

Platelets in defense against bacterial pathogens

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Received: 5 November 2009 / Accepted: 5 November 2009 / Published online: 15 December 2009
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Abstract Platelets interact with bacterial pathogens through a wide array of cellular and molecular mechanisms. The consequences of this interaction may significantly influence the balance between infection and immunity. On the one hand, recent data indicate that certain bacteria may be capable of exploiting these interactions to gain a virulence advantage. Indeed, certain bacterial pathogens appear to have evolved specific ways in which to subvert activated platelets. Hence, it is conceivable that some bacterial pathogens exploit platelet responses. On the other hand, platelets are now known to possess unambiguous structures and functions of host defense effector cells. Recent discoveries emphasize critical features enabling such functions, including expression of toll-like receptors that detect hallmark signals of bacterial infection, an array of microbicidal peptides, as well as other host defense molecules and functions. These concepts are consistent with increased risk and severity of bacterial infection as correlates of clinical abnormalities in platelet quantity and quality. In these respects, the molecular and cellular roles of platelets in host defense against bacterial pathogens are explored with attention on advances in platelet immunobiology.

Keywords Platelets · Host defenses · Bacteria · Pathogen · Interactions

Platelets: the other white cells

Invertebrates contain specialized dual-function cells called hemocytes, which act to maintain hemostasis and defend against microbial pathogens. These unique cells limit hemolymph loss upon tissue injury, engage microorganisms introduced by trauma, and coordinate tissue remodeling and wound repair [1–3]. In higher organisms including mammals and marsupials, platelets, leukocytes, and lymphocytes are often viewed as independently mediating hemostasis, inflammation, and immune regulation, respectively [4, 5]. Of course these cells interact with one another in many ways. Nonetheless, while mammalian platelets may be best known for their maintenance of hemostasis, these understudied cells have retained features of archetypal inflammatory cells corresponding to multiple functions in antimicrobial host defense.

Platelet roles in host defense have been the topic of recent reviews [1, 6–11]. Thus, the focus of the following discussion is to provide a framework of understanding the antimicrobial structures and functions of platelets in defense against bacterial pathogens.

Platelets are ancient granulocytes

Platelets are small (2–4 μm), discoid cells that originate from megakaryocyte lineage [12–16]. Platelets have a life span in the peripheral bloodstream of 5–10 days. These anucleate cells can perform protein translation using stable mRNA templates originating from their megakaryocyte precursors [1, 6–9]. Mature platelets have three distinct types of cytoplasmic granules. Dense (δ) granules are rich in mediators of vascular tone, such as serotonin, adenosine diphosphate (ADP), calcium, and phosphate [1, 17]. In contrast, alpha (α) granules store proteins specialized to

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hemostatic functions, including adhesion (e.g., fibrinogen, thrombospondin, vitronectin, laminin, and von Willebrand factor), coagulation (e.g., plasminogen and α_2 -plasmin inhibitor), and endothelial cell repair [e.g., platelet-derived growth factor (PDGF), permeability factor, and transforming growth factors α and β (TGF- α and TGF- β)] [1, 6–9]. Platelet α -granules also store an array of microbicidal proteins (see below). Lysosomal (λ) granules contain enzymes that modulate thrombus dissolution [6–9, 17]. When activated, platelets respond in specific ways that emphasize their multiple roles in antimicrobial host defense, including: (1) activated metabolic status; (2) metamorphosis from discoid to amoeboid shape; (3) expression of receptors mediating increased adhesivity to injured or infected tissues; (4) vectored motility toward and intensification at sites of tissue injury or infection; (5) generation of reactive oxygen species including superoxide, peroxide, and hydroxyl radicals; (6) extension of pseudopodia that interact with microbial pathogens as well as host cells; (7) cytoskeletal remodeling to facilitate granule mobilization and organization; and (8) degranulation and processing of preformed granule molecules, including host defense peptides (see below). The distinct platelet granules are subject to synchronous or asynchronous release, depending upon agonist specificity and potency. For example, low levels of thrombin or ADP induce δ and α degranulation, whereas λ granules are not secreted until such agonists accumulate to much higher concentrations [6–9, 18–20].

Platelets serve roles that recapitulate and complement cell-mediated immune effector cells. *In vitro* and in experimental animal models, platelets intensify at sites enriched in microbial stimuli such as *N*-formyl-methionyl-leucyl-phenylalanine (*N*-*f*-met-leu-phe) [29] or complement proteins C3a and C5a [1, 7, 25, 30]. Experiments by Czapigaa et al. show that platelets express the formyl peptide receptor (FPR), and surfaces of activated platelets specifically ligate *N*-formyl peptides, followed by calcium mobilization [31]. Gradient-mediated chemotaxis of platelets occurs in response to formyl peptides. Notably, endogenous *N*-formyl peptides liberated from mitochondria of necrotic host cells evoke platelet chemotaxis via FPR recognition; however, cells undergoing apoptotic cell death do not appear to induce platelet chemotaxis. Such relationships further implicate archetypal phylogenetic origins of platelets in host defense against infection.

The concept that platelets contribute to host defense against infection is substantiated by a line of evidence relevant to platelet structure and function. For example, platelets and professional phagocytes such as neutrophils and monocytes share common surface antigens: the 40-kDa Fc γ RII receptor [21], Fc ϵ RI receptor for IgE [22], C-reactive protein receptor [23], and the thrombospondin

receptor CD36 (platelet GPIV) [24]. Platelets also express complement CR3 receptor [25], receptors for C3a and C5a products of complement fixation, and sense and respond to cytokines such as tumor necrosis factor- α (TNF- α), interleukin-1 (IL-1), and IL-6 in a manner similar to leukocytes [26, 27]. Clemetson et al. [28] reported that human platelets express functional chemokine receptors, including CCR1, CCR3, CCR4, and CXCR4.

As discussed below, platelets interact directly and indirectly with a broad variety of microbial pathogens, and can internalize microorganisms, likely enhancing pathogen clearance from the bloodstream [1, 32, 33]. Platelets stimulated through interactions with microbes or their signals generate reactive oxygen species that exert direct antimicrobial activity. Similar to neutrophils and macrophages, granules of activated platelets are mobilized by microtubule assembly, and subsequently secrete. However, in contrast to leukocyte degranulation directed to intracellular phagolysosomes, platelet degranulation directs the majority of granule constituents to the extracellular milieu. Taken together, these characteristics strongly support the concept that platelets are ancient granulocytes that recognize signals and organisms relevant to settings of infection, and respond at these sites to confer important host defense functions.

Platelets bridge innate and adaptive immunity

Platelets make an array of constitutive and inducible receptors that confer these cells the ability to sense and respond to hallmark signals of infection. To do so, platelets express several toll-like receptors (TLRs) that recognize cognate pathogen-associated molecular patterns (PAMPs). Thus, platelets function as sentinel cells to detect the presence of potentially harmful microbes. For example, quiescent CD41(+) platelets express low levels of TLR2, TLR4, and TLR9 on their surface. Expression of these TLRs is significantly increased upon platelet activation [34]. For example, thrombin activation induces significant upregulation of TLR9 expression, and human platelets express TLR1 and TLR6, particularly in settings of vascular lesions potentially mediated by bacterial infection [35]. In addition, bacterial LPS causes increased expression of functional TLR4 *in vivo*, and such activation and TLR expression appears to promote tumor-necrosis factor alpha (TNF- α) expression [36]. Cognasse et al. [37] recently showed that LPS stimulates platelet release of cytokines, platelet microbicidal proteins, and kinocidins by way of TLR4. Of note, incubation of isolated platelets with a synthetic TLR1/TLR2 agonist directly induces platelet aggregation and collagen adherence [38]. Stimulation of platelet TLR2 also increased platelet surface expression of P-selectin and integrin glycoprotein IIb/IIIa, generation

of reactive oxygen species, and, generation of platelet–neutrophil aggregates in whole human blood.

Despite such structural and functional evidence relating to roles in innate immunity, platelets have traditionally been viewed only as cellular fragments incapable of complex molecular pathways. This view is currently evolving. It is now known that platelets express constituents of the lipopolysaccharide (LPS) receptor–signaling complex, including toll-like receptor-4 (TLR4), TLR2, TLR9, CD14, MD2, and MyD88. Zhang et al. [39] have demonstrated that LPS evokes platelet activation and secretion conferred by a TLR4-mediated process. Here, LPS caused platelet δ - and α -degranulation as shown by ATP release and P-selectin expression, respectively. The platelet response to LPS was eliminated in the presence of α -TLR4 antibody, or using platelets from TLR4 knockout animals. Further, LPS-induced platelet aggregation and thrombus formation were mitigated in MyD88 null mice. This LPS-mediated cGMP upregulation was inhibited by α -TLR4 or TLR4 deficiency, implicating that platelets respond to LPS through a cGMP/protein kinase G pathway linked to TLR4. These findings suggest that platelets are indeed capable of TLR-mediated sense and response functions important to effector roles in host defense.

Interestingly, platelet TLR4 has emerged in a putative new mechanism of host defense. Clark et al. [40] have suggested that platelet TLR4 detects cognate ligands in blood, inducing platelet adhesion and subsequent neutrophils adherence. This sense and response relay yields vigorous neutrophil activation and subsequent formation of neutrophil extracellular traps (NETs). The formation of bacterial-capturing NETs was predominant in fenestrated tissues such as the liver sinusoids and pulmonary capillaries, where bacterial trapping is most efficient. Hence, it is possible that platelet TLR4-mediated responses to bacteria lead to platelet–neutrophil interactions that enhance bacterial clearance.

It should be noted that thrombocytes, ancestral nucleated platelets from vertebrates earlier than mammalian or marsupial, also express TLRs that mediate important host defense functions. For example, Scott et al. [41] found that avian thrombocytes express IL-6 and COX-2 mRNA in response to LPS via a TLR4 pathway. As a result, bioactive prostaglandin PGE₂ is generated. Importantly, the inhibition of IKK-2 mitigated COX-2 expression, yielding reduced PGE₂ accumulation. These findings suggest that MAP kinase pathways are in play in COX-2 and PGE₂ production, but not IL-6 expression in these cells. In related studies, platelets have been shown to enhance neutrophil oxidative burst in response to Gram-positive as well as Gram-negative bacteria [42]. Moreover, Bennett et al. demonstrated that platelets participate in the regulation of B- and T-lymphocyte- and immunoglobulin-mediated

immune responses via CD40/ligand–CD40 interactions [9]. Similarly, Blumberg et al. [43] hypothesized that soluble CD40 ligand (CD154) supernatants of stored platelets can induce cytokines, chemokines, and lipid mediators through activation of CD40-receptor-bearing cells. Although preliminary, such themes support the emerging concept that platelets actively participate in bridging the innate and adaptive immune response to infection.

Taken together, the expression of various TLRs, and an ability to bridge interactions with neutrophils and perhaps T and B cells suggests mechanisms through which platelets may directly or indirectly detect bacterial pathogens by recognition of molecular patterns and early warning signals of infection, and then contribute to optimization of immune response.

Platelets sense bacterial infection of endothelium

Beyond their ability to detect microbes themselves, platelets are exquisite sensors for signals of endothelial infection. Endothelial cells damaged by infection undergo changes in surface protein repertoires and secretory profiles that activate platelet responses. Distinct platelet receptors sense agonists and bind to ligands on the surface of activated endothelial cells, or exposed subendothelium due to infection or tissue invasion. Signals detected by platelet membrane glycoprotein (GP) receptors include: collagen [(GPVI and $\alpha 2\beta 1$ (VLA-2 or GPIIb-IIIa)), fibronectin ($\alpha 5\beta 1$ or VLA-5), laminin ($\alpha 6\beta 1$ or VLA-6), von Willebrand factor (GPIb-IX-V), vitronectin (integrin $\alpha V\beta 3$), and thrombin] [1, 6–10]. Contact with blood or microbes induces tissue factor release from subendothelial stroma, thereby catalyzing thrombin production [1, 44–46]. Thrombin is a potent platelet agonist [1, 7, 17], which induces expression of ensuing platelet receptors, including the fibrinogen receptor [integrin $\alpha IIb\beta 3$ (GPIIb-IIIa)]. Activated platelets also undergo metamorphosis from a discoid to amoeboid form, along with microtubule assembly and granule reorganization [47, 48]. Degranulation follows, releasing an array of bioactive molecules, including ADP from δ -granules, and generating thromboxane A₂ and platelet-activating factor (PAF) through activation of membrane phospholipase A₂ [1, 7, 17]. These potent agonists trigger subsequent waves of platelet activation at sites of tissue colonization, promoting an accumulation of antimicrobial proteins from α -granules ([49]; also see below). In addition, P-selectin (CD62P) is exposed on the activated platelet plasma membrane, potentiating leukocyte rolling and eventual recruitment to these sites.

Platelets accumulate at sites of tissue infection

Platelets are the earliest and most numerous cells to accumulate at sites of vascular infections [50–60],

including: infective endocarditis, suppurative thrombophlebitis, mycotic aneurysm, septic endarteritis, catheter and dialysis access site infections, and infections of vascular prostheses and stents. The rapid and intensive accumulation of platelets at these sites has long been known. In 1886, Osler [61] reported perhaps the first observation of platelets rapidly coating filaments introduced into animal blood vessels. Subsequently, Cheung and Fischetti [62] and others demonstrated that platelets are the first cells to adhere to indwelling vascular catheters. Similarly, platelets rapidly adhere to cardiac valve prostheses and vascular stents [60], and are abundant cells in early phases of endocarditis vegetations in rabbits and humans. Once again, such rapid and targeted responses to sites of tissue injury or microbial invasion support a specific role for platelets in host defense.

Subversion of platelet functions by bacterial pathogens

Vascular infections caused by virulent bacterial pathogens such as *Staphylococcus aureus* have traditionally been considered to proceed through multiple phases: (1) adherence of hematogenous pathogens to normal or compromised vascular endothelium (e.g., rheumatic conditions, proximal to artificial device such as a stent or prosthetic valve, etc.); (2) endothelial cell expression of selectins and secretory agonists that promote platelet activation, adhesion, and platelet/fibrin matrix formation [1, 63, 64]; and (3) ensuing deposition of circulating platelets in response to successive waves of agonists (e.g., thromboxane A₂, ADP, PAF), or ligands (e.g., integrin α IIb β 3) associated with secondary platelet activation [1, 7, 17]. Less virulent pathogens, such as viridans streptococci, are thought to require pre-existing abnormalities or injury to endothelial surfaces to initiate endovascular infection through indirect pathways: (1) fibrinogen-mediated adherence to intact or abnormal vascular endothelium or non-bacterial thrombotic vegetations [1, 54]; (2) vascular endothelial cell expression of platelet ligands and agonists (see above); and (3) further platelet/fibrin deposition and evolution of the lesion [1, 7, 10]. In either case, platelets localize to sites of infection of the vascular endothelium, and represent the most rapid and abundant inflammatory cells that respond to endothelial cell damage or microbial colonization. Thus, platelets represent the earliest opportunity for inflammatory responses to intercede in host defense against vascular infection.

Studies over the last two decades have clearly demonstrated that platelets have explicit roles in antimicrobial host defense. Yet, historically, platelets were viewed as promoting the establishment and evolution of endovascular infection [1, 7, 53, 65, 66]. Early studies were interpreted to suggest that platelets facilitate microbial adhesion to fibrin matrices or endothelial cells in vitro [66]. Subsequent

data demonstrated that bacterial and fungal pathogens can aggregate human and rabbit platelets in vitro [67–69]. Herzberg et al. [71] hypothesized that streptococcal binding to and aggregation of platelets may increase virulence of these strains in experimental animal models of endocarditis. Platelet aggregation has also been hypothesized to be detrimental to the host, since massive endovascular vegetations can be associated with clinical complications such as emboli and infarcts [72]. Finally, some investigators have suggested that platelet aggregation and enclosure of microorganisms in platelet–fibrin matrices may protect pathogens from antibiotics or clearance by neutrophils or other leukocytes [73].

Platelets have known antimicrobial structures and functions, and interact with bacteria and other pathogens directly and indirectly. Moreover, no studies substantiate the concept that platelets inherently facilitate microbial pathogenesis or inhibit the immune response. Rather, evolutionary adaptation would have almost certainly selected against such cells. Therefore, the consequences of platelet interaction with bacteria or other pathogens likely rests on the efficacy platelet antimicrobial mechanisms or their interaction with other immune defenses versus the ability of a pathogen to circumvent or survive in the face of such mechanisms.

Platelets interact with bacterial pathogens

Platelets interact with all kingdoms of microbial pathogens, including viruses, bacteria, fungi, and protozoa. However, platelets interact with distinct types of pathogens in different ways. Such interactions may be direct or indirect, and can yield significant antimicrobial responses and mechanisms. As thorough reviews of such interactions have been previously compiled [1, 6–10, 17], the following discussion focuses on interactions between platelets and human bacterial pathogens. These interactions are viewed with an emphasis on key themes and recent advances in understanding this host–pathogen relationship.

Key events in platelet–bacterial relationships

Platelets engage bacterial pathogens directly and indirectly in vitro and in vivo. The earliest methodical studies of such relationships were those of Clawson and White [73–76]. In their studies, bacterial pathogens were shown to adhere to, aggregate, activate, and prompt degranulation of human and other mammalian platelets. Typically, the sequence of events associated with platelet–bacterial interaction proceeds through distinct and progressive phases: (1) direct contact, (2) morphogenesis, (3) initial aggregation, and (4)

irreversible aggregation [74]. As this process evolves, platelets undergo a significant shape change from resting discoid cells to amoeboid forms that form multiple pseudopodia. This shape transition typically occurs well in advance of platelet aggregation, leading to platelet microtubule organization such that granules are mobilized from the platelet perimeter to the cytoplasm [75]. This organization of granules precedes platelet degranulation [76] and secretion of an array of host defense molecules (also see below).

Diverse bacterial genera, and even different species of the same genus, may differentially adhere to, activate, aggregate, and induce platelet degranulation. For example, Clawson and White, and many platelet scientists since, have shown that *Streptococcus pyogenes*, *Staphylococcus aureus*, *Enterococcus faecalis*, and *Escherichia coli* rapidly adhere to and promote aggregation of human platelets in vitro [73–76]. Beyond these organisms, diverse pathogenic species interact with and aggregate platelets in vitro, including *Fusobacterium* [77], *Listeria* [78], *Mycobacterium* [79], *Pseudomonas* [80], *Salmonella* [81, 82], and *Yersinia* [83]. In contrast, bacteria that gain entry into the bloodstream, including *Staphylococcus epidermidis* and *Streptococcus pneumoniae*, do not trigger irreversible platelet aggregation [74]. Thus, the consequences of bacterial interactions with platelets adherence, activation, and antimicrobial response profile likely significantly influence host defense versus virulence of a given pathogen. The following section considers platelet interactions with staphylococci, streptococci, and notable Gram-negative pathogens such as prototypic bloodstream pathogens in these respects.

Staphylococcal interactions with platelets

Species of the genus *Staphylococcus* are currently the most frequent etiologic agents of infective endocarditis ([84–86]), and the second most frequent agents of bacteremia [87–89]. The incidence of *S. aureus* invasive infections continues to increase sharply [90], and now occurs in up to 20,000 new patients per year in the United States alone [91–93]. Even with modern advances in anti-staphylococcal therapy and endovascular surgery methods and devices, the 1-year mortality of these infections approximates 40% [86–93]. More concerning still is the global burgeoning in *S. aureus* resistance to conventional antimicrobial agents and strategies [94, 95].

Aside from *Streptococcus* spp., the interactions of platelets and with staphylococci, and in particular *S. aureus*, have been the most well-characterized. Traditionally, investigations focused on two distinguishable but complementary facets of this relationship: (1) toxins and other

secretory and capsular components; and (2) surface protein mediators of platelet activation.

Perhaps the most thoroughly studied *S. aureus* toxin or secretory factor interacting with platelet function is alpha (α) toxin. The expression of this exotoxin is accessory-gene regulon (*agr*)-regulated, and targets platelets as well as other host cells. The toxin is believed to interact with the cell membrane of platelets, erythrocytes, and even some leukocytes, inducing injury and ultimately cellular lysis. Notably, Bayer et al. [96] found a paradoxical relationship between *S. aureus* α -toxin expression and virulence in vivo, specifically pertaining to platelet interactions. These authors evaluated the impact of α -toxin in experimental endocarditis in vivo using isogenic strains differing in expression of functional versus mutant forms of α -toxin. In vitro, the parental α -toxin induced platelet lysis and secretion of platelet microbicidal proteins (PMPs; see below) active against *S. aureus*. In experimental endocarditis, the *S. aureus* strains producing either minimal α -toxin or non-functional α -toxin were significantly less virulent in vivo than wild-type strains. Paradoxically, an isogenic strain constructed to overexpress α -toxin was also significantly less virulent than its parental counterpart in this animal model. Moreover, increased α -toxin expression correlated with greater PMP release by exposed platelets. Together, these outcomes indicated that expression of excessive α -toxin leads to increased PMP release secondary to either platelet secretion or lysis, thus increasing anti-staphylococcal platelet responses and reduced virulence in experimental endocarditis. This phenomenon is referred to as a “Goldilocks Effect” in *S. aureus*–host relationships.

Regarding non-soluble mediators, most studies focusing on interactions of platelets with *S. aureus* have focused on surface proteins expressed by the pathogen. In early experiments, Hawiger et al. [97] demonstrated that *S. aureus* triggers platelet morphogenesis, aggregation, and degranulation in a pathway involving *S. aureus* protein A, host IgG, and platelet Fc receptors. Considerable work since then has revealed that this organism employs several factors to interact with platelets when it is advantageous to do so. For example, *S. aureus* surface proteins that mediate direct or indirect platelet interactions include clumping factors A and B (*ClfA/ClfB*), fibronectin-binding proteins A and B (*FnbA/FnbB*), protein A (*Spa*), serine-aspartate-repeat protein E (*SdrE*), serine-rich adhesin for platelets (*SraP*), extracellular fibrinogen-binding protein (*Efb*), and other molecules. A meritorious review of these *S. aureus* surface proteins, and homologous factors of other pathogens has been compiled by Fitzgerald et al. [10]. Hence, the following comments focus on fundamental themes regarding such surface proteins and recent studies of their platelet interactions.

In complementary studies, Sullam et al., Yeaman et al., and Bayer et al. used classical aggregometry and multi-color flow cytometry to investigate the molecular and cellular interactions between staphylococci and platelets [98–100]. These studies revealed that staphylococci adhere directly to human or rabbit platelets in vitro, in the absence of plasma. Notably, the *S. aureus*-to-platelet ratio directly influences the extent and velocity with which platelet aggregation follows after initial contact [99]. Additionally, rapid adherence to platelets occurs via saturable and reversible kinetics that are indicative of receptor–ligand interactions. As multiple *S. aureus* surface proteins can mediate platelet interactions, such findings suggest that each receptor–ligand interaction may occur independently as well as cooperatively. Recently, Siboo et al. [101] characterized a serine-rich protein, *SraP*, that mediates adhesion of *S. aureus* to platelets. Moreover, a mutant deficient in *SraP* has a reduced virulence phenotype in rabbit models of infective endocarditis. This protein is 227 kDa and, similar to its streptococcal homolog *GspB*, is predicted to contain unusual N-terminal signal sequences, two serine-rich repeat domains, and a C-terminal motif likely required for cell wall anchoring (LPDTG; also see below).

In related studies, Niemann et al. [102] reported that platelet quality influences *S. aureus*–platelet interactions. In their investigation, platelets obtained from patients with specific inherited deficiencies in platelet granule contents (e.g., α - δ storage pool disease or Gray platelet syndrome) were assessed for interactions with *S. aureus*. Interestingly, CD36 (thrombospondin receptor), GPIIb/IIIa (a major platelet integrin receptor), and CD62 (P-selectin) did not play significant roles as receptors for *S. aureus*. Further, platelet activation by ADP did not promote *S. aureus* adhesion, although platelets were found to be bound by fibrinogen. On the contrary, soluble fibrin (but not fibrinogen), clumping factor A and thrombospondin-1 increased platelet binding to *S. aureus*. Thus, these latter molecules likely act as bridging matrices that promote *S. aureus*–platelet interactions.

Such findings highlight an emerging theme increasingly evident in interactions between staphylococci and platelets: *S. aureus* and other staphylococci exploit plasma cofactors to facilitate interaction with platelets when advantageous, or limit interactions to subvert platelet antimicrobial responses. For example, *S. aureus* interacts with vascular endothelium and platelets via fibrinogen bridging [103, 104]. Bayer et al. [100] found that *S. aureus* can induce aggregation of rabbit platelets via a fibrinogen-dependent mechanism independent of GPIIb/IIIa. Moreover, fibronectin is not significantly involved in staphylococcal adhesion to quiescent platelets. In contrast, fibronectin does appear to facilitate *S. aureus* interactions with activated

platelet–fibrin clots or stimulated endothelium [105]. Likewise, Hermann et al. [106] found that *S. aureus* exploits thrombospondin to adhere to activated platelets and extracellular matrices. Loughman et al. [107] further demonstrated that cognate immunoglobulin directed against clumping factor (*ClfA*) can modulate *S. aureus*–platelet adherence. Alternatively, *S. aureus* has been shown to bind to platelets through mechanisms involving protein A and the platelet C1q complement receptor, gC1qR/p33 [108]. As gC1qR/p33 is expressed upon activated but not quiescent platelets, *S. aureus* may specifically exploit adhesion to activated platelets. Such examples highlight the emerging theme that *S. aureus* and platelets interact directly and indirectly through multiple soluble and cell-associated bridging ligands.

Other new evidence indicates that the serine-aspartate-repeat protein G (SdrG), a fibrinogen-binding protein present in a majority of clinical *S. epidermidis* isolates, is sufficient to enable this organism to adhere to and aggregate platelets [110]. Brennan et al. employed surrogate genetics to show that *Lactococcus lactis* expressing SdrG induces platelet aggregation in a manner dependent on both fibrinogen and IgG, and on the platelet IgG receptor Fc γ RIIa. Pre-exposure of SdrG-expressing *L. lactis* to β -chain fibrinopeptide reduced platelet aggregation; substantiating the interpretation that fibrinogen is a bridging intermediate. Interestingly, in their studies, such aggregation was inhibitable by GPIIb/IIIa antagonists as well as by aspirin.

Lastly, a new role for *S. aureus*–platelet interactions has recently been put forth. Youssefian et al. [109] reported that platelets can actively phagocytose *Staphylococcus aureus*. Internalization of the organism corresponded to morphological transition consistent with platelet activation. In turn, platelet activation recursively enhanced *S. aureus* internalization. Although these findings support the emerging view that platelets are active host defense cells, it remains to be seen whether platelet antimicrobial responses are related to internalization, or result from degranulation of microbicidal proteins to target extracellular pathogens (see below).

Streptococcal interactions with platelets

Pathogenic members of the genus *Streptococcus* are typically viewed as human commensal organisms that can gain entry to the bloodstream. Until recently, streptococci (e.g., *Streptococcus sanguis*) were viewed as the most common etiologic agents of infective endocarditis (IE); *S. aureus* has now assumed that role. Nonetheless, the fact that most humans experience transient streptococcal bacteremia regularly without the onset of endovascular infections or infective endocarditis suggests there are

highly efficacious host defense interactions between platelets and streptococci. In this respect, a substantial body of literature has been published investigating the ability of various streptococcal species to interact with human platelets. The following section illustrates comparative themes and recent insights into this relationship.

Traditionally, streptococci have been found to interact with platelets through a variety of mechanisms and molecular intermediates. Herzberg et al. reported that certain viridans streptococci such as *Streptococcus sanguis* adhere directly to the platelet surface via a 150-kDa two-domain adhesin [70, 71, 111]. Zimmerman et al. [112] showed that *St. pneumoniae* aggregates platelets only in the presence of cognate anti-pneumococcal antibody in vitro. As in *S. aureus*, Ford et al. [113] demonstrated that *St. sanguis* aggregates platelets through a fibrinogen pathway involving platelet integrin GPIIb/IIIa receptor. Similarly, platelet aggregation and degranulation prompted by *St. pyogenes* can be mediated by fibrinogen [114], while Johnson and Bowie [115] reported that some group C streptococci indirectly with platelets through the von Willebrand factor.

New evidence derived from contemporary methods in microbial genetics and flow cytometry have greatly advanced our understanding of streptococcal–platelet interactions. As seen in *S. aureus*, such findings illustrate how streptococci interact with platelets directly and indirectly. For example, although *St. gordonii* has been known to interact with platelets, new insights are emerging indicating that very specific intermediaries result in its adherence to and activation of platelets. Using surrogate genetics, Kerrigan et al. [116] demonstrated that overexpression of *St. gordonii* SspA or SspB (antigen I/II family proteins) in null *Lactococcus lactis* enabled the latter organism to induce platelet aggregation, but not adhere to platelets. This finding suggests that the Ssp-family proteins may mediate soluble activation of platelets. In contrast, *St. gordonii* Hsa protein (a homolog of GspB) facilitated platelet adhesion. Thus, *St. gordonii* appears to have distinct molecular mechanisms for platelet adhesion and platelet aggregation. Given its ability to cause IE in humans, it is intriguing to speculate that regulated expression of these or other platelet interaction molecules may determine how *St. gordonii* or other streptococcal pathogens interact with platelets to exploit these host cells, or avoid platelets if it is advantageous to subvert antimicrobial responses of these cells.

Perhaps the most novel insights into streptococcal–platelet interactions in recent years have emerged from important studies performed in the Sullam laboratory. Prior studies by Sullam et al. found that viridans group streptococci aggregate human platelets via direct platelet binding, as well as through plasma cofactors [68, 98, 117]. For

example, the aggregation of human platelets by some streptococci requires organism-specific IgG, which presumably interacts with the platelet 40-kDa Fc γ RII surface receptor [117]. Ensuing studies by Bensing et al. [118] identified two genetic loci in *St. mitis* that appeared to mediate binding to human platelets in vitro. These discoveries suggest that interactions between streptococci and platelets occurs via specific receptor–ligand interactions. More recently, this group demonstrated that two platelet-binding adhesins from *St. mitis*—PbIA and PbIB—are encoded as a result of lysogenic prophage integration [119]. Interestingly, translocation of these proteins to the bacterial cell wall for surface display requires holin or lysin gene expression. The export of these proteins apparently requires membrane permeabilization, enabling anchoring to cell wall choline rather than conventional anchoring motifs. More importantly, loss of PbIA and PbIB expression yields decreased platelet binding in vitro, and reduced virulence in experimental IE. More recent reports from this group indicate that PbIA and PbIB proteins target α 2-8-linked sialic acids on ganglioside GD3 for adhesion to human platelets [120]. Thus, this body of work reveals how streptococci appear to interact with human platelets through highly sophisticated molecular and cellular determinants.

In parallel to their studies above, the Sullam laboratory investigated the role of cell surface glycoprotein GspB in *St. gordonii* adherence to platelets [121]. Isogenic strains of *St. gordonii* M99 with or without gspB expression were evaluated in models of infection in rats, independently and in strain competition studies. When infected independently, cardiac vegetation, kidney, and spleen tissues had significantly lower densities of the null mutant as compared with the wild-type strain. Further, in competition studies in which both strains were co-infected, the wild-type strain achieved significant CFU densities in these tissues, while the gspB mutant was not detectable. These findings strongly suggest that GspB confers an important platelet binding function to this organism that may be important to virulence in vascular pathogenesis. It is also noteworthy that prior studies by this laboratory demonstrated that glycosylation of GspB is mediated in part by Gly and Nss proteins encoded in the secY2A2 locus [122]. Complementary genetic and protein analyses revealed that Gly, Nss, Gtf, and Orf4 are involved in the intracellular glycosylation and solubility of GspB. Thus, extracellular access and glycosylation state appear to be key to the ability of GspB to promote adhesion of *St. gordonii* to human platelets.

Bacteria must circumvent or subvert the significant antimicrobial mechanisms of platelets to exploit these cells in virulence strategies. Alternatively, it is possible that profusive platelet responses to streptococci may contribute

to adverse outcomes not necessarily related to virulence. For example, Ohba et al. [123] demonstrated in mice that *St. intermedius* or *St. constellatus* injected intravenously triggered platelet-mediated responses mirroring septic shock. In animals primed with muramyl dipeptide, whole organisms introduced into the vascular compartment caused a rapid anaphylactoid-like shock response. However, in non-primed animals, little or no sign of shock was observed. Interestingly, the cardinal signs of shock response were preceded by platelet degranulation and 5-hydroxytryptamine (5HT) secretion, as well as complement fixation yielding activated C5. The septic-shock-like paradigm was mitigated by a complement-C5 inhibitor. Collectively, these results suggest that platelets respond to streptococci by activating host defense mechanisms including degranulation and complement fixation. However, certain pathogens may hyperstimulate platelets such that activation, degranulation, complement fixation, or other immune responses occur to an excessive degree that results in non-optimal host outcomes.

The studies highlighted above illustrate the emerging theme that distinct streptococcal species and phenotypes vary in interactions with human platelets. It is highly likely that distinct pathogens have co-evolved with hosts so as to optimize their interactions with platelets for fitness advantages. For example, early studies demonstrated that *St. pyogenes* can prompt platelet aggregation perhaps by a molecular mimic of collagen [124]. In contrast, *St. pneumoniae* appears to optimally induce aggregation by way of an antibody-dependent mechanism [112]. In contrast, *St. agalactiae* expresses a fibrinogen-binding protein known as FbsA that confers adhesion and aggregation of platelets mediated by fibrinogen and IgG [125]. These and other theme-and-variation examples illustrate how pathogens such as streptococci or staphylococci that often encounter platelets and other host defenses in the bloodstream have necessarily evolved diverse mechanisms to interact with platelets. It is conceivable these some adaptations may provide advantages to enable pathogens to circumvent or subvert platelet-mediated host defense mechanisms.

Gram-negative pathogen interactions with platelets

Relatively little is known about platelet interactions with Gram-negative pathogens as compared with Gram-positive counterparts. Other reviews offer comprehensive perspectives of such comparisons [1, 6–10]. Here, the following discussion highlights the current view that Gram-negative pathogens capable of entering the bloodstream have important interactions with platelets that likely contribute to the difference between virulence and host defense.

One of the best examples of this theme can be considered from studies assessing interactions between platelets and *Pseudomonas aeruginosa* or *Yersinia* species. Coutinho et al. [126] reported that the major phospholipase C from *P. aeruginosa* caused platelet aggregation in a dose-dependent manner. Interestingly, the use of heat-denatured enzyme, or nitrophenol-phosphoryl-choline (inactive substrate of phospholipase C), revealed that enzymatic function of phospholipase C was required for platelet activation. Furthermore, ADP, epinephrine, collagen, or ristocetin were unable to inhibit phospholipase C activation of platelets, and lack of inhibition by aspirin or nordihydroguaiaretic acid suggested that the mechanism was independent of prostaglandin or lipooxygenase pathways.

In recent studies, Machado et al. [127] examined the role of *P. aeruginosa* phospholipase A2 on platelet activation in a mouse model of infection. Pseudomonas phospholipase A2 is encoded by the *ExoU* gene, and is a well-established cytotoxin. In a pneumosepsis model, mice instilled intratracheally with ExoU-producing *P. aeruginosa* caused a significantly higher mortality rate, reduced leukocyte concentration, higher platelet concentration, and higher hematocrit than animals infected with the *exoU* null mutant. Moreover, platelets from mice infected with the wild-type strain were significantly more activated than the null mutant, as indicated by P-selectin expression, platelet-derived microparticle detection, and immunoassay detection of thromboxane A2 (TXA2). Lung and kidney histopathology of wild-type-infected mice revealed thrombus formation that was absent in null-infected animals. Based on these observations, it appears that activation and thrombus formation in response to phospholipase A2-producing strains of *P. aeruginosa* may contribute to pathogenesis.

Yersinia species also appear to interact with platelets in ways that are likely to influence to the consequences of pathogenesis versus host defense. Recent studies by Shepel et al. [128] examined interactions of *Y. enterocolitica* and *Y. pseudotuberculosis* with platelets in vitro. From prior studies, *Y. pseudotuberculosis* is known to be capable of entering platelets and inducing platelet aggregation via a mechanism involving expression of the *inv* invasin gene. In the recent studies, both *Yersinia* strains were engineered to express green fluorescent protein to enable real-time assessment of interaction with platelets by flow cytometry. While both strains adhered to platelets, only *Y. pseudotuberculosis* caused platelet activation and aggregation. Moreover, *Y. enterocolitica* bearing virulence plasmid pYV+ interacted with platelets, while a pYV– strain did not. Transmission electron microscopy revealed that *Y. pseudotuberculosis* expressing the *inv* gene was able to invade platelets. However, *Y. enterocolitica* or

Y. pseudotuberculosis devoid of the *inv* gene remained on the extracellular surface of platelets.

An interesting recent study, Bhat et al. [129] analyzed potential correlates of organism-specific infection, platelet response, and thrombocytopenia in low birth weight neonates with sepsis. Over a 5-year period, very low birth weight neonates (<1,500 g) in a level-three intensive care unit were prospectively evaluated for sepsis. Gram-positive sepsis occurred in 20% of these patients, Gram-negative in 71%, and fungal sepsis in 8.6%. Thrombocytopenia was observed in 67% of patients overall. The frequency and duration of thrombocytopenia were significantly greater in Gram-negative and fungal infections; persistent bacteremia, multiorgan failure, and mortality were significantly greater in thrombocytopenic patients. Importantly, multiorgan failure and mortality were directly correlated with duration of thrombocytopenia. On multiple logistic regression analysis, the worst prognostic indicators were a severe drop in platelet count at onset of sepsis, a low platelet nadir, prolonged duration of thrombocytopenia, and need for platelet transfusion. Given the majority of poor outcomes correlating with Gram-negative infections, molecular interactions of platelets with such organisms, and the virulence versus host defense consequences of these interactions, remain areas that merit considerable focused research.

Platelet mechanisms of host defense against pathogenic bacteria

Chronologic overview

Historically, platelets have been viewed as contributing to microbial pathogenesis. However, it is now evident that platelets have explicit structures and functions that contribute significantly to host defense against infection. Over the past decade, contemporary techniques in host–pathogen relationships, protein chemistry, and molecular biology have been applied to identify and define the molecular effectors and mechanisms of platelet host defenses. Platelets interact rapidly, directly, and specifically with bacterial pathogens, liberate an array of direct antimicrobial molecules, and shape adaptive immune responses. Detailed reviews of such platelet mechanisms of molecular and cellular antimicrobial defense have been reviewed elsewhere [1, 6–10].

Perhaps the earliest mention of platelet antimicrobial function was in 1887, when Fodor reported a heat-stable bactericidal capacity of serum, termed β -lysin, distinct from heat-labile or α -lysin complement proteins [130]. Later, Gengou demonstrated that β -lysin bactericidal activity of serum derived from cells involved coagulation,

and was independent of complement [131]. In 1938, Tocantins reviewed studies that suggested possible immune functions of platelets [5]. Subsequent studies by Hirsch demonstrated that platelets, not classical leukocytes, conferred bactericidal activity to rabbit serum [132]. Eventually, many other noted investigators, including Jago and Jacox [133], Weksler [134], Kahn et al. [135], Czuprynski and Balish [78], and Miragliotta et al. [136] affirmed that platelets exert in vitro bacteriostatic and/or bactericidal effects versus many pathogens, including *Bacillus*, *Staphylococcus*, *Listeria*, *Salmonella*, and many other organisms.

In subsequent studies, numerous investigators studied platelet-specific molecule(s) that might be associated with the antimicrobial action of serum [133, 137–144]. Among the earliest of these reports, Myrvik [140] demonstrated that two platelet-derived serum factors were responsible for killing of *Bacillus subtilis*. Jago and Jacox [133] and Myrvik and Leake [141] confirmed these findings. Later, Donaldson et al. [142], Johnson et al. [143], and other groups isolated putative β -lysins from rabbit serum that exerted *S. aureus*- or *B. subtilis*-specific killing. In parallel, Weksler and Nachman [144] isolated two cationic proteins from platelets with molecular masses of 10 and 40 kDa that were bactericidal in vitro versus *B. subtilis* and *S. aureus* strains, respectively. In each of the above studies, the platelet antimicrobial effector molecules were identified as being small and cationic, with masses ranging from 6 to 40 kDa. Tew et al. [145] found that β -lysin proteins were released from rabbit platelets by stimulation with thrombin, establishing a potential link between tissue injury and platelet liberation of host defense effectors. Likewise, Dankert et al. [146, 147] also suggested that platelets release platelet-associated bactericidal substances following thrombin stimulation. Darveau et al. [148] identified peptide fragments of human platelet factor-4 with antimicrobial activity in combination with antibiotics against certain Gram-negative bacteria.

Platelet microbicidal proteins and kinocidins

In recent years, the molecular effectors of platelet antimicrobial response have been the topic of intensive investigation. By convention, the term platelet microbicidal proteins (PMPs) is used to include PMPs, thrombin-induced PMPs (tPMPs), and derivatives thereof termed thrombocidins. Importantly, a subset of PMPs have been discovered to be classical chemokines that have direct microbicidal activity. These polypeptides are termed kinocidins, reflecting their dual chemokine and microbicidal effector functions [149]. Important investigations by Cole et al. [150], Yang et al. [151], and others have subsequently reinforced this discovery. Detailed structures and mechanisms of PMPs and kinocidins are available in other reports

[152–163]. However, it is helpful to emphasize herein that PMPs and kinocidins differ in several important respects from classical antimicrobial peptides such as defensins.

The family of PMPs and kinocidins can be found in human and other mammalian platelets [1, 7, 152–163]. Two predominant PMPs (tPMPs 1 and 2) are liberated from platelets upon thrombin stimulation [154–156]. These, like PMPs and kinocidins, exist as native species that appear to undergo proteolytic cleavage in contexts of infection. For example, Yount et al. [156, 157] and Yeaman et al. [157–159] have shown that PMPs are cleaved into autonomous but complementary functional domains by context-relevant proteases such as thrombin. Likewise, Anderson et al. [164] have confirmed these previous findings that fragments of mammalian platelet factor-4 and related molecules can also have antimicrobial efficacy. The fact that thrombin is generated at sites of vascular infection, and is a strong inducer of platelet degranulation, illustrate how PMP and kinocidin release is coordinated with response to endothelial host defense [159]. Mass spectroscopy has demonstrated that PMPs and kinocidins range in size from approximately 6.0 to 9.0 kDa [1, 158, 181]. Cloning and amino acid analyses affirm these molecules to have a net cationic charge, mediated by an enrichment of basic residues lysine and arginine. Molecular mass, sequence, lysine content, and other features distinguish PMPs and kinocidins from classical defensins [1, 154, 158] overall. However, recent discoveries have identified a multidimensional signature—termed the γ -core—that unifies these and other host defense molecules by way of physicochemical themes as distributed across their 3D shape [161, 163]. To summarize a considerable body of recent literature, PMPs, kinocidins, fragments and synthetic congeners thereof have repeatedly been shown to exert strong *in vitro*, *ex vivo*, and *in vivo* efficacy against Gram-positive and Gram-negative bacteria and fungi [1, 158, 165]. Of note, Azizi et al. [166] and Bayer et al. [96] have also shown that specific pathogens or purified staphylococcal α -toxin prompt specific PMP or kinocidin release profiles from platelets *in vitro*. These and other aspects of PMPs and kinocidins have been reviewed elsewhere [1, 158]. Thus, the remainder of this report focuses on key themes and potential correlates of these molecular effectors of host defense against bacterial infection.

PMPs and kinocidins derive from distinct genetic lineages

The repertoire of PMPs and kinocidins is now known to represent multiple molecular species representing several genetic lineages. Tang et al. [152] first isolated and identified antimicrobial peptides from human platelets that are homologous to those in other mammals. Today, an array of

host defense effector peptides is known to be liberated from human platelets upon thrombin stimulation. The array of PMPs and platelet kinocidin molecules includes: platelet factor-4 (PF4), platelet basic protein (PBP) and its proteolytic derivatives, connective tissue-activating peptide-3 (CTAP-3) and neutrophil-activating peptide-2 (NAP-2), RANTES (“released upon activation, normal T cell expressed and secreted”), thymosin- β -4 (T β -4), and fibrinopeptides A and B (FP-A and FP-B) [152, 153]. Importantly, these antimicrobial effector proteins arise from five genetically-distinct lineages in humans and other mammals: (1) PF-4 and variants; (2) PBP and its proteolytic derivatives CTAP-3 and NAP-2; (3) RANTES; (4) T β -4; and (5) fibrinopeptides. Therefore, potential defects or deficiencies in one given lineage are not likely to negatively impact others. Such redundancy may have played an important role in evolution of mammalian immunity against bacterial and other microbial pathogens.

Kinocidins can be classified relative to their chemokine nomenclature. For example, PF-4, PBP, CTAP-3, and NAP-2 are α -kinocidins, as they possess CXC-chemokine motifs. In contrast, RANTES is a β -kinocidin, containing a CC-chemokine motif. The direct antimicrobial activities of these proteins were initially identified by Tang et al. [152] and Yeaman et al. [155]. Krijgsveld et al. [167] subsequently suggested that carboxy-terminal di-amino acid truncated versions of NAP-2 or CTAP-3 also exerted antimicrobial activity *in vitro*. Native PMPs and kinocidins can be further processed in the context of infection or tissue injury after liberation from platelets. Thrombin (a serine protease), platelet-derived proteases, and proteases activated by tissue injury, phagocytes (e.g., cathepsin G), or inflammation (e.g., plasmin) may process native PMPs and kinocidins, generating multiple antimicrobial peptide subspecies [1, 157, 161]. Recently, molecular studies of structure–activity relationships among PMPs and kinocidins have discovered fascinating new insights into the likely roles of these molecules in optimizing host defense [156, 161, 162]. For example, amino acid sequencing and mass spectrometry have demonstrated distinct N-terminal polymorphism variants of rabbit PMP-1 from unactivated versus thrombin-stimulated platelets, respectively, that arise from a single common PMP-1 propeptide [156]. Alternatively, PMPs and kinocidins respond to microbial virulence factors such as proteases by deploying domains with optimized antimicrobial efficacy versus cognate pathogens in contexts of infection [1, 157]. Yeaman et al. invented a novel platform of anti-infective agents that integrate the architecture and processing of PMP and kinocidin proteins which have autonomous yet complementary antimicrobial effector domains that are deployed via disassembly in context [157, 168]. Supporting this concept, Sieprawska-Lupa et al. [169] have

also shown that *Staphylococcus aureus* exoenzymes aureolysin and V8 protease cleave the antimicrobial peptide LL-37. Finally, discoveries of structural, functional, and evolutionary relationships among host defense peptides, classical toxins, and like molecules now afford a new understanding of host defenses, and opportunities to develop anti-infective peptides with minimal host cytotoxicity [170].

It is now known that monocytes have been shown to express PMPs and kinocidins with antimicrobial activity. For example, the PMP and α -kinocidin platelet basic protein (PBP) is also expressed in human monocytes [171]. Most recently, studies have demonstrated that exposure of human monocytes to bacteria or LPS evokes a significant increase in expression of monocyte PBP and derivatives thereof [171]. This response involves activation of proteinase-activated receptors (PARs) by thrombin or synthetic peptide ligands such as SFLLRN. Importantly, two bactericidal derivatives of PBP were seen to enter phagolysosomes, ostensibly to act therein to aid in intracellular inactivation and killing of pathogens. These findings reinforce the prior discoveries that PBP and other PMPs or kinocidins are multifunctional antimicrobial effector proteins as discussed below.

Kinocidins integrate complementary antimicrobial defense systems

Kinocidins orchestrate key events that provide innate anti-infective defenses in settings of infection, and bridge adaptive immunity as optimized to control specific pathogens. Kinocidins have well-established chemotactic functions known to recruit and activate antimicrobial mechanisms of leukocytes and lymphocytes. Yeaman and Yount have recently described how these molecules are optimized to defend against infection of distinct physiologic and anatomic contexts, and in dynamic contexts, as they change over time in response to infection [161, 163, 170]. This paradigm of immunocoordination is termed the AEGIS model of host defense effector peptides: Archetype Effectors Governing Immune Syntax [157]. This model derives from another recent advance in understanding host defense immunobiology—antimicrobial peptide immunorelativity [157, 163]. This concept is a functional concatenation of molecular regulation and physicochemical optimization of host defense peptide structures and functions in particular anatomic or physiologic context to defend against cognate pathogens. Thus, PMPs, kinocidins, and leukocytes recursively potentiate one another in host defense against infection. The AEGIS model of immunocoordination as applied to kinocidins is exemplified through an integration of multiple lines of evidence.

Modular architecture

Kinocidins exhibit modular functional domains that can be deployed to act autonomously to facilitate cooperative, synergistic host defense functions. For example, kinocidins effect immediate and direct antimicrobial actions, recruit leukocytes to sites of infection, and potentiate the antimicrobial mechanisms of these cells [1, 6–9]. Excellent studies by the Gallo laboratory [172] have also suggested that certain human cathelicidins also encode multifunctional capacity for host defense.

Immune syntax

The coordinated deployment of multiple and complementary defense mechanisms such as those encoded in kinocidins in sequence, place, and time to optimize host defense has been termed immune syntax. This process is believed to occur through a corresponding series of events [157]: (1) hallmark signals of infection evoke host cell release of kinocidins in cognate physiologic, anatomic, and microbiologic contexts in response to microbial signals or tissue injury; (2) affinity for microbial cells then concentrates native kinocidins, and their antimicrobial domains begin to exert immediate antimicrobial activity; (3) proteolytic cleavage yields further diversity of functions, including protease inhibition to extend peptide lifespan; (4) diffusion of kinocidin chemotactic domains establishes gradients for leukocyte chemonavigation, and potentiates their antimicrobial mechanisms en route; (5) in turn, pre-decoration by kinocidin opsonic domains promotes pathogen phagocytosis by recruited leukocytes, and in turn potentiates leukocyte; and (6) in the restricted context of the acidic phagolysosome, certain kinocidins or domains thereof have enhanced microbicidal properties. Thus, through immune syntax, kinocidins contribute to host defense through coordinated deployment of direct and indirect antimicrobial functions as optimized to context.

Molecular mechanisms of PMP and kinocidin response

Platelets can be viewed as vehicles, which sense molecular signals of tissue infection or injury and respond by delivering anti-infective molecules and wound-healing factors. Thus, the protective roles of PMPs and kinocidins rely on platelet detection of warning signals, and prompt mobilization and release of these molecules to act in innate host defense. As detailed above, PMPs and kinocidins are released from platelets upon exposure to infection-relevant stimuli, such as thrombin, *S. aureus* α -toxin, or microbial pathogens, including viridans group streptococci, *S. aureus*, and *C. albicans*. Recent studies have provided new insights into the molecular mechanisms through which

platelets release PMPs and kinocidins in response to pathogens. Trier et al. [173] demonstrated that platelets release PMPs and kinocidins through specific molecular pathways following interaction with *S. aureus*. At platelet-*S. aureus* ratios of 10:1 or greater, platelet activation occurred and resulted in release of staphylocidal levels of PMPs and kinocidins. Apyrase (ADP degradation), suramin (a general P2 receptor antagonist), pyridoxal 5-phosphonucleotide derivative (a specific P2X₁ antagonist), and cangrelor (a specific P2Y₁₂ antagonist) mitigated the staphylocidal response against both strains, correlating with reduced levels of PMP and PK release. Specific inhibition occurred in the presence and absence of homologous plasma. In contrast, inhibition of thromboxane A₂, COX-1 or -2, or phospholipase C pathways, or

hindrance of surface adhesion receptors, did not impede anti-*S. aureus* responses. Taken together, this pattern of data suggests a multifactorial platelet anti-*S. aureus* response mechanism involving: (1) platelet activation in response to *S. aureus*; (2) ensuing degranulation of PMPs, kinocidins, and ADP/ATP; (3) ADP activation of P2X₁/P2Y₁₂ receptors on adjacent platelets; and (4) recursive amplification of PMP and kinocidin release from successive platelets. Thus, PMP and kinocidin response to *S. aureus* occurs via an active, rapid and direct mechanism, amplified through autocrine pathways in which platelet ADP/ATP release prompts successive waves of platelet degranulation via the platelet P2X and P2Y₁₂ adenosine nucleotide receptor array (Fig. 1). Sharma et al. showed that outer membrane constituents of *Porphyromonas*

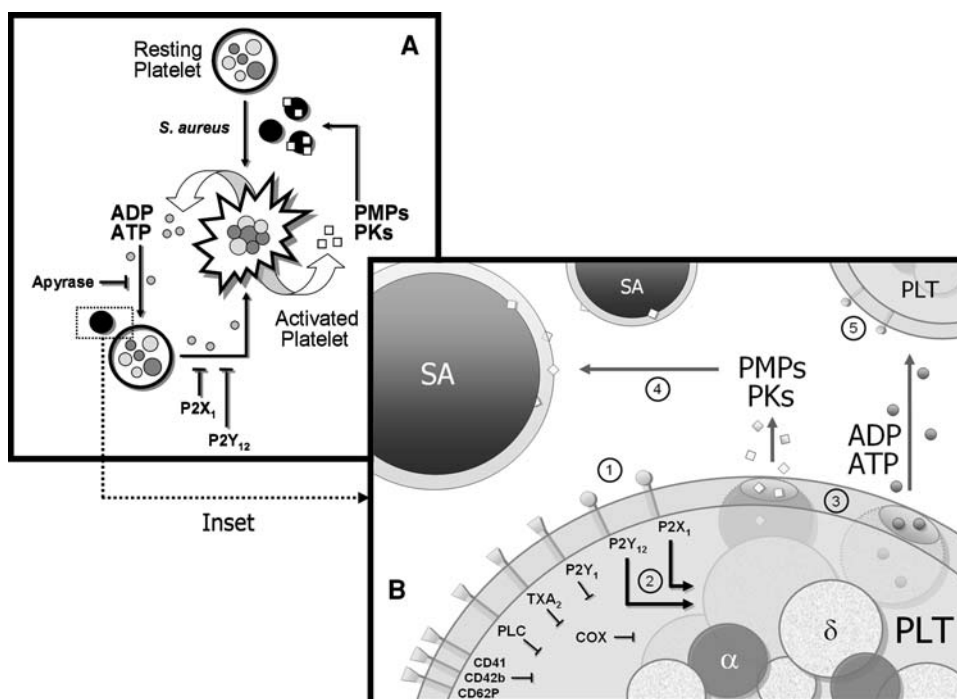


Fig. 1 Model of platelet interactions with *Staphylococcus aureus*. Based upon recent evidence, the model illustrates how platelets may sense and respond via parallel pathways that promote anti-staphylococcal host defense. **a** On the cellular level, interactions with *S. aureus* evoke: (1) platelet activation via specific receptors; (2) liberation and processing of PMPs and PKs which exert direct microbicidal effects, and (3) secretion of adenosine nucleotides (ADP/ATP) triggering a recursive cascade for activation of adjacent platelets. Note that inhibitors of the purinergic pathway of activation preclude anti-staphylococcal responses. **b** On the molecular level, specific sense and response pathway components are illustrated. Purinergic agonists such as ADP are known stimulants of strong platelet activation. Thus, degradation of extracellular ADP by apyrase (APY) or the inhibition of P2X or P2Y₁₂ adenosine nucleotide receptors by suramin (SUR; general P2 inhibitor), pyridoxal-5'-phosphonucleotide derivative (PND; high-affinity P2X₁ inhibitor), or cangrelor (CNG; high-affinity P2Y₁₂ inhibitor) specifically prevents platelet (PLT) sense and response activation for staphylocidal

efficacy. In contrast, antagonism of the P2Y₁, phospholipase C (PLC), thromboxane A₂ (TXA₂), or cyclooxygenase (COX) pathways, or inhibition of the CD41, CD42b, or CD62P platelet adhesion receptors do not impede the sense and response activation of platelets versus *S. aureus*. Thus, the anti-staphylococcal efficacy of platelets involves a self-amplifying, recursive sense and response mechanism: (1) direct or indirect interactions of platelets and *S. aureus* (SA); (2) platelet activation, via autocrine or intercrine P2X₁ or P2Y₁₂ receptor-mediated signal transduction prompting granule mobilization; (3) degranulation and liberation of ADP/ATP from δ -granules; (4) deployment of direct antimicrobial effector molecules (PMPs and PKs) from α -granules; and (5) adenosine nucleotide-mediated activation of adjacent platelets, with the ensuing amplification of antimicrobial responses. Such interactions are likely influenced by platelet-to-*S. aureus* ratios. In this respect, staphylocidal efficacy appears to involve a threshold platelet ratio to generate and sustain an intercrine platelet cascade required to achieve PMP/PK concentrations for staphylocidal efficacy. Adapted from [173]

gingivalis activate murine platelet aggregation and degranulation through similar pathways [174].

Antibacterial efficacy of platelets, PMPs, and kinocidins

The specific antimicrobial mechanisms of PMP and kinocidin actions against bacterial or other pathogens are detailed in other publications (e.g., [1, 175]). In brief, these peptides are rapidly microbicidal, with mechanisms that are distinguishable from classical antimicrobial peptides. For example, the staphylocidal mechanisms of PMPs and kinocidins against *S. aureus* differ from those of human defensin NP-1 (hNP-1) in several important respects: involvement of membrane potential ($\Delta\psi$), permeabilization, and bactericidal kinetics. Therefore, the following discussion highlights the novel and relevant themes in platelet, PMP, and kinocidin antibacterial response.

Efficacy of PMPs and kinocidins in vitro

A substantial body of literature now exists demonstrating that PMPs and kinocidins effect direct, rapid, and potent efficacy against many microbial pathogens in vitro. For example, PMPs and kinocidins exert potent and synergistic in vitro microbicidal activities against *Bacillus subtilis*, *S. aureus*, *S. epidermidis*, *E. coli*, and other bacteria including *Pseudomonas* species, as well as non-bacterial pathogens [152–159]. The microbicidal effects of these molecules are exerted in nanomolar to low micromolar concentrations (1–5 $\mu\text{g/ml}$), and across physiological ranges of pH (5.5–8.0). PMPs and kinocidins appear to act synergistically with one another against microbial pathogens in vitro [152, 153]. Importantly, PMPs and kinocidins have also been shown to interact with one another to effect synergistic antimicrobial activity [152]. Thus, antimicrobial activities of PMPs and kinocidins observed in vitro and ex vivo are relevant to conditions which exist in vivo.

Efficacy of PMPs and kinocidins ex vivo

Multiple lines of evidence indicate that PMPs and kinocidins exert antimicrobial activities in relevant settings of infection ex vivo. The host defense roles of platelets of PMPs and kinocidins appear to be involved in limiting infection of vascular biomatrices. Mercier et al. [176] found that platelet activation by thrombin 30 min prior to bacterial inoculation correlated with a significant staphylocidal effect in an ex vivo model of infective endocarditis. Inhibition of *S. aureus* was greatest in simulated infective endocarditis vegetations during the post-colonization and proliferation phases of infection. Reinforcing this interpretation, synthetic mimetics of platelet kinocidins exert significantly greater

antimicrobial efficacy in human blood and plasma ex vivo than in artificial medium, even after 2 h incubation in the biomatrices prior to inoculation of the target organisms [165]. Mercier et al. [177] also demonstrated that PMPs and kinocidins act in consort with conventional antibiotics to limit the evolution of simulated human infective endocarditis vegetations. In these studies, activated platelets alone, or in combination with antibiotics, inhibited the proliferation of *S. aureus* in model fluid or simulated vegetations over the initial 4-h period ($P < 0.05$ vs controls). Nafcillin regimens were inhibitory beyond 4 h against ISP479C in both model phases. Interestingly, introduction of activated platelets inhibited glycopeptide-intermediate-susceptible *S. aureus* (GISA) proliferation in vegetations, but not in the chamber model fluid. The combination of platelets plus nafcillin or vancomycin significantly inhibited proliferation of the GISA strain in vegetations as compared to platelets or antibiotics alone. However, platelets were not significantly effective with or without nafcillin or vancomycin against a PMP-resistant strain of *S. aureus*.

Complementary data suggest that platelets protect against endovascular infections such as infective endocarditis by modulating the responses of adjacent platelets. For example, PMPs and kinocidins appear to modify *S. aureus*-induced platelet aggregation in vitro [100]. Exposure to sublethal concentrations of PMPs reduces the velocity and magnitude of platelet aggregation by *S. aureus* [99, 178, 179]. It is not yet known whether these peptides do so by modulating bacterium-to-platelet binding, or through other mechanisms. Filler et al. [180] have also demonstrated that platelets protect human umbilical vein endothelial cells (HUVECs) from in vitro injury due to *C. albicans*. In these studies, $^{51}\text{chromium}$ -release from HUVECs due to *C. albicans* was reduced by 45% in the presence of a platelet-to-fungus ratio of 20:1. Moreover, HUVEC protection by platelets was associated with a 37% reduction in germ tube length in *C. albicans* after a 2-h exposure. Thus, it is conceivable that platelets defend vascular endothelium from bacterial injury or infection.

Efficacy of PMPs and kinocidins in vivo

Evidence from a body of recent literature demonstrates that platelets activate immediately and in high quantity in response to pathogens, as well as by soluble mediators and surface ligands expressed by infected host cells. As detailed herein and in previous comprehensive reviews [1, 6–10], platelets also contribute other key host defense effector functions, including: generation of microbicidal reactive oxygen species; facilitate complement fixation on microbes; internalization and clearance of pathogens from the bloodstream; generate and release of cytokines and

chemokines that shape adaptive immune responses; execute antibody-dependent cell cytotoxicity against pathogens; and potentiation of antimicrobial mechanisms of leukocytes [181].

Previous investigations have established that PMPs and kinocidin levels in human plasma increase dramatically (four to sixfold) in settings of microbial infection *in vivo*. For example, the plasma levels of kinocidin PF4 increase in bacterial septicemia [182], streptococcal nephritis [183], and many other microbial infections. Similarly, Wilson et al. [184] demonstrated that endotoxin prompts a marked increase in circulating levels of soluble CD62, an indicator of platelet α -degranulation. Interestingly, unlike most other markers of inflammation, plasma concentrations of this biomarker progressively increased for up to 8 h after endotoxin injection in human volunteers. Therefore, PMPs and kinocidins are likely released immediately and over a window of time from platelets in response to relevant signals of inflammation and infection, as well as microorganisms themselves.

Consistent with these perspectives, platelet transfusion is now well established to have clear beneficial effects in a number of clinical situations, including infection and sepsis. It is known that sufficient platelet quantity and quality are important to homeostasis and host defense. For example, inherited platelet disorders including Wiscott–Aldrich Syndrome, May–Hegglin Anomaly, and Gray-Platelet Syndrome, strongly correlate with morbidity and mortality due to *S. aureus* and other infections [1, 4]. However, such conditions often represent a convergence of multiple disorders in cell-mediated immunity, making definition of platelet-specific roles in host defense difficult to interpret. Nonetheless, evidence for the role of platelets in immune defense against infection in humans comes from diseases of platelet dysfunction as highlighted below. Thus, the specific roles of platelets in host defense have been investigated *in vivo* using complementary approaches. The following discussion illustrates host defense roles of platelets in light of broader themes and recent examples of such clinical conditions.

Deficiencies in platelet quantity

It is now becoming clear from experimental as well as human studies that a threshold platelet count is an important determinant in the host defense repertoire against infection. Sullam et al. [185] used an experimental animal model to examine the role of platelets in defense against infective endocarditis. Viridans streptococci were used to induce endocarditis in animals with normal platelet counts, as compared with animals in which thrombocytopenia was conferred by platelet-specific antibody. Importantly, these study groups had no differences in leukocyte quantity or

quality, or complement activity. Thrombocytopenic animals had significantly higher streptococcal densities in vegetation as compared with their counterparts with normal platelet counts. Other reports by Dankert et al. [146, 147, 186] likewise emphasize the concept that platelets act in host defense against infective endocarditis.

In humans, thrombocytopenia is now increasingly recognized as a significant, independent correlate of morbidity and mortality due to infection. For example, in oncological conditions and cancer chemotherapy, thrombocytopenia is an independent predictor of increased risk of morbidity and mortality due to bacterial or fungal infection [187–191]. Even in the absence of neutropenia, thrombocytopenia is positively correlated with incidence and severity of bacterial pneumonia in the elderly [188]. Similarly, Chang et al. [189] recently demonstrated thrombocytopenia to be an independent predictor of increased infection and related morbidity in liver transplant recipients. In this group, 50% of patients with platelet counts of $\leq 30 \times 10^9/L$ presented with infection within 30 days of transplantation, as compared with only 17% of patients with nadir platelet counts above this threshold. Likewise, Santolaya et al. [191] also evaluated five potential factors for risk of invasive bacterial infection: C-reactive protein serum level, hypotension, leukemia relapse, thrombocytopenia ($\leq 50 \times 10^9/L$), or chemotherapy. Thrombocytopenia was found to be an independent risk factor for bacterial infection in 12% of children in this cohort study (sensitivity, specificity, positive and negative predictive values of 92, 76, 82, and 90%, respectively). In a similar investigation, Yoshida et al. [192] employed multivariate analysis to find that reduced platelet count is an independent predictor of severe bacteremia in healthcare settings. Substantiating these data, Mavrommatis et al. [193] distinguished platelet function from overall coagulation responses to infection in humans. In the initial state, uncomplicated sepsis correlates with increases in blood levels of fibrinopeptide-A, a PMP, and the kinocidin PF4. Increased levels of such molecules were temporally associated with a reduction in platelet count, indicating clearance of activated upon degranulation. However, platelet responsivity is often reduced as coagulation factors are depleted in sepsis, as in other severe infections.

Deficiencies in platelet activation

The above studies strongly suggest that a threshold platelet count is an important barrier to infection. Beyond their number, the ability of platelets to sense and respond to soluble or cellular signals of infection appears to play a significant role in host defense. While platelets can sense and respond to pathogens, key activators of platelets are host signals emanating from injured or infected tissues. For

example, tissue infection often results in the elaboration of tissue factor, which in turn activates the cleavage of thrombin from pro-thrombin. In a recent investigation, Sun et al. [194] reported that deficiencies in thrombin generation increase host susceptibility to group A streptococcal infection. In these studies, mice made deficient in factor V (FV) either as circulating in plasma or contained in platelet compartments, were challenged with group A streptococcal infection. A reduction of FV in either pool was associated with marked increases in infection-caused mortality, in comparison to mice with normal levels of FV. Moreover, fibrinogen deficiency also yielded a significant increase in mortality to infection relative to controls. These findings support the concept that constituents mediating platelet signaling and response in local platelet activation and thrombosis contribute to host defense against bacterial pathogenesis.

Future perspectives

Immune system cells have evolved highly sophisticated molecular and cellular systems with which to detect and defend against infection. Platelets represent a highly specialized member of this repertoire. Although these cells have no nucleus, they retain unambiguous structural and functional attributes of host defense effector cells. Platelets are now known to express TLRs and many other typical characteristics of innate immune effector cells that play key and multifaceted roles in host defense against infection. Evidence substantiating this role derives from an integration of findings over the last decade, including: (1) the discovery of PMPs and kinocidins released from platelets; (2) experimental animal models in which platelets significantly contribute to host defense; and (3) increased susceptibility to infection in clinical deficiencies of platelet quantity or quality. Thus, platelets are sentinel cells that can detect infection and deliver antimicrobial effector molecules in innate defense and which potentiate the efficacy of leukocytes and other host defense systems. In turn, bacterial pathogens have evolved myriad surface proteins and other mechanisms by which to interact with platelets. In some pathogens, adherence and activation of platelets appear to subvert platelet responses and contribute to virulence. These advances emphasize the importance of future studies of human diseases and animal models to elucidate the roles of platelets in prevention or limitation of infection. Moreover, studies of specific platelet inhibitors may reveal important insights into platelet function in human infections, and lead to new anti-infective agents or strategies. For example, novel anti-infectives designed upon PMPs and kinocidins have already demonstrated efficacy against antibiotic-resistant

pathogens in vivo. In addition, such molecules or immunobiotics appear to amplify the efficacy of conventional antimicrobial agents, and therefore might be useful to restore or enhance or efficacies of conventional anti-infective against resistant pathogens.

Acknowledgments This paper summarizes a body of information that derives from a significant effort on the part of numerous investigators over many years of work. Although not every report could be cited in the space of this review, genuine appreciation is extended to all of the talented individuals working in this area. M.R.Y was supported in-part by grants AI39001 and AI48031 from the National Institutes of Health.

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