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PLATELET ENDOCYTOSIS: ROLES IN HEMOSTASIS AND INNATE IMMUNITY

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PLATELET ENDOCYTOSIS: ROLES IN HEMOSTASIS AND INNATE IMMUNITY

DISSERTATION

A dissertation submitted in partial fulfillment of the Requirements for the degree of Doctor of Philosophy in the College of Medicine at the University of Kentucky

By
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2017
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Abstract of Dissertation

PLATELET ENDOCYTOSIS: ROLES IN HEMOSTASIS AND INNATE IMMUNITY

Endocytosis is key to fibrinogen (Fg) uptake, receptor trafficking of integrins (αIIbβ3, αvβ3) and purinergic receptors (P2Y1, P2Y12), and thereby for normal platelet function. However, platelet endocytosis could potentially be critical for actively sensing changes in vascular microenvironments and responding accordingly to what is being taken up. This is a more dynamic view of platelets as active surveyors of the vasculature; extending the importance of platelet endocytosis beyond granule biogenesis and perhaps even hemostasis. The mechanistic underpinnings of endocytosis, its importance in platelets, and the molecular machinery required and possible trafficking routes are however understudied, in part due to a lack of experimental tools. The work presented here, puts forth new players that regulate platelet endocytosis and mediate cargo loading and hemostasis as well as provides a novel mechanistic understanding of how endocytosis allows platelets to act as immune cells and become the first responders to pathogens in the vasculature.

Previously we showed the importance of ADP-ribosylation factor 6 (Arf6), which regulates αIIbβ3-mediated Fg uptake/storage and affects acute platelet functions e.g., clot retraction and spreading. To further identify elements of this endocytic machinery, we examined the role of a vesicle-residing Soluble N-ethylmaleimide Factor Attachment Protein Receptor (v-SNARE) called Cellubrevin/Vesicle-Associated Membrane Protein-3 (VAMP-3) in platelet function. VAMP-3 KO mice had less platelet-associated Fg, indicating a defect in Fg uptake/storage. Loss of VAMP-3 led to a defective uptake of fluorescently-tagged Fg and low molecular dextran in platelets though it had a greater negative effect on receptor-mediated Fg uptake than on the fluid-phase marker uptake. Additionally, we followed the time-dependent trafficking of Fg and dextran into platelets using 3D-Structured Illumination Microscopy. Wild-type platelets endocytosed both cargoes but quickly sorted them into distinct compartments with partial overlap occurring only at early time points. Sorting was unaffected in VAMP-3 KO platelets. The VAMP-3 loss did affect some acute platelet functions leading to enhanced spreading on
Fg and faster clot retraction compared to wild-type. Additionally, the rate of JAK2 phosphorylation, initiated through the thrombopoietin receptor (TPOR/Mpl) activation, was affected in VAMP-3 KO platelets.

The idea that platelets can act as immune cells and contribute to innate immunity has been increasingly gaining ground. Groups have correlated thrombocytopenia with clinical outcomes of viremia and bacteremia. Chronic viral infections, e.g., HIV-1, severely increase the risk of acute myocardial infarction (MI), possibly via some level of platelet activation, contributing to increased thrombotic potential. Platelets do endocytose viruses and bacteria, but the molecular machinery is ill-defined. In nucleated cells, responses to HIV-1 are mediated by virus phagocytosis/endocytosis, degradation to release Toll-like Receptor ligands, and subsequent receptor activation. Is this process recapitulated in platelets? Here we show that platelets indeed use VAMP-3 and Arf6-dependent pathways to endocytose HIV-1 virions, degrade retroviral particles to release TLR ligands, which initiate platelet activation and secretion. HIV-1 uptake and subsequent activation is abolished in VAMP-3 and Arf6 KO mice.

Collectively, our studies shed light on how platelets act at the early stage of pathogen recognition and are able to process them to initiate an immune response.

**KEYWORDS:** Platelets, endocytosis, VAMP-3, Arf6, endosomal TLRs, HIV-1
PLATELET ENDOCYTOSIS: ROLES IN HEMOSTASIS AND INNATE IMMUNITY

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April 10th, 2017
To my grandparents, Ma and Baba and my brother
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Chapter One: Introduction

Subsection One: Platelets and their roles

Platelet biology overview

While Max Schultze, in 1865, and William Osler, in 1874, were the first to discover platelets as a “normal constituent of blood”, it was Giulio Bizzozero in 1882, who first coined the term “blood plates” to describe platelets as “morphological elements of blood” important for their roles in coagulation and thrombosis [1]. Mammalian platelets are small (~2-3 µM in diameter), anucleate, circulatory cell fragments that are derived from the cytoplasm of megakaryocytes, the only polyploid hematopoietic cell within the bone marrow. They are the smallest blood cells and are second only to erythrocytes in number, ranging from 150,000-450,000 platelets/µL of blood, making them the most abundant “responsive” cell type in the bloodstream due to their ability to respond to various kinds of damage in the circulation. The primary physiological role of platelets is to sense vascular damage and seal the breach in the vessel endothelium to prevent blood leaks by initiating hemostasis. Upon vascular injury, circulating platelets get exposed to collagen or other extracellular matrix (ECM) proteins and become activated upon binding via their myriad surface receptors and adhere to the site of injury. This resulting activation leads to increases in cytosolic [Ca^{2+}] levels followed by shape change culminating into secretion from the intracellular granule stores, leading to further platelet recruitment and activation, eventually forming the so-called “platelet plug” at the site of injury. This, in conjunction with the activation of the coagulation cascade and thrombin generation which converts fibrinogen to fibrin, allows platelets to form a fibrinous thrombus which arrests blood loss. As such, platelets, their activation and secretion, are essential for thrombosis and hemostasis. A growing body of evidence now suggests that platelet functions extend beyond hemostasis and thrombosis including promoting inflammatory and immune responses [2], maintaining vascular integrity [3] and contributing to wound healing [4]. Additionally, platelets have also been shown to contribute to pathophysiological conditions such as atherosclerosis [5, 6], ischemic cerebrovascular diseases [7], and angiogenesis [8-10] as well as cancer and tumor metastasis [11-13]. Platelets have a
diverse array of granule cargo as well as several unique receptors that enable them to oversee the aforementioned functions in health and disease. While exocytosis of vesicular (from granules and compartments) cargo is central to a majority of platelet functions, endocytosis of circulating exogenous cargo also occurs in platelets and is vital to at least some functions. As vascular sentries, platelets constantly sample the bloodstream contents and this surveillance mechanism could be mediated by endocytosis. Uptake of small particles and solutes by human platelets was first reported by Zucker-Franklin in 1981 [14] into a unique membrane structure called the open canalicular system (OCS) by a phagocytosis-like process. Further studies using electron microscopy and cytochemistry provide evidence for endocytosis of circulating proteins such as plasma fibrinogen [15, 16] into platelets. Platelet endocytosis is important for granule cargo loading (such as fibrinogen and VEGF) [9, 17] and contributes to thrombus formation by controlling the surface expression of several key proteins such as the integrins, chiefly α_{IIb}β_{3}, the major platelet glycoprotein receptor [18]. Integrin trafficking modulates platelet spreading on endothelial surfaces and clot retraction [19]. Endocytosis of purinergic receptors such as P2Y_{1} and P2Y_{12} mediates their resensitization and response to ADP [20]. CLEC-2 and Thrombopoietin receptor (Mpl/TPOR) endocytosis regulates their surface levels and downstream signaling responses [21, 22].

Clearly, platelets are far more multi-functional than we give them credit for. For normal physiological responses, it is imperative for both arms of membrane trafficking, exocytosis, and endocytosis, to occur under tight regulation. Disruption in any of these processes leads to severe platelet dysfunction. Hyperactive platelets cause spurious thrombosis which precipitates occlusive events such as heart attack and stroke, which collectively accounts for 1 in 3 deaths from non-communicable diseases, worldwide (World Health Organization, 2012 statistics). Hypoactive platelets or platelets from patients suffering from granule biogenesis disorders have a bleeding diathesis [23]. Thus, regulating platelet functions is crucial to developing therapeutics to prevent cardiovascular diseases (CVD). While targeting platelet activation and exocytosis remains the cornerstone for developing anti-platelet drugs, understanding the mechanisms of platelet endocytosis enables us with an
alternative approach to load platelets with therapeutics that can then modulate platelet responses paving the way to effectively treat cardiovascular diseases.

**Platelet structures**

Platelets are formed from the cytoplasm of megakaryocytes (MKs), their large precursor cells, in the bone marrow [24]. MKs release platelets through a multi-step and tightly regulated process involving the complex interplay of transcription factors, signaling regulators, extensive cytoskeletal rearrangements and the bone marrow microenvironment [25]. MKs become polyploid by endomitosis and undergoes maturation wherein their cytoplasm gets packaged into long pseudopodial projections, termed proplatelets [26]. Platelets form selectively at the tip of the proplatelets and derive granule and organelle content from the MK cell body [27]. Once the proplatelets extend into sinusoidal blood vessels, they undergo fission to release platelets into the circulation. Each megakaryocyte produces 5000-10,000 platelets. While the process of platelet production appears to be similar in mice and humans, human platelets have a lifespan of 10 days while mouse platelets survive for 4-5 days [28]. Mouse platelets are similar to human platelets, but they are smaller in size (mean platelet volume ~4.7 vs. ~7.5-10 fl) and more numerous (~1.1 X 10^6/µL of blood) [28, 29].

In the quiescent state (see adjoining figure), platelets are a discoid shape with smooth surfaces. Microtubules, located beneath the cell membrane of resting platelets, together with the actin cytoskeleton, help in maintaining this discoid shape [30, 31]. Anucleate platelets chiefly contain three major kinds of granules; dense core granules, alpha granules, and lysosomes. These granule stores contain a multitude of different kinds of cargo molecules that form the bulk of the platelet secretome, upon release. Alpha granules are the largest and most abundant secretory granule in platelets and based on electron microscopy of platelet thin sections; there are 50-80 of these per platelet with a diameter of 200-500 nm [32-35]. Alpha granules contain bioactive molecules such as growth factors (e.g. Platelet-Derived Growth Factor (PDGF), Transforming Growth Factor β (TGFβ), Thrombopoietin (TPO) etc.), chemokines (e.g. Platelet Factor 4(PF4), Regulated on activation, normal T cell expressed and secreted (RANTES) etc.), various pro-
Vascular Endothelial Growth Factor (VEGF) and anti-angiogenic molecules (e.g. Endostatin), adhesion molecules (e.g. von Willebrand factor (vWF), P-Selectin) and coagulation factors (e.g. fibrinogen). Some of these cargo molecules such as vWF, PF4, and P-Selectin, a granule membrane protein, are synthesized in the megakaryocytes while cargo molecules such as fibrinogen are endocytosed via the α\textsubscript{IIb}β\textsubscript{3} integrins [17]. Some alpha granules and their precursor multivesicular bodies (MVBs), which are present in platelets [41, 42], contain small vesicles called exosomes, that are about 40-200 nm in range and are released upon exocytosis [36]. Dense core granules number around 5-8 per platelet [37] and contain small molecules such as calcium, serotonin, ADP, and ATP, etc. Lysosomes are another type of secretory granule in platelets. Though largely considered as degradative compartments, yet there exists strong evidence suggesting the release of cargo upon platelet activation both \textit{in vitro} and \textit{in vivo} [38, 39]. Platelets contain only a few lysosomes (~2-3) [40] which contain degradative enzymes, including β-hexosaminidase, cathepsins D and E, and acid hydrolases [43-45]. Platelets also contain the lysosomal membrane proteins LAMP1, LAMP-2, and CD63 (LAMP-3) [46]. Additionally, platelets contain mitochondria as well as other organelles such as Endoplasmic Reticulum (ER), an ER-derivative dense tubular system (DTS) and Golgi Apparatus [48] and recent evidence suggests that cargo contained in these organelles may also be released upon activation [49, 50].

Upon activation, rapid re-organization of the actin cytoskeleton allows platelets to extend protrusions called filopodia to finally generate lamellipodia, resulting in a dramatic increase in the platelet surface area [51]. It is thought that this generous amount of extra membrane comes from a membrane reservoir, called the open canalicular system (OCS), unique to platelets. The OCS, which is an extension and invagination of the platelet plasma membrane, serves as the pathway for transport of substances into the cells as well as channels for the release of granule cargo upon activation-induced exocytosis [52, 53]. However, the exact roles of the OCS remain controversial. While it is widely thought that alpha granules dump their cargo into the OCS upon activation [54], recent evidence suggests otherwise. Tomographic analysis from scanning transmission electron microscopic images of quiescent
platelets shows alpha granules and the closed canalicular system to be discrete membrane-bound structures. In the early stages of platelet activation, the closed canalicular system fuses with the plasma membrane and becomes open, while the alpha granules fuse with the plasma membrane forming long tubular connections [55]. It could be speculated that these OCS, are in fact, some sort of endosomal compartments, that could get fused with the plasma membrane during platelet activation. Recent data from our lab provides evidence for the presence of two distinct populations of endosomes, Rab4-positive early endosomes and Rab11-positive recycling endosomes in platelets [19]. These new findings suggest that platelets are in fact more complex with what could be an extensive endomembrane system that allows greater capacity for intracellular trafficking than previously thought to exist, an avenue that will definitely warrant future studies.
Figure 1

vWF  TSP-1  PDIP  ERp57  Platelet Factor IV  β- hexosaminidase  
SERCA3  TGN46  Calreticulin  Actin  Microtubule

α Granule  Lysosome  Dense Granule  Golgi Complex  Dense Tubular System  ER fragment  Mitochondria
Figure 1 Resting and activated platelet structures. Mature quiescent platelets are discoid shaped and contain various cargo molecules within the different secretory granules and ER-derived and intracellular membrane compartments as depicted in the legends. Upon activation, platelets undergo a dramatic shape change and become stellate, followed by granule/compartment-plasma membrane fusion drives surface mobilization and release of luminal cargo into the extracellular space.
Platelet surface receptors and their downstream signaling pathways: roles in hemostasis and thrombosis

Platelets detect and respond to their external environment via a multitude of surface receptors on their plasma membrane. The majority of the platelet surface receptors contribute to the primary function of hemostasis; by either platelet activation or adhesion to the damaged endothelium and in facilitating thrombus formation. Platelets also express several other receptors that have seemingly non-hemostatic functions namely in pathogen recognition and immunological defense. Several transmembrane receptors span the platelet surface, including many integrins (αIIbβ3, α3β1, α5β1, α6β1, αvβ3), leucine-rich repeats (LRR) receptors (Glycoprotein [GP] Ib-IX-V, Toll-like Receptors), G-protein coupled receptors (GPCRs) (PAR-1 and PAR-4 thrombin receptors, P2Y1 and P2Y12 purinergic receptors, TPα and TPβ thromboxane A2 (TxA2) receptors), proteins belonging to the immunoglobulin superfamily (GP VI, FcyRIIA), C-type lectin receptors (CLEC-2), tyrosine kinase receptors (thrombopoietin receptor TPOR/Mpl) and other miscellaneous receptors [56]. While platelets do share certain receptors with other cells present in the vasculature, there are some that are unique to platelets only, e.g., αIIbβ3. As such, they have become the hotspots for therapeutic intervention. Most of the surface receptors, whether they are engaged in hemostasis or non-hemostatic functions, give rise to downstream signaling cascades that crosstalk significantly in platelets.

Platelet adhesion, recruitment and activation leading to thrombus formation at the sites of vascular damage is dynamic and requires a series of time-dependent concerted events that can be broadly classified as: (i) “initiation phase” when the first layer of platelets descend on the exposed endothelium and get activated; (ii) “extension phase” when the first activated platelets release ADP, TxA2, which act as autocrine and paracrine agonists and help in the recruitment and activation of additional platelets; (iii) “stabilization phase” when the forming platelet plug is maintained to arrest blood loss and prevent its premature dislodgement until wound healing commences [58]. Different platelet receptors come into play at each of these phases, in an order, leading to a complex but coordinated interplay of receptor-
ligand interactions and downstream signaling that is tightly regulated to prevent uncontrolled thrombosis at the site of injury.

In this section, the major platelet receptors will be discussed in order of their engagement in forming the growing thrombus.

**GPIb-IX-V complex (GPIb complex)**

At the vascular site of injury, the first contacts between surveilling platelets and the damaged endothelium, underflow, is made through the interaction between the glycoprotein (GP) Ib-IX-V complex and collagen receptors, GP VI and α2β1 integrin, on the platelet surface, and by von Willebrand Factor (vWF) and fibrillary collagen in the exposed endothelium [57]. This initial tethering and firm adhesion of platelets allows further platelet recruitment and drives activation. Platelet adhesion to the vessel wall components that are exposed to damage largely depends on the shear rates [57]. In the microvasculature (small arteries), where platelets encounter high shear conditions (>1000 s⁻¹), GPIb-IX-V-mediated adhesion to the vessel wall is chiefly vWF-dependent [59]. In the veins and larger arteries, where platelets encounter low shear rates (~500 s⁻¹) adhesion to the vessel wall primarily involves binding to fibrillar collagen, fibronectin, and laminin [60]. The GPIb-IX-V complex contains four distinct subunits that each span the lipid bilayer once: the two disulfide-linked subunits of GPIb (GPIbα[CD42b] and GPIbβ[CD42c]), GPIX (CD42a) and GPV (CD42d), expressed in the ration of 2:2:2:1 respectively [61]. Each subunit of this complex has one or more leucine-rich repeats of about 24 amino acids: GPIbα has 7 repeats, GPIbβ and GPIX have 1 each, and GPV has 15 repeats [61]. Aside from the hydrophobic membrane-spanning segments of this complex, GPIbβ and GPIX are palmitoylated at cysteine residues to further anchor them in the plasma membrane [62].

In addition to vWF, GPIb-IX-V complex can also bind to P-Selectin, expressed on activated platelets and activated endothelial cells [63], and to the integrin αMβ₂ (Mac-1) expressed on leukocytes [64]. GPIbα also binds to coagulation factors from the intrinsic pathway such as high molecular weight kininogen, FXII and FXI and thrombin generated from the activation of the extrinsic or intrinsic coagulation cascades [65-67]. The cytoplasmic tail of GPIbα interacts with filamin, calmodulin,
14-3-3ζ, which in turn allows it to interact with signaling proteins such as phosphatidylinositol 3-kinase (PI-3K), focal adhesion kinase (FAK), Src-related tyrosine kinases, GTPase-activating protein and tyrosine phosphatases (PTP1b and SHPTP10) [68, 69]. The GPIbα-vWF interaction drives the initial platelet activation by causing intracellular calcium transients followed by PLCγ2, ERK-1/2 and Syk phosphorylation, TxA2 generation and ADP release from dense granules, ultimately leading to the activation of the integrin αIIbβ3 [69].

Deficiency of functional GPIbα on human platelets causes a rare autosomal recessive disease, called Bernard-Soulier syndrome, characterized by thrombocytopenia, large platelets and mild to severe bleeding phenotype [70], stressing the importance of this receptor complex in mediating hemostasis and thrombosis.

**Glycoprotein VI (GPVI)**

At the site of damaged endothelium, collagen is present, which facilitates the initial platelet adhesion. Fibrillar collagen is better suited to bind to platelets than monomeric collagen [71]. Platelets utilize GPVI, a member of the immunoglobulin superfamily, and an integrin, α2β1, to bind directly to collagen.

Glycoprotein VI (GP VI) is a platelet and megakaryocyte-specific, a transmembrane protein, consisting of two immunoglobulins (Ig)-like domains in the extracellular region connected to a highly glycosylated linker, a transmembrane domain, and a cytoplasmic tail [72, 73]. This cytoplasmic tail can bind to Fyn and Lyn Src kinases [74]. GP VI exists as a complex with the homodimeric Fc receptor γ chain (FcRγ with a possible stoichiometry of 2 GP VI molecules and 1 FcRγ dimer) on the platelet surface, which bears an immunoreceptor tyrosine-based activation motif (ITAM) acting as a signal-transducing subunit of the receptor [75]. Studies deciphering the role of GP VI in activating platelets have often used agonist surrogates such as Collagen-related peptide (CRP) consisting of (Gly-Pro-X)n or (Gly-X-Hyp)n sequences organized in triple helices [76] or the snake venom C-type lectin, convulxin, [77, 78] that are thought to work by clustering GP VI molecules on the platelet surface. When the GP VI receptor is cross-linked by binding to collagen or CRP or convulxin, the constitutively bound Src kinases (Fyn and Lyn) phosphorylate the ITAM sequence in the FcR γ-chain allowing the assembly and activation of Syk and initiating activation.
of a downstream signaling pathway that has many similarities with that employed by immune cell receptors, mainly T-cell receptors. Activation of this signaling cascade leads to the formation of the signalosome, composed of various adaptors and effector proteins, such as LAT (Linker for Activation of T-cells), SLP-76 (SH2 domain-containing leukocyte protein of 76 kDa), which associates to and activates PLCγ2, PI3K, and small molecular weight G proteins, leading to full platelet activation [79-81].

GP VI blockade, or its depletion, has been shown in mice to result in complete protection against arterial thrombosis, without significant prolongation of bleeding time. Blockade of GP VI results in impaired platelet response to collagen and may, therefore, present a useful antithrombotic target [82-84].

**Integrin α2β1**

Integrin α2β1 also called GP Ia/IIa, VLA-2 or CD49b/CD29, plays a role in the adhesion of platelets to collagen and subsequent platelet activation [75]. Loss of α2 subunit or β1 subunit in mice does not cause bleeding defects, although these platelets show minor defects in platelet adhesion and aggregation to collagen [85-86]. Studies on these knockout mice showed variable phenotypes in in vivo models of arterial thrombosis ranging from normal thrombus formation [87] to mildly delayed, reduced and unstable thrombi [88, 85]. Similar to other integrins, conformational activation of α2β1 increases its affinity for collagen and requires GP VI and collagen-dependent inside-out signaling [89]. However, combined deficiency of α2β1 and GPVI in mice causes complete inhibition of thrombus formation while isolated deficiency of each receptor leads to a partial defect in thrombus formation [90]. Thus, it is widely accepted that α2β1 and GPVI function synergistically for optimal platelet adhesion and activation by collagen.

After the initial platelet adhesion and deposition on vWF and collagen exposed surfaces, the next step in thrombus formation is the recruitment of additional platelets from the flowing blood to form the platelet plug, called the “extension phase”. This process requires the localized accumulation of soluble agonists that are secreted by adherent-activated platelets, including ADP, TxA2, epinephrine, and thrombin. The final step is activation of αIIbβ3, causing a conformational change that
enables it to bind fibrinogen and vWF, allowing stable bridges between platelets that form large aggregate at the site of vascular damage. GPCRs or G-protein coupled receptors are important seven-transmembrane spanning receptors that play crucial roles in this extension phase.

**Platelet ADP receptors: P2Y<sub>1</sub>, P2Y<sub>12</sub>, P2X<sub>1</sub>**

When the adherent platelets get activated and release their cargo content, the dense granules which are pre-docked on the plasma membrane, are the first ones to be exocytosed [91]. ADP is stored in platelet dense granules and gets released upon activation at the site of injury. Red blood cells also release some ADP. ADP, though being a weak agonist, still causes the full range of platelet activation events including intra-platelet Ca<sup>2+</sup> influx, thromboxane A<sub>2</sub> synthesis, protein phosphorylation, shape change, granule secretion, and activation of α<sub>IIbβ3</sub>, and aggregation. These events are driven by ADP interacting with two types of purinergic GPCRs namely P2Y<sub>1</sub> and P2Y<sub>12</sub> coupled to G<sub>q</sub>α and G<sub>i</sub>α, respectively [92].

**P2Y<sub>1</sub> receptor**

P2Y<sub>1</sub> receptor couples with G<sub>q</sub>α to mobilize calcium and is responsible for initiation of platelet aggregation and shape change when ADP binds to it [93]. P2Y<sub>1</sub> can also couple to G<sub>12/13</sub> and can activate platelets via Rho Kinase and Calcium [94]. A3P5PS, a P2Y<sub>1</sub>-specific inhibitor, blocks shape change and calcium signaling in response to 2MeSADP, a stable ADP analog, without affecting cAMP downregulation [95]. Platelets from P2Y<sub>1</sub>-deficient mice are unable to aggregate in response to usual concentrations of ADP and display defective aggregation to other agonists, while high concentrations of ADP (together with serotonin) induce platelet aggregation without shape change. These mutant mice do not present a bleeding diathesis but are resistant to thromboembolic mortality induced by injecting ADP or collagen and adrenaline [96].

**P2Y<sub>12</sub> receptor**

ADP activation of P2Y<sub>12</sub> results in amplification and sustenance of aggregation, and secretion but plays no role in ADP-induced shape change [98]. In addition to amplification of P2Y<sub>1</sub>-mediated platelet responses, P2Y<sub>12</sub> plays an essential role in potentiating platelet responses initiated by other important platelet activators such
as thromboxane A₂ and thrombin and therefore, is a critical regulator of hemostasis and thrombosis [92]. This second ADP receptor couples to Gᵢ to inhibit adenylate cyclase and cause platelet aggregation [97]. Activation-based signaling downstream of the P₂Y₁₂ receptor potentiates agonist-induced dense granule release as well as alpha granule release of P-selectin [99, 100]. P₂Y₁₂ coupling to Gᵢ also leads to activation of PI3K, Akt, Rap1b and potassium channels [99]. Both Rap1b and Akt are signaling mediators that contribute to platelet aggregation and are activated in a PI3K-dependent manner. Inhibiting P₂Y₁₂ receptor reduces thrombus formation without affecting its stability [101, 102]. In P₂Y₁₂-deficient mice, ADP does not induce aggregation and platelet responses to other agonists are dramatically reduced; these mice have a bleeding phenotype as well as reduced thrombus formation and resistance to thromboembolism [102, 104]. While congenital P₂Y₁₂ receptor deficiency is a rare autosomal recessive disorder, it presents with a bleeding diathesis, though the severity of it varies from patient to patient [103].

Given its well-established importance in hemostasis and thrombosis, the P₂Y₁₂ receptor is the target of a host of anti-platelet drugs such as the thienopyridine class of drugs (ticagrelor, clopidogrel, cangrelor, elinogrel, and prasugrel) [105]. These antagonists bind to the receptor irreversibly and reduce platelet function. Some of these are already in use as antithrombotic while others are in development. These drugs are routinely used together with aspirin which inhibits TxA₂ synthesis in platelets and thus provides additional benefits of reducing platelet function. Of these, clopidogrel, marketed as Plavix, remains the most widely used anti-thrombotic to prevent vascular events in patients with cardiovascular diseases, especially in the case of coronary stent insertion.

An important aspect of both the P₂Y₁ and P₂Y₁₂ receptors is their ability to become desensitized to ADP. This serves to regulate platelet reactivity and control thrombus formation. Once initially activated by ADP, there is a transient loss of platelet responsiveness to a second stimulation by ADP by selective desensitization of the P₂Y₁ receptor [106]. The P₂Y₁₂ receptor remains functional and conserves its ability to amplify the platelet aggregation induced by other agonists, suggesting that the two receptors are regulated differently [107]. Platelets, at the site of vascular injury,
use receptor desensitization as means to regulate responses when ADP, released from red blood cells, is the sole agonist in the medium, a situation when P2Y₁ comes to play. Then, when platelets become refractory to ADP, P2Y₁₂ still remains viable and can respond to other agonists thus ensuring platelets’ responsiveness and preserves the hemostatic function of the platelets [92]. Furthermore, surface levels of P2Y receptors and consequently ADP responsiveness is regulated through endocytic trafficking [148]. Endocytic trafficking of P2Y receptors allows platelets to be desensitized and resensitized to ADP. It has been shown that P2Y₁ and P2Y₁₂, upon ADP stimulation, rapidly desensitize in a kinase-dependent manner [149]. Endocytosis of P2Y₁ is dependent on protein kinase C (PKC) activity, whereas P2Y₁₂ requires G protein-coupled receptor kinase (GSK). After removal of agonist, P2Y receptors recycle back to the cell surface, a process that is called resensitization. ADP-ribosylation factor 6 (Arf6), a small GTPase that is present in platelets and is key for integrin recycling [19], has been suggested to be important for P2Y₁₂ internalization by regulating Nm23-H1, an NDP (nucleoside diphosphate) kinase which facilitates dynamin-dependent fission of clathrin-coated vesicles [150].

**P2X₁ receptor**

In addition to P2Y₁ and P2Y₁₂ receptors, platelets express a third purinergic receptor P2X₁. This receptor is actually an ATP-gated cation channel [108] that is unable to trigger platelet aggregation by itself, but under high shear conditions acts as a positive regulator of platelet responses to collagen and thus plays a significant role in thrombus formation [109]. P2X₁-deficient mice have normal bleeding times but are resistant to thromboembolism upon collagen-epinephrine injection as well as to localized laser-induced injury of the vessel walls of mesenteric arteries [109]. Conversely, mice overexpressing the human P2X₁ receptor are more prone to increased systemic thrombosis [110]. The P2X₁ antagonist NF449 [111] inhibits platelet function *ex vivo* and *in vivo* thrombus formation [112]. Together, these data strongly suggest the effectiveness of P2X₁ antagonists as potent anti-thrombotic drugs especially in patients with severe stenosis where shear forces are high. Amongst the soluble agonists secreted at the site of injury, thromboxane A₂, is one of the key prostanoids secreted by activated platelets through the sequential
activities of cyclooxygenase (COX) and TxA₂ synthase enzymes [117]. TxA₂, an unstable arachidonic acid metabolite, is a vasoconstrictor and a potent platelet activator causing shape change, phosphoinositide hydrolysis, calcium mobilization, protein phosphorylation, secretion and aggregation [113]. Once synthesized, it diffuses through the plasma membrane and activates other recruited platelets, thus allowing the platelet plug formation. The receptor that TxA₂ binds to on platelets is the thromboxane A₂ receptor TP [114].

**Thromboxane A₂ (TP) receptor**

TP exists in two splice variants (TPα and TPβ) which differ mostly in their C-terminal cytoplasmic domains though their ligand-binding properties are the same. TPα was cloned from the placenta [115] while TPβ was cloned from endothelial cells [116]. Platelets contain both these isoforms [118]. TPs in platelets couple to G_qα and G_{12}α activating pathways, but not to G_oα, activating the phosphoinositide hydrolysis (leading to IP₃ formation), mobilization of calcium and activation of Rho GTPases when activated by the thromboxane A₂ analog, U46619 [119, 120]. Mice deficient in TP are unable to respond to TxA₂ analogs, have reduced response to collagen and have prolonged bleeding times as well as altered vascular responses to arachidonic acid a TxA₂ [121]. Blocking TxA₂ synthesis using antagonists/inhibitors especially those that inhibit cyclooxygenase enzymes and thromboxane A₂ synthase, such as aspirin (irreversibly inhibits COX-1 and modifies the enzymatic activity of COX-2) have been used for decades to manage high cardiovascular risk [122]. Aspirin still remains one of the most widely used drugs for preventing and managing cardiovascular diseases. Aspirin’s importance was honored when John Robert Vane, a British pharmacologist, won the Nobel Prize in Physiology or Medicine in 1982, for his landmark discovery in showing the inhibitory effects of aspirin on thromboxane and prostaglandin production, essentially being one of the most important anti-platelet drugs to be discovered.

The next major and a most important agonist that comes into play at promoting and stabilizing the growing thrombus at the site of damage under all shear conditions is thrombin [58]. Thrombin is a multifunctional serine protease that has both anticoagulant and procoagulant properties, being the only one of all the coagulation
cascade proteins to have the ability to regulate normal hemostasis and spurious uncontrolled thrombosis [147]. Thrombin is generated by the action of several members of the coagulation cascade on the surface of the platelets, namely tenase complex (FIXa in complex with FVIIa, which activate FX) and by the prothrombinase complex (FXa in complex with FVa, which activate FII) [123]. These coagulation factors together with the activated platelet surface which provides exposed phosphatidylserine, creates a procoagulant surface that allows thrombin generation to occur [124].

Thrombin is the most potent platelet activator and generates full blown platelet activation in terms of platelet shape change, secretion, TxA2 generation, Ca2+ mobilization, protein phosphorylation and aggregation [58, 56]. Even low concentrations of thrombin (0.1 nM) can activate platelets [56]. Thrombin can efficiently couple to PLCβ activation, leading to rapid increase in cytosolic Ca2+ levels, triggering downstream events such as activation of PLA2 [125]. The thrombin-induced platelet responses are mediated partially by the GPIb-IX-V complex [126, 127, 68-69]. However, thrombin acts on platelets mainly through the action of two protease-activated receptors (PARs), PAR-1 and PAR-4 in humans and PAR-3 and PAR-4 in mice [128].

**Protease-activated receptors (PARs)**

PARs belong to the seven transmembrane domain containing G-protein coupled receptors that are present on the surface of platelets and are sufficient to activate platelets by themselves in the presence of thrombin. To date, four PAR receptors have been identified and characterized, three of which PAR-1, PAR-3, and PAR-4 are substrates for thrombin while PAR-2 is activated by mast cell tryptase, trypsin and factor Xa but not by thrombin [134-140]. There are four PAR members on platelets, PAR-1 and PAR-4 in human platelets while PAR-4 is the major mouse platelet PAR receptor with PAR-3 acting as a co-receptor [129]. Since thrombin generation requires some level of platelet activation (usually ADP, TxA2-mediated), PAR-mediated activation occurs after platelet adhesion and activation.

PAR-mediated activation requires irreversible proteolytic cleavage within the first N-terminal extracellular loop of the seven transmembrane domain receptors that
contain a putative thrombin cleavage site LDPR/S [130]. Upon cleavage by thrombin, a new N-terminus is exposed that now serves as a tethered ligand. Studies were done on PAR-4 knockout mice, PAR-4 being the major thrombin receptor in mouse platelets, showed prolonged bleeding times and were protected in the FeCl₃ injury model of arterial thrombosis; the platelets from these mice were unresponsive to thrombin, even at higher concentrations, with no observed shape change, ATP release from dense granules or aggregation [131]. In PAR-3 knockout mice, thrombin responses were abnormal as in low levels of thrombin did not elicit any response in terms of ATP release and aggregation, while higher concentrations elicited markedly delayed responses; bleeding times were normal in these mice [132]. Interestingly, platelets from PAR-3 knockout mice responded normally to a PAR-4 activating peptide mimetic, suggesting that while PAR-3 is necessary for normal responses, it is not the major thrombin receptor in mouse platelets. Furthermore, it leads to a dual-receptor model for platelet activation by thrombin that has important implications for developing anti-thrombotic drugs.

Understanding the role of the thrombin receptors on human platelets becomes key to developing therapeutic strategies against myocardial infarction and stroke. Similar to mice, a dual-receptor system for thrombin-induced platelet activation is also present in humans, mainly through the actions of PAR-1 and PAR-4. PAR-1 is the major thrombin receptor in human platelets [134]. The PAR-4 activating peptide can also activate human platelets suggesting that PAR-4 also plays a secondary but important function. Blockade of PAR-1 with antibodies or specific antagonists ablates platelet function even at low concentrations of thrombin, whereas similar blockade of PAR-4 at low thrombin concentrations has no inhibitory effect [133]. Blocking or inhibiting both PAR-1 and PAR-4 receptors results in complete ablation of platelet response to even very high concentrations of thrombin [133]. Further support for this dual receptor model in human platelets can be gleaned from studies on intracellular cytosolic calcium transients using PAR agonists and antagonists. Human platelets have a biphasic calcium response, the first phase being a rapid phase where intracellular cytosolic calcium levels spike in response to PAR-1 activation (by thrombin) and the second more sustained phase of calcium transient is maintained.
by PAR-4 activation [141]. Studies using PAR-1 knockout mice revealed that disruption of the murine PAR-1 gene causes 50% embryonic lethality at E9-10 days, while the other half of mice developed normally with no hemorrhagic diatheses. PAR-1 knockout platelets normally respond to thrombin and have normal hemostasis, mainly because PAR-3 and PAR-4 thrombin responses are unaffected. However, these mice have defective vasculogenesis in their yolk sac and cardiovascular collapse leading to early embryonic lethality [142]. While PAR-3 and PAR-4 knockout mice are protected against thrombosis and demonstrate defective thrombus formation, direct PAR-1 inhibition in primate models also abolishes arterial thrombosis [131, 143-144]. PARs provide an important link between hemostasis and inflammatory pathways [145] due to the pro-inflammatory nature of thrombin. PARs have been implicated in thrombin-mediated activation of endothelial cells by triggering release of vWF from the Weibel-Palade bodies (granules that store vWF within the endothelial cells) and surface expression of P-selectin that facilitates rolling and adhesion of platelets and leukocytes on the endothelial surface as well as release of interleukins-6 and 8, thereby mediating inflammation [128, 145-146]. PARs have also been implicated in atherothrombosis [128].

After thrombin-mediated PAR activation and engagement in the formation of the growing thrombus, the last phase that finally leads to a stable thrombus at the site of injury to arrest blood loss, is called the “stabilization phase”. In this phase, activated platelets within the developing platelet plug come into close contact with each other, aided by fibrin fibers that form bridges closing the gap between nearby platelets and facilitating the formation of the thrombus. Once aggregation has started, a series of late signaling events sets in to consolidate the stable thrombus and prevent its embolization. These contact-dependent signaling events that drive this late phase of thrombus formation is mainly mediated by the outside-in signaling through the platelet integrins, particularly α\textsubscript{IIb}β\textsubscript{3}.

**Integrins**

The integrins are a major class of adhesive and signaling receptors that are present on most cell types consisting of two subunits, α subunit and β subunit [151]. Vertebrates have 18 α subunits and 8 β subunits, generating 24 heterodimers that
are non-covalently associated [152]. Integrins have a large extracellular domain, a single transmembrane domain from each subunit, and small cytoplasmic tail, although α and β subunits have distinct domain structures. Both subunits contribute to the ligand-binding site in their extracellular domain, with specific binding ligand for individual integrin. Integrins exist in two affinity states, low and high, which are altered by cytoplasmic signaling and phosphorylation of their cytoplasmic domains [153].

Platelets express integrins from three families (β1, β2 and β3) and in total six different integrins: α2β1, α5β1, α6β1, αIIbβ3, and αIβ3 [154].

**β3 family (αIIbβ3 and αvβ3)**

Integrin αIIbβ3, or the GPIIb-IIIa complex, is the only integrin that is exclusively expressed on platelets and megakaryocytes [155]. There are about 80,000 copies of αIIbβ3 on the surface of quiescent platelets and additional heterodimers in the membranes of platelet granules are translocated to the surface during platelet activation [156-157]. αIIbβ3 is the major receptor for fibrinogen, vWF, fibronectin and vitronectin and is essential for platelet aggregation [158]. As a result, absence or deficiency of αIIbβ3 prevent platelet aggregation and leads to sever bleeding disorder called Glanzmann’s thrombasthenia [159]. Conversely, the thrombi formed during arterial circulation result from αIIbβ3-mediated formation of platelet aggregates [160]. Given the importance of αIIbβ3 in mediating hemostasis and thrombosis, it is one of the most studied integrins and most targeted receptor for anti-thrombotic therapies.

Integrin αvβ3

This integrin is present in platelets in small amounts (several hundred copies per platelet) and is known to mediate platelet adhesion to osteopontin and vitronectin in vitro [161-162]. Integrin αvβ3 supports adhesion on fibronectin and promotes the formation of filopodia but not lamellipodia or downstream focal adhesion kinase (FAK) phosphorylation [163]. However, the in vivo role of integrin αvβ3 is unknown.

**β1 family (α2β1, α5β1, and α6β1)**

Integrin α2β1
Also known as GPIa-IIa and on lymphocytes as VLA-2, it is the second most important integrin after GPIIb-IIIa on platelets [164]. There are 2000-4000 copies per platelet and is the major collagen receptor on platelets as well as other cell types.

Integrin $\alpha_5\beta_1$

The extracellular matrix protein, fibronectin, is the major ligand for the integrin $\alpha_5\beta_1$ and supports static platelet adhesion on fibronectin [163, 165]. Soluble fibronectin binds to integrin $\alpha_{\text{IIb}}\beta_3$ on thrombin-activated platelets and this binding is not observed with platelets from Glanzmann’s thrombasthenia patients that lack $\alpha_{\text{IIb}}\beta_3$. However, these platelets can still retain the ability to bind to fibronectin-coated surfaces as adhesion to fibronectin does not require platelet activation and is in fact inhibited by soluble fibronectin [166]. Another hallmark of platelet-fibronectin interactions is that it is shear stress-dependent and as a result platelet adhesion to fibronectin is thought to occur in large arterioles where the shear stress is considerably less [167].

Integrin $\alpha_6\beta_1$

$\alpha_6\beta_1$ is thought to be the principal laminin receptor on platelets and has a supplementary role in platelet adhesion at injury sites [170, 174]. Platelet adhesion to laminin strongly depends on the presence of divalent cations like Ca$^{2+}$ and Mg$^{2+}$ and low shear rates (about 800/s) but does not lead to aggregation although filopodia and lamellipodia formation does occur [168-169]. Laminin stimulates platelet spreading through $\alpha_6\beta_1$-dependent activation of the collagen receptor, GPVI [174]. Lamellipodia formation on laminin is completely inhibited in the absence of GPVI, while filopodia formation remains viable and is thought to be mediated via $\alpha_6\beta_1$ [174].

$\beta_2$ family ($\alpha_4\beta_2$)

Integrin $\alpha_4\beta_2$

Quiescent platelets express low to variable expression levels of the integrin $\alpha_4\beta_2$, also known as CD11a/CD18 (LFA-1). However, activation of platelets leads to a marked increase in the levels of this integrin, suggesting that this integrin is expressed on granule membranes, which upon granule fusion with the plasma membrane gets exteriorized, in a manner similar to the alpha granule marker, P-selectin [171].
Studies done on mice further revealed that stimulation of thrombocytopoiesis (production of new platelets) leads to loss of β₂, but not β₁ or β₃ integrins, from the newly released platelets and there appears to be a selective loss of β₂ integrin from platelets in circulation [172]. Further studies using the β₂ integrin knockout mice, demonstrated that β₂ modulates caspase activation and consequently platelet life span and response to inflammatory cytokines such as Tumor Necrosis Factor (TNF) [173].

Platelet outside-in signaling, mainly mediated through fibrinogen binding to αᵢλβ₃, triggers key events for thrombus growth and stabilization, such as cytoskeletal reorganization, formation and stabilization of large platelet aggregates, development of a procoagulant surface and clot retraction that helps in bridging the gaps between platelets and increase the local concentration of soluble platelet agonists [175]. At the heart of this growing thrombus, where the concentrations of the soluble agonists (mainly thrombin) is highest is called the core and consists of fully activated platelets, while the surrounding region is overlaid by what is called the shell comprising of minimally activated platelets [176-178]. While the integrins are the main players in promoting the continued growth and stability of the hemostatic thrombus, other participants include cell adhesion molecules (junctional adhesion molecules, JAM-A and JAM-C) that provide cohesive and signaling interactions between adjacent platelets and leukocytes favoring thrombus stabilization [179]; receptor tyrosine kinases such as the Ephrin family of kinases (EphA4, EphB1, and ephrinB are present in human platelets) that cluster to cause platelet adhesion on fibrinogen [180]; secreted agonists and protein fragments that are shed from the surface of activated platelets such as CD40 ligand (CD40L, CD154). CD40L is shed from activated platelets to produce a soluble form, sCD40L that can bind to αᵢλβ₃ through the Arg-Gly-Asp motif and favors outside-in signaling that drives thrombus growth and stability [181].

**Receptors involved in the negative regulation of platelet activation and thrombus growth**

To prevent uncontrolled thrombus growth at the site of injury that grows into an occlusion that covers the entire blood vessel, several negative regulators or passivators of platelet activation exists. Chief among them are nitric oxide (NO) and
prostacyclin (PGI$_2$). PGI$_2$, produced by the vascular endothelium, is a labile metabolite of arachidonic acid and acts as a potent vasodilator in coronary circulation as well as dampens platelet activation by inhibiting platelet adenylyl cyclase and preventing formation of cAMP [182]. Endogenous nitric oxide has been shown to inhibit platelet adhesion to the vascular endothelium and protects against platelet aggregation in stenosis and endothelium-injured arteries [183-184]. Platelet activation can also be blocked by the adhesion molecule, platelet endothelial cell adhesion molecule, PECAM-1 or CD31, a member of the Ig superfamily that contains an immunoreceptor tyrosine-based inhibitory motif (ITIM) that gets phosphorylated upon platelet activation. PECAM-1 plays a vital role in attenuating thrombus formation involving GP VI, GP Ib, thrombin-mediated activation of platelets as well as $\alpha_{\text{IIb}}\beta_3$-mediated platelet activation [185-187]. Another mechanism to arrest unregulated thrombus growth is by the action of the matrix metalloprotease ADAMTS13, which cleaves highly adhesive large vWF multimers that are formed on the surface of the growing thrombus. ADAMTS13 works as an anti-thrombotic by down-regulating both platelet adhesion to damaged endothelium and thrombus formation in injured arterioles [188].

**Platelet exocytosis**

Activation-dependent platelet exocytosis, also called the “platelet release reaction”, is at the core of several physiological and pathophysiological processes such as thrombosis, wound healing, angiogenesis, and inflammation among others [245]. Activated platelets release their granule contents that consist of ADP from dense granules (activates other platelets in the immediate vicinity in a paracrine manner), fibrinogen and vWF from alpha granules that modulates thrombus formation and stability, various cytokines, chemokines and growth factors that allows communication with other cell types (endothelial cells, leukocytes etc.) as well as several lytic enzymes from lysosomes that are thought to modulate clot remodeling. Platelet secretion, its kinetics, extent, and release composition, appears to have no functionally thematic pattern, with only agonist potency driving these parameters for the release reaction [91]. However, there are distinct kinetic patterns or waves in cargo release with dense granule cargo being the fastest and most important in
mediating hemostasis, followed by the secondary wave of alpha granule release and then by lysosomal release being the slowest. Distinct kinetic patterns of the granule releasates allow platelets to control their microenvironment. Platelet granule secretion provides a concentrated release of key effector molecules at the site of injury that allows platelets to carry out their functions. Secretion of more than 300 proteins and bioactive small molecules [189-190] from activated platelets requires a cascade of complex protein-protein interactions that are tightly regulated and govern this release reaction. The Soluble N-ethylmaleimide Sensitive Fusion Protein Attachment Protein Receptor (SNARE) proteins are the core machinery [194] that drives the process of granule-plasma membrane fusion in platelets, using mechanisms similar to those found in other secretory cells such as neurons, chromaffin cells, mast cells, neutrophils etc. Platelet exocytosis involves the movement of granules to juxtapose with the plasma membrane, leading to granule-plasma membrane fusion, and release of intracellular contents. Within the platelet itself, granule-granule fusion may occur in a process called compound fusion which may or may not precedes fusion with the plasma membrane [191-192]. SNAREs can be classified into two groups based on their location, v-SNAREs for the ones residing on the granules and vesicles, t-SNAREs for target-membrane localization. Extensive sequence analysis led to a further classification of the SNAREs based on amino acids present within their SNARE motifs, called the QabcR system [193]. All SNAREs contain 1-2 amphipathic, heptad-repeat-containing cytosolic domain called the SNARE motif consisting of 60-70 amino acids. Cognate v- and t-SNAREs interact with each other by binding to these SNARE domains. Specificity of binding is conferred by a highly conserved 16 amino acids stretch which lay up- and down-stream of an ionic “zero” layer. This zero layer invariably consists of three glutamines (Q) and an arginine (R) residue. The v-SNAREs also called Vesicle-Associated Membrane Proteins (VAMPs) make up the majority of the R-SNAREs while the t-SNAREs are mostly Q-SNAREs. The t-SNAREs can be subdivided into two classes of proteins; the Syntaxins (Qa-SNAREs) and the Synaptosome Associated Proteins (SNAP-25/23 proteins) (Qbc SNAREs) [195-196]. SNARE-driven exocytosis in platelets proceeds in conformation to the SNARE hypothesis which states that members of the v- and t-SNAREs form
cognate pairs on the basis of binding specificity and should be essential and sufficient for vesicle docking in vivo [197]. Accordingly, four SNARE motifs, one each from Qa and R and two from Qbc families form a functional four-helical complex that undergoes a \textit{cis} to \textit{trans} conformational change that brings the granule membrane and plasma membrane in close physical apposition and drives fusion [197]. Previous studies done by our lab and others have established the importance and contribution of several of these platelet SNARE proteins and their contributions to hemostasis and thrombosis.

\textbf{Platelet v-SNAREs}

SNAREs were first reported by our group when NSF, SNAP and Syntaxin-2 and Syntaxin-4 were detected in platelet lysates [198]. Platelets contain multiple isoforms of the VAMPs (VAMP-2,-3,-4,-5,-7,-8), based on data gleaned from proteomic analyses and quantitative Western blotting [199-200]. In terms of a number of copies per platelet, VAMP-8 and VAMP-7 are the most abundant in human platelets while VAMP-8, VAMP-2, and VAMP-3 are the major ones in mouse platelets. VAMP-8 which is the most abundant in both mouse and human platelets is the primary v-SNARE to mediate secretion. Loss of VAMP-8 attenuates secretion from all three granules, causes defective thrombosis (thrombus growth is slower and lessened in comparison to WT) with no bleeding diathesis [199, 201]. Loss of VAMP-3 by itself had no defect in secretion and hemostasis proceeded normally as measured by tail bleeding assay [202]. Loss of VAMP-2 is embryonic lethal [201]. Treatment of tetanus toxin which specifically cleaves VAMP-2 and VAMP-3 showed that platelet secretion is unaffected as long as VAMP-8 and VAMP-7 are present [201]. Loss of VAMP-7 does not lead to a bleeding diathesis or defects in platelet accumulation at sites of injury in the laser injury model of arterial thrombosis, although alpha granule secretion is attenuated in these platelets [203]. Collectively, these data implies there appears to be an order of ranking amongst the isoforms in mediating secretion with some amount of redundancy. VAMP-8 is primary with VAMP-2 and VAMP-3 playing secondary roles. VAMP-7 on the other hand may be less involved in secretion but mainly play a part in platelet spreading due to its interactions with the actin cytoskeleton via VPS9-domain Ankyrin-repeat containing protein (VARP) and Arp2/3.
Immunofluorescence studies have shown VAMP-7-dotted structures to be localized to the platelet periphery while VAMP-8 and VAMP-3 have been shown to be more centrally localized within the granulomere in spread platelets [204]. The central localization of VAMP-3 concurs with studies done in our lab where we reported the intracellular punctate staining of VAMP-3 containing structures that resist being targeted to the plasma membrane during thrombin activation [205]. These could very easily be VAMP-3 positive endosomes in platelets. Additionally, VAMP-8 and VAMP-7 could potentially mediate differential cargo release or may be differentially responsive to various kinds of agonists. However, future studies need to be done to parse this distinction.

**Platelet t-SNAREs**

While SNAP-25 is the major Qbc SNARE in neuronal cells, most hematopoietic cells including platelets rely on SNAP-23 as the functionally relevant t-SNARE [206-207]. Platelets also contain SNAP-25 [200] and SNAP-29 [208] but to lesser amounts. Platelets contain Syntaxins 2, 4, 6, 7, 8, 11, 12, 16, 17 and 18 based on proteomic analyses and functional studies [200]. Of the Syntaxins or Qa-SNAREs, Syntaxin 11, is the most important for platelet secretion. Platelets isolated from Familial Hemophagocytic Lymphohistiocytosis type 4 (FHL4) patients, which lack Syntaxin 11, were deficient in secretion from all three granules [209]. Co-immunoprecipitation analyses showed that upon stimulation, Syntaxin 11, complexed with SNAP-23 and VAMP-8 to form the fusogenic SNARE complexes. While previous secretion studies in permeabilized platelets using inhibitory antibodies and peptides showed the importance of Syntaxin 2 and 4 in platelet secretion [206-207, 210-212], both Syntaxin 4 single knockout mice and Syntaxin 2/4 double knockout showed no secretion defect [209, Ye unpublished data]. The previously reported incorrect results were due to the original Syntaxin 2 antibodies cross-reacting with Syntaxin 11 to inhibit secretion [209]. As mentioned before, Syntaxin 8 is present in platelets and has been shown to regulate dense core granule release, platelet aggregation and thrombus stability [213].

**SNARE regulatory proteins**
Several regulatory proteins affect the functions of the SNAREs by controlling their fusogenicity either temporally or spatially. Some of these SNARE regulators either function as chaperones or tethering/docking factors or modulate the SNAREs via post-translational modifications.

SNARE chaperones

**Munc18b**: Members of the Sec1/Munc 18 (SM) gene family regulate interactions between the various SNAREs. The Sec1/Munc 18 proteins bind to the Syntaxins and form a Syntaxin-Munc18 complex that regulates vesicle priming and potentially modulates the conformation of the SNARE proteins [214-215]. Platelets contain Munc18a, Munc18b, Munc18c (STXBP1, 2, 3) with Munc18b being the most abundant of these three [216]. Platelets isolated from Familial Hemophagocytic Lymphohistiocytosis type 5 (FHL 5) patients, which have defective Munc18b, had severe secretion deficits showing the importance of Munc18b in mediating secretion from all three different granules [216]. In platelets, Munc18c has been shown to interact with Syntaxin 4 and thereby contribute to platelet secretion [217]; however, platelets from heterozygous Munc18c mice showed normal secretion indicating that Munc18c loss does not affect secretion to a great extent [218]. Munc18s have also been reported to be phosphorylated in platelets and this is thought to affect their interactions with Syntaxins [219-220]. Platelets also contain Vacuolar Protein Sorting-associated protein 33A and B, VPS33A and VPS33B, which are members of the SM family and are important for dense and alpha-granule biogenesis respectively [221-222]. VPS33B has been recently shown to bind to the integrin β subunit and can modulate αβ-mediated fibrinogen endocytosis, platelet activation, aggregation, spreading, clot retraction and *in vivo* thrombosis and hemostasis [223].

**STXBP5/Tomosyn 1**: Syntaxin Binding Protein 5 or Tomosyn 1 is a t-SNARE regulator that interacts with the t-SNARE heterodimers (e.g., Syntaxin 11/SNAP-23) through its v-SNARE-like domain, and is thought to be a “place-holder” for the t-SNAREs [224-225]. In platelets, STXBP5 binds only to t-SNAREs and loss of this protein causes platelet secretion in mostly alpha granules and lysosomes, with a little defect in dense granule secretion. STXBP5 knockout mice also have a severe bleeding defect [226].
**Tethering/docking factors**

**Rab27**: Platelets contain both the isoforms of Rab27, Rab27a, and Rab27b, which are small GTPases that mediate granule docking and tethering in secretory cells [227]. Loss of Rab27a in mouse platelets does not cause any bleeding defects. When these mice were crossed with a Rab27b knockout, both the double knockout and the Rab27b single knockout mice had a robust bleeding phenotype as well as a reduced secretion from dense granules. Additionally, the number of dense granules per platelet and the serotonin content within the dense granules was reduced in these Rab27b and Rab27a/b double KO strains suggesting a role for Rab27 in dense granule packaging at the megakaryocyte level [228]. Rab27a could partially compensate for the secretion deficit but not the reduced granule number, indicating there exists some level of compensation between these two homologs within platelets.

**Munc13-4**: Protein unc-13 homolog D, commonly known as Munc13-4, belongs to a family of multi-domain proteins that contain the Munc Homology Domain (MUN) domain and two calcium/lipid-binding C2 domains and act as “priming or docking factors” in driving SNARE-mediated fusion. Munc13-4 is highly expressed in cells of the immune system and platelets where it acts as a Rab27a-binding protein that regulates dense-core granule release [229]. Munc13-4 was the first identified priming factor that promotes Ca^{2+}-dependent SNARE complex formation and SNARE-mediated liposome fusion [230]. Cytotoxic T lymphocytes deficient in Munc13-4 from familial hemophagocytic lymphohistiocytosis type 3 (FHL3) patients fail to exocytose docked cytotoxic granules, suggesting a role for Munc13-4 in granule priming [231]. Munc13-4 knockout platelets have a robust secretion defect as well as severe bleeding phenotype and acts as a limiting factor in driving fusion of docked granules [232]. Loss of Munc13-4 has a more pronounced effect on dense granule release than on alpha granules and lysosomes. Furthermore, the addition of ADP has shown to override the apparent alpha granule secretion deficiency in Munc13-4 knockout platelets [233], implicating the role of ADP as an autocrine factor that modulates alpha granule and lysosome release.

**NSF and SNAPs**: N-ethylmaleimide-sensitive factor (NSF), an AAA\(^+\) ATPase, and its adaptor proteins, Soluble NSF attachment proteins (SNAPs), are proteins that
disassemble spent SNARE complexes for recycling [234-237]. NSF and SNAPs are present in platelets [198]. Inhibitory peptides and antibodies that block alpha-SNAP-stimulated ATPase activity of NSF impede secretion from permeabilized platelets [238]. Nitric oxide inhibits platelet secretion partly by the reversible S-nitrosylation of NSF [239]. Since platelet secretion is a terminal event with no discernible need to recycle exhausted SNARE complexes, the role of platelet NSF is unclear. However, in light of new studies that depict platelets to be more proficient at intracellular trafficking and membrane fusion, especially in the context of autophagy and endocytic trafficking, NSF and SNAPs may certainly become more relevant and functional than previously thought [240, 19].

Post-translational modifications of SNAREs: Of the platelet SNAREs, SNAP-23 has been shown to be phosphorylated by both Protein Kinase C in vitro [241] and by IκB Kinase β (IKKβ or IKK2) [242]. Our group has shown that IKKβ, but not PKC, is the proximal kinase that drives SNAP-23 phosphorylation in platelets both in vivo and in-vitro. IKKβ-mediated phosphorylation affects SNARE complex formation and platelets from either IKKβ knockout mice or those treated with IKKβ inhibitors shown severe secretion deficiency from all three granule types and robust bleeding phenotype [242]. Agonist-induced activation causes SNAP-23 to get phosphorylated at serine 95 and serine 120 by IKKβ in platelets. Data generated in this thesis shows that this phosphorylation event holds true for most platelet agonists (thrombin, convulxin, ADP, etc as well as immune agonists such as Toll-like Receptor ligands) implicating that this is an essential step that precedes granule secretion. Phospho-SNAP-23 can thus be used as readout for activation-induced secretion in platelets. Acylation is another common post-translational modification of SNARE proteins. Thioester-linked fatty acids attached to cysteines present within several platelet proteins have been reported in proteomic analyses [243] and this active acylation appears to be agonist-induced and important for platelet function and in vivo thrombus formation [244]. Two potential SNAREs that could be acylated in platelets are Syntaxin 11 and SNAP-23. Both are membrane-anchored and yet lack classical transmembrane domains. The presence of a cysteine-rich domain at their C-terminus that can get readily acylated, allows them to be appropriate candidates for using
acylation to tether to the plasma membrane and disruptions in acylation leads to defects in platelet secretion and aggregation [Zhang J, in prep, 2017].

**Signaling events during activation-exocytosis coupling**

Platelets exocytosis is uniquely coupled to signaling events triggered by activation. Resting platelets contain *cis*-SNARE complexes that prevent the non-specific release of cargo in the absence of any activating stimulus. However, when platelets are activated by agonists such as thrombin, collagen, ADP, thromboxane A₂, phosphatidylinositol-4, 5-bisphosphate (PIP₂) present in the platelet membranes gets cleaved to form diacylglycerol and inositol-1, 4, 5-trisphosphate (IP₃). Diacylglycerol (DAG) activates several forms of Protein Kinase C while IP₃ increases the intracellular Ca²⁺ concentration, triggering platelet secretion [246-249]. Increases in intracellular Ca²⁺ are sufficient to induce platelet secretion but PKC also plays a key role, both being critical second messengers in this process and use of PKC inhibitors has been shown to block secretion [250]. How calcium regulates platelet secretion still remains a mystery, simply because no definite calcium sensors (Synaptotagmins) haven been detected in platelets. Although several proteins containing calcium-binding C2 domains (synaptotagmin-like domains) are present and can potentially work as calcium sensors, yet definitive studies are yet to be done.
Subsection Two: Intracellular trafficking in platelets

Eukaryotic cells are surrounded by a plasma membrane that serves as a partition between the inside of the cell and its outside. Membrane-bound compartments within the cells can transport cargo molecules (proteins and lipids) both into and out of the cell at the plasma membrane interface in order to communicate with their external environment. This process of cargo trafficking is key to the normal function, growth, and survival of cells. These membrane-bound compartments or vesicles traffic cargo via two major pathways: the outwards or the exocytic pathway that carries intracellular contents to the outside, and the inwards or endocytic pathway, that allows uptake/internalization of cargo from the external environment to the inside of the cell. Vesicular transport, thus, is a major process by which compartments within the cell, communicate with each other and connect the cell with its external environment. Cargo-loaded vesicles can form at a donor compartment with the help of specific coat and adaptor proteins (e.g., COPI, COPII and clathrin), and then targeted to their appropriate acceptor compartments, followed by tethering and vesicle fusion to said compartments, using an expansive array of proteins including the SNAREs that oversee this highly regulated process. As proposed by the SNARE hypothesis [197], intracellular membrane fusion reactions require a specific set of SNARE proteins from one membrane (donor) to bind to their cognate SNARE proteins associated with the other acceptor membrane, to drive this process. Although there are several regulatory proteins that participate in this collaborative process, it is widely accepted that the membrane-bridging SNARE complexes are the main drivers of the cellular fusion machinery [251].

Platelets are secretory cells that release their cargo contents, upon activation, to oversee their primary functions of hemostasis and thrombosis. In light of this, platelet exocytosis has been extensively studied, with the SNAREs being the major players executing the platelet release reactions. Platelet endocytosis, on the other hand, has been largely recognized in the context of cargo loading into granules, and receptor-mediated uptake of fibrinogen and it’s biological ramifications in the context of platelet cell biology and function. The role of SNAREs in mediating platelet endocytosis, though implicitly acknowledged given its necessity, remains largely
understudied. My thesis project aims to make important inroads into this facet of SNARE-mediated endocytic trafficking in platelets.

**General overview of endocytosis**

The routes that carry cargo inside a cell from the cell surface start with the process of endocytosis, by which cells internalize macromolecules, particulates and in some cases even dead cells and pathogens. Small portions of the cell membrane invaginate and engulf the material to be endocytosed, pinching off to form endocytic vesicles that allow internalization of said material. Two main types of endocytosis are distinguished on the basis of the size of the endocytic vesicle formed. Phagocytosis or “cell eating”, involves the ingestion of large particles such as microbes or dead cells via large vesicles called phagosomes (generally >250nm in diameter) [252]. The other process is called pinocytosis or “cell drinking” and involves ingestion of fluid and solutes via small pinocytic vesicles (about 100nm in diameter) [253]. Pinocytosis is generally thought to be a constitutive process with cells ingesting small solutes and fluids continually. The third kind of endocytosis, which allows uptake of specific macromolecules from the extracellular fluid, is called receptor-mediated endocytosis. In this process, specific macromolecules bind to their complementary transmembrane receptor proteins, accumulate in coated pits, and then enter the cell as receptor-macromolecule complexes in coated vesicles [254].

**The pathways of endocytosis**

**Phagocytosis**

Phagocytosis involves ingestion of large particles such as invading microbes or dead cells and is a critical mode of response of the innate immune system. Phagocytosis in eukaryotic cells serves to provide nutrition and is required for clearing apoptotic bodies, which is key for maintaining cell turnover, tissue homoeostasis and remodeling [255]. Phagocytosis of foreign bodies and microbes such as bacteria, fungi, and viruses can be cleared from infection sites by professional phagocytes-macrophages, neutrophils, and dendritic cells, thus forming the first line of defense against infection. Phagocytosis is central to innate immunity; however, professional phagocytes can present processed/degraded phagocytosed antigens to the lymphoid
cells and initiate an adaptive immune response [256-258]. While the immune phagocytes can engulf microbes and initiate a pro-inflammatory response, phagocytosis by other non-professional phagocytes such as fibroblasts, epithelial cells, and endothelial cells mount an anti-inflammatory response. These cells, however, cannot ingest microbes, but they contribute in clearance of apoptotic bodies and help in clearance of dead cells and thereby serve to contain tissue damage [259]. Phagocytosis is a receptor-mediated event and as such numerous receptors are present on the cell surface that can engage with foreign bodies and mediate uptake. Some of the key receptors are the pattern recognition receptors that detect unique pathogen-associated molecular patterns (PAMPs) on microbes that are never found in higher eukaryotes (e.g., mannose receptor CD206, dectin-1, CD114, scavenger receptor CD204, CD36 etc.), opsonic receptors that allow opsonization of microbes (e.g., FcγRI, FcγRIIa, FcγRIIc, FcγRIIIa receptors that bind to the different IgGs), apoptotic corpse receptors that bind to apoptotic bodies and help clear them (e.g., TIM-1 and TIM-4, CD36 receptors etc.) amongst others [255].

The presence of FcγRIIa on platelet surface enables platelets to be uniquely capable of doing phagocytosis. This receptor is crucial for immune complex clearance and platelets have been shown to phagocytose immune complexes in a FcγRIIa-dependent manner [260-261]. Platelets can phagocytose bacteria such as P. gingivalis [262] and S. aureus [263-264] and it is usually thought to be inside a membrane-bound compartment or the OCS. Platelets respond to phagocytosed bacteria by generating antimicrobial peptides (defensins) that could potentially kill the bacteria. Nevertheless, what happens to phagocytosed bacteria remains far from clear. Theories have been put forth suggesting that the bacteria remain trapped within these membrane-bound compartments but remain viable [265]. This is could be a potential mechanism to evade the immune system and may play a role in disease pathogenesis.

Platelets can also phagocytose inert molecules such as latex beads that cause platelet aggregation due to the release of ADP [266]. Interestingly, longer incubations of platelets with latex beads led to the loss of granule integrity [266], though the mechanism of this is not clear.
Macropinocytosis

Another regulated form of endocytosis that mediates the non-selective uptake of solutes, nutrients and antigens. It is one of the clathrin-independent endocytosis processes and is an actin-dependent process initiated from surface membrane ruffles that give rise to distinct heterogeneous endocytic vesicles called macropinosomes that are much larger than clathrin-coated vesicles [253]. Macropinocytosis is important for cell motility which becomes key in the case of tumor metastasis due to its role in Actin-dependent membrane ruffling [267]. In the immune system, micropinocytosis becomes a way for dendritic cells and macrophages to sample their microenvironment and endocytose antigens that are then processed and presented with major histocompatibility complex (MHC) class II or class I molecules to T cells to mount an adaptive immune response [268-270]. Data generated in this thesis and others [271] have shown that platelets present antigen in the context of MHC class I. Though there are no definite reports of micropinocytosis occurring in platelets, platelets acting as antigen presenting cells could potentially utilize an endocytic mechanism similar to micropinocytosis to engulf pathogens and respond accordingly.

Clathrin-mediated endocytosis

Clathrin-mediated endocytosis also called receptor-mediated endocytosis is the most ubiquitous endocytic pathway and it requires the use of clathrin, a coat protein, which plays a major role in the formation of coated vesicles [272]. Clathrin forms a triskelion-shaped structure composed of three heavy chains and three light chains. The three heavy chains form the structural backbone of the clathrin lattice while the three light chains are thought to regulate the formation and disassembly of the polyhedral clathrin lattice. The clathrin lattice builds around the small vesicles where cargo is transported from the outside to the inside of the cell. These vesicles are called clathrin-coated vesicles (CCVs) and are formed at specialized domains of the plasma membrane called the clathrin-coated pits (CCPs) [273-274]. Based on ultrastructural and cell biology data, CCV formation occurs in five stages: initiation, cargo selection, coat assembly, scission, and uncoating. Several adaptors and accessory proteins are involved in the process. In the first stage of vesicle
budding, or nucleation, a clathrin-coated pit, in the form of a membrane invagination if formed and is triggered when the highly conserved adaptor protein 2, AP2, is recruited to the plasma membrane [275]. AP2 can bind to the cytoplasmic tails of receptors for the specific cargo being endocytosed and plasma membrane specific lipids such as phosphatidylinositol-4, 5-bisphosphate (PtdIns (4,5)P₂) and facilitates in cargo selection [276, 279-280]. Nucleation promotes polymerization of clathrin into curved lattices which aid in the formation and constriction of the vesicle neck, bridging the two membranes at the neck into close apposition. The F-BAR domain of the FCH domain only containing proteins (FCHO) proteins, which come into play during nucleation, provides the membrane-bending activity that is needed to generate curvature at the neck for the membrane to bridge close to each other [277]. Cargo selection is mainly mediated by AP2 at the plasma membrane, while other isoforms such as AP1, AP3, and AP4 are found to aid in cargo selection at the internal membranes [278]. AP2 binds clathrin and most other accessory proteins and helps in the maturation of the forming clathrin-coated vesicle. During the assembly process, clathrin triskelia get recruited at the pits where AP2 concentrations are higher. The absence of clathrin recruitment at these sites prevents maturation of the coated vesicle [277]. After the mature coated vesicle has formed, Dynamin, a small molecule GTPase, is recruited to this site by BAR-domain-containing proteins such as amphiphysin, endophilin, and sorting nexin 9 (SNX9), which have SRC homology 3 (SH3) domains that bind to the proline-rich domains of Dynamin [278-285]. Dynamin forms a helical polymer around the neck, constricting it. Clathrin polymerization facilitates a conformational change in Dynamin powered by GTP hydrolysis which mediates scission of the mature coated vesicle [286-289]. The clathrin-coated vesicles are subsequently released by proteins such as the ATPase heat shock cognate 70 (HSC70) and its cofactor, auxilin, that allows the uncoated vesicle to now travel to its targeted endosome [290-291]. Additionally, alterations in phosphoinositide composition of the clathrin-coated vesicles mediated by the phosphatase, synaptojanin, are required for the uncoating process [292]. Eventually, uncoating allows disassembly of the clathrin machinery and its adaptor proteins
from the membrane back to the cytosol for recycling in subsequent rounds of clathrin-dependent endocytosis.

Clathrin-independent endocytosis

Several endocytic pathways have now been identified that traffic cargo inside cells without using clathrin and these are collectively grouped under the clathrin-independent endocytic pathways. These pathways can be further classified based on their use of Dynamin. Some clathrin-independent (CI) pathways use Dynamin, such as those involving caveolae and Rho families of GTPases, while there are others that use members of the CDC42 and ADP-ribosylation factor (Arf) families of small GTPases to mediate vesicle scission, which is central to subsequent vesicle internalization [293].

Of the Dynamin-dependent CI pathways, caveolae-mediated endocytosis is the most studied process. It utilizes formation of caveolae at the plasma membrane, which is 50-80nm flask-shaped membrane invaginations marked by the Caveolin family of proteins [294-295]. Caveolae are enriched in cholesterol, sphingolipids, signaling proteins and glycosyl phosphatidylinositol-anchored proteins (GPI-APs) [296, 297]. Cargo transported by caveolae includes lipids, proteins, and lipid-anchored proteins. The second type of Dynamin-independent CI pathways utilizes the GTPase, RhoA, as noted in internalizing transmembrane receptors such as the interleukin-2 receptor β chain in fibroblasts and immune cells [298]. It is thought that the actin machinery is recruited through the actions of RhoA, to mediate this type of endocytosis [295].

Dynamin-independent CI pathways use either the Rho family member CDC42 or the Arf family of small GTPases, chiefly Arf6. Most GPI-anchored proteins are transported via this process using CDC42 [299]; while Arf6 mediates endocytosis of class I MHC, β1 integrins, carboxypeptidase E, E-cadherins and some other GPI-anchored proteins [300-301].

How cargo enters cells through the different CI pathways is not clear when compared to clathrin-mediated processes, simply because no well-defined adaptors or accessory proteins have been identified yet. Ubiquitination of cargo has been suggested as a possible mode to tag cargo proteins for entry via this pathway, for example, receptor tyrosine kinases such as epidermal growth factor (EGF), by the E3
ubiquitin ligase c-Cbl, preferentially enters cells via the caveolae [302]. Flotilin-1 and -2, are integral membrane proteins that mediate endocytosis of GPI-anchored proteins and cholera toxin b (CtxB) in cells that lack Caveolin [303] and can form dynamic punctate structures that allow endocytosis at sites that are distinct from clathrin and Caveolin sites at the plasma membrane [304]. Despite the discovery of many proteins that mediate the different CI pathways, the exact mechanisms are still poorly understood.

The components of the endocytic pathway

In mammalian cells, distinct membrane-bound compartments called endosomes, serve as carriers of internalized cargo molecules. They shuttle cargo from the plasma membrane to the inside (early endosomes) or recycle internalized cargo back to the cell surface (recycling endosomes), or sort them to degradative compartments such as the late endosomes or lysosomes.

**Early endosomes:** It is the starting point for cargo entry into the cell [305] irrespective of the endocytic route used (clathrin-dependent, -independent, caveolar, or Arf6-dependent pathways) [293]. They are small and have a characteristic tubulovesicular structure ranging up to 1µm in diameter and patrol the cell periphery close to the plasma membrane along microtubules [306]. The limiting membrane of early endosomes contains various subdomains that vary in their composition and function [307]. Early endosomes are identified by the presence of Rab5, Rab4 GTPases, with Rab5 being key in maintaining organelle identity, together with its effector molecule VPS34/p150, a phosphatidylinositol 3-kinase (PI3K) complex that generates phosphatidylinositol-3-phosphate (PtdIns3P) [307-308]. Rab5 oversees early endosomal maturation and eventually becomes the main regulator for conversion of early endosomes to late endosomes. Early endosomes are weakly acidic in nature (pH 6.8-5.9) [309] and contain low levels of Ca\(^{2+}\) within them [310]. The subdomains located in the tubular extensions of the early endosomes are the epicenters for molecular sorting and generate vesicles either targeted to the other cell organelles or the plasma membrane, recycling endosomes, late endosomes or the Trans-Golgi network (TGN) [311].
Recycling endosomes: Endocytic uptake and recycling are key to ensure the plasma membrane composition. Cargo that is endocytosed faces one of two fates; either they are targeted to the lysosomes for degradation or they are recycled back to the cell surface [311]. Most peripheral early endosomes are capable of recycling cargo; however, most cells have a distinct subset of endosomes, called the recycling endosomes or endosomal recycling compartment (ERC), located along the perinuclear microtubule-organizing center (MTOC) [312]. These recycling endosomes have a slightly higher pH of ~6.4 [315] and are incredibly heterogeneous in morphology and composition due to dynamic trafficking to and fro between early endosomes and plasma membrane [313-314]. Rab11 is the most important GTPase that adorns recycling endosomes [316]. Rab11 interacts with the Ral/Exocyst complex (made up of RalA/B GTPases and the exocyst complex comprising of Sec5, Sec6, Sec8, Sec10, Sec15 and Exo70 proteins), a multi-protein complex, that is essential for polarized targeting of vesicles in most cells including epithelial cells [317-319]. Recycling back to the cell surface occurs either through the faster Rab4-dependent short-loop (allows trafficking back to the plasma membrane) or the slower Rab11-dependent long loop (where cargo moves from early endosome to endosomal recycling compartment (ERC) and then recycles back to the plasma membrane) [320]. Arf6, a small GTPase belonging to the mammalian Arf family of proteins, has a distinctive plasma membrane and peripheral localization that plays a key role in endocytic recycling back to the plasma membrane [321].

Late endosomes: Cargo that is destined to the lysosome passes through the late endosomes en route, arriving either from early endosomes, trans-Golgi network, or the phagosome. Mature late endosomes are round or oval shaped with a diameter of 250-1000 nm and their pH ranges from 6.0-4.9 [309]. Limiting membranes of these endosomes contain several lysosomal membrane glycoproteins such as LAMP-1 and numerous acid hydrolases are present in their lumen. Late endosomes localize to the perinuclear region of the cell and can sometimes fuse with each other to form larger late endosomes or can fuse with the lysosomes or with endosomes to form hybrid endolysosomal organelles [322]. Rab7-positive late endosomes are derived from the vacuolar domains of the early endosomes and starts with the generation of
a Rab7 domain in a process facilitated by Rab5-GTP [323]. Post GTP hydrolysis, the Rab5-to-Rab7 switch occurs with the GDP-bound Rab5 dissociating with its effectors from this newly formed late endosome. Rab5 (early) and Rab7 (late) are the master regulators of the endocytic pathway and provide the most important organelle identity markers for their respective compartments. Rab7 and its effector molecules determine the functions of late endosomes and lysosomes [324].

**Lysosomes:** They are the final and largest compartment, sized at 1-2 µM, of the endocytic pathway where cargo is degraded. Lysosomes utilize a host of acid hydrolases to accomplish the process of cellular waste turnover to generate new building blocks for the cell. Inside the lumen of the lysosomes, the pH drops to ~4.5 [309]. Lysosomes are also important in energy metabolism, plasma membrane repair, cell signaling and secretion [325].

**Multivesicular bodies:** An intermediate intracellular organelle that incorporates cargo that gets sorted for degradation and operates in the endosome-to-lysosome route of endocytosis. Multivesicular bodies (MVBs) arise during maturation of the early to late endosome process. Cargo that is destined for lysosomal degradation is ubiquitinated and then incorporated into the intermediate intraluminal vesicle (ILV) that buds from the limiting membranes of the early endosome, giving rise to the MVB, which can eventually fuse with or mature into lysosomes [368-369]. Cargo trafficking to MVBs provides a mechanism for damaged or misfolded proteins to be degraded, especially plasma membrane receptors (*e.g.*, EGFR) [370] that undergo downregulation or clearing from the cell surface as part of their regulation. MVBs perform diverse functions within the cell. They are the major sorting hub for damaged proteins as well as serve as signaling nodes within the cell. MVBs recruit ubiquitinated cargos, with Ubiquitin serving as a positive sorting signal for MVB-directed degradative pathway, using the endosomal sorting complexes required for transport (ESCRT) machinery made up of cytosolic protein complexes: ESCRT-0, ESCRT-I, ESCRT-II, and ESCRT-III, together with a host of accessory proteins including the AAA-ATPase vacuolar protein sorting-associated 4 (Vps4) and endosome-specific phosphatidylinositol-3-phosphate (PtdIns3P) [371-372]. In addition to degradation, MVBs also function as non-degradative storage organelles as seen when von
Willebrand Factor (vWF) gets packaged into the lumen of alpha granules in platelets [41-42] or the Weibel-Palade bodies of endothelial cells [373]. Proteins such as the transferrin receptor, which are recycled back to the plasma membrane post-internalization, are incorporated into the MVBs for secretion at the cell surface [374]. Numerous cell types including neurons, epithelial cells, mast cells, tumor cells, melanocytes, platelets as well as other cell types within the hematopoietic lineage are known to generate extracellular vesicles called exosomes, which are derived from MVBs [375]. MVBs have a slightly acidic pH of 6.0-5.0 [309].

Role of endocytosis in platelets

As mentioned earlier, platelet endocytosis has been largely studied in the context of granule cargo packaging and $\alpha_{IIb}\beta_3$-mediated uptake of fibrinogen. Uptake of small particles and solutes by human platelets was first reported by Zucker-Franklin in 1981 [14] into a unique membrane structure called the open canalicular system (OCS) by a phagocytosis-like process. Further studies using electron microscopy and cytochemistry provide evidence for endocytosis of circulating proteins such as plasma fibrinogen [15, 16] into platelets. The integrin $\alpha_{IIb}\beta_3$-mediated endocytosed fibrinogen eventually ends up in alpha granules [340]. Platelet endocytosis is important for granule cargo loading (such as fibrinogen and VEGF) [9, 17] and contributes to thrombus formation by controlling the surface expression of several key proteins such as the integrins, chiefly $\alpha_{IIb}\beta_3$, the major platelet glycoprotein receptor [18]. Integrin trafficking modulates platelet spreading on endothelial surfaces and clot retraction [19]. Endocytosis of purinergic receptors such as P2Y1 and P2Y12 mediates their re-sensitization and response to ADP [20]. CLEC-2 and Thrombopoietin receptor Mpl/TPOR endocytosis regulates their surface levels and downstream signaling responses [21, 22].

Coated vesicles and membranes are central to intracellular trafficking and clathrin-mediated endocytosis in almost all eukaryotic cells. The first report of the presence of such coated vesicles in platelets came from studies done on aldehyde-fixed human platelets back in the early 1980s. The concomitant addition of alcian blue and tannic acid to human platelets during glutaraldehyde fixation allowed for the
visualization of coated membranes within platelets [326]. These coated membranes were found either as coated pits or vesicles or dotted around the plasma membrane and the secretory granules (alpha granules and lysosomes). Further evidence using fluid-phase markers showed that the coated pits are lined with the plasma membrane and open canalicular system (OCS) where the coated vesicles either existed by themselves or fused with the secretory granules [327]. These studies first demonstrated that an active endocytosis and intracellular trafficking process occurs in platelets. Following that, a series of experiments done by Bainton and colleagues provided evidence for endocytosis of plasma proteins such as albumin, IgG and most importantly $\alpha_{\text{IIb}}\beta_3$ receptor-mediated uptake of fibrinogen into both megakaryocytes and platelets, thereby laying the groundwork for platelet endocytosis and its importance [15-17, 328-329]. In support of these studies, transmission electron microscopy of antibody-stained ultrathin frozen sections of platelets showed that plasma proteins such as fibrinogen and von Willebrand factor (vWF) are taken up and translocate to storage granules (alpha granules in this case) [330]. Klinger et al. used post-embedding immunocytochemistry on platelets using the acrylic resin, LR white, to first show the presence of clathrin-coated vesicles colocalizing with fibrinogen, vWF, fibronectin on the cytoplasmic faces of the alpha granules, open canalicular system and the plasma membrane [331]. Morphological studies on quiescent platelets using fibrinogen-stabilized colloidal gold as a receptor-mediated endocytosis marker and acid phosphatase as a lysosome marker demonstrated that both clathrin-dependent and clathrin-independent endocytic pathways occur in platelets [332]. While fibrinogen uses a clathrin-mediated route to enter the alpha-granules, acid phosphatase uses a clathrin-independent degradative endo-lysosomal route to reach the lysosomes. This report also proposes the presence of a lysosomal autophagy system that is tasked with degradation in platelets and could be linked to platelet senescence [332]. Many platelet surface receptors such as integrin $\alpha_{\text{IIb}}\beta_3$, glycoprotein Ib are internalized and cycle back to the plasma membrane [334-338]. In unstimulated platelets, an internal storage pool of these receptors exists which can be targeted to the plasma membrane upon platelet activation, thereby increasing their surface expression. Stimulation by agonists such as thrombin or ADP
often leads to re-distribution on the plasma membrane as can be noted in the case of glycoprotein IV (CD36) [339]. ADP-stimulation causes an enhancement in fibrinogen internalization in platelets [333]. Megakaryocytes but not platelets can endocytose plasma-derived factor V, a member of the coagulation cascade, via a clathrin-dependent and most likely receptor-mediated process [342], although factor V is a known to platelet protein part of the alpha granule repository, where it colocalizes with fibrinogen [341].

Quantitative proteomic analyses have shown platelets to carry most of the proteins that are part of the clathrin-mediated endocytic machinery, including all three isoforms of the mechanochemical small GTPase Dynamin, Dynamin1 (DNM1), Dynamin2 (DNM2) and Dynamin3 (DNM3) [200, 343]. Human platelets contain all three isoforms, DNM1, DNM2 and DNM3 while mouse platelets predominantly express DNM2. Dynamin plays a key role in clathrin-coated vesicle fission. Interestingly they have been shown to play a role in megakaryopoiesis, especially DNM3 [344-345]. DNM3 co-localizes to the demarcation membrane system (DMS), which serves as a membrane reservoir during proplatelet formation in the megakaryocytes [345]. In several human macrothrombocytopenia disorders, the nonmuscle myosin IIA (MYH9) protein important for cytoskeletal reorganization, appears to be mutated [346] and DNM3 has been shown to bind MYH9 [345]. In vitro use of pan-Dynamin inhibitor such as Dynasore in megakaryocytes impaired proplatelet formation [347]. In fact, GWAS studies corroborate the importance of DNMs in regulating platelet size and formation when a single nucleotide polymorphism (SNP) within the DNM3 gene promoter was detected to be associated with variable mean platelet volume in humans [347].

Platelets have an abundance of DNM2 [200] and mutations in DNM2 have been associated with thrombocytopenia and hematopoietic diseases, most notably Charcot-Marie-Tooth disease [348-349]. Using platelet-specific DNM2 knockout mice, the role of DNM2 in thrombopoiesis was determined by Bender et al. [22]. These mutant mice had severe macrothrombocytopenia with giant platelets but increased clearance from circulation leading to low platelet counts. Bone marrow megakaryocytes had altered demarcation membrane system packed with
abnormally high numbers of clathrin-coated vesicles in addition to increased emperipolesis (presence of intact nucleated cells within the MK cytoplasm). Compared to control MKs, the clathrin-mediated endocytic pathway was also impaired in this DNM2-null MKs as noted by mislocalization of early endosomal markers such as Early Endosomal Antigen 1 (EEA1) and Adaptor protein, Phosphotyrosine interacting with PH domain and Leucine Zipper 1 (APPL1). Proplatelet formation from DNM2-null MKs was also impaired as was Thrombopoietin receptor (TPO/Mpl) endocytosis in DNM2 null platelets leading to constitutive phosphorylation of downstream Janus Kinase 2 (JAK2). Constitutive JAK2 signaling is consistent with defective endocytosis that is seen in these platelets. Additionally, DNM2 null mice developed MK hyperplasia, myelofibrosis, extramedullary hematopoiesis and splenomegaly with a concomitant increase in hematopoietic stem progenitor cells [22].

DNM1 levels are very low in platelets and it is mainly expressed in the brain where it functions in synaptic vesicle endocytosis [350].

Dynamin-related protein-1 (Drp1) belongs to the Dynamin family of small GTPases and plays a key role in mediating fission and fusion during mitochondrial biogenesis [352]. Platelets contain Drp1, which gets phosphorylated upon activation [351]. Drp1 plays a key role in fusion pore stability and granule exocytosis. Inhibition of Drp1 affects platelet accumulation during thrombus formation in vivo. Any role of Drp1 in mediating endocytic events in platelets is currently unknown.

Disabled-2 (Dab2) is a clathrin, AP-2, and cargo binding adaptor protein that is involved in endocytic trafficking of many cell surface receptors and modulating intracellular signaling [353]. Alternate splicing generates two isoforms, p82-Dab2 and p59-Dab2 [354]. Dab2 is present in megakaryocytes and platelets and plays a key role in facilitating megakaryocytic differentiation [355, 358] with p82-Dab2 mainly expressed in human platelets while p59-Dab2 is predominant in mouse platelets [356]. In human platelets, p82-Dab2 is present in the cytosol and alpha granules and interacts with the cytoplasmic tail of αIIbβ3, gets phosphorylated by thrombin and upon thrombin stimulation of platelets, gets secreted but remains tethered to the αIIb subunit, regulates fibrinogen binding and platelet aggregation [357]. In mouse
platelets, p59-Dab2 is required for platelet aggregation, fibrinogen uptake, RhoA-ROCK activation, secretion of ADP and α\textsubscript{IIb}β\textsubscript{3}-mediated platelet activation [356]. Platelet-specific Dab2-deficient mice have a bleeding diathesis and defective thrombus formation \textit{in vivo} [356]. Dab2 is a key modulator of integrin inside-out signaling in platelets and plays key roles in fibrinogen uptake; however the exact mechanistic underpinnings of how Dab2 acts, which proteins of the clathrin-mediated endocytic pathways it interacts within platelets, remains unknown and needs to be further explored.

ADP-ribosylation factor 6, Arf6, is a small GTPase from the ARF family of proteins that oversee endocytic trafficking and receptor recycling and has been shown to be involved in several steps of trafficking such as recruitment of coat proteins, coated pit assembly, vesicle fission, and vesicle route [359, 366]. Our group was the first to show the presence of Arf6 in platelets [360]. There are six members of the Arf family, with Arf1, Arf3, and Arf6 being present in platelets [200, 360]. In platelets, ASAP1, a member of the family of ArfGTPase-activating proteins (Arf-GAPs), proteins that induce hydrolysis of GTP bound to Arf, was detected and shown to be recruited to peripheral focal adhesions via CrkL, an SH2 and SH3 adaptor protein for WASP, Syk and STAT5 [361, 364, 367]. Previous work by our group has shown that in resting platelets, Arf6 is present in the GTP-bound form, and upon platelet activation, Arf6-GTP is quickly converted to Arf6-GDP [360]. This transition is regulated by two waves of platelet signaling pathways, primary signaling (e.g. PAR receptor, GPVI) and contact-dependent signaling (e.g. α\textsubscript{IIb}β\textsubscript{3} integrin) [363]. This was in corroborations to previous studies showing the involvement of Arf6 in regulating integrin function in platelets [361-362]. Blocking integrin α\textsubscript{IIb}β\textsubscript{3} outside-in signaling could partially reverse the loss of Arf6-GTP, suggesting a regulation of Arf6 by signaling downstream of integrin α\textsubscript{IIb}β\textsubscript{3}. Arf6 is downstream of several key platelet kinases, like PLC, PI3K, PKC, and Syk, indicating its importance in platelet signaling [364]. Using an inhibitory, myristoylated Arf6 N-terminal peptide, loss of Arf6-GTP could be blocked in human platelets, probably due to the inhibition of the Arf6 GAP activity by the peptide. Pretreatment with this peptide inhibited platelet aggregation, adhesion and
spreading on the collagen-coated surface, and activation of Rho family members, suggesting the central role of Arf6 in platelet activation [363].

The endocytic trafficking function of Arf6 has been widely recognized in other cells [366]. In platelets, Arf6 was suggested to be involved in P2Y12 receptor internalization required for receptor desensitization and resensitization to ADP. It was shown that P2Y activation by ADP could stimulate Arf6 activation, and activated Arf6 stimulates Nm23-H1, a nucleoside diphosphate kinase, which in turn promotes dynamin-dependent internalization of P2Y receptors [150]. It was also proposed that Arf6-GTP in resting platelets is maintained by cytohesin-2, an Arf–guanosine exchange factor (Arf-GEF) protein, and upon platelet activation, PKC-mediated phosphorylation of cytohesin-2 decreases the interaction between Arf6-GTP and cytohesin-2, leading to loss of Arf6-GTP and platelet secretion [365]. However, it should be noted that these studies used Myr-Arf6 peptide and pharmacological inhibitors, and the dynamics of Arf6-GTP changes were not consistent with previous reports.

To better understand the role of Arf6, a platelet-specific (PF4-Cre)-Arf6 conditional knockout mice were generated [19]. Arf6 null platelets have defective αIIbβ3-mediated fibrinogen uptake in vitro and in vivo. Although resting and activated levels of αIIbβ3 were unchanged, Arf6 null platelets demonstrated enhanced spreading on fibrinogen-coated surfaces and the rate of thrombin-induced clot retraction, in the presence of fibrinogen, was faster than controls. Arf6 null platelets had no overt morphological defects, no secretion defects or aggregation defects. Tail bleeding times were normal and no occlusion defect was noted in the FeCl3-induced carotid artery injury model of arterial thrombosis. Taken as a whole, the noted defects in the Arf6 null mice could be largely attributed to altered integrin trafficking and serves as a viable model to study endocytic trafficking in platelets and megakaryocytes. While Arf6-mediated integrin trafficking largely affects platelets’ roles in acute platelet functions such as clot retraction and spreading, it remains to be seen what other platelet functions are influenced by Arf6.
Role of integrin trafficking in platelets

As mentioned previously, integrins are one of the major platelet receptors and their function is essential for hemostasis and thrombosis. While trafficking of integrin heterodimers between the plasma membrane and the intracellular endosomal compartments is a key aspect of regulating integrin function [376], most notably in the context of cell migration [382-383], how much of this process occurs in platelets, what are the regulators for said process and the potential biological ramifications of integrin trafficking in platelet function still remains to be fully explored.

Mechanistically, integrin endocytosis occurs both in a clathrin-dependent and a clathrin-independent manner. α5β1 and αvβ3 integrins endocytose via both clathrin-dependent endocytosis [377-379] and caveolin-dependent endocytosis [380] while αIIbβ3 mainly prefers a clathrin-dependent endocytic pathway [381]. After internalization, integrins are recycled back to the plasma membrane via two temporally and spatially distinct mechanisms, a short-loop and a long-loop recycling. After reaching early endosomes, integrins such as αvβ3, selected for short-loop recycling are sorted to particular subdomains of these endosomes and then rapidly returned to the plasma membrane in a Rab4-dependent manner [385]. Alternatively, integrins β1, αvβ3, and α6β4 may pass from EEs to the recycling endosomes, from where they return to the plasma membrane under control of the Rab11 GTPase, referred as long-loop recycling [384]. These trafficking routes are under the control of several regulators such as Arf6 and signaling kinases such as PKB/Akt, GSK3β as well as several isoforms of Protein Kinase C [384].

Integrin trafficking plays an important role in cell adhesion and migration. As such, inhibition of long-loop recycling events by targeting Rab11, Arf6 etc., impairs cell migration and motility in a number of different cell types [386]. Selective inhibition of the short-loop recycling of integrin αvβ3 by using dominant-negative Rab4 leads to impaired spreading on vitronectin-coated matrices in an ERK1-dependent manner [387]. Additionally, it has been demonstrated that treating cells with primaquine inhibits receptor recycling and leads to accumulation of integrins within endosomes with the consequent detachment of cells from the substratum [385]. Integrin trafficking also plays a key role in tumor cell invasiveness with several reports of
regulators such as Arf6 and Protein Kinase D1 abounds in the invadopodia of aggressive tumor cells [388-389].

In platelets, studies regarding integrin recycling have been mainly focused on how $\alpha_{\text{IIb}}\beta_3$ is trafficked in and out of platelets. While the concept of $\alpha_{\text{IIb}}\beta_3$ transiting back and forth between the plasma membrane and intra-platelet compartments has been largely recognized for close to three decades, the mechanistic understanding is still far from complete. Fibrinogen, part of the alpha granule repository in platelets, enters via binding to $\alpha_{\text{IIb}}\beta_3$, transits through the multivesicular bodies and ends up in alpha granules [17, 41]. Therefore, fibrinogen content in $\alpha$-granules is a good indicator of trafficking of integrin $\alpha_{\text{IIb}}\beta_3$. This has been exploited to determine the intricacies of the process of integrin $\alpha_{\text{IIb}}\beta_3$ trafficking in platelets. One of the first insights into this process came from the studies using the molecule c7E3 Fab fragment, an antagonist of integrin $\alpha_{\text{IIb}}\beta_3$, commercially available as abciximab [390]. One of the startling findings of this study was that trafficking of $\alpha_{\text{IIb}}\beta_3$ in platelets is very fast where they showed that abciximab or c7E3 Fab, could be detected within 3 hours post-infusion into a patient. Consistently, it requires much shorter time for abciximab to be detected in vitro after incubation with washed platelets. Open canalicular systems (OCS) were stained as early as 1 min showing that indeed trafficking of $\alpha_{\text{IIb}}\beta_3$ is very fast. This is in agreement with the widely held theory in the platelet field that the open canalicular system, which serves as a membrane reservoir for platelets, is the entry site for the endocytosis of various exogenous substances [391]. Studies using monoclonal antibody AP6, which binds to integrin $\alpha_{\text{IIb}}\beta_3$ only when the receptor is occupied by intact fibrinogen, suggested the presence of a pool of ligand-bound $\alpha_{\text{IIb}}\beta_3$ within the $\alpha$-granules [392]. Platelet activation by ADP or thrombin receptor activation peptide (TRAP) significantly increases the actin cytoskeleton-dependent internalization of $\alpha_{\text{IIb}}\beta_3$ [335]. Studies using an anti-Ligand-induced binding site (anti-LIBS) antibody, which activated $\alpha_{\text{IIb}}\beta_3$ by directly binding to it, showed that enhanced integrin internalization directly modulates platelet aggregation [393]. Our recent studies have now added to this growing body of integrin trafficking and its importance in platelet function. Arf6, a Ras-like, small guanosine triphosphate (GTP)-binding protein called adenosine 5’-
diphosphate ribosylation factor 6 (Arf6), which is important for intracellular trafficking of integrins and membrane receptors, mediates fibrinogen endocytosis and trafficking of $\alpha_{\text{IIb}}\beta_3$ in platelets [19]. Loss of Arf6 leads to defective fibrinogen uptake and storage which manifests in enhanced platelet spreading and clot retraction. This could potentially be due to faster short-loop recycling of $\alpha_{\text{IIb}}\beta_3$, which could explain why $\alpha_{\text{IIb}}\beta_3$-bound fibrinogen resides for less time (compared to wildtype) within the intra-platelet compartments that are manifested as lower fibrinogen content of Arf6 KO platelets. Altered dynamic routing of $\alpha_{\text{IIb}}\beta_3$ could lead to enhanced surface presence of the integrins which may allow more areas of contact with fibrinogen-coated matrices corresponding to enhanced spreading. This coupled with faster thrombin-induced clot retraction could predispose Arf6 KO mice to become hyper thrombotic in nature. While this hyper thrombotic phenotype could not be detected in standard models of arterial thrombosis due to technical redundancies of the assays involved, it does shed some light on the global importance of integrin trafficking and recycling on hemostasis and thrombosis. Arf6 KO mice do not have a secretion defect or a bleeding phenotype, yet altered integrin trafficking leads to defective cargo uptake and a predisposition to hyperthombosis (though this remains to be confirmed yet). In summary, Arf6-mediated integrin $\alpha_{\text{IIb}}\beta_3$ trafficking occurs constitutively in platelets and appears to play an important role in the deposition of $\alpha$-granule cargo, like fibrinogen, and in the modulation of other integrin-mediated platelet functions, like spreading, clot retraction. In my thesis, Arf6 null mice are used as a model system to understand how endocytosis and potentially integrin trafficking influences other platelet functions e.g., mediating innate immune responses against pathogens.
Figure 2 Potential platelet endocytic routes. Cargo can enter platelets either via clathrin-dependent endocytosis, requiring GTP hydrolysis by Dynamin and using specific surface receptors (e.g., αIIbβ3-mediated fibrinogen entry) or via clathrin-independent endocytosis that may require Dynamin (via caveolin- or RhoA-dependent pathways) or may not (via Arf6- or Cdc42-dependent pathways). Internalized cargo then transits through Rab4 GTPase-positive early endosomes, where it can be sorted to recycling endosomes (Rab 11-positive) for a return to the plasma membranes or to multivesicular bodies and ultimately into α-granules for storage (e.g., fibrinogen, vWF, thrombospondin-1). Alternatively, cargo can move into late endosomes, either directly from early endosomes or through multivesicular bodies. Cargo from late endosomes can transit into dense granules or into lysosomes where it may be degraded or stored. The complexity of these pathways in platelets has not been studied in sufficient detail.
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*Listed are human platelet proteins that may contribute to endocytosis and/or subsequent cargo-sorting events.

*Included are copy numbers, estimated based on quantitative proteomic analyses [200].

ND = Not determined.
Subsection Three: Vesicle-associated membrane protein 3 (VAMP-3/Cellubrevin)

VAMPs in platelets

Platelets contain multiple isoforms of the Vesicle-associated Membrane Proteins or VAMPs (VAMP-2,-3,-4,-5,-7,-8), based on data gleaned from proteomic analyses and quantitative Western blotting [199-200]. In terms of a number of copies per platelet, VAMP-8 and VAMP-7 are the most abundant in human platelets while VAMP-8, VAMP-2, and VAMP-3 are the major ones in mouse platelets. VAMP-8 which is the most abundant in both mouse and human platelets is the primary v-SNARE to mediate secretion. Loss of VAMP-8 attenuates secretion from all three granules, causes defective thrombosis (thrombus growth is slower and lessened in comparison to WT) with no bleeding diathesis [199, 201]. Loss of VAMP-3 by itself had no defect in secretion and hemostasis proceeded normally as measured by tail bleeding assay [202]. Loss of VAMP-2 is embryonic lethal [201]. Treatment of tetanus toxin which specifically cleaves VAMP-2 and VAMP-3 showed that platelet secretion is unaffected as long as VAMP-8 and VAMP-7 are present [201]. Loss of VAMP-7 does not lead to a bleeding diathesis or defects in platelet accumulation at sites of injury in the laser injury model of arterial thrombosis, although alpha granule secretion is attenuated in these platelets [203]. Collectively, these data imply that there is a ranking amongst the isoforms in mediating secretion with some amount of redundancy. VAMP-8 is primary with VAMP-2 and VAMP-3 playing secondary roles. VAMP-7 on the other hand may be less involved in secretion but mainly play a part in platelet spreading due to its interactions with the actin cytoskeleton via VPS9-domain Ankyrin-repeat containing protein (VARP) and Arp2/3 [203].

Immunofluorescence studies have shown VAMP-7-dotted structures to be localized to the platelet periphery while VAMP-8 and VAMP-3 have been shown to be more centrally localized within the granulomere in spread platelets [204]. The central localization of VAMP-3 concurs with studies done in our lab where we reported the intracellular punctate staining of VAMP-3 containing structures that resist being targeted to the plasma membrane during thrombin activation [205]. These could very easily be VAMP-3 positive endosomes in platelets. Additionally, VAMP-8 and VAMP-7 could potentially mediate differential cargo release or may be differentially
responsive to various kinds of agonists. However, future studies need to be done to parse this distinction.

**VAMP-3 and its cellular localization**

Synaptobrevins are small, highly conserved integral membrane proteins that are part of the Vesicle-Associated Membrane Protein (VAMP) family and are abundantly found in synaptic vesicles within the brain [394-395]. VAMP-3 is a type of synaptobrevin that is ubiquitously expressed in all cells and was thus named cellubrevin as the cellular homolog of synaptobrevins [396]. Like synaptobrevins, which are targets of clostridial neurotoxins such as tetanospasmin, a protein from Clostridium tetani, and botulinum toxin, from Clostridium botulinum [397-398], cellubrevin/VAMP-3 is also cleaved by tetanus toxin substrate [396]. Using immunofluorescence and subcellular fractionation techniques, VAMP-3 was shown to be intracellularly localized to all parts of the cell with a localized concentration in and around the Golgi complex. Using transferrin receptor (Tfn-R) as a marker for a recycling receptor, it was shown that while all transferrin-positive vesicles stained for VAMP-3, not every VAMP-3-positive vesicles stained for transferrin [396]. This suggested that VAMP-3 is a component of receptor-mediated endocytosis pathway and is present on coated vesicles that recycle transferrin as well as other similar vesicles. In Chinese hamster ovarian (CHO) cells transfected with synaptophysin, VAMP-3 was shown to colocalize with synaptophysin, which in turn associated with the transferrin receptor and is targeted to the receptor-mediated endocytic pathway [396, 399-400]. VAMP-3 was also shown to reside in insulin-sensitive GLUT4 glucose transporter vesicles in 3T3-L1 adipocytes and is a part of the insulin-regulated membrane trafficking pathways [401]. Furthermore, subcellular fractionation of pancreatic acinar cells revealed VAMP-3 to be present on the smooth microsomal fraction consisting of small vesicles and condensing vacuoles that originate from the Golgi complex, indicating a possible role in the maturation of secretory granules within these cells [402]. Transferrin receptor recycling has been instrumental in shedding light regarding the subcellular localization of VAMP-3. Following the entry routes of Tfn-R in CHO cells using immunofluorescence microscopy, showed that upon internalization, Tfn enters the early endosomes that stain positive for both
VAMP-3 and Rab4, which marks early endosomes. Soon after that, Tfn moves into a subset of pericentriolar cluster or tubules and vesicles that are now referred to as the recycling endosomes, which stain positive for VAMP-3 but not Rab4. These endosomes instead stained positive for another Rab GTPase called Rab11. These Rab11-VAMP-3 double positive vesicles transiently accumulate Tfn inside the cells on its route back to the plasma membrane [403].

Role of VAMP-3 in receptor trafficking

VAMP-3 has been shown to be concentrated in vesicles which recycle transferrin receptors but its role in membrane trafficking and fusion was demonstrated by the use of tetanus toxin, a metallo-endoprotease that cleaves VAMP-3 (and VAMP-2) which partially blocked transferrin release in CHO cells [404]. Like Tfn-R, the intracellular glucose transporter 4 (GLUT4)-containing vesicles dock and fuse with the plasma membrane to facilitate insulin-controlled glucose uptake in muscle and fat cells. GLUT4 colocalizes with both VAMP-3 and VAMP-2 (both synaptobrevin type vesicle SNAREs that are tetanus toxin-sensitive) and tetanus toxin treatment inhibited insulin-stimulated GLUT4 translocation to the plasma membrane [405]. Furthermore, overexpression of VAMP-3, but not VAMP-2, in HL-1 atrial cardiomyocytes, prevented lipid-induced inhibition of insulin-stimulated GLUT4 translocation from the intracellular stores to the plasma membrane, in an in vitro model of cardiac insulin resistance [406].

VAMP-3 has also been implicated in the trafficking of a host of other plasmalemma receptors such as translocation of Na+/K+/2Cl− co-transporter (NKCC2) to the apical surface of the renal cells of the thick ascending limb in kidney and is required for NaCl absorption and regulation of blood pressure [407]. VAMP-3 regulates recycling of glutamate transporters in mouse cortical astrocytes, which express VAMP-3 but not VAMP-2, and as a result, plays a key role in Ca2+-independent recycling of these receptors to the surface and glutamate uptake by astrocytes [408]. VAMP-3, in association with the small GTPase, Rab8, regulates recycling of T-cell receptors (TCR) by interacting with IFT20, a component of the intraflagellar transport (IFT) system that modulates ciliogenesis and immune synapse assembly in non-ciliated T cells [409]. In dendritic cells, cross-presentation of peptides derived from phagocytosed
microbes, infected cells, or tumor cells to CD8+ T cells requires the action of VAMP-3/VAMP-8/Rab11-positive endosomal recycling compartments that carry major histocompatibility complex I (MHC class I) receptors to fuse with phagosomes, which drives Toll-Like Receptor activation in response to infection [410]. Endosomal TLRs such as TLR7 and TLR9 that bind viral nucleic acids traffic to distinct endomembrane systems, namely VAMP-3-positive and LAMP-positive endosomes, for signaling [411]. This bifurcation of the TLRs to separate compartments allows for cytokine and type I interferon gene induction. Studies in RAW264.7 cells showed the involvement of the phosphatidylinositol 3P 5-kinase, PIKfyve, to mediate selective trafficking of TLR9 to VAMP-3 positive endosomes. Blocking PIKfyve activity of disruption of VAMP-3 by tetanus toxin, lead to a reduction in trafficking of TLR9 and consequent reduction in type I interferon induction [412].

**Role of VAMP-3 in vesicle trafficking**

As discussed earlier, VAMP-3 dots the early and recycling endosomes and is essential for recycling of several plasma membranes and endomembrane receptors. On the same vein, VAMP-3 is also crucial for targeting of vesicles to specific locations within the cell. Autophagosomes are specialized double-membraned organelles where degradation of intracellular macromolecules, RNA and protein turnover as well as damaged organelles takes place [413]. Cargo that is destined for the autophagosomes passes through the sorting hubs or the Multivesicular bodies (MVBs) and several reports have shown that these MVBs can fuse with autophagosomes to form a pre-lysosomal hybrid organelle called the amphisome [414]. A mature amphisome can then fuse with the lysosome to form the autolysosomal organelle where the actual degradation of cargo occurs. Several SNAREs have been implicated in mediating one or more steps of these concerted vesicle trafficking. Key among those is VAMP-3 that oversees fusion of the MVBs with the autophagosomes to generate the mature amphisome. VAMP-7, another key v-SNARE involved in vesicle trafficking, comes into play after amphisome formation step and is responsible for fusing the amphisome to the lysosome. VAMP-3, however, does not play any role in this step [415].
Role of VAMP-3 in phagocytosis

Phagocytosis or the engulfment of pathogens is an important immune response. This mode of entry of cargo (pathogens) can be receptor-mediated as it is in macrophages and require membrane expansion at the site of phagocytosis and several key intracellular trafficking steps. Not only is membrane trafficking in macrophages required for pathogen phagocytosis but it also stimulates proinflammatory cytokine secretion. VAMP-3 heads a trafficking pathway that oversees both these actions thereby ensuring efficient immune responses in macrophages; this pathway follows tumor necrosis factor α (TNF-α) being trafficked from the Golgi complex to the VAMP-3-positive recycling endosomes. TNF-α containing recycling endosomes are then targeted to the plasma membrane where the phagocytic cup forms allowing the concomitant rapid release of TNF-α and expansion of the membrane to engulf the pathogen [416]. VAMP-3 has also been shown to play a role in phagosome maturation which causes phagosomes containing pathogens to fuse with the lysosomes to form the phagolysosomes, using *Mycobacterium tuberculosis* var. *Bovis* BCG, an organism that causes phagosomal maturation arrest upon entering phagosomes. This allows the bacterium to evade lysosomal degradation. Inhibition of VAMP-3 leads to disruption in mycobacterial phagosomes, overcoming this arrest and thereby causing degradation of the pathogen in the lysosomes and protecting against mycobacterial infection [417]. In a process similar to phagocytosis, bacterial invasion of cells, also involves membrane expansion at the host cell plasma membrane where the bacteria invade. In *Salmonella typhimurium* infections, VAMP-3 has been shown to accumulates at sites on the plasma membrane where the bacteria invades and this accumulation leads to membrane protrusions in an N-ethylmaleimide-sensitive factor (NSF)-independent manner [418]. Though VAMP-3 does indeed play a role in phagocytosis and recycling of receptors, bone-marrow derived macrophages (BMM) from VAMP-3 null mice showed no defects in phagocytosis of IgG-beads, complement-opsonized particles, or latex microspheres although zymosan internalization was found to be significantly slower than wild-type [419]. In fact, in primary mouse embryoblast isolated from VAMP-3 null mice, rates of transferrin internalization (endocytosis), transferrin
release (recycling) and horse-radish peroxidase (HRP) uptake (pinocytosis) were essentially unchanged compared to wild-type [420]. Insulin-dependent glucose uptake in isolated primary adipocytes as well as insulin-, hypoxia-, and exercise-stimulated glucose uptake in isolated skeletal muscle cells from VAMP-3 null mice were unchanged compared to wildtype [420]. Taken as a whole, it appears that VAMP-3 is necessary for phagocytic uptake of pathogens as well as cargo (receptors/macromolecules) in some cells while in others, other VAMPs such as VAMP-2 can play compensatory roles to tide over any possible defects caused by loss of VAMP-3.

**Role of VAMP-3 in exocytosis**

VAMP-3, being a v-SNARE, partners with cognate SNAREpins such as the t-SNAREs (SNAP-23/SNAP-25 and syntaxins) to mediate vesicle fusion during intracellular trafficking events as well as during granule/vesicle exocytosis. VAMP-3 usage for the sake of release of granule cargo varies from cell to cell. Some cells require VAMP-3 for exocytosis while others do not. In mast cells, VAMP-7 or VAMP-8, but not VAMP-3 or VAMP-2, are required for high-affinity IgE receptor-mediated histamine release [421]. Trafficking and secretion of matrix metalloproteinases (MMPs) determine cellular remodeling of the extracellular matrix (ECM). Studies done in invasive human fibrosarcoma cell lines HT-1080 showed that inhibition of SNAP-23 and VAMP-3 by using dominant-negative forms of these SNAREs, RNAi treatment or Tetanus toxin-mediated cleavage of VAMP-3 leads to a reduction in the secretion of MMP2 and MMP9 as well as impaired trafficking of membrane type I MMP to the cell surface [422]. Blocking of these SNAREs also led to less invasiveness of the HT-1080 cell lines on gelatin substrates *in vitro*.

In endothelial cells, Weibel-Palade Bodies (WPB) are the storehouses of the thrombogenic glycoprotein von Willebrand Factor (vWF). At the site of damage, endothelial cell activation triggered by thrombin or histamine leads to Ca$^{2+}$-, and SNARE-dependent exocytosis of WPBs to release vWF into the vessels that in turn leads to platelet adhesion via the GPIb-IX-V complex and thrombus formation. Both VAMP-3 and VAMP-8 are present on WPBs; yet only VAMP-3 is required for forming stable SNARE complexes with plasma membrane syntaxin 4 and SNAP-23 to drive
membrane fusion of WPB to release vWF. Using mutant VAMP-3 and VAMP-8 comprising of the cytoplasmic domains of the proteins, it was noted that only VAMP-3 but not VAMP-8 inhibits with vWF release in permeabilized endothelial cells [423].

Cytotoxic granules containing granzymes and perforin are important for killing viral infected and transformed host cells by natural killer (NK) cells. The release of inflammatory cytokines such as TNF-α and IFN-γ is also mediated by exocytosis of these cytotoxic granules through SNARE-mediated pathways. Studies in NK cells have shown that granules that contain perforin and granzymes are different from the ones that contain cytokines such as TNF-α and IFN-γ. The vesicles that contain these cytokines are actually recycling endosomes and release of cytokines from these is mediated by Rab11 and VAMP-3 [424]. Inactivation of recycling endosomes or mutating Rab11 or inactivating VAMP-3 by tetanus toxin treatment or mutant versions of VAMP-3 impaired TNF-α and IFN-γ release from NK cells. VAMP-3, together with SNAP-23, has also been shown to be involved in the release of IL-6 and TNF-α in an SNARE-dependent, IL-1β-stimulated exocytosis event from human synovial sarcoma cell lines SW982 [425].

**Role of VAMP-3 in cell migration**

Cell migration is a highly-regulated pathway of cell polarization that involves both endocytosis and exocytosis to drive membrane protrusion at the leading edge of the cell, adhesion of the leading edge and de-adhesion at the cell body and rear end followed by cytoskeletal contractions to pull the cell and move it in the direction of the stimulus. As with any membrane trafficking/fusion events, SNAREs play a key role in mediating membrane trafficking in cell migration. Inhibiting SNARE-mediated membrane traffic by using a dominant-negative form of the enzyme NSF and Tetanus toxin-mediated cleavage of VAMP-3 leads to reduced serum-induced migration of CHO-K1 cells in transwell migration assays with a concomitant reduction in cell surface α5β1 integrins [426]. This study was closely followed by Thierry Galli’s group who also showed that Tetanus toxin-mediated cleavage of VAMP-3 impairs epithelial cell migration and β1 integrin-dependent cell adhesion [427]. Tetanus toxin cleavage of VAMP-3 also led to enhanced adhesion of epithelial cells (CHO cells) to collagen, laminin, fibronectin and E-cadherin matrices with altered spreading on collagen-
coated surfaces due to impaired recycling of β1 integrins. siRNA-induced silencing of VAMP-3 in CHO cells also leads to a similar reduction in chemotactic cell migration without affecting cell proliferation [428] agreeing with previous reports. Recycling of adhesion receptors is crucial for lamellipodia formation at the leading edge of a migrating cell. VAMP-3 containing recycling endosomes is incorporated into the leading edge to bring about the expansion of the lamellipodia membrane in an SNARE-dependent membrane fusion event that also requires SNAP-23 and syntaxin 4. Inhibition of VAMP-3 by tetanus toxin showed that fusion of recycling endosomes is necessary for lamellipodia formation and delivery of the α5β1 integrins present on the recycling endosomes to the cell surface necessary for efficient cell migration [429]. VAMP-3 together with SNAP-23 and syntaxin 4 has also been shown to regulate the formation of ring-like podosome structures that accumulate in the leading edge of spreading cells such as macrophages migrating on fibronectin. Lowering VAMP-3 levels or disrupting VAMP-3 function (or SNAP-23 or syntaxin 4) leads to disrupted trafficking of the recycling endosomes to the plasma membrane, impaired polarization of these podosome structures at the leading edge and reduced adhesion and spreading on fibronectin [430]. Since integrins are the major adhesion receptors, VAMP-3 mediated trafficking of integrins plays an essential role in regulating cell migration and by extension proliferation and survival. Surface expression of integrins is controlled by SNARE-mediated endo-exocytic recycling. While VAMP-3, by virtue of being present on recycling endosomes, does play a key role in this process, what determines the specificity of regulating one integrin trafficking event versus another often depends on the t-SNARE partners it pairs with. This also determines whether a particular integrin recycles directly back to the plasma membrane from the recycling endosomes or traverses through the trans-Golgi network (TGN) before it reaches its cell surface destination. For example, syntaxin 6, a t-SNARE located in the trans-Golgi network, pairs with VAMP-3 to mediate trafficking of α3β1 integrins from the recycling endosomes to the TGN before being recycled to the plasma membrane surface in epithelial cells [431].
**VAMP-3 and its role in platelets**

The presence of VAMP-3 in human platelets was first reported by our group [205]. Termed as human Cellubrevin (Hceb) was present in detergent-solubilized platelet membranes and participated in α-SNAP-dependent 20S SNARE complex formation and ATP-dependent 20S SNARE complex disassembly thereby functioning as a v-SNARE in human platelets. Immunofluorescence microscopy showed an intracellular punctate staining in human platelets, megakaryocytes and HEK-293 cells agreeing with previous reports of how cellubrevin/VAMP-3 is located on endosomes/vesicles within the cell. This was confirmed when thrombin-stimulated human platelets resisted targeting of these Hceb-positive punctate structures to the plasma membrane even though all dense core and most alpha granules had been released. This was the first indication showing that VAMP-3 could be present on an endosomal organelle that is not part of the exocytic pathway in platelets. This was further corroborated by immunogold labeling which showed that almost 80% of VAMP-3 in resting human platelets was localized to platelet granule membranes including alpha granules [206]. The role of VAMP-3 in mediating platelet secretion was first monitored by introducing anti-VAMP-3 antibody in streptolysin-O-permeabilized platelets which resulted in a 50% reduction of Ca\(^{2+}\)-induced alpha granule secretion, an effect that could be reversed by using a VAMP-3 blocking peptide [206]. These studies were further expanded upon by others to demonstrate the role of VAMP-3 in mediating secretion in human platelets. VAMP-3 and VAMP-8 were both shown to coimmunoprecipitate with syntaxin 4 and SNAP-23 in human platelets using microcapillary reverse-phase high-performance liquid chromatography-nano-electrospray tandem mass spectrometry [433]. Introducing soluble recombinant VAMP-3 into streptolysin-O-permeabilized platelets led to a dramatic reduction in P-selectin exteriorization from alpha granules as measured by flow cytometry and C\(^{14}\)-serotonin from dense granules as measured by radioactivity. This led to the idea that VAMP-3 together with SNAP-23 and syntaxin forms fusogenic SNARE complexes that drive granule secretion in human platelets.

Insulin largely recognized for its role in glucose homeostasis, acts as a vasodilator in the vasculature in a nitric oxide synthase (NOS)-dependent manner, in part by
enhancing platelet-endothelial NOS activity [434]. Insulin works by inducing ATP, adenosine, and serotonin release from dense granules in an NO-dependent manner, a response that is abolished in NOS null platelets [433]. Similarly, insulin stimulation of human platelets requires SNAP-23/syntaxin t-SNARE heterodimers to associate with VAMP-3 to form the functional SNARE complexes that drive ATP release from dense granules via an NO-G kinase-dependent signaling cascade that is independent of αIIbβ3 activation [433]. Surprisingly, platelets isolated from VAMP-3 null mice showed no defect in secretion from all three granules (dense core, alpha granules, and lysosomes) tested in response to thrombin and collagen in both kinetic (time-course) and dosage assays [202]. Additionally, loss of VAMP-3 did not cause any change in levels of other SNARE proteins. Agonist-induced platelet aggregation was also unchanged in the VAMP-3 null platelets as was bleeding time measurements. Collectively, these data suggests that there is some level of heterogeneity in VAMP usage in mouse platelets versus human platelets; VAMP-3 is essential for secretion in human platelets while VAMP-3 plays a subordinate role and is not required for platelet secretion in mice. However, what other roles VAMP-3 plays in mouse platelets needs further exploration.
Subsection Four: Role of platelets as immune cells

Overview
Platelets have been classically studied for their roles in hemostasis and thrombosis. What has become increasingly clear is that platelets are, in fact, multifunctional and can carry out several important physiological and pathophysiological processes e.g., wound healing, inflammatory and immune responses. From an evolutionary perspective, lower invertebrates such as birds, reptiles, amphibians, and fishes, which have an open vasculature, only contain a single primordial cell type called thrombocytes or hemocytes. Hemocytes are nucleated and perform both hemostasis and innate immune responses within the hemolymph [435]. Invertebrate hemocytes mediate immune responses by virtue of bearing Toll-like Receptors (TLRs) on their surfaces which allow pathogen recognition, phagocytosis, and secretion of antimicrobial peptides [436]. They can also mediate wound healing and induce hemolymph coagulation and clotting at sites of tissue damage in a process that is similar to how mammalian platelets form clots. It is thought that as vertebrates climbed higher up the evolutionary tree, their hemocytes lost the nuclei, retained most of their hemostatic and thrombosis functions and evolved into the modern-day mammalian platelets, while the immune-related functions were taken over by the professional immune cells. As recent studies over the past decade bring to light more immune-related functions of platelets, it essentially solidifies this concept of platelets being the evolutionary link between the coagulation and immune responses, two key facets of the vascular system. How mammalian platelets mediate immune responses will be covered in the following sections.

Platelet Toll-like Receptors (TLRs)
Pathogen-sensing and subsequent innate immune responses are carried out by a specialized class of pattern-recognition receptors (PRR) that recognize molecules called pathogen-associated molecular patterns (PAMPs) broadly expressed by pathogens distinct from host molecules (see Table 1) and are expressed on immune cells such as dendritic cells, monocytes/macrophages, neutrophils, T and B lymphocytes. Toll-like receptors are type I transmembrane proteins possessing: leucine-rich repeat (LRR) ectodomains folded into beta sheets, enabling PAMPs
recognition, a transmembrane domain and a cytoplasmic Toll-Interleukin-1 receptor (TIR) domain that does signal transduction [437]. To date, 10 TLRs in humans and 13 TLRs in mice have been identified that can recognize PAMPs such as lipids, lipoproteins, proteins or nucleic acids derived from pathogens (viruses, bacteria, parasites, fungi etc.) or from endogenous sources, either at the cell surface or within intracellular compartments (endosomes, lysosomes, and endolysosomes), triggering distinct downstream signaling cascades based on the PAMP recognized [438].

Several reports have demonstrated the expression of TLR1-9 in both human and mouse platelets [439-442] and have shown that some of these TLRs (TLR2, TLR4, TLR7, and TLR9) are functional and can modulate platelet responses during infection and thus, will be discussed in depth.

**TLR2**

TLR2 primarily resides on the plasma membrane and can recognize bacterial lipopeptides, peptidoglycans, and lipoteichoic acid from Gram-positive bacteria, mycobacterial lipoarabinomannan, yeast zymosan, and viral hemagglutinin [437]. TLR2 can heterodimerize with TLR1 to form TLR2/1 complexes that bind triacylated lipopeptides or with TLR6 to form TLR2/6 that preferentially binds diacylated lipopeptides [437]. Platelet TLR2 binds to the adaptor protein MyD88 to trigger its downstream signaling cascades. In platelets, TLR2 engagement using triacylated lipopeptides such as Pam3CSK4 leads to activation of the PI3K/Akt signaling pathway [444-445] and platelet $\alpha_{IIb}\beta_3$ activation, aggregation, adhesion, membrane expression of P-selectin from alpha granules and platelet-neutrophil aggregates formation [443, 446]. These responses can be blocked by treatment with PI3K inhibitor, LY294002, or using anti-TLR2 antibodies or TLR2 knockout mice [447]. Platelet TLR2 stimulation by Pam3CSK4 also leads to increases in cytosolic $Ca^{2+}$ concentrations, the release of ATP and TxA$_2$ generation [443]. Platelet TLR2 has also been implicated in enhanced phagocytosis of periodontopathogens such as P. gingivalis by neutrophils with a concomitant increase in TLR2-activated platelet-neutrophil aggregates and CD40L expression in platelets [448-449]. Platelet TLR2 has also been implicated in platelet-viral interactions chiefly with human
cytomegalovirus. Human cytomegalovirus interacts with platelets via TLR2, giving rise to pro-inflammatory cytokine production by platelets [450].

A pivotal study by the Freedman group using the TLR2 ligand, Pam3CSK4, was the first to shed light on the concept of differential stimulation of platelets. Platelets have been traditionally thought to be fast responders to any kind of damage though thrombotic damage which leads to hemostasis has only been extensively studied. However, when platelets encounter immune damage such as those related to TLR activation, the response generated is both slower and more gradual than the rapid and maximal thrombotic stimulation of platelets [447]. Stimulation of platelets by Pam3CSK4 leads to a more gradual phosphorylation kinetics of Akt, p38, and ERK that are present downstream of TLR2 in platelets; compared to rapid and substantial phosphorylation kinetics of the same under thrombin stimulation. This differential stimulation of platelets that we have provisionally termed “hemostatic” versus “immune-activation” agrees with data generated in my thesis and supports this shifting view of platelets being more diverse in their response curves to various stimuli than the originally held idea of rapid activation and maximal release of cargo to all kinds of stimuli from platelets.

**TLR4**

TLR4 is the most abundant Toll-like receptor on platelets [441] and binds to lipopolysaccharides (LPS), a major component of the cell membrane of Gram-negative bacteria. TLR4, in a complex with an adaptor protein MD-2, binds to LPS, in the presence of CD14 and LPS-binding protein (LBP) [451].

LPS-induced thrombocytopenia and tumor necrosis factor-α (TNF-α) production *in vivo* is mediated by platelet TLR4-dependent pathways [439]. LPS induced a 60% reduction in platelet counts as early as 4 hr post-injection in wildtype mice compared to a 20% reduction in TLR4 KO mice. Drop in platelet counts is often due to the formation of platelet-neutrophil and platelet-monocyte aggregates that get trapped in the lungs and are thus removed from the circulation [475]. Another possible mechanism by which platelet numbers drop during inflammation is due to increased platelet destruction caused by phagocytosis [453].
LPS-induced TLR4-dependent functions of platelets have been largely studied in the context of bacterial sepsis. Endotoxin-induced or bacterial-induced sepsis often leads to severe thrombocytopenia [476]. Platelet activation during sepsis contributes to disseminated intravascular coagulation (DIC) or the formation of microthrombi, which impedes blood flow to vital organs causing death by multiple organ failures [477]. Platelet activation during sepsis leads to the production of both pro- and anti-inflammatory cytokines, which triggers immune responses in the circulation [478]. Increased platelet and leukocyte activation, often times correlate with severity of DIC-induced multiple organ failures during sepsis [479]. To further probe the role of platelets in endotoxin-induced sepsis, experimental thrombocytopenia was induced in mice by the in vivo administration of anti-glycoprotein Ibα monoclonal antibody [480] in LPS-induced endotoxemia and a bacterial infusion mouse sepsis model [481]. This caused increased plasma levels of pro-inflammatory cytokines such as TNF-α and IL-6, followed by widespread organ failure in the liver, lungs, and spleen and increased mortality, an effect that could be reversed significantly by transfusion of healthy platelets in these septic mice [481]. This was the first detailed and conclusive study that showed that platelets protect from septic shock, via a mechanism that involves inhibiting the macrophage-dependent inflammation via the cyclooxygenase 1 signaling pathways. In another similar study the role of platelets and specifically how thrombocytopenia affects the outcomes in sepsis was determined [493]. It was shown that thrombocytopenia caused, led to worsened outcomes that manifested as increased mortality strongly correlating with increased bacterial burden [493]. Titrating platelet counts by in vivo GPIbα administration to mice showed the diverse effects caused on Klebsiella pneumoniae-induced sepsis; when platelet counts were reduced to 5 X 10^9/L in blood of mice infected with Klebsiella through the airways, they showed impaired survival with increased bacterial burden in lungs, blood and in distant organs such as liver as well as causing severe hemorrhaging at primary sites of infection. However, this led to increased cytokine production which exacerbated the pro-inflammatory sepsis caused by Klebsiella. On the other hand, when platelet counts were titrated to 5-13 X 10^9/L, hemostasis could be maintained even within the infected lungs. Taken together, these studies showed the threshold platelet...
counts required to maintain hemostasis in a mouse model of Klebsiella-induced sepsis.

Another possible mechanism by which platelets provide protection during severe sepsis is by activating the neutrophil extracellular traps (NETs) in a TLR4-dependent mechanism that trap bacteria in septic blood [482]. Activated neutrophils release granule proteins and chromatin material from their nucleus in vivo that form these mesh-like extracellular DNA fibers containing histones and antimicrobial proteins and effectors called neutrophil extracellular traps (NETs) that bind and kill Gram-positive and Gram-negative bacteria [484]. NET formation is called NETosis. In a series of imaging experiments in liver sinusoids and pulmonary capillaries, platelets were shown to bind activated neutrophils via their TLR4 to release NETs to entrap the bacteria [482-483]. The complex interactions between platelets and neutrophils in the context of NETs were also shown to be important for thrombus formation [485]. NETs caused platelet activation, adhesion, and aggregation by providing a scaffold that triggers thrombus formation. DNase treatment which degrades the meshwork of DNA fibers that form the NETs or treatment with the anticoagulant heparin can disrupt pre-formed NETs and thus inhibit thrombus formation. Furthermore, markers of NETs including histones were found to be inside thrombi within veins that also contained red blood cells, giving rise to red thrombi. This led to further studies showing the importance of NETs in the propagation of deep vein thrombosis [486-487]. In neutrophils, peptidyl arginine deiminase 4 (PAD4) mediates citrullination of histones, a process that is required for chromatin decondensation that releases the DNA fibers and NETs formation [488]. Studies using PAD4 KO mice showed that only 10% of these mice formed venous thrombi 48 hr after inferior vena cava stenosis compared to 90% of wild-type mice that formed venous thrombi, suggesting that PAD4-mediated chromatin decondensation in neutrophils is responsible for pathological deep vein thrombosis.

Another key NET mediator is platelet P-selectin. NET formation by neutrophils induced by thrombin-activated platelets can be inhibited by anti-P-selectin glycoprotein ligand-1 (PSGL-1) inhibitory antibody or by the use of anti-P-selectin aptamers. The NET formation is also abolished when platelets from P-selectin KO
mice are used to induce neutrophils [489]. Platelet-induced NET formation by neutrophils has now become an increasingly growing field of research, providing new insights into how NETs promotes tumor growth and contribute to cancer-associated thrombosis [490-491].

While platelet TLR4 is essential in mediating inflammatory responses by platelets in vivo, reports elucidating the exact mechanism of how LPS stimulates TLR4 in ex vivo and in-vitro assays have been confusing. Platelets lack CD14, an essential co-receptor that engages with LPS-bound TLR4 to initiate proper downstream signaling. While all other TLR4-expressing cells have both CD14 and LPS-binding protein, LBP, unless added otherwise in the form of free plasma or recombinant versions of CD14 and LBP to washed platelets, platelets do not respond to LPS [492]. This has led to controversial reports where some suggest that LPS does not stimulate platelet activation when measuring P-selectin expression [441] while others have reported otherwise [454]. However, since platelets express MyD88, the adaptor protein that is required for TLR4 signaling, in amounts similar to those found in leukocytes [454], platelets do seem to share signaling kinases that are common to TLR4 signaling pathway in immune cells. These include TBK-1, IRAK-1, JNKs, MAPKs, TRAF3, TRAF6, IRF-3, IKK complex, NF-κB p65, Akt among others [452, 492]. LPS has also been shown to initiate splicing of mRNA encoding IL-1β in a TRAF6-Akt-JNK activation-dependent manner [492].

LPS has also been shown to increase in vivo thrombopoietin (TPO) levels by influencing megakaryocyte TLR4, this led to a concomitant increase in circulating reticulated platelets (young platelets) and enhanced platelet-neutrophil aggregates [494]. LPS via TLR-4 also induced bone marrows to produce more TPO leading to more platelet production [495-496].

TLR7

One of the key viral sensors as part of the host’s innate immune defenses is the endosomal Toll-like Receptor 7 that binds to single-stranded RNA [497]. TLR7 stimulation by ssRNA causes a type I interferon (IFN) response. Many ssRNA viruses (HIV-1, VSV, and influenza viruses) bind to cell-surface receptors and gain entry into the cell via endocytosis. Once the viruses get trafficked to an endosome, it fuses with
the endosomal membrane to release its capsid, followed by endosomal acidification that causes degradation of the envelope via TLR7-MyD88 dependent signaling cascade to cause IFN production; both steps being required for effective responses [498-499]. TLR7, being an endosomal TLR7, has been studied in the context of self-vs-non-self-recognition of PAMPs; with ssRNA not being a part of the host, but clearly abundant in pathogens such as ssRNA-containing viruses that infect hosts. Endosomal localization may be a strategy to prevent recognition of self-nucleic acids, a feature that is common to most nucleotide-sensing endosomal TLRs such as TLR8, TLR9 and TLR3 [499]. ssRNA engagement of TLR7 initiates MyD88-dependent responses that signal via an IRAK4/IRAK1-TRAF3/TRAF6 pathway that leads to activation of the IRF7 transcription factors that cause interferon production [500].

In platelets, the first report about the presence of TLR7 was shown by the Freedman group. TLR7, which resides in an intracellular compartment and recognizes single-stranded RNA, was found to be present in platelets [411, 437, 472]; and is required for mediating platelet counts and host survival in a mouse model of encephalomyocarditis virus (EMCV) infection. Viruses such as EMCV, HIV-1, and influenza virus as well as small guanosine analogs such as Loxoribine and Imiquimod (R837) have been shown to activate TLR7 signaling pathways [437]. EMCV-induced or Loxoribine-induced TLR7 activation led to PI3K/Akt, p38-MAPK phosphorylation events in platelets and these effects were largely abolished in the TLR7 KO platelets. In this dissertation, the role of the endosomal TLR7 in platelets and how it affects platelets responses to ssRNA-containing viruses is further explored.

**TLR9**

Like TLR7, TLR9 is also an endosomal TLR that senses unmethylated 2’-deoxyribose cytidine-phosphate-guanosine (CpG) DNA motifs that are present in bacteria, viruses and other pathogens [501]. In platelets, TLR9 is present both intracellularly and on the plasma membrane [442] and increased expression of the receptor occurs upon CpG ODN stimulation of human platelets [502]. It was reported that in both human and mouse platelets, TLR9 is present in a specific platelet granule, called the T granule, which is different from the other kinds of granules present within platelets (namely dense, alpha and lysosomes) [502]. In fact, TLR9 transcripts are upregulated
during proplatelet formation in the megakaryocytes [503]. Within the T granules, TLR9 has been shown to colocalize with protein disulfide isomerase (PDI) and is associated with either VAMP-7 or VAMP-8. These v-SNAREs allow targeting of the T granules to the plasma membrane during platelet activation and is thought to put TLR9 on the surface. However, recent studies have shown that PDI is located in an ER-type compartment within platelets, present near the inner surface of the platelet plasma membrane, which is different from the secretory granules found in platelets [47]. These compartments are targeted to the plasma membrane via an actin polymerization process and are independent of SNARE-mediated vesicle fusion. TLR9 resides in the endoplasmic reticulum of resting dendritic cells and macrophages [505] and gets trafficked in COPII coated vesicles to an endo-lysosomal organelle [504]. CpG DNA, on the other hand, enters the cells through the early endosomes and has to be trafficked to a TLR9-containing organelle, where it partners with MyD88 for proper signal transduction to be initiated [504]. Hence, in platelets, given that PDI is present in ER-like compartments and TLR9 colocalizes with PDI, these T granules could very easily be ER-remnants instead of being a specialized separate compartment as previously thought.

In platelets, TLR9 has been shown to bind to derivatives of polyunsaturated fatty acid oxidation such as carboxy-alkyl-pyrroles (CAPs), generated during oxidative stress, and activates platelet via the PI3K-Akt pathway leading to aggregation and secretion [506].

While CpG motifs bearing oligonucleotides have been used as activators of TLR9 in dendritic cells and macrophages, a recent study, discovered an unexpected new mechanism of how CpG ODNs activates platelets [507]. When oligonucleotides were first discovered as potent therapeutics, measures had to be taken to prevent rapid degradation of these short single-stranded DNA molecules from cellular and plasma nucleases, to ensure pharmacostability of the drugs inside the host system. One such way was to introduce a phosphorothioate modification where the nonbridging oxygen molecules are replaced by sulfur [508]. This phosphorothioate modification of TLR9 agonists causes GP VI-dependent activation, adhesion, and aggregation of platelets, a response that was unchanged in TLR9 KO platelets, suggesting that this
mechanism of activation of platelets proceeds in a TLR9-independent manner [507]. These findings stress the importance of developing novel alternative chemical modifications of oligonucleotides used as drugs, such that they do not have spurious platelet activation side-effects.

**Platelet cytokines/chemokines**

Platelets store several different kinds of cytokines and chemokines within their secretory granules (mostly alpha granules) such as Platelet Factor 4 (PF4), RANTES, β-thromboglobulin, MIP-1α, TGF-β [91]. Transforming growth factor-β (TGF-β) is a potent immunomodulatory molecule and platelets contain large amounts of it within their granules [509] and have been shown to regulate blood levels of TGF-β. In patients with immune thrombocytopenia (ITP), levels of TFG-β were markedly reduced and only reached normal levels upon platelet transfusions [510-511]. The release of cytokines and chemokines by platelets allows them to modulate their microenvironments in the vasculature. Platelets are the largest source of PF4, which can render monocytes apoptosis-resistant and induce differentiation into macrophages [512]; enhance neutrophil adhesion on endothelial surfaces and degranulation [513] among others. Platelet-derived CXC chemokines such as β-thromboglobulin, that is stored in platelet alpha granules and released upon activation, can be stimulatory or can be inhibitory as in the case of neutrophil function [514]. Platelets release the chemokine Macrophage Inflammatory Protein-α (MIP-α) upon activation which can then stimulate granulocytes such as basophils and mast cells to release histamines [515]. CCL5 or RANTES, the second-most abundant chemokine after PF4, is released from platelets upon activation and can stimulate proplatelet production by the megakaryocytes [516].

One of the most important inflammatory mediators expressed by platelets is CD40L or CD154 that can bind to CD40, the costimulatory protein found on antigen presenting cells [517]. Membrane-anchored CD40L is expressed by platelets and upon activation, release most of it in its soluble form, as sCD40L; which makes up for the majority of circulating sCD40L in the plasma [518].

One of the key functions of platelet-derived CD40L has been in linking the innate immunity with adaptive immunity by enhancing CD8+ cytotoxic T cell responses in
response to *Listeria monocytogenes* infection [519] as well mediating CTL-induced liver damage in response to Hepatitis B infection [520]. Platelets have been shown to bind dendritic cells (DC) in a CD40L-dependent manner to suppress DC differentiation, downregulate pro-inflammatory cytokine production of TNF, IL-12p70 while upregulating IL-10 production [521]. In addition to affecting dendritic cells, platelet-derived CD40L can modulate B cell differentiation and antibody class switching [522]. Taken together, platelet-derived cytokines, chemokines and immune mediators such as CD40L allow platelets to modulate the effector functions of other immune cells, thereby forming an important link between immune systems and the coagulation cascades [523].

**Platelet-bacterial interactions**

In addition to the Toll-like receptors, which are the premier innate immune receptors to interact with bacteria and bacterial products, platelets express a wide range of potential receptors such as complement receptors, FcγRII as well as the conventional hemostatic receptors such as αIIbβ3, GPIbα, which allow bacteria to directly or indirectly interact with platelets via fibrinogen, fibronectin, complement C1q, vWF among others [524]. Bacteria can interact with platelets in three ways: (i) direct binding of bacteria to platelet receptors, (ii) direct binding of bacterial products or toxins to platelet receptors, and (iii) indirect binding of bacteria to proteins that are ligands of platelet receptors. Of the hemostatic receptors that are known to bind bacteria either directly or indirectly by binding to their cognate ligands bound to bacteria, αIIbβ3 and GPIbα, are the most important. *Staphylococci* express surface proteins that can bind to fibrinogen and fibronectin, ligands for αIIbβ3 [525-527]. Protein A from *Staphylococcus aureus* can bind to vWF, which in turn interacts with GPIb β, allowing the bacteria to adhere to platelets [528-530]. Platelets also express members of the complement factor receptors such as gC1q-R, the receptor for the complement factor C1q, which allows opsonization of C1q complement-coated bacteria [531]. This occurs during platelet activation when there is an increase in C1q-R surface expression. P-selectin, which is expressed upon platelet activation on the surface, has been shown to bind to the complement C3b that can act as an opsonin for bacteria [532].
Platelets also express the immune receptor, FcγRII, which recognizes the Fc domain of IgG and enables pathogen phagocytosis and engulfment of immune complexes [533]. Heparin is often administered to patients as a potent anticoagulant. However, this occasionally leads to thrombocytopenia, which predisposes these patients towards thrombosis. This is the basis of heparin-induced thrombocytopenia (HIT), a condition where autoantibodies are generated by the body against heparin-platelet factor 4 complexes. Heparin acts as a hapten and thus can be recognized by IgG antibodies in the bloodstream. It complexes with PF4 in circulation to form PF4-heparin complexes that can then bind to FcγRIIA on the platelet surface, leading to strong platelet activation, followed by internalization of these platelet-bound complexes by phagocytes leading to increased clearance of activated platelets [534-535].

Platelets have been shown to harbor bacterial pathogens such as *Staphylococcus aureus* within their vacuole-like compartments [263] that are separate from the OCS. These compartments could be potential endosomes, though this has not been confirmed yet. *Porphyromonas gingivalis* has also been similarly found within platelet vacuoles [536]. IgG-mediated opsonized particles or bacteria can be internalized by human platelets via FcγRIIA, resulting in activation and degranulation of sCD40L and RANTES, in a process that requires actin cytoskeletal rearrangements [537].

What happens to bacteria that have been internalized by platelets? While this remains an active area of ongoing research, some insights are available [538]. Bacteria such as *Streptococci* and *Staphylococci* that bind via αIIbβ3 or to GPIb via vWF, activate platelet aggregation, release ATP from dense granules and generate TxA2 [539]. *Streptococcus sanguinis* has been shown to activate platelets via ERK and p38 MAPK phosphorylation [540]. An interesting feature of note in the context of bacteria-induced platelet activation is the difference in kinetics of responses as compared to hemostatic responses [538]. Bacteria-induced platelet aggregation proceeds after a lag time, dependent on the bacterial density that lasts between 3-20 min, as compared to thrombin or ADP-mediated hemostatic responses which reach maximal activation in less than 3 min. This harks back to this overarching
hypothesis that platelets do indeed interpret different stimuli and respond accordingly. Thus, there exists a differential response in reacting to “immuno agonists” vs “hemostatic agonists”. Data generated in this thesis further consolidates this idea.

**Platelet-viral interactions**

As in the case with severe bacteremia, thrombocytopenia is commonly associated with viral infections. Platelets get activated by viruses in the circulation, present due to the ongoing viremia, leading to a platelet-leukocyte aggregate formation that causes sequestration of platelets from the circulation, possibly by macrophages that contribute to lowering the platelet counts. Viruses have also been found inside platelets, with the first electron micrographs of platelets containing influenza virus, is the first evidence that platelets can indeed internalize viruses [541]. Since platelets can internalize FcγRIIA-bound complexes, viruses that can bind to FcγRIIA are often found inside platelets [535]. Platelets isolated from patients infected with viruses such as HIV-1 [263], influenza [469], dengue virus [471] and hepatitis C virus [470], have been shown to harbor viral particles. Some of these cause functional responses, while for others the same is yet to be discovered.

**Human Immunodeficiency Virus-1 (HIV-1)**

HIV-1 is a lentivirus containing single-stranded RNA belonging to the Retroviridae family. It causes HIV infection that eventually becomes Acquired Immunodeficiency Syndrome (AIDS) over time. AIDS is a cohort of immune dysfunction related conditions that happens when HIV infection has significantly impaired the immune system to the point where opportunistic pathogens wreak havoc in an already immunocompromised individual. This creates infections to become life-threatening as well as increased propensity for cancers to thrive. In total, death is often caused due to one or more complications arising from this plethora of infections in HIV+ individuals than due to the actual HIV infection. HIV-induced AIDS is a global killer with estimates of over 22 million people worldwide having been killed by this disease ever since its discovery in the 1980s.

The first report of platelets and megakaryocytes internalizing HIV-1 was obtained from electron micrographs [542] suggesting that direct infection of these cells could
perhaps play a role in the disease progression of HIV-related AIDS. Youssefian et al. have previously shown that platelets isolated from HIV-AIDS patients with severe thrombocytopenia contain virions inside endocytic vesicles lying close to the plasma membrane [263]. This observation was further clarified using genetically modified non-replicative HIV and vesicular stomatitis virus (VSV), which were found to be present within endocytic vesicles in platelets [543]. Electron micrographs showed that these virus-containing vesicles colocalized with alpha granules markers and were trafficked to the surface-connected canalicular system (SCCS) where degradation of the lentiviral particles occurred when in contact with alpha granule proteins. In cultured megakaryocytes, lentiviral particles were also found in endocytic vesicles and then trafficked into acid-phosphatase-containing Multivesicular bodies (MVBs).

HIV-1 entry into platelets is mediated by a host of surface receptors. While megakaryocytes express CD4, which is the main receptor for viral entry, megakaryocytes also express other co-receptors such as CXCR1, 2, 4, and CCR3 [544-545]. HIV-1 viruses can be transferred from CXCR4+ megakaryocytes to CXCR4− cells, aiding in viral dissemination [546]. Platelets, on the contrary, do not express CD4 but only express CXCR1, 2, 4 and CCR1, 3 and 4 [546-549]. HIV-1 can also bind the dendritic cell-specific intercellular adhesion molecule-3-grabbing non-integrin (DC-SIGN/CD209 and the C-type lectin-like receptor 2 (CLEC-2) to enter platelets [550].

In patients with HIV-1 infection, increased plasma levels of PF4, increased platelet-leukocyte aggregates as well as increased sCD40L levels, all suggest platelet activation [551]. HIV-1 trans-activator of transcription protein (Tat) directly activates platelets to release sCD40L in a CCR3 and β3 integrin-dependent manner [552]. Various soluble endothelial activation markers like sVCAM-1, sICAM-1, and von Willebrand Factor levels are higher in HIV-1 infected patients [544].

Anti-retroviral therapies have been successful in lessening deaths caused due to AIDS. This has also led to a decreased severity in thrombocytopenia by containing the bone marrow damage caused by HIV-1 replication, reduction in pro-inflammatory cytokines and festering of opportunistic infections [553]. Highly active anti-retroviral
therapies (HAART) have also reduced markers of endothelial and platelet activation such as P-selectin and CD40L levels in HIV-infected patients [554].

HIV-1 infects platelets and megakaryocytes. Yet how it enters and much of what it does upon entry remains unclear. The bigger questions remain whether infection of platelets is beneficial or detrimental to HIV viral dissemination. Do platelets encounter the virus at the early stages of infection or does it occur, when the infection is truly systemic and widespread in the circulation? Do platelet responses to the virus facilitate immune clearance by allowing detection of virus-containing platelets by the macrophages, monocytes, dendritic cells, T-cell populations etc.? Conversely, do platelet responses to the virus exacerbate the process of viral infection? Further studies will help answer some of these questions.

**Hepatitis C Virus (HCV)**

Like HIV-1 infections, HCV infections also cause thrombocytopenia in addition to hepatic complications like liver cirrhosis [555]. Hepatitis C virus is a small enveloped positive-sense, single-stranded RNA virus belonging to the Flaviviridae family and is known to cause Hepatitis C infections as well as some liver cancers such as hepatocellular carcinoma, although the mechanisms are unclear. Thrombocytopenia in Hepatitis C patients strongly correlates with hepatic dysfunction, HCV viral load (HCV RNA) and severity of cirrhosis and is thought to be a consequence of bone marrow damage, inhibition of thrombopoietin production by the liver, coupled with autoimmune activation that leads to increased platelet clearance [555-556]. Platelets bind to HCV via their envelope-residing glycoprotein E2, in a CD81-independent manner [557]. The E2 glycoprotein of HCV has been known to bind CD81 to allow viral attachment and entry to a target cell [558]. CD81-lacking platelets still seem to bind HCV, although the mechanisms of viral attachment and entry remain unclear. The presence of HCV-bound platelets isolated from HCV patients indicates that there are other potential receptors that could perhaps mediate HCV binding; what those may remain to be seen. Liver sequestration of HCV-bound platelets has been thought to be a way by which HCV evades the immune system, facilitating viral dissemination and persistence in the body [557]. Further studies are required to fully understand how HCV binds platelets and modulates platelet functions in causing
thrombocytopenia, in an effort to develop antiviral strategies to combat this infection.

**Dengue Virus**

Another member of the positive-sense, single-stranded RNA virus of the Flaviviridae family, is Dengue virus, which causes dengue hemorrhagic fever. Thrombocytopenia and platelet dysfunction go hand-in-hand with dengue viral infection and are related to the clinical outcomes of this disease [559, 563]. Platelets isolated from dengue hemorrhagic fever (DHF) have increased levels of P-selectin expression, lysosomal CD63 expression, and $\alpha_{\text{IIb}}\beta_3$ activation compared to healthy control platelets [561]. In dengue patient platelets, the intrinsic pathway of apoptosis is activated with concomitant elevation in phosphatidylserine exposure, mitochondrial depolarization, and activation of caspase-3 and caspase-9. This phenotype is strongly correlated with increased platelet activation. Studies have further shown that increased platelet activation during DHF leads to increased production of pro-inflammatory cytokines as well as increased vascular permeability [560]. *In vitro* exposure to dengue virus caused increased expression of the pro-inflammatory cytokine, IL-1$\beta$, in platelets, a phenotype mimicking *in vivo* analyses of platelets and platelet-like microparticles isolated from DHF patients that showed elevated levels of IL-1$\beta$ synthesis. The nucleotide-binding domain leucine-rich repeat containing protein (NLRP3) inflammasomes, which are present in platelets, are turned on by dengue virus infection. NLRP3 activation leads to caspase-1 activation that mediates secretion of IL-1$\beta$ from platelets [560]. Dengue virus infects human dendritic cells via DC-SIGN (CD209) receptor [562]; dengue virus also uses DC-SIGN to infect platelets [561]. Dengue virus has been shown to replicate inside the platelet cytosol producing infectious viral particles, upon DC-SIGN and heparan sulfate proteoglycan-mediated viral binding and entry [565]. qRT-PCR and ELISA assays confirmed the presence of newly synthesized ssRNA as well as presence of the highly antigenic viral NS1 protein, with copies of about 800 viruses bound per platelet, when platelets were incubated with dengue virus *in vitro* at 37°C. This was the first report to describe an active process of viral replication inside platelets. This replicative ability becomes a serious issue while storing platelet transfusion units, which might be already unknowingly
(due to lack of testing) infected with the dengue virus. Dengue virus can persist and replicate, albeit at a much lower level in these storage units. Upon entering a new host, during platelet transfusion, dengue virus can then infect the host and disseminate in circulation to cause dengue fever [566].

Dengue hemorrhagic fever is commonly associated with elevated plasma levels of pro-inflammatory cytokines such as MIP-1β, RANTES, IL-7, IL-12, IL-5, and PDGF, caused due to increased platelet activation [564]. Clearly, DHF caused by dengue virus leads to a complex disease etiology, that oversees a dynamic interplay between dengue virus, platelets and other participating immune cells and future studies will aim to better understand how to ameliorate some of the platelet-related dysfunctions prevalent in dengue viral infections.
Thesis overview

Ever since their discovery, platelets have almost exclusively been studied in the context of hemostasis and thrombosis. Recent research over this past decade has challenged this canonical view of platelets and has provided insights into how platelet functions extend beyond hemostasis and thrombosis. Platelets have been shown to be important for immune and inflammatory responses, wound healing, angiogenesis, tumor progression and cancer metastasis among others. Platelet activation and exocytosis remain the cornerstone for mediating platelets’ functions in hemostasis and thrombosis. Endocytosis, on the other hand, has been shown to be involved in granule cargo loading (e.g., fibrinogen and VEGF), receptor trafficking of integrins (αIIbβ3, αvβ3) and purinergic receptors (P2Y1, P2Y12), and thereby for normal platelet function. However, platelet endocytosis could potentially be critical for actively sensing changes in vascular microenvironments and responding accordingly to vascular damage and exogenous substances that are being taken up by platelets (i.e. pathogens, PAMPS and DAMPS). We posit that endocytosis confers platelets with the ability to become vascular sentries providing active and dynamic “immune surveillance” against circulating pathogens and “hemostatic surveillance” against prothrombotic vascular damage. The mechanistic underpinnings of endocytosis, its importance in platelets, and the molecular machinery required and possible trafficking routes are however understudied, in part due to a lack of experimental tools. The work presented here puts forth new players that regulate endocytosis and mediate cargo loading and hemostasis as well as provides a novel mechanistic understanding of how endocytosis allows platelets to act as immune cells and become the first responders to pathogens in the vasculature.

Work presented in the first part of the thesis identifies a v-SNARE protein called Cellubrevin/VAMP-3 as a key mediator of endocytosis in modulating platelet functions. Together with another previously identified endocytic regulator, Arf6, a small GTPase that mediates cargo uptake and integrin trafficking in platelets, we probe the varied roles of endocytosis in mediating the innate immune responses of platelets to circulating pathogens in the bloodstream, in the second part of the thesis. Taken together, we uncover novel mechanistic understandings of how endocytosis is
important for platelet functions, shedding light on some of the critical regulators of the process, in general, and how it allows platelets to act as effector immune cells.
Chapter Two: Reagents and methods

Reagents and antibodies

Rabbit anti-cellubrevin/VAMP-3 antibody (Ab) was obtained from Novus Biologicals (Littleton, CO). FITC-anti-CD41/61, PE-anti-Jon/A, FITC-P-selectin, PE-LAMP1 Abs were from Emfret Analytics (Eibelstadt, Germany). Rabbit anti-Integrin β3, anti-β-actin Ab, rabbit anti-Akt, rabbit anti-phospho-Akt (Ser32), rabbit anti-TLR7, rabbit anti-TLR9, mouse anti-IRA4 were from Cell Signaling (Danvers, MA). Rabbit anti-fibrinogen Ab was from Dako (Glostrup, Denmark). Rabbit anti-vitronectin Ab was from Molecular Innovation (Novi, MI); mouse anti-fibronectin and rabbit anti-TPOR Abs were from Abcam (Cambridge, MA). Anti-rab4, anti-rab11, anti-dynamin 2, and anti-clathrin heavy chain Abs were from BD Transduction Laboratories (San Jose, CA). Anti-JAK2 and anti-phospho (Tyr 1007/1008) JAK2 Abs were from EMD Millipore (Darmstadt, Germany). Rabbit anti-murine PF4 Ab, ELISA kit for mouse PF4 detection and thrombopoietin were from R&D Systems (Minneapolis, MN). Rabbit anti-RabGDI-Ab was generated by our laboratory using recombinant RabGDIα as antigen and described in [202]. The anti-SNAP-23 antibody was used as described in [202]. Anti-SNAP-23-pSer95 antibody that specifically recognizes phosphorylation at the Ser95 site in mouse SNAP-23 was previously characterized [457] and was generously gifted to us by Dr. Paul Roche (NCI). Rabbit Anti-Arf6 serum was homemade as described before [360]. FITC-Fibrinogen, Dextran (10,000 MW) Oregon Green 488, Alexa 647-Fibrinogen were obtained from Molecular Probes, Invitrogen (Carlsbad, CA). Prolong Diamond Antifade Reagent was from Invitrogen (Carlsbad, CA). Human fibrinogen, fibronectin, poly-D-lysine, hirudin, ammonium chloride and apyrase were all from Sigma (St. Louis, MO). Thrombin, collagen, and ADP were from Chrono-log (Havertown, PA). Prostacyclin (PGI2) was from Cayman Chemicals (Ann Arbor, MI). Trypan Blue was from Thermo Scientific (Waltham, MA). [3H]-tryptamine was from Perkin Elmer (Waltham, MA). RGDS peptide was from Anaspec (San Jose, CA). Apyrase, human fibrinogen, poly-D-lysine, hirudin, paraformaldehyde, and proteinase K were from Sigma (St. Louis, MO). Thrombin, Collagen, ADP, and CHRONO-LUME reagent were from Chrono-log (Havertown, PA). U46619 and prostaglandin I2 (PGI2) was from Cayman (Ann Arbor, MI). A23187 was from
Calbiochem (San Diego, CA). Convulxin was from Centerchem (Norwalk, CT). PAR1/PAR4-peptide was from Bachem (Bubendorf, Switzerland). Fibronectin-free fibrinogen was from Enzyme Research Laboratory (South Bend, IN). DNA ligation Kit was from Enzymax (Lexington, KY). Fura-2/acetoxyethyl ester (AM) was from Molecular Probes (Eugene, OR). Calcein-AM was from BD Biosciences (San Jose, CA). Complete, EDTA-free protease inhibitor cocktail was from Roche (Indianapolis, IL). Acid citrate dextrose (ACD) blood collection tubes were from BD Diagnostics (Sparks, MD). All other reagents used were of analytical grade.

**Mice**

The following mice were used for the experiments enlisted in this thesis. All work with animals was under the supervision of the University of Kentucky Institutional Animal Care and Use Committee (IACUC) as described in the approved protocol 884M2005.

**WT mice** (C57BL/6 background), initially purchased from Jackson Laboratory and bred in our animal vivarium.

**IKKβ**\(^{fl/fl}\)/PF4-Cre\(^{+}\) and **IKKβ**\(^{fl/fl}\) generated by Dr. Zubair Karim in our lab [242]. Polymerase Chain Reaction (PCR)-based genotyping was performed using genomic DNA isolated from tail tip of an individual mouse. For **IKKβ** gene, the PCR analysis was carried out using following primer set:

Forward primer, (MB1F) 5'-ATGTCTTGTGCCCTCTGGAAAGA-3',

Reverse primer, (IKK49 antisense) 5'- TCCTCTCCTCGTCATCCTTCG-3'.

The PCR yields a 436 bp DNA product for WT allele and a 533 bp one for floxed allele. The PCR conditions for **IKKβ** reactions were: 94°C for 7 min for 1 cycle, followed by 40 cycles of 94°C for 30 sec, 60°C for 30 sec and 72°C for 1 min and lastly 72°C for 10 min for 1 cycle.

**Global VAMP-3 KO** mice were bred and genotyped as described [202].

Forward primer, (3F) 5’-CACAGGCACCTCTGGTGCATT-3’,

Reverse primer, (6R) 5’-CCACACAGGCTCTGATCTT-3’,

Neo primer, (mutant allele) 5’-GAGCAGCCGATTGTCTGTGG-3’.

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The PCR reactions were run for WT (with 3F WT and 6R reverse) and for KO (with Neo and 6R reverse). The PCR yields a 500 bp DNA product for WT allele and a 750 bp one for the mutant allele.

The PCR conditions for VAMP-3 reactions were: 94°C for 7 min for 1 cycle, followed by 30 cycles of 94°C for 1 min, 60°C for 1 min and 72°C for 90 sec, and lastly 72°C for 10 min for 1 cycle.

Arf6^{fl/fl}/PF4-Cre^{+} and Arf6^{fl/fl} was generated in our lab by Dr. Yunjie Huang [19]. For Arf6 gene, the PCR analysis was carried out using following primer set:
Forward primer, 5'-GACCCCATGAGTGTTGTCAC-3',
Reverse primer, 5'-GGGATACATAGAGAAACCTTGTCTCAGG-3'.
The PCR yields a 240 bp DNA product for WT allele and a 270 bp one for floxed allele.
The PCR analysis for the Cre transgene was carried out using the following primer set:
For the wild-type allele,
Forward primer 5’-CCCATAACGACACCTTTTG-3’,
Reverse primer 5’-GAAACAACAGGCCCAGAAGC-3’;
For the Cre allele,
Forward primer 5’-CCCATAACGACACCTTTTG-3’,
Reverse primer 5’-TGCACAGTCAGCAGGTT-3’.
PCR yielding a 450 bp DNA product indicates the presence of PF4-Cre gene.

PCR conditions for both Arf6 gene and PF4-Cre gene are 94°C for 10 min for 1 cycle, followed by 30 cycles of 94°C for 1 min, 54°C for 45 sec and 68°C for 30 sec, and lastly 68°C for 7 min for 1 cycle.

Global TLR23479-KO mice were a generous gift from Dr. Jayakrishna Ambati, University of Virginia, VA.

Genomic DNA isolation from mouse tail tip
Mouse tail tips (3-5 mm) were digested overnight (O/N) at 55°C in 400 µl tail lysis buffer (50 µM Tris/HCl, pH 7.5, 100 µM EDTA, 100 µM NaCl, and 1% SDS after autoclave) containing 20 µl 10 mg/ml proteinase K,. Protein/peptide was precipitated by mixing with 200 µl saturated 6 M NaCl and centrifuged at 13,000 x g for 30 min at room temperature (RT). The supernatant containing DNA was
harvested and mixed with equal volume of 100% ethanol. After incubation for 10 min at RT, DNA was pellet down by centrifuge at 13,000 x g for 10 min. DNA pellets were washed once with 1 ml 70% ethanol and then dried in a vacuum centrifuge for around 20 min. Genomic DNA was finally dissolved in 100 µl autoclaved ddH₂O.

**Whole blood count**

Blood was drawn via cardiac puncture from VAMP-3 KO and control WT littermates and whole blood counts were performed using a Hemavet (Drew Scientific, Dallas, TX). Mean Platelet Volume (MPV) measurements were taken using the Z2 Coulter Counter (Beckman Coulter, Inc., Miami, FL). Statistical analyses were done using the statistical software program in SigmaPlot (v13.0).

**Preparation of mouse platelets**

Mouse blood was harvested via cardiac puncture into 0.38% sodium citrate (final) and diluted (1:1 v/v) in PBS, incubated with 0.2 U/mL apyrase and 10 ng/mL prostacyclin (PGI₂) and centrifuged at 237 x g at room temperature (RT). Platelet-rich plasma (PRP) was recovered and platelets were pelleted at 657 x g. The pelleted platelets were resuspended in HEPES Tyrode buffer (pH 6.5, 20 mM HEPES/KOH, 128 mM NaCl, 2.8 mM KCl, 1 mM MgCl₂, 5 mM D-glucose, 12 mM NaHCO₃, and 0.4 mM NaH₂PO₄), supplemented with apyrase, PGI₂, and 1 mM EGTA, and recovered by centrifugation at 657 x g. Pelleted platelets were finally resuspended in HEPES Tyrode buffer (pH 7.4) and concentrations were measured using Z2 Counter (Beckman Coulter, Inc., Miami, FL).

**Preparation of fresh human platelets**

Fresh whole blood was drawn from an informed healthy donor using acid citrate dextrose (ACD) collection tubes (BD Biosciences). Whole blood was diluted in HEPES Tyrode buffer (pH 6.5) with 1:1 ratio in the presence of 0.2 U/ml apyrase and 10 ng/ml PGI₂. After centrifugation at 215 x g for 20 min at RT, the supernatant (PRP) was harvested. To remove residual red blood cells and white blood cells, PRP was subjected to centrifugation at 155 x g for 10 min at RT. Platelets were recovered from the resulting PRP by centrifugation at 750 x g for 13 min at RT and then resuspended in HEPES Tyrode buffer (pH 6.5) in the presence of 0.2 U/ml apyrase, 10 ng/ml PGI₂ and 1 mM EGTA. Platelets suspensions were subjected to centrifugation
again at 750 x g for 13 min at RT and the resulting pellet was resuspended in HEPES Tyrode Buffer (pH 7.4) supplemented with 1 mM CaCl₂. Platelet concentrations were determined by using Z2 Counter (Beckman Coulter, Inc., Miami, FL) and adjusted to 4 X 10⁸/ml with HEPES Tyrode buffer (pH 7.4) unless otherwise indicated. Platelets were incubated at RT for 30 min before use.

**Platelet aggregometry and ATP release from dense granules**

Washed platelets were freshly prepared and adjusted to the desired concentration with HEPES Tyrode buffer (pH 7.4) containing 1 mM CaCl₂. Platelets suspensions (250 or 500 µl) in a siliconized glass cuvette (Chrono-log, Havertown, PA) was warmed to 37°C under stirring conditions (1200 rpm) for 2-3 min in a Model 460Vs Lumi-dual aggregometer (Chrono-log). After pretreatment with the indicated reagents, platelets were stimulated with the indicated agonists to initiate platelet aggregation. A model 810 Aggro/Link computer interface and Aggro/Link software (Chrono-log) were used to monitoring platelet aggregation traces. Buffer alone had been used to set baselines.

ATP release from platelets dense-core granules was monitored using Chrono-Lume reagents (Chrono-log) while measuring platelet aggregation. Chrono-Lume reagents containing luciferin and luciferase enzyme were pre-incubated with platelet suspension (1:20 ratio) for 1 min prior to addition of agonists. Luminescence traces were recorded using the same interface and software as platelet aggregation. Graphs of platelet aggregation and ATP release was processed and assembled into figures using Adobe Photoshop CS5 (Adobe Inc. San Jose, CA).

**Platelet lysate preparation and western blotting**

Platelet lysates were prepared in SDS-PAGE sample buffer supplemented with 2 mM Na₃VO₄, 2 mM PMSF, protease inhibitor cocktail, and phosphatase inhibitor cocktail (PhosStop, Roche). Platelet proteins were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to Immobilon-P PVDF membranes (Millipore Corp., Bedford, MA). Transferred proteins were probed with suitable primary Abs and visualized using appropriate alkaline phosphatase-conjugated secondary Abs and Amersham™ enhanced chemifluorescence (ECF) substrate (GE Healthcare Life Sciences, Piscataway, NJ). Images were recovered with
a Typhoon 9500 Variable Mode Imager and quantified using ImageQuantTL software (GE Healthcare Life Sciences, Piscataway, NJ).

Flow cytometry analysis
To measure the surface levels of total and activated GPIIb/IIIa, washed platelets (5 x 10^7/mL) were either kept resting or stimulated with 0.1 U/mL thrombin for 1 min. The reactions were stopped with hirudin (2-fold excess) and incubated with FITC-anti-CD41/61 antibody and PE-Jon/A antibody. The mixture was analyzed by flow cytometry and geometric mean fluorescence intensity (GMFI) was measured.

Washed platelets from WT and KO mice were freshly prepared and adjusted to 5 X 10^7/ml in HEPES Tyrode buffer (pH 7.4) in the presence of 1 mM CaCl_2. Platelets (20 µl) were either kept resting or stimulated with 0.1 U/ml thrombin for 1 min or with any of the TLR agonists (Loxoribine, CpG ODN2395, LPS, Pam3CSK4 or HIV-1 pseudovirions (HIVpp)). The reactions were stopped by adding a 2-fold excess of hirudin or HT pH 6.5 wash buffer in the case of TLR and HIVpp agonists. Samples were then incubated with fluorophore-conjugated antibodies (2.5 µl each) for 15 min at RT. FITC-anti-CD62 antibody and PE-anti-LAMP1 antibody were used to measure P-selectin and LAMP1. The mixture was transferred to polystyrene Falcon™ tubes (BD Biosciences, San Jose, CA) containing 200 µl HEPES Tyrode buffer (pH 6.5). Unlabeled platelets were used as background controls. Samples were analyzed at the flow cytometry facility at the University of Kentucky. Flow cytometry analysis was performed using FACScan™ flow cytometer (BD Biosciences). Platelet populations were determined by a combination of forward light scattering (FSC) and side light scattering (SSC). CellQuest™ (BD Biosciences) was used to monitor platelet fluorescent intensities. A total of 10,000-20,000 platelet events were analyzed and the geometric mean of the fluorescent intensity was determined and analyzed using the SigmaPlot (v13.0) statistical software.

To measure steady-state FITC-fibrinogen binding, platelets (1.0 x 10^9/mL) were incubated on ice for 20 min, and then with 0.06 or 0.12 mg/mL FITC-fibrinogen for an additional 20 min. Platelets were fixed with 2% paraformaldehyde (PFA) and analyzed by flow cytometry. The difference of geometric mean fluorescence intensity (GMFI) before and after 0.04% Trypan Blue addition was used to assess
surface fibrinogen binding (0.04% Trypan Blue quenched >95% of free FITC-fibrinogen [19]).

To study activated state FITC-fibrinogen endocytosis, washed platelets (1.0 x 10^9/mL) were incubated with or without ADP (10 µM) in the presence of 0.12 mg/mL FITC-fibrinogen at 37°C for 30 min. The platelets were then put on ice for 20 min, fixed with 2% PFA (final conc.), and GMFI measurements were taken by flow cytometry before and after addition of 0.04% Trypan Blue.

**Whole blood platelet-leukocyte aggregation assay**

Whole blood (in ACD) was pre-treated for 10 min with an anti-mouse CD16/CD32 antibody (Biolegend, San Diego, CA) to block the FcγRIII/II receptors on immune cells to prevent antibody-antigen immune complexes. This antibody was a generous gift from Dr. Ahmad Al-Attar, University of Kentucky. After pretreatment with the indicated reagents, platelets were kept quiescent or stimulated with the indicated agonists (Loxoribine, CpG ODN2395, LPS, Pam3CSK4, HIV-1 pseudovirions or thrombin) to initiate platelet-leukocyte aggregation. To 50 µL of whole blood in a polystyrene Falcon™ tubes (BD Biosciences, San Jose, CA), add 5 µL each of anti-platelet CD41-FITC conjugated antibody, anti-neutrophil Ly6G-APC/PE-conjugated antibody or anti-monocyte CD14-PE conjugated antibody and vortex gently while incubating for 15-30 min in the dark at RT. Add 500-1000 µL of 1X BD FACS/Lyse Solution (BD Biosciences Cat No. 349202) and vortex gently while incubating for 10 min in the dark at RT. Centrifuge the tube contents at 500 x g for 7 min to remove the supernatant. This removes all the lysed red blood cells. Finally, samples were resuspended in 500 µL of 1X PBS pH 7.4 and take the tubes for FACS analysis. Samples were run on the BD LSRII machine and 500,000 events were captured per sample with gating around 50,000 granulocytes. Data was then analyzed using the FlowJo software (v7.6.5) and plotted and analyzed using the SigmaPlot (v13.0) statistical software.

**Static platelet adhesion**

Washed platelets (4 x 10^8/mL), labeled with 10 μM calcein AM (BD Bioscience) and 0.2% of Pluronic F-127 (Invitrogen), were seeded onto 96-well opaque plates, precoated with either 50 µg/mL human fibrinogen or 5% bovine serum albumin (BSA) in
buffer (25 mM Tris, 137 mM NaCl, pH 7.4). After incubation for 1 hr at 37°C, the wells were washed thrice with HEPES Tyrode Buffer (pH 7.4) and adherent platelets were measured by fluorescence using a SpectraMax plate reader (excitation/emission/cutoff: 485/538/515 nm). Standard curves were generated using labeled WT and KO platelets.

**Platelet spreading**

Fibrinogen-coated slides were prepared by incubating human fibrinogen (50 µg/ml in PBS) in the wells of a Nunc Lab-Tek II chamber slide (Thermo Scientific) overnight at 4°C. WT and KO platelets (2 X 10^7/mL) were suspended in HEPES Tyrode buffer (pH 7.4) containing 1 mM CaCl2, seeded into the fibrinogen-coated wells, and incubated at 37°C for the indicated times. Unbound platelets were removed by washing and the adherent platelets were fixed with 2% PFA overnight at 4°C. Images of spread platelets were taken using DIC microscopy and quantified with ImageJ software (v1.48, NIH).

**Clot retraction**

Acrylamide pads (polymerized 10% solution) were added to siliconized cuvettes and washed thrice with HEPES Tyrode buffer pH 7.4. Thrombin (0.05 U/mL) was used to stimulate clot formation and retraction in washed WT and KO platelets (500 µL; 3 x 10^8/mL) supplemented with 1 mM CaCl2 in the presence of human fibrinogen (0.5 mg/mL; Sigma). Images were taken at increasing times and clot sizes were measured using ImageJ software (v1.48, NIH). The percent of clot sizes relative to initial suspension volumes were determined and analyzed using SigmaPlot (v13.0).

**Plate assay to measure endocytosis**

Opaque/black 96 well polystyrene plates (Corning, USA) were coated with 5% bovine serum albumin (BSA) in Phosphate Buffered Saline (PBS) overnight at RT. Wild-type (WT) and knockout (KO) washed platelets (100 µL; 5 X 10^7/mL), pre-incubated with 1 mM CaCl2 for 5 min, were added to each well and either incubated with varying concentrations of FITC-Fg or low molecular weight (10 kDa) Oregon Green 488-dextran for increasing times at 37°C. Fluorescence intensities were measured using a SpectraMax plate reader (Molecular Devices; excitation/emission: 495/520 nm) before and after addition of 0.04% Trypan Blue (0.04% Trypan Blue quenched >95%
of Oregon green 488-dextran, data not shown) to stop reactions at various time points. Standard curves were generated using serial dilutions of FITC-Fg or Oregon Green 488-dextran.

**Cargo uptake *ex vivo***

Washed platelets (1 X 10^9/mL) from WT and KO mice were incubated with FITC-Fg and Oregon Green 488-dextran (1 µM each) at 37°C for increasing times up to 30 min. Platelets were fixed with 2% PFA and mixed with 0.1% Trypan Blue prior to imaging. Platelets were visualized using epifluorescence and Differential Interference Contrast (DIC) microscopy on a Nikon Eclipse E600 microscope (Nikon, Melville, NY) fitted with an AxioCam MR camera (Zeiss, Germany) and processed using the Zen 2011 Digital Imaging software (blue edition, Zeiss). The number of FITC-positive or Oregon Green 488-positive puncta per platelet in both WT and KO samples were counted and plotted using SigmaPlot software (v13.0).

**Cargo uptake *in vivo***

WT and KO mice (n= 3, each) were injected with Alexa 647-Fg and Oregon Green 488-dextran (2 µM each) through the retro-orbital sinus and, 24 hr post-injection, mice were euthanized and blood was drawn directly into fixative (0.38% sodium citrate containing 2% PFA and 0.05% glutaraldehyde). Platelets were harvested, refixed with 2% PFA, and prepared for imaging.

**3D-SIM super-resolution microscopy (3D-SIM)**

Washed WT and KO platelets (1.0 x 10^9/mL) were incubated *ex vivo* with Alexa 647-Fg and Oregon Green 488-dextran (1 µM each) at 37°C, then fixed with PFA. Centrifugation (700 x g for 5 min) was used to remove fixative and platelet pellets were re-suspended in PBS. The platelets were fixed a second time and then allowed to settle onto poly-D-lysine (0.1mg/mL) coated coverslips for >90 min at RT in a humid chamber. To quench fixative, coverslips were incubated cell-side down in 50 mM NH4Cl in PBS for 30 min at RT. Coverslips were washed and mounted in Prolong Diamond Antifade Mounting medium and cured for 2 days at RT in the dark.

For *in vivo* experiments, 24 hr post-injection of Alexa 647-Fg and Oregon Green 488-dextran, mice were euthanized and platelets were directly fixed during the blood draw. Platelets were then isolated and allowed to settle onto poly-D-lysine coated
coverslips. Imaging was done using the Nikon Ti-E N-STORM/N-SIM super-resolution microscope at the University of Kentucky Imaging Core. Images were processed using NIS-Elements v3.2 N-SIM/STORM software.

**TPO-induced JAK2 signaling**

Washed WT and KO platelets (4 x 10⁸/mL) were pre-incubated with 1 mM CaCl₂ and then with 50 ng/mL of thrombopoietin (TPO; R&D Systems) for 0 to 30 min at 37°C. Platelet extracts were prepared in SDS-PAGE sample buffer (supplemented with protease inhibitors) and analyzed by western blotting using anti-JAK2 and anti-phospho JAK2 (Tyr 1007/1008) and anti-TPOR antibodies.

**Measurement of platelet granule cargo release (secretion assays)**

Platelet-rich plasma (PRP) from WT and KO mice were prepared and labelled with 0.4 μCi/mL [³H]5-HT (serotonin, Perkin Elmer, Waltham, MA) for 30 min at 37°C. After washing, platelets were resuspended in HT buffer pH 7.4 fortified with 0.7mM CaCl₂ for 5 min prior to stimulation with thrombin or TLR agonists (LPS, Pam3CSK4, Loxoribine, CpG ODN) for indicated times or doses. 2X Hirudin was added to stop the reaction for thrombin-treated samples while centrifugation at 13, 800 x g for 2 min was done to stop the TLR agonist reactions. For each time point or dose reaction, samples were prepared in triplicates. The supernatant and pellet was separated post-centrifugation, kept on ice and the pellets were lysed with 1% Triton X-100 solution in 1X PBS on ice for about 45 min. The supernatant and pellet samples were vortexed and centrifuged one final time (13, 800 x g for 1 min) before doing the individual granule assays.

For dense granule assay, 25 µL of supernatant or pellet is added to 3 mL of scintillation cocktail (Econo-safe™, Research Products International Corp, Mt. Prospect, IL) and the radioactivity is read as counts per minute using a Tri-Carb 2100TR liquid scintillation analyzer (Beckman, Fullerton, CA). For alpha granule assay, sandwich enzyme-linked immunosorbent assay (ELISA) is used to detect the release of platelet-specific alpha granule cargo, Platelet Factor 4, PF4, a chemokine, in the supernatant and pellet fractions of resting and activated platelets. The assay is performed according to the manufacturer’s instructions (R&D Systems, Minneapolis, MN). For lysosomal secretion, a colorimetric assay to measure the release of β-
hexosaminidase in the pellet and supernatant fractions is measured by analyzing its enzymatic activity on its substrate p-nitrophenyl-N-acetyl-β-D-glucosaminide (Sigma). Optical density from each sample (pellet and supernatant) is measured at 405 nm using a Biotek Elx808 plate reader and data is collected using the Softmax Pro 6 software (Molecular Devices, Sunnyvale, CA). The percent release for each marker at each data point was calculated by using the supernatant fraction measurements divided by the total measurements (supernatant + pellet values) multiplied by 100.

**TLR activation of platelets**

Washed WT and KO platelets or freshly isolated human platelets (4 X 10^8/mL) were pre-incubated with 1 mM CaCl2 and then with increasing times or increasing concentrations of various TLR compounds such as TLR2 agonist (Pam3CSK4), or TLR4 agonist (LPS O111:B4), or TLR7 agonist (Loxoribine) or TLR9 agonist (CpG ODN2395 Class C) at 37°C. In some instances, platelets were either pre-treated with ammonium chloride or Dynasore (to inhibit endocytosis) prior to TLR stimulation. Platelet extracts were prepared in SDS-PAGE sample buffer (supplemented with protease inhibitors) and analyzed by western blotting using anti-Akt, anti-phospho-Akt (Ser473), anti-SNAP-23, anti-phospho-SNAP-23 (Ser95), anti-IRAK4, anti-phospho-IRAK4 (Thr345) (Abbomax), anti-IκBα and anti-phospho-IκBα (Ser32/36) antibodies (in the case of human platelets).

**Preparation of HIV-1 pseudovirions**

**Plasmids**

Viral vectors to make the HIV-1 pseudovirions were a generous gift from Dr. Gregory Melikyan, Emory University. These were: (i) A chimeric Ecph-TM expressing vector containing the ecliptic pHluorin, derived from the original ecliptic pHluorin construct [455] and transmembrane (TM) domain of ICAM-1 (intercellular adhesion molecule 1). The pCDM8 ICAM-1 vector (Addgene) was used as the template for the TM domain of ICAM-1 while the MLV (murine leukemia virus) Gag-EcpH-expression vector was used as a template for the EcpH [456]. (ii) The HIV-1 Gag-mCherry expression vector was constructed by cloning the Gag fragment of the p96ZM651gag-opt vector [National Institutes of Health ARRRP (AIDS Research and Reference Reagent Program)] into pcDNA3.1zeo (+) plasmid (Invitrogen) together
with the mCherry fragment, which was obtained from pRSET-BmCherry (from Dr. R. Tsein, University of California, San Diego). The other two vectors were (iii) HIV pR8 delta Env and (iv) pCAGGS.HXB2 Env.

**Pseudovirion Preps**

The vectors were first amplified in E. coli expression systems by Midi-Prep. Viral preps were made by Dr. Gabriel J. Popa and Dr. Michael D. Mendenhall at the University of Kentucky Genetic Core. Pseudovirions were then prepared by transfecting 293-LTV cell lines with all four viral vectors in the following proportions [HIV pR8 delta Env (30%), HIV-Gag-mCherry (12.5%), 3x FLAG-EcpH-ICAM (12.5%) and pCAGGS.HXB2 Env (45%)] using polyethyleneimine (PEI). Cells were transfected and after 5-6 hrs, the transfection medium was withdrawn and fresh medium (DMEM+ fortified with 10% FBS) was added. Cells were grown for 48-72 hrs depending on GFP and mCherry expression. Viruses were then harvested and concentrated by centrifugation at 4°C over a 20% sucrose cushion. Viral titers in the concentrates were quantified by measuring amount of p24 using the ZeptoMetrix HIV-1 p24 antigen ELISA kit (Cat No. 0801111) (ZeptoMetrix, Franklin, MA).

**Ex vivo uptake of HIV-1 pseudovirions**

Washed WT and KO platelets were resuspended at concentrations of 1 × 10^9/ml in supernatants from the 293-LTV cells (containing the HIV-1 pseudovirions) or in HPEPS Tyrode Buffer containing 1% BSA and 1 mM CaCl₂ that was supplemented with 100X concentrated pseudovirions. Platelets (100 µl) were incubated for varying times at 37°C in the CO₂ incubator. Aliquots were drawn at different intervals and fixed with 2% paraformaldehyde at 4°C overnight. Platelets were recovered by mild centrifugation and resuspended in 15 µl HEPES Tyrode buffer. Microscopic slides with 5 µl platelets mixed with 1 µl Trypan Blue (stock 4%) were prepared and visualized using Nikon Eclipse E600 microscope (Nikon, Melville, NY) with a 100X/1.40 numeric aperture DIC H oil objective lens (Nikon). Images were taken using Zeiss camera (AxioCam MR, Germany). Images were processed using Zen 2011 (blue edition, Zeiss). As a metric of viral uptake, the number of GFP-mCherry double-positive puncta per platelet was counted. To eliminate non-specific fluorescence signal from the EcpH-TM-labelled particles that are probably membrane vesicles
lacking the Gag-mCherry, only the double-positive virions were counted. The presence of double-positive pseudovirions within the platelets should thus reflect virus internalization.

**In vivo uptake of HIV-1 pseudovirions**

Mice from WT and KO groups (age-matched and sex-matched) were anesthetized under isoflurane inhalation and HIV-1 pseudovirions were injected using a 27G needle (BD Biosciences) through the retro-orbital sinus. 24-hr post-injection, mice were euthanized, blood was drawn directly into fixative and platelets isolated as previously described. Microscopic slides with 5 µl platelets mixed with 1 µl Trypan Blue (stock 4%) were prepared and visualized using Nikon Eclipse E600 microscope (Nikon, Melville, NY) with a 100X/1.40 numeric aperture DIC H oil objective lens (Nikon). Images were taken using Zeiss camera (AxioCam MR, Germany). Images were processed using Zen 2011 (blue edition, Zeiss). As a metric of viral uptake, the number of GFP-mCherry double-positive puncta per platelet was counted.

**HIV-1 pseudovirion activation of human and mouse platelets**

Washed WT and KO platelets or freshly isolated human platelets (4 X 10^8/mL) were pre-incubated with 1 mM CaCl₂ and then with increasing times or increasing concentrations of HIV-1 pseudovirions at 37°C. In some instances, platelets were either pre-treated with ammonium chloride or Dynasore (to inhibit endocytosis) prior to HIV-1 pseudovirion stimulation. Platelet extracts were prepared in SDS-PAGE sample buffer (supplemented with protease inhibitors) and analyzed by western blotting using anti-Akt, anti-phospho-Akt (Ser473), anti-SNAP-23, anti-phospho-SNAP-23 (Ser95), anti-IRAK4, anti-phospho-IRAK4 (Thr345) (Abcamax), anti-IκBα and anti-phospho-IκBα (Ser32/36) antibodies (in the case of human platelets).

**Study approval**

All animal procedures were done under an approved protocol (884M2005) from the University of Kentucky Institutional Animal Care and Use Committee (IACUC). Human blood drawn from healthy donors was obtained at the Kentucky Clinic Lab by a phlebotomist.
Statistics

Where appropriate, Student’s t-test or Mann-Whitney U test (from the SigmaPlot statistical software) were used to determine statistical significance. The data from secretion assays and FACS-based experiments were analyzed by two-way ANOVA. A one-tailed Student’s t-test was used to analyze the properties of platelets from KO mice. In all cases, the p values are indicated.
Chapter Three: VAMP-3-mediated endocytosis and trafficking regulate platelet functions

Introduction

Blood platelets respond to repair vascular damage via activation and subsequent granule exocytosis [458]. Platelet endocytosis is essential for cargo packaging (e.g., fibrinogen (Fg)) [14, 9, 340]. However, platelet endocytosis could be critical for actively sensing changes in vascular microenvironments and responding accordingly to what is being taken up. This is a more dynamic view of platelets as active surveyors of the vasculature; extending the importance of platelet endocytosis beyond granule biogenesis and perhaps even hemostasis. The mechanistic underpinnings of endocytosis and its importance in platelets are however understudied, in part due to a lack of experimental tools.

Endocytosis refers to cargo uptake and receptor trafficking to and from the plasma membrane through distinct membrane-bound compartments. Seminal studies, from the 1980s, provided the first glimpses into the presence of an active endomembrane system in platelets. Platelets have clathrin-coated vesicles [326, 331]; can endocytose several plasma proteins (e.g., albumin, IgG, Fg, von Willebrand Factor (vWF), fibronectin) and translocate them to α-granules [340, 15-17, 328-330]. Both clathrin-mediated and clathrin-independent endocytosis has been reported in platelets [327]. Additionally, several platelet surface receptors such as integrin α_{iib}β_{3} and glycoprotein Ib are internalized and then recycled back to the plasma membrane [334, 336-337]. Platelet CLEC-2 is internalized in a Syk-independent manner whereas Arrestin and Arf6 have been shown to be crucial to endocytic trafficking of the P2Y_{12} receptors [21, 459]. ADP and thrombin stimulation leads to translocation of internal pools of receptors and surface re-distribution [333, 335-336, 338-339]. Consistent with these reports of platelet endocytosis, platelets do contain key endocytic proteins and regulators including the vesicle scission-inducing large GTPase, Dynamin, Dynamin-related protein 1 (Drp1), and adaptor proteins such as Disabled-2 (Dab-2) [22, 344, 347, 351, 356-357]. Recently, we highlighted the role of ADP-ribosylation factor 6 (Arf6), a small GTP-binding protein, that specifically regulates α_{iib}β_{3}-mediated Fg uptake/storage and hence modulates acute platelet functions.
such as clot retraction and spreading [19]. These studies are supported by “omics” analyses [200, 343] that highlight the burgeoning importance and complexity of endocytosis in platelets.

Here we describe a role for Cellubrevin/Vesicle-Associated Membrane Protein-3 (VAMP-3) in mediating endocytosis and trafficking in platelets. This v-SNARE has been reported to be localized to intracellular punctate structures within platelets [30]. Using a knockout (KO) mouse, with normal hemostasis and whose platelets had no aggregation or secretion defects [202], we determined that VAMP-3 plays a role in Fg uptake and governs platelet endocytosis. VAMP-3 deletion caused defective α\textsubscript{IIb}β\textsubscript{3}-mediated Fg uptake and accumulation while fluid phase pinocytosis, as monitored by dextran uptake, was marginally affected, both \textit{in vivo} and \textit{ex vivo}. Platelet spreading on Fg and thrombin-stimulated clot retraction was faster in the VAMP-3 KO platelets. Additionally, we demonstrate that platelets sort endocytosed cargo into discrete compartments \textit{in vivo} and \textit{ex vivo}. Taken together, our work presents VAMP-3 as a regulator of platelet endocytosis and this knockout mouse adds to the repertoire of tools for studying endocytic trafficking in platelets while maintaining normal exocytosis and hemostasis.

\textbf{VAMP-3 KO platelets were fibrinogen-deficient}

Given past reports of fibrinogen (Fg) endocytosis by platelets [340, 329] and the intracellular localization of VAMP-3 [205], we asked if VAMP-3 played a role in Fg uptake and/or accumulation. In Figure 1A-B, VAMP-3 KO platelets, isolated from 3 individual mice, had less associated Fg (~40% less, \(p \leq 0.001\)) than their corresponding WT littermate controls, indicating a defect in uptake and/or accumulation of Fg. Plasma Fg levels were unchanged suggesting that loss of VAMP-3 did not affect Fg production (Figure 7). The major platelet integrin that binds Fg [337], α\textsubscript{IIb}β\textsubscript{3}, was also unaffected by loss of VAMP-3 as evidenced by the unchanged levels of β\textsubscript{3} in the KO platelets (Figure 3C). Ligands for other platelet integrins, \textit{e.g.}, fibronectin (binding to α\textsubscript{5}β\textsubscript{1} and α\textsubscript{v}β\textsubscript{3}) and vitronectin (binding to α\textsubscript{v}β\textsubscript{3}), were either unchanged or slightly elevated in KO platelets (Figure 3D). Of note, vitronectin levels were also slightly increased in the Arf6 KO platelets as compared to WT [19]. Since VAMP-3 is thought to localize on both early and recycling endosomes [403, 458],
Rab4 (early) and Rab11 (recycling) levels were measured and found to be unchanged in KO platelets (Figure 3C-D). Other platelet endocytic markers e.g., Dynamin-2, Clathrin Heavy Chain and Disabled-2 (Dab2) were also unchanged (Figure 3C-D). Levels of de novo synthesized cargo such as platelet factor 4 (PF4) and other platelet SNARE proteins were unaltered in the VAMP-3 KO platelets, consistent with normal granule secretion [202]. Defective Fg endocytosis did not alter the gross morphology of VAMP-3 KO platelets. Moreover, the KO mice had normal platelet, RBC and WBC counts (See Table 2), although their platelet size was ~5% smaller than age-matched WT littermate controls. Thus the reduced intra-platelet Fg observed in KO platelets was not due to alterations in any endocytic proteins and regulators or due to decreased levels of β3 integrins and circulating plasma Fg, suggesting that VAMP-3 is essential for uptake and/or accumulation of Fg in platelets.
**Figure 3 VAMP-3 KO platelets had lower fibrinogen levels.** (A) Fg levels in washed platelet extracts from 3 WT and KO mice were measured by western blotting. β-actin was used as loading control. (B) Quantification of Fg levels in (A) was done using ImageQuantTL and plotted with SigmaPlot (v 13.0). (C) Comparison of protein levels by Western blotting between WT and KO platelets. Washed platelet extracts (5 X 10^7 platelets/lane) were loaded, and the indicated proteins were probed by western blotting. (D) Quantification of protein levels was done using ImageQuantTL and data was plotted as the ratio of KO over WT. The dashed line represents the ratio 1 of KO/WT protein levels. Statistical analysis was done using Student’s t-test where *** represents p-value of ≤0.001. Data for (C) and (D) is representative of platelets pooled from 2-3 mice in at least 2 independent experiments.
Surface $\alpha_{\text{IIb}}\beta_3$ levels and Fg-binding was unchanged on the VAMP-3 KO platelets

Reduced levels of internalized Fg could be due to decreased surface and/or activated $\alpha_{\text{IIb}}\beta_3$. By flow cytometric analysis, levels of surface $\alpha_{\text{IIb}}\beta_3$, under resting conditions, were unchanged in the KO platelets (Figure 4A i-iii). As expected [19], thrombin-induced activation mobilized an internal pool of $\alpha_{\text{IIb}}\beta_3$ integrins. We observed a $\sim$25% increase in the total surface $\alpha_{\text{IIb}}\beta_3$ levels upon stimulation. While this $\sim$25% increase was noted in both WT and KO platelets, there was no significant difference between the two. Similarly, in Figure 4B (i-iii), levels of activated $\alpha_{\text{IIb}}\beta_3$, as measured by JonA binding, were unchanged in VAMP-3 KO platelets, consistent with normal aggregation [202]. Defective Fg uptake, despite normal levels of $\alpha_{\text{IIb}}\beta_3$, could be attributed to defective surface-binding of Fg to $\alpha_{\text{IIb}}\beta_3$. Under steady-state conditions, FITC-Fg binding at two different concentrations (0.06 and 0.12 mg/mL) was unaffected in the VAMP-3 KO platelets (Figure 4C). Taken together, these results indicated that the lower Fg levels in KO platelets were not due to defective binding or reduced surface levels of $\alpha_{\text{IIb}}\beta_3$. 
Figure 4

A (i) Surface αIIbβ₃
- Control
- WT
- KO

(ii) Surface αIIbβ₃
- Resting
- Thrombin

B (i) Activated αIIbβ₃
- Control
- WT
- KO

(ii) Activated αIIbβ₃
- Resting
- Thrombin

(iii) Activated αIIbβ₃
- Resting
- Thrombin

C

FITC-Fibrinogen Bound (GMFI)

Fibrinogen (mg/mL)

0.06
0.12
Figure 4. Total surface and activated $\alpha_{\text{IIb}}\beta_3$ and fibrinogen-binding were unchanged in VAMP-3 KO platelets. (A) Using FITC-anti-CD41/61 antibody, surface levels of total $\alpha_{\text{IIb}}\beta_3$ between WT (dark gray, solid lines) and KO platelets (light gray, dotted lines) (5 X $10^7$ platelets/mL) and (B) Using PE-Jon/A antibody, activated $\alpha_{\text{IIb}}\beta_3$, in resting state (i) or thrombin-stimulated (ii) were compared using flow cytometry. Unlabeled platelets (black, solid lines) were used as background controls. (iii) Quantification of data shown in Ai or Bi and Aii or Bii expressed as geometric mean fluorescence intensity (GMFI) was plotted. WT is depicted in black while KO is depicted in gray. (C) Fg-binding to $\alpha_{\text{IIb}}\beta_3$ between WT (black) and KO (gray) platelets was compared. Washed platelets (1 X $10^9$ platelets/mL) were held on ice for 20 min, and then incubated with FITC-Fg (0.06 mg/mL and 0.12 mg/mL) for a further 20 min on ice. Platelets were fixed with 2% PFA and analyzed using flow cytometry. Data expressed as GMFI was quantified as shown. Data shown here is representative of at least 3 independent experiments (mean ± SEM) for (A), (B) and (C).
VAMP-3 KO platelets had defective Fg uptake

ADP-stimulation enhances internalization of Fg-bound α_{Iibβ3} into platelets [333, 335]. We verified this observation using flow cytometric analysis to measure FITC-Fg uptake in resting and ADP-stimulated WT and VAMP-3 KO platelets. Similar to Huang et al. 2016 [19], we used 0.04% Trypan Blue (TB) to quench external FITC fluorescence (including the fluorescence that is trapped within the open canalicular system) and measured TB-resistant fluorescence of internalized cargo as a metric of endocytosis. Platelets were incubated with FITC-Fg, either in the presence or absence of ADP, at 37°C, and then chilled on ice to arrest further endocytosis, fixed with 2% PFA, analyzed using flow cytometry prior to (darker bars) and after (lighter bars) addition of TB. In the absence of TB, geometric mean fluorescence intensity (GMFI) measurements represent the total fluorescence intensity of FITC-Fg present both inside the platelet and on the surface. In Figure 5A, under resting conditions, the total fluorescence intensity prior to the addition of TB was equivalent between WT and KO platelets, confirming equal amounts of FITC-Fg bound in each sample. Post-TB quenching, KO platelets had ~50% less FITC-Fg signal (p ≤ 0.03 at 0.06 mg/mL and p ≤ 0.006 at 0.12 mg/mL) indicating that under steady-state conditions, VAMP-3 loss affected platelet internal accumulation of Fg. In Figure 5B, activation by ADP (10 µM) stimulation increased overall net fluorescence for both WT and VAMP-3 KO platelets by ~30 fold, indicative of α_{Iibβ3} activation caused by ADP receptor signaling [333, 335]. Although VAMP-3 KO platelets had enhanced Fg association comparable to WT, less FITC-Fg was resistant to TB addition, consistent with a ~50% deficit in internalized FITC-Fg (p ≤ 0.039 at 0.06 mg/mL and p ≤ 0.023 at 0.12 mg/mL). Collectively, these data suggest that VAMP-3 deletion leads to impaired endocytic uptake and/or accumulation of Fg into platelets in both resting and activated states.

Endocytosis defects in VAMP-3 KO platelets: receptor-mediated vs. fluid-phase endocytosis

Platelets can endocytose diverse cargo e.g., VEGF [459], bacteria, and viruses [[263] and Banerjee, et al. (in preparation)] etc., thus we asked whether loss of VAMP-3 affected other cargo. Uptake of fluorescently tagged, receptor-mediated cargo (Fg) and fluid-phase pinocytosis cargo (low mol. wt. dextran) was tracked in a plate-based
Unsurprisingly, fluid phase endocytosis/pinocytosis occurs constitutively in WT platelets and showed a linear increase in dextran uptake with time (Figure 5D) and concentration (Figure 5F). Loss of either VAMP-3 or Arf6 renders platelets nominally defective in dextran uptake, but there was still a time- and dose-dependence similar to WT. Our data agrees with reports of normal pinocytosis of small molecules such as horseradish peroxidase in VAMP-3 KO mouse embryonic fibroblasts (MEFs) when compared to wildtype MEFs [419-420]. Interestingly, from a quantitative perspective, more of the fluid phase marker (dextran) was endocytosed than Fg ($10^{18}$ vs. $10^{15}$ molecules/platelet over 60 min). These data were consistent with fluid phase pinocytosis having a higher overall capacity than receptor-mediated uptake. Arf6 KO platelets had only a modest decrease in dextran uptake as compared to WT (Figure 10B, D). Collectively, our data suggests that both VAMP-3 and Arf6 are more important for receptor-mediated uptake of Fg in platelets than for fluid-phase pinocytosis.

We used fluorescence microscopy to further confirm these results. WT and VAMP-3 KO platelets were incubated with 1 µM of FITC-Fg for 30 min, fixed, treated with 0.04% TB to quench external fluorescence, and imaged by epifluorescence. WT platelets had more FITC-Fg$^+$ puncta than VAMP-3 KO platelets after 30 min (Figure 5G). Quantification of total fluorescent puncta present per platelet showed that over 80% of VAMP-3 KO platelets (vs ~30% in WT) had no puncta consistent with defective Fg uptake (Figure 5H; overall $p \leq 0.01$). Only WT platelets had $\geq 3$ puncta compared to 2 or less in KO platelets. Consistent with our ex vivo plate-based assay, quantification
of dextran+ fluorescent puncta (indicative of dextran uptake) in WT and VAMP-3 KO platelets showed no significant differences (Figure 11A-B).

Collectively, these data show that Fg uptake and/or accumulation is dramatically reduced in the VAMP-3 KO platelets and comparatively more affected than dextran uptake and/or accumulation. Since α\textsubscript{\text{IIb}}β\textsubscript{3} activation and Fg-binding to α\textsubscript{\text{IIb}}β\textsubscript{3} remained unaffected in the VAMP-3 KO platelets, it appears that VAMP-3 could be mediating membrane trafficking step(s) that are downstream of Fg binding and internalization but required for accumulation of Fg inside platelets.

**Platelets sorted different classes of endocytic cargo**

In VAMP-3 and Arf6 KO mice, Fg uptake and/or accumulation was more affected than dextran uptake. This suggested that VAMP-3 and Arf6 are more important for receptor-mediated uptake of Fg than for fluid-phase pinocytosis of dextran. Could the two different cargos be endocytosed differently and access different compartments upon internalization? We used 3D-Structured Illumination Microscopy (3D-SIM) to address this question. *Ex vivo* platelets were co-incubated with equimolar concentrations of Alexa 647-Fg and Oregon Green 488-dextran and imaged over time. In Figure 6A, at 1 min, Fg (depicted in magenta) was found on the platelet plasma membrane (possibly via binding to α\textsubscript{\text{IIb}}β\textsubscript{3}), while dextran (depicted in green) already appeared to be internalized within punctate structures inside the platelet. Surface staining for dextran was minimal; most likely because no specific dextran receptor is present on platelets. At 5 min, while surface staining was still visible, Fg\textsuperscript{+} puncta started to appear, with a concomitant increase in dextran\textsuperscript{+} puncta. By 30 min, more Fg-positive puncta appeared, and the surface localization was minimal. Strikingly, Fg and dextran puncta did overlap but in a very limited way, suggesting that the two cargo might be in distinct compartments. This implies that platelets can distinguish and perhaps sort their internalized cargo. Consistently, VAMP-3 KO platelets have fewer Fg puncta compared to WT while dextran uptake was less affected. Loss of VAMP-3, however, did not completely compromise cargo sorting as discrete punctate localization of Fg and dextran was still seen in the KO platelets, similar to WT.
To further confirm the defects in the accumulation of Fg and dextran in the VAMP-3 KO platelets *in vivo*, equimolar amounts of Alexa 647-Fg and Oregon Green 488-dextran were injected via the retro-orbital sinus of WT and VAMP-3 KO mice. Platelets were harvested 24 hr post-injection and imaged by 3D-SIM. In both WT and VAMP-3 KO platelets, Fg and dextran were localized to distinct compartments (Figure 6B). VAMP-3 KO platelets had a robust defect in Fg accumulation with very few fluorescent puncta visible. Surprisingly, dextran⁺ puncta were also diminished in KO platelets, suggesting that loss of VAMP-3 may have a greater effect on accumulation over longer incubation times (24 hr; *i.e.*, when in circulation), than during acute uptake at shorter times (up to 60 min). This would argue that VAMP-3 is essential for both acute uptake and long-term accumulation/storage of receptor-mediated cargo (Fg) whereas it preferentially affects accumulation/storage over acute uptake of fluid-phase pinocytic cargo. The integrity of the fluorescently-tagged dextran was not evaluated so its degradation could also explain our findings.
**Figure 5 VAMP-3 KO platelets had defective fibrinogen uptake.** WT and KO platelets (1.0 x 10^9/mL) were either kept resting (A) or stimulated with ADP (10 µM) (B), then incubated with FITC-Fg (0.06 mg/mL or 0.12 mg/mL) at 37°C for 30 min. The platelets were then put on ice for 20 min, fixed with 2% PFA and GMFI measurements were taken by flow cytometry before and after addition of 0.04% Trypan Blue (TB). Quantification of data shows both GMFI measurements before addition of TB (WT/KO –TB) which gives the total fluorescence and after addition of TB (WT/KO + TB) which gives the measure of internal fluorescence. As explained in “Methods” under plate assay to measure endocytosis, WT and KO platelets were added to each well and either incubated with FITC-Fg (0.1-5 µM) (E) or low mol. wt. (10 kDa) dextran-Oregon green 488 (1-100 µM) (F) or for times 0 to 60 min (C-D) at 37°C. Reactions were stopped at given time points and fluorescence was measured before and after addition of 0.04% TB. Data were plotted using SigmaPlot (v13.0) and graphed as number of molecules of Fg or dextran endocytosed per platelet. (G) Washed WT and KO platelets (1 X 10^9/mL) were incubated with FITC-Fg at final concentrations of 1 µM at 37°C for increasing times up to 30 min. Platelets were fixed with 2% PFA (final conc.) and mixed with 0.1 % TB prior to imaging. Platelets were visualized as in the Methods section. Exposure times for DIC were 100 msec while for FITC laser were 500 msec. Scale bars are 5 µM. (H) Quantification of the number of FITC+ puncta/platelet in both WT and KO samples and plotted using Sigma Plot software (v13.0). Statistical significance (**) denoted by p-value ≤0.01. Data is representative of 3 independent experiments (mean ± SEM) for all.
Figure 6

Alexa 647-Fibrinogen/Oregon Green 488-Dextran

A

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Figure 6 Platelets sorted endocytosed cargo. (A) Washed WT and KO platelets (1.0 x 10^9/mL) were incubated ex vivo with Alexa 647-Fg and Oregon green 488-dextran at final concentrations of 1 µM each and incubated at 37°C for 1 min to 30 min and prepared for 3D-SIM imaging as described in “Methods”. (B) WT and KO mice were injected with Alexa 647-Fg and Oregon Green 488-dextran, at a concentration of 2 µM each, through the retro-orbital sinus. 24 hours post-injection, platelets were harvested and prepared for 3D-SIM analysis. Slides from (A) and (B) were then imaged using the Nikon Ti-E N-STORM/N-SIM super-resolution microscope and images were processed using the NIS-Elements v3.2 N-SIM/STORM suite software. Scale bars are 5 µm. Data is representative of at least 2 independent experiments.
### Table 2 Characteristics of blood Cells from WT and KO mice

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<td>RBC (M/µL)</td>
<td>7.68 ± 1.72</td>
<td>7.57 ± 1.59</td>
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<td>Platelets (K/µL)</td>
<td>878.62 ± 520.7</td>
<td>945.8 ± 496.1</td>
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<tr>
<td>Mean Platelet Volume (fL)</td>
<td>4.36 ± 0.32</td>
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Loss of VAMP-3 caused faster platelet spreading on Fg and enhanced clot retraction

VAMP-3 has been shown to modulate β₁ integrin-dependent cell adhesion and migration in epithelial cell lines [426-427]. We asked whether VAMP-3 plays a similar role in αIIbβ₃-mediated platelet spreading. Consistent with our data in Figure 4C where there was no change in Fg-binding to αIIbβ₃ in the VAMP-3 KO platelets, there was no statistically significant difference in static adhesion to Fg between WT and KO platelets (Figure 7C). To determine whether VAMP-3 loss affected platelet spreading, we monitored WT and KO platelet spreading on Fg-coated surfaces from 0 to 120 min at 37°C. VAMP-3 deletion caused faster spreading on Fg-coated surfaces with significantly larger platelet surface areas covered in as early as 30 min (ρ ≤ 0.05), and continuing to later time points [45 min (ρ ≤ 0.001), 60 min (ρ ≤ 0.001) and 90 min (ρ ≤ 0.01)] (Figure 7A-B). The rate of spreading was 82% faster in the KO platelets (rate of 0.80 μm²/min for KO vs 0.44 μm²/min for WT; assuming spreading is a linear function over time). Consistent with increased spreading, clot retraction was also faster in KO platelets compared to WT (Figure 7D-E). Unlike spreading, which is a linear function, clot retraction proceeded exponentially over time, with the decay constant for WT being 0.011 % of initial clot size/min and that for KO is 0.015 % of initial clot size/min. This led to a 36% enhancement in the rate of clot retraction in the KO platelets as early as 30 min (ρ ≤ 0.05) and continued at 60 min (ρ ≤ 0.05) and 120 min (ρ ≤ 0.05). Note that exogenous Fg was added in excess and was not limiting in these assays. Thus, loss of VAMP-3 led to enhanced platelet spreading and clot retraction. These alterations, however, do not affect platelet aggregation and hemostasis on a global scale, as both were normal in the VAMP-3 null mice which also have no bleeding diathesis [202].
Figure 7

A

WT 60 min KO

B

Platelet Area (µm²)

WT KO

0 20 40 60 80 100 120 140

Time (min)

C

Adherent Platelets (X 1000)

BSA Fibrinogen

WT WT1 WT2 WT3 KO KO1 KO2 KO3 n.s.

WT WT1 WT2 WT3 KO KO1 KO2 KO3 n.s.

D

WT KO

WT KO

WT KO

Thrombin

0 min 30 min

60 min 120 min

E

% of Initial Clot Size

WT KO

0 30 60 120

Time (min)

*
Figure 7 VAMP-3 KO platelets spread faster on Fg and had enhanced clot retraction. 

(A) Representative images of WT and KO platelets allowed to spread for 60 min are shown. Images of spread platelets were taken using DIC Microscopy as described in “Methods”. Exposure times for DIC images were 100 msec. (B) Quantification of platelet surface area from WT and KO Fg-spread platelets for indicated times. Data is representative of 2 independent experiments (mean ± SEM). (C) Quantification of static adhesion on 50 μg/mL human Fg and 5% BSA-coated surfaces for WT and KO calcein-labeled platelets harvested from 3 different KO and WT mice. Adherent platelets were measured by fluorescence using a SpectraMax plate reader. Data was plotted using SigmaPlot (v13.0). (D) Representative images of thrombin-stimulated (0.05U/mL; denoted by +) clot retraction in WT and KO platelets at increasing times. (E) Clot sizes were measured and the percent of clot size relative to initial suspension volume (measured at time 0 and set as 100%) were determined and plotted. Three different KO and corresponding WT littermate controls were used in this experiment. Statistical significance (*) denoted by p-value ≤0.05, (**) denoted by p-value ≤0.01, (*** ) denoted by p-value ≤0.001, and n.s stands for not significant.
Loss of VAMP-3 led to increased JAK2 phosphorylation downstream of TPOR/Mpl receptor

Since VAMP-3 mediates trafficking of several transmembrane receptors such as the Transferrin receptor in other cell types [404], we asked what other platelet receptors could rely on VAMP-3-dependent trafficking step(s). Thrombopoietin (TPO) binding to its receptor Thrombopoietin Receptor (TPOR/Mpl) induces internalization of the receptor in a Dynamin-2 and clathrin-mediated process that initiates signaling via Janus Kinase 2 (JAK2) phosphorylation [22, 460]. We asked whether VAMP-3 contributes to this process. In contrast to Dynamin-2 KO platelets, VAMP-3 KO platelets did not exhibit constitutive JAK2 phosphorylation. Instead, they showed a time-dependent increase in phospho-JAK2 levels similar to WT. However, at each time point, phospho-JAK2 levels were markedly elevated in VAMP-3 KO vs in WT platelets (Figure 8A-B) with statistically significant differences at 15 min ($p \leq 0.05$) and at 30 min ($p \leq 0.05$). The levels of total JAK2 appeared to be lower in the VAMP-3 KO platelets compared to WT and the levels of total TPOR were unchanged (Figure 8A). Our data suggests that while TPOR levels may be unaffected, there exists a VAMP-3-mediated membrane trafficking step(s) that occurs upon TPO-bound TPOR internalization, which affects JAK2 phosphorylation. Given that Arf6 is important for integrin trafficking, we asked whether it could play a role in signaling through the TPOR-JAK2 axis. Arf6 KO platelets demonstrated normal JAK2 phosphorylation upon TPO induction similar to WT platelets, with no change in TPOR levels (Figure 12A-B). Arf6, thus, may be less important than VAMP-3 or Dynamin-2 in regulating TPOR/Mpl-JAK2 signaling.
Figure 8 Loss of VAMP-3 led to increased JAK2 phosphorylation downstream of TPOR/Mpl receptor. (A) Platelet extracts were prepared from washed WT and KO platelets (4 X 10^8/mL) were incubated with 50 ng/mL of thrombopoietin (TPO) for 0 to 30 min at 37°C. Western blotting was performed using anti-JAK2 and anti-phospho JAK2 (Tyr 1007/1008) and anti-TPOR antibodies. Images were taken on a Typhoon 9500 Variable Mode Imager and quantified using the ImageQuantTL software. (B) Volumetric quantification of phospho-JAK2 levels over total JAK2 levels was plotted as a ratio of pJAK2/JAK2 (arbitrary units) over time of TPO incubation. Statistical significance (*) denoted by p-value ≤0.05 and is representative of at least 3 independent experiments (mean ± SEM).
Figure 9

WT1  WT2  KO1  KO2

Fibrinogen
Figure 9 Plasma Fg levels were unaffected in VAMP-3 KO mice.

Western Blotting showing levels of plasma Fg from 2 different VAMP-3 KO and corresponding WT littermate controls. Equal amounts of protein 30 µg/lane, quantified by protein BCA assay, were loaded.
Figure 10 Arf6 KO platelets had a greater negative defect in Fg uptake than in dextran uptake.

100 µL of WT and Arf6 KO washed platelets (5 X 10^7/mL), pre-incubated with 1 mM CaCl2 for 5 min, were added to each well in a 96-well black opaque plate and either incubated for increasing times (0-60 min) (A-B) or with varying concentrations of FITC-Fg (0.1-5 µM) (C) or low mol. wt. (10,000 MW) dextran-Oregon green 488 (1-100 µM) (D) at 37°C. Reactions were stopped at given time points and fluorescence intensity was measured using a SpectraMax plate reader (Molecular Devices; excitation/emission at 495/520 nm) before and after addition of 0.04% Trypan Blue. Data were plotted using SigmaPlot (v13.0) and plotted as number of molecules of Fg or dextran endocytosed per platelet. Data are representative of at least 2 independent experiments.
Figure 11 VAMP-3 deletion did not affect dextran uptake. Washed platelets (1 X 10^9/mL) from WT and KO mice were incubated with Dextran-Oregon Green 488 at final concentrations of 1 μM each at 37°C for increasing times up to 30 min. (A) Representative images at 30 min are shown here. Platelets were fixed with 2% PFA (final conc.) and mixed with 0.1 % Trypan Blue prior to imaging. Platelets were visualized using the using Differential Interference Contrast Microscopy on a Nikon Eclipse E600 microscope (Nikon, Melville, NY) fitted with an AxioCam MR camera (Zeiss, Germany) and processed using the Zen 2011 Digital Imaging software (blue edition, Zeiss). Exposure times for DIC was 100 ms while for FITC laser was 500 ms. Scale bars are at 5 μM. (B) Quantification of the number of Oregon Green 488-positive puncta per platelet in both WT and KO samples that were counted and quantified using Sigma Plot software (v13.0). Data are representative of 2 independent experiments.
Figure 12

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B

Plot: pJAK2/JAK2 (A.U.) vs. Time (min)

- WT
- Arf6 KO
Figure 12 Arf6 KO platelets had normal TPO-induced JAK2 phosphorylation. (A) Washed WT and KO platelets (4 X 10^8/mL) were pre-incubated with 1 mM CaCl2 for 5 min and incubated with 50 ng/mL of Thrombopoietin (TPO; R&D Systems) for 0 to 30 min at 37°C. Platelet extracts were prepared in SDS-PAGE sample buffer (supplemented with protease and phosphatase inhibitor cocktail mix). Western blotting was performed using anti-JAK2 and anti-phospho JAK2 (Tyr 1007/1008) and anti-TPOR antibodies. Images were taken on a Typhoon 9500 Variable Mode Imager and quantified using ImageQuantTL software (GE Healthcare Life Sciences, Piscataway, NJ). (B) Volumetric quantification of phospho-JAK2 levels over total JAK2 levels was plotted as a ratio of pJAK2/JAK2 (arbitrary units) over time of TPO incubation. Data shown here are representative of 2 independent experiments (mean ± SEM).
Conclusions

Endocytosis is key to fibrinogen (Fg) uptake, receptor trafficking of integrins (α\textsubscript{IIb}β\textsubscript{3}, α\textsubscript{v}β\textsubscript{3}) and purinergic receptors (P2Y\textsubscript{1}, P2Y\textsubscript{12}), and thereby for normal platelet function. However, the molecular machinery required and possible trafficking routes are still ill-defined. Using VAMP-3 KO mice, that are otherwise unaffected in their activation, aggregation and secretion responses, we examined the role of a vesicle-residing Soluble N-ethylmaleimide Factor Attachment Protein Receptor (v-SNARE) called Cellubrevin/Vesicle-Associated Membrane Protein-3 (VAMP-3) in platelet function. In this chapter, we explored the role of VAMP-3 in mediating endocytosis in platelets. Platelets from VAMP-3 KO mice had less platelet-associated Fg, indicating a defect in Fg uptake/storage. This defect was not attributed to any alterations of endocytic machinery components, surface expression levels of α\textsubscript{IIb}β\textsubscript{3}, or reduced plasma Fg levels. Loss of VAMP-3 led to a defective uptake of fluorescently-tagged Fg and low molecular dextran in platelets though it had a greater negative effect on receptor-mediated Fg uptake than on the fluid-phase marker uptake. Additionally, we followed the time-dependent trafficking of Fg and dextran into platelets using 3D-Structured Illumination Microscopy. Wild-type platelets endocytosed both cargoes but quickly sorted them into distinct compartments with partial overlap occurring only at early time points. This is the first demonstration of platelets being able to sort cargo upon endocytosis. Sorting was unaffected in VAMP-3 KO platelets. The VAMP-3 loss did affect some acute platelet functions leading to enhanced spreading on Fg and faster clot retraction compared to wild-type, suggesting that VAMP-3 is involved in endocytic trafficking of integrin α\textsubscript{IIb}β\textsubscript{3}. Additionally, the rate of JAK2 phosphorylation, initiated through the thrombopoietin receptor (TPOR/Mpl) activation, was affected in VAMP-3 KO platelets. Collectively, our studies show that platelets are capable of a range of endocytosis steps with VAMP-3 being pivotal in these processes. This is the first report of v-SNARE being involved in endocytic trafficking in platelets. How VAMP-3-mediated endocytosis affects other platelet functions will be explored in the next chapter.
Chapter Four: Endocytosis mediates innate immune responses by platelets in the vasculature

Introduction

How multifunctional platelets can act as immune cells, bridging the gap between coagulation cascades and immune systems in the vasculature, has in recent years become an active field of research [464]. Being the most abundant “responsive” cell type in circulation, it is hardly surprising when studies showed that platelets are, indeed, the first responders to trap bacteria and present them to leukocytes, even before the professional immune cells encounter the pathogen [463]. In fact, studies within activated high endothelial venules (HEVs) showed that neutrophils hone in on inflammatory damage by actively seeking P-selectin-bearing activated platelets to mediate responses [611].

Systemic viral and bacterial infections have long been associated with severe thrombocytopenia especially in the cases of sepsis [465], viral hemorrhagic fevers [466], hepatitis C virus infections [467], and human immunodeficiency virus-1 infections [468] among others. The severity of thrombocytopenia often predicts the clinical outcomes of viremia and bacteremia. Platelets isolated from patients infected with viruses such as HIV-1 [263], influenza [469], dengue virus [471] and hepatitis C virus [470], have been shown to harbor viral particles, yet the molecular machinery that is required for viral entry is unclear.

Platelets express several Toll-like Receptors (TLRs) that are pattern-recognition receptors that can recognize viral and bacterial pathogen-associated molecular patterns (PAMPS) or damage-associated molecular patterns (DAMPS) [437, 439-442]. Of the ones that are present in platelets, the plasma membrane-residing TLR2 and TLR4 and the intracellular-residing TLR7 and TLR9 have been found to be functionally relevant [411]. Recently, TLR7, which resides in an intracellular compartment and recognizes single-stranded RNA, was found to be present in platelets [411, 437, 472]; and is required for mediating platelet counts and host survival in a mouse model of encephalomyocarditis virus (EMCV) infection. Viruses such as EMCV, HIV-1, and influenza virus as well as small guanosine analogs such as Loxoribine have been shown to activate TLR7 signaling pathways [437]. In plasmacytoid dendritic cells,
responses to HIV-1 are mediated by virus phagocytosis/endocytosis, degradation to release Toll-like Receptor ligands, and subsequent receptor activation [473]. Whether all or some of this process is recapitulated in platelets is unknown. Towards this direction, we queried the molecular machinery that is required for HIV-1 entry into platelets and how does viral endocytosis affect platelets in determining the clinical outcomes in these HIV-1^+ patients. We first examined platelets’ responses to two specific nucleotide-based, TLR agonists, loxoribine (TLR7) and unmethylated CpG oligonucleotides (TLR9). Using secretion assays to measure release from all three classes of granules (alpha, dense and lysosomes), we found that both TLR agonists elicited a small (compared to hemostatic agonists, i.e., thrombin) secretory response that was sufficient to form platelet-leukocyte aggregates as measured by FACS. Additionally, both activated IKK and PI3K-Akt pathways as measured by immunoblotting. Inhibitor studies (using known inhibitors of endocytic trafficking such as Dynasore, ammonium chloride) and analysis of platelets from two different endocytosis mutants (VAMP-3 KO and Arf6 KO) demonstrated the importance of platelet endocytic trafficking in regulating responses to these intracellular TLRs. To further understand this process, we used HIV-1 pseudovirions that contain ssRNA which can be recognized by the endosomal TLR7, showing that platelets did endocytose and degrade retroviral particles to release TLR agonists, which initiate platelet activation and secretion. Fluorescence micrographs showed uptake of HIV-1 pseudovirions in both in-vitro and in vivo analyses of wild-type platelets. Consistently, HIV-1 uptake and subsequent activation were abolished in endocytosis-deficient VAMP3 KO or Arf6 KO platelets. Taken together, our data supports that platelets can act as early damage responders by recognizing pathogenic viruses and mount an appropriate immune response to them.

**Platelets responded differentially to different stimuli: “immuno-activation” versus “hemostatic activation”**

Given that platelets express TLR7 (binds to ssRNA) and TLR9 (binds to DNA, unmethylated CpG motifs) [472, 442], we asked how these TLRs activates platelets. Using bioactive, pathogen-mimetics such as Loxoribine a guanosine analog that
specifically activates TLR7 and CpG oligonucleotides Class C (CpG ODN2395) that specifically turns on TLR9; we measured platelet secretion as an indicator of activation. Wildtype platelets when stimulated with thrombin (0.05 U/mL), which is the most potent activator of platelets, showed rapid and robust secretion that reached maximum saturation levels within 3 min of stimulation, from all three types of granules (Figure 13A i, ii, iii). When platelets were stimulated with TLR7 agonist Loxoribine (1 mM) and TLR9 agonist CpG ODN2395 (5 µM), the kinetics of release were much slower and lower in terms of percent secretion. Thrombin stimulation of platelets causes 80-90% secretion of [3H]-serotonin from dense granules, 50-60% secretion of platelet factor 4 (PF4) and 20-30% secretion of β-hexosaminidase from lysosomes. Compared to that, both Loxoribine and CpG ODN2395 stimulated roughly 30-40% release of [3H]-serotonin from dense granules, 15-20% secretion of platelet factor 4 (PF4) and hardly any secretion of β-hexosaminidase from lysosomes. It is to be noted here that lysosome secretion is usually the weakest of the three granules. This lower extent of release held true even for longer times of incubation with the respective agonists. Thrombin reaches its maximum saturation within 3-5 mins while it takes much longer for the TLR agonists to reach even half of thrombin’s maximum release percentages.

P-selectin (CD62P), which is exteriorized by platelets upon exocytosis of alpha granules during activation, functions as a cell adhesion molecule by binding to the leukocyte receptor, P-selectin glycoprotein ligand-1 (PSGL-1) that are essential for forming the platelet-leukocyte aggregates and serve as markers for thromboinflammation in the circulation [474]. We measured whether these TLR agonists could stimulate P-selectin exteriorization from platelet alpha-granules. In Figure 13B, Loxoribine at two different concentrations (0.1 mM and 1mM) and CpG-ODN (at 2.5 µM and 5 µM) stimulated platelets, when incubated for 30 min at 37°C, to exocytose P-selectin, that was higher than resting levels (p values ≤ 0.05) but was markedly lower than the P-selectin levels expressed by platelets upon thrombin stimulation (0.1 U/mL for 3 min). TLR-stimulated P-selectin levels (at least in the case of Loxoribine) were even lower than those exteriorized by sub-optimal doses of thrombin (25 mU/mL for 3 min). Consequently, we measured platelet-neutrophil
aggregates in whole blood using anti-CD42b antibodies (platelets) and anti-CD11b antibodies (neutrophils and monocytes), in response to Loxoribine (1 mM) and CpG ODN (5 µM) stimulated for 30 min and compared that with thrombin (0.1 U/mL) stimulation for 3 min. As expected, TLR agonists stimulate low levels of CD11b-CD42b aggregates compared to thrombin stimulation (Figure 13C). Taken together, we show using three different assays, that platelets respond differently (slower and lower levels of activation) to “immune agonists” than they do to “hemostatic agonists” such as thrombin, where responses are extensive and robust.

**Responses to endosomal TLRs required Dynamin-mediated endocytosis and acidic compartments**

TLRs 3, 7, 8, and 9 are present in intracellular compartments. Response to these endosomal TLRs requires Dynamin-dependent endocytosis of their specific ligands, followed by trafficking to an acidic endosomal compartment, where receptor binding and signaling occurs [411]. We asked whether the response to these endosomal TLRs also proceeds via similar endocytic trafficking routes in platelets. Washed platelets were pre-incubated for 30 min, at 37°C, with either Dynasore (80 µM), a small molecule Dynamin inhibitor [577], at concentrations that do not inhibit ATP secretion from platelets [351] or with ammonium chloride (20 mM), which neutralizes the pH of acidic compartments to determine whether activation-induced signaling downstream of TLR7 and TLR9 was affected. Loxoribine stimulation activates PI3K-Akt pathways in platelets [472], while TLR9 stimulation also activates PI3K and MAPK pathways in platelets [506]. IKK-β-mediated SNAP-23 phosphorylation drives SNARE complex formation and granule secretion [242]. We have a phospho-peptide specific antibody that detects SNAP-23 phosphorylation, at Ser95, that allows us to measure SNAP-23 phosphorylation as a metric of secretion. This antibody only works in mouse platelets [242]. In Figure 14A, Akt and SNAP-23 phosphorylation was measured in platelets in response to Loxoribine (1 mM) (TLR7), CpG ODN2395 (5 µM) (TLR9) and HIV-1 pseudovirions (100x) (TLR7) stimulation, incubated for 30 min, either in the presence or absence of NH₄Cl (20 mM). In Figure 14B, Akt phosphorylation was reduced in the presence of NH₄Cl, in response to Loxoribine, CpG ODN and HIV-1 pseudovirions (HIVpp), suggesting the need for
trafficking into acidic compartments for efficient TLR-induced signaling. In Figure 14C, SNAP-23 phosphorylation was affected in CpG ODN and HIVpp stimulated platelets, while NH₄Cl-mediated inhibition of Loxoribine-stimulated SNAP-23 phosphorylation appeared to be much weaker than the others. In a similar vein, we monitored signaling responses to these TLR ligands in response to Dynasore-mediated Dynamin inhibition. In Figure 15A, Akt and SNAP-23 phosphorylation was measured in platelets in response to Loxoribine (1 mM) (TLR7), CpG ODN2395 (5 µM) (TLR9) and HIV-1 pseudovirions (100x) (TLR7) stimulation, incubated for 30 min, either in the presence or absence of Dynasore (80 µM). In Figure 15B, Akt phosphorylation was reduced in the presence of Dynasore, in response to Loxoribine, CpG ODN and HIV-1 pseudovirions (HIVpp), suggesting the need for Dynamin-dependent endocytosis for efficient trafficking to TLR-containing compartments to initiate TLR-induced signaling. In Figure 13C, SNAP-23 phosphorylation was also affected in Loxoribine-, CpG ODN- and HIV-1pp-stimulated platelets. Collectively, our data suggests that responses to endosomal TLRs proceed via Dynamin-mediated pathways, followed by trafficking into acidic compartments, where engagement of the TLR ligand with its receptor initiates downstream signaling responses. To determine whether platelet activation by these TLR ligands indeed occurs through the actions of TLRs, we probed platelet activation by measuring Akt and SNAP-23 phosphorylation in response to LPS (TLR4), Pam3CSK4 (TLR2), Loxoribine (TLR7) and CpG ODN (TLR9) in TLR23479 mutants. In Figure 21, we see the loss of signaling (both Akt and SNAP-23 phosphorylation) in response to each of these agonists in the quintuple mutants, indicating that signaling through these ligands indeed proceeds via their cognate TLR receptors. Of note, thrombin-induced responses are unchanged in these mutants suggesting that defective Akt and SNAP-23 phosphorylation is due to inability to induce signaling upon ligand binding and not due to any other hemostatic or platelet activation defects.
Figure 13 Platelets responded differently to “hemostatic activation” vs “immuno-activation”. (A) Washed platelets (2.5 X 10^8 platelets/mL) were stimulated with Loxoribine (1 mM), CpG ODN2395 (5 µM) and Thrombin (0.05 U/mL) for the indicated times and percent secretion (i) [3H]-Serotonin from dense core granules, (ii) PF4 from alpha granules and (iii) β-hexosaminidase from lysosomes was measured. (B) anti-FITC-P-selectin antibody was added to platelets (5 X 10^7/mL) stimulated with Loxoribine (0.1 mM and 1 mM), CpG ODN2395 (2.5 µM and 5 µM) for 30 min and thrombin (0.025 U/mL and 0.1 U/mL) for 3 min and geo mean fluorescence intensity was plotted for each condition. (C) Anti-APC-CD11b and anti-FITC-CD42b antibodies were added to 50 µL of wildtype whole blood, stimulated with Loxoribine (1 mM), CpG ODN2395 (5 µM) stimulated for 30 min and Thrombin (0.1 U/mL) for 3 min or buffer control (for unstimulated) and samples with analyzed for double-positive CD42b-CD11b stained conjugates using BD LSRII Flow Cytometer. Data was then analyzed using FlowJo (v5.6.7 software) and plotted as percent of aggregate formation. Data is representative of at least three independent experiments with statistical significance represented by p≤0.05 (*).
Figure 14

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$\text{NH}_4\text{Cl}$

phospho-Akt

Akt

phospho-SNAP-23

SNAP-23

B

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\text{\% Akt phosphorylation normalized over total Akt}
\]

C

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\text{\% SNAP-23 phosphorylation normalized over total SNAP-23}
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Figure 14 Responses to endosomal TLR7, TLR9, and HIV-1 pseudovirions required endosomal maturation. (A) Washed platelets (4 × 10^8 platelets/mL) were pre-incubated with or without NH_4Cl (20 mM) for 30 min and then stimulated with Loxoribine (1 mM), CpG ODN2395 (5µM), HIV-1 pseudovirions (HIV-1pp) for 30 min at 37°C. Western blotting was performed using anti-phospho-Akt (Ser473), anti-Akt, and anti-phospho-SNAP-23 (Ser95) and anti-SNAP-23 antibodies. Images were taken on a Typhoon 9500 Variable Mode Imager and quantified using the ImageQuantTL software. (B) Volumetric quantification of the percent of phospho-Akt relative to total Akt and (C) of phospho-SNAP-23 over total SNAP-23. Statistical significance (*) denoted by p-value ≤0.05, (**) denoted by p-value ≤0.01 and is representative of at least 3 independent experiments (mean ± SEM).
Responses to endosomal TLRs required VAMP-3 and Arf6-mediated endocytosis

Our data suggests that platelets utilize endocytosis to mediate responses to endosomal TLR ligands. Using fibrinogen uptake as a metric for endocytosis, we have identified two key endocytic regulators in platelets. Both Arf6 and VAMP-3 loss causes a robust defect in fibrinogen uptake. Arf6, the Ras-like small GTPase, that is involved in integrin trafficking and Fg uptake in platelets has also been shown to be involved in the pathogenic invasion of various cell types [587-590]. Inhibition of Arf6 in RAW264.7 macrophages by siRNA knockdown or dominant-negative mutants, show impaired uptake of CpG oligonucleotides, reduced PI3K/Akt signaling and impaired TLR9-dependent immune responses [612]. On the other hand, data presented in this thesis demonstrates VAMP-3 is critical for receptor-mediated uptake of fibrinogen. VAMP-3/Cellubrevin has also been shown to be critical for phagocytosis of pathogens and various particles [416-417]. However, the importance of both Arf6 and VAMP-3 in mediating uptake of endosomal TLR ligands in platelets remains to be determined. We queried whether platelets utilize a common endocytic machinery to internalize granule cargo and pathogens or “immunogenic cargo”.

Towards this direction, we incubated washed WT and VAMP-3 KO platelets with either TLR7-Loxoribine (1 mM) or TLR9-CpG ODN2395 (5 µM) for 0 to 60 min at 37°C and platelet lysates were prepared and probed for Akt and SNAP-23 phosphorylation as metrics of activation. As shown in Figure 16, in WT platelets, both Loxoribine and CpG ODN induced Akt and SNAP-23 phosphorylation in a time-dependent manner, with the VAMP-3 KO platelets showing robust attenuation in Akt and SNAP-23 phosphorylation at each time point measured. There appears to be a lag in generating appreciable phosphorylation (usually about 15 min) in platelets treated with Loxoribine and CpG oligonucleotides, possibly due to the time taken by respective ligands to internalize and traffic to the right compartment that has the endosomal TLRs. Similarly, as shown in Figure 17, time-dependent Loxoribine and CpG ODN stimulation were also affected in the Arf6 KO platelets. It would appear that while both TLR7-Loxoribine and TLR9-CpG ODN signaling responses are significantly affected in VAMP-3 KO platelets while as expected, TLR9-CpG ODN
stimulation induced Akt and SNAP-23 phosphorylation is more affected in the Arf6 KO platelets than the TLR7-Loxoribine induced responses. This would suggest that there exists some heterogeneity in endocytic regulator usage by these TLR ligands to target them to their respective TLR-bearing endosomal compartment. VAMP-3 may be more central in coordinating trafficking to both TLR7⁺ and TLR9⁺ endosomes, while Arf6 may be preferred for trafficking cargo into TLR9⁺ endosomes and to a lesser extent to TLR7⁺ endosomes. While this diversity in trafficking routes and regulator usage is indeed interesting in platelets, at this point it is unclear whether platelets have discrete TLR7⁺ and TLR9⁺ endosomes or whether both TLRs co-exist in the same endosome. Immunofluorescence studies will shed more light on this complex trafficking system. What other potential endocytic proteins and regulators are involved in this process in platelets remains to be seen.

The observed reduction in Akt and SNAP-23 phosphorylation in response to TLR7 and TLR9 stimulation in the Arf6 or VAMP-3 mutants could be due to loss of any or both of the TLR, unstable receptors (prone to degradation) or some aberrant dysfunction that could potentially explain the impaired signaling responses. To exclude this possibility, we probed for both TLR7 and TLR9 in platelet extracts from the mutants and compared them to littermate-matched controls. In nucleated cells, processing of the endosomal TLRs (7, 9, etc.) occurs in the endoplasmic reticulum, where the full-length receptor gets cleaved to generate the functional component of the TLR (that which initiates ligand-binding and downstream activation) that gets trafficked into the appropriate endosome [411]. Platelets, much like the nucleated cells, have both the full-length and the cleaved ligand-binding functional form and western blotting showed that the levels of both forms appear to be unchanged in the VAMP-3 and Arf6 mutants (Figure 20A-B). This holds true for both TLR7 and TLR9. This argues that the impairment of signaling in these mutants is possibly due to defective endocytosis of ligands and not due to altered levels of the TLRs. However, the possibility of the presence of both full-length and cleaved, but functionally inactive, forms of the TLR7 and TLR9 receptor in the VAMP-3 and Arf6 mutants, that could arguably give similar defects in signaling responses, cannot be excluded at present. Further experiments will need to confirm this possibility.
Figure 15

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<td>Dynasore</td>
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B

% Akt phosphorylation normalized over total Akt

C

% SNAP-23 phosphorylation normalized over total SNAP-23
Figure 15 Responses to endosomal TLR7, TLR9, and HIV-1 pseudovirions required Dynamin. (A) Washed platelets (4 x 10^8 platelets/mL) were pre-incubated with or without Dynasore (80 µM) for 30 min and then stimulated with Loxoribine (1 mM), CpG ODN2395 (5µM), HIV-1 pseudovirions (HIV-1pp) for 30 min at 37°C. Western blotting was performed using anti-phospho-Akt (Ser473), anti-Akt, and anti-phospho-SNAP-23 (Ser95) and anti-SNAP-23 antibodies. Images were taken on a Typhoon 9500 Variable Mode Imager and quantified using the ImageQuantTL software. (B) Volumetric quantification of % of phospho-Akt levels over total Akt levels and (C) of phospho-SNAP-23 levels over total SNAP-23 levels. Statistical significance (*) denoted by p-value ≤0.05, (**) denoted by p-value ≤0.01 and is representative of at least 3 independent experiments (mean ± SEM).
Endocytosis of HIV-1 pseudovirions required VAMP-3 and Arf6

Like the small bioactive TLR ligands, the more physiologically relevant agonist, HIV-1 pseudovirions also require endosomal uptake and processing to generate TLR ligands. In plasmacytoid dendritic cells, Dynamin-dependent endocytosis internalizes HIV-1 virions upon CD4-mediated entry; virus gets trafficked through endosomal compartments and eventually reaches the late endosomes and lysosomes, where it gets degraded. We asked how much of this process is recapitulated in platelets. Figure 14 and 15, indicates that response to HIV-1 pseudovirions requires dynamin-mediated uptake and trafficking to acidic compartments, where the viral particles get degraded to release TLR ligands that might then activate Akt and SNAP-23 phosphorylation. Additionally, Arf6 is also required for HIV-1 endocytosis in T-cells [586]. Use of siRNA against Arf6, or inhibiting Arf6 function by using GDP-bound or GTP-bound inactive mutants, led to a decrease in HIV-1 viral membrane fusion, entry and infection of T lymphocytes. Furthermore, Arf6 inhibition impaired cell-to-cell transmission of HIV-1 viral particles in CD4+ T lymphocytes. On the other hand, VAMP-3/Cellubrevin has been critical for phagocytosis of pathogens and various particles [416-417]. However, the importance of VAMP-3 in mediating HIV-1 endocytosis is unclear. We sought to determine, whether VAMP-3 and Arf6 play a role in mediating viral uptake.

Using our plate assay to measure endocytosis as described in Methods, we incubated WT, VAMP-3 KO and Arf6 KO platelets with two different concentrations of FITC-conjugated CpG ODN2395 (5 µM and 10 µM) at 37°C for 30 min. Reactions were stopped by addition of 0.04% Trypan Blue. Similar to Huang et al. 2016 [19], we used 0.04% Trypan Blue (TB) to quench external FITC fluorescence (including the fluorescence that is trapped within the open canalicular system) and measured TB-resistant fluorescence of internalized cargo as a metric of endocytosis. In Figure 18A, WT platelets internalized more CpG ODN in a dose-dependent manner, while VAMP-3 KO and Arf6 KO platelets had robust defects in uptake. This argues that the signaling that we measure in terms of Akt and SNAP-23 phosphorylation is caused due to endocytosis of CpG ODN molecules and this response is significantly affected in the endocytosis mutants. To determine whether the same holds true for the HIV-1
pseudovirions, we tested viral endocytosis in our plate assay. Figure 18B shows a schematic of the HIV-1 pseudovirion construct that was obtained from Dr. Gregory Melikyan’s lab [613]. These genetically modified non-infectious HIV-1 viral particles, provisionally called “pseudovirions” retain most of the actual HIV-1 viral backbone, with modifications introduced in the form of double fluorescent tags such that the green fluorescent protein is fused to the plasma membrane-residing intercellular adhesion molecule-1 (ICAM-1) and the viral core has an mCherry fluorescent tag encoded with the viral Gag protein. Viral expression in 293-LTV (lentiviral derived) cell lines generated red-green double-tagged pseudovirions that were produced by the Genetics Core Facility, here at the UK. Pseudovirions obtained were incubated with WT, VAMP-3 KO and Arf6 KO platelets at increasing concentrations (1x and 2x) at 37°C for 30 min and Trypan Blue-resistant fluorescence intensities were measured. In Figure 18C, WT platelets internalized more HIV-1pp in a dose-dependent manner, while VAMP-3 KO and Arf6 KO platelets had robust defects in uptake. These data provide the first indication that VAMP-3 and Arf6 may be involved in viral uptake.

We used fluorescence microscopy to further confirm these results. WT, VAMP-3 KO, and Arf6 KO platelets were incubated with HIV- pseudovirions (100x concentrated) at 37°C, for 0 to 6 hr, fixed with PFA, treated with 0.04% trypan blue to quench external fluorescence, and imaged by epifluorescence. WT platelets had more GFP-mCherry double positive puncta than either of the endocytosis mutants (Figure 16D). Based on Miyauchi et al 2011 [613], the GFP-ICAM-1 molecule often gets incorporated into the membrane to form membrane vesicles; these are not virions and hence were excluded from measurements. Only double-positive red-green viruses were considered whole virions and were counted to denote viral uptake inside platelets. Quantification of total fluorescent puncta present per platelet showed that over time, WT platelets accumulated more HIV-1 pseudovirions and the number of red-green double-positive puncta increased, while the VAMP-3 KO and Arf6 KO platelets failed to do to any great extent, even up to 6 hr of incubation with HIV-1pp (Figure 18E), consistent with our ex vivo plate-based assay.

To further confirm the ex vivo defects in the endocytosis of HIV-1pp, age-matched WT and VAMP-3 KO mice were intravenously injected with the pseudovirions (100X)
via the retro-orbital sinus of the mice. Platelets were harvested 24 hr post-injection and imaged by fluorescence microscopy. As shown in Figure 18F-G, quantification by counting the number of double-positive viral particles in WT and VAMP-3 KO platelets showed viral particles were present in WT platelets, even 24 hr post-injection while there were hardly any detectable viral particles within VAMP-3 KO platelets. Our data indicates that VAMP-3 and Arf6 are not only involved in the acute uptake of HIV-1pp (over shorter times) but also on chronic accumulation (24 hr; i.e., when in circulation) of the viral particles. In an essence, uptake of HIV-1pp in WT platelets and its lack thereof in the endocytosis mutants mirrors that of Fg uptake in both acute and chronic phases of endocytosis. Given that HIV-1pp requires a surface receptor (CLEC-2, DC-SIGN or any other potential receptor) to internalize, it is hardly surprising that its endocytosis proceeds in a similar fashion to $\alpha_{\text{IIb}}\beta_3$-mediated Fg binding and uptake. Further experiments are in the works to clarify this theory. Given that CLEC-2 and DC-SIGN are the potential HIV-1-binding receptors on platelets (which lack CD4), defective endocytosis of the pseudovirions in the VAMP-3 and Arf6 mutants could be due to decreased levels of one or more of the receptors. We, thus, probed for DC-SIGN levels in the VAMP-3 KO and Arf6 KO platelets. Only 15% of platelets express DC-SIGN [543] which explains the low levels of detection of the protein by western blotting. However, the levels of DC-SIGN appear to be unaltered in the mutants (Figure 18E). The other receptor, CLEC-2, remains unaltered in the Arf6 KO platelets (Figure 18D). Surprisingly, VAMP-3 KO platelets showed lower levels of CLEC-2 than WT platelets (Figure 18C). While this could partly explain, why VAMP-3 platelets have such a severe phenotype in their ability to internalize the virions; the fact that Arf6 KO platelets are still defective in viral uptake despite expressing normal levels of CLEC-2, suggest that viral uptake by platelets may have multiple components with viral-binding by CLEC-2 being one aspect of it. Low levels of CLEC-2 on VAMP-3 KO platelets portend towards a biogenesis defect at the megakaryocyte level. What this entails will require further experiments at probing the role of VAMP-3 in platelet formation, cargo biogenesis and/or packaging in the megakaryocytes.
Collectively, these data show that HIV-1pp uptake and/or accumulation is dramatically reduced in the VAMP-3 KO and Arf6 KO platelets suggesting the need for endocytosis to internalize the virus. Our data provides the first mechanistic understanding as to the molecular machinery required for HIV-1 endocytosis in platelets.

Endocytosis of HIV-1 pseudovirions triggered platelet activation

What happens to the viral particles once it gets endocytosed within platelets? In Figure 19A-B, washed WT, VAMP-3 KO, and Arf6 KO platelets were incubated with HIV-1pp (100X) for 30 min at 37°C and probed for activation markers. HIV-1pp stimulated Akt and SNAP-23 phosphorylation in the WT platelets but had a significant loss of activation in the endocytosis mutants. This indicates that endocytosis of the HIV-1 pseudovirions, mediated by VAMP-3 and Arf6, leads to platelet activation.

Activation-induced signaling through PI3K/Akt pathway and IKKβ-mediated SNAP-23 phosphorylation converges to drive platelet granule secretion. We measured HIV-1pp-induced platelet secretion by probing for P-selectin exteriorization by flow cytometry, as a metric of alpha granule secretion. In Figure 19C, HIV-1pp caused P-selectin exteriorization from WT platelets. Loss of either VAMP-3 or Arf6 significantly inhibited P-selectin exteriorization from platelets (p ≤ 0.01). Similarly, Loxoribine (1 mM) and CpG ODN (5 µM) stimulated P-selectin from platelet alpha-granules and this response was also impaired in the mutants. Consistent with the activation-induced Akt and SNAP-23 phosphorylation, defects in endocytosis leads to severe of HIV-1pp-induced immune responses in platelets.

P-selectin exteriorization by activated platelets acts as a “beacon of damage” for patrolling leukocytes, alerting them to existing inflammatory or immune-induced damage in the circulation. As a result, these platelet-leukocyte aggregates serve as “units of inflammation” and initiates host immune responses [611]. Patients with active HIV-1 infection often have increased levels of platelet-leukocyte aggregates, which strongly correlated with platelet activation [614]. Based on these reports, we sought to determine whether endocytosis of HIV-1 pseudovirions triggers platelet-leukocyte aggregate formation. In Figure 19D, while compared to unstimulated
controls, thrombin-induced robust platelet(CD41/61)-neutrophil(Ly6G) aggregates, as measured by flow cytometry, in whole blood and was unaffected in the VAMP-3 and Arf6 mutants (these mutants are not secretion-deficient to hemostatic agonists). Loxoribine, CpG ODN and HIV-1pp all induced aggregate formation in WT, but showed significant reductions in the mutants. Of note, the level of platelet-neutrophil aggregate formation in response to thrombin (hemostatic activation) is almost two-fold higher than all the other TLR ligands, including HIV-1pp activation (immuno-activation). This harkens back to our initial findings that platelets do interpret and respond differentially to “immune agonists” than they do to stronger and more potent “hemostatic agonists”. While agonist potency drives rates and extents of secretion [91], platelets appear to interpret between different classes of agonists and respond accordingly. How this mechanism will warrant future studies to parse the distinguishing signaling aspects that lead to variable activation patterns observed in platelets. Conceivably, these platelet-neutrophil aggregates generated by HIV-1pp, get recognized by professional phagocytes and are cleared from the circulation, dropping platelet counts, which may, in part, explain the severe thrombocytopenia observed in HIV-1+ patients.

Our studies provide a novel mechanistic understanding of how viral particles enter platelets and why platelets from HIV-1+ patients with chronic HIV-1 infection show signs of platelet activation.

To determine whether platelet activation by HIV-1 pseudovirions occurred through the actions of TLR7, which is the physiological sensor for viral ssRNA, we probed platelet activation by measuring Akt and SNAP-23 phosphorylation in response to HIV-1pp in TLR23479 mutants. Unlike Loxoribine, which binds to TLR7 and shows loss of signaling in the mutant platelets, HIV-1pp induced activation only shows a partial loss of Akt and SNAP-23 phosphorylation, suggesting that there may be a TLR-independent component (in addition to a TLR-dependent component) of HIV-1pp-induced platelet activation (Figure 21). Other potential TLR ligands that may be generated by viral degradation inside platelet endosomes, or activation by surface binding to any surface TLR, such as TLR2, 4, 3 or 9 has been already excluded in these mutants. As a result, we posit that viral activation may be through some other
platelet receptor that leads to signaling responses. Once such receptor that binds to HIV-1pp and can induce signaling downstream in platelets is the hem(ITAM)-receptor, CLEC-2, that leads to activation of Syk and SFK, PLCγ and activates Akt and eventually causes platelet secretion. Use of specific inhibitors such as PP2 (which inhibits Src-family kinases (SFK)) or Dasatinib, another Src family tyrosine kinase inhibitor, or use of CLEC-2 null platelets, may be useful in determining whether this partial loss of signaling is indeed due to CLEC-2 activation or not.
Figure 16

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B

(i) TLR7

(ii) TLR7

C

(i) TLR9

(ii) TLR9
Figure 16 VAMP-3 was required for signaling through TLR7 and TLR9 in platelets. (A) Washed platelets (4 × 10^8 platelets/mL) from WT and VAMP-3 KO mice were incubated with TLR7-Loxoribine (1 mM) or TLR9-CpG ODN2395 (5 µM) for the indicated time points at 37°C. Western blotting was performed using anti-phospho-Akt (Ser473), anti-Akt, and anti-phospho-SNAP-23 (Ser95) and anti-SNAP-23 antibodies. Images were taken on a Typhoon 9500 Variable Mode Imager and quantified using the ImageQuantTL software. (Bi-ii) Volumetric quantification of % of phospho-Akt levels over total Akt levels and of phospho-SNAP-23 levels over total SNAP-23 levels in response to Loxoribine. (Ci-ii) Volumetric quantification of % of phospho-Akt levels over total Akt levels and of phospho-SNAP-23 levels over total SNAP-23 levels in response to CpG ODN2395. Statistical significance (*) denoted by p-value ≤0.05, (**) denoted by p-value ≤0.01 and is representative of at least 3 independent experiments (mean ± SEM).
Figure 17 Arf6 was required for signaling through TLR7 and TLR9 in platelets. (A) Washed platelets (4 × 10^8 platelets/mL) from WT and Arf6 KO mice were incubated with TLR7-Loxoribine (1 mM) or TLR9-CpG ODN2395 (5 µM) for the indicated time points at 37°C. Western blotting was performed using anti-phospho-Akt (Ser473), anti-Akt, and anti-phospho-SNAP-23 (Ser95) and anti-SNAP-23 antibodies. Images were taken on a Typhoon 9500 Variable Mode Imager and quantified using the ImageQuantTL software. (Bi-ii) Volumetric quantification of % of phospho-Akt levels over total Akt levels and of phospho-SNAP-23 levels over total SNAP-23 levels in response to Loxoribine. (Ci-ii) Volumetric quantification of % of phospho-Akt levels over total Akt levels and of phospho-SNAP-23 levels over total SNAP-23 levels in response to CpG ODN2395. Statistical significance (*) denoted by p-value ≤0.05, (**) denoted by p-value ≤0.01 and is representative of at least 3 independent experiments (mean ± SEM).
Endocytosis of HIV-1 pseudovirions in human platelets required Dynamin-mediated internalization and trafficking to acidic compartments

Given that endocytosis of HIV-1pp causes platelet activation, we asked whether this process occurs in human platelets. Freshly isolated human platelets, from healthy donors, were prepared and stimulated with HIV-1pp, in the presence of Dynasore or NH₄Cl, to inhibit either Dynamin-mediated endocytosis or neutralize endosomal maturation process required for normal processing and signaling. In Figure 22A, human platelets endocytose viral particles, much in the same way as mouse platelets do. Human platelets lack CD4; hence probable viral binding occurs through CLEC-2 or DC-SIGN. In Figure 22B, use of Dynasore (80 µM) inhibits phosphorylation of IκBα, that is used as a surrogate metric for IKK-β-mediated SNAP-23 phosphorylation and hence platelet activation. NH₄Cl (20 mM), also inhibited phosphorylation of IκBα. In Figure 22C, formation of platelet (CD41/61)-neutrophil (Ly6G) aggregates in response to HIV-1pp was also significantly inhibited in the presence of Dynasore and NH₄Cl. Taken together, our data suggests that Dynamin-dependent endocytosis, targeting to acidic compartments and activation of signaling cascades, in response to the HIV-1 pseudovirions, can be recapitulated in human platelets, indicating that human platelets probably utilize similar VAMP-3 and Arf6-dependent pathways for viral endocytosis and response.
Figure 18 Endocytosis of HIV-1 pseudovirions required VAMP-3 and Arf6. (A, C) Washed WT, VAMP-3 KO and Arf6 KO platelets (5 x 10^7/mL) were incubated with FITC-CpG ODN (5 µM or 10 µM) or with GFP-HIV-1pp (1X and 2 X concentrations) at 37°C for 30 min as described in the Methods section, under endocytosis plate-based assay. (B) Schematic of the HIV-1pp viral constructs showing the GFP-fused ICAM-1 on the surface and mCherry-fused gag protein inside the viral core. (D) Representative micrographs from washed WT, VAMP-3 KO, and Arf6 KO platelets (1 X 10^9/mL) that were incubated with red-green HIV-1pp (100X) for indicated time points at 37°C. Platelets were fixed with 2% PFA (final conc.) and mixed with 0.1 % Trypan Blue prior to imaging. Platelets were visualized as described in the Methods section. Exposure times for DIC were 100 msec, for FITC laser were 1000 msec and for the mCherry was 500msec. Scale bars are 5 µM. (E) Quantification of the number of GFP⁺-mCherry⁺ puncta/platelet in WT, VAMP-3 KO, and Arf6 KO samples and were plotted using Sigma Plot software (v13.0). Data is representative of 2 independent experiments. (F) Age-matched WT and VAMP-3 KO mice were injected with HIV-1pp (100X) through the retro-orbital sinus and platelets were harvested 24 hr post-injection, fixed and mixed with 0.1 % Trypan Blue prior to imaging. Platelets were imaged using the Nikon E600 Eclipse fitted with a Zeiss Axiocam MR camera and visualized under 100X oil-immersion lens. Representative micrographs show endocytosed viral particles within platelets. (G) Quantification of the number of GFP⁺-mCherry⁺ puncta/platelet in WT and VAMP-3 KO samples and were plotted using Sigma Plot software (v13.0). 394 platelets (for WT) and 323 platelets (for VAMP-3 KO) over 6 different fields were counted.
Figure 19 Endocytosis of HIV-1 pseudovirions triggered platelet activation. (A) Washed platelets from WT, VAMP-3 KO, and Arf6 KO mice were either kept resting or stimulated with HIV-1pp (100X) for 30 min at 37°C and lysates were prepared for western blotting using anti-phospho-Akt (Ser473), anti-Akt, and anti-phospho-SNAP-23 (Ser95) and anti-SNAP-23 antibodies. Images were taken on a Typhoon 9500 Variable Mode Imager and quantified using the ImageQuantTL software. (B) Volumetric quantification of % of (i) phospho-Akt levels over total Akt levels and (ii) of phospho-SNAP-23 levels over total SNAP-23 levels. (C) Anti-FITC-P-selectin antibody was added to WT, VAMP-3 KO and Arf6 KO platelets (5 X 10⁷/mL) stimulated with Loxoribine (1 mM), CpG ODN2395 (5 µM) or HIV-1pp (100X) for 30 min at 37°C and geo mean fluorescence intensity was plotted for each condition. (D) Whole mouse blood from WT, VAMP-3 KO, and Arf6 KO mice was either kept unstimulated or stimulated with thrombin (0.1 U/mL) for 3 min, Loxoribine (1 mM), CpG ODN (5 µM), or HIV-1pp (100X) for 30 min. Using BD LSRII, platelet (FITC-CD41/61)- neutrophil (PE-Ly6G) aggregates were acquired by gating around 50, 000 granulocytes and percentage of double-positive events were plotted over total granulocytes. Data was analyzed using FlowJo (v.5.6.7). Statistical significance is denoted by (*) p≤0.05, (**) p≤ 0.01. Data is representative of at least three independent experiments.
Endocytosis of Eco-HIV required Dynamin-mediated internalization, trafficking to acidic compartments and proteolytic degradation

Our overarching hypothesis is to determine how platelet endocytosis contributes to viral dissemination and disease etiology in HIV-1 infections. Towards this goal, we have acquired viral constructs that produce Eco-HIV virions from Dr. David Volsky at Mt. Sinai Icahn School of Medicine. These chimeric viruses have the gp120 of HIV-1 replaced by the gp80 of the murine leukemia virus to render it infectious to rodents only. The virus behaves similar to its human counterpart, infects mouse T cells, generating viral infection with the production of pro-inflammatory cytokines [602]. The Eco-HIV virus is neuroinvasive and causes blood-brain barrier (BBB) disruption. We have Preliminary studies performed with the virus was to determine, whether it behaves like the pseudovirions and does viral endocytosis of Eco-HIV lead to platelet activation.

In Figure 23A, Eco-HIV (100X) induced platelet activation by phosphorylating Akt and SNAP-23, in a time-dependent manner. Dynasore treatment-, NH₄Cl treatment- and protease inhibitor cocktail treatment of mouse platelets, incubated with Eco-HIV virions, showed robust inhibition. In Figure 23B-C, quantification of Akt and SNAP-23 phosphorylation shows that protease inhibition caused the most dramatic inhibition, possibly due to the prohibition of degradative processing of the virus that is essential for signaling. Dynasore and NH₄Cl inhibition follow the pattern observed in mouse platelets (and human platelets) in response to HIV-1pp. Taken together, our data suggests that Eco-HIV virions get internalized by platelets using Dynamin, get trafficked into acidic compartments where proteolytic processing of the virus leads to activation-induced signaling in platelets.

In a future series of experiments, we will determine whether VAMP-3 and Arf6-mediated endocytosis affect infection and neuroinvasiveness in mice infected with Eco-HIV viruses.
Figure 20 Receptor levels of TLRs in VAMP-3 and Arf6 mutant platelets. Lysates were prepared from platelets (4 x 10^8/mL) isolated from WT and VAMP-3 KO and Arf6 KO mice and western blotted for the indicated protein using specific antibodies. (A) TLR7 levels in WT, VAMP-3 KO, and Arf6 KO platelets. β-actin was used as a loading control. (B) TLR9 levels in WT, VAMP-3 KO, and Arf6 KO platelets. β-actin was used as a loading control. (C) CLEC-2 levels WT and VAMP-3 KO platelets. RabGDI was used as a loading control. (D) CLEC-2 levels WT and Arf6 KO platelets. RabGDI was used as a loading control. (E) DC-SIGN (CD209) levels in WT, VAMP-3 KO and Arf6 KO platelets with RabGDI as a loading control. Data is representative of at least two independent experiments.
Figure 21
Figure 21 Response to Loxoribine, CpG ODN required TLR signaling. Washed platelets (4 x 10^8 platelets/mL) were from WT and TLR23479 KO platelets were either kept resting or stimulated with thrombin (0.1 U/mL), LPS (100 ng/mL), Pam3CSk4 (10 µg/mL), Loxoribine (1 mM), CpG ODN2395 (5 µM), HIV-1pp (100X) for 30 min at 37°C. Lysates were prepared and western blotting was performed using anti-phospho-Akt (Ser473), anti-Akt, and anti-phospho-SNAP-23 (Ser95) and anti-SNAP-23 antibodies. Images were taken on a Typhoon 9500 Variable Mode Imager and images were processed using the ImageQuantTL software.
Figure 22
Figure 22: Endocytosis of HIV-1 pseudovirions in human platelets required Dynamin-mediated internalization and trafficking to acidic compartments. (A) Representative fluorescent micrographs of washed human platelets containing red-green HIV-1pp showing platelets with endocytosed pseudovirions (denoted by arrows). (B) Human platelets were incubated with HIV-1pp for 30 min either in the presence or absence of Dynasore (80 µM) or NH₄Cl (20 mM) and lysates were prepared for western blotting with anti-phospho-IκBα and anti-IκBα antibodies. (C) Whole human blood was pre-incubated with either Dynasore (80 µM) or NH₄Cl (20 mM) and stimulated with HIV-1pp for 30 min. Using BD LSRII, platelet (FITC-CD41/61)-neutrophil (PE-Ly6G) aggregates were acquired by gating around 50,000 granulocytes and percentage of double-positive events were plotted over total granulocytes. Data was analyzed using FlowJo (v. 5.6.7). Statistical significance is denoted by (*) \( p \leq 0.05 \), (**) \( p \leq 0.01 \). Data is representative of at least three independent experiments.
Figure 23

(A) Western blot analysis showing the expression levels of various proteins under different conditions. The conditions include WT, Dynasore, NH₄Cl, and PIC. The time points are 0, 30, and 60 minutes.

(B) Bar graph showing the ratio of pAkt/Akt (x100) for each condition at the indicated time points.

(C) Bar graph showing the ratio of pSer95-SNAP-23/SNAP-23 (x100) for each condition at the indicated time points.
Figure 23 Eco-HIV-stimulation of platelets required Dynamin-mediated internalization, trafficking to acidic compartments and proteolytic degradation. (A) Washed platelets (4 × 10^8 platelets/mL) were stimulated with Eco-HIV (100x) for the indicated time points at 37°C or pre-incubated with Dynasore (80 µM), or NH₄Cl (20 mM) or protease inhibitor cocktail mixture (PIC) (100X) for 30 min and then stimulated with Eco-HIV (100X) for 30 min at 37°C. Western blotting was performed using anti-phospho-Akt (Ser473), anti-Akt, and anti-phospho-SNAP-23 (Ser95) and anti-SNAP-23 antibodies. Images were taken on a Typhoon 9500 Variable Mode Imager and quantified using the ImageQuantTL software. (B) Volumetric quantification of % of phospho-Akt levels over total Akt levels and (C) of phospho-SNAP-23 levels over total SNAP-23 levels. Data is representative of one experiment.
Conclusions

It is becomingly increasingly clear that platelets bridge coagulation cascades with inflammation. Platelets respond to changes in the vasculature and modulate their response accordingly. Platelets also interact with pathogenic bacteria and viruses to mediate immune responses against them. Infection-related platelet activation can be strongly correlated with thrombocytopenia that leads to further cardiovascular complications that can prove to be detrimental to prognosis and cure. Chronic viral infections, e.g., HIV-1, severely increase the risk of acute myocardial infarction (MI), possibly via some level of platelet activation, contributing to increased thrombotic potential. Platelets do endocytose viruses and bacteria, but the molecular machinery is ill-defined. In nucleated cells, responses to HIV-1 are mediated by virus phagocytosis/endocytosis, degradation to release Toll-like Receptor ligands, and subsequent receptor activation. Is this process recapitulated in platelets? Here we show that platelets indeed use VAMP-3 and Arf6-dependent pathways to endocytose HIV-1 virions, degrade retroviral particles to release TLR ligands, which initiate platelet activation and secretion. HIV-1 uptake and subsequent activation are abolished in VAMP-3 and Arf6 KO mice.

Collectively, our studies shed light on how platelets act at the early stage of pathogen recognition and are able to process them to initiate an immune response. Our overarching hypothesis is to determine how platelet endocytosis contributes to viral dissemination and disease etiology in HIV-1 infections. Future experiments planned with the Eco-HIV viruses in a mouse model of HIV infection using our endocytosis mutants will go a long way in elucidating the relevance of platelet endocytosis in mediating disease outcomes.
Figure 24 Model diagram showing the role of Dynamin-, VAMP-3- and Arf6-mediated endocytosis of HIV-1 pseudovirions and TLR ligands in platelet activation. Platelets can internalize bacterial and viral ligands via Dynamin-, VAMP-3- and Arf6-dependent endocytosis. Viruses such as HIV-1 or TLR ligands such as unmethylated CpG oligonucleotides, Loxoribine etc. can then be trafficked to an endosomal compartment bearing their respective Toll-like receptors. Viruses require acidic compartments to allow degradation of the viral capsid, releasing ssRNA or TLR ligands, which can then bind to TLR7 and initiate a response. Receptor-ligand interactions lead to the recruitment of the adaptor protein MyD88, which then triggers downstream signaling through TRAF6, TAK1, and IRAK-1 activation, phosphorylating IRAK-4. Concomitant PI3K activation also leads to Akt activation, driving IKK activation. IKK then activates SNAP-23, which drives SNARE complex formation and ultimately granule secretion. TLR and HIV-1 pseudovirion activation of platelets leads to P-selectin exteriorization from alpha granules, which can then form platelet-leukocyte aggregates with PSGL-1-bearing leukocytes in the circulation.
Chapter Five: Endocytosis in platelet function: roles in hemostasis and innate immunity

Through the work presented in this thesis, we have taken several key steps in understanding how endocytosis modulates platelet functions in the context of hemostasis and innate immunity. We have identified VAMP-3 to be a critical regulator of fibrinogen (Fg) uptake in platelets. Loss of VAMP-3 causes defective fibrinogen uptake, which manifests itself as dysfunctional integrin trafficking of αIIbβ3 that translates into increased platelet spreading and clot retraction. However, these defects do not cause obvious alterations in thrombus formation or hemostasis. Additionally, using 3D super-resolution microscopy, we present the first report of differential sorting of receptor-mediated endocytic (fibrinogen) and fluid-phase pinocytic (low mol. wt. dextran) cargos in platelets. Future research will determine the intricacies of the different routes and endosomal compartments utilized by the two kinds of cargos in platelets. How platelets respond to pathogenic viruses and bacteria that determine the disease outcomes in viremia and bacteremia is still unclear. Using both Arf6 KO and VAMP-3 KO mutants as tools to probe the importance of endocytosis in platelets, we posited that platelet endocytosis could potentially be critical for actively sensing pathogenic damage in the vascular microenvironments and allowing platelets to act as immune cells. Using non-replicative, genetically modified HIV-1 pseudovirions, we show that platelets endocytose HIV-1 via VAMP-3 and Arf6-mediated endocytic pathways, degrade viral particles, causing platelet activation, as measured by PI3K/Akt and IKKβ-mediated SNAP-23 phosphorylation that drives secretion of P-selectin from alpha granules to form platelet-neutrophil aggregates. HIV-1 uptake and subsequent activation are abolished in VAMP-3 and Arf6 KO mice. This mechanism of viral endocytosis and subsequent activation also occurs in human platelets and may, in part, explain some of the platelet-associated phenotypes seen in HIV-1-infected individuals.

VAMP-3 is required for accumulation of fibrinogen in platelets

Fibrinogen, made by hepatocytes and not megakaryocytes [461], is an alpha-granule cargo because it is taken up via a αIIbβ3-mediated process. VAMP-3 deletion results in defective intra-platelet Fibrinogen accumulation (Figures 3A-B, 5). Data presented in
this thesis is the first report of this v-SNARE protein being involved in internal membrane trafficking steps in platelets. These trafficking steps are needed for $\alpha_{\text{IIb}}\beta_3$-mediated Fg uptake, endocytic trafficking of cargo, platelet spreading, clot retraction, and proper regulation of JAK2 phosphorylation downstream of TPOR. Global loss of VAMP-3 did not affect the gross hematological profile of the mutant mice, with whole blood counts unaltered in the KO (Table 2). Electron micrographs depicted normal platelet ultrastructure in the VAMP-3 KO platelets [202]. Western blotting analysis showed that loss of VAMP-3 did not cause overt defects in levels of other endocytic cargo, endosomal markers, or proteins involved in endocytosis (Figure 3C-D). Levels of total surface and activated $\alpha_{\text{IIb}}\beta_3$ were unaltered in resting and thrombin-stimulated platelets (Figure 4A-B) as was the steady-state binding of Fg (Figure 4C). ADP-stimulated WT platelets showed enhanced Fg-binding and increased uptake of Fg; however, ADP-stimulation of VAMP-3 KO platelets was unable to override the defect in Fg internalization (Figure 5A-B). In sum, the loss of VAMP-3 had no gross effects on many aspects of platelet function, yet it affected Fg accumulation. Of note, the ~40% deficit in Fg seen in platelets from KO mice was not sufficient to affect hemostasis [202].

Using TB as a quencher of external fluorescence of FITC/Alexa 488, we could monitor cargo uptake by platelets. Our plate-based endocytosis assay allows more flexibility for testing a variety of conditions and more sensitivity than flow cytometry-based assays. Using this assay, we showed that Fg internalization reached saturation in WT platelets and demonstrated classical receptor-ligand saturation kinetics over the range of concentrations tested (Figure 5C, E). Conversely, uptake of small molecules usually occurs via fluid-phase pinocytosis and is generally a non-specific process. In platelets, dextran uptake appeared to occur constitutively and rapidly. In as early as 1 min, dextran uptake was detected and it linearly increased over time and dose (Figure 5D, F). In platelets, dextran uptake had at least a 1,000-fold higher capacity (as measured by total molecules internalized) than Fg uptake. This argues that fluid-phase pinocytosis is more efficient in platelets, but further studies with other cargo are needed to strengthen that conclusion. Fluorescence micrographs depicting endocytosed FITC-Fg showed that VAMP-3 KO platelets indeed had a robust defect in
Fg uptake/accumulation. The number of fluorescent puncta was significantly lower in
the KO platelets (Figure 5G-H). Dextran uptake, alternatively, did not show any
robust differences between WT and VAMP-3 KO platelets, both in the plate-based
endocytosis assay (Figure 5D, F) and in the fluorescence images (Figure 11). This
argues that, in platelets, VAMP-3 is important for an endocytic trafficking step(s) that
is part of the path taken by Fg bound to α<sub>IIbβ3</sub>; more so than cargo internalized by
constitutive pinocytosis. However, whether VAMP-3 is involved in mediating uptake
of cargo via other types of platelet endocytosis processes remains to be determined.

**Platelets can sort different kinds of endocytic cargo**

Can platelets decipher and sort endocytosed cargo or is it passively internalized via a
common endocytic route? Using 3D-SIM microscopy, we demonstrated that platelets
actively sort endocytosed cargo, exhibiting a more complex endocytic system than
previously imagined. Fg binds to surface α<sub>IIbβ3</sub>, at initial time points and then
proceeds to accumulate inside platelets as discrete puncta within 30 min. At this
time, surface binding was less apparent (Figure 6A). Fg is thought to enter platelets,
transit through two classes of multi-vesicular bodies, and end up in α-granules [41].
Platelets contain roughly 50-80 α-granules [34-35], yet we did not see equivalent
numbers of Fg puncta at 30 min or even after the overnight incubation in vivo (Figure
6B). The global distribution of Fg into α-granules, seen in recent immuno-electron
microscopy studies, would seem at odds with our data [55]. While unclear at present,
our observations could mean that only specific α-granules are readily loaded with
endocytosed cargo during circulation and that most cargo loading occurs at the
megakaryocyte stage. Our previous studies are not totally consistent with that
scenario since we failed to observe FITC-Fg in bone marrow megakaryocytes, 24 hr
post injection [19]. Alternatively, our data could imply the Fg loading into all α-
granules requires more time, perhaps the lifetime of a platelet. It is equally possible
that larger amounts of FITC-Fg are needed to fully label all the granules. In contrast,
dextran uptake occurred very quickly and platelets accumulate more dextran+
puncta than Fg+ puncta over time (Figure 6A-B). Both cargoes showed partial overlap
at early time points but mostly existed as discrete puncta. VAMP-3 deletion did not
compromise the platelets’ ability to sort cargo but did affect their ability to
accumulate it. Surprisingly, when dextran was injected for an overnight period, less dextran accumulated in the VAMP-3 KO platelets (Figure 6B) compared to WT. This was in contrast to Figure 6A, where no clear defect in dextran uptake was seen in the VAMP-3 KO platelets up to 30 min of incubation and in the plate-based endocytosis assay (Figure 5D). This could indicate that loss of VAMP-3 causes a defect in chronic accumulation of cargo, irrespective of the initial endocytic pathway employed.

**Potential role of VAMP-3 in integrin αIIbβ3 endocytic trafficking in platelets**

Our previous studies of Arf6 KO platelets suggested that Fg uptake and integrin recycling are important for some acute platelet functions e.g., clot retraction and spreading [19]. VAMP-3 KO platelets had a similar phenotype. VAMP-3 is important for epithelial cell migration, particularly mediating trafficking at the lamellipodia and it also localizes to focal adhesions [426-427]. Tetanus toxin-mediated cleavage of VAMP-3 reduced epithelial cell migration but enhanced adhesion to collagen, laminin, and fibronectin; perhaps due to impaired recycling of β1 integrins [427]. In Figure 7A-B, the rate of platelet spreading on immobilized Fg was faster in VAMP-3 KO than in WT platelets. This, together with the Arf6 KO phenotype, reinforces the notion that altered αIIbβ3 trafficking affects platelet spreading. Unlike epithelial cells where migration is directional, platelet spreading on coated surfaces is not vectorial but seems to occur in all directions. In our assays, loss of VAMP-3 enhanced platelet spreading. If VAMP-3 controls spatially-specific membrane fusion steps that facilitate vectorial platelet spreading in a thrombus, its loss could make random, multidirectional fusion more efficient leading to enhanced spreading in all directions on a coated surface. Alternatively, loss of VAMP-7, a key v-SNARE in platelets, leads to granule exocytosis defects and reduced spreading [203]. VAMP-3 KO platelets had no defect in exocytosis; however, its deletion causes a modest secretion rate enhancement [202]. Given that VAMP-7-mediated exocytosis is important for spreading, enhanced secretion could enhance spreading. Loss of VAMP-3, which could be acting as an inhibitory SNARE (i-SNARE) and competing with VAMP-7 [462], could increase the efficacy of exocytic SNARE complex formation (containing VAMP-7, or -8, Syntaxin-8 or -11, and SNAP-23) and thus increase the fusion needed for spreading. These two potential mechanistic explanations for our phenotypes are
difficult to distinguish; but, comparing platelets from VAMP-3 and VAMP-7 KO mice will be invaluable in understanding how membrane trafficking affects platelet spreading. The enhanced spreading and clot retraction phenotype is subtle and might be expected to predispose these mice towards a more pro-thrombotic phenotype. However, that was not detected in the tail-bleeding or FeCl3-injury ([19] and Joshi, et al. in preparation) models that we have tested nor was an effect seen with the VAMP-7 KO mice [203]. More refined assays will be needed to parse the biological ramifications of these observations. Additionally, Fg deficiency causes bleeding diathesis in both mouse and humans [607-608]. Both VAMP-3 and Arf6 KO mice have about 50% defect in Fg levels, yet have normal hemostasis with no bleeding defects. Thus it is not clear whether platelet-derived Fg is functionally important for physiological processes or not; and if it's, how much Fg do platelets contribute to form the fibrinous thrombus at the site of injury.

“Immuno” versus “hemostatic” stimulation of platelets: differential kinetics and extent of responses

How platelets respond to “immune agonists” such as those tested in this thesis as well as in previously published reports, have challenged the canonical view of platelets being “all-or-none” type responders to vascular damage. Platelets need to be able to respond to hemostatic damage rapidly in order to contain blood loss. This is usually achieved via rapid degranulation of the already docked dense core granules, which secrete ADP and ATP that allow paracrine activation of additional platelet to form the platelet plug. The dense core granules are the first to secrete their granular contents [91]. Agonist potency determines the extent and rate of cargo release, stronger agonists such as thrombin and collagen prompted rapid release that was extensive and maximal compared to weaker agonists such as PAR4-peptide that elicited a lower degree of release. Agonist-induced secretion was random and heterogeneous in nature bearing no thematic pattern in the distribution of cargo within the releasates [91]. However, reports explaining the differential release of proangiogenic and antiangiogenic factors in response to specific agonists suggest that the observed platelet release heterogeneity could be a function of partial platelet activation [567-569]. Platelet stimulation by the “immune agonists”
such as the Toll-like receptor ligands and HIV-1 pseudovirions, are markedly slower in their kinetics and have a less extensive release, irrespective of the dose of agonist or time of stimulation, a phenomenon that could be attributed to partial platelet activation. Our findings are corroborated by previous reports showing differential activation of signaling pathways, differences in alpha granule release, and unique protein-protein interactions when platelets were stimulated with thrombin versus when they were stimulated with Pam3CSK4, a TLR2-specific agonist [447]. Additionally, bacteria-induced platelet aggregation proceeds after a lag time, dependent on the bacterial density that lasts between 3-20 min, as compared to thrombin or ADP-mediated hemostatic responses which reach maximal activation in less than 3 min [538]. Taken together, these data suggest that platelets can interpret between various kinds of activating stimuli and respond accordingly. While we have not yet tested the platelet “secretome” in response to TLR/HIV-1 agonists using antibody arrays similar to the ones used in [91], it could be possible that response to “immuno-agonists” elicits differential releasates compared to hemostatic agonist-stimulated releasates. While hemostatic-stimulation gives rise to factors and mediators that allow containment of vascular damage by sealing the breached endothelial vessel walls, thereby initiating a cascade of responses that lead to hemostasis and thrombus formation; immune-stimulation may elicit platelets to release immunomodulators that either allow platelets to directly annul the pathogenic threat (ways how these works have been explained in the introduction under platelet-bacterial/viral interactions) or alert the surveilling professional immune cells to eliminate the infectious damage. A low-grade of activation may be sufficient to generate an “immunomodulatory phenotype” for platelets, while at the same time preserving its inherent hemostatic activities. This feature harkens back to the evolutionary nature of platelets, having been descended from the primordial hemocytes that were capable of hemostasis, coagulation and immune functions [435]. How this differential response works, in terms of what signaling pathways it activates, potential differences in calcium transients (which effectively determine agonist potency in mediating aggregation and eventual thrombus formation [570])
etc, are some of the potential future questions that will help uncover the basis of this differential response.

Differential usage of the SNARE-machinery may provide useful insights into differential responses of the platelet release reaction to “immune” versus “hemostatic” stimuli. Major SNAREs that are essential for release from all three classes of granules are VAMP-8 [201], SNAP-23 [39, 198, 210-212] and syntaxin 11 [209]. Loss of VAMP-3 does not affect platelet secretion [202] while the loss of VAMP-7 preferentially affects alpha granule release [203]. Loss of VAMP-8 has less effect on dense core granule but profoundly affects alpha granule and lysosomal secretion [201]. Loss of syntaxin 11 affects serotonin release from dense granules and PF4 release from alpha granules more than it affects β-hexosaminidase release from lysosomes [209]. Loss of SNARE regulator, Munc18b, has a similar phenotype to syntaxin 11 [216]. These differences in affecting release reactions from the three granules suggest heterogeneity between fusogenic SNARE complexes. While other SNAREs can participate in forming fusogenic SNARE complexes, the efficacy of secretion is maximal when VAMP-8/SNAP-23/syntaxin 11 complexes are formed. Can platelets utilize different SNARE complexes to mediate differential secretion in response to different secretagogues (“immune” versus “hemostatic”)? Future investigations will be required to answer this question in greater details.

Platelets endocytose HIV-1 using Dynamin, VAMP-3, and Arf6-mediated pathways

Platelets and megakaryocytes have been shown to endocytose HIV-1 viruses [542, 263] into an endosomal-like vacuole, thought to be part of the OCS in platelets [263]. Megakaryocytes utilize CD4 to bind to HIV-1 [547] whereas platelets, which lack CD4, utilize CLEC-2 and DC-SIGN to bind the virus [550]. However, the molecular machinery required to mediate viral endocytosis is unknown in platelets. Data presented in this thesis provides the first report on the elements of the platelet endocytic machinery that mediate HIV-1 uptake in platelets. Our studies show that Dynamin, VAMP-3, and Arf6 are at least some of the proteins that mediate viral endocytosis.

Enveloped viruses, such as HIV-1, require endocytosis for entry into cells. Surface viral glycoproteins such as Env (gp120), interact with CD4 and coreceptors such as
CCR5 or CXCR4 to allow initial binding [571]. Except for CD4, platelets express members of the CXCR family including CXCR4 [549]. Following binding, it is thought that HIV viruses fuse with endosomes and macropinosomes [572-573]. Clathrin-mediated endocytosis is thought to play a role in viral uptake as inhibition of this route of endocytosis reduces the efficacy of viral fusion and infection in HeLa-derived cells [574]. Use of dynasore, the small molecule inhibitor that blocks Dynamin activity [577], inhibits HIV-1 uptake, fusion, and infection in TZM-bl cells [575]. Using similar conditions as in Miyauchi et al. 2009 [613], we treated mouse platelets with Dynasore (80 µM) to determine the effects on HIV-1 mediated platelet activation. It is to be noted here, Dynasore concentrations were selected so as to not affect platelet granule secretion [351 and data not shown]. Dynasore treatment inhibited HIV-1 pseudovirion-mediated activation of platelets, leading to a reduction in levels of phospho-Akt and phospho-SNAP-23. This result indicates that, like monocytes and T-cells, HIV-1 requires Dynamin-mediated activity to enter platelets, possibly via clathrin-mediated endocytosis. Dynamin2 has been specifically shown to be required for the enhancement of HIV-1 infection by directly interacting with HIV-1 virulence factor Nef [585]. Inhibition of Dynamin2 activity, using either dominant-negative DNM2 or siRNA against DNM2, inhibited Nef-mediated HIV-1 infectivity of 293T cells. Incidentally, Dynamin2 is rather abundant in mouse platelets and also present in human platelets [343], hence it could very easily play a role in HIV-1 endocytosis in platelets and possibly contribute to subsequent activation.

Plasmacytoid dendritic cells utilize a similar Dynamin-dependent endocytosis to uptake HIV-1 virions and upon CD4-mediated entry; virus gets trafficked through endosomal compartments that stained positive for EEA1, Rab5, Rab7, Rab9 and LAMP-1 [576]. A study in plasmacytoid dendritic cells shows time-dependent trafficking of the virions starting from the early endosomes (EEA1-positive and Rab5-positive compartments) to late endosomes (Rab7-positive and Rab9-positive compartments) and eventually to lysosomes (LAMP-1-positive) [576]. HIV-1-mediated plasmacytoid dendritic cell activation as measured by interferon-α production was significantly reduced upon dynasore treatment. It is thought that HIV-1 viral particles get trafficked to the late endosomes and lysosomes, where it
gets degraded. As a result, blocking lysosomal degradation and/or endosomal acidification process leads to increased viral infection and concomitant IFN-α production [473, 578-582]. Viral particles colocalize with Rab7 and Rab9 and LAMP-1, indicating endolysosomal compartments, where the virus gets degraded, releases viral nucleic acids (in this case ssRNA) that can then interact with endosomal Toll-like receptors like TLR7 [473, 583, 584, 580].

How much of this process occurs in platelets? Platelets contain various RabGTPase markers such as Rab4, Rab11, Rab7, and Rab5 among others. We have shown the presence of two distinct endosomal populations, Rab4+ early endosomes and Rab11+ recycling endosomes [19]. TLR7 is present in platelets [472]. Treatment of platelets with ammonium chloride, which inhibits the acidification of endosomes, leads to a reduction in Akt and SNAP-23 phosphorylation in mouse platelets and a reduction in IκB phosphorylation and subsequent platelet-neutrophil aggregate formation in human platelets and human whole blood respectively. Given that most of the endocytic machinery and signaling components required for HIV-1 entry and subsequent IFN-α production in plasmacytoid dendritic cells are present in platelets, ongoing and future experiments will determine whether HIV-1 follows a similar route of endocytosis in platelets or not.

Arf6, the Ras-like small GTPase, which is involved in integrin trafficking and fibrinogen uptake in platelets has also been shown to be involved in the pathogenic invasion of various cell types [587-590]. Arf6 is also required for HIV-1 endocytosis in T-cells [586]. Use of siRNA against Arf6, or inhibiting Arf6 function by using GDP-bound or GTP-bound inactive mutants, led to a decrease in HIV-1 viral membrane fusion, entry and infection of T lymphocytes. Furthermore, Arf6 inhibition impaired cell-to-cell transmission of HIV-1 viral particles in CD4+-T lymphocytes. On the other hand, VAMP-3/Cellubrevin has been critical for phagocytosis of pathogens and various particles [416-417]. However, the importance of VAMP-3 in mediating HIV-1 endocytosis is unclear.

In our ex vivo assay, endocytosis of HIV-1 pseudovirions is dramatically impaired in both VAMP-3 and Arf6 KO platelets. This is the first report to show the importance of two critical endocytic regulators in platelets that drive pathogen internalization. Wild
type platelets endocytose more pseudovirions in a time-dependent manner. On the other hand, KO platelets have hardly any pseudovirions in them and viral endocytosis remains impaired even over prolonged periods of time. Consistently, when HIV-1 pseudovirions were intravenously injected through the retro-orbital sinus of VAMP-3 KO mice, a severe defect in viral uptake was noted in these platelets even after 24 hours post-injection. Taken together, our data suggests that both VAMP-3 and Arf6 are required for HIV-1 entry into platelets.

Since HIV-1 binds to CLEC-2 and DC-SIGN on platelets and requires Dynamin-dependent clathrin-mediated endocytosis, we posit that HIV-1 entry utilizes similar receptor-mediated endocytic routes that Fg uses in platelets and thereby could colocalize with Fg. In fact, preliminary data obtained from 3D super-resolution microscopy confirms this hypothesis (data not shown). Colocalization of HIV-1 pseudovirions with Fg increases over time while colocalization with low mol. wt. dextran (fluid-phase pinocytosis marker) is very limited, corroborating our earlier observations of differential sorting of receptor-mediated and fluid-phase cargos. However, immunofluorescence experiments to demonstrate colocalization with endosomal and platelet granule markers still need to be performed to pinpoint specific endocytic routes of HIV-1 in platelets. It is interesting to note, that platelets somehow can interpret between two different physiological cargos and respond accordingly, despite using a similar route of endocytosis. For example, Fg enters platelets and eventually traffics through the early endosomes, to multivesicular bodies and ends up in alpha granules. Fg uptake does not activate platelets or cause any overt physiological changes, yet HIV-1 endocytosis activates platelets enough to cause Akt and SNAP-23 phosphorylation and mediate granule secretion in the form of P-selectin exteriorization. How platelets make this distinction, pose an interesting question, and add to the budding complexity of the platelet endomembrane system, than previously imagined.

**Mechanisms of platelet activation upon HIV-1 endocytosis**

Patients with HIV-1 infections show up in the clinics with severe thrombocytopenia that is commonly associated with increased plasma markers of platelet activation [468], including increased plasma levels of PF4, increased platelet-leukocyte
aggregates, and increased sCD40L levels [551]. HIV-1 trans-activator of transcription protein (Tat) directly activates platelets to release sCD40L in a CCR3 and β3 integrin-dependent manner [552]. Various soluble endothelial activation markers like sVCAM-1, sCAM-1, and von Willebrand Factor levels are higher in HIV-1 infected patients [544]. In a simian immunodeficiency virus (SIV)/macaque model, acute SIV infection causes increased surface expression of P-selectin, CD40L, and major histocompatibility complex class I [591], with a drop in circulating platelet counts. Acute infection led to an increase in monocyte counts, which led to increased platelet-monocyte aggregates in the bloodstream, contributing to the observed thrombocytopenia. Infection with HIV-1 is associated with a 50% increase in risk of acute myocardial infarction in a Veterans Aging Cohort Study Virtual Cohort of 82,459 participants that were HIV-1+, beyond that explained by all standard Framingham risk factors such as HIV status, age, sex, race/ethnicity, hypertension, diabetes, dyslipidemia, smoking, hepatitis C infection, body mass index, renal disease, anemia, substance use, CD4 cell count, HIV-1 RNA, antiretroviral therapy, and incidence of acute myocardial infarction (MI) [592]. Given that platelet hyperactivity contributes to acute MI, this study provided important insights into how prolific platelet hyperactivation is in chronically infected HIV-1 patients. In essence, as antiretroviral therapies prolong lifespans of HIV-1+ patients, it makes them increasingly susceptible to cardiovascular-related ailments such as acute MI. The severity of HIV-1 infection strongly correlates with thrombocytopenia and platelet activation, yet the molecular mechanisms of how this activation occurs remain unclear.

In nucleated immune cells such as plasmacytoid dendritic cells, responses to HIV-1 are mediated by virus phagocytosis/endocytosis, degradation to release ssRNA (in the case of HIV-1), and subsequent TLR7 receptor activation. While platelets use more or less similar strategies to endocytose and respond to the viral particles, much to our surprise, ablating TLR7, only causes a partial reduction in platelet activation. Clearly, HIV-1 activation of platelets utilizes both TLR-dependent and TLR-independent pathways. A possible explanation for this partial reduction in platelet activation, as measured by Akt and SNAP-23 phosphorylation, could be due to activation of the CLEC-2 receptor and its downstream signaling pathways, that in and
of itself, are potent platelet activation receptors. HIV-1 binding to CLEC-2 could activate these pathways leading to Akt phosphorylation and SNAP-23 phosphorylation [593]. DC-SIGN, the other HIV-1 binding receptor on platelets, does not induce any downstream activation signals [543]. The CXCR family of coreceptors that can bind to HIV-1 and are present on platelets do not elicit any platelet activation responses either [549]. There could be some other signaling cascade being turned on by HIV-1 endocytosis in platelets that we do not know of, as yet. However, it does indicate that HIV-1-mediated activation of platelets is indeed, a complex interplay of multiple signaling pathways. This poses as a double-edged sword because, on one hand, therapeutic interventions to target one signaling pathway over another allows partial disruption of activation, thereby dampening levels of platelet activation markers while preserving some normal functions of platelets; this is advantageous. However, the flipside to this problem is the challenge to design drugs that do not have any adverse side-effects on other cell types, which perhaps need those signaling pathways to function properly.

**Role of autophagy in mediating platelet activation upon HIV-1 endocytosis**

Platelets have been shown to contain autophagosomal structures and autophagy plays vital roles in mediating proper platelet function and hemostasis [240]. Autophagy is induced upon platelet activation. Agonists such as thrombin, convulxin initiate platelet signaling cascades that lead to loss of autophagy marker LC3II and requires proteolytic degradation, endosomal acidification and SNARE-membrane fusion (involvement of VAMP-2, VAMP-3, and VAMP-8) [240].

Several recent reports have shown autophagy induction upon TLR stimulation [594-598]. LPS causes TLR4-mediated autophagy induction in mouse macrophages as demonstrated by the GFP-LC3 puncta formation, with LPS inducing an increased amount of lipidated LC3II in these cells [597]. TLR7 stimulation by ssRNA and imiquimod also induced autophagy in RAW264.7 macrophages, leading to increased puncta formation (denoting autophagosomes) and LC3II formation in the presence of pH-tropic drugs like Bafilomycin A (that blocks autophagosomal maturation by inhibiting LC3II degradation) [595]. TLR7-mediated autophagic induction requires MyD88 and is thought to utilize Beclin-1, a critical regulator of autophagy, (and is
present in platelets) [595]. It is thought that TLR7-mediated autophagic induction proceeds in a similar fashion to starvation-, rapamycin- or LRG47-induced autophagy [599]. In HeLa cells and plasmacytoid dendritic cells infected with HIV-1, autophagy was induced as measured by LC3II formation, upon binding of ssRNA to TLR7 [595, 598]. The speculative model is that viral PAMPs (HIV-1 ssRNA) are introduced to TLR7+-endosomes via autophagic degradation of the viral nucleocapsid. Once TLR7 binds to ssRNA, it signals through MyD88 and IRF7 to trigger activation of type I IFN and other cytokine genes, which play critical roles in innate immunity and pathogenesis of HIV-1 [498, 600].

How much of this process recapitulates in platelets remains to be seen? Whether platelets utilize the autophagosomal machinery to respond to HIV-1 also remains to be seen. Ongoing experiments are investigating whether HIV-1 viral particles, upon endocytosis, gets trafficked to autophagosomes and colocalize with GFP-LC3 puncta. Does HIV-1 stimulation of platelets cause an induction of autophagy, leading to more GFP-LC3 puncta and formation of LC3II? Does TLR7 stimulation (by Loxoribine and Imiquimod) of platelets leads to increased LC3II levels and increased puncta formation? Using GFP-LC3 mice, we can answer these questions.

In mouse platelets, preliminary studies show that Loxoribine (TLR7) and CpG ODN (TLR9)-mediated activation leads to a time-dependent phosphorylation of Akt and SNAP-23 and loss of Atg7 or Beclin-1 had no effect on these activation profiles, whereas HIV-1 pseudovirion stimulation of Atg7 KO platelets led to a partial reduction in Akt and SNAP-23 phosphorylation. While LC3II levels were not yet measured in these assays, these results suggest that viral degradation in platelets possibly require autophagosomal machinery but some of the other TLR7 ligands might not. Is this due to the differential sorting of cargo? Small molecules like Loxoribine and Imiquimod could utilize the fluid-phase endocytic pathway in platelets to reach TLR7+-endosomes for active signaling and thus may bypass trafficking through autophagosomes. Contrary to that, HIV-1 pseudovirions use the receptor-mediated pathway and require some degree of degradation in order to generate functional ligands for signaling in TLR7+-endosomes and hence are directed to the autophagosomes? Further investigation using immunolocalization studies will
help answer these questions. Whatever might be the case, these results will further shed light on how complex endocytic trafficking and cargo sorting might be in platelets.

**Global importance of platelet endocytosis in HIV-1 infection**

Our current work clearly suggests that HIV-1-mediated activation of platelets requires endocytosis. Using endocytosis mutants (VAMP-3 and Arf6 KO) that are affected by one or more steps of endocytic uptake and trafficking in platelets, we demonstrate for the first time that endocytosis of physiological cargo leads to activation of platelet signaling cascades that cause granule secretion and formation of platelet-leukocyte aggregates, considered to be a marker of active inflammation in the bloodstream. Since activation-induced platelet sequestration from circulation leads to thrombocytopenia, a common occurrence in HIV-1 infections, it stresses the importance of endocytosis in mediating platelet responses to HIV-1 infection. Our studies would indicate that platelets come into play in the initial stages of viral infection, required for early detection of the viral particles in the bloodstream via their surveillance mechanisms. This could happen when HIV-1 enters a new host through blood transfusions, sharing needles with HIV-1 infected individuals etc. Platelets take up the virus when they encounter it and process it to initiate a response. This response may simply be manifested by P-selectin exteriorization on its surface, which acts as a “beacon of damage” and alerts patrolling immune cells. It is the immune cells *e.g.*, T cells, dendritic cells, macrophages, monocytes and neutrophils (or cells that express the cognate PSGL-1) that detect P-selectin-bearing platelets and do the bulk of the immune responses. As a result, platelet endocytosis may be an essential necessity for mounting appropriate immune responses against the virus. Viruses such as HIV-1 often develop strategies to avoid interactions with endolysosomes, where viral degradation occurs to initiate responses, by hijacking components of the endocytic machinery and trapping themselves inside endosomal organelles to evade immune-mediated detection and killing [601]. Viruses hiding within endosomes may then utilize the host cell as a “Trojan Horse” to facilitate cell-to-cell transmission and viral dissemination throughout the vasculature. This portends that viral endocytosis may have deleterious effects and devise ways to
interrupt endocytosis may have beneficial effects in mitigating viral spread and infection. Whether the same occurs in platelets is unclear, but our endocytosis mutants may provide some useful insights into the global importance of platelet endocytosis in HIV-1 viremia and infections.

A mouse model of HIV-1 infection that mimics most of the viral etiology and disease progression in humans has become increasingly beneficial in understanding HIV-1 infection, viral replication, control and pathogenesis [602]. These murine HIV-1 viruses, called Eco-HIV viruses can be made to target mouse cells by replacing the coding region of gp120 in HIV-1/NL4-3 viruses with that of gp80 from the ecotropic murine leukemia virus, a ssRNA-bearing retrovirus that targets only mice and rats [602]. This chimeric Eco-HIV virus was shown to infect mouse lymphocytes only and replication within mouse cells led to the production of infectious viruses that caused neuroinvasiveness by breaching blood-brain barrier (BBB). Eco-HIV viruses are detected in spleen and brain as early as 3 weeks post-inoculation of the virus into mice and lead to expression of pro-inflammatory infectious response genes such as MCP-1, STAT-1, and IL-1β etc. Co-incidently, studies done by Jones et al. [603] showed that mice infected with Eco-HIV showed increased blood-brain barrier permeability as early as 2 weeks post-injection. These mice also had increased plasma levels of sCD40L and PF4, both signs of platelet activation. Inhibiting platelet function using eptifibatide (that antagonizes normal αIIbβ3 function), restored BBB permeability, sCD40L, PF4 levels and P-selectin expression, indicating platelet activation is important for the neuroinflammatory phenotype and breach of BBB in Eco-HIV infected mice. These studies will be the basis for our planned future experiments to answer whether platelet endocytosis of Eco-HIV is beneficial or exacerbates viral infection and neuroinvasiveness.

We have obtained the Eco-HIV viral constructs from Dr. Volsky’s group and have prepared the Eco-HIV viruses. We seek to determine the effects of Eco-HIV infection in our VAMP-3 and Arf6 KO mice. We will inject Eco-HIV viruses into VAMP-3 and Arf6 and littermate controls and probe for platelet activation markers (plasma PF4 and inflammatory cytokine levels, platelet-leukocyte aggregates), BBB permeability (using fluorescent tracers such as sodium fluorescein), signs of viral replication (via
measuring p24 antigen levels) in plasma and tissues, platelet counts to determine thrombocytopenia at different timed intervals (24 hr, 2 weeks, 4 weeks and 6 weeks). At the end, survival measurements will also be done. Preliminary ex vivo analyses show that Eco-HIV infects mouse platelets and activates them. Eco-HIV induced Akt and SNAP-23 phosphorylation in a time-dependent manner similar to HIV-1 pseudovirions. This phosphorylation was also inhibited by Dynasore-, ammonium chloride-, and protease inhibitor cocktail, suggesting that activation responses to Eco-HIV require Dynamin-dependent endocytosis, trafficking to acidic compartments and some sort of proteolytic degradation to break down the viral capsid to release ssRNA, which then activates platelets. So far, Eco-HIV behaves in a similar fashion to HIV-1 pseudovirions, at least in the ex vivo analyses. Our proposed studies will, therefore, be instrumental in answering the global importance of platelet endocytosis in HIV-1 viral infection. The Arf6 KO mice are of special importance in these assays because they are platelet-specific mutants and hence will be useful in isolating the platelet endocytosis contribution to the HIV-1 disease progression and outcomes.

Given that chronic HIV-1 infection increases risk of acute MI [592], it would be interesting to test the Eco-HIV infected VAMP-3 and Arf6 KO mice and their wildtype counterparts, in various models of arterial thrombosis (FeCl3-induced carotid artery injury model, laser-induced vessel wall injury model) and coronary artery ligation models to induce MI [604], to determine whether chronic viral infection leads to a prothrombotic state and whether modulating endocytosis alters the experimental outcomes.

**Targeting platelet endocytosis for therapeutic interventions**

Jones *et al.* 2016 [603] have shown that use of the antiplatelet drug, eptifibatide (marketed commercially as Integrilin) reduces platelet activation associated outcomes in Eco-HIV infection in mice. This would indicate that antiplatelet drugs ameliorate the disease outcomes in HIV-1 infections. However, if platelets are involved in the initial recognition of HIV-1 viruses in the bloodstream and help the other immune cells in mediating immune responses to the virus, inactivating platelet function might be imprudent. This becomes a challenge, especially in patients with a
chronic HIV-1 infection that show an increased propensity to acute MI or stroke, where administration of antiplatelet and antithrombotic drugs is necessary. Suffice to say, role in platelets in HIV-1 infection is complex and our current intervention strategies to mitigate aberrant platelet activation should be re-evaluated. While anti-retroviral therapies (HAART) have been successful in reducing markers of endothelial and platelet activation such as P-selectin and CD40L levels in HIV-infected patients [554], in some instances it has been shown to cause bleeding problems in patients, in part, due to aberrant dysfunction of platelets by some of the nucleosidase reverse transcriptase inhibitors (NRTIs) type anti-retroviral drugs [468]. For example, NRTI drugs, like Abacavir, which bear structural similarities with endogenous purine bases, induce platelet adhesion to endothelial cells by directly affecting the ATP-binding P2X7 purinergic receptors on endothelial cells. Abacavir activates endothelial cells and causes endothelial cell ICAM-1 to bind to surface-expressed αIIbβ3, GPIbα and also with P-selectin on platelets. This helps in recruiting more platelets, facilitating adhesion and aggregation [610]. These studies indicate that drug-induced platelet-endothelium dysfunctions may exacerbate cardiovascular complications in HIV-1 patients on ART. Platelets in these patients already have a heightened state of activation with a predisposition to thrombosis-related problems such as MI and stroke. Hence, care must be taken to determine drug side-effects in HIV-1 patients to prevent platelet-related cardiovascular complications. pH-tropic drugs like chloroquine have proven to be beneficial in ameliorating systemic T-cell immune activation [605]. When hydroxychloroquine was administered to 20 HIV-1 infected patients, it reduced immune activation, production of pro-inflammatory cytokines (IL-6 and TNF-α) and helped increase T-regulatory cell counts and CD4+ T cell counts [606]. Chloroquine is not thought to adversely affect platelet functions, except for inhibiting acidification of endosomal compartments and autophagosomes [240]. Thus, if platelet endocytosis of HIV-1 virus exacerbates the severity of infection, treating platelets with chloroquine provides a useful strategy to modulate immune responses targeted towards HIV-1, without affecting the hemostatic functions of platelets.
Finally, understanding how platelet endocytosis works will prove to be beneficial in uncovering strategies for novel therapeutics. Current antiplatelet and antithrombotic therapies dampen platelet function in general; it is unclear whether the use of these drugs is also dampening platelets’ role in other normal and essential aspects of platelet functions, namely immune-related functions. Both arms of platelet vesicle trafficking, endocytosis, and exocytosis, is important for platelet function. Exocytosis is more important for hemostasis and thrombosis while it appears that endocytosis is more crucial in mediating non-hemostatic functions of platelets, such as immune-related and cargo trafficking functions. However, designing therapeutics that specifically affect one arm of trafficking without affecting the other might prove to be beneficial in modulating cardiovascular diseases. Thus, understanding the mechanisms and routes of platelet endocytosis enables us with an alternative approach to load platelets with therapeutic cargos that can then modulate platelet responses paving the way to effectively treat cardiovascular diseases.

Final comments
While platelet endocytosis has been known to be important for granule cargo loading since the 1980s, other physiological functions have not been clearly defined. Work presented here shows for the first time, that platelets actively endocytose physiological cargo (Fg) and pathogens such as HIV-1 viruses, interpret and sort between different classes of cargos and can then mount appropriate responses. Whether other viruses or bacteria can utilize VAMP-3 and Arf6-mediated endocytosis to infect platelets remains to be seen. It has been known for decades that certain cargo molecules in platelet granules are internalized and packed in platelets through either receptor-mediated endocytosis (e.g. fibrinogen), or pinocytosis (e.g. immunoglobulin, albumin). Therefore, endocytosis is a key mechanism for modulating platelet granule content and thus may be important for other platelets functions. How important endocytosis is in mediating platelet activities in wound healing, tumor metastasis, and cancer, angiogenesis etc. also remains to be seen.

Endocytosis is a multi-step process that utilizes several routes of entry-transit-exit of cargo. Our data suggests that many of these paths are present and active in platelets.
Clearly, VAMP-3 and Arf6 mediate some, but not all of the key steps that facilitate α\textsubscript{IIb}β\textsubscript{3}-mediated Fg uptake, integrin trafficking, and the dynamic processes that affect contact-based signaling \textit{i.e.}, spreading and clot retraction. Endocytosis may also allow platelets to actively interact with their environment and interpret damage signals in the form of circulating pathogens and infectious agents. VAMP-3 and Arf6, both appear to be critical for mediating these responses. Platelets’ ability to endocytose and sort between different kinds of cargos provides them with the ability to differentially respond to them. In essence, platelets act as the “vacuum cleaners” of the vasculature, surveilling for damage (physiological and pathological) and respond in a graded manner, much like a rheostat. There is clearly a robust fluid-phase pinocytosis activity in platelets for taking in a host of small molecules. Future studies will uncover even more kinds of cargo molecules that platelets can internalize. Future studies will also uncover other potential endocytic regulators in platelets that modulate platelet functions.

Our studies provide unique mechanisms of how platelets utilize endocytosis as a tool to monitor their micro-environments. Platelet endocytosis for acute physiological functions, \textit{i.e.}, detecting pathogenic bacteria and viruses, damage molecules (DAMPs) champions novel functions of platelets that extend beyond cargo packaging and granule biogenesis and perhaps even hemostasis. Endocytosis may be a way for platelets to communicate not just with other cells in the circulation \textit{viz.} neutrophils, monocytes, macrophages, T and B lymphocytes but also with its parental megakaryocytes, to modulate thrombopoiesis and megakaryocyte functions. The VAMP-3 KO mice described here and the Arf6 described previously [19] offers us means to answer some of these questions and allows us to parse the complexity of platelet endocytosis and to define its physiological relevance.
# Appendices

## ABBREVIATIONS

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Full Form</th>
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<tbody>
<tr>
<td>ADP</td>
<td>Adenosine diphosphate</td>
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<tr>
<td>Arf6</td>
<td>ADP-ribosylation factor 6</td>
</tr>
<tr>
<td>ATP</td>
<td>Adenosine triphosphate</td>
</tr>
<tr>
<td>CLEC-2</td>
<td>C-type Lectin domain family member-2</td>
</tr>
<tr>
<td>DAB-2</td>
<td>Disabled-2</td>
</tr>
<tr>
<td>DAMP</td>
<td>Damage-associated molecular pattern</td>
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<tr>
<td>EC</td>
<td>Endothelial cells</td>
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<tr>
<td>ECM</td>
<td>Extracellular matrix</td>
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<tr>
<td>EE</td>
<td>Early endosome</td>
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<tr>
<td>ER</td>
<td>Endoplasmic reticulum</td>
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<tr>
<td>ERC</td>
<td>Endocytic recycling compartment</td>
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<tr>
<td>Fg</td>
<td>Fibrinogen</td>
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<tr>
<td>FAK</td>
<td>Focal adhesion kinase</td>
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<tr>
<td>GAP</td>
<td>GTPase-activating protein</td>
</tr>
<tr>
<td>GDP</td>
<td>Guanosine diphosphate</td>
</tr>
<tr>
<td>GEF</td>
<td>Guanine nucleotide exchange factor</td>
</tr>
<tr>
<td>GPCR</td>
<td>G protein-coupled receptor</td>
</tr>
<tr>
<td>GPI</td>
<td>Glycosylphosphatidylinositol</td>
</tr>
<tr>
<td>GPVI</td>
<td>Glycoprotein VI</td>
</tr>
<tr>
<td>GSK</td>
<td>G protein-coupled receptor kinase</td>
</tr>
<tr>
<td>GT</td>
<td>Glanzmann thrombasthenia</td>
</tr>
<tr>
<td>GTP</td>
<td>Guanosine triphosphate</td>
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<tr>
<td>HIV-1</td>
<td>Human Immunodeficiency Virus-1</td>
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<tr>
<td>ITIM</td>
<td>Immunoreceptor tyrosine-based inhibitory motif</td>
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<tr>
<td>Abbreviation</td>
<td>Full Form</td>
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<tr>
<td>ITAM</td>
<td>Immunoreceptor tyrosine-based activation motif</td>
</tr>
<tr>
<td>LPS</td>
<td>Lipopolysaccharide</td>
</tr>
<tr>
<td>LC3</td>
<td>Microtubule-associated protein 1A/1B-Light Chain 3 (LC3)</td>
</tr>
<tr>
<td>LC3II</td>
<td>LC3-Phosphatidylethanolamine conjugate (LC3II)</td>
</tr>
<tr>
<td>MAPK</td>
<td>Mitogen-activated protein kinase</td>
</tr>
<tr>
<td>MHC</td>
<td>Major Histocompatibility Complex</td>
</tr>
<tr>
<td>MVB</td>
<td>Multivesicular bodies</td>
</tr>
<tr>
<td>MI</td>
<td>Myocardial Infarction</td>
</tr>
<tr>
<td>NETs</td>
<td>Neutrophil extracellular DNA traps</td>
</tr>
<tr>
<td>NSF</td>
<td>N-ethylmaleimide-sensitive factor</td>
</tr>
<tr>
<td>OCS</td>
<td>Open Canalicular System</td>
</tr>
<tr>
<td>PAMP</td>
<td>Pathogen-associated molecular pattern</td>
</tr>
<tr>
<td>Pam3CSk4</td>
<td>Palmitoylated (tri)CysSerLys4</td>
</tr>
<tr>
<td>PDGF</td>
<td>Platelet-derived growth factor</td>
</tr>
<tr>
<td>PF4</td>
<td>Platelet factor 4</td>
</tr>
<tr>
<td>PGI₂</td>
<td>Prostacyclin I2</td>
</tr>
<tr>
<td>PI(4,5)P2</td>
<td>Phosphatidylinositol 4,5-bisphosphate</td>
</tr>
<tr>
<td>PI3K</td>
<td>Phosphoinositide 3-kinase</td>
</tr>
<tr>
<td>PIP5K</td>
<td>Phosphatidylinositol 4-phosphate 5 kinase</td>
</tr>
<tr>
<td>PKC</td>
<td>Protein Kinase C</td>
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<tr>
<td>PKD</td>
<td>Protein kinase D</td>
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<tr>
<td>PLC</td>
<td>Phospholipase C</td>
</tr>
<tr>
<td>PLD</td>
<td>Phospholipase D</td>
</tr>
<tr>
<td>PNRC</td>
<td>Perinuclear recycling compartment</td>
</tr>
<tr>
<td>PPP</td>
<td>Platelet poor plasma</td>
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<td>PRP</td>
<td>Platelet rich plasma</td>
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<td>Abbreviation</td>
<td>Full Form</td>
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<tr>
<td>RBC</td>
<td>Red blood cells</td>
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<tr>
<td>RGDS</td>
<td>Arginine-glycine-asparagine-serine acid</td>
</tr>
<tr>
<td>RANTES</td>
<td>Regulated on activation, normal T cell expressed and secreted</td>
</tr>
<tr>
<td>SFK</td>
<td>Src family kinase</td>
</tr>
<tr>
<td>SH2</td>
<td>Src homology 2</td>
</tr>
<tr>
<td>SNAP</td>
<td>Soluble NSF attachment proteins</td>
</tr>
<tr>
<td>SNARE</td>
<td>Soluble N-ethylmaleimide-sensitive fusion protein attachment receptor</td>
</tr>
<tr>
<td>SNPs</td>
<td>Single nucleotide polymorphism</td>
</tr>
<tr>
<td>TLR</td>
<td>Toll-like Receptor</td>
</tr>
<tr>
<td>TNF-α</td>
<td>Tumor Necrosis Factor-alpha</td>
</tr>
<tr>
<td>TfR</td>
<td>Transferrin receptor</td>
</tr>
<tr>
<td>TPOR</td>
<td>Thrombopoietin receptor</td>
</tr>
<tr>
<td>TXA2</td>
<td>Thromboxane A2</td>
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<tr>
<td>VAMP</td>
<td>Vesicle-associated membrane protein</td>
</tr>
<tr>
<td>VEGF</td>
<td>Vascular endothelial growth factor</td>
</tr>
<tr>
<td>vWF</td>
<td>von Willebrand Factor</td>
</tr>
<tr>
<td>WBC</td>
<td>White blood cells</td>
</tr>
<tr>
<td>3D-SIM</td>
<td>3-dimensional structured illumination microscopy regulatory complex</td>
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</tbody>
</table>
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Education

University of Kentucky
Ph.D. Student in Integrated Biomedical Sciences Program, College of Medicine 2010-2011
Ph.D. Student in Department of Molecular and Cellular Biochemistry 2011 - 2017
Defense on April 10\textsuperscript{th}, 2017
Dissertation: Platelet endocytosis: roles in hemostasis and innate immunity.

Lady Brabourne College
B.S. (Hons.) Microbiology
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2005 - 2008

University of Calcutta
M.S. (Hons.) Biophysics and Molecular Biology
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2008-2010

St. Joseph’s High School
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1991-2005

Research Experience

University of Calcutta
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Summer Research Fellow; Advisor: Dr. Javed Iqbal
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Project Title: Targeting the menaquinone biosynthetic pathway in \textit{M. tuberculosis} for discovering novel anti-tuberculosis drugs.

University of Kentucky (Lab Rotations)
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Graduate Research Assistant;
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Fall 1, 2010
Graduate Research Assistant;
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Teaching Experience
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Teaching Assistant in Biochemistry 401(G)
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Publications


Banerjee M and Whiteheart SW. The ins and outs of endocytic trafficking in platelet functions. (In press, Current Opinion in Hematology)


Awards and Honors

**Young Investigator Award**, at the 16th Midwest Platelet Conference, Memphis, TN, 2016.

1st in Graduate Student Poster Presentation, Dept. of Biochemistry Retreat, Lexington, KY, 2016.

**Max Steckler Fellowship** from University of Kentucky Graduate School, 2014.

2nd in Graduate Student Poster Presentation, Dept. of Biochemistry Retreat, Lexington, KY, 2013.

**Graduate Student Travel Award** from the American Society of Molecular Biology and Biochemistry for Experimental Biology Meeting, 2012.

**Science Academies’ Summer Research Fellowship Award** from the Indian Academy of Sciences-Indian National Science Academy-The National Academy of Sciences (IAS-INSA-NASI), India, 2009.

Conference Presentations

Banerjee M, Huang Y, Popa GJ, Mendenhall MD, Melikyan GB, Whiteheart SW. *Endocytosis of HIV-1 triggers platelet activation*. **Selected for student talk** at the 16th Biennial Midwest platelet conference in Memphis, TN, October 2016.; Poster presentation delivered at 19th Annual Gill Heart Institute Cardiovascular Research Day in Lexington, KY, November 2016; Poster presentation delivered at Departmental Retreat in Lexington, KY, May 2016. Adjudged **Best Poster**.


Banerjee M, Whiteheart SW. *Toll-like receptor signaling in mediating platelet activation*. Poster presentation delivered at Departmental Retreat in General Butler State Resort, KY, August 2013; **Adjudged 2nd in graduate student poster competition**. Poster presentation at the 16th Annual Gill Heart Institute Cardiovascular Research Day in Lexington, KY, October 2013.

presentation delivered at Departmental Retreat in General Butler State Resort, KY, August 2012; Poster presentation at the 15th Annual Gill Heart Institute Cardiovascular Research Day in Lexington, KY, October 2012.