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Our Expanding View of Platelet Functions and Its Clinical Implications

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Abstract Platelets are the primary cell mediator of thrombosis. A deficiency of platelets can result in severe bleeding defects. "Overactive" platelets contribute to life-threatening outcomes in diseases such as heart attack, stroke, and cancer. The use of platelet inhibitors for thrombosis prevention must therefore seek a delicate balance between inhibiting platelet activation and an associated increased bleeding risk. There are currently few platelet inhibitors clinically available, making the search for novel anti-platelet drug targets a major research priority. Several newly identified pathways of platelet activation may hold hope in this area. In addition, important roles for platelets beyond hemostasis have been discovered. Platelets have recently been described as mediators of diverse inflammatory diseases such as arthritis, hepatitis, malaria, and atherosclerosis. This has heightened the need to broaden our understanding of platelet functions and platelet-derived inflammatory mediators. It has also heightened interest in a continued search for new platelet inhibitors and presents new opportunities for platelet inhibitors to be used in a wide array of disease treatment strategies.

Keywords Platelet · Inflammation · Thrombosis · Aggregation · Activation · Therapy

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Introduction

Platelets are small (~1–2 μm), anucleate, megakaryocyte-derived circulating blood cells. Typical human platelet counts are 100,000–200,000/μL, making platelets the second most numerous blood cells. The most well understood function of platelets is its role as the primary cell mediator of thrombosis. A lack of platelets (thrombocytopenia), or defects in platelet function, can result in major life-threatening bleeding. Untoward or unregulated platelet activation can also have severe outcomes by initiating thrombosis and loss of blood supply to major tissue beds. Cardiovascular disease and stroke are leading causes of morbidity and mortality in the Western world, and although many processes play a role in the development of vascular disease, thrombosis is the primary event that precipitates stroke and acute coronary syndromes. There are currently few platelet-specific anti-thrombotic agents available, making direct platelet inhibitors, such as aspirin and clopidogrel (Plavix), mainstays in the long-term treatment of cardiovascular patients.

Thrombosis is the result of a complex set of interactions between platelets, coagulation factors, and the vessel wall. Thrombosis proceeds through what is often described as a stereotypic set of steps: Platelets are first activated, adhere to the vessel wall, and then aggregate with other platelets to form a stable thrombus [1, 2]. There are also many intermediate steps important in efficient thrombus formation, such as loose contacts between platelets before firm adhesion. This creates micro-environments with high local concentrations of pro-thrombotic mediators [3]. Platelets can be activated by vessel wall exposure of extracellular matrix components, by activation of the coagulation cascade generating platelet agonists (e.g., thrombin), or by factors released from activated endothelial cells and

platelets (e.g., ADP, thromboxane A2, and vWF) [4–8]. Activation of receptors on resting platelets triggers a variety of intra-platelet signaling pathways, leading to subsequent steps of platelet activation, including conformational changes in receptors (e.g., GPIIb/IIIa), granule exocytosis, and the secretion of vasoactive mediators.

Platelets have an obvious vital function in hemostasis, but they also have an underappreciated immune function. This has been most studied in the context of platelet interactions with innate immune cells where platelets are known to recruit and activate neutrophils and monocytes by contact-dependent and contact-independent mechanisms [9–13]. Platelets also can influence T-cell and B-cell functions, but this is less well explored and is mainly via platelet-derived cytokines and chemokines [14–16]. Many platelet granule constituents and secreted molecules have primary roles as immune mediators (Table 1) [9, 17–19]. These platelet-derived immune mediators recruit and activate leukocytes both at the site of platelet deposition and systemically. Platelets are not only activated by a denuded endothelium, but platelets are also activated by an intact inflamed endothelium and deposit at the site of vascular inflammation without forming a complete obstructive thrombus. We have demonstrated using two separate vascular inflammation based mouse models (a transplant model and a cerebral malaria model) that thrombi are present, lining the lumen of inflamed blood vessels, without occluding the vessels (Fig. 1). Interactions between an inflamed endothelium and platelets may be mediated by increased endothelial adhesion molecule expression (e.g., P-selectin, ICAM, and VCAM) serving to localize platelets and induce platelet secretion. These events perpetuate the cycle of inflammatory interactions between platelets, endothelial cells, and leukocytes.

Activated platelets can also initiate interactions with quiescent endothelial cells and leukocytes through both contact-dependent and contact-independent mechanisms. Platelet inflammatory mediators include surface expressed

Table 1 Short list of prominent platelet inflammatory molecules

Category	Molecule	Function	Location
Adhesion	P-selectin (CD62)	Rolling, WBC attachment	Alpha granule
	vWf	Platelet and WBC adhesion	Alpha granule
Chemokines	MIP-1	Granulocyte recruitment and activation	Alpha granule
	PF4	WBC recruitment and activation	Alpha granule
	RANTES	WBC recruitment and activation	Alpha granule
	MCP	WBC recruitment and activation	Alpha granule
	PPBP/β-TG/NAP-2	Granulocyte recruitment and activation	Alpha granule
	SDF-1	Recruitment of WBC and stem cells	Alpha granule
Cytokines	IL-1α and β	Pro-inflammatory effects on WBC, endothelial cells	Alpha granule
	TGF-β	T-cell development, vascular remodeling	Alpha granule
	TNF-α	Pro-inflammatory effects on WBC, endothelial cells	Alpha granule
Growth Factors	PDGF	Angiogenesis, immune activation	Alpha granule
	VEGF	Angiogenesis, endothelial inflammation and cytokine induction	Alpha granule
Other	GAS6	Promotes WBC trafficking	Alpha granule
	Complement	Complement cascade	Alpha granule
	Glutamate	Cyclooxygenase activation	Dense granule
	ADP/ATP	Effects on T-cells and endothelial cells	Dense granule
	Serotonin	Effects on T-cells and VSMC	Dense granule
	Epinephrine	Augment WBC responses	Dense granule
Integrins	GPIbα	Binds Mac-1 (CD11b/CD18)	Membrane
	GPIIb/GPIIIa	Binds fibrinogen	Membrane
	ICAM-2	Binds WBC LFA-1	Membrane
	JAM-1 and JAM-3	Bind integrins such as LFA-1 and Mac-1	Membrane
Scavenger	CD36	Receptor for Ox-LDL and malaria iRBC surface antigen	Membrane
	ApoER2 (LRP8)	Beta2-GPI binding	Membrane
			Membrane
Induced enzymes	Cyclooxygenase	Prostaglandins and thromboxane	Cytoplasm

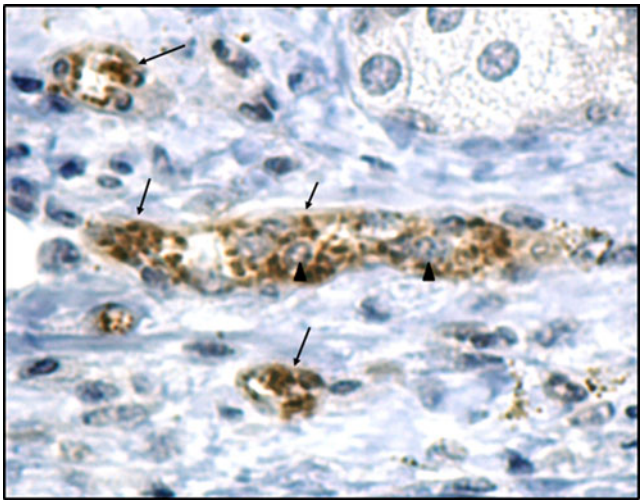

Fig. 1 Platelet-rich thrombi stained with anti-vWF antibody in mouse kidney with ischemia reperfusion injury (*left side*) and skin grafts (*right side*). *Arrows* point out thrombi; *arrowheads*, leukocytes within the thrombi

adhesion molecules (P-selectin and integrins), secreted small molecules (ATP/ADP, serotonin, and glutamate), as well as chemokines, and cytokines (Table 1). Platelet-derived chemokines and cytokines, including platelet factor 4 (PF4/CXCL4), pro-platelet basic protein (ppbp and its breakdown product CXCL7/NAP-2), RANTES/CCL5, IL-1α/β, and TGF-β can recruit and activate leukocytes distant from their site of deposition [20]. Other secreted mediators such as serotonin, ADP, and prostaglandins tend to exert pro-inflammatory effects in the local environment. Exteriorized or activated adhesion molecules on the surface of adherent platelets also assist in arresting leukocytes, providing a contact-dependent mechanism for platelets to exert pro-inflammatory effects.

A complete appreciation for both the thrombotic and pro-inflammatory functions of platelets, and the concept that platelets initiate, participate in, and sustain inflammation beyond the clot, has been slow to develop. This may be due in part to a misunderstood idea that because platelets are anucleate, they cannot adapt to "environmental changes." Because of their easily defined role in hemostasis, a long-standing focus of platelet researchers has been on platelet activation signaling pathways and integrin regulation. Recently, the focus of platelet research has broadened to include new signaling mediators and previously unrecognized roles for platelets in vascular inflammatory diseases. We will highlight a few recent discoveries with the potential to lead to the development of new anti-thrombotics and impact the current use of platelet inhibitors. We will also highlight the current state of clinical anti-thrombotic therapy.

Our Changing View of Platelet Functions

Platelets were once viewed as a unique set of cells with their own distinct functions and signaling pathways. We now know that platelets share much in common with other seemingly dissimilar cell types, such as neurons, and share many immune cell functions, presenting new pathways to target for anti-thrombotic drug development. Platelets and the synaptic termini of neurons are surprisingly similar in their composition. On the surface, these are very different cells with very different functions. However, platelets and neurons share many similarities in receptor composition and molecular constituents. Each cell expresses proteins found in few other cell types such as synucleins, ADP/ATP receptors, TPO receptors, serotonin receptors, and glutamate receptors [16, 21–25]. Most platelet dense granule constituents are also neurotransmitters (glutamate, ADP, ATP, serontonin, dopamine, Ca^{2+}, etc.). This similarity may arise from both platelets and synaptic termini having unique functional demands due to their great distance from the "nuclear center": the megakaryocyte in the bone marrow and the neuron cell body. Our lab has found that platelets, like neurons, express functional ionotropic glutamate receptors [25, 26]. Glutamate is a major neurotransmitter that binds to NMDA, AMPA, and kainate (KA) receptors. Glutamate induces a Ca^{2+} influx through NMDA receptors and a Na^{+} influx when glutamate binds AMPAR and KAR. Others have described that platelet-dense granules store and release glutamate, but a role for glutamate in platelet activation had not been described [27, 28]. We found that glutamate is released in large concentrations within a developing thrombus and that AMPAR and KAR signaling amplifies platelet activation. Mice that lack AMPAR and KAR subunits or mice that are treated with receptor inhibitors have prolonged time to thrombus formation in vivo. Importantly, these mice do not have any bleeding diathesis but form a delayed thrombus. This makes glutamate receptors an attractive target for the development of new anti-thrombotics. An important quality for a platelet inhibitor is an ability to blunt, but not totally block, platelet activation so as to not pose a large bleeding risk. We have found that AMPAR and KAR antagonists each prolong bleeding times and delay thrombus formation in mice and also reduce human platelet aggregation [25, 26]. Many glutamate receptor antagonists have gone through clinical trials for treatment of stroke, but none to date has proven particularly effective. It remains to be seen through clinical trial if any of these existing glutamate receptor antagonists with established safety and toxicity data can find a new use as platelet inhibitors.

Another major functional adaptation in common between platelets and neurons is that there are only two known places where pre-mRNA splicing occurs outside the nucleus: in

synaptic termini and in platelets [29]. Platelets have stable pre-mRNA, and upon platelet stimulation, this pre-mRNA is spliced into mature mRNA that can be translated into protein. The importance of this discovery goes beyond just the demonstration of RNA splicing outside the nucleus; it provided a clear demonstration that despite the lack of a nucleus platelets have the ability to directly respond to environmental changes by altering their protein composition. Before this discovery, it was known that platelets had RNA, but its significance was not appreciated, and it was largely viewed to be a by-stander effect of platelet budding from the megakaryocyte. With this paper, the group headed by Andrew Weyrich helped to greatly advance a broader understanding that despite their anucleate status, platelets have a clear ability to up- or down-regulate protein expression. The relevance of this finding to health and disease remains to be demonstrated; but nonetheless, it represented a major advance in focusing platelet research in new directions. Recent publications, such as one demonstrating active platelet microRNA, have continued to build on this concept of peripheral platelet translational regulation [30].

The use of mouse models has demonstrated that in addition to forming a coronary artery occlusion at the time of myocardial infarction (MI), platelets may have a prominent role in promoting the pathogenesis of atherosclerosis. Ley and colleagues demonstrated in an $ApoE^{-/-}$ mouse model that platelets deposit RANTES at the site of atherosclerotic lesions, one of the first clear mechanistic descriptions of how platelets may accelerate atherosclerosis [31, 32]. This work and that by others in the mouse model have shown that platelets may be important early in atherosclerotic lesion development and its progression towards an unstable plaque. One of the major issues with mouse models of atherosclerosis is that mice do not form unstable plaques, making an appreciation for the role of platelets in MI risk in humans difficult to extrapolate. However, there have been several human clinical studies indicating that platelets may drive lesion progression and platelets may be activated at sites of a growing atherosclerotic lesion [9, 33], helping to validate this mouse model-based research.

Atherosclerosis is now seen as an immune-mediated disease, with high cholesterol and oxidized lipid products well established risk factors at the heart of its pathogenesis. Recent work by Podrez et al. has found that not only may platelets be activated at the site of atherosclerotic lesion development but also oxidized lipids may directly activate platelets via platelet CD36 [34]. This study puts platelets in the center of atherothrombosis by indicating that oxidized lipid mediated platelet activation may contribute immune mediators that promote lesion development locally and at sites distant from the actual lesion. Subsequent work by authors from this study has shown that the CD36-dependent platelet activation pathway functions through mitogen activated protein kinase (MAPK) signaling [35]. This knowledge helps to identify two new potential targets for anti-platelet drug development in the prevention and treatment of atherosclerosis: CD36 and MAPK members. However, both CD36 and MAP kinases are expressed by many cells, making the development of platelet-specific antagonists targeting these molecules potentially difficult.

Platelet factor 4 (CXCL4) was the first CXC class chemokine identified and is the most abundant protein found in platelet α-granules. PF4 is prominently known for its role in heparin-induced thrombocytopenia; but through the use of knockout mice, its pro-inflammatory role is becoming better understood. $PF4^{-/-}$ mice on an $ApoE^{-/-}$ background have reduced atherosclerotic lesion development compared with control $ApoE^{-/-}$ mice [36]. This study indicates an important role for this platelet-specific chemokine in the pathogenesis of atherosclerosis. Other studies have taken this knowledge into a potentially clinically relevant therapeutic development direction. PF4 forms a complex with RANTES and together create a highly pro-inflammatory environment. Through the use of peptides that disrupt PF4–RANTES associations, investigators were also able to reduce lesion development in $ApoE^{-/-}$ mice [37].

With a more broad appreciation for platelet functions has come a more broad appreciation of the disease processes in which platelets may have a pathogenic role. Platelets may have a central role in the development of cerebral malaria. A large percentage of children infected with *Plasmodium falciparum* develop stroke-like symptoms resulting in long-term neurologic complications or death. We have found that platelet depletion of mice after infection with *Plasmodium berghei* ANKA leads to increased survival compared with control infected mice, and that this is in part mediated by PF4 [15]. Compared with WT mice, $PF4^{-/-}$ mice have improved survival, decreased inflammation, and less cerebral trafficking of T-cells and monocytes [10, 15]. These data, and that of other labs, suggest that platelet antagonists may have potential utility in the treatment and prevention of cerebral malaria. Caution is needed, however, as others have demonstrated that platelets may be able to directly kill intraerythrocytic parasites in uncomplicated infections [38]. Perhaps platelets have differential roles in response to different *Plasmodium* species or physiologic outcomes of infection.

Rheumatoid arthritis (RA) is a debilitating inflammatory joint disease. The pathogenesis of RA is not well understood and likely multi-factorial. In a recent paper, platelets were shown to help drive the pathogenesis of RA in a mouse model [39]. In particular, platelet-derived microparticles were recovered from RA joints and contributed IL-1 to incite the inflammatory response. Blocking the signaling of IL-1 reduced lesion development. Similarly, transplant rejection

begins at the vascular–immune interface and platelets may have a functional role in driving graft rejection. Platelet-derived soluble CD154 in a cardiac allograft model has been shown sufficient to initiate cardiac allograft rejection independent of any cellular source for this molecule [40]. We have also found that platelets sustain vascular inflammation and recruit leukocytes to the transplant vasculature [41]. Can platelet inhibitors be useful in the treatment of RA and as adjunctive therapy for the prolongation of graft survival? More study is needed to address these potential clinical utilities for platelet inhibitors.

Current Clinical State of Anti-platelet Therapies

While investigators have made great strides in identifying new platelet activation pathways and relevant disease processes, clinically, anti-platelet therapy has continued to focus on acute coronary syndromes. As more basic science evidence accumulates on the importance of some of these newly identified platelet signaling pathways and the broad disease implications of platelet functions, we may see new directions in clinical platelet research. The search for agents that interfere with platelet aggregation and thrombus formation is currently driven by percutaneous coronary innovations, with a particular focus on preventing thrombosis following stent implantation. We will briefly discuss the current state of common anti-platelet therapies as well as emerging and investigational agents.

Aspirin

Aspirin is the current cornerstone of anti-platelet therapies. It acts by permanently inactivating prostaglandin H synthase 1 and synthase 2, or cyclooxygenase-1 and cyclooxygenase-2 as they are otherwise known. This inhibition leads to decreased synthesis of thromboxane A_2 (TxA_2) and subsequently TxA_2-induced platelet aggregation [42]. Numerous studies have demonstrated the effectiveness of aspirin in the treatment and prevention of acute coronary syndromes, including myocardial infarction, stroke, and peripheral artery disease [43].

An important issue in aspirin therapy is the concept of "aspirin resistance," a term coined to describe the lack of a measureable change in platelet function ex vivo, inhibited TxA_2 synthesis in vivo, or preventing thrombotic events in individuals [42]. Such patients are at higher risk of ischemic events. It has been suggested that these select patients may require more than the recommended 81 mg of aspirin daily to achieve an ideal protective effect, although it has also been observed that other factors may be at play such as drug interactions and TxA_2 production from other sources [42, 43].

Thienopyridines

Along with aspirin, thienopyridines are the other common class of anti-platelet agents in clinical use today. After activation by the cytochrome P450 system, these drugs bond with the $P2Y_{12}$ receptor on the platelet surface. ADP has an important role in the amplification of platelet aggregation, and thienopyridines block the binding of ADP to the $P2Y_{12}$ receptor. This class of drugs includes ticlopidine, clopidogrel, and prasugrel [43].

Ticlopidine is the prototype thienopyridine, and its use was shown to be effective in treating stroke and myocardial infarction, particularly in combination with aspirin. However, it was also found to be linked to thrombotic thrombocytopenia purpura, neutropenia, and bone marrow aplasia [43, 44]. Thus, it has since given way to the newer thienopyridine clopidogrel. The Clopidogrel Aspirin Stent Interventional Cooperative Study (CLASSICS) demonstrated clopidogrel to be as effective as ticlopidine with a lower incidence of side effects, and it has since become the first-line thienopyridine for dual anti-platelet therapy with aspirin [45].

As with aspirin, there is a subset of patients who harbor "resistance" or "non-responsiveness" to clopidogrel. The prevalence of clopidogrel resistance has been estimated by various sources to be between 8% and 30%, but depends on dose and time from ingestion. This is associated with insufficient levels of the active clopidogrel metabolite, possibly due to variations in cytochrome P450 (CYP) activity, as well as a genetic predisposition to decreased intestinal absorption [46, 47]. There are polymorphisms in the genes coding for CYP enzymes, with common alleles in CYP2C19 having reduced function resulting in lower clopidogrel active metabolite concentrations [48, 49]. Carriers of a reduced-function *CYP2C19* allele have diminished platelet inhibition and a higher rate of adverse cardiovascular events than do noncarriers [50, 51]. There is some evidence that a higher loading and maintenance dose of clopidogrel may be an effective strategy to counter clopidogrel resistance [52, 53].

Prasugrel is a recently FDA-approved third-generation thienopyridine for use in acute coronary syndromes treated with PCI. Phase I and II studies have shown that prasugrel has rapid effect onset and generation of active metabolites superior to clopidogrel with less non-responsiveness and greater ADP inhibition. Prasugrel was approved after the TRITON-TIMI 38 trial revealed that it was superior to clopidogrel when combined with aspirin in reducing the primary endpoints of cardiovascular death, nonfatal MI, and nonfatal stroke [43, 54, 55]. However, it is associated with a significantly increased bleeding risk.

Glycoprotein IIb/IIIa Inhibitors

GPIIb/IIIa is a platelet receptor that has a central role in aggregation by cross-linking platelets via fibrinogen and is the

target of a potent class of anti-thrombotic agents. These inhibitors are administered by intravenous route only and are limited to acute settings, typically patients with acute coronary syndromes or who are undergoing PCI. The three drugs in this class are abciximab (a monoclonal antibody against the IIb/IIIa receptor), and eptifibatide and tirofiban (peptide and non-peptide receptor inhibitors, respectively) [56]. All three of these agents have been tested extensively in clinical trials, and meta-analyses have shown benefit versus placebo. The TARGET trial is one of the few studies to directly compare these agents, having compared abciximab and tirofiban directly in patients undergoing PCI. Although abciximab was found superior at 30 days in various composite end points, at 6 months, there was no significant difference. A sub-group analysis found that abciximab's benefit was seen primarily in patients with acute coronary syndrome undergoing PCI at both 30 days and 6 months, but not at 1 year. This has been attributed to the dosing regimen of abciximab (a large bolus followed by continuous infusion) used in the trial, as well as its high degree of activity in the first hour after initiation, a critical period following PCI [56, 57]. There is no evidence currently that abciximab is superior to tirofiban over the long term, although it does have more data from large clinical trials supporting its use. Eptifibitide's advantage lies in its cost and its early onset of anti-platelet action similar to abciximab. Bleeding is a major complication with this class of drugs. Thrombocytopenia is the other major problem associated with these drugs, which appears to be more associated with abciximab than tirofiban or eptifibitide [58].

Emerging Therapies

There are currently compounds in development and clinical trials as platelet antagonists. Elinogrel is an investigational $P2Y_{12}$ receptor inhibitor, which unlike other thienopyridines, is reversible in its blockade of ADP-mediated platelet aggregation with near complete reversal within 24 h of administration [59]. Likewise, ticagrelor is a cyclopentyl-triazolopyrimidine derivative currently awaiting FDA approval that also reversibly acts on the $P2Y_{12}$ receptor [60]. Protease-activated-receptor-1 (PAR-1) is another target of new drug development. In addition to its functions in the coagulation cascade, thrombin activates platelets by cleavage of PAR receptors. There are now two PAR-1 inhibitors under investigation, SCH530348 and E5555. SCH530348 is currently in two large, multi-center phase III trials, while E5555 in currently under phase II study [61, 62]. It remains to be seen if the promises of either compound gain approval for general clinical use.

The omega-3 fatty acids, eicosapentaenoic acid and docosahexaenoic acid, are found in oily fish and are compounds known to have anti-platelet effects. Although previous research into their potential anti-platelet effects has been inconclusive, a possible synergistic effect with standard dual anti-platelet therapy, namely aspirin and clopidogrel, may exist. Thus far, a few small studies have shown that the addition of omega-3 fatty acids to clopidogrel and aspirin or aspirin alone were able to potentiate platelet response to these agents as measured by ex vivo platelet assays [63–65]. A retrospective study looked at patients treated with high-dose fish oil, aspirin, and clopidogrel and found no statistically significant association with bleeding events compared with matched controls [66]. The approach of combining these omega-3 fatty acids with aspirin and clopidogrel appears to be safe and may represent a simple new means to increase responses to standard anti-platelet therapies without a large increase in bleeding risk.

The major complication of any platelet inhibitor is an increased bleeding risk, making platelet antagonists that can be reversed by a defined antidote a large step forward in clinical therapy. The use of aptamers with complementary antidotes as platelet adhesion antagonists is currently in research development and early clinical trial [67, 68]. Aptamers are single-stranded nucleic acid molecules that directly and specifically inhibit protein functions [69–71]. Rationally designed aptamers that specifically target a part of the VWF A_1 domain (the platelet GPIbα binding domain) with an antidote molecule that quickly reverses the aptamer function has shown promise in this area [72].

Summary

Our knowledge of platelet activation pathways and platelet functions has greatly expanded in the past decade. The emerging role of platelets in driving vascular inflammation has likewise seen a great increase in significant publications. These findings will continue to fuel the development of platelet antagonists and the application of these drugs to many new disease processes.

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