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The platelet Fc receptor, FcγRIIa

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Summary: Human platelets express FcγRIIa, the low-affinity receptor for the constant fragment (Fc) of immunoglobulin (Ig) G that is also found on neutrophils, monocytes, and macrophages. Engagement of this receptor on platelets by immune complexes triggers intracellular signaling events that lead to platelet activation and aggregation. Importantly these events occur *in vivo*, particularly in response to pathological immune complexes, and engagement of this receptor on platelets has been causally linked to disease pathology. In this review, we will highlight some of the key features of this receptor in the context of the platelet surface, and examine the functions of platelet FcγRIIa in normal hemostasis and in response to injury and infection. This review will also highlight pathological consequences of engagement of this receptor in platelet-based autoimmune disorders. Finally, we present some new data investigating whether levels of the extracellular ligand-binding region of platelet glycoprotein VI which is rapidly shed upon engagement of platelet FcγRIIa by autoantibodies, can report on the presence of pathological anti-heparin/platelet factor 4 immune complexes and thus identify patients with pathological autoantibodies who are at the greatest risk of developing life-threatening thrombosis in the setting of heparin-induced thrombocytopenia.

Keywords: *platelet, thrombocytopenia, glycoprotein, metalloproteinase*

Introduction

The primary function of platelets is to minimize blood loss in situations of vascular trauma; however, platelets circulate through the vascular system in numbers that hover around half a million per microliter of blood, quantities that vastly exceed platelet numbers required for routine maintenance of vascular hemostasis. It is possible that the platelet count is maintained at such high levels because in addition to this central role in normal hemostasis, platelets make important contributions to host inflammatory and immune responses required in response to tissue injury or infection (1). Understanding the roles performed by platelets is crucial because under patho-physiological conditions where platelet function is not tightly controlled, platelets play critical roles in pathogenic processes underlying cardiovascular

disease, uncontrolled inflammation, coagulopathy, and in tumor metastasis (2). While platelets are notorious for their pathogenic role that underpins thrombosis, stroke, and myocardial infarction (3), new and equally important roles for platelets in chronic inflammation and tumorigenesis have emerged (4, 5). To carry out these hemostatic and pathogenic roles, platelets utilize an array of surface adhesion and signaling receptors which orchestrate platelet responses to a range of dynamic vascular conditions including blood rheological conditions, chemokine concentrations, and exposure of thrombogenic surfaces. Targeting key interactions involving platelet receptors provides new therapeutic opportunities for platelet-related thrombotic and other diseases (6).

Human platelets express FcγRIIa (7), the low-affinity receptor for the fragment constant (Fc) portion of immunoglobulin (Ig) G. Platelets can coat an Ig-bound (opsonized) entity such as a bacterium via FcγRIIa, and this binding triggers platelet activation and release of secondary mediators resulting in an amplification of the platelet response to a wide range of bacteria (8, 9). Interestingly, under certain experimental conditions, this involvement of platelets may also be the advantage of certain bacteria, where platelet cloaking of the invading pathogen can minimize detection and aid survival (10, 11). The interactions between specific bacteria and platelets via FcγRIIa engagement have been recently discussed in excellent reviews (8, 12–14).

Molecular aspects of FcγRIIa and platelet function

FcγRIIa (also known as CD32a) is a low-affinity receptor for monomeric IgG that readily binds IgG immune complexes and is broadly expressed on platelets, monocytes, and macrophages as well as other cell types (15–17). Human platelets carry 1000–4000 copies of this receptor, which when considered in the context of platelet abundance, makes platelets the richest source of FcγRIIa in the body (18). FcγRIIa protein expression is limited to higher primates, and an equivalent to the *FCGR2* gene is not found in the murine genome. Roles for FcγRIIa have been identified in mediating interactions between platelets and immune complexes, specific strains of bacteria (8, 9), and innate pentraxins such as the acute phase proteins serum amyloid P component and C-reactive protein (19). When the respective ligand-binding partners of FcγRIIa are considered *in toto*, the expression of FcγRIIa on platelets may represent an example of evolutionary overlap between the innate and adaptive immune systems.

FcγRIIa is a Type I transmembrane protein of approximately 40 kDa and consists of two extracellular Ig-like domains, a single transmembrane domain, and a cytoplasmic tail that bears an immunoreceptor tyrosine-based activation motif (ITAM) domain (16) with dual YXXL amino acid consensus sequences (*Fig.* 1). The Ig-binding region of FcγRIIa resides within the second Ig-like domain (20, 21). FcγRIIa is one of the three ITAM-bearing receptors on platelets, a select group that includes the platelet-specific collagen

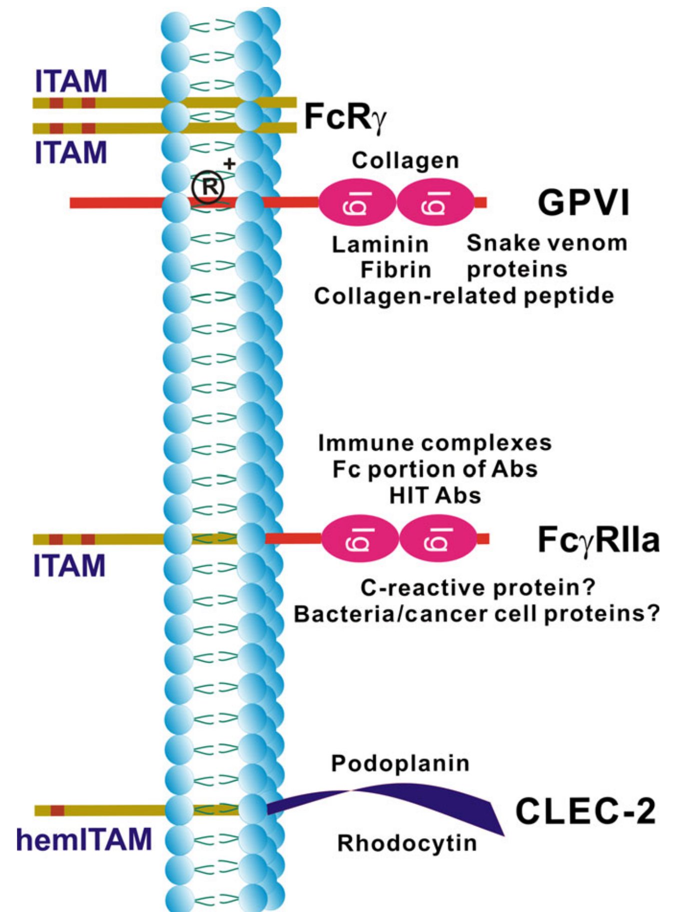

***Fig.* 1. Immunoreceptor tyrosine-based activation motif (ITAM)-containing receptors on platelets and their respective ligands.** Platelet ITAM-bearing receptors include the GPVI/FcRγ-chain complex (a positively charged arginine (R) in the transmembrane region of GPVI mediates this association via formation of a salt bridge. ITAM motifs (YXXL motif in dark red) are located within the cytoplasmic tails of the FcRγ-chain), the Fc receptor FcγRIIa, and the C-type lectin-like receptor CLEC-2 which contains a single (hemITAM) ITAM. The known ligands for GPVI include collagen, fibrin, laminin, collagen-related peptide, and snake venom proteins (convulxin, alborhagin). FcγRIIa binds IgG monomer with low affinity but avidly binds the Fc portion of antibodies involved in immune complexes. Platelet FcγRIIa may directly engage ligands on cancer cell lines as well as C-reactive protein (demonstrated for FcγRIIa on other cell types). Podoplanin and the snake venom protein rhodocytin are identified as CLEC-2 ligands that cause platelet activation.

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receptor glycoprotein (GP) VI that does not contain ITAMs within its cytoplasmic tail but forms a complex with the ITAM-containing Fc receptor γ (FcRγ) chain, required for GPVI surface expression. GPVI/FcRγ mediates collagen-induced platelet activation. C-type lectin-2 (CLEC-2), a receptor for the lymphatic endothelial cell protein, podoplanin, also contains a single cytoplasmic tail with ITAM sequence (22, 23) (Fig. 1). Regardless of the cell type, engagement of FcγRIIa by the Fc portion of antibodies, particularly as part of an immune complex induces phosphorylation of the ITAM within the cytoplasmic tail, and activation of ITAM-dependent signaling pathways involving subsequent phosphorylation of sarcoma (Src) family kinases such as Fyn and Lyn, spleen tyrosine kinase (Syk), and phosphatidylinositide 3-kinase (PI3-kinase) pathways (24, 25). Interestingly, similar to GPVI which also contains two extracellular Ig-like domains (Fig. 2), the tertiary structure of the ectodomain region of FcγRIIa favors receptor dimerization (20, 26), and signaling is optimal when either molecule is able to cluster and dimerize on the membrane (27–29). In case of both of these platelet receptors, rapid dimerization occurs in response to ligand binding (27, 28, 30).

In experimental models involving simultaneous deletion of GPVI/FcRγ or CLEC-2 or both, it has been demonstrated that these receptors may show functional redundancy, involving downstream ITAM-dependent signaling (31). Whether ITAM-bearing FcγRIIa on human platelets contributes synergistically to ITAM signaling is not certain, but may have significant implications for platelet responses to immune, hemostatic, and inflammatory functions of platelets. On the human platelet surface, there are several reports of co-operative functional association between FcγRIIa and other unique platelet receptors including GPIbα (32) of the GPIb–IX–V complex that binds von Willebrand Factor (VWF) via a shear-sensitive mechanism, and αIIbβ3 (33, 34), the primary platelet-specific integrin that binds fibrinogen or VWF. In both cases, ligand-mediated activation of these abundant platelet receptors triggers the activation of components of the FcγRIIa signaling cascade, thus amplifying intracellular signals (33, 35, 36). At threshold levels of ligand, FcγRIIa also contributes to platelet signaling mediated by G-protein-coupled receptors including the thromboxane receptor and protease-activated receptor (PAR) 1 and PAR4 (36, 37). The extent of physical co-association between FcγRIIa and either GPIbα or αIIbβ3 is unclear; however, the cooperation between FcγRIIa and other platelet receptors is likely to depend on the relative density of each receptor within membrane microdomains (38).

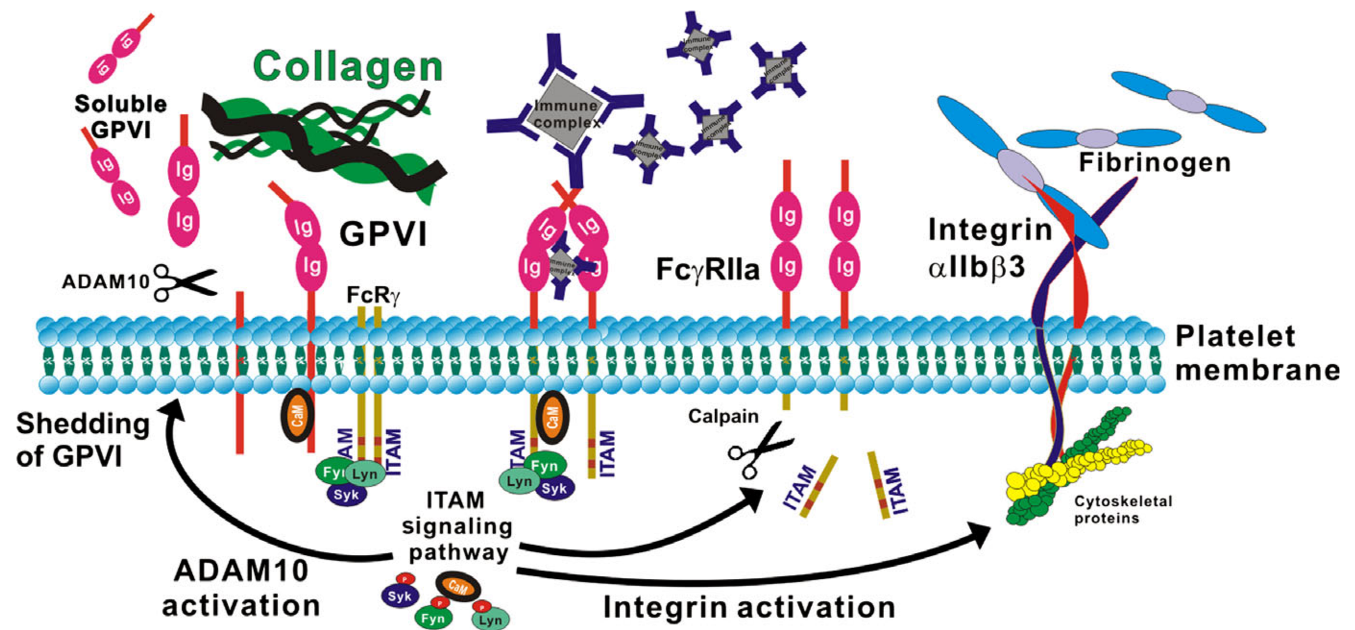

Fig. 2. Signaling and proteolysis of platelet immunoreceptor tyrosine-based activation motif (ITAM) receptors. Ligand binding to either GPVI or FcγRIIa triggers a powerful platelet activation cascade, driven by the ITAM signaling pathway, including dissociation of calmodulin (CaM) and sequential activation/phosphorylation of Src family kinases Fyn and/or Lyn, as well as Syk family kinases leading to integrin activation and binding of fibrinogen. The ITAM signaling pathway simultaneously induces activation of a member of the A Disintegrin And Metalloproteinase (ADAM) family, and intracellular calpain, leading to ADAM10-mediated extracellular cleavage (shedding) of GPVI and calpain-mediated intracellular proteolysis of FcγRIIa.

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Functional outcomes of engagement of platelet FcγRIIa

In platelets, activation of FcγRIIa by anti-platelet autoantibodies triggers Ca^{2+} mobilization, release of platelet factor (PF) 4, degranulation (9) and upregulation of surface levels of P-selectin, generation of intraplatelet reactive oxygen species (39), and integrin-dependent platelet aggregation (23, 40). FcγRIIa-dependent platelet activation is induced either by autoimmune anti-platelet antibodies or by murine monoclonal antibodies against platelet membrane glycoproteins that bind in an orientation permitting the interaction between the Fc portion of the antibody and FcγRIIa. Examples include 14A2 against tetraspannin CD151 (41, 42), VM58 against CD36 (GPIV) (43), and Raj-1 against αIIbβ3 (44). In addition, there is an important functional link between FcγRIIa and GPVI on human platelets, as ligands acting at either receptor activate dual proteolytic regulatory pathways: one leading to ectodomain metalloproteolysis of GPVI, the other leading to inactivation of FcγRIIa by intracellular proteolysis mediated by the Ca^{2+}-sensitive cysteine protease, calpain (42) (*Fig.* 2). This latter cleavage results in the detachment of the ITAM sequences from the cytoplasmic domain of FcγRIIa that contains presumably ablating signaling as a result. It will be interesting to investigate whether this cleavage event occurs *in vivo*, for example in patients with platelet-related autoantibodies where immune complex formation results in FcγRIIa-mediated platelet activation, for example in heparin-induced thrombocytopenia (HIT) or immune thrombocytopenia (ITP) which are discussed below. In this regard, however, the intracellular cleavage of FcγRIIa could be induced in donor platelets treated with purified immune complexes isolated from a HIT patient (42) or HIT patient serum (45).

Aside from Fc portions of antibodies, platelet FcγRIIa may have additional binding partners. An unidentified ligand on human prostate cancer cells was shown to cause platelet degranulation and augment platelet aggregation via a pathway that required functional platelet FcγRIIa and ITAM receptor signaling (46). Additionally, the acute phase protein, C-reactive protein (CRP) has been reported to bind FcγRIIa on monocytes and macrophages (47), and CRP has been shown to enhance clearance of IgG-opsonized platelets (48); however, evidence that CRP directly engages platelet FcγRIIa is currently lacking.

Polymorphisms in FcγRIIa influence receptor function

FCGR2, the gene encoding FcγRIIa, is found within a cluster of Fcγ receptor genes located on chromosome 1q23 that are all polymorphic. Several alleles of *FCGR2* are known and are typically expressed codominantly (49). Each allele of the gene encoding FcγRIIa is transcribed and translated generating equal amounts of protein with sequence matching the genetic code in each allele. Age- and gender-independent variation in levels of expression of FcγRIIa in the healthy population has been described (50, 51) and levels of FcγRIIa are increased in patients with acute myocardial infarction, unstable angina, diabetes, or ischemic stroke (52). A polymorphism, resulting from the amino acid substitution from arginine (R) to histidine (H) at position 131 in the second Ig-like ligand-binding domain of FcγRIIa, has been shown to alter the binding affinity of the receptor for different IgG subclasses (53, 54). Crystallography studies indicate that the H131R position is on the contact interface between receptor and IgG (20, 27). FcγRIIa-H131 binds to human IgG2 with higher affinity than FcγRIIa-R131; however, FcγRIIa-R131 has a higher binding affinity for murine IgG1 than FcγRIIa-H131. Small but significant differences in the interaction of these variants with IgG1 and IgG3 also exist leading to functional difference between the two allotypes (55). The FcγRIIa-R allotype binds more poorly to IgG (especially to IgG2) than the ancestral H allotype. Effector cells of homozygous individuals for FcγRIIa-H are more effective in recognizing and clearing IgG2–antigen complexes than FcγRIIa-R homozygous individuals. Heterozygous individuals (FcγRIIa-HR) have effector cells bearing both allotypes of the receptor and are referred to as 'intermediate immune responders' with respect to FcγR functions. FcγRIIa-R allotype also binds other IgG subtypes weaker than H allotype. In the healthy population, the frequency of distribution is 28% (131HH), 24.5% (131RR), and 47.5% (131HR) (56). Norris and colleagues (57) reported an additional FcγRIIa genotype, with a point mutation (C to A) in the codon encoding amino acid position 127 resulting in an amino acid substitution from glutamine (Q) to lysine (K), in one healthy individual. Their following studies demonstrated that when compared with homozygous QQ127 RR131, the K127 substitution increased the affinity of FcγRIIa-R131 to bind to human IgG2 subclass, leading to enhanced phagocytosis by monocytes and neutrophils. Several studies have demonstrated that heterozygous expression of the FcγRIIa polymorphism (H/R131) is associated with immune or inflammation-related disease and with cardiac diseases due to differential IgG binding affinities at FcγRIIa (58–61). A further polymorphism of glutamine (Q) or tryptophan (W) in FcγRIIa at position 27 is apparently not linked with the polymorphism at position

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131 and in isolation did not affect receptor function or IgG binding (62); however, it is conceivable that point mutations within the first Ig-like domain of FcγRIIa may have a greater effect on ligand binding to dimeric FcγRIIa where the first Ig-like domain is likely spatially proximal to the second Ig-like domain of an adjacent FcγRIIa (27). The important relationship between genotypes at positions 131, 127, and 27 with regard to receptor–ligand interactions and FcγRIIa function remains to be addressed experimentally.

Platelet FcγRIIa and thrombocytopenia

Platelets have an average lifespan of 5–10 days in the human circulation and around 10^{11} platelets are produced by megakaryocytes every day by a healthy adult to maintain normal peripheral blood counts ($150–400 \times 10^9$/l) (63). Aged platelets are cleared from the circulation by spleen macrophages and possibly hepatocytes in a regulated multifactorial process (64) that involves platelet glycoprotein modification (65) and apoptotic machinery (66).

Thrombocytopenia (broadly defined as a platelet count below 100×10^9/l) can potentially result from abnormally low platelet production or exacerbated clearance, or both. Normal platelet production can be affected by a wide range of hematological defects affecting megakaryocytes, including congenital abnormalities affecting the formation or stability of platelets, or malignancy (67). In addition, platelet production can be transiently affected by treatment with drugs affecting normal bone marrow hematopoiesis, for example, chemotherapy or drug treatment prior to bone marrow transplants (68). Alternatively, platelet consumption or clearance can also be associated with congenital or acquired causes. The latter includes immune clearance related to autoimmune disease (69), or can be drug-induced (67, 70). Clearly, the cause of the thrombocytopenia can impact on the effectiveness of treatments involving stimulation of platelet production (71), transfusion of donor platelets (72), and/or immunosuppression. Necessity and effectiveness of treatments, and propensity to bleeding vary greatly among individuals and are currently difficult to predict (73). What, if any, is the role of FcγRIIa in these events is yet to be determined.

The relative contribution of platelet FcγRIIa to platelet clearance and to reduced production of platelets in autoimmune thrombocytopenia remains unclear, due in part to the lack of experimental animal models that globally recapitulate the human Fc receptor system (74, 75), complicating the interpretation of studies of autoimmune thrombocytopenia pathogenesis (76, 77). This has in part been addressed with the development of mice engineered to express the full set of human Fc receptors (78) as well as the use of large non-human primate models of antibody-mediated thrombocytopenias (79, 80), although these models are also not without limitations (81–83). Interestingly, macaques have been reported to suffer spontaneously from acquired thrombocytopenias (84) suggesting that immune-based systems for platelet clearance, similar to mechanisms found in humans, exist in these species, supporting their use in studies of platelet lifespan and turnover.

Immune thrombocytopenia

ITP is an immune thrombocytopenic disorder with a complex heterogeneous pathogenesis and a bleeding phenotype that does not necessarily correlate with platelet count. In ITP, there is a loss of tolerance of the immune system to self-antigens on platelets and megakaryocytes; platelets are opsonized by autoantibodies produced by B cells resulting in clearance from the circulation by macrophages in the reticuloendothelial system and decreased platelet production (67, 85). Anti-platelet autoantibodies in up to 50% of patients can be detected by specialized laboratories in plasma using monoclonal antibody-specific immobilization of platelet antigens and on the platelet surface using flow cytometry (86–88). The predominant platelet receptor autoantigens that trigger autoantibody production by B cells are GPIbα and GPIX of the GPIb–IX–V complex and integrins α2β1 and αIIbβ3 (89, 90); however, autoantibodies to other receptors such as GPVI have also been reported (91, 92). Sustained autoantibody production, mainly of the IgG classes in ITP, requires interactions between B cells, T cells, and antigen-presenting cells that predominantly reside in the spleen (93). ITP affects women more than men, and is relatively common in both adult and pediatric patient populations (94, 95). There is a current lack of reliable platelet-related tests relevant to the diagnosis or monitoring treatment in ITP, as accurate diagnosis is complicated by the heterogeneity of pathogenic antibodies, lack of suitable, standardized laboratory tests, and difficulties in distinguishing ITP from other causes of thrombocytopenia (96, 97).

Clinical management of ITP

Immune thrombocytopenia is a disease mediated by both increased platelet Fc mediated destruction in the reticuloendothelial system mainly the spleen and liver and a decrease

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in megakaryocyte production and release of platelets from the bone marrow. Initial treatment for ITP is based on either using high dose corticosteroids and/or intravenous immunoglobulin (IVIg). Splenectomy, rituximab (anti CD20 humanised monoclonal antibody), thrombopoietin mimetics such as Romiplostim or Eltrombopag (98–100) and other immunosuppressive drugs are used in refractory/non responsive cases (101, 102). Inhibition of Syk activation using an oral inhibitor R788 which is likely to ablate Fc receptor ITAM signaling and disrupt monocyte/macrophage phagocytosis has also shown promise in a phase 2 treatment of refractory ITP patients (103). However, the prediction of recovery in the platelet response to treatment remains problematic, as recovery of platelet count in responsive individuals may take 4 weeks or longer, meaning it is difficult to assess response to treatment or identify non-responsive individuals at earlier time points. Nonetheless, promising new approaches using flow cytometry may aid the evaluation of bleeding risk in chronic sufferers of ITP (104).

FcγRIIa polymorphisms in ITP

It is difficult to assess the impact of FcγRIIa polymorphisms on development of ITP as the disease etiology is not well understood but involves antibody engagement of Fc receptors (FcγRIIa as well as other Fc receptors) on macrophages in order to mediate platelet phagocytosis and clearance from the circulation as well as activation of the complement cascade (105, 106). Numerous studies have evaluated the association of FcγRIIa polymorphism with susceptibility to ITP with generally conflicting results probably relating to small sample size, differences in ethnicity, and clinical definitions of ITP (107). However, three studies analyzing Caucasian childhood-onset ITP cases identified that 131H allele carriers were at higher risk (108–110). Evidence that platelet FcγRIIa contributes to ITP pathology is less clear except in isolated cases (91, 92) where anti-platelet autoantibody-mediated activation of platelets triggered loss of platelet GPVI that was FcγRIIa-dependent, and an acquired bleeding syndrome. It is likely that other FcγRIIa-independent mechanisms also contribute to platelet clearance in ITP. In a mouse model of ITP with no FcγRIIa present, anti-GPIbα antibodies could induce platelet activation and clearance, and interestingly a GPIbα receptor desialylation which resulted in enhanced platelet clearance by the liver and intriguingly, supported the use of neuraminidase inhibitors that block receptor desialylation as a potential therapy for ITP patients specifically with anti-GPIbα autoantibodies (111).

Heparin-induced thrombocytopenia

In contrast to ITP, there is a clear role for platelet FcγRIIa in the pathogenesis of HIT. Heparin is the most common and widely used intravenous anticoagulant in the hospital setting as it is fast-acting, reversible, has a short half-life, and is relatively inexpensive. Notwithstanding these clear benefits, heparin has some serious adverse effects. HIT is the most common drug-induced, antibody-mediated cause of thrombocytopenia and thrombosis affecting 1–5% of patients who are treated with heparin (112–114). Some 20–50% of the patients affected by HIT develop limb- or life-threatening thrombosis (115), leading to amputation, new thromboembolic events, and longer hospital stays, and there is a mortality rate of 10–20% observed in people with HIT (116). Despite low-molecular weight heparin (LMWH) gradually replacing heparin, a prospective cohort study detected HIT (with associated anti-PF4/heparin antibodies) in 0.8% of the medical patients treated with LMWH, a similar prevalence to that observed with heparin (117), most likely because of the highly stable nature of the heparin/PF4 antigenic complexes that can be formed with heparins of different sizes (118). HIT remains a significant clinical concern that is difficult to diagnose and to rule out as around 20% of hospitalized patients who were exposed to heparin have anti-PF4/heparin antibodies making antibody testing alone unhelpful unless negative. Delays in diagnosis and treatment are associated with an initial 6% daily risk of thromboembolism, amputation, and death. Misdiagnosis, in contrast, unnecessarily exposes patients without HIT to costly alternative anticoagulants and their attendant 1% daily risk of major bleeding (112).

Patients with HIT have circulating autoantibodies (predominantly IgG1 class), typically involving an antigen complex of heparin and PF4 on platelets (114) (*Fig.* 3). In this regard, HIT is reminiscent of a bacterial host defense mechanism and in fact other polyanions can substitute for heparin and couple with PF4 to induce similar autoimmune responses (119, 120). Antibodies bind to distinct epitopes within PF4 (120) and engage FcγRIIa on platelets as well as Fc receptors on monocytes (122, 123). Thrombocytopenia and thrombosis, both of which are potentially deleterious for the patient, can result from these immune complexes binding and activating platelets *via* FcγRIIa (124, 125). Some of the clinical variations and complexity of HIT pathogenesis are outlined in *Fig.* 4. Clinical diagnosis of HIT remains a key challenge in hematology care, underscoring the demand for new and improved methods of laboratory testing for HIT (126).

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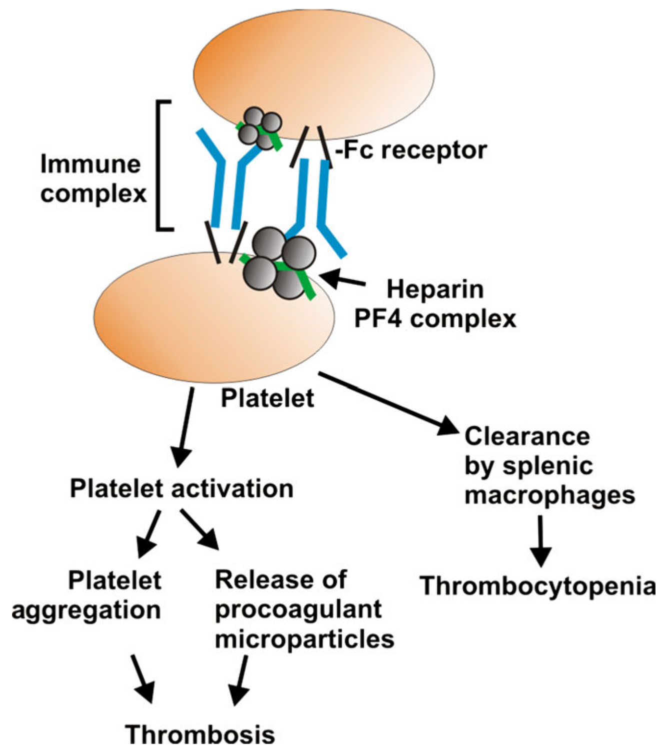

Fig. 3. **Heparin-induced thrombocytopenia: a syndrome mediated by FcγRIIa.** In heparin-induced thrombocytopenia (HIT), IgG autoantibodies that recognize PF4 in complex with heparin engage FcγRIIa primarily on platelets. This leads to significant consumption of platelets via uncontrolled platelet activation and clearance from the circulation within 4–10 days of exposure to heparin. Antibody Fc domains within the immune complex bind to the platelet receptor FcγRIIa, and activate platelets leading to platelet aggregation (thrombosis) and clearance. Platelet count may rebound with removal/replacement of heparin with an alternative anticoagulant; however, the patient is significantly at risk of developing a thrombosis in the following days, particularly if re-exposed to heparin.

FcγRIIa polymorphisms in HIT

There are discordant results on whether FcγRIIa allele specificity influences the predisposition of an individual to suffer HIT. Platelet counts are lower in FcγRIIa 131RR patients with antibodies to PF4/heparin after cardiac surgery (127). Several reports have identified a higher risk of thrombosis in HIT patients homozygous for the FcγRIIa 131R allele (56, 58, 128). In two reports, no association between FcγRIIa 131RR and thrombosis was found in patient cohorts who had thrombosis not associated with HIT, suggesting that this association may be HIT-specific (56, 58). The authors proposed that the increased risk of prothrombotic complications found with FcγRIIa 131RR genotype was due to either reduced clearance of immune complexes and prolonged activation of endothelial cells and platelets (58) or due to increased cell activation by antibodies to PF4/heparin as well as a reduction in IgG2-mediated competitive binding (56). Plasma levels and ratios of IgG1 and IgG2 were stable and consistent for both healthy donors and patients with HIT suggesting that the receptor polymorphism rather than level of antibody was controlling platelet responsiveness (56). In a third study (128), association of 131RR with HIT was identified only when this genotype was combined with one or more risk alleles within PECAM-1 and αIIbβ3, and in the fourth study (129), no association between FcγRIIa allele frequency and HIT-related thrombotic complications could be identified. Meta-analysis of the compendium of studies suggests that overall there is no consistent effect of FcγRIIa polymorphism in HIT (61), a finding that is consistent with the complex, temporal, and multifactorial pathogenesis of this disease. Similarly, in a recent genome-wide association study, associations between the development of HIT and single nucleotide polymorphisms in FcγRIIa and other Fc receptors could not be identified (130). However, interestingly links between circulating levels of heparin/PF4 antibodies or development of HIT and presence of *HLA-DR* alleles were found, which may help explain some of the complexity around predisposition to develop HIT (130).

The detection of a HIT antibody alone in a patient with thrombocytopenia does not equate to the diagnosis of HIT (126). This is because such antibodies occur with high frequency in, for example postsurgical and trauma hospital patient groups (131); the majority of heparin/PF4 antibodies are not pathological, and there are many possible causes for thrombocytopenia in hospitalized patients. Generally, HIT will be confirmed in only a small subset of these patients as the assessment of prothrombotic propensity of blood from patients with suspicion of HIT requires fresh platelets from a donor with a 'high responder' platelet phenotype for immune complexes, and analysis in a specialist laboratory (112, 132). There is a significant unmet need for a simple, reliable, and readily available assay that detects platelet-activating antibodies that can be used to confirm the diagnosis of HIT (133–135).

sGPVI as a marker of a pathological HIT antibody?

Previous studies demonstrated that engagement of platelet FcγRIIa by anti-platelet antibodies induces metalloproteinase-mediated ectodomain shedding of the platelet collagen receptor GPVI generating a 55-kD ectodomain fragment termed soluble GPVI (sGPVI) in plasma (91). In the context of HIT, donor platelets rapidly metalloproteolytically shed GPVI from the surface of platelets that had been mixed with

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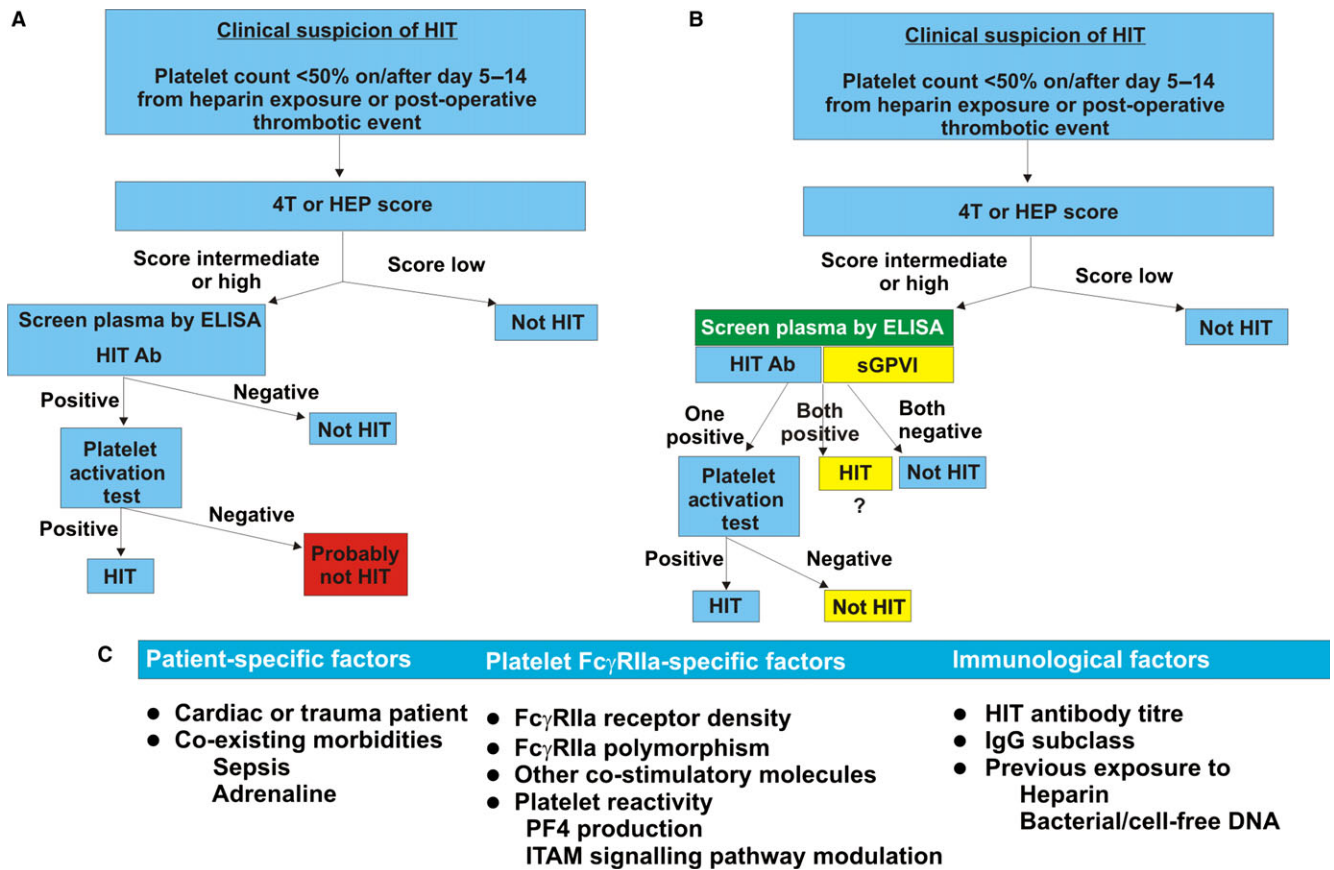

Fig. 4. Complexities in clinical diagnosis of heparin-induced thrombocytopenia (HIT), and a possible refinement using a patient platelet-specific marker of platelet FcγRIIa engagement. (A) A possible algorithm for the management of patients at risk of developing HIT, adapted from reference 125. Following assessment of HIT propensity using a clinical assessment tool (4T or HEP score) plasma samples from patients are analyzed for heparin/PF4 antibodies (HIT Ab) by ELISA. Based on ELISA optical density measurement which provides an indication only of antibody titer, positive plasma samples are assessed for their ability to activate donor platelets (from a 'high responder' individual of with appropriate FcγRIIa genotype). Samples that are positive for antibody but negative in a platelet activation test indicate a patient is less likely to have HIT; however, this is not conclusive as assays involving donor platelets do not report on the patient-specific pathology of the HIT antibody. These assays are also generally batch-analyzed, meaning clinical information is often significantly delayed. (B) The value of integrating a second ELISA measurement of a platelet-specific plasma protein, such as sGPVI which is released from platelets upon engagement of FcγRIIa, would permit simultaneous measurement of HIT antibody titer and a patient-specific marker of HIT antibody pathology. This may aid a more rapid risk stratification of patients, particularly those with an intermediate score and clinical suspicion of HIT, although this remains to be formally tested in a prospective study of HIT diagnosis. (C) Some disparate factors that contribute to the significant complexity when considering a clinical diagnosis of HIT, and that can ultimately impact on the clinical outcomes.

IgG purified from a patient with confirmed HIT (43) (Fig. 2). The release of sGPVI required small amounts of heparin and was either competitively blocked by high concentrations of heparin, or was blocked by an inhibitory antibody against FcγRIIa, or by inclusion of metalloproteinase inhibitors or inhibitors of ITAM signaling pathways (43) (Fig. 2). Under similar experimental conditions, an intracellular cleavage of the FcγRIIa cytoplasmic tail could also be detected (43, 46). Together, these findings (i) identify GPVI receptor shedding as a consequence of FcγRIIa-dependent platelet activation; (ii) provide a mechanism for irreversible inactivation of both GPVI/FcRγ and FcγRIIa on platelets; and (iii) suggest potential novel mechanisms for dampening clinical sequelae associated with ITAM-dependent signaling in HIT patients. For example, an inhibitor of Syk prevented both HIT immune complex-induced thrombocytopenia and thrombosis, without causing bleeding in a transgenic HIT mouse model (136) implying that inhibitors of Syk that have been approved for human use, may ultimately aid in the management of patients with a pathological heparin/PF4 antibody (137), as well as other autoimmune diseases with pathological roles for Fc receptors and ITAM signaling (138).

To address whether elevated levels of sGPVI could be detected in HIT patients consistent with engagement of FcγRIIa, we used an enzyme-linked immunosorbent assay

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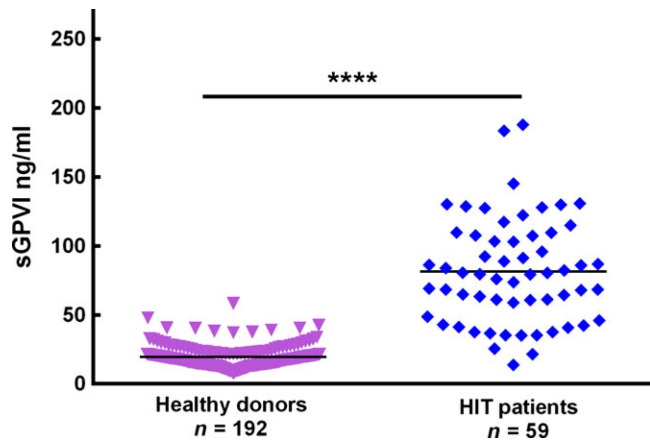

Fig. 5. sGPVI is elevated in plasma of patients with confirmed heparin-induced thrombocytopenia (HIT). Levels of sGPVI in samples of double-spun and frozen/thawed plasma were evaluated by ELISA. Significantly elevated levels of sGPVI were detected in plasma from patients with confirmed HIT (positive for HIT antibody and positive by functional assay as described in the text). Unpaired t test with $^{****}P < 0.0001$.

(ELISA) (139) to measure sGPVI in plasma samples from 59 patients with confirmed HIT. A diagnosis of HIT was defined as a drop in platelet count within 5–10 days of exposure to heparin with no other explanation for thrombocytopenia, the presence of a heparin/PF4 antibody, and a positive result by one or more functional assays (heparin-dependent donor platelet aggregation, or heparin-dependent release of serotonin from donor platelets). sGPVI presents as an excellent candidate plasma marker of platelet activation because (i) GPVI is a platelet-specific receptor; (ii) levels of the receptor on normal circulating platelets are stable; (iii) plasma levels of sGPVI are not influenced by age, gender, smoking, occurrence of Type II diabetes or GPVI polymorphism (140); and (iv) GPVI shedding is a measurable consequence of treatment of human platelets with FcγRIIa agonists (42). Our initial analysis revealed elevated levels of sGPVI in plasma samples from patients with confirmed HIT ($P < 0.0001$) and compared with sGPVI levels in a control cohort of healthy donors (*Fig.* 5). Future investigations can address the specific association of these results with levels of HIT-related antibody, the timing of changes in antibody/sGPVI levels relative to heparin exposure, and the value of immune-assay of plasma sGPVI in conjunction with other factors and current/future tests in at-risk HIT groups (141).

Concluding comments

Platelets have physiological roles that extend well beyond their pivotal role in thrombosis and hemostasis. Platelets interact with leukocytes and the endothelium and are rapidly activated by invading pathogens or their products that may have already been targeted by the adaptive immune system. Platelets play a critical role in recruiting neutrophils to sites of injury and infection, and also contribute to the localization and activation of neutrophils via engagement of neutrophil and endothelial cell receptors and release of chemokines. Platelets release proinflammatory modulators that mediate recruitment of additional cells to a site of infection and amplify the innate protective response. FcγRIIa, on human platelets, controls much of the observed platelet activation in response to multiple strains of *Staphylococcus* and *Streptococcus* bacteria as well as viral infection to trigger the release of secondary mediators, platelet-derived microparticles and chemokines, thereby amplifying platelet activation. Because it plays a minor (if any) role in platelet adhesion, platelet FcγRIIa remains an attractive therapeutic target because ablation of FcγRIIa is less likely to result in bleeding side effects.

For reasons mentioned earlier, platelet FcγRIIa and the role it plays in human disease may well be under-studied due to shortfalls in established rodent models of disease, as well as the complexities conferred by polymorphisms within FcγRIIa that influence IgG binding and impact on platelet and other cell responses. In clinical trials, the contribution of platelet FcγRIIa both as a main driver of a response or as an auxiliary receptor has been difficult to assess, particularly relative to input from FcγRIIa on other cell types. Finally, in part supported by our findings showing elevated plasma sGPVI in HIT (above), future research could aim to counter FcγRIIa function possibly in a platelet-specific manner, in individuals at risk of thrombotic complications, or with autoimmune complications.

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