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5B9, a monoclonal anti-platelet factor 4 /heparin IgG with a human Fc fragment that mimics heparin-induced thrombocytopenia antibodies

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Key words: heparin, thrombocytopenia, monoclonal antibody, platelet activation.

Running title: 5B9, a monoclonal HIT antibody with a human Fc.

Essentials

- No humanized monoclonal antibody was available to study heparin-induced thrombocytopenia (HIT).
- We developed the first anti-platelet factor 4 (PF4)/heparin antibody with a human Fc fragment.
- This antibody (5B9) fully mimics the effects of human HIT antibodies.
- 5B9 binds two regions within PF4 that may be critical for the pathogenicity of HIT antibodies.

SUMMARY

Background: The diagnosis of heparin-induced thrombocytopenia (HIT) is based on clinical and biological criteria, but a standard is lacking for laboratory assays. Moreover, no humanized HIT antibody is available for pathophysiological studies. *Objective*: To characterise 5B9, a chimeric monoclonal antibody, which fully mimics the effects of human HIT antibodies. *Methods/Results*: 5B9, a chimeric anti-PF4/H IgG1 antibody was obtained after immunizing specific transgenic mice. 5B9 induced heparin FcγRIIA-dependent platelet aggregation and tissue factor mRNA synthesis in monocytes. It also induced significant thrombocytopenia and thrombin generation in mice expressing human PF4 and FcγRIIA receptors. The binding of 5B9 to PF4/H complexes was inhibited by 15 of 25 HIT plasma samples and only 3 of 25 samples containing non-pathogenic anti-PF4/H antibodies. KKO, a murine IgG2b HIT antibody, also inhibited the binding of 5B9 to PF4/H, suggesting that epitopes recognized by both antibodies are close.

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A docking analysis based on V_H and V_L sequences of 5B9 showed that binding of 5B9 Fab to PF4 involved 12 and 12 residues in B and D monomers, respectively, including 7 previously identified as critical to the formation of a PF4/KKO complex. Two regions (Asp-7 to Thr-15 and Ala-32 to Thr-38) therefore appeared important for the binding of 5B9 and KKO on PF4 modified by heparin.

Conclusions: 5B9 is the first anti-PF4/H monoclonal antibody with a human Fc fragment, which induces similar cellular activation as HIT antibodies. Moreover, 5B9 binds epitopes within PF4 that are likely critical for the pathogenicity of HIT antibodies.

INTRODUCTION

Heparin-induced thrombocytopenia (HIT) is a frequent drug-adverse event caused in most patients by platelet-activating antibodies (Abs) directed against complexes of heparin (H) bound to platelet factor 4 (PF4) [1, 2]. Most HIT Abs are IgG, which are potentially pathogenic because they can activate platelets with heparin *via* FcγRIIA receptors. The diagnosis of HIT is based on clinical and pathological criteria. The first category of laboratory HIT tests commonly used is immunologic and detects the binding of Abs to PF4 immobilized with heparin or polyvinyl sulfonate. The second category of assays is functional, more specific for pathogenic IgG Abs and therefore is necessary for confirming the diagnosis of HIT in many patients. Nevertheless, these functional assays require strict quality controls, (*i.e.*, testing a weak positive HIT serum and using an appropriate negative control, as well as checking that platelet activation is inhibited by a high heparin concentration) [3]. Functional assays performed with platelet-rich plasma (PRP) or whole blood are less sensitive than methods with washed platelets such as serotonin release assay, with high frequency of false-negative results [4, 5]. In addition, despite improvements in HIT laboratory assays, a standard is still lacking for both immunological and functional assays.

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In addition, platelets from healthy donors also show wide variability in response to HIT antibodies, and HIT does not develop in many patients with significant levels of antibodies against PF4/H. The mechanisms that regulate the pathogenicity of HIT antibodies have not been fully defined, although recent studies found that variations in *CD148*, *FCGR2A* and *UBASH3B* genes might have a significant effect on platelet activation induced by HIT antibodies [6-8]. Several studies of the pathophysiology of HIT involved KKO, a murine monoclonal anti-PF4/H antibody that activates platelets and monocytes by cross-linking FcγRIIA and behaves like human HIT IgG [9-12]. However, KKO is an IgG2b antibody, a subclass absent in humans [13]. Moreover, IgG anti-PF4/H antibodies present in HIT plasma samples are predominantly IgG1. Also, human and mouse Fcγ receptors (FcγRs) have shown large differences in binding abilities [13]. Indeed, mouse FcγRs efficiently bind human IgG subclasses, whereas human FcγRs do not or poorly bind mouse IgG subclasses. FcγR-IgG interactions are highly critical in the pathogenesis of HIT [14], so we aimed to develop a monoclonal anti-PF4/H antibody with a human Fc fragment by using transgenic mice homozygous for the Cγ gene of a G-class human immunoglobulin that directly produce chimeric IgG1 antibodies. We then studied the characteristics of the obtained monoclonal antibody, 5B9, and demonstrated that it fully mimicked the cellular effects of human HIT antibodies.

MATERIALS AND METHODS

Development of monoclonal antibodies to PF4/heparin complex

To develop an anti-PF4/H antibody with a human Fc fragment, we used transgenic Balb/c mice homozygous for the Cγ gene of a G-class human immunoglobulin that produce chimeric IgG1 antibodies composed of murine light chains and chimeric heavy chains with a murine variable region and a human constant region (GammaPrim™, B Cell Design, Limoges, France). Four 6- to 8-week-old mice were injected intraperitoneally on day 0 with a 50 µL sterile solution of phosphate buffer saline (PBS, Sigma-Aldrich, Saint-Quentin Fallavier, France) containing

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purified human PF4 (50 µg, Hyphen BioMed, Neuville-sur-Oise, France), unfractionated heparin (UFH 2 IU; Heparin Choay; Sanofi), and 25µL Freund's complete adjuvant [15]. Then, the same preparation containing preformed PF4/heparin complexes in PBS with Freund's complete adjuvant was injected intraperitoneally or through the tail vein in mice on days 15 and 30. Finally, 10 days after the later injection, a boost of human PF4/heparin complexes in PBS with Freund's incomplete adjuvant was injected intravenously in all mice 3 days before they were sacrificed. The synthesis of anti-PF4/heparin antibodies was monitored using a homemade ELISA, adapted from Arepally *et al* [15] with microplates coated with purified human PF4 (2.5 µg/mL, Hyphen Biomed) with or without 0.05 IU/mL of heparin. Three mice positive for anti-PF4/H antibodies were killed and their spleens were removed for fusion procedures. Hybridomas were then obtained from chemical fusion of B cells with SP2/0 AG14 myeloma cells using a method adapted from Kölher and Milstein [16]. Hybridomas were cultured for 13 days, and supernatants screened by ELISA to detect antibodies to PF4/H and PF4. Wells considered positive were then sub-cloned by limiting dilutions, and positive ELISA results were confirmed for 8 clones. Finally, 4 clones were isolated: 3 produced antibodies that equally bound PF4/H complex and PF4 alone on ELISA (1C12, 1E12, 2E1; supplemental Figure S1). The other clone produced an IgG1 named 5B9 that was mainly specific to PF4/H and was isolated from ascitic fluid by using affinity columns.

The reactivity of 5B9 was evaluated with 3 commercial IgG-specific ELISA (Asserachrom HPIA IgG; Stago, Asnieres, France, Zymutest HIA IgG; Hyphen Biomed and PF4 IgG; Immucor GTI, Waukesha, WI) involving different antigen targets with modified PF4 (PF4/H, PF4/H/protamine and PF4/polyvinyl sulfonate complexes, respectively).

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Platelet activation and aggregation tests

Whole blood from healthy donors was collected after informed consent in acid-citrate-dextrose supplemented with prostaglandin E1 (0.1mM, Sigma-Aldrich), according to the Helsinki Declaration principles and International Society on Thrombosis and Haemostasis recommendations [17]. Platelets were washed and suspended at a final count adjusted to 350 G/L. In addition, whole blood from healthy donors was collected on 0.129 M sodium citrate and PRP was isolated. Platelet aggregation tests (PAT) with PRP and washed platelets were performed in an APACT 4 aggregometer (ELITech Group, Puteaux, France) with different concentrations of 5B9 (50, 20 and 10 µg/mL) and heparin (0, 0.5 and 50 IU/mL).

Serotonin release assays (SRA) were performed [18], with the same concentrations of 5B9, with or without addition of exogenous PF4 (10 µg/mL).

Assays of tissue factor mRNA levels after stimulation by 5B9 antibody

Monocytes were isolated from whole blood collected in healthy donors on 0.129 M sodium citrate with the Monocyte Isolation Kit II (Miltenyi Biotec, Paris, France) [11]. P24 plates were seeded with $5x10^5$ cells/well and various concentrations of purified human PF4 and heparin were then added, followed by the addition of 5B9 (20 µg/mL). After incubation at 37°C for 2 hours, total RNA was isolated using the RNeasy Mini kit (Qiagen, Courtaboeuf, France) and mRNA levels specific for tissue factor (*TF*) and *CD14* genes were measured by qPCR as described [7].

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Human neutrophil degranulation assay

Blood neutrophils were isolated as described [19]. After priming with cytochalasin B (5 µg/mL) and human tumour necrosis factor α (10 ng/ml), neutrophils (10^6 cells for each assay) were stimulated with various concentrations of heparin, human PF4 (5 µg/mL), and 5B9 (20 µg/mL), or phorbol myristate acetate (100 ng/mL, Sigma-Aldrich) as positive control. After incubation for 90 min, supernatants were collected and neutrophil activation was assessed by using a fluorogenic substrate of neutrophil proteases (Abz-HPV-PVYAFSPQ-Yno2, 25µM, *GeneCust Europe,* Ellange, Luxembourg) [19]. For each condition, maximal hydrolysis speed of the substrate (Vmax) was measured and ratio of values under stimulated and basal conditions was calculated.

Stimulation of human whole blood with 5B9 antibody

Whole blood from healthy donors on 0.129 M sodium citrate was incubated 60 min with 5B9 (20µg/mL), with and without heparin (0.5 IU/mL) at 37°C. After centrifugation, plasma was collected and clotting time was measured with the Procoag-PPL assay (Stago), which is sensitive to the presence of phospholipid microparticles [7]. Total RNA was isolated from cells using the Qiamp RNA blood Mini kit (Qiagen) and mRNA levels from *TF* and *CD14* genes were measured by qPCR.

ELISA competition assays

The ability of 5B9 and KKO to bind PF4/H-coated wells in the presence of plasma from patients with anti-PF4/H antibodies was evaluated using the Asserachrom HPIA® assay (Stago) with modification. Diluted plasmas were first incubated for 50 min at room temperature (RT) in wells coated with PF4/H complex. Then, 5B9 or KKO were added to wells without removing diluted plasma samples and incubated for 10 min at RT. The concentrations of 5B9 and KKO

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used (10 and 0.7 µg/mL, respectively) were defined in specific ELISA experiments and provided 75% of the maximal binding of each antibody to PF4/H complexes. After washings, alkaline-phosphatase-conjugated goat anti-murine κ chain (1/500; Jackson ImmunoResearch, Suffolk, UK), was added and incubated for 1h at RT. After washings, pNPP substrate was added, and the reaction was stopped with NaOH 3N before absorbances were read at 405 nm. The reference value (OD ref) was measured after incubation of 5B9 or KKO without plasma, and the percentage binding inhibition was obtained as: $[(OD_{ref} - OD_{test}) / OD_{ref}] \times 100$.

For 5B9/KKO competitive ELISA, 5B9 was first incubated 10 minutes at 2.5µg/ml in wells with PF4/H, and then KKO was added at different concentrations (0, 5, 10, 15 and 20 µg/mL). Finally, 5B9 binding was revealed using an anti-human Fc antibody.

In vivo effects of 5B9 in HIT mouse model

All studies in mice were conducted in accordance with the guidelines and approval of the Institutional Animal Care and Use Committees of Thomas Jefferson University and Portola Pharmaceuticals. Both facilities are accredited by the Association for Assessment and Accreditation of Laboratory Animal Care. The transgenic HIT mice (human FcγRIIA, hPF4-transgenic, mPF4 knockout) were assigned to 2 groups receiving 5B9 or KKO. At day 0, platelet counts were obtained for baseline. Mice were injected with KKO (30 µg/g body weight, intraperitoneally; n = 3) or 5B9 (15.8 µg/g; n = 3) on day 0. At days 1, 2, and 3, 1400U/kg heparin were injected subcutaneously using 28G ½ needles, then 4 hours later, platelets were counted by using a Hemavet Analyser (model 850, CDC Technologies) and monitored over time to assess thrombocytopenia. Level of thrombin–antithrombin complex (TAT) was also measured as a marker of thrombin generation *in vivo* by using ELISA as described [20].

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Docking analysis of 5B9-PF4 complex

5B9 cDNA was sequenced using the Sanger method, and a model of the 3D structure of 5B9 antibody was then built by using MODELLER [21]. The structures used as templates for homology modelling were PDB:1F58 for the variable heavy (VH) chain and constant domains and PDB:5B9L for the variable light (VL) chain. The resulting 3D model was docked on PF4 complexed with fondaparinux (PDB:4R9W) [22], using the method PRIOR optimized for antibody/antigen complexes [23]. The models for the assembly between heparin, three tetramers of PF4 and 3 antibodies were built manually, with the structure of heparin heptasaccharide (PDB:1SR5) used as a guide. Structures were imaged using the PyMOL Graphics System (v1.8).

Statistical analysis

Student t-test was used to compare data obtained after stimulating monocytes, neutrophils, or whole blood with 5B9 and during studies *in vivo*. $P < 0.05$ was considered statistically significant.

RESULTS

5B9 specifically binds to PF4/H complexes

Homemade ELISA revealed that 5B9 mostly bound to the PF4/H complex with no significant interaction with PF4 alone (Figure 1A). Also, the binding of 5B9 to PF4/H complex was optimal with concentrations of UFH and PF4 of 0.1 IU/mL and 5 µg/mL, respectively, (Figure 1B) and was inhibited with higher concentrations of heparin (5 and 25 IU/mL). The binding of 5B9 to modified PF4 was evaluated with 3 commercial IgG specific ELISA (*i.e.* Asserachrom HPIA® IgG, Zymutest HIA® IgG and Lifecodes PF4® IgG) testing different complexes

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with modified PF4. The 3 assays produced excellent correlation (R^2> 0.98), but the binding of 5B9 to modified PF4 seemed lower with the Zymutest HIA® than the 2 other immunoassays. Therefore, the mean absorbance measured with 5 µg/mL of 5B9 was 1.3 with the Zymutest HIA®, and above 2.0 with HPIA® and Lifecodes PF4® (Figure 1C).

5B9 induces heparin-dependent platelet activation and aggregation

5B9 induced heparin-dependent platelet activation with maximal release of ^{14}C serotonin achieved with 0.1 IU/mL UFH, which was similar to the pattern observed with most of HIT plasma samples (Figure 2A). As expected, platelets were not activated by 5B9 when heparin was absent or was present at a high concentration (10 IU/mL). With exogenous PF4 (10 µg/mL) in the reaction mixture, 5B9 induced strong platelet activation without requiring heparin, but with complete inhibition with 10 IU/mL UFH (Figure 2B). The release of serotonin was always inhibited by the monoclonal IV.3 antibody whatever the conditions, with or without exogenous PF4. 5B9 also induced heparin-dependent platelet aggregation with PRP and washed platelets from healthy donors. This response (Figure 2C and D) was observed with 50 or 20 µg/mL 5B9 and 0.5 IU/mL UFH, with no aggregation obtained with 10µg/mL. Similar results were obtained with 5B9 tested in the presence of enoxaparin, a low- molecular-weight heparin (data not shown).

5B9 induces monocyte and neutrophil activation

5B9 with exogenous PF4 (5 µg/mL) increased tissue factor mRNA synthesis from isolated monocytes, effect which was potentiated by a low concentration of UFH (0.1 IU/mL) and fully inhibited by 10 IU/mL UFH (Figure 3A). In addition, after incubation of whole blood from healthy donors with 5B9 and UFH (0.5 IU/mL), TF mRNA level was 3.2 fold increased than without heparin (P<0.05), and this effect was inhibited by a high concentration of heparin (50

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IU/mL) (Figure 3B). This TF gene induction was associated with procoagulant activity because plasma clotting time was shortened after incubation of whole blood with 5B9 and UFH (39 vs. 60 sec without UFH; Figure 3C). Also, 5B9 incubated with PF4 and without UFH led to strong release of protease activity from isolated neutrophils (with a 5.9 fold increase in maximal hydrolysis speed compared to the non-stimulated condition) (Figure 3D). But, this effect was potentiated (8.9-fold) by a low UFH concentration (0.1 IU/mL) and inhibited with 10 IU/mL UFH.

5B9 induces thrombocytopenia in the hFcγRIIA/hPF4 transgenic mouse model

In vivo, 5B9 injected with UFH at 15.8 µg/g in transgenic mice expressing human FcγRIIA and PF4 induced thrombocytopenia with a 38% to 40% decrease in platelet count as compared to the basal value (Figure 4A). This effect of 5B9 was similar to that obtained with KKO injected at 30 µg/g (decrease in platelet count between 29% and 35%). Plasma level of TAT was measured at times that coincided with platelet nadirs in each experimental group. TAT plasma levels significantly increased after injection of UFH in all treated mice, but this effect was more pronounced on day 1 with 5B9 (mean 47 *vs*. 5 ng/mL at day 0) than with KKO (33 *vs*. 11 ng/mL). This difference was no longer observed 2 days after injection of UFH, but TAT levels remained high in all mice studied (36 and 33 ng/mL with 5B9 and KKO, respectively).

5B9 binds an epitope close to that recognized by human HIT antibodies

Cross-competition experiments were performed to determine whether 5B9 and pathogenic HIT antibodies could recognize overlapping epitopes in the PF4/H complex. The binding of 5B9 to PF4/H complexes was inhibited (*i.e*. inhibition > 20%) by 60% of HIT plasma samples tested (15/25) and only 12% of plasma samples containing non-pathogenic anti-PF4/H antibodies (3/25)(Figure 5A). Noticeably, there was no significant difference in the main clinical (4T's, thrombosis, treatment) and biological (A450 value, maximal serotonin release) features in

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HIT patients with inhibitory and non-inhibitory antibodies (supplementary table). Similar cross-competition experiments were performed with KKO: its binding to the PF4/H complex was inhibited by 32% of HIT plasma samples (8/25), and 0% of non-HIT samples. Levels of 5B9 and KKO binding inhibition to PF4/H by HIT plasma samples were also highly correlated (R^2= 0.85), which suggests that epitopes recognized by both antibodies are similar. In addition, competitive assays with KKO and 5B9 showed that KKO inhibited in a concentration-dependent manner the binding of 5B9 to the PF4/H complex, with absorbance varying from 2.05 without KKO to 0.55 with 20 µg/mL KKO (Figure 5C).

Modelling the 5B9 scFv/PF4 complex by docking

After 5B9 cDNA was sequenced, we defined a docking model based on the V_H and V_L sequences of 5B9 (scFv) obtained, and with recently described data for the crystal structure of PF4 tetramer modified by fondaparinux [22]. According to this model, 5B9 appeared to bind B and D monomers of PF4 (Figure 6A) and this interaction could stabilize the PF4 tetramer and then macromolecular complexes composed of several PF4 tetramers bound to extended glycosaminoglycan chains (Figure 6B). The epitope of 5B9 was predicted to involve 24 amino-acids, 12 in monomer B and 12 in monomer D. Comparatively, KKO mostly interacts with monomer B, with a binding site involving 22 residues including Gln9 and Val13-Thr15 (VKT) that are also recognized by 5B9 (Figure 6C and D). Comparatively, KKO bound only 7 amino acids of monomer D, but 3 of these (i.e., Gln9-Cys10 and Thr38) also appeared to contribute to the epitope of 5B9. Five other residues, (i.e., Ala32-Pro34 or AGP and Cys36-Pro37) either in monomer B or monomer D, were also showed to interact with KKO and 5B9, respectively (Figure 6D).

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DISCUSSION

In this study, we characterized the first chimeric monoclonal HIT antibody that contains a human Fc fragment, and showed that it behaves similarly to human IgG anti-PF4/H antibodies. 5B9 was obtained after injecting purified hPF4/H complexes into a transgenic mouse able to produce IgG antibodies with a human constant region. Among the clones obtained, only 5B9 produced an IgG1 antibody specific to the PF4/H complex with very low reactivity against PF4 alone.

Two other HIT activating antibodies have previously been developed. The first is KKO, a monoclonal antibody that also mainly binds PF4/H complexes and weakly PF4 alone [15]. However, KKO is a murine $IgG2_k$ antibody, a subclass absent in humans, and therefore does not interact with FcγRs similar to HIT human IgG antibodies. More recently, Asada *et al.* developed a monoclonal murine IgG1 antibody, but it bound PF4/H complex and PF4 alone similarly and induced platelet activation without heparin [24].

Importantly, 5B9 was shown to induce heparin- and FcγRIIA-dependent platelet activation similar to that observed with plasma samples from HIT patients, whether tested with PRP or washed platelets. We recently demonstrated that the platelet response to 5B9 widely varied among donors, particularly when testing PRP [7]. This variability was similar to that observed with human HIT plasma samples and likely explains the relatively low sensitivity of platelet aggregation tests for the diagnosis of HIT [25]. Hence, 5B9 may be used as a positive control in functional assays to determine whether healthy platelet donors are good or poor responders to HIT antibodies. In addition to activating platelets, 5B9 can also activate monocytes inducing TF mRNA synthesis even in the absence of exogenous heparin as previously reported [26, 27], and procoagulant activity, which *in vivo* likely contributes to the risk of thrombosis [11, 28]. 5B9 also activates neutrophils with a significant release of a protease activity, which confirms previous data obtained with HIT plasma samples [29], but the pathophysiological significance of this effect remains undefined.

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When 5B9 was tested in ELISA, the concentration allowing about 75% of maximal binding was 10 µg/mL, although less than 1 µg/mL KKO was enough to achieve a similar binding. This finding suggests a lower affinity of 5B9 for PF4/H complex than KKO. However in functional assays, 20 µg/mL 5B9 were sufficient to induce platelet activation with heparin, but without addition of exogenous PF4. Comparatively, 80 µg/mL KKO were used to induce heparin-dependent platelet activation with 10 µg/mL PF4 [15, 30]. Our *in vivo* experiments also demonstrated that 5B9 induced thrombocytopenia after intravenous injection with heparin in transgenic mice expressing human FcγRIIA and PF4, an effect similar to that obtained with a higher dose of KKO. In addition, thrombin-antithrombin level, validated as a surrogate marker of thrombosis in patients with HIT [31], and of thrombosis in mouse models [32, 33], was significantly higher in mice having received 5B9 than in those injected with KKO.

The class of 5B9 and KKO and the origin of their Fc portion are not the same, these variations probably explaining differences in platelet reactivity to these IgG antibodies *in vitro* and *in vivo*. 5B9 is indeed composed of murine light chains and chimeric heavy chains with a murine variable region and a human IgG1 constant region, and IgG1 is the most frequently represented IgG subclass among human HIT antibodies [4, 34]. In contrast, KKO is a full murine $IgG2b_{k,}$ a class absent in our species, and weakly binds human FcγRIIA. Indeed, the affinity of murine IgG2b for human FcγRIIA receptor is very low (association constants or K_A = 1.2 and 0.9 x 10^5 M^{-1} for 131H and 131R isoforms, respectively) as compared with those of human IgG1 (5.2 and 3.5 x 10^5 M^{-1}) [35]. Moreover, Fc receptors other than FcγRIIA likely contribute to the pathogenic effects of HIT antibodies [14], particularly FcγRIIIA, which does not bind murine IgG2b [36]. In contrast, human IgG1 subclass shows relatively high affinity ($K_A > 10^6$) for this receptor [37] and an association between FcγRIIIA V158F polymorphism and the risk of HIT was previously demonstrated in patients with high titre of anti-PF4/H antibodies [38].

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In clinical practice, anti-PF4 antibodies in HIT patients are detected by different immunoassays based on variable PF4 complexes. 5B9 appeared to react similarly with PF4 bound to heparin (Stago assay) or polyvinyl sulfonate, a non-heparin polyanion (Immucor GTI assay), but its binding to modified PF4 seemed lower when evaluated by the Hyphen BioMed assay. In this assay, PF4 (and likely other heparin-binding antigenic proteins) is provided by a platelet lysate, and binds to heparin immobilized by protamine during the incubation step. Therefore, 5B9 (as well as human HIT antibodies) likely interacts differently with PF4 modified by protamine-bound heparin (i.e., in the Hyphen BioMed assay) and PF4/H or PF4/PVS complexes used as antigens in the other methods. We also showed that 5B9 mainly bound to PF4 modified by heparin, and this binding was strongly reduced by a high concentration of heparin, similar to findings with KKO [15] or most HIT human antibodies [39].

Importantly, 5B9 binding to PF4/H complexes was decreased by human antibodies present in 15/25 HIT plasma samples, but this inhibitory effect was obtained with only 3 of 25 samples containing non-activating human anti-PF4/H antibodies. Hence, human HIT pathogenic antibodies may bind PF4 epitopes that are close to those recognized by 5B9, but different from those targeted by non-platelet-activating antibodies. In similar experiments with KKO, we found no significant inhibition of this antibody binding to PF4/H in the presence of non-pathogenic anti-PF4/H antibodies, but a significant inhibitory effect with 8 of 25 HIT plasma samples. These results agree with those from Cuker *et al* [40], and confirm that the KKO binding site on modified PF4 likely overlaps epitopes recognized by some of the pathogenic antibodies in HIT patients. Several HIT human antibodies also decreased significantly the binding of both 5B9 and KKO to PF4/H complex. Moreover, high concentrations of KKO also strongly inhibited 5B9/PF4 interaction. Accordingly, our predictive docking model suggested that the epitopes of 5B9 and KKO largely overlap. However, differences in these epitopes, notably because 5B9 seems to bind to both B and D monomers whereas KKO mostly interacts with the B monomer [22], could explain some of the variation observed in ELISA competitive assays performed with both antibodies and HIT plasma samples. Several of these residues, including Pro34, Pro37 and the

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sequence Asp7-Gln9 were previously found critical in the epitopes recognized by human HIT antibodies [41] and KKO [42]. Also, the amino-acid region that binds both KKO and 5B9 is likely important to promote heparin-dependent platelet activation and thrombocytopenia. As recently demonstrated, KKO stabilizes the ternary complex KKO/PF4/H [22], and initiates the oligomerization of PF4 tetramers [43]. Our functional studies also strongly suggest that like KKO, 5B9 probably also favours the formation of ultra-large immune complexes on cell surfaces, a prerequisite for potent FcγRIIA-dependent cell activation.

Our docking model was developed based on data obtained after crystallization of KKO Fab with PF4 and fondaparinux, which is a homogeneous molecule. We therefore cannot exclude the possibility that therapeutic glycosaminoglycans other than fondaparinux, are inducing other changes in PF4 antigenicity [44].

In conclusion, 5B9 is the first monoclonal antibody specific to PF4-heparin complex with a human Fc fragment, and could be used as a positive control and standard reagent for laboratory HIT assays. Moreover 5B9 fully mimics the effects of human antibodies and hence could be a useful specific tool when studying FcγR-dependent cell activation in the pathophysiology of HIT.

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ADDENDUM

J. Rollin and C. Kizlik-Masson performed and designed the research, analyzed the data and wrote the paper. Y. Gruel designed the research, analyzed the data, and wrote the paper. S. E. McKenzie and C. Pouplard analyzed the data, and wrote the paper. A. Poupon, Y. Zhou, C. Vayne and G. Champier performed research and analyzed the data.

DISCLOSURE OF CONFLICTS OF INTERESTS

G. Champier is an employee of B Cell Design. Other authors have nothing to disclose.

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Legend to figures

Figure 1: **Binding of 5B9 to PF4/heparin complex. Panel A:** Absorbances obtained with different concentrations of 5B9 added to wells coated with purified PF4 alone or PF4/heparin complex. **Panel B:** Absorbances obtained in ELISA with 5B9 (10 µg/mL) incubated in wells coated with PF4 (5µg/mL) and various concentrations of UFH. **Panel C:** Binding of 5B9 to modified PF4 evaluated by Asserachrom HPIA IgG, Zymutest HIA IgG and Lifecodes PF4 IgG (n=5). Data are mean ± SEM.

Figure 2: **Platelet activation and aggregation induced by 5B9. Panel A:** Serotonin release from washed platelets (n = 10 donors) after incubation with 5B9 or HIT plasma samples and different concentrations of UFH. **Panel B:** Serotonin release induced by 5B9 (20µg/mL) with and without exogenous PF4 (10µg/mL) and IV.3 (10 µg/mL), a monoclonal antibody inhibiting FcγRIIA. **Panel C:** Representative platelet aggregation profile after addition of 5B9 (50, 20 and 10 µg/mL) and heparin (0.5 IU/mL) to platelet-rich plasma (PRP) from one healthy donor. **Panel D**: Representative platelet aggregation induced by 5B9 (20 µg/mL) in the absence or presence of heparin (0.5 or 10 IU/mL) and inhibitory effect of monoclonal IV.3 antibody (10 µg/mL). Data are mean ± SEM.

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Figure 3: **Activation of blood cells with 5B9.**

(**A**) TF mRNA levels (Mean ± SEM) after stimulation of isolated monocytes with 5B9 without or with UFH and purified PF4. (**B**) TF mRNA synthesis and (**C**) shortening of plasma clotting time (Mean ± SEM) after addition of 5B9 (20µg/mL) and heparin to whole blood. (**D**) Mean (± SEM) of Ratio of maximal speed (Vmax) of substrate hydrolysis after stimulation of isolated neutrophils with 5B9 without or with UFH and purified PF4.

p values were calculated using t-test; * = p value < 0.05

Figure 4 : Transgenic HIT mice (hFcgRIIA, hPF4 transgenic, mPF4 knockout) were injected with KKO (30µg/g body weight, n = 3) or 5B9 (15.8µg/g; n = 3) on day 0.

(A) At day 1, 2, and 3, 1400U/kg heparin were injected and 4 hours later, the relative decrease in platelet count compared to day 0 was evaluated. **(B)** Thrombin–antithrombin complex (TAT) levels were measured in ELISA at days 0, 1, 2 and 3.

Data are mean ± SEM. ** = p value < 0,01

Figure 5: Competition enzyme immunoassays. Panel A: Inhibition of 5B9 and KKO binding by human anti-PF4/H antibodies in plasma samples from HIT patients (i.e., pathogenic antibodies, dark symbols) or from asymptomatic patients undergoing cardiac surgery (i.e., non pathogenic antibodies, clear symbols). An inhibition of binding > 20% (horizontal line) was considered as significant. **Panel B:** Correlation between inhibition levels of 5B9 and KKO binding to PF4/H complex by pathogenic HIT antibodies. **Panel C:** Inhibition of 5B9 binding to PF4/H complex with increasing concentrations of KKO. p values were calculated using t-test; ***= p value < 0.001

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Figure 6: Panel A: Docking model of 5B9/PF4 complex. 5B9 is shown in cartoon (VH in dark red, VL in light red), PF4 tetramer is shown on the surface (A chain: light cyan, B chain: cyan, C chain: sky blue, D chain: marine blue), and fondaparinux is in small spheres. **Panel B:** Model of three PF4 tetramers complexed with three 5B9 Fab regions, and bound to heparin. **Panel C:** Comparison of KKO and 5B9 epitopes. PF4 tetramer is shown on the surface (A chain: white, B chain: light grey, C chain: medium grey, D chain: dark grey), and fondaparinux is in small spheres. 5B9 epitope is red, KKO epitope is cyan, and residues common to both epitopes are in yellow. **Panel D:** Sequences of PF4 monomers: residues involved in the interaction with 5B9 and KKO are boxed.

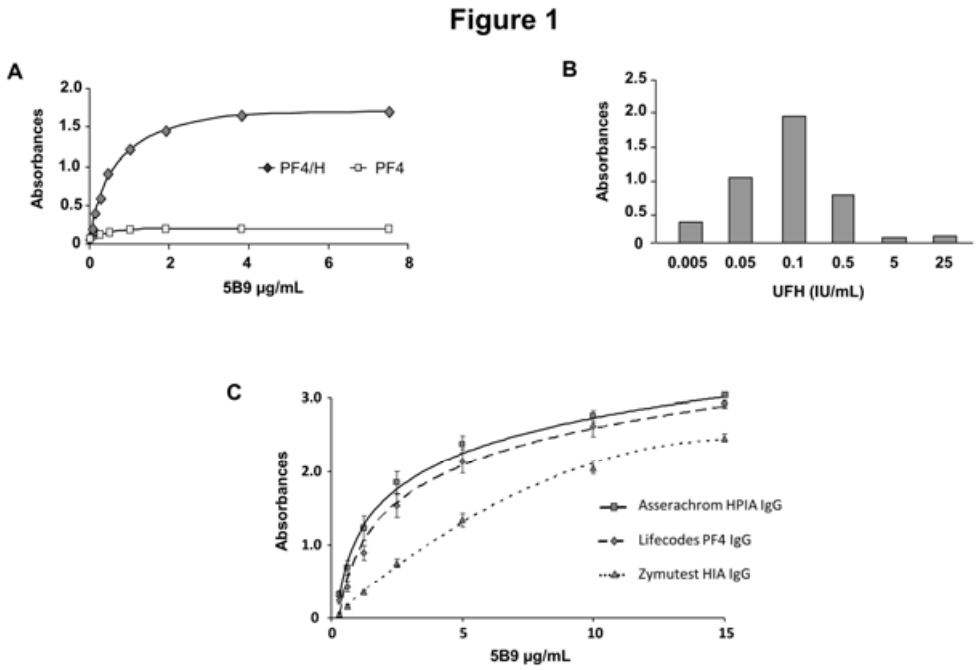

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Figure 2

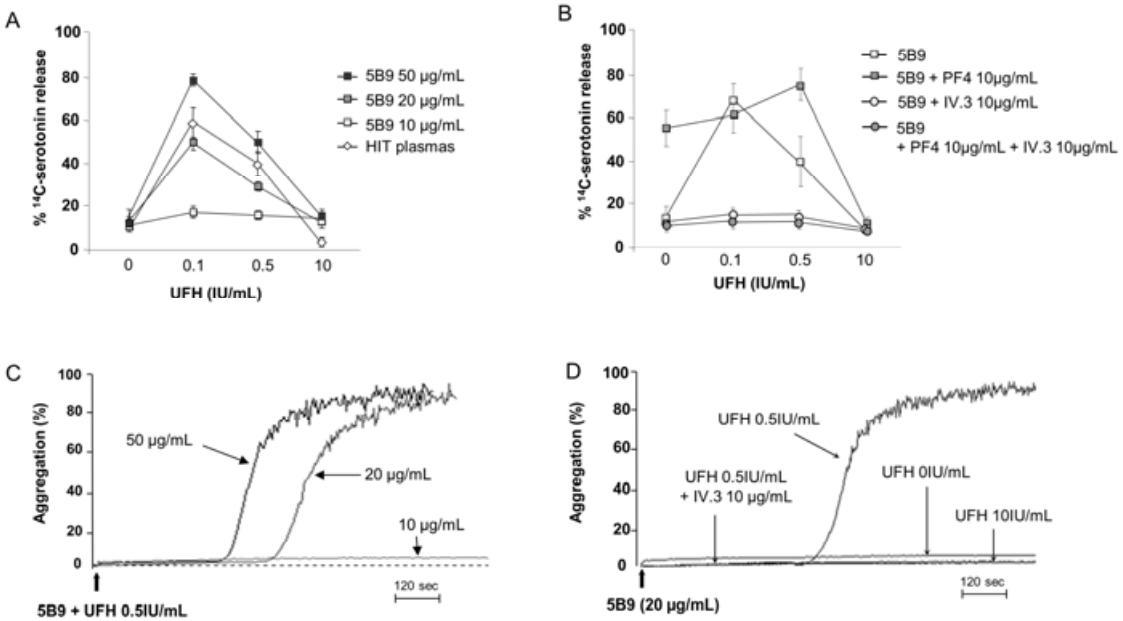

Figure 3

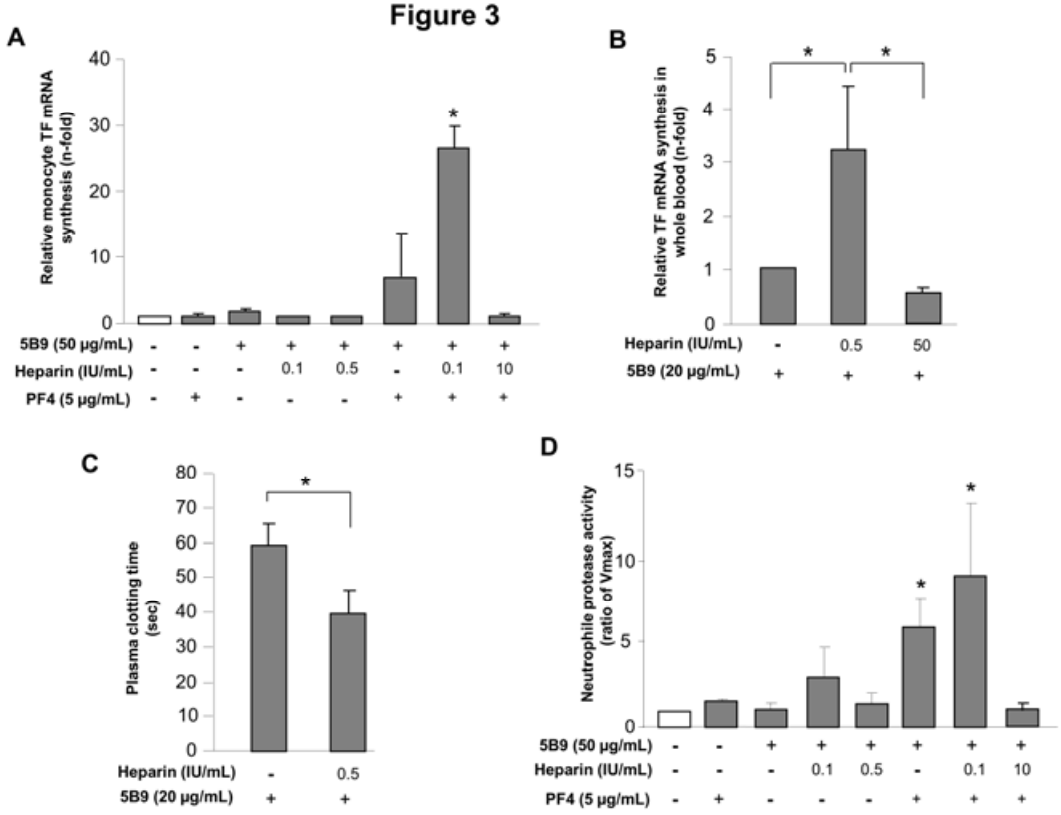

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Figure 4

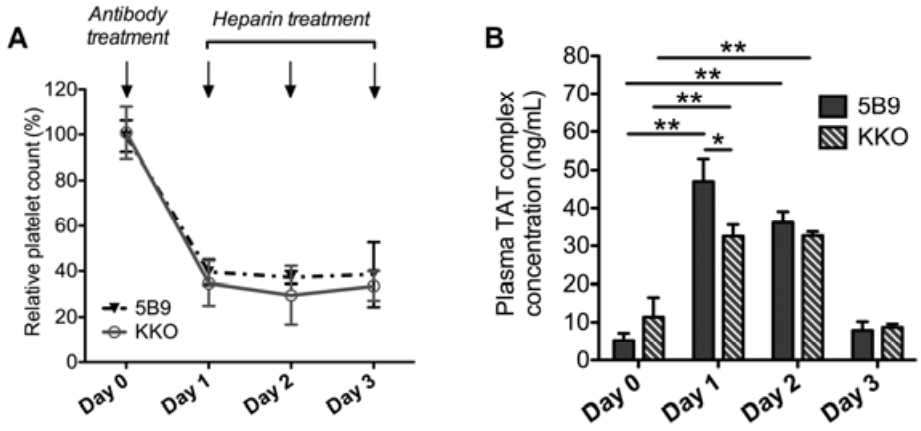

Figure 5

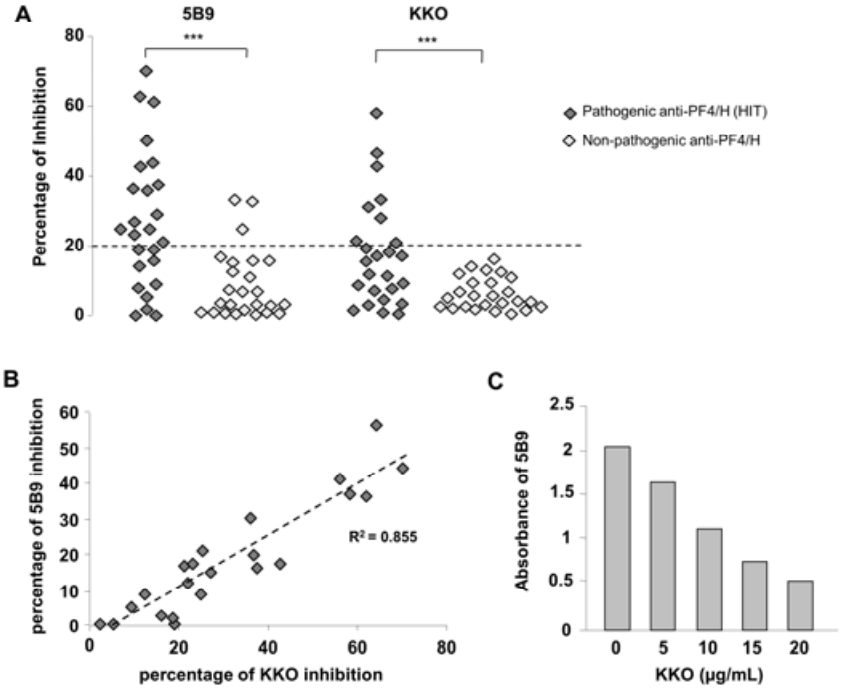

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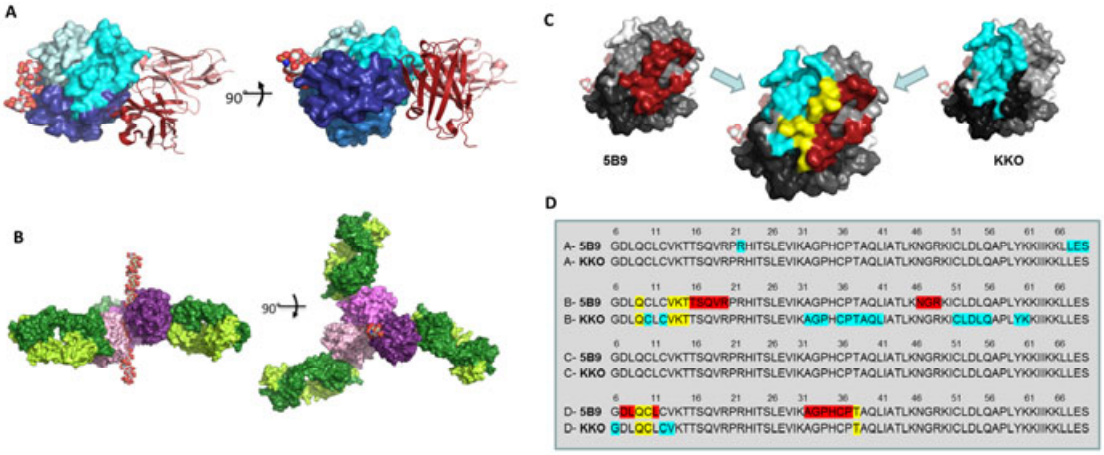
Figure 6



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