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Michael Ian Edward Quach Date

Platelet Clearance and Mechanosensation via GPIb-IX-V: How Platelets Use the Force

By

Michael Ian Edward Quach
Doctor of Philosophy

Graduate Division of Biological and Biomedical Science
Biochemistry, Cell, and Developmental Biology

_____

Renhao Li, Ph.D.
Advisor

_____

Graeme L. Conn, Ph.D.
Committee Member

_____

John R. Hepler
Committee Member

_____

David G. Lynn
Committee Member

_____

Brian G. Petrich
Committee Member

Accepted:

_____
Lisa A. Tedesco, Ph.D.
Dean of the James T. Laney School of Graduate Studies

_____
Date

Platelet Clearance and Mechanosensation via GPIb-IX-V: How Platelets Use the Force

By

Michael Ian Edward Quach

B.S., Muhlenberg College, 2014

Advisor: Renhao Li, Ph.D.

An abstract of a dissertation submitted to the Faculty of the

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Abstract

GPIb-IX-V is the primary mechanosensitive receptor complex on the platelet surface. All ligands targeting the complex bind to the GPIbα subunit and unfolding of a membrane proximal mechanosensory domain (MSD) in GPIbα leads to receptor activation. Despite this, expression of the other subunits of GPIb-IX-V (GPIbβ and GPIX) is required for expression on the platelet membrane. I present evidence that antibodies targeting GPIbβ modulate the activity of GPIb-IX-V, supporting a model wherein conformational changes in GPIbβ are part of the transduction pathway for mechanical signals through GPIb-IX-V. Many patients suffering from immune thrombocytopenia are resistant to first-line immunosuppressive treatments, a prognosis associated with autoantibodies targeting GPIb-IX-V. However, the mechanism of this resistance is unknown. Here, we present evidence that antibodies against the ligand binding domain (LBD) of GPIb-IX-V activate the receptor in a shear-dependent manner. Single-molecule optical tweezer experiments demonstrate that this effect depends on and antibody's unbinding force from the LBD. Antibodies with unbinding forces too low to unfold the MSD in GPIbα cannot activate the receptor, nor can they induce IVIG resistant ITP. Imaging flow cytometry and light transmission aggregometry reveal that activating antibodies can sustain crosslinking of platelets via GPIb-IX-V under shear, suggesting a mechanism by which anti-LBD antibodies exert force on GPIb-IX-V via crosslinking, activate GPIb-IX-V by unfolding its MSD, and induce Fc-independent platelet clearance.

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CHAPTER I

INTRODUCTION

I. Introduction

"The task of science is to stake out the limits of the knowable, and to center consciousness within them." – Rudolf Virchow

A. Stop! In the Name of Blood: Circulation and Hemostasis

It was the father of analytical psychology, Carl Jung, who said that history is not contained in books, but in our very blood. Although Jung was likely referring metonymically to one's familial ancestors, blood and the circulatory systems through which it flows tell a history different in kind but equally rich, not only of man but of our phylogenetic ancestors stretching back to early metazoans. Through a brief survey of the emergence and evolution of circulatory systems, we can glean a deeper understanding of the structure and function relationships in our own blood vascular system. Multicellular organisms of the smallest order can rely on the energetically conservative process of diffusion for the distribution of oxygen, waste products, and nutrients. However, as body geometries become larger and more functionally distributed, there quickly arises a need for more sophisticated transport and interchange (that is, circulatory) systems. Although diploblasts, some of the simplest animals, have "circulatory systems" that function mainly to push seawater through their body, more complex triploblastic organisms employ what we might more commonly think of as a blood vascular or circulatory system[1]. Almost all triploblasts (including humans) have a body cavity between their ectoderm (outer body wall) and endoderm (their digestive tract) called a coelom along with a circulatory system to distribute fluid throughout the different segments of the body cavity. These systems consist of spaces (vessels, compartments, pumping organs) filled with blood or another circulatory fluid[2] and fulfill a host of functions not only related to

nutrient transport but storage, thermoregulation, immunity, waste removal, and wound healing.

The description of triploblastic circulatory systems thus far may incorrectly conjure an image of a unified design where, in truth, there is great diversity. In arthropods and mollusks, open circulatory systems bathe organs directly in a substitute for blood called hemolymph[3]. Lobsters (which are arthropods) have a well-developed heart which expands to passively fill with hemolymph, then empties into 7 major arteries before spilling into the body cavity, called the hemocoel[4]. Insects have a main pump somewhat analogous to a heart but not as developed as a lobster's, called the dorsal vessel, which circulates hemolymph through the hemocoel. In addition to the main pump, hemolymph is moved along by a series of distributed muscular pumps or "auxiliary hearts"[5] and despite a lack of discrete vessels, studies in drosophila show that circulatory fluid follows certain "preferred routes"[6]. Along with the lamprey, the hagfish is one of the oldest (likely *the* oldest) extant vertebrates[7]. Given that all vertebrates are derived from a common ancestor, the hagfish's circulatory system gives us insight into the earliest prototypical design of our own blood vascular system. Hagfish have a two-chambered heart with one atrium and one ventricle, along with a secondary heart which functions to move blood between the gut and liver[8]. As is the case with all vertebrates, the hagfish circulatory system is made up of blood vessels lined with endothelial cells which are phenotypically different based on the vascular bed they are found in[2,9]. This is in contrast to the closed circulatory systems seen in invertebrates, made up of spaces between epithelial cells lined only with matrix. The specialization of the endothelial lining to the particular vascular bed they are found in or "endothelial heterogeneity" is a well-studied phenomenon and extends to macro-level architecture, morphological differences, changes in inflammatory

propensity, regulation of transport, and the tightness of junctions[9-12]. Its presence in hagfish indicates that it likely evolved as an early feature of vertebrate circulation.

The human circulatory system is characterized by a powerful, central propulsive pump in the heart which propels blood through a series of distributing and collecting vessels which are lined with a tightly controlled barrier of specialized endothelial cells. One of the benefits of our endothelium-lined vasculature is regulation of the transport of plasma proteins and other components between the circulation and interstitial fluid. In the vertebrate circulatory system, blood is separate from the interstitial fluid and exchange between the two occurs in specialized capillary beds[2]. When specific tissues are active (muscle groups during exertion, the gastrointestinal tract during digestion, etc.) they require an increased supply of nutrients and oxygen and produce more waste. Additionally, blood flow to certain tissues is of special systemic importance; control of blood flow to the skin determines heat loss and thus thermoregulation and delivery of sufficient plasma to the kidneys allows them to excrete waste and modulate concentrations of electrolytes. Instead of the heart pumping much harder or slowing down significantly, local vascular beds can vasodilate or vasoconstrict to meet individual tissue needs[13]. Along with the nervous system, endothelial cells exert control over vascular tone (the extent to which a vessel is constricted) by altering the activity of vascular smooth muscle (VSM) via paracrine signaling through molecules like nitric oxide (NO)[14,15].

Throughout the development of the circulatory system, specific vascular beds are formed by a system of vessels that follows a hierarchy of vessel diameter wherein larger vessels branch into smaller and more numerous vessels that deliver blood to different tissues[16]. It is said that

evolution proceeds via the path of least resistance, but resistance (that of vessels to shear stress) may have played an important role in the evolution of the branched vascular circulatory system. In the early 20th century, the physiologist Cecil Murray proposed a theoretical relationship between the radii of a vessel immediately upstream of a branch point, called a parent artery, and its resultant daughter arteries[17]. He operated under a few key assumptions: (i) it costs energy to make vessels and the blood contained in them (ii) it costs energy to move the fluid through the vessels (iii) the energy cost of moving the fluid follows Poiseuille's law for flow in a tube[18,19] (iv) blood is a Newtonian fluid moving in fully developed laminar flow. Based on these assumptions, Murray's law describes the optimal relationship to minimize the energy cost as such:

$$r_0^3 = r_1^3 + r_2^3 + \cdots + r_n^3$$

Surprisingly simple, the law states that the parent artery's radius cubed (r_0^3) is equal to the sum of the cubed radii of n daughter arteries that branch from it. Although it would later be revealed that some of Murray's assumptions didn't hold true (blood has many non-Newtonian characteristics, such as shear-thinning[20,21]), his function is surprisingly well reflected in nature. When put to the test in human coronary arteries[22], rat arteries[23], and even astrorhizae. the branched system of canals of several species of sponge[24], circulatory systems from phylogenetically diverse organisms seem to be in good agreement with Murray's law. Although it makes intuitive sense that the most energetically conservative vascular architecture would be the most evolutionarily favored, Murray did not provide any hypotheses for how vessel radius might be regulated biologically. Nearly a century later, the most commonly proposed mechanism states that shear force (frictional force exerted on the vessel by blood flow) can trigger circumferential growth in vessels when wall shear passes a threshold[25]. This mechanism is sometimes referred to as "shear force remodeling", or SFR.

Aberrantly high shear can cause many issues, including mechanical damage to cells and disruptions to laminar flow that are energetically unfavorable and associated with atherosclerosis[26]. As such, it is reasonable to assume that vessels can adjust their radii to compensate and avoid these deleterious effects. SFR has significant support from modeling studies[27,28], which demonstrate that it is possible to arrive at Murray's law from a fluid dynamics framework. Furthermore, it has long been established that the endothelium reacts to shear differentials with changes in protein secretion and membrane potential[29-32]. In 2002, evidence began to emerge that an endothelial stretch-activated cation channel or channels might mediate the response to hemodynamic forces[33]. Within the last 5 years, a series of several studies narrowed down the identity of this postulated channel to the shear-sensing Piezo 1 channels. Expression of these channels in endothelial cells is necessary for the proper development of the vasculature; total deletion in mice is embryonic lethal and mutations in human PIEZO1 gene lead to severely abnormal lymphatic vascular development, indicating a large contribution of shear sensation by the endothelium to vascular development and ongoing remodeling[34-36]. This is a fascinating demonstration of one of the many vital functions that shear has in the biology of the blood vascular system. The coevolution of our branched, endothelialized vasculature and the blood that flows through it make them unique compared to other tissues due to the challenges and biophysical opportunities presented by shear flow. Going forward, it is important that we not overlook the potential contributions of shear to the many interactions and processes that take place in the blood.

Blood is a complex milieu of ions, biological molecules, cells, and cell fragments. Blood cells are broadly of myeloid (erythrocytes, megakaryocytes, granulocytes/monocytes) or lymphoid (T-cells, B-cells, and NK cells) lineages[37], and serve a variety of functions including transport,

inflammation, immunity, and hemostasis. The last of these, hemostasis, is the process by which our bodies ensure minimum blood loss upon vessel injury, and as it turns out, our circulatory systems function best when blood remains inside, rather than outside of our bodies. The process of hemostasis is not only necessary to seal up grievous wounds or shallow cuts, but also to close the thousands of small ruptures that occur daily in the microvasculature. The endothelial lining of blood vessels plays several major roles in hemostasis. Endothelial control of vascular tone helps to ensure laminar blood flow and minimize cross-current and turbulent flow. Turbulence often leads to clotting pathologies like atherothrombosis and increased cardiovascular mortality[26,38,39]. Under normal conditions the intact endothelium can also actively repress thrombosis (pathological, aberrant blood clotting) via secretion of inhibitors of platelet activation and coagulation[40,41]. However, perhaps the most important role of the endothelium in hemostasis is in absentia, during traumatic vessel damage.

Following vascular injury, disruption of the endothelial layer triggers a cascade of events that stop the blood from extravasating. Hemostasis is achieved by (i) vessel constriction, (ii) platelet arrest, activation, and formation of a platelet plug, (iii) platelet aggregation and blood coagulation, and (iv) growth of fibrous tissue into the clot space to permanently seal the vascular breach[13]. Immediately following vessel trauma, the VSM contracts, causing local vasoconstriction to mitigate blood loss from the damaged vessel. This early response is triggered by local myogenic spasm in response to endothelial damage[42,43], nervous reflex initiated by pain, and eventually by release of the vasoconstrictor thromboxane A_2 (TXA2) by platelets[44,45]. Under normal circumstances, the endothelial layer acts as a barrier between platelets and the agonist-rich sub-endothelial matrix. When vascular damage disrupts the

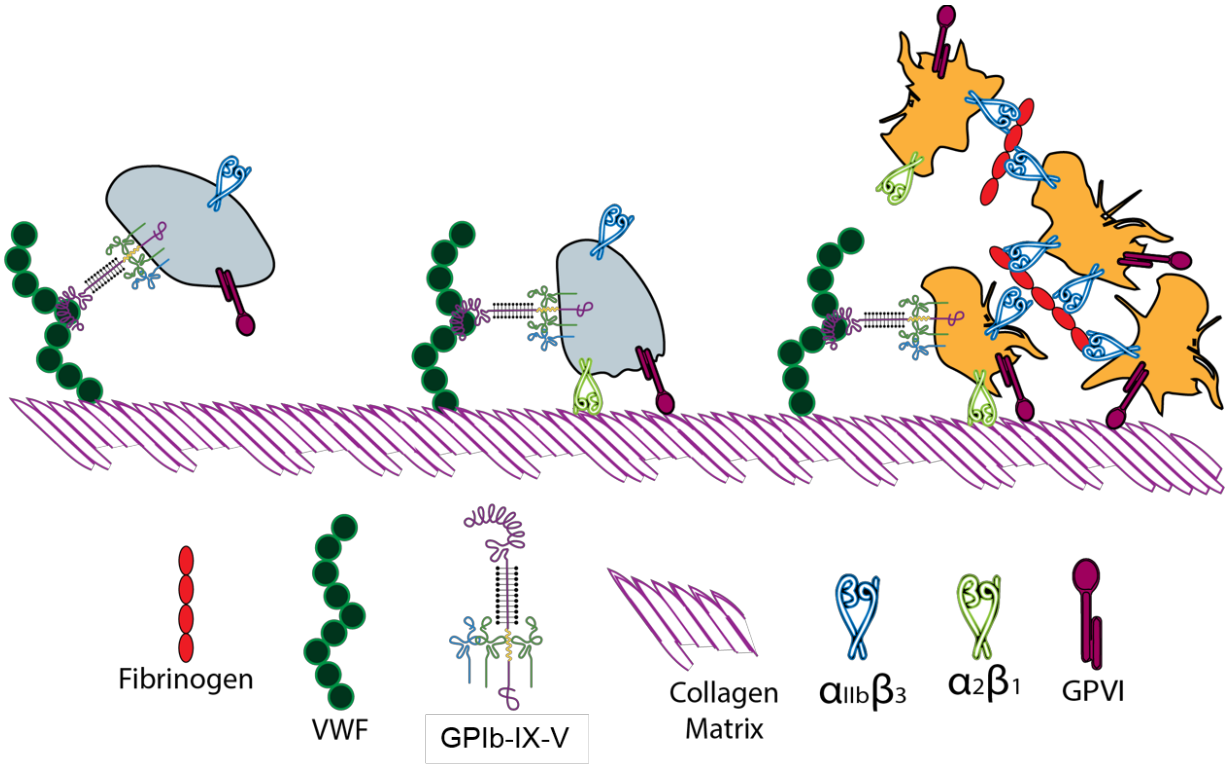

Figure I.1 Platelet adhesion in primary hemostasis. The early steps of platelet adhesion and platelet plug formation. Initial adhesion to the exposed matrix collagen is mediated by GPIb-IX-V's interaction with shear-activated VWF. As GPIb-IX-V binds to VWF, it puts a brake on circulating platelets, which begin to "roll" along the matrix. Once platelets begin to roll, other collagen receptors like GPVI and $\alpha_2\beta_1$ arrest platelets. Shear-induced activation of GPIb-IX-V, in combination with some activation through GPVI and other receptors, leads to platelet activation (depicted by orange platelets undergoing morphological changes). Platelets then expose active $\alpha_{IIb}\beta_3$, which binds fibrinogen and VWF, facilitating aggregation of platelets and formation of the platelet plug.

endothelial layer, the blood is exposed to the components of the matrix (proteoglycans, fibronectins, laminins, collagen IV, among others)[46,47]. Matrix collagen binds to von Willebrand Factor (VWF), a large multimeric protein secreted into the plasma by endothelial cells. VWF multimers span a range of sizes and are made up of disulfide-linked subunits each over 2,000 residues long[48]. Under normal circumstances, VWF circulates in a compact, globular form[49]. However, when tethered to collagen under flow conditions, VWF undergoes force-induced unfolding to expose its A1 domain, normally masked by an autoinhibitory module, which mediates binding to the platelet surface glycoprotein (GP)Ib-IX-V[50-52]. Through this mechanism, inert VWF can circulate without binding platelets, only becoming active when localized to the site of injury. Platelets are thus arrested at the site of injury via VWF's interaction with GPIb-IX-V. Many other platelet receptors participate in adhesion to the subendothelial matrix, but these receptors cannot initiate thrombus formation in regions of high blood flow rate without the initial interaction between GPIb-IX-V and collagen-tethered VWF[50,53]. Under arterial shear rates (upwards of 1000 s^{-1}), platelet adhesion cannot occur at all without the engagement of GPIb-IX-V[54]. Once bound to VWF under flow, GPIb-IX-V's membrane-proximal mechanosensory domain (MSD) is unfolded, triggering early platelet activation[55,56]. This is another example of how the blood-vascular system exploits the force generated by blood flow, this time to accomplish both the priming of an important hemostatic ligand (VWF) and activation of its receptor (GPIb-IX-V) on platelets. In addition to the indirect platelet-collagen interaction mediated by GPIb-IX-V and VWF, several platelet receptors interact with directly with collagen; chief among these receptors are GPVI and α_2-β_1[57]. GPVI signaling through the immunoreceptor tyrosine–based activation motif (ITAM) on the tightly associated FcR γ chain contributes to platelet activation[58-60], although the relatively mild effect of GPVI mutations and deficiency on hemostasis has called into question the

importance of its role in the process[61,62]. Other platelet contributors to subendothelial matrix adhesion include integrin $\alpha_6\beta_1$, which recognizes laminins, and $\alpha_5\beta_1$ which binds to fibronectins[63].

Once the matrix-tethered platelets are activated, they secrete a slew of signaling molecules from granules. Platelets contain two main types of granules, α granules and δ or "dense" granules, which fuse with the platelet membrane upon activation. Two key agonists released by adherent platelets are adenosine diphosphate (ADP) and TXA2, which activate platelets through the $P2Y_1$ and $P2Y_{12}$ purinergic receptors, and the aptly named thromboxane receptor, respectively[64,65]. This propagating step in platelet activation is a common target for anti-platelet therapies such as $P2Y_{12}$ inhibitors like clopidogrel (trade name, Plavix) or cangrelor[66,67] and aspirin, which blocks the synthesis if TXA2[68-70]. Although the term "platelet activation" is a sometimes used as a catchall phrase encompassing the release of granules from the platelet membrane, changes in morphology from discoid to protruding filopodia and lamellipodia, and various adhesive events, one of the most important hallmarks of platelet activation is the inside-out activation of platelet surface integrins such as $\alpha_{IIb}\beta_3$, $\alpha_v\beta_3$, and $\alpha_2\beta_1$. Perhaps the most critical of these integrins is $\alpha_{IIb}\beta_3$, also known as GPIIbIIIa or CD41/61. Expressed mainly on platelets and megakaryocytes[71-73], it represents 3% of the total protein content of platelets[74], with each platelet bearing as many as 100,000 copies[75]. $\alpha_{IIb}\beta_3$ is absolutely essential for platelet hemostatic function, as it is responsible for binding to several matrix proteins including fibrinogen, fibronectin, vitronectin, thrombospondin-1, and VWF[76]. $\alpha_{IIb}\beta_3$ recognizes a simple Arg-Gly-Asp (RGD) motif which is present in many of these ligands and which is also recognized by several other integrins[77]. Once platelets adhere to the site of vascular damage, signaling downstream of GPIb-IX-V activates $\alpha_{IIb}\beta_3$, which binds to VWF and fibrinogen,

leading to robust platelet aggregation[78-80]. As with $P2Y_{12}$ and thromboxane receptor, $\alpha_{IIb}\beta_3$ antagonists have been used to block thrombosis[81], but their use has begun to be phased out in favor of other anti-platelet or anti-coagulant strategies. Overall, during this stage of hemostasis (sometimes called *primary hemostasis*), growing numbers of platelets accumulate along the exposed subendothelial matrix and recruit/activate additional platelets to form a platelet plug. The plug is held together loosely by platelet adhesion to "sticky" proteins like VWF (via GPIb-IX-V and $\alpha_{IIb}\beta_3$), fibrinogen (via $\alpha_{IIb}\beta_3$) and collagen (via GPVI and $\alpha_2\beta_1$), but if the breach is small enough, it may be sufficient to stop blood loss.

Following (and in parallel to) the development of the initial platelet plug, secondary hemostasis involves the deposition of fibrin, stabilization of a thrombus, and blood coagulation. Although secondary hemostasis is remarkably complex, involving several dozen soluble factors participating in intersecting cascades, the nexus of this network centers around the conversion of prothrombin to thrombin, which in turn cleaves soluble fibrinogen into fibrin[78]. Fibrin chains then form a covalently-linked, polymeric fibrin net that stabilizes a clot[82]. The fundamental role of fibrin-generation in hemostasis is literally an *ancient* concept, dating back more than two millennia to the Greek philosopher Plato, who first observed and described the fibrinous nature of clotted blood and even coined the term "fibrin". By the early 1900s, a five factor (tissue factor, prothrombin, thrombin, fibrinogen, fibrin) model had been advanced[82], and while this better captured the reality of coagulation cascades, it was still too simple. Fast forward to our current understanding of coagulation which, while it too may be overly simplistic, involves cascades of zymogen and cofactor clotting factors which iteratively activate the next factor in line to form two intersecting pathways for coagulation. Before these two cascades are covered in more detail, it will be useful to include a small note on

nomenclature. As is often the case in biology, not all factors follow these rules and many have colloquial names in the literature. Nonetheless, clotting factors by and large follow a three-part naming system. The shorthand for each factor begins with a letter "F" (factor) followed by a roman numeral specific to each factor, and if it is a zymogen in its active form, a lowercase "a" (active) is added to the end. For example, the inactive form of thrombin, prothrombin, is referred to as "FII" and thrombin as "FIIa".

While it is not inclusive of all regulators and cofactors, Figure I.1 outlines the extrinsic and intrinsic coagulation pathways. The extrinsic pathway is sometimes referred to as the initiation phase of coagulation or the tissue factor pathway[82]. The latter name is a reference to the initiating step of this pathway, which begins when traumatized subendothelial tissues like fibroblasts or VSM expose a transmembrane glycoprotein called tissue factor (TF or FIII) on their membranes. TF is the receptor/cofactor for the serine protease FVII[83]. Although fibroblasts and VSM express a large amount of TF on their surfaces, it appears not to be in a pro-coagulant state until cells are challenged with other agonists like Ca^{2+}[84] and there the interaction between TF and FVII leads to changes in TF phosphorylation[85]. In the case of hemostasis, it is likely that the trigger for an increase in TF procoagulant activity is damage to the cells it is expressed on[84,86]. TF acts as a cofactor for FVII, prompting its conversion to FVIIa, and this complex then cleaves/activates both FIX and FX, earning it the moniker "extrinsic Xase" (pronounced "tennase")[78,87]. FXa joins with FVa to form the prothrombinase complex[88] which generates thrombin from prothrombin. In this phase, the amount of thrombin generated from FXa is minimal, as the presence of plasma protease inhibitors like tissue-factor pathway inhibitor (TFPI) antagonize FXa and antithrombin (AT) tempers the activity of many coagulation proteases including FXa, FIXa, thrombin[89,90]. The FIXa generated

by the intrinsic Xase complex is not targeted by TFPI. It is this factor which is free to then diffuse to other cells and participate in the intrinsic pathway, discussed below. Although the thrombin generated by the tissue factor pathway alone is insufficient to kick off robust fibrin polymerization, this promiscuous workhorse protease of the coagulation system has other targets to cleave. Thrombin also activates the FV in the prothrombinase complex, which accelerates thrombin generation[91,92]. Additionally, thrombin activates components of the intrinsic pathway, converting FVIII to FVIIIa and FXI into FXIa[78]. The activation of these factors by thrombin is sometimes referred to as the amplification phase of coagulation.

In our current understanding of the tissue factor (extrinsic) pathway, initiation of hemostasis via prothrombinase occurs mostly along the surface of TF-expressing extravascular cells. However, in order for fibrin polymers to enmesh and stabilize the clot, thrombin generation must first proliferate throughout the growing platelet plug. The intrinsic pathway of coagulation spatially extends the processes set into motion by the TF pathway and propagates thrombin generation throughout the forming clot. For this reason, you may see it referred to as the propagation phase, although it may be reductive to think of the intrinsic pathway merely as a propagation of what was initiated by the TF pathway. While the extrinsic pathway takes place on TF-rich surfaces, the intrinsic pathway occurs on procoagulant phospholipids found on the surface of activated platelets and microparticles. When platelets are activated by thrombin or collagen, they expose procoagulant phospholipids, specifically phosphatidylserine (PtdSer), on the outside of their membranes[93]. The composition of phospholipids in membrane bilayers is asymmetrical, and PtdSer normally sits on the inner leaflet of the membrane. This skew in composition is maintained by proteins called flippases, which “flip” PtdSer from the extracellular to the cytosolic side of a membrane[94]. During

platelet activation, increases in intracellular calcium activate TMEM16F, a phospholipid scramblase that exposes PtdSer on the outer membrane of platelets[95], providing the surface necessary for the intrinsic pathway. Indeed, without platelet TMEM16F, TF-induced thrombin generation is significantly suppressed[96]. Recall that, in the amplification phase, both FXIa and FVIIIa are activated by the initial thrombin generated in the tissue factor pathway (Fig. I.2). FXIa cleaves and activates FIX to FIXa. In addition to FIXa generated by extrinsic Xase, this newly formed FIXa associates with FVIIIa on the phospholipid surface to form "intrinsic Xase", and generate FXa[97]. Generation of intrinsic Xase is a pivotal step in hemostasis, and absence or deficiencies in either FVIII or FIX lead to severe bleeding disorders (hemophilia A and B, respectively)[98]. Intrinsic Xase mediated generation of prothrombinase throughout the clot then results in a widespread burst of thrombin generation, driving cleavage of fibrinogen into fibrin monomers. Fibrin monomers undergo hydrogen bond mediated polymerization into an initially weak and highly extensible network[78,99]. In a final stabilizing step, thrombin-activated FXIIIa covalently crosslinks fibrin to yield an elastic, stable, polymerized fibrin clot which retains platelets and erythrocytes[100-102].

Earlier in this section I alluded to a more complex role for the members of the intrinsic pathway than just a multiplicative expansion of the extrinsic pathway. Our traditional understanding of the interplay between coagulation cascades has initiation of coagulation resting firmly on the shoulders of the extrinsic pathway, which leads to a temporally downstream induction of the intrinsic pathway. A more nuanced view of the pathways of coagulation indicate that the intrinsic and extrinsic pathways can be activated more or less simultaneously. This view is supported by the identification of three physiological triggers to the intrinsic pathway, aka the contact activation pathway. Blood contact with collagen[103], long

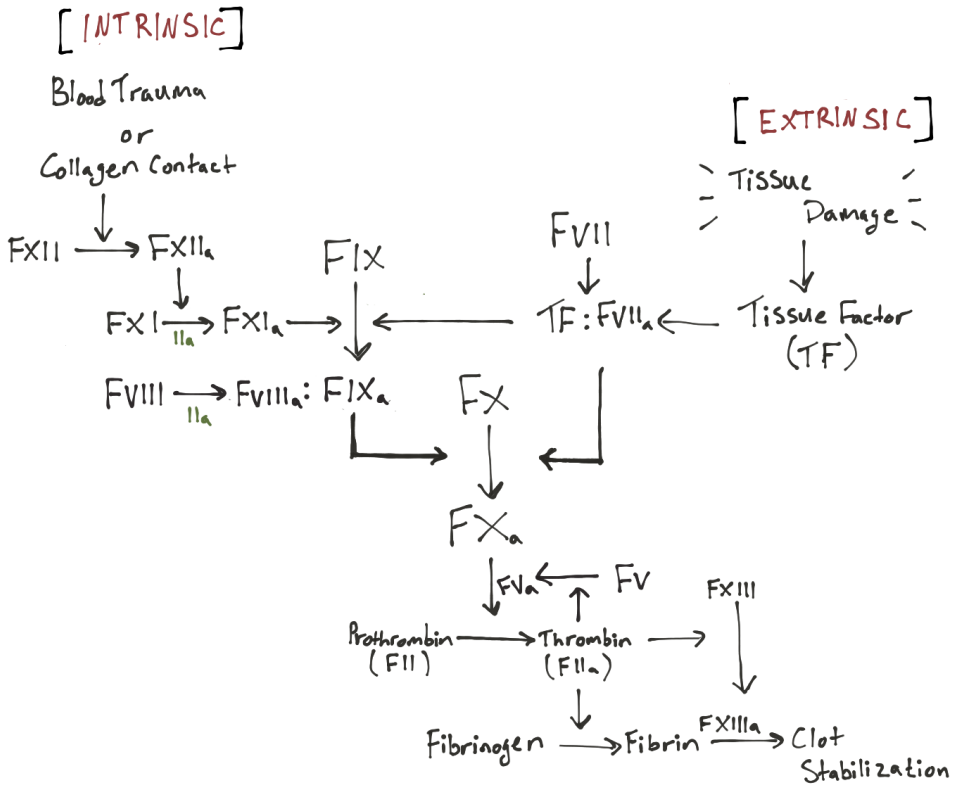

Figure I.2. The intrinsic and extrinsic pathways of coagulation. Activation cascades of the extrinsic and intrinsic pathways of coagulation. Briefly, in the extrinsic pathway, exposed tissue factor and FVIIa form extrinsic Xase, activating FX. FXa, together with FVa, forms prothrombinase which activates thrombin (FIIa). Thrombin then cleaves FV, FXI, FVIII, FXIII and fibrinogen. In the intrinsic pathway, FXIIa activates FXI, FXIa activates FIX. Together with FVIIIa, FIXa forms intrinsic Xase. Intrinsic Xase also activates FX to form prothrombinase. FXIII covalently links fibrin to stabilize the clot. Activation of zymogen factors facilitated in part by thrombin sometimes indicated by "IIa" below the arrow.

polyphosphate chains[104], and neutrophil extracellular traps (NETs)[105,106] have all been implicated in activation of hemostasis and in pathological thrombosis. Thusfar, I have not addressed the activation of the coagulation factor atop the cascade of the intrinsic pathway, FXII (Hageman factor). While thrombin can activate the FXIa/FIXa circuit of the intrinsic pathway, FXIIa also serves this role by cleaving FXI to FXIa. Deficiency or inhibition of FXII leads to defective thrombus formation[107], and blocking its activity may be effective against thrombosis[108,109]. In the last decade or so, evidence has emerged that collagen, exposed at in the subendothelial matrix, is also a trigger for the intrinsic pathway via FXII, even in the absence of platelets[103]. Many studies also highlight a role for platelet-derived polyphosphate, a linear chain of inorganic phosphates released from platelet dense granules upon activation[110], in activation of FXI and FXII[111,112]. These triggers for the intrinsic pathway (exposure of collagen and activation of platelets) occur during formation of the platelet plug, contemporaneous with exposure of TF-rich tissues. Together, these results paint a picture of ongoing additive contributions of the extrinsic and intrinsic pathways to thrombin generation, coagulation, and hemostasis.

When injury first occurs, the robust amplifying cascades described herein are important in ensuring a rapid clotting response that minimizes blood loss. However, unchecked coagulation can lead to thrombosis in a wide range of pathologies including embolisms, disseminated intravascular coagulation, stroke, etc.. For this reason, extensive negative regulation of coagulation is also essential. Some inhibitors like TFPI and antithrombin (AT) have already made an appearance, putting the brakes on thrombin generation by targeting the heart of the coagulation cascade, prothrombinase and Xase. In the same vein, other negative regulators of coagulation, like the protein C/protein S pathway, target FVIIIa and

FVa, the cofactors for Xase and prothrombinase, respectively. When thrombin concentrations increase during coagulation, thrombin interacts with an endothelial membrane protein called thrombomodulin, a cofactor permitting thrombin's activation of protein C to activated protein C (APC)[113,114]. APC is bound to endothelial cells via its receptor, the somewhat unimaginatively named endothelial protein C receptor (EPCR). Once activated, APC complexes with circulating protein S to proteolytically inactivate FV and FVIII, effectively shutting down the spread of coagulation to non-disrupted endothelia[115,116]. Other circulating protease inhibitors include heparin cofactor II and C1 inhibitor[82]. Heparin, a negatively charged polysaccharide, is a common anticoagulant in the clinic. When bound to antithrombin III, heparin exponentially increases thrombin inactivation[117].

During hemostasis, platelet adhesion and aggregation and fibrin polymerization function in tag-team roles to seal up an injury. Early in this section I positioned thrombin generation at the nexus of coagulation pathways. Thrombin generation is the vital step in fibrinogen cleavage and therefore clot stabilization. It also serves in a positive feedback loop with many upstream coagulation zymogens (Fig. I.2). However, thrombin's work is not quite done. The crown protease of hemostasis also plays a role in platelet activation. Although there are multiple receptors for thrombin on the platelet surface, the classic targets through which it activates platelets are the protease activated receptors (PARs)[118]. In total, three thrombin-responsive PARs have been identified: PAR1 and PAR4 in human platelets and PAR3 and PAR4 in mouse platelets[119-122]. The PARs are G-Protein Coupled Receptors (GPCRs) which have a unique mechanism of activation. Their extracellular N-termini are cleaved by a protease (thrombin, for example) and the cleaved fragment then binds intramolecularly to activate the receptor[123]. In humans, PAR1 appears to mediate the primary response to

thrombin, while PAR4 generates a long-lasting response to higher levels of thrombin[124]. The PARs interact intracellularly with $G\alpha_q$ and $G\alpha_{13}$. $G\alpha_q$ stimulates formation of inositol triphosphate (IP3) and diacylglycerol (DAG), leading to intracellular calcium mobilization and protein kinase C (PKC) activation[125,126]. $G\alpha_{13}$ mediates activation of RhoA, and RhoA kinase (ROCK) [127]. The end result of the signaling events downstream of both PAR1 and PAR4 are the platelet degranulation and shape change essential for robust platelet activation[122,128-130].

B. <u>If I Only Had a Brain: Platelets and Other Thrombocytes</u>

Platelets are anucleate cell fragments originating from the cytoplasm of megakaryocytes in the bone marrow[131-133], although there is also some evidence for pulmonary megakaryocytes contributing to platelet genesis[134,135]. As the primary thrombogenic cell type in humans, I have already touched on their role in hemostasis in section I.A. (Due to their lack of a nucleus, there is some discussion in the literature regarding whether to refer to platelets are cells or not and whether to refer to them as "cell fragments", or "anucleate cells"[136,137]. I will herein use these terms interchangeably.) Proplatelets are the morphological intermediate between megakaryocytes and platelets. Protruding from the megakaryocyte membrane, proplatelets are long (as large as millimeters) extensions made of several platelet-sized fragments connected by thin cytoplasmic linkers[138]. Although initially challenged by other models of thrombopoiesis (platelet genesis), the vast majority of studies now support this model, with proplatelets being observed *in vitro* and *in vivo*[139,140], in several mammalian species[141-143], and adjacent to the sinusoids, where they release into the circulation for further fragmentation into platelets[144]. *in vivo*, each megakaryocyte can release thousands of platelets into the bloodstream[135]. Thrombopoietin (TPO) is a key cytokine in thrombopoietic signaling. Although it is generally thought of as stimulating platelet production, it is not actually vital to

the final stages of thrombopoiesis[145,146]. Instead, through its interactions with the TPO receptor, Mpl, it leads to active differentiation of megakaryocytes from their progenitors[147]. Thus, it is perhaps more appropriate to think of TPO as a trigger for megakaryopoiesis. It has been demonstrated by several groups that another key trigger for platelet biogenesis is blood flow-dependent shear stress[133,148,149]. Specifically, shear may facilitate the release of proplatelets into the bloodstream[133]. The importance of this shear stress, and perhaps even the directionality of flow[150], is especially evident in the development of methods for *in vitro* platelet production, where it is well established that shear force is necessary for the recapitulation of the megakaryocyte niche[151,152]. Prior to the recognition that shear is a natural part of the megakaryocyte environment, megakaryocytes were cultured under static conditions, where they generated platelets with very low efficiency[148]. In this, we see yet another example of the central role of shear force in the blood vascular system.

Megakaryocytes themselves arise from bone marrow resident hematopoietic stem cells (HSCs)[153]. The classical model of hematopoiesis is hierarchical, with HSCs at the top (Fig. I.3a). In this model, the pool of HSCs undergoes robust self-renewal so as to continue regenerating the blood system, while some HSCs split off down paths of increasingly restrictive lineage commitment. HSCs gradually become multipotent progenitors (MPPs), and undergo the first significant split in the hematopoietic tree that separates the lymphoid and myeloid branches (of which megakaryocytes belong to the latter)[154,155]. Focusing on the committed myeloid progenitor (CMP) lineage, megakaryocyte-erythrocyte progenitors (MEPs) split from granulocyte-macrophage progenitors (GMPs) and eventually go on to generate unipotent precursors for individual cell types[154,156,157]. Although the classical model of hematopoiesis is attractive in its simplicity, its neat, stepwise clonal dynamics have faced several challenges. In

the late 90s and early 00s (even as the classical model was still being fleshed out), new studies proposed more nuanced progenitor populations, including some that maintain high proliferative capabilities and lympho-myeloid differentiation potential, but lose their ability to adopt erythroid and megakaryocyte lineage fates[158,159]. These observations suggest a model where loss of megakaryocyte/erythrocyte potential is among the first steps in HSC commitment. HSC-enriched bone marrow cells express VWF, which is also a reliable marker of megakaryocyte lineage cells[160,161]. However, more rigorous single-cell analyses have revealed two HSC populations: one with and one without VWF expression[161]. Lymphoid-primed MPPs (LPMPs), the most primitive multipotent progenitor lacking megakaryocyte potential, do not express VWF[162]. On the other hand, VWF-expressing HSCs can actively self-renew and give rise to both megakaryocyte (VWF^+) and lymphoid (VWF^-) primed lineages. The classical model suggests that HSCs give rise to MPPs which give rise to myeloid progenitors, which give rise to MEPs, which give rise to unipotent megakaryocyte progenitors. Instead, these data place megakaryocyte-primed HSCs at the apex of the HSC hierarchy. A more updated model includes a route by which HSCs quickly commit to unipotent megakaryocyte progenitors not originating from MEPs (Fig. I.3b)

Anucleate platelets and the polyploid megakaryocytes from which they come are a special feature of the mammalian blood system. In other organisms, thrombogenic blood cells are nucleated and referred to herein by the generic term “thrombocytes”. Many invertebrate circulatory systems have only one main type of circulating cell. This cell-of-all-trades participates in some defensive roles, as well as prevention of blood loss, accomplished via aggregation[163]. This may imply that aggregation to maintain the integrity of blood vascular systems is an early function of circulating blood cells. This should not be surprising, as it

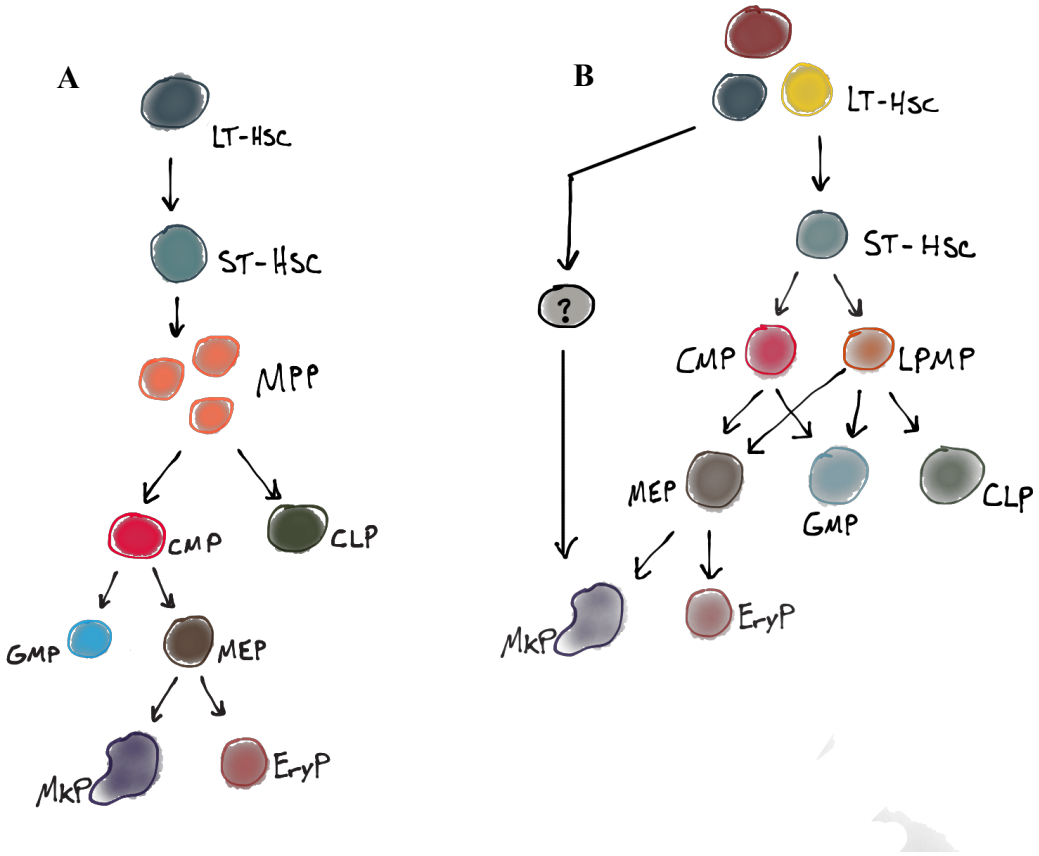

Figure I.3. Models of hematopoiesis. (A) In the classic model of hematopoiesis, differentiation is linear and myeloid and lymphoid lineages are strictly separated (further differentiation from lymphoid progenitor not shown here). Megakaryocyte progenitors (MkP) arise from megakaryocyte-erythrocyte progenitors (MEP). (B) More recent models of hematopoiesis include a heterogenous population of hematopoietic stem cells (HSCs) with regard to lineage potential, a more flexible fate map for myeloid and lymphoid progenitors, and the possibility of a megakaryocyte-biased HSC which circumvents intermediates to give immediate rise to MkPs. Other abbreviations: Long-Term Hematopoietic Stem Cell (LT-HSC), Short Term Hematopoietic Stem Cell (ST-HSC), Multi-Potent Progenitor (MPP), Committed Myeloid Progenitor (CMP), Committed Lymphoid Progenitor (CLP), Lymphoid-Primed Multipotent Progenitor (LPMP), Granulocyte-Macrophage Progenitor (GMP), Erythroid Progenitor (EryP). Adapted from Machlus et al. 2019[164].

makes sense that a common thread in circulatory systems would be a cell purposed with preventing blood (or hemolymph) loss. Although the evolutionary link between the aggregating, thrombotic cells of the invertebrate hemolymph and our own anucleate platelets remains broken, several features of their aggregation functions bear striking resemblance to the same function in platelets. For example, some mollusk hemocytes (a generic term for blood cells) aggregate in contact with foreign surfaces outside circulation, in a manner dependent on divalent cations, under regulation of adenosine[165,166]. Amoebocytes, a circulating hemocyte in horseshoe crabs, release enzymatic factors upon activation to promote coagulation[167]. Reminiscent of mammalian megakaryocytes, thrombocytoids, a type of hemocyte in some insects, break into small anucleate cell fragments which undergo aggregation[168,169].

Higher up the evolutionary tree (or closer to humans, at least), we find the first dedicated thrombocytes in nonmammalian vertebrates, spindly blood cells specializing in hemostasis[170]. As observed by light microscopy, the ultrastructure of thrombocytes is described as spindle shaped and spiked, and spherical or ovular, containing a number of cytoplasmic granules, along with a nucleus[171]. Mammalian platelet membranes contain a system of densely invaginated canals and channels called the open canalicular system (OCS), a system mirrored in form and nomenclature by the surface-connected canalicular system (SCCS) in the thrombocytes of some birds, amphibians, reptiles, and fish[172-175]. One of the oldest extant vertebrates, the Atlantic hagfish, has recently been shown to express VWF in both its thrombocytes and endothelial cells, despite a somewhat simpler domain structure[176]. Thus, both VWF and dedicated thrombocytes seem to have arisen early in vertebrate hemostasis. Curiously, these early vertebrate thrombocytes do not express GPIb-IX-V, a primary VWF

receptor in human platelets. With regard to functional receptors, avian thrombocytes express a version of $\alpha_{IIb}\beta_3$ similar enough to human $\alpha_{IIb}\beta_3$ to be recognized by the same monoclonal antibodies[177,178]. These thrombocytes also undergo thrombin-induced aggregation in the presence of fibrin, which can be blocked by anti-$\alpha_{IIb}\beta_3$ antibodies[179]. qPCR studies on chicken also reveal the expression of Mpl and the GPIb-IX-V complex[180].

Regarding organisms closely related to humans, similarities between their thrombocytes and our platelets may be less surprising. However, there may be valuable insights to be mined by harkening back to more distant relatives of human blood components, such as the horseshoe crab's amoebocytes (aka amebocytes). Arising over 450 million years ago, the horseshoe crab is a living fossil, an extant representative of ancient organisms. Their amoebocytes are one of the most extensively studied invertebrate hemocytes, and share a remarkable number of features of their hemostatic functions with platelets[181]. With what we already know about platelets in primary and secondary hemostasis in mind, consider that amoebocytes have at least two different kinds of granules, which contain the necessary activating components of coagulation[182,183] and degranulate upon activation[184,185]. The normally discoid cells also undergo shape change when activated, extending small membrane protrusions and developing pseudopodia[186]. Curiously, the similarities between amoebocytes and platelets are not restricted to hemostatic behavior. As mentioned near the top of this section, many invertebrate systems involve only one type of circulating cell possessing an arsenal of many tools to meet all the needs of the blood system. Indeed, there is evidence that platelets, too, serve an array of purposes not directly tied to hemostasis. Platelets act as rudimentary immune phagocytic or inflammatory cells, capable of interacting with bacteria, viruses, fungi,

and other pathogens. Platelets are also involved in development/angiogenesis and, pathologically, in tumor growth and metastasis.

Hemostasis and inflammation are intimately intertwined processes, each capable of amplifying the other[187-189]. As a two-pronged defensive strategy, the physiological benefits of this linkage manifest in the potential for immobilization and destruction of pathogens that may otherwise enter the bloodstream via a wound[190]. Of course, dysregulation of this relationship may also lead to pathological activation and disease states[191]. Many studies suggest that platelets, already localized to the site of injury and incorporated in thrombi, are key effectors in the physiological and pathophysiological confluence of hemostasis and inflammation[192,193]. Human platelets' lack of a nucleus permits a small average size (~4μm diameter), which allows them to exist at a high concentration in the blood and making them arguably the most numerous blood cell with a role in immunity. As mentioned in section I.A, when platelets are activated by a number of stimuli, they release diverse granules containing many stimulatory molecules. α granules are the most numerous platelet granules[194,195]. By some proteomic analyses, α granules contain upwards of 200 different proteins! These include the membrane proteins P-selectin, integrin $\alpha_{IIb}\beta_3$, and GPIb-IX-V, along with fibrinogen, albumin, VWF, platelet factor (PF) IV, coagulation factors V and VIII, various growth factors, SDF-1, RANTES, and ppbp[196,197]. Although many of the contents of α granules function in platelet adhesion and activation, others serve primarily as chemokines and cytokines that recruit and activate other immune cells or induce endothelial cell inflammation[198]. Dense granules are about 1/10th as abundant as α granules, and are named as such due to their electron-dense appearance when platelets are visualized by electron microscopy[199]. Dense

Molecule		Role
α granules	PF4	monocyte, neutrophil, and T-cell recruitment; Th differentiation
	ppbp	neutrophil activation and recruitment
	P-selectin	leukocyte adhesion, complement activation
	CD40L	antigen-presenting cell activation, B-cell responses, endothelial cell activation
	TGF-β	cell proliferation, T-cell differentiation, B-cell and macrophage phenotype regulation
	PDGF	cell growth and differentiation, monocyte/macrophage differentiation
	CD63	transmembrane adaptor for leukocyte recruitment
	SDF-1	T-cell/monocyte chemotaxis
	Thrombospondins	endothelial cell inflammation
	MIP-1α	neutrophil and eosinophil activation, B-cell immunoglobulin production
	MMP-2, MMP-9	extracellular matrix breakdown, platelet-leukocyte aggregate formation
Dense granules	Serotonin	dendritic cell and T-cell functions
	Glutamate	T-cell trafficking
	Polyphosphates	inflammatory response amplification
	ADP	leukocyte activation
	Histamine	vessel reactivity and degranulation
Constitutively Expressed	IL-1β	acute phase immune response, leukocyte and endothelial activation
	Thromboxane	T-cell differentiation, monocyte activation
	GPIbα	binds Mac-1 on leukocytes

Table I.1. Immune/inflammatory modulators in platelets. Platelet factor 4 (PF4), proplatelet basic protein (ppbp), CD40 ligand (CD40L), transforming growth factor β (TGF-β), platelet derived growth factor (PDGF), stromal cell derived factor (SDF), macrophage inflammatory protein (MIP), matrix metalloproteinase (MMP), adenosine diphosphate (ADP), interleukin (IL), glycoprotein Ibα (GPIbα). Adapted from Morrell et al. 2014[198] (License 1006148-1).

granules contain ATP, ADP, Ca^{2+}, serotonin, histamine, glutamate, and polyphosphates[195,200]. Although many of these molecules stimulate platelets, some also target receptors on immune cells. ATP signaling through T-cell P2X7 increases differentiation toward a proinflammatory cell type[201], glutamate induces T-cell migration[202,203], and serotonin stimulates monocyte differentiation into dendritic cells[204] and T-cell activation[205]. For an expanded list of platelet-derived inflammatory mediators and immune modulators, see Table I.1.

Platelets also participate in immune responses by acting as crude pathogen-binding cells and phagocytes. Naturally, the mechanism through which platelets bind to pathogens varies depending on the specific pathogen, but common mechanisms involve pathogen interaction with $\alpha_{IIb}\beta_3$ or GPIb-IX-V using some plasma protein as a bridge[206]. This may point to some conserved evolutionary role for these platelet surface glycoproteins in immunity, although it is also likely that interactions with these receptors are common due to their high expression on platelets. Platelets possess an Fc receptor, FcγRIIa, which recognizes the Fc region of antibodies. When a pathogen is recognized and opsonized (marked for phagocytosis) by antibodies, platelet FcγRIIa binding to their outward facing Fc regions leads to platelet activation and aggregation, thereby neutralizing the pathogen[207,208]. Factors released during degranulation also fulfill many anti-microbial functions by suppressing pathogen growth, and stimulating inflammatory responses in other immune cells[209]. Although platelets themselves do engage in phagocytosis, they are somewhat limited in the size of particles they can consume by their own modest diameter. This disadvantage is partially offset by the high surface area to volume ratio gained from the OCS. Platelets unfold these deep stores of membrane to envelop (if not fully engulf into their cytoplasm) pathogens like bacteria[210]. Platelets are naturally poised by virtue of their ubiquity and sheer number to act as tripwires

for a rapid response to pathogens. Their adhesion to endothelial damage places them on the front line of the defense against pathogens that would otherwise enter the bloodstream through a wound. To this end, platelets have recently been shown to collect and bundle bacteria trapped in fibrin meshwork, potentially making the pathogens more "visible" to other immune cells[190]. Like macrophages, platelets detect pathogen associated molecular patterns (PAMPs) through the Toll-Like Receptor (TLR) superfamily of receptors[211,212]. Platelets can dynamically enhance this response by upregulating expression of TLR1 and TLR6 at vascular injuries containing bacteria[213].

So far, we have seen how platelets orchestrate early immune responses by bringing crowd control and physical neutralization of pathogens into harmony with signaling to inflammatory immune cells. Being directly proximal to the endothelium, platelets are well positioned for a role in vascular development or angiogenesis. Indeed, platelets contain many pro-angiogenic factors in their granules[214]. When new vessels are just forming, they are somewhat less well developed and thus considered "leaky". This leakiness manifests in exposure of collagen in the subendothelial matrix, which acts as bait for platelets to localize to new vasculature[215,216]. Once platelets adhere to developing angiogenic vessels, they stabilize the vessel and support angiogenesis by releasing factors like platelet derived growth factor (PDGF), vascular endothelial growth factor (VEGF), and angiopoietin-1[217]. Platelets' contribution to angiogenesis is especially clinically relevant in the case of many cancers, which must remodel their microenvironment to support growth. Tumors represent zones of constantly active endothelia, presenting surfaces that can activate platelets and bring them in contact with cancer cells. When cancer cells and platelets interact, platelets can be directly activated by factors released from the tumor cells, which exploit the platelets' proangiogenic

effects[216,218-221]. Some of these factors released from tumors include classic agonists like ADP, thrombin, and tissue factor[222-225]. Whether through secreted agonists or via direct interaction with cancer cells[226,227], tumor cell-induced platelet aggregation (TCIPA) occurs in several types of cancer. TCIPA itself, apart from bringing platelets together to promote angiogenesis, also has an influence on tumor progression and metastasis. Platelets contain transforming growth factor β (TGF-β), a multifunctional cytokine that controls many vital oncogenic processes including proliferation, differentiation, apoptosis, angiogenesis, epithelial-mesenchymal transition (EMT), metastasis, and immunosuppression[228]. When cancer cells undergo EMT, they transdifferentiate from an epithelial to a mesenchymal phenotype and acquire more motile and invasive qualities[229]. This process occurs during cancer progression, facilitates metastasis, and the best-characterized driver is TGF-β[230,231]. Once in the bloodstream, circulating tumor cells (CTCs) can recruit platelets, which in turn will aggregate and protect CTCs with a "platelet cloak" that thwarts immune detection[232].

Despite the rather sinister outlook on the role of platelets as "cancer helper cells", there are some bright sides to the platelet-cancer axis. Due to the close interaction of platelets with tumor cells both in tumors and throughout circulation, platelets take on some of the molecular signatures of these cells. These "tumor educated platelets" can be used in advanced blood-based liquid biopsies to accurately diagnose several different types of cancers[233,234]. Some groups have also hypothesized that platelets could be used to deliver drugs or to prime cancer cells for improved immunotherapy[235]. Many traditional chemotherapy drugs suppress bone marrow cells and lead to low platelet count as a result. As such, anti-platelet agents are contraindicated by treatments that many cancer patients are already on. However, as modern therapies move toward targeted approaches and immunotherapy, it may be possible to take

the straightforward path of inhibiting or depleting platelets with common anti-platelet drugs so as to mitigate their role in cancer immune evasion and tumor growth[236-238].

Of course, while the pathogenic and therapeutic roles of platelets in cancer are relatively new clinical pursuits, the study of platelets and their contributions to human disease states dates back to the initial descriptions of platelets as hemostatic/thrombotic cells by Bizzozero in the early 1880s, and Osler's establishment of platelets' contribution to thrombosis some 5 years later[239]. During the next 80 years, what Barry Coller refers to as the "descriptive period" of platelet research[240], many foundational discoveries of clinical platelet disorders and cell-level events in hemostasis were made, including descriptions of immune thrombocytopenia, thrombotic thrombocytopenic purpura, Glanzmann thrombasthenia, von Willebrand disease, and Bernard-Soulier syndrome. During this period, extensive research into mechanisms underlying platelet deficiencies (thrombocytopenia) was undertaken, notably by Erich von Willebrand (on the eponymously named disease), and by Harrington on immune thrombocytopenia (discussed further in section I.D). In the next section, I will explore many established mechanisms underlying the endogenous destruction of platelets.

C. Symphonies of Destruction: Mechanisms of Platelet Clearance

Portions of section I.C are adapted from

Quach ME, Chen W, and Li R. Mechanisms of platelet clearance and translation to improve platelet clearance. *Blood.* 2018; 131(14): 1512-1521

Platelets are one of the most prevalent cell types in the blood, with a normal count of 150-400k per μl of blood[78]. Maintaining a steady-state platelet count is a dynamic process involving

robust destruction of old or activated platelets and the production of new platelets. All in all, our bodies recycle as many as 10^{11} platelets every day. In section I.B, I discussed the generation of new platelets from megakaryocytes, aka thrombopoiesis. The destruction or "clearance" of platelets is an equally important and perhaps even more complex process. Dysregulation of clearance can result in either thrombocytosis or thrombocytopenia. Platelets are cleared based on their activity, age, and in many pathological disorders. We will discuss the many different determinants of a platelet's lifespan in the bloodstream including apoptotic regulation, immune clearance, recognition by glycan receptors, and the regulation of clearance by GPIb-IX-V.

In order to study platelet clearance, we must monitor platelet count and age, and in order to do this, we must first understand how platelet count is interrogated. There are several techniques used to measure platelets, including manual counting via microscopy, flow cytometry, and optical light scattering or fluorescence based commercial analyzers. Developed in the early 1950s[241], manual platelet counting via phase contrast microscopy in a hemocytometer was long considered to be the gold standard reference for platelet count. More recently, the wide availability of commercial analyzers that can readily perform complete blood count (CBC) analysis on whole blood has made the automated method widespread in research and the clinic. These machines rely on a variety of technologies to count cells accurately including detection of specific electrical resistances, optical light scattering, and fluorescent dyes[242,243].

There are three main ways to monitor platelet clearance. The first method tracks a compound's ability to clear platelets in circulation. A blood count is taken prior to and at set

time intervals following administration of the compound. These values, typically expressed as a percent of the normal count taken before administration, are plotted to produce a curve that demonstrates the compound's initial clearing effect[244]. In a second method, pulse labeling with fluorescence or radiolabeling, tracks the lifespan of endogenous platelets. This method places the average lifespan of human platelets at 7-10 days, and murine platelets at 5 days[245,246]. The third method tracks the *in vivo* survival of transfused platelets. Platelets are labeled prior to transfusion with radioisotopes or chromophores[247], then the ratio of transfused to endogenous platelets is plotted as determined by periodic counts. This allows us to track the effects of treatments or storage conditions on the transfused platelets' clearance.

Platelets contain many components of the apoptotic machinery. Similar to many nucleated cells, intrinsic platelet apoptosis depends on the balance between pro- and anti-apoptotic molecules (Fig. I.4). In intrinsic apoptosis, the anti-apoptotic Bcl-2 family proteins function to restrain the pro-apoptotic molecules Bak and Bax. Inhibition or loss of Bcl-2 family proteins disinhibits Bak and Bax, leading to apoptosis. Several Bcl-2 family proteins, including Bcl-2, Bcl-w, and Bcl-x_L are expressed in both human and murine platelets[248,249]. However, platelet-specific knockout (KO) of Bcl-2 and systemic KO of Bcl-w do not alter platelet lifespan[250,251]. Furthermore, treatment with the drug ABT-199, which specifically inhibits Bcl-2, does not induce thrombocytopenia[252]. Alternatively, specific pharmacological inhibition[253] or Cre-mediated deletion of Bcl-x_L,[254] or broad inhibition of Bcl-2-family proteins such as by ABT-737[255], lead to platelet apoptosis and thrombocytopenia. Further, double deletion of Bak and Bax prolongs platelet lifespan, and can rescue thrombocytopenia caused by loss of Bcl-x_L[249,256]. Together these data indicate Bcl-x_L as the primary Bcl-2 family protein controlling apoptosis

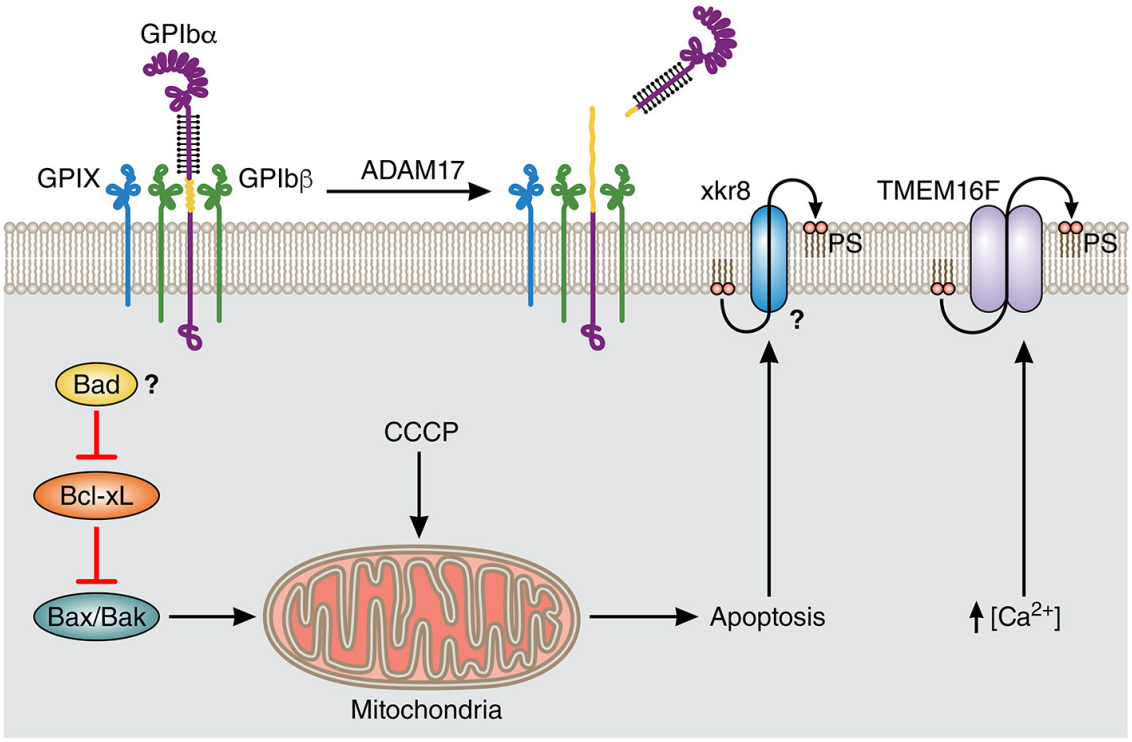

Figure I.4. The platelet apoptotic machinery. The anti-apoptotic Bcl-xL restrains proapoptotic Bax/Bak in platelets. Mitochondrial damage induced by CCCP, an ionophore, leads to robust ectodomain shedding of GPIba. If inhibition by Bcl-xL is blocked pharmacologically, Bax/Bak will induce mitochondrial damage, leading to the apoptotic cascade. The BH3-only initiator of apoptosis, Bad, may also affect platelet lifespan, though further study would help to elucidate its role. Apoptotic cells redistribute PS from the inner to the outer leaflet of their plasma membranes. One calcium-independent pathway may involve Xkr8. Another pathway present in platelets is facilitated by TMEM16F, a calcium-activated phospholipid scramblase. Adapted from Quach et al. 2018[260].

in platelets (Fig. I.4) In terms of the pro-apoptotic death molecules, single deletions reveal that Bak is likely the major regulator of lifespan while Bax plays a smaller role[256,257].

In many apoptotic cells, Bcl-2 family anti-apoptotic proteins are inhibited by "BH3-only" *initiators* of apoptosis, so named because they contain a homologous BH3 region of just nine amino acids[258]. This inhibition ultimately leaves Bax/Bak free to initiate mitochondrial membrane damage and trigger the apoptotic cascade. Of the 4 BH3-only proteins expressed in platelets (Bid, Bim, Bad, and Bik), genetic deletions of Bid or Bim did not alter the platelet count in mice[249]. Loss of Bad leads to a modest increase in platelet count and lifespan[259]. Even though these data are somewhat uninspiring, the expression of BH3-only proteins in platelets may yet imply their involvement in regulating intrinsic apoptosis. Future studies are needed to fully elucidate their roles. Platelets express certain components of the extrinsic apoptosis pathway as well, including caspase 8, but the limited data so far do not support a critical role for this pathway in regulating platelet lifespan[261,262].

In many cells undergoing apoptosis, the redistribution of phosphatidylserine (PS) from the inner to the outer leaflet of the plasma membrane serves as a molecular cue for engulfment and clearance by phagocytes. While lactadherin and scavenger machinery can mediate clearance of platelet-derived PS-expressing microvesicles,[263] whether they mediate clearance of apoptotic platelets (along with the identity of the "clear-me" sign on apoptotic platelets) remains to be fully elucidated. Earlier studies have ruled out several markers of platelet activation, such as P-selectin, as "clear-me" signs for platelet clearance[264]. Platelets possess two distinct pathways through which they expose PS on their surface[265,266] (Fig. I.4). One is dependent on intracellular Ca^{2+} and TMEM16F, a Ca^{2+}-activated phospholipid scramblase and

ion channel[95,96]. (Recall the discussion of TMEM16F's involvement in platelet activation in section I.A). The other is associated with apoptosis, and may involve Xk-related protein 8 (Xkr8), a 10-transmembrane domain scramblase, instead of TMEM16F[265]. Earlier studies suggest that apoptosis-associated morphological changes in platelets, such as PS exposure and recognition by phagocyte scavenger receptors, are not inhibited by broad-spectrum caspase inhibitor zVAD-fmk[267]. Whether or how Xkr8 and/or TMEM16F are involved in regulating the platelet lifespan and mediating its clearance in a caspase-independent manner remains unclear. In most apoptosis pathways mitochondrial outer membrane permeabilization (MOMP) is a critical step, resulting in decrease of the mitochondrial electrochemical gradient and release of cytochrome C (CytC). Carbonyl cyanide 3-chlorophenylhydrazone (CCCP) is a lipid-soluble protonophore and oxidative phosphorylation uncoupler that induces mitochondrial permeabilization and loss of membrane potential[268] (Fig. I.4). When platelets are pre-treated bulk of CCCP-treated platelets are cleared rapidly *in vivo*[269].

Those that are not cleared rapidly do not have reduced lifespan. Current thought is that mitochondrial damage itself can lead to platelet clearance, and MOMP triggered by CCCP may not be representative of endogenous platelet apoptosis. It is noteworthy that while CCCP treatment induces modest PS exposure, it does lead to significant ectodomain shedding of platelet GPIbα (CD42b)[269] (Fig. I.4). This links mitochondrial damage to another (not caspase-dependent) potential mechanism of accelerated platelet clearance with the shedding of GPIbα as a key step.

Recent studies have highlighted the role of glycan modifications on platelets in mediating their clearance. In circulation, loss of terminal sialic acid (a derivative of neuraminic acid)

from the platelet surface has been linked with senescent platelet removal[270]. Neuraminidases (sialidases) are glycoside hydrolase enzymes which remove the terminal sialic acid residues on glycans, a process known as desialylation. Injection of neuraminidase into animal models leads to rapid platelet clearance[271], and certain bacterial infections marked by a release of pathogen-derived neuraminidase, are often accompanied by thrombocytopenia[272]. Patients with congenital defects affecting sialic acid expression on platelets demonstrate megakaryocyte abnormalities and macrothrombocytopenia, which suggests a defect in platelet production[273]. Some recent studies have shown that endogenous, platelet-derived neuraminidase also plays a role in the problematic fast clearance of refrigerated platelets[274,275]. Many anti-platelet antibodies that cause rapid clearance also induce platelet surface presentation of lysosomal neuraminidase (neu1), and thus desialylation of platelets[276], a process which also naturally occurs downstream of platelet activation. Treatment with 2,3-dehydro-2-deoxy-N-acetylneuraminic acid (DANA), a neuraminidase inhibitor, reduces this desialylation and ameliorates the associated thrombocytopenia[276].

It is common for the terminal residue in both N- and O-glycans to be sialic acid, which is in turn linked to a penultimate β-galactose (β-gal). Desialylation of platelets therefore leads to the increased exposure of β-gal (Fig. I.5)The exposed β-gal on platelet surface is recognized by the Ashwell-Morell receptor (AMR), a multimeric endocytic receptor complex also known as the asialoglycoprotein receptor[277], expressed on the surface of hepatocytes and liver macrophages (Kupffer cells). Binding facilitates the recognition of desialylated platelets, and induces clearance of these platelets from circulation[274,278,279]. Mice lacking the AMR have elevated platelet count (mild thrombocytosis), and fast clearance of platelets in response to neuraminidase injection is abolished in them[280,281]. On the other hand, *St3gal4*$^{-/-}$ mice, which have deficiencies in terminal sialic acid residues on platelet surface proteins due to genetic

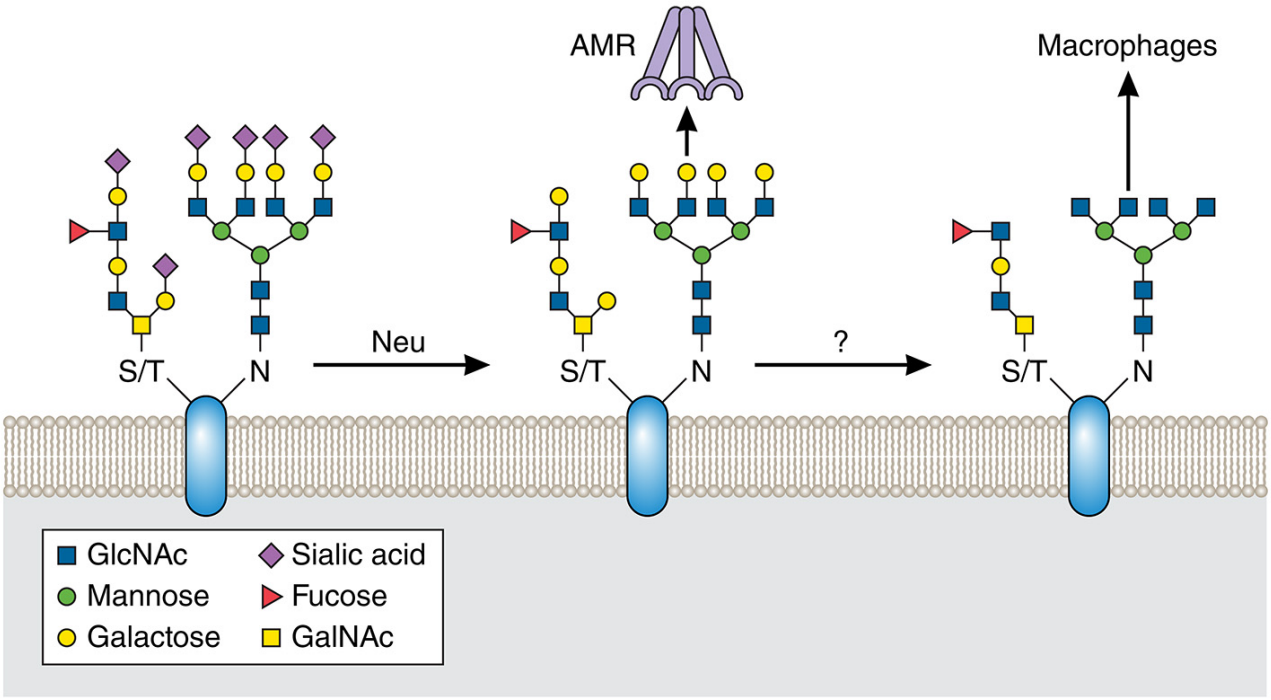

Figure I.5. Protein desialylation as a "clear-me" sign on platelets. Over the platelet lifespan, surface glycoproteins lose the terminal sialic acid residues in their glycans, a process associated with clearance. Neuraminidases are glycoside hydrolases that can remove terminal sialic acid from glycans. Neuraminidases are found in platelets, which present neuraminidase on their surface downstream of GPIb-IX-V complex signaling. In many glycans, desialylation leads to exposure of the penultimate galactose residues on glycans. These can in turn be recognized by the AMR. Further deglycosylation leads to exposed GlcNAc residues, which may be recognized by other carbohydrate receptors and potentially mediate their uptake by macrophages.

loss of an important sialyltransferase, suffer from thrombocytopenia as a result of accelerated platelet clearance via the hepatic AMR[278]. In addition to mediating clearance, the AMR may lead to stimulation of platelet production, forming a clearance/thrombopoiesis feedback loop for maintaining platelet homeostasis[281].

In addition to the interaction between galactose and the AMR, other carbohydrate receptors may also play a role in platelet clearance (Fig. I.5). It was reported that integrin αMβ2 recognizes refrigerated platelets via binding to exposed GlcNAc on the platelet surface and mice lacking the αM subunit show a small increase in platelet count[282,283]. Clodronate depletion of macrophages alleviates thrombocytopenia in a mouse model of von Willebrand disease (VWD) type 2B[284]. Furthermore, pre-injection of GlcNAc into guinea pigs prior to induction of antibody-induced thrombocytopenia partly protects against depletion of platelets[285]. However, while galactosylation of GlcNAc residues via treatment of uridine 5'-diphosphogalactose (UDP-galactose) results in the normal survival of short-term refrigerated platelets, it does not ameliorate the survival of long-term (48 hours) refrigerated human and murine platelets[286]. The various degrees of contribution of each glycan moiety to platelet clearance remains murky.

Platelet GPIbα is heavily decorated with sialic acid residues, accounting for as much as 70-80% of the total sialic acid on the platelet surface. Unlike human GPIbα, the murine GPIbα amino acid sequence lacks any N-glycosylation consensus sequences. Grewal et al. showed recently that even in mice lacking GPIbα, neuraminidase treatment leads to platelet clearance, albeit at a slower rate than in WT mice[280]. This suggests that the glycans on GPIbα are necessary to set off a rapid rate of AMR-dependent clearance, but the exposed galactoses on other platelet

glycoproteins may also be counter-receptors for the AMR. GPIbα, and the GPIb IX-V complex in general, sit at the intersection of many different mechanisms of platelet clearance, including VWF-platelet agglutinated complexes[284,287,288], Fc-independent anti-GPIb-IX-V antibody-induced clearance[244,276], platelet surface desialylation[278,280], and ectodomain shedding of GPIbα during platelet storage[269,289]. GPIb-IX-V-mediated clearance is covered in greater detail in section I.E

Another mechanism of platelet clearance involves opsonization by anti-platelet antibodies, Fc-receptor mediated recognition, and subsequent clearance. Infusion of monoclonal antibodies (MAbs) targeting the N-terminal ligand-binding domain (LBD) of GPIbα in particular causes fast depletion of nearly all platelets from animals[290-293]. In patients with immune thrombocytopenia (ITP), autoantibodies targeting platelet surface glycoproteins, primarily GPIIb-IIIa ($\alpha_{IIb}\beta_3$) and GPIb-IX-V, lead to Fc-dependent clearance via macrophages[294].

D. <u>My Own Worst Enemy: Platelet Clearance in Immune Thrombocytopenia (ITP)</u>

Although descriptions of the characteristic red-purple skin lesions (purpura) that are a hallmark of immune thrombocytopenia date back over thousands of years of human history to ancient Greece, it was in 1735 that German physician Paul Gottlieb Werlhof first described the clinical presentation of what would now be recognized as ITP in two young girls[295]. It would be almost another 150 years before Brohm and Kraus would link a low platelet count (thrombocytopenia) to the symptoms described by Werlhof[296]. Just a few years later, in his seminal 1892 medical textbook, Sir William Osler described the same condition, which he

referred to as "purpura haemorrhagica". His account included a description of common symptoms, epidemiological notes, and some distinction of acute and chronic disease forms[297].

> *Under this heading may be considered the cases of very severe purpura with haemorrhages from the mucous membranes. The affection...is most commonly met with in young and delicate individuals, particularly in girls...In favorable cases the affection terminates in from ten days to two weeks.*
>
> *-Osler 1892 (318-319)*

By the early 1900s, the mechanism of ITP remained almost as elusive as it was to Werlhof. At that time ITP was commonly referred to as "idiopathic thrombocytopenic purpura" rather than the identically abbreviated "immune thrombocytopenia", reflecting the field's uncertainty regarding the pathophysiology of the condition. However, some reports began to postulate an immune mediated mechanism for ITP. In 1916 a medical student in Prague, Paul Kaznelson, proposed that ITP was caused by increased platelet destruction in the spleen. He convinced his mentor Hermann Schloffer to perform a splenectomy on a woman presenting symptoms indicative of ITP. Following the procedure the patients platelet was rapidly increased, a result that was soon replicated in two other patients[298]. Due to the procedure's marked success, splenectomy soon became a common treatment for persistent ITP. In 1938, Troland and Lee demonstrated through a series of experiments that a substance extracted from ITP patient spleens (which they called "thrombocytopen") could induce thrombocytopenia in animals. Upon injection with the splenic extract, rabbits' platelet count dropped significantly and recovered over the next 24-36 hours[299]. The clinical records of at least one of the patients involved in this study indicated that splenectomy was successful in

ameliorating her bleeding symptoms. This was the first demonstration of a transmissible spleen-derived factor underlying ITP.

Contemporaneously, a debate had been ongoing in the literature regarding whether the thrombocytopenia in ITP was caused by elevated peripheral clearance of platelets or impaired platelet production by megakaryocytes. In 1946, in the second paper ever to appear in the journal *Blood*, Dameshek and Miller conducted an observational study of the bone marrow aspirate of several ITP patients and healthy controls via bone marrow and blood smears. Their study concluded that, although megakaryocyte numbers were elevated in ITP patients, the megakaryocytes showed "greatly diminished productivity of platelets" and that following splenectomy, platelet production by megakaryocytes was restored[300]. The authors therefore concluded that ITP was primarily a disorder of platelet production by megakaryocytes and added to the growing body of evidence linking the spleen to ITP pathogenesis. Just a few years later, in 1951, two hematology fellows at Barnes Hospital in St. Louis, Harrington and Hollingsworth, conducted a landmark study in both the pathogenesis of ITP and the heroic pursuit of scientific clout through self-experimentation. In light of reported cases of a mother with ITP giving birth to a child that also presented with ITP, they sought to test the hypothesis that some causative factor in peripheral blood could be passed from mother to child. Earning his first authorship in a most dramatic fashion, Harrington agreed to receive a transfusion of blood from an ITP patient whose blood type matched his own. Upon transfusion, Harrington's platelet count dropped dramatically from a healthy 250,000 per μl to 10,000 per μl and he suffered a seizure[294]. Although he recovered from the seizure quickly, he was hospitalized with mucous membrane bleeding and purpura, the hallmarks of ITP, and was believed to be at risk of intracranial hemorrhage. Essentially, Harrington had developed acute

ITP from a transfusion of ITP patient blood (which he would go on to recover from within a week). Analysis of Harrington's bone marrow aspirate showed no abnormalities in his megakaryocytes, and the results of his transfusion with patient blood were recapitulated in all suitable members of the hematology division of the hospital, who volunteered for the study even despite witnessing Harrington's seizure and hospitalization[301]. Harrington and Hollingsworth's study established that ITP was caused by a circulating component in the blood, and it may have caused many in the field to overlook suppression of megakaryocytes as a key factor in ITP. Indeed, the role of decreased thrombopoiesis in ITP took a back seat to that of accelerated platelet clearance for decades, until the 1980s, when studies on platelet turnover/lifetime using radioisotope labeling once again raised the question of a dual increased-clearance, decreased-production pathophysiology[302,303]. More recently, stimulation of thrombopoiesis was recognized as an effective treatment strategy when several clinical studies demonstrated the therapeutic efficacy of TPO mimetics in ITP[304-306].

Once it was identified that ITP was caused by a platelet-depleting, circulating, transmissible factor in patient blood, the field did not have to wait long for the first identifications of these factors: IgG class anti-platelet antibodies. In 1965, testing hypotheses put forward in part by Harrington and Hollingsworth as well as Robert Evans, Schulman et al. presented an impressive study in which they demonstrated that known anti-platelet antibodies had dose-dependent platelet clearance effects similar to those of ITP patient plasma when injected into healthy controls, and that radiolabeled anti-platelet antibodies localized to spleen and liver upon infusion. Finally, they showed that the 7S gamma-globulin fraction of ITP patient plasma was necessary and sufficient to cause the observed effects[307]. They were convinced that these results showed that "...ITP is an immune disorder, and leave little doubt that the ITP

factor is an antibody". Thus, together with the Harrington-Hollingsworth experiment, these discoveries changed the I in ITP from idiopathic to "immune"[296,301], although the former persisted in the literature for quite some time afterwards, perhaps due to uncertainty over the etiology of the immune response in ITP, the role of thrombopoiesis, and the resistance of many cases of ITP to immunosuppressive treatments. The last of these will be further discussed below.

In the mid 70s, studies went on to reveal that indeed, ITP patient plasma contained elevated levels of platelet-binding antibodies and that levels of platelet surface bound IgG predicted a patient's disease severity and response to different treatments[308]. In their 1982 study, van Leeuwen et al. reported that one of the major targets of these antibodies was likely platelet surface glycoproteins, specifically glycoprotein (GP) IIbIIIa (aka CD41 or integrin $\alpha_{IIb}\beta_3$). Conducting experiments with Glanzmann thrombasthenia patients, they had found that ITP patient sera only bound to their platelets, which lack $\alpha_{IIb}\beta_3$, ~25% of the time[309]. In some cases of ITP, it is difficult to detect any anti-platelet antibodies, and in the 90s and early 00s some groups reported T-cell abnormalities associated with ITP, including a reduction in the functional pool of regulatory T-lymphocytes[310,311] and increased cytotoxic T-cell mediated platelet lysis[312].

Buttressed by the series of pathophysiology-defining studies in the latter half of the 20th century, it became possible for researchers to more strategically approach the development and application of therapies for ITP. Although splenectomy, which we recall first entered the armory of treatment options over 100 years ago[298], remains a common and effective treatment in adults with ITP[313,314], it is not without downsides or restrictions. Patients who undergo

splenectomy have an increased risk of infection and sepsis[315] and a two to four fold increased risk of venous thromboembolism[316], and old age and certain coexisting conditions contraindicate the operation for some ITP patients[315]. Additionally, 30-50% of ITP patients do not have a long-lasting response to splenectomy[317] and there is no reliable way to determine who will fall into that group prior to performing the surgery. Combined with the emergence of many medical (non-surgical) therapies for ITP, these drawbacks have led to a significant decrease in its frequency[318], especially as a first line treatment option.

Beginning in the 1960s, once the autoimmune nature of ITP was widely accepted, immunosuppressive drugs became commonly used therapies, especially corticosteroids such as prednisone or dexamethasone. Both high-dose pulses of dexamethasone or longer courses of prednisone/prednisolone are very effective in the short term, with ~70-80% of patients responding favorably. However, once steroids are withdrawn most patients' ITP relapsed[319,320], with as little as 20-50% of steroid-treated patients maintaining a durable response, depending on the specific drug and treatment course. Long-term treatment with corticosteroids is not an option, because prolonged exposure to corticosteroids is not recommended due to their negative side effects and accumulation[315]. Many ITP patients do not require intervention, and treatment is primarily recommended in patients who are actively suffering from bleeding or who have a dangerously low platelet count[321]. Therefore, despite the response to corticosteroids being transient, they remain the most common first-line treatment for ITP due to their good initial response. In the early 80s, intravenous immunoglobulin (IVIG) treatment emerged as another first-line immunosuppressive treatment with a similar response to corticosteroids[322,323], and the two are often used in tandem to greater effect[324]. The introduction of IVIG as a treatment option for ITP was especially impactful for cases where corticosteroids

cannot be used, such as in children, one of the main populations in whom ITP commonly occurs. As with corticosteroids, the response to IVIG is short-lived[322].

Absent a response to first-line treatments, or once a patient has relapsed, second-line alternatives must be considered. Among these are immunomodulators like the B-cell depleting drug rituximab and TPO mimetics such as eltrombopag and romiplostim. Rituximab is currently not approved by the FDA for use in ITP, but is often used off-label. Although most patients on rituximab still relapse eventually, the response rate is high and more than 50% of patients have a prolonged increase in their platelet count[325,326]. Lastly, TPO mimetics are a recent addition to the canon of second-line ITP treatments, and elicit an increased platelet count in 75-80% of patients who were resistant to prior treatments[304,327].

From Werlhof's first recorded patients to the modern state of our understanding of the disorder, elucidating the complete pathophysiology of ITP has proven itself a formidable challenge. Even today, heterogeneity in disease presentation, severity, and etiology stand in the way of a unified mechanism. Nevertheless, three load-bearing pillars of the disorder have coalesced from the current body of literature: rapid platelet clearance induced by circulating anti-platelet antibodies, immune-mediated abnormalities in thrombopoiesis, and changes in T-cell populations and activity. Of these, by far the most well-documented is antibody-mediated clearance. It is therefore unsurprising that the bulk of treatments for ITP seek to address this phenomenon. Canonically, autoantibodies target platelet antigens, opsonizing platelets and resulting in clearance by macrophages in the liver and spleen[307,328,329]. By and large, the most common antigens are the highly expressed platelet surface receptors GPIIbIIIa and GPIb-IX-V[330-332]. Several lines of evidence suggest that the primary target antigen in ITP is

predictive of disease severity and response to different treatments. Specifically, the presence of anti-GPIb-IX-V antibodies is associated with more severe ITP, and ITP that is refractory to many immunosuppressive treatments including corticosteroids and IVIG[333-335]. This implies that, in addition to the expected macrophage-mediated pathway, anti-GPIb-IX-V antibodies may clear platelets in an immune-independent manner. Indeed, platelet clearance via anti-GPIb-IX-V monoclonal antibodies proceeds with or without their Fc-region[244] and some anti-GPIb-IX-V antibodies induced murine ITP that did not respond to IVIG pre-treatment[336].

The complex pathophysiology of ITP is a puzzle that has engaged hematologists for hundreds of years, and although several great leaps and even more small steps have been made, many questions remain for the enterprising investigator. The remaining issues to be addressed are both mechanistic and clinical in nature.

A significant portion of the patients currently diagnosed with ITP have no detectable anti-platelet antibodies. Together with emerging evidence for a T-regulatory role in ITP, it is possible that what is being diagnosed as a single disorder may arise from diverse mechanisms. Further investigation into changes in T-cell populations in ITP will be vital to our evolving understanding of platelet clearance in the context of the disorder. Convinced that impaired thrombopoiesis contributes to thrombocytopenia in ITP, investigators should also seek to identify the status of megakaryocytes in the bone marrow.

There are now a host of tools at our disposal for the treatment of ITP, having gone from the catchall splenectomy to a decision tree incorporating broad immunosuppressants, more specific immunomodulatory compounds, TPO mimetics, Fc-receptor inhibitors, and other

developing therapies. However, there is a dearth of randomized control trials and other clinical investigations comparing one therapy to another in terms of efficacy, safety, and patient-centered outcomes like cost effectiveness and quality of life. Additionally, we lack reliable diagnostic tests for ITP and prognostic assays to predict response to specific treatments, leading to significant trial-and-error or guesswork in the clinic. While the link between the primary antigen of anti-platelet antibodies and clinical outcomes is well documented, knowledge regarding the mechanistic underpinnings is sparse, specifically the difference between the clearance effects of antibodies against the glycoproteins IIbIIIa and Ib-IX-V.

E. <u>The Force Theme: GPIb-IX-V and Platelet Mechanosensation</u>

When the endothelium suffers damage, the wound must be quickly sealed. To prevent significant blood loss, one of the first problems that the circulatory system must solve is a mechanical one, that of deceleration. How can a platelet, moving through the vasculature at an average of 33 cm/s[13], arrest and remain in place long enough to begin forming a platelet plug while experiencing significant shear from blood flow? Second, how can platelet activation be quickly initiated once tethered, while platelets in circulation remain inert? In mammals, the platelet surface receptor complex GPIb-IX-V coordinates solutions to both problems.

Much like precious follicles of hair in a receding hairline, GPIb-IX-V was not fully appreciated until its absence was felt. The first recognition of GPIb-IX-V's importance in hemostasis dates back to 1948 and the description of the rare but severe bleeding disorder, Bernard–Soulier syndrome (BSS)[337], where patients' GPIb–IX–V is either absent, expressed at low levels or

dysfunctional. Although the syndrome was first documented by its eponymous discoverers in '48, it is perhaps more accurate to place the actual recognition of GPIb-IX-V in the 1970s when it was identified as the missing component in BSS platelets[338,339]. Expressed exclusively on platelets and megakaryocytes, GPIb-IX-V is the second most prevalent platelet surface receptor (after $\alpha_{IIb}\beta_3$). Some homolog of GPIb-IX-V is expressed on the anucleate platelets of all mammals, and good sequence conservation surrounding N-terminal and TM domains suggests a generally conserved structure. Although GPIb-IX-V is probably best known as the primary receptor for VWF, the two do not seem to have coevolved[176]. Unlike GPIb-IX-V, which has a much more limited phylogenetic distribution, VWF has deep evolutionary roots going back to the earliest vertebrates, and even back to some invertebrates, where its evolution may predate its role as an important multimeric hemostatic scaffold in the vertebrate circulatory system[340]. In some organisms that express VWF but not GPIb-IX-V, the VWF domain structure includes the GPIbα binding region, and snake venoms known to induce VWF binding to GPIbα can also induce aggregation of their thrombocytes, alluding to the existence of a protein binding partner that fulfills the role of GPIbα[176]. In other words, VWF did not evolve as a ligand to GPIb-IX-V, and it may be the case that GPIb-IX-V did not evolve as the receptor for VWF either. Interestingly, GPIb-IX-V binds to several other functional ligands in circulation, including thrombin[341], P-selectin[342], $\alpha_M\beta_2$[343], FXI[344], and FXII[345].

GPIb-IX-V is a heteropentameric receptor complex containing four unique subunits: (1)GPIbα, (2)GPIbβ, (1)GPIX, and (1)GPV (Fig. I.6). Each subunit is an independently expressed transmembrane protein with a short cytoplasmic tail, a single transmembrane domain, and a glycosylated extracellular domain. GPIb-IX-V is held together by disulfide linkages between

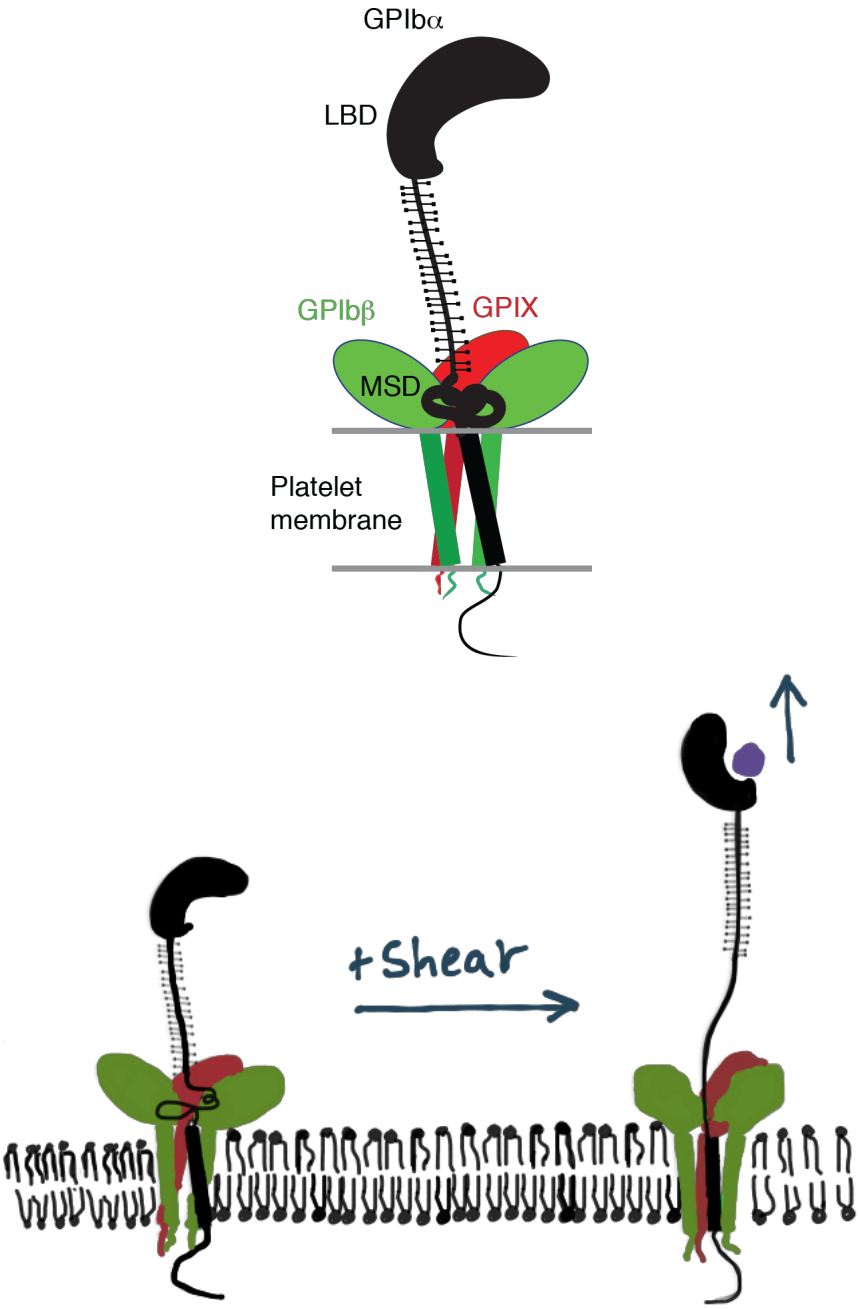

Figure I.6 The GPIb-IX-V complex Top: one GPIbα, two GPIbβ, one GPIX, and one GPV (not shown) subunit make up the heterotetrametric receptor. Key features of GPIbα include its membrane proximal mechanosensory domain (MSD), a long, glycosylated stretch called the sialomucin region, and a membrane distal ligand binding domain (LBD). Bottom: sketch of the trigger model. Ligand binding under shear extends the MSD, leading to GPIb-IX-V activation and possible conformational change in GPIbβ.

GPIbα and two GPIbβ subunits, and noncovalent interactions with GPIX and GPV[346-348]. Efficient expression of the complex on the platelet membrane depends on co-expression of GPIbα, GPIbβ, and GPIX, but not GPV[349,350].

GPIbα is the "business end" of the complex, by far the largest subunit, and responsible for binding to all the previously mentioned ligands of the complex. Starting at its extracellular N-terminus, GPIbα contains a ~45 kDa leucine-rich repeat (LRR) domain, also known as the ligand-binding domain (LBD) (Fig. I.6). The C-terminal portion of the LBD contains a small "thumb" region which is vital for binding VWF[351]. Following the LRR domain is a short anionic sequence involved in binding to thrombin[352]. After this anionic sequence is a flexible stalk region known as the macroglycopeptide or sialomucin region. The sialomucin region spans 30-40 nm, potentially behaving like the length of rope tied to a grappling hook (LBD), swinging it high above the platelet membrane to facilitate latching to ligands and counter-receptors in circulation. The sialomucin region is so named due to its extensive O-glycosylation, which contributes to over 30% of the entire molecular mass of GPIbα. In fact, according to some estimates, GPIbα alone accounts for as much as 80% of the entire sialic acid content on the platelet surface[353]. Following the sialomucin region is a quasi-stable mechanosensory domain (MSD) which is central to GPIb-IX-V's function as a mechanoreceptor. Although the MSD's structure is not fully characterized, the overrepresentation of Ser and Thr residues in its sequence suggest that, like the rest of the sialomucin region, it also bears some O-glycosylation. Single molecule optical tweezer studies indicate that the MSD unfolds under ~15 pN of force[55,354], triggering receptor activation and platelet clearance[56]. Some early results suggest that glycosylation of the MSD may be important for regulation of MSD stability, and thus unfolding. This putative regulatory

mechanism could function as a cog in a positive feedback system wherein neuraminidase release downstream of platelet activation primes GPIb-IX-V for further activation. Further investigation of the dynamics of MSD unfolding in glycosylated and deglycosylated states would be necessary to establish this relationship. Upon shear-induced unfolding, the MSD adopts an extended conformation[55]; assuming 3-4 Å per residue, ~10 residues of the MSD would be in direct contact with the extracellular domains of GPIbβ and GPIX[355,356]. This ~10 residue stretch in the MSD is known as the trigger sequence, and compared to the MSD overall (which has poor sequence conservation across species), it has much higher sequence conservation. Based on studies in mice with chimeric GPIbα extracellular domains, exposure of the trigger sequence is the "trigger" for GPIb-IX-V activation[56,357]. GPIbα's extracellular domain is referred to as "glycocalicin". Glycocalicin is continuously shed from circulating platelets via cleavage by the metalloproteinase ADAM17[358,359].

The extracellular domains of GPIbβ and GPIX are smaller than that of GPIbα, although they also both contain an LRR. The domains exhibit significant sequence homology to one another, and likely participate in GPIX's association with GPIbβ[355]. The physical transduction pathway of mechanosensation through the GPIb-IX-V complex has yet to be comprehensively described. Initial force applied to the molecule via pulling on the LBD of GPIbα unfolds the MSD (Fig. 1.6), exposing a trigger sequence within, but the conformational changes following these steps are still poorly understood. Some recent data generated I have generated addresses an interesting theory, that the extracellular domains of GPIbβ and/or GPIX may also participate in GPIb-IX-V signal transduction. Due to the proximity of the GPIbβ and GPIX extracellular domains to the MSD[55], it stands to reason that, rather than simple accessories to GPIbα expression, the other subunits also participate in signal transduction.

Indeed, GPIbβ and GPIX can change conformations in response to an alteration in inter-subunit contacts[355,360], and their participation in detecting or propagating the signal initiated by MSD unfolding would help to justify why these two subunits are of such important to the receptor complex's surface expression. This hypothesis is discussed further in chapter V.

The extracellular domain of GPV also contains LRRs, along with a cleavage site for thrombin[361]. The transmembrane (TM) domains in GPIb-IX-V are highly conserved, and together form an α-helical bundle[362]. The TM domains of GPIbα and GPIbβ contain the cysteine residues which participate in disulfide linkages between the subunits[346], and the TM domains by themselves are sufficient to assemble into a native-like complex. While the contributions of the complex's TM domains and the extracellular domains of GPIX and GPIbβ to quaternary structure are documented, their part in the signaling mechanism of GPIb-IX-V remains an outstanding question in the field.

GPIb-IX-V is an integral factor in platelet adhesion via its interaction with VWF. While it is by no means the sole linkage between platelets and the subendothelial matrix, the GPIb-IX-V/VWF linkage initiates adhesion. Without this interaction, other adhesive events are insufficient to mediate platelet arrest and initiate formation of a platelet plug[50,53]. Recall from section I.A that, under normal circumstances, VWF must undergo a shear-induced conformational change to enable binding to GPIbα and recruitment of platelets[50-52]. However, under many abnormal or pathological conditions, VWF may associate with GPIbα independent of shear. VWF is released from specialized compartments in endothelial cells called Weibel-Palade bodies, in which VWF exists as long multimers called ultra large VWF (ULVWF). ULVVWF is normally cleaved by ADAMTS13 (a metalloproteinase) and released

into circulation as smaller multimers[51]. Prior to cleavage, ULVWF can spontaneously associate with GPIbα[363]. VWF association to GPIbα can also be induced by exogenous mediators like the bacterial peptide ristocetin[364] or a component of snake venom, botrocetin[365]. In patients with type 2B Von Willebrand disease (VWD), mutant VWF exhibits spontaneous association to GPIbα without the requirement of shear[366]. Type 2B VWD patients present with accelerated platelet clearance and thrombocytopenia of varying severity, depending on the underlying mutation[56,367,368]. Further, transgenic mice expressing type 2B VWF exhibit thrombocytopenia due to clearance of large VWF-platelet complexes in the liver and/or spleen[284]. Indeed, thrombocytopenia is observed in all of the aforementioned contexts where VWF spontaneously associates with GPIbα. Ristocetin (which was used as an antibiotic) was pulled from use in the clinic because it caused thrombocytopenia[287], and injection of botrocetin causes acute thrombocytopenia in animals[288,369]. In the bleeding disorder thrombotic thrombocytopenia purpura (TTP), elevated levels of ULVWF resulting from ADAMTS13 deficiency lead to thrombocytopenia[370]. Thrombocytopenia is also observed in many patients who have received implantations of left ventricular assist devices[371], which generate abnormal shear flow conditions and may potentially induce VWF association with GPIbα. Under normal circumstances, binding of plasma VWF to GPIbα at the site of injury induces shear-dependent GPIb-IX-V signaling and platelet desialylation (via neu1) which causes platelet clearance via the glycan-mediated mechanism discussed in section I.C[56]. It is likely that this same process underlies in the cases where VWF spontaneously associates with GPIbα.

The ADAM17 cleavage site of GPIbα is located in the MSD[56,372]. Although the site is still accessible when the MSD is folded (consistent with the constant shedding of glycocalicin, even from resting platelets), MSD unfolding induced by ligand binding under shear may

further expose the ADAM17 shedding cleavage site, thereby boosting shedding of GPIbα[56]. On the other hand, upon shedding of GPIbα and subsequent separation of glycocalicin from the platelet, the structure of the MSD is disrupted, and the membrane-proximal trigger sequence is unprotected. Thus, it is conceivable that shedding of GPIbα may achieve MSD unfolding and induce GPIb-IX-V signaling. This is consistent with the observation that mutations in the MSD that cause MSD unfolding can induce ligand-free signaling from GPIb-IX-V[56].

In addition to its adhesive functions, GPIb-IX-V-dependent signaling is vital to the initiation of platelet activation. VWF-dependent GPIb-IX-V activation leads to inside-out activation of $\alpha_{IIb}\beta_3$[373-375] (which mediates stable platelet adhesion, spreading, and aggregation), formation of platelet microparticles[376,377], TXA2 synthesis and release[378,379], degranulation[379,380], desialylation via neu1[276], among other procoagulant phenomena. The intracellular signaling pathways that link GPIb-IX-V activation to these phenomena are not yet fully elucidated. Several downstream signaling mediators of GPIb-IX-V have been identified including: Ca^{2+}[375,381-383], Src family kinases[378,384]; PLCγ2[385], PI3K[384,386]; mitogen-activated protein kinase (MAPK) pathway[378,387]; and LIM kinase 1 pathway[388]. Numerous studies have been published investigating the role of the regulatory protein 14-3-3ζ, which binds to the cytoplasmic tails of both GPIbα and GPIbβ[389-391]. These reports conflict regarding 14-3-3ζ's effect on GPIb-IX-V signaling, though some suggest that 14-3-3ζ has a regulatory effect on GPIbα binding to VWF[392-394]. It has also been reported that 14-3-3ζ's association with GPIbα is involved in platelet apoptosis[395,396]. The distinctions between GPIb-IX-V signaling pathways leading to platelet clearance and platelet activation remain unclear.

F. <u>No Line on the Horizon: What We Know and What We Hope to Learn</u>

In this first chapter I have endeavored to assemble and present all the myriad lines of inquiry required to contextualize the body of work laid out in future sections. Through a fast-flowing review of the development of circulatory systems, I introduced the importance of the endothelium in regulating blood flow, coagulation, and triggering hemostasis. In our exploration of Murray's Law, we were first introduced to the concept of shear force, and how it factors into many of the processes that occur in our blood-vascular system. This concept of shear-sensing and shear-directed processes is one that recurs through this chapter as I discuss PIEZO1 and shear force remodeling, the activation by elongation of globular VWF when anchored under flow, shear-induced unfolding of the mechanosensory domain in GPIb-IX-V to initiate platelet activation at the site of injury, high-shear induced thrombotic pathologies, and the importance of specific shear flow to the production and release of proplatelets from megakaryocytes. In Chapter II I will explore some examples of mechanoreceptors in the body. We will cover some common techniques currently used to investigate mechanoreceptors, and I will detail one specific workhorse assay for examining the effects of shear on activation of platelet GPIb-IX-V.

The latter half of section I.A introduced primary and secondary hemostasis. In primary hemostasis, platelet surface receptors and platelet agonists coordinate to slow down platelets in blood flow, adhere them to the subendothelial matrix, and form the platelet plug. Secondary hemostasis is the domain of zymogen coagulation factors, which participate in intersecting cascades to generate thrombin and stabilize the growing thrombus in a fibrin net. Section I.B properly introduces platelets, their evolutionary context, their genesis from megakaryocyte progenitors, and several features of their biology. The simplicity of these 4 μm cell fragments is rivaled only by the fascinating diversity of processes they have their hands

(filopodia?) in. In addition to their most recognized and central role in primary hemostasis, platelets act as innate immune cells, pro-inflammatory agents, and facilitators of angiogenesis (a function sometimes exploited by interacting tumor cells).

While section I.B covered platelet generation aka thrombopoiesis, section I.C asks how platelets are removed from circulation by the many mechanisms of platelet "clearance". Many factors extrinsic and intrinsic to platelets control their lifespan. Platelet apoptosis is mediated by the anti-apoptotic Bcl-x_L as it restrains the death molecule Bak from inducing mitochondrial depolarization. Aged and activated platelets are cleared via glycan-mediated processes. Platelet surface O-glycans lose sialic acids during aging or activation, exposing β-galactose, the ligand for the hepatic Ashwell-Morell receptor. Removal of sialic acid or "desialylation" is a consequence of GPIb-IX-V activation, proceeding through the sialidase neu1. Lastly, mostly in a pathological context, autoantibodies against platelet surface receptors lead to platelet opsonization and clearance in the spleen.

Autoimmune platelet clearance is the defining feature of immune thrombocytopenia, a highly prevalent bleeding disorder covered extensively in section I.D. Our understanding of immune thrombocytopenia has developed greatly over the last 150 years. It has transitioned from an idiopathic disorder with little characterization outside of a demographic profile to an autoimmune condition with a sophisticated mechanism underlying accelerated clearance and decreased thrombopoiesis. As the molecular basis was iteratively elucidated, so too did therapeutic options expand. As immune thrombocytopenia is an autoimmune disorder, many therapies target the immune system, including the oldest targeted treatment (splenectomy) as well as more modern first-line treatments (IVIG and corticosteroids). However, transient

responses and overall refractoriness to these treatments remains a persistent challenge to treatment. To compound these troubles, our current understanding of the underlying mechanism of resistance to these treatments is lacking. As a result, there are no reliable techniques to determine what a given patient's response will be. In chapter III, I present data establishing a biophysical mechanism for IVIG resistance and suggest approaches for determining treatment responsiveness. Without putting the cart before the horse, the findings of chapter III suggest some unique possibilities for alternative ligands to GPIb-IX-V. In chapter IV I will dive deeper into this paradigm, exploring what may be a shift in thinking about what binding partners to GPIb-IX-V can trigger mechanosensation.

At the origin of the hemostatic response is the interaction between the two shear-sensitive proteins VWF and GPIb-IX-V. In section I.E, I expand on the structure, function, and activity of GPIb-IX-V. The trigger model of GPIb-IX-V activation identifies the unfolding of the membrane-proximal mechanosensory domain as the key mechanosensitive step in receptor signaling. Through the lens of this model, many observations regarding the requirement of shear for platelet activation through GPIb-IX-V are clarified. However, many parts of the picture remain to be brought into focus. This is to be expected due to the fairly recent discovery and characterization of the mechanosensory domain and trigger model, over the last 5 years. One pressing question regarding the mechanism of GPIb-IX-V activation is the contribution of subunits other than GPIbα to receptor signaling. As discussed briefly in section I.E, no role for GPIbβ or GPIX in signal transduction has been identified, despite the fact that all three subunits are required for expression on the surface of platelets. Beyond MSD unfolding, the next steps in propagation of the mechanical signal are unknown. In

chapter V, I present intriguing data regarding the participation of GPIbβ in GPIb-IX-V activation, moving toward a more comprehensive trigger model.

CHAPTER II

A UNIFORM SHEAR ASSAY FOR HUMAN PLATELET AND CELL SURFACE RECEPTORS VIA CONE-PLATE VISCOMETRY

M. Edward Quach, Anum K Syed, Renhao Li

Aflac Cancer and Blood Disorders Center, Children's Healthcare of Atlanta and Department of Pediatrics, Emory University School of Medicine, Atlanta, GA 30322

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Abstract

Many biological cells/tissues sense the mechanical properties of their local environments via mechanoreceptors, proteins that can respond to forces like pressure or mechanical perturbations. Mechanoreceptors detect their stimuli and transmit signals via a great diversity of mechanisms. Some of the most common roles for mechanoreceptors are in neuronal responses, like touch and pain, or hair cells which function in balance and hearing. Mechanosensation is also important for cell types which are regularly exposed to shear stress such as endothelial cells, which line blood vessels, or blood cells which experience shear in normal circulation. Viscometers are devices that detect the viscosity of fluids. Rotational viscometers may also be used to apply a known shear force to fluids. The ability of these instruments to introduce uniform shear to fluids has been exploited to study many biological fluids including blood and plasma. Viscometry may also be used to apply shear to the cells in a solution, and to test the effects of shear on specific ligand-receptor pairs. Here, we utilize cone-plate viscometry to test the effects of endogenous levels of shear stress on platelets treated with antibodies against the platelet mechanosensory receptor complex GPIb-IX.

II. A Uniform Shear Assay for Human Platelet and Cell Surface Receptors via Cone-plate Viscometry

A. More Than a Feeling: Detecting the Effects of Force on Mechanoreceptors

Mechanoreceptors are proteins that respond to mechanical stimuli, such as pressure, shear, or mechanical perturbation/deformation. In the case of some mechanoreceptors, sensing mechanical perturbations is explicit to the function of the cell types in which they are expressed. Take, for example, the stretch receptors or "baroreceptors" of the arterial pressure control system; these mechanosensitive ion channels regulate blood pressure by sensing vascular "stretch"[397,398]. Modulation of circulatory fluid pressure is a fundamental feature of circulatory systems, and this job role is filled by baroreceptors in organisms as distant from humans as fish and amphibians, and perhaps even further, in some invertebrates[399]. In humans, these receptors are embedded in the walls of many large arteries, especially the carotid artery and the wall of the aorta[13] and control blood pressure via the arterial baroreflex[400]. Dysfunction of these vessel-wall mechanoreceptors is associated negative prognosis in patients with arrhythmia, cardiovascular disease, and diabetes and can lead to sudden cardiac death[401-403]. Some other examples: In the inner ear, ion channels on hair cells detect mechanical deformations caused by sound waves[404] and cutaneous low-threshold mechanoreceptors facilitate the transmission of tactile information instrumental in our sense of touch[405].

Mechanoreceptors also have diverse functions in cell types not specialized for mechanosensation. Here, they may transmit important information regarding a cell's physical microenvironment, adhesive state, and cell-cell interactions. Some cells can sense the rigidity of their local environment, and may rely on contractile forces exerted via the actin

cytoskeleton and integrins to dictate growth or spreading[406,407]. A substrate's "stiffness" (the relationship between force applied per unit area and the corresponding deformation of that substrate) can be described by a mechanical property called Young's modulus. This stiffness sensing is especially important in many developmental programs. For example, altering the Young's modulus of a hydrogel growth substrate to match that of endogenous muscle tissue greatly improves muscle stem cell self-renewal and regenerative capabilities[408]. A similar functional responsiveness to substrate stiffness is observed in neural stem cells[409,410] and mesenchymal stem cells[411]. Some studies implicate integrin-cytoskeleton linkage in focal adhesions as the mechanism of mechanosensation in these cases[412,413]. In section I.A, I discussed how another class of mechanoreceptors, Piezo channels, direct the development of the vasculature[34-36]. While that function of the PIEZO1 channel is localized to endothelial cells, a recent study has uncovered a curious role for PIEZO1 in mechanosensitive stimulation of inflammation in myeloid cells of the innate immune system[414]. Although many of these examples center around human physiology, responses to mechanical stimuli via mechanoreceptors is a ubiquitous phenomenon in the various disparate clades of life from man to arthropods[415] to nematodes[416,417], all the way to prokaryotes[418]. As mechanosensitive components of more and more processes come to light, it is increasingly important to develop and adapt new approaches to study mechanosensation itself. When studying receptor-ligand interactions in cell or tissue-based models, basic biochemistry training primes us to consider the effects of altering temperature, pH, ligand concentration, membrane potential, and many other parameters which can vary *in vivo*. Common assays therefore exist which can detect the effects of these parameters on receptor activation/signaling. However, these assays may fall short when it comes to detecting the contribution of mechanical force to receptor activation.

Whether cells are sensing the rigidity of their microenvironment, detecting sound waves, or responding to stretch, one thing the aforementioned mechanoreceptors have in common is that they are participating in interactions where the ligand, receptor, or both, are anchored to a surface. Many of the assays developed to test the effects of mechanical forces on receptor interactions reflect this paradigm. For example, microfluidics and flow chambers are used to study the effects of shear flow on cells and receptors[419,420]. In these assays, ligands can be immobilized on a surface, and cells may be passed over them in a flowing solution. These types of experiments have the advantage of fine-tuned of shear rates, which may be calculated from flow speeds and vessel dimensions. In this way, cells can be exposed to specific shear rates reflective of endogenous or pathological conditions. Other techniques employ fluorescent molecular probes to detect forces applied by cells on ligand-rich surfaces, yielding an accurate readout of the magnitude and orientations of forces involved in the interaction[421,422].

In addition to mechanosensation occurring where one or both partners are anchored to a surface, shear stress may affect proteins and cells in solution. This is often observed in blood cells/proteins which are constantly in the circulation, and may manifest via activation of mechanoreceptors that are normally surface-anchored, or through exposure of target sequences which would be occluded under static conditions, as with VWF[423]. However, relatively few techniques assay the effects of shear force when ligand and receptor are both in solution. Some such approaches introduce shear via vortexing cells in fluid suspension with varying speed and duration, although these approaches may not allow a very precise determination of the shear stress generated. Rotational viscometers measure viscosity by applying a specific shear force to fluids. More quantitative studies on platelet responses to

shear use a cone-plate viscometer (a type of rotational viscometer) to examine shear force dependent substrate selection[424], and to apply shear to whole blood or platelet rich plasma[425]. The benefits of using a cone-plate viscometer include specific control over the intensity and duration of shear, and uniform application of laminar shear throughout a sample, whereas other methods may lead to differential shear regimes and turbulence throughout the sample.

Recall from chapter I that VWF engagement to its binding site in GPIbα under physiological shear stress induces unfolding of the MSD in, which in turn activates GPIb-IX-V[56]. VWF also responds to force, unmasking its binding site for GPIbα only when tethered and exposed to shear[50-52], although it is probably better described as a force-sensitive ligand than as a mechanoreceptor itself. Under normal, healthy circumstances, this allows globular VWF to circulate without being "sticky" to platelets. However, in pathological situations such as Type 2B VWD and in the presence of agonists like botrocetin and ristocetin, VWF-platelet aggregates form, with the eventual result being thrombocytopenia[284,287]. Platelet-antibody aggregates have also been hypothesized to clear platelets via a GPIb-IX-V-dependent mechanism (see chapter III). GPIb-IX-V is a shear-sensitive mechanoreceptor, so to detect the effect of soluble ligands like VWF with type 2B VWD mutations or anti-GPIb-IX-V antibodies on the complex, we have exploited the approach of introducing shear force via cone-plate viscometry, paired with specific flow cytometry-based detection methods (Fig II.1). In the remainder of this chapter we describe our protocol and workflow for determining the effects of specific laminar shear rates on platelets (or other cell types), especially when treated with a soluble ligand.

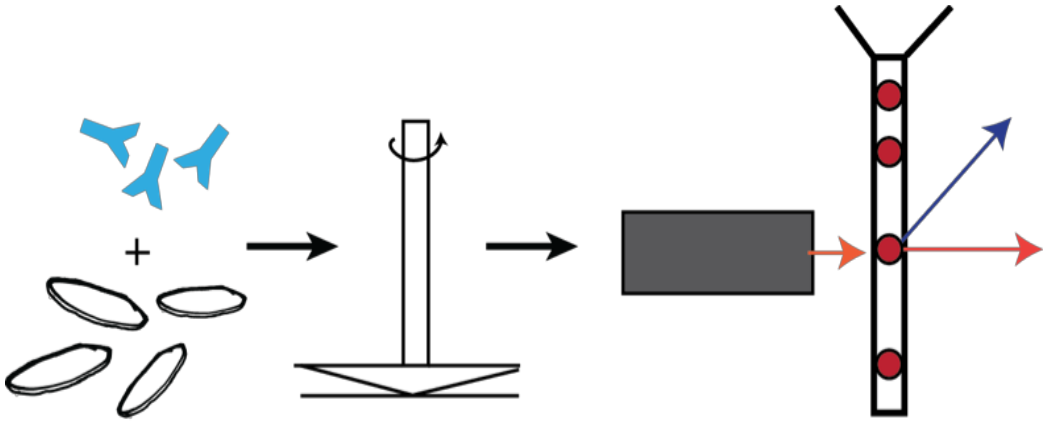

Figure II.1 Schematic of Uniform Shear Assay. Proceeding from left to right, cells are isolated (steps 1.3-1.5) and mixed with antibodies or a ligand of interest (step 2.1). Cells are sheared on a cone-plate viscometer at a specific rate (steps 2.3-2.4). Lastly, the fixed samples which have been treated with markers of receptor activation (steps 2.6 & 2.7) are interrogated via flow cytometry (steps 3.1 & 3.2).

B. Protocol

1. Blood Draw and Platelet Isolation

All methods using donor-derived human platelets described herein were approved by the Institutional Review Board of Emory University/Children's Healthcare of Atlanta.

1.1) Draw human blood from consenting healthy adult donors via venipuncture on the day of the experiment. Blood should be drawn into 3.8% trisodium citrate. One 4.5mL tube of blood is sufficient to yield enough platelet rich plasma (PRP) for 20-25 conditions in donors whose platelet counts are close to $250x10^3$ per µL. Avoid drawing blood via narrow gauge needles (smaller than 21 gauge).

1.2) Prepare PRP via centrifugation at 22 °C and 140 g for 12 min with a long brake. This will result in two distinct layers, with red blood cells on the bottom and the light colored PRP on top.

1.3) Isolate the top layer, cloudy, yellow layer of PRP, via careful pipetting through a pipette tip cut on a 45° angle and obtain the platelet count via CBC

1.4) If necessary, wash platelets in PIPES buffered saline (150 mM NaCl, 20 mM PIPES) in the presence of prostaglandin E1 (PGE1) and resuspend in Tyrodes buffer (134 mM NaCl, 0.34 mM Na2HPO4, 2.9 mM KCl, 1 mM MgCl2, 5 mM glucose, 12 mM NaHCO3, 20 mM HEPES, pH 7.35) with 5 mM glucose. Briefly,

1.4a) Adjust PRP volume to 10mL with PIPES saline and 0.6µM PGE1

1.4b) Centrifuge for 8 minutes at 1900 g, then discard supernatant and let platelet pellet sit in 400 µL Tyrodes+glucose for 5 minutes

1.4c) Gently resuspend the platelet pellet and let rest for 30 minutes

Otherwise, proceed to step 1.5.

1.5) Adjust the platelet count to ~250x10^3 platelets per µL with pooled human platelet poor plasma (PPP) and maintain the suspension at 22°C at rest or under gentle rotation.

2. Ligand and uniform shear treatment

All steps in section 2 that require pipetting should be done slowly, so as not to introduce shear

2.1) Add desired antibody or ligand to PRP or washed platelets and mix gently by pipetting up and down or stirring with the pipette tip. Let rest at room temperature for 5-10 minutes. Add an equivalent volume of PPP or Tyrodes buffer to a negative control.

2.2) Turn on the cone-plate viscometer and set the plate temperature to 22 °C and allow time for the plate to reach this temperature.

2.3) Pipette the treated PRP or washed platelets onto the temperature-controlled cone-plate viscometer directly on the center of the plate. Ensure that all of the sample is between the cone and plate when they come into contact, and not on the outside of the cone's rim.

2.4) Shear at an appropriate rate and duration. Calculate shear as indicated by the viscometer manual, or as previously shown[56,426]. Briefly, determine shear rate from viscosity and desired shear stress via Newton's law of viscosity; $\tau = \mu \frac{du}{dy}$; plasma viscosity is 1.5-1.6 centipoise (cP)[427]. For reference, a normal shear range for human circulation is 5-30 dyn/cm^2 and shear should be applied on the single digit minute time scale.

2.5) Lift the cone off of the plate by a small margin (approximately 2 mm) so that the sample remains in contact with both the plate and cone and use a gel-loading or other long pipette tip to collect 5-10 µL from the center of the sample volume.

2.6) Incubate the sheared samples with the desired markers for 20 min at room temperature.

For markers of phosphatidylserine, β-galactose, and P-selectin exposure use Lactadherin C2 domain (Lact-C2) (0.08 μM)[428], Erythrina cristagalli lectin (ECL) (6.25 μg/mL), and anti-P-selectin antibody (20 μg/mL), respectively.

2.7) Fix samples in 2% P-formaldehyde for 20 minutes at RT prior to dilution or cold storage and proceed to 3.1 or store samples at 4 °C, not longer than 12 hours.

3. Detection of surface markers and crosslinking via flow cytometry

3.1) Analyze the sample via flow cytometry, collecting at least 20,000 events for each condition.

3.2) Quantitate signal strength of the fluorescent markers using the height value for the intensity of each fluorophore, or the geometric mean fluorescence intensity (gMFI).

3.3) If aiming to detect platelet-ligand aggregate formation following shear treatment, analyze the sample on an imaging-capable flow cytometer and quantitate aggregates using area and aspect ratio parameters. Briefly, plot a histogram of area and/or aspect ratio. Use a negative control with BSA or vehicle to draw a gate excluding most fully circular events (this gate is usually drawn at an aspect ratio ~0.8[429,430]) and quantitate the percentage of events inside of this gate. Events with a lower aspect ratio are more likely to be multiplatelet aggregates.

C. Notes on Performing This Assay

The protocol described above allows quick and versatile assessment of the effect of laminar shear on platelet and cell surface receptors in solution. The goal of this iteration of the uniform shear assay is to examine how the effects of soluble ligands on cell-surface receptors can be affected by shear flow. In addition to this application, a uniform shear assay has broad applications in observing shear-dependent effects. In the absence of a known ligand-receptor pair, a uniform shear assay can also detect the effects of shear on factors such as cell shape

and signaling, especially paired with flow cytometric analyses.

Although the assay is accessible as written, there are a few key steps which should be performed with extra care. Foremost among these is being careful not to introduce shear from external sources other than the actual shear treatment itself. If the sample is exposed to unnecessary vortexing, mixing/perturbation, drawing through narrow passages, vigorous pipetting, or any other external forces, it may introduce noise into analysis of the data. When drawing blood, as mentioned in step 1.1 of this protocol, it is important to draw through proper gauge needles. For human blood collection via venipuncture from consenting adult donors, this can be accomplished with a 21 gauge blood collection set. If the centrifuge used to isolate PRP can be programmed to break at different speeds it is best to choose a slower break to minimize disturbance of the PRP layer and application of unnecessary force during this step. Once PRP is isolated or the cell type of interest is in fluid suspension, avoid pipetting any mixtures too quickly. Instead, aspirate and expel samples in a slow and steady fashion especially during step 2.5, where the sample is drawn through a particularly narrow pipette tip. For samples volumes which can accurately be pipetted via pipette tips of different sizes, it is advisable to use the largest among them. For especially sensitive samples, the very end of the pipette tip can be cut on an angle to widen the opening through which the sample is to be drawn. Ultimately, the uniform shear assay provides a reasonably cheap, quantitative, and specific method for applying laminar shear to cells and cell fragments in solution, and can be used upstream of many detection methods.

CHAPTER III

Fc-INDEPENDENT IMMUNE THROMBOCYTOPENIA VIA MECHANOMOLECULAR SIGNALING IN PLATELETS

M. Edward Quach,[1] Matthew A. Dragovich,[2] Wenchun Chen,[1] Anum K. Syed,[1] Wenpeng Cao,[2] Xin Liang,[1] Wei Deng,[1] Simon F. De Meyer,[3] Guangheng Zhu,[4] Jun Peng,[5] Heyu Ni,[4] Carolyn M. Bennett,[1] Ming Hou,[5] Jerry Ware,[6] Hans Deckmyn,[3] X. Frank Zhang,[2] and Renhao Li[1]

[1]Aflac Cancer and Blood Disorders Center, Children's Healthcare of Atlanta, Department of Pediatrics, Emory University School of Medicine, Atlanta, GA, 30322

[2]Department of Mechanical Engineering and Mechanics, Bioengineering Program, Lehigh University, Bethlehem, PA, 18015

[3]Laboratory for Thrombosis Research, Katholieke Universiteit Leuven Campus Kulak Kortrijk, Kortrijk, Belgium

[4]Toronto Platelet Immunobiology Group and Department of Laboratory Medicine, Keenan Research Center for Biomedical Science, St. Michael's Hospital, Toronto, ON, Canada

[5]Department of Hematology, Qilu Hospital, Shandong University, Jinan, China

[6]Department of Physiology and Biophysics, University of Arkansas for Medical Sciences, Little Rock, AR, 72701

Blood

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Abstract

Immune thrombocytopenia (ITP) is a prevalent autoimmune disease characterized by autoantibody-induced platelet clearance. Some ITP patients are refractory to standard immunosuppressive treatments such as intravenous immunoglobulin (IVIG). These patients often have autoantibodies that target the ligand-binding domain (LBD) of glycoprotein Ibα (GPIbα), a major subunit of the platelet mechanoreceptor complex GPIb-IX-V. However, the molecular mechanism of this Fc-independent platelet clearance is not clear. Here, we report that many anti-LBD monoclonal antibodies such as 6B4, but not AK2, activated GPIb-IX-V in a shear-dependent manner and induced IVIG-resistant platelet clearance in mice. Single-molecule optical tweezer measurements of antibodies pulling on full-length GPIb-IX demonstrated that the unbinding force needed to dissociate 6B4 from the LBD far exceeds the force required to unfold the juxtamembrane mechanosensory domain (MSD) in GPIbα, unlike the AK2-LBD unbinding force. Binding of 6B4, not AK2, induced shear-dependent unfolding of the MSD on the platelet, as evidenced by increased exposure of a linear sequence therein. Imaging flow cytometry and aggregometry measurements of platelets and LBD-coated platelet-mimetic beads revealed that 6B4 can sustain crosslinking of platelets under shear, whereas 6B4 Fab and AK2 cannot. These results suggest a novel mechanism by which anti-LBD antibodies can exert a pulling force on GPIbα via platelet crosslinking, activating GPIb-IX-V by unfolding its MSD and inducing Fc-independent platelet clearance.

III. Fc-independent immune thrombocytopenia via mechanomolecular signaling in platelets

A. Shadowboxin' - Fighting Immune Thrombocytopenia in the Dark

As I covered extensively in section I.D, ITP is a common bleeding disorder characterized by increased platelet clearance, primarily via anti-platelet autoantibodies[328,431,432]. Diagnosis of ITP has historically been a diagnosis by exclusion, and this is still the case today. Even in the last decade, a diagnosis of ITP is commonly defined by a platelet count below 100,000 per μl in the absence of other conditions or disorders that may be the cause of the thrombocytopenia[431,433]. Although other conditions may be ruled out via blood smear, CBC, hemoglobin count, etc., there is no positive diagnostic test for ITP[315]. As previously mentioned, autoantibodies are detectable in only ~60% of ITP patients[434], likely due in part to limits in assay sensitivity and untested antigens going undetected[435,436], and as a result assaying for anti-platelet antibodies is not recommended for reliable diagnosis[433]. Within this information-poor paradigm, it is also possible that many patients' *immune* thrombocytopenia may be better categorized as *idiopathic* thrombocytopenia, as the disorder has been called in the past.

Although some patients with acute ITP can simply be monitored to determine whether their platelet count will correct in time, for cases with particularly low platelet count or situations where a severe bleeding phenotype develops, treatment is necessary. The first-line treatments for ITP are currently immunosuppressants like IVIG or corticosteroids. However, as much as 30% of patients treated with corticosteroids are refractory and up to half may have only a fleeting response[319]. Furthermore, corticosteroids in general are not an advisable long-term treatment option and are not used in children. In pediatric cases, IVIG treatment is more common, but it is estimated that 20% of patients are refractory to IVIG[437]. The underlying

mechanism of refractoriness to these immunomodulatory treatments is not entirely clear and thus there are no clinical tests that can make a prediction of the success of a given treatment[322,433]. Treating ITP is therefore a "try and see" endeavor. In some of the worst cases of ITP, splenectomy may be recommended. It is one of the oldest ITP treatments, and has a decent success rate. Keeping in mind that most patients who now have splenectomy recommended to them have already tried some immunosuppressive first line treatment, 30-50% of these patients don't have a durable response to splenectomy either[317]. Notably, there is correlation between refractoriness to IVIG or steroids and the presence of anti-GPIb-IX antibodies in patient sera[333-335]. Additionally, infusion of monoclonal antibodies (MAbs) targeting the N-terminal ligand-binding domain (LBD) of GPIbα causes fast depletion of nearly all platelets from animals[290-293]. Clearance by these anti-LBD MAbs is Fc-independent and largely unaffected by IVIG treatment[244,336], consistent with the findings that these MAbs can activate GPIb-IX-V, which leads to deglycosylation and subsequent platelet clearance by hepatocytes or macrophages[276,285,438]. Many studies corroborate a remarkable similarity between the effects of ITP patient sera antibodies and MAbs targeting GPIb-IX-V on platelets. Markers of GPIb-IX-V activation, especially platelet desialylation, have been seen in MAb-induced ITP as well as in ITP patients, and the extent of desialylation correlates with patient response to first-line treatments[276,439,440].

Despite these findings, the mechanism by which these antibodies activate GPIb-IX-V has yet to be elucidated. Regarding the mechanism, four key observations have coalesced from the literature. First, the $F(ab')_2$ but not the Fab fragment of an anti-LBD MAb can induce platelet clearance[244,293]. This indicates that the bivalent structure of the antibody is required for activating GPIb-IX-V. Second, many antibodies against GPIb-IX-V's LBD clear platelets

rapidly in animal models, regardless of their epitope in the LBD[276,290-293]. This implies that the mechanism of clearance does not involve steric occlusion of any binding pockets or regions of interest within the LBD itself. Third, most MAbs targeting the LBD, but not other domains in GPIb-IX-V, induce Fc-independent clearance[292,441-443] (Fig. III.1a). This indicates that there is something unique about the LBD that permits activation of the receptor and/or clearance of platelets in an Fc-independent manner. Lastly, while almost all anti-LBD antibodies induce platelet clearance, one anti-LBD MAb, VM16D does not[285] (Fig. III.1a). To date, no unified model has been proposed to fully explain these four observations.

A GPIbα "clustering model" has been proposed as the mechanism of GPIb-IX-V activation[444,445] and applied to explain the observed effects of anti-LBD MAbs. In this model, a multimeric ligand (in this case, a dimeric antibody) binds two copies of GPIbα, inducing lateral dimerization or "clustering", and thereby transmitting a signal into the platelet that subsequently leads to fast clearance[285]. VWF, being a multimeric ligand, is also capable of clustering GPIb-IX-V[444,445]. The clustering model can explain the first and second observations about anti-LBD MAbs. However, it is difficult to conceive how the clustering model accounts for the third and fourth observations. If the only requirement was dimerization, it would be anticipated that MAbs against not only the LBD, but other regions of GPIbα and other subunits of the complex, would be able to cluster GPIbα and induce activation or Fc-independent clearance. Recently, it was demonstrated that 3D1, a MAb targeting the MSD of GPIbα, binds to two copies of GPIbα on the platelet, but induces neither platelet activation in vitro nor thrombocytopenia in mice[443]. Moreover, the requirement of shear in VWF-mediated GPIb-IX-V signaling is well documented[446,447] but remains to be addressed by the clustering model.

In sections I.A and I.E we discussed the current model for GPIb-IX-V activation, the trigger model, which has gained significant supporting evidence since its proposal in 2015-2016[55,56,448,449]. This model helps to explain the mechanosensory functions of GPIb-IX-V. Briefly, under physiological shear, binding of VWF to the LBD generates a pulling force on GPIbα, and induces MSD unfolding on the platelet surface, exposure of the membrane-proximal Trigger sequence therein and subsequent receptor activation which leads to exposure of several markers of platelet activation and clearance. It may help to visualize the trigger model through the lens of a gas-powered lawn mower: the handle represents the LBD, the cord itself represents the sialomucin region, and the coiled portion of the cord represents the MSD. When the handle/LBD is pulled on, the coiled cord (MSD) unfolds and triggers activation. This model of GPIb-IX-V activation accounts for the requirement of shear force, as well as potentially all aforementioned observations regarding antibody-induced signaling. The dimeric structure of activating ligands is utilized to crosslink platelets via GPIb-IX-V and induce MSD unfolding[450]. In the trigger model, the defining characteristic of an activating ligand to GPIb-IX-V is its ability to bind tightly to the LBD under sustained shear[56,450]. Thus, it is conceivable that ligands with similar binding affinities and binding sites but disparate mechanical properties, such as different anti-LBD MAbs or VWF bearing different type 2B mutations[368,450], may differentially activate GPIb-IX-V and induce platelet clearance. In this chapter we report evidence that, like VWF, anti-LBD MAbs induce platelet signaling in a shear-dependent manner that entails MSD unfolding, which leads to a new mechanomolecular mechanism to account for all four observations. Our findings have mechanistic implications for GPIb-IX signaling, and clinical implications in the context of IVIG-resistant ITP and other thrombocytopenic diseases.

B. Methods

Mice Transgenic mice expressing only human GPIbα (hTg) have been previously described.[451] All experimental procedures were approved by the Institutional Animal Care and Use Committee (IACUC) of Emory University and were carried out in accordance with the NIH Guide for the Care and Use of Laboratory Animals. Mice between the ages of 6 and 10 weeks were used for experiments and both male and female mice were equally represented. Murine PRP was prepared from whole blood that was obtained via cardiac puncture under isoflurane anesthesia and the platelet count was standardized to 200-300k per μl using mouse platelet-poor plasma (PPP).

Human platelets All procedures using donor-derived human platelets were approved by the Institutional Review Boards at Children's Healthcare of Atlanta/Emory University and Qilu Hospital. Written, informed consent was received from participants prior to their inclusion in studies. Venous blood was obtained from healthy adult volunteers by venipuncture into 3.8% trisodium citrate. Human PRP was prepared via centrifugation (12 min, 140g) of whole blood. Platelets were counted with a Sysmex XP-300 automated hematology analyzer and adjusted to $250x10^3$ platelets per μl with pooled human platelet poor plasma (PPP) and maintained at 22°C. Flow cytometry, aggregometry, and uniform shear treatment were performed as previously described[289,428].

Uniform shear treatment Platelets in PRP or washed platelets reconstituted in PPP were incubated with noted antibodies at a final concentration of 5 μg/ml or with plasma for 5 min in a 96-well plate. The mixture was then uniformly sheared for 5 min on a Brookfield Cap 2000+

cone-plate viscometer at 22°C. Shear-treated PRP was labeled with antibodies or lectins for detection of surface markers [Lact-C2 (0.08μM), ECL (6.25 μg/ml), anti-P-selectin (20μg/ml)] for 20 minutes before fixation in 2% *p*-formaldehyde. This protocol is described in further detail in chapter II.

Flow cytometry Flow cytometry of platelets were performed on a Becton-Dickinson (San Jose, CA) FACS Canto II instrument. Signal strength was quantitated using the height value for the intensity of each fluorophore or the geometric mean fluorescence intensity (MFI) as indicated. At least 20,000 events were collected for each sample and recorded for each sample and data were analyzed by FlowJo and BD FACSDIVA software.

ELISA ELISA on patient serum was performed at room temperature in Costar 96-well assay plates coated with purified human GPIb-IX^{428} (5μg/ml) and blocked with BSA (1% w:v). 25μl undiluted plasma was incubated for 1hr and washed 2x (0.1% Tween 20). Binding of human sera antibodies was detected via HRP-conjugated goat anti-human Fc antibody (1:2000 dilution) (Southern Biotech). Following a 3x wash, wells were incubated with 50μl SureBlue TMB Peroxidase Substrate and read on a Molecular Devices Spectramax Plus plate reader.

Platelet aggregometry Human PRP was isolated via centrifugation as described above. PPP obtained from Precision BioLogic (Dartmouth, Nova Scotia, Canada) was used to adjust the platelet count in each sample to 2.5×10^8 platelets/ml in the 250-μl sample cuvette. Antibodies were added to PRP to a final concentration of 5μg/ml and ristocetin to a final concentration of 1.5μg/ml. PRP aggregation was analyzed in a CHRONO-LOG Model 700 aggregometer. Aggregation was monitored by optical density, which was converted into percentage activity.

Data were collected over 8 minutes with agitation.

Platelet clearance 60 µl whole blood was drawn from hTg mice for platelet counting via Sysmex hematology analyzer, before the mice were injected intraperitoneally (IP) with 2mg/g IVIG (Gammagard 10%, McKesson) or HSA (Sigma)[336]. After 24 hours, platelet counts were measured again, and these values were treated as 100%. Mice were injected retro-orbitally with anti-platelet antibodies (IgG, 6B4, AK2) at 15µg for each animal. Blood was collected from each mouse at 24, 48, 72 and 96 hours post antibody injection, and the platelet count measured and normalized.

Single-molecule force spectroscopy Single-molecule force measurement of pulling MAbs on GPIb-IX was performed as described[55]. Recombinant biotinylated GPIb-IX, and MAb coupled to one end of the DNA handle, were prepared as described. The pulling speed was 200 nm/s, and the spring constant of the optical trap was approx. 0.1 pN/nm. Data were analyzed as described before[55]. Lifetimes were obtained by transforming the histograms of unbinding or unfolding forces[452]. Assuming the histogram has N bins of width $\Delta F = (F_{max} - F_{min})/N$, let the number of counts in the ith bin be c_i, $1 \le i \le N$, then the total counts is $C = \sum_{i=1}^{N} c_i$, resulting in the probability $P(F_i) = c_i/C$, and the density $p(F_i) = c_i/(C \cdot \Delta F)$. Thus, the force-dependent lifetime is: $\tau(F_i) = \dfrac{\left(p(F_i)/2 + \sum_{k=i+1}^{N} p(F_i) \right) \cdot \Delta F}{\dot{F}(F_i) \cdot p(F_i)}$, where $F_i = F_{min} + (i - 1/2)\Delta F$, and the loading rates were calculated with the simple expression $\dot{F} = dF/dt = Kv$, where K is the cantilever's spring constant, and v is the pulling speed[55,453].

Platelet mimetic beads Recombinant human LBD (His1-Arg300) was cloned onto a ReNeo mammalian expression vector[454] with an N-terminal FLAG tag, and C-terminal biotin acceptor peptide sequence[455] and decahistidine tag. The protein was expressed in BHK cells and purified via nickel-NTA affinity followed by anion exchange chromatography (resource Q column, GE Healthcare). Purity was assessed by SDS-PAGE. Avidin-conjugated polystyrene beads (3.0-3.9μm diameter, Spherotech), were incubated with recombinant biotinylated LBD under agitation for 1 hour at 4°C. Beads were isolated via centrifugation (1000g for 10min) and resuspended in working buffer (either Tyrode's buffer or PBS). LBD-conjugated beads were adjusted to $250x10^3$ beads per μl and incubated with 5μg/ml antibody on a plate shaker. After 20 minutes, beads were labeled with APC conjugated goat anti-mouse antibody (Biolegend). Beads were analyzed via flow cytometry as described above.

Statistical analysis Statistical analysis was performed in Graphpad Prism software. Unless otherwise indicated, significance was determined by one-way ANOVA or student's T-test. Flow cytometry data was analyzed using FlowJo, BD FACSDIVA (BD Biosciences), and IDEAS software (Amnis), as indicated. Differences were considered statistically significant when $P < 0.05$.

C. Results

Activation of GPIb-IX by anti-LBD MAbs is epitope-independent but shear-dependent.

A previous report suggested that antibodies targeting an N-terminal portion of the LBD activate GPIb-IX more readily than those targeting other epitopes in the LBD.[285] To this end, we analyzed the ability of several representative anti-LBD MAbs to activate GPIb-IX-V. MAbs

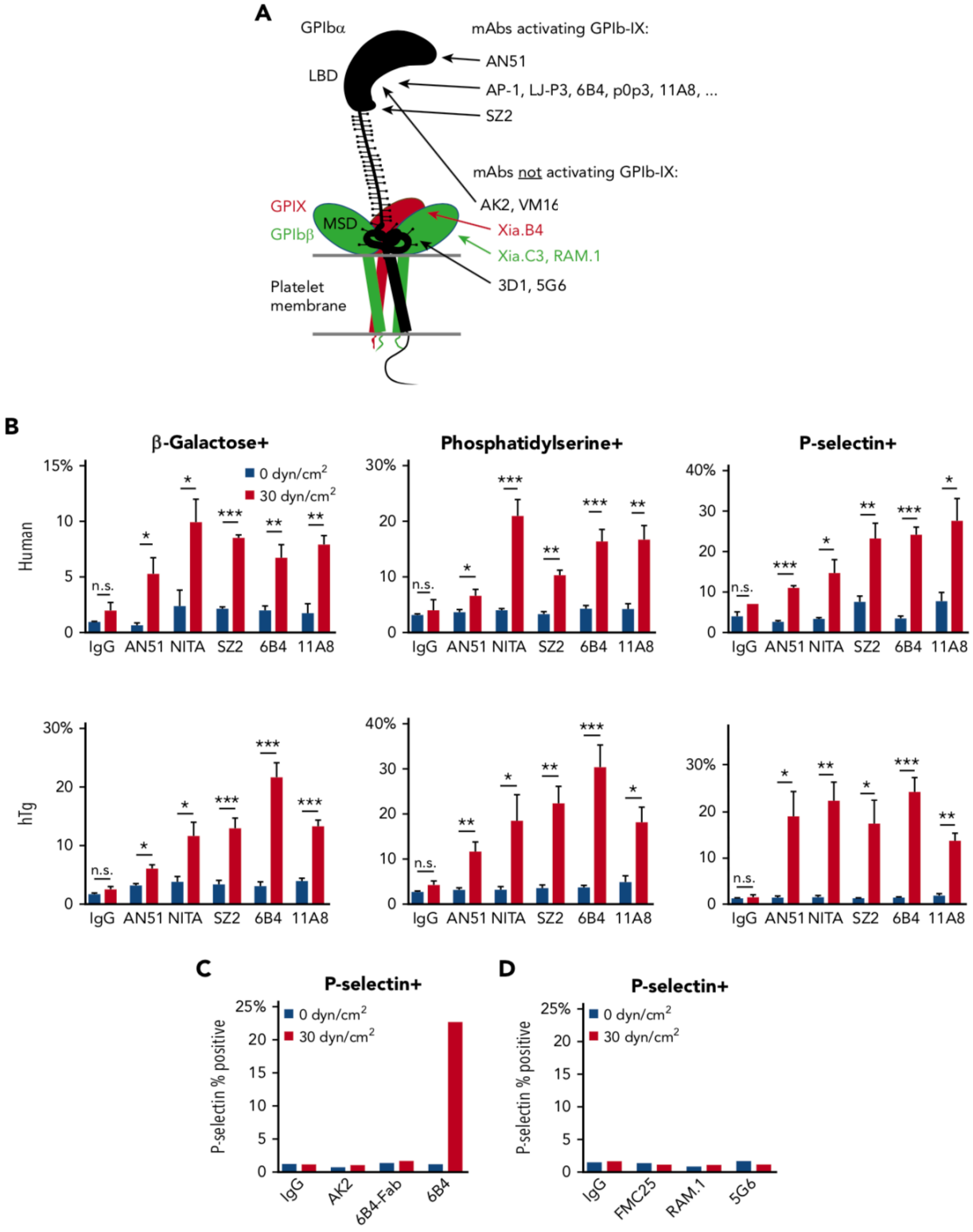
A
GPIbα
LBD
mAbs activating GPIb-IX:
AN51
AP-1, LJ-P3, 6B4, p0p3, 11A8, ...
SZ2
mAbs not activating GPIb-IX:
AK2, VM16
GPIX
GPIbβ
MSD
Xia.B4
Xia.C3, RAM.1
3D1, 5G6
Platelet membrane
B
β-Galactose+
Phosphatidylserine+
P-selectin+
0 dyn/cm2
30 dyn/cm2
Human
hTg
n.s.
IgG AN51 NITA SZ2 6B4 11A8
C
P-selectin+
P-selectin % positive
IgG AK2 6B4-Fab 6B4
D
P-selectin+
P-selectin % positive
IgG FMC25 RAM.1 5G6

Figure III.1. Anti-LBD MAbs, but not others, induce shear-dependent signaling. (A) Illustration of GPIb-IX domains and differential effects of mAbs. Locations of mAb epitopes are noted by arrowheads. (B) Graphs of percentage of positive events for β-galactose, phosphatidylserine, and P-selectin exposure in human (top) and hTg mouse (bottom) PRP treated with control IgG, AN51, NITA, SZ2, 6B4, or 11A8 as indicated under static or sheared conditions. (C) Graph of P-selectin exposure in hTg mouse PRP treated with control IgG, AK2, 6B4-Fab, or 6B4 under static or sheared conditions. (D) Graph of P-selectin exposure in human PRP treated with control IgG, FMC25, RAM.1, or 5G6 under static or sheared conditions. For all graphs, gray bars represent 0 dyn/cm^2 and red bars represent 30 dyn/cm^2. *P≤.05; **P≤.01; ***P≤.001. n.s., not significant.

AN51 and AK2 bind to the N-terminal portion of the LBD, 6B4 to a site in the middle (residues 230-262), and SZ2 to the anionic sulfated tyrosine region in the C-terminal portion of the LBD[456,457]. Although the epitopes of NIT-A[276] and 11A8[458] are not determined, both can inhibit VWF binding[276]. MAb-treated human platelet-rich plasma was exposed to static (0 dyn/cm^2) and uniform arterial (30 dyn/cm^2) shear stress on a cone-plate viscometer. After shear treatment at 22˚C for 5 min, several markers of GPIb-IX activation (surface exposure of β-galactose, phosphatidylserine, and P-selectin) were detected by flow cytometry via FITC-conjugated *Erythrina cristagalli* lectin (ECL), GFP-conjugated lactadherin C2 domain (Lact-C2), and APC-conjugated anti-P-selectin antibody, respectively. GPIb-IX activation was observed by all anti-LBD MAbs tested (Fig. III.1B, top). The effect was shear-dependent, as GPIb-IX signaling was absent in static samples. These experiments were also performed in PRP containing murine hTg platelets, which produced very similar effects (Fig. III.1B, bottom). In agreement with previous reports that bivalency is required for MAb-induced GPIb-IX activation,[244,293] the monovalent 6B4 Fab did not induce signaling (Fig. III.1C). Given the diversity of epitopes for the MAbs tested, these data indicate that the ability of an anti-LBD MAb to activate GPIb-IX is not determined by its precise epitope in the LBD. On the other hand, MAbs FMC25, RAM.1, and 5G6, targeting GPIX, GPIbβ, and the MSD of GPIbα, respectively,[56,350,428] did not induce P-selectin exposure in human platelets under either static or sheared conditions (Fig. III.1D), confirming the difference between them and most anti-LBD MAbs in their abilities to activate GPIb-IX.[428,441]

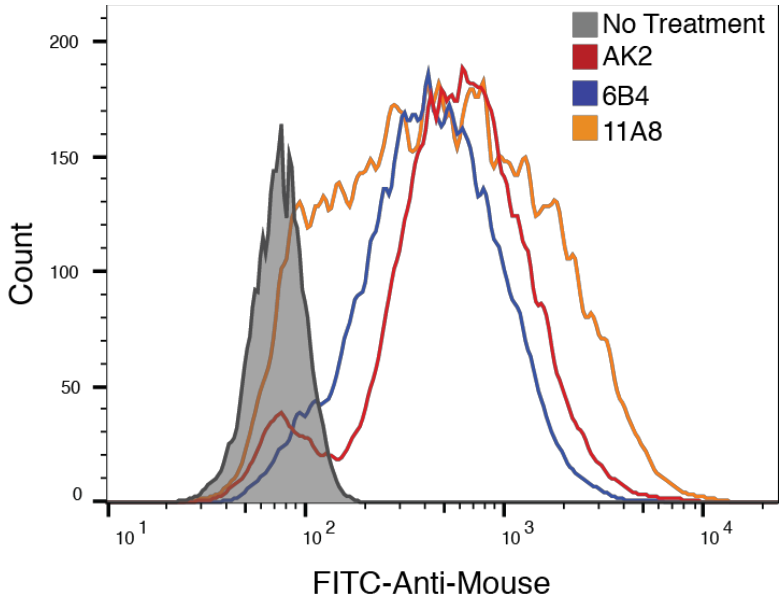

Figure III.2. Anti-LBD antibodies AK2, 6B4, and 11A8 bind to platelets. Binding of mouse anti-LBD antibodies AK2, 6B4, and 11A8 to platelets was detected by a fluorescently labeled anti-mouse Fc antibody. Flow cytometry histograms depicting anti-mouse associated fluorescence for AK2 (red), 6B4 (blue), and 11A8 (orange).

Anti-LBD MAb AK2 neither activates GPIb-IX nor induces IVIG-resistant clearance of platelets.

In our initial screen of anti-LBD MAbs, we found that one, AK2, behaved differently from the others (Fig. III.1C). Additional measurements were carried out to characterize and compare the effects of AK2 with 6B4, a canonical anti-LBD MAb well-documented to induce platelet clearance[293]. Both MAbs bind GPIbα (Fig. III.2) and block VWF binding to GPIbα[293,457,459,460]. In both human and hTg platelets treated with 6B4, expression of all three markers of GPIb signaling increased in a shear-dependent manner, an effect not observed in samples treated with IgG or AK2 (Fig. III.3a). It has been previously observed that many anti-LBD antibodies can clear platelets in an Fc-independent and IVIG-resistant manner[244,336]. To compare the abilities of AK2 and 6B4 to induce platelet clearance, hTg mice were treated with intraperitoneal injection of IVIG or human serum albumin (HSA) 24 hours prior to intravenous injection of either AK2 or 6B4. Platelet counts in these mice were measured prior to MAb injection and over a 4-day period following induction of thrombocytopenia by either MAb. As anticipated[293], 6B4 induced robust and long-lasting thrombocytopenia, and its effect was not ameliorated by IVIG pre-treatment (Fig. III.3b). In contrast, AK2 induced clearance to a lesser extent, which was significantly attenuated by IVIG pre-treatment. Overall, these results demonstrate that not all anti-LBD MAbs can effectively activate GPIb-IX and induce IVIG-resistant platelet clearance.

6B4 but not AK2 induces shear-dependent unfolding of MSD in GPIbα on the platelet.

Given the shear requirements of anti-LBD MAb-induced platelet signaling that was similarly observed for VWF, we next tested whether MAbs induce GPIb-IX activation via unfolding of the MSD. In order to detect MSD unfolding on the platelet surface, MAbs 5G6, which binds a linear epitope in the MSD,[428,461] and WM23, which binds the macroglycopeptide region of

GPIbα, were used. When the MSD is unfolded, 5G6 has greater access to its epitope while WM23 binding remains the same, making the ratio of 5G6 binding to WM23 binding a proxy for the extent of MSD unfolding, as previously established.[56] Human PRP was pre-treated with fluorescently labeled 5G6 or WM23 in the presence of EDTA, an inhibitor of metalloproteinases that cleave GPIbα. Following incubation with 5G6 or WM23, platelets were treated with either 6B4, AK2, or control IgG under static or shear conditions. Under static conditions, neither 6B4 nor AK2 increased 5G6 binding above the baseline established by control IgG. Under shear conditions (30 dyn/cm^2), 6B4 induced a greater than two-fold increase in 5G6 binding while AK2 induced little change (Fig. III.4a-b). WM23 binding did not change between static and sheared samples for all MAbs, indicating that any changes in 5G6 binding were not due to alterations in overall GPIbα surface expression (Fig. III.4b). These results suggest that binding of 6B4, but not AK2, induces shear-dependent unfolding of the MSD on the platelet.

Differential unbinding forces of 6B4 and AK2 underlie disparate abilities to unfold MSD of GPIb-IX. Faced with the differential abilities of 6B4 and AK2 to unfold the MSD in response to shear, we next measured the unbinding forces between MAbs and LBD (*i.e.* the force required to pull a MAb apart from the LBD) and their effects on MSD unfolding by single-molecule force spectroscopy. As described earlier[55], recombinant biotinylated GPIb-IX was immobilized on a streptavidin bead held by a fixed micropipette, and the Fab fragment of MAbs (AK2, 11A8 or 6B4) was coupled to a DNA handle-attached bead that was controlled by an optical laser trap. Under typical conditions with contact force of 2 pN, contact time of 0.1 s, and pulling speed of 200 nm/s, adhesion frequencies of 10-20% were detected between MAb- and GPIb-IX-coupled

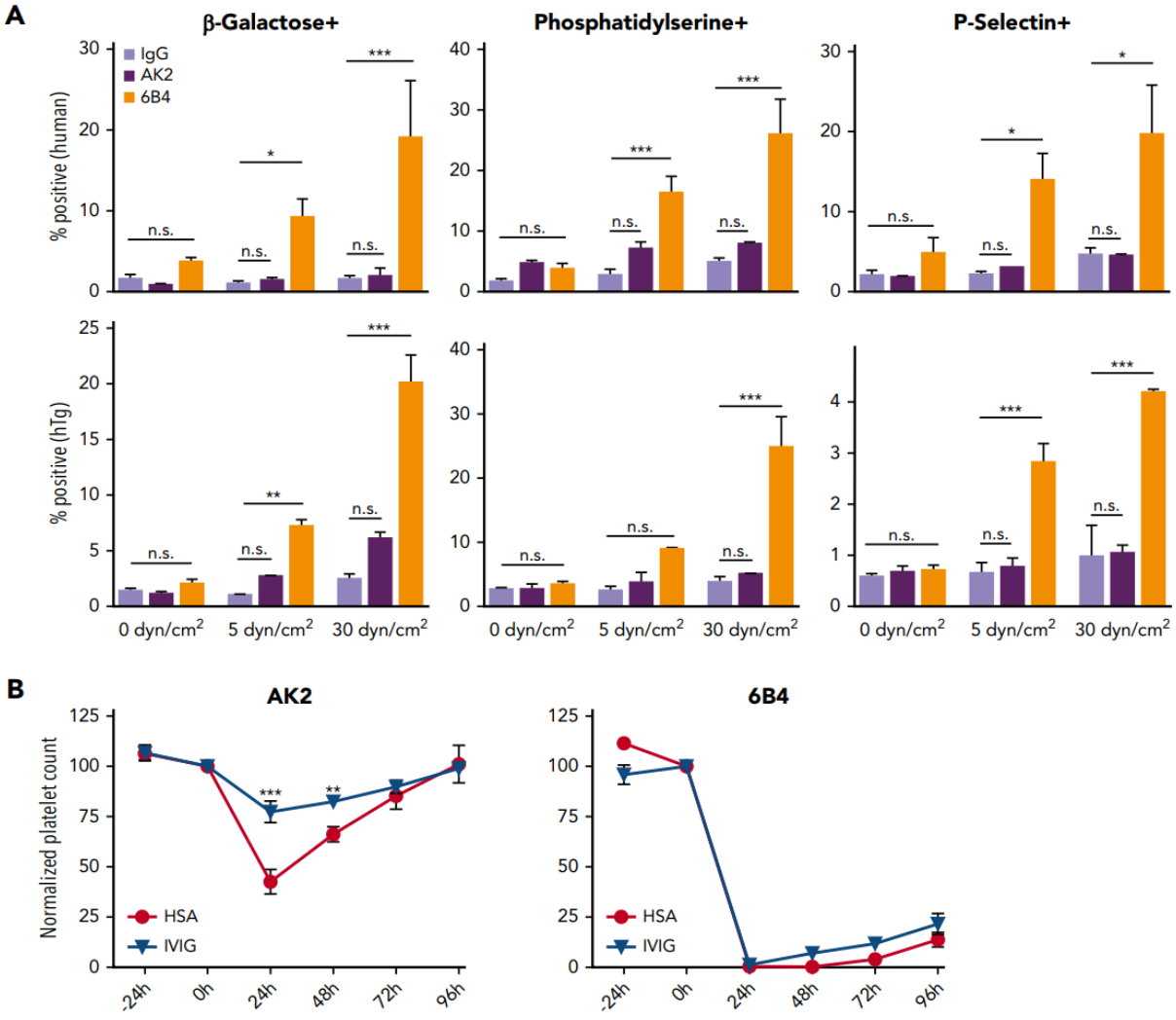

Figure III.3. 6B4, but not AK2, induces shear-dependent platelet signaling and IVIG-resistant platelet clearance in mice. (A) Graphs of percentage of positive events for ECL, Lact-C2, and P-selectin exposure in human (top) and hTg (bottom) PRP treated with control IgG (white), AK2 (gray), or 6B4 (black). Samples were exposed to 0, 5, or 30 dyn/cm² as indicated (n=5). *p≤0.05, **p≤0.01, ***p≤0.001. (B) Platelet survival curves for hTg mice injected retro-orbitally with AK2 or 6B4 24 hours following intra-peritoneal IVIG treatment. Blood was drawn from mice at the time of IVIG administration (-24h), time of antibody injection (0h) and every 24 hours following until 96h post antibody injection. Platelet count determined by CBC analysis. *p≤0.05, **p≤0.01, ***p≤0.001.

beads. In comparison, adhesion frequency was 1% between an uncoupled bead and a GPIb-IX-coupled beads, and undetectable between two uncoupled beads or between a MAb-coupled bead and an uncoupled bead. Under our experimental condition, almost 90% of the observed adhesion events should be mediated by a single-molecule bond between the MAb and the LBD in GPIb-IX[462,463]. Bond lifetimes and force distributions of the MAb-LBD interactions were measured and compared to those of MSD unfolding, obtained from our earlier study[55] (Fig. III.4c-e). Among these, the AK2-LBD interaction has the weakest unbinding force, displaying the shortest lifetime under any given force. The force required for MSD unfolding is slightly stronger than the AK2-LBD bond strength, but much weaker than 11A8-LBD or 6B4-LBD. The 6B4-LBD interaction is the strongest, with 5% of the traces beyond the detection limit of our optical tweezer instrument (about 80-100 pN) as they exhibited DNA overstretching. In comparison, pulling the biotin-streptavidin bond on the same instrument produced DNA overstretching in >80% of the traces.

At a given force, the 6B4-LBD bond exhibits a 5-10-fold greater lifetime than MSD unfolding (Fig. III.4c). Thus, the MSD unfolding event was observed in most pulling traces of 11A8 or 6B4 (Fig. III.4d). In contrast, MSD unfolding was rarely observed when AK2 was used to pull. Together these data indicate that AK2 is much less likely than 6B4 and other anti-LBD MAbs to activate GPIb-IX-V due to its inability to sustain the interaction necessary to exert enough force to unfold the MSD. This is consistent with our observations that AK2 induced much lower and less frequent platelet signaling or clearance of platelets than 6B4.

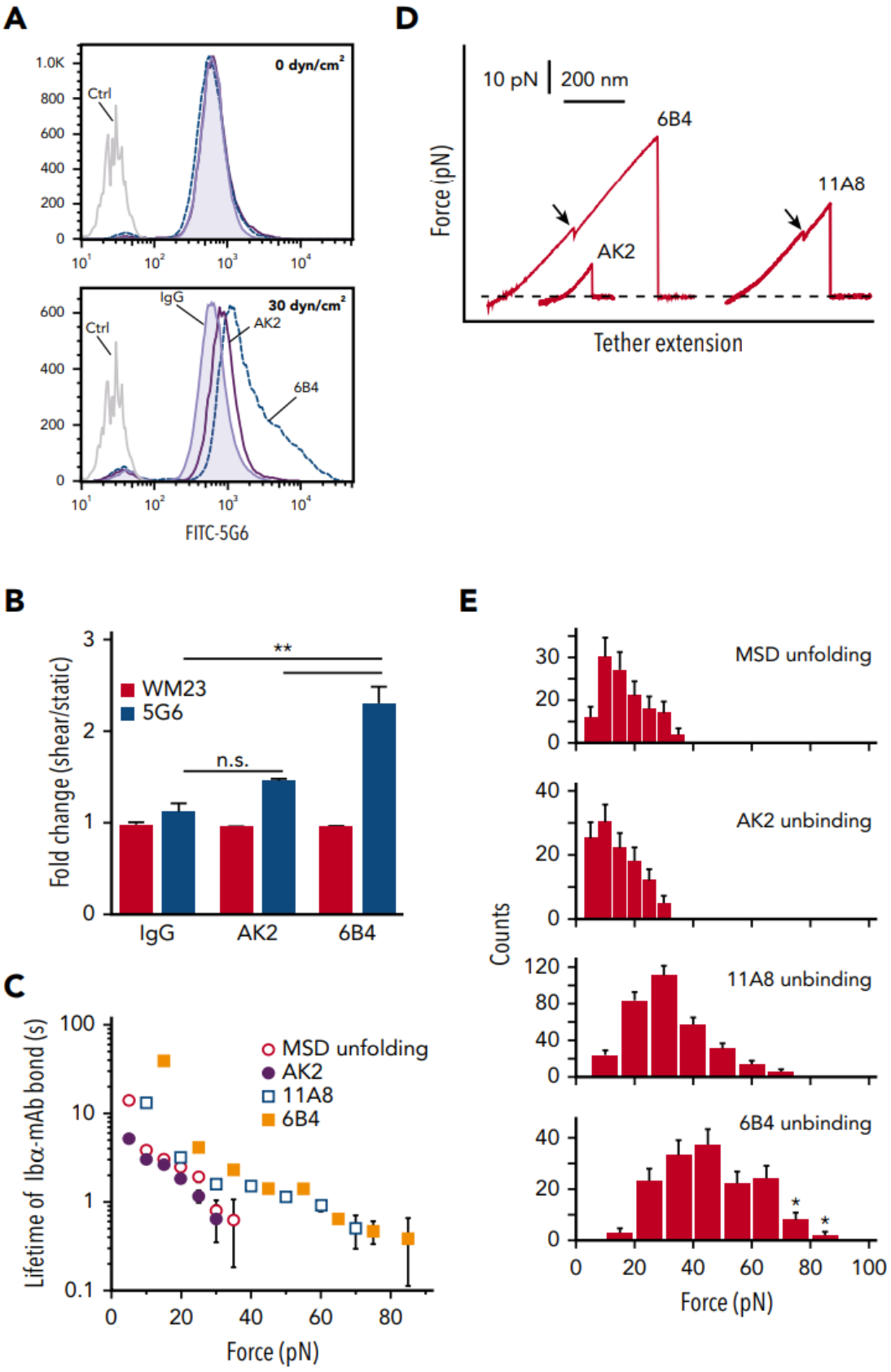
A
0 dyn/cm²
Ctrl
30 dyn/cm²
IgG
AK2
6B4
FITC-5G6
D
10 pN
200 nm
6B4
11A8
AK2
Force (pN)
Tether extension
B
WM23
5G6
**
n.s.
Fold change (shear/static)
IgG
AK2
6B4
C
MSD unfolding
AK2
11A8
6B4
Lifetime of Ibα-mAb bond (s)
Force (pN)
E
MSD unfolding
AK2 unbinding
11A8 unbinding
6B4 unbinding
Counts
Force (pN)

Figure III.4. Platelet signaling is triggered by MSD unfolding. (A) Representative histograms illustrating 5G6 binding in PRP treated with control IgG, 6B4 or AK2 under 0 dyn/cm² (top) and 30 dyn/cm² (bottom) of uniform shear. EDTA was added to prevent GPIbα shedding via metalloproteinases. (B) Graphs of fold increase (sheared/static) in 5G6 (black) and WM23 (gray) binding for PRP treated with IgG, AK2, or 6B4. *p≤0.05, **p≤0.01, ***p≤0.001 (C) Plots of lifetimes of noted bonds as a function of force. Lifetimes were obtained by transforming the histograms of unbinding or unfolding forces, using the method developed by Dudko, et al.[452] (D) Overlaid force-distance traces for pulling AK2, 11A8 or 6B4 on GPIb-IX. (E) Force distributions of MSD unfolding, or unbinding between LBD MAbs and GPIb-IX. Loading rate was ~10 pN/s. Error bars are Poisson noise. Asterisks indicate an underestimation of unbinding force, due to strong bonds that were above the detection limit of our optical tweezers

Anti-sera from a chronic ITP patient produces 6B4-like effects.

To verify whether human antibody-induced effects are similarly shear-dependent to those of murine MAbs such as 6B4, plasma was obtained from 12 chronic ITP patients and the presence of anti-GPIb-IX antibodies was assayed via ELISA. Among these, one patient, patient 11, appeared positive for anti-GPIb-IX antibodies (Fig. III.5a). Washed healthy human platelets were reconstituted in plasma from a healthy donor, ITP patient lacking anti-GPIb-IX Abs (patient 02), or ITP patient with anti-GPIb-IX Abs (patient 11) to a normal human platelet count of 150-450x10^3 platelets per microliter, and exposed to either static (0 dyn/cm^2) or sheared (30 dyn/cm^2) conditions. Subsequent testing for platelet signaling showed that healthy donor or patient 02 plasma did not induce shear-dependent expression of P-selectin or phosphatidylserine. In contrast, plasma from patient 11 significantly increased expression of both markers in a shear-dependent manner (Fig. III.5b). To assess whether ITP patient anti-sera could induce MSD unfolding as 6B4, the remaining reconstituted PRP samples were pre-treated with fluorescently labeled 5G6 or WM23 in the presence of EDTA. In sheared platelets treated with plasma from patient 11, the 5G6 binding increased by ~16 fold with respect to that under static conditions, which is markedly larger than the ~1.3 fold change observed for healthy donor plasma (Fig. III.5c). For comparison, WM23 binding was largely unchanged for both samples. Overall, these results indicate that human anti-sera from chronic ITP patients can produce shear-dependent platelet signaling and induce MSD unfolding in a similar manner to murine MAbs.

6B4, but not AK2, can crosslink platelets under shear.

In order to exert force on platelet GPIb-IX-V under blood flow, VWF can be anchored to the

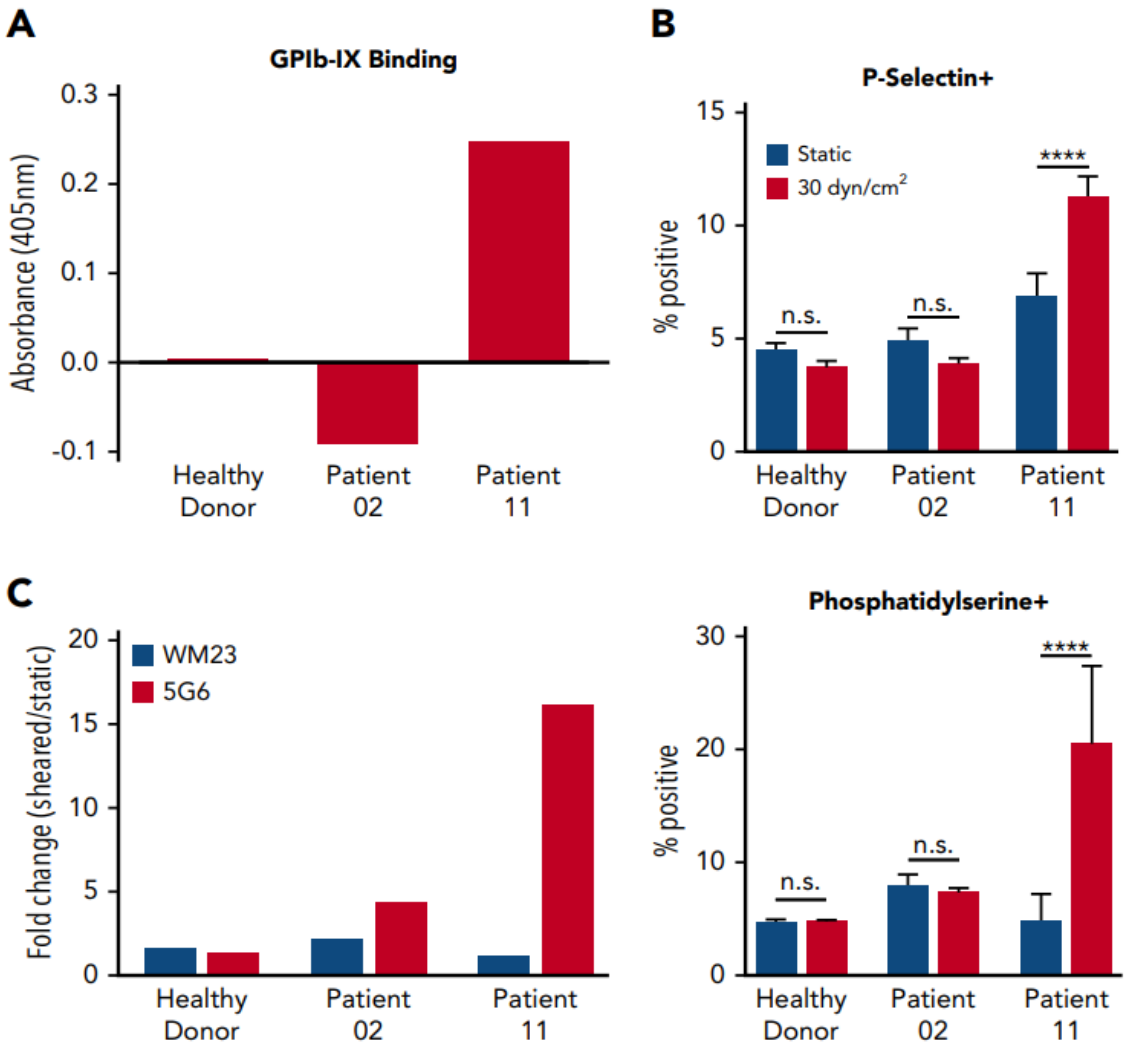

Figure III.5. Sera from chronic ITP patients with anti-GPIb-IX antibodies induce MSD unfolding and shear-dependent platelet signaling. (A) ELISA absorbance values for representative patient samples binding to GPIb-IX. Bars represent the values for plasma binding to purified GPIb-IX+BSA minus the values for BSA binding alone. (B) Graphs of percentage of positive events for P-selectin (top) and phosphatidylserine (bottom) exposure in healthy donor platelets reconstituted in plasma from healthy donors, patient 02, or patient 11 under sheared (black) or static (gray) conditions. (n=4-5) *p≤0.05, **p≤0.01, ***p≤0.001, ****p≤0.0001 (C) Graphs of fold increase (sheared/static) in 5G6 (black) and WM23 (gray) binding to healthy donor platelets reconstituted in plasma from healthy donors, patient 02, or patient 11. Bars represent the mean of duplicate (n=2) values for each condition.

site of injury, but anti-LBD MAbs clearly produce their shear-dependent activity in solution. This raises the question of how anti-LBD MAbs exert a pulling force on GPIb-IX in platelets. Unlike the platelet, which could exert a dragging force upon attachment to the immobilized VWF under shear flow,[51] the size of an anti-LBD MAb is too small to create sufficient "drag" in shear flow. To test the possibility that anti-LBD MAbs crosslink platelets under shear flow, we analyzed the abilities of AK2, 6B4, 6B4 Fab, SZ2, and control IgG to induce platelet aggregation or agglutination by platelet aggregometry. Compared to the full platelet aggregation induced by ristocetin, 6B4 and SZ2 induced a moderate response, leading to platelet agglutination reflected by ~20% aggregation. 6B4 Fab did not induce any observable agglutination (Fig. III.6a), suggesting that the dimeric structure of the antibody is utilized to crosslink platelets. Anti-LBD MAbs may induce platelet agglutination, in part, by activating integrin $\alpha_{IIb}\beta_3$[285]. In order to separate the crosslinking effects of anti-LBD MAbs and integrins, we applied EDTA, a broad inhibitor of integrin binding. The addition of EDTA reduced the extent of anti-LBD MAb induced agglutination (Fig. III.6a), but did not completely block it, confirming that platelet crosslinking is initiated by anti-LBD MAb binding. It should be noted that unlike 6B4 or SZ2, AK2 did not induce any observable agglutination. Platelet crosslinking via anti-LBD MAbs was directly visualized and quantitated via imaging flow cytometry. Human platelets treated with 6B4 or AK2 at various shear levels were labeled with fluorescently labeled anti-$\alpha_{IIb}\beta_3$ antibody. Each fluorescent particle interrogated by the cytometer was imaged and categorized by the aspect ratio, the ratio of the height and width dimensions of the particle. Single platelets have aspect ratios close to 1, whereas clumps of two or more platelets have lower aspect ratios, typically below 0.8[429,430] (Fig. III.6b). Under static conditions, platelets treated with IgG, AK2, or 6B4 contained the same percentage of clumped platelets. When exposed to physiological shear, 6B4 crosslinked a higher percentage of

platelets (Fig. III.6c). This effect is not due to increased mixing, as neither IgG nor AK2 had the effect. Further, we tested whether anti-LBD MAbs could crosslink platelet-sized beads coated with recombinant LBD under shear conditions. By flow cytometry plots, LBD-coated beads incubated with IgG or AK2 produced two major populations where the larger was roughly double the size (represented by forward scatter, FSC) of the smaller (Fig. III.6d). These two populations likely represent single beads and two beads stuck together, a phenomenon which occurs at a certain frequency regardless of treatment. In addition to these two populations, incubation with 6B4 produced a third major population of beads with even larger size, which represents more highly crosslinked clumps of beads (Fig. III.6d). Overall, these results suggest that through its binding to the LBD 6B4 crosslinks significantly more platelets under shear than AK2 does. When crosslinking the platelets, 6B4 must use each of its two Fab fragments to engage a copy of GPIb-IX-V on opposing platelets (Fig. III.7). In this scenario, a platelet is of sufficient size to generate drag on a linked platelet, thus allowing exertion of tensile force on GPIb-IX-V and subsequent unfolding of the MSD. It is conceivable that due to the relatively weak AK2-LBD unbinding force, AK2-crosslinked platelets or bead complexes are not stable under shear. On the other hand, 6B4 and other anti-LBD antibodies with a strong unbinding force to the LBD can sustain crosslinking of platelets under shear in the bloodstream, allowing tensile force to be exerted on GPIb-IX-V and leading to GPIb-IX-V-mediated signaling (Fig. III.7).

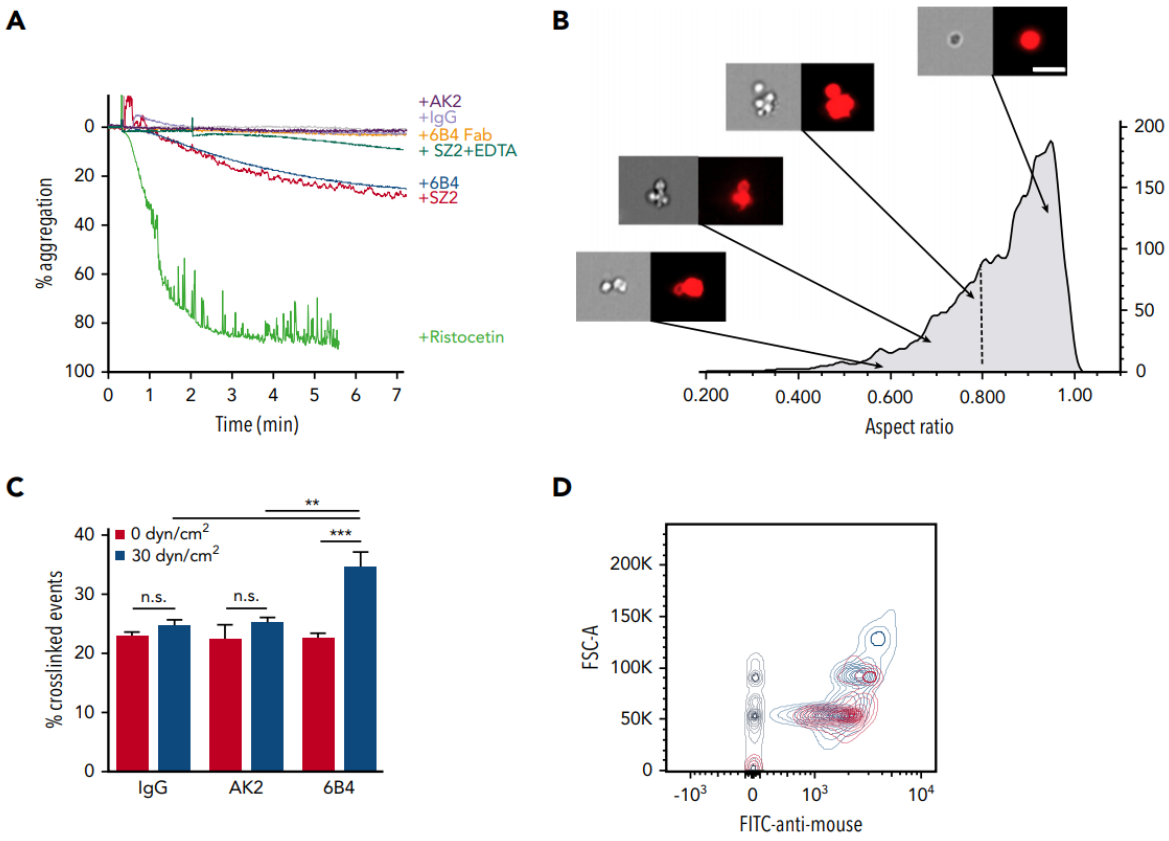

Figure III.6. MAbs induce platelet signaling via crosslinking platelets. (A) Percentage aggregation of human PRP treated with AK2 (purple trace), IgG (black trace), 6B4 Fab (orange trace), SZ2+EDTA (dark green trace), 6B4 (blue trace), SZ2 (red trace), or ristocetin (light green trace), determined via aggregometry (B) Representative images of platelets (right: bright field, left: anti-CD41) in PRP treated with 6B4 at low shear. Images obtained via imaging flow cytometry. Arrows indicate the aspect ratio to which each set of images corresponds. Dotted line demarcates the gate used to identify crosslinked events. Scale bar = 10μm. (C) Frequency of crosslinked events (events with aspect ratio <0.8) in PRP treated with IgG, AK2, or 6B4 at static (white bars) or sheared (black bars) conditions. (n=3) (D) Contour plots of forward scatter (FSC-A) vs anti-mouse antibody fluorescence for platelet mimetic beads treated with AK2 (red), 6B4 (blue), and IgG (gray) under sheared conditions. Contour lines = 5%

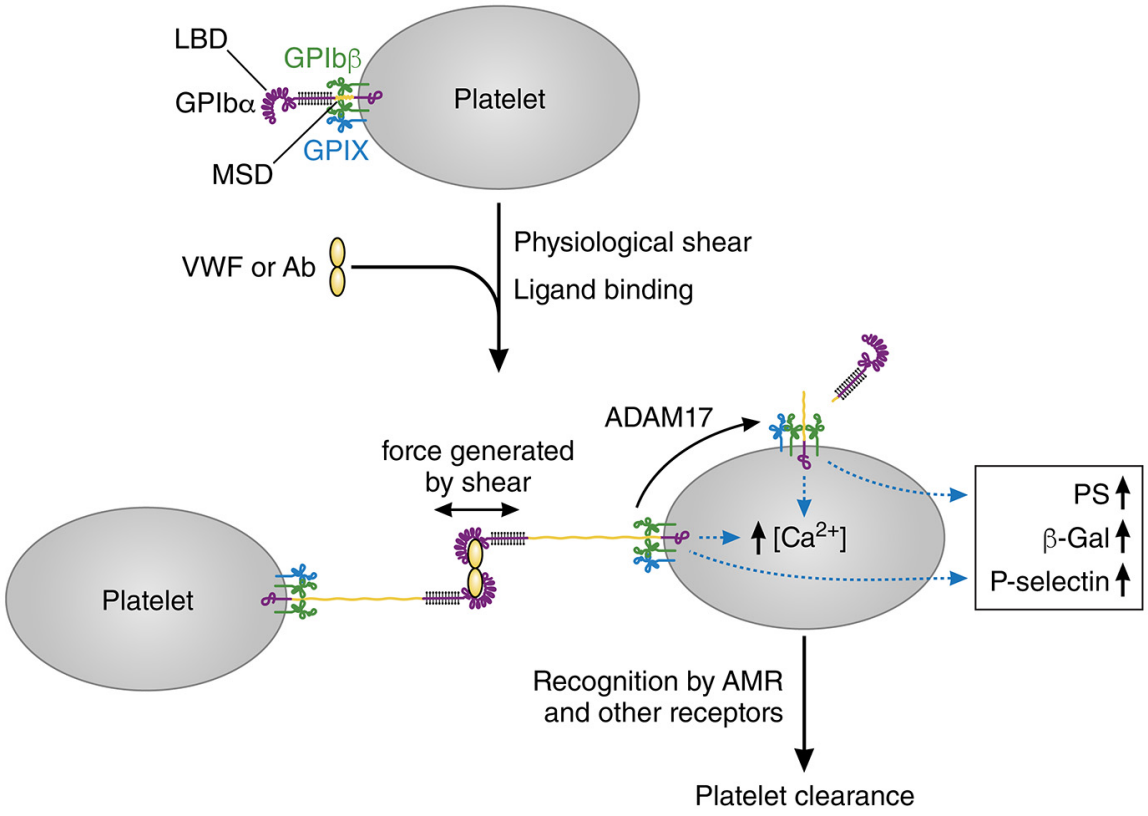

Figure III.7. A model of GPIb-IX-V activation via crosslinking by antibodies against the LBD. In this model, a soluble, multimeric ligand such as plasma VWF or an anti-LBD antibody binds to two copies of GPIb-IX on opposing platelets, thereby crosslinking them. Under physiological shear, the crosslinking can generate a pulling force on GPIbα and induce unfolding of the MSD therein. This induces platelet signaling as illustrated, including desialylation (the exposure of β-galactose), leading to rapid clearance of platelets. The ability to crosslink platelets under shear depends on a sufficiently high unbinding force between the antibody and its epitope in the LBD. Antibodies with low unbinding force to the LBD, like AK2, cannot effectively crosslink platelets and thus cannot exert a shear force to unfold the MSD of GPIb-IX-V.

D. Living in the World Today: A Discussion of Results at Hand and Future Directions

In this chapter, we provide new evidence that shear is required for anti-LBD antibody-induced GPIb-IX-V signaling. Following this critical observation, the binding and functional properties of anti-LBD MAbs, particularly those of AK2 and 6B4, were characterized. While 6B4 and AK2 are similar in their high-affinity binding to the LBD, they differ significantly in their abilities to activate GPIb-IX-V and induce IVIG-resistant clearance of platelets (Figs. III.1 and III.2). Further, we demonstrate for the first time that their difference in function is correlated with a difference in their unbinding force for the LBD, and consequently in the ability to sustain platelet crosslinking under shear and induce MSD unfolding (Figs. III.4 and III.6). These results suggest a new mechanomolecular mechanism for IVIG-resistance in immune thrombocytopenia dependent on Fc-independent platelet clearance induced by anti-LBD MAbs. In this mechanism, anti-LBD antibodies crosslink platelets through binding of both Fab domains to copies of GPIbα on opposing platelets and generate a pulling force on GPIb-IX-V. For most anti-LBD antibodies, their unbinding force for the LBD is sufficiently strong to sustain platelet crosslinking, induce unfolding of the MSD under shear, and subsequently activate GPIb-IX-V and result in rapid platelet clearance (Fig. III.7).

While numerous studies have confirmed GPIb-IX-V as the endogenous VWF receptor and characterized its structure, the mechanism by which antibodies and ligands activate the complex is less well defined. An early electron microscopy study indicated that GPIb-IX-V is uniformly distributed on the surface of the resting platelet, and it undergoes receptor clustering in platelets activated by ristocetin/VWF and thrombin[464]. Further work suggested that clustering of GPIb-IX-V leads to its migration or partition into platelet glycosphingolipid-enriched microdomains and promotes activation of GPIb-IX-V[444,445]. These data led to a

"clustering model" for the mechanism of GPIb-IX-V activation[465]. Regarding antibody-induced GPIb-IX-V signaling, the clustering model could explain the requirement of bivalency (which is required if an antibody is to laterally dimerize the receptor), and the lack of a specific activating epitope within the LBD. However, it could not adequately account for the lack of activation by certain anti-LBD (*i.e.* AK2), anti-GPIbβ, anti-GPIX-V, and anti-MSD antibodies, which should also be capable of lateral dimerization[292,441-443]. In comparison, the platelet-crosslinking model we propose here is the first model to fully explain the four observations about anti-GPIb-IX-V antibodies, in addition to the shear requirement of anti-LBD antibody-induced signaling (Fig. III.7). First, the dimeric structure of an antibody, but not the Fc region, is needed to crosslink platelets, which explains the requirement of bivalency and the feature of Fc-independence[244]. Second, the location of the binding epitope is not the defining feature of an activating anti-LBD antibody, which explains why these antibodies can have non-overlapping epitopes. Third, the defining feature is instead an unbinding force for the LBD that is sufficiently large to induce MSD unfolding, which explains why certain anti-LBD antibodies such as AK2 are not as effective in activating GPIb-IX-V as 6B4 (Fig. III.4). Fourth, shear is required to generate a pulling force through an anti-LBD antibody on GPIb-IX-V and to induce unfolding of the MSD. Lastly, compared to anti-LBD antibodies, antibodies targeting the other portions of GPIb-IX-V are not positioned to exert a pulling force on the MSD, which explains why these antibodies generally do not activate GPIb-IX-V.

Overall, we provide evidence that the bond strength or the force resistance of an antibody to the LBD of GPIb-IX-V, rather than the location of its binding epitope in the LBD, is the determinant of whether the antibody induces GPIb-IX-V signaling and thrombocytopenia. The mechanical strength of antibody-antigen interactions is used by the immune system in

order to identify high-affinity antibodies in B-cells[466] and as a feature of T-cell signaling through T-cell receptor (TCR)[467]. However, this study is likely the first example of the mechanical features of an antibody being the defining pathological feature in disease. Our results provide insight to the mechanism of IVIG-resistance in ITP and inform potential new diagnostic and therapeutic approaches. One such prognostic assay for response to immunosuppressive treatment would involve platelet-mimetic beads like the ones coated by recombinant LBD used in Figure III.6. Based on what we have shown in this chapter, in order to induce immune independent platelet clearance, MAbs need to bind to the LBD (or at least above the MSD in GPIbα) and have a strong enough unbinding force to unfold the MSD via crosslinking. Thus, an assay for these types of antibodies must take both factors into account. The LBD-coated platelet mimetic beads are the same size as a platelet, and only coated with the LBD, excluding antibodies against other subunits of GPIb-IX-V. Further, we have demonstrated that under shear, 6B4 sustains more crosslinking of the beads than AK2. This indicates that the beads can discriminate between antibodies which cause IVIG resistant ITP and those which don't. Therefore, combining patient serum with these beads should provide a quick flow cytometric readout of whether or not their blood contains strong-binding anti-LBD antibodies, and therefore their likelihood to respond to immunosuppression.

CHAPTER IV

RETHINKING LIGANDS OF GPIb-IX-V

IV. Rethinking Ligands of GPIb-IX-V

A. Come Together: Divalent Ligands for GPIb-IX-V

As established in the previous chapter, anti-LBD antibodies can crosslink platelets via binding of two GPIbα subunits on opposing platelets. Provided that the unbinding force of this interaction is sufficiently high, this will activate GPIb-IX-V in a shear-dependent manner (under flow, for example). Likewise, when plasma VWF spontaneously associates with the LBD (as is the case with type 2B VWD, ristocetin, botrocetin, etc.) its multimeric chains make it possible to bind to multiple copies of GPIbα. Multimeric VWF linked to several platelets at a time could allow them to exert force on one another in blood flow. This paradigm neatly explains the thrombocytopenia observed in all these circumstances, as platelet desialylation and subsequent clearance are both downstream of GPIb-IX-V activation. Although crosslinking of platelets by spontaneously associated VWF has not been demonstrated in as much detail as the crosslinking via MAbs demonstrated in chapter III, many observations suggest that it may occur. Recall from section I.E that type 2B VWD patients present with accelerated platelet clearance/thrombocytopenia, and that patient plasma (containing mutant VWF) can induce shear-dependent desialylation and intracellular calcium spikes[56,367,368].

Type 2B VWD is an autosomal dominant disorder, meaning that only one copy of the gene needs to bear the mutation in order to cause a disease phenotype. The crosslinking hypothesis offers a theoretical explanation for this observation, given that only some of a patient's total VWF would need to spontaneously bind to and crosslink platelets in order to induce clearance. This paradigm parallels some of what we see in the IVIG resistant ITP described in chapter III. Although ITP is not a genetic disorder and therefore wouldn't be classified as dominant or recessive, ITP patients have autoantibodies targeting many platelet surface

receptors[468], but just one clone with an epitope in the LBD is sufficient to induce robust Fc-independent clearance as we see with monoclonal antibodies (Fig. III.3). Further, transgenic mice expressing VWF with type 2B VWD mutations exhibit thrombocytopenia, decreased platelet lifespan, and large VWF-platelet aggregates in the liver[284]. Together, these data reveal a potential set of core requirements for the phenomenon of GPIb-IX-V activation by soluble ligands: a divalent (or multivalent) ligand that binds GPIbα's LBD and can sustain a pulling force high enough to unfold the MSD (~15 pN[55,354]).

B. (F)X(I) Gon' Give it to Ya: The Curious Case of FXI and GPIb-IX-V

In the early 2000s, several studies indicated that coagulation Factor XI (FXI), bound to platelets via an interaction with GPIb-IX-V[469]. This interaction with GPIb-IX-V can be competed off with the VWF A1 domain[344] (which binds to the LBD), indicating that the binding site for FXI was also in the LBD or adjacent anionic stretch. FXI is the zymogen of FXIa, which activates FIX in the intrinsic pathway (Fig. I.2). Although FXI deficiency causes relatively mild bleeding[470], it has recently gained renewed interest due to studies indicating that FXI is instrumental in thrombosis[471] and ischemia-reperfusion injuries[109,472]. FXI is and obligate homodimer that circulates in a complex with its non-enzymatic cofactor, high molecular weight kininogen (HK)[473-476]. It has also been reported that GPIb-IX-V binds to HK[477]. Together, these observations appear to implicate FXI as a potential crosslinking, activating ligand for GPIb-IX-V, warranting further investigation of the FXI/HK-GPIb-IX-V interaction. However, it should be noted that a series of retractions and errata on studies regarding the interaction of FXI and GPIb-IX-V from the Walsh group around the same time period cast a reasonable amount of doubt on some of these findings[478-480]. Specifically, many.

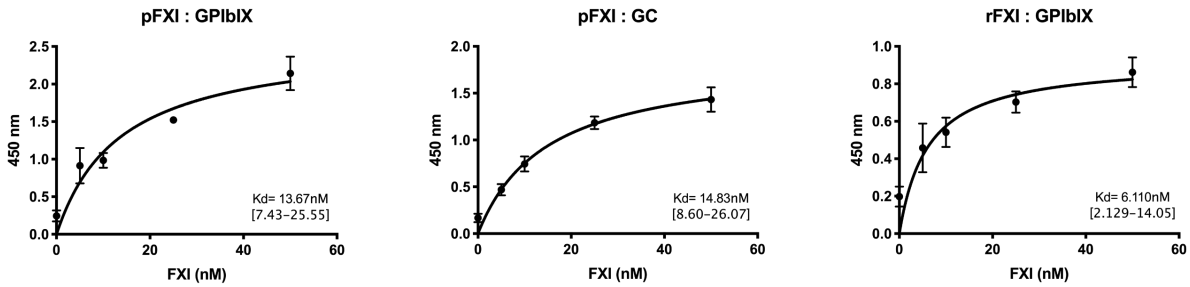

Figure IV.1. Coagulation factor XI binds GPIbα with high affinity. Aggregate ELISA data and nonlinear fits for the binding of plasma FXI (pFXI) to immobilized GPIb-IX complex, pFXI to glycocalicin (GC), or recombinant FXI expressed in CHO cells (courtesy of Dr. David Gailani) to immobilized GPIb-IX complex. Experiments performed in triplicate; brackets denote 95% CI. All R squared values≥0.80.

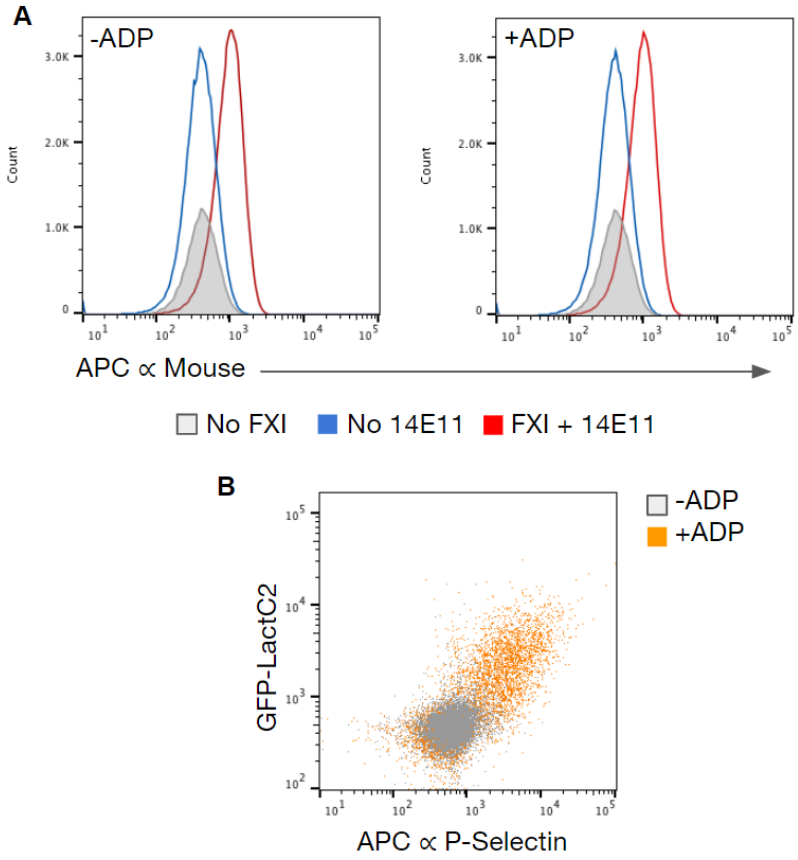

Figure IV.2. FXI binding to platelets is not altered by activation state. (A) FXI binding to platelets under resting (left) or ADP stimulated (right) conditions. Histograms of mean fluorescence intensity for 14E11 binding to FXI on platelets. 14E11 was detected by an APC conjugated anti-mouse secondary antibody. (B) Dot plot graphing P-selectin exposure against phosphatidylserine exposure (detected by GFP-Lact-C2). Events in the upper right quadrant represent activated platelets expressing both markers.

of these corrections concerned the disputed observation that activated platelets had higher affinity for FXI than non-activated

We confirm the interaction of GPIb-IX and plasma FXI (pFXI) (obtained commercially) via ELISA, establishing a Kd of 13.6 nM, in good agreement with previous reports[481] (Fig. IV.1). pFXI also had similar affinity for glycocalicin, the extracellular domain of GPIbα, indicating that the binding site is likely within the GPIbα subunit (Fig. IV.1). This is consistent with reports that VWF competes with FXI for GPIbα. When obtaining pFXI commercially, we cannot totally rule out the presence of some HK contamination, given that FXI always circulates with HK in the plasma. Thus, it may be difficult to confirm that the observed interaction is entirely due to the FXI-GPIb-IX-V interaction without the participation of HK. Courtesy of Dr. David Gailani, we obtained recombinant human FXI (rFXI) generated from CHO cells. Given that this sample was prepared entirely outside of the plasma, there is no concern of HK contamination.

We then demonstrated that rFXI binds to GPIb-IX with high affinity, better even than the pFXI (Kd = 6.1 nM) (Fig. IV.1), indicating that either our pFXI is not contaminated, or HK does not alter the binding significantly (only an ~2-fold change in Kd). Previous studies also indicate the clear requirement of μM Zn^{2+} for low nM binding[481,482], but on the contrary, our binding buffers contained no added zinc. It should, at this point, be noted that some of the studies that showed this requirement or their supporting studies have been retracted. So far we can confirm the claim that FXI binds to GPIb-IX-V, and contradict the dependence on zinc and/or HK.

To assess the importance of platelet activation on FXI binding, we treated PRP with 8 μM ADP for 10 min to induce activation. Following this, we incubated 25 nM FXI in PRP for 5 min before using 14E11, an anti-FXI antibody to detect FXI binding to platelets. There appears to be little difference in platelet association with FXI between ADP-treated and resting platelets (Fig. IV.2a), despite ADP inducing robust activation in PRP (Fig. IV.2b) as measured by P-selectin and phosphatidylserine exposure. These data confirm that platelet activation state does not influence FXI binding. Further investigation is required in order to determine whether dimeric FXI's unbinding force from GPIb-IX-V is sufficient to crosslink platelets and activate GPIb-IX-V under shear.

C. Rules of Engagement: A MAb Against the Sialomucin Region Activates GPIb-IX-V

So far, our unofficial rules for a crosslinking ligand of shear-induced GPIb-IX-V activation have been: a multivalent ligand, with high unbinding force, that associates with the LBD of GPIbα. However, harkening back to our useful gas-powered lawn mower pull cord metaphor, we may be able to call into question one of the assumptions of these rules, specifically the assumption that a ligand must target the LBD, and not any other portion of GPIbα. Of course, this assumption is not without experimental precedent. In chapter III we demonstrated that a host of MAbs targeting the LBD at diverse epitopes all induced shear-dependent GPIb-IX-V activation, while MAbs targeting GPIX, GPIbβ, or the MSD in GPIbα did not (Fig. III.1). This is either because steric hinderance prevents antibodies against those regions of the complex from crosslinking (recall that the long sialomucin region raises the LBD as much as 40 nm above the platelet surface, increasing accessibility), or more likely, because applying force to these regions does not directly pull on the MSD. Likewise, if you pull on the lawn mower by its wheels, or blades, or even by the coiled-up cord, you are not likely to "activate" the engine.

However, in both our tongue-in-cheek metaphor and within the trigger model of GPIb-IX-V activation, the ligand theoretically only needs to bind GPIbα somewhere above the MSD in order to exert a pulling force. WM23 is an anti-GPIbα antibody with an epitope in the sialomucin region, between the MSD and LBD[483]. WM23 has been extensively characterized in the literature[389,484,485]. Most pertinently, the biophysical properties of its interaction with GPIbα have been studied. In optical tweezer experiments, the WM23-GPIbα bond withstood several cycles of stretching and relaxing without WM23 becoming detached, indicating a high unbinding force[354]. Thus, WM23 fulfills most of the requirements for crosslinking and activating GPIb-IX-V. If specific binding to the LBD is not a requirement, we might expect to see WM23 induce shear-dependent GPIb-IX-V signaling much in the same way as MAbs targeting the LBD. To that end, human PRP was incubated with control IgG, 6B4, WM23, or AK2 and sheared at 20 dyn/cm^2 on a cone-plate viscometer over a time course of 0-300 seconds. After shear treatment at 22˚C, platelets were fixed and surface expression of phosphatidylserine and P-selectin was detected via flow cytometry with GFP-Lact-C2 and APC-conjugated anti-P-selectin antibody, respectively. Compared to AK2, WM23 generated higher expression of both P-selectin and phosphatidylserine. The response in platelets incubated with WM23 increased over time, as opposed to AK2-treated samples or control IgG. On the other hand, 6B4 induced activation in more platelets over a shorter period of shearing than all other antibodies tested (Fig. IV.3).

These data indicate that WM23 can behave like anti-LBD antibodies by inducing shear-dependent signaling. Whether this signaling is explicitly due to crosslinking via bivalent WM23 remains to be seen. Nevertheless, the data alone are valuable in their proof of the

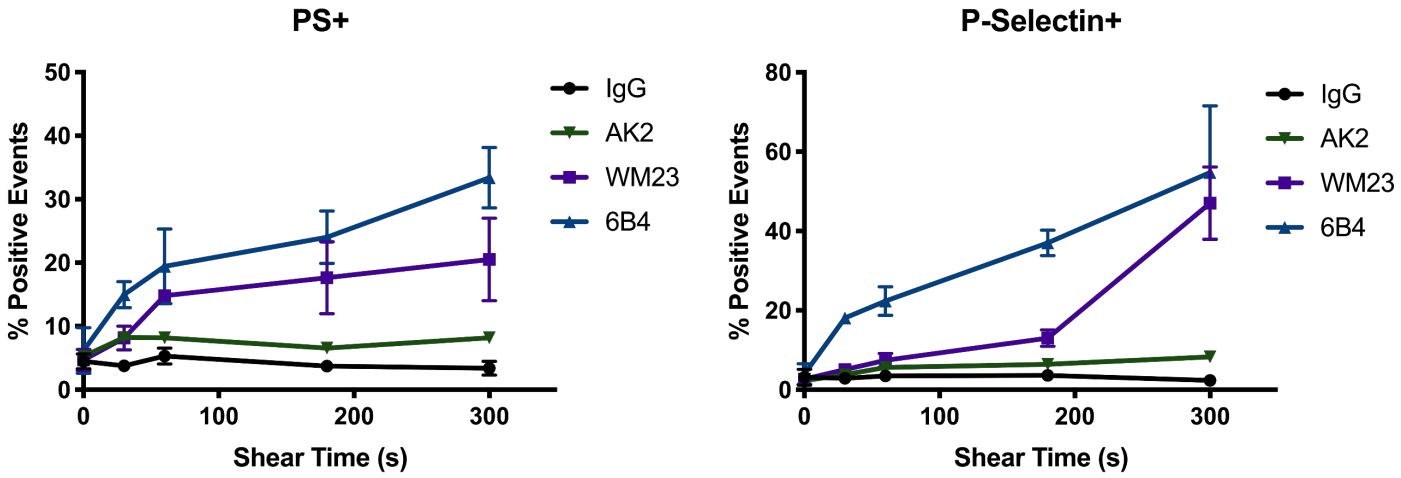

Figure IV.3. WM23 activates platelets in a shear-dependent manner. Time course of shear exposure in PRP incubated with control IgG, AK2, WM23, or 6B4. Shown is the percentage of events positive for either phosphatidylserine (PS) or P-Selectin exposure, based on a negative control sample without secondary antibody.

principle that any divalent ligand with a strong unbinding force which binds GPIbα above the MSD should be able to activate GPIb-IX-V.

D. Hold On (We're Going Home): Prototype of a Crosslinking Ligand

A crosslinking hypothesis details how anti-LBD MAbs can hold on to two copies of GPIbα on opposing platelets, and activate GPIb-IX-V by exploiting force generated by blood flow as these platelets tumble through solution (Fig III.7). In figure III.7, I depict one antibody (or other multivalent ligand) crosslinking two platelets in solution, and the subsequent unfolding of the MSD in two opposing copies of GPIbα. This is a simplification for illustrative purposes. In reality, given that GPIb-IX-V is a highly expressed surface receptor with ~30,000 copies per platelet[486,487], it is likely that the region of platelet membrane that comes into contact with another platelet actually contains a high density of GPIb-IX-V complexes. Presuming that the concentration of antibody in the blood is high enough, this means that two platelets that are crosslinked in the manner described above would not be held together by just one crosslinking event, but rather by a zipper-like association of many copies of GPIb-IX-V bound together by ligand. In other words, although one single event must be first, avidity may assist in more robust crosslinking and the activation of several GPIb-IX-V complexes. Not only could two platelets be extensively crosslinked via GPIb-IX-V, but in the case of a multivalent ligand like VWF containing a type 2B VWD mutation, platelets bind one each to the multiple linked VWF monomers in a multimer, forming the aggregates seen in mice modeling type 2B VWD mutations[284]. These caveats or modifications to our mental image of platelet crosslinking expand our concept of how crosslinking via GPIbα occurs.

Coagulation factor XI is a dimeric zymogen that has been reported to compete with VWF for GPIbα, implying that FXI binds to the GPIbα's LBD. While the LBD has many endogenous binding partners (reviewed in section I.E), the dimeric nature of FXI likens it to spontaneously associating VWF or bivalent anti-LBD MAbs. Unfortunately, the literature surrounding the claims of FXI binding to platelets is riddled with retractions. In this chapter, I present data bringing some clarity to the question. I demonstrate that FXI binds to GPIb-IX-V and isolated GPIbα extracellular domain (glycocalicin) with low nanomolar affinity. This binding was also observed with recombinant FXI. Together these data indicate that FXI does bind to GPIbα, and that FXI's endogenous binding partner, HK (which also is reported to bind GPIbα[477]), is not necessary for the association (Fig. IV.1). Therefore, FXI is an intriguing candidate for another crosslinking ligand to GPIbα. Previous studies also indicated that zinc was necessary for strong binding, and that platelet activation by ADP enhanced FXI binding to GPIb-IX-V[480]. The data presented here stand in contradiction to those claims, as our buffers did not contain zinc, and activated platelets did not bind more FXI than resting (Fig. IV.2). Due to the uncertain nature of the previous literature, it was necessary to re-establish the conditions of binding and whether or not binding occurs at all. Further investigation will determine whether FXI can crosslink platelets, and whether it can sustain a bond long enough for shear to unfold the MSD. Pushed to speculate, one might expect that FXI does not naturally interact with GPIb-IX-V with a strong, shear-resistant bond, given that crosslinking could lead to significant platelet clearance, and FXI is not reported to regulate platelet lifespan or clearance.

Up to this point, I have only presented data wherein anti-LBD antibodies with strong unbinding forces activate GPIb-IX-V. However, it is theoretically possible for an antibody targeting a non-LBD epitope to exert a pulling force on GPIbα's MSD, provided that the

epitope for this antibody is between the MSD and the end of the LBD. WM23 is a monoclonal antibody targeting the sialomucin region of GPIbα. In figure IV.3, I demonstrate that WM23 is capable of inducing shear-dependent signaling largely in the same fashion and over the same time scale as 6B4, an anti-LBD antibody. These data increase our confidence that the ability to unfold the MSD is the key factor in activating GPIb-IX-V. The effects predicted by the trigger model are reflected in this experimental setting. They also show that, while the LBD provides a convenient "handle" to pull on, it is not necessary that ligands target the LBD and there is likely no specific conformational change that need be induced in the LBD in order to activate GPIb-IX-V. From this we can broaden our expectation for ligands of GPIb-IX-V to include those that bind in the anionic stretch below the LBD, the sialomucin region, and perhaps even portions of the MSD.

Crosslinking of GPIb-IX-V complexes on opposing platelets under shear leads to receptor activation and subsequent clearance. By borrowing insights from ligands to GPIb-IX-V in disease states that may reflect this relationship (like MAbs in IVIG-resistant ITP and VWF in type 2B VWD), we can determine some core characteristics of ligands that activate GPIb-IX-V in solution. In this chapter I propose that FXI may fulfill the requirements of a platelet crosslinking ligand to GPIbα. I also demonstrate that a previously held assumption, that only ligands binding the LBD could activate GPIb-IX-V, is not supported by data showing activation of GPIb-IX-V by WM23.

CHAPTER V

ANTI-GPIbβ ANTIBODIES REGULATE ACTIVATION OF THE PLATELET GPIb-IX-V COMPLEX

V. Anti-GPIbβ antibodies regulate activation of the platelet GPIb-IX-V complex

A. Dancing in the Dark: Pulling Back the Veil on GPIbβ's Contribution to GPIb-IX-V Activation

As discussed in section I.A, platelets serve a vital role in primary hemostasis as first responders at the site of injury. Platelets' initial recognition of endothelial damage proceeds through the GPIb-IX-V complex. GPIb-IX-V is not only important for initiating adhesion to collagen via its interaction with immobilized VWF, but also for its intracellular signaling, which initiates several events in platelet activation. Upon VWF binding under shear, the MSD unfolds, exposing the trigger sequence and leading to activation. Across the membrane, GPIb-IX-V activation induces an intracellular signaling cascade that leads to inside-out activation of $\alpha_{IIb}\beta_3$[373-375], spikes in intracellular calcium[375,381-383], release of platelet agonists from granules[378,379,387], etc. (GPIb-IX-V signaling is reviewed in more detail in section I.E). However, despite the establishment of MSD unfolding as the key mechanosensitive step in GPIb-IX-V activation and a growing understanding of various intracellular mediators of signaling, there is still a "black box" surrounding the steps in the physical transduction of the signal through GPIb-IX-V.

While it is established that expression of GPIbα, GPIbβ, and GPIX are required for robust surface trafficking of the complex[349,350], there remains a significant knowledge gap regarding the possible contributions of GPIbβ, GPV and GPIX to signaling. Although it is possible that GPIbβ and GPIX serve solely to stabilize the GPIb-IX-V complex, they also likely have roles in receptor signaling. Some reports utilizing mutational analyses and/or antibodies targeting the GPIbβ subunit suggest that it participates in GPIb-IX-V signaling and platelet activity[441,488]. However, it is difficult to disentangle these functional effects from the importance of GPIbβ to

organization of the complex. Each copy of GPIbβ in a GPIb-IX-V complex makes different contacts with the extracellular domain of GPIX, and also potentially with the MSD of GPIbα[360]. It has been demonstrated GPIbβ's extracellular domain can undergo conformational changes based in response to changes in its inter-subunit contacts[355,360]. While no binding sites for endogenous ligands have been identified on the extracellular domains of GPIbβ or GPIX, in the early 2000s a novel MAb against the murine GPIbβ extracellular domain, RAM.1, was developed. RAM.1 was reported to alter GPIb-IX-V's affinity for VWF under flow[489], although the specific study examined adhesion of cells transfected with the complex, rather than the protein-protein interaction itself. Later work using GPIb-IX reconstituted in phospholipid bilayer nanodisks demonstrated that RAM.1 did not modify VWF binding[490]. Nevertheless, although RAM.1 appears not to influence GPIb-IX-V binding, it does appear to modulate the receptor's signaling. Chinese hamster ovary (CHO) cells expressing human GPIb-IX (CHO-Ib-IX) undergo morphological changes and extend filopodia when the receptor is activated[491]. While RAM.1 doesn't affect the adhesion of these cells to VWF, it does significantly inhibit filopodia formation. In addition to preventing morphological changes, RAM.1 inhibits the intracellular Ca^{2+} spikes characteristic of GPIb-IX-V activation and blocks collagen-induced thrombin generation and thrombus formation[441].

In this study, we report a novel anti-GPIbβ antibody, 3G6. In the presence of GPIb-IX-V activating antibodies, 3G6 dramatically increases platelet activation. We also further characterize the ability of RAM.1 to inhibit GPIb-IX-V signaling. Our results are the first evidence of an amplifying or "potentiating" modulator of GPIb-IX-V. These data strongly suggest that a change in orientation or conformation in the GPIbβ extracellular domain underlies force-induced activation of GPIb-IX-V.

B. Methods

Materials: Sources for FITC-conjugated ECL, RAM.1, 6B4, AK2, and WM23 are described in chapter III. MAb 3G6 was obtained courtesy of Dr. Bernhard Nieswandt. CHO cells expressing human GPIb-IX have been previously described[56].

Uniform Shear Assay: The uniform shear assay and subsequent flow cytometry analyses were performed much as described in chapters II and III. For this study, instead of adding one agonist, occasionally both RAM.1/3G6 and another antibody were combined, as indicated. Unlike anti-LBD antibodies, which are routinely incubated at 5 μg/ml, RAM.1 and 3G6 were added at 10 μg/ml, owing to twice the number of epitopes being present as with antibodies against other subunits.

Microscopy and Filopodia Assay: Glass cover slips were coated with ligand of interest at 2-4 μg/ml in PBS overnight at 4°C. Coverslips were blocked with 1% bovine serum albumin at 22°C for at least 1 hour. CHO cell expression of GPIb-IX was maintained by growth in selection media every other passage, and surface GPIb-IX levels were checked every 10 passages. CHO-Ib-IX cells were pelleted and resuspended in modified Tyrodes buffer (134 mM NaCl, 0.34 mM Na2HPO4, 2.9 mM KCl, 1 mM MgCl2, 5 mM glucose, 12 mM NaHCO3, 20 mM HEPES, pH 7.35) containing 5mM EDTA. Cells were then incubated on coverslips for 25 minutes at 37°C. Nonadherent cells were washed away with PBS. Cells were fixed for 10 minutes in 4% PFA, permeabilized with 0.1% Triton X-100 for 15 minutes, and stained with 1 μg/ml TRITC-Phalloidin (Thermo-Fisher) for 30 minutes. Images were collected on an Olympus FluoView FV1000 confocal microscope.

Software and Statistics: Unless otherwise indicated, statistical analyses were performed in Graphpad Prism, Microsoft Excel, and Fiji (ImageJ). Significance determined by one-way ANOVA or student's T-test. Image analysis performed in Fiji (ImageJ). Flow cytometry data was analyzed with FlowJo. Differences considered statistically significant when $P \leq 0.05$.

C. Results

RAM.1 and 3G6 bind the extracellular domain of GPIbβ

3G6 is a previously unpublished rat anti-human MAb raised against GPIbβ provided courtesy of the Nieswandt group at the Rudolf Virchow Center at University of Wurzburg. In order to confirm both RAM.1 and 3G6's binding to GPIbβ, recombinant GPIbβ extracellular domain (GPIbβE) was run on SDS-PAGE under reducing and nonreducing conditions and transferred to a nitrocellulose membrane. GPIbβE appears as a neat band at ~20 kDa. While both RAM.1 and 3G6 were able to blot for the extracellular domain, binding was abolished under reducing conditions (Fig. V.1a), suggesting that the epitopes of both antibodies rely on disulfide bond-mediated structures within the protein. In addition to western blot analysis, several ELISAs were done to determine the apparent Kd of RAM.1 and 3G6's interaction with GPIbβE as well as GPIb-IX. Both 3G6 and RAM.1 bound to immobilized GPIbβE with low nanomolar affinity (3G6 = 20.34 nM; RAM.1 = 11.54 nM) (Fig. V.1b). Interestingly, affinity for the full GPIb-IX complex was higher by an order of magnitude for both MAbs. In order to preclude any possibility of a second binding site in GPIbα, glycocalicin was immobilized on the plate and challenged with 11A8 (an anti-LBD MAb) or 3G6, both at 20 nM. As expected, 3G6 had no affinity for the extracellular domain of GPIbα alone (Fig. V.1c). Together, these data suggest a conformational epitope for both RAM.1 and 3G6. It is possible that the increase in affinity for the full GPIb-IX complex over GPIbβE is due solely to avidity (once a MAb binds one copy of

GPIbβ, GPIb-IX has another copy immediately available). It may also be the case that the epitope for either of these MAbs involves stretches where two subunits meet, or preferentially bind to GPIbβ in a conformation it is held in by the other members of the complex.

RAM.1 and 3G6 have functional effects on shear-dependent signaling

RAM.1's documented effects on filopodia formation in CHO cells expressing GPIb-IX along with inhibiting other markers of GPIb-IX-V activation[441], and unpublished reports that 3G6 also had an effect on filopodia formation indicated that both of these anti-GPIbβ antibodies showed promise in dispelling some of the darkness surrounding GPIb-IX-V signal transduction. The functional effects of RAM.1 and 3G6 were therefore assessed in the uniform shear assay. The assay was performed as described extensively in chapter III, and in this case, we also added samples with just RAM.1, just 3G6, or with either of them as a modulator of another established ligand. PRP incubated with the indicated MAb or MAbs was sheared for 5 minutes at increasing rates from static conditions up through 30 dyn/cm^2. P-selectin and β-galactose were used as readouts of activation.

As established, 6B4 responded to increased shear with higher and higher percentages of total platelets expressing P-selectin or exposing β-galactose on their glycans. This effect was all but abolished by co-incubation with RAM.1 (Fig. V.2a). Both RAM.1 and 3G6 alone had little to no effect. Curiously, when co-incubated with 3G6, 6B4 generated a much more robust platelet activation phenotype, essentially doubling percentage of events positive for both readouts of activation (Fig. V.2a). In chapter III we demonstrated that AK2's unbinding force from the LBD was very low, often lower than the force required to unfold the MSD for any period.

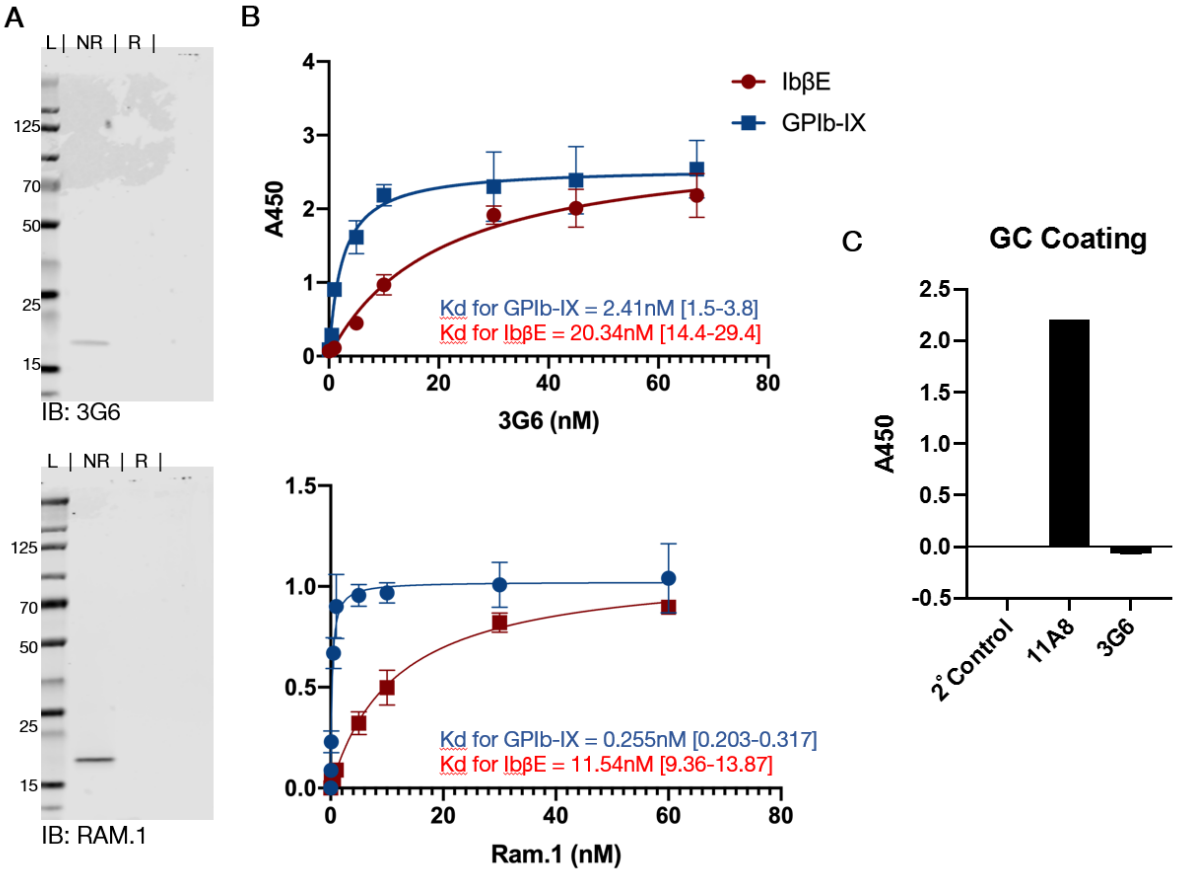

Figure V.1. Both Ram.1 and 3G6 bind GPIbβ with high affinity. (A)Western blot analysis of purified human GPIbβ extracellular domain blotted with 3G6 (top) or RAM.1 (bottom), run under non-reducing (NR) or reducing (R) conditions. L; Ladder. (B) ELISA with immobilized GPIb-IX or GPIbβE. Brackets are 95% CI. (C) ELISA with immobilized glycocalicin (GC) challenged with 20 nM 11A8 or 3G6.

However, if 3G6 can "potentiate" 6B4's shear-dependent signaling, then even the meager 5-8% positive events generated by AK2 at its peak may be amplified by 3G6 as well. Indeed, when 3G6 was added to AK2 and exposed to shear, it increased the percentage of platelets expressing P-selectin over three-fold and doubled the percentage of events positive for both readouts (Fig. V.2b).

Like VWF, anti-LBD MAbs can activate GPIb-IX in CHO cell filopodia assay

One of the primary observations regarding RAM.1's ability to inhibit GPIb-IX signaling came in the context of CHO cells expressing human GPIb-IX. In these cells, activating ligands to GPIb-IX trigger morphological changes including filopodia formation and spreading of the CHO cell membrane when the cells are seeded on a surface coated with ligand. So far, this assay has been employed with VWF coated surfaces, exploiting the snake venom botrocetin, which forces spontaneous interaction of VWF and GPIb-IX[56,441]. Theoretically, this should be possible without a serpentine middleman if instead the ligand coating the surface was an anti-LBD MAb. Using MAbs removes many concerns about other interactors with VWF on CHO cells or platelets and it can be used to determine the force-sensitivity of interactions with anchored ligands. If filopodia formation is downstream of GPIb-IX activation, and activation must proceed through MSD unfolding, then there must be some MAbs that are better than others at inducing the phenotype. Glass slides were coated with AK2, WM23, or 6B4 overnight.

As we know, WM23's unbinding force from GPIbα is particularly high, and our pilot experiments in chapter IV indicate that WM23 is capable of shear-dependent activation of

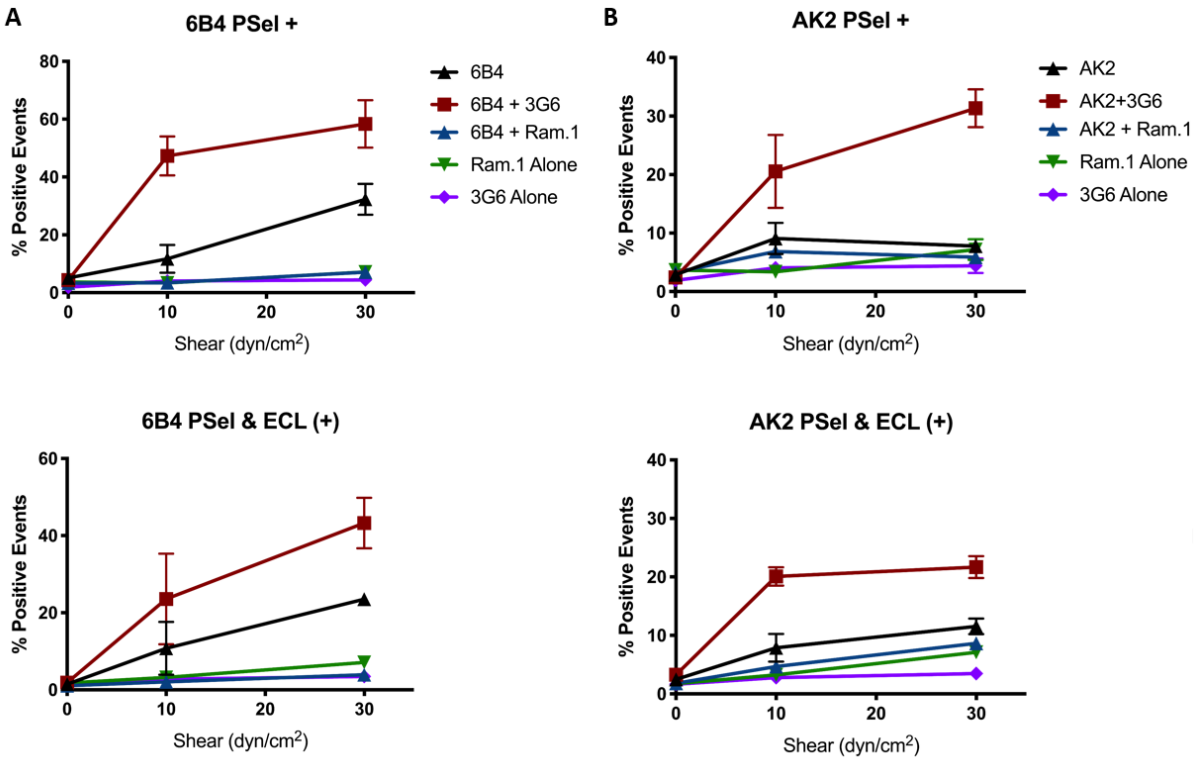

Figure V.2. RAM.1 inhibits and 3G6 potentiates shear-dependent GPIb-IX-V signaling. (A) Graphs of percentage positive events for P-selectin exposure or double exposure of P-selectin and β-galactose in PRP treated with 6B4, 6B4+3G6, 6B4+RAM.1, Ram.1, or 3G6. Shear stress ranges from 0 dyn/cm² (static conditions) to 30 dyn/cm². (B) Graphs of percentage positive events when PRP was incubated instead with AK2, AK2+3G6, AK2+Ram.1, Ram.1, or 3G6. Percentage positive events based on a gate drawn around a no secondary MAb control.

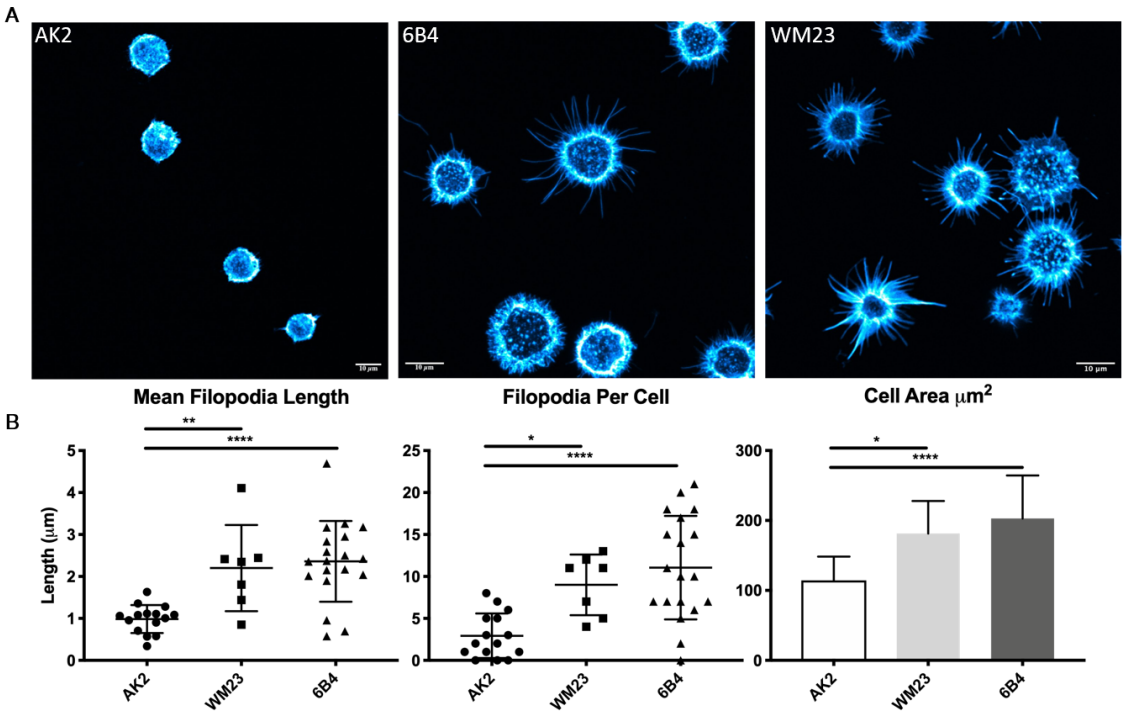

Figure V.3. Ligand induction of filopodia formation in CHO-Ib-IX cells depends on unbinding force. CHO cells expressing human GPIb-IX were seeded on surfaces coated with AK2, 6B4, or WM23, then fixed and stained with TRITC-phalloidin. (A) Representative images for each coating condition. (B) Quantitation of mean filopodia length, # of filopodia ≥1.5 μm per cell, and cell area.

GPIb-IX-V. When CHO-Ib-IX cells were seeded on the indicated surfaces, both WM23 and 6B4 induced robust filopodia formation, while AK2 generated notably less filopodia and filopodia of shorter length (Fig. V3). Another interesting feature of the MAbs with strong binding was that the cells seeded on those surfaces (WM23 and 6B4) tended to be larger. This is likely due in part to the early spreading phenotype the CHO cells adopt. The images generated in this assay were analyzed by a semi-automated macro in Fiji/ImageJ. The text of this macro is included as an appendix. Briefly, the macro identifies a cell body, subtracts it from the image, thresholds the filopodia, then counts and measures them. In this way, the effect of ligands and modulators on filopodia formation can be more precisely quantified.

RAM.1 inhibits filopodia formation, 3G6's effects mixed

Now that it has been established that anti-LBD MAbs can activate GPIb-IX on transfected CHO cells, triggering filopodia formation, the next question brings us back to where we began our line of inquiry. Can we use this assay to further characterize the potentiating effect of 3G6 and the inhibitory effect of RAM.1 with respect to GPIb-IX-V signaling? Coverslips were coated with AK2, 6B4, and 6B4 Fab, along with either RAM.1 or 3G6. CHO-Ib-IX cells were seeded on these surfaces and filopodia were quantified. Compared to AK2 alone, 3G6 generated a modest increase in the length and number of filopodia generated by AK2 as well as the mean cell area (Fig. V.4). Compared to the 2-3 fold increases in percentage of activated platelets seen in the uniform shear assay, these results are more subdued. RAM.1 successfully inhibited filopodia formation and cell area growth generated by all MAbs (Fig. V.4).

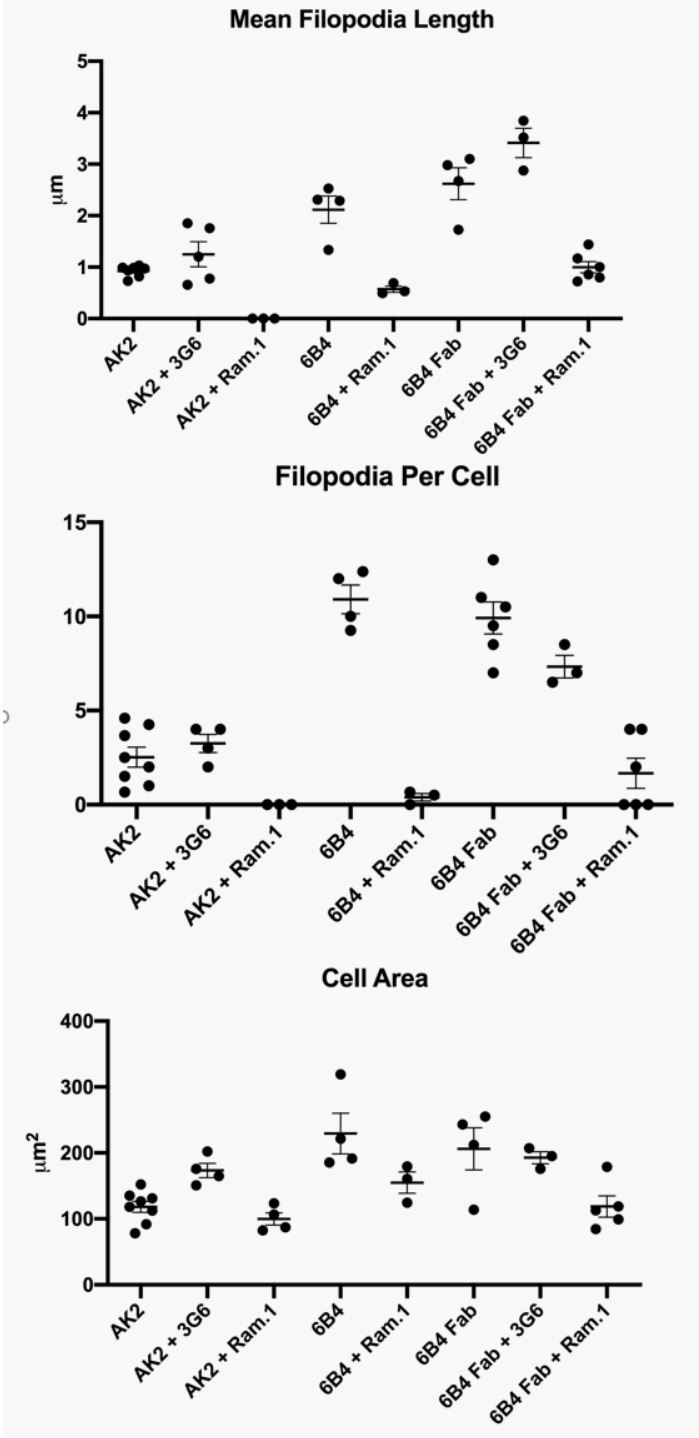

Figure V.4. Ram.1 inhibits filopodia formation. Effect of 3G6 unclear. Surfaces were coated with AK2, AK2+3G6, AK2+RAM.1, 6B4, 6B4 + RAM.1, 6B4 Fab, 6B4 Fab +3G6, and 6B4 Fab + RAM.1. Each data point represents an experimental replicate. Within each replicate, at least 3 regions of the surface were imaged and at least 20 cells were quantified by the macro. In cases where there are two antibodies listed, both were coated onto the coverslip during the same incubation.

Interestingly, 6B4 Fab was able to induce filopodia formation and cell area expansion on par with the effect of full-length 6B4. This underscores the difference in mechanism from in-solution multimeric ligands which crosslink platelets, and anchored ligands. Under the Trigger model, so long as the unbinding force of the ligand is strong enough to sustain the interaction while the MSD unfolds, signaling can occur. On the other hand, this ability of an anchored Fab to activate GPIb-IX is difficult for a clustering model to explain mechanistically.

The disconnect between 3G6's effect on GPIb-IX-V signaling in platelets under laminar flow (Fig. V.2) is initially difficult to square. It is possible that having both 3G6 and an activating antibody coating a surface, rather than having 3G6 in solution, prevents it from properly accessing its binding epitope. It may also be the case that there are some other CHO-specific factors at play, or that anchored ligands "pull" on GPIb-IX-V differently than when in solution.

D. Discussion

Portions of this section adapted from my contributions to Zhang et al. 2019[492]

The key step in GPIb-IX-V signaling is the unfolding of the membrane proximal, quasi-stable MSD. However, despite new evidence supporting this model of GPIb-IX-V activation continuing to develop, the link between MSD unfolding and intracellular signaling is not yet understood. How is it that the physical unfolding of the MSD results in the effects we observe? GPIbβ's extracellular domain is proximal to the MSD, and when the MSD unfolds, the trigger sequence within the MSD is still estimated to be able to contact GPIbβ[355,493]. In studies with chimeric GPIbβ and GPIX extracellular domains, GPIbβ adopts different conformations based on its interactions with different portions of the GPIX extracellular domain[360], and it is not difficult to imagine that some similar changes occur based on contact with the MSD.

In this chapter, I demonstrate that RAM.1, an antibody against the extracellular domain of GPIbβ (Fig. V.1) can inhibit GPIb-IX-V signaling induced by anchored ligands, in partial confirmation of some prior reports. I also demonstrate that the unbinding force-dependent activity of anti-LBD MAbs observed in chapter III is recapitulated when these MAbs are anchored (Fig. V.3). I go on to show that 3G6, a previously unpublished antibody that also targets the GPIbβ extracellular domain has an opposite effect on GPIb-IX-V signaling induced by in-solution anti-LBD MAbs. When added to the activating antibodies 6B4 (Fig V.2) or 11A8 (not shown), 3G6 amplifies the activation of the receptor, as evidenced by an almost 100% increase in the percent of platelets expressing P-Selectin and β-galactose on their surface. On the other hand, while RAM.1's effects on crosslinking-mediated activation of GPIb-IX-V was recapitulated in CHO-cell experiments with anchored ligands to the LBD (Fig. V.3 and V.4), 3G6's effect was rather small, not nearly on the same scale as the effect seen in platelet flow cytometry in figure V.2. Nevertheless, the identification of a second anti-GPIbβ MAb with modulatory effects on GPIb-IX-V's mechanotransduction presents exciting new evidence that may elevate GPIbβ from a glorified molecular chaperone to a participant in platelet mechanosensation.

One attractive mechanism for 3G6's potentiation of antibody-induced signaling is that the MAb may interfere with MSD re-folding, sterically or allosterically. While the biophysical properties of MSD unfolding and the link between unfolding and GPIb-IX-V activation are becoming clearer[55,56], refolding of the MSD, along with the spatio-temporal events in transduction of the signal, is less understood. From experiments done in CHO cells expressing GPIb-IX, we know that signaling through GPIb-IX (at least in CHO cells) is fairly localized,

given that filopodia form only in the regions of the cell where GPIb-IX is in contact with the surface coated ligand[56,491]. When we picture platelets "rolling" over a collagen-VWF matrix, we can imagine that as they roll, GPIb-IX-V all over the platelet is being iteratively activated. Thus, it likely requires a not insignificant "critical mass" of receptors to be activated before a platelet undergoes the full range of what we expect from GPIb-IX-V signaling, which is already not full platelet activation without the engagement of other platelet surface receptors! It is unclear, however, whether GPIb-IX-V signaling is reversible, and whether a sustained amount of shear is required to unfold this critical mass of GPIb-IX-V complexes.

As we recall from chapter III, the monoclonal antibody 5G6 specifically recognizes a linear epitope in the MSD. As shown in figure III.4, while 5G6 binds to GPIb-IX-V on the surface of resting platelets, its binding is significantly increased when platelets are stimulated by GPIbα ligands under shear stress, and the MSD is more extended. Like 5G6, the Fab fragment of 5G6 exhibits higher binding when the MSD is unfolded, and has overall better sensitivity, likely due to its smaller size. An increase in 5G6 Fab binding, as detected by flow cytometry, was observed when it was premixed with platelets before shear stress was applied (Fig. V.5a). Interestingly, when 5G6 Fab was added to platelets shortly (~10 sec) after the shear stress treatment, its binding only marginally increased compared to that with no shear treatment and was significantly less than that of the premixed sample (Fig. V.5a). This observation suggests that the MSD may refold once shear stress is removed.

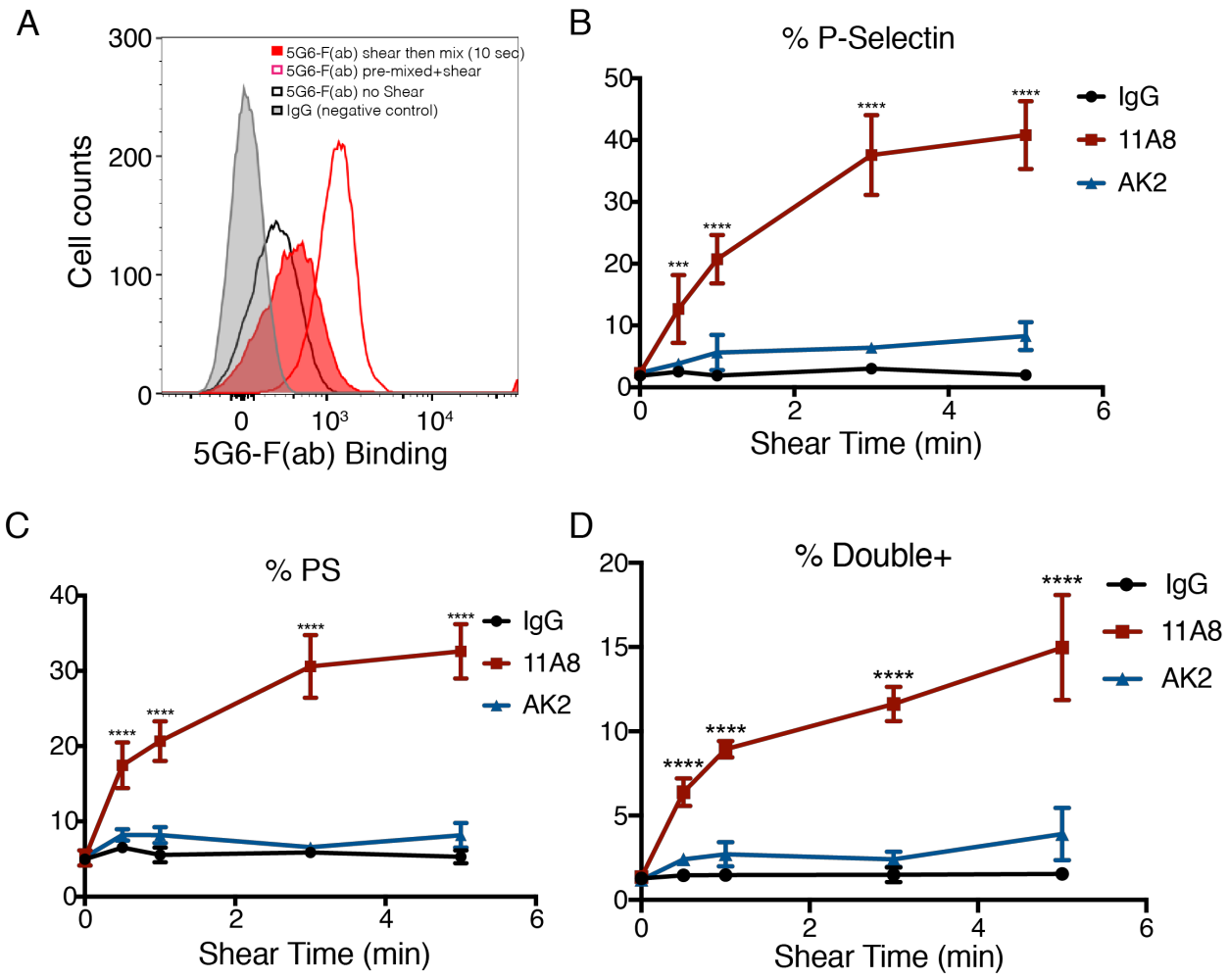

Figure V.5 Platelet activation increases with shear time. (A) Flow cytometry histograms comparing the binding of 5G6 Fab to platelets under the indicated conditions, in the presence of botrocetin. (B-D) Shown is the percentage of platelets positive for (B) P-selectin exposure, (C) phosphatidylserine (PS) exposure, or (D) both on human platelets treated with control IgG, 11A8, or AK2 under 20 dyn/cm2 of shear force at the indicated time points. n = 3; significance determined by one-way analysis of variance with Tukey test for multiple comparisons. *p < 0.05; **p < 0.01;***p < 0.001; ****p < 0.0001.

11A8, an antibody with an average unbinding force of ~30 pN is sufficient to sustain the 15 pN of force required to unfold the MSD, unlike AK2, with an unbinding force <15 pN. Here, the two antibodies of differing unbinding forces were incubated with PRP under shear. When incubated with 11A8, platelet activation increased with shear duration, an effect not observed with AK2, which did not trigger activation at any shear duration tested. The extent of platelet activation, as measured by surface expression of P-selectin and phosphatidylserine (PS), after brief shear exposure of ~30 sec was markedly less than after longer exposures of >3 min (67% and 45% less for P-selectin and PS, respectively) (Fig. V.5b-d). Increasing shear time to 5 minutes did not lead to significant increase of activation signals. These results indicate that activation of GPIb-IX-V was dependent on the duration of shear exposure. They are consistent with a recent observation in which pulling on the LBD via engaged VWF for at least 2 seconds was required to trigger sustained calcium influx in platelets [382], and support the idea that applying tension on GPIb-IX-V for a period of time may be needed to sustain unfolding of the MSD and to activate GPIb-IX.

Under this model, where the MSD unfolds to turn on signal and refolds without constant shear, turning off the signal, then it is possible to imagine a modulator causing the receptor to favor the unfolded state. This would increase GPIbα's "mean dwell time" in the extended conformation. Whether or not 3G6 operates in this manner remains to be seen, but the data presented in this chapter would be consistent with a model wherein 3G6 binds to a region on GPIbβ which participates in stabilizing the folded MSD. We have established that the folded and unfolded MSD likely come into contact with GPIbβ's extracellular domain. It is possible that 3G6 amplifies MSD unfolding induced by other ligands in a manner such as: (1) a ligand like 6B4 or 11A8 induces MSD unfolding under shear conditions, (2) unfolding of the MSD

increases the exposure of the 3G6 epitope on GPIbβ and 3G6 binds to it, (3) when the MSD would normally refold, instead it is physically blocked by 3G6, forcing the MSD to remain open for longer. As we saw in figure V.2, 3G6 alone has very little effect on GPIb-IX-V activation, which would be the case if it acted to block MSD refolding (the MSD would need to be unfolded in the first place).

I expect that, with further studies on 3G6 and RAM.1's epitopes, effects on the kinetics of GPIb-IX-V signaling, and overall mechanism of action we will be able to pull back the veil on precisely how the complex transduces signal, and how platelets sense the shear force of their environment.

CHAPTER VI

CONCLUSION AND CLOSING REMARKS

A. Back at One

Our circulatory system is an evolutionary heirloom passed down to us from simple multicellular organisms to sponges, through the crustaceans, insects, early vertebrates, apes, people who eat pizza with a knife and fork, and finally, to mankind. Throughout my discussion of the development of circulation, I have stressed the importance of shear generated by circulatory fluids (recall Murray's law and the role of shear force remodeling on our branched vasculature) as a component of interactions that occur in blood vascular systems. From the development of vasculature to the maintenance of vascular integrity through the process of hemostasis, shear is Shah. In primary hemostasis, which involves initial platelet adhesion and formation of the platelet plug, shear primes VWF to bind to platelet GPIb-IX-V. Once the two are associated, shear also activates GPIb-IX-V through its MSD. In secondary hemostasis, coagulation cascades lead to fibrin polymerization and stabilization of a clot.

Platelets, our primary thrombotic cells, are first formed from their parent cells, the polyploid bone-marrow resident megakaryocytes. Shear is a vital factor in elongation and release of proplatelets into the bloodstream as well as for recapitulation of the thrombopoietic niche in many *in vitro* platelet production schemes. Platelets' evolutionary roots are as fascinating as they are tangled and meandering. Aside from the thrombocytoid fragments of some insects, anucleate thrombocytes are largely a mammalian feature, with other vertebrates boasting dedicated thrombocytes with nuclei. Although most of the data I present in chapters III-V relates to the clearance and hemostatic function of platelets, it is important that we remember platelets' other diverse roles, a potential evolutionary holdover from the multipurposed

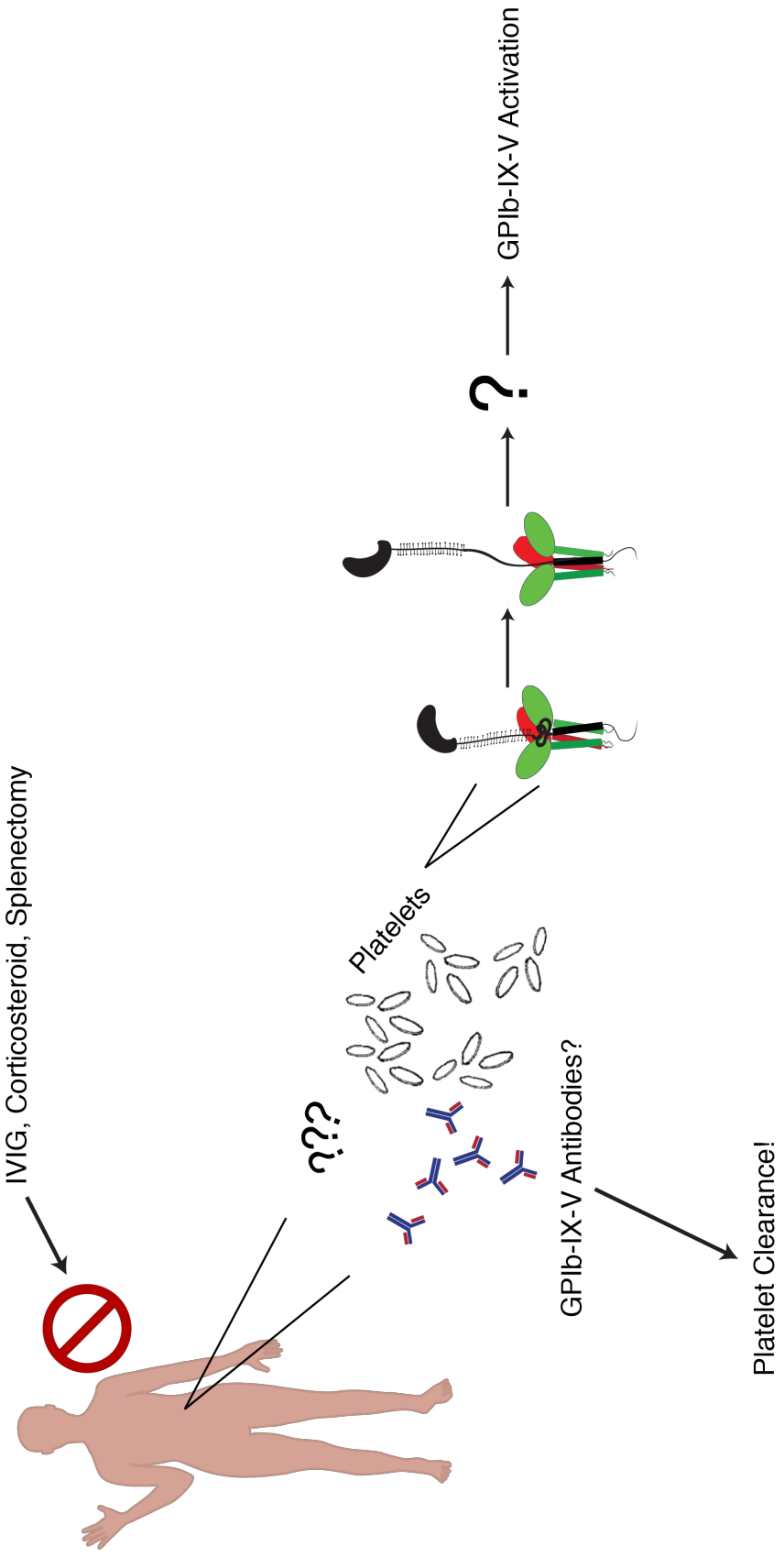
IVIG, Corticosteroid, Splenectomy
¿¿¿
Platelets
GPIb-IX-V Antibodies?
Platelet Clearance!
?
GPIb-IX-V Activation

Figure VI.1 Open questions prior to this work Many ITP patients are refractory to immunosuppressive treatments (IVIG, corticosteroids, splenectomy). Although there is some association between presence of anti-GPIb-IX-V antibodies in patient sera and response to treatments, the underlying mechanism is unknown (left). On the surface of platelets, GPIb-IX-V is activated when force applied through ligand binding to the LBD unfolds the MSD. However, the conformational changes in the complex following MSD unfolding and upstream of receptor signaling are unknown (right).

hemocytes of a common ancestor. Platelets behave as rudimentary immune cells in innate immunity, as pseudo antigen-presenting cells, proinflammatory cells, and proangiogenic cells.

Part of the beauty of platelets' myriad jobs in circulation is their simplicity and their ubiquity. At ~350,000 platelets per μl of blood, they are an extremely prevalent cell type. Platelets are turned over in our bodies at an astounding rate of 10^{11} platelets every day. This destruction/renewal axis requires robust processes for platelet clearance to balance out thrombopoiesis. Above, I discuss three main avenues to destruction for platelets: apoptotic pathways regulate platelet lifespan through Bcl-x_L, desialylation (either as a result of activation or senescence) facilitates platelet clearance by the hepatic AMR, and in disease states, autoantibodies clear platelets in the spleen and liver.

Immune-mediated clearance of platelets is the hallmark of immune thrombocytopenia (ITP). Possibly the oldest blood disorder to be described on record, humans have been diagnosing (and in the last 100 years, effectively treating) ITP for a long time. Although many cases of ITP are mild and resolve on their own, some are particularly severe and carry life-threatening risks. In other words, the population receiving treatment for ITP often already is showing some risk factor like bleeding events, or a dangerously low platelet count (all ITP patients have reduced platelet count, to an extent). Effective, targeted therapies for ITP began about 100 years ago with the use of splenectomy to eliminate the primary clearance site for autoantibody tagged platelets. Since then, the first line treatments have evolved to oral or intravenous immunosuppressants like corticosteroids and IVIG. However, both corticosteroids and IVIG are short-term treatments, and even then, are ineffective in a significant percent of patients. It is currently unclear what mechanism underlies such

widespread refractoriness, and there are no reliable ways to predict how patients will respond to the treatments (Fig. VI.1). Given that the patients being treated are already the most severe cases, waiting and trying different approaches may cost valuable time in the absence of actionable insights. Indeed, one of the only clues as to prognosis for different treatments is that resistance to first line immunosuppresants is associated with ITP mediated by antibodies against the GPIb-IX-V complex.

The question of resistance to IVIG is of both clinical and basic mechanistic interest. Naturally, disease etiology is a vital part of treatment decisions, and the apparent ability of antibodies against GPIb-IX-V to induce clearance in an immune independent manner raises the question of whether the antibodies activate GPIb-IX-V themselves. In chapter II I outline a uniform shear assay that uses cone-plate viscometry to apply uniform shear across a sample. This technique is not entirely novel, as many groups have used cone-plate or other types of rotational viscometers to apply shear to biological samples. Here we are using it to test the effect of a putative ligand or ligands (the MAbs) on a mechanoreceptor (GPIb-IX-V)

In chapter III, this technique allowed us to demonstrate that antibodies targeting the LBD of GPIb-IX-V activate the complex in a shear-dependent manner that proceeds through the unfolding of the mechanosensory domain. We show that antibodies which activate GPIb-IX-V can induce robust IVIG resistant ITP in mouse models, while an antibody against the LBD which does not respond to shear also does not induce IVIG resistant ITP. These findings have mechanistic implications for GPIb-IX-V signaling. VWF is normally anchored to collagen when it engages GPIb-IX-V, but we demonstrate a new crosslinking mechanism by which an antibody can bind to two copies of GPIbα on opposing platelets in order to generate the force

to unfold both MSDs (Fig. VI.2). By demonstrating that dimeric anti-LBD MAbs in ITP can activate GPIb-IX-V and induce clearance, we provide a mechanism for resistance to IVIG and other immunosuppressive drugs. Our findings outline factors that would predict resistance/refractoriness to IVIG prior to trying the treatment. In addition to the burden of treatment and financial toll, this can help to quickly select the proper treatment for a given patient's ITP.

Once we demonstrated that anti-GPIb-IX-V antibodies in ITP could induce immune-independent clearance via GPIb-IX-V signaling, it became clear that this same mechanism didn't depend on the ligand being an antibody. Indeed, any molecule that was multivalent, bound the LBD, and could sustain enough force to unfold the MSD (~15 pN), could theoretically trigger activation of GPIb-IX-V. For example, multimeric VWF bearing a type 2B VWD mutation. In chapter IV I investigate a putative dimeric ligand to the LBD, FXI and characterize binding to the GPIb-IX complex. I also challenge one of the assumptions about crosslinking ligands, that they need to target the LBD. Using an antibody against the sialomucin region with significant unbinding force, I show force-dependent GPIb-IX-V signaling. In figure III.1 we demonstrated that several antibodies with non-overlapping epitopes in the LBD could induce shear-dependent activation. Here the epitope is outside the LBD entirely, but still in a position to exert a pulling force on the MSD if crosslinking platelets under shear.

GPIbα, GPIbβ, and GPIX are all necessary for robust surface expression of the receptor (sorry GPV), but most investigation centers around GPIbα (Fig. VI.1), likely because it contains the LBD, much of the total platelet surface glycosylation and the MSD. In other words, it's the

business end of the molecule. However, in chapter V, I highlight two antibodies targeting GPIbβ: RAM.1, which has been shown to inhibit GPIb-IX-V signaling in the literature, and another unreported antibody, 3G6. While RAM.1 totally shuts down signaling through GPIb-IX-V, 3G6 appears to amplify the signal. Although RAM.1 has been in the literature for over a decade, its mechanism of inhibition has not yet been elucidated. Now, another anti-GPIbβ antibody which has a starkly different effect on signaling indicates that GPIbβ's extracellular domain is highly likely to participate in signal transduction in GPIb-IX-V (Fig VI.2).

When I first approached this body of work, I was a biochemist in search of a receptor to chase. In a narrow sense of receptor-ligand interactions, a soluble ligand like a drug or hormone binds to its receptor, induces a conformational change which alters the physical orientation of some intracellular segment and leads to a chemical change like phosphorylation or release of a GTPase. Changing this frame of reference to think of shear as a ligand (or a cofactor!) opens many more lines of inquiry, and often requires a unique set of assays. The trigger model of GPIb-IX-V activation was established in a series of two papers within a year of each other, which characterized the MSD, identified the region of GPIbα containing this domain, and linked MSD unfolding to platelet signaling. This novel mechanism of activation for one of the most important (that is, prevalent and necessary for adhesion and activation!) platelet receptors meant that there were potentially previously unappreciated roles for this receptor, especially in human disease states. My work forwards the field in both clinical and mechanistic ways. In one story, my co-authors and I link observations about GPIb-IX-V autoantibodies' association with IVIG resistant ITP to the molecular mechanism of the trigger model. By providing a mechanistic explanation for resistance to many first line treatments, I inform potential new diagnostic and therapeutic approaches, even outlining a potential

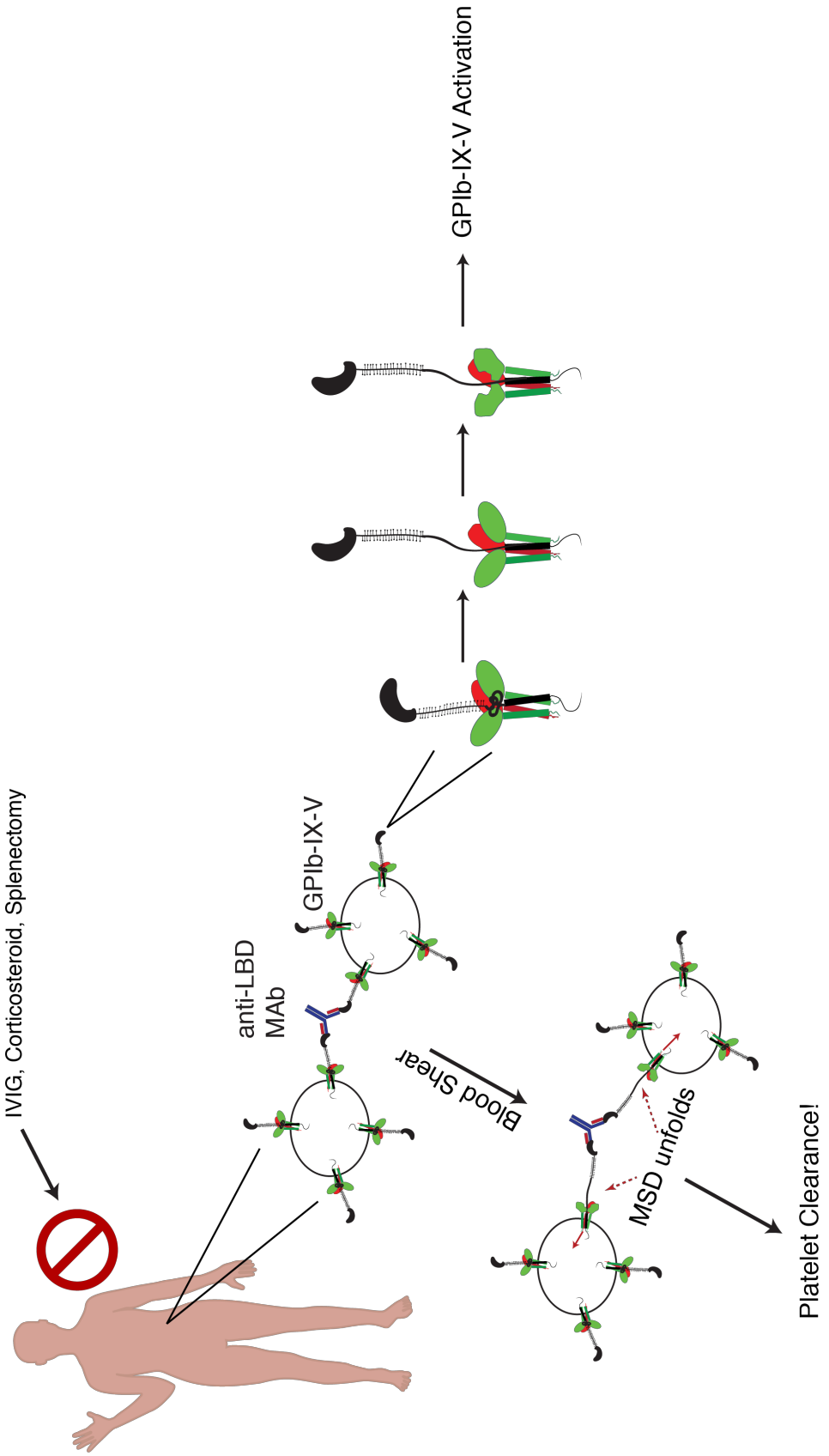
IVIG, Corticosteroid, Splenectomy
anti-LBD MAb
GPIb-IX-V
Blood Shear
MSD unfolds
Platelet Clearance!
GPIb-IX-V Activation

Figure VI.2 Contributions from this body of work. Previously, there was no mechanism explaining why certain ITP patients were refractory to immunosuppressive treatments. In chapter III we establish that platelet crosslinking by bivalent antibodies binding to copies of GPIbα on opposing platelets can generate the force necessary to unfold the MSD in GPIb-IX-V. This leads to platelet clearance via GPIb-IX-V activation. Identification of this mechanism suggests prognostic approaches to determining response to first-line treatments in ITP. Activation of GPIb-IX-V proceeds through unfolding of the MSD, but no contributions of GPIbβ and GPIX to signal transduction had been identified aside from a GPIbβ antibody which could inhibit activation. I demonstrated that this antibody inhibited activation in solution and adherent conditions, and find that another unpublished antibody can amplify signaling, confirming that GPIbβ conformational changes are involved in transduction of the mechanical signal.

prognostic bead assay for immune thrombocytopenia (P-BAIT). This insight may be the first situation where the mechanical strength and force resistance of an antibody's bond (not the specific epitope) underlies the pathology of a disease. I also present a unique mechanism for multivalent ligands to activate GPIb-IX-V in solution, as opposed to the anchored ligand (VWF) which activates it under normal circumstances. I demonstrate the ability of antibodies against GPIbβ to modulate GPIb-IX-V function, supporting a role for GPIbβ in the propagation of signal initiated by MSD unfolding.

These data paint a more complete picture of mechanosensation via GPIb-IX-V. There is clear therapeutic potential to treat disorders of VWF and GPIb-IX-V to be gained from a unified mechanism for the trigger model. The nuances of how shear acts on platelets are vital to an understanding of platelets' many functions in circulation. With further insights into pathological receptor activation and the roles of GPIbβ, GPIX, and GPV to the complex's signal transduction, we are closer to a complete understanding of how platelets use the force.

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Appendix

Filopodia Macro

*Our gratitude to Neil Anthony of the Emory University Imaging Core for providing much of the base code needed to build out this macro and for his advice and guidance during analysis.

```
//  Analyze filopodia

//  get file path and title; remove file ext
title0 = getTitle();
path = getInfo("image.directory");
dotIndex = indexOf(title0, ".");
if (dotIndex > 0) {
	title = substring(title0, 0, dotIndex);
}
else {
	title = title0;  //  in cases where it's already been taken off
}
masktitle = title + "_mask";
rename(title);

str = "title=" + masktitle;
run("Duplicate...", str);
run("Median...", "radius=35");
setAutoThreshold("Li dark");
run("Convert to Mask");

//  dilate three times; change setting and then change back to 1 iteration
run("Options...", "iterations=3 count=1 do=Nothing");
run("Dilate");
run("Options...", "iterations=1 count=1 do=Nothing");

//  convert to real binary and invert
run("Divide...", "value=255");
run("XOR...", "value=1");
run("Enhance Contrast", "saturated=0.35");

//  apply binary to original image
imageCalculator("Multiply create", title, masktitle);

//clean and theshold filopodia
run("Remove Outliers...", "radius=1.8 threshold=50 which=Bright");
```

```
setThreshold(380, 65535);
run("Convert to Mask");

//  analyze skeleton
run("Skeletonize");
run("Analyze Skeleton (2D/3D)", "prune=none show");

//  save and clean up
selectWindow("Branch information");
savStr = path + title + "_branch_info.csv";
saveAs("Results", savStr);
run("Close");

selectWindow("Results");
savStr = path + title + "_filo_results.csv";
saveAs("Results", savStr);
run("Close");

str = "Result of " + title;
selectWindow(str);
savStr = path + title + "_filo_results.png";
saveAs("PNG", savStr);
close();

selectWindow(masktitle);
savStr = path + title + "_mask.png";
saveAs("PNG", savStr);
close();

selectWindow("Tagged skeleton");
savStr = path + title + "_filo_tagged.png";
saveAs("PNG", savStr);
close();
```

Table of Abbreviations

Abbreviation	Definition
ADP	Adenosine Diphosphate
APC	Activated Protein C
BSS	Bernard-Soulier Syndrome
CBC	Complete Blood Count
CHO	Chinese Hamster Ovary
CTC	Circulating Tumor Cell
ECL	Erythrina cristagalli lectin
ELISA	Enzyme-Linked Immunosorbent Assay
EMT	Epithelial-Mesenchymal Transition
GP	Glycoprotein (as in GPIb-IX-V or GPIIbIIIa)
HSA	Human Serum Albumin
HK	High-molecular-weight Kininogen
ITP	Immune Thrombocytopenic Purpura or Immune Thrombocytopenia (formerly Idiopathic Thrombocytopenic Purpura)
IVIG	Intravenous Immunoglobulin
LBD	Ligand Binding Domain
LRR	Leucine-Rich Repeat
MAb	Monoclonal Antibody
MSD	Mechanosensory Domain
PDGF	Platelet-Derived Growth Factor
PPP	Platelet-Poor Plasma
PRP	Platelet-Rich Plasma
PtdSer	Phosphatidylserine
SFR	Shear Force Remodeling
TCIPA	Tumor Cell Induced Platelet Aggregation
TF	Tissue Factor
TFPI	Tissue Factor Pathway Inhibitor
TGF	Transforming Growth Factor
TM	Transmembrane
TPO	Thrombopoietin
TTP	Thrombotic Thrombocytopenic Purpura
TXA_2	Thromboxane A_2
ULVWF	Ultra-Long vonWillebrand Factor
VEGF	Vascular Endothelial Growth Factor
VSM	Vascular Smooth Muscle
VWD	vonWillebrand Disease
VWF	vonWillebrand Factor