PLATELETS AND IMMUNE RESPONSES DURING THROMBOINFLAMMATION

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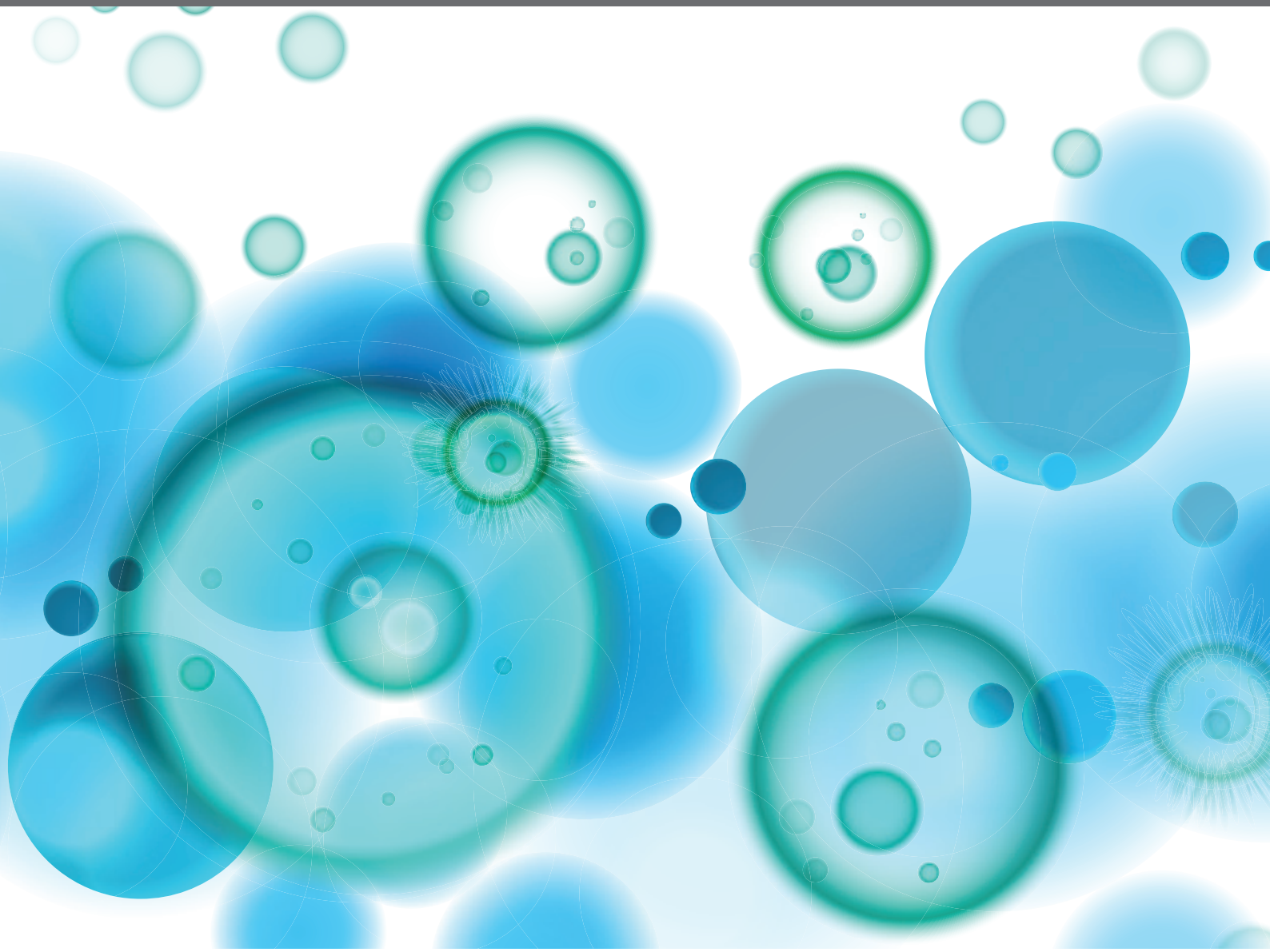

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PLATELETS AND IMMUNE RESPONSES DURING THROMBOINFLAMMATION

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EDITORIAL
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Editorial: Platelets and Immune Responses During Thromboinflammation

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Keywords: platelets, thromboinflammation, neutrophils, endothelial cells, inflammation, sepsis, cancer

Editorial on the Research Topic

Platelets and Immune Responses During Thromboinflammation

The word thromboinflammation appeared in 2004 to describe the interactions and cooperation between platelets and neutrophils in the context of arterial in-stent restenosis (1). Almost two decades later, multiple sources of evidence clearly show that the interplay between thrombosis and inflammation involves several pathways and occurs in various pathophysiological situations such as sepsis, disseminated intravascular coagulation (DIC), stroke, cancer, stress and rheumatoid arthritis, among others. Thromboinflammation is driven by mutual interactions and reciprocal activation between endothelial cells, subendothelium, leukocytes, platelets, and the humoral innate immune system, involving the complement, coagulation, and fibrinolytic signaling cascades.

In many respects, this linkage between inflammation and thrombosis is not entirely surprising. Some of the earliest examples of innate immunity center on the use of "clotting" or "coagulation" to respond to infectious agents and tissue damage. In some invertebrates, this "clotting" response occurs in the hemolymph and is facilitated by hemocytes, the evolutionary ancestor to the vertebrate platelet (2). In these systems, exposure of hemocytes, or hemolymph to bacteria results in rapid coagulation of the hemolymph, trapping the pathogen, and limiting its dissemination (3). This early basic response has evolved to become more specialized, creating distinct roles of hemostasis, inflammation, and immunity; however, the evolutionary overlap in these processes remain and continue to play a role in a wide variety of pathophysiological conditions and human disease.

In this Special Research Topic issue on the recent advances in Thromboinflammation, we compiled 15 reviews and one original article which provide comprehensive basic, and clinical insights on the current view of thromboinflammation. These articles also address the development and use of novel therapeutics in both experimental settings as well as in clinical trials, moving our understanding of the topic forward within the context of treating human disease.

Long before the term thromboinflammation was coined, interactions between neutrophils and platelets had been suspected to play a pathophysiological role in both myocardial and cerebral ischemia-reperfusion injury (4–7). Schanze et al. and Stegner et al. present early and recent findings on the crucial contribution of platelets to the course of ischemia-reperfusion injury and discuss how our current understanding of the underlying molecular mechanisms could translate into the clinics. A particular focus on the role of the von Willebrand factor (VWF)/glycoprotein Ibα (GPIbα) axis in the thromboinflammatory response during ischemic stroke is given by Denorme et al.. This work explores the "thrombo-inflammatory" environment surrounding VWF-platelet interactions within

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the context of cardiovascular diseases and how these players may serve as both important clinical markers of disease and also key therapeutic targets for the treatment of stroke patients.

Recent years have seen a growing interest for the impact of the gut microbiome on the development of diseases with an immune and/or thrombotic component, such as cardiovascular diseases. In this context, Bayer et al., highlight the fact that antibiotics-based protocols commonly used in this research field can significantly alter host physiology, notably by impacting the cardiovascular system and the actors of the thromboinflammatory cascade. The authors emphasize the importance of instead considering the use of germ-free mouse models, which remain the state-of-the-art approach for studying host-microbe and microbe-microbe interactions.

Beyond cardiovascular health, inflammation is often associated with the development and progression of cancer. Emerging in this field is the view that considers malignant tumors as wounds that do not heal. In this scenario, platelets are intimately involved in the vicious circle of activating cancer cells which, in turn, activate platelets. This mutual cell activation can ultimately end in the well-recognized cancer-associated thrombosis or can further fuel the inflammatory microenvironment which favors tumor growth and metastasis. Palacios-Acedo et al. give a detailed description of the molecules and mechanisms in these events, while Marin Oyarzún and Heller summarize current knowledge about the role of platelets in the thrombotic complications of chronic myeloproliferative neoplasms.

The crossroad of inflammation and thrombosis is very well-exemplified during envenomation. Several characterized proteins in particular from viper snake venoms can activate the innate immune and/or hemostasis systems. Teixeira et al. offer and extended review of these molecules and its mechanisms of action in this elaborate interplay between predator venom and host hematology.

Platelets and coagulation are often centrally involved in the host response to infectious disease as well. Manifestations of dysregulated coagulation, such as DIC, are frequently associated with the systemic immune response, such as in the case of sepsis, and are almost invariantly linked to worse patient outcomes. In this issue, reviews by Kerrigan et al. and by McDonald and Dunbar explore how this dysregulated response is initiated in the septic patient and how its manifestation impacts both the infection itself (protective) and vascular health (pathogenic). In a complementary review, Assinger et al. give a timely update on experimental models of sepsis, highlighting both the interest and limitations of animal models to study platelet-related functions in sepsis.

Additional contributions address the role of the platelet as an immune sentinel, detecting and initiating the response to infection. Guo and Rondina address the broad ability of platelets to respond to Pathogen Associated Molecular Patterns (PAMPs), complement and antibodies and how activated platelets in turn drive and modulate the host immune response. The authors also address how this host immune response is intimately intertwined with both the hemostatic and thrombotic pathways, directly impacting patient outcomes in infectious disease. Ramirez et al. build on this theme, shedding light on the direct interplay between platelets and neutrophils within the context of chronic inflammation. Importantly, this work also addresses both platelet production within the bone marrow of patients with chronic disease and the role of platelet-derived microparticles within chronic inflammation. The role of the platelet as an innate immune cell is further discussed in the review by Eriksson et al., in which the interactions between activated platelets and the complement system, as well as their clinical implications, are thoroughly presented. Cognasse et al. broaden our perspective of the immunomodulatory properties of platelets by presenting how platelets can themselves release non-infectious danger signals during storage of platelet concentrates, thus contributing to adverse immune reactions to platelet transfusion.

Additionally, a review by Mezger et al. gives an overview of several non-hemostatic functions of platelets, including the regulation of angiogenesis, tissue remodeling, and other processes contributing to the progression of various thromboinflammatory conditions like cancers and cardiovascular diseases.

Finally, in an original article and by using a pre-established mouse model of oral acute Chagas disease, Antunes et al. demonstrate for the first time that *Trypanosoma cruzi* infection leads to a decrease in platelet count, increased bleeding and coagulation time, host responses that correspond to the peak of parasitemia. Importantly, circulating IL-6 levels seem to be involved in these hematological changes during oral Trypanosoma cruzi infection. These data may help elucidate the mechanism of oral acute Chagas disease pathogenesis and provide additional insight on the interaction between inflammation and coagulation in the context of infectious diseases.

Overall, this collection of manuscripts expands our understanding of the non-hemostatic roles of platelets, highlighting the central role thrombocytes play in linking coagulation, inflammation, and immunity by participating in a spectrum of immunothrombotic responses and directly impacting host responses and disease outcomes.

AUTHOR CONTRIBUTIONS

All authors listed have made a substantial, direct and intellectual contribution to the work, and approved it for publication.

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ORIGINAL RESEARCH
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Oral Route Driven Acute *Trypanosoma cruzi* Infection Unravels an IL-6 Dependent Hemostatic Derangement

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Oral transmission of *Trypanosoma cruzi*, the etiologic agent of Chagas disease, is presently the most important route of infection in Brazilian Amazon. Other South American countries have also reported outbreaks of acute Chagas disease associated with food consumption. A conspicuous feature of this route of transmission is presenting symptoms such as facial and lower limbs edema, in some cases bleeding manifestations and risk of thromboembolism are evident. Notwithstanding, studies that address this route of infection are largely lacking regarding its pathogenesis and, more specifically, the crosstalk between immune and hemostatic systems. Here, BALB/c mice were orally infected with metacyclic trypomastigotes of *T. cruzi* Tulahuén strain and used to evaluate the cytokine response, primary and secondary hemostasis during acute *T. cruzi* infection. When compared with control uninfected animals, orally infected mice presented higher pro-inflammatory cytokine (TNF-α, IFN-γ, and IL-6) serum levels. The highest concentrations were obtained concomitantly to the increase of parasitemia, between 14 and 28 days post-infection (dpi). Blood counts in the oral infected group revealed concomitant leukocytosis and thrombocytopenia, the latter resulting in increased bleeding at 21 dpi. Hematological changes paralleled with prolonged activated partial thromboplastin time, Factor VIII consumption and increased D-dimer levels, suggest that oral *T. cruzi* infection relies on disseminated intravascular coagulation. Remarkably, blockade of the IL-6 receptor blunted hematological abnormalities, revealing a critical role of IL-6 in the course of oral infection. These results unravel that acute *T. cruzi* oral infection results in significant alterations in the hemostatic system and indicates the relevance of the crosstalk between inflammation and hemostasis in this parasitic disease.

Keywords: Chagas disease, oral transmission, cytokine, platelet, coagulation, interleukin-6

INTRODUCTION

The hemoflagellate protozoan *Trypanosoma cruzi* is the causative agent of Chagas disease or American trypanosomiasis which is transmitted primarily through contact with feces of triatomine insects after biting (1). Transmission can also occur through blood transfusion (2), organ transplantation (3), congenitally (vertical transmission) (4), laboratory accidents (5) and by ingestion of contaminated food/juices (6, 7). Currently, oral infection is the most frequent route of transmission in Brazil and other Latin American countries (7–9). Mortality rates in these orally infected patients are higher (8–35%) when compared with the classical vectorial transmission (<5–10%) (10). The acute phase of the disease is a critical period often accompanied by non-specific clinical symptoms, such as fever, asthenia, face and limb edema, headache, myalgia, and others. Minor bleeding manifestations, most commonly from nose, skin petechiae, or bruising, are apparent in some patients with oral acute Chagas disease (ACD) and occasionally, risk of thromboembolism is reported and digestive bleeding may cause death (7, 11–13). These clinical/hematological signs have a frequency of 4.9% in orally-transmitted ACD outbreaks (14), although higher values were described in some cases (12, 13). Moreover, the association between anemia and thrombocytopenia in the ACD was already envisioned by Carlos Chagas in 1909 (1).

The knowledge of immunological events that occur during ACD are mainly based on studies using murine models. *Trypanosoma cruzi* experimental infection leads to pathogen-associated molecular patterns (PAMPs) activation in macrophages and dendritic cells with IL-12 secretion. Furthermore, synthesis of interferon-γ (IFN-γ), tumor necrosis factor-α (TNF-α), and nitric oxide (NO) by macrophages contributes to parasite clearance (15). In a mouse model of *T. cruzi* oral infection with the Tulahuén strain, it was shown that the major source of TNF in infected tissues are macrophages and high levels of this cytokine are associated to cardiac, hepatic and spleen injuries as well as toxic shock in infected BALB/c (16, 17). IL-6 is also involved in host protective response since IL-6-/- mice presented 3-fold higher parasitemia and died earlier than wild-type *T. cruzi* infected animals, by the subcutaneous route (18). Interestingly, Th1 cytokines are involved in an intense crosstalk between immune and hemostatic systems. Acute inflammation, as a response of an infection can modulate the systemic activation of the coagulation cascade and impair physiological anticoagulant pathways (19–22).

Tissue factor (TF), the integral transmembrane protein that initiates coagulation, is strongly induced by pro-inflammatory cytokines and C reactive protein on monocytes, fibroblasts and endothelial cells surface allowing further interaction with factor VII to form the complex TF-factor-VIIa, ultimately resulting in fibrin formation (19, 23). Under normal conditions, cells in direct contact with circulating blood do not express physiologically active TF (24). The traditional coagulation cascade includes intrinsic and extrinsic pathways that lead to the activation of different coagulation factors converging at the activation of factor X to factor Xa. Factor Xa forms a complex with factor Va to activate prothrombin into thrombin. Thrombin then converts fibrinogen to a fibrin network forming the clot (22).

Baboons lethally challenged with *Escherichia coli* and infused with recombinant antithrombin (protease inhibitor of thrombin and factor Xa) at high concentrations, had lower IL-6 and IL-8 plasma levels and the mortality was markedly reduced (25). Furthermore, blockade of IL-6 with a monoclonal antibody, in a primate model of sepsis, attenuated the LPS-induced coagulation (26). This effect was independent of TNF, since abrogation of this cytokine with recombinant TNF receptor IgG fusion protein or a neutralizing TNF antibody in healthy humans or LPS injected chimpanzees had no effect in coagulation activation (26, 27). To the best of our knowledge, there are no studies focusing on the inflammatory and hematological crosstalk as well as their mechanisms in oral ACD. The few studies addressing this interaction in Chagas disease in literature focus on chronic *T. cruzi* infection and have controversial results regarding the existence of a prothrombotic status in *T. cruzi*-infected patients (28–30).

Here, by using a pre-established mouse model of oral ACD, we demonstrate that infection leads to a decrease in platelet count, increased bleeding and coagulation time, mainly in the peak of parasitemia. Importantly, circulating IL-6 levels seem to be involved in these hematological changes during oral *T. cruzi* infection. This information may help elucidating the mechanism of oral ACD pathogenesis and provide an additional view on the interaction between inflammation and coagulation in the context of infectious diseases.

MATERIALS AND METHODS

Animals and Infection

Male BALB/c mice were obtained from ICTB Oswaldo Cruz Foundation animal facilities (Brazil) and maintained in SPF conditions. Mice (6–8 weeks old) were infected via oral cavity by pipetting 50 all of excreta into their mouth with 5×10^4 *T. cruzi* insect-vector (*Triatoma infestans*)-derived metacyclic forms (Tulahuén strain, TcVI). A different group of mice received the same number of trypomastigotes by the subcutaneous route (SC) after a single inoculation in the dorsal region. Before the infection, mice were maintained starving 4 h and at least 15 min after inoculation.

Ethics Statement

This study was performed in strict accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of the Brazilian National Council of Animal Experimentation and the Federal Law 11.794 (10/2008). The Institutional Ethics Committee for Animal Research of the Oswaldo Cruz Foundation (CEUA-FIOCRUZ, License: L-028/2016) approved all the procedures used in this study.

Parasitemia

Parasitemia was detected at different dpi by counting trypomastigotes in 5 μL of tail blood and parasite number was calculated using the Pizzi-Brener method.

Cytometric Bead Array (CBA)

Mice were anesthetized, bled by cardiac puncture and euthanized by exsanguination at 3, 7, 14, 21, 24, and 28 dpi. Each bleeding point represents one mouse. To measure IL-6, IFN-γ, and TNF-α cytokines in the serum, we used the BD Mouse Th1/Th2/Th17 Cytokine CBA kit (BD Biosciences, USA). The assays were performed according to manufacturer's instructions. Sera were stored frozen at −70°C until used.

Blood Cell Analysis

The mice were anesthetized and blood was collected into EDTA BD microteiner® tube by cardiac puncture. Blood cell count was automatically determined using the Poch 100- iV DIFF hematology analyzer (Sysmex, Japan).

Clotting Assays and Measurements of Coagulation Factors

Activated Partial Thromboplastin Time (aPTT) and Prothrombin time (PT) were evaluated on a STart 4 stagocoagulometer (DiagnosticaStago, USA). For the aPTT, plasma (50 μL) was incubated in the coagulometer for 5 min at 37°C. Then, 50 μL of pre-warmed aPTT reagent (STA PTT; DiagnosticaStago, France) was added and further incubated for 2 min $CaCl_2$ (50 μL at 25 mM) was added to start reactions. For determining the PT, plasma (50 μL) was incubated in the coagulometer for 5 min at 37°C. Then, 100 μL of the PT reagent (NEOplastine CI plus; DiagnosticaStago, France) was added. Time for clot formation was recorded in duplicates.

Serum levels of coagulation factors (FV, FVII, FVIII, and APC) and D-dimer were determined using commercial enzyme linked immunoabsorbent assay (ELISA) kits according to manufacturer's protocol (ElabScience Biotechnology, China).

Tail Bleeding Assay

Bleeding propensity was evaluated as previously described (31). Mice were anesthetized with intramuscular xylazin (16 mg/kg) and ketamine (100 mg/kg). After 15 min, the distal 2 mm segment of the tail was removed and immediately immersed in 40 mL distilled water warmed to 37°C during exactly 30 min. The samples were properly homogenized and the absorbance was determined at 540 nm in order to estimate the hemoglobin content.

Anti-IL6R and Anti-TNF Treatment

Orally infected BALB/c mice were treated intraperitoneally with a monoclonal antibody against the interleukin-6 receptor (IL-6R) (8 mg/kg, Tocilizumab, Actemra®, Roche, Switzerland), IgG control antibody (8 mg/kg, chrompure IgG Jackson Immunoresearch Labs, USA) or with a chimeric anti-TNF protein (0.83 mg/Kg, Etanercept Enbrel®, Wyeth Pharmaceuticals, USA). The treatment began at the 14 dpi with 48 h subsequent doses in the case of anti-IL-6R antibody or IgG control antibody and with another dose at 18 dpi for the anti-TNF reagent. The control group received normal saline solution at the same volume (100 μL) and frequency as described for orally infected mice.

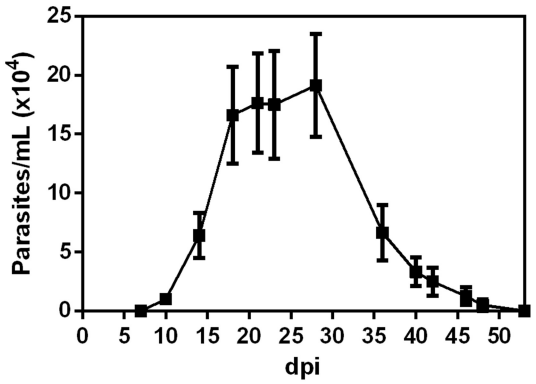

FIGURE 1 | Parasitemia development. Male BALB/c mice were infected with 5 × 10^4 insect-derived metacyclic forms of *T. cruzi* within the oral cavity. Parasitemia (mean and SEM) was assessed during the acute phase and is expressed as parasites per milliliter. Parasites were counted by light microscopy and parasitemia calculated by the Pizzi-Brener method. n: 7 dpi = 21, 10, and 14 dpi = 15, 18 dpi = 18, 21 dpi = 11, 23 dpi = 6, 28 dpi = 8; 36, 40, 42, 46, 48, and 53 = 4. The total number of animals in each time point was obtained from different experiments.

Statistical Analysis

Data were subjected to the D'Agostino-Pearson normality test to determine whether they were sampled from a Gaussian distribution. If a Gaussian model of sampling was fulfilled, parametric test (one-way ANOVA with Tukey's multiple comparison test) was used. If the samples deviated from a Gaussian distribution, non-parametric test (Kruskal–Wallis with Dunn's multiple comparison test) was applied. All statistical analysis was done in GraphPad Prism 6 (GraphPad Software Inc.). $P < 0.05$ were considered statistically significant.

RESULTS

Increased Pro-inflammatory Cytokine Secretion Parallels Parasitemia Elevation in Acute *T. cruzi* Oral Infection

We initially determined the parasitemia of BALB/c mice orally infected (OI) with 5 × 10^4 *T. cruzi* metacyclic trypomastigotes. As shown in **Figure 1**, circulating parasites were first detected at 10 dpi. Furthermore, the animals showed higher numbers of parasites between 21 and 28 dpi. We next evaluated if *T. cruzi* infection caused transient changes in serum concentrations of pro-inflammatory cytokines. Accordingly, OI animals presented increased levels of TNF (**Figure 2A**), IFN-γ (**Figure 2B**) and IL-6 (**Figure 2C**) when compared with non-infected animals (NI). During 14–24 dpi, all cytokines exhibited high levels concomitantly to the increased numbers of circulating parasites (**Figure 1**). In contrast, there were no statistically significant differences in concentrations of TNF, IFN-γ, and IL-6 in the initial stages of infection (3–7 dpi), when parasitemia is not detected. Statistically significant differences in the Th2 and Th17 cytokine levels were not detected (**Figure S1**).

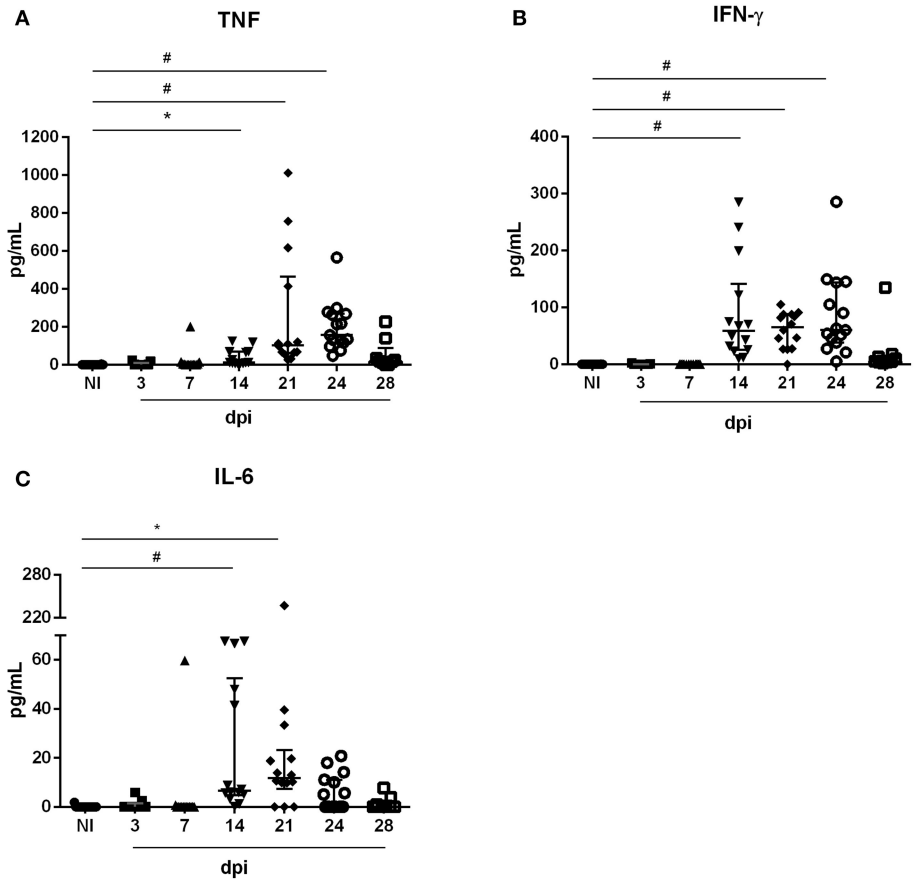

FIGURE 2 | Serum cytokine levels during oral acute *T. cruzi* infection. Male BALB/c mice were infected with 5×10^4 insect-derived metacyclic forms of *T. cruzi* within the oral cavity. In the course of the acute infection, serum was isolated and levels of TNF **(A)**, IFN-γ **(B)**, and IL-6 **(C)** were quantified in non-infected (NI) and infected mice by the CBA method. Values represent the median with interquartile range for each group/day post-infection and are representative of two independent experiments. Results were analyzed using Kruskal–Wallis with Dunn's multiple comparisons test (*$0.0001 < p < 0.05$, #$p < 0.0001$). Significant differences not displayed in the graph: TNF: 3, 7 dpi ≠ 21, 24 dpi; IFN-γ: 3, 7 dpi ≠ 14, 21, 24 dpi; IL-6: 3 dpi ≠ 14 dpi e 7 dpi ≠ 14, 21 dpi. n: NI = 16; 3 e 28 dpi = 9; 7, 14 e 21 dpi = 14; 24 dpi = 15. n: NI = 16; 3 and 28 dpi = 9; 7, 14, and 21 dpi = 14; 24 dpi = 15.

Mice Orally Infected With *T. cruzi* Exhibit Signs of Disseminated Intravascular Coagulation, Including Thrombocytopenia and Increased Bleeding

We analyzed several hematological parameters in NI and OI mice on 7, 14, 21, and 28 dpi. As shown in **Table 1**, OI induced thrombocytopenia. On day 14 and 21, the platelet counts were 775.4 (±62.54) × $10^3/\mu$L and 840.8 (± 83.74) × $10^3/\mu$L for infected mice, respectively. There were no significant changes in red blood cell count, hemoglobin concentration, hematocrit and mean corpuscular volume when compared to NI. In addition, orally infected mice exhibited leukocytosis on 21 and 28 dpi (**Table 1**).

In order to evaluate the impact of acute infection-induced thrombocytopenia, the tail transection method was employed to evaluate the bleeding tendency. As seen in **Figure 3A**, OI mice showed a marked increase in bleeding at 21 dpi when compared with the NI counterparts.

The elevated pro-inflammatory cytokine profile concomitant with the decrease in platelet count suggests that oral infection may course with disseminated intravascular coagulation. In order to test this hypothesis, plasma from mice were collected and used for determination of *ex vivo* aPTT and PT coagulation tests. Oral infection with *T. cruzi* prolonged the aPTT (**Figure 3B**) but did

TABLE 1 | Blood counts of non-infected (NI) and orally *T. cruzi* infected (OI) mice.

	NI	7	14	21	28
WBC	6.6 ± 0.86	9.1 ± 0.41	7.3 ± 0.60	16 ± 2.1*	18 ± 1.4*
RBC	9.8 ± 0.27	11 ± 0.20	9.5 ± 0.21	9.1 ± 0.27	10 ± 0.46
HGB	14 ± 0.33	16 ± 0.35	14 ± 0.41	13 ± 0.45	13 ± 0.49
MCV	52 ± 0.97	52 ± 0.42	49 ± 0.47	53 ± 0.35	49 ± 0.69
HMT	51 ± 0.58	58 ± 1.1	47 ± 1.3	48 ± 1.6	49 ± 1.6
PLT	1195 ± 81.71	1313 ± 101.0	775.4 ± 62.54*	840.8 ± 83.74*	1282 ± 17.44

Blood cells were measured on the Poch 100- iV DIFF hematology analyzer. Values are presented as mean ± SEM for each group/day post-infection and are representative of two independent experiments. Results were analyzed using the Kruskal-Wallis with Dunn's multiple comparisons test (≠ NI and $0.0001 < p < 0.05$. Significant differences not displayed in the graph: WBC: 7, 14 dpi ≠ 21, 28 dpi; RBC: 7 dpi ≠ 21 dpi; HGB: 7 dpi ≠ 21, 28 dpi; MCV: 14 dpi ≠ 21, 28 dpi; HMT: 7 dpi ≠ 14, 21 dpi. n = 5). HGB, total hemoglobin (g/dL); HMT, hematocrit; MCV, mean corpuscular volume (fL); PLT, number of platelets (10^3 cells/μL); RBC, number of red blood cells (10^6 cells/μL); WBC, number of white blood cells (10^3 cells/μL).*

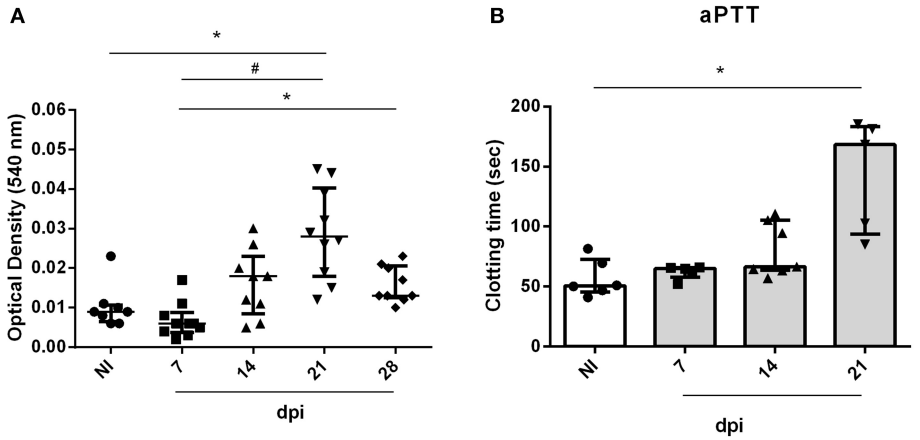

FIGURE 3 | Bleeding from the tail of BALB/c mice and *ex vivo* PT and aPTT measurements during oral infection. Male BALB/c mice were infected with 5×10^4 insect-derived metacyclic forms of *T. cruzi* (Tulahuén strain) within oral cavity. **(A)** Bleeding was caused by a tail transection in NI, 7, 14, and 21 dpi OI mice. Absorbance at 540 nm (hemoglobin concentration) was used to estimate blood loss. **(B)** NI or OI plasma samples were obtained by cardiac puncture followed by addition of aPTT or PT reagent as described in the "Methods" section. Clotting time was estimated using a coagulometer. **(A,B)** Values represent the median with interquartile range for each group/day post-infection and are representative of two independent experiments. Results were analyzed using the Kruskal-Wallis with Dunn's multiple comparisons test (*$0.0001 < p < 0.05$, #$p < 0.0001$).

TABLE 2 | Serum contents of D-dimer during acute phase of oral *T. cruzi* infection.

Group	Mice with D-dimer>0 ng/mL
NI	0/6
7 dpi	0/5
14 dpi	3/6
21 dpi	2/6

Male BALB/c mice were infected with 5×10^4 insect-derived metacyclic forms of T. cruzi within the oral cavity. NI or OI sera were obtained by cardiac puncture and used to measure levels of D-dimer by ELISA. The mean and SEM of animals with detectable levels of D-dimer were 25.7 ± 7.59 and 45.1 ± 11.0 for 14 and 21 dpi, respectively.

not affect the PT (data not show). This is compatible with a derangement in the intrinsic pathway of the coagulation cascade.

Comparison of the coagulation factor levels between NI and OI showed statistically significant differences for FV, FVII, and FVIII (**Figure S2**) with a consumption of FVIII at 14 dpi. D-Dimer, a fibrin degradation product that marks fibrin generation, degradation and reflects the turnover of the coagulation system, was also measured in NI and OI. The concentration of this biomarker was detected on 14 and 21 dpi in OI (**Table 2**).

Blocking of IL-6 Signaling Prevents Hematological Changes in the Murine Model of *T. cruzi* Oral Infection

Oral infected mice had higher pro-inflammatory cytokine serum levels, especially between 14 and 24 dpi (**Figure 2**). Therefore, we hypothesized that blockade of IL-6R or soluble TNF could attenuate hemostatic changes observed in OI mice. To test this hypothesis, we treated OI mice with Tocilizumab (T), a monoclonal antibody that targets IL-6 signaling by competing for IL-6R (**Figure 4A**) or with a quimeric anti-TNF protein, etanercept (Enbrel®) (**Figure S3A**). Both treatments started at 14 dpi. Treated OI mice group (OI+T) had a significant drop in bleeding compared with vehicle alone (saline) OI+V (**Figure 4B**). Moreover, OI+T had lower aPTT than OI+V and OI+isotype and showed no significant differences between non-infected controls (NI+V or NI+T) (**Figure 4C**). In contrast, values recorded for aPTT and bleeding assays remained unaltered after anti-TNF treatment (**Figures S3B,C**). These results suggest that blocking the IL-6R attenuates changes in the hemostatic system under *T. cruzi* oral infection whereas TNF blockade did not influence those alterations.

Hematological Disturbances Are Also Observed in Subcutaneously Infected Mice

Orally infected mice showed hemostatic alterations in the acute phase. However, we wondered whether these changes were related to the oral transmission or with *T. cruzi* presence in the bloodstream. To answer this point, mice were infected with the same inoculum by the SC route. As demonstrated in **Figure 5**, SC infected mice also showed a significant increase in aPTT (**Figure 5B**) but not in bleeding tendency or PT (**Figures 5A,C**). Unlike OI mice, changes in aPTT of SC animals started at 14 dpi, prior to changes in OI animals (21 dpi).

At the SC route, infection killed all animals between 15 and 16 dpi.

DISCUSSION

Previous studies indicate a relationship between inflammation and coagulation in infection (20, 21). Some patients with oral ACD present symptoms related to hemostatic alterations such as facial and lower limbs edema, minor bleeding manifestations, most commonly from nose, skin petechiae or bruising and risk

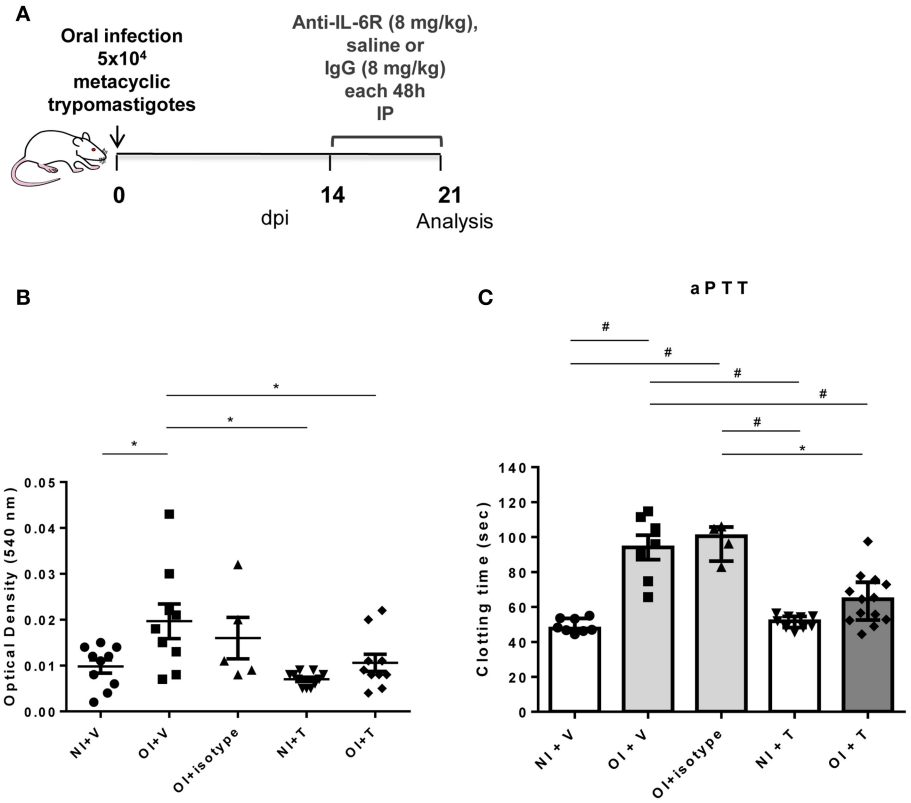

FIGURE 4 | Experimental design, coagulation test aPTT and tail bleeding assay in NI+V, OI+V, OI+isotype, NI+T and OI+T mice. **(A)** Male BALB/c mice were infected with 5×10^4 insect-derived metacyclic forms of *T. cruzi* (Tulahuén strain) within the oral cavity. Anti-IL6R or IgG treatment began after 14 dpi and was performed in each 48 h. **(B)** Bleeding was caused by a tail transection in NI+Vehicle (NI+V), OI+V, OI+isotype, NI+treatment (NI+T) and OI+T. Absorbance at 540 nm (hemoglobin concentration) was used to estimate blood loss. **(C)** NI+V, OI+V, OI+isotype, NI+T and OI+T plasma were obtained by cardiac puncture followed by addition of the aPTT reagent as described in the "Methods" section. Clotting time was estimated using a coagulometer. **(B,C)** Values are presented as mean ± SEM for each group/day post-infection and are representative of three independent experiments. Results were analyzed using one way ANOVA with Tukey's multiple comparisons test (*$0.0001 < p < 0.05$; #$p < 0.0001$).

of thromboembolism (7, 11–13) indicating the need of exploring the crosstalk between immune and hemostatic systems. In the present study, we examined if the systemic inflammation led to hemostatic abnormalities in oral ACD and how blocking of IL-6 signaling pathway can modulate these changes.

We found that OI mice had significantly higher TNF, IL-6, and IFN-γ circulating levels than controls, thus demonstrating that *T. cruzi* infection leads to a potent pro-inflammatory systemic response. This is consistent with previous data showing that Tulahuén strain of *T. cruzi* induces TNF and IFN-γ production in BALB/c and C57BL/6 following subcutaneous (32) or OI (16). Moreover, high levels of TNF are involved in the toxic shock seen in IL-10-deficient mice infected intraperitoneally with 50 blood trypomastigotes of the Tulahuén strain of *T. cruzi* (33) as well as in cardiac, hepatic and spleen injury (16, 17). IFN-γ and IL-6 control parasite multiplication and confers host resistance (18, 34, 35). Furthermore, chronic patients with Chagas disease have elevated circulating levels of IL-6 when compared to healthy individuals (29, 36).

Interestingly, proinflammatory cytokines play a central role in the differential effects upon the coagulation and fibrinolysis pathways. TF is strongly induced after inflammatory stimuli mainly on monocytes and endothelial cells (23). Cytokines that have the ability to increase TF expression are TNF, IL-1β, IL-6, IFN-γ and the chemokine CCL2 (19, 37). Injection of low doses of LPS in healthy volunteers induced endotoxemia and TF mRNA had a 125-fold increase in whole blood cells (38). Blocking IL-6 with a monoclonal antibody in a primate model of sepsis, largely prevented LPS-induced coagulation activation once decreased significantly levels of prothrombin fragment 1+2 (F1+2) and thrombin-antithrombin complex (26). Contrarily, the same treatment in humans did not reduce LPS-induced TF mRNA or plasma concentrations of the same markers of coagulation activation showing that results obtained from primates may not automatically be transferable into humans. IL-6 is also involved in platelet thrombogenicity, once after addition of this cytokine to whole blood samples of healthy individuals, a marked spreading and clumping of the platelets was induced indicating an hyper-activation state (39).

Although the coagulation alterations mentioned above have been demonstrated to occur *in vivo* as a general response to pro-inflammatory stimuli, it is likely that other hematological changes

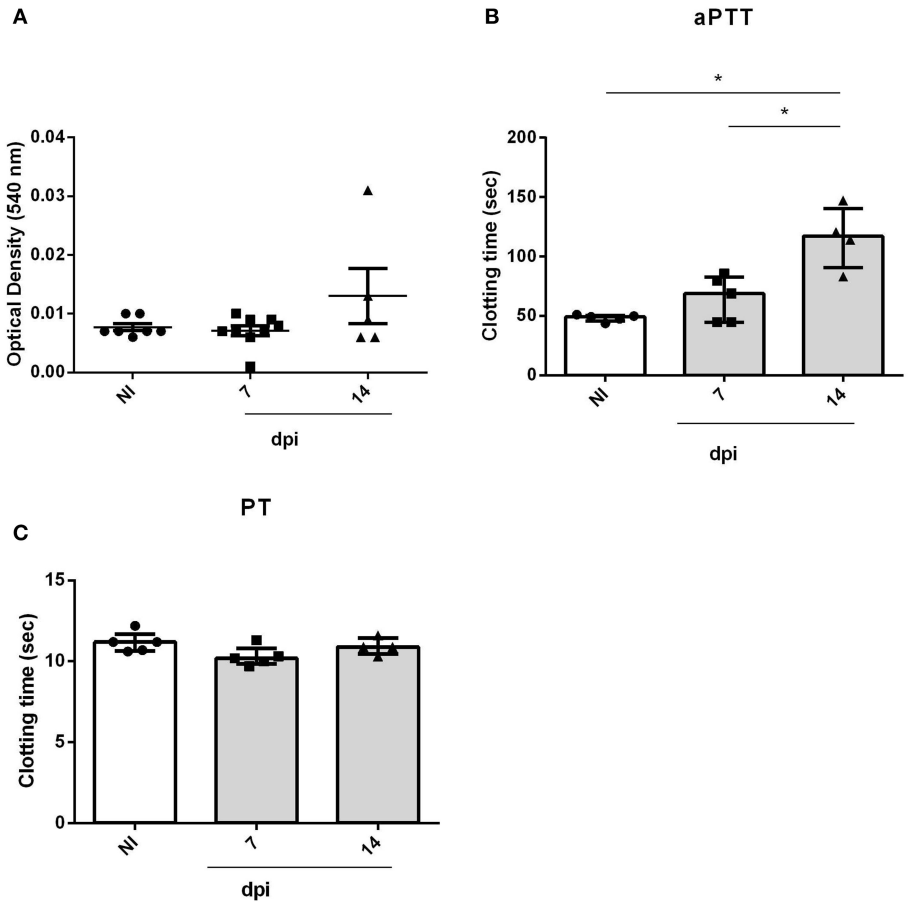

FIGURE 5 | Bleeding from the tail and *ex vivo* PT and aPTT measurements of BALB/c mice during subcutaneous infection. Male BALB/c mice were subcutaneously infected with 5×10^4 culture-derived *T. cruzi* trypomastigotes (Tulahuén strain). **(A)** Bleeding was caused by a tail transection in NI, 7 and 14 dpi SC mice. Absorbance at 540 nm (hemoglobin concentration) was used to estimate blood loss. **(B,C)** NI or SC plasma samples were obtained by cardiac puncture followed by addition of aPTT or PT reagent. Clotting time was estimated using a coagulometer. **(A-C)** Values are presented as mean ± SEM for each group/day post-infection and are representative of one experiment. Results were analyzed using one way ANOVA with Tukey's multiple comparisons test (*$0.0001 < p < 0.05$).

may occur. *Trypanosoma cruzi* infection in humans is associated with anemia, thrombocytopenia and leukocytosis, mainly during the acute phase of disease (1, 40). Studies in inoculated mice with different *T. cruzi* strains also revealed anemia and thrombocytopenia during the acute phase of infection (41, 42). The mechanisms underlying these changes in blood count are not fully understood, but bone marrow suppression in hemopoiesis may be involved (42). In a second vein, the parasite trans-sialidase may induce the accelerated clearance of the platelets after depleting their sialic acid content that could lead to the thrombocytopenia observed during ACD (43). In a review of 31 published studies concerning hematological alterations in non-human hosts infected with *T. cruzi* (44), half of the studies reported anemia. However, we did not find significant changes in the erythrogram. An explanation could be the different route of inoculation.

Additionally, increased numbers of leukocytes were observed on 21 and 28 dpi. This alteration is also described in patients and in animal models (44–47). At 21 dpi, when platelet counts were still significantly lower, the normal platelet plug formation was affected as ascertained by tail bleeding assay. Notwithstanding, thrombocytopenia in humans with functional platelets generally does not induce or induces only minor bleeding symptoms, with the exception of life-threatening hemorrhages (48, 49). By contrast, mice with severe thrombocytopenia and inflammation resulted in spontaneous hemorrhage in different organs (50). Also at 21 dpi, beyond reduction in platelet plug formation, coagulation changes were seen with increased aPTT. Interestingly, production of D-dimer was evaluated and was detected at 14 and 21 dpi and FVIII levels were very low at 14 dpi.

Taken together, the disturbances mentioned above characterize the clinical syndrome of disseminated intravascular coagulation (DIC). This syndrome corresponds to a derangement of hemostasis with hemorrhage being the most common presentation consisting of widespread production of thrombin, which in turn leads to microvascular thrombosis, organ failure, and a consumptive coagulopathy related to a systemic

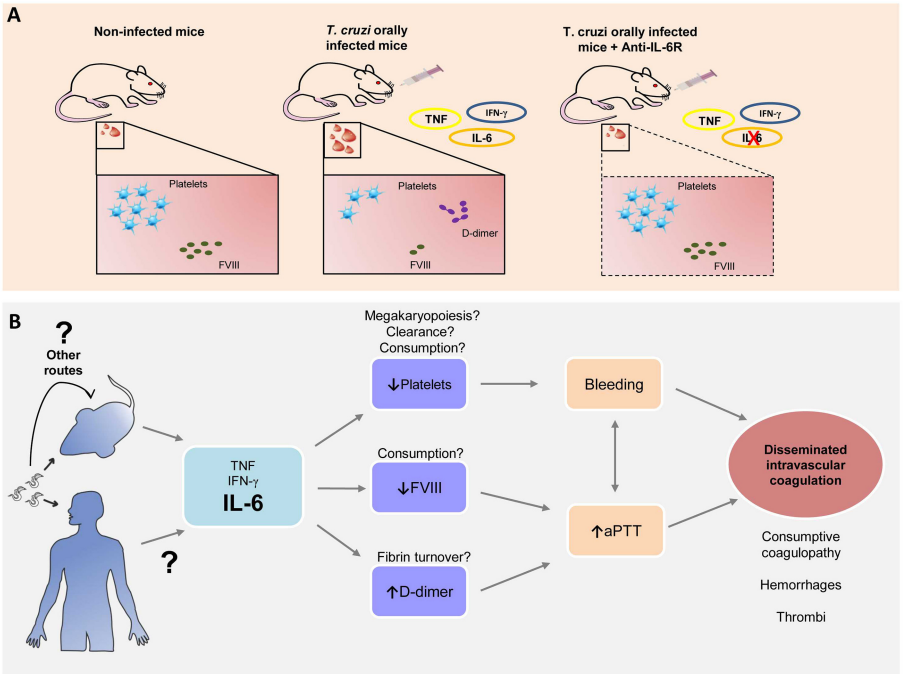

FIGURE 6 | *Trypanosoma cruzi* oral infection causes hemostatic derangement linked to a systemic inflammation. **(A)** *Trypanosoma cruzi* orally infected mice have thrombocytopenia, Factor VIII depletion and increased D-dimer levels, linked to a systemic inflammation. Blocking of IL-6 signaling restores normal hemostasis. **(B)** Hypothesis: Acute *T. cruzi* infection leads to increase of pro-inflammatory cytokines. IL-6 is associated with a decrease with platelet count, possibly due to megakaryopoiesis disturbances, platelet clearance and/or consumption; reduction in serum FVIII levels and high levels of D-dimer related to fibrin turnover. Altogether these processes are involved in the increase of bleeding tendency and aPTT, signs of disseminated intravascular coagulation that leads to microhemorrhages and microthrombi as previously reported (7, 13, 16).

inflammation (19). In fact, we previously showed formation of thrombotic masses in the liver of OI mice (16). Patients with DIC have higher IL-6 levels with the cytokine increase being paralleled by the severity of the disease (51). Therefore, we hypothesized that blockade of the IL-6 signaling would protect or minimize the hematological disturbances observed at 21 dpi. Yet, there was an impressive reduction in inflammation associated changes comprising significant reduction in coagulation time, aPTT and bleeding time. Both did not differ from control levels. However, anti-TNF treatment did not change these parameters, suggesting that TNF is not affecting directly the coagulation cascade, as observed in sepsis (26, 27).

Thus, based on these early findings, targeting the immune system, more specifically IL-6R, during the acute phase of oral infection, can prevent a hemostatic derangement (**Figure 6A**). Since any long-term benefits and liabilities of the intervention still remain uncertain, it will be interesting to explore whether long-term benefits are also gained in our model of *T. cruzi* oral infection.

This study instigates many questions to explore. The hematological disturbances also develop in a parenteral route of transmission such as subcutaneously, suggesting that inflammatory response to parasite presence in the blood triggers this process. Moreover, whether there is an influence of the genetic variability of the parasite, i.e., *T. cruzi* DTUs (named as TcI to TcVI) correlated with more severe hematological changes. TcI, TcII, TcIII, TcIV, and TcVI genotypes had been reported in oral outbreaks (52–54) even though in the Brazilian Amazon prevails TcI (55) and in this study we used Tulahuén strain (TcVI). Ultimately, if there is any dysregulation of megakaryopoiesis leading to thrombocytopenia, changes in platelet clearance or consumption, deep alterations in fibrinolytic system remains undetermined (**Figure 6B**).

In conclusion, we show for the first time that oral ACD promotes a hemostatic derangement linked to systemic inflammation. This process is associated to low platelet count, bleeding and increased coagulation time, in parallel with high parasitemia. Blocking IL-6 signaling pathway ameliorates all these changes. Our studies open a new paradigm of looking to the hemostatic system when evaluating a patient infected with *T. cruzi* and suggest that translation of these results may be possible in the near future.

ETHICS STATEMENT

This study was carried out in accordance with the recommendations of the Guide for the Care and Use of Laboratory Animals of the Brazilian National Council of

Animal Experimentation and the Federal Law 11.794 (10/2008) Institutional Ethics Committee for Animal Research of the Oswaldo Cruz Foundation (CEUA-FIOCRUZ). The protocol was approved by CEUA-FIOCRUZ, License: L-028/16.

AUTHOR CONTRIBUTIONS

Conceived and designed the experiments: DA, RM, and JdM. Performed the experiments: DA, AM-D-S, MR, BM, CM, and DF-d-O. Analyzed the data: DA, WS, and JdM. Wrote the manuscript: DA, WS, RM, and JdM. All authors read and revised the manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2019.01073/full#supplementary-material

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Platelet Contributions to Myocardial Ischemia/Reperfusion Injury

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Obstruction of a coronary artery causes ischemia of heart tissue leading to myocardial infarction. Prolonged oxygen deficiency provokes tissue necrosis, which can result in heart failure and death of the patient. Therefore, restoration of coronary blood flow (reperfusion of the ischemic area) by re-canalizing the affected vessel is essential for a better patient outcome. Paradoxically, sudden reperfusion also causes tissue injury, thereby increasing the initial ischemic damage despite restoration of blood flow (=ischemia/reperfusion injury, IRI). Myocardial IRI is a complex event that involves various harmful mechanisms (e.g., production of reactive oxygen species and local increase in calcium ions) as well as inflammatory cells and signals like chemokines and cytokines. An involvement of platelets in the inflammatory reaction associated with IRI was discovered several years ago, but the underlying mechanisms are not yet fully understood. This mini review focusses on platelet contributions to the intricate picture of myocardial IRI. We summarize how upregulation of platelet surface receptors and release of immunomodulatory mediators lead to aggravation of myocardial IRI and subsequent cardiac damage by different mechanisms such as recruitment and activation of immune cells or modification of the cardiac vascular endothelium. In addition, evidence for cardioprotective roles of distinct platelet factors during IRI will be discussed.

Keywords: myocardial infarction, ischemia reperfusion injury, platelets, reperfusion, ischemia

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INTRODUCTION

Myocardial infarction (MI) is the leading cause of death in the western world (1). The role of platelets in the progression of coronary plaques and the thrombotic occlusion of coronary vessels leading to ischemia and MI is well understood (2). Besides this, microembolization, and platelet accumulation within the affected microcirculation of the myocardium during late ischemia and reperfusion (IR) lead to a secondary tissue damage (2). Rapid restoration of blood flow after MI is the primary goal of state-of-the-art therapy in order to limit the damage of cardiac tissue caused by the lack of oxygen supply. Paradoxically, reperfusion itself also causes adverse effects and increases infarct size (1, 3). This phenomenon is called ischemia/reperfusion injury (IRI) and might account for nearly 50% of the final myocardial damage in acute MI (1). IRI can cause myocardial stunning, the no-reflow phenomenon, reperfusion arrhythmia and also lethal reperfusion injury (reviewed in (4). As underlying mechanisms oxidative stress, calcium overload and pH shifts have been described (1). In this context, mitochondrial damage including disruption of ATP production and opening of the mitochondrial permeability transition pore with subsequent necrotic and apoptotic cell death play an important role (5). Additionally, ischemia–reperfusion induced alterations in the regulation of cytosolic osmolality and cell volume cause cellular and interstitial edema that

are associated to microvascular obstruction, cell dysfunction and death (6, 7). Reperfusion has repeatedly been shown to be an inflammatory state that is accompanied by leukocyte infiltration (8, 9). While a certain degree of inflammation is necessary for cardiac repair after IRI it may also develop in an unwanted direction and extend injury (9, 10). Already since the 1980s an involvement of platelets in the inflammatory reaction associated to IRI has been recognized. The damage caused by IR in the vascular endothelium causes activation of circulating platelets, resulting in the release of their immunomodulatory contents (11). The degree of platelet activation is related to the duration of the preceding ischemia and the extent of reperfusion injury (5). This mini review aims to summarize mechanisms of platelet contributions to myocardial IRI.

THE IMMUNOMODULATORY EQUIPMENT OF PLATELETS

Platelets are small anucleate cells that circulate through the bloodstream in large numbers. Beyond their primary role in hemostasis platelets have been recognized as immunomodulatory cells that fulfill their tasks through a variety of mechanisms ranging from secretable factors to stably or variably expressed surface receptors (12). Specifically, platelets secrete or expose adhesion proteins like fibrinogen, fibronectin, von Willebrand factor, thrombospondin or P-selectin that are involved in cell-cell interactions (e.g., with leukocytes or endothelial cells) during inflammation (13–20). Mitogenic factors released by platelets such as platelet-derived growth factor (PDGF) and transforming growth factor beta (TGFβ) target monocytes, macrophages as well as T-cells, for example to achieve wound healing or immunosuppression (21–24). And chemokines like RANTES/C-C motif ligand (CCL)5, platelet factor 4 (PF4)/ C-X-C motif ligand (CXL)4 or serotonin (5-hydroxytryptamine) are involved in leukocyte recruitment to sites of inflammation (25–29). Moreover, platelets can trigger the complement activation and play a role in localizing inflammatory areas (30). Already with these few examples of immunomodulatory platelet functions in mind, it is obvious to implicate these cells as key contributors to the myocardial IR associated inflammatory response.

EXPERIMENTAL MODELS OF ISCHEMIA/REPERFUSION INJURY

In the past years a number of experimental models of IRI were applied in various species by different research groups. The following table briefly summarizes the models mentioned in this review with their respective modes of platelet activation (**Table 1**).

PLATELET MEMBRANE PROTEINS IN IRI

Collagen Receptor

The combination of glycoprotein (GP) VI with Fc receptor (FcR)γ forms the platelet collagen receptor. Takaya et al. demonstrated in 2005 that collagen-dependent activation of platelets can drive the extension of myocardial IRI. In their study, knock-out (k.o.) of FcRγ improved outcome of mice after myocardial IR with the infarct size being significantly smaller than in wildtype (WT) mice. Mechanistically the FcR$\gamma^{-/-}$ mice displayed less platelet aggregation and occlusive microthrombi, less platelet spleen tyrosine kinase (Syk) activation as well as reduced myeloperoxidase (Mpo) activity in the injured area (51). A more recent study confirmed a role of the collagen receptor in IRI by targeting GPVI with a monoclonal antibody derivative in a murine *in vivo* model of left anterior descending artery (LAD) ligation followed by reperfusion. Anti-GPVI treatment significantly reduced infarct size vs. control, which was again primarily based on an improved microperfusion (52).

Adenosine Diphosphate (ADP) Receptor

The platelet $P2Y_{12}$ receptor is responsible for mediating the sustained ADP-dependent platelet aggregation response. In a dog coronary thrombosis model, ticagrelor, a reversibly binding $P2Y_{12}$ receptor antagonist, was shown to significantly decrease infarct size and rapidly restore myocardial tissue perfusion by inhibition of ADP-mediated platelet aggregation and recruitment (50). Supporting results were demonstrated by infusing platelets from acute MI (AMI) patients (untreated or treated with 10 μM of the $P2Y_{12}$ inhibitor cangrelor) into isolated rat hearts that were subjected to 40 min of ischemia with subsequent 60 min of reperfusion. Platelets from AMI patients significantly augmented myocardial injury while $P2Y_{12}$ blockage with cangrelor reduced infarct size and attenuated the adverse effects of platelet infusion on cardiac function (48). Besides direct inhibition of aggregation, protective effects of cangrelor during IRI could in part be mediated by inhibition of platelet P-selectin expression and platelet-leukocyte interactions (53). Furthermore, in a rabbit model of myocardial IR, coapplication of individual inhibitors revealed a role for adenosine A_{2B} receptors, ERK, Akt, redox signaling, and mitochondrial KATP channels in mediating the protective cangrelor effect independent of its antiaggregatory properties (37).

Glycoprotein-IIb/IIIa-Receptor

Given its important role in platelet activation GPIIb/IIIa is predestined as a contributor to IRI. Indeed, inhibition of GPIIb/IIIa reduces platelet induced aggravation of IRI in isolated rat hearts. Infusion of platelets from AMI patients worsened myocardial injury in hearts subjected to IR, as measured by left ventricular (LV) developed pressure, higher maximal LV end-diastolic pressure and coronary resistance as well as increased lactate dehydrogenase (LDH) release and infarct size. Pretreatment of platelets with the GPIIb/IIIa inhibitor abciximab greatly attenuated these effects (48). Mechanistically, in addition to interrupting platelet adherence to the reperfused endothelium through GPIIb/IIIa-fibrinogen binding, abciximab also interferes with other mechanisms of platelet adhesion including the vitronectin receptor or leukocyte Macrophage-1 antigen (Mac-1) (48, 54, 55). In contrast, a publication from 2016 did not confirm a significant effect of GPIIb/IIIa antagonization on infarct size using a specific monoclonal antibody derivative in a murine *in*

vivo model of left coronary artery ligation (52). Yet, another study confirmed the adverse effect of GPIIb/IIIa dependent intracoronary platelet retention during low flow ischemia on cardiac function using the GPIIb/IIIa inhibitor tirofiban. This was partially attributed to tirofiban-induced blockage of platelet adherence to the vessel wall mediated by an interaction of the GPIIb/IIIa-receptor and von Willebrand-factor (56). However, in this study no GPIIb/IIIa effect on cardiac function was observed during the reperfusion phase (56), suggesting that conflicting results between studies might at least partially be based on different time points of inhibitor application during the course of IRI, especially since platelet adhesion to reperfused endothelium and platelet-mediated myocardial damage have been described to occur very early after reperfusion (48). Additionally, the use of different experimental models which induce different modes of platelet activation are a decisive factor for experimental outcome (see **Table 1**).

P-Selectin

P-selectin is an adhesion molecule expressed on the surface of activated platelets and mediates cell-cell interactions involving platelets, e.g., platelet-neutrophil-complexes which have been associated with many inflammatory conditions (14, 57). Platelet P-selectin expression is increased after IR in several animal models as well as in humans (48, 58). Infusion of platelets activated in this manner into isolated rat hearts subjected to ischemia and reperfusion strongly increases myocardial LDH release representing cardiomyocyte damage (58). In loss of function approaches it was demonstrated that the chemical or small molecule dependent blockade of platelet P-selectin has beneficial effects on platelet mediated reperfusion injury after myocardial IR in pigs and rats, respectively (38, 43). Additionally, a study using genetically modified mice confirmed these results as significantly smaller infarct sizes after myocardial IR were observed in P-selectin k.o. mice or mice transfused with P-selectin k.o. platelets as compared to wild-type (32). Based on these studies the role of activated platelets in the process of myocardial IRI seems to depend at least in part on their activation status as represented by platelet-derived P-selectin. Mechanistically, enhanced P-selectin expression on platelets increases adherence to the reperfused endothelium as well as postischemic leukocyte adhesion, thereby aggravating the inflammatory reaction associated to IRI. However, in terms of leukocyte recruitment it needs to be taken into account that endothelial P-selectin expression can contribute to the observed effects of P-selectin antagonization in some of the reported experimental models, too (59).

G-Proteins

G proteins are involved in transmitting signals from a variety of stimuli outside of a platelet to its interior. $G_{\alpha q}$ k.o. in mice eliminates platelet function in terms of aggregation and secretion of cytokines. In these mice infarct size to area at risk ratio was significantly smaller as compared to WT after 30 min of regional myocardial ischemia (LAD ligation) followed by 24 h of reperfusion. Additionally, $G_{\alpha q}$ k.o. improved fractional shortening in this model. The beneficial effects were resembled by transplantation of $G_{\alpha q}$ k.o. bone marrow into WT mice (34). The effect of $G_{\alpha q}$ k.o. outperformed the protection of sole inhibition of platelet aggregation and was accompanied by reduced expression of the fibrinogen receptor CD41 and P-selectin as well as secretion of platelet-derived growth factor after platelet activation (34). Likewise, the platelet Gi protein $G\alpha_{i2}$ is an essential mediator of thrombo-inflammatory organ damage in mice. This was shown in mice lacking $G\alpha_{i2}$ in megakaryocytes and platelets ($Gnai2^{fl/fl}$/PF4-Cre) that developed a dramatically reduced reperfusion injury that correlated with diminished formation of ADP-dependent platelet neutrophil complexes after myocardial IR (35).

Na^+/H^+ Exchanger Isoform 1 (NHE1)

NHE1 is an integral membrane protein that removes one intracellular H^+ for one extracellular Na^+ protecting cells from intracellular acidification. NHE1 activation in cardiomyocytes is known to contribute to injury and arrhythmias during IR by promoting calcium overload via the sodium-calcium exchanger (60–62). But not only cardiomyocyte NHE1 activation is a driver of IRI. NHE1 is also expressed on platelets and is involved in the regulation of the platelet's intracellular pH, platelet volume as well as cell signaling and platelet activation (63, 64). In a rat model of myocardial IR the NHE1 blocker KR-32568 dose-dependently inhibited NHE-1-mediated rabbit platelet swelling and dose dependently reduced infarct size when applied 10 min before ischemia (39).

MEDIATORS RELEASED BY PLATELETS

As part of their pleiotropic actions platelets can rapidly secrete a wide array of inflammatory mediators, either from their granules or in a granule independent manner (12) which are reasonable candidates for mediating inflammation during IRI.

Reactive Oxygen Species (ROS)

ROS occur as byproducts of certain enzymatic reactions (e.g., catalyzed by xanthine oxidases, cytochrome P450 or NADPH oxidase). ROS play important roles in cell signaling and homeostasis but are also involved in the pathogenesis of several diseases including cardiovascular disease (65, 66). In guinea pig hearts exposed to low-flow ischemia with following reperfusion activated human platelets administered in the beginning of reperfusion significantly reduced the recovery of external heart work (REHW). Coapplication of the radical scavenger enzyme superoxide dismutase improved REHW during reperfusion indicating a role of ROS in the provoked IRI. Interestingly, by coapplication of the GPIIb/IIIa-blocker tirofiban the authors could show that the platelet-induced ROS-dependent myocardial dysfunction in their experimental model was independent of intracoronary platelet adhesion (49, 56, 66). In a follow up study, by applying a platelet pretreatment with diphenyliodonium chloride Seligmann et al. elegantly proved that the shown cardiodepressive effects were mediated by ROS released from platelets and not the heart itself (49). ROS-induced effects on reperfused myocardium

TABLE 1 | Experimental models of ischemia/reperfusion injury.

Model	Species	Characteristics	Modes of platelet activation	References
LAD ligation	Mouse, rat, rabbit, minipig, and sheep	Intact heart with physiological blood flow Localized ischemia induction via ligation of LAD No occlusive thrombus formation Reperfusion with whole blood via reopening of LAD ligature	Accumulation of procoagulants due to stasis Activated endothelium due to stasis, hypoxia and subsequent reperfusion Cytokines and chemokines released in the ischemic myocardium To some extent by the invasive surgical procedure	(31–44)
Isolated working heart preparations	Guinea pig and rat	Perfusion with physiological cell-free buffer Induction of global ischemia and reperfusion with cell-free buffer Optional perfusion with washed platelets or supernatants of aggregated platelets	Activated endothelium Cytokines, chemokines released in ischemic myocardium Optional pre-activation by chemical agents Optional pre-activation by IRI in AMI patients	(45–48)
Isolated working heart preparations, low flow ischemia	Guinea pig	Perfusion with physiological cell-free buffer Induction of global low flow ischemia (1 ml/min) and subsequent reperfusion Optional perfusion with platelets	Exogenous thrombin activation during low flow ischemia and beginning of reperfusion	(49)
Coronary thrombosis induction	Dog	Coronary thrombus induction by electrolytic injury Localized ischemia Reperfusion by thrombolysis with tissue plasminogen activator	Injured endothelium (e.g., GPVI interaction with exposed subendothelial collagen, PSGL-1/P-selectin interaction etc.) Shear stress Activated endothelium due to ischemia and reperfusion Cytokines, chemokines released in ischemic myocardium	(50)

are based on several mechanisms including calcium overload by interference with myocardial calcium transport, damage to membranes and proteins, as well as opening of the mitochondrial permeability transition pore and subsequent apoptosis (4, 49).

Serotonin

Serotonin (5-hydroxytryptamine) is a biogenic amine present in circulation and non-neuronal cells as peripheral hormone and in the central nervous system as neurotransmitter. In the periphery it is stored in high concentrations in dense granules of platelets. Myocardial IRI is accompanied by elevated serotonin plasma levels in mice (33). In 1994 Hohlfeld et al. demonstrated that nexopamil, a combined Ca^{2+} and serotonin antagonist, reduced infarct size and improved functional cardiac parameters in minipigs subjected to 1 h of LAD occlusion with a subsequent 3 h reperfusion. Besides calcium channel blocking activity, inhibition of ischemia-induced neutrophil activation and enhanced endogenous PGI_2 formation were claimed to be factors contributing to the beneficial effects of nexopamil (42). Later, serotonin's harmful mode of action in IRI was partially attributed to oxidative stress caused by mitochondrial MAO-A activity. MAO-A is responsible for serotonin degradation with H_2O_2 production. Evidence was presented that the oxidative stress induced by this enzymatic reaction was responsible for receptor-independent apoptotic effects of serotonin in cardiomyocytes and postischemic myocardial injury (40). We found recently that absence of platelet serotonin improves outcome of mice after myocardial ischemia and reperfusion, i.e., a 30% smaller infarct size and less compromised LV function and ejection fraction, which were accompanied by reduced neutrophil infiltration within the infarcted tissue. Mechanistically, platelet-derived serotonin induced neutrophil degranulation with release of myeloperoxidase and H_2O_2 as well as increased surface expression of the adhesion molecule CD11b, leading to enhanced inflammation in the infarct area and reduced myocardial salvage (33).

Platelet Activating Factor (PAF)

PAF is a phosphoglyceride produced by platelets, leukocytes and endothelial cells which acts as an autocrine and paracrine mediator on different cell types, e.g., cardiomyocytes, endothelial cells and platelets (67). During IRI high quantities of PAF (1–10 nmol/L) are released and can exert negative effects on coronary and cardiac functions, including arrhythmogenic effects (68–70). Negative effects of PAF can be mediated either by the generation of secondary mediators, or through the activation of inflammatory cells like platelets and neutrophils (67). For example, it was demonstrated that administration of a specific PAF receptor antagonist immediately before reflow in an intact sheep model reduces myocardial reperfusion injury—an impact which was partially attributed to its anti-platelet effect (44). Furthermore, PAF has been shown to stimulate NHE1 in neutrophils and platelets (63, 71). Negative consequences of NHE1 activation in the context of IRI involve platelet swelling and calcium overload in cardiomyocytes (see above). However, PAF seems to play a dual role in IR depending on its local concentration. Potential cardioprotective effects of PAF are described later in this review.

LEUKOCYTE-PLATELET-INTERACTIONS IN IRI

It has long been known that the interaction between platelets and neutrophils is associated to MI (57). Already in 1997 Neumann et al. showed that in peripheral venous blood samples of patients with AMI leukocyte platelet adhesion was increased and claimed that this was part of the regulation of the inflammatory response in acute MI (72). A causative connection between platelet neutrophil interactions and IRI was supported by several studies showing worsened cardiac functions in isolated heart models of global ischemia and reperfusion after simultaneous perfusion with both neutrophils and platelets compared to perfusion with either platelets or neutrophils alone (45, 73) as well as attenuation of these adverse effects by inhibition of platelet neutrophil complex formation (74). These relationships seem logical, especially as neutrophils interact strongly with platelets to regulate the performance of their immune cell functions (75). However, there are opposing studies as well. Seligmann et al. did not observe an additional effect of simultaneous applications of platelets and neutrophils over sole application of either of both in a study using isolated guinea pig hearts (76). In contrast, indications for a role of platelet neutrophil interactions in IRI were also found by different inhibition approaches of the P-selectin-P-selectin glycoprotein ligand 1(PSGL1) axis that led to alleviation of myocardial IRI. Several animal studies in different species demonstrated beneficial effects of P-Selectin neutralization, e.g., via small molecule inhibition (38), chemical blockade (43), or antibody blocking (77) on IRI which were all associated with an antiplatelet effect accompanied by less neutrophil infiltration or platelet-neutrophil adhesion in the infarcted region. In addition to P-selectin, also platelet GPIIb/IIIa is claimed to contribute to postischemic leukocyte adhesion (36) and also the disintegrin-dependent attenuation of platelet induced myocardial IRI was shown to be accompanied by reduced neutrophil infiltration (41).

PLATELET-ENDOTHELIAL-INTERACTIONS IN IRI

IR induces cellular responses on microvascular endothelial cells and circulating platelets become activated (5). At the same time the adhesion and activation of platelets goes along with the release of various proinflammatory and promitogenic substances which change chemotactic, adhesive and proteolytic properties of the endothelial cells in the local surrounding (2). The inhibition of GPVI-mediated platelet-endothelial interaction via recombinant soluble GPVI-Fc was shown to reduce platelet

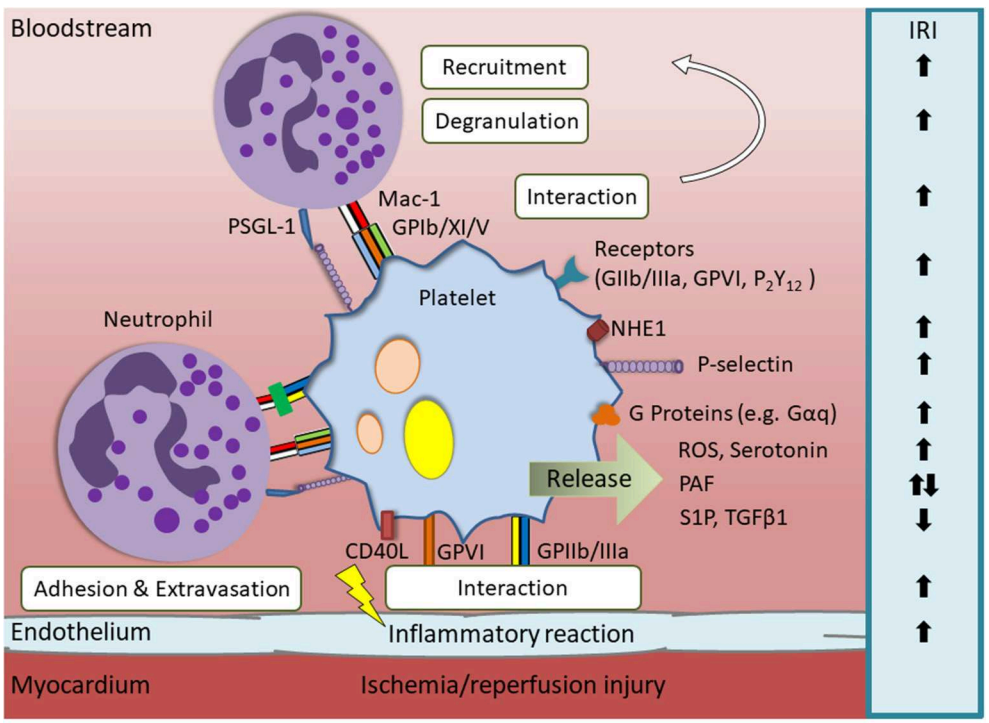

FIGURE 1 | Overview of relevant platelet derived mediators and their influence on myocardial ischemia/reperfusion injury (IRI). Endothelial damage caused by IRI leads to activation of platelets. This is accompanied by upregulation of surface proteins and the release of immunomodulatory contents that influence the progression of IRI via different mechanisms. Platelet receptors that are involved in IRI aggravation are glycoprotein (GP) IIb/IIIa, GPVI and P2Y12. Additionally, platelet membrane proteins, such as sodium-hyodrogen-exchanger 1 (NHE1), Gαq, Gαi2, and P-selectin, or secretable factors, e.g., reactive oxygen species (ROS) and serotonin, worsen the cardiac outcome after myocardial infarction. Cardioprotective effects are for example exerted by platelet-derived sphingosine-1-phosphate (S1P), low concentrations of platelet activating factor (PAF) and transforming growth factor beta 1(TGFβ1).

degranulation and the release of proinflammatory cytokines. This lead to decreased infarct size and improved cardiac function due to a reduced inflammatory response of the infarcted myocardium in a mouse model of IRI (31). Furthermore, CD40 ligand on activated platelets triggers an inflammatory reaction of endothelial cells (78) and dual neutrophil and platelet infiltration leads to enhanced P-selectin expression on the coronary microvascular endothelium in rat hearts subjected to IR (73).

CARDIOPROTECTIVE EFFECTS OF PLATELETS IN IRI

Besides the negative effects induced by platelet adhesion and aggregation as well as the platelet dependent leukocyte infiltration into the infarcted myocardium, constituents released by platelets may have beneficial effects on the integrity of the coronary endothelium and on cardiac function after IRI (79, 80). In isolated rat hearts perfusion with platelets or supernatant of aggregated platelets was shown to exhibit cardioprotective effects (46) that were partially attributed to serotonin, thromboxane A2 or adenin nucleotides and their ability to induce the release of cardiac microvascular endothelial NO and its associated tissue protecting effects (81, 82). The platelet α granule contents transforming growth factor-beta 1 (TGF-β 1) and stromal cell-derived factor (SDF)1-α were also shown to be cardioprotective (83–85)—an effect most probably mediated by enhanced cardiomyocyte proteinkinase C (PKC) activity as a prosurvival signaling mechanism (85). Also platelet-derived sphingosine-1-phosphate (S1P) seems to facilitate protection from IRI. Although platelet derived S1P can have both pro- and anti-aggregatory effects via G-protein coupled receptors on platelets it was shown to directly induce myocardial protection. S1P signals through S1P receptors of cardiomyocytes with concomitant activation of pro-survival signaling, namely the reperfusion injury salvage kinase (RISK) and the survivor activating factor enhancement (SAFE) pathway (68). SAFE and RISK were both shown to be protective on cardiomyocytes when acutely activated at the time of reperfusion, most probably through inhibition of the opening of the mitochondrial permeability transition pore (86, 87). PAF which was already mentioned as an IRI causing factor earlier in this review is cardioprotective in very low concentrations. This effect involves activation of the RISK pathway, including protein kinase C, AKT, and nitric oxide synthase (47, 67). Another described protective mechanism of platelets during IRI is the process of mitophagy which removes damaged mitochondria. Hypoxic mitophagy in platelets leads to extensive degradation of mitochondria and reduces IRI by diminishing platelet activity (5).

CONCLUSIONS

The pathophysiology of IRI and the contribution of the involved immune cells to its progression is complex and only partially understood. However, during the past years critical roles of platelets in the origin and course of IRI as well as several underlying molecular mechanisms have been unraveled and **Figure 1** summarizes most of them. Although single-agent approaches targeting these mechanisms have not yet entered clinical practice, a better understanding of platelet mechanisms in IRI could provide the basis for new and effective treatment strategies aimed at further improving protection of the myocardium during reperfusion in the future.

AUTHOR CONTRIBUTIONS

NS did literature research, designed figures, and wrote the first draft of the manuscript. DD contributed the idea of the manuscript, wrote sections of the manuscript, and provided critical feedback. CB supported writing and provided critical feedback. All authors contributed to manuscript revision, read, and approved the submitted version.

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Platelets as Mediators of Thromboinflammation in Chronic Myeloproliferative Neoplasms

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Chronic myeloproliferative neoplasms (MPN) are stem cell disorders driven by mutations in *JAK2*, *CALR,* or *MPL* genes and characterized by myeloid proliferation and increased blood cell counts. They encompass three closely related conditions, including essential thrombocythemia, polycythemia vera, and primary myelofibrosis. Elevated levels of cytokines released by clonal and non-clonal cells generate a chronic proinflammatory state that contributes to disease pathogenesis. Thrombosis represents the most common cause of morbidity and mortality in MPN, although paradoxically, patients may also present with a bleeding diathesis. The mechanisms leading to thrombosis are complex and multiple and include increased blood cells together with qualitative abnormalities of red cells, leukocytes, and platelets that favor a prothrombotic activated phenotype. The functional interplay between blood cells, the clotting cascade, and dysfunctional endothelium contributes to hypercoagulability and this process is perpetuated by the effect of inflammatory cytokines. In addition to their well-known function in hemostasis, platelets contribute to innate immunity and inflammation and play a key role in MPN thromboinflammatory state. *In vivo* platelet activation leads to platelet aggregate formation and exposure of adhesion molecules which favor their interaction with activated neutrophils and monocytes leading to circulating platelet-leukocyte heterotypic aggregates. Platelets are recruited to the activated endothelium further enhancing the reciprocal activation of both cell types. Crosstalk between activated cells drives cytokine production, further fuelling the self-reinforcing thromboinflammatory loop. In addition, MPN platelets provide a procoagulant scaffold which triggers the coagulation cascade and platelet-derived microparticles amplify this response. Markers of platelet, leukocyte, endothelial and coagulation activation are increased in MPN patients although prospective studies are required to determine the potential value of these parameters for identifying patients at increased thrombotic risk. Thrombosis remains the main complication of MPN patients, with a high risk of recurrence despite adequate cytoreductive and antithrombotic treatment. Deeper insight into the mechanism favoring thrombosis development in this setting may lead to novel therapeutic approaches for MPN thrombosis. Considering the critical role of inflammation in the vascular risk, concomitant targeting of inflammatory pathways could potentially impact on primary or secondary prevention strategies.

Keywords: chronic myeloproliferative neoplasms, platelets, JAK2, thrombosis, inflammation, innate immunity, bleeding

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INTRODUCTION

Philadelphia-negative chronic myeloproliferative neoplasms (MPN) are clonal hematopoietic stem cell disorders characterized by excessive production of myeloid progenitors and mature blood cells. They comprise three closely related disorders, including essential thrombocythemia (ET), which is characterized by megakaryocyte proliferation and thrombocytosis, polycythemia vera (PV), which is defined by predominant erythroid expansion and increased red blood cells, frequently associated with high leukocyte and platelet counts, and primary myelofibrosis (PMF), featured by increased numbers of dysplastic megakaryocytes and granulocyte progenitors together with variable degrees of bone marrow fibrosis (1). Hyperactivation of JAK2-signaling is a common feature in MPN pathogenesis and is driven by mutations in three genes, including *JAK2*, *CALR,* and *MPL*. The *JAK2*V617F mutation is the most frequent molecular abnormality and may be found in over 95% of patients with PV and 50–60% of those with ET and PMF (1, 2). Defects in calreticulin (*CALR*) represent the second most frequent abnormality, which can be detected in 20–30% of ET and PMF patients (1, 2). Calreticulin mutants interact abnormally with the Mpl receptor leading to its activation and persistent JAK2 signaling (3). Finally, *MPL* mutations can be found in a low proportion (1–10%) of ET and PMF patients and generate constitutive receptor activation. None of the above-mentioned mutations are detected in 10–25% of ET and PMF cases, so-called triple-negative patients (1, 2).

Thrombosis is the main cause of morbidity and mortality in MPN and develops in around 20–35% of patients with PV, 15–30% in ET, and 10–15% in PMF (4, 5). Arterial thrombosis accounts for 60–70% of all vascular complications, and include stroke, cardiovascular events, and peripheral artery disease, whereas venous events include deep venous thrombosis and pulmonary embolism, but may also occur at unusual sites, such as the splachnic circulation. Indeed, MPN are the most frequent underlying disorders leading to Budd-Chiari syndrome and non-cirrhotic portal vein thrombosis, which may develop even in the absence of overt MPN (4, 5). A population-based study on the causes of death in MPN patients showed that cardiovascular and cerebrovascular disease accounted for high risk of death at all ages, particularly in younger patients. The most common cause of death was cardiovascular disease in patients with PV and ET, whereas patients with PMF had an increased probability of dying from hematologic malignancies (6). In addition to large-vessel thrombosis, transient platelet aggregates may clog small vessels and lead to microvascular disturbances, such as erythromelalgia and visual abnormalities, seen typically, but not exclusively, in ET (7). Paradoxically, MPN patients may also suffer from bleeding complications, which also substantially contribute to morbidity in these disorders.

THROMBOTIC RISK FACTORS

The main risk factors for thrombosis include age over 60 years and a previous history of thrombosis. According to the presence or absence of these factors, patients are stratified into low- or high-risk groups in order to guide treatment recommendations and the use of cytoreductive therapy (1). More recently, the IPSET-thrombosis model, which includes cardiovascular risk factors and the *JAK2*V617F mutation, has been proposed to better predict the thrombotic outcome in ET (8), although this score has not been yet incorporated into clinical practice. The influence of the *JAK2*V617F mutation in the thrombotic risk has been established by several studies (1, 9) and confirmed by a meta-analysis, which revealed a two-fold increase in vascular events (10). Interestingly, individuals harboring *JAK2*V617F-positive clonal hematopoiesis of indeterminate potential, in the absence of overt MPN, have an increased thrombotic risk, highlighting the relevance of the *JAK2* mutation in the thrombotic predisposition (11, 12). Conversely, *CALR*-positive patients are at lower risk of thrombosis (1).

PATHOGENESIS OF MPN THROMBOSIS

The pathogenesis of thrombosis in MPN is multifactorial and results from the complex interplay among blood cells, the endothelium and the clotting system. Increased numbers of red cells, leukocytes and platelets coupled to qualitative abnormalities that favor a prothrombotic phenotype contribute to the hypercoagulable state (4, 5). Hyperviscosity due to increased red cell mass clearly plays a role in the thrombotic predisposition of PV and, moreover, PV red cells display enhanced adhesion to endothelial laminin (13). In addition, high hematocrit favors platelet margination and accumulation at sites of vascular injury (14). In recent years, growing evidence has highlighted the key role of leukocytes in the prothrombotic state and leukocytosis has been shown to be an independent risk factor for thrombosis (15). In addition to increased numbers, there is evidence of *in vivo* neutrophil activation, as revealed by CD11b expression and elevated elastase and myeloperoxidase in circulation (16, 17), and of monocyte activation, as shown by elevated CD25 (18). Another element contributing to the thrombotic tendency involves endothelial dysfunction, which renders a pro-adhesive and proinflammatory surface, favoring leukocyte and platelet tethering and activation (4, 5).

It is currently well-established that the MPN clone induces a systemic inflammatory response, reflected by elevated levels of a wide spectrum of proinflammatory cytokines, such as IL-6, IL-1, IL-8, and TNFα (19). Inflammation and hemostasis are closely connected processes and, recently, the link between the innate immune system and coagulation as a host defense strategy against pathogens has led to the concept of immunothrombosis (20). Dysregulation of this mechanism may drive vascular disease and contribute to arterial and venous thrombosis in several disease conditions (20). Emerging work highlights the contribution of chronic inflammation to MPN hypercoagulable state, as demonstrated by the association of elevated C-reactive protein and thrombosis (5, 21). Inflammatory mediators favor the activation of both malignant and non-malignant blood cells, induce microparticle generation and elicit vascular damage, fuelling the thrombotic process (5).

ROLE OF PLATELETS IN MPN THROMBOSIS

Platelet Activation

Platelets are essential players in MPN thrombosis. Their role in this process and interplay with other elements of the procoagulant network represents the focus of this review. Both increased platelet numbers and *in vivo* activation may be involved in the prothrombotic phenotype. However, considerable controversy exists regarding the role of thrombocytosis in the thrombotic risk, as no correlation has been shown between platelet counts and vascular complications (22). Furthermore, extreme thrombocytosis (>1,000–1,500 $\times$ 10^9/L) is associated with increased bleeding rather than thrombosis. Indeed, ET patients with extreme thrombocytosis, in the absence of leucocytosis, carry a lower thrombotic risk (23). Nonetheless, reduction in platelet counts by cytoreductive therapy is useful at preventing thrombotic and bleeding complications (24–26) and current recommendations suggest that the goal of cytoreduction is to target the platelet count at <400 $\times$ 10^9/L (27). Notwithstanding controversy regarding the role of high platelet counts, there is unequivocal evidence supporting the contribution of platelet activation to the procoagulant state. *In vivo* platelet activation has been demonstrated in ET, PV and also in PMF (17, 28–31), as revealed by the finding of platelet activation markers, such as surface and soluble P-selectin and CD40L (17, 28, 29), raised β-thromboglobulin, platelet factor 4 and PDGF in plasma (32, 33) and urinary TXB2 metabolite as a reflection of thromboxane biosynthesis (30).

The mechanisms leading to platelet activation involve both intrinsic platelet abnormalities derived from disturbed hematopoietic stem cell function and linked to driver mutations that lead to hyperactive JAK2-dependent signaling (34), and extrinsic factors, such as cellular interaction with activated leukocytes, endothelial cells and soluble mediators, which may trigger activation of platelets derived not only from clonal cells, but also from megakaryocytes not involved in the malignant clone (**Figure 1**). In addition, increased platelet turnover leading to higher proportion of newly-formed platelets, which are known to display enhanced platelet reactivity, may also contribute to the hyperactivated state (29, 35). Previous data suggest that changes in megakaryocyte gene expression profile might give rise to circulating platelets with an altered hemostatic or inflammatory function in infectious or inflammatory conditions (36). Whether changes in megakaryocyte transcriptome may be associated with a similar phenotype in MPN platelets remains an intriguing possibility. In this regard, downregulation of several genes involved in thrombin signaling and platelet activation has been demonstrated in *CALR*- vs. *JAK2*V617F-positive patient samples, correlating with lower thrombotic predisposition in the former (37).

Among MPN disorders, platelet hemostatic abnormalities have been most thoroughly studied in ET. Despite the presence of basal platelet activation, conflicting results have been published regarding the *ex vivo* response to classical agonists, such as ADP and TRAP. Whereas, some studies showed normal or enhanced response to stimuli (17), others described an impaired hemostatic function, as shown by reduced P-selectin, CD63 expression and PAC-1 or fibrinogen binding triggered by classical agonists (38, 39). Similarly, light transmission aggregometry may reveal spontaneous platelet aggregation together with impaired response to different agonists, particularly ADP and epinephrine (40), although it has been suggested that the *in vitro* aggregation defect may be partly due to a laboratory artifact (41) or depend on analytical conditions (42). *In vivo* release of platelet granule contents due to spontaneous activation and secondary storage pool deficiency may explain the platelet function defect found *ex vivo*. Coexistence of platelet activation and dysfunction may contribute to the paradoxical occurrence of both thrombotic and bleeding complications. In addition, adsorption of high molecular weight multimers of von Willebrand factor (vWF) to platelet GPIbα leading to loss of large vWF multimers and acquired von Willebrand disease contributes to the bleeding diathesis (43).

Activated platelets mediate several functional responses which contribute to MPN prothrombotic state. In addition to the classical platelet hemostatic properties, growing body of evidence over the last decade highlights the relevance of alternative platelet functions, such as their role as effectors of inflammation and essential players in the innate immune response (44). The contribution of platelets as mediators of thromboinflammatory responses in MPN and their interplay with other components of the prothrombotic and proinflammatory circuit is discussed below and summarized in **Figure 2**.

Platelet-Leukocyte Cross-Talk

Platelet interaction with leukocytes is central to MPN prothrombotic scenario. Platelets bind to both neutrophils and monocytes through adhesion molecules such as P-selectin, which recognizes the PSGL-1 counterreceptor, and through GPIbα and GPIIbIIIa (via fibrinogen), both of which engage CD11b/CD18 (Mac-1). In accordance to elevated P-selectin, increased levels of platelet-neutrophil and platelet-monocyte aggregates have been shown in circulation in ET (17, 28), PV (28), and PMF (31). Crosstalk between platelets and leukocytes triggers the reciprocal activation of both cell types, contributing to the activated phenotype.

Several leukocyte functional responses are enhanced in MPN neutrophils, including the release of intracellular proteases, such as elastase and catepsin G, which activate platelets and trigger the clotting cascade by inactivating coagulation inhibitors (16). In addition, MPN neutrophils produce high levels of reactive oxygen species (45, 46), which lead to endothelial injury and modify coagulation factors. Besides these classical neutrophil functions, more recently, neutrophils have been shown to release extracellular traps (NETs), which are networks of DNA, histones and granular components which promote thrombus formation (47). Although deregulated NET formation underlies several prothrombotic conditions (47), the role of NETs in MPN remains controversial. Whereas, MPN neutrophils seldom undergo spontaneous NETosis *ex vivo* (11, 46), their response to stimuli was shown to vary according to the experimental setting. In this regard, enhanced response to ionomycin was shown in one study (11), while NETosis triggered by inflammatory

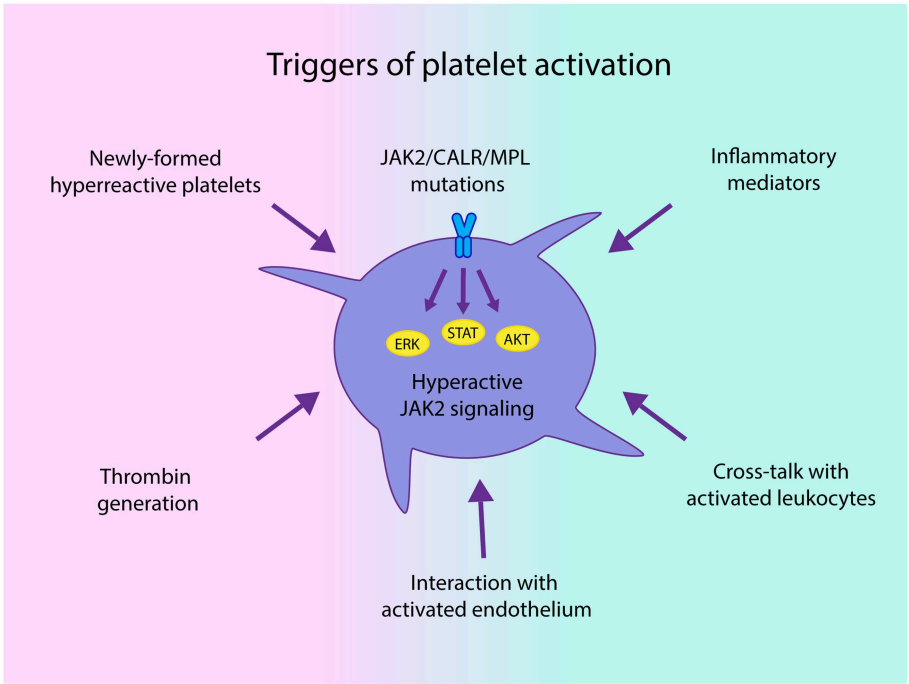

FIGURE 1 | Triggers of platelet activation in chronic myeloproliferative neoplasms (MPN). Both intrinsic abnormalities derived from the MPN clone, such as JAK2-dependent hyperactivation of signaling pathways and hyperreactive newly-formed platelets, as well as extrinsic signals driven by enhanced interaction with activated leukocytes and endothelial cells and soluble mediators, including classical platelet agonists, such as thrombin generated by the hypercoagulable state and inflammatory factors may all converge to trigger platelet activation in MPN.

cytokines and PMA was normal or impaired in another study (46), suggesting differential response to diverse NET inducers. Considering that activated platelets are a known trigger for NET formation, it might be relevant to study platelet-induced NETosis in these conditions. Likewise, platelet cross-talk with monocytes may prime monocyte functions, including tissue factor expression (48), which is increased at baseline in MPN (17), and cytokine synthesis (49), which has been found to be constitutively upregulated in PMF monocytes (50), thus perpetuating MPN prothrombotic and proinflammatory loop.

Platelet-Endothelial Interaction

The functional interplay between activated platelets and endothelial dysfunction plays an important role in the prothrombotic state. Evidence for endothelial activation in MPN is well-established, as reflected by elevated vWF antigen, soluble thrombomodulin and E-selectin (16, 29). Activated endothelial cells exhibit a pro-thrombotic phenotype which fosters platelet and leukocyte recruitment. Release of vWF from Weibel-Palade bodies tethers and activates platelets, leading to the surface translocation of platelet CD40 ligand (CD40L), which binds endothelial CD40. Cleavage of membrane CD40L generates a soluble fragment (sCD40L), which is increased in MPN plasma (29). Several factors contribute to endothelial activation in MPN, including interaction with blood cells, reactive oxygen species and inflammatory cytokines. Intriguingly, the *JAK2*V617F mutation has been detected in mature endothelial cells from selected organs, such as the spleen of PMF patients and the liver of PV patients with Budd-Chiari syndrome, suggesting the potential involvement of endothelial cells in the malignant clone (51, 52). Endothelial-like cells differentiated from MPN patient-derived induced pluripotent stem cells (53) and *JAK2*V617F-transduced HUVECs exhibit pro-adherent properties *in vitro* (54). Moreover, increased thrombus formation has been demonstrated in mouse models expressing *JAK2*V617F only in the endothelial compartment (54), overall suggesting that *JAK2*-mutant endothelial cells could contribute to the prothrombotic phenotype.

Procoagulant Potential of Platelets

MPN patients show several laboratory abnormalities indicative of chronic low-grade activation of the clotting system, such as elevated thrombin–antithrombin complexes, prothrombin fragment 1 + 2 and D-dimer levels (16, 29). Platelets are endowed with coagulation factors and activated platelets expose phosphatidylserine on their membrane, providing a catalytic substrate for the assembly of coagulation complexes and thrombin generation. Indeed, increased baseline phosphatidylserine expression on the platelet membrane has been shown in some MPN patients and overall platelet procoagulant potential was increased, as revealed by the finding of elevated platelet-induced thrombin generation (55). In addition, MPN platelets express higher surface levels of tissue factor, which represents the main initiator of blood coagulation, further enhancing the procoagulant activity (56).

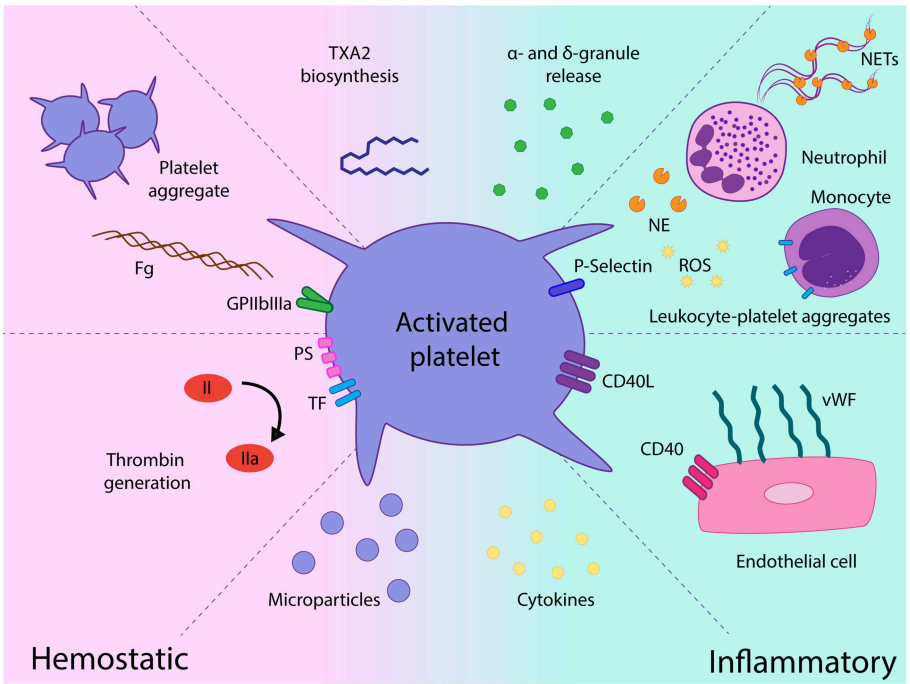

FIGURE 2 | Role of platelets as mediators of hemostatic and proinflammatory responses in chronic myeloproliferative neoplasms (MPN). Platelet activation leads to increased GPIIbIIIa activation and platelet aggregate formation, platelet procoagulant response promotes thrombin generation on the platelet surface and platelet-derived microparticles futher fuel coagulation. Release of a miriad of α- and dense granule hemostatic and inflammatory mediators may contribute to the prothrombotic and proinflammatory loop. Enhanced interaction with leukocytes mediated by P-selectin leads to leukocyte-platelet heterotypic aggregates and may trigger several leukocyte responses, such as release of proteases, production of reactive oxygen species, and expression of tissue factor. Exposure of CD40L favors platelet recruitment and adhesion to the endothelium which, in turn, elicits endothelial cell activation, featured by vWF release from Weibel-Palade bodies. Elevated cytokines in the MPN milieu may promote platelet activation and, reciprocally, platelets may represent a potential source of inflammatory cytokines and chemokines. TXA2, thromboxane A2; Fg, fibrinogen; TF, tissue factor, PS phosphatidylserine; NE, neutrophil elastase; ROS, reactive oxygen species; NETs, neutrophil extracellular traps; vWF, von Willebrand factor, EC, endothelial cell.

Platelet-Derived Microparticles

Patients with ET harbor higher numbers of circulating microparticles of platelet, endothelial and leukocyte origin, with the former comprising the vast majority of the microparticle population (57). Remarkably, a subset of microparticles co-expressing platelet and endothelial markers were also detected in ET, suggesting their bilineage origin. Platelet-derived microparticles are rich in tissue factor and phospholipid-dependent procoagulant activity and may deliver platelet-derived cytokines and chemokines, thus amplifying platelet proinflammatory and procoagulant signals.

Platelets as Immune Cells

Platelets play a key role in innate immunity and inflammation through their interaction with other immune cells and the release of proinflammatory mediators (44) and thereby participate in several disease conditions characterized by acute or chronic inflammation, such as infection, autoimmune disorders, and atherosclerosis. MPN patients display raised levels of a broad array of cytokines and chemokines in circulation, which are aberrantly secreted by multiple cell populations, including monocytes, neutrophils and hematopoietic stem cells (58). Activated platelets release pro-inflammatory chemokines stored in their α-granules, such as RANTES (CCL5) and platelet factor 4 (PF4) (CXCL4) and may undergo *de novo* cytokine synthesis following agonist-triggered RNA splicing, as shown for IL-1β. The potential contribution of platelets as a source of cyto/chemokines and inflammatory mediators in MPN has not been explored. Alternatively, platelet interaction with monocytes could deliver signals that upregulate monocyte proinflammatory gene expression, thus contributing to elevated cytokine secretion (50). Reciprocally, elevated proinflammatory cytokines might contribute to platelet activation in MPN. In this regard, IL-1β has been shown to foster hemostatic responses in normal platelets (59).

Patients with MPN carry a significant risk of second malignancies, including both solid tumors and lymphomas (60). Platelets promote tumor growth and invasiveness through several mechanisms, including the release of growth factors, cytokines, and regulators of angiogenesis. Moreover, thrombocytosis in solid tumors is associated with inferior survival supporting the role of platelets in tumor progression. On this basis, it is tempting to consider the possibility that elevated platelet counts could contribute to tumorigenesis in the setting of MPN second cancers (61). Furthermore, although the contribution of platelets to innate immunity has been more extensively studied,

platelets also influence adaptative immune responses through their interaction with T-cells, NK-cells and dendritic cells (44). Platelet-coated tumors may evade NK destruction by inhibiting NK cytotoxicity (62). In this setting, elevated platelet counts might also have implications in tumor immune surveillance and immunoregulation.

Relationship Between Platelet Activation and Clinical Features

Several factors account for the higher frequency of thrombosis observed in *JAK2*-positive ET patients, including higher hemoglobin and leukocyte counts, lower platelet counts and older age compared to *CALR*-positive and triple-negative patients. In addition, *JAK2*-positive patients display higher levels of platelet activation markers (17, 29, 56, 63), as well as leukocyte and endothelial activation and circulating microparticles (29, 56, 64), which may represent additional elements favoring thrombosis development in this subset. In addition, although sP-selectin and sCD40L were shown to correlate with a previous history of thrombosis in one study (29), currently, the value of platelet activation markers to estimate the vascular risk remains uncertain. Prospective studies assessing the role of platelet, together with leukocyte, endothelial and coagulation activation parameters in the same MPN cohort would be required to adequately address this issue and to establish whether one or more of these parameters might be useful to predict the risk of thrombosis in this setting.

On the other hand, despite the usefulness of cytoreductive therapy in preventing thrombosis development (24–26), controversy exists regarding its influence on platelet reactivity. Whereas, no difference in platelet activation was shown between patients with and without cytoreductive treatment in two different studies (16, 17), analysis of sequential samples of patients treated with hydroxyurea demonstrated a decrease in platelet-neutrophil aggregates (65) and this drug was shown to block P-selectin-triggered platelet-aggregate formation *in vitro* (65). Similarly, despite the relevance of JAK2-dependent signaling in MPN cellular abnormalities and the fact that the JAK1/2 inhibitor ruxolitinib reduces the thrombotic risk (66), no decrease in platelet activation markers was shown in patients treated with this drug (67). In another study, normalization of circulating platelet-derived microvesicles was noted in ruxolitinib spleen-responders (68), pointing out that the impact of JAK2 inhibitors in hemostatic parameters deserves further evaluation.

ANTIPLATELET THERAPY

The efficacy of low-dose aspirin for the prevention of thrombotic complications in MPN highlights the key role of platelets in this setting (69). Patients with extreme thrombocytosis (>1,000 $\times$ 10^9/L) are at higher risk of bleeding under aspirin, mainly attributed to acquired von Willebrand disease. Screening for ristocetin cofactor activity and withholding aspirin therapy if <30% is suggested in this scenario (70). Current guidelines recommend aspirin for primary prevention in PV, high-risk ET and low-risk *JAK2*-mutated ET patients who have no contraindications for antiplatelet therapy (70). On the other hand, considering that aspirin treatment of *CALR*-positive low-risk ET patients does not reduce thrombosis and may actually increase bleeding, it is not routinely recommended for primary prophylaxis in this subgroup (71). Aspirin has also been shown to reduce thrombosis recurrence after both a first arterial or venous event (72). However, this risk remains high even after combined cytoreductive plus anticoagulant or antiplatelet therapy (73), highlighting the need for novel therapeutic antithrombotic strategies. Persistent thromboxane biosynthesis due to accelerated platelet production may lead to aspirin resistance in MPN (74). On this basis, a twice-daily schedule has been proposed for optimal platelet inhibition in patients with very high-risk features (70).

Statins have been shown to decrease the risk of both atherothrombotic events and venous thromboembolism. Among their pleiotropic actions, statins exert potent anti-inflammatory effects on the vascular wall and suppress platelet, endothelial and leukocyte activation (75). Due to these properties, statins might be relevant agents in MPN, although their role to prevent thrombotic events in this setting remains to be established (76).

CONCLUDING REMARKS

Thrombosis remains the main complication of MPN patients, with a high risk of recurrence despite adequate cytoreductive and antithrombotic treatment (73). Multiple and concomitant factors converge to increase the thrombotic risk in MPN, although the relative role of each factor may vary in the individual patient. Although progress has been made in unraveling the mechanisms underlying the thrombotic predisposition, the role of platelet and cellular activation markers in identifying patients at increased risk of thrombosis has not been established and prospective studies are needed to address this issue. Finally, the impact of new therapies on platelet and cellular activation and the potential development of novel therapeutic approaches to manage MPN coagulopathy could contribute to thrombosis prevention and result in improved patient outcome. Considering the critical role of inflammation in the vascular risk, simultaneous targeting of inflammatory pathways could potentially impact on primary or secondary prevention strategies.

AUTHOR CONTRIBUTIONS

CM contributed to the writing of the article and prepared the figures. PH wrote the article. Both authors contributed to manuscript editing and final approval.

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REVIEW
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Platelet Inflammatory Response to Stress

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Blood platelets play a central hemostatic role, (i) as they repair vascular epithelial damage, and (ii) they play immune defense roles, as they have the capacity to produce and secrete various cytokines, chemokines, and related products. Platelets sense and respond to local dangers (infectious or not). Platelets, therefore, mediate inflammation, express and use receptors to bind infectious pathogen moieties and endogenous ligands, among other components. Platelets contribute to effective pathogen clearance. Damage-associated molecular patterns (DAMPs) are danger signals released during inflammatory stress, such as burns, trauma and infection. Each pathogen is recognized by its specific molecular signature or pathogen-associated molecular pattern (PAMP). Recent data demonstrate that platelets have the capacity to sense external danger signals (DAMPs or PAMPs) differentially through a distinct type of pathogen recognition receptor (such as Toll-like receptors). Platelets regulate the innate immune response to pathogens and/or endogenous molecules, presenting several types of "danger" signals using a complete signalosome. Platelets, therefore, use complex tools to mediate a wide range of functions from danger sensing to tissue repair. Moreover, we noted that the secretory capacity of stored platelets over time and the development of stress lesions by platelets upon collection, processing, and storage are considered stress signals. The key message of this review is the "inflammatory response to stress" function of platelets in an infectious or non-infectious context.

Keywords: platelets, innate immunity, transfusion, cytokine/chemokine, inflammation

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INTRODUCTION

Several reviews have recently been published discussing the role of the interaction between platelets and both vascular endothelial cells and leukocytes during hemostasis and the initiation of the vascular repair process (1–3). This review focuses on the interactions between platelets and their environment beyond hemostasis, particularly in inflammation. It has been suggested that platelets detect and respond to local dangers such as infectious pathogens accidentally introduced into the bloodstream at the site of wounds. To achieve both goals (hemostasis /vascular repair and danger sensing), platelets use both membrane-bound and secreted products that interact with other cell types, including leukocytes.

PLATELETS AS KEY PLAYERS IN INFLAMMATION

Although platelets are regarded primarily as cells associated with hemostasis, it has now become clear that platelets play a wide variety of roles. Endothelial wall alteration or disruption exposes the sub-endothelial matrix rich in prohemostatic proteins. The engagement of platelet surface receptors with these matrix proteins leads to (i) platelet adhesion at the sites of lesions and (ii) the initiation of a complex intracellular signaling process (4). This process results in the formation and release of transcellular mediators, the exocytosis of adhesive and inflammatory proteins and the expression of both additional adhesive receptors and a procoagulant surface (5). Developing platelets retain cytoplasmic granules from their precursors during megacaryocyte differentiation and platelet production, the contents of which are secreted during platelet activation. Exocytosis of the platelet granule contents requires granule membranes to be fused with plasma membranes or open canalicular system (OCS) membranes. The OCS provides a transportation pathway for the release of platelet granule contents (6, 7). In general, these contents fall into three types. First, dense (δ) granules, which are rich in ADP, ATP, calcium, and serotonin, play an important role during hemostasis. Second are alpha (α) granules, which contain a variety of proteins, including adhesive proteins such as thrombospondin, von Willebrand factor, and fibronectin; growth factors such as insulin-like growth factor (IGF), transforming growth factor beta (TGF-β), and platelet-derived growth factor (PDGF); platelet factor 4 (PF4); and a variety of pro-inflammatory/modulatory chemokines and cytokines (1). In addition, α-granule exocytosis results in the expression of P-selectin (CD62P) on the external surface of platelets (5). Interestingly, there is now sound evidence to support the fact that α-granules are heterogeneous, in terms of both their contents and their exocytotic regulation (8–10). Third are platelet-enclosed lysosomes, which secrete hydrolases after activation (11). More recent reports have described a possible new type of granule termed a T-granule (11, 12). Resting platelets express basal surface TLR9 levels that increase significantly after thrombin activation, suggesting that although the majority of TLR9 is expressed intracellularly, some is relocalized to the plasma membrane upon agonist exposure (11–13).

The influence of the cytoskeleton on granule secretion has been a matter of discussion, with studies suggesting that reorganization of microtubules does not affect granule secretion (14, 15). Other studies suggest that the cytoskeleton does not facilitate the secretion of granules and that F-actin disassembly may be required for normal secretion of granules (16). In addition, the function of α-granules is dependent on inflammation, atherosclerosis, angiogenesis, wound healing, antimicrobial host defense, and platelet function in malignant hematological disorders. However, there is little knowledge about the cellular processes that help platelets release α-granule contents. Kamykowski et al. attributed the segregation of α-granule contents previously observed by investigators to the compartmentalization of cargo within single α-granules (17), while other reports showed that angiogenic factors are localized to different α-granules and released by different agonists upon stimulation (18, 19). Lastly, van Nispen tot Pannerden et al. showed that high spatial protein gradients exist within platelet α-granules and propose that tubular α-granules have different secretory capacities than conventional spherical granules and that the spatial segregation of cargo within tubular subtypes may result in differential release of their contents (20). A greater understanding of the dynamics of the fusion pore may illuminate the ways in which platelets drive the release of granule content with disparate platelet functions.

It has been known for more than three decades that platelets release arachidonic acid from membrane phospholipids (21). Arachidonic acid is converted into thromboxane A2 (TxA2), which has both prothrombotic and vasoconstrictive properties. This pathway serves as the target for aspirin, the primary antithrombotic currently in use. As outlined above, if an injured vessel is exposed to subendothelial structures (e.g., collagen), circulating platelets respond quickly, convert to an activated state, adhere and start to form the characteristic hemostatic clot (3). Among other processes, repair of the damaged vessel involves activated endothelial cells. Several reports describe the involvement of various cell adhesion molecules (e.g., P-Selectin, GPIb, GPVI, GPIaβ1, GPIIbβ3, CD40L, TNSF14; JAM-A, PSGL-1, P-Selectin, αvβ3, ICAM-1, CD40, TNSF14R, and JAM-A) acting at the interface between platelets and endothelial cells (19, 22, 23). P-selectin is a well-characterized endothelial and platelet adhesion receptor mediating the interaction of activated platelets and endothelial cells with leukocytes. Platelets release various growth factors (e.g., TGF-β, PDGF, and EGF) that influence endothelial cell physiology and vice versa and activate endothelial signalosome signaling (24).

PLATELET INTERACTION WITH THE ADAPTIVE IMMUNE RESPONSE

Platelet interaction with leukocytes can facilitate the activation of adaptive immune responses. Platelets can promote dendritic cell maturation and NK cell and monocyte/macrophage responses, which themselves affect specific T and B cell responses. Furthermore, platelets can directly affect B cell isotype switching and $CD8^+$ T cell proliferation (25). Czapiga et al. demonstrated that platelet-derived CD40L induces the maturation of immature dendritic cells, professional antigen-presenting cells, via the upregulation of co-stimulatory molecules and IL-12/p40 production (26). Kaneider et al. showed that platelets trigger dendritic cell maturation independently of cyclo-oxygenase-derived arachidonic acid metabolites by mechanisms involving CD40L (27). Lastly, our data indicate first that platelets secrete a soluble dendritic cell-activating factor that was shown not to be sCD40L, as was expected from previous *in vivo* and *in vitro* studies, but instead a nucleotide, and second, that cell-to-cell contact does not induce the maturation of dendritic cells, possibly since nucleotide release by platelets was prevented by direct contact with dendritic cells (28, 29).

Adhesive interactions between platelets and monocytes deliver specific signals that initiate inflammatory gene expression, as described by Dixon et al. (30), showing that activated platelets induce COX-2 synthesis in monocytes by signaling at the transcriptional and post-transcriptional levels (30). Moreover, the formation of platelet-monocyte complexes and the detection of platelet-bound CX(3)CL1 on inflamed smooth muscle cells suggest that the CX(3)CL1-CX(3)CR1 axis contributes significantly to platelet and monocyte concentration in atherosclerotic arterial injury (31). Wong et al. (32) showed that platelets interacted with Kupffer cells in the liver sinusoids and that those interactions quickly changed to firm adhesion after specific microbes were captured by Kupffer cells. Elzey et al. reported that platelet-derived sCD40L increases serum IgG levels and germinal center formation under conditions where antigen-specific $CD4^+$ T lymphocyte amounts are limiting (33). Regarding T lymphocyte activation and platelets, platelet reduction was shown to decrease intrahepatic accumulation of virus-specific cytotoxic T lymphocytes (CTLs) and organ injury in mouse models of acute viral hepatitis. Moreover, activated platelets contribute independently of their procoagulant function to CTL-mediated liver immunopathology (34). Zamora et al. investigated the proliferation and cytokine release of $CD36^+$ $CD4^+$ lymphocytes. Flow cytometric analysis and immunofluorescence microscopy indicated that $CD36^+$ platelets were responsible for CD36 recognition on $CD4^+$ lymphocytes. Moreover, Zamora et al. described that IL-17 and IFN-γ production was reduced in $CD4^+$ lymphocytes with bound platelets (35). Furthermore, CD40L-positive T lymphocytes stimulated platelet activation through a CD40-dependent interaction with RANTES release, which activated endothelial cells, and facilitated T cell recruitment (36). Chapman et al. in Craig Morrell's laboratory at the University of Rochester, provided evidence that murine and human platelets express MHC class I molecules and that platelets activate T cells in an MHC class I-dependent manner. This interesting report suggests a novel hypothesis that platelets participate in the initiation of the acquired immune response (37).

PLATELETS AS SENSORS IN INNATE IMMUNITY IN RESPONSE TO INFECTIOUS STRESS

It is becoming increasingly clear that platelets have inflammatory functions and can influence both adaptive and innate immune responses. Below are discussed some of the mechanisms by which platelets contribute to the innate immune response.

Platelets express the transmembrane protein CD40 ligand (CD40L, CD154), a member of the TNF receptor family. CD40L engages CD40, a second member of the TNF receptor family also present on B cells, monocytes, macrophages, carcinoma cells, dendritic cells, Kupffer cells and vascular endothelial cells, as well as on non-hematopoietic cells such as endothelial cells, smooth muscle cells, fibroblasts and keratinocytes (38). Platelets also express CD40. CD40 and CD40L are instrumental in both innate and adaptive immunity, with complex functions. Platelet activation leads to the surface expression and secretion of a wide range of proteins. P-Selectin (CD62P) is present on the inner leaflet of platelet granules and, following exocytosis, is expressed on the external leaflet of the plasma membrane. The ligand for CD62P is P-selectin glycoprotein ligand-1, which is expressed on a variety of leukocytes, notably neutrophils, eosinophils, lymphocytes, and monocytes. The GPIIbIIIa complex is present on the surface of quiescent platelets in an inactive, closed configuration (36). However, upon activation, there is a structural change in GPIIbIIIa to an active open state, which allows its binding to ligands. Although the natural ligands for GPIIbIIIa are von Willebrand factor and fibrinogen, the HIV surface protein gp120 can be bound by GPIIIa (along with other receptors) on platelets.

TLR adapters and signaling proteins downstream of TLR activation are potential targets for therapeutic drugs in eukaryotic cells (2); however, a more complete understanding of the platelet signaling complex is necessary. An increasing number of studies report both that platelets participate in the inflammatory process and that they may have an impact on pathogen clearance and the pathogenesis of bacteraemia, sepsis and, potentially, severe sepsis (39–41). However, a recent study presents the opposite view, in which a dual-track clearance mechanism balances innate and adaptive immunity during bacteraemia. Liver macrophages mediate fast clearance of intravascular *Listeria. monocytogenes* via scavenger receptors, in contrast to platelets, whose binding shifts *L. monocytogenes* clearance from "fast" to CRIg-dependent "slow" clearance pathways (42). Of critical importance to immunity and inflammation are Toll-like receptors (TLRs). TLRs are sensors of pathogen-associated molecular patterns (PAMPs), molecular determinants generally expressed by pathogens, specifically infectious pathogens. Several groups have described the presence and functionality of TLRs in mice and humans on both the membrane (TLR2/TLR1/TLR6/TLR4 and TLR9) and within platelets (TLR9); and TLR3 and TLR7 have also been identified (11–13, 43–48). Several recent studies suggest that TLR2,-4 and -9 are targets for bacterial-platelet interactions during severe sepsis and that they provide interesting targets for pharmacological analysis. Clark et al. suggested platelet TLR4 to be a threshold switch for bacterial trapping in severe sepsis. LPS-activated neutrophils, in combination with TLR4-activated platelets, were found to lead to the formation of neutrophil extracellular traps (NETs), which were able to ensnare bacteria in the blood flow for targeting immune clearance events (46). The addition of septic plasma but not control plasma to healthy neutrophils and platelets in the presence of DNA dyes evidenced the formation of NETs. Moreover, the author showed that platelet TLR4 mediates NETosis by decreasing the neutrophil DNA release time from 2 to 3 h (generally observed when neutrophils are stimulated) to ∼10 min (when platelets are present). Therefore, it has been suggested that inhibition of platelet activation with TLR4 inhibitors, such as eritoran, may reduce NET formation and limit tissue damage (46). Evidence is also emerging that certain TLRs play a major role in the pathogenesis of infectious and/or inflammatory diseases (49). Sabroe et al. reported that stimulation by natural ligands of TLR2 (Pam3CSK4) or TLR4 (LPS) did not cause any changes

in platelet aggregation, the surface levels of CD62P or the intra-platelet calcium levels (44). These data are consistent with the absence of direct effects on platelet activation as a result of the engagement of TLR2 or TLR4; the authors therefore concluded that these receptors are non-functional residues from megakaryocytes. Similar data were obtained by Jayachandran et al. (50) who showed that LPS did not affect platelet responses. In contrast, other groups, including our own, have found that the TLRs on platelets are functional and that their engagement evokes a variety of platelet responses (1, 2, 36, 40). In contrast to other cell types involved in immune responses (e.g., macrophages and dendritic cells), any possible link formed by platelets between innate immunity and adaptive immunity has yet to be proven (1–3, 33, 36, 40). Recently, Panigrahi et al. showed that physiological platelet agonists, primed by either suboptimal concentrations of thrombin receptor-activating peptide (TRAP) or the weak agonist ADP, act synergistically with TLR9 ligands by inducing TLR9 expression on the platelet surface and that the platelet TLR9 receptor is a functional receptor linking oxidative stress, innate immunity, and thrombosis (12). Thon et al. further demonstrated that TLR9 is located in a newly identified intracellular compartment in platelets and described a new organizational and signaling mechanism for TLR9 in human platelets (11). Finally, Hally et al. observed that platelet TLR9 expression was significantly elevated in subjects with acute coronary syndromes (ACSs) compared to that in healthy subjects, which may result in increased sensitivity to TLR9 agonists. Platelet activation caused increased expression of TLR9 in healthy platelets. We suggest that platelet activation, which occurs as part of ACSs, is a potential mechanism explaining the increased expression of platelet TLR9 observed in ACS patients (51). We provided evidence for differential signaling in platelets exposed to various TLR ligands leading to cytokine and chemokine secretion (52, 53). This difference indicates that platelet TLRs are functional, as they not only engage intracellular signaling pathways but also select among distinct adaptors (MyD88 vs. TRIF) to terminate NF-kB phosphorylation. The correlation between platelet TLR2 and TLR4 stimulation *in vitro* and the NF-kB/TRIF/Myd88 adaptor and signaling molecules is currently under investigation. In addition, studies showing the expression of TLR3 (54) and TLR7 (48) in human platelets have been published recently. Human platelets express TLR3 and are able to respond to poly I:C, indicating that these cells influence the innate immune response after exposure to viral dsRNA (54). Encephalomyocarditis virus (EMCV) infection rapidly reduces platelet count, and this phenomenon is credited to platelet Toll-like receptor 7 (TLR7) (48). Interestingly, Koupenova et al. demonstrated that platelets express all TLR transcripts and that these transcripts are more important in women with regard to cardiovascular risk and inflammatory markers (55).

Platelets are the major physiological contributor of sCD40L in plasma (56, 57). As outlined above, these soluble molecules can interact with the epithelium lining cells or mononuclear circulating cells that constitutively express CD40 counter receptors. It has been suggested that platelets can alter the binding of CD40/sCD40L, which is essential to inflammation. Platelets are cells that co-express surface CD40 and sCD40L molecules (58–63) in a platelet activation-dependent manner (CD40L is expressed and secreted only after activation, unlike CD40, which is constitutively expressed and not upregulated). However, while sCD40L is characteristic because of its quantitative and qualitative importance, this molecule is just one of the many secretory platelet molecules that contribute significantly to both hemostasis and immune modulation. In addition to releasing molecules that alter immune responses, platelets are involved in antimicrobial responses; indeed, platelets aggregate when exposed to certain bacteria (such as *Staphylococcus aureus*) and viruses (such as HIV), which may trigger responses to danger signals (39, 40, 64). In support of this finding, platelet degranulation, endocytic bacterial, and viral engulfment, and the release of antibacterial/antifungal proteins have been observed in conjunction with platelet aggregation events. Several studies have attempted to determine the involvement of platelets in immune responses dependent on CD40/CD40L and to determine the interactions between platelets and peripheral B cells. Platelets and B cells (in an *in vitro* co-culture model) were mutually activated, as validated by the increased expression of membrane platelet CD62P and B cell CD86. Platelet and B cell interactions were accompanied by changes in the membrane expression of CD40 and CD40L by both platelets and B cells. Differentiated B lymphocytes increased their production of IgG1, IgG2 and IgG3 but not IgG4, IgA, or IgM after a 3-days incubation with platelets *in vitro* (65). Another example of indirect interactions is at the interactions between platelets and macrophages in innate immunity and inflammation (66).

Platelets respond rapidly to changes in their environment, as they express surface receptors for a variety of ligands, such as the subendothelial proteins von Willebrand factor and collagen, as well as soluble agonists, such as thrombin, ADP and TxA2. This activation process leads to a variety of changes in platelets, including the extension of pseudopodia, the secretion of granule contents and PMPs, the synthesis and secretion of TxA2 and IL-1β, the formation of a procoagulant surface and the surface expression of a range of adhesive proteins, either by the exposure of granular membrane proteins on the plasma membrane or by structural changes in surface proteins from inert to active conformations (3).

While it is not yet generally accepted that platelets are also stimulators of immunity and inflammation, several recent reports argue in favor of acknowledging platelets as sensors in innate immunity and players in inflammation (2, 67–71). Platelets exert immune functions by acting to remove pathogen-infected host cells by binding directly to bacteria, viruses, and fungi (1, 39) and by mediating interactions between target cells and these infectious agents to potentiate the immune response (3, 33, 72). As a consequence, platelets have been linked with various inflammatory pathologies, such as cardiovascular disease (73), sepsis (72), and arthritis (36). A variety of mechanisms are involved in the contribution of platelets to the inflammatory process, including the increased expression of receptors for various immune mediators such as cytokines and chemokines and the exocytosis of a range of soluble factors,

immunomodulatory factors, growth factors, biological response modifiers, etc., from α-granules (74–79). Several proteins are associated with the inflammasome family. These proteins are divided into two groups depending on the domains they contain: NLRPs contain pyrin domains and NLRCs contain caspase recruitment domains (CARDs). Hottz et al. noted that platelets constitutively express the inflammasome components NLRP3 and ASC (apoptosis-associated speck-like protein containing a caspase recruitment domain) and can use them to assemble functional inflammasomes, activate caspase-1, and process IL-1β (80).

In addition, in patients with dengue or after platelet exposure to dengue virus *in vitro*, increased expression of IL-1β in platelets and platelet-derived microparticles was observed. Infection with dengue virus results in NLRP3 inflammation, caspase-1 activation, and caspase-1-dependent IL-1β secretion. IL-1β derived from platelets is released mainly as microparticles through mechanisms dependent on inflammatory NLRP3 triggered by mitochondrial ROS. Activation of IL-1β-rich microparticles by the inflammasome and platelet shedding is correlated with increased vascular permeability. These findings show that platelets contribute to the increased vascular permeability in dengue virus infection by the inflammation-dependent release of IL-1β (80).

Moreover, Dr. Craig Jenne group are interested by the infections mediated by multidrug-resistant *S. aureus*. Recently, Surewaard et al. demonstrated (81), using an elegantly intravital imaging, that alpha toxin targets platelets directly, resulting in circulation detrimental aggregation. Moreover, neutralizing alpha toxin during infection of *S. aureus*, while escaping microvascular damage, does not interfere with beneficial platelet responses. In this context, Platelets are always able to recruit macrophages and participate to the eradication of *S. aureus*. Considered platelets as sensors in innate immunity in response to infectious stresses (1, 2, 36, 39, 82–94) contributes to the understanding of the interrelationship between infection, inflammation, and coagulation.

PLATELETS AS SENSORS OF STORAGE LESIONS AS A NON-INFECTIOUS STRESS

Platelet concentrates for transfusion are living cell products with a certain life span that degrade in a physiological mechanism-dependent manner via mechanisms that may be accelerated by mechanical production and storage mechanisms (95). Platelets prepared for transfusion are subject to stress injury upon collection, preparation and storage (96). Under these types of stress, platelets undergo morphologic/metabolic changes likely to lead to platelet activation and an increase in the concentration of BRMs (82). *Ex vivo* platelet processing can have an effect on BRM secretion (97). These BRM-promoting events lead to negative changes and a gradual deterioration in platelet viability, structure, and function.

When stored as PCs, platelets can undergo changes that are mainly related to the storage solutions and conditions (platelet agitation and storage temperature and time).

In general, PCs—especially those prepared for prophylactic usage—are stored for an average of 5 days at a maximum temperature of 22 ± 2°C under constant, gentle agitation to prevent platelet aggregation. Additionally, buffy coat-derived pooled platelet concentrates (PPCs) and single-donor apheresis platelet concentrates (SDA-PCs) are stored in suspension in 35% donor plasma and 65% platelet additive solution (PAS). Compared to platelet storage in autologous plasma, platelet storage in an additive solution has satisfactorily improved platelet function preservation. PASs are generally used as plasma replacements to (i) reduce the quantity of plasma transfused; (ii) avoid the transfusion of large volumes of plasma to reduce the incidence of adverse reactions and circulatory overload; (iii) enable certain photochemical treatments for pathogen inactivation; and (iv) maintain storage conditions (98). Platelet storage lesions include the appearance of platelet morphological changes, activation markers, GPIbα expression loss, α granule secretion, and mitochondrial dysfunction (99). Platelet concentrate storage can lead to the secretion of several BRMs, such as sCD40L, PDGFAA, RANTES, IL1β, IL6, IL7, IL8, PF4, IL13, OX40L, IL27, and TGFβ (2, 82). Generally, extended PC storage is accompanied by increased BRM production, which may be related to an increase in the percentage of adverse events (AEs) observed according to PC storage time. To minimize AEs, it would be preferable to transfuse PCs as early as possible. It is fitting, however, to consider this conclusion in light of the PC production and issuing constraints on blood establishments according to the demand for the product in hospital banks. In particular, it has been demonstrated that from the 3rd day of PC storage, there is a significant increase in the concentration of BRMs, especially sCD40L (96). These observations suggest that storage lesions play a role in the inflammation caused by PC. sCD40L induces the production of reactive oxygen species (ROS) during PC storage, leading to an increase in the production and release of pro-inflammatory substances (100).

Additionally, the type of PC processing used during the preparation and storage process can have an effect on platelet activation. Leitner et al. showed that platelets stored in an InterSol™ solution exhibited significantly higher initial activation levels, as indicated by CD62P expression, than platelets stores in other additive solutions (Composol® and SSP+®) (101). However, platelet storage in an additive solution has demonstrated a certain number of benefits, especially a reduction in serious adverse reactions (102). Although the different types of PCs are of comparable quality, there is debate about their safety. Daurat et al. showed that AEs were less commonly related to PPCs than to SDA-PCs (103). These results challenge the widespread use of SDA-PCs and suggest that these concentrated should be prescribed for specific indications. Evaluation of the risk/benefit balance of transfusing different types of PCs would enable the prescription of the optimal product according to the medical indication.

BRMs contained in PC are also transfused. It has been shown that BRMs can induce immune responses (33) and post-transfusion reactions (104) and can affect hemostasis (105) and inflammation in the recipient (106). Storage lesions triggered by extrinsic factors (preparation methods) or intrinsic mechanisms

(plasma and platelet factors, residual leukocytes) could be largely responsible for both reducing the therapeutic efficacy of PC transfusion and inducing AEs (76). In addition to blood platelets, BRMs contained in PC are also transfused to the recipient. Among these molecules, sCD40L is described as being partly responsible for febrile non-hemolytic transfusion reactions (FNHTRs) after platelet transfusions (58, 107). In addition to its role in inflammation, CD40L seems to play a role in AEs. sCD40L is found in PCs, and its concentration increases during storage (96). Numerous studies have shown that sCD40L is involved in PC transfusion reactions (106, 108). In addition, we showed that other soluble factors, such as IL27 and sOX40L, are involved in FNHTR (104). Several soluble factors with high predictive value for the occurrence of AEs, such as sCD40L, IL13, and MIP1α, have been identified, primarily via machine learning algorithms (109). Indeed, this study shows a correlation between the concentrations of sCD40L and IL13 and the onset of AEs. Additionally, the concentration of MIP1α found in the supernatants that induced AEs seems to be able to differentiate the type of AEs, FNHTR or allergies. PCs also contain mitochondrial DNA (mtDNA), which is associated with adverse effects (96, 110). Boudreau et al. showed that activated platelets release mitochondria, in both encapsulated microparticles and membrane-free organelles. Extracellular mitochondria are found at higher levels in transfused PCs that caused acute reactions (FNHTR, cutaneous, and cardiovascular signs) in transfused patients than in those that did not (99, 110, 111).

It is clearly acknowledged that the increased levels of cytokines and chemokines in platelet concentrates developed during storage, in the absence of detectable exogenous stimuli, can contribute to AEs (96, 112). In addition to cytokines/chemokines, platelet Extracellular vesicles (EVs), and platelet microparticles (PMs), which are important mediators of inflammation and immune response regulation also seem to be involved in the onset of AEs (113, 114). EVs are a heterogeneous group of structures and comprise a large group of particles, including exosomes and microvesicles, and are released from virtually all cell types. EVs can be divided according to their size into microparticles (MPs) or microvesicles (MVs) that vary in size between 0.1 and 1 μM, and exosomes in size of 30–100 nm (115). Platelet exosomes strongly expressed tetraspanin CD63, CD9, CD63, TSG101, ALIX, CD31, CD41, CD42a, P-selectin, PF4, and GPIIb/IIIa. Platelet exosomes might play a lesser role in procoagulant activity than PMPs. Platelet exosomes can directly stimulate target cells by providing ligands that increase the secretion of various signaling molecules, e.g., growth factors or cytokines. They can also transfer membrane receptors and molecules of adhesion. In addition, Platelet exosomes provide proteins, mRNA, and transcription factors that cause target cell epigenetic reprogramming (116).

PMPs containing microRNA can also be involved in a pathophysiologic response and AE induction following PC transfusion. Additionally, studies have shown that pathogen reduction technologies aimed at reducing the potential risk of transfusion-transmitted infections induce platelet activation and a reduction in the mRNA (117) and microRNA levels (118). These RNA changes are correlated with an increase in the PMP concentration. As a result, it seems likely

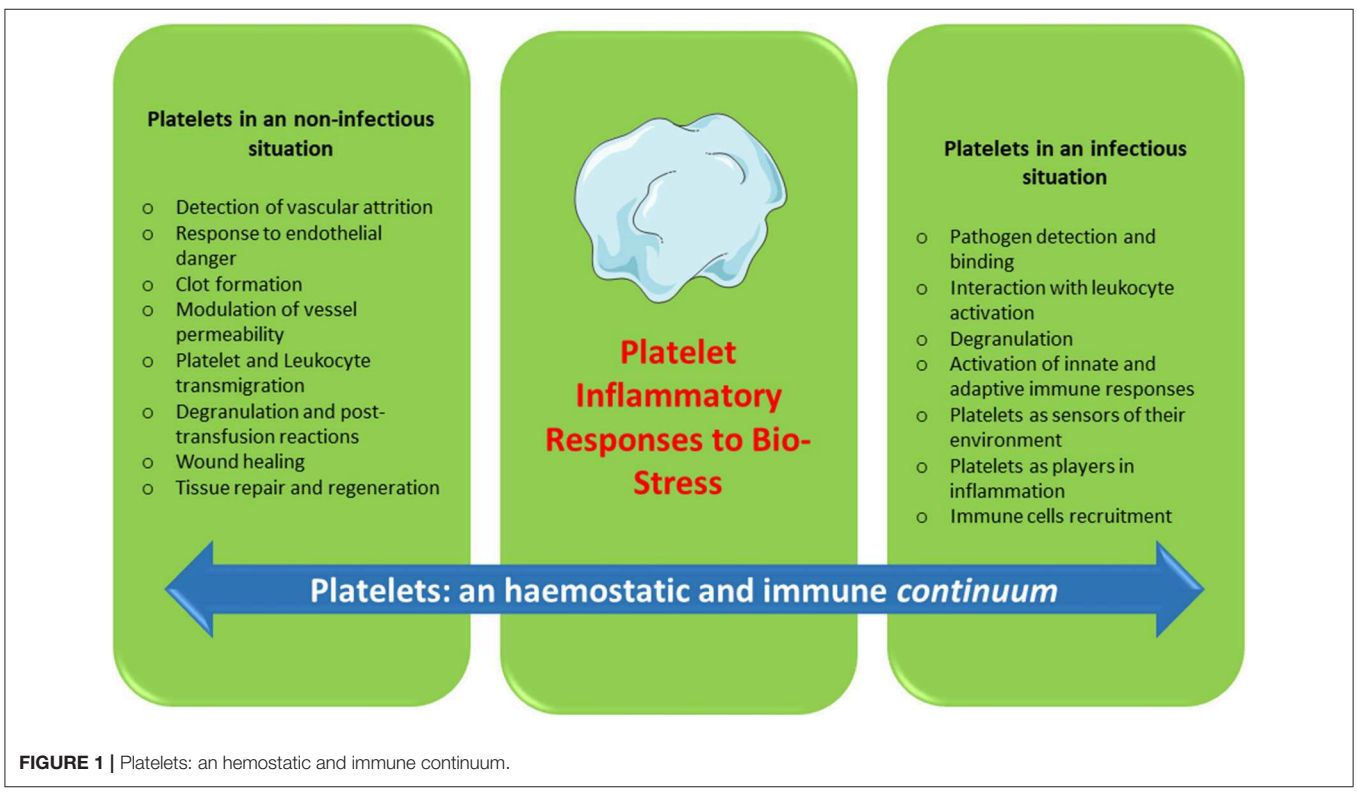

FIGURE 1 | Platelets: an hemostatic and immune continuum.

that pathogenic agent reduction technologies can increase PMP formation in PCs (118). Given the pro-inflammatory properties of PMPs, it is reasonable to presume that they can aggravate acute and chronic inflammatory reactions in blood vessels, such as those associated with platelet transfusion and atherosclerosis (119).

CONCLUSION AND FUTURE DIRECTIONS

Platelets contain and secrete numerous cytokines and other immunomodulatory proteins, which may be candidates for innovative targeted therapeutic approaches. For instance, there is now sound evidence that the platelet-activating factor (PAF)/PAF-receptor pathway is a promising target for pharmacological involvement in acute coronary syndrome (120), central nervous system diseases (121), autoimmune diseases (122), and rheumatoid arthritis (123). Platelet-related CD40L, IL-1β, PF4, and RANTES are currently under consideration as molecular targets against inflammation and hypercholesterolemia syndromes (124). Platelets continue to motivate much biological research. To date, a substantial amount of data has been collected over several decades on the hemostatic properties and immunogenic competence of platelets, yet much about the roles of platelets in physiological and pathological processes remains to be clarified. Furthermore, if platelets are immune sentinels with the capacity to bridge innate and adaptive immunity, preformed products from platelets, and products resulting from platelet neosynthesis can act on cell-cell interactions (platelet-dendritic cell, platelet-B cell and platelet-T cell) and play a major role in immune responses. Several reports have investigated "platelet physiology" as an immune cell concept (2, 71), and a notable number of papers (67–70, 90, 125, 126) have recently detailed this new concept. Indeed, future directions for research concern the critical role of platelets as an immune cell in the host immune response. It is now clear, therefore, that in addition to their roles in hemostasis and thrombosis, platelets have a large range of other functions, notably playing key roles in the inflammatory process, immune responses (2, 82, 84), regenerative medicine and host defense against pathogens (**Figure 1**). The challenge for therapeutic intervention in pathological processes will be to identify drugs that block specific targets involved in the complex contribution of platelets to inflammation/immunity without affecting their hemostatic function.

AUTHOR CONTRIBUTIONS

FC, SL, PB, TB, HM, PM, OG, and HH-C have made a substantial, direct and intellectual contribution to the work, wrote and approved it for publication.

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REVIEW
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The Human Platelet as an Innate Immune Cell: Interactions Between Activated Platelets and the Complement System

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Platelets play an essential role in maintaining homeostasis in the circulatory system after an injury by forming a platelet thrombus, but they also occupy a central node in the intravascular innate immune system. This concept is supported by their extensive interactions with immune cells and the cascade systems of the blood. In this review we discuss the close relationship between platelets and the complement system and the role of these interactions during thromboinflammation. Platelets are protected from complement-mediated damage by soluble and membrane-expressed complement regulators, but they bind several complement components on their surfaces and trigger complement activation in the fluid phase. Furthermore, localized complement activation may enhance the procoagulant responses of platelets through the generation of procoagulant microparticles by insertion of sublytic amounts of C5b9 into the platelet membrane. We also highlight the role of post-translational protein modifications in regulating the complement system and the critical role of platelets in driving these reactions. In particular, modification of disulfide bonds by thiol isomerases and protein phosphorylation by extracellular kinases have emerged as important mechanisms to fine-tune complement activity in the platelet microenvironment. Lastly, we describe disorders with perturbed complement activation where part of the clinical presentation includes uncontrolled platelet activation that results in thrombocytopenia, and illustrate how complement-targeting drugs are alleviating the prothrombotic phenotype in these patients. Based on these clinical observations, we discuss the role of limited complement activation in enhancing platelet activation and consider how these drugs may provide opportunities for further dissecting the complex interactions between complement and platelets.

Keywords: complement, platelets, lectin pathway, disulfides, phosphorylation, thiol isomerases, innate immunity

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INTRODUCTION

The Complement System

The complement system functions as an intravascular surveillance system and constitutes one of the branches of the innate immune system. It consists of >50 circulating and cell-bound activating proteins and regulators and a number of receptors expressed on vascular cells (1). Most of the plasma-based complement components are synthesized in the liver, although the importance of

local production of complement proteins in tissues has recently received renewed interest. The complement system has two major functions; (i) to purge the vascular system of foreign pathogens and substances and (ii) to remove debris and dead cells. It accomplishes these tasks by tagging foreign surfaces with powerful opsonins, by generating potent pro-inflammatory mediators at sites of activation, and by directly lysing targets cells through cell-surface attachment of the membrane attack complex (MAC).

Three major pathways initiate complement activation. The classical and lectin pathways are triggered by pattern-recognition molecules (PRMs) that bind to foreign targets or altered self surfaces, whereas the alternative pathway is continuously activated at a low level in the fluid phase and can be rapidly amplified when an activating foreign surface is present. Each of these pathways converges in the activation of complement component C3, a powerful opsonin and a component of the C5 convertase; this convertase, in turn, activates the terminal complement pathway, leading to MAC assembly by activated C5b, C6, C7, C8, and multiple copies of C9 (C5b-9) (2). Apart from direct lysis of target cells by the MAC, the major mechanisms undergirding the effects of complement are opsonization and generation of pro-inflammatory mediators. C3 and C4 possess a reactive thiol ester group that is buried inside the molecule in the native state but becomes exposed on the surface as a part of a larger conformational change that occurs after activation. The exposed thiol ester allows C4b or C3b to attach to surfaces via a covalent chemical bond, thereby tagging pathogens and debris for clearance by phagocytic cells. A potent inflammatory response is achieved by release of anaphylatoxins, i.e., the N-terminal fragments C3a and C5a that are generated when C3 and C5 are proteolytically activated.

The classical pathway is triggered by C1q, which recognizes the Fc portion of IgM or clusters of IgG fixed to antigens. C1q is also a true PRM in that it directly recognizes foreign surfaces such as patterns of proteoglycans e.g., chondroitin sulfate (serglycin). When C1q binds a target, the C1q-associated proteases C1r and C1s become activated via a conformational change, and they in turn activate C2 and C4, which eventually form the classical pathway C3 convertase. The lectin pathway is evolutionarily older than the classical pathway and has a larger repertoire of PRMs, which likely occurred first as a kind of primitive antibody. These PRMs include three ficolins (ficolin-1, -2, and -3), mannose-binding lectin (MBL) and two collectins (collectin-10 and collectin-11) that recognize pathogen- and damage-associated molecular patterns on foreign microbes and altered self surfaces (3). The lectin pathway PRM genes each encode a monomeric subunit, and these subunits polymerize to form a multimeric protein whose constituent parts are mainly held together via disulfide bonds; this multimerization is necessary to attain sufficient binding avidity for biological activity. Whereas, a single monomer has low affinity for its ligands, the multivalent binding of an assembled multimer leads to high avidity and a binding affinity in the low nanomolar range (4, 5). In an analogous manner to the classical pathway, the lectin pathway PRMs form circulating complexes with serine proteases (MBL-associated serine proteases 1, 2, and 3 [MASP-1, MASP-2, and MASP-3, respectively]) that are activated upon target binding and cleave C2 and C4 to generate a C3 convertase. Ficolin-3 is the quantitatively dominating PRM of the lectin pathway, with a serum concentration of $\sim$39 μg/ml, as compared to $<$5 μg/ml for ficolin-2, ficolin-1, and MBL and $<$1μg/ml for the collectins (6) (**Table 1**). In addition to being the most abundant PRM in the lectin pathway, ficolin-3 is also the strongest complement activator (9).

The alternative pathway serves as an amplification loop that can be triggered by C3b generated by the classical or lectin pathways. In addition, as mentioned above there is continuous C3 turnover in the fluid phase as a result of its spontaneous hydrolysis to C3(H_2O). Although the C3(H_2O) thiol ester is no longer reactive after hydrolysis, significant conformational changes enable C3(H_2O) to bind factor B and form a C3 convertase that can initiate the alternative pathway. The structure of C3(H_2O) resembles that of the proteolytically activated C3b, by analogy to the C3b-like functional properties of C3(H_2O) (10).

To achieve a selective targeting of foreign surfaces, complement activation is tightly controlled by fluid-phase and membrane-bound regulators, collectively called the regulators of complement activation (RCA) family of proteins. These protect host cells from complement-mediated tissue injury and damage, the hallmarks of diseases caused by insufficient complement regulation. RCA proteins show significant homology and are characterized by repeated domains with a β-sandwich arrangement, so called complement control protein (CCP) or sushi domains (11). Among the membrane-bound regulators, CD46 (membrane co-factor protein, MCP) and CD55 (decay-accelerating factor, DAF) inhibit C3 convertase formation by catalyzing the degradation of C3b by factor I (MCP), or accelerating the decay of any formed C3 convertase (DAF). A third membrane-bound regulator, CD59, acts instead on the terminal complement pathway to limit MAC assembly. These proteins are ubiquitously expressed on most cell types.

Soluble RCA proteins provide an additional mechanism for complement control; they act in the fluid phase or can be recruited to host-cell surfaces. Initiation of the lectin and classical pathways is controlled by the protease-inhibiting C1 inhibitor and C4b-binding protein (C4BP). C1 inhibitor is not specific for complement but is also a physiological inhibitor of the contact and plasma kallikrein systems. Indeed, C1 inhibitor deficiency mainly manifests itself as an overactive bradykinin/kallikrein system and not as a defect in complement regulation (12). The abundant plasma protein factor H serves to regulate the alternative pathway by two mechanisms, either by acting as a co-factor for the serine protease factor I, leading to the proteolytic processing of C3b, or by accelerating disassembly of the alternative pathway C3 convertase.

PLATELETS AND COMPLEMENT

Platelets

Platelets are circulating anucleate cells that form a thrombus and seal a wound when a breach forms in the vascular system. A more integrated view is emerging recently whereby the platelet is seen as a functional immune cell, active in a network with other

TABLE 1 | Comparison of serum concentrations of MBL and ficolin variants in humans and mice.

Human gene	Gene product (protein)	Serum conc (μg/ml) (humans) (6)	Mouse gene ortholog	Mouse gene product (protein)	Serum conc (μg/ml) (mice) (7, 8)	Mouse strain	-Fold difference: mice vs. humans
FCN1	Ficolin-1	2.42	FCNB	Ficolin-B	0.130	C57BL/6J	0.05
FCN2	Ficolin-2	2.75*	FCNA	Ficolin-A	3.50	C57BL/6J	1.3
FCN3	Ficolin-3	39.9	n/a	n/a		n/a	n/a
MBL2	MBL	1.69	MBL2	MBL-C	45	Serum pool	27
n/a	n/a	n/a	MBL1	MBL-A	7.5	Serum pool	n/a

Plasma concentration, human ficolin-2 measurements are unreliable in serum.

vascular cells and the cascade systems of blood (13). Platelets are activated by agonists that are located on the injured vessel wall or are generated when coagulation is initiated (14). Von Willebrand factor immobilized on exposed subendothelial collagen binds to GPIb—IX-V on platelets, and trace amounts of thrombin generated during coagulation initiation potently activate platelets through protease-activated receptors (PAR) 1 and 4, G protein-coupled receptors (GPCRs) that are activated through proteolytic cleavage. Thrombin is the most potent platelet agonist known of, and in addition to PARs it also binds the GPIb alpha chain of the GPIb-IX-V complex with high affinity, an interaction which is important for the platelet response to low doses of thrombin (15, 16). Additional platelet agonists that contribute to full activation include adenosine diphosphate (ADP) and thomboxane A2, which both bind to platelet surface GPCRs.

Dramatic changes in platelet morphology and function occur as a consequence of activation in order to rapidly produce a hemostatic plug and, subsequently, a stable thrombus (14). Flip-flop reactions of phospholipids in the platelet membrane lead to the exposure of phosphatidylserine and a negatively charged surface that provide a scaffold for the activation of coagulation factors. The main platelet integrin αIIbβ3 transitions to a high-affinity active state and mediates the formation of a platelet plug by forming cross-links with fibrinogen. A central event in platelet activation is the release of intracellular granules, which serve to amplify platelet activation and create a microenvironment permissive of chemical reactions that are essential for the cross-talk between platelets and protein-based cascade systems. The main types of platelet granules are alpha granules, containing organic macromolecules such as growth factors, coagulation factors, complement components and chondroitin sulfate; and dense granules, which are a source of inorganic compounds, including serotonin, ADP, adenosine triphosphate (ATP), and Ca^{2+}. **Figure 1** summarizes the key events that occur during platelet activation.

Platelet-Complement Interactions

Once considered a strictly fluid-phase cascade system, complement's involvement in interactions with vascular cells is now well-appreciated. In the case of platelets, several lines of evidence point to an intimate relationship with complement, with multiple touchpoints that serve to facilitate the host defense response and bridge innate immunity and the intravascular cascade systems. However, if insufficiently controlled, these interactions can result in excessive platelet activation and thromboembolic complications, eventually leading to human disease.

In a number of publications, we have reported our investigation of this cross-talk by using whole-blood experimental systems. As complement activation is dependent on the divalent cations Ca^{2+} and Mg^{2+}, any anticoagulant that chelates cations, such as EDTA or citrate, will severely affect complement function. To circumvent this problem, we have developed blood-based experimental systems that use the thrombin inhibitor lepirudin (recombinant hirudin) as anticoagulant, in which the final stage of blood clotting, i.e., the conversion of fibrinogen to insoluble fibrin by thrombin, is prevented (17). Lepirudin has an affinity for human thrombin in the picomolar range and is not known to inhibit any other protease or affect complement function (18). Since thrombin generation is disabled, fibrin generation, and spontaneous platelet activation are prevented, but platelets still can be fully activated by the addition of other agonists.

Using the lepirudin-based *ex vivo* whole blood system, we found that the glucosaminoglycan chondroitin sulfate, released from platelet alpha granules, acts as a potent trigger of complement activation in the fluid phase (19) (**Figure 2**, lower right). Activation of platelets leads to the generation of C3a and soluble C5b9 complexes, which can be abolished by adding chondroitinase. We identified C1q as the initiating PRM, given that chondroitin sulfate did not activate complement in C1q-depleted serum, and this failure could be corrected by the addition of purified C1q. Our further studies demonstrated that platelets also recruit a number of complement components to the surface upon activation, and this recruitment is partially dependent on chondroitin sulfate exposure (20–22). In an effort to reconcile these observations with previous studies suggesting that complement activation also occurs on the platelet plasma membrane despite its high expression of complement regulators (23, 24), we reassessed the mechanism by which complement factors bind to platelets. Paradoxically, we found that the platelet surface appeared to be protected from proteolytic complement activation (20). C3 was shown to bind to platelets in the form of $C3(H_2O)$, but in a manner independent of preceding complement activation. Instead, we posited that the platelet surface provides a scaffold for contact activation of C3 into $C3(H_2O)$ (**Figure 2**, upper left). We further showed that platelet-associated $C3(H_2O)$ can serve as a ligand for CD11b/CD18 expressed on leukocytes to promote the formation of platelet-neutrophil and platelet-monocyte aggregates (25).

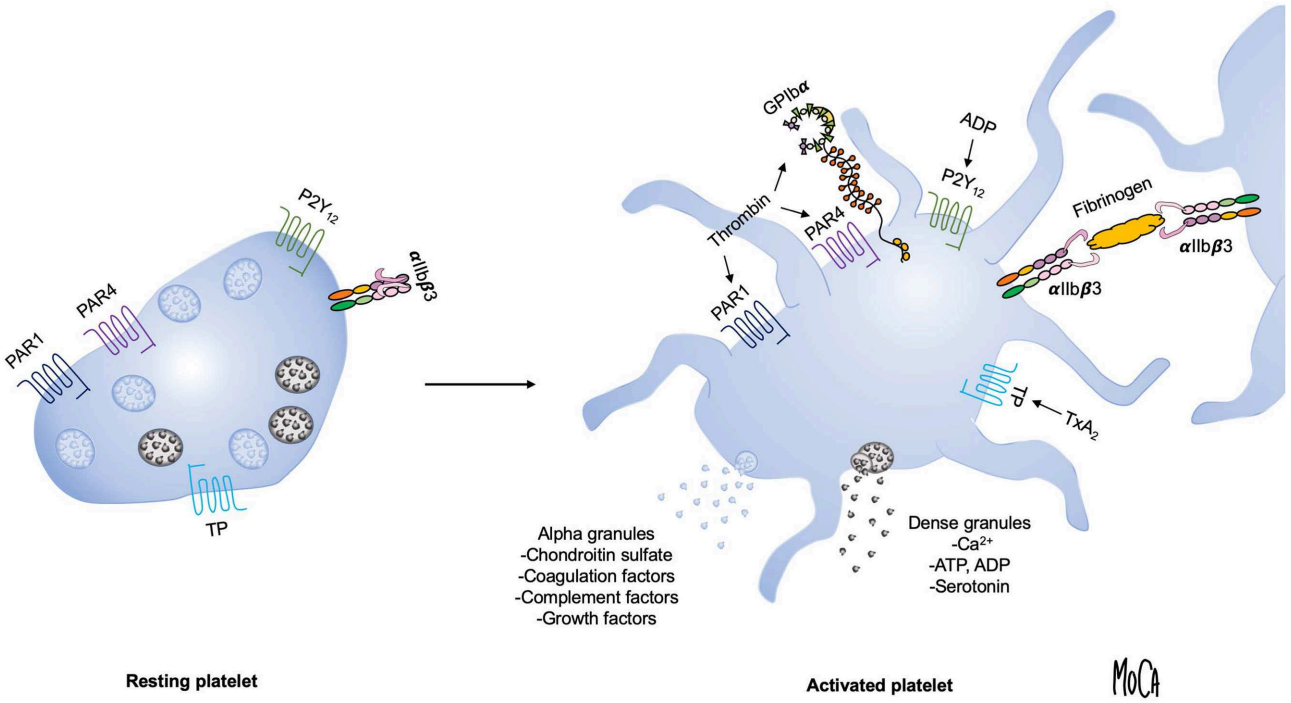

FIGURE 1 | Overview of platelet activation. Platelet activation triggers platelet shape change and granule exocytosis. Alpha granules (blue) contain growth factors, coagulation and complement components and chondroitin sulfate; release of dense granules (gray) provides a source of ADP, ATP, Ca^{2+}, and serotonin. Selected platelet agonists and their receptors are indicated. Thrombin binds to GPIbα and activates protease-activated receptor 1 and 4 (PAR1 and PAR4, respectively), ADP activates the P2Y12 receptor, and thromboxane A2 activates the thromboxane (TP) receptor. Agonist stimulation results in integrin $\alpha_{IIb}\beta3$ activation and the transition to an active conformation that binds fibrinogen and mediates platelet aggregation.

This finding is in line with results from Saggu et al. (26), who reported that physiological forms of properdin can bind to human platelets after activation and recruit C3(H_2O) to the surface. Hence, complement components bound to platelets may in some cases serve other functions than that of "classical complement" activation, such as mediating heterotypic interactions with leukocytes.

Although it was not immediately apparent, these findings were quite logical in view of the fact that all vascular cells express high numbers of complement regulators on their surfaces, an essential attribute to allow them to avoid complement-mediated damage. Membrane-integrated complement regulators expressed by platelets include CD46 (27), CD55 (28), and CD59 (29) (**Figure 2**, right). In addition, platelets bind and recruit soluble complement regulators such as factor H, C4BP, and C1 inhibitor. Both factor H (30, 31) and C1-inhibitor (32, 33) are stored in alpha granules and secreted from platelets, demonstrating a second reservoir of these proteins in addition to the pool circulating in the plasma (**Figure 2**, lower left). It has been suggested that the released pool of factor H serves to increase the local concentration on the activated platelet surface or in the context of a thrombus. However, the released amount has been quantified and found to constitute only 0.05% of the amount of factor H present in plasma (30), and a substantial amount is bound to the platelet surface (34). Similarly, the released amount of C1 inhibitor is very low when compared to its plasma concentration (33). An interesting possibility is that the platelet-derived pool has distinct functional properties, which would explain why release of such a comparably low amount could be of functional importance.

Complement and Platelet Microparticles

Another mechanism that may explain the apparent absence of activated complement factors on platelets is the rapid removal of membrane-inserted C5b-9 complexes through the shedding of microparticles (**Figure 2**, upper right). This process has been proposed as an additional complement-regulatory mechanism by which host cells can maintain membrane integrity when other complement-regulatory mechanisms are inadequate (35). By analogy to this concept, a number of studies from Sims and co-workers have detailed how C5b9 insertion into the platelet membrane can cause platelet activation through intracellular Ca^{2+} elevation and the release of platelet microparticles (36). These microparticles were shown to express binding sites for the FXa/FVa prothrombinase complex and to generate pro-coagulant activity (37). Hence, complement activation that has proceeded to its final stage, i.e., MAC formation, is a strong trigger of platelet procoagulant activity.

Given the apparent absence of complement activation on the platelet surface discussed above, the question remains: To what extent does this mechanism account for platelet microparticle formation under physiological conditions?

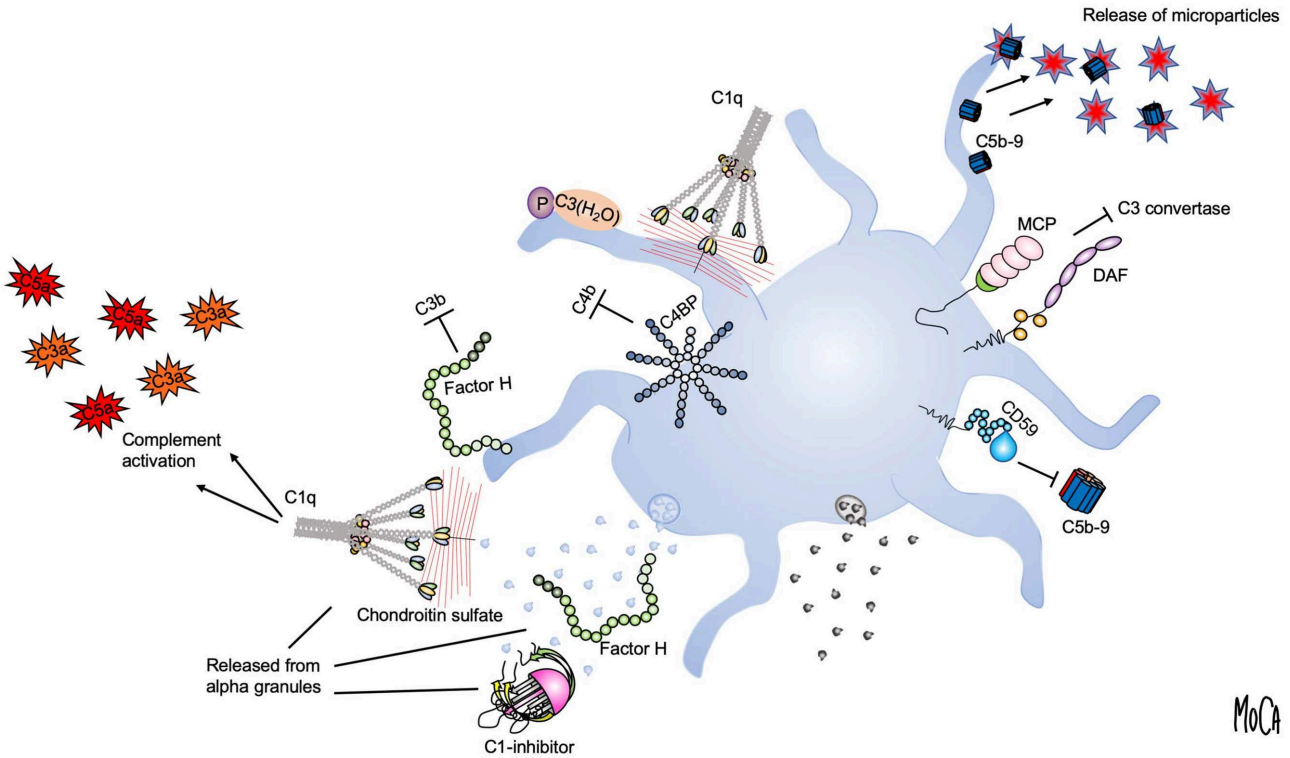

FIGURE 2 | Platelet-complement interactions. Interactions between complement components and activated platelets are shown. The platelet surface provides a binding scaffold for complement factors and supports contact activation of C3 to C3(H_2O), which interacts with platelet-bound properdin (P) (upper left). C1q recognizes chondroitin sulfate released from activated platelets or bound to the platelet surface and triggers complement activation in the fluid phase (left). Localized formation and surface attachment of sublytic concentrations of C5b9 complexes triggers rapid shedding of pro-coagulant microparticles (upper right). Platelet integrity is maintained by membrane-bound (MCP, DAF, CD59) (right) or fluid-phase complement regulators (C1-inhibitor, factor H, and C4-binding protein (C4BP) (left). C1-inhibitor and factor H are also released from alpha granules (lower left).

C3(H_2O) bound to activated platelets could potentially combine with factor B to form an alternative pathway C3 convertase to initiate the events leading to MAC formation, a model that has received experimental support (26).

Another potential mechanism for limited complement activation on platelets involves the transmembrane protein P-selectin, a platelet receptor localized to alpha granules that was initially discovered as an activation-specific platelet membrane antigen (38). Its ligand PSGL-1 is expressed on leukocytes, and the P-selectin-PSGL-1 interaction is essential for platelet-leukocyte aggregate formation. Interestingly, P-selectin contains several CCP domains and was proposed to act as a scaffold for complement activation by binding C3b (24). The significance of these observations are not fully determined, and C3 also binds to platelets in a P-selectin independent fashion (20, 26).

An attractive model based on these observations is that the limited C5b9 formed at sublytic concentrations is rapidly shed from the platelet surface in the form of released microparticles, which would maintain platelet integrity and explain the absence of detectable C5b9 at the platelet membrane. Indeed, in other cell types exposed to complement, sublytic C5b-9 concentrations have been shown to induce Ca^{2+} flux and cellular responses such as inflammasome activation concomitant with preservation of membrane integrity (39, 40).

Evaluation of Cross-Talk Between Complement, Coagulation, and Platelets in Mouse Models

Animal models constitute a valuable tool for studying biological processes in a more physiological context than can be reproduced in a test tube. Recent developments in gene editing technology provide nearly unlimited opportunities to design transgenic mice with specific genetic alterations. However, the use of rodent models to study the role of complement in human disease comes with the inherent limitation that the complement systems of mice and humans, and their immune systems in general, have diverged during evolution as result of adaptation to distinct repertoires of pathogens. There are also important differences in platelet biology between mice and humans, among which the ∼3-fold higher platelet count in mice and the absence of the thrombin receptor PAR1 on mouse platelets can be mentioned. In the absence of PAR1, mouse platelets

are instead activated by thrombin through a dual PAR3-PAR4 mechanism (41).

Despite these caveats, data from mouse models have lent consistent support to the notion that complement is important for platelet function. Both C3- and C5-deficient mice show impaired venous thrombus formation, albeit as the result of different mechanisms (42). Additional investigations in C3-deficient mice have revealed a modest reduction in PAR4-induced platelet aggregation, impaired thrombus formation *in vivo,* and a markedly prolonged tail-bleeding time (43). Studies have demonstrated the presence on platelets of functional receptors for the anaphylatoxin C3a that mediate platelet activation (44, 45). Since C3a is generated by the cleavage of C3, loss of C3a signaling could contribute to the prolonged bleeding time in C3-deficient mice.

The lectin pathway has recently received attention as a potential amplifying mechanism during thromboinflammation, and we have shown that the lectin pathway is triggered when platelets are activated or when fibrin is formed during blood clotting (22). Intriguingly, in addition to acting on their canonical complement substrates, the MASPs exhibit coagulation factor-like serine protease activity and cleave coagulation factors *in vitro* (46), although it should be noted that these observations have mainly been made using recombinant proteins. In comparison, MASPs exhibit much slower kinetics than true coagulation proteases in cleaving coagulation-related substrates (3). Nonetheless, data from mouse models have indicated that activation of the MBL branch of the lectin pathway can exacerbate atherothrombotic diseases and that pharmacological MBL inhibition is protective against stroke (47), thrombosis (48), and myocardial infarction (49). However, a noteworthy difference between the mouse and human complement systems is the repertoire and abundance of PRMs that initiate the lectin pathway.

Mice have two functional MBL genes, whereas humans have only one. Mouse MBL-C is the ortholog of the human MBL-2 gene, whereas the second mouse MBL variant MBL-A does not exist in higher primates. The circulating concentration of human MBL appears to be around 1.5 μg/ml (6), but the total MBL concentration in mice is >30-fold higher (7). Mice have two ficolins (A and B) that are orthologs of human ficolin-2 and ficolin-1, respectively (50), and their plasma concentrations are similar to those reported in humans (8) (**Table 1**). It is important to note that ficolin-3, which is the quantitatively and qualitatively dominant lectin pathway PRM in humans (9), does not exist in mice, since the FCN3 gene was inactivated to a pseudogene in the rodent lineage (51). Hence, mice have a strongly MBL-biased lectin pathway, whereas ficolins are the main lectin pathway PRMs in humans.

Population-based studies in humans have provided less conclusive results than have animal models regarding the role of MBL in thrombotic disease. The human MBL2 gene displays extensive genetic variability, and functional MBL deficiency is the most common human immunodeficiency. Genetic analyses have concluded that the high frequency of MBL variants is likely an effect of genetic drift, as opposed to positive selection, and that MBL may therefore be redundant in host defense in humans (52). While the outcome after ischemic stroke is improved in MBL-deficient patients (53), MBL deficiency is associated with an increased risk of arterial thrombosis (54), and myocardial infarction (55). Furthermore, circulating MBL serum concentrations have been found to predict a decreased likelihood of myocardial infarction (56). The favorable outcome associated with the presence of functional MBL in certain epidemiological studies is in apparent contrast to results from mouse models and may suggest an atheroprotective role of MBL in humans, possibly through increasing phagocytic removal of atherogenic vascular debris.

Direct cleavage of C5 by coagulation proteases such as thrombin and factor Xa has been suggested as potential mechanism for activating the terminal complement pathway in the absence of activation triggered at the PRM or C3 level (57). In C3-deficient mice, thrombin has been shown to fully substitute as a C5 convertase, as complement activation occurred normally but was sensitive to thrombin inhibitors. The physiological relevance of this observation for humans has not yet been fully determined, and it appears that mouse C5 is much more susceptible to cleavage by thrombin than that of humans. In general, high concentrations of active coagulation proteases are required for efficient complement factor cleavage *in vitro* (58, 59), and coagulation factors mainly circulate in their zymogen forms *in vivo* at concentrations that are considerably lower than those of most complement components. Furthermore, the ability of endogenous thrombin to cleave C5 has been studied in a human blood-based system anticoagulated with the fibrin polymerization-inhibiting peptide Gly-Asp-Arg-Pro (GDRP), which allows thrombin to be formed but inhibits the formation of a fibrin clot and is fully compatible with complement activation as Ca^{2+} and Mg^{2+} levels are not affected (60). While platelets were rapidly activated and consumed by thrombin in this system, C5a generation was not increased compared to lepirudin-anticoagulated blood, a finding which appeared to refute that thrombin is a physiological C5 cleaving enzyme (60). Similar conclusions were reached from induction of systemic coagulation under sterile conditions in a baboon model, where infusion of factor Xa or phospholipids generated robust thrombin generation as measured by thrombin-antithrombin complex levels, but no measurable increase in complement activation products (61).

THE ROLE OF ALLOSTERIC DISULFIDE BONDS IN PLATELET FUNCTION, THROMBOSIS AND COMPLEMENT ACTIVATION

Chemical modifications of proteins after they have been translated in the ribosome, i.e., post-translational modifications, constitute an important mechanism for regulating their function. Accumulating evidence provides solid support for the concept that such modifications can also occur in the extracellular milieu and that activation of cells provides a microenvironment with sufficient local concentrations of co-factors and a scaffold on which these reactions can be assembled. Here we discuss two

such modifications, cleavage of allosteric disulfide bonds and phosphorylation of amino acid side chains, and detail how these two types of modification regulate complement initiation and the role of activated platelets in facilitating these reactions.

The amino acid cysteine has the unique property of being able to form a disulfide bond with another cysteine via its sulfur group. Disulfide bonds stabilize a protein's tertiary and quaternary structure and are formed through an oxidation reaction in proteins destined for secretion during their synthesis and folding in the endoplasmatic reticulum. These reactions are catalyzed by thiol isomerases, a large group of endoplasmatic reticulum-resident enzymes, with protein disulfide isomerase (PDI) being the archetypal member. A general feature of thiol isomerases is their conserved active sites that include a thioredoxin-like fold harboring a Cys-X-X-Cys active site, with two vicinal cysteines separated by two amino acids (denoted X). The two cysteines of the active site can transition between an oxidized and a reduced state and render thiol isomerases capable of catalyzing redox reactions in their substrates (62).

An emerging concept for protein regulation is the modification of so-called "allosteric disulfide bonds," i.e., a cleavage or formation of a disulfide bond by a thiol isomerase that results in a change in protein function (63). Cleavage of disulfide bonds occurs either through reduction, which requires a source of electrons, or through thiol-disulfide exchange, when the disulfide bond is reshuffled through a reaction with an unpaired cysteine in close proximity (64). In contrast to peptide bonds that are irreversibly cleaved by proteases, disulfide bond cleavage is potentially reversible, since the bond can be reformed through oxidation.

An unresolved question regarding allosteric disulfide bonds is what drives redox reactions in the extracellular milieu. The endoplasmic reticulum contains millimolar concentrations of oxidized and reduced glutathione that constitute a redox buffer that permits efficient disulfide bond formation (65). The cytoplasm is a more reducing environment and features a continuous supply of electrons from NADPH formed during cellular respiration. In contrast, plasma is a comparably oxidizing environment and contains only micromolar amounts of low molecular weight redox pairs such as glutathione and cysteine (66). A possible explanation for these seemingly disparate observations is the ability of platelets to catalyze these reactions, by analogy to the way in which the platelet membrane provides a negatively charged surface for the assembly of coagulation factor complexes during blood clotting. Essex et al. have shown that platelets contain an endogenous glutathione-reducing activity that potentiates platelet aggregation and is hypothesized to originate from intracellular NAPDH (67). In an elegant study, a platelet-targeting redox sensor was devised by combining an anti-CD61 antibody with a quenched reporter (68); this methodology allowed the demonstration of disulfide-reducing activity on the surface on activated platelets in various i*n vitro* systems and during thrombus formation *in vivo*, lending strong support to the concept that platelets provide the necessary conditions for these reactions.

A subset of thiol isomerases can escape the endoplasmic reticulum through a poorly characterized mechanism and are actively secreted by vascular cells. Platelets and endothelial cells secrete a number of thiol isomerases, including PDI, endoplasmic reticulum protein 57 (ERp57), endoplasmic reticulum protein 72 (ERp72), and endoplasmic reticulum protein 5 (ERp5), a group of enzymes that have been termed the vascular thiol isomerases (69). Their presence in platelets and platelet releasates has been confirmed by unbiased proteomic investigations (70, 71). Intense interest in this group was ignited when it was shown that extracellular PDI is essential for thrombus formation and fibrin deposition in various animal models of thrombosis (72, 73). An important role in thrombosis has subsequently been confirmed for other vascular thiol isomerases, including ERp57 (74), ERp5 (75), and ERp72 (76). In addition, the use of chemical inhibitors and blocking antibodies has demonstrated an important role for thiol isomerases in platelet activation, possibly through the regulation of the αIIbβ3 and α2β1 integrins (77–80). A recent study described that platelet surface PDI enhances the ligand binding function of the platelet surface receptor GPIb alpha to promote platelet-leukocyte interactions (81). Apart from their postulated role in supporting platelet activation through these pathways, the specific substrates of extracellular thiol isomerases have mostly remained elusive.

To gain insight into the mechanisms of action of extracellular thiol isomerases, we have developed a substrate-screening strategy based on an engineered variant of the enzyme that forms stable complexes with substrates (82). The application of this methodology to ERp57 led to the unanticipated finding that ERp57 displays a striking selectivity for the lectin pathway of complement among extracellular substrates (83). Specifically, ERp57 attenuates the activation of the ficolin-3-dependent branch of the lectin pathway and inhibits ficolin-3 ligand recognition, the proteolytic activity of the ficolin-3/MASP1 complex, and downstream complement activation. Like other lectin pathway PRMs, ficolin-3 forms large multimers of a basic subunit through intermolecular disulfide bonds, and a range of multimers and oligomers is found circulating in plasma (3). As compared to MBL and ficolin-2, which mostly assemble into 9-mers and 12-mers, we readily detected ficolin-3 18-mers and 21-mers in human plasma. By biochemical assays and mass spectrometry, we identified the mechanism by which ERp57 inhibits ficolin-3 and showed that ERp57 specifically cleaves large ficolin-3 multimers into smaller biologically inactive oligomers. This cleavage is the result of reduction of the intermolecular disulfide bonds that mediates ficolin-3 multimerization (**Figure 3**, left).

Our results also indicated that the unique susceptibility of ficolin-3 to disulfide bond cleavage is related to its extensive multimerization. Disulfide bonds under mechanical stress are primed for reduction, since application of force destabilizes the oxidized state of the disulfide (84, 85). In the case of ficolin-3, the assembly of large multimeric complexes would impose significant strain on the connecting disulfide bonds. We provided experimental support for this concept by determining their redox potentials, a measure of the stability of a disulfide bond. A clear multimerization-dependent effect was observed, in which the large biologically active

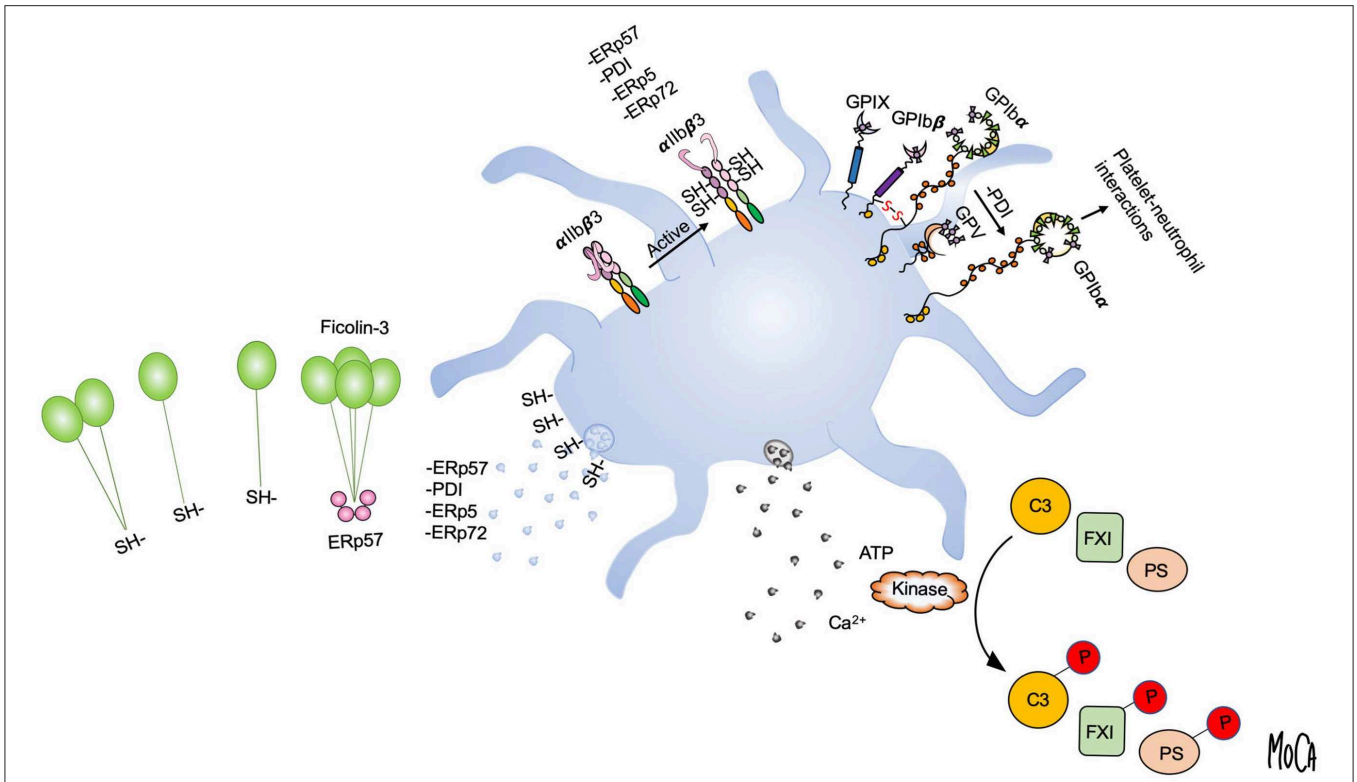

FIGURE 3 | Post-translational protein modifications in the platelet microenvironment. Platelet activation and granule release create a microenvironment in which post-translational protein modifications can occur in the extracellular environment, as exemplified by disulfide bond rearrangement and phosphorylation. Platelet intrinsic reductase activity leads to exposure of free thiols and may provide a regenerating system for thiol isomerases. Platelets release vascular thiol isomerases that rearrange allosteric disulfide bonds in protein substrates. Multiple thiol isomerases are required for full $\alpha_{IIb}\beta3$ activation and platelet aggregation. Platelet-derived ERp57 cleaves multimeric ficolin-3 by disulfide bond reduction to limit platelet recognition by the lectin pathway (left). PDI released from platelets reduces disulfide bonds in GPIbα of the GPIb-IX-V complex to increase GPIbα-mediated platelet-neutrophil interactions (upper right). Exocytosis of platelet dense granules elevates the local ATP and Ca^{2+} concentrations. Substrates including complement C3, coagulation factor XI (FXI), and protein S (PS) can then be phosphorylated by platelet-derived or circulating kinases (lower right).

ficolin-3 multimers appeared to be primed for reduction and displayed the highest redox potentials. Apart from explaining the preference of ERp57 for ficolin-3 among the lectin pathway PRMs, this multimer size-dependent redox potential may have general implications for the regulation of multimeric proteins.

The results detailed above were the first to describe a mechanism to regulate the lectin pathway at the PRM level, and how thiol isomerases can limit the recognition of platelets by PRMs. Thus, in addition to its role in thrombus formation, ERp57 appears to be a novel complement-regulatory protein, that could, for example, protect platelets from being targeted by the lectin pathway. Indeed, we have detected binding of ficolins and MASPs to activated platelets, with the ficolins tentatively recognizing epitopes that are exposed on the platelet membrane after activation (22). In combination with previous studies showing secretion of active C1-inhibitor from platelet alpha granules (33), our studies indicate that platelets appear to possess an intrinsic system to regulate their interactions with the lectin pathway.

PLATELET-MEDIATED EXTRACELLULAR PHOSPHORYLATION

Phosphate-Containing Plasma Proteins

Numerous plasma proteins, many of which are part of cascade systems, contain covalently bound phosphate *in vivo*. Using a colorimetric technique, we have found the amount of phosphate in plasma proteins to average 0.09 mol phosphate per mol of average protein in healthy blood donors (86, 87). Some of these plasma proteins were reported to be phosphoproteins decades ago, including fibrinogen (88), FV (89), complement component C3 (90–92), vitronectin (93), and human serum albumin (HSA) (94). Additional phosphoproteins have been identified only recently by mass spectrometric analysis of human plasma or serum samples (95–97). These include the complement components MASP-1, C5, C7, C8, and C9; the regulators factor I, factor H, C4BP, and clusterin; as well as the coagulation/fibrinolysis proteins FXIII, high molecular weight kininogen (HMWK), antithrombin (AT), and plasminogen (**Table 2**). Interestingly, several of these phosphoproteins were detected in serum but not in plasma, implying the involvement

TABLE 2 | Complement- and coagulation-related plasma phosphoproteins.

	Plasma phosphoproteins					
	Pre-proteomics		Plasma (96)	Serum (97)	Platelet-mediated phosphorylation	
COMPLEMENT						
C3	x	(90–92)	x	x	x	(98–100)
C5				x		
C7				x		
C8			x			
C9			x	x		
Factor I			x	x		
Factor H			x	x		
C4BP				x		
MASP-1			x	x		
Vitronectin	x	(101)		x	x	(86)
Clusterin				x		
COAGULATION/FIBRINOLYSIS						
Fibrinogen	x	(88)	x	x	x	(86)
FV	x	(89)	x	x		
FXIII				x		
HMWK			x	x		
AT			x	x		
Plasminogen			x	x		
FXI					x	(102)
Protein S					x	(103)
OTHERS						
HAS	x	(94)	x	x		(86)
α2-MG			x			

C4BP, C4b-binding protein; MASP-1, MBL-associated serine protease 1; F (coagulation) factor; HMWK, high molecular weight kininogen; HSA, human serum albumin; α2-MG, α2 Macroglobulin.

The table gives an overview of phosphorylated plasma proteins involved in complement activation and coagulation that have been reported in the literature. Pre-proteomic studies indicate targeted investigations performed before mass spectrometry became available. The table also shows phosphoproteins detected in unbiased mass spectrometry analyses of plasma and serum samples.

of platelets in the phosphorylation process, since serum is the end product of coagulation, a process that is initiated by platelet activation (see below).

Plasma ATP

The phenomenon of extracellular phosphorylation has been known for decades, and numerous extracellular kinases (ectokinases) have been identified; however, the physiological relevance of extracellular phosphorylation, at least in the plasma milieu, has been questioned. Nevertheless, the concept of extracellular phosphorylation has recently regained interest in the era of proteomics, since it has been demonstrated by high-throughput mass spectrometry and other techniques that numerous extracellular proteins, both in tissue and in body fluids, can exist in phosphorylated forms (104, 105).

A key point is whether, under normal conditions, plasma contains sufficient amounts of ATP. EDTA-plasma drawn from healthy volunteers who had received no medication for at least 10 days has been reported to contain between 0.2 and 1 μM ATP (98, 106). (EDTA inhibits ATP catabolism and is routinely used for ATP determinations). In blood collected in EDTA with other additives such as theophylline and dipyridamole to inhibit ATP release from the blood cells, the detected levels are even lower, typically 28–650 nM (98, 106–108). Since the Km for ATP for classical protein kinases such as cyclic AMP-dependent protein kinase (PKA) are in the range of 13–30 μM (109, 110), it is unlikely that any substantial amount of extracellular phosphorylation takes place in plasma in the absence of cellular activation and/or release. However, ATP and Ca^{2+} in high amounts [1–2 M (111)] are found in the dense granules of platelets. When these granules are released in response to physiological activation by agonists such as thrombin or ADP, the plasma concentration of ATP increases from 1 to 20 μM as measured in activated PRP (98), and similar levels are found in the blood after vascular damage (112). This concentration, which should be enough to support phosphorylation, would be expected to be even higher in close proximity to activated platelets. In addition, cells such as lysed erythrocytes (106) or dying or apoptotic cells (113, 114) may also show an increased plasma concentration of ATP; however, in contrast to platelet-mediated release, this increase is the result of a non-regulated process.

Regardless of its cellular origin, ATP and Ca^{2+} can be utilized by various protein kinases (either released from the platelets or already present in the plasma, originating from other cells or exposed on their surfaces) to phosphorylate plasma proteins in the micro-milieu adjacent to the platelets. In this context, it should be taken into account that a number of plasma proteins are phosphorylated by several protein kinases, with different impacts on protein function (see below).

Extracellular Protein Kinases

The nucleus of this review article concerns the interaction between activated platelets and the complement system. Therefore, in this section we will focus on protein kinases identified as being present in or released from platelets. The postulated origin of specific protein kinases found in the plasma that can mediate extracellular phosphorylation is discussed in detail in Yalak and Vogel (104) and Klement and Medzihradszky (105).

Protein kinase activities in the form of protein kinase A (PKA) (93), several isoforms of protein kinase C (PKC) (115), and casein kinases 1 and 2 (CK1 and CK2) (98–100, 102, 103, 107–115) have all been identified in the releasate from activated platelets. They have all been shown to mediate extracellular phosphorylation of specific plasma proteins, in particular proteins within the cascade systems, provided that ATP and Ca^{2+} are present in sufficient amounts. These protein kinases are all classified as serine/threonine kinases (116). Recently, a newly identified kinase, designated vertebrate lonesome kinase (VLK), was the first protein-tyrosine kinase shown to be released from TRAP-activated platelets (117, 118). Through proteomic approaches, protein kinases of all these classes have been identified in platelets (119). Information from platelet releasate is less conclusive regarding the identification of protein kinases (71,

120), suggesting that they may be associated with platelet-derived microparticles rather than being found in the fluid phase.

Examples of Plasma Proteins Whose Function Is Affected by Phosphorylation

Most of the initially identified human plasma phosphoproteins (fibrinogen, C3, vitronectin, FV) have been shown to be subject to phosphorylation *in vitro* by various protein kinases with different functional effects.

Fibrinogen is a substrate for PKA, PKC, CK1, and CK2 (121), and phosphorylation affects its activation by thrombin, leading to changes in the thickness of the resulting fibrin bundles. Similar alterations are found in plasma collected from patients after hip replacement operations, i.e., in a situation in which platelets have been activated *in vivo* (122).

Complement component C3 is a substrate for at least five different protein kinases (including platelet CK, which is discussed below). All of these covalently bound phosphate groups affect C3's activity in distinct ways. PKA, PKC, and an ecto-kinase from the parasite *Leishmanina major* all phosphorylate a serine residue in the C3a moiety, thereby protecting C3 from activation (123, 124). In contrast, C3 phosphorylated by CK2 derived from the leukemia cell line U937 (either intracellularly prior to release or extracellularly) is markedly more sensitive to proteolytic cleavage, generating iC3b (92).

Similarly, vitronectin is phosphorylated by PKA (93), PKC, and CK2. Phosphorylation by PKC attenuates its cleavage by plasmin (125), whereas phosphorylation with CK2 enhances cellular spreading (126).

Plasma C9 becomes phosphorylated by CK2 ectokinases on human leukemia cell lines such as U937 or K562, with the net effect of inhibiting complement-mediated cell lysis (127).

The phosphate content of fibrinogen, vitronectin, C3, and HSA has been shown to increase in the plasma after platelet activation (86), further underscoring the importance of platelets as mediators of extracellular phosphorylation but without addressing the question of whether their main effect is to provide protein kinase(s) or enough ATP to sustain phosphorylation, or a combination of both.

It is possible that HSA, the most abundant plasma protein and a substrate for multiple protein kinases (94), can act as a scavenger phosphorylation substrate in plasma.

Plasma Proteins Whose Activity Is Regulated by Platelet-Mediated Phosphorylation

A direct link between platelet activation, extracellular phosphorylation of plasma proteins, and alteration of the proteins function(s) has been demonstrated only in a few cases, such as complement component C3, coagulation factor XI (FXI), FVa, and, most recently, the anticoagulant effector protein S (**Figure 3**, right).

In a series of publications, we have reported that extracellular phosphorylation of C3 is mediated by a putative casein kinase associated with activated platelets. The main phosphorylation site was a serine residue in the C3d, g portion of the α-chain, and phosphorylation had dramatic effects on at least three of C3's functions, with the combined effect being an enhancement of the complement-mediated opsonization of immune complexes resulting from C3 phosphorylation. First, we found that the phosphorylation of C3b increases its resistance to cleavage by factor I to iC3b (98). Because C3b (but not iC3b) is a subunit of the alternative pathway C3 convertase, the activation of C3 is increased, as we were able to demonstrate in the form of an enhanced opsonization of model immune complexes (99). Lastly, we showed that bound phosphorylated C3b has a significantly higher affinity for CR1 than does its unphosphorylated counterpart (100), suggesting that phagocytosis of the opsonized immune complexes may be facilitated by platelet-mediated phosphorylation.

We further showed that the same platelet-derived casein kinase phosphorylates FXI in the coagulation cascade. Phosphorylation markedly enhanced FXI activation by FXIIa in particular, but also to a lesser extent by thrombin, suggesting that the phosphorylation enhances the activation of the coagulation cascade induced by the contact system and facilitates the thrombin-meditated amplification loop of the coagulation system, which involves the intrinsic components starting with FXI (102). The concept of increased activation was further supported by our observation that the levels of FXIa-AT complexes (which are generated secondarily to FXI activation) were higher in plasma from systemic lupus erythematosus (SLE) patients than in controls and that there was a clear correlation between the levels of FXIa-AT complexes and β-thromboglobulin, a marker for platelet activation (87).

Furthermore, it has been suggested that anticoagulation is regulated by platelet-mediated phosphorylation: Coagulation factor Va becomes more readily inactivated by activated protein C after phosphorylation by platelet casein kinase (128). More recently, platelet-secreted casein kinase(s) have been shown to phosphorylate protein S, thereby enhancing its activated protein C cofactor activity (103). In that publication, the authors postulated that extracellular platelet-mediated phosphorylation of protein S is a previously unrecognized mechanism for regulating its anticoagulant activity, and that role of phosphorylation most likely applies to all the substrates discussed here.

THE PRO-COAGULANT PLATELET PHENOTYPE IN DISORDERS OF COMPLEMENT REGULATION

Given the numerous and complex interactions between complement and platelets, it is interesting to note that conditions with a genetically determined deficiency in complement regulation include a pro-thrombotic platelet phenotype in their clinical presentation. Here we further discuss two such conditions, atypical hemolytic uremic syndrome (aHUS) and paroxysmal nocturnal hemoglobinuria (PNH), and consider what they can teach us about platelet-complement interactions.

Atypical Hemolytic Uremic Syndrome (aHUS)

aHUS is a thrombotic microangiopathy that presents with a symptom triad of intravascular hemolysis, thrombocytopenia, and acute kidney failure caused by complement-mediated tissue damage (129). aHUS appears to primarily be caused by a genetically determined impaired regulation of the alternative pathway, and heterozygosity for mutations in the alternative pathway regulator factor H is the most prevalent genetic lesion in aHUS. Mutations in factor I, CD46, factor B, and C3 have also been consistently observed, all of which have a common consequence, alternative pathway dysregulation (130, 131). The penetrance of aHUS causing mutations is not complete and often an external trigger such as an infection is needed to precipitate the disease in individuals with genetic predisposition (129).

Platelet consumption is an important but underappreciated feature of aHUS, where complement attack is postulated to cause platelet activation and formation of platelet-rich microthrombi and eventually thrombocytopenia. aHUS-associated factor H mutations cluster in the C-terminal part of the protein and impair binding to self surfaces, with insufficient protection from autologous complement attack as a result (132, 133). It has been shown that aHUS-associated factor H mutations directly impair platelet binding, resulting in increased complement deposition on platelet surfaces, increased baseline platelet activation, and formation of pro-coagulant microparticles (134). Further evidence for a prothrombotic state associated with platelet activation has been obtained in a mouse model of aHUS, in which a factor H point mutation found in aHUS patients has been introduced into mice (135). In addition to renal microangiopathy and kidney failure, mice harboring the mutant factor H variant developed an unexpectedly severe phenotype, with systemic thrombophilia, large vessel thrombosis, and multi-organ involvement.

Paroxysmal Nocturnal Hemoglobinuria (PNH)

PNH is a hemolytic anemia caused by complement-mediated hemolysis of red blood cells (136). It has a different genetic background than does aHUS and is caused by somatic mutations in clones of hematopoietic cells, leading to a deficiency of glycosylphosphatidylinotisol (GPI)-anchored proteins, and thus a lack of the GPI-linked complement regulators CD55 and CD59. The primary targets of complement attack in PNH are GPI-deficient red blood cells, which are continuously consumed by intravascular hemolysis. PNH is also associated with an increased risk of thrombosis, to which excessive complement-mediated platelet activation has been hypothesized to contribute. Platelets from GPI-deficient PNH clones still have some complement-regulatory capacity, since they express CD46 and recruit circulating factor H to the cell surface, as discussed above. Nonetheless, it appears that these soluble complement regulators are not sufficient to prevent complement attack in the context of the loss of CD55 and CD59, and there is clear evidence of complement-mediated platelet damage in PNH. Platelet counts are low, and both platelet dysfunction resulting from chronic hyperstimulation (137) and higher levels of baseline platelet activation have been observed (138). Interestingly, elevated numbers of platelet-derived microparticles are found in PNH patients, an observation that provides intriguing evidence of complement-mediated platelet microparticle formation (139).

Mechanisms of the Prothrombotic State in Disorders of Complement Regulation

Introduction of the anti-C5 antibody eculizumab (Soliris®), which blocks MAC formation and C5a generation, has constituted a therapeutic revolution for patients with aHUS and PNH. Anti-C5 treatment provides a clear benefit in these patients, and today, although very expensive, it is still the treatment of choice (140). Interestingly, eculizumab treatment rapidly normalizes suppressed platelet counts in both aHUS (141) and PNH (142) patients, indicating that platelet consumption in these conditions is complement-mediated. Because eculizumab also significantly reduces thromboembolic events, there appears to be a causal involvement of the terminal complement pathway in the hypercoagulable state observed in these patients (143).

What is the mechanism by which complement activates platelets and converts a quiescent vasculature to a prothrombotic state in these conditions? Direct complement-mediated damage to endothelial cells and platelets likely plays a major role, as well as sublytic concentrations of C5b-9 that potently triggers cellular activation. Apart from effects mediated by direct complement deposition, release of anaphylatoxins is also a potential contributor. C3a exerts stimulatory effects on platelets through the C3a receptor (44, 45). PAR1 and PAR4 have recently been identified as receptors for C4a (144), and although platelets were not directly tested in the same study, these PARs are strong agonistic platelet receptors that could implicate C4a as a mediator of platelet-complement cross-talk. The C5a-C5aR1 signaling axis mediates a potent pro-inflammatory response, but with the main target cells being endothelial cells and leukocytes. Blood clotting induced by intravascular invasion of pathogens has been shown to be dependent on C5 and the induction of tissue factor expression on monocytes. A strongly diminished response to LPS or whole bacteria was seen in blood from an individual with congenital C5 deficiency, a defect that was phenocopied in normal blood by addition of eculizumab or a C5aR1 antagonist, implicating signaling by soluble C5a rather than MAC deposition in this scenario (145, 146). Likewise, in a follow up study on an aHUS mouse model based on factor H deficiency, breeding of the mice onto a C5aR1 deficient strain rescued the large vessel thrombosis phenotype, while thrombotic microangiopathy and renal damage was MAC dependent. Interestingly, thrombocytopenia was seen in both scenarios, indicating that both C5a-dependent leukocyte activation and increased MAC formation contributes to a procoagulant platelet phenotype. Ongoing clinical trials of C5a-directed therapeutics and C5a receptor antagonists will undoubtedly provide important insights into these phenomena (140).

Collectively, aHUS and PNH demonstrate that in the context of a deficiency in its regulation, complement is clearly a very potent trigger of thrombotic events. The question remains

as to what extent complement activation is a physiological mechanism that supports platelet activation and thrombosis, and whether enough complement activity can be generated on cells to trigger these events under conditions of full complement-regulatory capacity.

CONCLUSIONS

There is now ample evidence to consider the platelet an immune cell and not limit its role to primary hemostasis and platelet plug formation. Platelets interact with the cascade systems of blood in a highly controlled fashion, with complement mediating platelet-leukocyte interactions, contributing to platelet activation, and helping to mount a pro-inflammatory response, all while preserving host cell integrity and avoiding complement-mediated damage to healthy cells.

The platelet phenotype of disorders of complement regulation involving excessive platelet activation and thrombocytopenia resulting from platelet consumption provide intriguing evidence regarding how complement can trigger platelet activation. However, the question remains: Do numerous complement regulators serve to totally prevent complement activation in the platelet microenvironment under physiological conditions? Investigators need to determine how this mechanism operates under physiological levels of complement-regulatory capacity and reconcile this model with the abundance of complement regulators associated with platelets.

Post-translational modifications of proteins, thought to occur exclusively in the intracellular milieu, are now gaining in interest as important regulatory mechanisms throughout the entire lifespan of a secreted protein. Mass spectrometry and proteomic approaches have been instrumental in characterizing and quantifying these mechanisms for fine-tuning protein function. In the context of platelets, we are beginning to unravel how these chemical reactions direct platelet activation and the interaction between platelets and the complement and coagulation systems. The concept of a "platelet microenvironment," in which exocytosis of platelet granules provides the co-factors and catalytic enzymes that drive these reactions, provides a model for how disulfide bond modifications and extracellular phosphorylation reactions proceed in the extracellular space, though many of the specific enzymes and substrates involved remain to be identified.

AUTHOR CONTRIBUTIONS

All authors listed have made a substantial, direct and intellectual contribution to the work, and approved it for publication.

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REVIEW
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Platelets in Sepsis: An Update on Experimental Models and Clinical Data

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Beyond their important role in hemostasis, platelets play a crucial role in inflammatory diseases. This becomes apparent during sepsis, where platelet count and activation correlate with disease outcome and survival. Sepsis is caused by a dysregulated host response to infection, leading to organ dysfunction, permanent disabilities, or death. During sepsis, tissue injury results from the concomitant uncontrolled activation of the complement, coagulation, and inflammatory systems as well as platelet dysfunction. The balance between the systemic inflammatory response syndrome (SIRS) and the compensatory anti-inflammatory response (CARS) regulates sepsis outcome. Persistent thrombocytopenia is considered as an independent risk factor of mortality in sepsis, although it is still unclear whether the drop in platelet count is the cause or the consequence of sepsis severity. The role of platelets in sepsis development and progression was addressed in different experimental *in vivo* models, particularly in mice, that represent various aspects of human sepsis. The immunomodulatory function of platelets depends on the experimental model, time, and type of infection. Understanding the molecular mechanism of platelet regulation in inflammation could bring us one step closer to understand this important aspect of primary hemostasis which drives thrombotic as well as bleeding complications in patients with sterile and infectious inflammation. In this review, we summarize the current understanding of the contribution of platelets to sepsis severity and outcome. We highlight the differences between platelet receptors in mice and humans and discuss the potential and limitations of animal models to study platelet-related functions in sepsis.

Keywords: sepsis, inflammation, platelets, thrombocytopenia, infection

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SEPSIS

Sepsis is a highly complex life-threatening syndrome of organ dysfunction caused by dysregulated inflammatory host response to an overwhelming systemic infection (1). Sepsis is typically manifested by an early dominant hyper-inflammatory phase, the systemic inflammatory response syndrome (SIRS), characterized by fever and hyper-metabolism, which can eventually lead to septic shock. This pro-inflammatory state is followed by or co-exists with a compensatory anti-inflammatory response (CARS) and immunosuppression, leading to secondary complications (2).

According to the Sepsis-3 guidelines, sepsis is diagnosed by the Sequential Organ Failure Assessment (SOFA) score, which assesses organ dysfunction and risk of mortality (1). Sepsis is

the primary cause of death from infection and occurs in 6–30% of patients in intensive care units (ICUs) (3). In-hospital mortality of patients with sepsis exceeds 10%, but increases to over 40% in severe cases which deteriorate into septic shock (1).

While early and effective medical interventions have managed to lower mortality over the last three decades (4), sepsis incidence is actually rising which might reflect the aging population in developed countries and increases the total number of patients suffering from or dying of sepsis.

Pathology of Sepsis

Normal host response to pathogen invasion involves a complex process to localize and confine microbes, while initiating repair processes of injured tissue. Microbial components can be recognized by germline-encoded pattern recognition receptors (PRR) on host immune cells, which bind pathogen-associated molecular patterns (PAMPs) of microorganisms or danger-associated molecular patterns (DAMPs) that are released from injured tissues during the inflammatory insult. PRRs can be further divided in toll-like receptors (TLRs), C-type lectin receptors (CLRs), nucleotide-binding oligomerization domain (NOD)-like receptors, and retinoic acid-inducible gene I (RIG-I)-like receptors (RLRs) (5).

Engagement of PRRs elicits various signaling cascades essential for the neutralization of pathogens. A central event in this process is the activation of cytosolic nuclear factor-κB (NF-κB). Activated NF-κB translocates from the cytoplasm to the nucleus, where it binds to transcription sites and induces activation of a plethora of genes involved in inflammatory host response, including pro-inflammatory cytokines, chemokines, adhesion molecules, and nitric oxide (NO) synthase (6).

As first line of defense to infection, the complement cascade, neutrophils and endothelial cells are activated, inducing the expression of adherence molecules on endothelial cells and promoting neutrophil and subsequent monocyte migration and extravasation to the site of inflammation. Monocytes differentiate into macrophages *in situ* and secrete a mixture of pro-inflammatory [e.g., tumor necrosis factor (TNF)-α and interleukin (IL)-1] and anti-inflammatory (e.g., IL-10) mediators. The ingestion of apoptotic neutrophils by inflammatory macrophages induces their switch to anti-inflammatory macrophages with repair properties (7).

These processes are responsible for the cardinal signs of local inflammation: warmth and erythema due to local vasodilation and hyperemia, protein-rich edema due to increased microvascular permeability and pain due to mediators released by innate immune cells.

A fine-tuned balance of pro-inflammatory and anti-inflammatory mediators regulates and restricts the inflammatory processes, the invading pathogens are cleared, homeostasis is restored and tissue repaired. The systemic dissemination of the immune response to uninfected remote tissue and failure to restore homeostasis, leads to a malignant intravascular inflammation called sepsis.

Sepsis is characterized by an aggravated, uncontrolled, and self-sustaining inflammation which spreads via the circulation. The exact mechanism by which an inflammation switches from being locally restricted to systemically spread is unclear and is likely to be multifactorial. Pathogens and their toxic products contribute to this process, as endotoxins are found in the blood of patients and associated with shock and multiple organ dysfunction. The most common causes of sepsis are infections of the respiratory system, followed by genitourinary and abdominal infections (8). While sepsis can be caused by infection with bacteria, virus and fungi, the most frequent pathogens in sepsis are gram-positive bacteria such as *Staphylococcus aureus* and *Streptococcus pneumoniae* (4, 9). However, almost half of all patients with sepsis have unknown infections and are pathogen culture negative. This form of sepsis is on the rise and associated with higher incidences of acute organ dysfunction and mortality (10).

Systemic Effects of Sepsis

The systemic immune response results in widespread cellular injury, which precedes organ dysfunction. The precise mechanism of cellular injury is highly complex and still incompletely understood. The main mechanisms involved include: cytopathic injury, which is mediated by direct cell injury by pro-inflammatory mediators and/or other products of inflammation, tissue ischemia due to insufficient oxygen supply, and an altered rate of apoptosis.

Tissue ischemia is caused by endothelial and microcirculatory lesions. Circulating inflammatory mediators activate the endothelium and induce loosening of tight junctions between endothelial cells, thereby increasing vascular permeability and leakage. As a consequence, systemic endothelial activation leads to hypotension and edema formation and thus to inadequate tissue oxygenation (11). Moreover, erythrocytes lose their normal ability to deform within the systemic microcirculation during sepsis (12), which impedes microcirculatory blood flow and depresses tissue oxygen supply. Microcirculatory lesions further occur as a result of imbalances in the coagulation and fibrinolytic systems, both of which are activated during sepsis.

Pro-inflammatory cytokines during sepsis promote cytopathic injury and also delay apoptosis in activated macrophages and neutrophils, thereby prolonging or augmenting the inflammatory response and contributing to the development of multiple organ failure. During the later phases of sepsis endotoxin tolerance (13) and extensive lymphocyte and dendritic cell apoptosis alter the immune response efficacy, decreasing the clearance of invading microorganisms and increasing the susceptibility to secondary infections (14).

Patients that die of sepsis show numerous overlapping mechanisms of immunosuppression involving both innate and adaptive immunity. Immune cells from spleens or lungs of septic patients, harvested shortly after the patients died, display a significant decrease in the production of pro- and/or anti-inflammatory cytokines and upregulated expression of inhibitory receptors including PD1. Also, the immunosuppressive cell populations of regulatory T cells and myeloid-derived suppressor cells are increased and CD28 and HLA-DR-mediated activation pathways downregulated (15).

During sepsis many cellular functions are affected, including mitochondrial functions which results in altered metabolic

substrate utilization, biogenesis and mitochondrial reactive oxygen species (ROS) production. Reduced autophagy of damaged mitochondria and autophagy exhaustion further enhances inflammatory dysregulation and tissue injury (16).

Sepsis further promotes a pro-coagulant and pro-thrombotic state of the host. Local and circulating endotoxins induce the expression of tissue factor (TF) on endothelial cells and monocytes, promoting intravascular fibrin deposition and vascular occlusion. Moreover, microbial pathogens trigger the release of neutrophil extracellular traps (NETs) from neutrophils which provide a negatively charged surface for the activation and assembly of coagulation factors (17). Activation of the coagulation cascade provides a positive feedback loop inducing platelet activation via generation of thrombin, thereby promoting microthrombosis in response to inflammation and infection. This so-called immunothrombosis is part of the anti-microbial host response and aims to entrap invading pathogens and prevent their spreading although this mechanism is likely pathogen- and organ-dependent (18, 19). Further, the activated endothelium increased the recruitment and adhesion of circulating platelets, thus contributing to the formation of microthrombi throughout the body (20). During sepsis platelets can be sequestrated in the capillary-rich microvasculature of the spleen and liver. However, the majority of platelets accumulate in the lung microvasculature (21–23).

Thus, excessive responses of the immune system during sepsis are often associated with exaggerated and dysregulated activation of coagulation and thrombosis, manifesting itself as disseminated intravascular coagulation (DIC). During DIC microthrombi readily form within small and medium vessels, leading to disturbed tissue oxygenation, multi-organ, and eventually circulatory failure (24).

Activated platelets promote the development and progression of sepsis via their involvement in inflammation and thrombosis. Sepsis is characteristically accompanied by a drop in platelet count, reflecting their sequestration and their consumption in microthrombi although many other mechanisms contribute to the severity and persistence of thrombocytopenia (see below) (20). Severe thrombocytopenia is associated with a dysregulated host response leading to an increase in cytokine levels and endothelial dysfunction (25, 26). Hence, sepsis is associated with increased systemic thrombosis and coagulation as well as with elevated risk of hemorrhage due to the consumption of coagulation factors and platelets (24). Thrombocytopenia was found to correlate with sepsis disease severity and is associated with increased mortality risk (27, 28).

Organ Specific Effects of Sepsis

Overwhelming systemic inflammation results in organ dysfunction. While no organ is protected from the consequences of sepsis, the most common complications are outlined below.

Circulation

During sepsis vasoactive mediators, including prostacyclin and NO, are released by endothelial cells. NO synthase can be induced by endotoxin and NO plays a central role in the vasodilation accompanying septic shock (29). Systemic NO release results in persistent vasodilation and diminishes the response to vasoconstrictors during sepsis (29). Furthermore, compensatory secretion of antidiuretic vasopressin is diminished during sepsis.

In the central circulation, decreased systolic and diastolic pressure diminishes the cardiac output. Low blood pressure also leads to disturbed blood flow in small vessels and restricts capillary functions in the microcirculation, leading to inefficient oxygen extraction due to edema, endothelial swelling and plugging by leukocytes.

Sepsis leads to endothelial dysfunction via direct and indirect interactions with pathogens. Degradation of the endothelial glycocalyx is associated with the upregulation of adhesion molecules (30), promoting the adherence of blood cells, which fosters mutual activation and further promotes inflammation and edema formation.

Gastrointestinal Tract

Hemodynamic abnormalities during sepsis depress the normal barrier function of the gut, allowing translocation of microbiota into the systemic circulation, further contributing to the progression of sepsis (31).

Lung

During sepsis microbial burden and/or increased inflammation results in endothelial injury of the pulmonary vasculature, leading to edema, increased leukocyte influx, and diminished oxygen supply. Excessive infiltration of innate leukocytes amplifies these processes by boosting inflammatory responses, causing injury to the lung tissue and hemorrhage, and thus causes loss of lung function. This may lead to the acute respiratory distress syndrome characterized by loss of the alveolar-capillary barrier function and increased vascular permeability, lung injury, and pulmonary edema (32). Clinical presentation thus includes diffuse bilateral pulmonary infiltrations and acute and persistent arterial hypoxia.

Liver

The liver works as a lymphoid organ in response to sepsis by mediating immune responses, leading to clearing of bacteria and toxins but also causing inflammation, immunosuppression, and organ damage. Attenuating liver injury and restoring liver function lowers morbidity and mortality rates in patients with sepsis (33).

Kidney

Acute kidney injury is a common phenomenon in sepsis and occurs in 40–50% of septic patients and increases mortality by 6–8-fold (34). The underlying mechanism is still incompletely understood. While previous notions regarded organ dysfunction solely as side effect of hypoperfusion, recent studies challenge this view and emphasize a role of heterogeneous areas of colocalized sluggish peritubular blood flow and tubular epithelial cell oxidative stress. Microvascular dysfunction, inflammation, and the metabolic response to inflammatory injury are crucial for the pathophysiologic mechanisms of kidney damage (34).

Brain

Sepsis is often characterized by an acute brain dysfunction which is associated with increased morbidity and mortality. Its pathophysiology is highly complex and involves inflammatory and non-inflammatory processes. Pathophysiological mechanisms include excessive microglial activation, impaired cerebral perfusion, blood-brain-barrier dysfunction, and altered neurotransmission. Systemic insults, such as prolonged inflammation, severe hypoxemia, and persistent hyperglycemia, may also contribute to aggravate sepsis-induced brain dysfunction or injury (35).

PLATELETS IN SEPSIS

Sepsis is characteristically accompanied by increased activation of platelets, small anucleate blood cells with pivotal functions in hemostasis. It is becoming increasingly apparent that platelets have also essential roles in immunity and modulate physiologic and pathologic responses to inflammation and infection.

Platelets are crucial regulators of leukocyte function and thus of inflammatory immune responses (36). They readily interact with innate immune cells and exert immunomodulatory effects directly via cell-cell contact or indirectly via the release of chemokines and cytokines (37). Platelets promote endothelial adhesion and extravasation of leukocytes at sites of inflammation while securing vascular integrity at the site of transmigration. They modulate cytotoxic neutrophil effector function and induce a pro-inflammatory phenotype of neutrophils by modulating their activation, phagocytosis, oxidative burst, and formation of NETs. Platelets are also involved in monocyte differentiation into macrophages and modulate their effector functions. Thereby platelets also contribute to excessive inflammatory host response during sepsis and promote the development and progression of sepsis via their involvement in both inflammation and thrombosis. On the other hand, platelets can inhibit inflammation and promote tissue repair in a receptor- and organ-dependent manner. Therefore, the balance between the pro-inflammatory and anti-inflammatory roles of platelets regulates the outcome.

Platelet Receptors in Sepsis

Platelets express various receptors that are involved in the initiation and progression of sepsis. They include receptors for pathogen recognition, immune cell activation and platelet activation. While a plethora of receptors are ubiquitously expressed on platelets, others are only found on platelet subpopulations [e.g., TLRs (38)]. Circulating platelets differ in age, maturation state, or density. It is currently unknown if receptor composition differs due to platelet maturation, differences in thrombopoiesis and receptor distribution during platelet formation (39) or if a subset of megakaryocytes produces immune-regulatory platelets which express e.g., TLRs, while others produce platelets that only fulfill thrombotic functions. Moreover, there are gender-specific differences in expression levels of some receptors. Women, for example, express more copy numbers of TLRs compared to men (40) and receptor expression correlates with distinct cardiovascular risk and inflammatory biomarkers (40). Interspecies differences in receptor expression and copy numbers are even bigger. Mice and humans not only strongly differ in platelet count but also show differences in receptor density and co-receptor expression (41–43). Moreover, some receptors are not expressed in mice, while they fulfill important immunomodulatory functions in humans (e.g., FcγRIIA). This makes it often difficult to translate results from animal models to the clinical situation. A comparison of human and murine receptors relevant to sepsis pathology is given in **Figure 1**.

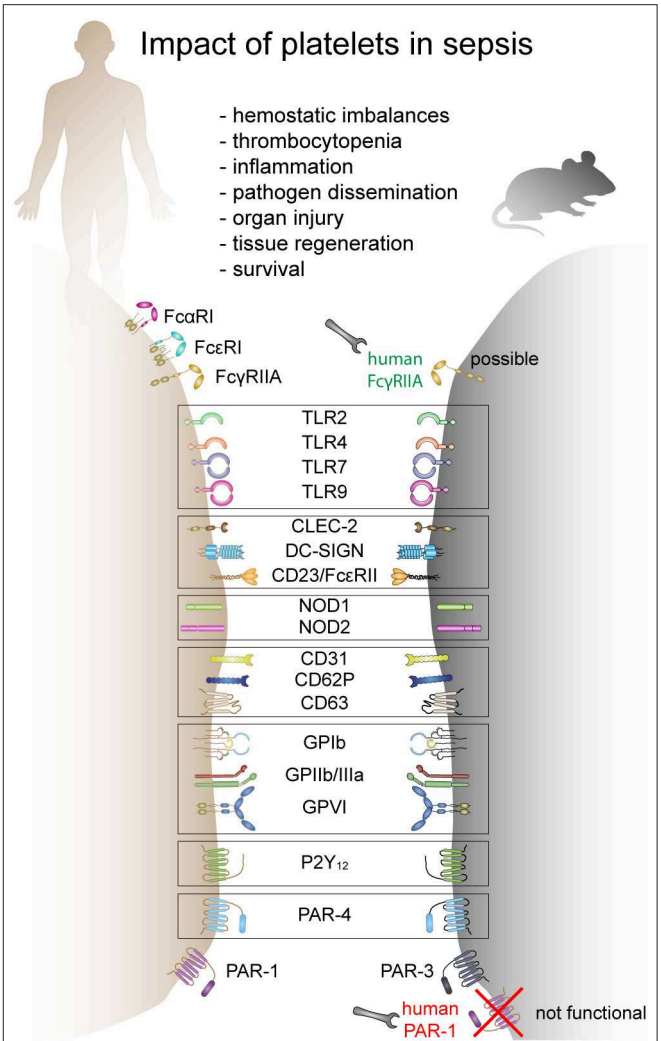

FIGURE 1 | The role of platelets in sepsis and receptors involved in mice and humans. Human and mouse platelets express a variety of immune receptors involved in thrombosis or/and inflammation during sepsis. Many receptors are conserved between mice and human and other receptors are species-restricted. FcαR, Fc-alpha receptor; FcεR, Fc-epsilon receptor; FcγR, Fc-gamma receptor; TLR, toll-like receptor; CLEC-2, C-type lectin-like receptor-2; DC-SIGN, dendritic cell-specific intercellular adhesion molecule-3-grabbing non-integrin; NOD, nucleotide binding oligomerization domain containing 1; GP, glycoprotein; $P2Y_{12}$, purinergic receptor $P2Y_{12}$; PAR, proteinase-activated receptor.

Toll-Like Receptors (TLRs)

TLRs are type I transmembrane proteins comprising of an ectodomain, which contains leucine-rich repeats that mediate the recognition of PAMPs, a transmembrane region, and a cytosolic Toll-IL-1 receptor (TIR) domain that activates downstream signaling pathways. TLRs are either expressed on the cell surface or associated with intracellular vesicles. Each TLR detects distinct PAMPs and therefore recognizes viruses, bacteria, mycobacteria, fungi, or parasites (44).

Human and murine platelets express functional TLRs. TLR2 regulates megakaryopoiesis (45), thromboinflammation (46), and bacterial phagocytosis (47). TLR7 stimulation triggers platelet degranulation and platelet-leukocyte aggregate formation and alters survival in virally infected mice (48). TLR9 regulates foreign DNA sequestration and CD62P surface expression in platelets (49). TLR4 is involved in rapid TNF-α induction (38), NET formation (50), and thrombocytopenia (38, 51). During gram-negative infection but not gram-positive infection, TLR4 activation induces the expression of neuraminidase, promoting alkaline phosphatase clearance and increasing LPS phosphorylation and toxicity (52–54). Interestingly, injection of neuraminidase post *Streptococcus pneumoniae* infection promotes survival and decreases fibrin clot frequency as well as liver and spleen injury in septic mice. Neuraminidase further induces moderate thrombocytopenia with platelet counts dropping by 70%, indicating that moderate thrombocytopenia might be beneficial in sepsis (55).

While TLR2 is only expressed on a small subset of platelets (10–20%), TLR4 is found on approximately 60% of human and 40% of murine platelets (23, 38). TLR9 is found on approximately 40% of resting human platelets and can increase to up to 60% in activated platelets indicating an intracellular storage pool of TLR9 (38, 49, 56). In mice, 60% of platelets are TLR9 positive and no further upregulation could be observed upon activation (38).

C-Tlatelet Antibodype Lectin Receptors (CLRs)

CLRs comprise a large family of receptors that bind to conserved bacterial structures via carbohydrate-recognition domains (CRDs) in a calcium-dependent manner. Based on their molecular structure, two groups of membrane-bound CLRs can be distinguished. Type I CLRs carry multiple CRDs or CRD-like domains, while Type II CLRs contain only a single CRD. CLRs are expressed on several immune cells. On platelets CD23 (FcεRII) and dendritic cell-specific ICAM-grabbing non-integrin (DC-SIGN) are expressed and functional (57–60). DC-SIGN is involved in lentiviral internalization by platelets (57).

Nucleotide-Binding Oligomerization Domain-Like Receptors (NOD-Like Receptors)

NOD-like receptors are cytoplasmic receptors with a strong structural similarity. All NOD-like receptors contain a central nucleotide-binding oligomerization domain (NOD), a C-terminal leucine-rich repeat domain and a variable N-terminal protein-protein interaction domain which interacts with downstream effectors. NOD1 and NOD2 are the two important NOD-like receptors fitting the typical structure with NOD1 containing one caspase recruitment domain (CARD), whereas NOD2 contains two CARD domains (61). While NOD1 recognizes d-glutamyl-meso-diaminopimelic acid primarily from gram-negative bacteria, NOD2 detects the muramyl dipeptide (MDP) motif in peptidoglycan from all bacteria. NOD1 is broadly distributed, whereas NOD2 is mainly expressed in innate immune cells (61) and platelets (62). In platelets NOD2 contributes to platelet activation and is possibly involved in arterial thrombosis during infection (62).

Glycoprotein Ib (GPIb)

GPIb is exclusively expressed on platelets and megakaryocytes and plays a crucial role in platelet adhesion under high shear. GPIb is part of the GPIb-V-IX complex, which binds von Willebrand factor (vWF), allowing platelet adhesion and platelet plug formation at sites of vascular injury. Many bacteria such as *Streptococcus sanguis* contain serine-rich protein A (SrpA) which is also recognized by GPIb and allows platelet-bacteria binding in a sialic acid-dependent manner (63). *Staphylococcus aureus* protein A (Spa) also binds vWF, which mediates indirect interaction with platelets via GPIb (64).

Integrin $\alpha_{IIb}\beta_3$ (GPIIb/IIIa)

Glycoprotein IIb/IIIa (GPIIb/IIIa) represents the most abundant platelet glycoprotein and is central for platelet aggregation via fibrinogen bridging. GPIIb/IIIa becomes activated via inside-out signaling, leading to a conformational change that uncovers an arginine-glycine-aspartic acid (RGD) sequence, allowing the binding of fibrinogen, vWF, fibronectin and vitronectin, and platelet aggregation (65). Several bacterial proteins are able to bind to the RGD sequence on GPIIb/IIIa, including SdrG (Fbe) from *Staphylococcus epidermis* causing platelet aggregation (66). *Borrelia burgdorferi* also binds human platelets via GPIIb/IIIa (67). Clumping factors (Clf) on *Staphylococcus aureus* bind fibrinogen causing platelet aggregation (68).

Glycoprotein VI (GPVI)

GPVI is an immunoreceptor tyrosine-based activation motif (ITAM) receptor that plays a crucial role in the collagen-induced activation and aggregation of platelets. By binding to exposed subendothelial collagen, GPVI mediates the sealing of vascular injuries and ensures integrity of the circulatory system.

Protease Activating Receptors (PARs)

PARs are a subfamily of related G protein-coupled receptors highly expressed on platelets and are activated by cleavage of part of their extracellular domain by thrombin. Human platelets express PAR-1 and PAR-4 with PAR-1 mediating strong thrombin-induced activation. In contrast, murine platelets express PAR-3 and PAR-4 (69) with PAR-4 being the most potent receptor for thrombin.

Fc Receptors

Human platelets are reported to express various Fc receptors including FcαRI (CD89) (70), FcεRI (71), FcεRII (CD23) (59), and FcγRIIA (CD32a) (72). In infectious settings, FcγRIIA mediates immune complex-induced platelet activation or killing of opsonized bacteria (73, 74).

P2Y Receptors

P2Y receptors are a family of purinergic G protein-coupled receptors, stimulated by nucleotides such as adenosine diphosphate (ADP). The ADP receptors, $P2Y_1$ and $P2Y_{12}$, are differentially involved in pro-thrombotic platelet activation as well as expression of platelet P-selectin in response to various agonists (75). $P2Y_{12}$ inhibitors are routinely used in the clinics for prevention of thrombotic complications.

P-Selectin (CD62P)

In resting platelets, CD62P is stored in the α-granule membrane and becomes exposed on the platelet surface upon activation. CD62P functions as adhesion molecule and is responsible for platelet interaction with activated endothelial cells and leukocytes via binding to its ligand P-selectin glycoprotein ligand 1 (PSGL-1), which enables platelets to fine-tune their cellular functions.

CD40 Ligand (CD40L)

Following activation platelets upregulate CD40L, promoting their interaction with innate immune cells, in particular monocytes. CD40L can be shed by metalloproteinase 9 (MMP9) which is upregulated during sepsis, resulting in a significant increase in soluble CD40L.

Platelet Activation in Septic Patients

Increased platelet activation is observed in septic patients and is further potentiated in septic shock. Platelet activation is associated with the upregulation of surface expression of CD62P, CD63, CD31, increased fibrinogen binding and soluble GPVI (76–79), particularly in patients with DIC (80). Moreover, an increase in thrombospondin expression on circulating platelets is observed in patients with multiple organ failure (77). Platelets from septic patients show spontaneous aggregation but the *ex vivo* response to platelet agonists is severely reduced (81–83). Interestingly, the impairment in platelet aggregation is not associated with DIC (82). Whereas, platelet activation in sepsis is not questioned, platelet aggregation data is more contradictory due to the low platelet count in these patients which is not taken into account in many studies (80). Platelet activation is also associated with increased platelet-neutrophil and platelet-monocyte aggregates in septic patients (84, 85) further potentiating the inflammatory response. Together these results suggest that septic patients' platelets circulate in an activated state, increasing their thrombotic potential (84).

Different mechanisms may contribute to direct and indirect platelet activation during sepsis, including platelet activation by the pathogen (86–90), pathogen- and inflammation-driven activation of the endothelium and leukocytes and complement activation-mediated platelet activation (91). The complexity of platelet activation in sepsis suggests the contribution of multiple receptors, making it likely that combined therapy might be required to inhibit platelet activation in sepsis. However, increased bleeding risk in these patients adds another layer of complexity for targeting platelets in septic patients.

Platelet Count in Septic Patients

The correlation between platelet count and sepsis severity and outcome has drawn increasing attention to the contribution of platelets to the pathophysiology of sepsis (92, 93). The critical role of platelets in sepsis is emphasized by the fact that platelet count is included in the SOFA score and is inversely associated with sepsis severity (94). Thrombocytopenia is often used to stratify patients with sepsis and septic shock based on the nadir of platelet count: mild thrombocytopenia (platelet count $< 100–150 \times 10^9$/L), moderate thrombocytopenia (platelet count between 50 and 100×10^9/L) and severe thrombocytopenia (platelet count $< 50 \times 10^9$/L), with severe thrombocytopenia being associated with worse outcomes (25). Another important parameter is relative thrombocytopenia, that represents a drop of the initial platelet count over 4 days (95). Indeed, the kinetics of platelets in sepsis is often biphasic, characterized by an initial drop within the first few days (day 1–4) followed by an increase in platelets and thrombocytosis (96). Lack of this biphasic response leads to persistent thrombocytopenia and is associated with poor prognosis and increased 28-day mortality (96, 97).

Thrombocytopenia may occur before admission at the hospital or during the ICU hospitalization (96, 98, 99). Of note, a single measurement of platelet count is not predictive of mortality (92, 96, 100). The initial platelet drop does not discriminate between survivors and non-survivors while late thrombocytopenia is more predictive for mortality. Indeed, stratification of septic patients revealed that not only the severity of thrombocytopenia but more importantly persistence of thrombocytopenia is associated with worse outcome. Severe thrombocytopenia is independently associated with disease severity and mortality at the ICU admission and is associated with a dysregulated host response (25).

In general, 20–58% of septic patients develop thrombocytopenia, of which 10% develop severe thrombocytopenia (28, 93, 96, 98, 101). The discrepancy in the reported values might arise from patient heterogeneity, different inclusion criteria, pathogens and other factors. Moreover, a decrease in immature platelets, indicating impeded thrombopoiesis, is associated with severe thrombocytopenia and 28-day mortality (102). One recent study showed that immature platelet fractions could predict sepsis occurrence in critically ill subjects (103). Interestingly, phosphatidylserine-expressing platelet microvesicles are decreased in non-survivors and correlate with thrombocytopenia but are not associated with DIC (104). Therefore, severity and persistence of thrombocytopenia as well as immature platelet fractions and platelet microvesicle composition are strong predictors of mortality in sepsis.

Causes of Thrombocytopenia in Septic Patients

The association between thrombocytopenia and clinical outcome does not reveal any causality as many parameters could be both the cause and/or the consequence of dropping platelet counts.

Thrombocytopenia can either occur due to diminished platelet production or increased platelet turnover. Platelet activation diminishes platelet life span as activated platelets are

rapidly cleared from the circulation. Animal models revealed that thrombocytopenia in sepsis is largely TLR4-dependent, which suggests that immune-mediated platelet activation represents a main cause for the drop in platelet count (38). Many mechanisms have been proposed for thrombocytopenia in sepsis although a combination of multiple mechanisms might occur in severely thrombocytopenic patients. An overview of these processes is given in **Figure 2**.

Platelet-Leukocyte Aggregation

The formation of platelet-leucocyte aggregates (PLA) in the blood depends on platelet activation and is an early phenomenon occurring in sepsis. Circulating PLA are increased in sepsis patients at an early phase, but significantly decrease in non-survivors and patients developing multiple organ failure, likely due to enhanced peripheral sequestration or sepsis-associated thrombocytopenia (84). Platelet-neutrophil aggregates can also potentiate thrombocytopenia through the release of platelet-activating NETs (105).

Pathogen-Induced Thrombocytopenia

Fungi and bacteria can interact with platelets and induce platelet activation and aggregation (86, 87, 89). Although many bacteria activate platelets in a GPIIb/IIIa- or FcγRIIA-dependent manner and involve plasma proteins such as IgG, complement proteins and fibrinogen, other bacteria directly bind and activate platelet receptors such as GPVI and TLRs, increasing platelet activation and PLA formation (46, 87, 106). Mechanisms of pathogen clearance by platelets may occur indirectly through the release of various antimicrobial peptides and platelet-derived mediators regulating the activation of the endothelium and immune cells. Some pathogenic bacteria, particularly blood stream infections, may also trigger apoptosis in platelets resulting in thrombocytopenia (107, 108). Thrombocytopenia occurs in 20–30% of patients infected with *Staphylococcus aureus*, *Escherichia coli,* or *Streptococcus pneumonia*, suggesting that platelet activation by pathogens contributes to thrombocytopenia but does not represent a major mechanism (109). TLR4-mediated responses reduce platelet counts in murine endotoxemia (38, 110), but the role of TLR4 in patients has never been investigated.

Tissue Injury-Mediated Platelet Activation

Infections are commonly associated with tissue injury and cell destruction that fuel inflammation. DAMPS and other mediators released from activated and injured cells such as histones and high mobility group protein B1 (HMGB1) can activate platelets and enhance agonist-induced platelet activation and granule secretion, potentiating thrombocytopenia and delaying its resolution (111–113).

Clearance Through Desialylation

Platelet desialylation is increased in septic patients with thrombocytopenia compared to patients without thrombocytopenia (114). Desialylation occurs during pneumococcal infections, leading to the release of neuraminidase and the exposure of galactose residues, increasing the clearance of platelets by the Ashwell Morrell receptors (AMR) on hepatocytes. GPIbα is the main receptor involved in platelet clearance and is desialylated by neuraminidase, although other glycoproteins are also susceptible to desialylation. Moreover, platelet desialylation increases platelet reactivity, thereby potentiating thrombocytopenia (115).

Anti-platelet Antibodies

Another possible mechanism that contributes to thrombocytopenia is the immune clearance and destruction of platelets. Anti-platelet antibodies (e.g., anti-PF4/heparin) are detected in patients with bacterial septicemia and their level increases in thrombocytopenic patients with no significant difference between gram-positive and gram-negative infection (116–118). Moreover, IgG-opsonization enhances the clearance of LPS-binding platelets in an Fc-dependent manner and further potentiates platelet clearance in gram-negative infection (51). Inflammation fosters immune thrombocytopenia as C-reactive protein, produced during the acute phase of inflammation, enhances antibody-mediated platelet clearance by FcγR-dependent phagocytosis (108). More recently a novel mechanism of platelet sequestration through FcγRIIA activation by immune complexes was suggested (119). These studies consolidate that different mechanisms contribute to thrombocytopenia during different infections.

Disseminated Intravascular Coagulation (DIC)

DIC is defined as an excessive fibrin deposition leading to the occlusion of blood vessels and organ damage that is associated with consumption of coagulation factors and platelets. DIC is often associated with low platelet count, abnormal coagulation and fragmented cells; however thrombocytopenia is not always associated with DIC (100). DIC was one of the first mechanisms suggested to explain thrombocytopenia in septic patients but the low association between thrombocytopenia and DIC (15–30% of patients with thrombocytopenia present with DIC) suggests that DIC might contribute to thrombocytopenia only in some cases of severely ill patients (96, 120). DIC is mostly observed in patients with septic shock, however the association between DIC and mortality largely depends on the study and the inclusion criteria (96). More recently, it was shown that although DIC occurs in septic patients and mice, the time course and the composition of thrombi differ between organs even within the same infection (18, 121). This might be due to the different susceptibility of the endothelium, the local and systemic generation of coagulation factors, fibrinolytic factors and extracellular matrix proteins as well as due to the activation of immune cells.

Platelet Production (Thrombopoiesis)

The presence of normal megakaryocyte counts in the bone marrow of septic patients with low platelet count suggests that thrombopoiesis remains unaffected (95). Moreover, the increase in immature platelet fraction, the absolute immature platelet count and the increase in thrombopoietin (TPO) levels consolidate this hypothesis (122, 123). Increased TPO levels might result from reduced platelet count or enhanced TPO production in the liver by inflammatory mediators. However, in some severely ill patients with advanced thrombocytopenia, a

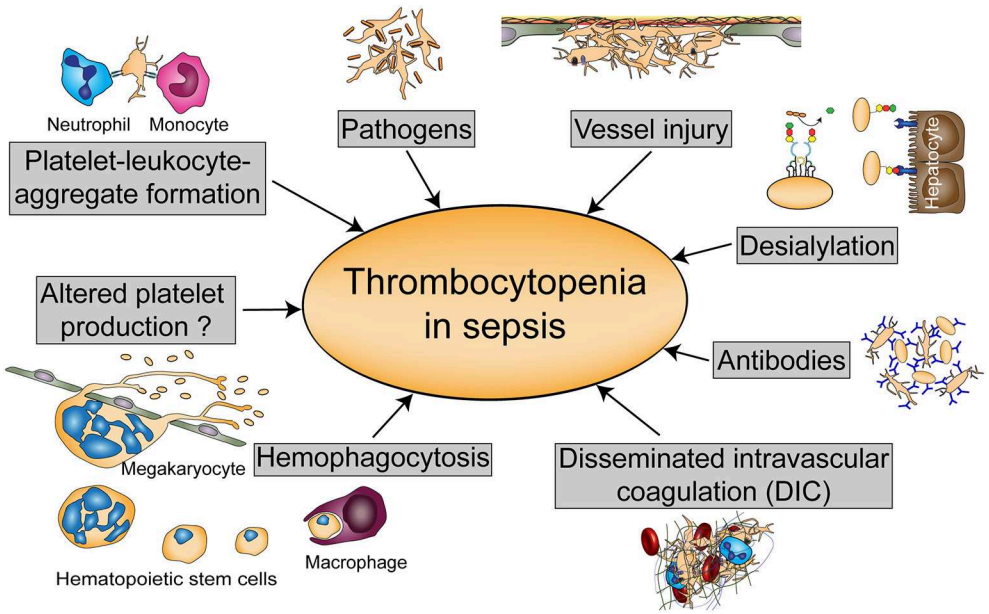

FIGURE 2 | Possible causes of thrombocytopenia in sepsis. Thrombocytopenia in sepsis might be either regulated by altered platelet production or hemophagocytosis, or by platelet scavenging in the circulation either due to platelet-leukocyte or platelet- pathogen interactions, vessel injury, or desialylation. Platelets can also be targeted by antibodies during sepsis or reduced due to disseminated intravascular coagulation (DIC).

defect in thrombopoiesis might occur, which could explain why these patients do not recover to a normal platelet count (102).

Cytokine-Driven Hemophagocytosis

An increase in the proliferation and activation of monocytes and macrophages in the bone marrow was observed in septic patients with thrombocytopenia. The uncontrolled proliferation is associated with an increase in macrophage-colony stimulating factor (M-CSF) which accelerates the ingestion of hematopoietic cells by macrophages and may contribute to thrombocytopenia (124).

Taken together, mild and moderate thrombocytopenia might result from one or the combination of different mechanisms, whereas severe and non-resolved thrombocytopenia involves concomitant mechanisms of platelet activation, sequestration and destruction.

Anti-platelet Drugs in Sepsis

Several observational and retrospective clinical studies have shown that anti-platelet agents such as aspirin (COX-1 inhibitor), platelet $P2Y_{12}$ receptor antagonists like clopidogrel or GPIIb/IIIa antagonists reduce mortality or complications in critically ill patients (99, 125, 126). In human experimental endotoxemia, $P2Y_{12}$ inhibitors reduce the pro-inflammatory and pro-thrombotic mechanisms (127). Further, in septic patients low dose aspirin is associated with a decrease in mortality during hospitalization (128). Patients on low dose aspirin have shorter in-hospital stays and reduced need for intensive care treatment. Administration of aspirin for 24 h at the time of SIRS recognition is associated with increased survival in a large cohort of over 5,000 septic patients (129).

Inhibition of platelet function in sepsis represents an attractive target due to their role in thrombosis and inflammation. However, as platelet receptors play different roles in thrombosis, inflammatory hemostasis and inflammation, precautions have to be taken when targeting platelets in infection (130). Large randomized controlled clinical trials with anti-platelet therapy in stratified patients are warranted to determine a conclusive beneficial effect of anti-platelet drugs in sepsis.

RODENT MODELS TO STUDY THE ROLE OF PLATELETS IN SEPSIS

In septic patients, disease progression before hospital admission often remains unknown. Therefore, it can be challenging to determine cause and effect of the clinical symptoms. Animal models help to unravel these early processes and the availability of genetically modified mouse strains contributed to the identification of distinct signaling pathways or genes as potential biomarkers or drug targets. Thus, rodent and especially mouse models have proven to be convenient and widely-used tools to study cellular and molecular mechanisms of sepsis in defined settings.

A number of different sepsis models have been developed that vary in technical complexity, controllability, and representativeness for the human sepsis patient setting. As sepsis commonly originates from infections of lung and genitourinary tract and to a less extend the abdomen (8), intraperitoneal or pulmonary routes are favored for primary induction and experimentally easy accessible. An overview of the

different animal models used to investigate the role of platelets and their receptors in sepsis is given in **Figure 3**.

Different Models to Study Platelet Function in Sepsis

Single PAMP

The most controllable *in vivo* model is the injection of a single bacterial or fungal PAMP via intravenous, intraperitoneal, or intranasal/intratracheal application. Most commonly LPS from gram-negative *Escherichia coli* or *Klebsiella* is used, which stimulates TLR4 on host cells including platelets (23). LPS represents a weak platelet agonist and primes platelets for stimulation with other agonists (131), though its effect on platelet degranulation may not be detectable in every experimental setting (23). LPS usually signals via MyD88, however lack of platelet MyD88 did not alter host responses to LPS including thrombocytopenia and immune cell recruitment (132). Intravenous challenge with low-dose (0.125–0.25 mg/kg) LPS leads to rapid but transient thrombocytopenia accompanied by platelet sequestration in capillary-rich organs such as lungs and liver (133). For intraperitoneal application doses commonly range between 1 and 10 mg/kg which induces prolonged thrombocytopenia, platelet sequestration and innate immune cell infiltration into lungs and liver, changes in the coagulation state and an inflammatory cytokine response involving TNF-α and IL-6, which may eventually lead to death (23, 134). As such, LPS triggers a strong but short-lived response favoring the innate immune system.

However, the intensity of platelet responses depends on the specific LPS serotype of the O antigen, the outermost polysaccharide domain of the molecule. LPS serotypes O8 and O9 readily induce platelet activation, thrombocytopenia, platelet sequestration in the lungs and liver as well as increased mortality, whereas O111 and LPS of the strain K-12, lacking an O antigen, trigger weaker responses (135). Additionally, LPS can be classified in smooth (full-length O chains) or rough (reduced or absent O chains). A comparison of eight different smooth and rough LPS serotypes from *Escherichia coli*, *Klebsiella*, *Salmonella minnesota,* and *Salmonella typhimurium* in mice identified LPS of *Klebsiella* O3 as most potent to induce a platelet response and shock, triggering a complement-dependent accumulation and degradation of platelets in lungs and liver (136).

In addition to LPS administration, cell-surface proteins from gram-positive bacteria such as the M1 protein of *Streptococcus pyogenes* can be used to mimic sepsis that does not engage TLR4, but rather stimulates the immune system via super-antigens or peptidoglycans (137). However, M1-induced sepsis represents an unfavorable model to study platelets in sepsis as M1 challenge leads to neutrophil-dependent organ damage independently of platelets (138).

Fungal sepsis can be investigated by injection of the yeast-derived cell surface glucan zymosan A (e.g., from *Saccharomyces cerevisiae*) which activates TLR2 and the alternative complement pathway. Unlike LPS, zymosan A induces a triphasic immune response which resembles the prolonged sepsis in humans. The first phase is characterized by a strong pro-inflammatory response with high levels of TNF-α and IL-6, followed by chronic low-grade inflammation which eventually culminates in organ damage and death (137, 139).

Nevertheless, screening of literature revealed that LPS from *Escherichia coli* O111:B8 appears to be the most commonly used agent for murine endotoxemia.

Single Live Pathogen

Injection of a single, live pathogen is more representative of infections in patients that originate from one infectious agent. While, by nature of live pathogens and their proliferation *in vivo*, this model has a lower level of controllability than injection of isolated PAMPs, the possibility of challenging animals with individual pathogens of choice provides the huge advantage of studying mechanisms underlying specific pathogens or bacterial strains.

Commonly used bacteria to study the role of platelets in murine sepsis models include *Escherichia coli*, *Klebsiella pneumoniae*, *Staphylococcus aureus*, *Streptococcus pyogenes,* and *Streptococcus pneumoniae* (140–144). Although intravenous injection of bacteria leads to strong bacteremia, complement-mediated host responses may prevent efficient colonization of organs and as such this model fails to accurately reproduce human sepsis (145). Additionally, due to the immediate effect on endothelial cells and the vasculature, intravenous administration triggers a potent, rapid, pro-inflammatory immune response that may be stronger than the host response induced by a local infection.

Nonetheless, challenge with single live pathogens represents a good model to study e.g., pneumosepsis, in which infections originate in the lungs before spreading systemically. Accordingly, intranasal infection with 10^4-10^6 colony forming units (CFU) of *Klebsiella pneumoniae* or *Streptococcus pneumoniae*, the most common gram-negative and gram-positive causative pathogens of community-acquired pneumonia, respectively (146), induces local pulmonary inflammation with accompanied cytokine response and infiltration of neutrophils and macrophages into the inflamed lungs. As local immunity becomes unable to contain the infection, bacteria disseminate into the bloodstream and can be detected in distant organs such as spleen, kidneys, and liver (141, 144).

Therefore, infection with live bacteria also allows studying of antibacterial host responses including phagocytosis, formation of NETs and release of antimicrobial agents, which play important roles in human sepsis.

In addition to live bacteria, experimental sepsis can also be induced by the human commensal fungus *Candida albicans* which may cause sepsis in humans upon breaching mucosal barriers of the gut e.g., following surgery or trauma (147). Innate immune recognition of *Candida albicans* involves various CLRs and TLRs, several of which are expressed on platelets (148). Infection of susceptible mice with live *Candida albicans* results in thrombocytopenia and decreased clotting times, indicating activation of primary and secondary hemostasis similar to bacterial sepsis (149).

		Single PAMP	Single live pathogen	Polymicrobial sepsis
Pathogen	Bacteria	LPS: Klebsiella spp., Escherichia coli, Salmonella spp. M1: Streptococcus spp.	Klebsiella pneumoniae, Escherichia coli (Gram negative) Staphylococcus aureus, Streptococcus pyogenes, Streptococcus pneumoniae (Gram positive)	Cecal ligation and puncture (CLP) Cecal slurry (CS)
	Fungal	Zymosan A	Candida albicans	–
	Viral	–	–	–
Stimulus	Trigger	Single known PAMP	Single known pathogen Multiple PAMPs	Multiple unknown bacteria Numerous PAMPs
	Intensity	Adjustable initial dosage	Adjustable initial dosage Variable exacerbation	Undefined initial dosage Variable exacerbation
Technique	Application	iv, in, ip	iv, in, ip	ip
	Species	Mouse, rat, rabbit, dog, pig, sheep	Mouse, rat, rabbit, dog, pig, sheep, baboon	Mouse, rat, pig, sheep
	Biosafety	Safe	Depends on pathogen	Safe
Host-response		Strong, innate LPS: short-lived Zymosan A: trimodal		CLP:innate & adaptive, persistent CS: innate, persistent strong initial infammation
Role of platelet receptors	Thrombo-cytopenia	↑ $P2Y_{12}$, GPIb, TLR4 – CLEC-2, GPVI, PAR-4, GPIIb/IIIa	– $P2Y_{12}$, CD62P, PAR-4	↑ $P2Y_{12}$ – CLEC-2
	Inflammation	~ $P2Y_{12}$ – TLR4, GPVI, PAR-4 ↓ CLEC-2	↑ GPVI	↑ CD40L, $P2Y_{12}$, CD62P ↓ CLEC-2, GPIb
	Bacterial burden		– $P2Y_{12}$, CLEC-2 ↓ GPVI, CD62P, PAR-4, TLR4	
	Disease severity	↑ GPIb, GPIIb/IIIa, TLR4 ~ $P2Y_{12}$ – GPVI, PAR-4 ↓ CLEC-2		↑ CD40L, $P2Y_{12}$, CD62P ↓ CLEC-2
	Thrombosis	↑ TLR4 – CD62P	↑ CLEC-2	
	Bleeding		↓ GPVI	↓ CLEC-2
Clinical relevance		Immune stimulation ✓	Immune stimulation ✓ Bacterial dissemination ✓ Antibacterial effects ✓	Immune stimulation ✓ Bacterial dissemination ✓ Antibacterial effects ✓ Endogenous pathogens ✓ Necrosis ✓

↑ increasing contribution – no contribution
↓ decreasing contribution ~ unclear contribution

FIGURE 3 | Overview of different mouse models to study sepsis. The role of platelets was addressed using single PAMP injection, single live pathogen or polymicrobial pathogens. Platelets regulate thrombosis, inflammation, bleeding, and sepsis outcome in a receptor- and pathogen-dependent manner. PAMP, pathogen-associated molecular pattern; LPS, lipopolysaccharides; M1, streptococcal M1 protein; iv, intravenous; in, intranasal; ip, intraperitoneal; $P2Y_{12}$, purinergic receptor $P2Y_{12}$; GP, glycoprotein; TLR, toll-like receptor; CLEC-2, C-type lectin-like receptor-2; PAR, proteinase-activated receptor.

Polymicrobial Infection

Models of polymicrobial infection most closely resemble human sepsis originating from an intestinal center of infection.

Cecal ligation and puncture (CLP) represents one of the most commonly used sepsis models as it most closely resembles sepsis in humans regarding biochemical, hemodynamic and immune responses, including hypotension, leukopenia, thrombocytopenia with a concomitant pro-thrombotic and pro-coagulatory phenotype, raised levels of pro-inflammatory cytokines as well as markers of organ dysfunction (150–152). Perforation of the cecum mimics a breach of intestinal barrier with subsequent dissemination of intestinal microbial flora into the peritoneum. In CLP this peritonitis is combined with necrosis of the ligated tissue and eventual dissemination into the periphery, resulting in septicemia and distant organ damage (152).

In addition to resembling the human sepsis situation, CLP has the advantage of being adjustable in severity based on the ratio of ligated tissue and the size and number of cecal perforations. However, CLP is prone to variation due to the operator and local immune responses may manage to contain bacteria in an abscess, thereby preventing the progression to septic shock (153). CLP-induced microvascular dysfunctions are not mediated by LPS (154), underlining the importance of gram-positive bacteria in this model. Compared to LPS challenge, the inflammatory cytokine response to CLP develops slower, even at similar mortality and morbidity (155), which may be due to the gradual disease progression.

Another model of polymicrobial sepsis is the cecal slurry (CS) method which comprises the intraperitoneal injection of a defined amount of donor feces and results in a stronger early inflammatory response than CLP (156). Curtailed variability in infectious dose and technical ease make CS a suitable model for polymicrobial infection in settings where surgery is problematic.

Underlying Mechanisms Identified by Mouse Models

The Impact of Platelet Depletion on Sepsis

Platelet depletion has detrimental effects on survival in gram-negative (*Klebsiella pneumoniae*) and gram-positive (*Streptococcus pneumoniae*) pneumosepsis, accompanied by increased pulmonary hemorrhage and clinical pathology score (141, 144, 157). While pulmonary neutrophil infiltration appears largely platelet-independent in live bacterial models, platelets facilitate leukocyte recruitment in LPS/zymosan-induced lung injury (158). Accordingly, platelets seem to contribute to tissue injury in the absence of live pathogens. In contrast, low platelet counts are associated with increased secondary hemostasis, liver and kidney damage as well as exacerbated bacteremia and systemic bacterial dissemination in bacteria-induced sepsis (140, 141, 144, 158, 159). In line with these findings, thrombocytopenia also exacerbates the inflammatory response in sepsis, raising plasma levels of TNF-α, IL-6, IL-10, myeloperoxidase (MPO), monocyte chemotactic protein 1 (MCP-1), and interferon-γ (IFN-γ) (141, 144, 159), potentially as a consequence of more severe infection.

Nonetheless, the role of platelets in sepsis is multi-faceted and current research has only begun to untangle the complex interplay of inflammation, thrombosis, and coagulation that occurs during sepsis. Indeed, the role of platelets may depend on the specific pathologic setting and thus experimental model, as platelet depletion in *Streptococcus pyogenes* actually ameliorates weight loss, decreases bacterial burden and dampens the inflammatory host response (143). The reason for this discrepancy with other reports is currently unknown.

Platelets Receptors in Sepsis

Platelet receptors regulate platelet activation as well as subsequent platelet-mediated modulation of immune responses during sepsis.

Platelet TLR4 mediates microvascular thrombosis and thrombocytopenia in response to LPS, thereby fostering tissue injury caused by vessel occlusion. Furthermore, in *Escherichia coli* infection platelet TLR4 contributes to bacterial trapping by supporting NET formation (38, 50, 110).

Additionally, platelet activation during sepsis may be induced by activation of secondary hemostasis with generation of thrombin or release of vWF from activated endothelial cells, respectively. Inhibition of PARs does not alter mortality, inflammation, or thrombocytopenia in endotoxemic mice, suggesting that thrombin formation is not the main cause of thrombocytopenia in this model (160). Contrarily, in *Streptococcus pneumoniae*-induced pneumosepsis PAR-4 limits bacterial growth and lung damage (161).

Direct cellular interactions of activated platelets with leukocytes or endothelial cells via surface expressed CD62P or CD40L contribute to the inflammatory host response in sepsis, fostering both bacterial clearance and organ damage (162–164). Elevated CD62P promotes formation of platelet-neutrophil aggregates in the circulation of septic mice, assisting pulmonary neutrophil infiltration independent of local chemokines, and thereby limiting bacterial dissemination but also contributing to lung damage in pneumosepsis or CLP (162, 163, 165). Reduced surface CD40L curtails direct platelet-leukocyte interaction and dampens neutrophil infiltration and tissue damage in bacterial sepsis (164).

Following LPS challenge, thrombocytopenia, thrombosis and mortality are decreased in IL-4R/Ibα mice that lack the extracellular part of GPIbα (166), showing a detrimental role of GPIbα in endotoxemia. The role of GPIbα involves its interaction with vWF as disruption of this axis confers the same protective effect (166). While platelets commonly have pro-inflammatory effects on leukocytes, GPIb seems to confer anti-inflammatory leukocyte modulation as it supports platelet-leukocyte interaction, but dampens the inflammatory cyto-/chemokine response (167).

Furthermore, blockade of GPIIb/IIIa using integrilin also reduces mortality but did not alter thrombocytopenia in LPS-challenged mice (166), suggesting that thrombocytopenia is not the main cause of mortality in this model.

Recent studies have also identified glycoprotein GPVI and CLEC-2 as novel modulators of inflammatory responses in gram-negative sepsis or CLP. GPVI contributes to local immunity

in pneumosepsis by enhancing platelet-neutrophil-aggregate formation and bacterial clearance (157). In contrast, CLEC-2 confers immune-inhibitory effects by dampening levels of pro-inflammatory cyto- and chemokines as well as limiting immune cell recruitment, inflammatory bleeding, and bacterial dissemination, thus ameliorating organ damage in endotoxemia and CLP (168). However, CLEC-2 also mediates inflammation-driven thrombosis in sepsis (169). Interestingly, while GPVI is not required for immune responses in endotoxemia, concomitant deletion of GPVI and CLEC-2 reverses the exaggerated inflammation and disease severity caused by lack of CLEC-2 alone. Thus, despite similar downstream signaling molecules, GPVI and CLEC-2 seem to play opposite roles during sepsis, most probably by regulating both the inflammatory response and thrombosis (168).

Platelet-Derived Soluble Mediators in Sepsis

In addition to direct cellular interactions, activated platelets secrete a plethora of soluble mediators from their granules that potentially modulate host responses to sepsis. Nbeal2-deficient mice, which lack α-granules, challenged with *Klebsiella pneumoniae*-induced pneumosepsis exhibit similar circulating platelet-leukocyte aggregates as wildtypes, but increased pulmonary leukocyte influx and elevated multi-organ damage. However, limiting Nbeal2-deficiency to the platelet compartment does not reproduce these results, suggesting that platelet granule content does not regulate host responses during *Klebsiella pneumoniae*-induced pneumosepsis (170).

Nonetheless, multiple studies focusing on specific mediators and using different sepsis models such as LPS-induced endotoxemia or CLP reported significant contributions of platelet granules proteins.

Platelet activation during sepsis triggers the release of RANTES (CCL5) and platelet factor 4 (PF4, CXCL4) and subsequent heteromer formation in the circulation. PF4 and RANTES in turn stimulate alveolar macrophages to produce the chemokines macrophage inhibitory protein-2 (MIP-2) and KC (CXCL1; homologous to human IL-8/CXCL8), thus promoting neutrophil recruitment but also edema formation (171–173). Moreover, PF4 accelerates generation of activated protein C, counteracting the increasing pro-coagulant state during sepsis which may promote DIC. Accordingly, PF4 has been found to increase survival in endotoxic shock (174).

In addition to cytokines and chemokines, serotonin released from dense granules upon platelet activation may play a role in sepsis. Using an FcγRIIA-humanized mouse model, it has recently been discovered that immune complexes cause platelets to transiently sequester to the lung, where they release serotonin before returning to the circulation (119). As serotonin activates endothelial cells, platelet-derived serotonin may subsequently promote the adhesion and extravasation of neutrophils (175). Additionally, platelet-derived HMGB1 has also recently been implicated in augmenting leukocyte recruitment and bacterial clearance in murine CLP (176).

The contribution of platelet release products to organ damage during sepsis may not be limited to support of neutrophil influx. During sepsis platelets carry intracellular granzyme B, probably due to transcriptional alterations in megakaryocytes, which causes local apoptosis at sites of platelet accumulation such as the lungs, spleen, and kidneys, contributing to multiple organ dysfunction and sepsis progression (177, 178).

These findings strongly underline the importance of platelet activation for essential host responses during bacterial sepsis such as immune cell recruitment, bacterial clearance, and organ dysfunction. Indeed, mice lacking PAR-4 show reduced levels of circulating PF4 48 h after infection with *Streptococcus pneumoniae*. In line with the protective effect of PF4 in endotoxic shock described above, these mice also suffer from increased bacteremia and bacterial burden in the lungs, as well as exacerbated pulmonary damage (161).

Pharmacological Platelet Inhibition During Sepsis

Given the availability of anti-platelet drugs, pharmacologic targeting of platelet function represents an attractive approach to mitigate platelet-assisted excessive inflammation that contributes to sepsis progression.

Interestingly, inhibition of cyclooxygenase-1 (COX-1) ameliorates thrombocytopenia and kidney dysfunction in endotoxemia (179), yet transfusion of COX-1-deficient platelets into platelet-depleted mice leads to worse survival than transfusion of wildtype platelets (140). The impact of $P2Y_{12}$ on murine sepsis remains subject to discussion and depends on the specific model used. Mice deficient in $P2Y_{12}$ show a protective role for $P2Y_{12}$ in endotoxemia by ameliorating inflammation and lung injury, although the results are not mirrored by the use of a $P2Y_{12}$ inhibitor (180). In contrast, blocking ADP feedback by $P2Y_{12}$ receptor antagonists, clopidogrel, prasugrel, or ticagrelor, appears to be beneficial during sepsis, as it inhibits platelet activation and binding to circulating neutrophils and monocytes during pneumonia and CLP (181–183). This is accompanied by diminished TNF-α and IL-1β levels, as well as impeded neutrophil infiltration and platelet sequestration, ultimately reducing lung and kidney injury, whereas bacterial clearance does not seem to be affected by $P2Y_{12}$ blockage (144, 181–184). Furthermore, clopidogrel failed to ameliorate thrombocytopenia in gram-negative pneumosepsis (185), whereas in a modified CS model, clopidogrel also improved sepsis-induced thrombocytopenia (186).

Thus, $P2Y_{12}$ receptor antagonists show promising results in pre-clinical studies to ameliorate sepsis pathogenesis, while the potential of COX-1 inhibition remains unclear. Further, the effects of anti-platelet medication on established sepsis as well as the impact of $P2Y_{12}$ blockers on bleeding risk have not been addressed in detail thus far. Therefore, large clinical trials are required to confirm if results from animal studies will be translatable to the human patient setting.

Translational Limitations of Rodent Sepsis Models

Mouse and rat models have proven to be valuable tools to investigate cellular and molecular processes in sepsis. However, animal models have inherent limitations independent of the specific sepsis model and species that need to be taken into consideration when evaluating and interpreting results.

Age and Sex

Despite efforts to optimize, murine models do not appropriately represent the archetypal septic patient which is an elderly person with one or more co-morbidities. In contrast, mice are typically used at the age of 6–12 weeks with most studies focusing only on males. A literature screen for studies on platelets in sepsis revealed an average age of 9 weeks with 60% of studies being performed on males, 7% on females, 12% on both sexes, and no specified information was available in 21% of studies. Similarly, studies using rats as model organism also mostly use adolescent males. Therefore, most *in vivo* studies are more representative of healthy young men regarding age and sex rather than heterogeneous patient populations. Time and financial constraints are contributing to this bias as aging animals are a costly investment. Further, reproducibility is higher in young cohorts that vary less in weight and exposure to environmental stressors.

Species

While a variety of species are currently used as animal models in sepsis research, rodent models are the preferred approach for studying platelets. LPS-induced endotoxemia and CLP are widely used in both rats and mice. Rats are favorable models for *in vitro* analyses of platelets as their larger body weight and thus blood volume allows various concomitant measurements. Indeed, rat models have unveiled a number of crucial intracellular responses of platelets to sepsis, including activation of the NLRP3 inflammasome (187), upstream regulation of NADPH subunit $p47^{phox}$-dependent ROS production (188) and the contribution of protein kinase C for platelet-mediated leukocyte infiltration and organ injury (189). Furthermore, rats are more commonly used when investigating effects of therapeutic intervention strategies on clinical parameters e.g., hemodynamics. However, genetic tools such as transgenic or knockout strains are rare in rats, but readily available for mice. Thus, despite limited sample material, murine models offer a wider range of experimental approaches which are invaluable for investigating underlying cell-specific molecular mechanisms. Of note, inflammatory responses appear to be stronger and peak earlier in mice than in rats (190). Therefore, interspecies differences have to be considered when interpreting findings of different animal models as well as their translation into the human setting.

Genetic Background

Another point to consider is the genetic background of mice as individual inbred strains vary in their immune competence due to polymorphisms and/or mutations, e.g., in TLRs or complement factors. While the most widely used strains C57BL/6 and BALB/c express functional TLR4 and are therefore sensitive to LPS, point mutations in the tlr4 gene have rendered some strains (e.g., C3H/HeJ, C57BL10/ScSr) resistant to LPS (191), making them useful tools to study endotoxin-independent host responses (154). However, C57BL/6 are more susceptible to fungal sepsis induced by zymosan or *Candida albicans* infection than outbred CF-1 mice, showing weaker Th1 response and poor survival (192). Further, mouse strains carrying loss-of-function mutations of complement factor C5 such as DBA/1 or DBA/2 display altered susceptibility to certain bacterial strains.

Genetic background not only impacts on innate but also on adaptive immunity. C57BL/6 mice tend to respond to pathogens with an enhanced Th1-type response, leading to increased phagocytic clearance of intracellular pathogens. In contrast, BALB/c mice are skewed toward Th2-type responses that support humoral immunity especially against extracellular parasites (191).

Timeline

With the exception of CLP, rodent sepsis models rarely mirror the timeline of sepsis pathogenesis in human patients. Injection of PAMPs or live bacteria does not represent the slow outgrowth and dissemination of bacteria from a center of infection, but rather a sudden, overwhelming infection that typically leads to death of the mice in a matter of hours or days. However, adjustment of the infectious dose may yield a transient, non-lethal infection that may be resolved within days. During this timeframe, immediate host responses involving platelets, coagulation and the innate immune system can be studied, as thrombocytopenia may occur within minutes after infection and induction of acute phase cytokines and neutrophil infiltration toward sites of acute inflammation can be observed within hours.

Additionally, experimental setups seldom reproduce the reality of sepsis in patients, where interventions have to be efficient in counteracting established sepsis (137). Contrarily, genetic modifications and drugs commonly take effect prior to induction of experimental sepsis in mice. Therefore, observations are only partly translatable to the patient situation.

Physiological Differences Between Mice and Humans

Most prominently, humans and mice differ considerably in their circulating immune cell composition. In humans, neutrophils constitute the most abundant leukocyte subpopulation (40–70%), whereas mice show up to 84% lymphocytes (193), which may impact on the relative contributions of innate and adaptive immunity to host responses. As already mentioned, distinct receptor expression regarding TLRs, PARs and Fc receptors determines immune cell capacity of platelets. This makes it often difficult to translate results from animal models to the clinical situation. To overcome this problem, mice expressing human receptors have been generated. While this was successful in some cases (FcγRIIA) (194), other attempts failed so far to lead to functional receptor expression (PAR-1) (195, 196). However, the role of FcγRIIA was never addressed in a murine sepsis model. Furthermore, while LPS challenge yields similar inflammatory responses in mice and men, including cytokine production and lymphopenia, humans are more sensitive to LPS than mice, which necessitate the use of LPS concentrations in mouse models that surpass those required to induce septic shock in humans about 1,000–10,000-fold (197, 198).

Nevertheless, despite their pitfalls mouse models have been immensely helpful to further our understanding of the role of platelets in sepsis and have shed light on cause and effect of, e.g., thrombocytopenia.

CONCLUSION

Although, it has been decades of research in sepsis, the gained knowledge did not lead to the discovery of an effective treatment approved in patients. Sepsis is a complex disease with multiple players resulting in a very heterogeneous patient population with different comorbidities, immune statuses, and susceptibilities to infection. Many strategies are currently under investigation to restore platelet count in sepsis patients. However, it is still not known whether thrombocytopenia is a cause or a consequence of sepsis severity and how platelets contribute to sepsis progression. Moreover, as platelet receptors regulate inflammatory hemostasis and infection in a stimulus- and organ-dependent manner, a better understanding of the receptors and the mechanisms involved is crucial for successful treatment. Another factor to take into consideration is the immune status of patients as different treatments might be required based on the immune profile of the patients. While patients with SIRS would benefit from an anti-inflammatory therapy, immune-suppressed patients might benefit from an immuno-adjuvant therapy. Anti-platelet therapies, in particular aspirin, seem promising in experimental sepsis, however the risk of bleeding has to be closely monitored. Currently two clinical trials address the role of aspirin in patients and the outcome of these studies are expected to further clarify the beneficial use of aspirin in septic patients.

The use of mouse models shed light on new mechanisms in sepsis, however many factors limit the translation to the human setting. One major concern when targeting platelets is their dual role in inflammation and hemostasis. Platelets are not only pro-inflammatory cells but they also contribute to the resolution of inflammation and tissue repair. Most of the studies performed in mice use wild-type mice that lack FcγRIIA on platelets, one of the major receptors on platelets regulating pathogen-mediated activation, raising the question if FcγRIIA transgenic mice are required to investigate infection-mediated sepsis in mice. Moreover, comorbidities, age, and other factors might need to be taken into consideration in experimental models to reflect the clinical profile of the patients. In this context, animal models associated with other comorbidities may provide a better understanding of sepsis pathophysiology. A deeper knowledge of the role of platelet receptors in sepsis along with randomized clinical trials will determine the beneficial potential of different anti-platelet therapies in patients.

AUTHOR CONTRIBUTIONS

All authors listed have made a substantial, direct and intellectual contribution to the work, and approved it for publication.

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REVIEW
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Platelets and Immune Responses During Thromboinflammation

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Besides mediating hemostatic functions, platelets are increasingly recognized as important players of inflammation. Data from experiments in mice and men revealed various intersection points between thrombosis, hemostasis, and inflammation, which are addressed and discussed in this review in detail. One such example is the intrinsic coagulation cascade that is initiated after platelet activation thereby further propagating and re-enforcing wound healing or thrombus formation but also contributing to the pathophysiology of severe diseases. FXII of the intrinsic pathway connects platelet activation with the coagulation cascade during immune reactions. It can activate the contact system thereby either creating an inflammatory state or accelerating inflammation. Recent insights into platelet biology could show that platelets are equipped with complement receptors. Platelets are important for tissue remodeling after injury has been inflicted to the endothelial barrier and to the subendothelial tissue. Thus, platelets are increasingly recognized as more than just cells relevant for bleeding arrest. Future insights into platelet biology are to be expected. This research will potentially offer novel opportunities for therapeutic intervention in diseases featuring platelet abundance.

Keywords: platelets, innate immunity, complement, inflammation, stroke, infection, tissue remodeling, EAE

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INTRODUCTION

In recent years, an increasing body of evidence demonstrates that platelets have several functions beyond hemostasis (1, 2). For instance, abundant evidence highlights the role of platelets for atherosclerosis, inflammation, and tissue regeneration. For example, platelets contribute to vascular inflammation of the brain during stroke or experimental autoimmune encephalitis (EAE) (2, 3). As a consequence, the question was raised whether platelets may even be considered as immune cells, despite the fact that they are not equipped with a nucleus (4). Most of the data available today originate from studies performed in small animal disease models and, thus, require validation in the course of the aforementioned diseases in patients. This is especially true in the context of immunology, since there are distinct differences between species (5). In this review, we seek to highlight the role of platelets for immune responses during thromboinflammation. In particular, we will address the relevance of platelet-associated mechanisms directly affecting the course of diseases in patients, as well as translational approaches.

PLATELETS ORIGINATE FROM MEGAKARYOPOIESIS LOCATED IN THE BONE MARROW

Like other hematopoietic cells, platelets are produced in the bone marrow (4). They are characterized by a small cell size and are missing a nucleus (4). Mean platelet volume in healthy individuals usually is in a range of 7 to 13 femtoliter (6). In the bone marrow, platelets derive from megakaryocytes. So far, one of the most important, but also best investigated factors known to be involved in megakaryopoiesis and platelet development is the cytokine thrombopoietin (TPO) (7). After binding of thrombopoietin to its counterreceptor Myeloproliferative Leukemia Virus Oncogene (c-MPL) on the megakaryocyte surface, intracellular signaling through Janus Kinase 2 (JAK2) is triggered (8, 9). The process of megakaryopoiesis is still incompletely understood, so far. For example, when thrombopoietin or the respective counterreceptor c-MPL is missing, the total amount of platelets is reduced to 10% of the normal platelet count (7). At the same time, platelet function as well as platelet morphology are not altered under these conditions (7) indicating that redundant factors besides thrombopoietin are involved in the process of terminal megakaryocyte maturation (10). Of note in myeloproliferative neoplasias (MPN), megakaryocytes are part of the malignant clone and source of inflammatory cytokines (11, 12), leading to chronic inflammation, constitutional symptoms and induction of fibroblast proliferation and bone marrow fibrosis (13) which are characteristic for primary and secondary myelofibrosis. In addition, this chronic inflammatory state might drive clonal evolution, cardiovascular disease and thrombohemorrhagic complications in these patients (14). The JAK1/2 inhibitor ruxolitinib attenuates inflammatory cytokines in myelofibrosis and clinical responses correlate with cytokine attenuation (15). Furthermore, there are hints pointing toward a role of megakaryocytes in antigen presentation through MHC-I leading to CD8 T-cell activation (16). Additionally, transfer of antigen loaded MHC-I from megakaryocytes to proplatelets has also been described (16). Vice versa, the inflammatory cytokine IL-6 was shown to be linked to increased plasma levels of thrombopoietin and an ultimately increased platelet number in a murine and human setting (17) and TPO was shown to augment platelet P-selectin (CD62P) expression stimulating platelet-leukocyte associations (18).

PLATELET RECEPTORS AND INTERACTIONS

Despite being small particles, platelets are equipped with a multitude of receptors to interact with themselves, with other cells, e.g., endothelial cells and cells of the immune system and, of course, with the extracellular matrix (**Figure 1**). In general, four types of receptors can be found on platelets: integrins, glycoproteins, selectins and receptors of the immunoglobulin type (**Figure 2**). First, after injury to the vessel wall has occurred, GPIbα on the platelet surface binds von Willebrand factor (VWF). This is especially important under conditions of high shear stress such as in the arterial branch of the vascular system (19). In addition, platelets bind to exposed subendothelial collagen fibers through glycoprotein VI (GPVI) (20–22), finally leading to a high affinity state of GPIIb/IIIa (23) (**Figure 2**). Subsequently, fibrinogen can be bound to GPIIb/IIIa on the platelet surface thereby crosslinking platelets with platelets and platelets with endothelial cells (24). Inhibiting GPIIb/IIIa was shown to be beneficial in myocardial infarction (25), however in stroke, clinical studies revealed an increased risk of bleeding (26). Ongoing thrombus formation is further supported by interaction of integrins on the platelet surface with fibrinogen and components of the extracellular matrix such as collagen and laminin. Interaction with fibrinogen is mediated through integrin α5β1 on the platelet surface, binding to laminin is mediated through integrin α6β1 (27). In addition, binding to collagen also involves integrin α2β1, which brings about platelet filopodia and lamellipodia formation (28). After platelet activation has happened, mediators from platelet granules are released further fueling platelet activation (29). Among these are P-selectin, VWF and fibrinogen from α-granules and ADP, calcium and serotonin from dense granules (30–32). Selectins were shown to be involved in platelet-endothelial and platelet-leukocyte interactions as well especially under conditions when the endothelial barrier is not disrupted (**Figure 3**). For instance, in ischemia-reperfusion injury platelets were shown to be involved in leukocyte recruitment since both adherent leukocytes as well as emigrated leukocytes were significantly reduced when either platelets were depleted through administration of platelet depleting serum or platelet receptors were blocked through administration of blocking antibodies (33). Besides platelets, P-selectin as well as VWF are stored within Weibel-Palade bodies in endothelial cells, too (34, 35). P-selectin on platelets was shown to interact with PSGL-1 expressed on leukocytes (36). The interaction of P-selectin on platelets with PSGL-1 was also demonstrated for platelet-neutrophil interactions (37, 38). In addition, platelets can interact with endothelial cells through binding of GPIbα on platelets to P-selectin expressed on endothelial cells (39). Under conditions of inflammation such as in atherosclerotic lesions, platelet-mediated recruitment of leukocytes through platelet P-selectin has been shown to be followed by platelet binding with CXCR3 to inflamed endothelium expressing CX3CL1 (40).

Another mechanism especially relevant for the recruitment of dendritic cells to the vascular wall was shown to be mediated by JAM-C (**Figure 2**), a member of the immunoglobulin family of receptors expressed on platelets, and MAC-1 on dendritic cells (41). Interaction of MAC-1 with platelets bound to endothelial cells has been shown for endothelial transmigration of neutrophils, too (42). Both the interaction of platelets with neutrophils as well as the interaction of platelets with DCs initially requires binding of platelet P-selectin to PSGL-1 on leukocytes (41, 42). Interestingly, MAC-1 was shown to be a binding partner of platelet GPIbα, too (43). Fascinatingly, MAC-1 deficiency goes along with delayed thrombosis although hemostasis seems not to be affected (43). This raises the question whether targeting MAC-1 could offer a way to efficiently inhibit thrombosis without hampering with hemostasis.

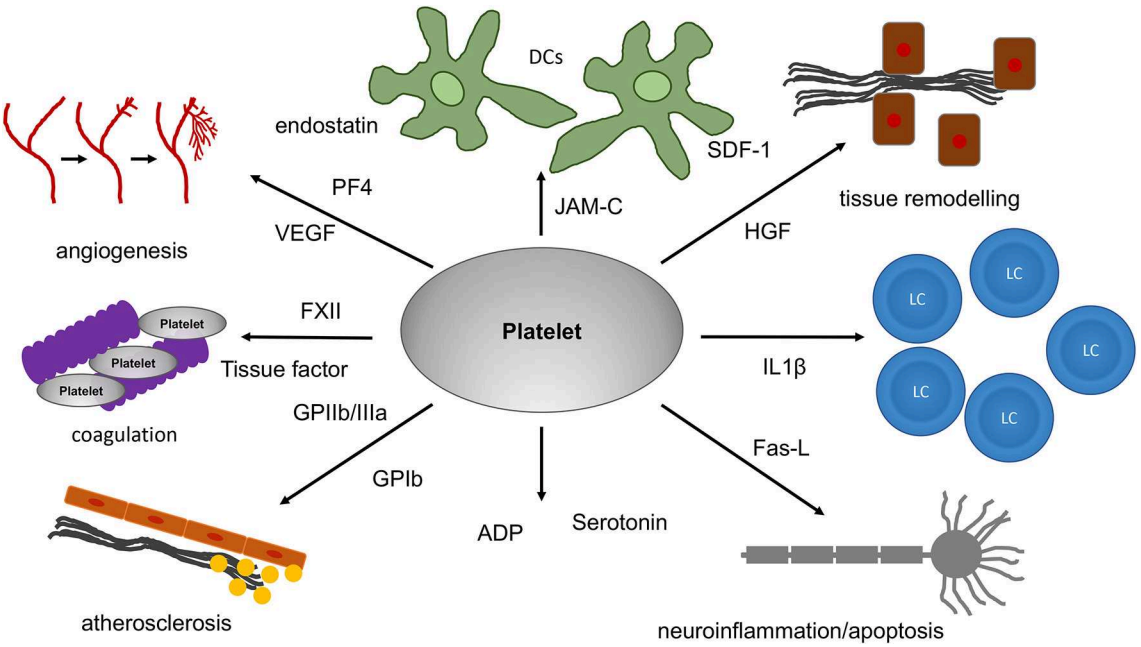

FIGURE 1 | The role of platelets for tissue remodeling, apoptosis and angiogenesis. Platelets can have an influence on tissue remodeling under different pathological and physiological conditions. For tissue remodeling as well as apoptosis and angiogenesis, platelets are equipped with a multitude of receptors. HGF and SDF-1, for example, are of relevance in tissue fibrosis. In addition, platelets can release a multitude of proteins. Among these are proteins with strong pro- or antiangiogenic effects. PF4 and endostatin are known to inhibit angiogenesis. In contrast, VEGF, which platelets can release as well, strongly increases angiogenesis. Since platelets are transported within the blood-stream they can reach almost all organs and tissues thereby even influencing processes associated with inflammation in the brain and systemic diseases like atherosclerosis. In addition, platelet mediated apoptosis through FasL on the platelet surface has been reported in the brain. VEGF, vascular endothelial growth factor; PF4, platelet factor 4; SDF-1, stromal derived factor; HGF, hepatocyte growth factor; JAM-C, junctional adhesion molecule C; IL1β, Interleukin 1β; FasL, Fas-ligand; ADP, adenosine diphosphate; FXII, coagulation factor XII.

Finally, platelets were shown to be involved in the recruitment of $CD34^+$ bone marrow cells and bone marrow progenitor cells to sites of vascular injury (44), linking platelets to tissue remodeling and neointima formation. In this context, platelet P-selectin as well as GPIIb/IIIa were shown to be involved (44). Blockade of platelet receptors with monoclonal antibodies abrogated recruitment of $CD34^+$ cells to sites of vascular injury, further underlining the relevance of platelets for recruitment of bone marrow cells to the vascular wall (44).

PLATELETS ARE CLOSELY LINKED TO THE PLASMATIC COAGULATION SYSTEM, THEREBY LINKING HEMOSTASIS AND THROMBOSIS TO INFLAMMATION

Platelets mediate thrombosis and hemostasis through the different receptors expressed on the platelet surface but also through soluble mediators released immediately after platelet activation. With respect to hemostasis, platelets operate in parallel to the plasmatic coagulation cascade. In a recent review, the ongoing debate on whether platelets can release tissue factor or not is delineated in a concise fashion (45). Some studies show that platelets can release tissue factor by themselves after activation (46), for instance on the surface of platelet microparticles (47). Tissue factor initiates the extrinsic coagulation cascade (48, 49). On top of that, procoagulant activity was significantly reduced after either tissue factor or F VII were missing in thrombin-activated platelets adhering to fibrinogen (46). Accordingly, in the presence of anti-tissue factor antibody an increased time was observed for clot formation (46). In contrast, other studies were not able to show a role of platelets for tissue-factor mediated coagulation. One study could only show enhanced tissue factor expression when monocytes were present besides platelets, too (48). In another study, no relevant tissue factor secretion could be detected after prolonged stimulation of platelets with lipopolysaccharides (LPS) (50). In the same study, stimulation of a monocyte cell line with LPS yielded large amounts of tissue factor leading to clot formation through activation of the extrinsic coagulation cascade (50), indicating that monocytes in contrast to platelets are responsible for tissue factor production.

There is also evidence that platelets interact with the intrinsic pathway of the coagulation system (**Figure 2**). Platelets contain polyphosphates that can be externalized onto the platelet membrane, thereby creating a large surface with negative charge triggering activation of coagulation factor XII (contact system of the coagulation cascade) (51). After activation, FXII activates FXI thereby further accelerating coagulation. Additional investigations regarding the role of FXII for coagulation revealed that FXII is necessary for stable thrombi in different models of arterial injury in mice

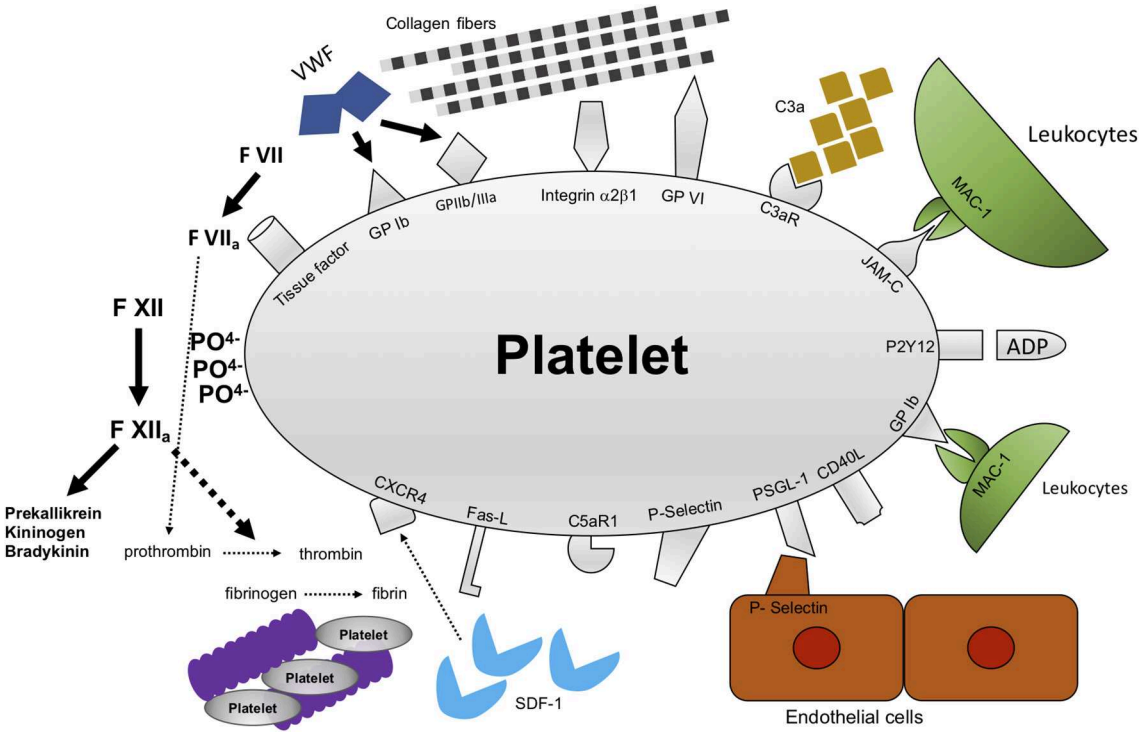

FIGURE 2 | Mechanisms of interactions between platelets and their microenvironment. Platelets were first recognized to be important for thrombosis and hemostasis after vessel injury has happened. In addition, there is accumulating evidence that platelet function goes beyond thrombosis and hemostasis. Platelets interact with a multitude of cells and proteins. Among these are receptors and proteins modulating thrombosis/hemostasis, e.g., GPIbα, GPIIb/IIIa, GPVI, and polyphosphates. In addition, platelets modulate inflammation, e.g., through C3aR, JAM-C, PSGL-1, P-selectin, CXCR4. GPIbα, glycoprotein Ibα; GPIIb/IIIa, glycoprotein IIb/IIIa; GPVI, glycoprotein VI; C3AR, receptor for complement factor 3; JAM-C, junctional adhesion molecule C; CXCR4, C-X-C-chemokine receptor type 4.

(52). Coagulation Factor XII, besides mediating coagulation, seems to have a role for driving inflammation, as well. After activation, FXII drives activation of the prekallikrein-kininogen-bradykinin-cascade whereby inflammation is triggered (53). Strikingly, in humans FXII deficiency is not associated with an increased bleeding risk. In contrast, in a study population of 74 patients suffering from FXII deficiency, two subjects had already suffered venous thromboembolism at an age <40 years (54). In an experimental design where FXII knockout mice as well as wildtype mice were subjected to transient middle cerebral artery occlusion (tMCAO), FXII knockout mice showed similar reduction in cerebral blood flow in MRI measurements 2 h after tMCAO (55). However, 24 h after tMCAO cerebral blood flow was markedly improved in FXII knockout mice compared to their wildtype counterparts (55). In addition, in tMCAO intravascular fibrin deposits leading to vessel occlusion were reduced in FXII knockout mice. In wildtype animals however, occluding thrombi contain both platelets and fibrin suggesting synergism between FXII mediated fibrin formation and platelets (56). Indeed, knockout mice for FXII showed impaired platelet rich occlusive thrombi in distinct arterial beds (57). This finding was also reported to be of particular relevance in the context of neurovascular inflammation since mice lacking FXII showed protection from ischemic brain damage (55).

PLATELETS, DESPITE LACKING A NUCLEUS, EXHIBIT TRANSLATIONAL ACTIVITY AND CAN RELEASE A MULTITUDE OF ACTIVE FACTORS

Early experiments in the 1960 by Warshaw et al. produced first evidence on protein synthesis by platelets since they could inhibit translational activity in platelets with puromycin (58). However, it took several decades and the power of PCR technology and proteomics to demonstrate that platelets are capable of protein synthesis since they incorporate RNA as well as a transcriptional and translational machinery (59). Interestingly, platelets can splice intronic interleukin-1β pre-mRNA generating fully mature interleukin-1β mRNA and interleukin-1β protein (60). Interleukin 1 comprises a group of 11 cytokines with both pro- and anti-inflammatory effects and is a good example demonstrating the role of platelets at an intersection between inflammation and thrombosis (61) (**Table 1**). The release of a multitude of factors from platelets makes them powerful tools for interaction with various cells and tissues (**Figure 1**, **Table 1**). Platelet proteomics could reveal that more than 300 proteins are secreted from human platelets after they become activated by thrombin (62). Among these were proteins responsible for coagulation including the factors FV or FXIII (62), but

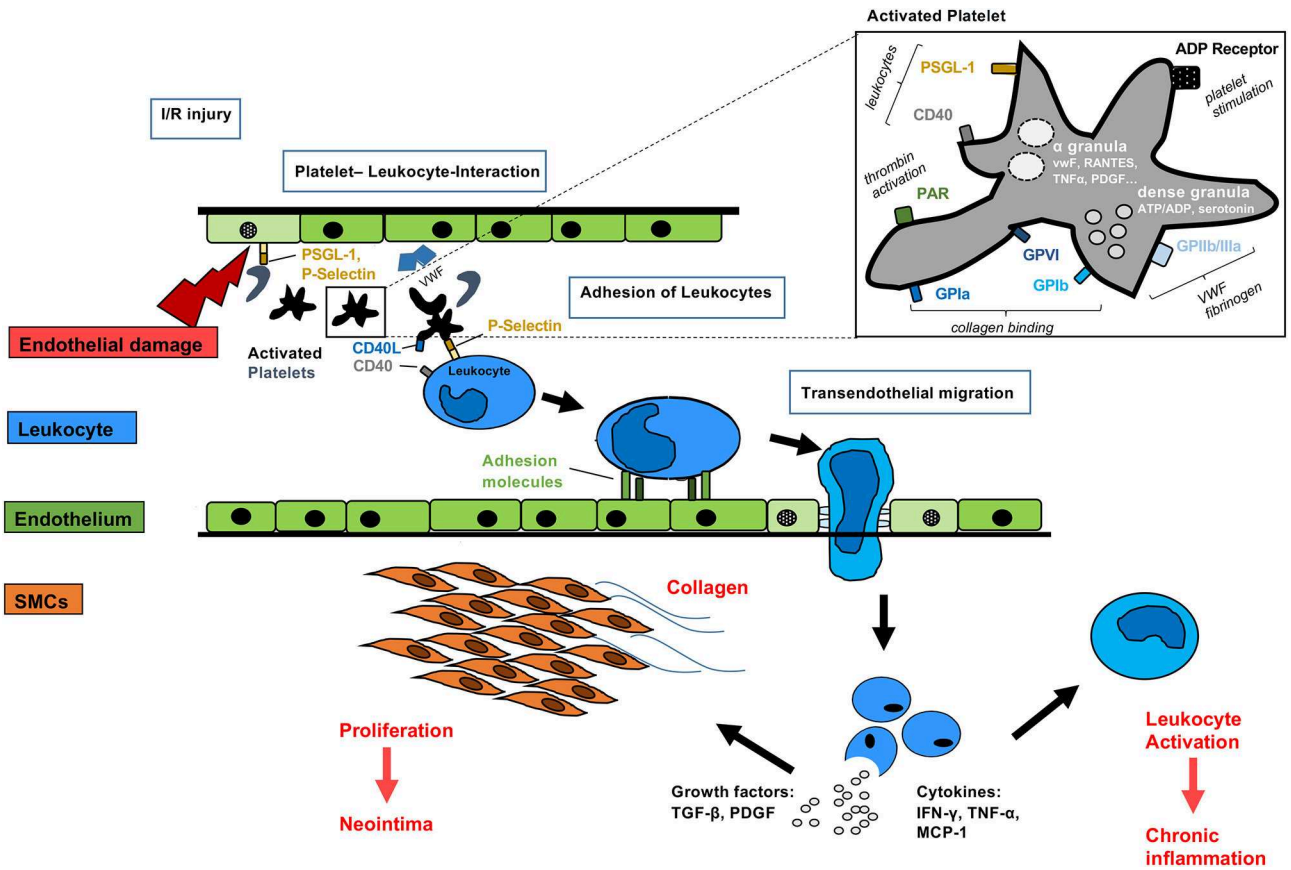

FIGURE 3 | Mechanisms of activated platelets after organ transplantation Platelets may be activated before, during and after allogeneic organ transplantation. Direct interaction with the immune system may occur via simultaneous P-selectin and CD40 binding to leukocytes. Non-nucleated platelets contain dense granules with serotonin, ADP and α-granules with pro-inflammatory cytokines, such as RANTES, TNFα, PDGF, and VWF. Surface receptors including glycoprotein receptors lead to collagen binding, VWF and fibrogen activation. Activated leukocytes adhere to the endothelium of transplanted grafts, migrate and release growth factors and pro-inflammatory cytokines finally leading to smooth muscle cell proliferation, collagen deposition and further leukocyte activation.

also proteins such as albumin, platelet factor 4 (PF4, CXCL4) or matrix metalloproteinase inhibitor 1 (62). Finally, factors responsible for tissue remodeling such as CXCL12 (SDF-1) and growth factors, for example hepatocyte growth factor (HGF), can be released by platelets (63, 66). In addition, a specialized way of how translational activity in platelets is modulated through altered ribosome function has been uncovered. It was observed that the platelet lifespan was linked to mRNA and ribosomal RNA content, both of which were reduced with increasing platelet age (78). A recent study could show that loss of the RNA surveillance factor Pelota is a decisive mechanism by which platelets stop decay of their limited pool of mRNA (79). This finding seems to be of relevance in a clinical context, too, since an association of platelet protein synthesis and cardiovascular disease could be demonstrated. Platelet WDR-1 (WD-40 repeat domain 1) mRNA and protein level was found to be significantly lower in patients suffering from cardiovascular disease compared to matched controls (80). WDR-1 is known to enhance actin depolymerizing factor activity (81), thereby altering the platelet cytoskeleton (82). In addition, WDR-1 mutations in mice were linked to defects in neutrophil cytoskeleton and defective platelet production and, finally, brought about macrothrombocytopenia and autoinflammation (83). Whether the autoinflammation observed in this model is attributable only to defective neutrophil function or if platelets are involved as well, e.g., through altered platelet-neutrophil interactions, still remains to be investigated in the future.

PLATELETS INTERACT WITH CELLS OF THE INNATE IMMUNE SYSTEM AND ENDOTHELIAL CELLS MAKING THEM IMPORTANT PLAYERS INVOLVED IN SEPSIS AND INFLAMMATION-INDUCED BLEEDING

When inflammation takes place, endothelial cells lining the inner surface of blood vessels become activated and change their receptor expression profile and their phenotype. Platelets as well as VWF were shown to be closely linked to inflammation in

TABLE 1 | Receptors and factors associated with platelets relevant for thromboinflammation.

Receptor/Factor	Relevant for	References
IL1β	Inflammation	(60, 61)
PF4	Tissue remodeling/angiogenesis	(62)
CXCL12(SDF)	Tissue remodeling	(63–65)
HGF	Tissue remodeling	(66, 67)
tissue factor	Coagulation	(46, 47)
polyphosphates/FXII	Coagulation/thromboinflammation	(51)
GPIbα/VWF	Coagulation/atherosclerosis/ neuroinflammation/ thromboinflammation	(19, 68, 69)
GPIIb/IIIA/fibrinogen	Coagulation/atherosclerosis/ neuroinflammation	(19, 68, 69)
GPVI/collagen	Coagulation	(20, 21)
ADP	Coagulation	(70)
thromboxane A2	Coagulation	(70)
serotonin	Coagulation/inflammation	(71, 72)
P-selectin	Inflammation/atherosclerosis/ thromboinflammation	(36, 44)
PSGL-1	Inflammation	(36)
JAM-C	Inflammation/apoptosis	(41)
C3aR	Inflammation/atherosclerosis/thrombosis	(73, 74)
C5aR	Atherosclerosis	(75)
VEGF	Tissue remodeling/angiogenesis	(76)
endostatin	Tissue remodeling/angiogenesis	(76)
FAS-L	Tissue remodeling/apoptosis	(77)

a neurovascular setting (84). For instance, antibody-mediated blockade of platelet GPIbα, the receptor for VWF, after induction of tMCAO was associated with improved cerebral blood flow in magnetic resonance imaging (85) leading to improved outcome (86). In addition, deficiency in VWF was paralleled with a protective phenotype in tMCAO, as well (87). In contrast, when VWF was reconstituted in VWR knockout mice, protection from cerebral ischemia was diminished (87). A common finding in severe generalized infections and sepsis is that platelet count is markedly reduced (88). In addition, a prolonged time span of thrombocytopenia is correlated with increased mortality in intensive care patients (88). Clinical data, although derived from retrospective studies, indicate that treatment with antiplatelet drugs is beneficial under conditions of severe inflammation/sepsis raising the question of how platelets might be involved in sepsis (89, 90).

Interestingly, in thrombocytopenia inflammatory mediators like LPS increased the risk of bleeding (91), underlining the importance of platelets not only for thrombosis and hemostasis but also for inflammation. This finding was demonstrated in various models of inflammation in mice. For instance, when mice were suffering from thrombocytopenia, induction of contact dermatitis resulted in increased bleeding compared to WT animals (91). Investigations using the dorsal skinfold chamber model, which enables *in vivo* observations of the vascular system, further confirmed the relevance of inflammation for bleeding in cases of thrombocytopenia (91). Mice suffering from severe thrombocytopenia as well as inflammation induced by application of LPS showed spontaneous intraalveolar hemorrhage (91). In addition, inflammatory bleedings under conditions of thrombocytopenia in the skin as well as in the lungs, were shown to be strongly dependent on neutrophil-endothelial interactions, thereby involving endothelial VE-Cadherin (92). In addition, platelet GPVI was shown to attenuate inflammation-induced bleeding mediated through neutrophils by binding to exposed subendothelial collagen (93). Recently, the maintenance of inflammatory hemostasis was shown to be organ- and stimulus-dependent, i.e., GPVI and GPIIb/IIIa were not required for prevention of intraalveolar bleeding after LPS challenge, while GPIbα attenuated inflammation-induced bleeding in the lung (94).

However, in sepsis adverse outcome is not only due to inflammation-induced bleeding. Instead, increased mortality could be observed irrespective of bleeding in cases when thrombocytopenia was present (95). Here, platelet transfusion was shown to be beneficial with respect to sepsis (95). The mechanisms behind the protective role of platelets observed, involved prostaglandin E2 biosynthesis within platelets through COX-1, and activation of prostaglandin receptors on macrophages (95). Furthermore, platelets have been demonstrated to protect from extracorporeal-circulation induced inflammatory lung injury. Here, platelet transfusion was associated with milder lung injury which also went along with decreased levels of TNFα and neutrophil elastase which were measured in the blood plasma (96). Blocking platelet GPIIb/IIIa through administration of Tirofiban reversed the observed effects (96). Finally, platelets were shown to limit neutrophil-induced endothelial damage by interaction with neutrophil elastase, as well (93).

Furthermore, the interplay of DCs and platelets was shown to be of paramount importance with respect to bacterial infections. Listeria monocytogenes is a bacterial infection threatening immunocompromised individuals as well as creating fetal infections that finally can lead to abortion (97, 98). Recently, new insights on the role of platelets for infection with Listeria monocytogenes were published. Platelets, with the aid of complement C3 and GPIbα, were shown to be directly involved in the transport of Listeria to splenic CD8α+ DCs, which then cross-present antigenic peptides via MHC-I to T-helper cells thereby creating an adaptive immune response (99). Besides Listeria, C3-mediated platelet association could be demonstrated for other gram-positive bacteria, e.g., Staphylococcus aureus (99). This further underlines the importance of platelets for any defense against bacteria and the development of adaptive immunity. In addition, blockade of GPIIb/IIIa had protective effects when sepsis was induced in mice (100). Furthermore, when GPIIb/IIIa was blocked, reduced levels of apoptosis in splenocytes in an *in vitro* approach were observed (100).

Another link of platelets to inflammation could be demonstrated *in vivo* in a mouse model of immune complex-mediated systemic shock, since interaction of platelets with immune complexes led to serotonin release from platelets (71) (**Table 1**). After platelet depletion, injection of immune

complexes did not create a systemic shock-reaction according to the clinical scores (71). Further investigations of the mechanisms underlying platelet response to immune complex-mediated shock revealed that GPIIb/IIIa is necessary in this context since dysfunctional receptor ligand interaction due to mutated fibrinogen abrogated immune complex-mediated systemic shock (71). In contrast, neither P-selectin nor GPIbα were required for immune complex-mediated shock (71).

PLATELETS INTERACT WITH THE COMPLEMENT SYSTEM

The complement system is a very old and well-conserved cascade of proteases produced in the liver, which are involved in clearance of dead cells as well as pathogens that have passed the natural barrier between the body and the environment (101, 102). The complement system is involved in various human pathologies associated with dysregulated platelet function and disseminated thrombosis in microvessels, e.g., hemolytic uremic syndrome (HUS) (103). In atypical HUS (aHUS), deposition of complement factors C3 and C9 could be verified on the platelet membrane (104). Furthermore, CD40L expression was increased on platelets, indicating platelet activation (104). Complement receptors for C3a and C5a are expressed on platelets, too (73, 75) (**Table 1**). Interestingly, in HUS renal dysfunction is caused through microthrombosis in the renal vascular system and mutations in complement C3 were shown to predispose to development of aHUS (105). Investigating the role of platelets for cardiovascular disease, we were able to show in a clinical study with patients suffering from coronary artery disease that expression of complement receptor for C5a on platelets (C5aR) could be correlated to markers of platelet activation (75). Interestingly, *in vitro* investigations of platelets with flow cytometry performed after platelets had been stimulated with oxidized low-density lipoprotein (oxLDL) revealed that expression of C5aR and P-selectin increased after platelet incubation with oxLDL (75). In the same study, an inverse correlation between platelet bound oxLDL and plasma C5a could also be observed (75). Another study performed in this field uncovered a strong correlation between expression of C3aR and GPIIb/IIIa on human platelets with known coronary artery disease (73), further highlighting the intimate connection between the complement system and platelets. When thrombi of cardiovascular patients were analyzed, coexpression of C3aR and GPIIb/IIIa was evident (73). Additional investigations *in vivo* in a mouse model deficient for either C3aR or C3 revealed that C3a affects not only bleeding time but also tissue injury after stroke, myocardial infarction and thrombosis (73). Reconstitution of mice deficient for C3aR with WT platelets, could reverse the observed protective effects of C3aR deficiency with respect to thrombosis-related ischemic injury (73). Bleeding was aggravated in the C3aR knockout mice, which could be reversed after transfusion of WT platelets (74). In addition there is increasing evidence that platelet P-selectin could be a receptor for C3b, underlining the close intersection between coagulation and inflammation (106). The crosstalk between platelet activation and the complement cascade is a good example of how closely platelets link inflammation to thrombosis and vice versa.

THE ROLE OF PLATELETS FOR TISSUE REMODELING, APOPTOSIS AND ANGIOGENESIS

The Role of Platelets for Tissue Remodeling

Besides the established function of platelets for coagulation, there is also increasing evidence for a function of platelets in tissue remodeling and angiogenesis (**Figure 1**, **Table 1**). For example, platelets are directly involved in the process of atherosclerosis, even before any thrombotic event. Massberg et al. could demonstrate in a mouse model of ApoE deficient mice suffering from severe atherosclerosis, that the development of atherosclerotic lesions was preceded by platelet adhesion to the endothelium through interaction of GP Ibα and GPIIb/IIIa with the arterial wall (68). Platelet adhesion was the first event preceding atherosclerotic plaque formation, followed by leukocyte adhesion to the vascular wall (68). There is conclusive evidence supporting a role of platelets not only for acute atherothrombosis, for instance in myocardial infarction, but also in the process of chronic vascular inflammation. For example, individuals suffering from familial hypercholesterolemia display elevated levels of platelet microparticles in the blood (107). These microparticles exhibit markers of platelet activation such as P-selectin or GPIIb/IIIa and tissue factor (107). Furthermore, MRI-imaging performed in these patients revealed increased atherosclerotic plaque burden reflecting dangerous lipid-rich cores prone to rupture, particularly in the case when tissue factor bearing microparticles were present (107). Increased levels of platelet microparticles were also reported in patients suffering from severe heart failure requiring cardiac assist device therapy (108). These patients often suffer both from bleeding disorders as well as thromboembolic complications (109) and management of coagulation is a major concern.

As already mentioned, platelets can release various factors already known from tissue remodeling processes (**Figure 1**). Among those factors released from platelets are CXCL12 and hepatocyte growth factor (HGF), respectively (44, 63, 66) (**Table 1**). HGF is highly relevant in tissue fibrosis and remodeling as investigated in an *in vivo* model, in which Syrian hamsters suffering from hereditary cardiomyopathy were treated with HGF (67). The animals showed severe cardiac dysfunction and fibrosis. After treatment with recombinant HGF for 3 weeks, cardiac fibrosis was ameliorated (67). This was accompanied by reduction of transforming growth factor β 1 (TGFβ1) and type I collagen (67). Interestingly, platelets themselves were shown to interfere with HGF, as well, since they could inhibit migration of mesenchymal stem cells to apoptotic cardiomyocytes. HMGB-1 released by platelets was directly involved in this process (110, 111). However, the inhibitory role of platelets for recruitment of stem cells has also been questioned since an inhibitory effect on recruitment of vascular endothelium has been reported, too (112).

CXCL 12 also known as SDF-1, which can be released by platelets, is involved in neointima formation after vascular injury through recruitment of vascular smooth muscle cell progenitors (64, 65). First, SDF-1 is released by media smooth muscle cells undergoing apoptosis after injury has happened (65). Subsequently, SDF-1 binds to platelets which then are attached to the vessel wall at sites of injury (65). This is followed by attachment of smooth muscle cell progenitor cells to the platelets mediated by P-selectin and CXCR4, the SDF-1 counterreceptor (65). With respect to cardiovascular disease it has been demonstrated that platelet expression levels of SDF-1 correlate with adverse outcomes (113).

Platelets were shown to be involved in experimental autoimmune encephalitis (EAE) as well, linking them to another field of neuroinflammation and tissue remodeling besides stroke (69, 114). Experimental autoimmune encephalitis is a preclinical model for the human disease multiple sclerosis (MS), during the course of which inflammation of the brain is induced through administration of central nervous tissue or myelin peptides (115). Both in the human as well as in the murine disease, platelet specific CD41 was shown to be upregulated in brain tissue (69, 116). In addition, the course of the disease was affected as a result of platelet depletion. When platelets were depleted through administration of platelet depleting serum in the effector phase of the disease, reduced microgliosis within inflamed brain tissue could be observed. Both interfering with platelet GPIbα as well as platelet GPIIb and the GPIbα counterreceptor on leukocytes, MAC-1, through administration of blocking antibodies was able to ameliorate EAE (69). Recently, the importance of the timing of platelet depletion for the course of the disease was further supported by another study (114). Platelet depletion in the immunization phase of EAE did not have an impact on the course of the disease (69, 114). Microarray analysis of the spinal cords after induction of EAE revealed several factors relevant for inflammation such as CCL2, CCL5, CXCR4, and IL1β which were downregulated significantly after platelet depletion (69). The question whether platelets themselves, are drivers of neuroinflammation in the context of experimental autoimmune encephalitis, or whether they contribute to experimental autoimmune encephalitis through recruitment of inflammatory leukocytes either by receptor-ligand interaction with leukocytes or by releasing inflammatory mediators is still open. Hopefully, future research will be able to resolve this question thereby significantly improving therapies for patients suffering from multiple sclerosis.

The Role of Platelets for Angiogenesis

Angiogenesis in general is a tightly regulated process that is modulated by a multitude of cells and soluble factors (117, 118). Angiogenesis can be beneficial, for instance with respect to wound healing and tissue regeneration (117, 118). However, angiogenesis can also be harmful since tumors need a so-called angiogenic switch to grow beyond a certain size (117, 118). Angiogenesis is tightly associated with inflammation (119). Some of the proteins released from platelets possess angiogenic potential either exerting pro- or antiangiogenic responses on endothelial cells (**Table 1**). Experimental approaches using Matrigel, an extracellular matrix from the Engelbrecht-Holm-Swarm (EHS) sarcoma, together with endothelial cells is an established way to investigate angiogenesis *in vitro* (120). In a Matrigel model, a proangiogenic effect of platelets could be demonstrated after endothelial cells and platelets had been added (121). Interestingly, platelets directly adhere to endothelial cells (121). Adding platelet supernatant to the Matrigel showed significantly reduced tube formation compared to adding platelets (121), further supporting that direct platelet-endothelial interaction is necessary for the observed proangiogenic effect *in vitro*. Regarding platelet physiology, differential release of pro- or antiangiogenic factors happens depending on the stimulus (122). There are hints that ADP as well as GPVI favor a proangiogenic phenotype of platelets (122). In addition, platelets can release VEGF after stimulation with ADP (76). In contrast, PAR-4 favors an antiangiogenic phenotype of platelets (122). This was also observed, when platelets were stimulated with thromboxane A2 since this triggered release of the antiangiogenic agent endostatin (76). Platelets have been recognized as a major source of vascular endothelial growth factor (VEGF) (123), one of the most important growth factors involved in angiogenesis (124). When VEGF is released, it can bind to a variety of growth factor receptors thereby directing proangiogenic effects (124). After VEGF has bound to endothelial cells, they start to proliferate and to form tubes, which results in the formation of new vessels with recruitment of pericytes as well as smooth muscle cells (124).

Furthermore, platelets have also been implicated in ischemia-induced revascularization after arterial occlusion, which is primarily achieved by arteriogenesis (125). Platelets have been shown to recruit bone marrow-derived cells in response to ischemia in mouse models of hindlimb ischemia or tumor implantation in mice (126). After platelet depletion through administration of an anti GPIbα antibody, levels of bone marrow cells within the tissue were significantly reduced (126). Among the different factors mediating platelet function, α-granules as well as the antiangiogenic protein thrombospondin were shown to be relevant for the observed effects (126). In addition, a role of platelet microparticles for angiogenesis has been reported, too (127).

These effects may be the reason why platelet-rich plasma (PRP) is a potent agent to foster wound healing. Using PRP in a patient collective suffering from dehiscent sternal wounds or necrotic skin ulcers has been shown to be beneficial since duration of hospital stay after administration of PRP was almost half of the time in patients with dehiscent sternal wounds compared to standard care (128). Unfortunately, detailed investigations of the underlying mechanisms were not undertaken, so far, and we are left with speculating on the molecular mechanisms involved. Nevertheless, a COCHRANE review has confirmed the clinical benefit of the administration of platelet-rich plasma in patients with diabetes and chronic wounds (129), offering promise for future platelet-based therapies in this field.

The Role of Platelets for Apoptosis

Recently, there were hints from preclinical studies in a murine model that platelets may be involved in the process of apoptosis (**Figure 1**, **Table 1**). Apoptosis in general can be induced both through external signaling as well as through internal pathways (130). The external pathway involves several factors (TNFα, FasL, TRAIL), which after binding to their respective receptor activate an intracellular signaling cascade finally leading to activation of a set of specialized enzymes the so-called caspases (130). Besides the external pathway there is an intrinsic way how apoptosis can be initiated as well. The intrinsic pathway relies on cytochrome c which activates caspases after their release from mitochondria (130). Finally the cells undergo a special program which finally leads to cellular clearance (130). Schleicher et al. could demonstrate that platelets can be found in the brain tissue after experimental stroke in the tMCAO stroke model. Apoptosis in the brain tissue was reduced after platelet depletion (77). Similar observations were made when GPIbα deficient mice were used (77). Further analysis revealed that platelets express FasL in their membrane thereby mediating apoptosis (77). In contrast, Bax/Bak signaling of the internal pathway of apoptosis was not required but additionally contributed to apoptosis (77). A previously unrecognized role of platelets for apoptosis was also identified in the context of platelet-DC interactions. JAM-C was shown to be directly involved in platelet-DC interactions mediating apoptosis of DCs (41). Platelets were directly responsible for the recruitment of DCs to the vessel wall. *In vivo*, when no vascular lesion was present in a model of carotid artery injury, no DCs were recruited to the vessel wall. In contrast, after vascular injury, the number of DCs adhering to the vessel increased markedly (41).

Finally, patients suffering from human immunodeficiency virus (HIV) were shown to have an increased rate of cardiovascular events (131), despite having achieved stable disease by means of combined antiretroviral therapy. In addition, *in vitro* investigations could show that markers of platelet activation, e.g., P-selectin were upregulated under this condition (132). Furthermore, activation of the intrinsic pathway of apoptosis was more prevalent in platelets from patients suffering from HIV infection despite being under viral control (132), suggesting dysregulated platelet function as one possible contributing factor to increased numbers of cardiovascular events. This was further underlined in a HIV positive patient collective suffering from acute coronary syndrome where, despite receiving Aspirin and P2Y12 inhibitor therapy, high residual platelet reactivity could be measured (133). However, the mechanism underlying the observed dysregulated platelet function in patients suffering from HIV is incompletely understood, yet. Altogether, the data reported strongly point to an intimate connection of platelets and inflammation and a function of platelets beyond thrombosis and hemostasis.

Platelets and Cancer

A common finding is that cancer is associated with thrombosis and embolism (Trousseau phenomenon). The increased risk of thrombosis in cancer can be attributed to a variety of mechanisms, including increased expression and release of procoagulant factors and microparticles by tumor cells and platelets, abnormal tumor vascularity and increased inflammation [reviewed in (134)]. An increasing body of evidence indicates that, in addition to increasing the risk of thrombosis, platelets can also contribute to tumor progression and metastasis by altering the tumor microenvironment, by expression of growth factors and proangiogenic factors and by assisting neoplastic cells to evade apoptosis (76, 135–137). In addition, platelets can promote metastasis by protecting tumor cells in circulation from immune surveillance and by assisting tumor cell adhesion and transmigation of the vascular endothelium (138, 139). Investigations in the field of platelets and cancer have shown that induction of thrombocytopenia by platelet-depleting antibodies increased the efficacy of paclitaxel therapy in a murine model of breast cancer, likely through increased tumor vascular permeability (140). In addition, platelet depletion caused intratumor hemorrhage in different tumor models in mice (141). Interestingly, no increase in intratumor hemorrhage could be observed when GPIbα was blocked (141). However, as expected, tail bleeding time was markedly increased after blockade of GPIbα (141). In patients suffering from ovarian cancer, the patients' platelet count had a prognostic relevance. Thrombocytosis was associated with reduced overall survival and resistance to chemotherapy (142, 143). Although these results point to a central role of platelets in cancer biology, these insights did not translate to therapeutic strategies exploiting the function of platelets in cancer progression and metastasis, so far. While the use of low-molecular-weight heparin has been demonstrated to reduce the rate of recurrent thrombosis and thus represents the current standard in patients with cancer who suffered venous thromboembolism (144), the use of antiplatelet therapies in patients with cancer remains controversial. Although retrospective analyses suggest protective effects of daily aspirin for some cancers (145), antiplatelet drugs have not entered cancer therapy.

Platelets Participate in Vascular Remodeling After Organ Transplantation

During the process of solid organ transplantation, platelets can be activated at multiple points. Activation and subsequent degranulation may already occur during graft procurement in organ donors (146). Brain death boosts a catecholamine storm resulting in organ malperfusion (147). Furthermore, blood or platelet transfusions administered perioperatively can promote platelet activation. Prolonged ischemia during organ procurement or transplantation results in platelet activation via P-selectin and CD40L (148, 149). Platelet activation in organ recipients can occur in patients with preexisting diseases such as atherosclerosis (150) or in contact with bioincompatible surfaces such as in dialysis patients, patients with a ventricular assist device and, of course, through contact with surfaces during extracorporeal circulation at time of transplantation (151, 152). Subsequently, activated platelets may trigger an inflammatory reaction of endothelial cells and interfere with leukocytes resulting in cellular rejection (153, 154) (**Figure 3**). These processes may lead to the development of cardiac allograft

vasculopathy and are the basis for a series of experiments regarding the role of platelets for chronic rejection after heart transplantation (155, 156). The experimental therapeutic approach of platelet inhibition with the P2Y12 ADP receptor blocker clopidogrel especially in combination with an mTOR inhibitor was very effective in a mouse aortic transplantation model, where allograft vasculopathy was almost abolished (157, 158). These findings paved the ground for a multi-center clinical trial called CEDRIC. However, the CEDRIC trial (Clopidogrel add on Certican: Effects on Coronary Diameter Reduction and Intimal Hyperplasia in Long-term follow-up after Cardiac Transplantation) had to be terminated due to recruiting problems and therefore further studies are necessary to ultimately validate this concept for a broad clinical application. Taken together, platelets play an important role in vascular remodeling after organ transplantation through both antithrombotic properties and the above-mentioned immune modulatory effects (**Figure 3**).

CONCLUDING REMARKS

Taken together there is increasing evidence for a role of platelets beyond hemostasis and thrombosis. Platelets are closely connected to inflammation. Contextual examples for this intimate connection between platelets and thromboinflammation are the plasmatic coagulation system as well as the complement system. Nonetheless, a lot of questions are still unanswered. One such question is how the beneficial effect of FXII deficiency on ischemic stroke can be explained. Solving this question might point the way how the outcome of this—sadly—very often disabling disease might be improved for patients. Another promising field of research is the close connection of the complement system and platelets to diseases featuring disseminated thrombosis, e.g., hemolytic uremic syndrome. Effective therapies for HUS are still missing, which raises the question, whether a clinically beneficial resolution of thrombus formation can be achieved by modulation of platelet function. Future research in platelet biology has the potential to show us even more novel, previously unexpected ways how platelets are directly involved in the most fundamental processes of health and disease.

AUTHOR CONTRIBUTIONS

MM, HN, RS, TG, CH, NvB, SE, and HL wrote parts of the manuscript. HL conceptualized and submitted the manuscript.

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Platelets, Thrombo-Inflammation, and Cancer: Collaborating With the Enemy

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Platelets are small anucleate cells present in the blood stream, their typical role in primary hemostasis has been well-described. However, new evidence suggests that they have critically important roles in cancer progression and inflammation. Cancer cells can activate platelets, thus using them as physical shields from blood shear forces and natural killer (NK) cells. The activated platelets may also regulate hematopoietic and immune cell migration toward the tumor site; therefore, contributing to the cancer-associated inflammation. The activation of platelets by cancer cells may also contribute to metastasis and cancer progression by stimulating deep venous thrombosis and neutrophil extracellular trap formations (NETs) that "hide" cancer cells. We strived to review the current literature to dissect the role of platelets in cancer-associated thrombosis and tumor microenvironment inflammation.

Keywords: platelets, thrombosis, inflammation, cancer, NETs

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PLATELET MORPHOLOGY AND PHYSIOLOGY

Platelets were first described by Bizzozero in 1882 who described them microscopically and established that platelets were the first component of the blood to adhere to damaged blood vessel walls *in vivo* and, *in vitro* (1). Since his discovery, platelets have been traditionally linked to hemostasis and thrombus formation (1–4). However, recent studies have shown that they are key players in tumor progression and metastasis, inflammation, atherogenesis, and antimicrobial host defense (1–4).

Platelets are arguably the most beautiful cells in the human body as they have an extraordinary capacity for morphological change and powerful secretion properties (5). As small, anucleate, discoid cells they are the smallest in blood circulation; measuring 2–5 μm in diameter with a thickness of 0.5 μm and a mean cell volume of 6–10 fl (5–7). Platelets are originated from big, nucleated cells called megakaryocytes that reside in the bone marrow and are part of the hemopoietic cell line (6). Platelets have an average lifespan of 5–7 days in the blood stream; where they endure such harsh conditions that as they age, they are reduced in size (5, 8). The average healthy human has 150,000–400,000 platelets per microliter (platelets/μl) in circulation at any given time, and changes in total platelet count and mean platelet volume are often related to pathological conditions and are used as an acute inflammatory marker (5, 9, 10).

The platelet membrane is covered in glycoproteins like GPIbβ-IX-V, GPVI, and GPαIIbβIII; which are essential for complete platelet aggregation and adhesion (11). The membrane also has protease activated receptors: PAR-1, PAR-4, and the P2Y family receptors that mediate activation

and aggregation (11). Platelets also contain three different kind of secretory granules: α-granules, dense granules, and lysosomal granules (12, 13).

The α-granules are the most represented and contain membrane-associated and soluble proteins that are expressed in the platelet membrane when it is activated (13). These membrane markers are involved in various processes; including cell adhesion, coagulation, inflammation, cell growth, and host defense (5, 13). They include P-selectin, fibrinogen, vonWillenbrand factor, epidermal growth factor, vascular endothelium growth factor, platelet-derived growth factor, and complement C3 and C4 precursors; to name a few (3, 7, 10).

Dense granules, on the other hand, are slightly rarer with just three to eight per human platelet (7, 13). They contain high concentrations of adenine nucleotides, specifically ADP and ATP; along with serotonin and histamine, which are released upon platelet activation (7, 13, 14). The third granule group, or lysosomes, is the least common with only 1–2 per cell (7, 13). They contain protein degrading enzymes like cathepsins, elastases, collagenases, and glucosidases as well as LAMP-1, LAMP-2, and CD63 (7, 13, 14).

Platelet activation may occur through contact with different agonists, the most predominant being: thrombin, ADP, von Willenbrand factor, and collagen (15). Thrombin is the most powerful platelet agonist; it acts on the GPIb-IX-V and the PAR receptors (7, 15). The PAR receptors have 4 subgroups: PAR2 is not present in the platelets and PAR3 functions only as a cofactor to PAR4 activation (15, 16). PAR1 is the most potent receptor for thrombin induced platelet activation, as it more sensitive to lower thrombin levels than PAR4 (15, 16).

Another strong platelet activator is adenosine diphosphate (ADP); it can be exogenous or released from the dense granules of activated platelets themselves; constituting an activation loop between converging platelets (10, 17). The ADP receptors in the platelets are the P2Y protein family (17). P2Y1 initiates ADP-induced platelet aggregation and is responsible for platelet shape change and P2Y12 amplifies and stabilizes the aggregation response (17). As the alpha granules contain ADP, this can constitute an activation loop between platelets that amplifies their aggregation (2, 17).

Von Willenbrand factor (vWF) is a large glycoprotein produced in the Weibel-Palade body of endothelial cells and by megakaryocytes; it is present in the platelet alpha granules and subendothelial connective tissue (7, 18, 19). It plays an essential role in primary and secondary hemostasis; as a mediator of platelet adhesion, and as a carrier for coagulation factor FVIII (18). The vWF is exposed in activated endothelial cells where it interacts with platelet GPIbα and supports platelet translocation to the subendothelium (19). The platelet αIIbβIII integrin also interacts with vWF, causing a cross linking of platelets that enables platelet aggregation and plug formation (18, 19).

When platelets have been activated, they expose negatively charged phosphatidylserine (PS) on their membrane through activation of scramblase (e.g., TMEM16F) (20). This acts as an anchor for the assembly of the prothrombinase complex which converts fibrinogen to fibrin (19). Activated platelets also contribute to the intrinsic pathway of coagulation by secreting Poly-P in their dense granules that activates fXII (19). Meanwhile, coagulation in itself will also activate platelets, as thrombin will cleave and activate PARs on the platelets; thus creating a positive feedback loop that greatly amplifies the hemostasis/coagulation process (20, 21).

PLATELETS, THROMBOSIS, AND CANCER

Tumor Cells Can Activate Platelets

The association between cancer and thrombosis has been known since 1865 when Armand Trousseau first described that localized cancers can induce venous thrombus formation at distant sites (21, 22). This malignant-associated thrombosis is one of the most common clinical manifestations in cancer patients and is associated to worse prognosis and survival (23). The major reason for the high thrombotic risk in cancer patients is that cancer cells can activate platelets and stimulate aggregation through direct and indirect mechanisms (19, 21). The tumor-cell induced platelet aggregation (TCIPA) has been demonstrated in various cell lines like pancreatic, colorectal, and kidney (24–26). Additionally, the TCIPA has been correlated to higher metastatic potential (19). There are several mechanisms involved in in platelet activation and TCIPA (20).

An important mechanism of TCIPA is the cancer cell secretion of thrombin (15, 27). Thrombin is a serine protease that converts fibrinogen to fibrin, but also activates coagulation factors V, VIII, XI, and XIII and the PAR receptors on platelets themselves (15, 19). Pancreatic and lung cancer in specific have been proven to activate platelets via thrombin secretion as well as thromboxane A2 secretion (28, 29). Tumor cells also express ADP, which activates platelets via the P2Y1 and P2Y12 receptors, making platelets release more ADP from their dense granules and thus activating other nearby platelets (30, 31). Interestingly; colon, prostate, and breast cancer cells can bind platelet FcγRIIa and induce dense granule secretion in the platelets (11). Different cancer types like squamous and germinal have also been proven to express podoplanin which binds to platelet-expressed CLEC-2 and induces platelet activation (32).

Tissue factor is arguably the main activator of the coagulation cascade once it comes into contact with activated factor VIIa in the blood stream (2, 5, 30). Tissue factor is often expressed in cancer cells and cancer derived microparticles (2, 6, 27). Elevated levels of TF in the serum has been evidenced in several types of cancer and in chemotherapy-induced thrombosis (28). Platelet as well as cancer derived microparticles have also been described to express Tissue Factors in their membrane, and thus contribute to platelet activations and cancer-related thrombosis (33–35).

There are other indirect mechanisms of platelet activation by the cancer cells. For example, cancer-cell expressed mucins can

Abbreviations: ADP, adenosine diphosphate; CLEC 2, C-type Lectin Like 2 receptors; CXCL, chemokine; F, coagulation factor; IL, interleukin; MHC, major histocompatibility complex I; MP, microparticle; NET, neutrophil extracellular traps; PAR, proteinase activated receptor; PDGF, platelet-derived growth factor; PECAM, platelet-endothelial cell adhesion molecule; PSGL-1, P-Selectin protein ligand 1; TF, tissue factor; TGF, transforming growth factor; TLR, toll like receptor; VEGF, vascular endothelial growth factor.

force platelets and granulocytes to interact (36). Subsequently, there is bidirectional signaling and Cathepsin G release by the granulocytes, which cleaves the platelet protease activated receptor-4 (PAR4) and activating G proteins (G_q and $G_{12/13}$) to induce shape change and platelet activation (36, 37). There are also malignancy-linked deficiencies of the vWF cleaving protease: ADAM13. Its deficiency causes large vWF multimers to circulate which can in turn activate platelets (11, 36, 38). A correlation between the presence of metastatic tumors and the concentration of vWF multimers in circulation, as well as aberrant ADAM13 in circulation has been previously demonstrated (39, 40).

The activation of platelets by cancer cells has a myriad of pro-cancerous effects like stimulating tumor growth, preparing the metastatic niche, and helping the metastatic cells survive in circulation. The induction of a cyclooxygenase 2 (COX-2) mediated paracrine signaling between the stromal and epithelial cells in the adenoma mediated by activated platelets can give the ensuing cancer cells a more aggressive phenotype (41–43). However, it has been shown that low-dose aspirin can have an antimetastatic effect by inhibiting COX-1 (43–45). This inhibition would decrease the cancer-mediated platelet activation and aggregation; thus, having an anti-metastatic effect on the tumor cells (41, 44).

Platelets Influence Tumor Growth

Platelets have a myriad of growth factors stored in their alpha (α) granules (5–7, 13). They are present in the tumor microenvironment outside of the vasculature where they can come into direct contact with the malignant cells (31, 46). When activated, they secrete transforming growth factor beta (TGF-β), vascular endothelial growth factor (VEGF), and platelet derived growth factor (PDGF) (47, 48). These factors not only induce tumor growth, but also promote angiogenesis and tumoral neovascularization (14).

It is important to also take into account that platelets also have anti-cancerous effects. Recently, platelet-derived microRNA has been identified as an important regulator of tumor development (49). Platelet-derived microparticles transfers miR-24 into cancer cells which targets mt-Nd2 and Snora75; triggering mitochondrial regulation and inhibiting tumor growth (49). This shows that platelet function and effects on cancer progression may be stage and context dependent (21, 49).

Platelet Receptors Mediate Distant Pre-metastatic Niche Preparation

Platelets are covered in membrane receptors that promote heterotypic cell interactions (27, 30, 31). These interactions play a crucial role in tumor growth and metastatic spread (11, 30, 31). Cancer cells that enter blood circulation during the metastatic process are exposed to high shear stress and to the immune system; to survive they use activated platelets to shield themselves (10, 27).

P-selectin is expressed on the surface of activated platelets and endothelial cells and is an important adhesion molecule (27). Cancer cells can bind to platelet P-selectin through TCIPA and form aggregates to protect themselves from the blood circulation and "hide" from NK cells (50). It has been proposed that platelet αIIbβ3 integrin can link fibrin with tumor αVβ3 integrin (19, 46, 47). The role of thrombin and integrin signaling is also very important in the platelet-cancer cell bonding mechanism (4, 27, 51). Thrombin increases the mRNA and protein levels of αVβ3 integrin and serves as a ligand to this receptor, it also increases the secretion of vascular-endothelial growth factor (VEGF) in human prostatic cancer cells (51).

Platelets also have an important effect on the preparation of the pre-metastatic niche (31, 52, 53). Primary tumors secrete metastasis-related proteins to the target organ that stimulate the migration of bone marrow-derived cells to create this pre-metastatic niche and stimulate neo-vasculogenesis (52, 53). Platelets have a role in managing the pre-metastatic communications; they secrete CXCL5 and CXCL7 upon contact with tumor cells to recruit granulocytes for the formation of the early metastatic niche (52–54). Activated platelets also release growth factors from their α granules, as well as metalloproteases that contribute to the degradation of the extracellular matrix and the preparation of the aforementioned metastatic niche (13, 54, 55).

Circulating Tumor Cell Survival and Arrest Is Mediated by Platelets

It is now widely accepted that increased platelet counts enhance cancer's metastatic power; while thrombocytopenia (low platelet count) may hinder the process (56). As previously stated, platelets have many adhesion molecules including integrins (αIIbβ3), selectins (P-selectin), leucine rich glycoproteins (P-selectin glycoprotein ligand -PSGL-1- and GPIb/V/IX), and immunoglobulin superfamily proteins (platelet-endothelial adhesion molecule -PECAM-1) (30, 31). These molecules allow them to form aggregates with cancer cells to protect them from the shear forces that would otherwise destroy their membranes (31, 50). These aggregates also serve to stabilize cancer cell arrest on the endothelial wall (27, 53, 55).

The TCIPA results in platelets coating the cancer cell and thus protecting it from the natural killer (NK) cells in the blood stream (57). They can also impair the NK cell mediated cytolytic/tumorilytic activity by secreting platelet TGF-β (57). The TGF-β impairs NK granule mobilization and interferon-γ secretion by downregulation the NKG2D immunoreceptor (50, 57). Another way that platelets aid the cancer cells escape the immune system is by membrane protein transfer (47, 48). In the midst of the platelet aggregate; the cancer cells can co-express platelet markers as well major histocompatibility (MHC) molecules to further camouflage themselves (58).

Platelets support cancer cell arrest in the same manner as it contributes to leucocyte arrest: by selectin (P-selectin) dependent rolling/tethering and integrin dependent adhesion (αIIbβ3, GP-Ibα, and vWF all contribute to firm adhesion) (21, 22). It is also important to note that many cancer cells express "platelet receptors" like αIIbβ3, αVβ3, or GP-Ibα (31, 58). These receptors not only help cancer cells escape the immune response but also mediate direct cancer-endothelial and cancer-leucocyte

interactions that promote cancer cell extravasation and prepare them for the colonization of the target tissue (31, 59).

Another important TCIPA effect on cancer endothelial transmigration is the release of ADP from the activated platelets' dense granules (31, 60, 61). ADP interacts with the endothelial PY2 receptor (P2Y1R), causing endothelial cell junctions to become laxer and enabling cancer cells to pass through more easily (60–62). Serotonin is also contained in the dense granules, and experimental studies have demonstrated that by blocking its receptor metastatic spread was inhibited (31, 63). Cancer patients that tend to have higher than average serotonin levels in the blood have a worse prognosis and survival (63).

PLATELETS, INFLAMMATION, AND CANCER

Malignant tumors have often been described as wounds that do not heal (64). Two of the most important tumor characteristics are their constitutive angiogenesis and perennial inflammation as well as the fibroblast infiltration and constant stroma regeneration (64). The vasculature in tumors is often fenestrated, facilitating the trans-endothelial transport, and exposing subendothelial factors like collagen and TF (14, 65). As we have previously stated, cancer cells can activate platelets through the various TCIPA mechanisms; with the added effect of the exposed subendothelial procoagulant factors there is a continuous platelet-activation loop (5, 19, 66). The activated platelets release their granule content that modulates the tumor microenvironment, including pro-inflammatory cytokines (67).

The proinflammatory cytokines released by the platelets are powerful recruiters and activators of leucocytes (67). These molecules include CXCL1, CXCL4, CXCL5, CXCL7, CXCL12 (SDF-1), and interleukin-8 (IL8) (67, 68). The CXCL12 chemokine attracts hematopoietic cells to the tumor site, stimulating tumor growth, and angiogenesis (69). Macrophages are also CXCR4 positive cells that are recruited to the tumor site by the platelet-expressed CXCL12 (67). On the other hand, CXCL5, and CXCL7 platelet secretion in distant sites to the primary tumor recruit granulocytes to prepare the pre-metastatic niche (5, 50, 52–54).

The activated platelets also express IL-1β (synthesized in the platelet from pre-mRNA) (70). The IL-1β induces TF expression in endothelial cells and stimulates expression of endothelial-leucocyte adhesion molecules (70). IL-1β also promotes platelet activation in an autocrine manner via the IL-1 receptor (67).

Transforming growth factor β (TGF-β) expressed and secreted by activated platelets in the tumor microenvironment has immunosuppressive properties and aids in the cancer cell escape

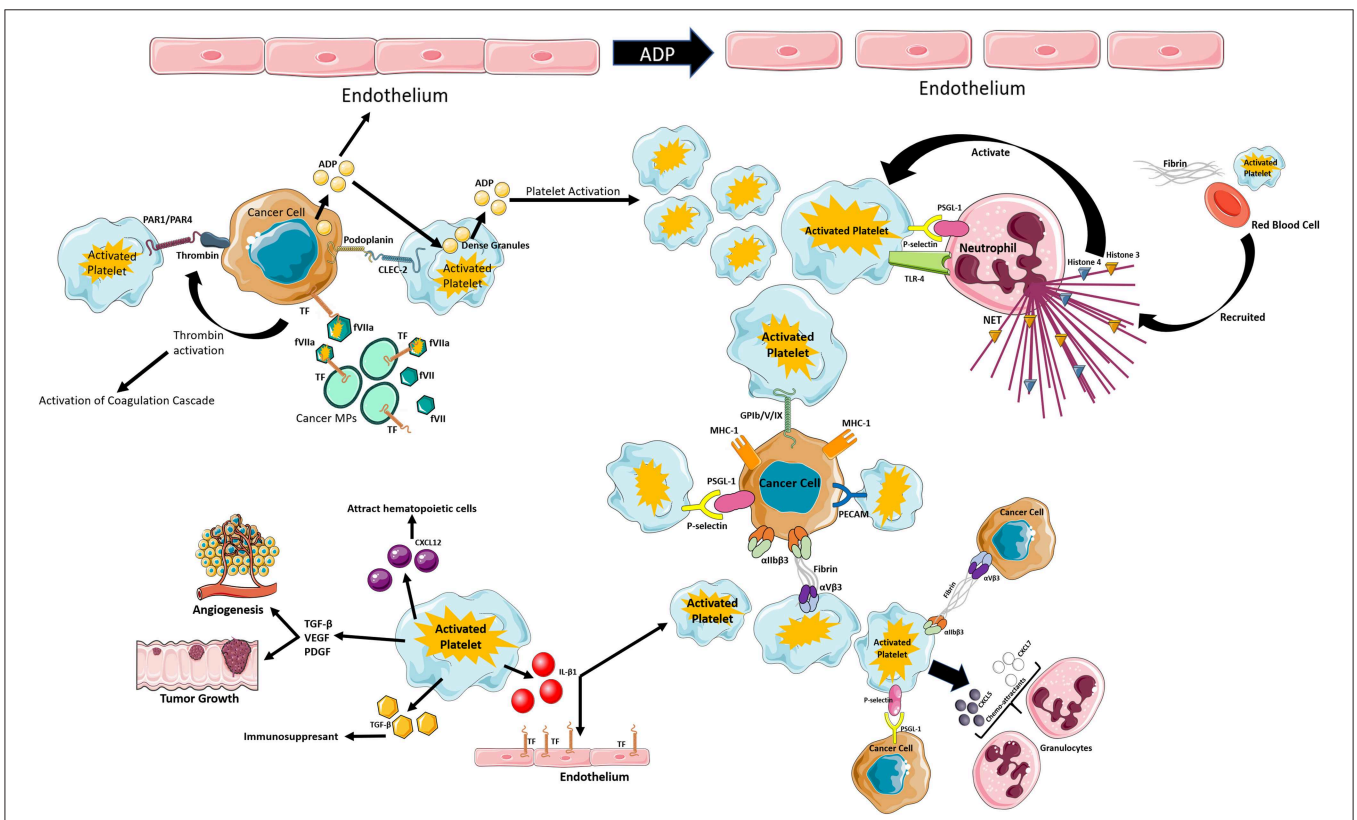

FIGURE 1 | Schematic review of the different mechanisms of platelet activation that can lead to thrombo-inflammation in cancer. Figure created using Servier Medical Art available at http://smart.servier.com/. Copyright Ana Luisa Palacios-Acedo.

from immune system recognition (71). TGF-β is also partially responsible for the transformation of the neutrophils toward a pro-tumorigenic phenotype (67).

PLATELETS AND NET FORMATION IN CANCER

Neutrophils are the body's first line of defense and have been traditionally characterized by two modes of action: pathogen engulfment and anti-microbial substance secretion (72). In recent years a new function has been identified: neutrophil extracellular traps (NETs) (72). The NETs are the result of the neutrophils' chromatin and granular content being expelled from the nucleus to form a web-like structure (67, 72). This structure can physically entrap and kill pathogens (67, 72). There are recent studies that suggest that NETs may also be involved in tumor progression, metastasis, and cancer-associated thrombosis (73).

Platelet TLR4 can trigger NETosis in activated neutrophils; and histones 3 and 4 released during the process can in turn, activate the platelets in a continuous loop (73, 74). The extracellular DNA in the NETs is capable of binding and activating coagulation factor XII as well as activating platelets directly (75). Additionally, activated platelet P-selectin can prime neutrophils through P-selectin glycoprotein ligand-1 (PSGL-1) activation and trigger NET formation (76, 77). These activation routes suggest that NETs are indeed a procoagulant factor as they provide a strong stimulus as well as a scaffold for thrombus formation (78). NETs promote fibrin deposition, recruit red blood cells and enhance platelet activation, and in turn, platelet activation promotes NET formation (76, 78).

Indeed, the link between NET formation and venous thromboembolism has long been established. In a baboon model of occlusion induced iliac thrombosis, researchers demonstrated an increase in circulation of NETs after 48 h that was maintained for 6 consecutive days; along with the presence of DNA markers in the thrombus (74). Another group demonstrated that plasma DNA is elevated in patients with deep vein thrombosis vs. healthy patients (79).

It is interesting to note that neutrophils originated from cancer patients are more prone to NETosis when exposed to PMA than those from healthy patients (80). This may be partially explained by the NET-activating properties of granulocyte colony-stimulating factor (G-CSF), and IL-8; which are locally secreted by tumor cells (67).

It has been proposed that tumor educated platelets may exert a pro-NETosis effect on the tumor-microenvironment neutrophils (80, 81). Cancer cells can allegedly use the NETs to protect themselves from shear stress in the circulation and from the immune system during the metastatic process (67, 81). The NET-induced platelet activation might play an important role in cancer progression, enhancing TCIPA, and the pro-thrombotic state (76). However, further research and information is needed to shed light on the contribution of platelets to the generation of NETs and their involvement in cancer progression.

CONCLUSION

Platelets are small but very powerful cells that interact with all components of the circulatory system. They are the main player in primary hemostasis but contribute to the secondary wave as well. As of recently, their involvement in the immune response was described, showing their power in regulating their environment. Their interactions with cancer cells and the tumor microenvironment are very complex and seem to have dual behaviors: pro and anti-cancerous, with the pro-cancerogenic effect out-numbering the anti-cancerous effects (**Figure 1**). However, it may seem that tumor education of platelets recruits them to the cancer cause, making them an ideal ally of tumor progression. This in turn, causes platelets to be continuously activated enhancing their thrombotic power and augmenting cancer-associated thrombosis. Moreresearch is needed in order to be able to establish the true power of these cells.

AUTHOR CONTRIBUTIONS

All authors listed have made a substantial, direct and intellectual contribution to the work, and approved it for publication.

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REVIEW
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Early Host Interactions That Drive the Dysregulated Response in Sepsis

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Sepsis is defined as life-threatening organ dysfunction caused by a dysregulated host response to infection. While many individual cells and systems in the body are involved in driving the excessive and sometimes sustained host response, pathogen engagement with endothelial cells and platelets early in sepsis progression, are believed to be key. Significant progress has been made in establishing key molecular interactions between platelets and pathogens and endothelial cells and pathogens. This review will explore the growing number of compensatory connections between bacteria and viruses with platelets and endothelial cells and how a better understanding of these interactions are informing the field of potential novel ways to treat the dysregulated host response during sepsis.

Keywords: sepsis, endothelial cell, platelets, hyper-activation, micro-organisms

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INTRODUCTION

Sepsis is defined as life-threatening organ dysfunction caused by a dysregulated host response to infection (Sepsis 3) and despite being the primary cause of in-hospital mortality there is little in the drug discovery pipeline for this disease (1). Treatment primarily focuses on the use of antibiotics but with the growing incidence of antibiotic-resistant strains of bacteria and the time it takes to diagnose sepsis there is clearly a need to discover novel approaches to treating sepsis. As the definition indicates that sepsis is a dysregulated host response (2) an obvious novel treatment strategy is to correct this dysregulated host response. Through significant advances in our understanding of the molecular interactions two possible theories have emerged that help explain the nature of the dysregulation. The platelet-pathogen theory suggests that pathogens bind to platelets activating them. These activated platelets bind to both endothelial cells and immune cells activating them which causes damage and disruption to the endothelial layer, leading to loss of barrier integrity, fluid leakage resulting in shock (**Figure 1**). Alternatively, the endothelial-pathogen theory suggests that pathogens bind to endothelial cells activating them. This leads to a release of granules and pro-inflammatory cytokines and chemokines, that recruit platelets to form a thrombus encasing the pathogens and immune cells and contribute to excessive thrombocytopenia and hyper-inflammatory response. Pathogen binding to endothelial cells also causes apoptosis which results in disruption of the endothelial layer leading to adherens junction disassembly, increased vascular permeability, fluid leakage, and shock (**Figure 2**). In this review, we will discuss each of these theories outlining the molecular mechanisms leading to each.

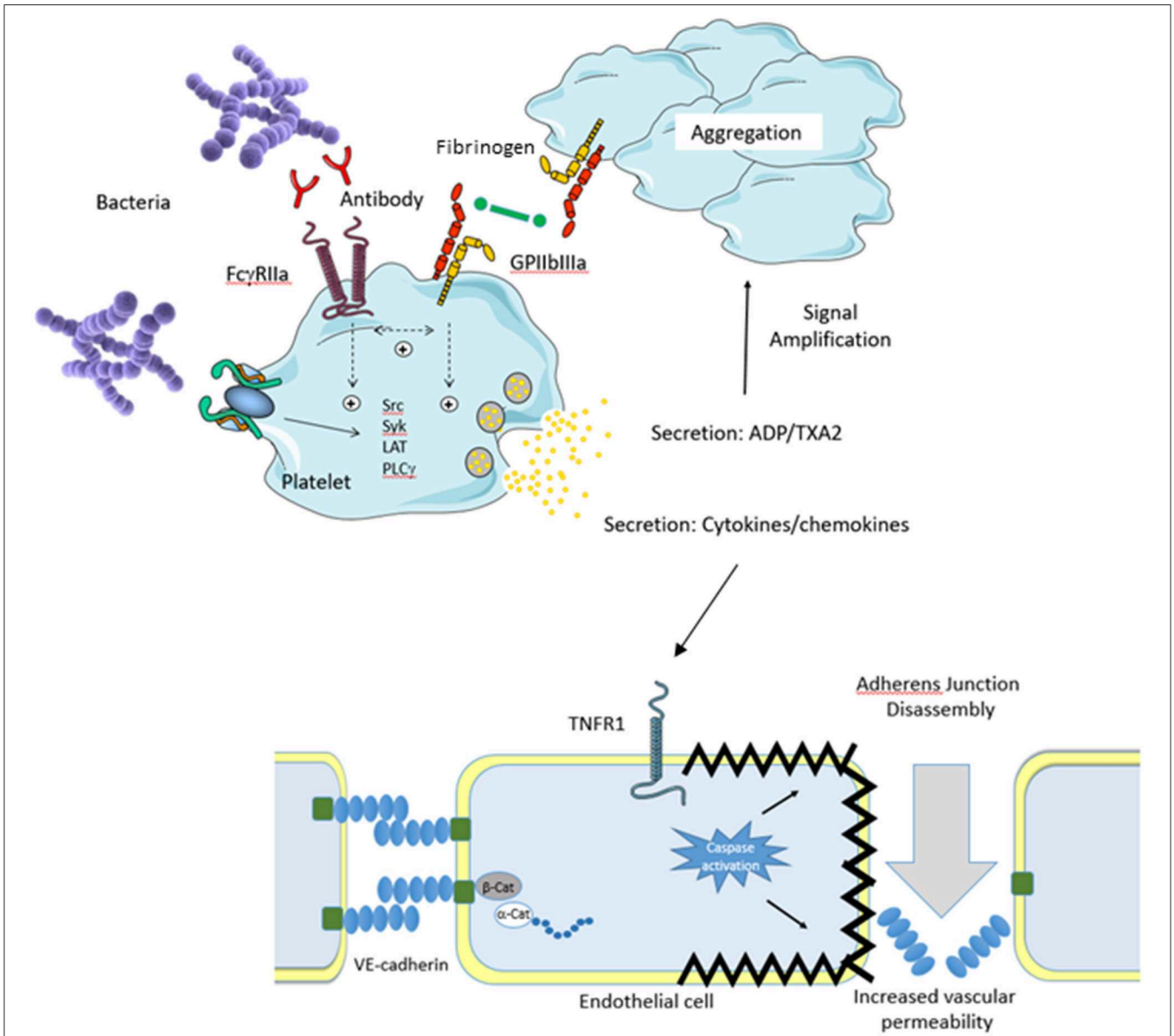

FIGURE 1 | Platelet Theory. An invading pathogen binds to platelets either directly (absence of plasma protein) or indirectly (presence of plasma protein). Binding results in platelet activation via Src kinases which results in prostanoid release, cytokine secretion, granule secretion, and activation of GPIIbIIIa. Release of ADP and thromboxane A2 (TXA2) serves to amplify the platelet response. In conjunction with this, activation of GPIIbIIIa allows fibrinogen binding resulting in platelet aggregation. Secretion of platelet cytokines and chemokines activates the vascular endothelium. For example, secreted TNFa activates the TNFR1 receptor on endothelial cells which triggers the death pathway resulting in apoptosis. This results in endothelial cell shrinkage and loss of barrier integrity leading to increased vascular permeability and shock. Separation of endothelial cells allows for pathogens to escape the bloodstream and infect major organs which eventually leads to multi organ failure.

PLATELET FUNCTION

After red blood cells platelets are the most numerous cell-like particle in the blood. Their total volume and surface area when combined together is larger than that of all the leukocytes taken together. They are not true cells as they have no nuclei and are in fact fragments of megakaryocytes (3). Their primary role is in hemostasis and they clump together to occlude any breach in the vasculature. They can be considered to have three distinct functions: adhesion, secretion and aggregation and platelet activation links these processes through multiple signaling pathways.

Adhesion

Platelets typically travel close to the endothelium patrolling for breaches in the vasculature. The resting endothelium is inert ensuring platelets do not adhere, however when the endothelial cell layer is damaged exposing sub-endothelial matrix

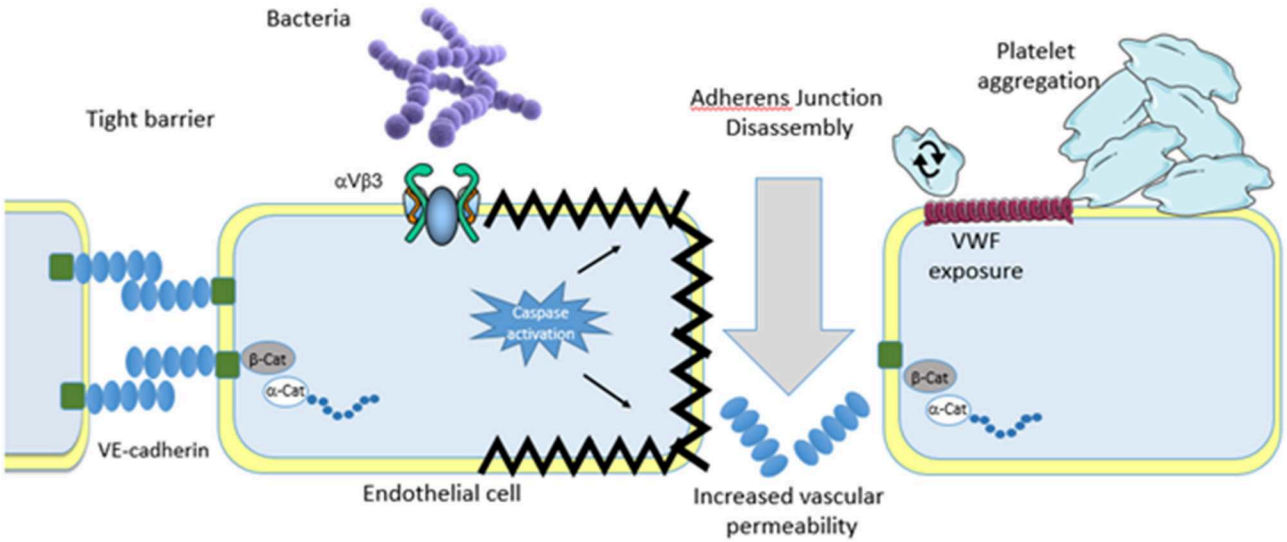

FIGURE 2 | Endothelial theory. Similar to platelets invading pathogens bind either directly or indirectly to the vascular endothelium. Binding results in endothelial cell activation that results in apoptosis. Although the pathway to apoptosis has not yet been defined apoptosis leads to endothelial cell shrinkage and loss of barrier integrity leading to increased vascular permeability and shock. Separation of endothelial cells allows for pathogens to escape the bloodstream and infect major organs which eventually leads to multi organ failure. Endothelial cell activation also leads to granule secretion which deposits vonwillebrand factor on the surface of the endothelial cells. This encourages platelet rolling, activation and aggregate formation. In addition, bacteria also roll on the deposited vonwillebrand factor eventually coming to a halt and firmly adhering. Passing platelets bind to the immobilized bacteria on the endothelial cells which leads to inappropriate thrombus formation and exacerbation of the dysregulation.

proteins (which as occurs with a cut), or becomes activated through inflammation the area becomes highly thrombogenic and platelets subsequently adhere. Key proteins in the matrix that support platelet adhesion are collagen and von Willebrand Factor (VWF). Adhesion is a highly controlled event and is mediated by platelet receptors such as integrin α2β1 and glycoprotein (GP) VI or collagen and GPIb/IX/V for VWF. The VWF-GPIb/IX/V interaction is shear-dependent and is primarily involved in adhesion under high shear stress such as in coronary arteries. See Nieswandt et al. for a review on platelet function (4).

Activation

While binding to either collagen or VWF facilitates adhesion of platelets to the site of injury it also triggers activation of the platelet leading to platelet secretion and aggregation. The adhesion process is not the only process for activating platelets as soluble mediators can also lead to platelet activation. Substances like adenosine diphosphate (ADP), thrombin, thromboxane A2, and adrenaline can also trigger platelet activation through their respective receptors. To further link all of the platelet functions the primary source of these soluble mediators are platelets in a process known as secretion. Most of these receptors mediate their effects through two different signaling pathways. Some receptors stimulate phospholipase (PL) A2 which ultimately leads to the production of thromboxane A2 while others trigger PLC activation (5).

Secretion

Once activated, platelets secrete the contents of their granules. Platelets contain different types of granules such as alpha and dense granules and lysosomes. The content of these granules plays an important role in haemostasis. Secreted ADP activates the surrounding platelets and this is critical in creating the growing clot. The granules also secrete adhesion molecules such as fibrinogen as well as up-regulating adhesion receptors such as GPIIb/IIIa (fibrinogen receptor) and p-selectin (CD62) to the platelet surface (6).

Aggregation

While platelet activation leads to granule secretion it also leads to activation of GPIIb/IIIa on the platelet surface. GPIIb/IIIa is an integrin (αIIbβ3) fibrinogen receptor and is usually present in a resting, non-binding conformation. Once activated it can bind soluble fibrinogen and as fibrinogen is a large bivalent molecule one fibrinogen molecule can bind to two GPIIb/IIIa molecules. If these GPIIb/IIIa molecules are on different platelets the effect is to link two platelets together. Considering that there are around 50,000 GPIIb/IIIa molecules per platelet this creates a platelet-rich clot cross-linked by fibrinogen. This process is known as aggregation and is critical for sealing a breach in the vasculature (7).

PLATELET SIGNALING

Platelet activation occurs through two processes—outside-in signaling and inside-out signaling and is reviewed in more detail by Stalker et al. (8). The classic example of outside-in signaling is the process by which soluble mediators such as ADP, thrombin, and thromboxane (Tx) A2 activate platelets. These bind to membrane receptors (in most cases G-protein-coupled receptors) triggering downstream events. Agonist binding to

these receptors trigger activation of one of two phospholipases (PL)—PLA2 or PLC. PLA2 is the primary PL and acts to release arachidonic acid from the inner membrane of the platelet. This arachidonic acid is a substrate for cyclooxygenase (COX). In platelets, the COX isoform is COX 1 and it converts arachidonic acid into prostaglandin (PG) H2. PGH2 is an intermediate in the signaling process and it is further metabolized to its active product by cell specific enzymes. In platelets the primary enzyme is thromboxane synthase which converts PGH2 to TxA2 which binds to receptors and triggers further platelet activation. This pathway is completely inhibited by COX 1 inhibitors such as aspirin.

The second signaling pathway uses a number of different isoforms of PLC which cleave inositol triphosphate (IP3) and diacylglycerol (DAG) from the membrane. IP3 binds to an intracellular Ca2+ channel which increases intracellular Ca2+ levels through release from the endoplasmic reticulum. DAG ultimately activates protein kinase C which activates further downstream events. This pathway is not inhibited by aspirin. Agonists are often divided into weak agonists which are PLA2-dependent and strong agonists which are PLC-dependent, although this is often concentration-dependent with low concentrations of agonists using PLA2 and high concentrations using PLC.

While soluble agonists activate platelets via PLA2/PLC this is not the only process for activating platelets. Platelet adhesion also leads to platelet activation however, the activation process for each of the adhesion receptors is receptor specific. One example is that of platelet activation in response to adhesion to fibrinogen. The platelet receptor involved is GPIIb/IIIa and initially it was considered that GPIIb/IIIa was merely an adhesion receptor as there were no obvious signaling pathways associated with it. However, it is now clear that GPIIb/IIIa can recruit signaling molecules [Src family kinases, focal adhesion kinase (FAK) etc.] and generate activating signals (9). GPIb/IX/V acts by recruiting 14-3-3ζ, actin binding protein, Src, FAK etc (10). Receptors that contain an ITAM (immunoreceptor, tyrosine-based activation motif) or ITAM-like domain such as Fc receptors and CLEC-2 recruit the tyrosine kinase syk when dimerized (11). Fc receptors can heterodimerise, that is they can dimerize with other receptors such as GPVI and GPIb. GPVI contains an SH3 domain that recruits the src family kinases and when dimerized with FcR-γ it triggers the recruitment of syk and phosphorylation of FcR (12).

The wave of outside-in activation is followed by a wave of inside-out signaling. This primarily involves talin binding to the β3 subunit of GPIIb/IIIa (13). This inside-out signaling is essential for full activation of the platelet.

Platelets also have inhibitory signaling pathways to counter the activating pathways. The primary pathway is mediated by prostacyclin (PGI2). When it binds to is receptor it increases cAMP which in turn activates PKA and inhibits platelet activation. A related mechanism is that of nitric oxide (NO) which directly enters the platelet and activates soluble guanylate cyclase increasing cGMP levels. Both prostacyclin and NO are produced by healthy endothelial cells to prevent clot formation.

In sepsis, thrombocytopenia develops in up to 50% of cases and is associated with poor outcome (14). This thrombocytopenia is likely to play a significant role in the pathogenesis of sepsis leading to development of multiple organ dysfunction syndrome (MOPS), disseminated intravascular coagulation (DIC) and/or massive bleeding as a result of platelet consumption and thrombus formation (15). It is well-established that innate immune cells (IIC) such as macrophages, natural killer cells (NK) cells, neutrophils, dendritic cells etc, release a plethora of pro-inflammatory mediators creating a so-called cytokine storm (16). We now also know that in addition to their hemostatic functions platelets also play a role in inflammation and regulation of inflammatory response by secreting cytokines, interferons, and chemokines. For example, *Staphylococcus* and *Streptococcus* spps can trigger platelet aggregation, cytokine release, and thrombocytopenia (17–19).

PLATELETS AND IMMUNITY

The critical role of platelets in the innate immune response is largely mediated by their ability to interact with other immune cells mainly neutrophils (20, 21). Platelets express receptors on their surface that are usually associated with immune cells such as FcγRIIa and Toll-like receptors (TLR) 2 and 4 (22). For example, in 2007 Clark et al., demonstrated that lipopolysaccharide (LPS) binds to platelet TLR4 which mediates attachment to neutrophils. Critical to this interaction is platelet activation which results in granule secretion, P-selectin expression on the surface of the platelet, and crosslinking to its counter receptor P-selectin glycoprotein ligand-1 on the leukocyte surface. Other studies have demonstrated that platelet GPIbα can bind VWF and crosslink the platelet to neutrophils via the β2 integrin (CD18). Neutrophil Extracellular Traps (NETs) are web-like structures composed of a chromatin backbone, histones and anti-microbial proteins and their main function is to trap and kill bacteria, virus, and fungi, avoiding their dissemination. While NET formation is a critical event in innate immunity, uncontrolled formation may exert significant tissue damage which contributes significantly to the already difficult to control host dysregulation (23). Dengue virus has been shown to activate platelets in a CLEC-2-dependent manner producing extracellular vesicles that induce NET formation (24). Regardless of the interaction, platelet attachment to the neutrophil results in rapid activation and most importantly the formation of NETs and together they play an important role in the pathogenesis of sepsis (25).

As platelet activation occurs during inflammation and infections such as sepsis there is also a need to control excessive platelet activation. One controlling factor is that of C-reactive protein (CRP) which is an acute phase protein synthesized in the liver in response to infection. It exists in a monomeric (mCRP) and pentameric (pCRP) forms which have opposing effects. pCRP is known to inhibit platelet aggregation by binding to GPIIb/IIIa and thus will act to reduce thrombus formation (26). Another agent that regulates the platelet response is nitric oxide (NO). NO is produced by the endothelium and is a potent vasodilator but also an inhibitor of platelet activation. During sepsis NO levels increase due to production by immune cells. This increased NO contributes to vasodilation and hypotension

as well as inhibiting platelet function (27, 28). Thus, platelet activation status during sepsis depends on the balance between activating and inhibiting factors.

Platelet Immune Receptors

Platelets express immunoreceptor tyrosine-based activation motifs (ITAMs)-containing receptors such as FcγRIIa, GPVI, and C-type lectin-like receptor (CLEC)-2 (29). The presence of FcγRIIa, a receptor for the Fc portion of IgG, on platelets is unusual as it is a receptor involved in phagocytosis and all other FcγRIIa-expressing cells are phagocytic (29). However, while not true phagocytic cells platelets do engulf bacteria in a manner that has some similarities to phagocytosis (30, 31). Platelet FcγRIIa is fully functional and can trigger platelet aggregation. Immune complexes [or even heat-agglutinated immunoglobulin (Ig) G] directly induce platelet aggregation in an FcγRIIa-dependent manner. Furthermore, bacteria that become coated in IgG can also induce platelet aggregation in an FcγRIIa-dependent manner (see below). The functionality of TLRs is more complex. While studies show that TLRs can mediate platelet activation others show that they don't (32). There is evidence to suggest that platelets activated by TLRs can engage with neutrophils and/or monocytes triggering their activation (33). Dendritic Cell-Specific Intercellular adhesion molecule-3-Grabbing Non-integrin (DC-SIGN), also known as CD209, is a C-type lectin which is usually expressed on macrophages and dendritic cells that is known to be involved in the phagocytosis of HIV and is also expressed on platelets (34). DC-SIGN and FcγRIIa are particularly implicated in platelet activation in Dengue virus infection (DENV) and incubation of platelets with anti-DC-SIGN antibodies prevented DENV-mediated platelet activation (35).

Other receptors shown to facilitate platelet interactions in response to pathogens are sialic-acid-binding immunoglobulin-like lectins (Siglecs)- a type I transmembrane proteins, that play role in regulating the host's immune responses to pathogen (36). In platelets Siglec-7 is most abundantly expressed and its function depends on the P2Y1 platelet receptor and of the GPIIb/IIIa integrin. It is proposed that Siglec-7 down-regulates pathogen-induced platelet activation by inducing apoptosis (37). Along with FcγRIIa, CLEC-2, and GPVI are ITAM receptors found on platelets. The ligands for CLEC-2 is podoplanin and for GPVI it is collagen, fibrinogen, and fibrin (38). These receptors have been found to play a role in the interaction with pathogens. Thus, CLEC-2 has been shown to bind to human immunodeficiency virus (HIV) and GPVI has been shown to bind to Hepatitis C virus (39, 40). GPVI has been shown to be important in *Klebsiella pneumoniae* sepsis models (41). CLEC-2 has been shown to drive thrombosis following Salmonella infection (42). Furthermore, the platelet CLEC-2-podoplanin interaction has been found to be an important modulator of inflammation during sepsis (43, 44).

Platelet Cytokines

Platelets release cytokines either directly into the bloodstream by de-granulation, or by secreting platelet-derived micro-vesicles (PDMV), which make up between 60 and 90% of extracellular vesicles (EV) in plasma and contribute to hemostatic and immune function of platelets (45–47). These "immuno-parcels" can elicit innate and adaptive immune responses at distant sites by delivering variety of immunomodulatory factors, such as CD154 (also known as soluble CD40 Ligand, sCD40). CD154 from PDMV is enough to activate antigen specific splenic B cell response in $CD154^{-/-}$ mice, in both T cell-dependent and independent manner (48). PDMV also contain a variety of nucleic acids including messenger and micro-RNAs (49). For example, platelets contain mRNA of pro-IL-1β, which upon platelet activation is translated *in situ* and fully synthesized pro-IL-1β is then released into circulation (50). IL-1β but not IL-1α binds to fibrinogen and it is the bound form of IL-1β that has enhanced action to induce monocyte chemoattractant protein 1 (MCP-1) and nitric oxide (NO) production by endothelial cells via NFκB pathway (51). Among other pro-inflammatory modulators released by platelets are: MCP-1, macrophage inflammatory protein (MIP)-1α, regulated on activation, normal T cell expressed and secreted (RANTES), IL-8, tumor growth factor (TGF)-β, angiogenesis and growth factors, and various immunoglobulins (48, 52–55).

PLATELET BACTERIAL INTERACTIONS

There are several platelet receptors that are involved in either direct interactions with pathogens either through direct interactions between microbial adhesins and platelet surface component or indirect associations via a bridging molecule (17, 20, 56, 57). The best studied interaction is that between *S. aureus* and platelets but the interaction with other Gram-positive and Gram-negative bacteria has also been described (17). The most significant causative agents of sepsis are *S. aureus* is the major cause (21%), *E. coli* (16%), *Staphylococcus epidermidis* (11%), and *S. pneumoniae* (4%) and these are also the best studied for their interactions with platelets (58).

Staphylococci-Platelet Interactions

S. aureus expresses several cell wall anchored surface proteins that enable binding of the bacteria to platelets (59). During the exponential growth phase *S. aureus* expresses Clumping factor (Clf) B, fibronectin-binding protein (FnBP) A and B; while ClfA is expressed during the stationary phase. These proteins bind to fibrinogen facilitating its binding to and activation of platelet GPIIb/IIIa (60–62). *S. aureus* surface protein A (SpA) is known to bind IgG but also binds to domain A1 on VWF which mediates binding to platelet GPIbα (63). However, these interactions are insufficient to activate platelets and all require simultaneous activation of FcγRIIa through engagement with opsonising IgG's on the surface of the bacterium. Engagement of integrins and FcγRIIa lead to platelet activation, aggregation, adenosine triphosphate (ATP) release, and thrombus formation. In the absence of plasma proteins *S. aureus* can also bind directly to GPIIb/IIIa via its iron-regulated surface determinant protein B (IsdB) inducing platelet adherence and aggregation (64). Four more platelet activating proteins that are secreted by *S. aureus* have been recently identified by Binsker et al: Extracellular adherence protein (Eap), the chemotaxis inhibitory protein of *S. aureus* (CHIPS), the formyl peptide receptor-like 1

inhibitory protein (FLIPr), and the major autolysin Atl (AltA) which were all shown to induce P-selecting expression, while Eap, CHIPS, and AltA also induced platelet aggregation (65). *Staphylococcus epidermidis* expresses serine–aspartate repeat protein (Sdr) G, a member of Microbial Surface Components Recognizing Adhesive Matrix Molecules (MSCRAMM), that engage with GPIIb/IIIa either via fibrinogen or directly (66).

Streptococcci-Platelet Interactions

A large number of streptococci have been shown to interact with platelets including *Streptococcus gordonii, Streptococcus sanguinis, Streptococcus oralis, Streptococcus agalactiae,* and *Streptococcus pneumoniae* (17). A common mechanism through which the streptococci interact with platelets is via a serine-rich repeat protein (Srrp). This Srrp is expressed on the surface of many streptococci (67–70). Numerous studies have demonstrated that the Srrp binds to platelet GPIbα and induces activation. Under low shear conditions ($<500\ s^{-1}$) platelets roll along immobilized streptococci using a mechanism characteristic of the interaction observed when platelets interact with VWF. Deletion of the Srrp ablates the interaction with platelets under both static and shear conditions (68, 70). *S. gordonii* have also been shown to bind GPIIb/IIIa. A common feature in this interaction is the presence of an RGD-like sequence in a large cell wall surface protein (3,500 amino acid residues) that mediates the binding called Platelet Adherence protein A (PadA) (71, 72). Binding resulted in src induced rearrangement of the platelet actin cytoskeleton leading to filopodia and lamellipodia formation resulting in platelet spreading over the bacteria (73). Protein analysis and site directed mutagenesis revealed that PadA contains two critical integrin-recognition motifs (383RGT and 484AGD) that mediate interaction with GPIIb/IIIa (73). *S. pneumoniae* has also been shown to bind to and induce platelet activation resulting in dense granule secretion in a TLR2-dependent manner. Although the bacterial component that interacts with platelet TLR2 was not identified a likely candidate is wall lipoteichoic acid (74).

Platelet-Gram-Negative Bacteria Interactions

Escherichia coli, *Helicobacter pylori*, *Porphyromonas gingivalis,* and *Brucella abortus* all have been shown to bind platelets (18, 75–78). *E. coli* O157:H7 interact with platelets via platelet TLR4 and P-selectin (CD62) leading to secretion of CD40L, increase in fibrinogen binding on platelets and the formation of aggregates (79). This platelet activation is both FcγRIIa- and GPIIb/IIIa-dependent, and requires opsonisation of bacteria with IgG (18, 32). Platelet activation induced by *H. pylori* has been shown to be FcγRIIa and GPIbα-dependent (77, 80). *B. abortus* binds directly to platelets in a dose-dependent manner, although platelet receptors for this interaction remain to be established. This interaction induced enhanced fibrinogen binding and P-selectin expression, and promoted infection of monocytes by delivering bacteria to them (75). *P. gingivalis* has also been shown to bind to platelets in an IgG-dependent manner. Depletion of IgG or pre-incubation of platelets with an anti-FcγRIIa antibody abolished platelet activation and aggregation (81).

Complement-Dependent Platelet Activation

While some bacteria have surface proteins that can interact with platelets many bacteria can activate platelets despite the absence of such proteins. *S. aureus* mutants which lack known platelet interacting proteins such as ClfA and ClfB and are thus unable to bind fibrinogen can still induce platelet activation. This is also true of strains of *S. sanguinis* that do not bind to platelets. However, the aggregation profile of these bacteria is quite different. While wildtype *S. aureus* induces aggregation within 2–3 min these non-interacting bacteria take more than 15 min to induce aggregation although the aggregation is still FcγRIIa-dependent. Bacterial-induced aggregation is usually mediated by FcγRIIa and a co-receptor such as GPIIb/IIIa and GPIb, in the case of non-binding bacteria the co-receptor is a complement receptor. This slow aggregation requires complement assembly most likely by the alternative pathway. The delay in onset of aggregation probably reflects the time required for complement membrane attack complex formation (82, 83).

PLATELET-VIRUS INTERACTIONS

Bacteria are not the only pathogens that affect platelet function during infection. Viral Haemorrhagic Fevers (VHF's) are very contagious zoonotic diseases that occur all over the world although more prevalent in tropical and warm climates (84). As name suggests VHF are associated with thrombocytopenia, hemorrhage, and fever caused by systemic inflammation. VHF viruses cause diseases such as Ebola, Lassa, Marburg, Yellow fever, and Dengue (69). Viruses interact with platelets mainly via FcγRIIa, integrins, DC-SIGN, and complement receptors (85). The best characterized of these interactions is with the Dengue virus which binds to DC-SIGN on platelets, causing their activation, mitochondrial dysfunction, and apoptosis via caspase-9 and 3 engagement thus contributing to systemic inflammation and platelet depletion (**Figure 3**) (35).

ENDOTHELIAL CELL FUNCTION

Endothelial cells make up a highly adaptive single cell layer displaying distinct apical and basolateral sides in blood vessels. They appear elongated in the direction of blood flow and form a tight cobblestone pattern. Endothelial cells are highly metabolically active and are constantly sensing alterations in the local extracellular environment (86). The endothelium receives and integrates information from hormones, neurotransmitters, pericytes, smooth muscle cells, leukocytes, platelets, viral or bacterial infection, proinflammatory cytokines, and oxygen tension (87). Endothelial cells also respond to vascular injury and high hydrodynamic shear stress. The primary function of the endothelium is to regulate systemic blood flow and maintain blood vessel wall permeability which selectively controls the movement of fluid, ions, and macromolecules between the circulating blood and the surrounding tissues.

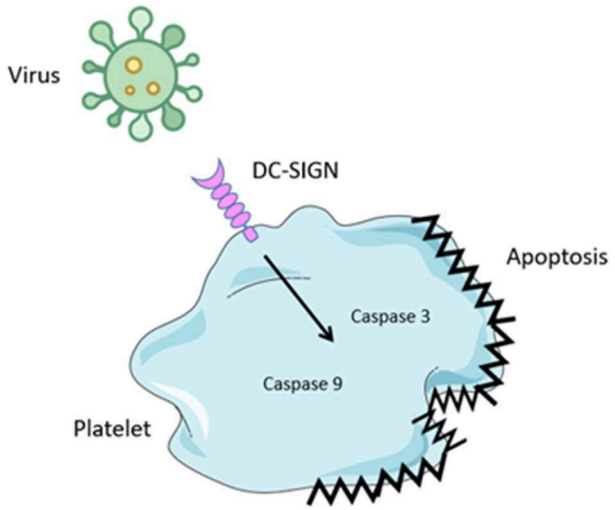

FIGURE 3 | Virus binding to platelets. Several virus have been shown to bind to platelets. Binding is mediated by attachment to the Dendritic Cell-Specific Intercellular adhesion molecule-3-Grabbing Non-integrin (DC-SIGN), a c-type lectin receptor. Engagement results in activation of caspase 3 and 9 leading to platelet apoptosis. This results in thrombocytopenia.

Barrier Integrity

The passage of molecules, cells, and fluid through the endothelial cell layer is a tightly controlled process. In the healthy state endothelial cells are held together to ensure barrier integrity through intracellular junctions called tight junctions and adherens junctions (87). Critical tight junction proteins include occludins and claudins which are localized at the apical area of the intracellular cleft. These tight junction proteins are predominantly responsible for control of permeability of solutes between blood and tissues (88). Adherens junction proteins on the other hand are predominantly responsible for maintaining endothelial cell-endothelial cell connection to ensure vascular integrity. The main adherens junction protein responsible for this is VE-cadherin which is localized at the basal membrane (89, 90).

Anticoagulant and Anti-thrombotic Surface

Under resting conditions the luminal surface of the endothelium is typically both anticoagulant and antithrombotic in order to maintain fluidity within the circulation (91). Healthy endothelial cells express a number of inhibitors to prevent the synthesis and activity of the key penultimate enzyme in the coagulation cascade, thrombin. Anti-coagulation is ensured when thrombin interacts with the endothelial cell integral membrane protein thrombomodulin. Engagement induces activation of protein C which forms a complex with protein S and results in inactivation of factor VIIIa and factor Va, critical co-factors for coagulation (92). Sustained or prolonged release of mediators from healthy endothelial cells inhibit activation and adhesion of platelets to the endothelium. Prostacyclin (PGI2) is released from resting endothelial cells and binds to the Gs-coupled prostacyclin I2 receptor (IP receptor) expressed on platelets (93). Receptor activation results in activation of protein kinase A which increases platelet cAMP, thus preventing platelet activation. Similarly, lipid-soluble nitric oxide is also released from resting endothelial cells and activates protein kinase G which increases cGMP, also preventing platelet activation. Increases in cAMP or cGMP inhibit platelet aggregation, platelet secretion and platelet adherence to the vessel wall (94).

Leukocyte Recruitment

The endothelium responds to tissue invasion by transporting leukocytes from the bloodstream to subendothelial compartments. Circulating leukocytes i.e., neutrophils and monocytes are recruited to the source of infection and release TNFα, a potent endothelial cell activator. TNFα induces the expression of adhesion molecules on the surface of endothelial cells facilitating leukocyte rolling-and-adhesion. MLK is involved in the release of Weibel-Palade bodies (WPBs) stored in the endoplasmic reticulum which harbor P-selectin (95). P-selectin is subsequently transported, by WPBs, and presented on the luminal side of the endothelium (96). The initial interaction between endothelium and leukocyte involves selectins (P- and E-selectins). These selectins recognize sialyl-Lewis-x moieties of leukocyte glycoproteins allowing reversible adherence to the endothelium (97). A second interaction between lymphocyte function-associated antigen 1 (LFA-1) and macrophage-1 antigen (MAC-1) on the surface of leukocytes with intracellular adhesion molecule (ICAM)-1 and ICAM-2 on the endothelium occurs, however, the initial interaction is weak which allows leukocytes to roll along the vasculature (98, 99). Chemokines, such as CXCL8 upregulated by NF-κB and AP-1, bind to receptors on leukocytes inducing a conformational change in LFA-1 and MAC-1 (CD18/CD11b) allowing them to adhere to ligands with high affinity (100). This facilitates firm adhesion of leukocytes to the endothelium arresting rolling movement. In a process known as extravasation, LFA-1, MAC-1 (CD18/CD11b), and platelet endothelial cell adhesion molecule (PECAM, CD31) enable the leukocytes to squeeze between endothelial cell junctions. The leukocytes migrate to the basement membrane (diapedesis) where enzymes break down the extracellular matrix (101). The leukocytes continue migrating through subendothelial tissue following a chemokine (CXCL8; CCL2) concentration gradient, a process known as chemotaxis, to the source of infection where they encounter pathogens.

ENDOTHELIAL CELL-BACTERIAL INTERACTIONS

Gram Positive Bacteria-Endothelial Cell Interactions

Bacterial interaction with endothelial cells is not well-defined and as a result few interactions have been identified. Lack of progression in this field can be attributed to poor models used to study the interactions. Endothelial cells exist in a dynamic environment surrounded by various circulatory cells (leukocytes, red blood cells, platelets) in plasma. To better reflect the physiological conditions during infection Cheung and Fischetti demonstrated that when endothelial cells are

grown in the presence of tumor necrosis factor α (TNFα), significantly more *S. aureus* bind to the endothelial cells. *S. aureus* binding was further increased when fibrinogen was added to the endothelial cells (102). These findings suggest that *S. aureus* binds to fibrinogen and cross-links the bacteria to the "activated" endothelial cell (103). Binding was abolished when *S. aureus* cells were treated with trypsin, suggesting that the bacterial adhesin was a cell wall protein. Using surface-biotinylated solubilized components of *S. aureus* revealed a critical role for protein A in binding endothelial cells (104). Protein A is known to bind a number of plasma proteins including IgG and VWF (63, 105). Subsequent studies demonstrated that upon endothelial cell activation, release of intracellular calcium causes mobilization of weibel palade bodies which results in deposition of VWF onto the surface of the cell, thus producing a binding site for protein A on *S. aureus*. Claes et al. identified a second VWF binding protein expressed on S. aureus called vwb (106). This protein typically acts as a coagulase and activates prothrombin to generate fibrin. Much similar to before deposition of VWF on the surface of endothelial cells following activation or injury provides a binding site for vwb. A *S. aureus* strain deficient in the vwb protein or an antibody against the A1 domain of vWf significantly reduced *S. aureus* adhesion to endothelial cells an *in vivo* model of blood stream infection. Collectively these data suggest that *S. aureus* use at least two different mechanisms to interact with surface deposited VWF after endothelial cell activation. While these observations are critical in our understanding of how *S. aureus* interacts with the endothelium it doesn't identify the primary interaction that triggers endothelial cell activation to lead to VWF deposition. Using a shear based model, McDonnell et al., identified a very early interaction that drives vascular dysregulation early in infection (107). Using primary human endothelial cells sheared at physiological shear rates experienced in the vasculature the authors demonstrated that the *S. aureus* ClfA binds plasma fibrinogen and crosslinks the bacteria to the major endothelial cell receptor αVβ3. Binding via this mechanism resulted in VWF deposition on the surface of the endothelial cells which will allow both bacterial and platelet attachment. Binding also resulted in a loss of barrier integrity as determined by an increase in vascular permeability and loss of VE-cadherin expression. Permeability changes is a common characteristic in sepsis patients and facilitates dissemination of infection to all major organs, thus contributing to organ failure. Blocking *S. aureus* attachment to αVβ3 prevented VWF deposition and loss of barrier integrity. S. aureus attachment also triggered significant cytokine and chemokine release contributing to hyper-inflammation and immune cell recruitment (108).

While it is evident that *S. aureus* has evolved to possess various mechanisms to attach to endothelial cells the functional significance of these interactions are still under investigation. One clear functional interaction demonstrates the ability of *S. aureus* to internalize into endothelial cells (109). Internalization likely occurs to evade immune or anti-microbial attack, as neither immune cells or antibiotics are capable of entering into endothelial cells. Internalization is mediated by Fnbp expressed on *S. aureus* which binds plasma fibronectin and cross links to endothelial cell receptor α5β1.

Gram Negative Bacteria-Endothelial Cell Interactions

In contrast to *S. aureus* interactions with endothelial cells which focused primarily on identifying bacteria proteins, research investigating the interaction between Gram negative bacteria interaction and endothelial cells has focused on its major cell wall component LPS and the downstream signaling as a result of this interaction. Possibly the best described interaction involves the Toll-Like Receptors (TLRs). For example, TLR4 recognizes LPS (110). TLR4 signaling begins with the formation of a TLR4/myeloid differentiation 2 (MD2) complex. Upon LPS binding, homodimerization of two TLR4/MD2 receptors occurs, inducing a conformational change that allows the Toll/interleukin-1 receptor-like (TIR) domains of TLR4 to recruit adaptor proteins for the activation of MyD88-dependent pathway at the plasma membrane. These adaptor proteins subsequently activate interleukin (IL)-1R associated kinases (IRAKs) and tumor necrosis factor (TNF) receptor associated factor 6 (TRAF6) (111, 112). This, in turn, activates transforming growth factor β-activated kinase 1 (TAK1) resulting in MAP kinase kinase (MKK) inducement of the MAPK signaling cascade (113). The MAPK signaling cascade activates nuclear transcription factors such as nuclear factor (NF)-κB and activator protein (AP)-1 (114). The activation of NF-κB and AP-1 induces the production of pro-inflammatory cytokines and chemokines driving the acute phase inflammatory response (115). Furthermore, LPS can stimulate a MyD88-independent pathway following internalization of the TLR4-MD2 complex (116). TLR4-MD2 complex utilizes adaptor proteins TIR domain-containing adaptor inducing IFN-β (TRIF), TIR domain-containing adaptor molecule-1 (TICAM-1), and TRIF-related adaptor molecule (TRAM) to activate TNF receptor associated factor 3 (TRAF3) (117–119). TRAF3 activates the kinase TBK1 and IKKε stimulating interferon regulatory factor 3 (IRF3) nuclear translocation, resulting in the production of type-I interferons (116). Type-I interferons are associated with upregulation of anti-inflammatory cytokines IL-10 and IL-27 which inhibit acute phase pro-inflammatory cytokine (TNFα and IL-1) and chemokine (CXCL1 and CXCL2) production (120–122). Nuclear upregulation of both pro- and anti-inflammatory genes corresponds to type-II endothelial activation.

While there is little doubt that LPS plays a key role in driving the inflammatory response during sepsis Gram negative bacteria i.e., *E. coli* can also bind to endothelial cells. McHale et al., demonstrated that the highly conserved outer membrane protein A (ompA) binds directly to aVb3 on endothelial cells in the absence of plasma proteins (123). In this unique interaction, the ompA protein contains the RGD integrin recognition motif that binds directly to the RGD binding site on αVβ3. Similar to *S. aureus* binding to αVβ3, *E. coli* attachment results in loss of barrier integrity causing an increase in permeability and loss of VE-cadherin expression.

ENDOTHELIAL CELL-VIRUS INTERACTIONS

While it generally accepted that virus are capable of binding to and dysregulating the endothelial cell barrier, the mechanisms through which they interact is not well-characterized. For example, the dengue virus envelope protein has been shown to bind to host cell Fc receptors, DC-SIGN (CD209), ICAM3 (CD-50), CD14, mannose receptor (CD206), HSP70/90, GRP78, and heparan sulfate proteoglycans (HSPGs), all of which are expressed on endothelial cells (124–129). In addition, hantaviruses have been shown to bind to endothelial cell αVβ3 which recruits VEGF receptor 2 to activate Src mediated internalization of VE-cadherin. Internalization causes loss of barrier integrity resulting in localized increases in vascular permeability and oedema (**Figure 4**) (130–134).

NOVEL TARGETS IN SEPSIS

As our basic understanding of the molecular mechanisms through which bacteria interact with either platelets or endothelial cells develops, key novel targets that drive dysregulation in both of these cells is becoming clear. While it can be argued that both platelets and endothelial cells may be innocent by-standers in these diseases there is strong evidence to support a role for them in driving the early signals that tips sepsis into a state of excessive and sustained host dysregulation. For example, if, as we have discussed above, platelets respond to pathogens by becoming activated they will aggregate forming micro-thrombi. These micro-thrombi can occlude the micro-circulation in many organs such as liver, kidney and brain. These occlusions cause ischemic damage which, as it accumulates, leads to organ failure. Furthermore, serotonin released from these activated platelets causes severe vasodilation leading to shock (135). The combination of organ damage and shock is what defines sepsis. On the other hand, several studies have demonstrated that upon entry to the bloodstream, bacteria bind to the vascular endothelium within minutes. Bacterial binding causes endothelial cell injury that results in loss of barrier integrity which causes the down-regulation of the critical adherens junction protein VE cadherin. This process facilitates bacterial dissemination to all major organs causing secondary infection and therefore contributing to organ failure. In addition, bacteria binding to the endothelium results in a significant cytokine and chemokine release driving the hyper-inflammatory response during sepsis.

As a result of our improved understanding of the molecular interactions that drive dysregulation in the bloodstream, it has led to identification of key novel targets that could control sepsis better. For example, a number of meta-analysis of retrospective studies showed that patients on aspirin or clopidogrel who develop sepsis have better outcomes than those not on an anti-platelet agent (136). This is despite the fact that patients on an anti-platelet agent are generally much older and sicker (usually anti-platelet agents are used post-MI) than those not on an anti-platelet agent. As conventional anti-platelet agents are designed to inhibit the hemostatic properties of platelets what about a new generation of anti-platelet agent that inhibits the interaction of the platelet with the pathogen? For instance a preliminary study

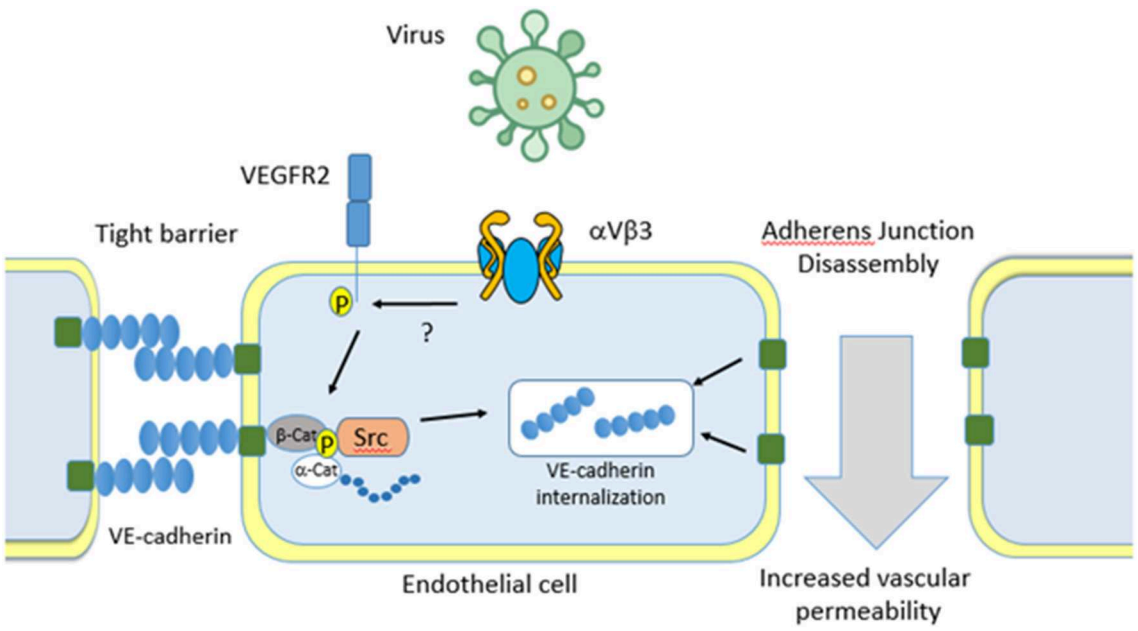

FIGURE 4 | Virus binding to endothelial cells. Virus can bind to the major endothelial cell receptor aVb3. Binding results in recruitment of the Vascular Endothelial cell Growth Factor Receptor 2 (VEGFR2) which activates Src kinase. This directs VE-cadherin internalization, dissociation of adherens junctions, and an increase in vascular permeability. Separation of endothelial cells allows for pathogens to escape the bloodstream and infect major organs which eventually leads to multi organ failure.

has shown benefit of targeting the CLEC-2 ligand podoplanin in mouse models of acute respiratory distress syndrome (44).

There are three approaches to targeting platelets in sepsis. The first is to target platelet activation. This can easily be achieved using existing anti-platelet agents—primarily aspirin and clopidogrel. The advantage is that there is a lot of experience with these agents and both are off patent and thus inexpensive. This is particularly important in treating sepsis in developing countries. There is good evidence to suggest that these agents have benefit in sepsis from retrospective studies and certainly a good quality prospective study is warranted (137, 138). The disadvantage with these agents is the potential for bleeding complications. This is especially true for patients with thrombocytopenia where there is a risk of preserving platelet number at the expense of platelet function. However, the meta-analysis suggests that this risk is more than compensated by the benefit. One factor with the meta-analyses is that patients were already on anti-platelet therapy prior to developing sepsis. This is the ideal situation as it prevents platelets being activated. However, when a patient is diagnosed with sepsis and then given aspirin it might be a bigger challenge as thrombocytopenia will already be established. Thus, they need to be given anti-platelet agents as early as possible to maximize their benefit.

A precision medicine approach can also be used where the platelet receptor that binds to the pathogen is targeted. For instance, GPIIb/IIIa is the receptor for *S. aureus* and thus GPIIb/IIIa antagonists have the potential to prevent platelet activation in *S. aureus*-mediate sepsis (139). However, the difficulty here is that the pathogen must be identified prior to treatment. The big challenge in sepsis is identification of the pathogen. Once identified antibiotic therapy is the only effective solution. Another difficulty is that many of these receptors are involved in haemostasis and thus their inhibition will lead to increased bleeding problems.

A third approach is to target platelet-immune receptors. This has the potential to prevent platelet activation induced by pathogens without compromising their hemostatic properties. Furthermore, they are pathogen-independent—or at least involved with many pathogens. One example of such a strategy is to target FcγRIIa on the basis that most bacteria use it as a co-receptor for platelet activation. Small molecules that inhibit FcγRIIa have been discovered and a monoclonal antibody against FcγRIIa is entering PI studies (140, 141). Such an agent could be given to patients prior to confirmation of sepsis. This would slow the progression of sepsis allowing time for appropriate antibiotic therapy to take effect. Not only would this improve survival it may reduce the incidence and severity of post-sepsis syndrome. Furthermore, as it does not impact haemostasis there is no risk of bleeding with patients and the identity of the pathogen is not necessary.

Similarly, given the unique and critical finding that a growing number of pathogens bind directly to the vascular endothelium using the same receptor, αVβ3, inhibition of this receptor may prevent endothelial injury thus preventing the patient from progressing to shock. In addition, by preventing pathogens from internalizing into endothelial cells may also help reduce the incidence of recurrent infection which is common in sepsis, a step is also partly mediated by αVβ3. Currently there are no drugs available to prevent bacterial attachment to the vascular endothelium and therefore endothelial cell dysregulation is difficult to control, however identification of the molecular interactions between bacteria and the endothelial cells makes it an attractive future target.

CLINICAL IMPLICATIONS—PERSPECTIVES

As described before, platelet activation is a key factor in the pathogenesis of sepsis, but what has been crucially lacking in this regard are (i) widespread acceptance and acknowledgment of the fundamental role of platelets in this area, (ii) the understanding that platelet activation can lead to microthrombi from platelet aggregation which can then lead to single or multiple organ failure, (iii) thrombocytopenia in sepsis is at least partly (or predominantly in authors' opinion) due to platelet aggregation in addition to decreased platelet production and destruction by the micro-organisms, and (iv) inadequacy of the trials using antiplatelet agents in conjunction with the standard therapies in the comprehensive management of sepsis. One of the fundamental issues in translational research in this area has been delineating when the platelet activation is protective in the fight against the infections from the destructive role by forming platelet aggregates and microthrombi. Clarification of the timing when the beneficial role changes to a damaging role can aid in targeting the antiplatelet therapy before organ failure has developed. In addition, identifying which specific receptors and molecular mechanisms are involved in the different infections and at different stages would help in selecting appropriate antiplatelet therapies rather than using the conventional antiplatelet drugs in all cases.

An additional area of interest is dealing with the platelet-endothelial interactions and how they may be perturbed in sepsis (142). Although there have been many studies on the topic in the setting of cardiovascular diseases, it is still early days for clinical interventions in sepsis. Two key experimental trials have shown promise by inhibiting histones and neutrophil extracellular trap formation, which are key players in the platelet-endothelial interactions. Esmon's group showed in an animal model of sepsis that they can protect the host from DIC by specifically blocking the protein, histone H4 (143). A more recent paper noted the formation of cell-free DNA and NETS in sepsis (144). They also correlated with sepsis severity. Importantly, the use of recombinant DNAse could cause the degradation of NETs which could attenuate organ damage in combination with antibiotics.

AUTHOR CONTRIBUTIONS

All authors listed have made a substantial, direct and intellectual contribution to the work, and approved it for publication.

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REVIEW
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Inflammation Induced by Platelet-Activating Viperid Snake Venoms: Perspectives on Thromboinflammation

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Envenomation by viperid snakes is characterized by systemic thrombotic syndrome and prominent local inflammation. To date, the mechanisms underlying inflammation and blood coagulation induced by *Viperidae* venoms have been viewed as distinct processes. However, studies on the mechanisms involved in these processes have revealed several factors and signaling molecules that simultaneously act in both the innate immune and hemostatic systems, suggesting an overlap between both systems during viper envenomation. Moreover, distinct classes of venom toxins involved in these effects have also been identified. However, the interplay between inflammation and hemostatic alterations, referred as to thromboinflammation, has never been addressed in the investigation of viper envenomation. Considering that platelets are important targets of viper snake venoms and are critical for the process of thromboinflammation, in this review, we summarize the inflammatory effects and mechanisms induced by viper snake venoms, particularly from the *Bothrops* genus, which strongly activate platelet functions and highlight selected venom components (metalloproteases and C-type lectins) that both stimulate platelet functions and exhibit pro-inflammatory activities, thus providing insights into the possible role(s) of thromboinflammation in viper envenomation.

Keywords: snake envenoming, venom-induced inflammation, toxins, platelet activation, thromboinflammation

INTRODUCTION

Thromboinflammation and Platelets

Coordinated activation of inflammation and hemostatic responses following tissue injury or invading pathogens is a defense mechanism with an essential role in homeostasis maintenance, leading to a thromboinflammatory response, elimination of pathogens, tissue remodeling, and repair (1). In general, thromboinflammation results from activation of the cascade systems of the blood (the complement, contact, coagulation, and fibrinolytic systems) and activation of a complex multicellular blood system comprising platelets, endothelial cells, and various subsets of inflammatory leukocytes. The functional interdependence among these systems has been largely described, and plentiful cross-talk occurring between different system cascades has been demonstrated (2–5). However, if not properly controlled, activation of these cascade systems will trigger severe thromboinflammatory responses, which can extend systemically and damage remote

organs. In diverse pathological conditions, a pro-inflammatory state combined with thrombotic events can produce multiorgan failure as a consequence of excessive platelet activation, coagulation, and fibrin deposition in the microvasculature (5, 6).

Platelets are the predominant cellular elements in the process of thromboinflammation. These anucleate cells are critical in maintaining hemostasis and in arresting blood loss. Platelets contain three types of secretory granules that are essential to maintain steady state hemostasis: α-granules, the most abundant organelle in platelets, contain proteins endocytosed from plasma, or synthetized by megakaryocytes, such as PF4; dense granules contain nucleotides, serotonin, polyphosphate, calcium and magnesium; lysosomes contain acid hydrolases, such as β-hesosaminidase; and T granules. At the site of a vascular injury, platelets develop a series of highly regulated functions, which include adhesion to endothelial and subendothelial structures, followed by activation and aggregation, constituting the called first wave of hemostasis (7–9). Once platelets become aggregated, the coagulation cascade is initiated by the intrinsic or extrinsic cascades, leading to activation of prothrombin and culminating in thrombin generation. Thrombin, in turn, activates platelets by cleaving protease activated receptors (PARs) expressed on platelets' surface, leading to thrombus formation (10, 11). Thrombin is considered a central mediator of thromboinflammation and plays a central role in propagating microvascular thrombosis and inflammation (12). This mediator affects the vasculature components by cleaving components of coagulation, complement, and fibrinolytic systems, as well as by activating endothelial cells, leukocytes, vascular smooth cells, fibroblasts, and platelets (13–15).

A number of receptors are found on the membrane surface of platelets, such as GPIb-V-IX, GPVI, 5HT2A, TP, α2A, P2Y1, P2Y12, integrin receptors, PARs, and toll-like receptors (TLRs) (16–21). Engagement of these receptors by their agonists triggers functional activities of platelets, being essential for the roles played by these cells in both physiological and pathological processes (22, 23).

Beyond hemostasis and thrombosis, platelets are involved in diverse physiological and pathological processes, including the innate immune response. Yet platelets, like many host defense cells, can detect pathogen associated molecular patterns (PAMPs) via TLRs expressed on their membrane (24, 25), promoting thrombus formation. In response to PAMPs presented via TLR4/MYD88, platelets release microbicidal products (26) and induce neutrophil degranulation and release of neutrophil extracellular traps (NETs) (27). In addition to anti-bacterial functions, NETs display pro-coagulant and pro-thrombotic activities and bind to von Willebrand Factor (vWF), inducing platelet recruitment. Further, neutrophil-derived serine proteases and extracellular nucleosomes induce thrombosis and fibrin formation (28), strengthening coagulation through local proteolysis of tissue factor pathway inhibitor (TFPI). Moreover, platelet-derived PDI promotes the decryption/activation of tissue factor by monocytes, contributing to fibrin generation (29). Therefore, platelets are immune effector cells since they are able to both recognize pathogens via TLRs and communicate with innate immune cells to enhance their prothrombotic functions. Moreover, they act as contact elements between the complement, coagulation, and contact activation systems (30). In this context, a variety of innate and adaptive immune responses and hemostatic disturbances are induced by animal venoms.

Snake Venom

Snake venoms consist of a complex mixture of bioactive molecules, known as toxins, which are delivered in their victims through bites and used for defense or predation. Toxins in turn exhibit a great diversity of chemical composition, including proteins, peptides, biogenic amines, lipids and polysaccharides, and exhibit strong activity and high specificity for their targets, which comprise virtually all physiological systems and tissues.

Venomous snakes belong to four main families, *Viperidae*, *Elapidae*, *Atractaspididae*, and *Colubridae*, and their venoms contain substances with diverse biochemical and biological activities. The *Viperidae* family comprises a medically important group of venomous snakes and accounts for the majority of death and morbidity worldwide compared to the others (31, 32). Snakes of this cosmopolitan family are currently arranged in three subfamilies: *Viperinae*, *Azemiopinae,* and *Crotalinae* (33). The *Crotalinae*, or "pit vipers," subfamily is the most diverse and widely distributed lineage of vipers and comprises species occurring both in the Old and New World (34). In general, viper snakebite envenomation causes systemic and prominent local effects in the victims. Systemically, these venoms induce hemostatic disturbances related to consumption coagulopathy via the action of their pro-coagulant toxins and bleeding, which may evolve into cardiovascular and respiratory failure and death (35, 36). Local effects include inflammation, with edema, pain and conspicuous leukocyte infiltration, hemorrhage, and myonecrosis.

Regarding systemic effects, there are clinical reports describing systemic and venous thrombosis and multiple cerebral, myocardial, and mesenteric infarctions in victims envenomed by *Bothrops* sp. snakes. In the case of envenomation induced by *B. lanceolatus*, histopathologic examination of cerebral, myocardial, and mesenteric small arteries and arterioles showed multifocal thrombotic microangiopathy and platelet aggregates, being endothelial cells found within microthrombi (37, 38).

Coagulopathy induced by viperid snake venoms is a consequence of activation of the clotting pathway by pro-coagulant toxins present in these venoms, resulting in clotting factor consumption. The enzymatic toxins interfering with coagulation are pro-coagulant proteases (prothrombin activators, thrombin-like enzymes, factor X, and factor V activators) and anticoagulant proteases (factor IX and X inhibitors, protein C activator, anticoagulant PLA_2s). The venom components acting on fibrinolysis are fibrinolytic enzymes and plasminogen activators (39–42). Finally, viperid venoms are known to act on platelets, and several venom components show high structural and functional similarities to different natural ligands of the platelet adhesion receptor, thus affecting platelet functions by different mechanisms, including (i) binding or degradation of vWF or platelet receptors, e.g., α2β3 integrin, (ii)

activation of protease-activated receptors (PARs) by thrombin-like enzymes, (iii) modulation of adenosine diphosphate (ADP) release, and (iv) modulation of thromboxane A_2 (TXA_2) formation. Both inhibition and activation of platelets by venom components contribute to venom-induced coagulopathy by depleting platelets, culminating in marked thrombocytopenia (39, 43–45).

Regarding activation of platelets, although the effects of isolated venom toxins have been investigated in detail, the potent activity of whole venoms of several species of *Viperidae* snakes to stimulate platelet function has not been well-established. *B. jararaca* snake venom has been demonstrated to activate platelet PARs, leading to platelet aggregation through the action of thrombin-like enzymes and prothrombin activators (46, 47). In addition, pro-secreting activity of *B. jararaca* on mouse and human washed platelets has been recently described. Release of PF4 and β-hexosaminidase from platelet α-granules and lysosomes, respectively has been demonstrated (45). Increased expression of TF at the site of *B. jararaca* venom injection and in lungs of rats, as well as decryption of this factor, were found in an experimental model of envenomation. In this condition, levels of TF were increased in plasma, indicating that disseminated intravascular coagulation syndrome may occur during *Bothrops* envenomation (48). Similarly, *B. atrox*, and *B. asper* snake venoms are also able to activate platelet PARs by virtue of their thrombin-like enzymes (49, 50). In the case of *B. asper*, the presence of pro-thrombin activators is also evidenced (51). In addition, *Crotalus durissus* snake venoms and those from diverse species of *Trimeresurus* genus have also been demonstrated to activate platelets via the action of their thrombin-like enzyme content (49, 52–54).

INFLAMMATION INDUCED BY VIPER SNAKE VENOM

Inflammation is largely recognized to be closely associated with the onset of local and systemic toxicity induced by *Crotalinae* venoms. Although still incomplete, studies on the inflammatory activities of snake venoms in the *Crotalinae* subfamily, mainly those of the *Bothrops* genus, are robust in comparison with others of the same family and subfamily. Therefore, in this review, emphasis will be placed on the present knowledge of inflammatory reactions induced by *Bothrops* sp. snake venoms.

Bothrops sp. snake venom induces a set of gross inflammatory events, including edema, leukocyte migration, and a complex network of released mediators. These events can progress to either resolution or an excessive and uncontrolled inflammatory response, depending on the volume of venom injected into the victim.

Severe local edema is a relevant clinical finding in *Bothrops* snake-bitten victims (55, 56) that frequently leads to ischemia and neural compression, contributing to tissue loss and disability (57–59). Experimental studies on the dynamics of local inflammation caused by *B. asper* venom have demonstrated early plasma extravasation from small venules and adjoining capillary segments a few minutes after exposure to venom, followed by stasis in the microcirculation (60). The edematogenic response to *Bothrops* venoms was shown to be dependent on local release of well-known synergistic inflammatory mediators, which cause increased vascular permeability and/or vasodilation. Accordingly, the roles of histamine via H1 and H3/4 receptors, serotonin, bradykinin (61–63), platelet activating factor (PAF) (64), prostaglandins (PGs) derived from both cyclooxygenase 1 and 2 pathways (64–66), neurokinins (67), nitric oxide (NO) (68), and α-1 and α-2 adrenergic mediators (61, 69) have been demonstrated in several experimental studies. Participation of anaphylatoxins, including the complement components C3 and C5, in edema formation has also been shown (70–74). The diversity of mediators participating in edema formation induced by *Bothrops* sp. venoms can be partially explained by the ability of these venoms to induce degranulation of mast cells (62, 63). Furthermore, the ability of *Bothrops asper* snake venom to contract collecting lymphatic vessels of mouse mesentery followed by halting the flow of lymph was demonstrated, suggesting that disturbance of the lymphatic system by venom may contribute to development of edema in envenomed tissues (75).

A conspicuous infiltration of leukocytes into the local snakebite is another important characteristic of inflammation triggered by the majority of *Viperidae* viperid snake venoms (55, 56). Leukocytes are essential cells in host defense, and they respond to injury by releasing inflammatory mediators, performing phagocytosis and inducing production of potent microbicidal products. Several experimental studies have demonstrated that *Bothrops* sp. venoms induce time-dependent infiltration of leukocytes into the site of the bite, characterized by an early neutrophil influx followed by a late mononuclear cell infiltrate, that accumulates at the site of venom injection and in adjacent tissues (66, 76–79). Moreover, studies on the mechanisms of *Bothrops* snake venom-induced leukocyte recruitment have supported species-dependent differences in the ability of these venoms to activate the intrinsic chemotactic activities of neutrophils. Whereas, *B. jararaca* neither triggers oriented cell locomotion nor modifies the intrinsic ability of neutrophils to migrate in response to a chemoattractant factor (80), *B. jararacussu* induces neutrophil chemotaxis *in vitro* by a direct mechanism (81). Such variability is explained by the presence of a lectin in *B. jararacussu* venom that recognizes glycoligands on neutrophil surfaces, leading to chemotaxis of neutrophils (82, 83). Moreover, the ability of *Bothrops* sp. snake venom to stimulate expression of the adhesion molecules L-selectin, LFA-1, ICAM- 1, PECAM-1, and CD18 was described in an *in vivo* experimental model in mice (84). This effect was related to the action of inflammatory cytokines and leukotrienes released by the venom acting on endothelial cells and/or leukocytes (84–86).

Cytokines and chemokines are important mediators of cell-cell communication and major mediators of the upregulation of adhesion molecule expression and chemotaxis of leukocytes (87). The involvement of cytokines and chemokines in *Bothrops* snake venom-induced recruitment of leukocytes has been suggested by many to be due to increased levels of Th1 class cytokines, the regulatory cytokine IL-10 and chemokines found at the

site of injection of distinct species of *Bothrops* snake venoms (64, 66, 77, 86, 88–92). Moreover, increased serum levels of IL-1β, IL-6, TNF-α, IL-10, and IFN-γ were found after injection of whole venoms from *B. jararaca* and *B. asper* into mice (93). Accordingly, increased levels of IL-6, IL- 8, TNF-α, MIP-1α, and RANTES were detected in the serum of patients bitten by *B. jararaca* and *B. asper* snakes (94). In addition, the direct action of venoms on leukocytes, primarily neutrophils, and macrophages, lead to production and release of mediators. Release of COX1- and COX2- derived prostaglandins, including PGE_2, PGD_2, and TXA_2 and the 5-LO-derived LTB_4, by neutrophils and macrophages has also been described (85, 86, 94–96). Further, formation of NETs upon stimulation by distinct *Bothrops* sp. venoms was also recently demonstrated (97).

A recent study demonstrated that *B. jararaca* and *B. jararacussu* venoms induce nuclear translocation of the transcription factor NF-κB in human monocytes and mouse alveolar macrophages, and this transcription factor participates in production of IL-1β. In addition, venom-induced activation of NF-κB was shown to be upregulated by PGE_2 but downregulated by LTB_4 (90). Although cytokines and eicosanoids are relevant for driving inflammatory events, the regulatory mechanisms underlying production of each group of mediators by *Bothrops* venoms and the cross-talk occurring between mediators has yet to be further investigated. Moreover, despite the importance of inflammasome assembly in the development of inflammatory processes (98), the potential role of this multiprotein complex in the inflammatory responses to snake venoms is still poorly understood. The beneficial role of the inflammasome in the host response to *Crotalus atrox* was demonstrated early (99), arguing for contribution of the inflammasome platform to the inflammatory effects of viper snake venoms.

In addition to the involvement of diverse inflammatory mediators, leukocyte infiltration induced by *Bothrops* snake venoms has been associated with generation of chemotactic fractions from the complement system. Participation of the C5a fraction of the complement system in *B. asper* venom-induced leukocyte migration was demonstrated in C5-deficient animals (100). The ability of several species of *Bothrops* snake venoms to activate the complement system, generating chemotactic fractions, has been demonstrated (70, 72, 74). In this regard, activation of the complement cascade by 19 different *Bothrops* species from South and Central America was demonstrated to occur by one or more pathways through their action either by directly cleaving C3 and C5 factors or by inactivating the soluble C1 inhibitor, C1-INH factor (71, 72). Moreover, activation of the complement system by *B. lanceolatus* snake venom in human serum has been reported (101).

Another important feature of *Bothrops* sp. venoms is related to their ability to stimulate leukocyte functions, primarily production of microbicidal substances and phagocytosis. Studies have showed generation of reactive oxygen species (ROS) by macrophages and peritoneal leukocytes stimulated with *Bothrops* sp. snake venoms (97, 102). Further, increased production of hydrogen peroxide and nitric oxide by macrophages followed by generation of peroxynitrites induced by *Bothrops* snake venoms (103) and the ability of *Vipera russelli*, a snake of medical importance in Asia, to activate production of ROS in neutrophils were also described. Prolonged or excessive release of leukocyte-derived peroxynitrites and ROS enhances inflammatory processes and potentiates tissue damage at inflammatory sites (104–106). Studies attempting to block the damaging effects of either *B. asper* or *Vipera russelli* snake venoms using different antioxidant compounds and/or inhibitors of NO production did not show similar protective effects (105, 107). Interestingly, the beneficial role of neutrophils in host defense against the noxious effects of snake venoms was demonstrated by studies showing that experimentally induced neutrophil depletion in mice reduced skeletal muscle regeneration after myonecrosis induced by *B. asper* snake venom and a myotoxic phospholipase A_2 isolated from this venom. Absence of these phagocytic cells impaired the late recruitment of macrophages into the damaged tissue, abolishing the clearance of necrotic *debris* (108). In this sense, the role of neutrophils in regulating production of NO and some cytokines and chemokines was further demonstrated in mice injected with *B. atrox* (88).

A number of surface and cytosolic receptors expressed in innate immune cells are able to sense PAMPs, damage-associated molecular patterns (DAMPs), or venom-associated molecular patterns (VAMPs) with high sensitivity and specificity, namely, TLRs (109, 110). Once engaged, these recognition receptors trigger signaling pathways that culminate in the transcription of inflammatory genes related to inflammatory mediators, including cytokines and eicosanoids. Adaptor proteins, such as the myeloid differentiation factor 88 (MyD88), mediate major TLR-activated intracellular signaling pathways (110, 111). Although the importance of TLRs in triggering inflammation is well-recognized, studies on their participation in snake venom-induced inflammation are scarce. Participation of TLR2 in inflammation via the MyD88 adaptor molecule induced by *B. atrox* snake venom was demonstrated in MyD88 knockout mice. These animals showed low infiltration of leukocytes into the site of venom injection and failed to produce Th1 and Th17 cytokines, CCL-2, PGE_2, and LTB_4 (112, 113). Moreover, TLR2 was shown to be involved in the release of PGE2, IL-1β, and IL-10 by macrophages stimulated with phospholipase A_2 (MT-III) isolated from *B. asper* snake venom (114). Consistent with the importance of inflammation in tissue regeneration, the involvement of TLR4 in protection against muscular damage induced by *B. jararacussu* snake venom in mice has been reported (115). Moreover, besides inflammatory mediators, the exudate generated in *Bothrops* venom-damaged tissue was shown to contain a large array of DAMPs derived from the affected cells and extracellular matrix (92). Among the DAMPs identified in exudates of mice injected with *B. asper* venom, several are known to play roles in the inflammatory reaction, coagulation and complement systems and may be critical elements for the overlap between inflammation and thrombosis, thus favoring thromboinflammation. For instance, among identified DAMPs is fibrinogen, a component of the coagulation system with the ability to stimulate chemokine secretion by macrophages through TLR4 engagement (116), potentially favoring thromboinflammation. Furthermore, considering the presence of TLRs on platelets' surface and formation of

DAMPS induced by viper venoms, it is plausible to suggest that thromboinflammation plays a role in the pathogenesis of viper envenomation. Hence, snake venom generates DAMPs by inducing inflammatory events and at the same time stimulating platelets and/or activating coagulation factors or endothelial cells, which may set up a pathway of thromboinflammation. This may thus culminate into formation of microthrombi, which may be critically associated to pathogenesis of snake envenomation.

Inflammation Induced by Snake Venom Toxins

To date, studies reporting venom components endowed with the ability to reproduce both the inflammatory and platelet activating effects induced by the whole venom are few. Among the best studied toxins are metalloproteinases and C-type lectin like proteins.

Snake Venom Metalloproteases

Coagulopathy following snake envenomation is triggered by pro-coagulant isolated toxins, in which metalloproteases play a relevant role by activating platelet function, the coagulation cascade, and fibrinolysis (48, 117–122).

The snake venom metalloproteases (SVMPs) comprise a subfamily of zinc-dependent enzymes of varying molecular mass and can be divided into three classes, depending on their domain organization: P-I, comprising only the metalloproteinase domain; P-II, having a metalloproteinase domain followed by a disintegrin-like domain; and P-III, comprising metalloproteinase, disintegrin-like, cysteine-rich domains and a lectin-like domain linked by disulfide bonds (123). Literature indicates that the precursor of SVMPs can be proteolytically cleaved at various interdomain sites, yielding molecules containing only the disintegrin or only the catalytic domain, followed by the disintegrin domain or even the complete SVMP containing all domains. Thus, the same venom may contain SVMPs with different molecular weights and variable domains (123, 124).

SVMPs have been reported to induce an intense inflammatory response, which has been associated with inflammation induced by the whole venom of *Bothrops* sp. snakes. In this context, Zychar et al. (125) demonstrated that the intensity of edema and hyperalgesia/allodynia, along with the interaction of leukocytes with endothelium, were significantly reduced in animals injected with *B. jararaca* snake venom pretreated with an inhibitor of SVMPs, confirming these enzymes as relevant contributors to the inflammatory reaction seen during *Bothrops* envenomation.

In vivo studies have demonstrated that SVMPs *per se* are able to induce increased levels of pro-inflammatory mediators and migration of leukocytes at the site of injection. After intraperitoneal injection, BaP1, a PI class SVMP from *B. asper* venom, induces increased levels of circulating neutrophils, followed by their infiltration into the site of injection. Participation of LECAM-1, CD18 and LFA-1 adhesion molecules in this effect was demonstrated. Moreover, increased levels of IL-1β and TNF-α in peritoneal washes collected from mice injected with BaP1 was shown (126). In addition, neuwiedase, another PI class SVMP from *B. neuwiedi* snake venom, was shown to induce inflammatory infiltration into gastrocnemius muscle and release of inflammatory mediators, such as KC, IL-1β, and IL-6, after intramuscular injection (127). Recently, the ability of the PI class BpirMP SVMP from *B. pirajai* snake venom to induce paw edema, recruitment of leukocytes and increased levels of nitric oxide, IL-6 and TNF-α in the peritoneal exudate has been demonstrated. Furthermore, these events were shown to be mediated by mast cell degranulation, histamine, prostaglandins, and cytokine production (128). Regarding PIII class SVMPs, the capability of jararhagin, isolated from *B. jararaca* snake venom, to induce infiltration of leukocytes into the mouse air pouch, dependent on activation of macrophages and the proteolytic activity of this SVMP, has been demonstrated (129).

The ability of SVMPs to activate different cell types and trigger pro-inflammatory responses *in vitro* has also been evidenced. Moojenactivase (MooA), a PIII class SVMP from *B. moojeni* snake venom, has been shown to stimulate production of the inflammatory mediators TNF-α, IL-8, and MCP-1 by peripheral mononuclear cells (122). After stimulation with neuwiedase, murine peritoneal adherent macrophages release significant levels of pro-inflammatory mediators, such as KC, IL-1β, and IL-6. Stimulation of satellite muscle cells (lineage C2C12) with neuwiedase caused release of the KC chemokine (127). In addition, HF3, a PIII class SVMP isolated from *B. jararaca* venom, induced phagocytosis of opsonized zymosan particles by eliciting macrophages in a process dependent on engagement of the αmβ2 integrin (130). Another PIII class SVMP, jararhagin, was shown to stimulate production of inflammatory mediators by murine macrophages, increasing mRNA translation for IL-6, TNF, and IL-1 (131). This SVMP also up-regulated gene expression of IL-8, IL-11, CXCL2, IL-1β, IL-6, and mammalian matrix metalloproteases (MMPs)-1 and 10 (132). Regarding activities on endothelial cells, berythractivase, a non-hemorrhagic PIII class SVMP, has been reported to induce an inflammatory state in these cells by upregulating expression of adhesion molecules, such as ICAM-1 and E-selectin, and inducing release of IL-8, NO, and vWF (133).

In addition, the ability of BaP1 to activate classic and alternative complement pathways, generating the chemotactic C5a factor, has been demonstrated. Accordingly, BaP1-treated serum developed the ability to induce chemotaxis of neutrophils *in vitro* (100). In line with these findings, participation of the complement system in the migration of inflammatory leukocytes into the site of injection of PI class SVMP from *B. atrox* snake venom has also been demonstrated (70).

In addition to pro-inflammatory activities, SVMPs can activate platelet functions by diverse mechanisms, including activation of the coagulation cascade, by inducing activation of prothrombin, vWF, factor X, and II and the complement cascade (C5a and C3a release), as well as engagement of platelet glycoprotein receptors. Prothrombin activating SVMPs are classified into A-D groups of prothrombin activators with the ability to cleave prothrombin, generating meizothrombin, which is then converted into α-thrombin, a potent mediator of microvascular thrombosis and inflammation (134–136). The first group of these factors comprise SVMPs isolated from *Bothrops* snake venoms of both PI and PIII classes. Examples

include bothrojaractivase, isolated from *B. jararaca* snake venom (120), insularinase A from *B. insularis* (119), cotiarinase from *B. cotiara* (121), and berythractivase, isolated from *B. erythromelas* (118). Of note, besides inducing release of vWF, berythractivase has also been demonstrated to upregulate tissue factor (TF) expression in endothelial cells *in vitro*, displaying potent systemic thrombogenic activity, which in association with a generation of thrombin and inflammatory mediators, may contribute to thromboinflammation by favoring formation of systemic microthrombi (137).

SVMPs that are able to activate factor X have also been isolated from viper snake venoms and encompass P-III class enzymes (138). Factors II and X, found in the plasma as zymogens, can be activated by SVMPs via proteolytic cleavage of specific peptide bond sites, resulting in blood clotting (139). In this sense, the SVMP moojenactivase (MooA) induces human plasma clotting *in vitro* by activating coagulation factors II (prothrombin) and X, which in turn generate α-thrombin and factor Xa, respectively. Additionally, MooA induces aggregation of washed platelets and expression of TF on the membrane surface of peripheral blood mononuclear cells (122).Tissue factor has been reported to be involved in both thrombosis and inflammation, and several mediators, including pro-inflammatory cytokines and thrombin, induce its expression (140, 141). In this context, MooA is another promising candidate as an inducer of thromboinflammation during *Bothrops* envenomation.

Platelet adhesion and aggregation may be initiated by engaging specific membrane receptors that lead to platelet activation. Botrocetin, an SVMP isolated from *B. jararaca* snake venom, is an example of a potent activator of platelet function because it induces vWF- and platelet glycoprotein Ib (GPIb)-dependent platelet agglutination *in vitro*, resulting in platelet agglutination. Botrocetin appears to act in a two-step manner; first, it binds to vWF, forming a complex, which then binds to GPIb, causing platelet agglutination (142, 143). Moreover, other SVMPs, such as jararhagin, a PIII class SVMP, jaracetin, a differentially processed form of jararhagin lacking the catalytic domain, and the one-chain botrocetin, which corresponds to the disintegrin and cysteine-rich domains of jararhagin from *B. jararaca* snake venom, are able to activate vWF and its binding to platelet GP Ib-IX-V (144, 145). Interestingly, both jararhagin and jaracetin, but not botrocetin, are able to block the adhesion of collagen to platelet integrin α2β1 (145). These data demonstrate that SVMPs of the same class can activate different molecular targets to induce their actions on platelets.

The diversity and abundance of SVMPs in *Viperidae* snake venoms and their ability to induce inflammatory and pro coagulation events make them one the most promising toxin families for studies related to the characterization of venom-induced thromboinflammation.

Snake Venom C-type Lectins

The C-type lectin family comprises proteins that recognize and bind carbohydrates in a Ca^{2+}-dependent manner (146, 147) and non-sugar-binding snake venom C-type lectin-related proteins (SV-CLRPs), also called snaclecs (148, 149). These proteins exhibit the C-type lectin domain (CTLD) but differ from it in a long loop that either contributes to the sugar-binding site or is expanded into a loop-swapping heterodimerization domain between two CLRP subunits. SV-CLRPs connect with a multitude of molecules implicated in hemostasis that are present on endothelial cells, coagulation factors, and receptors of platelets, having a role in thrombus formation and inflammation (150).

With regard to inflammation, interaction of SV-CLRPs with immune cells, such as peripheral mononuclear cells and neutrophils, has been shown with the galactose-binding lectins from the venoms of *B. jararacussu* and *B. leucurus* snakes (82, 151). In addition to inducing edema and increasing vascular permeability in murine experimental models (152), BJcul from *B. jararacussu* venom has been demonstrated to stimulate phagocytosis of zymozan particles by macrophages and increase the lysosomal volume in neutrophils. In addition, BJcuL delays apoptosis of neutrophils and stimulates peripheral mononuclear cells to produce superoxide anions and hydrogen peroxide (82, 151). In human monocytes, BJcul up-regulates expression of antigen presentation molecules and enhances TNF-α, GM-CSF, and IL-6 synthesis by macrophages. Under inflammatory conditions, BjcuL induces macrophages into the M1 state of functional activation and indirectly stimulates T cells to produce TNF-α, IFN-γ, and IL-6 in the presence of LPS (151, 153). Moreover, galatrox, a C-type lectin from *B. atrox* snake venom, has been reported to interact with LacNAc-bearing glycans on neutrophils and macrophages, as well as with extracellular matrix proteins, leading to the release of pro-inflammatory mediators, such as IL-6, TNF-α, and keratinocyte-derived chemokine (KC). In an *in vivo* experimental model, galatrox induced marked neutrophil migration and was shown to induce release of pro-inflammatory cytokines IL-1α and IL-6 in the peritoneal cavity of mice. These effects were mediated by activation of the TLR4-MyD88 signaling pathway by the galatrox carbohydrate domain (154).

Homeostasis of blood coagulation depends on platelet activation, a process closely linked to inflammation (4). In this context, SV-CLRPs have been shown a broad and partially overlap with the platelet receptor-binding spectrum (155–157). Activation of platelets by SV-CLRPs occurs in a non-enzymatic manner, leading to inadequate thrombus formation and vessel occlusion. Conversely, some SV-CLRPs inhibit binding of the physiological ligands, antagonistically preventing the receptor from eliciting signals and resulting in severe bleeding. SV-CLRPs reported to agglutinate platelets via binding to GPIb include agglucetin from *Agkistrodon acutus* venom (158, 159), alboaggregin-B from the *Viperidae* venom *Trimeresurus albolabris* (160–162), mucrocetin and mucetin from *Trimeresurus mucrosquamatus* (163, 164), and jerdonuxin from *Trimeresurus/Protobothrops jerdonii* (164, 165). Agglucetin and alboaggregin-B also bind vWF receptor, but neither increase intracellular Ca^{2+} ions nor trigger platelet degranulation (158, 159). This suggests that platelets can be cross-linked and agglutinated by SV-CLRP-mediated GPIb multimerization, whereas a physiological agonist (vWF) elicits an active signaling process. Stejnulxin from *Trimeresurus stejnegeri* venom has been reported to activate platelets via GPVI (156, 166), a receptor for collagen on the platelet surface. However, EMS16 from *Echis multisquamatus* (166–169) binds to the collagen receptor

of platelets, integrin α2β1, inhibiting the binding of collagen (170–172). Of note, α2β1 has been hypothesized to be responsible for strong adhesion of platelets to collagen, an event necessary for thrombus formation (172).

A platelet activation receptor, C-type lectin-like receptor 2 (CLEC-2), has been recognized as a platelet receptor activated by proteins isolated from snake venoms as evidenced by rhodocytin/aggretin (SV-CLRPs) from *Calloselasma rhodostoma* venom (173). CLEC-2 is expressed in immunocompetent cells, such as dendritic cells, monocytes, and neutrophils, and strongly expressed in platelets/megakaryocytes (174, 175). This receptor triggers strong activation of platelets through the tyrosine kinase dependent pathway. CLEC-2 has a single YxxL motif in its cytoplasmic tail, called hemi-ITAM because it resembles ITAM (tyrosine-based activation motif, YxxL-[X]10-12-YxxL), which has two YxxL motifs. ITAM is a signaling motif found in immune receptors, such as the T-cell receptor and the platelet collagen receptor GPVI–FcRc-chain complex. Engagement of CLEC-2 or GPVI triggers a signaling cascade culminating in platelet activation/aggregation (176) and thrombus formation (172, 177, 178).

Together, the above information evidences the high selectivity of various SV-CLRPs to platelet receptors, which are involved in activation of both platelet aggregation and inflammatory cascades, such as vWF and collagen receptor, known to be generated upon tissue injury.

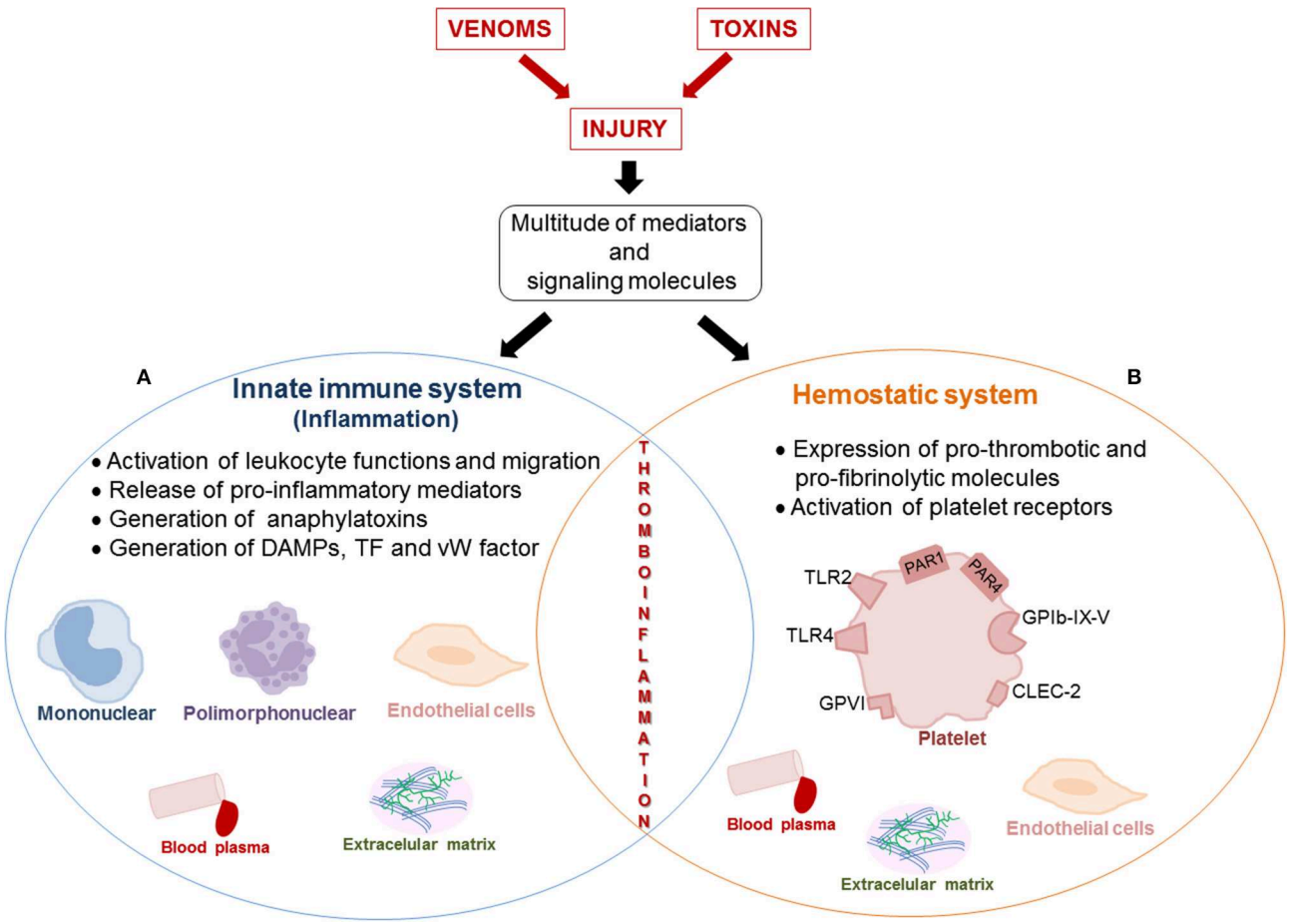

FIGURE 1 | Schematic representation of the interplay between inflammation and alteration of the hemostatic system induced by venom and toxins that can favor thromboinflammation. The viperid snake venoms- and toxins-induced tissue injury trigger defense mechanisms in the victims, characterized by inflammation and hemostatic responses. Release of a multitude of mediators and signaling molecules that simultaneously act in the innate immune and hemostasis systems occurs, suggesting an overlap between both systems during viper envenomation. This interplay between inflammation and hemostatic alterations can characterize thromboinflammation. **(A)** Inflammation induced by snake venoms and toxins is characterized by migration of leukocytes, activation of cells (leukocytes and endothelial cells), which produce and release local and systemic pro-inflammatory mediators (IL-1β, IL-6, IL-8, TNF-α, MIP-1α, NO, histamine, serotonin, PAF, bradykinin, PGE_2, TXA_2, LTB_4, and RANTES), generation of anaphylatoxins (C3 and C5) in blood plasma and DAMPs from extracellular matrix and cellular lysis. **(B)** Venoms and toxins induce hemostatic alterations, including platelet activation and aggregation, thrombin generation and suppression of the fibrinolytic system. Pro-coagulant toxins (prothrombin activators, thrombin-like enzymes, factor X and V activators), activate the expression of pro-thrombotic and pro-fibrinolytic molecules (vWfactor, tissue factor, fibrinogen, C5, and C3), which Interact with surface receptors (PAR-1, PAR-4, GPIb-IX-V, GPVI, TLR2, and TLR4) on platelets. Moreover, the activation of platelets by SV-CLRPs occurs in a non-enzymatic manner by interaction of toxins with the receptor CLEC-2. Several factors and signaling molecules of hemostatic system interfere with innate immune system, resulting in the amplification of inflammatory process and vice-versa.

CONCLUDING REMARKS

The inflammatory response is closely associated with the onset of local and systemic toxicity induced by viperid venoms and their toxins. To date, the mechanisms underlying inflammation and blood coagulation induced by *Viperidae* venoms have been viewed as distinct processes. However, studies on the mechanisms related to inflammation and hemostatic alterations induced by viperid envenomation have evidenced participation of a multitude of mediators and signaling molecules that can simultaneously act in the innate immune and hemostasis systems. Toxins involved in these effects have also been identified, and for some of them, molecular mechanisms of action have been revealed. Therefore, the available information strongly suggests that an overlap between inflammation and hemostasis alterations, regarded as thromboinflammation, may occur during envenomation by viperid snakes (**Figure 1**). In this context, pro-inflammatory cytokines, which increase in production during viper venom-induced inflammation, have been demonstrated to induce hemostatic alterations, including platelet activation and aggregation, endothelial activation, thrombin generation, and suppression of the fibrinolytic system in several pathological conditions. Reciprocally, components of the hemostatic system also interfere with the immune system, leading to inflammation and amplifying this process. Moreover, besides pro-inflammatory activities, some *Bothrops* sp. SVMPs have been described to activate the expression of pro-thrombotic and pro-fibrinolytic molecules by endothelial cells and leukocytes, thus enhancing the coagulopathy seen in viperid snakebites. Similarly, SV-CLRPs activate platelet receptors, which are involved in both platelet aggregation and inflammation cascades. Therefore, SVMPs and SV-CRLPs are venom components selective for the activation of platelet functions and thrombus formation and are potent inducers of inflammation. This additionally argues for development of thromboinflammation during viperid envenomation. However, despite the evidence presented herein, the potential interplay between inflammation and hemostatic alterations induced by viperid snake venoms has never been reported. Therefore, comprehensive studies on the crosstalk between disturbances of hemostasis and inflammatory processes displayed by viperid snake envenomation are stressed. The impact of thromboinflammation on the toxic effects induced by *Viperidae* venoms also requires further investigation. Finally, we believe that this new aspect on the research of snake venom activities may bring renewed understanding of the complex pathology triggered by viperid snake venoms and toxins and may allow the discovery of new therapeutic targets and procedures to confront envenomation mortality and morbidity.

AUTHOR CONTRIBUTIONS

CT, CF, EL, and AC-T contributed to conception and design of the review, wrote the first draft of the review, contributed to text revision, read and approved the submitted version.

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Antibiotic Treatment Protocols and Germ-Free Mouse Models in Vascular Research

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The gut microbiota influence host vascular physiology locally in the intestine, but also evoke remote effects that impact distant organ functions. Amongst others, the microbiota affect intestinal vascular remodeling, lymphatic development, cardiac output and vascular function, myelopoiesis, prothrombotic platelet function, and immunovigilance of the host. Experimentally, host-microbiota interactions are investigated by working with animals devoid of symbiotic bacteria, i.e., by the decimation of gut commensals by antibiotic administration, or by taking advantage of germ-free mouse isolator technology. Remarkably, some of the vascular effects that were unraveled following antibiotic treatment were not observed in the germ-free animal models and *vice versa*. In this review, we will dissect the manifold influences that antibiotics have on the cardiovascular system and their effects on thromboinflammation.

Keywords: platelets, germ-free mouse models, antibiotics, thrombosis, microbiota, vascular function

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INTRODUCTION

During the past decade, microbiome research has started to explore how the densely colonized gut resident ecosystem (microbiota) affects the host's vascular physiology (1, 2). This symbiotic microbial community, whose composition is highly dependent on nutrition, interferes with host metabolism and constitutes a chronic inflammatory stimulus. Nowadays, the various influences of the gut microbiome on vascular inflammatory phenotypes, such as atherosclerosis, myocardial infarction, arterial thrombosis, and stroke, are being increasingly recognized (3–6). Moreover, recent research with germ-free (GF) mouse isolator technology revealed a reduced tendency of arterial thrombus formation in different carotid artery mouse thrombosis models, arguing for a contribution of the gut microbiota to thromboinflammation (7–9).

Indeed, mounting evidence is linking the gut microbiota to the onset of cardiovascular disease and arterial thrombosis (10–12). Among microbial-associated molecular patterns (MAMPs), the microbiota-derived choline-metabolite trimethylamine (TMA) that is produced by gut bacterial TMA-lyases (cutC) and targets the liver, is just emerging as a risk factor for thrombotic manifestations (8, 13–15). Through the action of flavin-containing monoxygenase-3 (FMO3), TMA is oxidized into trimethylamine N-oxide (TMAO) (16), a metabolite that was reported to relieve agonist-induced platelet activation (7). Remarkably, both elevated TMAO plasma levels and the activation of Toll-like receptor (TLR) signaling by MAMPs were shown to accelerate atherogenesis in the apolipoprotein E (Apoe)-deficient mice, which are currently used as an animal model of atherosclerosis (17, 18). Clearly, the influences of the microbiota on atherosclerosis are not limited to the TMAO pathway and pattern-recognition receptors, as additional microbiota-derived factors, such as short chain fatty acids (SCFA), were recently identified (19).

Intriguingly, current research, investigating the impact of the gut microbiota on vascular phenotypes, has revealed discrepancies between broad-spectrum antibiotic treatment protocols and investigations on GF mouse models. To give an example, antibiotic treatment of (*Apoe*)-deficient mice starting at weaning (4 weeks of age) resulted in a reduced development of aortic root lesions at 20 weeks of age, when the mice were fed with a 1.0% choline-rich chow diet (17). Likewise, Kasahara et al. reported reduced aortic root plaque areas together with reduced macrophage and $CD4^+$ cell infiltration of the aortic sinus, when studying GF *Apoe*-deficient mice kept on an irradiated chow diet and analyzed at 20 weeks of age (20). To complicate the picture, the aortic root lesion size of GF *Apoe*-deficient mice kept for 12 weeks on 1.2% choline-rich chow diet starting at the age of 8 weeks was not different compared to conventionally raised (CONV-R) control mice (21). In the study conducted by Wright et al. no differences in aortic root lesion size was reported when GF and CONV-R *Apoe*-deficient mice were fed with a Western diet at weaning until 22 weeks of age (22). Finally, similar to the study of Lindskog Jonsson et al., Stepankova et al. reported increased atherosclerotic lesion sizes in the thoracic aortas of GF *Apoe*-deficient mice after feeding with a low cholesterol diet for 3–4 months (3, 21). These seemingly controversial results could be explained by apparently minor differences in the experimental protocols, e.g., the mouse line studied, the various diets used, the feeding time scheme applied, or the normalization of the measured lesion sizes. On the other hand, it is becoming increasingly clear that the interpretation of data collected after microbiota decimation experiments by antibiotics should be considered with caution, given the experimental variables and the several side effects of antibiotic treatment protocols. It should also be kept in mind that antibiotic decimation of the microbiota using broad-spectrum antibiotics represents a selective pressure that favors overgrowth of resistant bacterial taxa (23), which could in principle be causative at least for some of the described outcomes.

Moreover, recent research has implicated the intestinal microbiota in arterial thrombosis and it has been proposed that selective inhibition of the TMA-generating gut microbial enzymes could lower thrombotic risk (24). While TMAO has been shown to facilitate agonist-induced platelet activation (7), it was demonstrated that microbiota-derived TLR-ligands promote the activation of hepatic endothelial cells, triggering von Willebrand factor (VWF) synthesis and release (8, 15, 25). In mouse models, both pathways promote carotid artery thrombosis and thrombus growth was reduced in the GF mouse model, linking the gut microbiota mechanistically to arterial thrombosis (7, 8, 26). In the literature, in addition to atherosclerotic phenotypes, there are numerous other examples on microbiota-dependent vascular phenotypes that differ between gnotobiotic mouse models and depletion of the gut microbiota with antibiotics.

Here, we provide an overview on GF mouse isolator technology and antibiotic treatment protocols for microbiota depletion that are widely used to study vascular phenotypes. We will explain the limitations of these mouse models, but also describe the recently gained insights on microbiota-driven influences, affecting vascular physiology, cardiovascular disease development and thromboinflammation.

DIFFERENCES AND SIMILARITIES OF GERM-FREE AND ANTIBIOTIC-TREATED MICE

In 1885, Louis Pasteur claimed that a life without microbial associates is not possible (27). Several years later, in the 1940s, the first colonies of GF rodents were established. Since its beginning, the usage of this technology became a valuable model to understand how the microbiota impacts host physiology and disease processes (28). Gnotobiotic animals such as mice colonized with Altered Schaedler Flora (a defined bacterial community) or germ-free (axenic) mice, lacking all microorganisms, are animal models characterized by a defined colonization status (29). In particular, GF mice are bred and kept for their whole lifetime in sterile isolators to prevent their exposure to microorganisms. These animals are a biological model system to either study the complete absence of microbes, or to investigate the effects of colonization with selected and known microbial species (e.g., in the mono-association experiment) (30). However, the GF mouse model is a labor-intensive technology, which requires permanent controls for the hygiene status of the isolators and special facilities (30, 31). As an alternative and more basic method for the depletion of microbiota, administration of broad-spectrum antibiotics is commonly used. In contrast to GF mice, antibiotic application does not lead to the depletion of all microbes, but can selectively deplete different members of the gut microbiota (32). Furthermore, to prevent dehydration during treatment, Reikvam et al. recommended to gavage mice instead of delivering the antibiotics in the drinking water, or to combine both applications (33).

Importantly, decimation of the microbiota affects the anatomy and function of various organs such as liver and gut (34, 35) (summarized in **Figure 1**). One of the most evident anatomical changes is the enlarged cecum, observed both in GF and antibiotic-treated mice (30, 33). Furthermore, GF mice present elongated villus structures, a reduced villus width, and poorly developed capillary networks in small intestinal villi (1, 36, 37). In addition to altered organ morphology and physiology, immune cell populations are influenced by antibiotic treatment (38–40). Because the GF animal model and antibiotic decimation of the microbiota may result in different vascular phenotypes with respect to anatomy and function (8, 17, 41, 42), it is important to be aware of the limitations of these experimental models that enable the exploration of microbiota-host interactions.

ANTIBIOTICS AND THEIR INFLUENCE ON MYELOID CELL FUNCTION

In addition to the resulting selection of resistant bacterial taxa (23), antibiotics severely influence the development of the myeloid cell lineage (40), which may greatly hamper the interpretation of results on vascular physiology. It is firmly

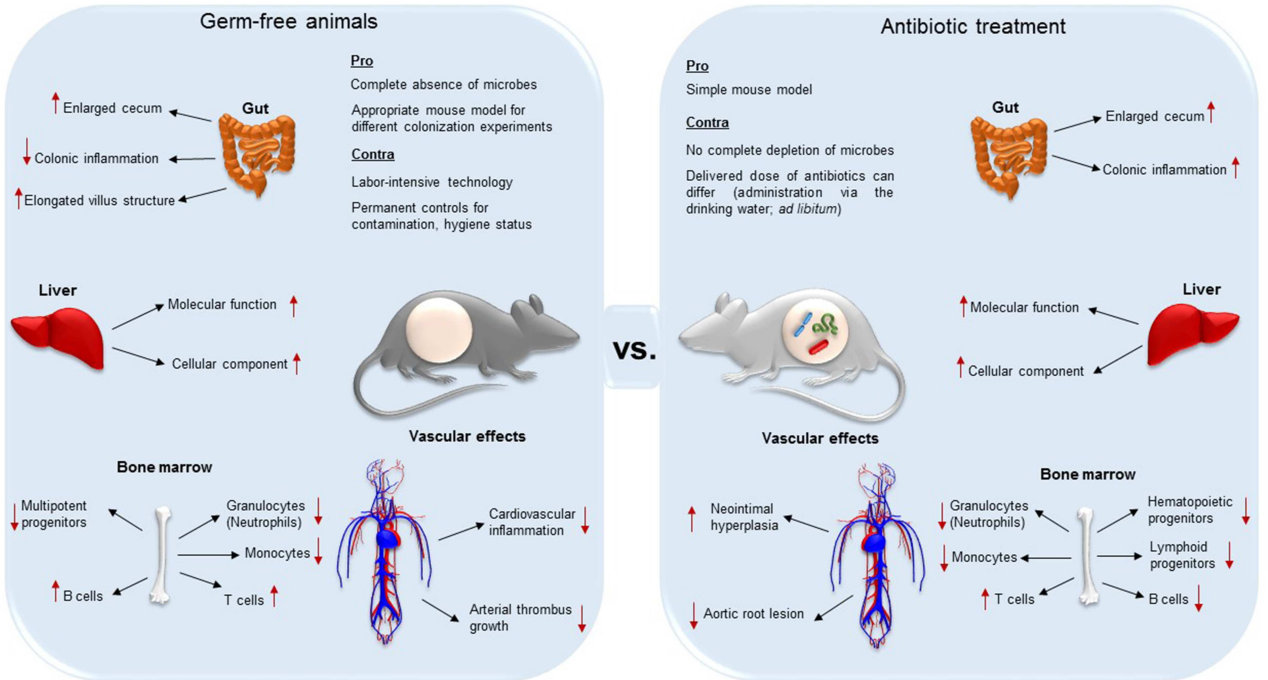

FIGURE 1 | Differences and similarities of germ-free (axenic) and broad-spectrum antibiotic-treated mice (decimation of microbes). While both techniques result in the aberrant enlargement of the cecum, GF mice are less susceptible for colonic inflammation, and present elongated villus structures compared to antibiotic treated animals. In both the animal models, absence of gut microbiota alters protein expression levels in the liver. In the bone marrow, the two mouse models present both reduced granulocytes, monocytes and progenitor cells, but higher T cell levels. On the other hand, while B cells in GF animals are increased, in antibiotic-treated counterparts they were reported to be decreased. Both the complete absence and the decimation of the gut microbiota influences vascular physiology and have an effect on vascular disease. Neointimal hyperplasia, a proliferation and inflammation response to arterial injury, was increased in antibiotic treated rats. Antibiotic treatment leads to a diminished development of aortic root lesion. Additionally, germ-free mice were protected from cardiac inflammation and arterial thrombus growth.

established that long-term treatment with beta-lactam antibiotics results in an inhibition of granulopoiesis (43). In this clinical study, the presence of granulocyte precursors with the lack of well-differentiated myeloid cells was observed in human bone marrow aspirates, discussed by the authors as a myelotoxic effect of beta-lactam antibiotics. Most important, in bone marrow cultures, Neftel et al. described a direct inhibitory effect of beta-lactam antibiotics on *in vitro* granulopoiesis. In addition, thrombocytopenia caused by beta-lactam antibiotic treatment was remarked. Likewise, reduced neutrophil counts were reported in the bone marrow of long-term antibiotics treated mice, which was explained by neutrophil aging promoted by MAMPs derived from the gut microbiota (40).

In contrast, Hergott et al. demonstrated that antibiotic treatment accelerates the turnover of circulating neutrophils and inflammatory monocytes, which showed decreased cell counts in the blood, the bone marrow and the spleen (44). In this work, it was described that long-term antibiotic treatment (i.e. 3–4 weeks) with the frequently used cocktail of neomycin, vancomycin, metronidazole, and ampicillin for microbiota decimation was associated with reduced survival of neutrophils and inflammatory monocytes, which was confirmed in the GF mouse model. This phenotype was explained by microbiota-induced sensing of peptidoglycan of nucleotide-binding oligomerization domain-containing protein-1 (NOD1), and by the production of interleukin-17A (IL-17A) in the ileum (44). The requirement of NOD1 sensing in mesenchymal stromal cells in the reduction of immune cells survival was confirmed in a subsequent study with GF mice, showing that these models presented an overall reduced numbers of hematopoietic stem and multipotent progenitor cells (39). By using the same antibiotic cocktail over 14 days and an alternative antibiotic cocktail containing ciprofloxacin and metronidazole, Josefsdottir et al. demonstrated that not only myeloid cell numbers were dramatically reduced, but also the peripheral blood counts of various lymphocyte subsets along with the counts of hematopoietic progenitors were diminished (B-cells, $CD4^+$ T-cells, $CD8^+$ T-cells) (38). Interestingly, it was demonstrated that the cell cycle activity in hematopoietic stem cells and myeloid progenitors was increased in the antibiotic treated group, excluding a direct myelotoxic effect of the antibiotics used by murine bone marrow culture experiments. Of note, there was no antibiotic effect on the hematopoietic progenitor population and on granulocytes in signal transducer and activator of transcription 1 (Stat1)-deficient mice, indicating that upstream pathways of this transcription factor may play a critical role.

While the obvious question for the fundamental differences between antibiotic treatment and GF housing state with regard to hematopoietic functions is apparent, these important studies clearly demonstrate that antibiotic intervention has manifold effects on innate immune cell types, which may lead to inconclusive mechanistic data in hematologic research. Therefore, gnotobiotic models based on GF knock-out mouse strains are essential to control for the validity of results obtained by antibiotic treatment protocols.

ANTIBIOTICS AND THEIR INFLUENCE ON CLOTTING, PLATELET FUNCTION AND THE VASCULATURE

Antibiotic Treatment Interferes With Vitamin K Metabolism and Clotting Factors

The lipophilic essential vitamin K has two sources: it can be found in green leafed plants as phylloquinone (vitamin K1) and as the microbial metabolite menaquinone (vitamin K2), which is produced by several bacterial species (45–48). Synthesis of clotting factors II, VII, IX, and X and their post-translational modifications in the liver are vitamin K-dependent (45, 46). The correlation between antibiotic treatment and increased bleeding risk due to vitamin K deficiency was already described in 1952 by Dam et al. (47). Over the years, more case reports of hypoprothrombinemic bleeding were published, involving several classes of antibiotics (46, 48–50).

In this context, since 1980s, N-methylthiotetrazole (NMTT) cephalosporins (for example cefoperazone, cefotetan, moxalactam, and cefamandole) administration has been frequently reported to result in impaired hemostasis (45, 46, 50–52). Here, several mechanisms and speculations are discussed in the literature, including: (a), interference of NMTT-side chain with vitamin K metabolism (45), (b), indirect inhibition of vitamin K-dependent blood coagulation (50, 53), and (c), reduction of menaquinone producing bacteria (50, 53). A more recent publication by Fotouhie et al. supports in part hypothesis (c), stating that several risk factors have to concur for the appearance of clinical symptoms (52). The following risk factors were identified: insufficient dietary intake of phylloquinone, modification of normal gut microbial communities via antibiotics, malabsorption of vitamin K, or chronic liver disease (48). Hence, antibiotic treatment protocols may interfere with the synthesis of vitamin K-dependent blood clotting factors.

Antibiotic Treatment and Platelets

Other reasons for impaired hemostasis and thrombus formation following antibiotics intake are thrombocytopenia or impaired platelet function. In the early 1970s, the antibiotic ristocetin got withdrawn from clinical use because it induced platelet aggregation (54). As a result, many research groups then replaced ristocetin administration with vancomycin, as the two molecules share many chemical properties. However, even if vancomycin itself is not able to directly trigger platelet aggregation, it was reported to induce precipitates in which platelets are incorporated (55, 56). A more recent publication revealed the underlying mechanism for vancomycin-induced thrombocytopenia. Towhid et al. described platelet apoptosis paralleled by mitochondria depolarization, activation of caspase-3, cell membrane scrambling and ceramide formation. Further tests revealed that Ca^{2+} is necessary for vancomycin to cause these effects. They hypothesized that the cell scrambling induces an accelerated clearance of platelets from the blood, resulting in the observed thrombocytopenia. Importantly, all the described side effects are induced by exploiting vancomycin concentrations normally reached during standard protocols of antibiotic treatments (57).

Penicillin antibiotics may also have an effect on platelets. Clinically relevant concentrations of penicillin G and carbenicillin seem to have a global effect on platelet membrane receptors, as platelets become less responsive to physiologic agonists and fail to aggregate with bovine factor VIIIa stimulation (45). Subsequently, Pastakia et al. have shown that penicillin G inhibits thrombin-induced upregulation of GPIb-IX levels on the platelet surface (58, 59). In contrast, other beta-lactam antibiotics (ceftriaxone, ceftazidime, and aztreonam) did not inhibit platelet aggregation (60). On the contrary, ceftriaxone (and to a lesser extent aztreonam) was shown to enhance platelet aggregation, but the underlying molecular mechanisms remain unclear. Another antibiotic presenting inhibitory effect on *in vitro* platelet aggregation, even after oral treatment, is metronidazole (61).

The underlying mechanisms for thrombocytopenia (i.e., a circulating platelet count inferior to 150,000/mm^3) are increased platelet consumption/destruction or reduced platelet production. Linezolid, an antibiotic commonly used to treat vancomycin-resistant enterococci and methicillin-resistant *Staphylococcus aureus* infections, is known to induce myelosuppression, but is more frequently associated with thrombo- than pancytopenia (62). It was described that linezolid has no direct toxic effects on platelets and it does not affect the differentiation of hematopoietic stem cells, but via phosphorylation of an enzyme relevant for platelet release, it induces thrombocytopenia (62). Although some influences of antibiotics on platelet functions were identified, the information on effects of antibiotic treatment on the coagulation system remains sparse.

Antibiotic Treatment and Vasomodulatory Effects

Since the 1950s, aminoglycoside antibiotics are known to have hypotensive effects and a negative inotropic effect on the heart (63). Gentamicin was reported to cause hypotension resulting from vasodilatation and relaxation of smooth muscle cells, thus yielding reduced vascular resistance, together with decreased cardiac contraction force and bradycardia. The proposed mechanism is an impaired Ca^{2+} influx. Neomycin, gentamicin and, to a lesser extent, streptomycin, and kanamycin were demonstrated to have vasorelaxant effects on the cerebral arteries of dogs (64). All four antibiotics inhibited vasoconstriction after administering depolarizing concentrations of potassium chloride (64). Two mechanisms were proposed: (a), the inhibition of

phospholipase C (an enzyme that catalyzes the production of the second messenger molecule inositol trisphosphate, which drives the release of Ca^{2+} from the sarcoplasmatic reticulum) and (b), direct interference with Ca^{2+} influx by blocking L-type voltage-dependent Ca^{2+} channels (64). Belus et al. revealed the mechanism for the aminoglycoside antibiotics neomycin, gentamicin, and streptomycin negative inotropic effect. The resting and transient intracellular Ca^{2+} levels of rat ventricular myocytes are decreased, leading to reduced contractility (65). Furthermore, high dosages of vancomycin (glycopeptide antibiotic) and tobramycin (aminoglycoside antibiotic) have relaxing effects on the vascular smooth muscles (66). Not only aminoglycoside antibiotics, but also beta-lactam antibiotics seem to have an influence on Ca^{2+}-influx in the vascular endothelium (60). This indicates that vascular function is significantly affected by the administration of several different antibiotics.

CONCLUSIONS

GF mouse models and antibiotic treatment protocols are frequently used as comparable methods to investigate the effect of gut microbiota in triggering inflammatory vascular phenotypes. In times of spreading antimicrobial resistance, every unnecessary use of antibiotics should be carefully weighed up with the expected findings (67). As pointed out in this review, antibiotic treatment results in significant changes of host physiology, but the underlying mechanisms remain often unclear. Since no standardized antibiotic treatment regimen exists (68) and residual surviving microbiota differs dependent on the animal facility and housing conditions, the reproducibility of this experimental procedure is at stake (69, 70). Therefore, all the functional studies on the gut microbiota exploiting antibiotic treatment should at least mention the limitations of this technique and point out what kind of controls were included. In contrast, the GF mouse model remains the state-of-the-art approach for studying host-microbe and microbe-microbe interactions, since mono-colonization, minimal microbial consortia or humanized microbial consortia are standardized experimental approaches (71). Therefore, if GF mouse technology or gnotobiotic animal models are available and applicable, the use of antibiotic treatment should certainly be reconsidered.

AUTHOR CONTRIBUTIONS

All authors listed have made a substantial, direct and intellectual contribution to the work, and approved it for publication.

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REVIEW
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The Era of Thromboinflammation: Platelets Are Dynamic Sensors and Effector Cells During Infectious Diseases

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Platelets are anucleate cells produced by megakaryocytes. In recent years, a robust body of literature supports the evolving role of platelets as key sentinel and effector cells in infectious diseases, especially critical in bridging hemostatic, inflammatory, and immune continuums. Upon intravascular pathogen invasion, platelets can directly sense viral, parasitic, and bacterial infections through pattern recognition receptors and integrin receptors or pathogen: immunoglobulin complexes through Fc and complement receptors—although our understanding of these interactions remains incomplete. Constantly scanning for areas of injury or inflammation as they circulate in the vasculature, platelets also indirectly respond to pathogen invasion through interactions with leukocytes and the endothelium. Following antigen recognition, platelets often become activated. Through a diverse repertoire of mechanisms, activated platelets can directly sequester or kill pathogens, or facilitate pathogen clearance by activating macrophages and neutrophils, promoting neutrophil extracellular traps (NETs) formation, forming platelet aggregates and microthrombi. At times, however, platelet activation may also be injurious to the host, exacerbating inflammation and promoting endothelial damage and thrombosis. There are many gaps in our understandings of the role of platelets in infectious diseases. However, with the emergence of advanced technologies, our knowledge is increasing. In the current review, we mainly discuss these evolving roles of platelets under four different infectious pathogen infections, of which are dengue, malaria, *Esterichia coli* (*E. coli)* and *staphylococcus aureus S. aureus*, highlighting the complex interplay of these processes with hemostatic and thrombotic pathways.

Keywords: platelet, sepsis, inflammation, infection, dengue, malaria, bacteria, thromboinflammation

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INTRODUCTION

Infectious diseases remain a leading cause of morbidity and mortality worldwide. Low platelet number, termed *thrombocytopenia*, is common in infectious diseases (also referred to at times as sepsis). The mechanisms are often multifactorial, but increased platelet clearance and/or decreased platelet production are common. Sepsis-associated thrombocytopenia has been recognized for many years and is a predictor of adverse outcomes.

Until more recently, the involvement of platelets in the pathogenesis of acute infectious diseases has been less often studied. Part of the reason might be the traditional dogma that platelets are primary effectors of hemostasis and thrombosis, rather than participating in host immune responses against infections. With the expansion of our knowledge in the last decade or so, it is increasingly recognized that hemostasis, thrombosis, and inflammation are tightly interconnected processes and that platelets are often the cell that bridge these three processes.

Classically, hemostasis is referred to as the process of clot formation under normal physiological situations to stop bleeding upon blood vessel damage (e.g., wound). Thrombosis is defined as clot formation under pathological situations (e.g., atherosclerosis plaque rupture, deep vein thrombosis, pulmonary embolism, and stroke – among other examples). Infections, particularly when acute, are known to increase the risk of thrombosis. For example, Kaplan et al. identified a cohort of septic patients, that approximately one-third of patients developed a deep vein thrombosis and/or pulmonary embolism (1–3). Although the reasons that infection trigger thrombosis are multifactorial and incompletely understood, inflammation is thought to be a contributing factor. In particular, there is increasing recognition that inflammation may directly activate the hemostatic systems, resulting in thrombosis (4, 5).

Inflammation is generally considered a process of various immune responses that causes clinical symptoms include heat, pain, redness, and swelling. Similar to thrombosis, inflammation can also be triggered by wounds, tissue damage, and pathogen invasion. The traditional separation of "thrombosis" and inflammation," while helping to understand the key physiological processes upon injury or infection, may hinder an understanding of the complete picture. Moreover, pursuits of novel therapeutics too focused on one aspect of the disease may not be successful (6).

In recent years, words like "thromboinflammation," "immunothrombosis," and "immunohemostasis" have been used to describe responses/mechanisms that are involved in both thrombosis and inflammation (7–11). To our knowledge, "thromboinflammation" was initially used by Blair et al. in 2009 to describe their discovery that the activation of platelet toll-like receptor 2 (TLR2), a receptor best known for its role in triggering inflammation, also promotes thrombosis (7). In 2013, Engelmann used the word "Immunothrombosis" to describe thrombosis "triggered by" or "involved with" innate immune responses (8). While not of our invention, we and others have used "immunohemostasis" to describe hemostatic responses that involves immune players under physiologic situations, as compared to the pathologic property of "thrombosis" (12). To clarify our discussion below, here we will define "immunohemostasis" as an integrated process that includes the classic coagulation, clot formation, as well as the immune responses for pathogen trapping upon blood vessel damage without pathological consequences. In contrast, "thromboinflammation" will refer to pathological responses within the vasculature following blood vessel injury, invasion by a variety of pathogens, or non-infectious inflammatory triggers. We hope that by this definition, the process of thromboinflammation be an umbrella that considers thrombus formation, coagulation system activation, and innate and adaptive immunity as an integrated detrimental process. Thus, thromboinflammation is associated with diseases that are historically under separated categories. Some examples include thrombotic diseases like stroke, deep vein thrombosis, and myocardial infarction, infectious diseases such as bacteremia, viremia, and parasitemia, cancer metastasis through blood vessels, and disseminated intravascular coagulation (DIC).

In the past decade, efforts in better understanding the pathogenesis of infectious diseases have led to new discoveries of the critical roles that platelets have in thromboinflammation. In some settings, platelets may be protective through limiting pathogen dissemination, directly killing pathogens, and eliciting timely and adaptive host immune responses timely. In other situations, however, platelet responses during infection may be harmful. In our current review, we will focus on the interactions between platelets and classic immune cells during infectious diseases. We use dengue, malaria, *Esterichia coli* (*E. coli)* and *Staphylococcus aureus* (*S. aureus*) infections as specific pathogen examples to illustrate the thromboinflammation as an integrated process in which platelets actively participate. Platelets are also involved in many other infectious diseases outside the scope of this review. Readers are referred to several other excellent summaries of this topic (13–19).

PLATELETS ARE VERSATILE PATROLLERS

Platelets are the smallest cells in blood circulation with a diameter of 2–3 μm under resting conditions (20). They are anucleate cells produced by fragmentation of the megakaryocyte extrusions into the vasculature, formed mostly in the bone marrow, although other sites of platelet genesis include the lung, spleen, and liver (21). Differentiated from hematopoietic stem cells or common myeloid progenitors (22–24), megakaryocytes and platelets share many myeloid lineage features, such as the expression of a panel of pattern recognition receptors (PRRs), phagocytosis of exogenous antigens, interactions with other immune cells, and the release of chemokines and cytokines upon activation (15, 20, 25, 26). Having a life span of 7–10 days in healthy humans and about 5 days in the mouse, it is estimated that about 100 billion platelets are produced every day in humans, with about 2 billion a day in mice (20). Platelets are mighty patrollers that constantly scan over the vascular endothelium and circulating leukocytes in a "touch and go" manner (27). Upon activation, platelets rapidly undergo massive plasma membrane extension (spreading), become activated, translocate and/or express multiple receptors on their surface that further enhance their aggregation with nearby platelets or leukocytes, or directly bind to and sequester extracellular pathogens (20). Platelets also degranulate and release pre-packed (or in some cases) newly-synthesized microbicide proteins and chemokines that facilitate pathogen destruction, signal immune cells, and promote inflammation (20). The reader is also referred to a recent review by Rossaint et al. that provides more detailed information on the platelet receptors and chemokines involved in inflammation (28).

Work from our group and others demonstrates that during pathogen invasion, platelets have alterations in their transcriptome and proteome that augment host defense

mechanisms (29–31), although these changes may also result in adverse outcomes in some settings. These and other features make platelets effective and dynamic sentinels against bloodborne pathogens.

DENGUE VIRUS

Dengue virus (DENV) is a mosquito-borne, positive sense, single-stranded RNA virus of the Flaviviridae family, with five serotypes documented so far (32, 33). The annual incidence of dengue is estimated to be 390 million globally, with about 100 million individuals having clinically apparent symptoms (34). While in most individuals the disease is self-limited with a high fever as the only symptom, about 10% of patients have thrombocytopenia and hemorrhagic symptoms (termed dengue hemorrhagic fever, DHF). In severe cases of dengue infection, patients may develop dengue hemorrhagic shock (DHS) (35). Morbidity and mortality rates in these last two types of dengue infection can be rather high. Emerging and established studies highlight that platelets are implicated centrally in the pathogenesis of the disease. **Figure 1** summarizes some of the responses of platelets during dengue infection.

Dengue virus can directly bind and activate human platelets via multiple receptors. The direct interaction was suggested from observations of dengue virus RNA and dengue-like particles in platelets from patients with dengue (36). Later studies demonstrated that the direct interactions between dengue and platelets are mediated through dendritic cell-specific intercellular adhesion molecule-3-grabbing non-integrin (DC-SIGN) and heparin sulfate proteoglycan receptors (HSPG) on platelets (37, 38). Intriguingly, the expression of DC-SIGN on platelets appears to decrease in some patients as dengue infection resolves (39). In addition, Chao et al. showed direct platelet activation by DENV nonstructural protein 1 (NS1) through interactions with TLR4 and TLR2 (40). Recently, Sung et al. showed DENV induces platelet activation via CLEC-2 (41). Following DENV binding, platelets become activated, undergo a series of changes that further amplify the thromboinflammation, which will be discussed later.

Similar to myeloid cells, platelets also express Fc receptors that are capable of binding IgG opsonized DENV complexes. This mediates, in part, the development of thrombocytopenia and DHF/DHS in patients infected with DENV. Fc receptors have recently been shown to play a central role in antibody-dependent enhancement (ADE) during dengue infection. ADE is the clinical setting whereby individuals previously exposed to (or immunized against) one dengue serotype have an increased risk of more severe dengue (e.g., DHF/DHS) when they become infected with a different dengue serotype (42, 43), suggesting an intimate relationship between antibody mediated inflammatory responses and platelets. The work by Katzelnick et al., which included a large cohort of more than 8,000 patients with dengue, reported that ADE and severity of dengue is associated with DENV-antibody titers between 1:21 and 1:80 (43). Moreover, during the early phase of DENV infection, the glycosylation of Fc regions and the ratio between IgG subclasses (IgG1/IgG2) appears to regulate platelet counts (42). Human platelets express FcγRIIA and FcγRIIIA receptors that can engage both IgG1 and IgG2 (31, 42, 44–46). Increased afucosylation (the absence of fucose on the Fc glycan) of the CH2 domain of the Fc region of both IgG1 and IgG2, together with an increased IgG1/IgG2 ratio, leading to altered binding affinity between IgG and FcγR receptors on platelets (42). In humanized FcR transgenic mice, this results in significant thrombocytopenia (42). Following binding of IgG immune complexes to platelets, platelets degranulate and release stored serotonin; this pathway is downstream of FcγRIIA signaling (47). In some settings, increased circulating levels of serotonin can contribute to systemic shock (47, 48).

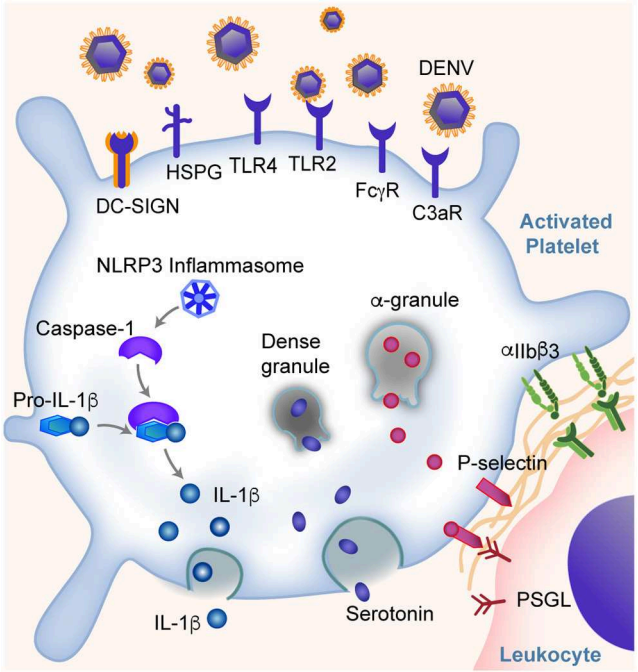

FIGURE 1 | Platelets are key sentinel and effector cells during dengue infection. Platelets can directly bind dengue virus (DENV) and virus: IgG immune complexes through DC-SIGN and other receptors as listed. This binding leads to altered gene and protein expression in platelets and the activation of platelets. Some of these responses include the expression of IFITM3, assembly of NLRP3 inflammasomes, production and release of IL-1β, secretion of α-granule and dense granule contents, translocation of P-selectin to the platelet surface (allowing platelet-leukocyte interactions and signaling), and integrin $\alpha_{IIb}\beta_3$ activation. Changes in platelets further trigger the platelet aggregation and thrombosis, endothelial inflammation and vascular leakage, and monocyte activation and cytokine production, and more. These responses span the classic pathogenesis of thrombosis and inflammation, and may contribute to hemorrhagic symptoms and shock in some patients.

Upon DENV infection, platelets can also be activated indirectly by complement C3, serotonin, or PAF. For example, platelets express complement receptor C3R (49, 50). Platelets from patients infected with dengue have increased IgG and C3 binding that is associated with increased platelet activation and clearance. This could not be completely blocked by FcγRIIA inhibition (51, 52). Moreover, platelets could be activated by serotonin via $5HT_{2A}$ receptors, which leads to increased platelet

clearance and development of thrombocytopenia (53). Recently, Masri et al. showed that the serotonin is mainly synthesized in mast cells during DENV infection and subsequently internalized by platelets via $5HT_2$ receptors (53). Similarly, PAF receptor (PAFR) deficient mice also exhibited elevated platelet counts and decreased vascular permeability after DENV-II inoculation, as compared to wild type (WT) mice, suggesting that PAFR is also involved in the development of thrombocytopenia (54, 55). This further supports our notion that platelets have many myeloid cell features that mediate thromboinflammation.

Following activation of platelets during dengue infection, the global gene expression, proteomic, and lipodomic profilings of platelets are all altered to facilitate viral clearance (56–58). Unbiased next-generation RNA sequencing of platelets from patients and in megakaryocytes infected *in vitro* with dengue virus by recent work from Campbell et al. (56) suggests that megakaryocytes sense dengue infection and/or agonists generated during infection, and in response alter the repertoire of mRNAs invested into newly produced platelets (56). One of transcripts significantly upregulated in megakaryocytes and platelets during DENV infection was interferon-induced transmembrane protein 3 (IFITM3). IFITM3 protein was also increased in platelets from patients infected with DENV and cultured megakaryocytes exposed to DENV *in vitro*. IFITM3 in other cells restricts viral replication, therefore enhancing resistance to DENV infection (56). Interestingly, IFITM3 induction in human megakaryocytes not only reduced DENV infection of megakaryocytes but also reduced DENV infection in stem cells in the surrounding bone marrow niche. This study highlights the ability of megakaryocytes to participate effective in innate antiviral immune responses.

As mentioned above, through DC-SIGN and HSPG, TLR4, CLEC-2, and other receptors, DENV could activate platelets, cause thrombocytopenia and thromboinflammation in patients. Platelet activation is associated with the severity of thrombocytopenia and increased risk of DHF in patients with DENV (37, 51, 59, 60). A hallmark of platelet activation is the conformational change of integrin $\alpha_{IIb}\beta_3$, which binds to fibrinogen and von Willebrand factor (vWF), triggering platelet adhesion to vascular endothelial cells and causing thrombosis (61). Activation of platelets also triggers degranulation and the release of a number of proteins including P-selectin, PAF, soluble CD40L (CD154), and serotonin—proteins that are often dichotomized into either pro-thrombotic or pro-inflammatory, but which could fall together under the term "thromboinflammation" (54, 55, 62, 63). P-selectin on platelets engage its ligand, P-selectin glycoprotein ligand (PSGL) on leukocytes, promoting proinflammatory cytokine production such as IL-1β and IL-8 by both platelets and monocytes (40, 62, 64). These cytokines induce enhanced permeability of endothelial cells and vascular leakage, and are associated with an increased risk of DHS in patients (64). P-selectin also tether platelets to PSGL on endothelial cells, and induces vascular endothelial damage (65, 66). Moreover, serotonin not only contributes to the development of thrombocytopenia. The stored serotonin could be released from a large number of platelets following platelet activation, lead to an increased concentration of serotonin in circulation, cause vasodilation and hypotension, and development of systemic shock (47, 48). Upon activation, platelets undergo apoptosis, with decreased mitochondrial potential, assembly of inflammasomes, increased phosphatidylserine (PS) exposure and P-selectin expression on the cell surface (37, 38, 67). This process further catalyzes thromboinflammation. For example, the apoptosis of platelets not only increases the phagocytosis of platelets by monocytes, but also triggers activation of the coagulation system, generation of thrombin, and formation of thrombi (68–70). In addition, during DENV infection, the release of angiopoetin-1 by platelets is reduced. This is associated with dampened inhibition of angiopoetin-2, and increased endothelial damage in patients with DHS (71).

Dengue activated platelets are associated with increased assembly of inflammasome NLRP3, which activates caspase-1 and triggers apoptosis within platelets, and promotes platelet aggregation (67, 72). This leads to a reduced platelet lifespan and contributes to the development of thrombocytopenia in dengue patients (63, 67). The NLRP3 inflammasome assembly in platelets also leads to the synthesis of IL-1β by platelets and its subsequent secretion into plasma and packaging into microvesicles (67). Platelet activation and apoptosis is higher in patients with DHF than without DHF, and correlates with *in vitro* phagocytosis of platelets by macrophages through a phosphatidylserine-recognizing pathway (37, 51). Furthermore, NLRP3 and FcγRIIIA have been shown to induce dengue-triggered hemorrhage in mice synergistically (73). NLRP3 has also been correlated with increased low-density lipoproteins (LDL) and decreased high-density lipoproteins (HDL), suggesting extravascular effects to host lipid metabolism following NLRP3 activation (74). However, the role of platelets in this pathological process remains unclear.

In addition to antibody-dependent platelet activation, platelets may also engage antigen specific T cells by presenting dengue antigens through MHC class I. For example, a proteomic study by Trugilho et al. revealed proteasome subunit proteins and HLA class I antigen presentation pathway proteins as the most significantly upregulated in platelets during dengue (57). In addition, platelets express several T cell co-signaling ligands, such as CD40, CD86, ICOSL, and upon activation, are capable of cross-presenting exogenous antigens *in vitro* and stimulating antigen specific T cell responses (75). It seems possible that following direct binding of virus or through FcγRIIA, platelets internalize dengue virus, degrade dengue antigens in their immunoproteasomes, and present antigen peptides through HLA class I for recognition by $CD8^+$ T cells.

Platelets are also involved in other pathological mechanisms of dengue infection. For example, platelets may increase endothelial barrier permeability due to decreased S1P levels, or promote DENV replication in monocytes through the release of platelet factor 4 (PF4, also known as CXCL4) (64, 76–78). Platelet-derived microparticles may also play a role in the pathogenesis of the disease, as they are notably increased in thrombocytopenic patients without bleeding, and decreased in thrombocytopenic patients with bleeding (67). A summary of some of these identified mechanisms can be found in **Figure 1**.

MALARIA

Malaria, also a mosquito-borne infectious disease, is caused by systemic infection with parasites of the *Plasmodium* group. In humans, five species of *Plasmodium* have been identified that cause malaria: *P. falciparum*, *P. malariae*, *P. ovale*, *P. vivax*, and *P. knowlesi*. Of these, *P. falciparum* is the most common (79). Malaria has been a major health concern worldwide for decades, with an estimated incidence of more than 200 million cases a year (79). The parasitic sporozoites are transmitted from the mosquito's saliva into human bloodstream, and subsequently infect hepatocytes where they mature into schizonts. Eventually, the infected hepatocytes rupture, releasing merozoites into the bloodstream. *Plasmodium* then invade erythrocytes, replicating until the cells burst. This cycle is repeated, typically causing fever each time the erythrocytes burst.

Common characteristics of malaria include headache, cyclical fevers, anemia, and thrombocytopenia. In the study by de Mast et al. healthy volunteers were infected with *P. falciparum* and developed thrombocytopenia at the earliest phase of blood-stage infection (80). Thrombocytopenia is so common in patients that platelet count has been proposed as an affordable and fast diagnostic test for malaria in low-income regions, with a reported sensitivity of 60% and specificity of 88% (81–83). Recently, Gardinassi et al. integrated plasma metabolomic data and whole blood transcriptomic data obtained from volunteers infected with *P. vivax* (84). They found that platelet activation together with changes in IFN signature modules and T cell signaling are the top three most significantly changed processes (84).

A major cause of morbidity and mortality in patients with malaria is cerebral malaria (CM) (85, 86). Increased platelet accumulation has been documented in the brain microvasculature of children who died of CM, as well as in animals with experimental cerebral malaria (ECM) (85–89). These platelets were often found aggregated with Plasmodium parasites or leukocytes, together with increased vWF staining (18, 90–92). Platelet activation and thrombosis may precede leukocyte infiltration (88). Moreover, recent proteomic studies performed on postmortem brain tissue obtained from CM patients revealed that proteins involved in platelet activation and coagulation were upregulated (93). Thus, platelets and leukocytes (as well as platelet-leukocyte aggregates) are associated with CM. **Figure 2** highlights a schematic representation of some of the interactions between activated platelets and other circulating blood cells during malaria infection.

Both platelets and platelet derived microparticles (PMPs) can directly bind *Plasmodium* infected RBCs (PRBCs) (88, 89, 95, 96). This binding is mainly mediated by CD31 (PECAM-1) and CD36 (GPIV) on platelets and the *P. falciparum* erythrocyte membrane protein 1 variant on PRBCs (89, 95, 96). After binding, platelets can directly kill the intraerythrocytic Plasmodium *in vitro* (97, 98). In patients, platelets can directly kill all major malaria parasites *in vivo*, and the decreased platelet count has partially been attributed to the increased binding to PRBCs (99). In some—but not all—studies, the severity of thrombocytopenia has been associated with increased parasitic density and adverse outcomes (98, 100, 101). The significance of platelets in parasite clearance remains somewhat uncertain, as the physiological platelet to RBC ratio ranges from ∼1:10 to 1:50, and platelet-bound PRBCs have been found in less than 5% of the total PRBCs in patients infected with malaria (98, 99, 102). Nevertheless, considering that about 100 billion platelets are produced daily (for comparison, about 200 billion RBCs are produced daily), and platelet-mediated parasite killing could be occur very rapidly (e.g., minutes), it remains plausible that platelets contribute to parasitic killing in humans, directly or indirectly. This remains an area of active investigation in the field.

One chemokine implicated in platelet-mediated parasite clearance is PF4. Following direct platelet-PRBC binding, platelets release PF4 which engage the Duffy antigen receptor (Df) on PRBCs. This engagement induces disruption of the parasite digestive vacuoles and terminal deoxynucleotidyl transferase deoxyuridine triphosphate nick-end labeling of parasite nuclei (TUNEL+), without lysing PRBCs (99, 103). Following this initial discovery, subsequent studies have shown that PF4-driven antiparasitic activity is mainly mediated by the C-terminus of PF4. A synthetic PF4 derived cyclic peptide, named cPF4PD, showed promising killing of parasites in PRBCs without lysing normal RBCs (104, 105). Recent work by Wang et al. showed that the host can upregulate PF4 production in malaria by activating the transcription factor E74 like ETS transcription factor 4 (ELF4) in megakaryocytes (106).

However, the PF4 released from platelets is not entirely protective, as in some settings PF4 release may trigger inflammation. For example, in a mouse model of experimental cerebral malaria (ECM), platelet accumulation and PF4 release in the brain led to significantly increased blood brain barrier permeability and T cell infiltration. In these models, PF4 deficient mice have decreased T cell infiltration and were protected from the development of ECM (89). Further studies showed that platelet-derived PF4 activated the transcription factor KLF4 in monocytes and promoted monocytes to produce proinflammatory cytokines such as IL-6 and TNFα, which are important in the pathogenesis of ECM (88).

Platelets also have been shown to participate in thromboinflammation during malaria infection through other mechanisms. For example, platelets are the major source of IL-1β, which protects against ECM development in experimental models of malaria (107). Additionally, plasma vWF levels (including ultra-large vWF multimers) are increased in patients with malaria (18, 92, 108, 109), also associated with increased GPIb shedding from platelets and the development of thrombocytopenia (110). The readers are referred to several excellent reviews on this topic (18, 81, 111).

GRAM-NEGATIVE BACTERIAL INFECTIONS

Bacteria are commonly classified into Gram-positive and Gram-negative species based on their cell wall structures. Gram-positive bacteria feature a thick layer of peptidoglycan, which is evident by Gram staining. Gram-negative bacteria have a thin layer of peptidoglycan covered by a layer of

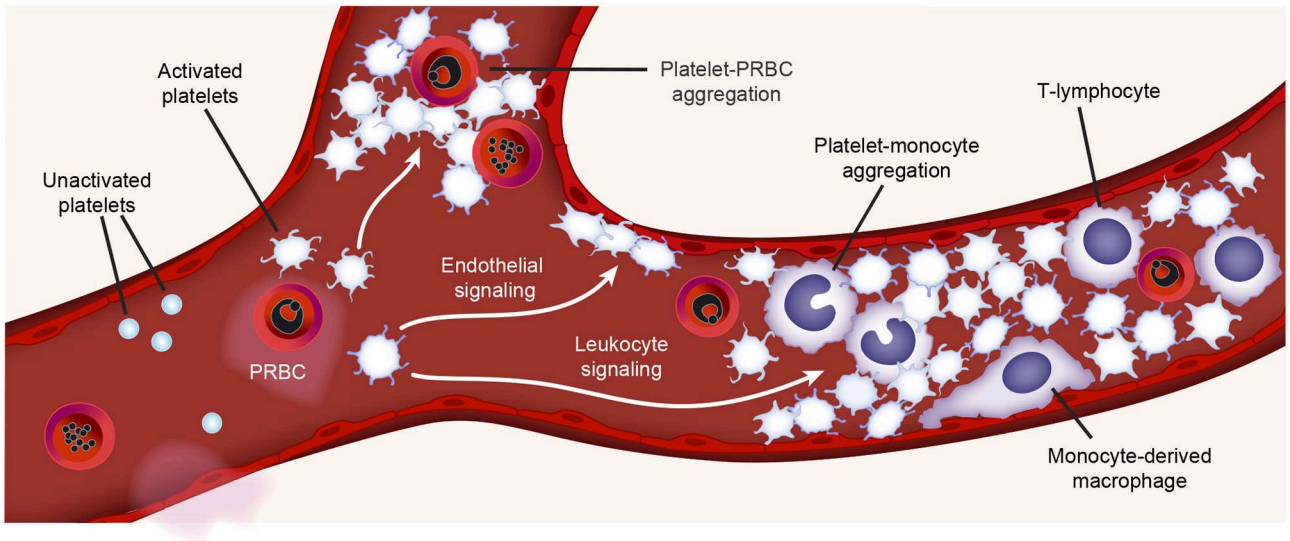

FIGURE 2 | Platelet activities during malaria infection. During malaria infection, platelets may be found in the vasculature closely surrounding plasmodium-infected RBCs (PRBCs) or interacting with monocytes (platelet-monocyte aggregates) or lymphocytes. Toxins generated during malaria infection (e.g., hemozoin) may active platelets. Platelet interactions with monocytes trigger pro-inflammatory cytokine synthesis by monocytes. Figure is adapted from Rondina et al. (94) with permission obtained from Elsevier.

outer membrane and lipopolysaccharides (LPS). Infection by pathogenic bacteria can cause thromboinflammation locally or systemically, such as via endothelium barrier damage and lung edema, intestine inflammation and diarrhea, platelet activation, and thrombocytopenia, coagulation activation and disseminated intravascular coagulation (DIC). In experimental models of infection, depleting platelets prior to infection often leads to increased mortality, suggesting an important role of platelets in host survival during bacterial infections (112–116).

The Gram-negative bacterium *E. coli* is a major cause of urinary tract infections. Strains of *E. coli*, as well as LPS purified from *E. coli* are widely used in research and these tools have improved our understanding of many host immune responses in platelets and other cells. We and others have shown direct interactions between platelets and *E. coli in vitro* (117). Direct interactions between platelets and *E. coli* have been captured *in vivo* recently using super-resolution microscopy (118). These investigators captured real-time, *in vivo* platelet migration toward *E. coli* in mouse liver sinusoids within hours after infection, as well as *in vitro* platelet migration toward and bundling of *E. coli* bacteria within minutes (118).

Human platelets express toll-like receptors (TLRs) 1–10 (at the mRNA and/or protein level), and mouse platelets express TLRs 1-8 (31, 119–121). Of these, TLR4, an LPS specific receptor, was the first TLR well characterized on platelets (122). In macrophages and dendritic cells, LPS activates the TLR4/MyD88 signaling, triggers inflammation (123, 124). In platelets, LPS activates the TLR4/MyD88 and signals downstream via cGMP/PKG-dependent pathway (125). Administration of LPS to rats resulted in platelet activation and adhesion to the endothelium through P-selectin and integrin GPIb receptors and the development of thrombocytopenia in rats within 30 min (122). Subsequently, Cognasse, Andoneigui and Aslam et al. showed independently that both human and mouse platelets have functional TLR4 on their surface (113, 121, 126).

Upon LPS stimulation and TLR4 activation, a number of events happen to platelets that cause thromboinflammation (127). LPS stimulation signals to human platelets to process tissue factor (TF) pre-mRNA into mature mRNA, with subsequent increases in TF protein, which is procoagulant (117, 128). This is a unique bacteria stimulated platelet response that has been not reported in any other infectious diseases. LPS also induces some other changes in platelets as we have mentioned above. Similar like activation during DENV infection, platelets can also be activated and undergo apoptosis upon LPS stimulation (129), and triggers the secretion of thrombo-inflammatory factors, including P-selectin at low LPS concentrations and CD40L, TNF-α, and PF4 at higher LPS concentrations (127, 130, 131). From an evolutionary perspective, this might serve as an adaptive rheostat to the host, perhaps by limiting the scale of thromboinflammation when the bacterial load is low (125, 126, 128, 131). Moreover, LPS stimulates platelets to opsonize IgG immune complexes and enhances the phagocytosis of platelets by macrophages, suggesting there may be a synergistic effect of TLR4 and FcR on platelets (132). LPS also stimulates platelets to interact with neutrophils through the interactions between P-selectin, PSGL-1, and lymphocyte function-associated antigen 1 (LFA-1) (112, 114, 133). Neutrophils reciprocally trigger platelet activation and thromboxane generation during *E. coli* infection (114). In addition, upon LPS stimulation, platelets maintain vascular integrity and prevent leukocyte infiltration via CLEC-2 (116).

Upon Gram-negative bacterial infections, platelets are actively involved in the generation of neutrophil extracellular traps (NETs) (112). NETs are extracellular lattices of chromatin, histones, and granule enzymes extruded by neutrophils upon activation (**Figure 3A**), via a unique process termed "NETosis" (134). NET formation is an efficient mechanism for trapping both Gram-positive and Gram-negative bacteria. Platelets mediate key aspects of NET formation. Although neutrophils can form NETs without platelets when infected with Gam-positive or Gram-negative bacteria, platelets are able to significantly expand the surface area of NETs *in vitro* (112). *In vivo* depletion of neutrophils or platelets impairs NET formation and significantly impedes bacterial clearance (112, 135).

Multiple surface receptors on platelets and secreted factors by platelets regulate NET formation. For instance, platelet-derived P selectin plays an important role in the early stage of NETosis, facilitating platelet-neutrophil direct interactions (114, 136). Platelet GPIbα, a well-studied receptor in hemostasis and thrombosis, has been found recently triggers the activation of neutrophils and extracellular vesicle release from neutrophils (114). In addition, another important receptor in hemostasis and thrombosis, the integrin αIIbβ3 on platelets, this also mediates NETosis, as the deficiency of integrin αIIbβ3 impairs NETs formation (135). Although NETs trap bacteria efficiently, exaggerated or misplaced NET formation may also be deleterious. Histones, serine proteases, and cathepsin G released by activated neutrophils can activate platelets, promoting coagulation, endothelial damage, and thrombosis (133, 137–139). Understanding the roles of NET formation in adaptive and maladaptive host responses remains an active area of investigation. We refer the reader to the recent review article by Zucoloto et al. for more detailed discussions on the platelet-neutrophil interactions that illustrate the intimate relationship between inflammation and thrombosis (19, 140).

Another example of the versatility of platelets and thromboinflammation as an integrated process is the synergistic signaling of FcγRIIA receptors and platelet integrin αIIbβ3 (a canonical hemostatic receptor) upon *E. coli* infection. In addition to activation through TLR4, platelets could also be activated by *E. coli* via FcγRIIA receptors, provided simultaneous integrin αIIbβ3 signaling (45). Either absence of IgG or blockage of αIIbβ3 signaling would abolish the aggregation of platelets when incubated with *E. coli* (45, 141). Recent work by Palankar et al. demonstrates that human platelets directly kill *E. coli* in mechanisms that require FcγRIIA and PF4 (142). As with malaria, PF4 is also central to effective *E. coli* killing. Disruption of platelet cytoskeletal functions also reduced the efficacy of *E. coli* killing by platelets. Moreover, the complement C3 opsonization of E. coli facilitates the formation of platelet-bacteria aggregates which is important in the induction of adaptive immune responses (143). Together, these findings suggest that platelets accumulate on bacteria, releasing antimicrobial α-granule contents that effectively kill *E. coli* (142). While not a central focus of this review, platelets interact with other leukocytes, including monocytes, forming stable platelet-leukocyte aggregates that promote the release of agonists from platelets and subsequent pro-inflammatory cytokine synthesis by monocytes (**Figures 3B,C**). Interestingly, in aging (where the risk of infection rises substantially), these interactions may be upregulated—potentially contributing to cytokine release injurious to the host (144). Whether these aging-dependent responses contribute to adverse clinical outcomes in sepsis remains an area of active investigation.

Bacteria also possess endogenous mechanisms to counteract host defenses. Some of these directly affect human platelet activities. As one example, work from our group demonstrated that pathogenic *E. coli* bacteria isolated from infected patients induced platelet apoptosis via calpain-mediated degradation of the cell survival protein Bcl-x_L (145). This was accompanied by impaired mitochondrial membrane potential and lateral condensation of actin. Degradation of Bcl-x_L was driven by alpha hemolysin, a pore-forming toxin produced by *E. coli*. Interestingly, clinical isolates of *S. aureus* (a Gram-positive bacteria discussed below) that produced alpha toxin (α toxin, also known as α hemolysin) degraded in Bcl-x_L platelets (145). These findings suggest a mechanism whereby bacterial pathogens contribute to thrombocytopenia.

GRAM-POSITIVE BACTERIAL INFECTIONS

Gram-positive bacterial infections, especially multidrug resistant pathogens, are a major global health challenge (146). *S. aureus*, *Listeria monocytogenes (L. monocytogenes),* and *Streptococcus pneumoniae (S. pneumoniae, or pneumococcus)* are common pathogens causing substantial morbidity and mortality. Here we highlight the mechanisms of platelet-mediated thromboinflammation during *S. aureus* infections, but readers are referred to the recent review by Anderson and Feldman on platelets in pneumonia and other excellent reviews as we have mentioned above (13, 14, 147).

For decades, *S. aureus,* including methicillin sensitive and resistant *S. aureus*, i.e., MSSA and MRSA, was known to interact with, and activate platelets. In 1964, Siegal et al. showed that a toxin from staphylococcal bacteria could induce platelet morphological changes observed by electron microscopy images and inhibition of platelet rich plasma clotting (148). In the 1970s, Clawson and White showed that among several strains of bacteria able to directly bind platelets and induce platelet aggregation and adhesion, *S. aureus* was the most potent (149, 150). Interestingly, at low bacterial numbers, platelets bind to bacteria without forming substantial platelet-platelet aggregates, suggesting there may be favored interactions between bacteria and platelets under these conditions. At higher bacterial numbers, platelet aggregation is induced and bacteria can be found encased in platelet-platelet aggregates (**Figure 4**) (149, 150). More recently, Wong et al. showed that upon MRSA infection *in vivo,* platelets scan liver Kupffer cells and rapidly (within minutes of infection) recognize MRSA on the surface of Kupffer cells (27). This triggers aggregation within sinusoids that limits bacterial spreading (27). Additionally, platelet depleted mice have significantly increased mortality upon MRSA infection *in vivo* (27).

There is heterogeneity for clinically isolated *S. aureus* species from patients to bind platelets (27, 152). When *S.*

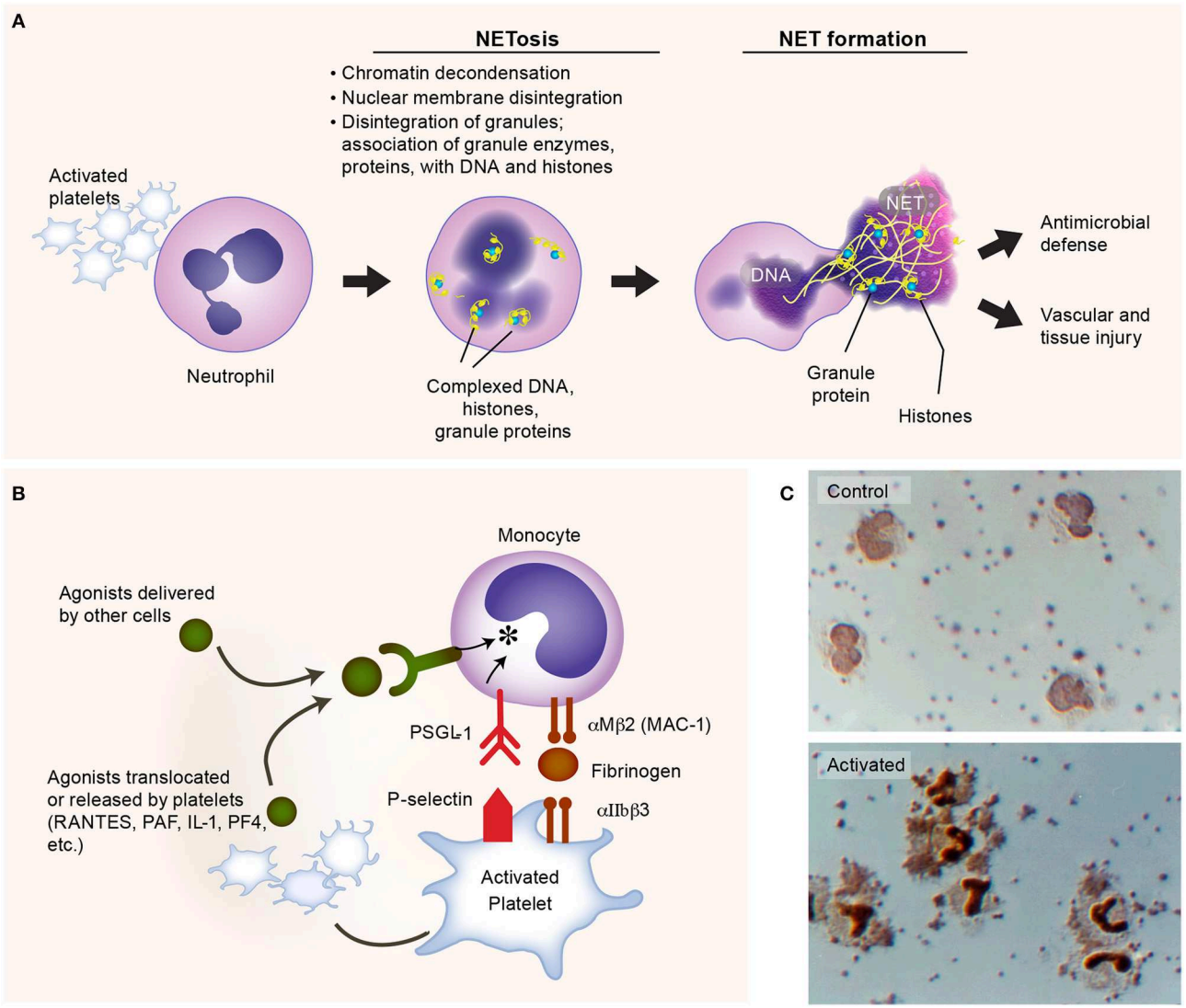

FIGURE 3 | Activated platelets induce formation of neutrophil extracellular traps (NETs). **(A)** Platelets are commonly activated during Gram-negative bacteria infections, and express multiple receptors that could facilitate the process of NET formation (NETosis), such as P-selectin. The lattices of chromatin, histones, and granule enzymes (NETs) play a critical role in pathogen clearance and may also induce thromboinflammatory responses, potentially contributing to vascular and tissue injury. **(B)** Activated platelets interact with monocytes, inducing the synthesis of inflammatory mediators. **(C)** Upper panel: isolated human platelets and monocytes incubated under control conditions. Lower panel: formation of platelet-monocyte aggregates and nuclear translocation of nuclear factor kappa B (NF-κB) in monocytes when the platelets were activated with nanomolar concentrations of thrombin. Figure is adapted from Rondina et al. (94) with permission obtained from Elsevier.

aureus binds to platelets, it appears to be in a saturable and reversible manner, suggesting there are receptor-ligand mediated interactions between platelets and *S. aureus* (153). Recently, platelets are shown to be capable of spreading and enclosing bacteria *in vitro* (118, 151).

Platelets can directly bind *S. aureus* antigens through multiple receptors. For example, activated human platelets express gC1qR, which could directly bind *S. aureus* protein A without complement opsonization (154). In addition, platelets could be directly activated by the α toxin, the Staphylococcal superantigen-like 5 (SSL5), extracellular adherence protein (Eap), chemotaxis inhibitory protein of S. aureus (CHIPS), the formyl peptide receptor-like 1 inhibitory protein (FLIPr) and other proteins of *S. aureus* (151, 155–158). Alpha toxin is the major cytotoxic virulent factor of *S. aureus*, capable of forming heptamers and develop pores on target cell membrane, causing cell death. Incubation of α toxin with washed platelets induced morphological changes of platelets in suspension, including visibly lysed shape, smaller in size, and decreased content of intracellular granules (159). The incubation of α toxin also impaired the spreading capability of platelets on collagen and fibrinogen (159). In addition, α toxin can form complex with ADAM10 on platelets, a widely expressed zinc-dependent metalloprotease, and activate the latter (160). The activated

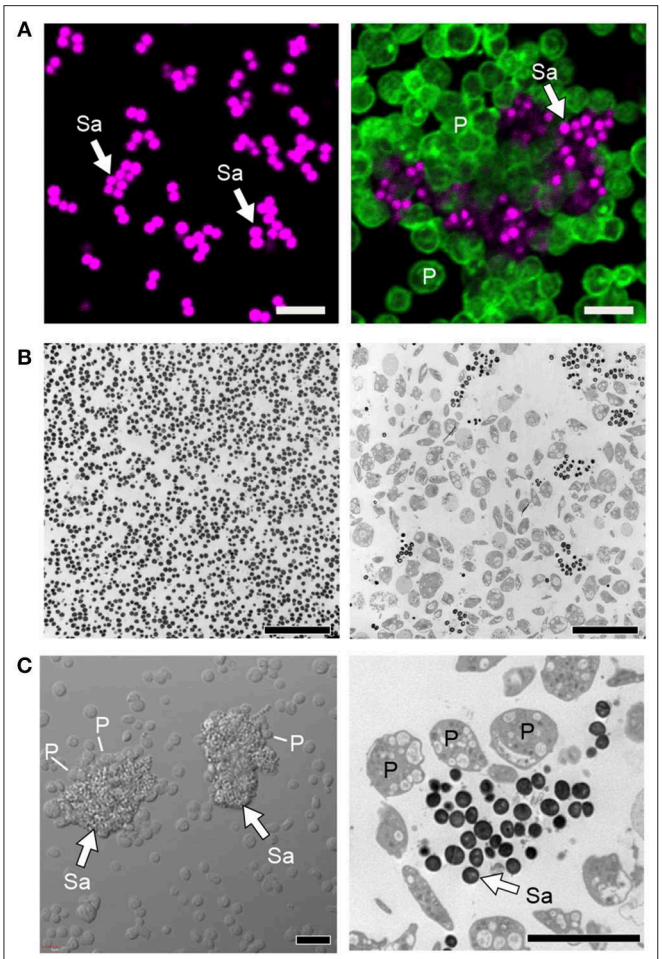

FIGURE 4 | Platelets sequester *S. aureus* and promote thromboinflammation. **(A)** Confocal and **(B)** transmission electron microscopy of cultured staphylococcus *aureus* (Sa) incubated in the presence (right panels) or absence of platelets (P, time = 240 min). **(C)** Differential interference contrast (left panel) and transmission electron (right panel) microscopy of clusters of *S. aureus* (Sa, white arrows) surrounded by platelets (P). Scale bars = 5 μm. After sequestration of *S. aureus*, platelets become activated, form aggregates *in vitro* and microthrombosis *in vivo,* interact with macrophages and neutrophils, trigger NETs formation, and also shuttle bacteria to splenic dendritic cells to activate $CD8^+$ T cell responses. Figure adapted from Kraemer et al. (151) with permission.

ADAM10 then proteolyze GPVI on platelets, and impede platelet adhesion to collagen, and *in vivo* reduce platelets activation and accumulation at sites of infection (155, 161). These mechanisms facilitate bacteria evasion and spreading. Furthermore, *in vivo*, the α toxin induced platelet aggregation in the liver and kidney and associated organ failure has been shown by Surewaard et al. (162). In contrast, platelets also evolved with protective responses following α-toxin encounters. For example, α toxin induces the release of multiple microbicidal proteins from platelets, such as thrombin-induced platelet microbicidal protein-1 (tPMP-1) and human β defensin-1, which significantly suppress bacterial growth and trigger NETs formation (151, 152). Alpha toxin also triggers integrin αIIbβ3 dependent platelet aggregation and increased protein synthesis of Bcl3, which promotes clot retraction, stabilizes thrombus within the vasculature (159). In addition, α toxin can indirectly bind platelets when opsonized by complement C3b, which promotes platelet aggregates as well as platelet-macrophage and platelet-neutrophil interactions (161, 162). These platelet-monocyte interactions can induce NLRP3 inflammasome and IL-1β production in monocytes; this may happen in platelets but as of yet remains unproven (163–165). SSL5 is a member of the SSL-family proteins that has been shown evolved in *S. aureus* evasion. It can directly bind either GPVI or GPIbα on platelets and signal downstream, induce activation of integrin αIIbβ3 and P selectin on platelets (157). Activated αIIbβ3 further promotes platelet aggregation, platelet-leukocyte aggregates, and adhesion to endothelial cells. Increased P-selectin also binds SSL5, as well as monocytes and neutrophils (156). In addition to direct binding, platelets can rapidly (within ∼1 min of infection) bind complement opsonized bacteria, including *S. aureus, in vivo* (166). This may play a critical role in shuttling bacteria to the splenic DCs to trigger host $CD8^+$ T cell responses (143, 166).

SUMMARY AND PERSPECTIVES

Small in size and abundant in number, platelets are effective sentinels constantly roaming the vasculature, quickly sensing and responding to invading pathogens. Many of the responses by platelets to pathogen invasion bridges hemostatic, inflammatory, and immune continuums: the activation of platelets leads to the expression of activated integrin $\alpha_{IIb}\beta_3$ and P-selection on the plasma membrane, formation of platelet aggregates and thrombosis, adherence and damage to the endothelium, increased interactions with macrophages and neutrophils, promotion of NETs formation, release of cytokines. These bridging features contributed to the evolution of the concept of thromboinflammation. Undoubtedly, the field will continue to see new discoveries expanding the armamentarium of platelet functions during infectious diseases. Exciting technological innovations are likely to continue to facilitate many of these discoveries. Sequencing techniques, such as next-generation RNA-sequencing and ribosomal footprint profiling, have already uncovered important new insights into the rich and dynamic nature of the platelet transcriptome and proteome (31, 167–170). Moreover, efforts to integrate sequencing data with machine-learning strategies may uncover new insights (84, 170). Proteomic studies of platelet lysates and granules have provided another layer of unbiased information about numerous proteins released by platelets and/or stored in their granules. Many of these include proteins synthesized within platelets, packaged from megakaryocytes, or internalized from the extracellular environment (84, 171–173). The application of super-resolution microscopy, including single-molecule localization microscopy (SMLM) and structured illumination microscopy (SIM), will provide unparalleled opportunities to visualize platelet granules and gather information about intracellular protein localization (174–176). The incredible evolution of our understanding of anucleate platelets in functions beyond just hemostasis has been

an exciting journey and further discoveries will likely continue to expand the role of platelets in infectious diseases.

AUTHOR CONTRIBUTIONS

LG and MR designed and wrote the paper. Both authors reviewed and critically edited the manuscript.

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REVIEW
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Platelets and Intravascular Immunity: Guardians of the Vascular Space During Bloodstream Infections and Sepsis

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Despite their humble origins as anuclear fragments of megakaryocytes, platelets have emerged as versatile mediators of thrombosis and immunity. The diverse spectrum of platelet functions are on full display during the host response to severe infection and sepsis, with platelets taking center-stage in the intravascular immune response to blood-borne pathogens. Platelets are endowed with a comprehensive armamentarium of pathogen detection systems that enable them to function as sentinels in the bloodstream for rapid identification of microbial invasion. Through both autonomous anti-microbial effector functions and collaborations with other innate immune cells, platelets orchestrate a complex intravascular immune defense system that protects against bacterial dissemination. As with any powerful immune defense system, dysregulation of platelet-mediated intravascular immunity can lead to profound collateral damage to host cells and tissues, resulting in sepsis-associated organ dysfunction. In this article, the cellular and molecular contributions of platelets to intravascular immune defenses in sepsis will be reviewed, including the roles of platelets in surveillance of the microcirculation and elicitation of protective anti-bacterial responses. Mechanisms of platelet-mediated thromboinflammatory organ dysfunction will be explored, with linkages to clinical biomarkers of platelet homeostasis that aid in the diagnosis and prognostication of human sepsis. Lastly, we discuss novel therapeutic opportunities that take advantage of our evolving understanding of platelets and intravascular immunity in severe infection.

Keywords: platelets, sepsis, intravascular immunity, infection, thromboinflammation

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INTRODUCTION

Expanding beyond their well-known role in primary hemostasis, much of the recent research in the field of platelet biology has focused on elucidating the contributions of platelets to host defense and immunity. It is now well-established that platelets are integral to the innate immune response to infection and inflammation, both as autonomous effectors as well as collaborative conductors of anti-microbial defenses (1–6). The orchestral role of platelets in host defense is exemplified by their contribution to the intravascular immune response that unfolds during acute systemic

infections, mediating host defense against microbial invaders while simultaneously contributing to organ dysfunction in sepsis (7). Below, we provide a comprehensive review of the cellular and molecular mechanisms of platelet functions in intravascular immunity as well as the pathogenesis of disease in sepsis. As detailed in the sections to follow, much has been learned from laboratory research and animal models of bacterial sepsis and endotoxemia, but it is helpful to begin with clinical observations from human sepsis that emphasize the critical importance of platelets in the pathogenesis of this systemic inflammatory disease.

PLATELETS AND SEPSIS: THE CLINICAL PERSPECTIVE

Abnormalities of platelet homeostasis are common during acute infections and sepsis, and clinical monitoring of platelet counts has emerged as one of the most important biomarkers in the management of septic patients (8). The most commonly monitored platelet parameter is the peripheral blood platelet concentration, which is measured routinely (often daily) in hospitalized patients with acute infections. While quantitative assessments of platelets may seem rudimentary (and lacking information about functional properties), many clinical, and epidemiological studies have identified the peripheral blood platelet count as a useful diagnostic and prognostic biomarker in sepsis (9–22).

An acute rise in the circulating platelet count (acute thrombocytosis) is generally interpreted as a manifestation of systemic inflammation, as may be seen in the context of acute infection (23). However, the most common perturbation seen in acute infection and sepsis is a reduction in circulating platelets, and the development of acute thrombocytopenia (24). In fact, observational studies of the incidence and prevalence of thrombocytopenia in critically ill patients with sepsis and septic shock have reported that low platelet counts occur in 15–50% of patients (13, 16, 25, 26). Furthermore, among patients with normal platelet counts on admission, up to 44% will subsequently develop thrombocytopenia during the course of their stay in the intensive care unit (16, 21).

In addition to being a common finding in sepsis, clinicians have long appreciated that thrombocytopenia represents an ominous sign in the setting of severe infection (9–11, 14–20, 22). This clinical acumen is supported by data from multiple studies finding strong associations between low circulating platelet counts and adverse clinical outcomes in septic patients (9, 16, 19, 21, 22, 25, 26). Likewise, failure of platelet counts to recover into the normal range during acute illness is also associated with increased mortality, whereas recovery of platelet counts is strongly associated with survival to ICU discharge (14, 17, 18). The importance of this link between thrombocytopenia and sepsis pathogenesis is now solidified by the inclusion of thrombocytopenia as a core criterion for the diagnosis of sepsis. The updated consensus definition of sepsis uses the Sepsis-related Organ Dysfunction Score (SOFA) score, in which platelet count represents 1 of 6 core parameters (27). Impressively, severe thrombocytopenia carries the same prognostic significance in the SOFA score as major organ failure requiring life-support interventions (e.g., respiratory failure requiring mechanical ventilation, circulatory shock requiring vasopressors, severe renal and hepatic failure, or coma). Clearly, circulating platelet counts are an important biomarker of disease severity and clinical outcomes in sepsis.

The strong epidemiological links between abnormalities of platelet homeostasis and outcomes strongly support a role for platelets in the pathogenesis of sepsis and septic shock. The development of thrombocytopenia in sepsis occurs primarily as a result of massive consumption of circulating platelets through interactions with immune cells in the vasculature (described below), with additional contributions from reduced thrombopoiesis, sequestration, thrombotic microangiopathy (disseminated intravascular coagulation, DIC), direct pathogen-induced thrombocytopenia, immune-mediated thrombocytopenia, drug induced thrombocytopenia, and hemophagocytosis [see recent reviews by (8) and (28)]. Although thrombocytopenia is also seen in other causes of critical illness (trauma, burns, and others), it has been found that critical ill patients with burns develop more severe thrombocytopenia if there is concomitant infection and sepsis which is also linked to an increased risk of death, suggesting that sepsis-associated thrombocytopenia may have unique mechanisms of pathophysiology (29, 30). While much of our understanding of platelet functions in the immunopathogenesis of sepsis comes from elegant animal models of disease, a number of clinical studies have also shed light on the linkages between platelets and immunity in sepsis. Claushius et al. analyzed associations between admission platelet counts and immune phenotypes in 937 consecutive patients with sepsis admitted to 2 ICUs in the Netherlands, and found a strong association between severe thrombocytopenia, disease severity (APACHE IV score), and increased 1-year mortality (26). Using a propensity-matching strategy (to limit potential confounding by disease severity), the investigators found that, compared to patients with normal platelet counts, those with severe thrombocytopenia had higher levels of proinflammatory cytokines as well as increased markers of endothelial cell activation (increased sICAM-1 and fractalkine) and vascular permeability. Similar findings were reported in a cohort of critically-ill septic patients in Greece, in whom platelet counts varied inversely with serum pro-inflammatory cytokines and soluble ICAM-1 levels (31). Using whole blood transcriptomics, it was also shown that severe thrombocytopenia was associated with up-regulation of both pro- and anti-inflammatory signaling pathways, TLR signaling, and suppression of leukocyte adhesion molecule genes (26). Together, these findings indicate that platelet consumption and the development of thrombocytopenia is intimately associated with the dysregulated immune response that defines sepsis (27). In the following sections, the cellular and molecular mechanisms underlying these clinical observations will be reviewed in detail to explore the biological role of platelets in the intravascular immune response to infection, as well as the pathogenesis of sepsis.

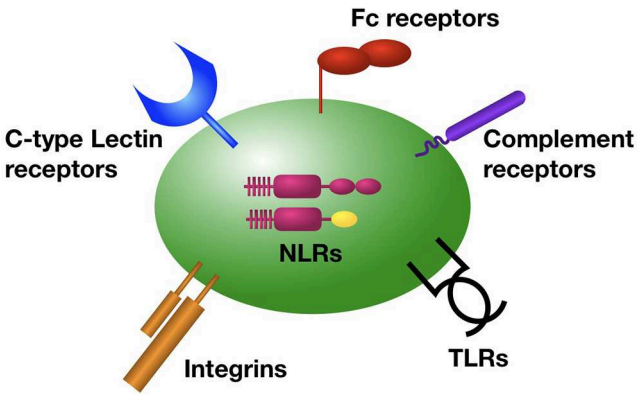

FIGURE 1 | Platelet receptors for the detection of bacterial pathogens in the bloodstream. Platelets are equipped with a diverse array of surface and intracellular receptors for pathogen detection, enabling their function as intravascular sentinel cells. These include receptors for the detection of bacterial produces and molecules (TLRs, NLRs, C-type lectins, integrins, GP1bα), as well as bacteria that have been coated by antibody (Fc recpetors), complement (complement receptors), or von Willibrand factor (integrins and GP1bα).

PLATELETS AND INTRAVASCULAR IMMUNITY IN SEPSIS

Platelets Are Immune Sentinels for Rapid Detection of Bloodstream Invaders

Intravascular immune sentinels are strategically positioned within the vasculature to rapidly detect and respond to bloodstream invaders (32). Platelets play critical, yet underappreciated roles in the detection of pathogens in the bloodstream, and the elicitation of a coordinated immune response. Platelets are equipped with a comprehensive set of receptors that rapidly respond to invading pathogens and pathogen-associated molecular patterns (PAMPs) (**Figure 1**).

Toll-Like Receptors

Like other immune sentinels, platelets express functional Toll-Like Receptors, including TLR 1, 2, 3, 4, 6, 7, 9 (33–40). However, activation of TLR signaling in platelets elicits a response that differs in many ways from other immune cells. First, platelets lack a nucleus, and therefore their response consists of an entirely post-transcription program. Platelet TLR signaling is incompletely understood, but appears to involve both MyD88-dependent and MyD88-independent signal transduction, including the activation of transcription factors such as NF-κB [recently review by (33, 41, 42)]. However, because platelets lack nuclei, these pathways lead to effector mechanisms that are independent of classical transcription programs, but the precise pathway details have not yet been fully elucidated [see (43) for a contemporary review]. Secondly, the activation thresholds for platelet TLRs have been found to differ from other immune cells in the context of sepsis and endotoxemia. The concentrations of LPS required to activate platelets via TLR4 *in vitro* is significantly higher than that required for activation of neutrophils (39). It has been hypothesized that this differential sensitivity to bacterial products allows platelets to reserve their arsenal for severe bloodstream infections with high concentrations of circulating PAMPs, thereby sparing the host from potential thromboinflammatory complications during minor infections (39). Thirdly, stimulation of platelets with LPS produces a functionally unique response compared to stimulation by other platelet activating substances (37, 39, 44). For example, Clark *et al.* found that stimulation of human platelets with classical activators like thrombin or PAF induced up-regulation of P-selectin, degranulation, and aggregation *in vitro*, while LPS did not (39). Instead, LPS induced a unique response linked to host defense, including adhesion to activated neutrophils and fibrinogen without marked aggregation or degranulation. Others have demonstrated a variety of additional effector functions elicited by TLR4 signaling in platelets, including the release of IL-1β and TNFα, tissue factor, and other immunostimulatory molecules, and augmented phagocytosis of platelets that are bound by autoantibodies (35, 36, 41, 45–47). Recently, it was shown that platelet TLRs can discriminate the nuances of specific PAMP characteristics, enabling tailored responses to different pathogens. Berthet et al. found that stimulation of platelets with structurally unique isoforms of LPS derived from *E. coli* or *Salmonella* yielded distinct responses (45). It was observed that incubation of PBMCs with supernatant from platelets stimulated with *S. enterica* LPS elicited significantly higher levels of IL-6, IL-8, and TNFα compared with supernatant from *E. coli* LPS-stimulated platelets. The mechanisms that enable platelet TLR4 signaling to discriminate between pathogen-specific LPS isoforms remains unknown, but these observations indicate that platelet sentinels possess the sensitivity to differentiate between microbes and induce tailored responses. Taken together, the versatile functions of platelet TLRs exemplify the sentinel characteristics of platelets in the bloodstream; poised to rapidly but precisely identify invading pathogens and initiate an appropriate and tailored response.

Other Pathogen Detection Mechanisms

In addition to TLRs, platelets are equipped with a variety of other receptors to detect pathogen invasion (**Figure 1**). Platelets have been shown to express functional intracellular pattern-recognition receptors (PRRs) including NOD2 and NLRP3, but their physiologic function in platelets remain to be defined (48, 49). Platelets can also recognize and respond to bacteria that have been opsonized by humoral mediators of innate and adaptive immunity (50). Fixation of C3b to the surface of bacteria can be recognized by platelets in a GP1b-dependent manner, resulting in the formation of circulating platelet-bacteria complexes that facilitate delivery of bacteria to professional phagocytes (51). Human platelets also express a range of functional immunoglobulin Fc receptors for IgG, IgA, and IgE to detect antibody-laden bacteria (50). Platelet GPIIbIIIa (αIIbβ3) and GP1bα can also facilitate binding to bacteria, either directly to bacterial surface proteins or via molecular bridges provided by fibrinogen, fibronectin, or von Willibrand factor (52). Platelets express C-type lectin receptors CLEC-2 and DC-SIGN that have been shown to mediate binding to viral

particles, and also contribute immunomodulatory effects during bacterial sepsis (53, 54). Lastly, collaboration between multiple receptor types may be required for platelets to detect and respond pathogens. For example, the response of human platelets to both Gram-positive and Gram-negative bacteria was shown to be dependent on FcγRIIA activation by IgG-bound bacteria, but only with concomitant engagement of GPIIbIIIa (αIIbβ3) (55, 56).

In addition to direct sensing of bacteria and bacterial products, platelets can become activated in response to inflammatory mediators liberated by other sentinels during acute infection. Platelets are decorated with a number of cytokine and chemokine receptors that detect prototypical signals of acute inflammation (57). Furthermore, platelets are potently activated by a variety of damage-associated molecular patterns (DAMPs) that are released from stressed and dying cells during acute infection. Interestingly, intravenous administration of purified danger signals such as histone proteins induces profound platelet activation and thrombocytopenia similar to that seen during endotoxemia and sepsis (58). Therefore, platelets are endowed with the machinery to detect and respond to both primary (bacteria and bacterial products) and secondary (DAMPs and inflammatory mediators) signals of acute infection, providing the necessary redundancy to function as effective sentinels within the bloodstream.

Lastly, in addition to their ability to detect passively circulating signals of pathogen invasion, platelets also conduct active surveillance of the microcirculation to enable a rapid and focused response to endovascular pathogens. Intravital imaging analysis of platelet behavior within the microcirculation of highly vascular organs (lungs, liver, brain) has revealed that platelets undergo transient touch-and-go interactions with the vascular endothelium and other intravascular immune cells such as Kupffer cells in the liver (59). Under homeostatic conditions, platelets were observed to instantaneously touch-down in a GP1b-dependent manner, and in the absence of bacteria would immediately release and return to the circulation (59). When pathogenic Gram-positive bacteria such as *Bacillus cereus* or *Staphylococcus aureus* were introduced into the bloodstream and captured by liver Kupffer cells, these touch-and-go interactions converted to adhesion and aggregation nucleated around the captured bacteria. Mice deficient in GP1bα, which were incapable of touch-and-go interactions, rapidly succumbed to overwhelming infection, demonstrating that platelet surveillance is essential for effective host defense against blood-borne bacteria (59). It remains unknown whether this active microvascular surveillance is required for control of other types of bloodstream infections (Gram negative, fungal, parasitic), and how this behavior is regulated to avoid overwhelming microvascular thrombosis. In addition, further research is required to define the molecular events that enable platelets to convert from surveillance to aggregation upon pathogen encounter, and whether dysregulation of this behavior contributes to disease pathogenesis in sepsis and other thromboinflammatory disorders.

Platelets and Microvascular Traffic Control in Sepsis

The early phase of sepsis is characterized by a vigorous systemic inflammatory response, during which platelets (together with leukocytes, primarily neutrophils) are recruited from the circulation and sequestered within highly vascular organs such as the lungs and liver, resulting in consumptive thrombocytopenia (37, 39, 60–65). Interestingly, although the spleen is a reservoir for large numbers of platelets, studies that have tracked radio-labeled platelets in the circulation of septic mice have found little contribution of the spleen to the development of thrombocytopenia in sepsis (37, 65). Once considered a non-specific and maladaptive reaction to severe infection, contemporary evidence suggests that the recruitment of platelets into the microcirculation of the liver and lungs is part of a highly coordinated intravascular immune response involving collaboration between platelets and leukocytes (7). Platelets have emerged as central regulators of the intravascular immune response, beginning with the orchestration of immune cell trafficking within the inflamed microvasculature. In this section, we will discuss the role of platelets in the coordination of immune cell recruitment to inflamed tissues during infection and inflammation, followed in the next section by a review of the role of platelets in regulating antibacterial functions of immune effector cells.

Platelets engage in a reciprocal relationship with neutrophils to coordinate their recruitment and function within the microvasculature. A number of studies have reported that depletion of neutrophils or inhibition of neutrophil recruitment prevents platelet consumption and the development of thrombocytopenia, suggesting that neutrophils are essential for platelet sequestration within the microcirculation (39, 62). Indeed, direct visualization of platelet-neutrophil dynamics in the pulmonary and hepatic microcirculation using intravital microscopy has shown that neutrophil adhesion is followed immediately by platelet binding and aggregation upon their surface (62, 66–68). Similar mechanisms of neutrophil-dependent platelet recruitment have been observed in a number of organ systems, and across a variety of sepsis models (37, 39, 62, 64, 66). A number of adhesion mechanisms can support platelet binding to neutrophils under flow conditions. First, selectin-mediated interactions can support adhesion between platelets and neutrophils *in vitro* and *in vivo* via both direct and indirect mechanisms. Interactions between selectins and selectin-ligands are best known for mediating transient low-affinity catch-bonds that support leukocyte rolling on activated endothelium (69, 70). In contrast, binding between platelet P-selectin and neutrophil PSGL-1 can mediate stable adhesion between these cells (61, 68, 71, 72). Given the low-affinity nature of their binding, it is likely that P-selectin—PSGL1 interactions between platelets and neutrophils are particularly effective within the low-shear environment of the pulmonary and hepatic capillaries. Furthermore, PSGL1 engagement can amplify platelet-neutrophil adhesion by stimulating "outside-in" signaling pathways in neutrophils (including Src-family kinase and MAP kinase pathways) that induce the activation of integrins (73–76). Neutrophil Mac-1 ($\alpha_M\beta_2$ integrin) can

mediate adhesion to platelets via multiple receptors, including GP1bα as well as GPIIbIIIa via a fibrinogen bridge (77, 78). Alternatively, it has been shown that binding between human neutrophils and platelets in response to plasma from septic patients can be mediated by engagement of LFA-1 ($\alpha_L\beta_2$ integrin) and ICAM-2 (62).

The multitude of adhesion mechanisms that support platelet-neutrophil binding reflects the complexity of their interactions *in vivo*. Studies using intravital microscopy have revealed that adherent neutrophils nucleate the formation of large, dynamic aggregates that fluctuate in size over time as a result of continuous binding and release of circulating platelets (62, 66, 67). Furthermore, these dynamic platelet aggregates migrate throughout the vasculature atop neutrophils as they crawl along the endothelial surface (62, 66, 67). The molecular mechanisms that regulate this dynamic aggregation and the functional role of continuous platelet recycling atop neutrophils are unclear, but are likely tightly controlled to avoid catastrophic microvascular thrombosis.

Under certain inflammatory contexts (primarily non-infectious), the recruitment of platelets and neutrophils within the microcirculation may follow the opposite sequence, that is, initial platelet accumulation upon the endothelium followed by subsequent neutrophil recruitment. This mode of platelet-dependent neutrophil recruitment has been observed in a number of models of inflammatory and thrombotic disease, including cytokine-induce cerebral inflammation (79), thermal liver injury (80), acute lung injury (61, 81), and venous thrombosis (82). In these contexts, platelets adhere and aggregate at sites of compromised vascular integrity, and then promote neutrophil recruitment either directly through P-selectin-PSGL-1 and/or integrin-mediated interactions, or indirectly through TXA1-mediated activation of endothelium (83). In instances where vascular integrity is severely disrupted, platelets can even be seen "paving" neo-vessel-like conduits through which neutrophils migrate to reach sites of inflammation (80). Thus, the trafficking and recruitment of platelets and neutrophils exists as a reciprocal relationship, in which neutrophils may recruit platelets, and platelets may recruit neutrophils. The mechanisms that dictate neutrophil-first vs. platelet-first recruitment are not well-understood. It is possible that the nature of the inflammatory stimulus (e.g., infectious vs. non-infectious), or the microvascular characteristics (endothelial integrity, adhesion molecule expression) may induce different patterns of cell recruitment to tailor the intravascular immune response to the inciting stimulus. As described in the sections below, this hypothesis is supported by evidence that neutrophil effector functions can differ in response to platelet-first compared to neutrophil-first recruitment interactions (80).

Following adhesion to the endothelium, immune cells migrate through the vasculature guided by a variety of chemotactic cues (84–86). The platelet payload includes a number of chemoattractant factors that can guide the chemotaxis of neutrophils and other leukocytes (4). In addition, platelets may directly influence neutrophil chemotaxis within blood vessels through contact-mediated interactions on specific microdomains of the neutrophil surface. Sreeramkumar et al. conducted a detailed *in vivo* investigation of neutrophil polarization (an essential pre-requisite for directional migration), and made the striking discovery that platelets dock to the leading-edge of adherent neutrophils in the vasculature, and that this polarized binding was crucial for directional migration (68). Following engagement of platelet P-selectin to PSGL-1 on the leading edge of neutrophils, outside-in signal transduction led to a redistribution of surface receptors Mac-1 ($\alpha_M\beta_2$ integrin) and CXCL2, generating polarized receptor microdomains that were essential for effective locomotion toward infection and injury. This discovery revealed that adherent neutrophils scan for activated platelets in the vasculature to enable physical interactions that steer neutrophil migration toward appropriate targets.

Lastly, like other innate immune cells, platelets themselves possess a rudimentary ability to migrate (undergo chemotaxis) in response to chemoattractant stimuli. Platelets express a variety of surface receptors for prototypical chemoattractants, as well as the necessary intracellular signal transduction and cytoskeletal machinery required for cell motility. Platelet chemotaxis remained an *in vitro* observation for many years (4), until a number of recent studies demonstrated evidence of platelet migration *in vivo* in mouse models of allergic pneumonitis and sepsis (87, 88). Directional migration of platelets *in vivo* relies on integrin-based interactions, as blockade of GPIIbIIIa (αIIbβ3) integrin inhibits intravascular platelet locomotion (88). The physiologic function of platelet locomotion remained a biological curiosity until recently, when Massberg et al. published a seminal study detailing a functional contribution of platelet migration to anti-bacterial host-defense *in vivo* (88). The authors observed that migrating platelets behaved as "mechano-scavengers" within the vasculature, piling-up substratum and other particles (including bacteria) as they moved. This mechano-scavenger behavior enabled platelets to collect bacteria that they encountered in their travels, bundling them within open cannalicular systems (OCS) for disposal by professional phagocytes. Therefore, migration of platelets appears to fill an important niche in the intravascular immune response by contributing to the collection and clearance of pathogens in collaboration with local phagocytes.

Platelets and Neutrophils: Partners in Intravascular Immunity During Sepsis

Platelets have emerged as versatile effectors of anti-bacterial immunity, contributing both autonomous bacteriocidal/bacteriostatic properties as well as synergistic partnerships with other innate immune cells. In co-culture experiments, platelets can autonomously inhibit the growth of various bacteria (89–91). The anti-bacterial properties of platelets have largely been attributed to their ability to produce anti-microbial peptides such as β-defensins and so-called "platelet-microbicidal proteins" (PmP) [reviewed by (50)]. In addition, it has been observed that platelets exhibit a rudimentary ability to engulf bacteria (92). However, the functional significance of these autonomous anti-bacterial properties in the context of *in vivo* host defense remains unknown.

In contrast, platelets have well-defined anti-bacterial functions *in vivo* that arise from synergistic partnerships with other immune cells (**Figure 2**). For example, platelets are crucial for survival in mouse models of *Bacillus cereus* and *Staphylococcus aureus* bacteremia due to their ability to collaborate with liver macrophages to clear circulating bacteria (59). During these infections, platelets were observed to aggregate upon bacteria-laden macrophages in the liver, providing an essential signal to macrophages that enabled clearance of bacteria from the circulation and protection against overwhelming sepsis (59). In addition, circulating platelets can scavenge blood-borne bacteria and enhance their delivery to phagocytes (51). However, it should be noted that under certain circumstances, platelet-mediated bacterial "opsonization" may actually impair clearance of bacteremia by diverting microbes toward less-efficient phagocytes, as is seen in *Listeria monocytogenes* infection (51).

The most extensively studied and perhaps most potent platelet-mediated anti-bacterial responses are generated through collaboration between platelets and neutrophils. Platelet-neutrophil interactions induce and/or augment a number of anti-bacterial neutrophil functions to enhance the clearance of bacteria from the bloodstream. First, neutrophil phagocytosis of extracellular bacteria is augmented by mediators released from activated platelets (93, 94). In addition, platelets augment intracellular killing of bacteria within neutrophils by promoting oxidative burst generation through both contact-dependent outside-in signaling as well the release of various soluble mediators (76, 95, 96). In addition to augmenting phagocytosis and intracellular killing, platelets help supply neutrophils with their prey through mechano-scavenging behavior in the microvasculature, bundling stray bacteria for efficient phagocytosis and clearance by neutrophils (and other phagocytes) (88).

Lastly, the most potent anti-microbial effector mechanism unleashed in response to platelet-neutrophil interactions is the neutrophil extracellular trap (NET). NETs are extracellular webs of decondensed chromatin laden with proteolytic enzymes and other anti-bacterial molecules that are expelled from activated neutrophils (97). NETs are capable of both capturing and directly killing extracellular microbes, including bacteria, fungi, parasites, and even display anti-viral properties (98). Although a number of activating stimuli can induce NETs release from neutrophils, activated platelets have emerged as one of the most potent stimuli for NETs release, and platelet-induced NETs have been observed in a variety of sepsis models as well as other non-infectious inflammatory conditions (39, 62, 63, 82, 99–101). During sepsis, neutrophils cast NETs into the bloodstream to filter pathogens from the circulation (39, 62, 63). This powerful and efficient bacterial clearance system is strategically positioned within the microcirculation of highly vascular organs (liver and lung), enabling maximal filtration of the cardiac output to protect against hematogenous dissemination of infection (62).

The molecular mechanisms controlling NETs release in response to platelet-neutrophil interactions are incompletely understood. Blocking the physical engagement of platelets with neutrophils inhibits the release of NETs, suggesting a contact-dependent induction pathway. However, while contact between neutrophils and platelets is essential for intravascular NETs release *in vivo*, it is not sufficient. Using a mouse model of sterile pulmonary inflammation, Rossaint et al. found that a second signal composed of platelet-derived CXCL4/CCL5 heterodimers was essential for NETs release in response to platelet-neutrophil binding (100). *In vitro*, β-defensin-1 released from activated human platelets was shown to induce NETs release from neutrophils, but this is yet to be confirmed *in vivo* (89). A full understanding of the molecular mechanisms of platelet-mediated NETs release during bacterial sepsis *in vivo* remains to be elucidated.

Finally, although platelet-neutrophil collaboration has been studied more extensively, it should be noted that platelets engage in functional interactions with other innate immune cells in the vasculature during sepsis. Platelet-monocyte complex formation has been observed in the blood of septic patients (102, 103), and the levels of circulating platelet-monocyte complexes may be a useful biomarker to predict adverse outcomes in older adults with sepsis (102). Functionally, platelets have been shown to aid in the recruitment of monocytes to foci of Listeria monocytogenes infection in mice, as well as modulate the inflammatory phenotype of monocytes during viral infection (104, 105). Platelets have also been shown to modulate the polarization, cytokine profile, and antibacterial effector mechanisms of macrophages in animal models of sepsis (59, 91, 106, 107).

Overall, there is strong evidence that collaboration between platelets, neutrophils, and other antibacterial effector cells within the microvasculature is crucial to protect against blood-borne infections. Unfortunately, as with most powerful immune defense mechanisms, dysregulation of the intravascular immune response during sepsis can result in tremendous collateral damage to host cells and tissue, resulting in organ dysfunction.

Platelets-Neutrophil-NETs Axis and Organ Dysfunction in Sepsis

The intricate interplay between platelets, neutrophils, and NETs in sepsis represents a classical example of a "double-edged sword"; providing protective host defense while simultaneously causing immune-mediated organ dysfunction (**Figure 3**). The importance of this axis in sepsis pathogenesis has been confirmed in a variety of animal models of acute infection, in which therapeutic blockade of the platelet-neutrophil-NETs axis reduced organ dysfunction and (in some models) improved survival (62, 67, 108–115). Platelets and neutrophils conspire together to induce microvascular dysfunction and tissue damage through a variety of mechanisms. In particular, the release of NETs in response to platelet-neutrophil engagement can be especially cytotoxic to host cells. During sepsis, NETs can be found throughout the vast microvasculature of the liver and other organs, exposing the endothelium and underlying parenchyma to a variety of potent cytotoxic mediators. First, extracellular histones proteins contained within the chromatin backbone of NETs are particularly cytotoxic to endothelial cells and parenchymal cells *in vitro* and *in vivo* (116). In fact, Esmon et al. revealed that antibody-mediated neutralization

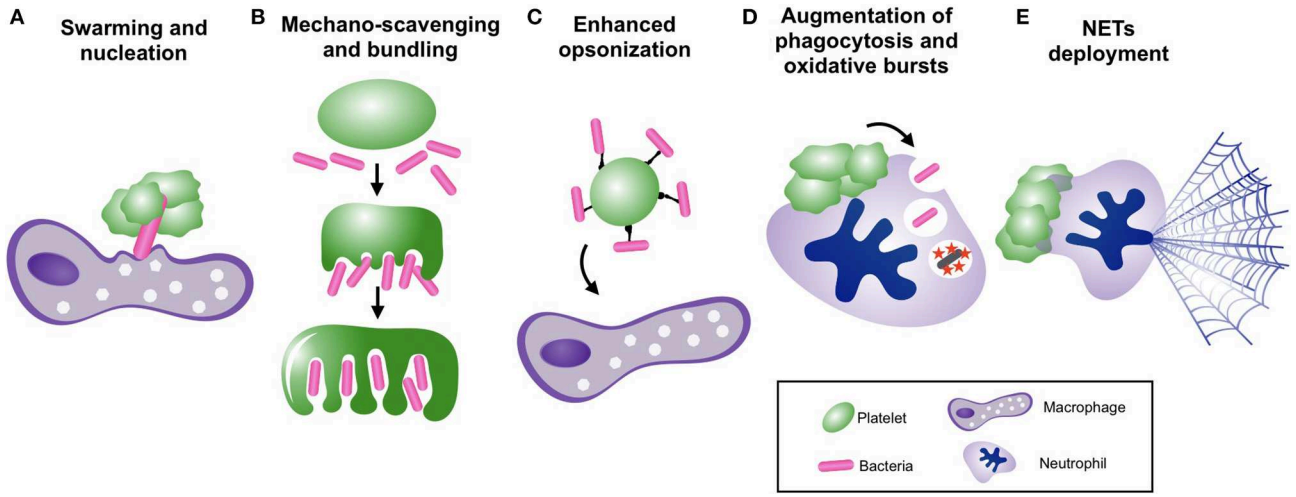

FIGURE 2 | Platelets collaborate with neutrophils and other immune cells to facilitate bacterial clearance from the bloodstream. Mechanisms of bacterial clearance facilitated by platelet-leukocyte interactions, including, **(A)** vascular surveillance and nucleation of bacterial invaders to promote phagocytosis by macrophages, **(B)** mechano-scavenging of bacteria within the microvasculature, **(C)** "opsonization" of circulating bacteria for clearance by phagocytes **(D)** augmentation of neutrophil-mediated phagocytosis and oxidative killing, and **(E)** induction of neutrophil extracellular trap (NET) release.

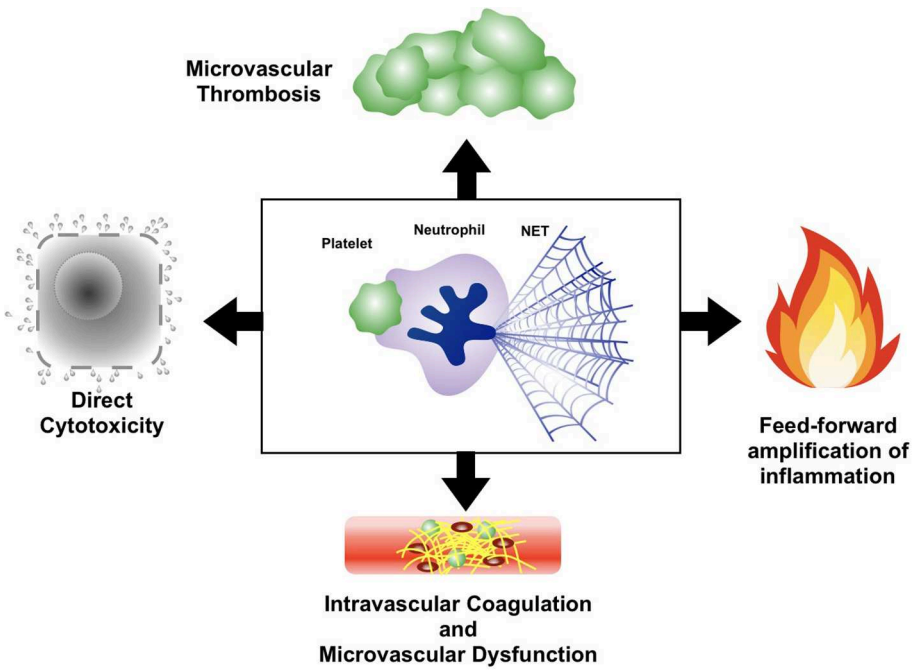

FIGURE 3 | Mechanisms of organ dysfunction in sepsis induced by the platelet-neutrophil-NETs axis. Intravascular collaboration between platelets, neutrophils, and NETs leads to cell and tissue damage as a result of directly cytotoxicity, induction of intravascular coagulation and microvascular dysfunction, and propagation of a dysfunctional thromboinflammatory response through feed-forward microvascular inflammation and thrombosis.

of histone proteins in a mouse model of polymicrobial sepsis protected against multi-organ dysfunction and death (116). Furthermore, NETs contain an abundance of proteolytic and antimicrobial proteins that can damage host cells and tissues. For example, genetic deficiency of neutrophil serine proteases such as neutrophil elastase, or blockade of their enzymatic activity with small molecule inhibitors, resulted in a dramatic decrease in biomarkers of tissue damage in mouse models of acute infection (115). Lastly, the fact that many NETs components can serve as pro-inflammatory DAMPs (DNA, histone proteins, proteolytic enzymes, antimicrobial peptides, and others) creates in a feed-forward system that propagates microvascular inflammation and amplifies tissue damage.

TABLE 1 | The role of platelets in host defense during bacterial infection in the bloodstream.

Host response in sepsis	Roles of platelets	Selected references
Pathogen detection	Direct detection of invading pathogens (see **Figure 1**)	(34, 35, 37, 39)
	Intravascular surveillance behavior	(59, 88)
Pathogen capture and killing	Direct platelet-mediated cytotoxicity	(89–91)
	Enhanced opsonization	(36, 51, 53, 59)
	Enhancement of leukocyte-mediated capture and killing	(51, 59, 62, 88, 93, 94)
	Induction of intravascular NETs	(39, 62, 63, 99)
Modulation of immune cell trafficking and function	Platelet trafficking and sequestration in microcirculations	(37, 39, 60–63, 65, 67)
	Neutrophil trafficking and function	(61, 68, 79, 81)
	Monocyte trafficking and function	(102–105)
	Macrophage polarization and function	(59, 91, 106, 107)
Modulation of systemic inflammatory response	Inflammatory mediator release from platelets	(36, 46, 47)
	Inflammatory-mediator production by innate immune cells	(26, 45, 106)
	Complement activation and anaphylatoxin production	(126, 127)
Microvascular thrombosis and coagulation	Activation and propagation of intravascular coagulation	(67, 112)
	Sepsis-associated DIC	(112, 117–119)

In addition to direct cytotoxicity, a growing body of literature implicates the platelet-neutrophil-NETs axis in sepsis pathogenesis through the induction of intravascular coagulation. Platelet-neutrophil interactions and the subsequent release of NETs coincides with diffuse activation of thrombin and fibrin deposition within the vasculature of multiple organs (67). Imaging of this process *in vivo* has revealed disseminated microvascular coagulation following NETs release, resulting in extensive obstruction of blood flow and ischemic injury to the affected organ (67). This widespread microvascular coagulation was largely NETs dependent, as mutant mice with severely reduced NETs production (peptidylarginine deiminase 4 [PAD4] deficient mice) have markedly diminished intravascular coagulation and organ injury, yet still have abundant neutrophil-platelet aggregation in the microvasculature (67). Conversely, studies using DNase-deficient mice have revealed that impaired clearance of NETs (leading to an overabundance of NETs in the vasculature) precipitates extensive microvascular coagulation and thrombosis. In fact, administration of endotoxin to DNase1-deficient or DNase-like-3 deficient mice resulted in multi-organ microvascular coagulation and microangiopathic hemolytic anemia characteristic of disseminated intravascular coagulation (DIC), followed by death within hours (112). There is also emerging evidence from human studies that NETs promote hypercoagulability in patients with sepsis, and that elevated levels of circulating NETs is associated with sepsis-related disseminated intravascular coagulation (117–119).

The pro-coagulant properties of NETs arise from a variety of components that are known to interact with the clotting cascade at multiple levels. For example, histone proteins have been shown to initiate coagulation through the upregulation of tissue factor as well as initiation of the contact-dependent pathway (63, 82, 120). Indeed, antibody-mediated neutralization of histone H4 within NETs in a mouse model of Gram-negative sepsis reduced intravascular thrombin generation in the liver and lung microcirculation (67). Furthermore, neutrophil serine proteases present within NETs (such as neutrophil elastase and cathepsin G) activate tissue factor- and factor XII-dependent coagulation pathways, and also promote platelet activation through protease-activated receptors (PARs) (63, 121). NETs may also acquire pro-coagulant factors from the bloodstream to bolster their ability to initiate and propagate microvascular coagulation. Circulating microparticles are captured by NETs that can augment thrombin generation via factor XII and the intrinsic pathway (122). Lastly, cross-talk between intravascular NETs and endothelial cells leads to upregulation of tissue factor expression and direct activation of the coagulation cascade within the vessel lumen (123).

Although a number of components within NETs have the ability to activate coagulation in isolation, emerging evidence suggests that the induction of intravascular coagulation *in vivo* requires the intact NETs macrostructure. Most notably, experiments using exogenous DNase to dissolve the DNA backbone of NETs have demonstrated marked inhibition of intravascular thrombin activation and fibrin production, despite the fact that individual pro-coagulant NETs components remain in the vasculature (67, 122). These findings suggest that intact NETs provide an essential catalytic scaffold for the induction of disseminated intravascular coagulation during sepsis.

Finally, in addition to stimulating NETs release from neutrophils, platelets also provide synergistic amplification of NET-induced coagulation. While the majority of platelets are sequestered in the microvasculature through interactions with neutrophils, platelets can also bind and aggregate directly on NETs. NETs-mediated platelet aggregation has been demonstrated in a variety of thromboinflammatory disorders, including sepsis, venous thromboembolic disease, and atherosclerotic vascular disease (67, 124, 125). During sepsis, platelets aggregate within intravascular NETs and amplify thrombin generation. The molecular crosstalk between platelets and NETs that propagates intravascular coagulation is incompletely understood, but one study identified platelet polyphosphate (released from dense granules) as an essential

signal for NETs-mediated thrombin activation *in vivo* (67), and polyphosphate has been identified as a cofactor for histone H4-mediated thrombin activation *in vitro* (120). Overall, the extensive cross talk between platelets, neutrophils, and NETs results in widespread intravascular coagulation, microvascular occlusion, and ischemic and cytotoxic organ damage that is characteristic of sepsis pathology.

CONCLUSIONS

Platelets are versatile mediators of antimicrobial immunity and host defense within the bloodstream during sepsis (**Table 1**). However, dysregulation of this platelet-mediated intravascular immune response leads to tissue damage, intravascular coagulation, and organ dysfunction. The pathological integration of immunity, thrombosis, and coagulation in sepsis provides a glimpse into why clinical trials of anti-inflammatory or anti-coagulant therapies alone have yielded underwhelming results, and underscores the importance of creating novel therapies that target keystone mechanisms in this complex pathological system. The integrated response of platelets, neutrophils, and NETs represents one such keystone mechanism, with a growing body of literature demonstrating therapeutic efficacy of targeting this system in animal models of sepsis and other acute inflammatory diseases. In humans, retrospective studies of patients with septic shock have found that anti-platelet agents may reduce the risk of end-organ dysfunction and even mortality (128–130). Furthermore, the upcoming AspiriN To Inhibit SEPSIS (ANTISEPSIS) trial will investigate the use of low-dose aspirin as a primary preventative measure to reduce sepsis-related mortality and organ dysfunction in elderly patients (131). Beyond prototypical anti-platelet agents, other novel therapeutic targets have emerged from our understanding of the cellular and molecular mechanisms of platelet function in sepsis. For example, an abundance of preclinical data described above supports the use of therapies targeting the platelet-neutrophil-NETs axis to protect against microvascular dysfunction, organ damage, and death in models of bacterial sepsis and endotoxemia. With a growing list of direct and indirect inhibitors of NETs production and/or function, clinical trials of NETs-blockade in sepsis are on the horizon. Of course, as with any immunomodulatory therapy, blockade of the platelet-neutrophil-NETs axis may produce unwanted side effects including defects in host defense. Therefore, continued research is needed to generate optimized treatment strategies that functionally uncouple the harmful pathogenic mechanisms from the protective immune properties of this intravascular immune response.

AUTHOR CONTRIBUTIONS

BM performed the literature review, wrote and edited the manuscript. MD contributed to the literature review, manuscript preparation, and figure development.

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Misunderstandings Between Platelets and Neutrophils Build in Chronic Inflammation

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Regulated hemostasis, inflammation and innate immunity entail extensive interactions between platelets and neutrophils. Under physiological conditions, vascular inflammation offers a template for the establishment of effective intravascular immunity, with platelets providing neutrophils with an array of signals that increase their activation threshold, thus limiting collateral damage to tissues and promoting termination of the inflammatory response. By contrast, persistent systemic inflammation as observed in immune-mediated diseases, such as systemic vasculitides, systemic sclerosis, systemic lupus erythematosus or rheumatoid arthritis is characterized by platelet and neutrophil reciprocal activation, which ultimately culminates in the generation of thrombo-inflammatory lesions, fostering vascular injury and organ damage. Here, we discuss recent evidence regarding the multifaceted aspects of platelet-neutrophil interactions from bone marrow precursors to shed microparticles. Moreover, we analyse shared and disease-specific events due to an aberrant deployment of these interactions in human diseases. To restore communications between the pillars of the immune-hemostatic continuum constitutes a fascinating challenge for the near future.

Keywords: platelets, neutrophil, inflammation, autoimmunity, systemic lupus erythematosus, systemic sclerosis, rheumatoid arthritis, vasculitis

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INTRODUCTION

The Immune-Hemostatic Continuum

The circulatory system provides functional integration to tissues throughout the body and constitutes a dynamic platform for tasks, such as immune patrolling and defense against threats (1–3). Consistently, abnormalities in blood cells and cardiovascular manifestations are disproportionately represented in systemic autoimmune diseases, reflecting a network of interactions among circulating elements in the blood (4–7). Humoral moieties, such as complement, opsonins, components of the coagulation cascade and regulators of the vascular tone lie at the lowest level of complexity in this system and provide immediate, stereotyped responses to abnormal changes in the environment, such as volume loss, vascular injury or pathogen invasion, besides supporting more elaborate, long-term tasks performed by cells or subcellular elements (8–11).

Cellular membranes allow the segregation of selected information in compartments and modulate their subsequent effects on the environment by integrating multiple stimuli. Circulating membrane-endowed players in the immuno-hemostatic network encompass leukocytes, platelets and microparticles, with the endothelium as a fourth static counterpart (12, 13). Platelets and neutrophils play a crucial role in the maintenance of vascular and tissue integrity and interact extensively and productively.

Consistently, alterations in platelet-neutrophil cross-talk have dramatic long-range effects on vascular and immune homeostasis (14–16) and are growingly appreciated as targets for therapeutic intervention (17, 18).

PATHOPHYSIOLOGY OF INTERACTIONS BETWEEN PLATELETS AND NEUTROPHILS

Characters on the Stage: Platelets, Neutrophils, and Microparticles

Hemostasis and inflammation counterbalance the effect of injuring external stimuli. Selective regulation and polarization of these pathways enhance homeostatic responses at sites of tissue or vascular injury and minimize the detrimental effects to the host. Multiple mechanisms have developed, including variability in the lifespan of players involved in the immune-hemostatic balance and availability of soluble or membrane-bound moieties. Emission of membrane-endowed subcellular particles fine-tunes cellular activation and converts locally concentrated high-intensity responses into a sum of smaller but widespread and reciprocally independent biological events. Generation of extracellular vesicles enables the extension of the total membrane area interacting with the environment as well as of the range of potential cellular targets. In addition, segregation of information in multiple signaling quanta discloses the possibility of independent interactions with distinct cellular counterparts according to the differential needs of target tissues. Platelets are anucleate cell fragments released by megakaryocytes. After a multi-stage process of cytoplasm compartmentalisation and concentration of bioactive compounds into granules taking place over the course of days, platelets are released as elongated precursors (proplatelets), which undergo multiple iterative fission events to reach their final size. Preplatelets are round-shaped precursors constituting a reversible intermediate stage in the transition from proplatelets to platelets (19). Small vessels of the bone marrow, spleen and lung might deliver signals that facilitate the final process of platelet maturation (20).

Increased platelet demand and/or consumption during acute systemic inflammation warrants adaptation of megakaryocytes. Inflammatory cytokines, such as IL6 promote megakaryocyte increase in ploidy and prompt thrombocytopoiesis through increased liver synthesis of thrombopoietin as part of the acute phase response (21). Platelets released under inflammatory stress are usually larger in volume, which correlates with an increased ischemic risk at a clinical level (22). Alternative sites of thrombocytopoiesis, such as the lung, might become activated under stress conditions in mice (23) and possibly in patients with lung cancer (24). In addition, stem-like megakaryocyte progenitors can be activated on demand during interferon-α-driven inflammatory responses (25). Platelets survive for 7–10 days in circulation, where they surrogate the damaged endothelium during vascular injury, recognize and control invading pathogens and release stimuli to promote tissue repair (12).

Controlled exocytosis or integration of bioactive compounds into platelet membrane is crucial for these tasks. Platelets are endowed with three classes of granules: alpha-granules; dense granules and few lysosomes. Some authors also described "T granules" equipped with Toll-like receptor 9 as a potential fourth platelet compartment (26–28). Besides being providers of bioactive compounds through exocytosis, platelets also produce microparticles. Platelet-derived microparticles (PDμP) constitute a substantial fraction of circulating microparticles in humans under physiological conditions (29). PDμP can present with a variety of sizes, contents and functions (30–32) that range from facilitation of coagulation through tissue factor (TF) and phospholipid (phosphatidylserine) scaffolds (33, 34) to angiogenesis, tissue repair (35–37) and defensive responses (38). Modulation of neutrophil behavior through delivery of nucleic acids (RNA) or inflammatory signal intercellular transfer also occurs under inflammatory conditions (39–42). In addition, mitochondria-enriched PDμP modulate target cell metabolism (32). Megakaryocytes release microparticles as well, influencing bone marrow homeostasis and synergising with PDμP (32, 43, 44).

Neutrophils constitute the most abundant leukocyte population in the blood and are in charge of the early innate effector response to noxious stimuli (45). Neutrophils express a vast array of oxidative and proteolytic enzymes, which are preformed and stored in ready-to-use granules. Activated neutrophils express TF, promoting isolation of injured tissues through thrombosis (46, 47) and contributing to immune-thrombosis upon interaction with platelets (2, 3). Neutrophils have a limited lifespan (45), which is mirrored by the timing of multiple acute or hyperacute clinical manifestations of infectious and immune-mediated diseases (48–50). Autophagy induction under inflammatory conditions might extend neutrophil survival, causing chronicization of tissue damage and facilitating autoimmunity (40, 51–55). In addition hematopoietic stem cells and myeloid progenitors respond to extreme inflammatory stimuli (thanks to the expression of innate germ-line encoded receptors, such as Toll-like receptors) causing massive granulopoiesis (56, 57).

Phagocytosis and digestion of invading pathogens constitutes the default-mode defensive task performed by neutrophils. However, frustrated microbial phagocytosis promotes the generation of extracellular traps (NETs) (58), i.e., the extracellular release of threads of decondensed chromatin and microbicidal moieties with or without loss of membrane integrity and cell vitality (suicidal vs. vital NET generation) (59). At least in cases leading to cell death, granule content is partially repurposed to facilitate chromatin remodeling and histone citrullination (60–63). Besides having a role in antimicrobial defense, NETs are also generated in response to unconventional stimuli, such as amorphous crystals, apoptotic bodies, cytokines, microparticles and changes in osmolarity (64, 65). Factors driving neutrophils to "choose" NET generation as opposed to phagocytosis are partially understood (66). Unsolicited formation and impaired clearance of NETs implies persistent exposure of self-antigens and inflammatory stimuli, which facilitates the development of autoimmunity (67–73).

Neutrophils also account for tissue and vascular damage in immune-mediated diseases including inflammatory bowel diseases, systemic lupus erythematosus (SLE) rheumatoid arthritis (RA) and vasculitides either directly or through NET-induced facilitation of thrombosis (2, 67, 68, 74–76). Neutrophils cooperate to regulate immune activation by secreting soluble pattern recognition receptors, such as pentraxin-3 (PTX3) or ficolins (3, 77, 78). Furthermore, they influence the activation and survival of other immunocompetent cells and tune the degree of systemic inflammation (45). Finally, neutrophils emit microparticles loaded with nucleic acids and/or digestive enzymes and armed with tissue factor (47, 79, 80). Neutrophil-derived microparticles prime endothelial cells, macrophages and neutrophils themselves to inflammatory activation (80, 81) and possibly destabilize genomic integrity in target tissues preventing resolution of the immune response (82). Elevated levels of such microparticles, possibly interacting with NET constituents are detectable in blood of patients with immune-mediated diseases (83, 84).

Weaving the Plot: General Features of Platelet-Neutrophil Interactions

Platelets interact productively with multiple cells types (12), although neutrophils constitute preferential partners (62) (**Figure 1**). P-selectin expression on platelet surface (which interacts with P-selectin granulocyte ligand 1, PSGL1, constitutively expressed by neutrophils) initiates platelet-neutrophil interaction (85, 86) and occurs after vessel injury/endothelial activation (87), pathogen recognition (88), or aging (89). P-selectin-dependent platelet-neutrophil interaction recruits downstream integrin-dependent pathways and culminates in neutrophil activation and facilitated extravasation (90). Neutrophils interacting with platelets can either: (a) phagocytose them, quenching their thrombogenic and inflammatory potential (85); (b) progressing to the generation of NETs (88), an event influenced by the neutrophil metabolic state (66, 91, 92).

Vital neutrophil internalization (emperilopolesis) into megakaryocytes occurs in the bone marrow. Engulfed neutrophils provide megakaryocytes with activating stimuli (causing a rise in platelet production) and donate membrane segments causing enhanced phosphatidylserine expression by chimeric daughter platelets (93). It is tempting to speculate that emperilopolesis holds the key to understanding the mechanisms of the later interactions between neutrophils and platelets in the circulation.

Platelets and megakaryocytes communicate long-range with neutrophils through exocytosed mediators and microparticles with enhancing actions on neutrophil activation (94, 95). The prototypic alarmin/damage-associated molecular pattern HMGB1, either as a soluble moiety or loaded into microparticles, is a potent promoter of extended neutrophil survival and of NET generation (40, 51, 96–99). Cooperation and bidirectional exchange of lipid metabolites between neutrophils and platelets through microparticles maximize the synthesis of prostaglandins, such as the pro-coagulant and vasoconstrictor signal, thromboxane A2 (100, 101) and to the activation of the complement cascade, which in turn promotes neutrophil recruitment and activation (102).

An Anthology of Recurring *Topoi*: Shared Events Linked to Platelet and Neutrophil Biology

The interaction between platelets and neutrophils impacts on multiple stereotyped pathological manifestations (**Table 1**). A first hint can be found in laboratory tests, such as blood cell counts. In most cases, platelet and leukocyte numbers roughly correlate with systemic inflammation (147), either due to disease activity or infections. By contrast, reduced blood cell counts are a hallmark of a minority of autoimmune conditions, including SLE and overlap syndromes and Felty's syndrome, but might dominate during sepsis, affecting survival. No specific evidence is available about the reciprocal interactions between platelets and neutrophils during severe thrombocytopenia as an isolated phenomenon. This fact might also be due to difficulties in uncoupling variations in platelet counts and neutrophil responses from shared inciting stimuli underlying both events at an experimental level. Nonetheless, consistent evidence from sepsis, idiopathic thrombocytopenic purpura, thrombotic thrombocytopenic purpura and thrombocytopenia induced by heparin or other drugs suggests that neutrophils are intravascularly activated and tend to generate NETs in association with low platelet counts, possibly contributing to dysregulated hemostasis and adverse clinical outcomes (148–153). Interestingly, viral infections causing thrombocytopenia seem to associate with the expansion of low-density neutrophils (which are thought to have a higher propensity to form NETs), as observed in autoimmune diseases, such as SLE (154). Pancytopenia is a distinctive feature of hemophagocytic lymphohistiocytosis, a severe disorder developing either as a complication of multiple autoimmune diseases or as a standalone disorder (155). Variations in cell volume are thought to correlate with cytoskeletal remodeling and changes in cell function and metabolism (156–159). Mean platelet volume (MPV) is a variable provided in the context of blood count, significantly susceptible to the effects of multiple confounders at the analytical and preanalytical level. Altered MPV has been detected in SLE, RA, large- and small-vessel vasculitides, systemic sclerosis (SSc) and chronic spontaneous urticaria (103, 104, 117, 118, 123, 129, 135, 136, 160). However, disease activity in these settings has been associated with high and low MPV values, preventing a straightforward translation of this laboratory tool into clinical practice (161). Gasparyan et al. (137) and Scherlinger et al. (97) offer a possible syncretistic perspective: high platelet volume might reflect chronic low-grade platelet activation (156), whereas low MPV can be consequent to more severe inflammation, causing extensive shedding of platelet microparticles (32). Neutrophils showing increased cellular volume and consequent lower granular density have long been recognized as a hallmark of systemic immune-mediated diseases (159), although they are also detectable in sepsis (162) and cancer (163). This cell population is characterized by enhanced ability to extravasate,

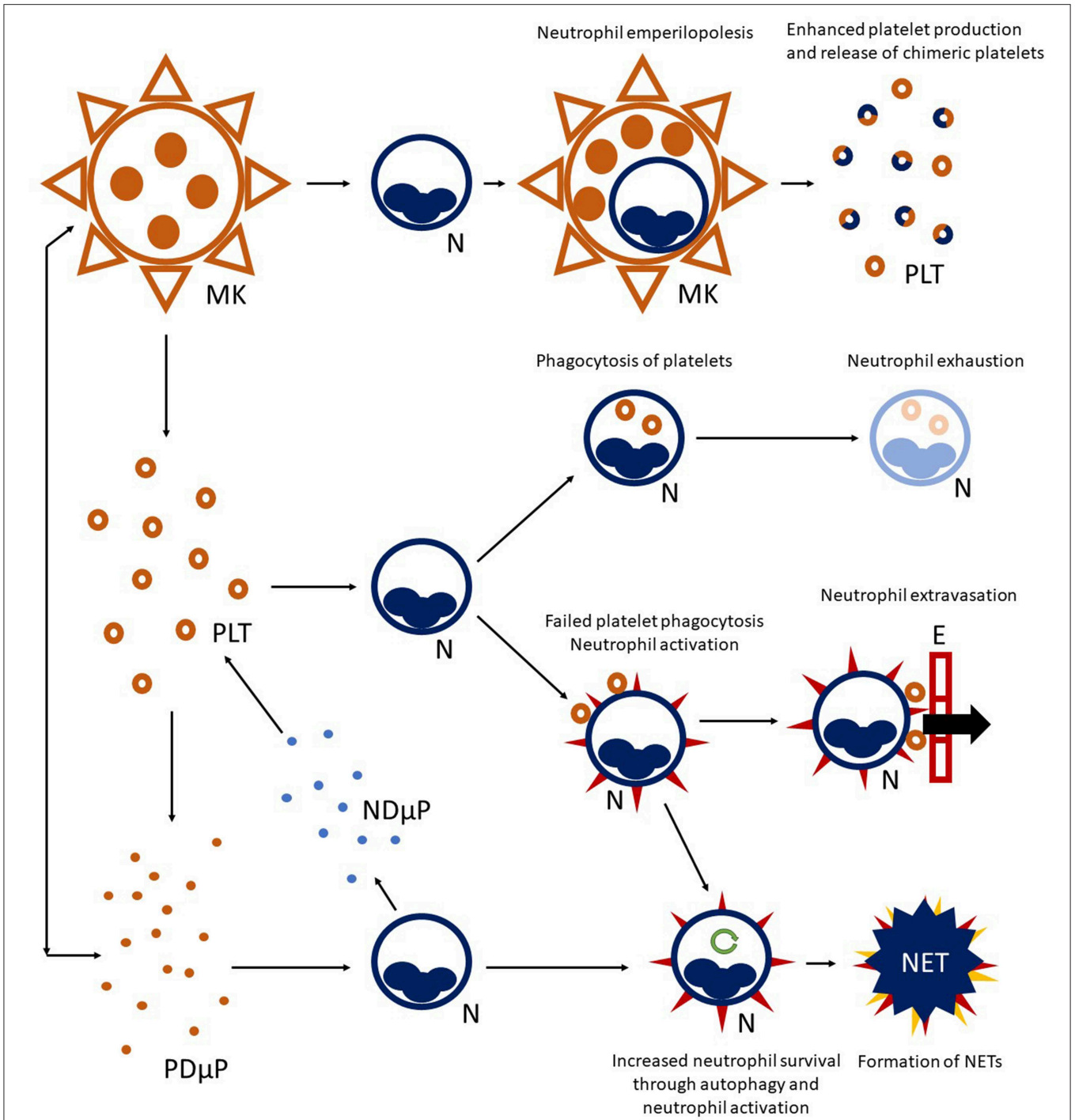

FIGURE 1 | Platelet-neutrophil crosstalk. A complex network of interactions connects platelet (PLT) to neutrophil (N) biology. Megakaryocytes (MK) are able to interact with neutrophils residing in the bone marrow and engulf them, preserving their vitality (a phenomenon called emperilopolesis). Neutrophils may eventually escape megakaryocyte engulfment after donating membrane components to the host cell. This event causes enhanced platelet production and release of chimeric platelets. Activated platelets and neutrophils can further interact in the circulating blood, either directly through cell-cell contact and/or through the exchange of soluble compounds or microparticles. Engagement of platelets by neutrophils through the P-selectin/PSGL-1 axis, and later on, integrin-mediated bonds can lead to platelet-phagocytosis, resulting in neutrophil exhaustion. Alternatively, failed platelet clearance can promote neutrophil activation (heralded by expression of surface markers, such as tissue factor and activation of integrins—red spikes in the figure) and facilitate neutrophil extravasation through the endothelial wall (E). Activated neutrophils can also enter autophagy programmes (green circular arrow), extending their survival, and progress toward the formation of neutrophil extracellular traps (NET). Platelet-derived microparticles (PDμP), especially when loaded with the damage associated molecular pattern HMGB1, are potent inducers of NET generation. Neutrophil-derived microparticles (NDμP) constitute and additional channel for platelet-neutrophil interchanges and are thought to have a role in lipid metabolism.

extended survival and by a tendency to form NETs (68, 158, 164). By contrast, consistent with the model of inverse association between NETosis and phagocytosis (**Figure 1**), low density neutrophils are less able to engulf substrates (159).

Patients with inflammatory diseases of the blood vessels (124, 130, 131), connective tissue diseases and chronic arthritides have higher risks of ischemic events (105, 106, 113, 135, 138, 139, 165–167). Accelerated atherosclerosis is a common finding in SLE (7, 106, 168–170), RA (7, 139, 165, 171), or systemic vasculitides (170). Inflammation-related atherosclerotic mechanisms are only partially understood (172). NETs are major determinants of endothelial dysfunction and have been detected in atherosclerotic lesions (173). Platelets are also involved in the early phases of atherosclerosis, where they facilitate leukocyte egress toward the subendothelial vascular layers (174, 175). Antiphospholipid antibodies (aPL) constitute a major independent risk factor in the antiphospholipid syndrome (APS), a condition characterized by arterial or venous thrombosis and/or pregnancy complications occurring as a standalone disorder or secondary to SLE and other autoimmune diseases (176). aPL promote HMGB1-related response in platelets and monocytes (177) and, in cooperation with platelet Toll-like receptor 4, induce NETosis (178), possibly contributing to immunothrombosis (76). In addition, they might impair microparticle scavenging by glycoprotein I, increasing the likelihood of pro-coagulant platelet-neutrophil activation (179). Besides atherosclerotic lesions, NETs have been detected into coronary thrombi (96, 180).

Aberrant coagulation is detectable in immune mediated diseases (34, 107, 108, 119). Altered coagulation cascade and increased cardiovascular risk are common in asthmatic patients (181, 182), while patients with chronic spontaneous urticaria show aberrant thrombin generation but suffer no excess prevalence of cardiovascular disease (125, 141–146).

Thrombotic microangiopathy, consisting in diffuse deposition of thrombi along small vessels due to widespread endothelial activation with hemolysis and platelet consumption, might complicate SLE, SSc, antiphospholipid syndrome and other immune-mediated diseases (183–185). Under intravascular hemolytic conditions, free hemoglobin favors platelet activation. In turn, activated platelet boost neutrophil activation, possibly further feeding the inflammatory cascade (186). In addition, sera from patients with thrombotic microangiopathies fail to degrade NETs, which, in turn, can trigger thrombosis (76, 187).

The lung is a major inflammatory target (188). Neutrophils and platelets undergo unique pathophysiological interaction with the lung vasculature, a reservoir for neutrophils that can thus easily respond to airborne infectious or sterile inflammatory stimuli (189). Unleashed neutrophil effector functions contribute to acute lung injury/acute respiratory distress syndrome (ARDS) during sepsis or vasculitis (50), to the long-term effects on bronchial tissue of chronic inflammation in cystic fibrosis (190, 191) and to interstitial fibrosis in SSc (40, 192). NET generation has a central role in neutrophilic lung injury (193, 194). The lung vasculature influences platelets since lung microvessels are (a) a site of maturation of platelet precursors from the bone marrow (20); (b) a niche for human and mouse megakaryocyte homing (23, 195); (c) a site of detoxification of platelet mediators,

TABLE 1 | Clinical and laboratory features potentially affected by altered platelet-leukocyte interactions in selected immune-mediated diseases.

	Increased ischemic risk	Abnormal thrombin generation	Thrombotic microangiopathy	Lung involvement	Leukopenia	Thrombocytopenia	Altered platelet volume	Low complement	Vasculitis	Urticaria	Vascular remodeling	Fibrosis	References
SLE	+	+	+/–	+	+	+	+	+	+/–	+/–	–	–	(34, 103–112)
APS	+	+	+/–	+	–	+/–	?	+/–	+/–	–	–	–	(113–116)
SSc	+	+/–	+/–	+	–	–	+	+/–	–	–	+	+	(117–122)
AAV	+	+	–	+	–	–	+	–	+	+/–	–	–	(123–128)
LVV	+	?	–	–	–	–	+	–	+	–	+	–	(126, 129–134)
RA	+	+	–	+/–	–	–	+	–	+/–	–	–	–	(119, 135–140)
CSU	–	+	–	–	–	–	+	+/–	+/–	+	–	–	(141–146)

AAV, ANCA-associated vasculitides; APS, anti-phospholipid syndrome; CSU, chronic spontaneous urticaria; LVV, large-vessel vasculitides; RA, rheumatoid arthritis; SLE, systemic lupus erythematosus; SSc, systemic sclerosis. +, common association/typical feature; +/–, possible manifestation; –, infrequent manifestation; ?, lack of sufficient data.

such as serotonin (196). Conversely platelets contribute to persistent vasoconstriction (potentially leading to pulmonary hypertension), interstitial fibrosis (197), and NET generation in acute lung injury (198).

ABNORMAL PLATELET-NEUTROPHIL INTERACTIONS IN SELECTED DISEASE SETTINGS

Systemic Vasculitides

Systemic vasculitides encompass a very large set of immune-mediated diseases characterized by vascular injury and downstream organ ischemia as the core pathophysiological event (199). They can be roughly classified into two main subsets, large- and small-vessel vasculitides, based on (prominent) sites of vascular involvement. From a pathophysiological point of view, vascular remodeling with immune infiltration, disassembly of the physiological extracellular architecture and aberrant proliferation of macrophages, fibroblasts and endothelial cells predominate in large-vessel vasculitides, resulting in stroke-like acute failure of large portions of tissues or entire organ due to vessel occlusion. By contrast, small-vessel vasculitis are characterized by necrosis associated or not to granulomatous inflammation and increased thrombotic risk (200). Altered cross-talk among platelet, neutrophils and microparticles might contribute to vascular injury (201).

Neutrophils cause vascular damage in small-vessel vasculitis, possibly reflected by the accumulation of leukocyte cellular debris in peri-vasculitic lesions (leukocytoclasia). In anti-neutrophil cytoplasmic antibody (ANCA)-associated vasculitides (AAV), neutrophil mediated vascular injury is part of a vicious circle linking exposure of myeloperoxidase (MPO) and/or proteinase-3 from granules to neutrophil surface or into the setting of NETs (202) to the development of ANCA, which in turn activate neutrophils and the vascular endothelium (3, 67). In patients with AAV neutrophil responses couple with platelet activation (126). Consistently, patients with active AAV show increased plasmatic levels of P-selectin (127) and HMGB1 (203, 204). Elevation of HMGB1 has also been detected in other vasculitic settings, such as IgA-vasculitis (formerly Henoch-Schonlein's purpura) and Kawasaki disease (205).

In large vessel-vasculitides, activation of platelets may contribute to promote vascular remodeling through the release of signals, such as VEGF (206). Platelets in large-vessel vasculitides show signs of activation (129, 201), and in giant cell arteritis are significantly increased in number during active disease (147) and form hetero-aggregates with leukocytes, possibly contributing to exacerbate ischemic risk (132, 201). This evidence provides a rationale for the use of aspirin in primary prevention (207) and for the employment of platelets as diagnostic surrogates (126). Results in the literature (208) and reports on a potential cyclooxygenase-independent mechanism for aspirin in giant cell arteritis (209) suggest however that the role of platelets should be interpreted with caution.

Little is known on platelet-leukocyte interactions in large vessel vasculitides, although relative depletion of neutrophil granule content has been reported in giant cell arteritis in association with platelet activation (126, 132). Notably, items of small-vessel (peri)vasculitis have been reported in large series of temporal artery biopsies from patients with giant cell arteritis (210) and might be secondary to stereotyped events resembling those observed in small-vessel vasculitides, possibly targeting the *vasa vasorum* and entailing aberrant platelet-neutrophil cross-talk.

Systemic Lupus Erythematosus

SLE is a multi-organ autoimmune disease with a wide spectrum of clinical manifestations and pathogenic mechanisms (211). Failure of clearance mechanisms and/or exposure of cell death debris in an inflammatory setting promotes autoimmunity and subsequent tissue damage (212). Hematological manifestations constitute a hallmark of SLE and are detected in >80% of patients (213). Cytopenia is the most frequent modality of presentation and affects either red blood cells, platelets and leukocytes. Bone marrow abnormalities are frequent, although no clear correlation can be established with disease activity (214–217). Accordingly, primary bone marrow failure is a rare cause of cytopenia (218), with most relevant mechanisms (besides drugs) being inflammation-induced iron deficiency and cytolysis. Neutropenia occurs in up to one third of SLE cases, in most cases due to antibodies (219), which is not apparently associated to infectious risk (109). Thrombocytopenia is also common in patients with SLE (220). Megakaryocyte number is generally increased during disease activity, reflecting extensive platelet production (214, 220). Patients with SLE and thrombocytopenia have an increased risk of a severe disease course and of mortality in large cohort studies (221).

Cardiovascular manifestations are frequent in patients with SLE and a cause of morbidity and mortality (222). Accelerated atherosclerosis, aPL and dysfunctional coagulation likely converge to determine this risk (106). Despite low absolute platelet counts, patients with SLE frequently show extensive platelet activation (223–227). Higher levels of P-selectin are detectable in urines from patients with lupus nephritis (228). Platelets also contribute to mesangial remodeling and renal vascular damage (229, 230).

Endothelial derived microparticles constitute the most abundant microparticle subset in patients with SLE and correlate with endothelial dysfunction and interferon-α signature (231, 232). However, PDμP also accumulate during active SLE (233, 234) and might impact on inflammation and hemostasis (34, 234). PDμP facilitate coagulation by providing phosphatidylserine scaffolds and intravascularly expressed TF. In addition, they promote neutrophil activation and NET generation being reservoirs of HMGB1 (96) and CD40L (234). Finally PDμP synergise with NETs as inducers of anti-nuclear immunity by constituting a source of mitochondria, which behave as potent damage-associated molecular patterns due to their bacterial origin (235).

Mechanistically, platelet activation in SLE might depend on circulating immunocomplexes, which are abundant in SLE patients biological fluids and are recognized on platelet surface by FcγRIIA and Toll-like receptor 4,7 (236). PDμP

themselves could take part in immunocomplexes, enforcing an inflammatory/immunogenic self-sustaining loop (235). Ensuing complement activation in turn amplifies and propagates neutrophil and platelet activation (102, 231, 234).

Systemic Sclerosis

SSc is a systemic autoimmune disease, characterized by unrelenting inflammation with a wound repair response consisting in mesenchymal extracellular matrix deposition leading to fibrosis, and by microvascular dysfunction and aberrant neoangiogenesis (120, 237, 238). Platelets and aberrant platelet-neutrophil interactions play a role in SSc (239). Possibly in response to microvascular damage, platelets of patients with SSc are constitutively activated and express signals driving neutrophil interaction (240, 241). P-selectin dependent cell-cell interactions seem to be relatively less represented in SSc, due to the lower leukocyte expression of PSGL-1 (242). Neutrophils have a pericellular distribution of granules and of their content, causing enhanced degradation of fibrinogen by exposed neutrophil proteases and eventually impairing fibrinogen dependent interactions between neutrophil CD11b/CD18 (also known as Mac-1 or αMβ2 integrin) and platelet glycoprotein IIbIIIa (40, 96). Indeed, platelet-neutrophil heterotypic aggregates are less frequently detected in SSc compared to other inflammatory conditions (40, 96, 242).

Activated platelets in SSc contribute to impaired vascular tone [due to altered arachidonic acid metabolism (197, 239)] and to fibrosis. In fact, platelets release multiple fibrogenic mediators, such as transforming growth factor beta, platelet-derived growth factor, CXCL4 (also known as platelet factor 4), beta-thromboglobulin, serotonin and HMGB1 (27, 97, 242–244). NETs promote fibroblast differentiation and function and might synergise with platelet in supporting fibrosis (192), also in light of the abundance of NET byproducts in the blood of patients with SSc (40). Synergistic NET/platelet-induced fibrosis is expected to be particularly significant in lung tissue, where neutrophil and platelets are abundant (189). Consistently, PDμP (retrieved from the plasma of patients with SSc) induce neutrophil granule mobilization and autophagy, culminating in extended neutrophil survival and generation of NETs through a HMGB1-dependent mechanism. Furthermore, neutrophils stimulated by SSc platelet microparticles migrate in murine lungs, associate with interstitial endothelial damage and promote lung fibrosis (40).

Rheumatoid Arthritis

Rheumatoid arthritis is a relatively frequent autoimmune disease characterized by prominent involvement of the synovial joints. Although extra-skeletal manifestations are relatively less frequent compared to other immune-mediated diseases, patients with RA show an increased ischemic risk, pointing to the existence of a core pathophysiological event linking inflammatory manifestations to vascular dysfunction (245).

Neutrophils undergoing NET generation might provide autoantigens in RA. Patients in fact frequently develop antibodies against citrullinated peptides (ACPA). Citrullination occurs thanks to the activity of deiminating enzymes, such as protein-arginine deiminase 4 (PAD4), abundantly expressed in neutrophils (246). Citrullinated histones constitute a fundamental component of chromatin threads within NETs (73, 247). Platelets might also contribute to ACPA formation due to their expression of vimentin, a preferential target of citrullination (248). ACPA sustain joint inflammation by perpetuating macrophage activation within the synovia, eventually causing chronically elevated levels of tumor necrosis factor alpha (TNFα), which in turn promotes synovial proliferation, bone reabsorption, neoangiogenesis and enhanced synovial infiltration through activated endothelium (249). Enhanced expression of TF on activated platelets and neutrophils, which coexist in the synovial fluid of inflamed joints in patients with RA, provides an interesting hint on potential mechanisms involved in RA-associated enhanced ischemic risk. Platelets and leukocytes from patients with RA are activated due increased plasmatic concentration of TNFα (140). Indeed, platelet respond to TNFα thanks to the expression of TNF receptors 1 and 2 (250) resulting in increased P-selectin expression, platelet degranulation, phosphatidylserine up-regulation and TF expression. TNFα-activated platelets prompt thrombin generation and activation of leukocytes due to P-selectin. Consistently, platelet and leukocyte activation is reduced in patients treated with anti-TNF agents (140), who also face relatively lower rates of cardiovascular events in the long-term (251, 252).

Platelets can be activated by collagen through the megakaryocyte lineage-specific glycoprotein VI and thus prompted to generate microparticles. Boilard and colleagues (253) showed that, following this mechanism, high concentrations of PDμP (possibly shuttled by engaging leukocytes) are detectable in synovial fluid of patients with RA and are required for arthritis development in a murine model. PDμP contain significant amounts of interleukin 1α and β, promoting synoviocyte proliferation, and of IL8, enhancing neutrophil recruitment and ensuring maintenance of inflammation (253).

CONCLUSION

Platelets and neutrophils are major determinants of the immune-hemostatic continuum and extensively interact based on cell-cell contact and/or exchange of soluble signals and microparticles to synergise in contrasting the noxious effects of endogenous or environmental stimuli toward vessel and tissue integrity and to promote physiological tissue renewal and homeostasis. These events, part of a set of simple, innate, but evolutionarily preserved stereotyped responses, are disproportionately active and self-sustained in patients with immune-mediated diseases, such as systemic vasculitides, SLE, SSc, RA and possibly allergic disorders and might account for the development of either some inflammatory manifestations and of cardiovascular complications. Patients with immune-mediated diseases consistently show signs of platelet (and/or PDμP) activation, possibly prompting either the formation of heterotypic aggregates with neutrophils (as in giant cell arteritis) or neutrophil activation toward enhanced survival and eventually

NET generation (as observed in small-vessel vasculitis, SLE, RA and to a higher extent SSc). Diagnostic and therapeutic strategies currently employed in the setting of autoimmune diseases to prevent disease progression and the occurrence of secondary complications are generally not targeted on these pathogenic mechanisms, suggesting the existence of a largely unexplored window of opportunity to improve survival and quality of life for patients by dampening sustained neutrophil-platelet interactions.

AUTHOR CONTRIBUTIONS

GR and NM collected the literature data, drafted, and revised the manuscript. AM revised the manuscript and provided critical analysis of intellectual content. All authors approved the final version of the manuscript and agree to be accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work will appropriately be investigated and resolved.

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REVIEW
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Platelets as Modulators of Cerebral Ischemia/Reperfusion Injury

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Ischemic stroke is among the leading causes of disability and death worldwide. In acute ischemic stroke, the rapid recanalization of occluded cranial vessels is the primary therapeutic aim. However, experimental data (obtained using mostly the transient middle cerebral artery occlusion model) indicates that progressive stroke can still develop despite successful recanalization, a process termed "reperfusion injury." Mounting experimental evidence suggests that platelets and T cells contribute to cerebral ischemia/reperfusion injury, and ischemic stroke is increasingly considered a thrombo-inflammatory disease. The interaction of von Willebrand factor and its receptor on the platelet surface, glycoprotein Ib, as well as many activatory platelet receptors and platelet degranulation contribute to secondary infarct growth in this setting. In contrast, interference with GPIIb/IIIa-dependent platelet aggregation and thrombus formation does not improve the outcome of acute brain ischemia but dramatically increases the susceptibility to intracranial hemorrhage. Here, we summarize the current understanding of the mechanisms and the potential translational impact of platelet contributions to cerebral ischemia/reperfusion injury.

Keywords: thrombo-inflammation, ischemic stroke, platelet, glycoprotein Ibα, platelet degranulation

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INTRODUCTION

Stroke is a leading cause of disability and death worldwide. The majority of strokes are caused by cerebral ischemia, only ∼20% of strokes are caused by primary intracerebral hemorrhage (1). The primary therapeutic goal in acute ischemic stroke is the rapid re-establishment of blood flow by thrombolysis or mechanical thrombectomy. The invention of thrombectomy has dramatically increased the rate of successful recanalization up to 80% and improved neurological sequelae, but the *number needed to treat* (NNT) for a good outcome is still high, around 4–6 (2, 3). It is known from experimental stroke models, that infarcts can grow despite successful reperfusion, which is commonly referred to as ischemia/reperfusion injury (I/RI). In humans, there are many possible reasons for unfavorable outcomes after recanalization such as advanced stroke development with low ASPECTS scores already at treatment entry or bleeding complications, but recent clinical trials testing immune-modulatory drugs in acute stroke indicate that infarcts can grow after restoration of cerebral blood flow also in humans (4, 5), although the occurrence of reperfusion injury in the human brain is less clear than in experimental animal models (6). However, the process of further tissue injury upon reconstitution of blood flow is known to apply to different other organ systems such as heart, liver and kidney (7). The middle cerebral artery (MCA) is the most commonly affected blood vessel in human occlusive/ischemic stroke. A broad range of MCAO models has been developed [reviewed in (8)] but no animal model perfectly reflects the disease under study and

each model has strengths and limitations trying to reproduce the complex heterogeneous nature of stroke in humans. To study the underlying pathomechanisms of I/RI in the brain, the transient middle cerebral artery occlusion (tMCAO) model of focal cerebral ischemia is widely used, mostly in rodents (9). Advantages and disadvantages of the tMCAO model have been discussed in a recent review (10). In this model, a filament is usually inserted via the internal carotid artery to occlude the MCA for defined time periods, most commonly for 1 h, resulting in complete infarction of the MCA territory. Importantly, infarcts are not fully developed immediately after recanalization but evolve in the reperfusion phase. Numerous experimental studies have established a contribution of platelets and immune cells, in particular T cells, in this cerebral I/RI (11). Therefore, ischemic stroke is now considered a thrombo-inflammatory disease (12). The contribution of neuro-inflammation to cerebral damage following ischemic stroke has been recently reviewed (11, 13, 14), thus, in this review, we focus on the contribution of platelets.

The initial capture of circulating platelets to exposed components of the extracellular matrix (ECM) at sites of vascular injury or inflammation is mediated by the interaction of the glycoprotein (GP) Ib-IX-V receptor complex with von Willebrand factor (vWF) (15). GPIbα-vWF interactions are essential for initial platelet tethering under high shear flow conditions, found e.g., in stenosed arteries, but are only transient and too weak to mediate firm platelet adhesion, but rather decelerate and recruit platelets from the blood stream, which is reflected by the "rolling" of platelets on the vessel wall (16). These "rolling" platelets can interact via their activatory platelet receptors with the corresponding ligands. Platelet activation is mainly triggered through two major signaling pathways, depending on the initial stimulus: signaling via G-protein coupled receptors (GPCRs) (17, 18) or the (hem)*immunoreceptor tyrosine-based activation motif* (ITAM)-bearing receptors. Both signaling pathways culminate in activation of intracellular signaling cascades involving a rise in cytosolic Ca^{2+} concentration, cytoskeletal rearrangements, mobilization of α- and dense granules and subsequent release of secondary platelet agonists and the conformational change of integrin adhesion receptors, most notably αIIbβ3 (GPIIb/IIIa), from a low to a high affinity state, thereby mediating firm platelet adhesion, aggregation and thrombus growth (19).

Efficient blockade of GPIIb/IIIa, leading to abolished platelet aggregation (20, 21), led to intracranial hemorrhage and the treatment did not reduce cerebral infarct sizes in mice (22). Similarly, in a clinical trial, anti-GPIIb/IIIa treatment of patients in acute ischemic stroke was not protective (23). These data exclude platelet aggregation as a critical pathomechanism underlying stroke progression after recanalization and demonstrate that cerebral I/RI cannot simply be explained by secondary thrombotic events in the microcirculation. This does not exclude the appearance of micro-thrombi in the cerebral microvasculature after ischemic stroke, as thrombo-inflammation might be associated with re-thrombosis but does not require these thrombotic events to cause tissue damage.

GPIbα-vWF INTERACTIONS

GPIbα is part of the GPIb-IX-V receptor complex that is expressed on the platelet surface. The GPIb subunit binds many ligands, including its major ligand vWF, P-selectin, macrophage antigen 1 (Mac-1), and the coagulation factors XI, XII, and thrombin (24). At high shear rates, prevailing in small arterioles or stenosed arteries, initial adherence of platelets to the ECM is mediated by the interaction of GPIbα and vWF (15). Blocking the vWF binding site on GPIbα (using Fab fragments of the p0p/B antibody) has been shown to abolish platelet adhesion to the injured carotid artery wall and protects mice from occlusive thrombus formation *in vivo* (25). Remarkably, treatment of mice with p0p/B Fab lead to reduced infarct sizes, sustained reperfusion and a better neurological outcome after tMCAO, irrespective whether treatment was given 1 h before or after vessel occlusion (see **Figure 1**) (22, 26). Although mice show a prolonged bleeding time after treatment with the GPIbα blocking Fab, MRI analysis excluded the presence of intracerebral hemorrhages (22). These findings could be reproduced using a transgenic mouse model, where the ectodomain of the human interleukin 4 receptor is expressed instead of the ectodomain of GPIbα (IL4Rα/GPIbα), leading to GPIbα deficiency without the loss of the receptor complex from the platelet surface (27–30). Likewise, vWF-deficient mice that were subjected to the tMCAO model had smaller infarcts and less neurological damage than the respective controls, without showing intracranial bleedings (31). Genetic reconstitution of plasma vWF levels restored the susceptibility of these mice to ischemic stroke (31). Of note, despite only constituting 20% of the total vWF protein (32), platelet vWF alone was sufficient to render mice susceptible to experimental stroke (33). Targeting of GPIbα downstream signaling also appears to be beneficial for the outcome after tMCAO, since deficiency or inhibition of phospholipase (PL) D1 and D2 protected mice from acute ischemic stroke (34–36). Further, GPIbα-blockade reduced cerebral damage in experimental stroke of aged and comorbid animals (e.g., atherosclerotic $Ldlr^{-/-}$, diabetic or hypertensive mice) indicating that GPIbα-blockade may be a therapeutic option in the future for patients with accompanying common cardiovascular diseases (37).

Studies performed in stroke patients revealed that genetic alterations in the *GP1bA* gene and increased serum levels of vWF are risk factors for stroke (38, 39), and a predictive factor for long term mortality after acute stroke (40). VWF is a large, multimeric protein (up to 20,000 kDa) synthesized in endothelial cells and megakaryocytes that is highly thrombogenic. In the plasma the enzyme *A disintegrin and metalloprotease with thrombospondin type 1 repeats* 13 (ADAMTS13) cleaves vWF multimers into smaller, less active fragments, thereby reducing vWF activity. Mice lacking ADAMTS13 suffered from severely worsened ischemic brain damage following experimental stroke (41, 42), while intravenous administration of recombinant ADAMTS13 prior to reperfusion reduced infarct size and improved functional outcome after focal cerebral ischemia (41). Autoantibodies against ADAMTS13 as detected in most patients with acquired thrombotic thrombocytopenic purpura (TTP), or mutations in

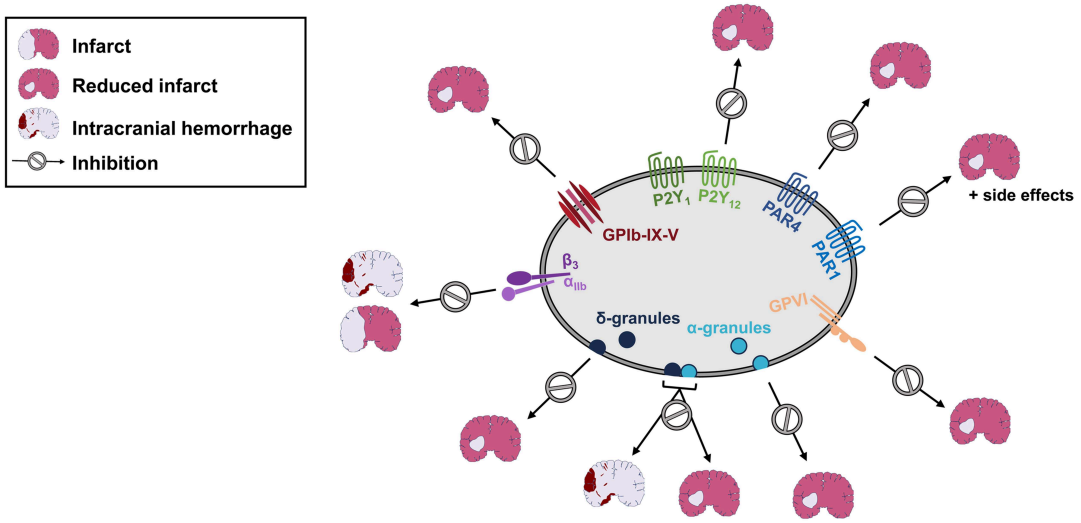

FIGURE 1 | Inhibition of platelet activation or degranulation, but not GPIIb/IIIa blockade results in reduced infarct sizes in experimental stroke. Blockade or genetic deficiency of GPIbα, GPVI, $P2Y_1$, $P2Y_{12}$, PAR1, or PAR4 reduces infarct sizes following cerebral ischemia/reperfusion injury. Likewise, lack of either dense (δ) or α-granule secretion improves the outcome following experimental stroke. In contrast, combined loss of dense and α-granule secretion or GPIIb/IIIa-blockade is accompanied by intracranial hemorrhage. See text for details.

the *ADAMTS13* gene as found in patients with hereditary TTP are associated with an increased risk of thrombotic occlusion of micro vessels in different organs, including the brain (43).

Collectively, these studies argue for the GPIb-vWF axis as an attractive target for stroke therapy. Inhibitory toxins and antibodies or aptamers targeting GPIb-vWF interactions have been designed and are currently tested in preclinical or clinical studies for different applications [reviewed as part of (44)]. Anfibatide, a snake venom-derived GPIb antagonist, led to a dose dependent reduction in infarct sizes without inducing intracranial hemorrhages when tested in mice using the tMCAO model (45) and has successfully completed a phase I clinical study (46). Although the anti-vWF nanobody caplacizumab was initially approved in 2018 as treatment for thrombotic thrombocytopenic purpura (47), it was also shown to be an effective therapy in a stroke model using guinea pigs (48), indicating that it might also be beneficial in stroke therapy. One additional promising feature of strategies targeting the GPIb-vWF axis is that they have been shown to exert thrombolytic activity (48–50). Thus, in addition to dampening thrombo-inflammation (22, 51), also the recurrence of stroke-causing thrombi might be reduced.

GLYCOPROTEIN VI

Glycoprotein (GP) VI is a platelet specific activatory receptor for collagen and fibrin. Upon ligand binding, signals are transduced via an immunoreceptor tyrosine-based activation motif (ITAM) which is contained in the non-covalently associated Fc receptor (FcR)γ-chain dimers that are part of the receptor complex (19, 52). Mice lacking GPVI, either by genetic deficiency of the *Gp6* gene or the FcRγ-chain or by antibody-mediated depletion from the platelet surface, display defective platelet responses to collagen but only a marginally impaired hemostasis, while they are profoundly protected from arterial thrombosis (53, 54) and experimental stroke (see **Figure 1**) (22). Morphological analysis of the infarcted brain areas and MR imaging could exclude the presence of intracerebral bleedings (22). The spleen tyrosine kinase (Syk) acts downstream of ITAM-coupled receptors and is therefore essential also for GPVI signaling. Upon ligand binding, cross-linking of GPVI brings Fyn and Lyn (two Src family tyrosine kinases) into contact with the FcRγ chain, which starts a tyrosine phosphorylation cascade via Syk, *linker of activated T cells* (LAT) and SLP-76 (SH2 domain containing leukocyte protein of 76 kDa) leading to the activation of most notably phospholipase (PL) C γ2 and phosphoinositide 3-kinases (PI_3K) (19). Syk not only acts downstream of GPVI but also CLEC-2 and, in human platelets, FcγRIIA. In line with the results from GPVI-deficient animals, Syk-deficient mice or mice that were treated with a Syk-inhibitor, were protected from arterial thrombosis and stroke but displayed only slightly increased bleeding times. Of note, the animals were still protected, when the treatment was given therapeutically, i.e., after induction of the infarction (55). In accordance with the data reported about the loss of ITAM-coupled receptors or signaling molecules, a simultaneous loss of the ITAM inhibitory proteins SLAP and SLAP2 dramatically aggravated neurological damage after tMCAO (56).

The protection from arterial occlusion and stroke by GPVI blockade without markedly increased risk of bleeding makes GPVI a potentially attractive target for the treatment of stroke patients in the clinic. Further, it could be shown that platelets of patients with transient ischemic attack or stroke show an elevated expression of GPVI on their surface when compared to control patients (57). To block GPVI-collagen interaction in the context of thrombosis, the GPVI-Fc fusion protein Revacept

was developed and successfully tested in animal models, where it protected mice from cerebral ischemia without inducing intracranial hemorrhages (58). In a first study in healthy humans, all doses were well-tolerated and neither prolonged bleeding time nor thrombocytopenia have been observed. Platelet response to collagen was inhibited in a time and dose-dependent manner, whereas platelet aggregation in response to ADP or TRAP was unaffected (59). The effect of Revacept in patients with carotid artery stenosis, transient ischemic attack (TIA) or stroke is currently tested in a phase II clinical study (NCT 01645306). A second phase II study in patients with coronary artery disease has been initiated and is in the phase of patient recruitment (NCT 03312855). Revacept binds to immobilized collagen and thereby indirectly prevents platelet adhesion and activation at sites of collagen exposure whereas it does not interact with fibrin (60). ACT017 (9O12), a humanized Fab fragment against GPVI, which was designed to directly inhibit GPVI on the platelet surface, was shown to inhibit collagen-induced platelet aggregation *ex vivo* and there were no signs of thrombocytopenia or excessive bleeding (61). In addition to blocking the interaction of GPVI with collagen, ACT017 has been shown to block spreading on fibrinogen of human GPVI transgenic mouse platelets (62) and reduces aggregate formation on fibrin in a model of venous thrombosis (63). A phase II clinical trial investigating the effects of ACT017 in patients with acute ischemic stroke is currently under way (NCT 03803007).

PROTEASE-ACTIVATED RECEPTORS (PARs) (THROMBIN RECEPTORS)

Thrombin activates platelets through the protease-activated receptors (PARs) 3 and 4 in mice (PAR 1 and 4 in humans), which transduce their signals via G-protein coupled receptors (G_q and $G_{12/13}$) thereby leading to platelet activation (64). Receptor expression is not restricted to platelets; PARs are expressed in a variety of tissues and cell types like bone marrow, spleen, gastrointestinal tissues, lung, placenta, thyroid, prostate tissues, and the brain, being involved in various cellular processes (65, 66). Thrombin at high concentrations can enter the brain as a result of increased BBB permeability and acts on PARs that are expressed in the central nervous system (on both, glial cells and neurons). Focal ischemia induces the expression of PAR-1 and PAR-3 on microglia and enhances PAR-4 labeling in the penumbra (67). Since PAR-1 is not expressed in mouse platelets, for initial experiments studying the role of platelet PAR-1, other model systems have been used. In preclinical models using cynomolgus monkeys, selective PAR-1 blockade led to potent inhibition of thrombin-induced platelet aggregation without impairing primary hemostatic function (68). A phase III clinical trial (NCT 00526474) showed that the PAR-1 inhibitor vorapaxar is beneficial in the secondary prevention of cardiovascular death or ischemic events, but increases the risk of bleedings in patients with a stroke history (69–71). Vorapaxar is approved for clinical use but its use is limited by a substantial bleeding risk (72). Another PAR-1 inhibitor, atopaxar has been shown to have a good safety profile in terms of bleeding while reducing the number of adverse ischemic events, but was not developed further because it had not tolerable side effects on heart and liver (73).

PAR4 is the only PAR member that is expressed on both, human and murine platelets, and when *Par-4*$^{-/-}$ mice were submitted to the tMCAO model, they displayed markedly reduced infarct sizes, less neurologic impairment and reduced BBB breakdown and cerebral edema formation (see **Figure 1**) (74). Since PAR-4 in humans is responsive at higher thrombin concentrations, it is hypothesized that initial platelet responses to low thrombin concentration via PAR-1 are important for hemostasis and later stages of platelet activation by thrombin are important for occlusive thrombus formation and more dependent on PAR-4. Targeting PAR-4 while maintaining PAR-1 function might be more selective in preventing thrombotic occlusion while maintaining hemostasis (75). A selective PAR-4 inhibitor (BMS-986120) was identified and tested in a monkey model of occlusive arterial thrombosis, where it showed highly efficacious antithrombotic activity, and although treated animals had a slightly increased risk for bleeding, this was still markedly reduced compared to clopidogrel (same study), abciximab or cangrelor treated animals (75, 76). BMS-986120 was investigated in phase I clinical trials (NCT02208882 and NCT02439190), where a reduction in *ex vivo* platelet activation, aggregation and thrombus formation could be observed without causing an increase in coagulation times or serious adverse events (77). For further clinical development, BMS-986120 is compared with standard therapy in a phase II study of stroke recurrence (NCT02671461) (78). Given the high abundance of PARs in various tissues and cell types and the regulation of the expression in pathological situations like ischemic stroke, targeting these receptors might always have an influence on other cell types either promoting the beneficial effect or, on the other hand, leading to undesired side effects.

ADENOSINE DIPHOSPHATE (ADP) RECEPTORS

ADP can activate platelets via three purinergic receptors, $P2Y_1$, $P2Y_{12}$, and $P2X_1$. $P2Y_1$ and $P2Y_{12}$ signal via G-protein coupled receptors ($G_{\alpha q}$ and $G_{\alpha i}$, respectively), and are important for platelet shape change, aggregation, thromboxane A_2 generation, procoagulant activity, thrombus formation and signal potentiation. $P2X_1$ is an ion channel, that causes Ca^{2+} influx upon activation but has negligible role for platelet plug formation (79). Both, the $P2Y_1$ and the $P2Y_{12}$ receptor would be potential targets for the treatment of platelet associated diseases. However, $P2Y_1$ is widely expressed, which increases the risk of side effects, whereas $P2Y_{12}$ expression is restricted to the platelet surface and the brain, which makes it a more specific target (79). $P2Y_{12}$ inhibitors can be divided into thienopyridines (like clopidogrel and prasugrel) and non-thienopyridines (like ticagrelor and cangrelor). Thienopyridines require metabolic activation and irreversibly inhibit the $P2Y_{12}$ receptor, whereas non-thienopyridines are already active and lead to a reversible receptor inhibition. Treatment of mice with ticagrelor inhibited

ADP induced platelet aggregation and reduced infarct sizes while improving neurological function after tMCAO to a greater extent than acetylsalicylic acid. Further, reperfusion of the infarcted brain area was better as increased cerebral blood can be measured in ticagrelor treated mice, compared to vehicle or acetylsalicylic acid (aspirin®) treatment (80). Likewise, $P2Y_{12}$-deficient mice displayed a slightly improved neurological outcome following 45 min tMCAO as compared to wild-type control animals, albeit the authors of that study ascribed the effect to $P2Y_{12}$ in glia cells (81).

In line with the data acquired for ticagrelor in mice, in a non-human primate model of photochemically induced thrombotic ischemic stroke, monkeys that received daily treatments with prasugrel had significantly smaller infarcts and a better neurological function without suffering from an increased risk of hemorrhage (82). In clinical trials assessing the risk of stroke recurrence subsequent to an initial thrombotic event, dual antiplatelet therapy (DAPT) comprising acetylsalicylic acid and clopidogrel was more efficient in reducing the risk of recurrent events in the acute treatment of non-embolic TIA or ischemic stroke (83, 84), but DAPT was not beneficial in the long-term treatment of patients [reviewed in (85)]. In another study, comparing ticagrelor with aspirin®, ticagrelor was not found to be superior to aspirin® in reducing the occurrence of stroke, myocardial infarction or death within the 90 days observation period (83). However, in a study with patients suffering from atherosclerosis, clopidogrel, and ticlopidine could provide a better protection from stroke, myocardial infarction or vascular death when compared to the aspirin® treated cohort (86).

GLYCOPROTEIN-IIb/IIIa-RECEPTOR

Platelet GPIIb/IIIa (integrin αIIbβ3) is the most abundant integrin on the platelet surface and mediates platelet adhesion on the ECM by binding to its main ligand fibrinogen as well as fibronectin and vitronectin. GPIIb/IIIa is essential for platelet aggregation and thrombus formation by bridging adjacent platelets via fibrinogen or, at high shear rates, vWF (24). Given this important role, several GPIIb/IIIa inhibitors have been developed to use its blockade in the prevention of ischemic cardiovascular diseases. There are three approved GPIIb/IIIa inhibitors, abciximab (a recombinant Fab fragment of a monoclonal anti-GPIIb/IIIa antibody), eptifibatide (small peptide), and tirofiban (small non-peptide compound) (87). Several randomized controlled trials could show that the currently available GPIIb/IIIa inhibitors are safe and efficiently prevent complications in perioperative periods, which is why they are recommended as routine emergency treatment for thrombotic complications in ischemic heart disease (88, 89). In the setting of ischemic stroke, however, blocking GPIIb/IIIa in an experimental stroke model markedly increased the risk of intracerebral hemorrhage and mortality and surviving animals still showed infarct volumes that were in the range of control treated mice. Decreasing the degree of receptor blockade improved survival and reduced bleeding complications, but had no impact on infarct size or neurological symptoms (see **Figure 1**) (22). Notably, these data also largely exclude thrombus formation (e.g., microthrombosis in cerebral vessels) as a central mechanism underlying thrombo-inflammation and infarct progression in the context of ischemic stroke.

Many clinical trials have been conducted assessing the use of anti-GPIIb/IIIa inhibitors in the treatment of acute ischemic stroke or the prevention of stroke [reviewed for tirofiban in (89)]. Although several studies verified the safety profile of tirofiban, results are highly variable concerning beneficial effects of anti-GPIIb/IIIa treatment for functional outcome and mortality. In the setting of rescue stenting in the acute treatment of ischemic stroke, stent patency correlated with tirofiban administration without increasing intracranial hemorrhage or mortality (89, 90). In other studies, however, GPIIb/IIIa inhibitors have been shown to induce a significant increase in fatal intracerebral hemorrhage as reported for tirofiban (91) and abciximab (23) and must therefore be used with caution.

PLATELET GRANULE SECRETION

Platelets contain three major types of granules, namely α-granules, dense granules and lysosomes, which secrete their content upon platelet activation. α-granules account for roughly 10% of the total platelet mass being the most abundant granule type (50–80 granules/mouse platelet) (92). They contain more than 300 different proteins that play roles in diverse processes such as coagulation, platelet adhesion, hemostasis, wound healing, angiogenesis, host defense and tumor growth (92). Dense granules are the second most abundant granule type (5–6 granules/mouse platelet) and their name results from a characteristic electron-dense spot seen in electron microscopic analysis due to high concentrations of Ca^{2+}, Mg^{2+}, ADP, ATP, and serotonin (93). All of these molecules act as second wave mediators and promote the activation of platelets. Lysosomes contain various proteolytic enzymes like proteases, glycosidases, and cationic proteins with bactericidal activity proteins (94).

Dense Granule Secretion

Platelet activation results in the release of dense granule content, such as ADP, ATP, serotonin, and calcium, amplifying the activation response and promoting thrombus formation (92, 95). Mice lacking functional Munc13-4 (*Unc13d*^{jinx}, *Unc13d*$^{-/-}$) display abolished platelet dense granule secretion resulting in defective hemostasis and impaired experimental thrombus formation (96–98). Following 1 h tMCAO, *Unc13d*$^{-/-}$ mice had smaller infarcts and a better neurological outcome after 24 h compared to control mice (see **Figure 1**), which was not accompanied by intra-cranial hemorrhages, indicating that platelet dense granule secretion is not required to preserve vascular integrity in the setting of acute cerebral ischemia (98).

Serotonin (5-hydroxytrytamine, 5-HT) is an important neurotransmitter that has key functions within the brain and in the periphery. Apart from the brain, 5-HT is synthetized by tryptophan hydroxylase 1 (TPH1) by the enterochromaffin cells of the gut (99). Platelets are the major pool for peripheral serotonin, despite being unable to synthesize it; however, they take serotonin up from the plasma and store it in their dense granules (100). *5Htt*$^{-/-}$ mice, which lack the serotonin transporter, cannot store serotonin in the platelet dense granules and display defective hemostasis and are protected in models

of arterial thrombosis (101). In experimental stroke, however, no differences were observed between *5Htt*$^{-/-}$ and control mice (101), indicating that abolished serotonin secretion is not responsible for the reduced cerebral damage observed in *Unc13d*$^{-/-}$ mice.

Given the well-established role of ADP/ATP in secondary platelet activation (102), it seems more likely that the reduced infarct sizes following brain I/RI in *Unc13d*$^{-/-}$ mice results from the decreased platelet activation due to missing ADP/ATP secretion in these animals. In line with this, ticagrelor-treated mice displayed reduced infarct sizes and better neurological outcome following tMCAO (80). Consequently, the primary contribution of platelet dense granule secretion to cerebral ischemia/reperfusion damage appears to be the amplification of platelet activation.

Alpha Granule Secretion

Platelet α-granules contain more than 300 different proteins involved not only in platelet adhesion but also inflammation, angiogenesis and wound healing (92). The gray platelet syndrome (GPS) is a rare, autosomal-recessive platelet disorder characterized by thrombocytopenia, large platelets lacking α-granules, and variable bleeding symptoms (103, 104). GPS has been linked to mutations in the neurobeachin-like 2 gene (*NBEAL2*) (105–107) and *Nbeal2*$^{-/-}$ mice mimic the hallmarks of GPS (108–110). They were protected in models of arteriolar thrombosis and ischemic stroke (see **Figure 1**) and displayed prolonged tail bleeding times (108). Of note, *Nbeal2*$^{-/-}$ mice did not display signs of spontaneous bleeding, nor was intracranial hemorrhage within 24 h after cerebral ischemia observed (108). We are not aware of any epidemiological studies that assessed the occurrence of ischemic stroke in GPS patients compared to the overall population. However, the results with *Nbeal2*$^{-/-}$ mice in experimental stroke indicate that α-granule proteins contribute to cerebral damage, but are not required for vascular integrity following I/RI of the brain.

Interestingly, *Unc13d*$^{-/-}$/*Nbeal2*$^{-/-}$ double KO mice, which are unable to secrete their granule content showed an impaired hemostatic response in the ischemic brain following tMCAO, causing increased intracranial hemorrhage and mortality (111). Platelet transfer experiments confirmed that the platelet granule content is required to prevent intracranial hemorrhage in these mice (111). Of note, *Unc13d*$^{-/-}$/*Nbeal2*$^{-/-}$ mice did not display a defective blood-brain barrier, nor did these mice bleed in experimental models of skin or lung inflammation, indicating that platelet granule content is of particular importance to maintain the integrity of the cerebral vasculature in the course of I/RI (111). The fact that *Unc13d*$^{-/-}$ platelets display up to 60% reduced α-granule secretion at threshold agonist concentrations (98) indicates that only a small amount of α-granule-derived mediators is sufficient to maintain vascular integrity during ischemic stroke. This is supported by studies on thrombocytopenic mice revealing that 5% of normal platelet counts were still sufficient to prevent intra-cranial bleedings in experimental models of focal cerebral ischemia, while only severely thrombocytopenic mice (peripheral platelet counts of <2.5% of control) suffered from intracranial hemorrhages (22, 112, 113). Interestingly, a small fraction of *Unc13d*$^{-/-}$/*Nbeal2*$^{-/-}$ mice did not display intracranial hemorrhages and these animals survived and had smaller infarcts as compared to controls (111).

The plethora of platelet α-granule proteins and the fact that the majority of them can also be released by other cells, makes it challenging to identify the key components that contribute to infarct progression following cerebral ischemia.

CONCLUSIONS AND PERSPECTIVES

The heterogeneity of stroke patients makes the development of safe antiplatelet treatments challenging. Nevertheless, experimental stroke studies suggest that targeting activatory platelet receptors might be a feasible strategy to reduce thrombo-inflammatory infarct progression following ischemic stroke. Due to the fact that the expression of GPVI and GPIbα is limited to platelets we consider these two receptors particularly interesting in this aspect, as side-effects of their inhibition on other tissues are unlikely. In particular, targeting the GPIbα-vWF interaction could be a very attractive approach to inhibit pathogenic platelet functions in acute ischemic stroke. According to this hypothesis, blocking GPIbα would prevent platelet adhesion and therefore the first step of platelet recruitment/activation in the reperfused cerebral microcirculation. Moreover, GPIbα-blockade also reduces the infiltration of immune cells into the infarcted area and thereby inflammation (51) while not inducing increased intracerebral hemorrhages, at least in the setting of experimental focal ischemia (22). In contrast, GPIIb/IIIa blockade is clearly a less favorable option as it is ineffective in reducing thrombo-inflammation and a full receptor blockade causes bleeding complications.

In the future, it will be of interest to investigate whether thrombo-inflammatory mechanisms already contribute to cerebral damage in the ischemic phase and whether targeting them before recanalization would further reduce infarct progression.

Likewise, treatment strategies that stimulate inhibitory signaling pathways in platelets could be considered in the context of ischemic stroke. Indeed, cilostazol an inhibitor of phosphodiesterase-3 (PDE3), which dampens platelet activity due to sustained levels of cyclic nucleotide monophosphate (cAMP and cGMP), has been shown to reduce recurrent strokes (114, 115). However, data from experimental studies argues that the protective effect of PDE3-inhibitors might be independent of platelet inhibition (116).

AUTHOR CONTRIBUTIONS

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REVIEW
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von Willebrand Factor and Platelet Glycoprotein Ib: A Thromboinflammatory Axis in Stroke

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von Willebrand factor (VWF) and platelets are key mediators of normal hemostasis. At sites of vascular injury, VWF recruits platelets via binding to the platelet receptor glycoprotein Ibα (GPIbα). Over the past decades, it has become clear that many hemostatic factors, including VWF and platelets, are also involved in inflammatory processes, forming intriguing links between hemostasis, thrombosis, and inflammation. The so-called "thrombo-inflammatory" nature of the VWF-platelet axis becomes increasingly recognized in different cardiovascular pathologies, making it a potential therapeutic target to interfere with both thrombosis and inflammation. In this review, we discuss the current evidence for the thrombo-inflammatory activity of VWF with a focus on the VWF-GPIbα axis and discuss its implications in the setting of ischemic stroke.

Keywords: von Willebrand factor, platelet glycoprotein Ibα, thromboinflammation, ischemic stroke, ADAMTS13

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VON WILLEBRAND FACTOR IN HEMOSTASIS: RECRUITMENT OF PLATELETS

In this section, we briefly summarize the synthesis, structure, and role of VWF in hemostasis and refer to more extensive reviews for further reading.

VWF is a large multimeric plasma protein that plays a major role in hemostasis (1–4). First, VWF recruits platelets to sites of vascular injury by forming a bridge between the damaged vessel wall and platelets. Second, VWF also serves as a carrier protein for coagulation factor VIII (FVIII) and hence protects FVIII from degradation, cellular uptake or binding to the surface of activated platelets and endothelial cells (5). VWF is produced exclusively by endothelial cells and megakaryocytes. VWF is synthesized as a pre-pro-VWF that consists of a 22 amino acid signal peptide, a 741 amino acid propeptide (D1-D2) and a mature subunit of 2,050 amino acids (6). The mature subunit is composed of different types of domains arranged in the following order: D′-D3-A1-A2-A3-D4-C1-C2-C3-C4-C5-C6-CK (**Figure 1**) (1, 7).

After removal of the signal peptide, pro-VWF monomers dimerize in the endoplasmic reticulum through disulfide linkage of the C-terminal CK domains. In the Golgi complex, complete multimerization of the dimers occurs via disulfide linkage of the N-terminal D3 domains, together with additional modifications such as removal of the propeptide, glycosylation, and sulfation. After synthesis, VWF is either constitutively secreted into the blood or is stored in endothelial Weibel-Palade bodies (WPB) and platelet α-granules, from which VWF is locally released *via* regulated secretion (8). Basolateral release of endothelial VWF leads to accumulation of VWF in the subendothelial matrix, which becomes exposed following damage to the vessel wall. Ultra Large-VWF (UL-VWF) that is released at the apical surface can

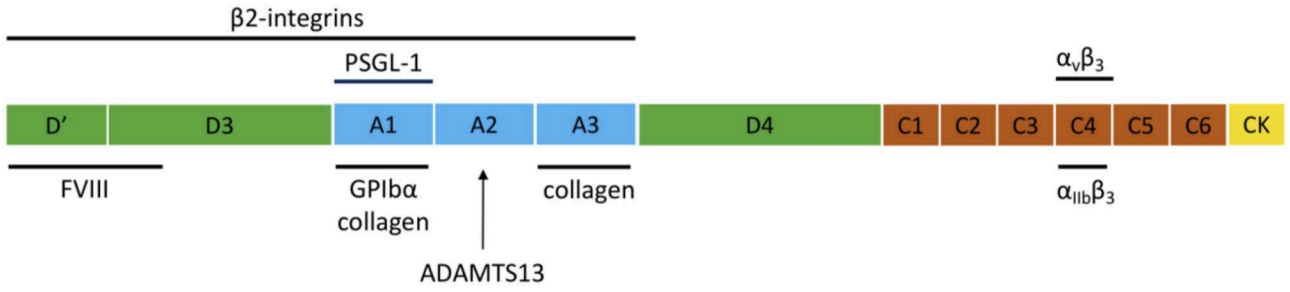

FIGURE 1 | Domain structure of VWF and the main interaction sites. The domain structure of VWF is given and the most important interactions for inflammation and hemostasis are indicated.

remain anchored on the surface of endothelial cells and form platelet-decorated strings (9). The structure of VWF is important for its function since several domains are essential for the hemostatic activity of VWF, such as the A1, A3, and C4 domains that mediate binding to GPIbα, collagen, and αIIbβ3, respectively (**Figure 1**).

The interaction of VWF with platelet GPIbα is crucial for initial platelet adhesion, especially in environments with high hemodynamic shear forces. GPIbα is a subunit of the platelet GPIb-IX-V complex that also contains the GPIbβ, GPIX, and GPV subunits, all of which are type I transmembrane proteins containing leucine-rich repeat domains. Under normal conditions, VWF circulates as a globular protein in which the binding site for GPIbα in the A1 domain is not accessible. However, upon blood vessel damage, VWF binds via its collagen binding sites (mainly in the A1 and A3 domains) to the exposed subendothelial matrix. Immobilization and flow shear forces then result in a conformational activation of the VWF A1 domain, enabling binding of the N-terminal domain of GPIbα (4). This force-induced regulation of the VWF-GPIbα interaction occurs via changes in intramolecular shielding of the VWF A1 domain by neighboring VWF sequences, possibly together with intrinsic changes in the affinity state of the VWF A1 domain itself (10).

The reversible nature of the VWF A1-GPIbα interaction permits platelets to roll and thus decelerate on immobilized VWF, ultimately allowing firm adhesion of platelets to the exposed subendothelial matrix via the platelet collagen receptors GPVI and integrin $\alpha2\beta1$. The GPIbα-VWF and GPVI/$\alpha2\beta1$-collagen interactions induce downstream intracellular platelet signaling leading to activation of platelet αIIbβ3, which mediates further stable adhesion and aggregation via binding to fibrinogen and VWF.

A central aspect of VWF activity is that larger VWF multimers are more active due to the presence of more monomeric subunits and the higher sensitivity for shear forces. UL-VWF multimers have a molecular weight of >10,000 kD and are highly reactive because the GPIbα binding sites in the VWF A1 domains are continuously exposed. As a result, spontaneous binding of platelets to VWF can occur. UL-VWF is stored in the endothelial WPBs from which it is released via both basal and regulated secretion pathways but also in platelet α-granules from which it is released only after agonist-induced stimulation (6). The local, regulated release of UL-VWF allows fast and confined hemostasis when needed at sites of injury. To prevent accumulation of prothrombotic UL-VWF, however, UL-VWF is cleaved by the VWF cleaving protease ADAMTS13 (A Disintegrin and Metalloprotease with ThromboSpondin type 1 repeats, number 13). Proteolysis of VWF by ADAMTS13 occurs in the VWF A2 domain and is dependent on conformational activation of the A2 domain to expose the cleavage site (11). Digestion of UL-VWF by ADAMTS13 results in smaller, less active VWF multimers (≤10,000 kDa) that adopt a folded conformation in which the platelet binding site in the A1 domain and the ADAMTS13 cleavage site in the A2 domain are cryptic. In the absence of ADAMTS13, spontaneous formation of VWF-platelet complexes leads to thrombotic complications as seen in patients with thrombotic thrombocytopenic purpura (12).

VON WILLEBRAND FACTOR IN INFLAMMATION: RECRUITMENT OF LEUKOCYTES

Besides its well-established role in hemostasis, VWF is recognized as an effective mediator of inflammatory responses as well. VWF can actively participate in the development of inflammatory processes by recruiting leukocytes at sites of vascular inflammation. Indeed, VWF deficiency or blockade has been shown to reduce leukocyte recruitment in various murine models of inflammation, including cytokine-induced meningitis (13), wound healing (13), atherosclerosis (14), cutaneous inflammation (15, 16), vasculitis (17), and peritonitis (18). When studying the inflammatory effects of VWF, it is important to keep in mind that VWF itself is essential for the formation of WPBs in endothelial cells (19). Alongside VWF, WPBs store also other molecules involved in inflammation and even angiogenesis (e.g., P-selectin, interleukin-6, interleukin-8, Eotaxin-3, Factor H, and angiopoietin-2). Failure of co-storage of inflammatory proteins in the endothelium of VWF-deficient mice can thus also cause defects in inflammation (20). However, recent research provided ample evidence for a direct role of VWF in inflammation, which might potentially be more important than co-storage of inflammatory proteins in the acute-phase response of the vessel wall.

When endothelial cells are activated by inflammatory mediators, UL-VWF is rapidly released from endothelial WPBs. As a consequence, increased levels of circulating VWF antigen has become a well-known marker of inflammation and endothelial activation. When secreted into the blood stream, released VWF can also remain anchored on the surface of endothelial cells through binding with P-selectin (21), integrin $\alpha V\beta 3$ (22), or the glycocalyx (23) and locally form platelet-decorated strings. VWF facilitates inflammatory processes by promoting leukocyte recruitment to inflamed tissues, either directly or indirectly after binding platelets.

An elegant study by Pendu et al. demonstrated that VWF can act as an adhesive surface for neutrophils and monocytes and that the adhesion process of these inflammatory cells involves various interactions that act in a concerted way (24). Direct adhesion of leukocytes occurs via multiple regions within the VWF molecule that interact with PSGL-1 and $\beta 2$ integrins on leukocytes (**Figure 1**). Whereas, PSGL-1 would be involved in initial rolling on VWF, $\beta 2$ integrins would be responsible for stable adhesion on VWF. $\beta 2$ integrins can interact with two distinct binding sites on VWF that are located in the D′D3 and A1-A2-A3 regions of VWF as well as to the Leu-Leu-Gly motifs found in the VWF D3 and the connecting region between the A1 and A2 domains (24, 25). The binding site for PSGL-1 is located in the VWF A1 domain (24). Similar for binding to GPIbα, the A1 domain needs to be in its active conformation to bind PSGL-1, which shares structural similarities with GPIbα (24).

Apart from binding directly to leukocytes, VWF can also indirectly promote leukocyte recruitment by forming VWF-platelet-leukocyte complexes, with a crucial role for the VWF-GPIbα axis. The exact mechanisms by which VWF-platelet complexes facilitate leukocyte diapedesis are not yet fully understood and might vary between different inflamed tissues. First, activated platelets bind to VWF and can then interact via P-selectin or GPIbα with leukocytes, thus promoting local adhesion of inflammatory cells (26). As such, immobilized VWF can function as a local matrix to recruit both platelets and leukocytes. Whereas, direct interactions between VWF and leukocytes might be sufficient under venous low-shear conditions, it is conceivable that platelets are needed for leukocyte recruitment under arterial high-shear conditions (26). Second, VWF-platelet complexes can regulate vascular permeability, leading to facilitated leukocyte extravasation. Indeed, using a model of thioglycollate-induced peritonitis, Petri et al. showed that leukocyte recruitment to the inflamed peritoneum was dependent on the presence of VWF and platelets and more specifically on the functional availability of GPIbα (18). In this study, the contribution of VWF-platelet complexes could be explained by destabilization of the endothelial barrier function rather than by increased leukocyte rolling and adhesion. The possible mechanisms through which VWF and platelets induce vascular leakage need further study. Endothelial permeability might be regulated by binding of the VWF RGD motif to endothelial $\alpha V\beta 3$ integrins (22), and platelets can release various soluble factors that influence endothelial junctions (27). A recent study also showed the involvement of microparticles in VWF-mediated vascular leakage (28).

Overall, the VWF A1 domain seems to be central for the participation of VWF in inflammatory processes. This was recently underlined by two studies from the group of Cécile Denis and Peter Lenting showing that specific inhibition of the VWF A1 domain leads to reduced vascular permeability and leukocyte recruitment (17), whereas a gain-of-function mutation in the VWF A1 domain results in increased leukocyte recruitment (16). Also clinically, the presence of an active A1 domain was shown to predict mortality in patients with systemic inflammatory response syndrome (29). Since an active A1 domain is a typical hallmark of UL-VWF in circulation, it might not be surprising that ADAMTS13 can exert anti-inflammatory activity by reducing the activity of VWF. By cleaving VWF, ADAMTS13 can remove VWF strings from the endothelial surface or reduce the size of reactive VWF to less adhesive VWF molecules. As a result, ADAMTS13 is able to attenuate VWF-dependent leukocyte rolling, adhesion, and extravasation under acute inflammatory conditions. The anti-inflammatory properties of ADAMTS13 have been demonstrated in various settings, including peritonitis (30), atherosclerosis (31), colitis (32), myocardial infarction (33–35), cardiac fibrosis (36), and ischemic stroke, as discussed further.

VWF-GLYCOPROTEIN IB MEDIATED THROMBOINFLAMMATION IN ISCHEMIC STROKE

Ischemic stroke occurs when a blood clot obstructs cerebral blood flow and causes ischemic brain damage. The primary objective in acute ischemic stroke care is achieving fast reperfusion of the occluded blood vessel to limit ischemic brain injury. Yet, sometimes progressive stroke still develops despite reperfusion of the affected brain tissue, a phenomenon attributed to "reperfusion injury" (37, 38). It has become clear that cerebral ischemia/reperfusion injury is a complex pathology that involves crosstalk between both thrombotic and inflammatory pathways, which has lead to the concept of thrombo-inflammation in stroke (39, 40). Given the dual role of VWF and GPIbα in both thrombosis and inflammation, the VWF-GPIbα axis has received quite some attention in the setting of ischemic stroke (41).

Evidence for the involvement of VWF in ischemic brain injury comes from mouse studies showing that absence of VWF is associated with a significant reduction in ischemic stroke brain injury and improved functional outcome (42, 43). The detrimental effects of VWF were later attributed to the specific involvement of the VWF A1 and A3 (but not C4) domains, indicating a key role for the VWF-GPIbα and VWF-collagen interactions (44). Of note, whereas platelet-derived VWF is largely dispensable for normal hemostasis and thrombosis in mice, we showed that it can actively contribute to ischemic brain injury via a mechanism that is GPIbα-dependent (45). In parallel with these studies on VWF, similar research demonstrated that also GPIbα is an important mediator of cerebral ischemia/reperfusion injury. Indeed, mice lacking functional GPIbα also develop smaller brain infarctions together with improved stroke outcome (46, 47), an observation that

was extended in a more translational setting using aged and comorbid (atherosclerotic, diabetic, and hypertensive) animals (48). Furthermore, anfibatide, a snake venom-derived GPIbα antagonist that specifically blocks platelet GPIbα binding to VWF had a potent protective effect in mouse models of ischemic stroke (49–52). As mentioned above, UL-VWF can spontaneously bind platelets and its reactivity can cause thrombotic events without proper regulation by ADAMTS13. Accordingly, experimental stroke studies showed that ADAMTS13-deficient mice developed larger brain infarctions and worse neurologic outcomes, whereas infusion of recombinant ADAMTS13 was able to attenuate ischemic brain damage (42, 53–56). Together, these studies highlight the pathophysiological involvement of VWF and GPIbα in cerebral ischemia/reperfusion injury, which can be counterbalanced by blocking the VWF-GPIbα interaction or by reducing the activity of VWF via ADAMTS13.

The precise mechanisms underlying the pathophysiological involvement of the VWF-GPIbα axis in ischemic brain injury are not yet fully elucidated but available data strongly points toward an intricate process that includes both thrombotic and inflammatory pathways. The cerebral microvasculature rapidly responds to brain ischemia leading to endothelial cell activation and endothelial denudation exposing subendothelial matrix components such as collagen. It has been long known that local platelet and leukocyte recruitment can lead to microvascular obstruction within the ischemic territory after occlusion and reperfusion, a process known as the "no-reflow" phenomenon (57–59). Given the fundamental role of VWF and GPIbα in thrombus development at sites of vascular damage, it is not surprising that the VWF-GPIbα axis is responsible for thrombotic events in stroke. In mouse models of cerebral ischemia/reperfusion injury, VWF deficient mice indeed showed less thrombosis in the cerebral microvasculature, as shown by reduced intracerebral fibrin(ogen) deposition in the affected brain tissue of these animals compared to wild-type mice (44, 45, 60). Remarkably, fibrin(ogen) deposition was considerably reduced in the ischemic hemisphere of the VWF deficient mice that were reconstituted with VWF defective in binding to fibrillar collagen or GPIbα compared with controls, again emphasizing the contribution of initial platelet adhesion interactions mediated by VWF (44). By specifically blocking the VWF-GPIbα axis, anfibatide reduced the number of fibrin(ogen)-positive blood vessels and microthrombi in the ischemic hemisphere (49, 51). In line with these results, anti-GPIbα treatment significantly reduced thrombus burden in the cerebral microvasculature, as measured by the number of GPIX-positive platelet aggregates and occluded brain vessels (61). Correspondingly, ADAMTS13 deficient mice showed an increased number of thrombi containing fibrin and VWF in the brain lesions after stroke (53). Recently, analogous observations were made in CD69 deficient mice (62). CD69 was identified as a negative regulator of endothelial VWF release, and in the setting of stroke, its absence resulted in a more severe stroke burden due to increased cerebral thrombosis (62).

Remarkably, whereas thrombus formation requires both platelet adhesion via GPIbα and GPVI and platelet aggregation via αIIbβ3, the latter does not seem to play a major role in acute ischemic stroke injury (44, 46, 48). Hence, platelets and VWF most likely contribute to stroke progression in a way that is not strictly related to thrombus formation. The most plausible explanation is the involvement of a corresponding inflammatory component mediated by the initial interactions between the damaged vessel wall, VWF, and platelets. Ample evidence for such an inflammatory reaction has been gathered in the last decade. Indeed, in mouse models of ischemic stroke, VWF deficiency is associated with reduced neutrophil infiltration in the ischemic hemisphere (55). In addition, expression levels of the pro-inflammatory cytokines IL-6, IL-1ß, and tumor necrosis factor-α are also decreased in the absence of VWF (55, 60). Interestingly, endothelial-derived rather than platelet-derived VWF seems to be the major determinant of these inflammatory effects (60). In line with the high activity of UL-VWF, elevated VWF-mediated inflammation is observed in the injured brain hemisphere of ADAMTS13-deficient mice. Increased myeloperoxidase activity, increased extravasation of neutrophils, and a higher expression of inflammatory cytokines high-mobility group box1, IL-6, and tumor necrosis factor-α were observed in ADAMTS13-deficient mice compared with wild-type controls (53–55). Interestingly, the increased brain damage and worsened neurological outcome observed in ADAMTS13-deficient animals were abrogated when neutrophils were depleted, indicating a causal role of neutrophils in the exacerbation of ischemic brain injury in the absence of ADAMTS13 (55). Blockade of GPIbα similarly led to decreased expression of IL-6, IL-1ß, and tumor necrosis factor-α (50, 61) and was also shown to lower the numbers of infiltrating T-cells and myeloid leukocytes (51, 61). The latter is in accordance with recent data from our group showing that inhibition of the VWF-GPIbα interaction results in significantly decreased recruitment of monocytes, neutrophils, and T-cells in the ischemic brain (63).

In summary, current evidence shows the involvement of the VWF-GPIbα interaction in a vicious circle of thrombotic and inflammatory responses in the ischemic stroke brain. Ischemia leads to endothelial damage, exposure of subendothelial matrix, upregulation of adhesion molecules, and release of UL-VWF. Local accumulation of VWF contributes to intravascular recruitment platelet and leukocytes, which can secrete proinflammatory cytokines that further stimulate inflammation. Aggregates of VWF, platelets, and leukocytes most probably plug brain capillaries, preventing efficient microcirculatory reperfusion. However, many aspects of the spatiotemporal involvement and molecular interactions between VWF and leukocytes in stroke remain to be elucidated. For, example, whether direct interactions between VWF and PSGL-1 and ß2 integrins are involved remains unanswered. Also, the potential effect of VWF and platelets on vascular permeability in the stroke brain needs further study. Initial results indeed indicate that interfering with VWF or GPIb can modulate the cerebrovascular integrity after stroke (51, 64). When blocking the function of GPIbα, it is important to realize that this platelet receptor contributes to arterial thrombosis *via* additional mechanisms that are independent of its binding to VWF (65). GPIbα also interacts with various other ligands such as thrombin, coagulation factors XI and XII, high molecular weight

kininogen, and thrombospondin-1. Hence, further studies are needed to generate a more complete picture of the involvement of GPIbα in ischemic stroke, besides binding to VWF.

New insights show that already very early during ischemia, neutrophils, and platelets are recruited to the ischemic brain and contribute to microvascular dysfunction (66, 67). Otxoa-de-Amezaga and colleagues recently visualized an early influx of neutrophils to the brain after stroke, predominantly located within the intravascular space already early after reperfusion (68). It would be interesting to further untangle the specific role of VWF during these very early responses in the ischemic tissue to better understand the involvement of VWF in the neurovascular unit.

TRANSLATIONAL ASPECTS

The clinical significance of the VWF-GPIbα interaction in stroke is suggested by an increasing number of human stroke studies showing the pathophysiological involvement of VWF in ischemic stroke (69–74). Furthermore, polymorphisms in the GPIBA gene that lead to enhanced VWF-GPIbα interactions are associated with an increased risk of ischemic stroke in humans (75). Intriguingly, increased VWF activity and/or reduced ADAMTS13 activity are associated not only with higher stroke occurrence, but also with worse long-term stroke outcomes (71, 76–78). Nonetheless, more clinical studies are needed to specifically address the contribution of VWF-mediated thromboinflammatory brain damage during ischemia and reperfusion in ischemic stroke patients. From a clinical perspective, it is promising that the first-generation of VWF-inhibitors is currently enrolled in clinical studies for thrombotic thrombocytopenic purpura, such as a specific inhibitor of the VWF-GPIbα interaction (79) and recombinant ADAMTS13 (80). Notably, we and others have demonstrated that targeting VWF can also promote blood clot dissolution in the setting of ischemic stroke (81–85), which could be of particular relevance to overcome thrombolysis resistance of platelet-rich blood clots in patients (86). Hence, compounds that target VWF could have the attractive potential to promote acute thrombolysis in the occluded blood vessel and attenuate ischemia/reperfusion injury in the microvasculature of the affected brain territory. The safety, especially in terms of bleedings, remains to be further investigated before clinical use. At least in preclinical animal research, targeting VWF via anti-VWF-GPIbα strategies or recombinant ADAMTS13 did not increase the risk of intracranial hemorrhaging in murine stroke models (42, 43, 49), even when combined with tissue-plasminogen activator (87, 88) or when treatment was delayed (56). Of note, ADAMTS13 therapy improved outcomes in murine models of intracerebral hemorrhage in a VWF-dependent way (89–92). More research, preferably also in larger animal models, is needed to bring the concept of blocking VWF-mediated thromboinflammation in stroke closer to the clinic.

AUTHOR CONTRIBUTIONS

All authors listed have made a substantial, direct and intellectual contribution to the work, and approved it for publication.

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