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Platelets as Cellular Effectors of Inflammation in Vascular Diseases

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Abstract

Platelets are chief effector cells in hemostasis. In addition, they are multifaceted inflammatory cells with functions that span the continuum from innate immune responses to adaptive immunity. Activated platelets have key "thromboinflammatory" activities in a variety of vascular disorders and vasculopathies. Recently-identified inflammatory and immune activities provide insights into the biology of these versatile blood cells that are directly relevant to human vascular diseases.

Keywords

Platelets; Inflammation; Immune Responses; Vasculopathy; Vessel Diseases

> "For blood clot turns into pus and excites inflammation, and prevents agglutination of the wound."
>
> – Cornelius Celsus [1]

Introduction

Platelets are highly specialized for hemostasis, and are first responders in vascular injury and endothelial disruption. This is traditionally the most well-known function of platelets, and platelet-dependent hemostasis is intensely studied [2]. Nevertheless, platelets are also inflammatory effector cells, and have activities across the spectrum from acute inflammation to adaptive immunity [3-6]. There is evidence that the inflammatory specializations of platelets – like their specializations for hemostasis – are evolutionarily-driven adaptations that facilitate host defense, tissue repair, and protection against pathogens[5, 6]. Furthermore, platelets provide key, central links between the inflammatory and hemostatic limbs of the host defense system, contributing to integration of vascular and wound repair and maintenance of tissue integrity [5, 7, 8]. The inflammatory activities of platelets also contribute to many organ-localized and systemic diseases with inflammatory components, however, based on existing evidence and reasonable inference where observations are incomplete [6]. It is intriguing that the ancient author Celsus, writing in Rome in the first century A.D.,

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described an apparent link between blood clots and pathologic inflammation (see introductory quote)[1], although platelets had not yet been discovered in his time [9] and delineation of their activities in vascular diseases and inflammatory syndromes is much more recent.

Human platelets respond with multiple innate immune effector activities when signaled by endogenous agonists generated or released in hemostasis and inflammation, by microbial toxins, or by many microbial pathogens. Key innate immune activities of platelets include release of chemokines, cytokines, and other mediators and interactions with endothelial cells, monocytes, and neutrophils, which are also critical responders in innate immunity and acute inflammation[3-6, 10-12] (Figure 1, Online Table I). (In this review we use "neutrophil" interchangeably with "polymorphonuclear leukocyte" and "PMN", although eosinophils and basophils are sometimes classified as polymorphonuclear leukocytes as well. In addition, however, activated human platelets operate across the immune continuum, and have a substantial armamentarium of adaptive immune activities[3-6]. These include synthesis and/or release of pleiotropic immune mediators including interleukin-1β, CD40 ligand (CD40L), and RANTES, and interaction with lymphocyte subclasses and dendritic cells (reviewed in [5, 6]).

Newly-recognized inflammatory activities of platelets, including toll-like receptor-mediated responses and the ability to trigger neutrophil extracellular trap (NET) formation (Figure 2), continue to emerge. Here we emphasize some of the recently-discovered and rapidly-evolving aspects of the biology of platelets in the immune continuum and the spectrum of inflammation, and provide an overview of inflammatory activities of platelets in vascular diseases and vasculopathies. Because of space limitations we telegraphically profile many topics that have been previously reviewed in comprehensive fashion. We also include additional online supplemental information to expand some of these issues.

The Inflammatory Repertoire of Platelets

Thrombosis and Inflammation: Linked Outcomes of Platelet Activation

Many functions of activated platelets that are induced by classic hemostatic agonists such as thrombin and ADP and that, for many years, were thought to exclusively mediate hemostasis and thrombosis are now known to also contribute to inflammatory and immune responses (reviewed in [5, 13]) (Figure 1; Online Table I). "Traditional" prothrombotic activities that are also proinflammatory include adhesion and aggregation [14], display of surface molecules that favor generation of thrombin[15], binding of fibrinogen [16, 17], and interaction with the fibrin mesh of clots[17] (Campbell R, Weyrich AS, manuscript in preparation). Thrombin and fibrinogen are key proinflammatory factors in addition to being essential components of the coagulation cascade[18, 19]. Activated platelets can also synthesize tissue factor (reviewed in [5]), which has complex roles in inflammation in addition to its critical procoagulant activities[20].

Platelets likely evolved from multifunctional defensive cells with the capacity to immobilize and kill pathogens, seal wounds, and repair tissues[3-5, 21]. "Modern" procoagulant and proinflammatory specializations of platelets, and the ability to link inflammation and hemostasis and sense pathogens[5, 21, 22], may thus have ancient origins in host defense. Nevertheless, "thromboinflammatory" activities of platelets contribute to a large number of vascular diseases.

Platelet Receptors, Activation Pathways, and Integrins

Platelets have a diverse array of surface receptors that are linked to intracellular signaling cascades and that trigger cellular activation and key functional responses[23, 24]. Many induce

and/or regulate hemostatic functions of activated platelets [23-27] and are targets for anti-platelet therapies in thrombotic diseases [26, 28, 29]. Many also link activation of platelets to proinflammatory responses[5, 6], in addition to triggering prothrombotic signaling cascades [23-29]. G protein-coupled receptors (GPCR) and their intracellular pathways[24, 26] are chief examples (Online Table I), but a variety of receptor-mediated pathways of platelets induce both inflammation and thrombosis [5, 23]. Inside-out signaling, also termed activation, of integrin $\alpha_{IIb}\beta_3$, a central downstream effector response that is essential for normal hemostasis[16], mediates pathologic thrombosis[25, 28, 29] and can amplify inflammation[5, 6]. Activation of integrin $\alpha_{IIb}\beta_3$ is induced by a signaling cascade terminating in RAP1, Kindlin 3, and Talin[24, 26, 30, 31]. Several other integrins also mediate adhesive activities of activated platelets[17, 23, 27] and may participate in inflammatory interactions[5, 31]. Integrin signaling is bidirectional, and components of the inside-out cascade that mediates activation of integrin $\alpha_{IIb}\beta_3$ [16, 30, 32], including Kindlin 3[33] and RAP1[34], participate in outside-in signaling. Outside-in signaling of $\alpha_{IIb}\beta_3$ triggers or amplifies functional responses of platelets that have both hemostatic[16, 24, 32] and inflammatory[5, 6] consequences.

Platelet Immunoreceptors and Toll-Like Receptors

Some platelet surface receptors have been termed immunoreceptors because of their ligand recognition specificities and/or features of their molecular structures[35]. Platelet Fc receptors (FcγRIIA, FcεRI, FcαRI), glycoprotein VI (GPVI), and C-type lectin-like receptor 2 (CLEC-2) are among these[5, 6, 23, 35]. Platelet Fc receptors bind immunoglobulins and immune complexes, providing direct mechanisms for immune recognition and signaling [23, 35, 36]. GPVI, which is uniquely expressed by platelets, is a major receptor for collagen with signaling activities in hemostasis and inflammation [6, 37] (Online Table I). CLEC-2, like GPVI, has emerging activities in platelet-dependent hemostasis, vascular integrity, and inflammation, including regulation of lymphangiogenesis in mouse models[6, 37].

Human platelets, and platelets and thrombocytes from other mammals and vertebrates, express TLRs (reviewed in [4-6, 23, 38, 39]). Intracellular and cell surface TLRs detect microbes and microbial products and transmit signals to inflammatory and immune transduction pathways. TLRs also recognize endogenous, host-derived “danger-associated molecular patterns” (DAMPs), in addition to microbial pathogen-associated molecular pattern (PAMP) motifs [40-42]. The presence of TLRs on platelets is persuasive evidence that they evolved as immune effector cells and provides a molecular basis for their activities as pathogen sensors [4, 5, 21, 38, 39]. Together with other classes of receptors with immune signaling activities[5, 23, 35], platelet TLRs provide a molecular basis for intravascular immune surveillance[43] and for pathologic inflammatory and immune activities[4-6, 44]. As with “prothrombotic” receptors[24-26], platelet TLRs may be engaged in parallel or in sequence with other receptors and may act cooperatively[5, 6, 36]. Megakaryocytic cell lines and megakaryocytes also express TLRs[38], indicating that the TLR signaling may regulate thrombopoiesis and other megakaryocyte functions in the marrow[4] and/or lungs[8].

Human and mouse platelets express multiple TLRs at the messenger RNA (mRNA) and/or protein levels[4-6, 38, 39]. TLR4 is the most extensively studied [6, 21, 23]. Early reports indicated that platelets from several species bind or respond to lipopolysaccharide (LPS), suggesting the presence of one or more receptors[6]. In vitro experiments with isolated human platelets indicated that LPS amplifies responses triggered via Fc receptors[36] and that the endotoxic backbone of LPS, Lipid A, can directly induce aggregation and serotonin secretion[45]. After molecular characterization of TLR4 – the major immune receptor for LPS [42, 46] – its expression was reported on human and murine platelets[47-50], although it was not detected in one early study[51]. In vitro experiments also demonstrated binding of LPS to platelet TLR4[50, 52, 53]. Binding of LPS to platelets may increase their consumption and phagocytosis

in hemolytic uremic syndrome and autoimmune thrombocytopenic purpura [52, 54]. Variable changes in surface expression of TLR4 on freshly-isolated platelets in response to thrombin have been reported[39, 50, 52]. One study indicated that binding of LPS to freshly-isolated, washed human platelets increases with thrombin stimulation and that TLR4 and P-selectin on the surfaces of activated human platelets cooperatively bind LPS[52]. Surface display of TLR4 on human platelets in stored concentrates was reported to decrease after activation[48].

An early report found no evidence for direct activation of human platelets by TLR4 or for modulation of GPCR-triggered platelet activation responses by LPS, and concluded that TLRs may be vestigial molecular remnants bequeathed to platelets by megakaryocytes[49]. Subsequent studies of platelet responses to LPS in vitro describe contradictory results that are both perplexing and intriguing (Supplementary Table 2). In some experiments LPS did not induce typical activation responses of human or murine platelets, including shape change, aggregation, or surface translocation of P-selectin and did not enhance activation triggered by classic agonists [47, 53, 55, 56] – similar to results in the previous survey[49]. In others, however, LPS potentiated activation responses triggered by subthreshold concentrations of thrombin or collagen, and directly induced actin cytoskeleton rearrangement, aggregation, binding of PAC-1 antibody (which recognizes activated integrin $\alpha_{IIb}\beta_3$), fibrinogen binding, degranulation of alpha and dense granule markers, display of CD40 ligand (CD40L), adhesion of platelets to cultured endothelial monolayers, platelet-neutrophil interaction and neutrophil extracellular trap (NET) formation, and splicing of pre-mRNAs coding for IL-1β and tissue factor and synthesis of these protein products[48, 52, 53, 56-59]. (Most of these functional activities will be discussed in later sections of this review.)There is as yet no clear explanation for these discrepancies, but some of the factors may include: variable potencies and structural features of LPS types from different bacterial sources [45, 52, 58]; delayed and more protracted responses of platelets to TLR4 engagement compared to activation via classic “prothrombotic” receptors (Online Table I)[57]; use of isolated, washed platelets versus platelet-rich plasma (PRP) or platelets supplemented with plasma, serum, or recombinant or purified CD14 and LPS binding protein (LBP) in key experiments[52, 57-59]. CD14 and LBP are blood proteins that facilitate recognition of LPS by TLR4 [42, 46], and are likely critical in the efficiency of binding of LPS to platelet TLR4 as with other cells that express this TLR family member. As examples, platelets in plasma but not washed platelets adhered to LPS-treated cultured endothelium[52], the concentration of LPS required to potentiate collagen-induced platelet aggregation was 5-10 fold lower in PRP than in washed platelets[58], and rCD14 and LBP facilitated pre-mRNA splicing by LPS-treated platelets [57, 59].

The signaling pathways that mediate responses of platelets to LPS have not been comprehensively defined, although key components that are required for TLR4 signaling in other cells are present. In addition to TLR4, MD2 – a lipoprotein binding protein family member that associates with TLR4 near its extracellular N-terminal domain[23] – is expressed on human platelets[58, 60]. CD14 has also been detected on the plasma membranes of resting human platelets in some, but not all studies, and may be primarily soluble CD14 derived from plasma[52, 58, 59]. Isolated human platelets or platelets in PRP did not bind anti-CD14 antibody in the resting state or when stimulated with thrombin, but binding of the antibody to platelets in PRP increased in response to pretreatment with LPS[52]. At the intracellular face, MyD88 – which is essential for downstream signaling by most TLRs [42] – is present in human platelets[58, 60], and small molecule inhibitors of MyD88 interrupt LPS signaling[59]. Further, MyD88, in addition to TLR4, is required for LPS signaling of platelets based on studies of mice with mutations in TLR4 that make them hyporesponsive or unresponsive to LPS, *TLR4*$^{-/-}$ mice, or *MyD88*$^{-/-}$ mice[47, 50, 52, 58, 59]. None of these experiments examined platelet-specific genetic modifications of *TLR4* or *MyD88*. Distal components of the canonical MyD88 pathway[42], including TIRAP, IRAK1 and 4, and TRAF6, are also present

in human platelets based on experimental inhibition of their activities and other approaches [22, 59, 60]. Downstream from the MyD88 cascade there is evidence for rapid phosphorylation of JNK and AKT (within minutes) that is associated with more delayed functional responses[59]. A TLR4/MyD88-dependent activating pathway involving cyclic GMP, protein kinase G, and nitric oxide synthase has also been described, and it was suggested that this mechanism can inhibit or activate platelets depending on the concentrations of LPS [58]. This might explain inhibition of platelet activities by LPS in some earlier reports[61, 62]. Taken together, the studies reported to date suggest that there are gaps in our understanding of signaling cascades triggered by platelet TLR4, and that novel pathways may be involved. It is unknown if recently-identified platelet signaling cascades such as those involving mammalian target of rapamcyin (mTOR) [63-65], ADP-ribosylation factor 6 (Arf6-GTP)[66-68], or others[24] mediate LPS-triggered responses.

In vivo experiments also indicate that LPS activates platelets, although complex signaling interactions involving parallel activation of myeloid leukocytes and endothelial cells occur as well. Challenge of mice or rabbits with LPS causes thrombocytopenia and time-dependent accumulation of platelets in the lungs and liver with the magnitude influenced by the microbial origin of the LPS [47, 50, 53, 69-73]. Platelet accumulation in microvessels of mice injected with LPS has been demonstrated using spinning-disc confocal microscopy and transgenic mice expressing endogenously-labeled integrin subunit α_{IIb} or in vivo labeling of platelets with anti-α_2 integrin subunit (CD49) antibody[73]. In other models, LPS induced accumulation of platelets in mesenteric vessels of rats [74] and cremasteric veins of mice [55, 73], and promoted platelet-dependent thrombus formation in ferric chloride-injured carotid arteries of mice[58]. These responses were abrogated in mice with disabling mutations of TLR4 or in animals genetically deficient in TLR4 or MyD88 [47, 50, 55, 58]. Maximal tumor necrosis factor α (TNFα) generation is dependent on platelet TLR4 in mice challenged with LPS[50]. Thrombocytopenia induced by LPS in mice may involve effects on megakaryocytes in addition to circulating platelets [75]. Administration of LPS to human subjects under controlled conditions causes thrombocytopenia, alterations in CD40L on platelets, and formation of platelet-monocyte aggregates [76-78], consistent with some of the studies with surrogate models and establishing translational relevance for them.

TLR2 on platelets has not been examined as extensively as TLR4, but it is clearly an important inflammatory receptor. TLR2, which heterodimerizes with TLR1 and TLR6, may be the most promiscuous of TLRs in terms of ligand recognition, and binds lipopeptides, lipoproteins, peptidoglycans, and lipoteichoic acids from gram positive bacteria, some LPS species, and some fungal and mycobacterial products[38, 58]. Human and mouse platelets express TLR2, and human platelets express TLR1 and TLR6[48-50]. Surface display of TLR2 may increase with cellular activation[39]. Most, but not all[49], reports indicate that treatment of human platelets with ligands for TLR2 triggers thromboinflammatory activities. These responses appear to be differentially regulated by intracellular pathways distinct from those activated by classic prothrombotic receptors (Online Table I), and include inside-out signaling of integrin $\alpha_{IIb}\beta_3$, aggregation, adhesion to collagen, P-selectin translocation, generation of reactive oxygen intermediates, and splicing of *IL-1β* pre-mRNA [57, 79-81]. In addition, the synthetic TLR2/TLR1 agonist PAM_3CSK4 induces formation of platelet-neutrophil aggregates in whole human blood[80]. PAM_3CYSK4 and a bacterium that is recognized by TLR2 triggered platelet activation in wild type mice that was reduced in $TLR2^{-/-}$ animals[80]. Engagement of TLR2 on murine megakaryocytes and a megakaryocyte cell line altered mechanisms that regulate megakaryocyte maturation; in addition, treatment of mice with Pam3CSK4 increased megakaryocyte maturation and platelet numbers in wild type animals but not those deficient in TLR2, indicating the TLR2 on megakaryocytes may regulate megaryopoiesis and thrombopoiesis in inflammation [38, 82].

TLR9 is the most intriguing member of the platelet TLR repertoire. TLR9 is basally expressed on the plasma membranes and in the cytoplasm of resting human platelets, and its surface display is increased when platelets are activated by thrombin[5, 48, 50, 83]. In other cell types, TLR9 is exlusively intracellular and is restricted to endosomal domains[40, 42]. Recent studies of mouse and human platelets and megakaryocytes demonstrate that TLR9 is distributed to a newly-identified compartment, termed the T granule, during proplatelet production and that Type IV collagen increases TLR9 surface expression. Certain oligodeoxynucleotides (ODN), which are known synthetic ligands for TLR9, induce P-selectin translocation and also increase TLR9 surface display [83]. TLR9 recognizes unmethylated CpG islands in viral and bacterial DNA[5, 83], suggesting a previously-unrecognized pathogen sensing system in platelets. In addition, platelet TLR9 was recently reported to bind a carboxyalkylpyrrole protein adduct that may act as a DAMP under conditions of oxidant stress, and to transmit signals triggering aggregation and degranulation via the MyD88 pathway[84].

Noncanonical Activities of Immune Transcriptional Regulators

In nucleated cells, signaling via the TLR/MyD88 pathway is a major mechanism of expression of cytokines and chemokines. Synthesis of many of these proteins is controlled by the nuclear factor kappa B (NF-κB) transcriptional regulatory system[41, 42]. Platelets are anucleate cells, however, and there is no evidence for transcriptional activity aside from mitochondrial transcription. Nevertheless, several NF-KB proteins are expressed by human platelets[60, 85-89] and one, BcL-3, is induced in human and mouse platelets as a result of signal-dependent translation of constitutive *BcL-3* transcripts[63, 64, 85] (Online Table III). Interestingly, levels of transcripts for some NF-κB pathway members and *TLR2* are altered in platelets from subjects with cardiovascular risk factors and obesity[90]. Together, these observations suggest that NF-κB family members have physiologic activities in anucleate platelets independent of their traditional roles in transcription[85, 86], although transcriptional regulation clearly occurs in megakaryocytes[38, 88]. Consistent with this prediction, Bcl-3 was found to be an intracellular regulator of clot retraction by human and mouse platelets[63]. More recently, a complex of NF-κB, IKB, and protein kinase A was reported to exert negative feedback activities that modulate cytoskeletal reorganization and aggregation in platelets stimulated by thrombin or collagen[87-89]. It is not yet known if pathways regulating – or regulated by – NF-κB are altered by upstream signaling from TLRs 2, 4, or 9 and MyD88 in platelets. Additional nuclear factors with noncanonical activities are also expressed by these cells [89].

Degranulation, Secretion, and Translocation: Inflammatory Signaling and Effector Mechanisms of Activated Platelets

Activated platelets have diverse mechanisms for inflammatory signaling and transcellular transfer of biologically-active factors and molecular information (reviewed in [3, 5, 6]). This is in part engendered by diverse mechanisms for release of signaling factors or their display on the platelet surface (Online Table III).

Rapid degranulation and secretion of preformed soluble factors are a classic activation responses of platelets [4, 5, 44, 91]. Over 300 proteins may be released, depending on the agonist. Alpha and dense granules are the most rigorously characterized intracellular storage organelles of platelets[91]. Distinct subpopulations of alpha granules have been reported in murine platelets[4, 92], but differential release of α granule cargo may predominantly be a kinetic variable when human platelets are stimulated[93]. Factors released from alpha and dense granules contribute to hemostasis, thrombosis, angiogenesis, endothelial barrier modulation, and inflammatory signaling [3-6, 8, 44, 91] (Online Table III). Examples of recently reported activities of secreted platelet factors include increased vascular permeability in

experimental arthritis mediated by serotonin[94], modification of inflammation-associated hemorrhage by TREM-like transcript 1[95], and modulation of angiogenesis [96].

Some factors that are degranulated by activated platelets are not immediately released into solution but are retained on the plasma membrane after surface translocation. P-selectin, which mediates platelet-leukocyte interactions and intercellular signaling is a key example (Online Table III)[5, 44]. Studies in mice and humans indicate that fibrinogen, another alpha granule constituent, contributes to maintenance of intracellular and cell surface of P-selectin[97, 98]. Additional constitutive or newly-synthesized factors can also mediate spatially-regulated juxtacrine intercellular signaling at the platelet surface. Platelet activating factor (PAF) – a phospholipid signaling molecule – CD40L, and IL-1β are examples[5, 6].

New platelet mediators, compartments, and intercellular signaling mechanisms continue to be identified. Human β-defensin 1 (hBD-1), an antimicrobial peptide not previously-known to be present in platelets, is released in response to staphylococcal α-toxin, induces formation of NETs (Figure 2), and kills *S. aureus* in vitro[99]. In resting human platelets, hBD-1 is found in an intracellular compartment distinct from classic granules[99]. As noted previously, TLR9 is also localized in a unique intracellular compartment[83].

Synthetic Pathways, the Platelet Transcriptome, and Signal-Dependent Alterations in the Platelet Proteome

The platelet is a synthetic cell when it is activated. Traditional synthetic pathways include those that regulate production of TXA_2 [13] PAF[100-102], and reactive oxygen species [13, 80, 103]. Most investigators discarded the possibility that platelets synthesize new proteins, however, because they are anucleate [104] – even though platelets were known to have messenger RNAs (mRNAs) in their intracellular arsenal [64, 105]. More recently, we and others found that many of these mRNA transcripts can be translated in a signal-dependent fashion by activated platelets and that this alters the platelet proteome in functionally significant ways [63, 64, 85, 106]. This led to the unexpected discovery that platelets also have pre-mRNAs and a spliceosome-dependent mechanism for their processing to mature transcripts and subsequent translation[107-109] (Online Table IV). The platelet transcriptome is now being comprehensively examined in the basal state and in disease using new approaches including genome-wide next generation RNA sequencing (RNAseq)[110]. Diversity in the constitutive platelet transcriptome continues to emerge, and it is now known that it includes microRNAs in addition to processed mRNA and pre-mRNA transcripts[108, 110, 111]. There is evidence that megakaryocytes differentially sort transcripts to platelets during thrombopoiesis and that this is a mechanism for regulating protein synthesis by the circulating cells[112]. In parallel, diversity in the post-transcriptional pathways for transcript processing and regulated translation has been identified in the platelet repertoire[64, 113]. There is evidence that these newly-discovered mechanisms influence the platelet proteome and functions in thrombosis, inflammation, and immune responses(reviewed in [5, 6, 64, 113]). There is also evidence that the platelet transcriptome is altered in infection and disease[5, 6, 56, 90, 110] (Rondina M, et al, manuscript in preparation).

Protein products with functional relevance in inflammation and hemostasis are synthesized in a signal-dependent fashion by activated human platelets (Online Table III) [56, 57, 59, 63, 64, 85, 106-109, 112-116]. Synthesis of IL-1β provides a physiologically-relevant example of intricate post-transcriptional pathways that are involved (reviewed in [5, 6, 64]). Quiescent human platelets have the *IL-1β* pre-mRNA but little or no processed mature *IL-1β* transcript or Il-1β protein[108]. When they are activated by thrombin or other classic agonists in the presence of fibrinogen they synthesize pro-IL-1β protein, some of which is processed to the mature IL-1β cytokine[107, 108]. Human platelets have key components of the inflammasome[6], which mediates conversion of pro-IL-1β to mature Il-1β[117]. Dissection of

the molecular steps upstream from IL-1β protein synthesis and processing yielded a regulatory mechanism unexpected in mammalian biology[118]. Key studies demonstrated evidence that megakaryocytes deliver the *IL-1β* pre-mRNA and a functional spliceosome to platelets during proplatelet formation and that spliceosome activity is triggered in a signal-dependent fashion in activated platelets[108]. The signal-dependent splicing pathway is controlled by Cdc-like kinase 1 (CLK1), a kinase not previously known to be present in platelets[57, 59, 109]. TLRs and immunoreceptors also activate this mechanism: LPS in the presence of CD14 and LBP (see previous section)[57, 59], engagement of TLR2[57], and engagement of FcαRI[114] – an immunoreceptor for IgA on platelets – trigger splicing of *IL-1β* pre-mRNA and synthesis of IL-1β protein. IL-1β is a pleiotropic cytokine that has been called the "gatekeeper of inflammation"[119]. Thus, this regulated synthetic pathway provides platelets with a powerful mechanism to influence inflammatory and immune events [5, 6, 107, 108]. In vitro and in vivo studies indicate that activated human platelets signal endothelial cells, leukocytes, and vascular smooth muscle cells by releasing Il-1β in solution or its transport in microvesicles (see below), or by juxtacrine signaling (reviewed in [6]). Recent in vivo observations also indicate that release of IL-1β in platelet microvesicles is one mechanism by which platelets communicate with and activate extravascular cells[120, 121].

An important feature of signal-dependent translation of proteins by platelets is that it can continue for many hours after they are activated [56, 85, 107-109, 112]. In addition, some proteins can be basally synthesized continuously over many days by platelets stored ex vivo[122]. These and other aspects of regulated post-transcriptional protein expression are features of the "new biology" of platelets[64, 121] and also have clinical relevance to thromboinflammatory diseases[6] and transfusion medicine[123]. Prolonged synthesis of proteins by platelets also raises interesting questions regarding their contributions to the biochemical milieu of clots and thromboinflammatory lesions in vivo. While the lifespan of platelets in the circulation and in vitro is known[124] their longevity in thombi and wounds has not been precisely determined, and they may persist and synthesize proteins and other factors for extended periods of time and in variable temporal patterns. In vitro studies indicate that thrombi may be reservoirs for IL-1β synthesized by platelets[6, 107] – suggesting a mechanism for inflammatory properties of clots proposed by Celsus[1].

Platelet-Leukocyte Interactions

Activated platelets interact with circulating myeloid leukocytes and with lymphocytes of several classes [5, 14]. Binding of activated platelets to leukocytes enhances their adhesion to endothelium and transmigration under some experimental conditions, and is an important mechanism that facilitates accumulation of neutrophils and monocytes in inflamed vessels and infected or injured tissues [5, 6]. As recent examples, platelets enhanced adhesion of $CD14^{high}CD16^{+}$ monocytes to endothelial cells in vitro[125], facilitated human and murine neutrophil transmigration across IL-1β-activated endothelial monolayers and in an inflamed cornea model[126], and selectively enhanced monocyte recruitment to endothelium in mouse and human models of vascular inflammation[127]. Alternatively, platelets were recruited to sinusoidal microvessels in the livers of mice injected with LPS primarily by interacting with neutrophils that were already adherent to the endothelial surface[73]. Platelet depletion reduced monocyte emigration and parasite killing in a mouse model of *Leishmania* infection[128]. These observations illustrate diverse defensive and pathologic consequences that may result from platelet-leukocyte interactions[5, 6]. In addition, platelet binding to exposed subendothelium is a mechanism to recruit myeloid leukocytes to injured vessels [6], and may be one that allows neutrophil accumulation at shear stresses that retard or prevent their adhesion to endothelium under flow[129].

Activated platelets also bind to leukocytes in circulating blood, forming multicellular "heterotypic" aggregates[5, 6, 44]. In each of these interactions, binding of P-selectin on the plasma membrane of the activated platelet to P-selectin glycoprotein ligand 1 (PSGL-1) on the leukocyte is a dominant molecular event (Figure 3), although other adhesive mechanisms have also been described depending on the specific leukocyte subtype, species of origin, and experimental condition[5, 6]. Binding of P-selectin on activated platelets to PSGL-1 on leuckoytes not only tethers the cells together but also delivers signals that induce integrin activation, trigger gene expression pathways, and induce other functional responses by the leukocytes [5, 6, 44] (see below). Studies using whole human blood indicated that reciprocal platelet activation also occurs[5, 6]. While such interactions of activated platelets with leukocytes have physiologic inflammatory consequences, there is also evidence that they contribute to microvascular occlusion, thrombosis, and propagation of inflammatory damage in disease[6, 44, 130, 131].

Interactions of platelets with monocytes and neutrophils have been studied the most. Platelet-monocyte aggregates are more stable than platelet-neutrophil complexes in part because of differential regulation of PSGL-1[132, 133], and formation of platelet-monocyte aggregates may be the most sensitive and durable index of in vivo platelet activation in experimental and clinical conditions[134]. Platelet-monocyte aggregates have been detected in the blood of humans with a variety of diseases [6] (Online Table V).

Early studies demonstrated that P-selectin mediates binding of activated platelets to monocytes[135, 136]. Both intercellular adhesion and signaling are consequences of this molecular interaction (Figure 3)[5, 44]. Signals delivered to the monocyte by ligated PSGL-1 can be amplified by, and integrated with, signaling by PAF, RANTES, or other factors from the activated platelet or autocrine signaling by the monocyte[136-141]. New expression of key proinflammatory cytokines and metalloproteinases, synthesis of tissue factor, and expression of cyclooxygenase 2 (COX-2) with generation of COX-2-dependent eicosanoids occur in response to signaling of human monocytes by platelets (Figure 3) [130, 137, 140-147]. Differential signaling and monocyte synthetic responses can be detected in model conditions mimicking ruptured atherosclerotic plaques [138]. Molecular interactions between activated platelets and monocytes provide specific targets for therapeutic interruption of thromboinflammatory activities in vascular diseases [28, 29, 125, 138, 140, 145]. As with monocytes, binding of activated platelets to neutrophils, eosinophils, and basophils via P-selectin/PSGL-1 induces signaling as well as intercellular adhesion[5]. Platelet-neutrophil aggregates have been detected in blood samples from patients with inflammatory syndromes[6], although as noted above they may be more transient than platelet-monocyte aggregates. PSGL-1 engagement on human neutrophils triggers kinase cascades and chemokine synthetic pathways [5, 44, 148], and in murine models induces activation of β_2 integrins on neutrophils[44, 149, 150]. Activation of leukocyte $\alpha_M\beta_2$ integrin in this fashion may stabilize aggregation of activated platelets and leukocytes by allowing $\alpha_M\beta_2$ to recognize GPIb on the platelet or fibrinogen bound to platelet integrin $\alpha_{IIb}\beta_3$ [44] . Activation of platelets in whole blood induces platelet-leukocyte aggregate formation and binding of tissue factor, factor Xa, and fibrinogen to the surfaces of neutrophils and monocytes; aggregates may thus be vehicles for local and systemic delivery of coagulation factors[151, 152]. In vitro modeling indicates that human neutrophils enhance fibrin deposition under flow in a fashion that is partly platelet-dependent[153]. Observations in humans and mouse models indicate that platelet-neutrophil aggregates contribute to increased pulmonary vascular permeability and a chronic inflammatory state in sickle cell disease[154]. Platelet-leukocyte interactions of this nature contribute to pathologic vascular inflammation and thrombosis in a variety of experimental and clinical syndromes [6] (Figures 4-6; Online Table V).

NETs are extracellular lattices of chromatin, histones, and granule enzymes extruded by neutrophils via a unique cell death pathway sometimes termed "NETosis"[155, 156]. NETs capture and kill a variety of microbes, but there is also evidence that they mediate infectious and sterile vascular injury in sepsis, immune vasculitis, transfusion-related acute lung injury (TRALI), venous thrombosis, and other syndromes[6, 10, 155, 156]. LPS-stimulated human platelets trigger NET formation under flow conditions and platelets are required for in vivo formation of NETs in mice challenged with LPS[53, 157]. Platelets and leukocytes accumulate and NETs are formed in lung microvessels in a murine model of TRALI, and platelet depletion, aspirin treatment, or blockade of integrin $\alpha_{IIb}\beta_3$ decreases NET formation and lung damage[158, 159] (Online Table V). The signals from activated platelets that induce NETosis are not completely defined. NET formation by human neutrophils is induced by hBD-1 from platelets activated by Staphylococcol α-toxin (Figure 2)[99], but this may not occur in mice because of species differences in defensin expression[160]

Interactions of activated platelets with lymphocytes, dendritic cells, and macrophages are key events with the potential to influence adaptive immune responses and contribute to chronic inflammatory and immune syndromes[3, 5]. Adaptive immune facets of the platelet repertoire complement the innate immune armamentarium of these cells. This has been progressively appreciated in recent evolution of the field[3-5]. Discovery that platelets express and release CD40L – a pivotal immune signaling molecule that is critical in T-cell-dependent isotype switching and generation of antibody subclasses by B cells, and for dendritic cell activation – has been central in recognition of platelets as adaptive immune effector cells[3, 5, 13, 39, 161]. Additional activities of platelets, such as synthesis of IL-1β, also have the potential to initiate, amplify, or modify adaptive immune pathways (reviewed in [5]).

Interactions of Platelets and Endothelium

Endothelial cells (EC) have potent mechanisms for retarding platelet activation at the uninjured vascular luminal surface, including enzymatic generation of prostacyclin and nitric oxide and degradation of ADP and ATP [13, 162]. There is also evidence that platelets signal uninjured endothelium and are critical for maintenance of basal endothelial barrier function in pulmonary and systemic vessels[6, 8]. Basal endothelial barrier properties regulate transvascular flux of water and solutes, contributing to the prevention of edema, and also prevent red blood cells extravasation and bleeding. In several experimental models thrombocytopenia leads to "leakiness" of vessels[8]. Increased endothelial permeability secondary to disrupted barrier properties is a cardinal feature of inflammation mediated by LPS, cytokines, and other factors. This often occurs in the absence of profound thrombocytopenia, but in some cases inflammation and thrombocytopenia negatively influence barrier function in concert[6, 95, 163]. In mice, dramatic reduction in platelet numbers (to <2-3% of control) sufficient to prolong tail clip bleeding times did not cause spontaneous bleeding; in contrast, spontaneous hemorrhage into inflamed tissues occurred when thrombocytopenic animals were subjected to the cutaneous arthus reaction, LPS-induced lung inflammation, or cerebral ischemia[163]. A relatively small increase in platelet numbers (~10%) afforded protection against spontaneous bleeding, and this was independent of classic mechanisms of platelet-dependent hemostasis mediated by integrin $\alpha_{IIb}\beta_3$, GPIb, GPVI, and Von Willebrand Factor[163]. This protective effect may result from local delivery of barrier stabilizing factors and/or inhibition of neutrophil-mediated endothelial injury by platelets[8, 164]. Changes in these components of the platelet repertoire in inflammatory syndromes have not been examined[8]. More recently, GPV1 and CLEC2 were reported to be required for prevention of inflammatory hemorrhage by murine platelets[165], at variance with some of the earlier observations[163, 164].

Paradoxically, platelets also contribute to disruption of endothelial barrier integrity in inflammation. Many of these observations come from models of acute lung injury and the

acute respiratory distress syndrome[8, 166] (Online Table V). In one study, formation of platelet-neutrophil aggregates and their interaction with endothelium mediated increased pulmonary vascular permeability and alveolar inflammation in murine models of acid aspiration and sepsis[167]. In other lung or systemic models, platelets or platelet lysates trigger inflammation and edema or are required for maximal vascular barrier disruption[6, 8, 94, 158, 159, 168, 169]. Serotonin delivered by platelets was recently reported to increase synovial vascular permeability in murine experimental arthritis[94]. Serotonin also had endothelial barrier destabilizing properties or – in contrast – promoted barrier stabilization in several earlier studies[8]. As outlined previously, activated platelets synthesize and release IL-1β, a potent mediator of endothelial barrier destabilization and increased endothelial permeability[5, 6]. Thus, platelets have multiple mechanisms by which they can influence endothelial barrier integrity in inflammatory diseases[6, 8].

Studies of cultured human EC, cell lines, and animal models indicate that activated platelets can signal endothelium, inducing the expression of adhesion molecules, chemokines, and other proinflammatory factors, and that platelets interact directly or indirectly with the endothelium and with the subendothelium of inflamed or injured vessels (Figures 4-6). Functional consequences, inflammatory responses, and molecular mechanisms involved in these interactions have been reviewed in detail[6, 14, 131, 170]. Relevant reports are also cited here[52, 53, 57, 58, 72-74, 107, 125-127, 129, 158, 159] (Online Table V). A recent study indicates that platelets may deliver RNA to endothelium and leukocytes[171], suggesting a potential mechanism for complex intercellular information transfer.

Platelet Microparticles: Intravascular and Extravascular Signaling

Microparticles shed by megakaryocytes and platelets are subcellular vehicles for delivery of biologically-active factors to vessels and to extravascular cells in hemostasis, thrombosis, and inflammation [3-5, 113, 172]. A variety of examples illustrate the potential for platelet-derived microparticles to drive inflammatory and immune events. Platelet microparticles mediate intercellular arachidonic acid transfer to other platelets and to EC, potentially amplifying activation responses[173] and modifing endothelial-monocyte interactions[174]. Platelet microparticles can deliver IL-1β [59, 107] and RANTES[175] to endothelium and transport newly-synthesized TF [109]. CD40L carried by platelet microparticles is a mechanism for lymphoid germinal center formation and antigen-specific IgG production[161, 176]. Microparticles from activated platelets bearing IL-1β and IL-1α signal synoviocytes and induce chemokine synthesis in vitro, and appear to fuel synovial inflammation in vivo[120, 121]. A variety of other experimental observations and clinical correlates indicate that microparticles are key components of the platelet repertoire in thromboinflammatory signaling and intravascular and extravascular communication[121, 172].

Platelets as Mediators of Inflammation in Vascular Diseases

There are many examples of inflammatory vasculopathies, which are syndromes that involve intravascular, intimal, and/or vessel wall inflammation. They range in frequency from extremely common to rare and arcane, in presentation from acute to chronic, and in topography from localized to systemic. Inflammatory vasculopathies are profiled in Online Table V.

Sepsis is a common, lethal, and informative example of acute systemic vasculopathy. Intravascular fibrin deposition and thrombosis, disrupted endothelial barrier function, and intravascular inflammation are key pathogenetic events[6, 177, 178]. Although pathogens, LPS, and other microbial toxins can directly induce the pathologic features of septic vasculopathy, clinical observations and animal studies support the central concept that molecular and

cellular responses of the host are key to the pathogenesis of sepsis [6, 177]. There is substantial evidence that platelets are pivotal cellular effectors (reviewed in [4-6, 10, 170, 179]) (Figure 6).

Each of the activities in the repertoire of activated platelets outlined in this review (Figure 1) have been implicated in the vasculopathy of sepsis[6] (Online Table V). Activated platelets and platelet-leukocyte aggregates are detected in clinical samples and in animal models (which, however, do not absolutely recapitulate clinical sepsis [6, 177]), and there is evidence that platelet sequestration and deposition of platelet-fibrin thrombi in microvessels are central mechanisms [6, 53, 69-73]. Sequestration of activated platelets bound to fibrin and leukocytes likely contributes to thrombocytopenia in sepsis, which is common and multi-factorial. TLR-dependent signaling of platelets by LPS and other bacterial factors provides one mechanism for platelet activation in sepsis [45-60, 69-81] (Online Table II). Activation of platelets by host factors generated in sepsis, including thrombin and PAF [6] (Online Table I), provides a second mechanism. Pathologic platelet responses including degranulation, secretion, and synthesis (Online Tables III, IV) may contribute to altered endothelial barrier function that is central to sepsis and to involvement of multiple organs, including the lungs, in septic syndromes [6, 8, 95, 163, 166]. Platelet synthetic responses (TxA_2, PAF, IL-1β) have the potential to drive pathologic interactions with endothelium and leukocytes and microvascular thrombosis (Figure 4)[6]. Platelet-neutrophil interactions and platelet-triggered NET formation (Figure 2) are mechanisms of endothelial barrier destabilization and injury based on animal models and in vitro experiments [10, 53, 58, 73, 80, 99, 167]. Clinical samples and human cell experiments indicate that platelet-monocyte interactions occur in sepsis[6], and animal models suggest that platelets regulate cytokine generation in this condition[50]. The platelet transcriptome is altered in sepsis [56] (Rondina M, et al, manuscript in preparation), potentially imparting pathologically-relevant changes in the platelet proteome in addition to diagnostic signatures. Platelet-derived microparticles may amplify systemic inflammation and thrombosis in sepsis [4]. Experimental and clinical observations establish a rationale for assessment of anti-platetlet therapies in human sepsis [179]. Nevertheless, studies of this nature will be challenging because sepsis is a group of syndromes with poorly-characterized subsets, a feature that often confounds clinical trials.

A number of other acute and subacute infectious vasculopathies (Online Table V) provide important but incomplete insights into inflammatory activities of platelets. Severe malaria involving the brain and lungs has features of acute vasculopathy similar to those of sepsis, including intravascular accumulation of platelets, leukocytes, and fibrin and endothelial barrier disruption. There is substantial evidence that platelets are key immune effectors in severe malarial vasculopathy [6, 180] (Online Table V). Intravascular interactions of platelets and monocytes may be central in this syndrome[6] (Figure 5). Dengue provides an example of an acute viral-induced vasculopathy in which platelets may be critical (Online Table V). The roles of platelets in many syndromes of infection-induced vasculitis and thrombosis [181] have not been comprehensively examined, however.

Atherosclerosis, which is chronic and affects vessels in a segmental fashion, is at the other extreme of the spectrum of inflammatory and immune vasculopathies (Figure 6). Inflammation of the vessel wall is central to atherogenesis. As in sepsis, most of the activities in the platelet inflammatory and immune repertoire (Figure 1) have been implicated in initiation and progression of atherosclerotic plaques and in thromboinflammatory consequences of plaque rupture, which frequently punctuates the natural history of atherosclerosis (reviewed in [13, 170, 182]) (Online Table V). Both innate and adaptive immune activities of platelets [3, 5] may contribute to the evolution of atherosclerotic lesions [182], although this is incompletely studied.

Evidence for contributions of activated platelets to initiation and progression of atherosclerotic lesions includes experimental and clinical observations that platelets commonly adhere to fatty streaks and established plaques and that platelets are activated by biochemical mediators thought to be important in atherogenesis, including oxidized low density lipoproteins and oxidatively-modified lipids (reviewed in [182]). Discovery that TLRs are in the platelet repertoire and that endogenous molecules and DAMPs with potential importance in atherogenesis may activate platelets via TLRs[84] – as with other cells[41, 42] – extends the potential initiating mechanisms. In *Apoe*$^{-/-}$ mice, a commonly-used model of atherosclerosis, platelets were found to adhere to the intimas of arteries before lesions were detectable, genetic deletion of the α_{IIb} subunit of integrin $\alpha_{IIb}\beta_3$ reduced lesional development, and circulating activated platelets secreted pro-inflammatory chemokines and formed platelet-monocyte aggregates in a P-selectin-dependent fashion, with leukocyte deposition on endothelial surfaces. These and other observations in murine models support key contributions of platelets to initiation and evolution of atherosclerotic plaques (reviewed in [131, 170, 182]). Clinical studies, including immunohistochemical analysis of atherosclerotic vascular tissue and assay of plasma and urine samples for markers of platelet release, suggest persistent platelet activation in atherosclerosis and in conditions that increase risk or accelerate the syndrome and that this drives atherosclerotic vascular inflammation[13, 182]. In parallel, many clinical and experimental observations indicate that platelet activation, cell-cell interactions, and inflammatory signaling are central in acute coronary syndromes and other thromboinflammatory complications of plaque rupture, and in responses to vascular intervention, including angioplasty and stent placement[13, 25-29, 134, 152, 182] (Online Table V). Thus, activated platelets may be ubiquitous effector cells in all phases of vascular inflammation in atherosclerosis. Acute ischemic stroke resulting from cerebral artery stenosis, thromboembolic vascular occlusion, and cerebral infarction is now recognized as a thromboinflammatory syndrome with platelets contributing to inflammation in addition to vascular occlusion (reviewed in [183]), as in acute coronary syndromes (Online Table V).

Many features of the inflammatory and hemostatic repertoire of activated platelets (Figure 1, Online Table I) seem to be common ininflammatory vasculopathies, yet the syndromes are unique (Figures 4-6; Online Table V). This in part clearly reflects the activities of other key cells [10-12]. It likely also reflects unique patterns of platelet responses to agonists that trigger and drive vascular inflammation – bacteria, fungi, and their products together with host factors in sepsis, parasitized erythrocytes and plasmodial toxins in malaria, oxidatively-modified lipids, metabolic DAMPs, and plaque components in atherosclerosis. It likely also reflects the fact that the platelet repertoire is not yet completely catalogued, and that new activities and mechanism will undoubtedly be identified.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abreviations

CD40L	CD40 ligand (CD154)
DAMP	danger-associated molecular pattern
EC	endothelial cell
IL-1β	interleukin 1β
LPS	lipopolysaccharide
NET	neutrophil extracellular trap
PAMP	pathogen-associated molecular pattern
PMN	polymorphonuclear leukocyte
PSGL-1	P-selectin glycoprotein ligand 1
RANTES	Regulated upon Activation, Normal T cell expressed presumed Secreted
TLR	toll-like receptor

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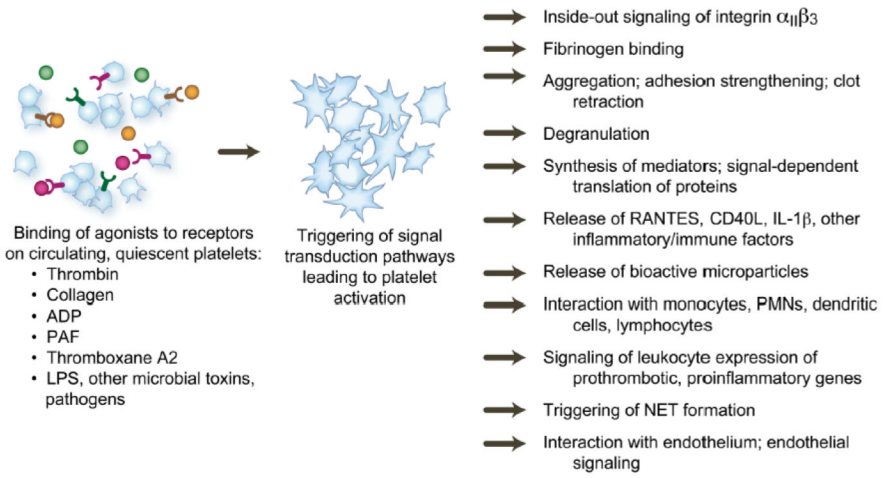

Figure 1.

The repertoire of activated platelets includes diverse mechanisms and molecular pathways that mediate inflammation and immune modulation in addition to hemostasis. Most functional responses shown contribute to both thrombosis and inflammation and to "thromboinflammatory" activities in vascular diseases. Each is discussed in the text.

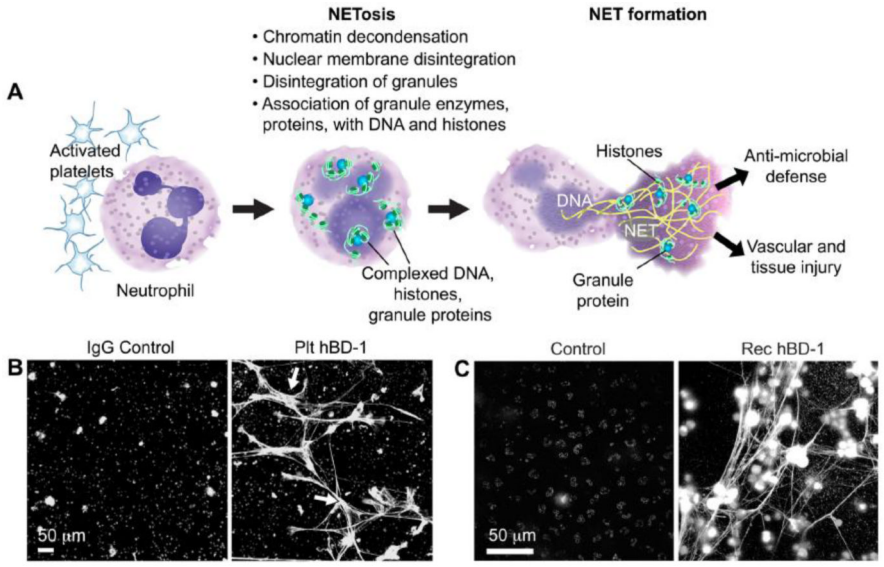

Figure 2.

Human neutrophils form NETs in response to signals from activated platelets in addition to other stimuli (A). NETs have antimicrobial functions, but also mediate inflammatory vascular and tissue injury. Human β-defensin1 immunocaptured from platelets (A) and recombinant hBD-1 (B) trigger NET formation, detected by extracellular DNA staining. Reproduced from [99] with permission of the publisher.

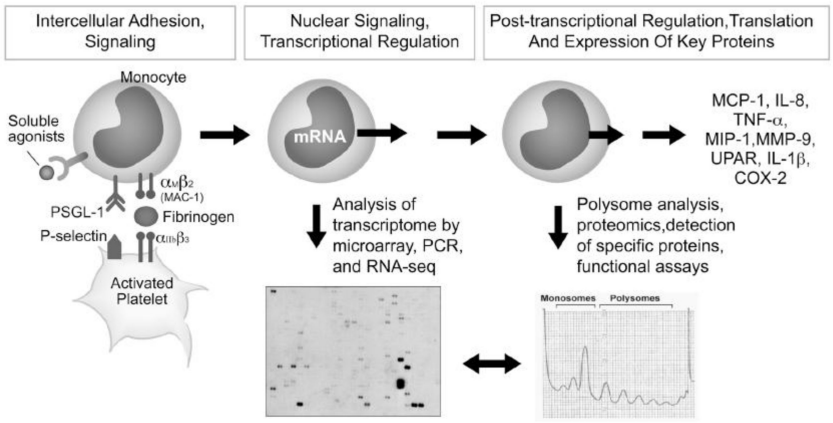

Figure 3.

Human platelets adhere to monocytes via P-selectin engagement of PSGL-1 and deliver signals that induce expression of pro-inflammatory gene products. From [5] with permission.

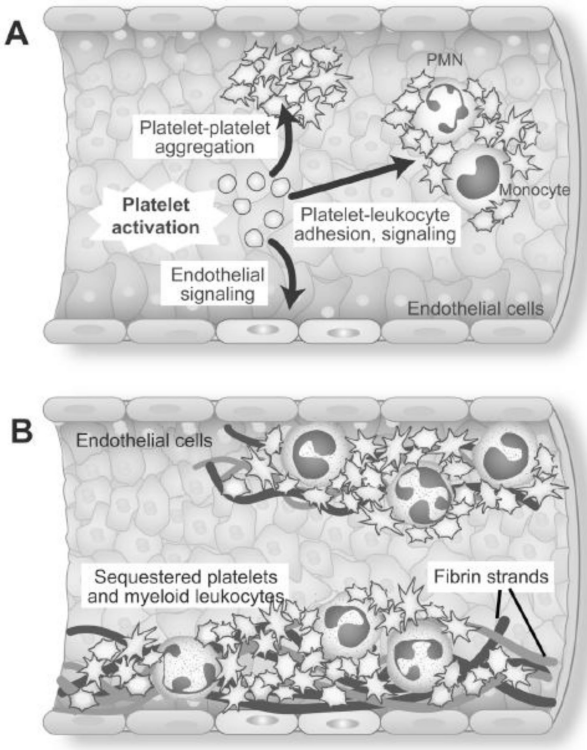

Figure 4.
Each of the effector responses of activated platelets (Figure 1) may be pivotal in the acute inflammatory vasculopathy of experimental and clinical sepsis. Reproduced from [184] with permission.

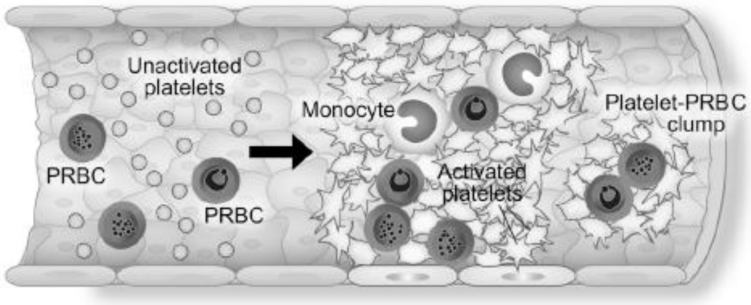

Figure 5.
Platelets, parasitized red blood cells (PRBC), and monocytes accumulate in cerebral and lung vessels in the vasculopathy of severe malaria. Platelet signaling of leukocytes and endothelium may be a key pathophysiologic mechanism. Reproduced from [6] with permission.

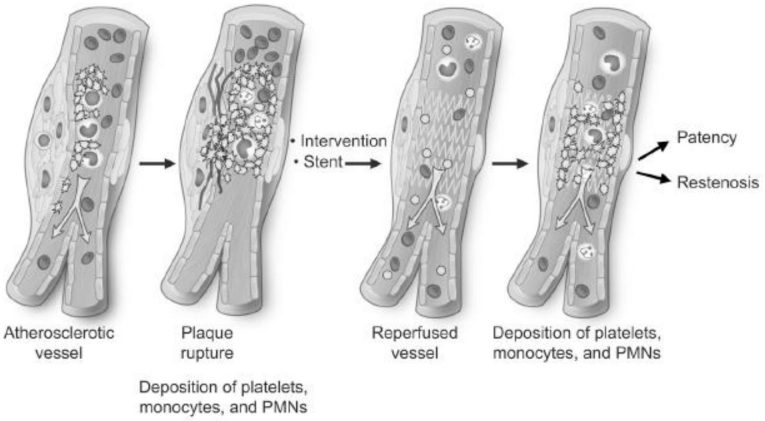

Figure 6.

Platelets are effectors of inflammatory and immune injury in the evolution of atherosclerosis, and in acute thromboinflammatory complications triggered by plaque rupture. Platelets also contribute to pathologic consequences of vascular stenting and angioplasty. From [182] with permission.