REGULAR ARTICLE

blood advances

Polyphosphate/platelet factor 4 complexes can mediate heparin-independent platelet activation in heparin-induced thrombocytopenia

Douglas B. Cines,[1,2] Serge V. Yarovoi,[1] Sergei V. Zaitsev,[3] Tatiana Lebedeva,[1] Lubica Rauova,[4] Mortimer Poncz,[4] Gowthami M. Arepally,[5] Sanjay Khandelwal,[5] Victoria Stepanova,[1] Ann H. Rux,[1] Adam Cuker,[1,2] Cecilia Guo,[6,7] Linnette Mae Ocariza,[8] Richard J. Travers,[9] Stephanie A. Smith,[9] Hugh Kim,[6,7,10] James H. Morrissey,[9] and Edward M. Conway[8]

[1]Department of Pathology and Laboratory Medicine, [2]Department of Medicine, and [3]Department of Pharmacology, Perelman School of Medicine, University of Pennsylvania, Philadelphia, PA; [4]Division of Hematology, Children's Hospital of Philadelphia, Philadelphia, PA; [5]Department of Medicine, Duke University School of Medicine, Durham, NC; [6]Faculty of Dentistry, [7]Faculty of Biochemistry, and [8]Faculty of Medicine and Centre for Blood Research, University of British Columbia, Vancouver, BC, Canada; [9]Department of Biochemistry, University of Illinois at Urbana-Champaign, Urbana, IL; and [10]Faculty of Molecular Biology, University of British Columbia, Vancouver, BC, Canada

Key Points

- Polyphosphates form antigenic complexes with PF4 that are recognized by HIT antibodies.
- Polyphosphate/PF4 complexes released by activated platelets can mediate platelet aggregation by HIT antibodies in the absence of heparin or cell-surface chondroitin sulfate.

Heparin-induced thrombocytopenia (HIT) is a thrombotic disorder initiated by antibodies to complexes between platelet factor 4 (PF4) and heparin. The risk of recurrent thromboembolism persists after heparin is cleared and platelet activation leading to release of PF4 has dissipated. We asked whether antigenic complexes between polyphosphates and PF4 released from activated platelets might intensify or sustain the prothrombotic phenotype of HIT. PF4 forms stable, ultralarge complexes with polyphosphates of various sizes, including those released from platelets, which are recognized by the HIT-like monoclonal KKO, an immunoglobulin G2bκ monoclonal heparin/PF4 binding antibody, and by human HIT antibodies. KKO helps to protect PF4/polyphosphate complexes from degradation by phosphatases. Complement is activated when HIT antibodies bind to PF4/polyphosphate complexes and PF4 reverses the inhibition of complement by polyphosphates. Polyphosphates and PF4 are stored primarily in separate granules in resting platelets, but they colocalize when the cells are activated. Platelets activated by subaggregating doses of thrombin receptor activating peptide release polyphosphates and PF4, which form antigenic complexes that allow KKO to further activate platelets in the absence of heparin and exogenous PF4. These studies suggest that thrombin- or immune complex–mediated release of endogenous antigenic PF4/polyphosphate complexes from platelets may augment the prothrombotic risk of HIT and perpetuate the risk of thrombosis after heparin has been discontinued.

Introduction

Heparin-induced thrombocytopenia (HIT) is a common drug-induced autoimmune disorder characterized by arterial and venous thromboembolism.[1] The thromboembolic complications (TECs) have been attributed in part to activation of platelets by immune complexes composed of platelet factor 4 (PF4), heparin, and immunoglobulin G (IgG) antibodies.[2,3] Thrombus formation is enhanced by monocytes and endothelial cells activated by HIT immune complexes, which are induced to express tissue factor and to generate thrombin, reinforcing immune-mediated platelet activation and procoagulant pathways.[4-6]

Submitted 26 August 2016; accepted 3 October 2016. DOI 10.1182/bloodadvances.2016000877.

The full-text version of this article contains a data supplement.
© 2016 by The American Society of Hematology

Recurrent TECs can occur even in the presence of thrombin and factor Xa inhibitors given in doses that predispose to bleeding,[7,8] and the risk of recurrent thrombosis can extend for weeks after heparin therapy has been stopped.[9] Heparin[10] and PF4[11] are cleared from the circulation and catabolized[12,13] within hours and heparin/PF4 complexes are endocytosed by monocytes and delivered to lysosomes in a similar time frame.[14] Circulating antigenic complexes would be expected to fall soon after the cessation of platelet activation. Further, inhibition of thrombin occurs within hours after institution of antithrombotic therapy. Anti–heparin-PF4 antibody can persist for many months,[15] but these antibodies rarely cause TECs in the absence of PF4 or heparin.[16,17] Therefore, the basis for the severe and protracted prothrombotic state is incompletely defined. Together, these findings suggest that HIT immune complexes and thrombin might initiate additional and as-yet unrecognized host responses that exacerbate and perpetuate the risk of TECs.

One clue toward identifying the pathways that might predispose to delayed thrombotic complications in patients with HIT is the finding that PF4 forms antigenic complexes with a variety of polyanions, including glycosaminoglycans, sulfated anticoagulants, lipid A from gram-negative bacteria, RNA, and inorganic polyphosphates (polyPs).[2,4,18-22] PolyPs are highly anionic linear polymers of orthophosphate linked by phosphoanhydride bonds.[23-25] Although found in all mammalian cells, polyPs are present in the dense granules of human platelets at millimolar concentrations and are released following activation.[26,27] In platelets, polyP polymers range in length from ~60 to 120 orthophosphate units,[26-30] and concentrations may exceed 1 to 3 μM in platelet-rich thrombi.[27] PolyPs provide an anionic surface to assemble factor XII, prekallikrein, and high-molecular-weight kininogen and trigger contact activation of coagulation.[31] PolyPs are prothrombotic and pro-inflammatory in in vivo mouse models[27,32] by affecting multiple steps in the coagulation cascade.[27,33-39] PolyPs also dampen activation of the complement system by interfering with assembly of the terminal membrane attack complex[40] and by binding to and potentiating the activity of C1-esterase inhibitor, which helps to control initiation of the classical pathway[41]; thus, changing the function of polyPs could modulate their participation in hemostatic and inflammatory pathways.

The biological effects of PF4 on the prothrombotic and pro-inflammatory effects of polyPs have not been fully investigated to our knowledge. Here we characterize the biophysical, antigenic, and platelet-activating properties of PF4/polyP complexes and ask whether activated platelets can generate endogenous polyP-containing antigenic complexes capable of exacerbating and perhaps perpetuating HIT in the absence of exogenous PF4 and heparin.

Methods

Materials

The following were purchased from commercial sources: thrombin receptor agonist peptide (TRAP), prostaglandin E1 (PGE1), and chondroitinase ABC from *Proteus vulgaris* (Sigma); calf intestinal alkaline phosphatase (CIP; New England Biolabs, Ipswich, MA); unfractionated heparin (UFH; Becton-Dickenson, Franklin Lakes, NJ); Hanks balanced salt solution (HBSS) and phosphate-buffered saline (Invitrogen/Life Technologies and GIBCO-Life Sciences, Grand Island, MI); gelatin veronal buffer, normal human serum (NHS) and human complement factors (Complement Technology, Inc., Tyler, TX); 96-well microplates (Corning, Amsterdam, Netherlands); 2,2′-Azino-bis(3-ethylbenzthiazoline-6-sulfonic acid) (Roche Diagnostics, Indianapolis, IN); 0.22-μM filters (Millipore); plastic cuvettes (Fisher); horseradish peroxidase (HRP)-conjugated goat anti-human IgG Fc and HRP-conjugated goat anti-mouse IgG Fc antibodies (Jackson ImmunoResearch; West Grove, PA); HRP-conjugated goat anti-human C3 antibody (MP Biomedicals; Santa Ana, CA) that recognizes native, hydrolyzed C3b, iC3b, C3c, and C3d; and chicken erythrocytes (Colorado Serum Company, Denver, CO). CIP was buffer exchanged using Micro Bio-Spin Columns with Bio-Gel P-6 (Bio-Rad**)**. Human PF4 was expressed in Schneider 2 cells (Invitrogen, Carlsbad, CA)[42] and was purified and characterized as described.[2,43] KKO, an IgG2bκ monoclonal heparin/PF4 binding antibody, and RTO, an isotype-matched monoclonal antibody that binds PF4 in the absence of heparin, were purified from hybridoma cell media.[44] TRA purified from hybridoma media PFHM-II (Gibco) and MOPC 141 (Sigma) is an isotype-matched murine monoclonal IgG control.

Polyphosphates

Heterogeneous, size-fractionated polyP preparations containing an estimated average of 14, 60, or 130 phosphate monomers ($NaPO_3$) (designated P_{14}, P_{60}, and P_{130}) were the kind gift of Toshikazu Shiba (Regenetiss Inc., Tokyo). All other polyP polymers were prepared by differential isopropanol precipitation of long chain polyP as previously described.[36] Their integrity was confirmed by electrophoresis on a 10% tris(hydroxymethyl)aminomethane-Borate-EDTA-urea gel, stained with 0.05% (wt/vol) toluidine blue for 20 minutes, followed by destaining overnight in water (not shown). Quantification was determined using the malachite green assay.[36] Final concentrations of polyPs are expressed in terms of monophosphate units. In some experiments, the effect of PF4 on the susceptibility of phosphates to digestion was studied (see the supplemental Methods for details).

Preparation of biotinylated PPXbd and PPX1

A plasmid containing the yeast exopolyphosphatase (PPX1) gene was a gift from Adolfo Saiardi, University College, London, United Kingdom. Biotinylated polyP binding domain (PPXbd) and 6xHis-tagged PPX1 were prepared as previously reported[41] (see the supplemental Methods for details).

Hemolytic assays

Chicken red blood cells (cRBCs) (3.3×10^8 cells/mL) were diluted in gelatin veranol buffer containing 10 mM EDTA to prevent upstream complement activation and generation of endogenous C5b,6. Hemolysis was initiated by adding purified C5b,6 and varying concentrations of PF4 and/or polyP for 30 minutes in the presence of 2% NHS as the source of C7, C8, and C9. Cells were pelleted at 600*g* and lysis, reflected by hemoglobin in the supernatant, was quantified by measuring absorbance at 405 nm. Percent lysis is expressed relative to 100% lysis with H_2O. The concentration of C5b,6 was determined in pilot studies to yield ~75% lysis at 30 minutes.

Immunolocalization of PF4 and polyP in platelets

Platelets were isolated from citrated platelet-rich plasma (PRP) by centrifugation at 800*g* followed by gradual deceleration. The platelet pellet was washed twice with 140 mM NaCl, 5 mM KCl, 12 mM trisodium citrate, 10 mM glucose, 12.5 mM sucrose, pH 6.0, and 1 μM PGE1 (Sigma, Oakville, Canada), resuspended in 10 mM *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid (HEPES), pH 7.4, and apyrase (Sigma) and allowed to rest for 30 minutes at 37°C.

Resting platelets were activated with the phorbol ester 12-myristate acetate (PMA; 100 nM) or with the calcium ionophore A23187 (1 μM) and plated onto glass coverslips, fixed immediately with 2% (vol/vol) paraformaldehyde, and permeabilized with 0.1% (vol/vol) Triton-X-100; unreactive sites were blocked with 1% (wt/vol) bovine serum albumin (BSA). Anti-PF4 antibody (Abcam, Cambridge, MA) was added for 1 hour at room temperature (RT) followed by fluorescein isothiocyanate (FITC)-conjugated secondary antibody for 1 hour. PolyPs were labeled with 40 μg/mL biotinylated PPXbd and 10 μg/mL tetramethylrhodamine isothiocyanate–conjugated streptavidin (DyLight, Life Technologies, Grand Island, NY). Confocal imaging was accomplished with a Nikon $C2^{+}$ confocal microscope; images were processed with NIS-Elements software and captured and processed using a Zeiss spinning disk confocal microscope and SlideBook software (Intelligent Imaging Innovations, Denver, CO). Confocal images supplied as grayscale were colorized in green or red using Adobe Photoshop.

Platelet activation: expression of P-selectin and binding of annexin V

Citrated whole blood diluted 1/50 in calcium-containing HEPES, pH 7.5, was incubated with PF4 (10 μg/mL) followed by addition of polyP_{130} (0-30 μM) and KKO (50 μg/mL) for 30 minutes at RT in the presence of allophycocyanin-labeled anti-CD41 and phycoerythrin-labeled anti–P-selectin antibodies (both BD Bioscience). Samples were diluted fivefold with calcium/HEPES buffer containing 5 μL FITC-Annexin V and assessed by flow cytometry (BD Fortessa) within 30 to 60 minutes. Platelets were identified based on their characteristic forward and side scatter profiles and CD41 fluorescence. Expression of P-selectin and binding of annexin V was expressed as the increase in mean fluorescence intensity over control.

Platelet aggregometry

Platelet aggregation was measured using light transmission aggregometry.[45] PRP was prepared by sequential differential centrifugation from citrated whole blood. Platelets were sedimented as described previously, washed once in HBSS supplemented with 0.1% BSA, pH 7.3, in the presence of 5 μM PGE1, and resuspended in HBSS supplemented with 0.1% BSA and fibrinogen (0.1 mg/mL) to a concentration of 250×10^{9}/L.

Five sets of experiments were performed in sequence. First, PRP was incubated with PF4 (10 μg/mL) or PF4 plus UFH (0.1 U/mL), each alone or followed by the addition of KKO or RTO (100 μg). Second, PRP was incubated with PF4 ± predetermined optimal concentrations of polyP_{14}, polyP_{60}, or polyP_{130} alone or followed by addition of KKO. Third, washed platelets were preincubated with chondroitinase ABC (2.5 U/mL) for 30 minutes at 37°C, shown previously to inhibit binding of KKO by 90% to 95%.[2] Fourth, platelets were stimulated with various concentrations of TRAP for 250 seconds to identify the minimum concentration that initiated a first wave but not an irreversible second wave of aggregation (~1 μM). Platelets were then stimulated with this subthreshold concentration of TRAP in the presence of 100 μg KKO and platelet aggregation was followed over the ensuing 250 seconds. Fifth, chondroitinase ABC, CIP (20 or 200 U/mL), PPX1 (2-10 μg/mL), or PPXbd (10-125 μg/mL) was added immediately before adding TRAP and KKO.

Results

Binding of a HIT-like monoclonal antibody to PF4/polyP complexes

We asked whether PF4 and polyPs form complexes capable of binding HIT antibodies as determined by enzyme-linked immunosorbent assay (ELISA). When PF4 was held constant, addition of polyP_{130} caused a concentration-dependent increase in the binding of KKO (Figure 1A), whereas binding of RTO was unaffected and the control monoclonal antibody MOPC1 did not bind under any conditions (data not shown). Binding of KKO to complexes of PF4 (3 μg/mL) and polyP_{130} (3 μM) met or exceeded binding to PF4 and a predetermined optimal concentration of heparin (0.1 U/mL) (Figure 1A). Higher concentrations of polyP_{130} impaired KKO binding to PF4, as is seen when concentrations of heparin exceed the optimal PF4/heparin ratio (Figure 1A).[2] PF4 formed antigenic complexes with polyPs of diverse chain lengths ($\text{polyP}_{14,\ 60,\ 100,\ 130,\ 155,\ \text{and}\ 675}$). Optimal binding was seen at the same molar ratio of PF4 to all polyPs with chain lengths ≥P60 (Figure 1B; other data not shown), whereas slightly higher concentrations of polyP_{14} were required to optimize binding (Figure 1B). At optimal concentrations, UFH (0.1 U/mL) and polyP_{130} (3 μM) increased binding of KKO to PF4 13.7 ± 2.7-fold and 30.9 ± 5.0-fold (mean ± SEM, n = 18), respectively. Addition of CIP to polyP for 30 minutes prevented formation of antigenic complexes but did not disrupt binding of KKO to preformed complexes of PF4 and polyP (Figure 1C). In contrast, when CIP was added before or after polyP was allowed to form complexes with PF4 in buffer containing dithiothreitol (to disrupt intramolecular disulfide bonds in PF4), binding of KKO was almost completely prevented (Figure 1C). These results show that antigen formation requires contact between polyP and PF4 and suggests PF4 partially protects polyP from digestion by phosphatases.

Analysis of PF4/polyP complexes by dynamic light scattering

PF4 (50 μg) and UFH form complexes that are sufficiently large (exceeding 5 nm) to be analyzed using dynamic light scattering (DLS). Therefore, we next used DLS analysis to compare the biophysical properties of complexes between PF4 (50 μg) and polyP_{130} with those formed with UFH. The DLS profiles of PF4/UFH complexes were nearly identical to those previously described[46] (Figure 2A-B). At low concentrations of UFH (0.1 U/mL), PF4 complexes were of moderate size (~220 nm) but with a high polydispersity index (PDI), indicating size heterogeneity. As the concentration of UFH was increased to 1.0 U/mL, which approached theoretic neutrality, the complexes enlarged up to 4500 nm, but heterogeneity persisted. Higher concentrations of UFH (10 U/mL) generated small negatively charged, highly unstable complexes (not shown).

The analogous experiments with polyP_{130} plus PF4 showed similar trends, but with some notable differences (Figure 2A-B). PF4 incubated with 2 μM polyP_{130} formed intermediate-sized complexes (~800 nm) with a high PDI. As the concentration of polyP was increased to 20 μM, complexes enlarged (~2300 nm) but retained a low PDI in clear contrast to UFH. Increasing the concentration of polyP further to 200 μM generated small (~150 nm) particles with a low PDI. Consistent with their biophysical homogeneity, these latter complexes were extremely stable over 72 hours (Figure 2B).

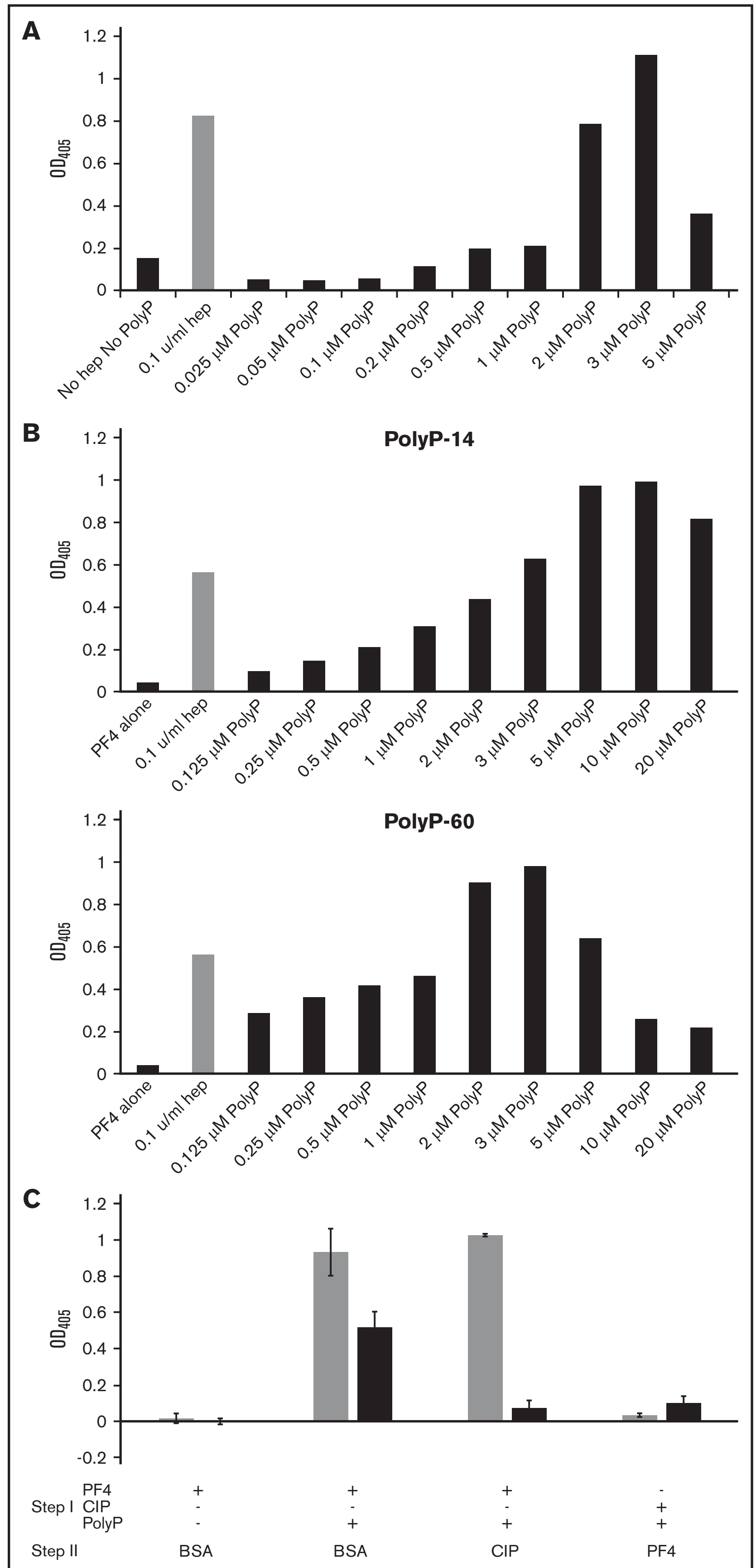


Figure 1. Binding of the HIT-like monoclonal antibody KKO to complexes of PF4 and polyP. (A) Effect of molar ratio of reactants. PF4 (5 µg/mL) was incubated with the indicated concentrations of UFH or polyP$_{130}$ and the binding of KKO was measured by ELISA. Results are representative of 6 such experiments. (B) Effect of chain length on the antigenicity of polyphosphates. PF4 (5 µg/mL) was incubated with the indicated concentrations of polyP$_{14}$ and polyP$_{60}$ and the binding of KKO was measured as in panel A. Binding of KKO to PF4 preincubated with polyP$_{100}$, $_{155}$, and $_{675}$ was essentially superimposable on the results shown for polyP$_{60.}$ (C) PF4 protects polyP from digestion by CIP. In step 1, PF4, PF4/polyP complexes, or polyP + CIP were incubated for 30 minutes in buffer containing (black) or not containing (gray) dithiothreitol. In step 2, CIP, PF4, or BSA was added for an additional 30 minutes, as indicated. Wells were coated with the mixture and the binding of KKO was measured by ELISA as in panel A. hep, heparin; OD$_{405}$, optimal density measured at 405 nm.

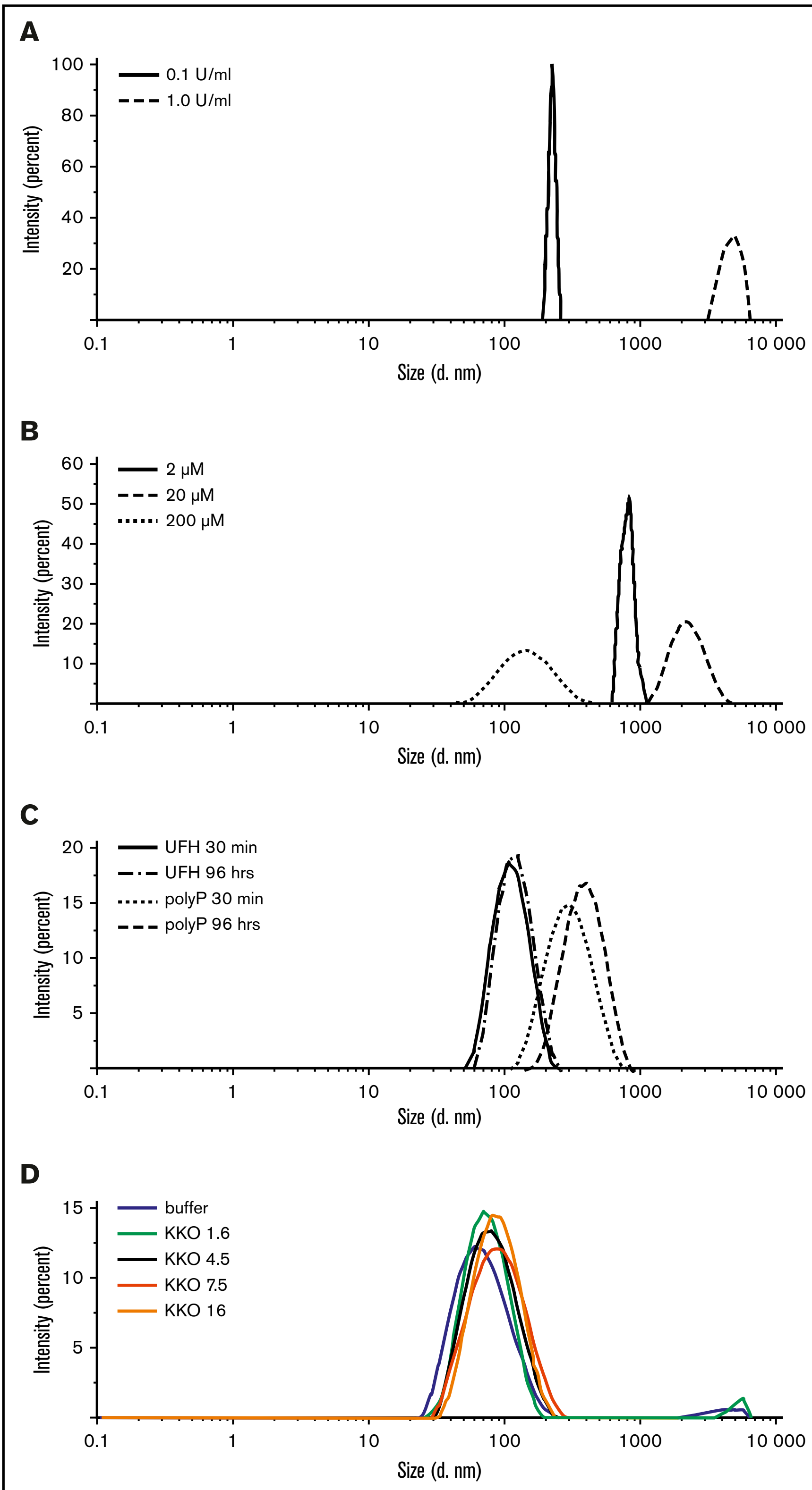
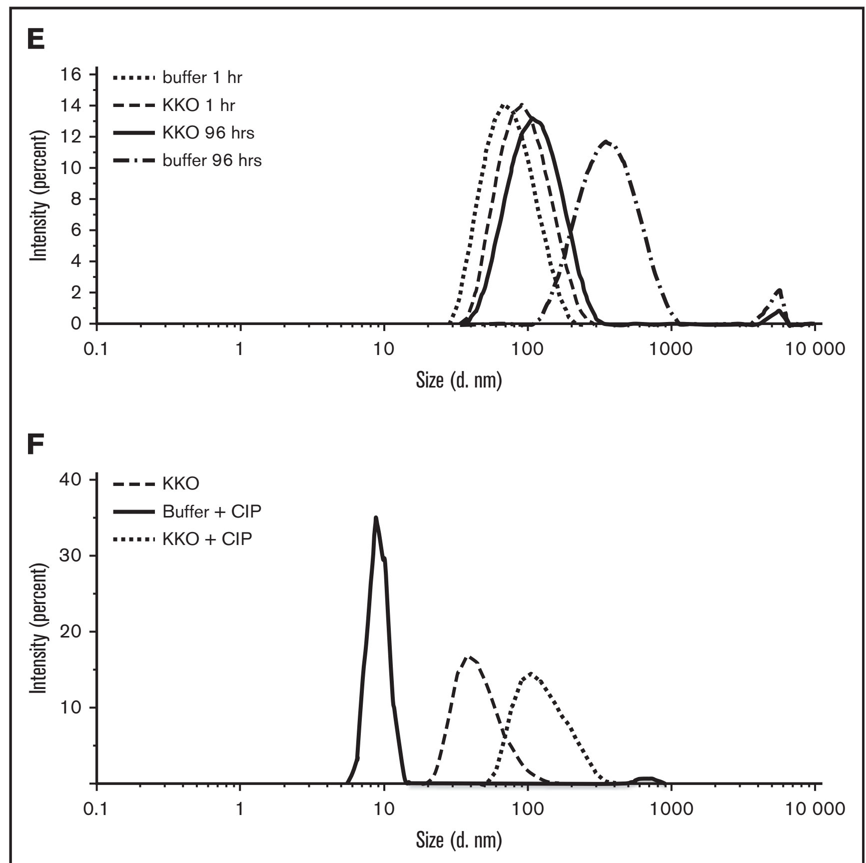


Figure 2. DLS analysis of PF4/UFH and PF4/polyP$_{130}$ complexes. (A) PF4/UFH complexes. PF4 (50 μg/mL) was incubated with a range of concentrations of UFH (0.1 U/mL, 1 U/mL, and 10 U/m) in HBSS for 30 minutes at RT to create theoretically net positively charged, neutral, and negatively charged complexes. The resultant complexes were analyzed for size and distribution by DLS. Complexes formed with 10 U/m heparin were too unstable for analysis. The data shown here and all subsequent figures are representative of 2 to 3 independent experiments. (B) PF4/polyP$_{130}$ complexes. The same experiment was performed with PF4 incubated with 2 μM, 20 μM, and 200 μM polyP$_{130}$, respectively. (C) Intrinsic stability of PF4/UFH and PF4/polyP$_{130}$ complexes. PF4 (5 μg/mL) was incubated with optimal antigenic concentrations of UFH (0.1 U/mL) or polyP$_{130}$ (3 μM) was incubated for 30 minutes at RT and the complexes were analyzed using DLS analysis 30 minutes or 96 hours later. Essentially identical protection was seen at 120 hours. (D) Binding of KKO to PF4/polyP$_{130}$ complexes. Complexes composed of PF4 (5 μg/mL) and polyP$_{130}$ (3 μM) were incubated for 1 hour with buffer alone or with KKO (1.6-16 μg/mL at RT before DLS analysis). (E) Effect of KKO on the intrinsic stability of PF4/polyP$_{130}$ complexes. Complexes were formed between PF4 (5 μg/mL) and polyP$_{130}$ (3 μM) as in panel D; DLS analysis was performed 1 and 120 hours later. (F) Effect of KKO on the susceptibility of PF4/polyP$_{130}$ complexes to phosphatases. Complexes were formed between PF4 (5 μg/mL) and polyP$_{130}$ (3 μM) as described in panel D. The complexes were then incubated with KKO (5 μg) or buffer for 30 minutes at RT; CIP (200 units) or buffer was added for 1 hour and the DLS analysis was repeated. d, diameter.

Figure 2. Continued.



We focused on complexes between 5 μg/mL PF4 and 3 μM polyP$_{130}$, the ratio that optimized antigen formation assessed by ELISA. The biophysical properties of these complexes followed a pattern similar to those between PF4 and UFH. PF4/polyP$_{130}$ complexes formed within 5 minutes, attaining a mean size of 295 nm and a PDI of ~0.227, whereas complexes with 0.1 U/mL UFH had a mean size of 122 nm and a PDI of ~0.208. The complexes increased only slightly in size during the ensuing 96 hours (Figure 2C).

Addition of KKO (7.5 μg/mL) to PF4/polyP$_{130}$ complexes for 1 to 120 hours caused a small stable increase in particle size from ~70 nm to ~90 nm (consistent with binding of multiple IgG antibody molecules) and lowered the PDI to ~0.130, excluding nonspecific particle agglutination through crosslinking PF4 (Figure 2D). In contrast to the time-dependent enlargement in PF4/polyP$_{130}$ complexes, complexes preincubated with KKO maintained their size (~70-100 nm) and PDI for up to 120 hours, indicating enhanced stability (Figure 2E).

In theory, complexes between PF4 and polyP$_{130}$ should be susceptible to degradation by plasma phosphatases unless "protected" by antibody; therefore, we examined degradation of preformed PF4/polyP$_{130}$ complexes in the absence of or following addition of KKO. Complexes were incubated with buffer or KKO for 1 hour and DLS analysis was performed. CIP (200 U/mL) was then added for 2 hours at 37°C and the DLS analysis was repeated. PF4/polyP$_{130}$ complexes were almost totally degraded by CIP in the absence of antibody (average size ~10 nM), whereas complexes preincubated with KKO increased slightly in size from ~70 nm to ~100 nm and showed minimal increase in polydispersity following addition of CIP (Figure 2F). The isotype control monoclonal antibody TRA had no effect on complex size, stability at 72 hours, or susceptibility to phosphatases. These data suggest that KKO may provide additional protection to PF4/polyP$_{130}$ complexes from phosphatases present in biologic milieus.

Endocytosis of PF4/polyP complexes by monocytes

Monocytes endocytose ultralarge complexes of PF4/heparin in a heparin-dependent manner.[46] To determine if PF4/polyP$_{130}$ ultralarge complexes are of sufficient size and stability to be processed similarly, monocytes were incubated for 24 hours with AF647-labeled PF4 (25 μg/mL) alone or with 10 to 100 μM polyP$_{130}$ and uptake was visualized by confocal microscopy. Uptake of PF4 alone was negligible (Figure 3A), whereas endocytosis of PF4/polyP$_{130}$ complexes was readily evident (Figure 3A). Endocytosis of the complexes was concentration dependent, reaching a maximum at 50 μM polyP$_{130}$ (Figure 3B).

PF4 prevents polyP$_{130}$-mediated suppression of complement

We previously showed that polyPs suppress the terminal pathway of complement activation in a concentration-dependent manner.[40] To examine the effect of PF4 on this property, exogenous purified C5b,6 (1250 pM) was added to cRBCs to achieve ~75% lysis after 30 minutes. PF4 alone (1-200 μg/mL) had essentially no effect on lysis (not shown). PolyP$_{130}$ (50 μM) reduced C5b,6-induced lysis to ~12%, which was used in subsequent experiments. PF4 at concentrations

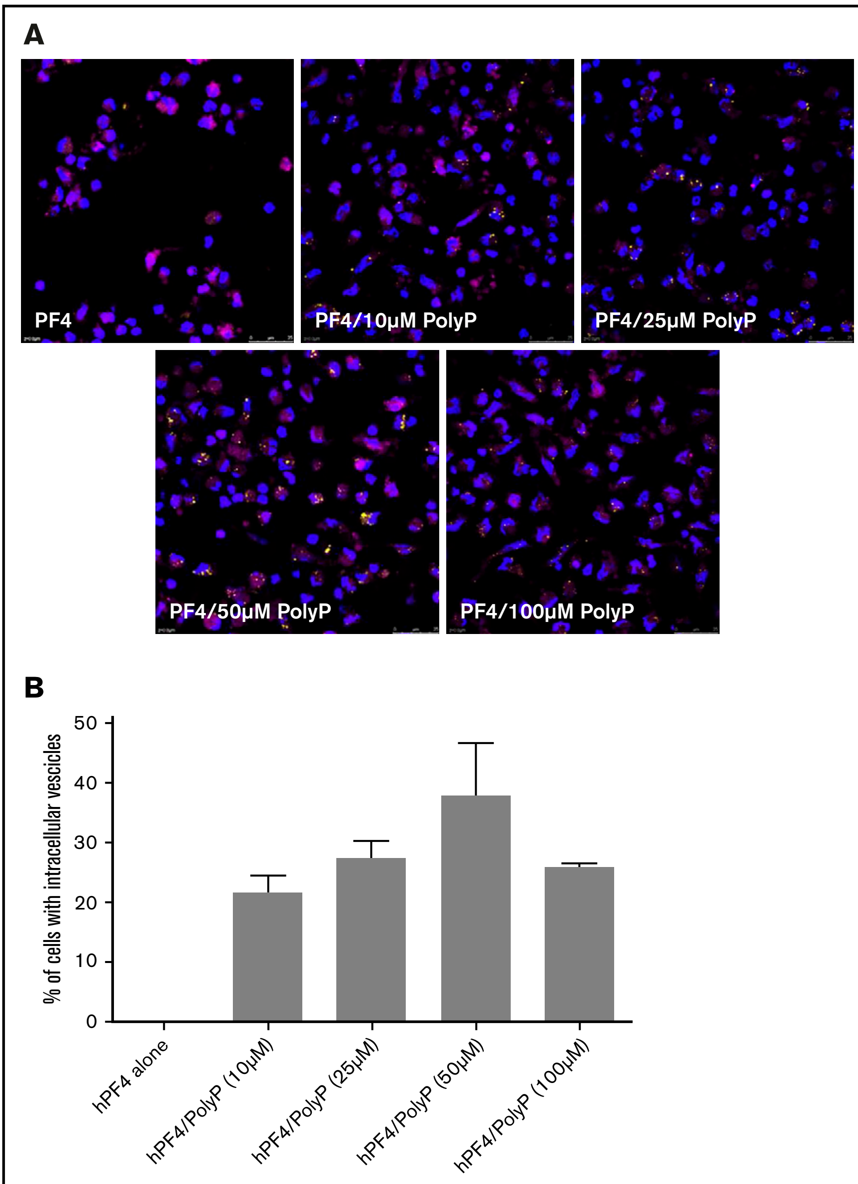


Figure 3. Internalization of PF4/polyP$_{130}$ complexes by monocytes. Peripheral blood mononuclear cells (PBMCs) were incubated with PF4-AF647 (25 μg/mL) and increasing concentrations of polyphosphate (10-100 μM). (A) Confocal images of PF4/polyP$_{130}$ uptake by PBMCs. The monocyte cell surface was stained with CD14-phycoerythrin (pink); DNA was stained with 4′,6-diamidino-2-phenylindole (blue). Internalized PF4/polyP$_{130}$ is identified with PF4-AF647 (yellow). (B) Quantification of PF4/PF4/polyP$_{130}$ uptake by PBMCs. The percentage of CD14^{+} cells with internalized fluorescent intracellular PF4/PF4/polyP$_{130}$-containing vesicles in each microscopic field is shown on y-axis as a function of polyP$_{130}$ concentration (x-axis). Images shown and results in both panels are representative of 3 independent experiments.

above ~12 μg/mL dramatically reversed the activity of polyP$_{130}$, which entirely lost its complement inhibitory properties (Figure 4A), whereas monophosphates had no effect (not shown). Moreover, addition of complexes between PF4 and polyP$_{130}$ to normal plasma activated complement directly, as we previously reported for complexes between PF4 and UFH,[47] with complete loss of activity following preincubation of polyP$_{130}$ with the exophosphatase (Figure 4B).

KKO and human HIT plasmas contain complement-fixing anti-PF4/polyP$_{130}$ antibodies

We also previously reported that HIT antibodies fix complement to platelets and to endothelial cells.[6,45] We asked if the alterations in complement activity by polyP we described might be reflected in complement activation initiated by HIT antibodies. To do so, we incubated KKO with PF4/polyP$_{130}$ or with PF4/UFH complexes in the presence of normal human plasma as a source of complement. Addition of KKO to PF4/polyP$_{130}$ complexes led to fixation of C3 to at least the same extent, as did PF4/UFH complexes (Figure 4C). Plasmas from patients referred for evaluation of HIT and a positive PF4/UFH ELISA[48] showed a clear relationship between IgG binding to each form of PF4/polyanion complexes (Figure 4D top). In the presence of human plasma as a source of complement, C3 deposition was at least as extensive, and perhaps greater, with PF4/polyP$_{130}$ as the target antigen (Figure 4D bottom). In contrast, none of the 8 plasmas with a negative ELISA result showed IgG binding to PF4/polyP$_{130}$ above the normal range, and only 1 caused increased C3 (data not shown).

Platelet activation by exogenous immune complexes composed of polyP, PF4, and KKO

KKO binds to complexes of PF4 released from activated platelets with heparin or cell-surface chondroitin sulfate, which activate platelets through FcRγIIA.[49,50] We asked whether immune complexes composed of polyP, PF4, and KKO activate platelets in an analogous manner. PRP was preincubated with PF4 (10 μg/mL) and optimized

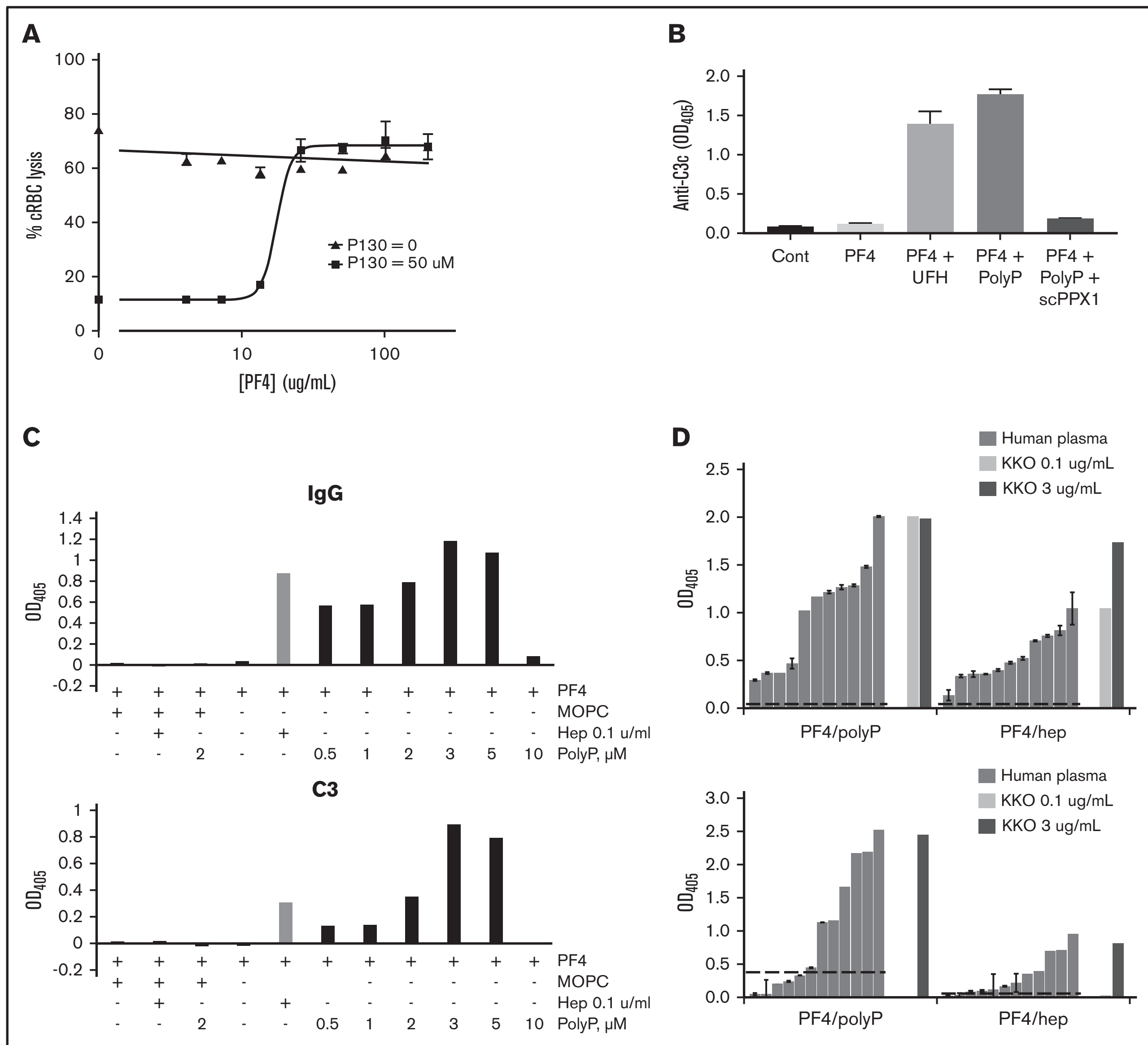


Figure 4. Effect of PF4/polyP130 and immune complexes on activation of complement. (A) Effect of PF4 on inhibition of the terminal pathway of complement activation by polyP$_{130}$. cRBCs were added to 2% NHS as the source of C7, C8, and C9; 10 mM EDTA was added to prevent upstream complement activation and generation of endogenous C5b,6. Lysis of cRBCs was triggered with purified C5b,6 at a predetermined concentration that yielded ~75% lysis (relative to water-induced lysis) after a 30-minute incubation at 37°C in the absence of PF4 or polyP. Varying concentrations of PF4 and/or polyP were added along with the C5b,6 to evaluate the effect on lysis. (B) Activation of complement by PF4/polyP$_{130}$ complexes. PF4 (25 μg/mL) alone or with UFH (0.25 U/mL) or with polyP (50 μM) was added to normal plasma for 1 hour at 37°C or polyP was preincubated with exophosphatase before addition to plasma supplemented with PF4. The complexes were captured on immobilized KKO. Deposition of C3c on PF4/heparin or PF4/polyP complexes was determined by ELISA using anti-C3c antibody. The mean ± standard deviation (SD) for triplicate measurements is shown. (C) Fixation of KKO and C3 to PF4/polyP$_{130}$ complexes. Microtiter wells were coated with PF4 (5 μg/mL) and incubated with the indicated concentrations of polyP$_{130}$. KKO was added and binding of IgG was measured by ELISA as in panel A (top). To another set of wells, a 1:80 dilution of normal human plasma was added as a source of complement, the wells washed, and the binding of anti-human C3 was measured by ELISA as in panel A (bottom). The interrupted line denotes the mean + 1 SD of the normal range. (D) Fixation of C3 to PF4/polyP$_{130}$ complexes by HIT antibodies. Plasma from patients referred for evaluation of HIT was assessed for binding of IgG (top) and C3 (bottom) to PF4/UFH and polyP$_{130}$, as described in Figure 1, using anti-human Fc antibody and anti-human C3 antibody as described in Figure 4B. The interrupted line denotes the mean ± 1 SD of the normal range. cont, control.

concentrations of polyP$_{130}$ (0.5 μM) or polyP$_{14}$ (8 μM) followed by KKO (or RTO as a negative control in the case of UFH). Addition of polyP$_{130}$ along with PF4 and KKO caused platelets to aggregate (Figure 5A). Similarly, addition of polyP$_{130}$ to PF4 mediated KKO-induced platelet activation in whole blood in a dose-dependent manner, assessed by surface expression of P-selectin and binding of annexin V (Figure 5B). The extent of activation followed a bell-shaped curve as seen with UFH,[2] with maximum activation at 3 μM polyP$_{130}$ (Figure 5B). PolyP$_{14}$ and polyP$_{60}$ also mediated platelet aggregation upon addition of PF4 and KKO; the concentration of polyP required was inversely related to chain length, similar to the relationship between chain length and the potency of heparin (Figure 5A).

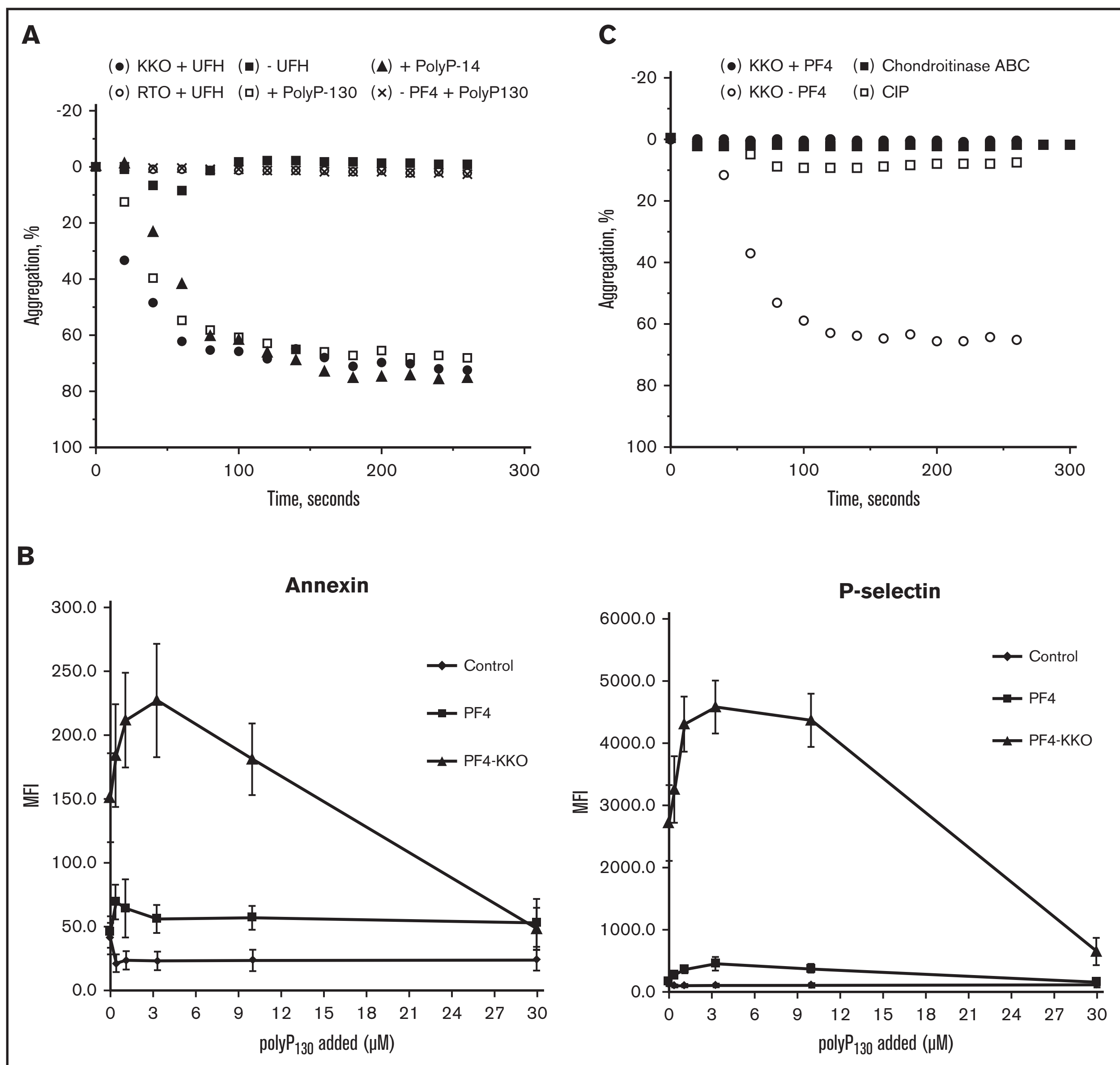


Figure 5. Aggregation of platelets in PRP by KKO plus exogenous PF4/polyP$_{130}$. (A) PRP was incubated with 10 μg/mL PF4 + 0.1 U/mL UFH followed by addition of 100 μg KKO (●) or RTO (○). Platelet activation was assessed by light transmission aggregometry. PF4 + KKO without heparin (■). PRP incubated with 0.5 μM polyP$_{130}$ (□) or 8 μM P14 (▲) followed by KKO as described previously. PF4 + KKO without polyP$_{130}$ (**X**). (B) Activation of platelets in whole blood assessed by flow cytometry. Whole blood was incubated with 10 μg/mL PF4 in the presence of increasing amounts (0-30 μM) of polyP$_{130}$ followed by 20 μg KKO (▲). Whole blood incubated with polyP$_{130}$ (▲) or PF4 and polyP$_{130}$ (■). Left, Annexin binding. Right, Expression of P-selectin shown as geometrical mean of florescence intensity of FITC-labeled Annexin or PE-labeled anti-P selectin binding to platelets. Mean ± standard error of the mean; n = 5. (C) Aggregation of washed platelets by exogenous PF4: effect of chondroitinase and CIP. Washed platelets were incubated with 2 μg/mL of PF4 (○) or buffer alone (●) followed by 100 μg/mL KKO. Preincubation of washed platelets with either chondroitinase ABC (2.5 U/mL, ■) or CIP (20 U/mL, □) abolished aggregation in response to PF4 + KKO.

We next asked if polyP must remain intact and form complexes with PF4 to permit antibody-induced platelet aggregation, as opposed to an indirect effect on platelet function. To do so, we used washed platelets and examined the effect of degradation of polyP by CIP. Platelet aggregation by PF4, polyP$_{130}$, and KKO was abolished in the presence of chondroitinase ABC or CIP (Figure 5C). This suggests that immune complexes containing exogenous polyP$_{130}$, exogenous PF4, and KKO activate platelets; activation requires persistent contact between the polyanion and PF4; and binding of PF4 to cell-surface chondroitin sulfate facilitates formation or stability of the resultant immune complexes.

Formation of endogenous complexes between polyP and PF4

PF4 is stored in platelet α granules, whereas polyPs are found primarily in dense granules (Figure 6A).[26,27] We asked whether PF4 and polyP form complexes potentially capable of mediating HIT antibody binding when platelets are activated. Using confocal

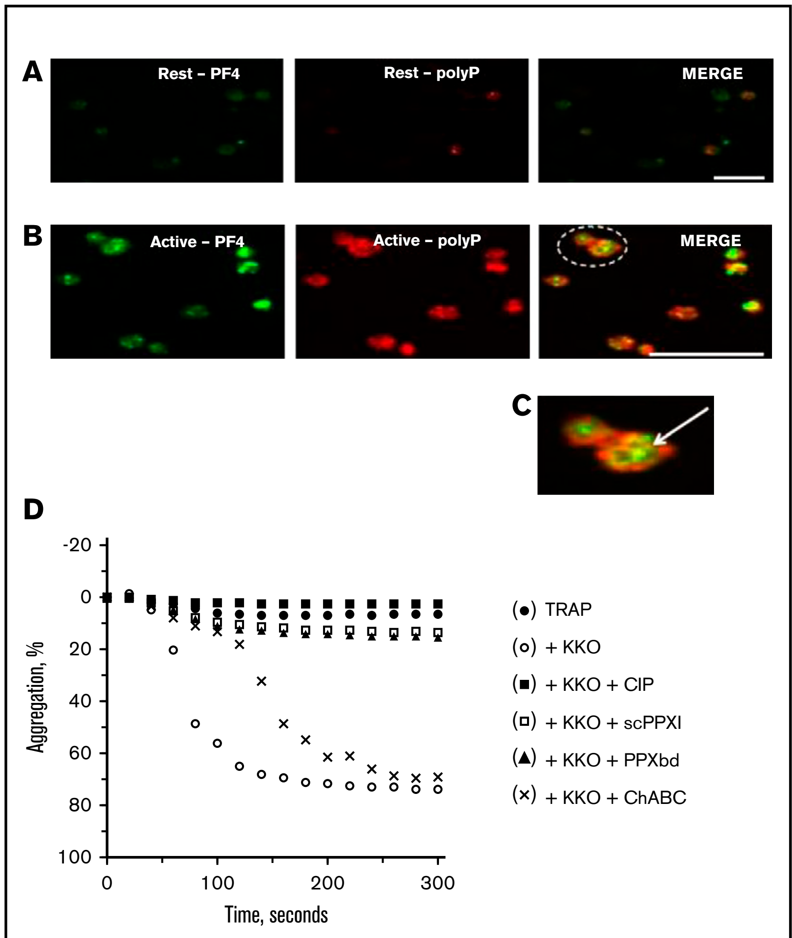


Figure 6. Subcellular localization of PF4 and polyP in resting and stimulated platelets. Human platelets were plated onto glass coverslips in the resting state (A, top) or after <1 minute of activation with 100 nM of the phorbol ester, PMA, and 1 μM of calcium ionophore A23187 (B, middle). After immediate fixation and permeabilization, the platelets were stained to detect PF4 (left, green) and polyP (middle, red) by confocal microscopy. The panels on the right reveal the merged images (yellow). In resting platelets, PF4 and polyP are clearly contained primarily within separate granules (A). After activation, PF4 and polyP coalesce toward the center of the platelets where they colocalize (B). Bars represent 10 μm. C, Higher magnification of the area indicated by the dotted circle in panel B, right panel, depicting centralization and colocalization of PF4 and polyP (arrow). (D) Washed platelets were incubated with chondroitinase ABC, CIP (20 or 200 U/mL), PPX1 (2-10 μg/mL), or PPXbd (10-125 μg/mL), followed immediately by addition of 1 μM TRAP ± 100 μg KKO. Platelet aggregation was followed over the ensuing 250 seconds. The data shown are representative of results of 3 separate experiments.

microscopy and direct immunolocalization, we independently visualized PF4 and polyP. PolyP was detected using biotinylated PPXbd, the specificity of which was confirmed by loss of signal when platelets were incubated with PPX1 (not shown). PF4 and polyP were distributed separately in resting platelets, confirming their localization in different organelles. When platelets were activated by PMA or the calcium ionophore A23817, polyP and PF4 appeared to coalesce and colocalize (Figure 6B). Because the platelets were permeabilized, we could not establish whether this occurred intracellularly and/or on the cell surface.

Platelet activation by exogenous complexes composed of polyP, PF4, and KKO

To address the question of localization, we asked whether PF4 and polyP, when released from activated platelets, form antigenic complexes on the cell surface in the absence of exogenous PF4 or exogenous heparin. To do so, we activated washed platelets with a subthreshold concentration of TRAP (ie, sufficient to initiate a first wave of aggregation but not an irreversible secondary wave) under the assumption that this would permit polyPs and PF4 to form complexes that would be released and expressed on the platelet surface where they could be recognized by KKO. Indeed, priming of platelets with TRAP sensitized them to KKO-induced activation, and activation was abrogated by CIP (Figure 6C). Because CIP can cleave ADP and potentially other platelet agonists, the experiments were repeated in the presence of the specific inhibitor PPXI, which does not degrade ADP. Either degradation of polyP by PPX1 or inhibition of polyP by including PPXbd totally prevented KKO-induced aggregation, whereas chondroitinase ABC minimally delayed, but did not prevent, complete aggregation (Figure 6C). These studies show that exogenous platelet activation is followed by formation of endogenous antigenic complexes capable of forming immune complexes that can support platelet activation in the absence of exogenous PF4, exogenous heparin, and cell-surface GAGs.

Discussion

These studies affirm that PF4 can form antigenic complexes with polyphosphates of diverse sizes ranging from those released by platelets to those released by bacteria[20] (Figure 1). The latter suggests a potential new link between the postulated binding of PF4 to bacteria and the genesis of anti-PF4 antibodies responsible for HIT.[51,52] The complexes were recognized both by the HIT-like monoclonal antibody KKO (Figure 1) that was generated by immunizing mice with PF4/UFH and by human HIT antibodies (Figure 4). PF4/polyP$_{130}$ complexes reach more than 1 μm in size, depending on the ratio of reactants, comparable in size and stability to those formed between PF4 and UFH,[46] but were more homogeneous (Figure 2), and were similarly susceptible to rapid internalization by monocytes (Figure 3). Binding of KKO protected

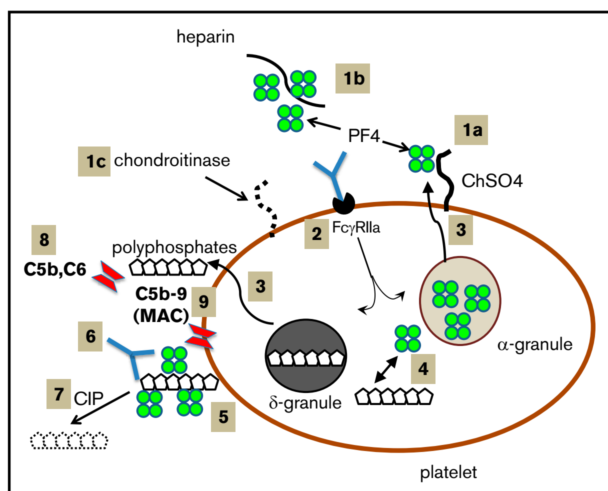


Figure 7. Model of platelet polyphosphates in the pathogenesis of HIT. (1) PF4 released from activated platelets binds to cell surface (1a) and forms complexes with heparin on the cell surface (1a) or in solution (1b). Cell surface complexes do not form when platelets are preincubated with chondroitinase ABC (1c). Binding of KKO to PF4/heparin or PF4/CS activates platelets through FcRγIIA (2). Polyphosphates (pentagon shapes) and PF4, which are found primarily in separate granules within resting platelets, are released (3) and can form antigenic complexes within the cell (4) and on the cell surface (5). PF4/polyP complexes are stabilized by KKO (6), which enhances and perpetuates platelet activation. This pathway is inoperative when platelets are preincubated with CIP (7). Dissociation of the C5bC6 pathway by polyP is blocked by PF4 (8), permitting HIT antibodies to generate C5b-9 membrane attack complexes (MAC) (9) that may further promote platelet activation. Alternatively, complexes of polyP, PF4, and HIT antibody might form in solution and bind directly to platelet FcRγIIA (not pictured). When platelets are exposed to exogenous PF4/heparin, as occurs early in patients with HIT, the polyP-mediated pathway augments the platelet response. When platelets are primed by thrombin, and presumably by other agonists, PF4 comes from within the cell. As plasma levels of heparin fall and thrombin is generated, the polyP pathway may make increasing contributions to exacerbating or sustaining HIT.

these complexes from rapid degradation by phosphatases, such as those present in plasma, suggesting they may endure for sufficient times to initiate or augment immune complex–mediated cell activation in vivo. PF4 neutralized the inhibitory effect of polyphosphates on activation of the terminal complement pathway and immune complexes formed between PF4, polyphosphates, and KKO or human antibodies activated complement and incorporated C3 to at least the same extent as PF4/UFH complexes (Figure 4).

Addition of PF4 and polyphosphates to PRP or whole blood initiated antibody-induced platelet activation (Figure 5). The polyphosphates had to remain intact for platelet activation to proceed, excluding an indirect or nonspecific effect on platelet function. The finding that chondroitinase ABC blocked platelet activation by exogenous PF4/polyphosphate complexes suggests that chondroitin sulfate may assist in the initial binding of PF4 to the cell and formation of the antigenic complexes. Endogenous PF4 and polyphosphates can also mediate antibody-induced platelet activation. We affirmed prior findings that PF4 and polyphosphates localize predominantly in discrete granules (Figure 6). When platelets are activated, these components coalesce, which we interpret as having the potential to form antigenic complexes. In support of this interpretation, platelets activated by a subthreshold concentration of TRAP released sufficient PF4 and polyphosphates to form complexes that mediate antibody-induced platelet activation in the complete absence of exogenous PF4 or exogenous heparin and following enzymatic digestion of cell-surface chondroitin sulfate (Figure 6). PolyPs released by other cell types as a result of inflammation or injury[53,54] might also complex with PF4 and participate in an autostimulatory loop involving antibody formation, platelet activation, perpetuation of thrombocytopenia, and the risk of thrombosis.

These findings suggest a model in which platelets initially activated by PF4/UFH-containing immune complexes in solution or PF4 bound to cell-surface chondroitin sulfate release additional PF4 and polyphosphates (Figure 7). Released PF4 and polyphosphates form antigenic complexes that bind to the cell surface and are capable of mediating platelet activation in the absence of exogenous PF4 or heparin. Polyphosphates released by platelets activated by immune complexes and later by thrombin or other agonists might thereby exacerbate PF4/UFH-mediated platelet activation and potentially perpetuate the prothrombotic state in patients with HIT after heparin has been metabolized and plasma levels of PF4 dissipate as the initiating inflammatory or postoperative state resolves. Additional studies are needed to elucidate how PF4/polyP binds to the platelet. Additional studies are also needed to determine whether polyPs released from bacteria or activated platelets predispose to formation of PF4/heparin antibodies upon exposure to high or low doses of heparin or after surgery[55] or might contribute to the development of "delayed" or "spontaneous HIT."[16,56] Inhibitors of polyphosphates might provide a new approach to interrupting the prothrombotic phenotype in HIT.[22,57-59]

Acknowledgments

The authors thank Connor Paez and Victor Lei for assistance in performing key experiments.

This study was supported by grants from the National Institutes of Health National Heart, Lung, and Blood Institute (grant HL128895) (D.B.C.), (grant HL110860) (D.B.C., L.R., M.P., and G.M.A.), and (grant R01 HL047014) (J.H.M.); Canadian Institutes of Health Research (CIHR) Operating Grant (OMH-134130) and Clinician-Scientist Salary Award (H.K.); University of British Columbia Faculty of Dentistry Postdoctoral fellowship (C.G.); and grants from the CIHR, the Natural Sciences and Engineering Research Council of Canada, and the Canada Foundations for Innovation (E.M.C.). E.M.C. holds a CSL Behring Research Chair and a Tier 1 Canada Research Chair in Endothelial Cell Biology, is an Adjunct Scientist with the Canadian Blood Services, and is a Canadian Venous Thromboembolism Trials and Outcomes Research Investigator.

Authorship

Contribution: R.J.T., S.A.S., and J.H.M. helped generate, characterize, and analyze experiments involving polyphosphates; L.R. and M.P. performed and interpreted the flow cytometry experiments; G.M.A. and S.K. designed and interpreted experiments involving complement activation and endocytosis by monocytes; A.C. identified and recruited patients with heparin-induced thrombocytopenia; H.K. and C.G. performed the confocal microscopy; S.V.Y., S.V.Z., V.S., T.L, and A.H.R. helped design and perform all other experiments; L.M.O. helped to design and implement and E.M.C. oversaw the complement inhibition experiments; D.B.C. is responsible for the overall design of project, integration of the results, initial preparation of the manuscript; and all of the authors read and contributed to preparation of the final manuscript.

Conflict-of-interest disclosure: R.J.T., S.A.S., J.H.M., and E.M.C. are coinventors on patents and patent applications on medical applications of polyphosphate and polyphosphate inhibitors. The remaining authors declare no competing financial interests.

Correspondence: Douglas B. Cines, Departments of Pathology and Laboratory Medicine and Medicine, Perelman School of Medicine, University of Pennsylvania, 513A Stellar-Chance Pavilion, 422 Curie Blvd, Philadelphia, PA 19104; e-mail: dcines@mail.med.upenn.edu.

References

1. Arepally GM, Ortel TL. Clinical practice. Heparin-induced thrombocytopenia. *N Engl J Med*. 2006;355(8):809-817.
2. Rauova L, Zhai L, Kowalska MA, Arepally GM, Cines DB, Poncz M. Role of platelet surface PF4 antigenic complexes in heparin-induced thrombocytopenia pathogenesis: diagnostic and therapeutic implications. *Blood*. 2006;107(6):2346-2353.
3. Rauova L, Poncz M, McKenzie SE, et al. Ultralarge complexes of PF4 and heparin are central to the pathogenesis of heparin-induced thrombocytopenia. *Blood*. 2005;105(1):131-138.
4. Rauova L, Hirsch JD, Greene TK, et al. Monocyte-bound PF4 in the pathogenesis of heparin-induced thrombocytopenia. *Blood*. 2010;116(23):5021-5031.
5. Tutwiler V, Madeeva D, Ahn HS, et al. Platelet transactivation by monocytes promotes thrombosis in heparin-induced thrombocytopenia. *Blood*. 2016;127(4):464-472.
6. Cines DB, Tomaski A, Tannenbaum S. Immune endothelial-cell injury in heparin-associated thrombocytopenia. *N Engl J Med*. 1987;316(10):581-589.
7. Kang M, Alahmadi M, Sawh S, Kovacs MJ, Lazo-Langner A. Fondaparinux for the treatment of suspected heparin-induced thrombocytopenia: a propensity score-matched study. *Blood*. 2015;125(6):924-929.
8. Koster A, Fischer KG, Harder S, Mertzlufft F. The direct thrombin inhibitor argatroban: a review of its use in patients with and without HIT. *Biologics*. 2007;1(2):105-112.
9. Warkentin TE, Kelton JG. A 14-year study of heparin-induced thrombocytopenia. *Am J Med*. 1996;101(5):502-507.
10. Hirsh J, van Aken WG, Gallus AS, Dollery CT, Cade JF, Yung WL. Heparin kinetics in venous thrombosis and pulmonary embolism. *Circulation*. 1976;53(4):691-695.
11. Rao AK, Niewiarowski S, James P, et al. Effect of heparin on the in vivo release and clearance of human platelet factor 4. *Blood*. 1983;61(6):1208-1214.
12. Dawes J, Papper DS. Catabolism of low-dose heparin in man. *Thromb Res*. 1979;14(6):845-860.
13. Rucinski B, Stewart GJ, De Feo PA, Boden G, Niewiarowski S. Uptake and processing of human platelet factor 4 by hepatocytes. *Proc Soc Exp Biol Med*. 1987;186(3):361-367.
14. Joglekar M, Khandelwal S, Cines DB, Poncz M, Rauova L, Arepally GM. Heparin enhances uptake of platelet factor 4/heparin complexes by monocytes and macrophages. *J Thromb Haemost*. 2015;13(8):1416-1427.
15. Warkentin TE, Kelton JG. Temporal aspects of heparin-induced thrombocytopenia. *N Engl J Med*. 2001;344(17):1286-1292.
16. Warkentin TE, Kelton JG. Delayed-onset heparin-induced thrombocytopenia and thrombosis. *Ann Intern Med*. 2001;135(7):502-506.
17. Rice L, Attisha WK, Drexler A, Francis JL. Delayed-onset heparin-induced thrombocytopenia. *Ann Intern Med*. 2002;136(3):210-215.
18. Jaax ME, Krauel K, Marschall T, et al. Complex formation with nucleic acids and aptamers alters the antigenic properties of platelet factor 4. *Blood*. 2013;122(2):272-281.
19. Brandt S, Krauel K, Gottschalk KE, et al. Characterisation of the conformational changes in platelet factor 4 induced by polyanions: towards in vitro prediction of antigenicity. *Thromb Haemost*. 2014;112(1):53-64.
20. Brandt S, Krauel K, Jaax M, et al. Polyphosphates form antigenic complexes with platelet factor 4 (PF4) and enhance PF4-binding to bacteria. *Thromb Haemost*. 2015;114(6):1189-1198.
21. Krauel K, Weber C, Brandt S, et al. Platelet factor 4 binding to lipid A of Gram-negative bacteria exposes PF4/heparin-like epitopes. *Blood*. 2012;120(16):3345-3352.
22. Smith SA, Choi SH, Collins JN, Travers RJ, Cooley BC, Morrissey JH. Inhibition of polyphosphate as a novel strategy for preventing thrombosis and inflammation. *Blood*. 2012;120(26):5103-5110.
23. Harold FM. Inorganic polyphosphates in biology: structure, metabolism, and function. *Bacteriol Rev*. 1966;30(4):772-794.
24. Caen J, Wu Q. Hageman factor, platelets and polyphosphates: early history and recent connection. *J Thromb Haemost*. 2010;8(8):1670-1674.
25. Rao NN, Kornberg A. Inorganic polyphosphate regulates responses of Escherichia coli to nutritional stringencies, environmental stresses and survival in the stationary phase. *Prog Mol Subcell Biol*. 1999;23:183-195.
26. Ruiz FA, Lea CR, Oldfield E, Docampo R. Human platelet dense granules contain polyphosphate and are similar to acidocalcisomes of bacteria and unicellular eukaryotes. *J Biol Chem*. 2004;279(43):44250-44257.
27. Müller F, Mutch NJ, Schenk WA, et al. Platelet polyphosphates are proinflammatory and procoagulant mediators in vivo. *Cell*. 2009;139(6):1143-1156.
28. Kornberg A. Inorganic polyphosphate: a molecule of many functions. *Prog Mol Subcell Biol*. 1999;23:1-18.
29. Brown MR, Kornberg A. Inorganic polyphosphate in the origin and survival of species. *Proc Natl Acad Sci USA*. 2004;101(46):16085-16087.
30. Brown MR, Kornberg A. The long and short of it - polyphosphate, PPK and bacterial survival. *Trends Biochem Sci*. 2008;33(6):284-290.

31. Smith SA, Mutch NJ, Baskar D, Rohloff P, Docampo R, Morrissey JH. Polyphosphate modulates blood coagulation and fibrinolysis. *Proc Natl Acad Sci USA*. 2006;103(4):903-908.

32. Bae JS, Lee W, Rezaie AR. Polyphosphate elicits pro-inflammatory responses that are counteracted by activated protein C in both cellular and animal models. *J Thromb Haemost*. 2012;10(6):1145-1151.

33. Müller F, Renné T. Platelet polyphosphates: the nexus of primary and secondary hemostasis. *Scand J Clin Lab Invest*. 2011;71(2):82-86.

34. Mutch NJ, Engel R, Uitte de Willige S, Philippou H, Ariëns RA. Polyphosphate modifies the fibrin network and down-regulates fibrinolysis by attenuating binding of tPA and plasminogen to fibrin. *Blood*. 2010;115(19):3980-3988.

35. Mutch NJ, Myles T, Leung LL, Morrissey JH. Polyphosphate binds with high affinity to exosite II of thrombin. *J Thromb Haemost*. 2010;8(3):548-555.

36. Smith SA, Choi SH, Davis-Harrison R, et al. Polyphosphate exerts differential effects on blood clotting, depending on polymer size [published correction appears in *Blood.* 2001;(12):3477]. *Blood*. 2010;116(20):4353-4359.

37. Smith SA, Morrissey JH. Polyphosphate as a general procoagulant agent. *J Thromb Haemost*. 2008;6(10):1750-1756.

38. Smith SA, Morrissey JH. Polyphosphate enhances fibrin clot structure. *Blood*. 2008;112(7):2810-2816.

39. van der Meijden PE, Heemskerk JW. Polyphosphates: a link between platelet activation, intrinsic coagulation and inflammation? *Expert Rev Hematol*. 2010;3(3):269-272.

40. Wat JM, Foley JH, Krisinger MJ, et al. Polyphosphate suppresses complement via the terminal pathway. *Blood*. 2014;123(5):768-776.

41. Wijeyewickrema LC, Lameignere E, Hor L, et al. Polyphosphate is a novel cofactor for regulation of complement by a serpin, C1 inhibitor. *Blood*. 2016; 128(13):1766-1776.

42. Schneider I. Cell lines derived from late embryonic stages of Drosophila melanogaster. *J Embryol Exp Morphol*. 1972;27(2):353-365.

43. Sachais BS, Rux AH, Cines DB, et al. Rational design and characterization of platelet factor 4 antagonists for the study of heparin-induced thrombocytopenia. *Blood*. 2012;119(25):5955-5962.

44. Arepally GM, Kamei S, Park KS, et al. Characterization of a murine monoclonal antibody that mimics heparin-induced thrombocytopenia antibodies. *Blood*. 2000;95(5):1533-1540.

45. Cines DB, Kaywin P, Bina M, Tomaski A, Schreiber AD. Heparin-associated thrombocytopenia. *N Engl J Med*. 1980;303(14):788-795.

46. Suvarna S, Espinasse B, Qi R, et al. Determinants of PF4/heparin immunogenicity. *Blood*. 2007;110(13):4253-4260.

47. Khandelwal S, Lee GM, Hester CG, et al. The antigenic complex in HIT binds to B-cells via complement and complement receptor 2 (CD21) [published online ahead of print 13 July 2016]. *Blood*. DOI: 10.1182/blood-2016-04-709634.

48. Cuker A, Arepally G, Crowther MA, et al. The HIT Expert Probability (HEP) Score: a novel pre-test probability model for heparin-induced thrombocytopenia based on broad expert opinion. *J Thromb Haemost*. 2010;8(12):2642-2650.

49. Reilly MP, Taylor SM, Hartman NK, et al. Heparin-induced thrombocytopenia/thrombosis in a transgenic mouse model requires human platelet factor 4 and platelet activation through FcgammaRIIA. *Blood*. 2001;98(8):2442-2447.

50. Kelton JG, Sheridan D, Santos A, et al. Heparin-induced thrombocytopenia: laboratory studies. *Blood*. 1988;72(3):925-930.

51. Krauel K, Pötschke C, Weber C, et al. Platelet factor 4 binds to bacteria, [corrected] inducing antibodies cross-reacting with the major antigen in heparin-induced thrombocytopenia. *Blood*. 2011;117(4):1370-1378.

52. Arman M, Krauel K, Tilley DO, et al. Amplification of bacteria-induced platelet activation is triggered by FcγRIIA, integrin αIIbβ3, and platelet factor 4. *Blood*. 2014;123(20):3166-3174.

53. Moreno-Sanchez D, Hernandez-Ruiz L, Ruiz FA, Docampo R. Polyphosphate is a novel pro-inflammatory regulator of mast cells and is located in acidocalcisomes. *J Biol Chem*. 2012;287(34):28435-28444.

54. Nickel KF, Ronquist G, Langer F, et al. The polyphosphate-factor XII pathway drives coagulation in prostate cancer-associated thrombosis. *Blood*. 2015; 126(11):1379-1389.

55. Bito S, Miyata S, Migita K, et al. Mechanical prophylaxis is a heparin-independent risk for anti-platelet factor 4/heparin antibody formation after orthopedic surgery. *Blood*. 2016;127(8):1036-1043.

56. Warkentin TE, Makris M, Jay RM, Kelton JG. A spontaneous prothrombotic disorder resembling heparin-induced thrombocytopenia. *Am J Med*. 2008; 121(7):632-636.

57. Jain S, Pitoc GA, Holl EK, et al. Nucleic acid scavengers inhibit thrombosis without increasing bleeding. *Proc Natl Acad Sci USA*. 2012;109(32): 12938-12943.

58. Travers RJ, Shenoi RA, Kalathottukaren MT, Kizhakkedathu JN, Morrissey JH. Nontoxic polyphosphate inhibitors reduce thrombosis while sparing hemostasis. *Blood*. 2014;124(22):3183-3190.

59. Zhu S, Travers RJ, Morrissey JH, Diamond SL. FXIa and platelet polyphosphate as therapeutic targets during human blood clotting on collagen/tissue factor surfaces under flow. *Blood*. 2015;126(12):1494-1502.