Role of monocytes and endothelial cells in heparin-induced thrombocytopenia

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Summary

Heparin-induced thrombocytopenia (HIT) is an autoimmune disorder characterised by thrombocytopenia and thrombosis. The mechanisms leading to platelet destruction are complex and the thrombotic complications of HIT appear to be due to multiple different intravascular targets. The dual binding of HIT antibodies to platelet surface PF4/GAG complexes and to FcγRIIA likely leads to both platelet clearance and to their direct activation. Monocytes and endothelial cells bind PF4 with higher avidity than platelets and are more resistant to competitive removal of surface-bound PF4 in the presence of heparin. Binding of HIT antibodies to PF4/glycosaminoglycan complexes on the surface on these cells leads to their activation and increased procoagulant activity. Binding of higher levels of PF4 released from activated platelets to the endothelium may lead to changes of the anticoagulant properties of the glycocalyx and target the endothelial cells for HIT antibodies. Pathogenic antibodies bound to endothelial cells further promote prothrombotic conditions by a mechanism that is independent of FcγR activation, yet not completely understood. A more detailed understanding of the role of monocytes and endothelium may identify new targets for intervention to mitigate the risk of thrombosis with less impact on systemic haemostasis than current approaches to treatment for this serious disorder.

Keywords

Thrombosis, coated platelets, FcγRIIA, glycosaminoglycans, PF4

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Introduction

Heparin-induced thrombocytopenia (HIT) is an autoimmune disorder characterised by thrombocytopenia that is often accompanied by devastating thrombotic complications. The mechanisms leading to platelet destruction are complex and likely extend beyond platelet activation, which, in contrast to most other immune platelet disorders, may account for the intensely prothrombotic nature of HIT. This article will highlight the role that monocytes and endothelial cells play in disease presentation.

HIT is mediated by cell activating antibodies reactive with a multimolecular complex of platelet factor 4 (PF4, CXCL4) modified by heparin (1) or assembled on the surface of target cells by membrane glycosaminoglycans (GAGs) (2, 3). Heparin is required to trigger the immune response and plays an essential role in altering PF4 to become immunogenic (4). Once the antibodies are formed, however, exogenous heparin is no longer necessary to produce the antigen targeted by these antibodies. Instead, cell membrane GAGs augment and may prolong the response to heparin through HIT IgG interaction with PF4 bound to cell surface GAGs.

PF4 is one of the most abundant proteins stored in platelet α-granules (5, 6) where it is found in complex with the chondroitin sulfate (CS) containing proteoglycan serglycin (7, 8). When platelets are activated, PF4/CS complexes are released from the granules and the PF4 binds to the platelet surface (9). However, haematopoietic and vascular cells also express GAGs that react with PF4 to produce structural changes recognised by HIT antibodies (10). GAGs consist of repeating disaccharide units, and their considerable biological variability arises from differences in chain lengths that range from 1 to 25,000 disaccharide units (11), chemical composition of the saccharide, mode of linkage, acetylation, and extent of N- and O-sulfation. The antigenicity of the PF4/GAG complex also depends on the length, chemical composition, and structure of the GAGs (12). The most abundant cellular GAG is heparan sulfate (HS), a polysaccharide that is expressed on most cells and that comprises 50% to 90% of the total GAGs associated with endothelial proteoglycans (13). The structures of HS are themselves highly variable, due largely to great diversity in sequence, patterns of sulfation, and size, ranging from 5–70 kDa (14). Heparin is a structurally similar GAG, that in addition to differences in underlying uronate composition, is generally shorter (~20kDa) than HS (mostly ~50 kDa), more highly sulfated and therefore more anionic (15). Other major classes of GAGs found on cell surfaces include CS and dermatan sulfate (DS), which are even less anionic

than HS (16). Many chemokines bind to heparin and other GAGs (17), but PF4 is the most abundant and binds with 10^{2-3}-fold higher affinity (18), which contributes to why this protein is by far the most commonly implicated in HIT.

PF4 forms antigenic complexes with endogenous GAGs on the surface of platelets (2), monocytes (19), neutrophils (20, 21) and endothelial cells (22, 23). Monocytes and endothelial cells, which express primarily high affinity HS and DS, may be the preferred target for forming immunogenic complexes of PF4 and GAGs compared with platelets, which express low affinity CS almost exclusively (10, 19), especially when PF4 concentrations are limiting. This helps to explain why the HIT-like monoclonal antibody KKO (24) shows considerably greater PF4-dependent binding to monocytes (19). However, this needs to be further studied using human HIT antibodies, which are polyclonal and heterogeneous, so additional mechanisms not revealed in this model might be operative.

Cell activation in HIT

Platelet activation is considered central to the pathogenesis of HIT, and platelet activating antibodies are the hallmark of laboratory confirmation of the diagnosis (25). However, monocytes, endothelial cells, and other cell types are also activated by HIT antibodies and contribute to the pathology, but the mechanism underlying their contribution is less well understood. HIT antibodies initiate platelet activation by engaging IgG-Fc receptor IIA (FcγRIIA) (26–29). Cross-linking of this low affinity receptor by multivalent immune complexes results in signal transduction leading to cell activation (30). In HIT, clustering of PF4 around a semi-rigid linear GAG chain might be essential for apposition of sufficient HIT antibodies to induce persistent activation of cellular FcγRIIA. In our recently published model based on the crystal structures of PF4 and KKO and that of PF4 with the pentamer fondaparinux (31), the GAG moiety binds to the 'closed' end of asymmetric PF4 tetramer. This stabilises the tetramer, which in turn orients the 'open' end for recognition by KKO. Optimal binding of the pathogenic monoclonal antibody KKO requires contact with three of four monomers within the PF4 tetramer. In this way, GAGs and the HIT-like antibody collaborate to "stabilise" the ternary immune complex. It appears that the biological difference between cell-activating (pathogenic HIT) and non-activating (nonpathogenic) antibodies is determined, at least in part, by differences in their ability to bind and stabilise ultralarge PF4/GAG complexes (ULCs) (32, 33). Binding of multiple HIT antibodies to each of the ULCs assembled on cell surfaces may facilitate the configuration of an array of multiple IgG antibodies with greater opportunity to stably crosslink or otherwise engage FcγRIIAs, thereby leading to cellular activation. Receptor engagement is followed by rapid tyrosine phosphorylation within their immunoreceptor tyrosine-based motif (ITAM) followed by multiple tyrosine phosphorylation events on nonreceptor spleen tyrosine kinase (Syk). In fact, inhibition of Syk signalling limits the thrombocytopenic and thrombotic manifestations *in vivo*, implying the central role of this pathway in HIT (34). In addition, it was shown recently that variation in platelet activation through FcγRIIA is at least partly attributable to differences in the expression of T-cell ubiquitin ligand-2 (TULA2), a protein tyrosine phosphatase, which inactivates Syk, with increased levels of TULA2 being associated with a reduced incidence of thrombosis (35).

Activation of platelets by HIT antibodies is accompanied by release of procoagulant microparticles and rapid clearance of antibody-bound platelets from the circulation (36, 37). The mechanism(s) that cause thrombocytopenia are not well defined and might change over the clinical course. Initially, hepatosplenic clearance may predominate, offering a protective mechanism to clear the triggering antigen and antibody from the circulation. As the disease progresses, activation of monocytes, endothelial cells and possibly other cell types contributes to thrombin generation typical for HIT (38), and platelet consumption within thrombi may play a greater clinical role.

The role of monocytes in thrombosis

Blood monocytes are bone marrow-derived leukocytes involved in the innate response that are functionally characterised and classified by their ability to phagocytose, produce cytokines, and present antigen. They play a pivotal role in tissue homeostasis, innate immunity, and both promotion and resolution of inflammation (39, 40), e.g. removal of apoptotic and necrotic cells (41, 42).

PF4 binding to monocytes

As stated above, platelets express mainly CS, while monocytes and endothelial cells express more highly sulfated GAGs such as HS that have greater affinity for PF4. It is possible that the low affinity of PF4 to CS permits facile local transfer of this chemokine to monocytes and endothelial cells in the circulation. The amount of PF4 that is ultimately deposited on the vascular endothelium and/or bound to the surface of platelets, monocytes and other haematopoeitic cells likely varies with the extent of intravascular platelet activation, which in turn contributes to the susceptibility to developing clinical disease.

HIT antigenic complexes form on monocytes at lower concentrations of PF4 than is required for their expression on platelets. Also, because of their higher natural affinity, PF4 complexed with monocyte-HS and DS is more resistant to dissociation by heparin. This implies that monocytes might remain targets for pathogenic antibodies after the levels of PF4 drop below those needed to form antigenic complexes on platelets. In addition, activation of monocytes by pro-inflammatory agents such as bacterial lipopolysaccharide and IL-1α triggers hypersulfation of monocyte GAGs and induces expression of additional HS-proteoglycans with sidechains that differ in length and structure. These changes in the monocyte surface GAG composition may further increase PF4 binding (43), underlying the reported link between inflammation and manifestation of HIT (44).

Monocyte activation in HIT

Binding of HIT antibodies to PF4 bound to the monocyte GAGs stimulates expression of tissue factor (TF) (19, 45, 46) and release of TF-expressing microparticles (47). This induction of TF expression by HIT antibodies may help to initiate or amplify intravascular coagulation, with the resultant generation of thrombin, leading to an explosive feed-forward system of platelet, endothelial cell, and leukocyte activation, and formation of fibrin. Monocytes or monocyte-derived particles are incorporated into thrombi, especially on the arterial side of the circulation, where they might directly contribute significantly to thrombus development (19). Depletion of monocytes from the circulation in a murine model of HIT attenuates thrombosis, but exacerbates thrombocytopenia (19). It is possible that in the absence of monocytes, more PF4, and consequently more HIT antibodies, may target the platelet surface, enhance their hepatosplenic clearance and reduce platelet availability to promote intravascular thrombosis. Indeed, the presence of monocytes *in vitro* in a "humanised" microfluidic model of HIT appears to be critical for platelet adhesion and aggregation as well as for fibrin deposition under flow (29). Depletion of monocytes from whole blood samples significantly decreases platelet accretion and fibrin deposition while repletion of monocytes to the physiologic level restores the ability of KKO to stimulate platelet accretion and fibrin deposition to levels similar to unmodified whole blood.

As mentioned, cellular activation by HIT antibodies involves intracellular signalling through the ITAM found on activating IgG-Fc receptor, which is rapidly phosphorylated upon Fc receptor engagement followed by phosphorylation of Syk (30). Inhibition of the Syk pathway using the specific inhibitor PRT318 blocks the development of thrombocytopenia and thrombosis in the murine model of HIT in vivo (34) and also clot formation in the microfludic model *in vitro* (29). Moreover, inhibition of Syk specifically in monocytes attenuated synthesis and expression of TF triggered by HIT antibodies *in vitro* (48) and also decreased whole blood thrombus formation in the microfludic model(29). Although both FcγRIIA, the only FcγR on human platelets, and FcγRI have been implicated in monocyte microparticle formation in HIT (47), experiments blocking individual Fcγ receptors by monoclonal antibodies as well as employment of transgenic mice monocytes showed that only the former is critical for the initial activation of monocytes by HIT immune complexes, similar to platelet activation (29). In addition, TF gene expression after stimulation with HIT IgG in whole blood was also inhibited by blocking the FcγRIIA, further signifying the role of this receptor in monocyte activation in HIT (49). However, activation through FcγRI may contribute to *de novo* synthesis of TF and microparticle release at later time points. Together these studies strongly suggest that monocytes play an important role in fibrin deposition and thrombosis in HIT, in part through the synthesis and surface expression of TF and generation of thrombin.

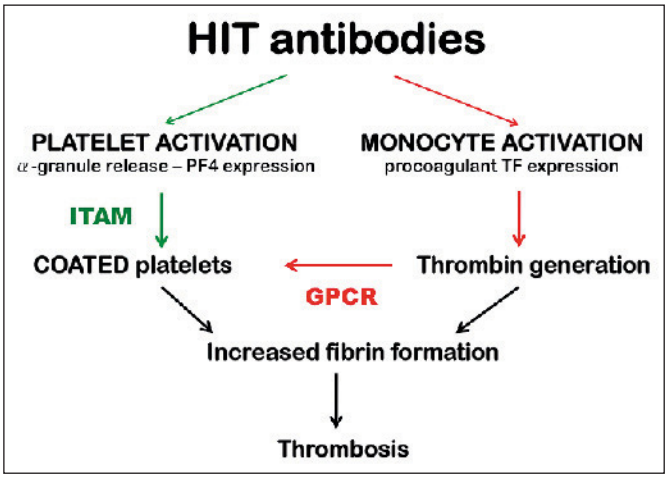

Figure 1: Role of monocytes in HIT. Monocytes activated by HIT antibodies express tissue factor (TF) and support thrombin generation. The thrombin generated acts as a second signal activating platelets through GPCR (G-Protein Coupled Receptors) pathway (PAR1 and PAR4, Protease-Activated Receptors 1 and 4) and, together with direct activation of platelets through the ITAM (Immunoreceptor Tyrosine-based Activation Motif) pathway by HIT antibodies, leads to the formation of coated platelets. These highly procoagulant platelets then contribute to increased fibrin formation and thrombosis.

Coated platelets in HIT

Thrombin generated on the surface of stimulated monocytes might also activate platelets via protease-activated receptor 1 (PAR-1) and PAR-4 (50–52) amplifying the signal induced by engaging of platelet FcγRIIAs. This dual activation of platelets through PARs, a G-protein–coupled receptor-dependent pathway, and FcγRIIA, an ITAM-dependent pathway, has been shown to lead to the formation of highly prothrombotic, coated platelets (53), which might contribute to thrombosis and platelet consumption within thrombi in HIT (▶Figure 1). This subpopulation, originally called COAT (Collagen And Thrombin) activated platelets is "coated" with high levels of several procoagulant proteins that together with surface exposure of phosphatidylserine (PS) support robust prothrombinase activity (54). It is possible that quantification of coated platelets remaining in the circulation might help to identify patients with HIT who are at higher risk for thrombosis, although the sequence and the relative contribution of a monocyte/macrophage-directed pathway vs direct platelet activation needs to be further elucidated. These findings may complement the reported procoagulant properties of microparticles released by platelets activated by HIT antibodies (37). This is a potentially important area for future investigation because traditionally, direct platelet activation through FcγRIIA is considered as an early event in the pathogenesis of HIT. However, monocytes, with their higher cell surface avidity for PF4 than platelets, may be a target for pathogenic HIT antibodies before substantial numbers of platelets are activated and while free PF4 is limited. When the level of available PF4 reaches a higher threshold level, activated platelets may become the predominant target for HIT antibodies, providing a second signal via direct platelet activation and clearance. This sequence of events would identify monocytes as an important target for early intervention that is not specifically addressed at present.

While the observations derived from the model above are very helpful to understand the pathophysiology of thrombosis in HIT, several pathways and factors cooperate *in vivo* and it may be difficult to dissect the sequence of cellular interactions leading to thrombosis and thrombocytopenia.

Role of monocytes in antigen presentation in HIT

Monocytes may also play an important role in antigen presentation and initiation of the antibody response in HIT. Monocytes internalise via an active endocytic pathway PF4–heparin complexes, which are then transported to late endosomes, where the complexes still express the antigen recognised by HIT antibodies. Heparin not only strikingly augments cellular uptake of PF4, but critically enhances the PF4 immunogenicity by rendering it a particulate antigen, which likely stimulates the immune response with far greater potency than soluble antigens (4).

The role of endothelial cells in thrombosis

Endothelial cells line the blood luminal surface of vessels. These cells play a pivotal role in regulating blood flow and vascular homeostasis. Quiescent endothelial cells are involved in the control of thrombosis and thrombolysis, platelet and leukocyte interaction with the vessel wall, and the regulation of vascular tone and growth of blood vessels. Changes in endothelial surface integrity induce diverse pathological responses that may predispose to the development of thrombosis (55).

Binding of PF4 to the endothelium

Under physiologic conditions, endothelial cells are covered with a relatively thick sheath of glycoproteins, proteoglycans, and GAGs referred to as the glycocalyx (56). HS, which has a high affinity for PF4, is the predominant endothelial GAG. Therefore, it is likely that endothelial cells would bind PF4 with greater affinity than platelets and even monocytes, making them an important target for HIT antibodies especially given their relative mass and surface area. Indeed, substantial amounts of PF4 are normally associated with endothelial cell proteoglycans (57). Plasma concentrations of PF4 increase 10– to 20-fold after heparin is infused intravenously (58), likely due to elution from the endothelium because the affinity of PF4 for endothelial cells is lower than to purified heparin (Kd = 2–3 μmol/l vs 2 nmol/l, respectively) (59). Binding of PF4 to the endothelium is attenuated by pretreatment with heparinase (59, 60) and independent of the pentasaccharide involved in the binding of antithrombin III (ATIII) (60). In addition, PF4 has 10– to 100-fold greater affinity for endothelial cell HS than does ATIII (61) and thereby markedly attenuates its anti-protease cofactor activity on intact vessels (62), which may impair the host response to thrombin generated on monocytes and on endothelial cells by HIT antibodies. PF4 released from platelets activated on injured endothelium might foster haemostasis through neutralisation of negatively charged GAGs located on cell surfaces, allowing closer approximation of platelets to each other and to other cells, e.g. endothelial cells (63). Heparin may stabilise or propagate thrombosis by neutralising the charge effects of "excess" cell-surface PF4 released in relatively large amounts from activated platelets, either due to constitutive overexpression or due to platelet-activating effects of atherosclerosis and vascular injury.

Effect of PF4 and HIT antibodies on activated protein C

The anticoagulant properties of PF4 on the vasculature are mediated in part through activation of protein C. The endothelial cell suface protein thrombomodulin (TM), which functions as a cofactor in the thrombin-induced activation of protein C, is post-translationally modified by addition of a CS-like GAG. This CS side chain provides TM with the capacity to bind cationic peptides at physiological pH. Binding studies using surface plasmon resonance (64) confirmed a strong interaction between PF4 and TM containing CS as well as PF4 and the Gla domain of protein C. Binding of PF4 increases protein C-cofactor activity 25–fold in a cell-free system (64, 65). This increase in generation of activated protein C by PF4 is dependent on both the Gla domain of protein C and CS side chain of TM. Addition of PF4 to cultured endothelial cells accelerates activated protein C generation approximately five- to 10-fold depending on vascular origin (66). Injection of PF4 into primates infused with thrombin increases activated protein C generation two- to three-fold and prolongs the baseline activated partial thromboplastin times (66). In mice, PF4 released from platelets enhances activated protein C generation in a model of thrombin infusion and increases survival of mice following lipopolysaccharide–induced endotoxaemia (67). The PF4-mediated acceleration of activated protein C generation exhibits the same bell-shaped profile seen with formation of HIT antigenic complexes of PF4 with heparin in solution or with GAGs on cell surfaces (2, 19, 68, 69). Pathogenic HIT antibodies block PF4's enhancement of activated protein C generation *in vitro*, which might further contribute to the prothrombotic state (70).

Binding of HIT antibodies

Binding of pathogenic antibodies to antigenic complexes of PF4 and GAGs expressed by the endothelium contributes to thrombosis. Several groups have shown that HIT antibodies bind to endothelial cells and induce procoagulant reactions. HIT sera induce TF expression on endothelial cells, and the expression of procoagulant activity was enhanced further in the presence of platelets (71). Antibody binding was reduced when the cells were pretreated with enzymes that degrade heparin or HS, whereas addition of chondroitinase was without effect (22, 23, 72). These observations were confirmed and extended by demonstration that the binding of HIT antibodies to human umbilical vein endothelial cells was dependent on PF4, but not on exogenous heparin (23). This is consistent with the concept that PF4, released from activated platelets, can form a competent antigenic complex on the pericellular matrix of the endothelium (73). Assembly of cell-

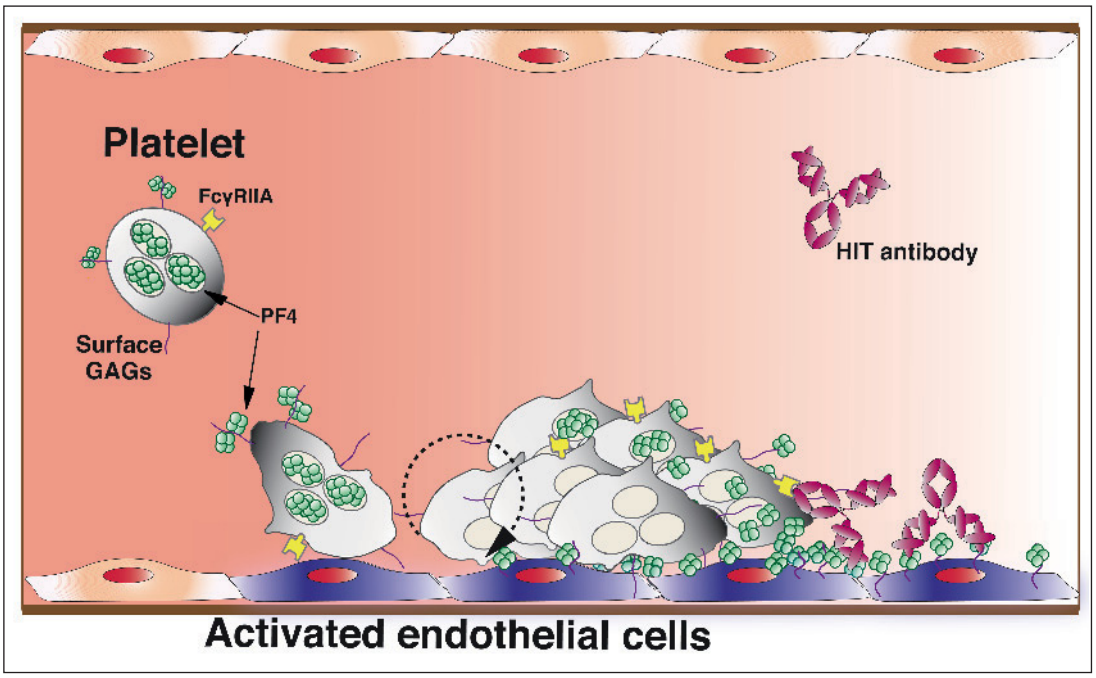

Figure 2: Role of endothelial cells in HIT. Platelets activated on the injured endothelium release PF4 from their α-granules, which, due to its high affinity, is immediately deposited on the endothelium, where it becomes a target for HIT antibodies. Binding of pathogenic antibodies contributes to enhanced endothelial activation, recruitment of more platelets and a feed forward loop of PF4 deposition and platelet activation, resulting in fibrin deposition and thrombosis.

surface antigenic complexes capable of binding HIT antibodies along the endothelium may contribute to the development of HIT when only low sensitising doses of heparin are employed and also contribute to persistence of the hypercoagulable state after heparin has been withdrawn. Alterations in the local vascular milieu and endothelial dysfunction may enhance PF4 binding, antibody binding and vascular response to immune injury, including greater adhesion of activated platelets and monocytes and binding other chemokines. In addition, different vascular beds may react differently to HIT antibody binding. In contrast to macrovascular endothelial cells (HUVEC) that required preactivation to interact with HIT antibodies, HIT antibodies in the presence of PF4 directly activated microvascular endothelial cells. This activation was characterised by IL-6 and von Willebrand factor release and expression of adhesion molecules that led to enhanced adhesion of monocytes (72). Together, these alterations in endothelial function likely promote proinflammatory and prothrombotic processes that contribute to the propensity for local thrombus formation, which is typical in HIT (►Figure 2). HIT antibodies may thus amplify local inflammatory and procoagulant processes by promoting monocyte binding to the endothelium (72) followed by generation of thrombin and by enhancing platelet-leukocyte aggregates (74).

Conclusions

In summary, unlike many other disorders caused by autoreactive platelet binding antibodies that cause thrombocytopenia and bleeding (e.g. idiopathic thrombocytopenic purpura, other drug induced thrombocytopenias, post-transfusion purpura and neonatal alloimmune thrombocytopenia, among others), the prothrombotic features of HIT appear to be due to multiple different targets. Platelet activation via the binding of HIT antibodies to platelet surface PF4/GAG complexes and to FcγRIIA likely leads to both activation of the platelets and acceleration of their clearance. However, monocytes and endothelial cell are also activated, which may be pivotal factors that lead to the highly prothrombotic nature of this disorder through the generation of thrombin and transactivation of platelets by monocytes, generating microparticles, promoting platelet adhesion and likely other processes that are not completely blocked by direct thrombin inhibitors. This may help to explain why antiplatelet therapy is not as efficacious as would be hoped if platelet activation alone were responsible. Moreover, monocytes and endothelial cells, having surface GAGs with higher affinity for PF4 than platelets and that are more resistant to competitive removal of surface-bound PF4 in the presence of heparin, may perpetuate the thrombotic condition after heparin is no longer present. Targeted intervention in monocyte FcR binding or activation might be an attractive therapeutic strategy to prevent bleeding complications but it is unknown if it will be sufficient to prevent treatment-associated thrombosis. On the other hand, overall inhibition of FcγR mediated cell activation by novel specific Syk inhibitors have been shown to be effective in HIT *in vitro* (48) and *in vivo* (34) as well as in inhibition of thrombosis and vascular injury responses without affecting haemostasis (75, 76). Therefore, a more detailed understanding of the role of monocytes and endothelium may identify new targets for intervention to mitigate the risk of thrombosis with less impact on systemic haemostasis than current forms of treatment for this serious disorder.

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Conflicts of interest
None declared.

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