INTRODUCTION

Platelet accumulation at a site of vascular injury is a dynamic process that integrates chemical and physical cues to promote cellular responses that are coordinated in time and space. Extensive studies of platelet intracellular signaling pathways over the past several decades have greatly enhanced our understanding of platelet function. In general, these signaling pathways start with an extracellular input acting on a platelet surface receptor. Inputs may either promote (e.g., thrombin) or dampen (e.g., prostacyclin) platelet activation, depending on the pathways involved and physiological context. Receptor activation leads to intracellular signaling, which in the case of platelet activation culminates in the release of intracellular Ca\(^{2+}\) stores, α\(_{Ibb3}\) integrin activation, platelet aggregation and granule exocytosis. The signaling pathways that are required have been reviewed extensively [1–4].

As many of the major players in platelet signaling have been identified, we and others have begun to try to understand how multiple signaling inputs are coordinated in time and space to achieve an optimal hemostatic response. An optimal response is defined as one sufficient to stop bleeding and promote wound healing, but not so robust that unwarranted vascular occlusion and ischemia occurs. In addition to traditional platelet signaling studies that are typically conducted in vitro, recent efforts have taken a systems approach, combining in-vitro, computational and, most importantly, in-vivo approaches that rely on animal models. Indeed, it is only by examining platelet function in the complex milieu of an intact vasculature that we can begin to understand how the platelet signaling network is integrated along with contributions from other cells, the vessel wall and local hemodynamic conditions in order to generate a hemostatic plug. This review summarizes some of those efforts and considers a model that arises from them. Observations of platelet response heterogeneity and the
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**KEY POINTS**

- Platelet activation at sites of vascular injury is heterogeneous.
- Heterogeneity of platelet activation arises from spatiotemporal regulation of agonist distribution within the evolving platelet mass.
- The physical characteristics of a platelet mass contribute to the development of agonist gradients.
- A systems approach is required to gain a better understanding of how multiple platelet signaling pathways are integrated to produce an optimal hemostatic.

underlying mechanisms responsible for such heterogeneity will be discussed.

**HETEROGENEITY OF PLATELET ACTIVATION DURING THE HEMOSTATIC RESPONSE**

It has long been recognized that platelet activation within a hemostatic plug or thrombus is not necessarily uniform. Early evidence demonstrating this point comes from transmission electron microscopy studies of experimental or human pathological thrombi showing platelets with varying degrees of shape change and granule release [5–7]. As these studies were static in nature, it was impossible to determine whether platelets that appeared less activated represented a distinct subpopulation, or rather were merely newly arrived platelets that had simply not yet been fully activated. In recent years, the utilization of fluorescence intravital imaging, pioneered by the Furie laboratory [8–10] and others [11–13], has permitted the visualization of platelet accumulation and activation at sites of vascular injury in vivo in real time. Studies using this approach in animal models, as well as flow chamber studies using human blood, have demonstrated that both the platelet and coagulation response to vascular injury are indeed heterogeneous in time and space. With regard to platelet activation in vivo, studies have visualized heterogeneity in shape change [14–16], calcium signaling [13,17,18], granule exocytosis [19*,20–22] and phosphatidyserine exposure [23,24], at the level of individual platelets and platelet subpopulations. Further, thrombin activity [25*] and fibrin deposition [8,19*,20,26] are not uniform throughout a platelet plug. The following is a description of some of the evidence demonstrating heterogeneity of platelet activation following vascular injury.

**Discoid platelet aggregation**

Platelet activation studies performed in vitro have long recognized that one of the initial platelet responses upon activation is shape change from a round or discoid morphology to one with extended filopods. Thus, it was somewhat surprising when investigators reported the accumulation of discoid platelets during the hemostatic response in vivo [14–16], as formation of platelet aggregates was assumed to require platelet activation. The formation of discoid platelet aggregates has been reported to depend on rheological factors (discussed more below), with some degree of platelet activation required as this aggregation was blocked by treating platelets with prostaglandin E1 or the prostacyclin2 analog iloprost [15,27**].

**Heterogeneity of intracellular Ca2+ mobilization**

Multiple studies have examined intracellular Ca2+ mobilization at the level of individual platelets using both in-vitro flow chamber systems and in-vivo models. In-vitro systems are best suited to tease out the Ca2+ response of individual platelets to distinct stimuli. For example, von Willebrand factor (vWF) engagement by glycoprotein (GP) Ibα on the platelet surface results in a transient rise in intracellular Ca2+ [28,29], ADP signaling results in oscillatory Ca2+ signals [28,30–33] and collagen/GPVI signaling results in a sustained rise in intracellular Ca2+ [34]. The in-vivo setting is more complex as multiple inputs are integrated. However, studies have observed heterogeneous Ca2+ signals in individual platelets within a platelet plug. For example, van Gestel et al. [13] found that platelets with a transient rise in Ca2+ are more likely to embolize, whereas stably adherent platelets exhibit a sustained elevation of intracellular Ca2+.

**Granule exocytosis**

One of the most commonly used markers of platelet activation in intravital imaging studies is P-selectin, a marker of α-granule secretion. In response to focal injuries in the microcirculation, expression of P-selectin on the platelet surface lags behind platelet accumulation temporally, propagates initially from the site of injury and remains restricted to a subpopulation of platelets adjacent to the site of injury that is overlaid by a shell of P-selectin-negative platelets [19*,20,21]. These observations (and others) led to the description of a hemostatic plug as having a heterogeneous architecture composed of a stable core of fully activated, densely packed platelets with an outer shell of less-activated, loosely
associated platelets (Fig. 1a). The spatiotemporal localization of dense granule secretion during the hemostatic response in vivo is less well understood because of the lack of imaging reagents indicative of this event. However, the regulation of dense granule release may be inferred from studies of ADP signaling (discussed below), as dense granules represent the primary storage pool of ADP released during the hemostatic response.

Phosphatidylserine exposure

Phosphatidylserine exposure on the outer platelet membrane surface has long been recognized as a heterogeneous response at the level of individual platelets, as only a subpopulation of platelets will bind the phosphatidylserine marker annexin V in response to physiologic agonists in vitro [35,36]. Phosphatidylserine exposure is thought to be a critical event during hemostasis and thrombosis.

**FIGURE 1.** A model for the interaction of local conditions with the platelet signaling network resulting in heterogeneous platelet plug architecture. (a) A description of the heterogeneous architecture of the platelet plug observed in intravital imaging studies following focal injuries in the microcirculation. The platelet mass is composed of at least two distinct regions, termed the core and outer shell, identified based on the secretion of the α-granule marker P-selectin. Additional properties of these regions are also indicated. Recent observations suggest that the endothelium is an important source of procoagulant membranes (yellow). (b) Data suggest that the development of the heterogeneous architecture described in (a) is the result of partially overlapping agonist gradients within the platelet mass emanating from the site of injury TxA2, thromboxane A2. This figure was adapted from Blood. Stalker TJ, Traxler EA, Wu J et al.; Hierarchical organization in the hemostatic response and its relationship to the platelet-signaling network. Blood 2013; 121(10): 1875–1885. © American Society of Hematology.
to facilitate the assembly coagulation factor complexes on phosphatidylserine(+) procoagulant membranes for subsequent thrombin generation. A number of studies have demonstrated unique localization of phosphatidylserine(+) platelets at the periphery of platelet aggregates formed under flow in vitro [24]. The spatiotemporal regulation of platelet phosphatidylserine exposure in vivo is less well understood and perhaps context dependent, although studies have shown annexin V-positive platelets at the base of platelet plugs in the region of the thrombus core [23,24,37]. A recent study by Ivanciu et al. [38**] used fluorescently-labeled factors Va and Xa rather than annexin V in order to specifically determine sites of procoagulant complex assembly. They observed factors Va and Xa binding to membranes localized at the base of the thrombus near the site of injury as well as on surrounding endothelial cell surfaces in a microcirculation focal injury model [38**].

SPATIOTEMPORAL REGULATION OF PLATELET SIGNALING

The recognition that platelet activation is not uniform during the hemostatic response leads to the question of how such a heterogeneous architecture develops and whether there are implications for both the use of existing antiplatelet therapies and the development of new ones. One possible explanation is that platelets with different degrees of activation represent subpopulations of circulating platelets with distinct properties. Although intriguing, there is at present little experimental evidence to support this hypothesis. Instead, the available evidence suggests that heterogeneity of platelet activation reflects non-uniformity in agonist distribution (Fig. 1b). Here, we will discuss what is known about each of the major platelet agonist signaling pathways with regard to their contribution to the spatiotemporal regulation of platelet activation.

Thrombin

Thrombin is a key regulator of robust platelet activation in response to vascular injury. In mouse models, inhibition of thrombin generation or activity or genetic deletion of the platelet thrombin receptor (protease activated receptor-4 on mouse platelets) results in significantly impaired platelet accumulation and a near complete lack of intracellular calcium mobilization [17,18] and P-selectin expression [19*,20] as markers of platelet activation. Thus, thrombin activity is critical for the development of a stable core composed of fully activated platelets. However, the localization of thrombin activity in time and space is limited. As described above, thrombin generation is localized to procoagulant membranes of platelets and endothelial cells at or immediately adjacent to the site of injury, thus limiting the distribution of thrombin within a platelet mass [23,24,38**]. Once generated, thrombin distribution is limited by its ability to diffuse away from the site of generation (discussed in more detail below) and by plasma-borne inhibitors that either directly inhibit its activity (e.g., antithrombin) or generation (e.g., tissue factor pathway inhibitor and activated protein C). This combination of factors limits thrombin activity to the core region of the hemostatic plug as demonstrated by studies using a fluorogenic thrombin sensor bound to the surface of platelets [25*] and studies showing the localization of fibrin formation restricted to the core [8,19*,20].

Adenosine diphosphate

ADP is released from damaged cells at the site of injury as well as from dense granules of activated platelets. It acts on two platelet receptors, P2Y1 and P2Y12, to reinforce platelet activation in a paracrine or autocrine fashion [39–44]. The importance of P2Y12 in platelet activation is highlighted by the efficacy of P2Y12 antagonists in inhibiting platelet activation and protecting against thrombotic events in humans. This role for P2Y12 signaling is recapitulated in animal models, in which deletion of P2Y12 [40,45] and the introduction of P2Y12 receptor antagonists have been shown to attenuate thrombus formation [46]. In general, these studies have ascribed a role for P2Y12 in regulating thrombus stability [45,47,48]. Viewed from the perspective of spatiotemporal regulation of platelet activation, this effect of P2Y12 signaling on thrombus stability is due to the importance of this signaling pathway in platelet recruitment and retention in the outer layers of a developing platelet plug, a region where thrombin activity rapidly declines. Indeed, we have shown that inhibition of P2Y12 activation greatly reduces platelet accumulation in the outer platelet shell, whereas a gain of function mutation in Gi2α, the principal G protein coupled to P2Y12 receptors, leads to an expansion of the shell [19*]. In contrast, a P2Y12 antagonist had no effect on robust platelet activation in the thrombus core, in which thrombin activity is high [19*]. This latter finding may help to explain the relative safety of P2Y12 antagonists used clinically.

Thromboxane A2

Like ADP, thromboxane A2 (TxA2) generated and released by activated platelets acts to reinforce
platelet activation in an autocrine and paracrine fashion. TxA2 is generated via the aspirin-sensitive cyclooxygenase-1 pathway in platelets. Upon release, it binds its receptors (TxA2 receptors α and β) on the platelet surface to provide an activating signal. Its importance in platelet activation in vivo is demonstrated by a number of large clinical studies demonstrating the efficacy of aspirin treatment in the prevention of platelet-mediated cardiovascular events (i.e., myocardial infarction and stroke) [49]. Thrombus formation is also attenuated in TxA2 receptor-deficient mice [50]. The spatiotemporal distribution of TxA2 within a growing hemostatic plug is not yet well defined. As a highly diffusible molecule, its localization will primarily be determined by its source (activated platelets) and its rapid metabolism in plasma to inactive metabolites. However, additional studies are required to ascertain the precise contribution of TxA2 signaling to heterogeneous platelet activation.

Collagen
In contrast to soluble platelet agonists, collagen is an insoluble component of the vessel wall and extravascular tissue. As such, its direct contribution to platelet signaling via activation of the collagen receptor GPVI is restricted to platelets in contact with the damaged vessel wall and those that escape into the extravascular compartment. The contribution of GPVI signaling in experimental models is, therefore, highly dependent on the mechanism and extent of injury, as well as the amount of thrombin generated [51]. This likely explains varying reports of GPVI being a critical regulator of platelet accumulation and activation in some settings, yet completely dispensable in others [51–54]. Thus, although collagen is clearly a potent activator of platelets in vitro, its contribution to hemostasis and thrombosis in vivo is likely much more context-dependent than other platelet agonists, such as thrombin, ADP and TxA2.

Contact-dependent signaling
In addition to the contribution of traditional platelet agonists, recent studies have elucidated a role for multiple contact-dependent signaling pathways in regulating platelet activation. These include outside-in signaling via the major platelet integrin αIIbβ3, surface receptor/ligand pairs, such as semaphorin 4D, ephrin/eph kinases and Gas6/Tyro-3/Axl/Mer receptors, as well as junctional adhesion molecules, such as platelet and endothelial cell adhesion molecule-1 and endothelial cell-specific adhesion molecule. It would be predicted that signaling pathways dependent on close contact between platelets would exert their effects primarily in the thrombus core, as this is where platelet packing density is the highest, as compared with the loosely associated platelets within the less stable platelet shell. Of the pathways studied to date, this appears to be true, although not necessarily via the same mechanism in each case. For example, genetic deletion of semaphorin 4D significantly impairs full platelet activation in the core region of a platelet plug [19*], presumably due to its reinforcement of platelet activation via Syk tyrosine kinase activation [55]. Tyr phosphorylation of β3 integrin cytoplasmic tails following αIIbβ3 ligand binding instead regulates platelet retractile mechanisms [56]. Disruption of this signaling pathway in mouse platelets by mutating β3 integrin Tyr to Phe results in impaired platelet mass contraction, leading to diminished local thrombin activity and platelet activation in the core via effects on the molecular transport properties of the uncontracted platelet plug [57*].

INFLUENCE OF LOCAL HEMODYNAMICS
Several investigators have demonstrated the importance of local hemodynamic conditions on platelet accumulation at sites of injury in vivo and in flow chambers ex vivo. In particular, changes in flow patterns around an obstruction or in a stenotic region, such as when blood flows over a developing platelet mass or through a stenotic atherosclerotic artery, lead to the development of shear deceleration zones [14,15,27**]. Platelet aggregation in these zones on the downstream side of a stenosis is dependent on vWF/GPIb interactions as well as αIIbβ3 integrin [14,15,27**]. This ‘shear-dependent’ aggregation is also dependent on platelet activation, as it is blocked by inhibitors of TxA2 generation and ADP signaling [15,27**]. These studies highlight the importance of the interaction between platelet aggregates and the bulk flow in shaping the response to vascular injury. As platelets accumulate within the lumen of a vessel, they alter the flow characteristics creating local microenvironments in which conditions are favorable for aggregation. Such a phenomenon could be particularly important at sites of stenosis because of atherosclerotic plaque formation in which platelet accumulation may be exacerbated. Importantly, even in the case of severely stenosed blood vessels, platelet accumulation is not spontaneous but rather still requires an initial injury to the vessel wall [14]. This differs from the vWF-dependent, activation independent platelet aggregation observed at extreme shear rates (>20 000 s−1) in vitro [58].
THE INTRATHROMBUS MICROENVIRONMENT SHAPES AGONIST DISTRIBUTION

As described above, agonist distribution within a platelet mass is a critical factor responsible for the observed differences in the extent of platelet activation (Fig. 1b). The distribution of each agonist depends on the location of its source, its ability to move and its stability over time. For example, collagen/GPVI signaling is limited to platelets in contact with the site of injury where collagen is exposed. In contrast, the distribution of soluble agonists is highly dependent on the physical architecture of the platelet mass as it evolves. Specifically, the movement of molecular species within and/or around a pile of platelets is dependent on the size of the pores (i.e., plasma volume) between platelets, the spatial distribution, connectivity and tortuosity of the pore space, the plasma velocity and the size of the solute. Several of these parameters have now been estimated using computational approaches based on experimental data, or have been directly measured either in vitro or in vivo wherever possible. For example, a mature platelet mass formed under flow conditions in vitro was found to have a permeability approaching that of an intact endothelial flow conditions. For example, a mature platelet mass formed under flow conditions in vitro was found to have a permeability approaching that of an intact endothelial

Why is it important to consider solute transport as a regulator of platelet activation? Transport has long been recognized as important for platelet mass formation as a mechanism for delivery of coagulation factors to the injury site, as well as elution of platelet agonists from within the platelet mass [66–68]. Coagulation is affected by solute transport as limited delivery of coagulation factors restrains thrombin generation, whereas limited transport of thrombin out of the platelet mass increases its effective concentration. Microfluidic assays have demonstrated that platelets create a physical barrier that covers up tissue factor surfaces and excludes plasma-borne coagulation factors, altering the rate of fibrin deposition [68–70]. Increasing platelet packing density can lead to the retention of larger, less mobile solutes (e.g., thrombin), whereas small solutes (e.g., ADP) can more easily escape to the edges of the platelet mass [57*,63,65*]. These effects tend to concentrate protein solutes within the core region of the hemostatic mass. Concentration of thrombin within the thrombus core helps to drive further platelet activation and greater fibrin accumulation [25*,57*,63,65*]. Interestingly, within the platelet mass formed in mice deficient in platelet retraction, solute transport within the core region is elevated resulting in reduced thrombin activity and platelet activation [57*]. These findings suggest that the reduced transport in the core is important for thrombin retention, as opposed to prevention of continued thrombin production.

CONCLUSION

In summary, consideration of platelet signaling pathways as an integrated network that responds to differences in agonist distribution, and the growing hemostatic mass as an obstacle to molecular transport, provides new insights into how an optimal hemostatic response is achieved. It also helps to better understand the safety and efficacy profiles of antiplatelet agents. Considerable work remains to be done, however. Much of what is currently known about platelet signaling mechanisms has been determined ex vivo with a focus on individual pathways, rather than with a view to understanding how different pathways are invoked and interact in time and space in vivo. Understanding the spatial and temporal relationships between the platelet signaling network and the altered transport environment within a growing hemostatic mass or thrombus is the realm of systems biology. We propose that the overall themes discussed in this review will be universally relevant, even if some of the details turn out to differ in different areas of the circulation or when pathologic thrombosis
is compared with the hemostatic response. Much remains to be learned as new tools are brought to bear.

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Conflicts of interest
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REFERENCES AND RECOMMENDED READING

Papers of particular interest, published within the annual period of review, have been highlighted as:
* of special interest
** of outstanding interest

2. Von Gestel MA, Reitsma S, Slaaf DW, et al. ADP and thrombin regulate arterial thrombus stabilization and embolization, but are not involved in initial hemostasis as induced by micropuncture. Microcirculation 2007; 14:193–205.
4. This study demonstrated the role of platelet agonist gradients in the spatio-temporal regulation of platelet activation following vascular injury in vivo.
11. The authors (T.J.S. and L.F.B.) have received research funding from The Medicines Company ( Parsippany, NJ, USA).
This study demonstrates the role of platelet retraction in regulating intrathrombus solute transport and localization of thrombin activity.
This study used a combination of in-vivo and computational approaches to examine the impact of a fibrin network on protein transport within a thrombus.
This computational study showed that hindered transport of platelet solutes within the intrathrombus microenvironment profoundly affects coagulation factor distribution, which may ultimately limit thrombus size.
This article characterizes for the first time intrathrombus solute transport in vivo and demonstrates how the distribution of soluble agonists is regulated by the internal architecture of a platelet mass.