregation of whole blood under arterial shear on nidogen.

Nidogen-1 supported platelet activation of GPIb-adherent platelets under shear. **A.** Recalcified human whole blood was perfused over capillary surfaces coated with nidogen-1 (100 µg/mL, ND), vWF (50 µg/mL), or fibrillar collagen (100 µg/mL, CL) at a shear rate of 1000 s^{-1} for 10 min. Total surface coverage and surface area per aggregate were recorded for 3 fields of view and expressed as mean ± SEM from at least 3 different experiments. Scale bar, 10 µm. **B.** In select experiments, recalcified human whole blood was pretreated with ACT017 (20 µg/mL) or 6D1 (10 µg/mL) prior to perfusion over capillary surfaces co-coated with nidogen-1 (100 µg/mL) and vWF (50 µg/mL) at a shear rate of 1000 s^{-1} for 10 min. Total surface coverage and surface area

per aggregate were recorded for 3 fields of view and expressed as mean ± SEM from at least 3 different experiments. *, ** $P < 0.05, 0.01$ with respect to platelet aggregation in the absence of inhibitors.

3.4 Discussion

Here we report a hemostatic role for the extracellular matrix protein nidogen-1 in supporting platelet adhesion, activation and thrombus formation. Moreover, our data adds nidogen-1 to the triumvirate of GPVI ligands present in the ECM including collagen and laminin. Nidogen exhibits a modular structure containing three globular domains, G1-3, separated by a linker region between G1 and G2 and a longer rod-like region located between G2 and G3.{Fox, 1991 #12} Common to other ECM proteins, nidogen contains an epidermal growth factor-like (EGF) module crosslinked to a β-barrel domain within the G2 globule; this complex is responsible for mediating interactions with both perlecan and collagen type IV. The rod domain between G2 and G3 contains another four EGF-like repeats, the first of which contains an RGD binding motif known for potentiating integrin interactions.{Dong, 1995 #28} The six LDL receptor LY modules present in the G3 globule of nidogen mediate interactions with laminin via its single laminin γ1 EGF-like repeat III4. The laminin-nidogen complex and nidogen alone but not laminin alone, are known to bind collagen, resulting in ternary complex formation, permitted by the fact that the G2 domain of nidogen contains the binding site for nidogen-collagen interactions. The binding site for nidogen is predominantly located within the triple helix region of collagen; it is the triple helical structure of collagen that is thought to promote the dimerization of the platelet receptor GPVI to induce signaling through receptor tyrosine kinases.{Aumailley, 1989 #65} An alternative mechanism for GPVI-mediated dimerization and activation may be higher-order receptor clustering as a result of increased ligand density. This may underlie the mechanism by which the polymers of fibrin and laminin and nidogen dimerize GPVI to induce platelet activation. How these ligands are recognized by GPVI remains to be established. Yet, the fact that soluble laminin and nidogen are incapable of activating platelets via GPVI in solution but rather require immobilization lends credence to the concept that the ECM microenvironment plays a critical role in congregating GPVI ligands to ensure hemostasis at sites of vascular injury.

Platelet adhesion and spreading was supported by nidogen-1 in static conditions, and further investigation led us to determine the individual contributions of $\alpha_{IIb}\beta_3$ and GPVI. Specifically, platelets treated with integrilin exhibited limited morphological shape change and filopodia extension, while formation of lamellipodia was nearly abolished. This is in agreement with previous studies demonstrating that lamellipodia formation on the ECM protein fibronectin is dependent on $\alpha_{IIb}\beta_3$.[88] In contrast, inhibition of $\alpha_{IIb}\beta_3$ did not affect platelet adhesion suggesting platelets bound to nidogen via another receptor. Our data demonstrates that treatment of platelets with the blocking antibody ACT017 exhibited a significant reduction in adhesion and spreading on nidogen-1, suggesting impaired GPVI-mediated platelet adhesion and activation. However, this inhibitory effect on adhesion was not observed on collagen surfaces, as platelets adhered via $\alpha_2\beta_1$ to collagen in the absence of functional GPVI, as previously demonstrated using the anti-GPVI antibody JAQ1 and GPVI-depleted platelets.[59] Together these results indicate platelets adhere to nidogen-1 via GPVI and that spreading is dependent upon $\alpha_{IIb}\beta_3$ activity.

As previously documented,[59, 113] platelet adhesion to collagen and laminin is supported by or dependent on binding of β_1 integrins, in combination with GPVI. In regard to nidogen-1, inhibition of β_1 had no effect on platelet adhesion and spreading, as nidogen-1 is known to interact with integrin $\alpha_3\beta_1$, absent from platelet membranes, and $\alpha_v\beta_3$, the vitronectin receptor.[72, 74] It is unknown whether platelet $\alpha_v\beta_3$ and nidogen interact, however it has been demonstrated that nidogen contains and RGD domain that is capable of inducing neutrophil adhesion and chemotaxis.[114] These results potentially indicate a second platelet adhesion receptor for nidogen, however the presence of GPVI-meditated signaling suggests nidogen may have a larger role in platelet activation. This is evidenced by the absence of platelet adhesion of whole blood under arterial shear flow to nidogen surfaces, in contrast to vWF, to which platelets adhered without activating. The activation of platelets exposed to both nidogen and vWF resulted from

platelet adhesion to GPIb and platelet activation by GPVI, supporting platelet aggregate formation. This is consistent with current models of platelet adhesion in vivo, where platelets initially tether to GPIb-GPIX-GPV and become activated through GPVI-mediated signaling.[55]

The importance of tyrosine phosphorylation in platelet signaling has been deeply studied, however, there remains much complexity surrounding the proteins involved and their specific interactions. The transcription factor NF-κB is well known for its genomic role in DNA transcription, yet the discovery of the NF-κB/IκB kinase complex in anuclear platelets suggests it may have non-genomic roles as well. Previous studies have identified that inhibiting NF-κB reduces platelet spreading on immobilized fibrinogen, as well as ADP-induced platelet aggregation.[109] In addition, thrombin stimulated platelets exhibited decreased activity, evident by reduced ATP release, thromboxane formation and P-selectin expression. Furthermore, phosphorylation of IκB kinase, releasing NF-κB from its inactive complex, has been shown to mediate platelet secretion and is potentially involved in additional platelet activation pathways.[108] Inhibition or genetic knockout of IκB kinase in vivo resulted in prolonged tail bleeding times of mice, indicating the importance of the NF-κB/IκB kinase complex in hemostasis. Our results further demonstrate that inhibition of the dissociation of this complex restricts platelet signaling and limits platelet shape change in response to agonists, suggesting these proteins play a critical role in platelet activation.

Recent proteomic analysis of the platelet releasate, the secreted components of α- and dense-granules, of thrombin activated platelets determined that nidogen-1 and nidogen-2 were part of a core of 277 proteins (out of 894) identified consistently among 32 healthy volunteers.[81] Based on label-free quantification of relative protein levels, nidogen-1 had the 42nd highest intensity, in comparison to fibrinogen α-, β-, and γ-chains (15, 22, 23 of 277), fibronectin (71 of 277), and vitronectin (85 of 277). The presence and release of nidogen from platelets suggests a potential

role for the protein upon platelet activation. Based on the interactions of nidogen with other ECM proteins and plasma proteins, this role of nidogen is potentially involved in hemostasis, thereby enhancing $\alpha_{IIb}\beta_3$ platelet aggregation of recruited platelets, or vascular integrity, promoting ECM deposition and platelet spreading in the absence of secondary mediators.[75, 86, 88, 115]. Recent reports have demonstrated that GPVI is a non-essential hemostatic receptor, due to the ability of integrins and secondary mediators ADP, TxA_2, and thrombin to sufficiently activate platelets and prevent bleeding.[115] Interestingly, only in cases of thrombocytopenia was GPVI required for prevention of bleeding in mice. The results of the current study suggest that the GPVI-nidogen interaction may have a larger role in maintaining vascular integrity rather than contributing to hemostasis and thrombosis.

3.5 Conclusion

In conclusion, this study demonstrates that nidogen-1 stimulates GPVI-mediated platelet adhesion and activation of platelets, inducing platelet spreading and aggregation under static and shear flow conditions respectively. This is the first report of nidogen-1 supporting the hemostatic potential of platelets and indicates a conserved role of GPVI-mediated platelet activation in response to exposed ECM proteins. It remains to be determined whether GPVI-mediated platelet activation by nidogen has a role in vascular health and disease.

Chapter 4. Conclusions and Future Directions

4.1 Overall Conclusions

The work presented within this thesis provides new insight into the interactions between platelets and the ECM, identifying nidogen-1 as a ligand for GPVI activation. In addition, this work further supports the mechanism of platelet adhesion under flow that is dependent on initial platelet tethering to vWF. Subsequent platelet binding via integrins and GPVI leads to platelet activation and secretion of granular contents for additional recruitment of platelets.

4.2 Future Directions

4.2.1 Nidogen-1

The studies described here provide evidence that nidogen-1 induces activation of platelets via GPVI. Further experimentation is needed to expand on the results presented to elucidate the signaling mechanism underlying nidogen-1 activation of platelets and to validate GPVI as a platelet receptor for nidogen-1. Additional, work is warranted to address the potential role of platelet integrin $\alpha_v\beta_3$ in platelet binding of nidogen-1, and to determine the extent of this interaction in platelet adhesion. Lastly, these studies demonstrate that nidogen-1 induced activation of platelets enables platelet spreading dependent on $\alpha_{IIb}\beta_3$ and indicate binding of nidogen-1 contributes to platelet secretion of fibrinogen. This necessitates the need to characterize platelet spreading on nidogen-1 surfaces to determine if secretion of platelet granules mediates spreading through the presence of fibrinogen. The experiments described here are suggested for future directions to fully characterize and define the interaction of platelets with the ECM protein nidogen-1.

To further characterize nidogen-1 induced platelet activation and the signaling pathway involved, tyrosine phosphorylation of proteins downstream of GPVI can provide evidence of protein activation and identify key proteins involved in the signaling mechanism. Previous studies investigating the role of ECM proteins and the platelet response generated have confirmed the contribution of platelet receptors and defined the signaling pathways involved through immunoprecipitation of platelet lysates after incubation with ECM surfaces.[53] These studies were able to quantify an increase in tyrosine phosphorylation of proteins that play key roles in the GPVI signaling cascade, such as Syk, PLCγ2, and the FcR γ chain. These methods provide clear identification of tyrosine phosphorylation and activation of proteins and have potential to define key proteins and mechanisms involved in nidogen-1 induced platelet adhesion activation.

In response to the proteomic analysis of the platelet secretome that discovered the presence of nidogen-1 in platelets, further investigation is required to determine the physiologic role of this finding, as it remains unclear whether platelets express nidogen-1 and how this expression may impact platelet function. In contrast, the presence of fibrinogen within platelets is well known to play a significant role in $\alpha_{IIb}\beta_3$ dependent spreading of adherent platelets, through the formation of lamellipodia, and the aggregation of platelets under shear flow. In an effort to determine if nidogen-1 plays a role in the propagation of fibrinogen mediated platelet activity, it is necessary to determine whether nidogen-1 is secreted from platelets in response to agonists. As such, investigation of platelet spreading on fibrinogen surfaces in the presence and absence of thrombin may reveal whether nidogen-1 is localized in platelet α-granules and if thrombin activation induces secretion of nidogen-1. To accomplish this, immunofluorescence staining of platelets using antibodies specific for nidogen-1 can be visualized by structured illumination microscopy. This method has proved effective in similar studies investigating the localization of proteins within platelets adherent to fibrinogen and is a promising technique to further define the nidogen-1 platelet interaction. [116]

Recent studies have employed microprinting of ECM proteins to confine surface coatings to pre-determined dimensions called microdots. This has proved valuable for the characterization of platelet spreading by constraining platelet adhesion within the geometric boundaries of printed microdots and enabling evaluation of platelet spreading beyond the edge of the microdot. This technique is advantageous in that the diameter of microdots varies, from the size of a single platelet to multiple platelets (2-10 μm). In particular, these studies have provided insight into the mechanism of platelet spreading and determined that platelets are able to sense the underlying ECM and respond by secreting α-granules to further platelet spreading in regions devoid of adhesive substrates.[117] This technique can provide meaningful evidence into the mechanism of how nidogen-1 induces platelet spreading of adhered platelets.

4.2.2 Development of an endothelialized flow chamber

Recent studies have revealed the advantages of the development and utilization of microfluidic devices for the investigation of the platelet-endothelium interface. Microfluidic devices have been used in a reductionist method to determine the role of individual components of the platelet-endothelium interface, simplifying the complex interactions that exist in *in vivo* models. The incorporation of endothelial cells and ECM proteins enabled investigation of the dynamic setting of the platelet-endothelium interface and provided insight into the mechanisms and processes regulating blood cell interactions. These studies have proved invaluable in their determination of the role of shear flow in endothelial cell morphology and platelet adhesion. Moreover, microfluidic devices offer a high-throughput platform and allow for real-time observation and quantification of biochemical mechanisms and responses. Lastly, studies using microfluidic devices hold potential for the determination of how the interaction between blood and endothelial cells regulates vascular damage and disease.

The work below describes initial adaption and optimization of an endothelialized microfluidic chamber for the investigation of the platelet-endothelium interface and proposes experiments to build off these results in order to validate the function of the device and to fully evaluate its potential impact for future studies.

4.2.3 Types of microfluidic devices for investigating blood coagulation

The first microfluidic models to investigate platelet function were introduced in the 1970's and consisted of a single channel created from inverted sections of blood vessels called annular perfusion models. These devices were developed to study platelet adhesion under flow and demonstrated the importance of platelet interaction with vWF and vWF-FVIII for proper platelet adhesion.[118, 119] Importantly, these models used native tissue of blood vessels, however this limited the amount of experiments and required sample preparation for histological staining in order to analyze the results.

An important step forward in the development of microfluidic devices for the study of the platelet-endothelium was the inclusion of an endothelial cell culture within optically-clear devices, such as parallel-plate flow chambers or later, borosilicate glass capillaries.[120, 121] These devices offered a simpler method of preparation than previous devices and allowed for real-time investigation of the interaction between blood and endothelial cells. The use of these devices has provided considerable insight into the mechanism of platelet adhesion under flow and in particular, demonstrated the shear-dependent binding of GPIb to vWF and its contribution to thrombus formation.[122] In addition, studies have utilized parallel-plate flow chambers to demonstrate the importance of laminar flow and shear stress in mechanotransduction by

endothelial cells, specifically their ability to migrate towards damaged or denuded endothelium, or the upregulation and expression of platelet inhibitors and anticoagulant molecules.[123, 124] Parallel-plate devices present a simpler experimental model and allow for specific surface composition, however these devices are limited by the stiffness of glass and are gas-impermeable materials.

A major advance in the field of microfluidic devices was the adoption of polydimethyl siloxane (PDMS), a compliant polymer that allows for the fabrication of optically-clear casts that are inert and gas permeable. The use of PDMS enables precise and controlled organization of ECM proteins and adhesive substrates for defined spatial interaction with cells.[125] In addition, studies have utilized PDMS to design deformable devices exhibiting specific channel geometry or desired shear rates and stresses.[126] These devices are easily formed via plasma bonding of the PDMS cast to microscope slides and are cost effective. These devices enable culturing of endothelial cells after coating the inside of the channel with adhesive substrates for cell attachment. Alternatively, microfluidic PDMS devices have also been developed to incorporate a 3D ECM for investigation of cancer metastasis, specifically extravasation and intravasation, demonstrating the potential for investigation of similar mechanisms dependent on the ECM and endothelial cells.[127] The establishment of a 3D ECM within the PDMS housing also enables increased solute permeability and deformation of endothelial cell layer.[128] Importantly, these channels form a cylindrical shape for recapitulating physiological geometry and fluid dynamics experienced by endothelial cell layers of smaller vessels.[129] The major biological concern of PDMS devices is the possible absorbance of hydrophobic molecules that may lead to toxic conditions during longer incubation. In contrast, PDMS devices are unable to recreate conditions of high Reynolds numbers, limiting their application to situations of laminar flow where viscous forces dominate.

The platelet-endothelium interface is a complex setting of cells and tissue that respond to mechanical, chemical, and biological stimuli to maintain vascular homeostasis. As such, the dysregulation of this interface has detrimental effects that can promote and increase progression of vascular disease. In particular, TNF-α is a cytokine that has been implicated in inflammation and thrombosis. Studies have demonstrated TNF-α promotes endothelial cell activation, resulting in increased permeability and expression of TF.[130-132] The work described below is focused on the interactions of the platelet-endothelium interface after stimulation by TNF-α.

As demonstrated by early microfluidic studies, blood rheology and shear flow play critical roles in the regulation of endothelial cell activity and inhibition of platelet adhesion.[133] The ability of endothelial cells to respond to changes in these properties through gene and protein expression further highlights the importance of microfluidic devices for the investigation of vascular function.[124]

In this study, we have developed a model to study the platelet-endothelium interface under shear flow by incorporating human umbilical vein endothelial cell (HUVEC) cultures within a 3-D type I collagen matrix. This model provides advantages over our 2D *in vitro* flow model by incorporating a cylindrical blood vessel geometry within a 3-D ECM matrix. This allows for visualization of platelet aggregation and quantification of the permeability across the endothelium. A similar 3-D matrix model has been used for investigation of vascular permeability during cancer metastasis, in the absence of blood flow.[127]

Polydimethyl siloxane (PDMS) has been used for the development of microfluidic chambers due to the material's desirable properties to form specific material geometries. This new method allows for production of optically clear rectangular and cylindrical channels for investigation of

shear-dependent interactions. Accordingly, we have developed a 3-D shear-flow cell culture model using type 1 collagen to from a stiff matrix housing a cylindrical channel that can be seeded with HUVECs to form a confluent endothelial cell layer. The major advantage of our model is the real-time observation of vascular permeability during shear-flow perfusion by recording intensity of fluorescent markers, which cannot be observed in classic platelet adhesion models. In addition, HUVECs are grown under shear flow to recreate the most physiologic environment for cellular response to mechanical stimuli, unlike static cell culture monolayers.

4.2.4 Fabrication of Endothelialized Microfluidic Device

Reagents were purchased from Sigma-Aldrich (St Louis, MO, USA) unless specified otherwise. Sylgard 184 Elastomer (DOW Corning), human umbilical vein endothelial cells (ProCell). Fabrication of the endothelialized microfluidic device required assembly of a sealed, perfusable housing and establishment of a type I collagen matrix. The body of the microfluidic device is made from Sylgard 184, a mixture of dimethyl siloxanes commonly known as polydimethyl siloxane (PDMS). PDMS was cured at a 10:1 (base: curing agent) ratio by weight in a custom aluminum mold at 80°C for 40 minutes. The cured product formed straight rectangular channels with dimensions 1.2 mm × 1.5 mm × 5 cm (W×H×L). Individual casts were cut to expose the channel and subsequently punched from the top to form two cylindrical holes on each end that would serve as ports for perfusion. The PDMS cast and a Superfrost microscope slide were treated with a BD-20AC corona treater for 90 seconds to activate the surfaces and then the corona treated surfaces were firmly pressed together to form a plasma bond. A 9 cm long piece of no-kink super-elastic nitinol wire with a diameter of 150 μm was then threaded through the channel and suspended from coverslips adhered on each end of the microscope slide. The rod was then straightened out and centered in the channel before being glued in place. Lastly, teflon tubing was

glued into each of the 4 ports of the device to allow for attachment of tubing and perfusion through the device.

Once assembled, the device was then prepared for matrix establishment and cell seeding. Poly-L-lysine (1 mg/mL) was incubated within the channel to enhance adhesion of the collagen matrix to the PDMS channel. The device was then washed to establish a neutral pH within the channel to allow for curing of the collagen solution added later. Type I collagen was chosen to create the ECM of the device based on its prevalence in human tissue and its desirable structural and biochemical properties for cell adhesion and formation of an endothelial cell monolayer. For this purpose, a high concentration rat tail type I collagen was mixed with NaOH and PBS to create a basic solution with a concentration of 7 mg/mL of collagen that would initiate curing once added to the PDMS channel. The collagen solution was injected into the channel and excess material aspirated, leaving the channel filled and the nitinol rod surrounded by collagen. Devices were initially cured at room temperature for 10 mins to avoid the formation of bubbles within the matrix, and then transferred to a 37°C incubator to promote polymerization of the collagen matrix. After 1 hour, the collagen matrices became optically transparent matrix with a slight opaqueness. To reinforce the type I collagen matrix, high strength agarose was added to the centermost ports flanking the channel in order to prevent the matrix from dislodging or deforming upon introduction of shear stress. The nitinol rod was carefully removed and replaced by cell culture media, leaving a straight cylindrical channel. The device was then placed in the 37°C incubator to allow the cell culture media to equilibrate throughout the collagen matrix prior to cell seeding.

Cells were then seeded into the device at a concentration of 6-10 $\times$ 10^6 cells/mL and allowed to adhere under low shear rate. After, an initial layer of cells began to adhere, extra volume was added to the inlet of the device to increase the shear rate and transport more cells through the

channel. After 2 hours, the initial layer of endothelial cells began to exhibit morphological changes, exhibiting a cobblestone shape. Devices were then connected to perfusion systems fed by gravity flow and placed in the incubator at 37°C and 5% CO_2. HUVECs were cultured using EBM growth media and confluent monolayers formed after two days, evident by the containment of phenol red within the cylindrical channel.

4.2.5 Investigation of thrombus formation during whole blood perfusion

Confluent HUVEC monolayers were serum starved for 2 hours prior to treatment in the presence or absence of TNF-α (10 ng/μL) for an additional 4 hours. Sodium citrate anticoagulated blood was recalcified by addition of Ca^{2+}/Mg^{2+} (7.5 mM and 3.75 mM) prior to perfusion at venous (100 s^{-1}) and arterial (1000 s^{-1}) shear rates. Platelet adhesion and aggregation to the endothelial cell layer was visualized using DIC microscopy and fluorescent markers to identify HUVECs, adherent platelets and fibrin incorporation.

Initial experiments performed using the endothelialized microfluidic chamber investigated the interaction of flowing blood with endothelial cells after treatment with TNF-α, as described in the methods above. As seen in Figure 9, the vehicle device was able to prevent platelet adhesion after 10 mins of blood perfusion, in contrast to the treated chamber which resulted in visual platelet adhesion and aggregation. There was also a significant difference observed in the diameter of the endothelialized chamber, as treatment with TNF-α induced vasoconstriction of the endothelium. At present, the ECM only consists of type I collagen and results in potent platelet adhesion and activation via GPVI. Future development of the platform should aim to incorporate BM proteins for a physiological environment beyond the endothelial cell layer.

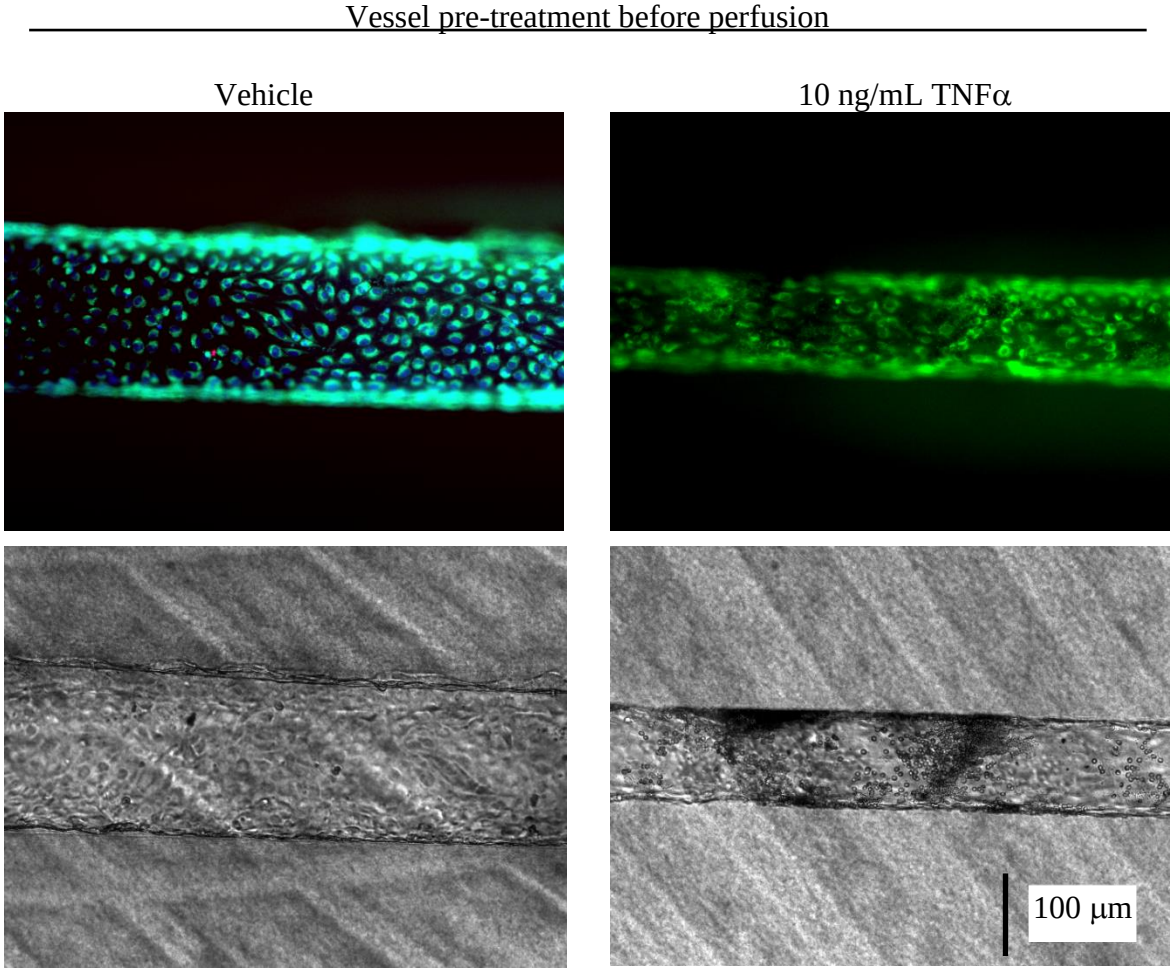


Figure 9. Blood Perfusion of an Endothelialized Microfluidic Chamber

Endothelialized microfluidic chambers were treated in the presence and absence of 10 ng/mL TNF-α for 4 hours prior to perfusion of human blood at 300 s^{-1} for 10 minutes and imaged using DIC and fluorescent microscopy. Vehicle conditions (left, bottom) did not support platelet adhesion. In contrast, TNF-α treated devices (right) supported platelet adhesion and aggregation (right, bottom).

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