Supplementary Materials

Monoclonal Gammopathy of Thrombotic/Thrombocytopenic Significance

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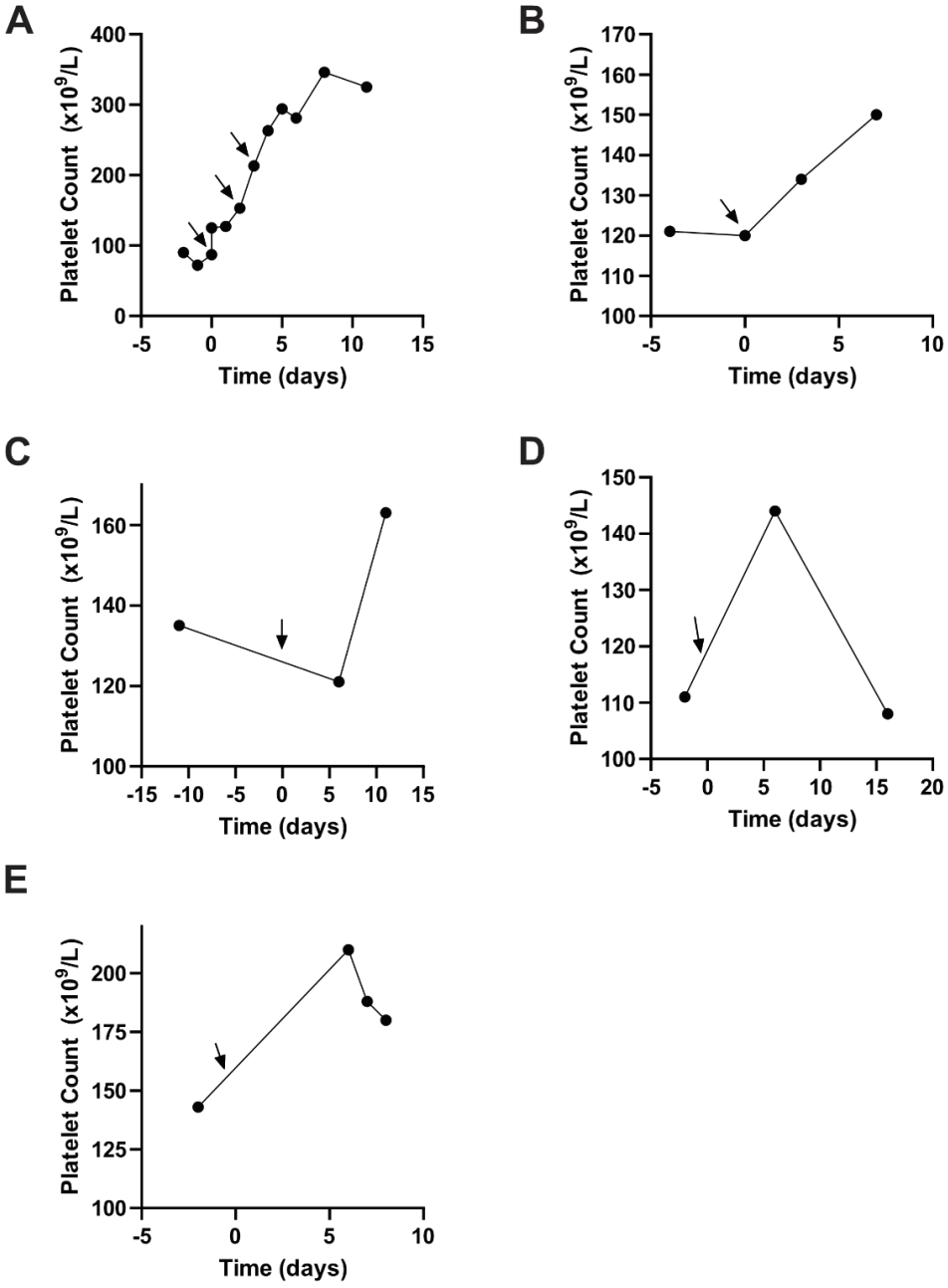
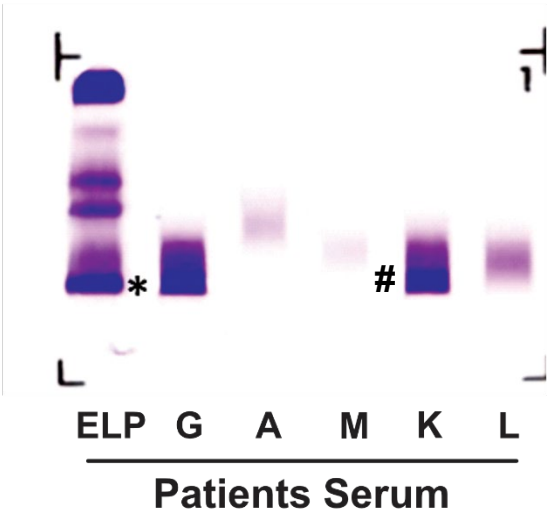
Supplemental Figure S1



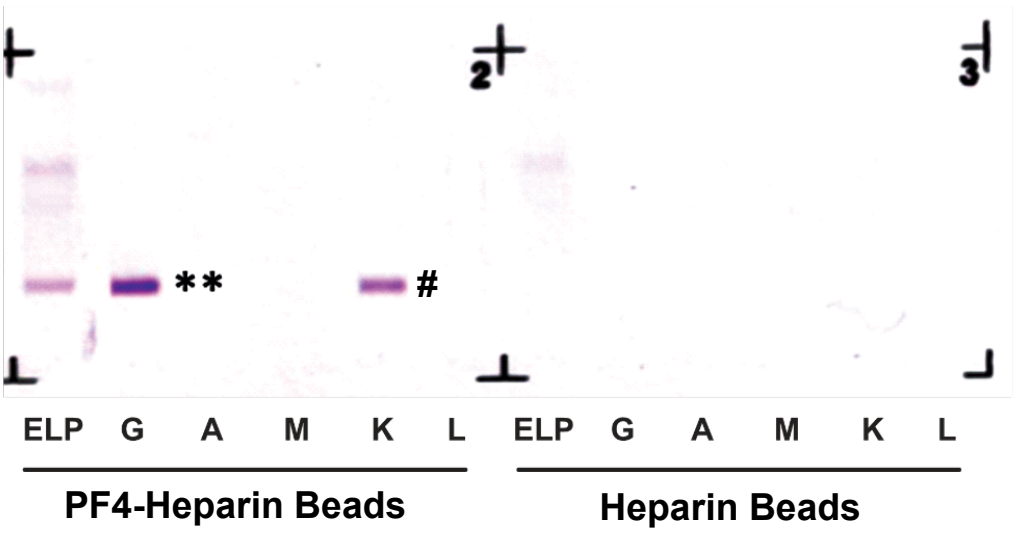
Supplemental Figure S1. Intravenous Immunoglobulin (IVIg) therapy results in an increase in platelet count. (A-E) Platelet counts are displayed on the y-axis and time relative to the day of IVIg infusion is displayed on the x-axis. Five of six IVIg treatments that the patient underwent had platelet counts obtained within 7 days of therapy and are presented here.

Supplemental Figure S2



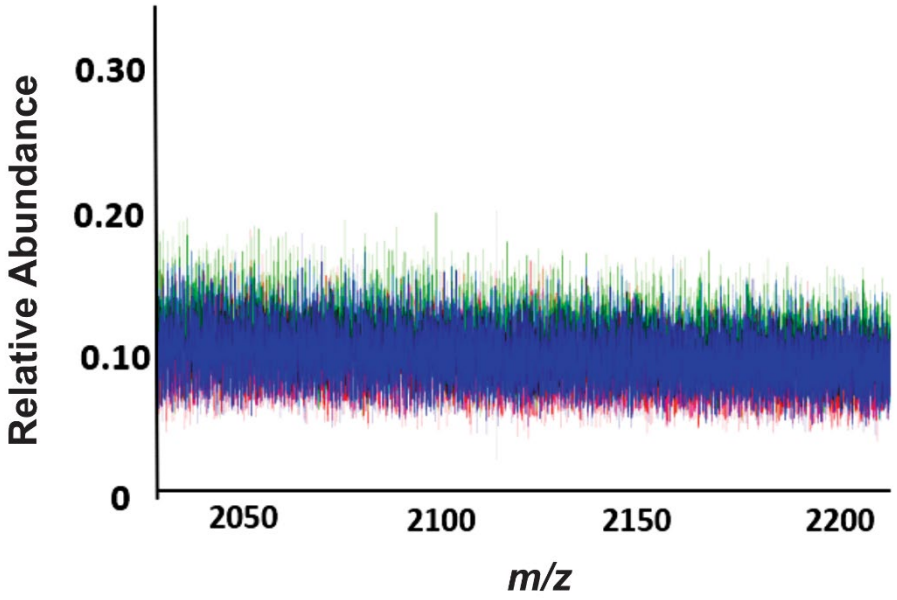
Supplementary Figure S2. Immunofixation electrophoresis of patient serum demonstrates a monoclonal antibody. Patient samples were run by electrophoresis with immunofixation to detect IgG, IgA, IgM, and kappa/lambda light chains. From left to right the gel lanes presented are: Protein fixative (ELP), IgG (G), IgA (A), IgM (M), kappa light chain (*k*), and lambda light chain (*L*). The monoclonal band present is represented by an asterisk (G) and a pound sign (*k*), respectively.

Supplemental Figure S3



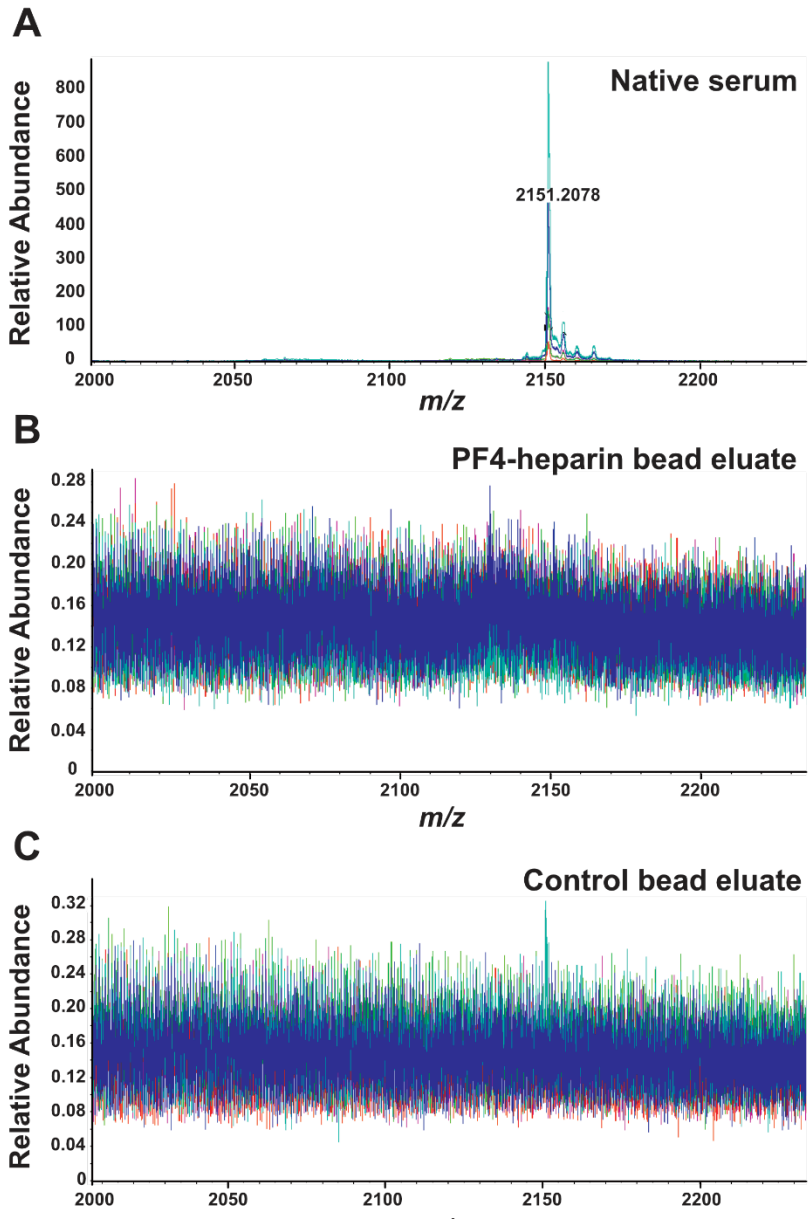
Supplementary Figure S3. Immunofixation electrophoresis of the isolated anti-Platelet Factor 4 antibody demonstrates a monoclonal immunoglobulin. Following PF4/heparin sepharose or heparin sepharose (control) bead-based antibody enrichment, samples were run by electrophoresis with immunofixation to detect IgG, IgA, IgM, and kappa/lambda light chains. From left to right the gel lanes presented are: Protein fixative (ELP), IgG (G), IgA (A), IgM (M), kappa light chain (*k*), and lambda light chain (*L*). The monoclonal band present is represented by asterisks (*G*) and a pound sign (*k*), respectively.

Supplemental Figure S4



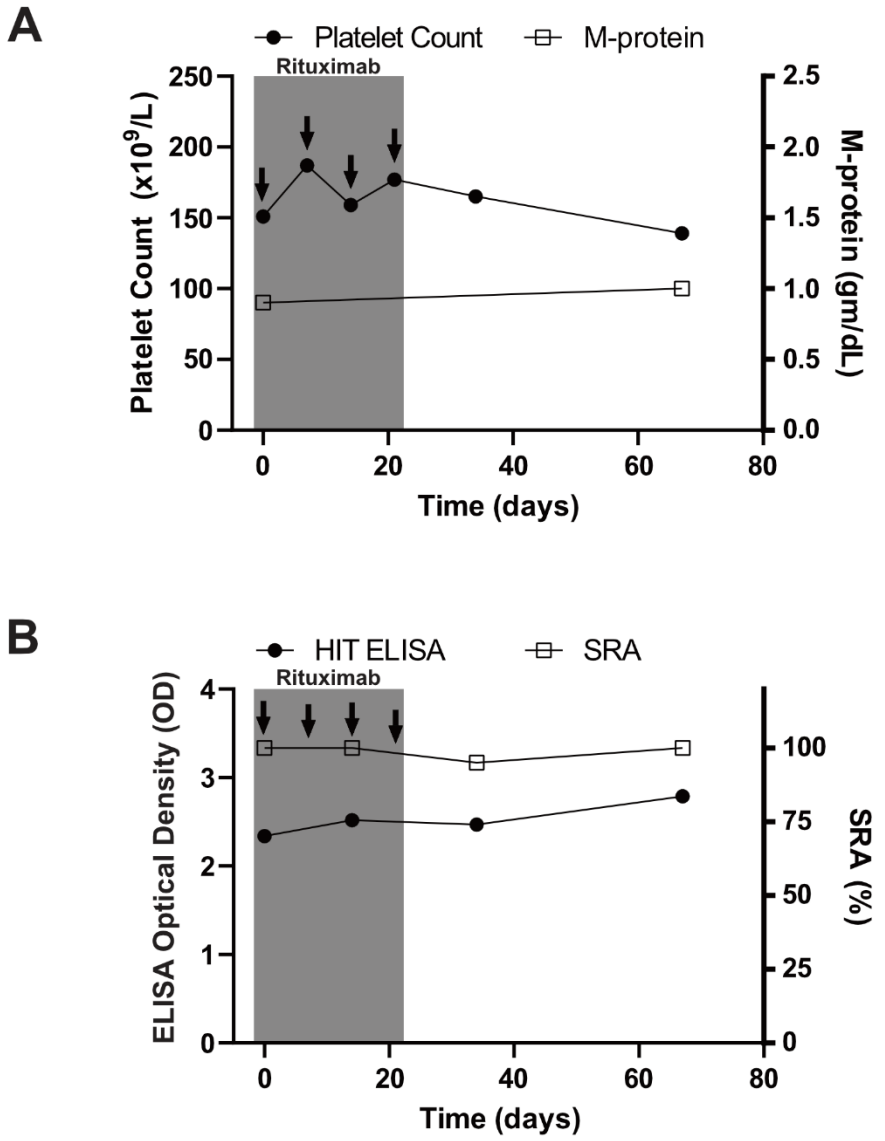
Supplementary Figure S4. The patient's anti-PF4/polyanion monoclonal antibody does not non-specifically bind to heparin sepharose beads. Displayed are LC-ESI-QTOF MS light chain +11 (m/z, mass to charge) distributions of the eluate heparin sepharose beads incubated with patient sample (i.e. no PF4 were added to the beads). In the spectra, green represents the distribution of all *lambda*-containing immunoglobulins (Igs), red represents the +11 m/z distribution of all *kappa*-containing Igs, and blue represents the +11 m/z light chain distribution of *kappa* and *lambda* light chains associated with an IgG heavy chain. The x-axis depicts mass/charge ratios, and the y-axis presents the relative abundance of antibodies identified.

Supplemental Figure S5



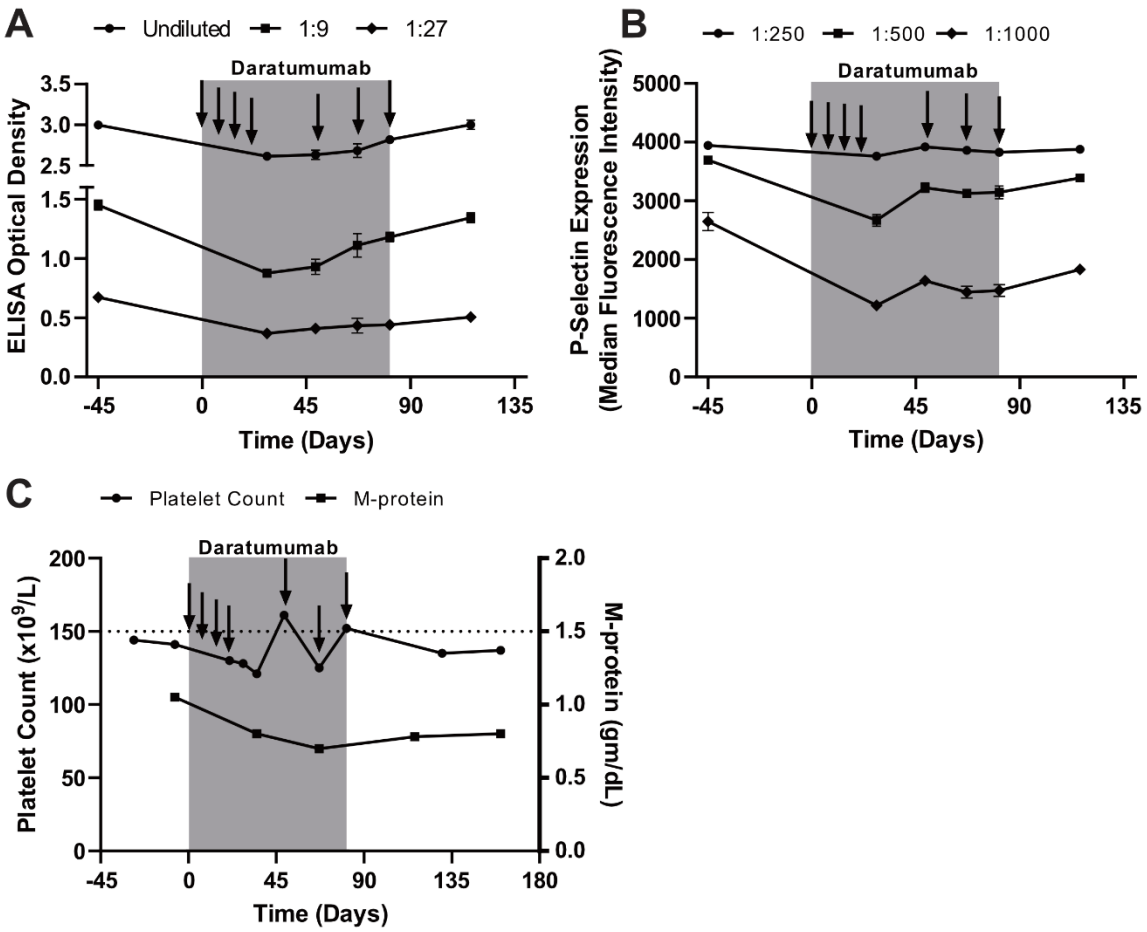
Supplemental Figure 5. Irrelevant monoclonal antibodies do not bind PF4-heparin sepharose beads. Displayed are LC-ESI-QTOF MS light chain +11 (m/z, mass to charge) distributions from (**A**) Native serum from a multiple myeloma patient undergoing therapy (with IgG *lambda* M-protein level of 0.4g/dL), (**B**) Eluate from PF4-heparin sepharose beads incubated with patient serum and (**C**) Eluate from control heparin sepharose beads (i.e. no PF4 was added to the beads) after incubation with patient serum. In the spectra, teal represents the distribution of all *lambda*-containing immunoglobulins (Igs), and blue represents the +11 m/z light chain distribution of *kappa* and *lambda* light chains associated with an IgG heavy chain. The x-axis depicts mass/charge ratios. The y-axis presents the relative abundance of antibodies identified.

Supplemental Figure S6



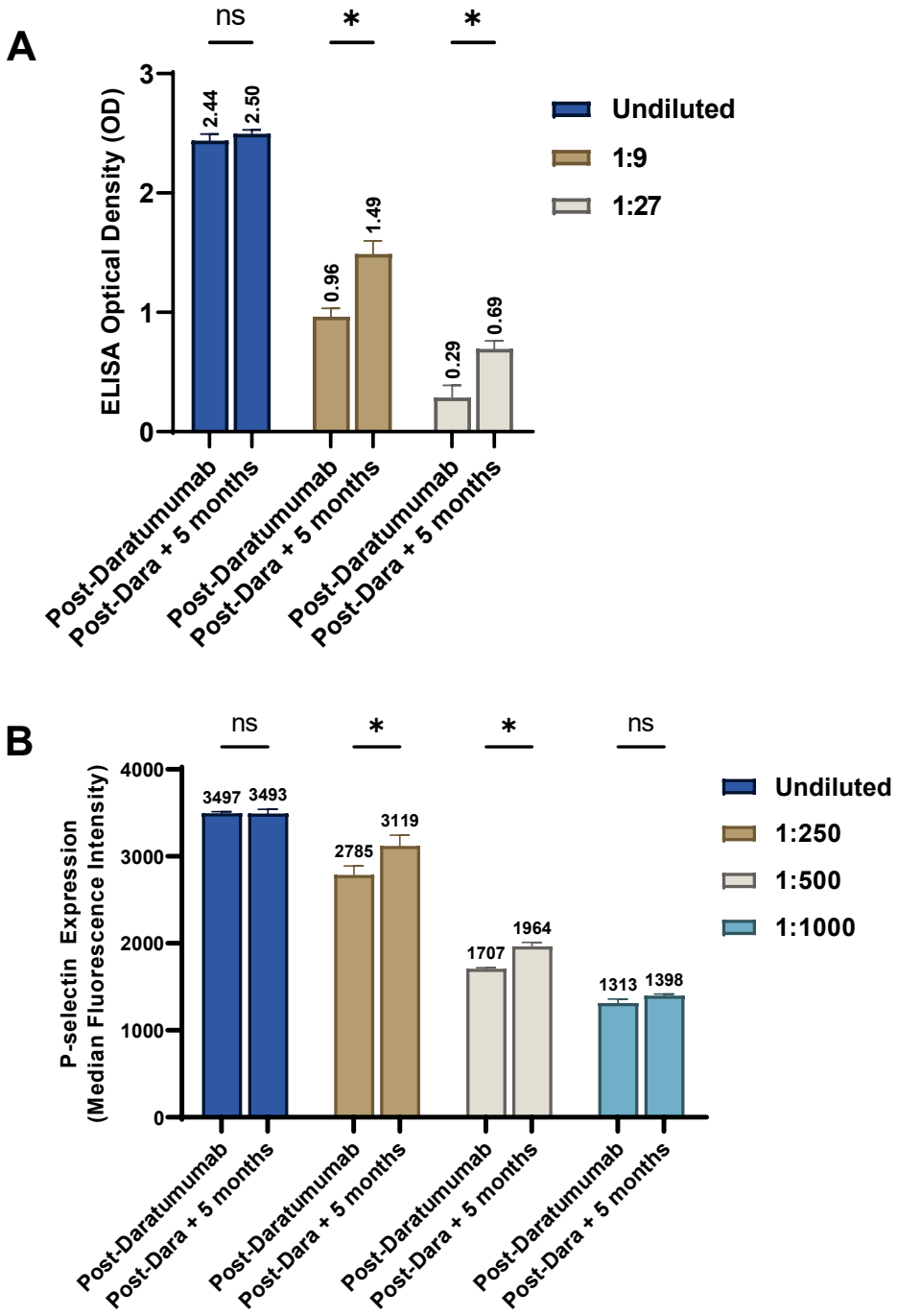
Supplemental Figure S6. Impact of Rituximab treatment on platelet counts, M-protein and HIT serologic testing. (A) Platelet counts (left y-axis; closed circles), M-protein levels (right y-axis; open squares), and time (x-axis) relative to the day of rituximab infusion (black arrows) are displayed. **(B)** The impact of rituximab treatment on anti-PF4/polyanion antibodies was evaluated by HIT ELISA (left y-axis; closed circles) and Serotonin Release Assay (SRA; right y-axis; open squares). Time relative to the start of rituximab treatment is displayed on the x-axis.

Supplemental Figure S7



Supplemental Figure S7. Daratumumab-dexamethasone treatment results in a modest reduction of M-protein level and anti-PF4/polyanion antibody profile. The impact of daratumumab-dexamethasone therapy on **(A)** HIT ELISA reactivity, **(B)** Platelet-activating antibodies, and (**C**) Platelet count (closed circle) and M-protein (closed square) were evaluated. Down-facing black arrows indicate the timing of daratumumab infusion. The dotted line represents the lower limit of the normal platelet reference range.

Supplemental Figure S8.



Supplemental Figure S8. Anti-PF4/polyanion antibody levels increase after cessation of Daratumumab therapy. HIT ELISA reactivity (**A**) and activation of platelet targets in the PEA **(B)** were evaluated in blood samples drawn from the patient shortly after final infusion of daratumumab (~1 month after the 7th infusion; "Post-Daratumumab") and compared to a sample obtained ~5 months after the final infusion ("Post-Dara + 5 months"). Two-way ANOVA was used to compare OD values and P-selectin expression. *p<0.05.

Tables

Table S1. Results of thrombophilia and related testing.

Laboratory Testing[a]	Result	Reference Range
Prothrombin time	10.7 sec	9.3-12.0 sec
Activated partial thromboplastin time	28.6 sec	22.0-30.0 sec
Antithrombin activity	105%	75-125%
Protein C activity	124%	80-150%
Protein S free antigen	76%	75-150%
Activated protein C resistance ratio [b]	3.1	>1.69
Dilute russell viper venom time screen	43.5 sec	0-48.0 sec
Hexagonal phospholipid neutralization	Negative	Negative
Factor VIII assay	180%	50-150%
Beta-2 glycoprotein 1-IgG, IgM, and IgA	<2, 2, and 11	<20 U/mL
Cardiolipin antibodies IgG, and IgM [c]	<2, and 11	<20 GPL/MPL
JAK2 mutational analysis of exons 12 and 14	Negative	Negative
Flow cytometry for paroxysmal nocturnal hemoglobinuria	Negative	Negative

[a] Prothrombin gene G20210A testing was not performed. By the time the patient was evaluated by hematology he had experienced recurrent thrombotic episodes and this testing was not anticipated to change management, and his venous/arterial thrombotic picture was felt to be inconsistent with Prothrombin G20210A mutation.

[b] The APC resistance ratio is highly sensitive for detecting the presence of a Factor V Leiden mutation. Due to a negative results in this assay, DNA analysis for Factor V Leiden was not undertaken.

[c] This test was repeated on multiple occasions (total of 10 times) across three health system laboratories and was weakly positive in two of the ten tests and negative in the rest (Weak positive results: Cardiolipin IgM antibodies at 22.6 MPL and 19.2 MPL; Reference range <15 MPL for testing laboratory), and thus was deemed to be negative.

Methods

Patient Samples

Blood samples were obtained from the patient following informed consent. Research studies were approved by the Institutional Review Board of Mayo Clinic.

Anti-PF4/polyanion antibody isolation

Anti-PF4/polyanion antibodies were isolated as described recently[1]. Briefly, heparin sepharose beads (200 µL, Cytiva Lifesciences) were washed with phosphate-buffered saline, pH 7.4 (PBS), and incubated with 200 µL (1 mg/mL) of recombinant PF4 (Protein Foundry) or an equal volume of PBS (control condition) for one hour. Beads were incubated with 500 µL of patient sample (serum) for one hour. Beads were thoroughly washed with PBS, and elution from the PF4/heparin sepharose or PBS/heparin sepharose beads was performed with 500 µL of 2M NaCl. Eluates were dialyzed against PBS before being evaluated by HIT enzyme-linked immunosorbent assay (ELISA), PF4-dependent P-selectin expression assay (PEA), and mass spectrometric studies.

HIT ELISA

Patient samples were evaluated in Lifecodes PF4 IgG (Immucor) ELISA, an FDA-approved in vitro diagnostic assay that employs PF4-polyvinyl sulfonate (PVS) targets. Lifecodes PF4 IgG (Immucor) was used according to the manufacturer's instructions. In brief, patient serum/plasma was incubated with PF4-PVS coated platelets, stringently washed, and incubated with an alkaline phosphatase labeled anti-human IgG antibody. After antibody incubation, pNPP substrate was added and colorimetric detection was performed after 30 minutes, with optical densities of 405 and 492 nm recorded.

Functional platelet studies

The PEA was performed as previously described[2,3]. Briefly, prostaglandin E1 was added to citrated whole blood obtained from healthy volunteers at a final concentration of 50 ng/mL and centrifuged at 200 x g for 15 minutes to obtain platelet-rich plasma (PRP). Platelets were then isolated from PRP by centrifugation at 1,000 x g for 15 minutes, after which the platelet pellet was resuspended in phosphate-buffered isotonic saline (pH 7.4) supplemented with 1% bovine serum albumin. Platelets (1×10^6) were treated for 20 minutes at room temperature with PF4 (37.5 μg/mL). After PF4 incubation, ten microliters of patient sample was added to 40 μL of PF4 treated platelets and incubated for one hour at room temperature, yielding a final PF4 concentration of 30 μg/mL. Early experiments were performed by addition of labeled anti-P-selectin (Anti-CD62P Clone HIP8, Invitrogen) and anti-GPIIb (Anti-CD41 clone AK-4, Invitrogen) antibodies. Subsequent experiments were performed using the addition of labeled anti-P-selectin (monoclonal antibody HB-299, ATCC) and anti-GPIIIa (monoclonal antibody HB-242, ATCC) antibodies. Platelet events were gated for GPIIb or GPIIIa positivity in flow cytometry, and P-selectin expression (median fluorescence intensity, MFI) was recorded. The SRA was performed in various CLIA-approved reference testing laboratories, as determined by the treating physician.

VITT (un-complexed PF4) ELISA

Un-complexed PF4 ELISAs were performed as previously described[4]. Briefly, ELISA plates (Thermo Scientific) were incubated with 50uL of recombinant PF4 (Protein Foundry; 10 μg/mL), and plates were washed three times with phosphate-buffered saline pH 7.4 (PBS)/0.1% Tween-20 and blocked with Superblock T20 (Thermo Scientific). Sera were incubated at 1:50 dilution for 60 minutes followed by four washes with PBS/0.1% Tween-20. After a 45-minute incubation with 50 μL of alkaline phosphatase-conjugated goat anti-human IgG Fc antibody (Jackson Immunoresearch) at a dilution of 1:5000, four additional washes were performed using PBS/0.1% Tween-20. Colorimetric detection was performed using p-nitrophenyl phosphate

(pNPP) substrate, and the optical density, OD (405nm minus 492nm) at 30 minutes, was recorded.

Platelet Binding Studies

Briefly, prostaglandin E1 was added to citrated whole blood obtained from healthy volunteers at a final concentration of 50 ng/mL and centrifuged at 200 x g for 15 minutes to obtain platelet-rich plasma (PRP). Platelets were then isolated from PRP by centrifugation at 1,000 x g for 15 minutes, after which the platelet pellet was resuspended in phosphate-buffered isotonic saline (pH 7.4) supplemented with 1% bovine serum albumin (PBS-BSA). Platelets (1×10^6) were treated for 20 minutes at room temperature with (37.5 µg/mL) or without PF4. After incubation, ten microliters of patient sample/normal serum were added to 40 µL of PF4-treated platelets and incubated for one hour at room temperature, yielding a final PF4 concentration of 30 µg/mL. Platelets were centrifuged at 1000 x g for 15 minutes, and the platelet pellet was resuspended in 50 µL PBS-BSA containing PE-labeled goat anti-human IgG gamma heavy chain (Jackson Immunoresearch), APC-labeled mouse anti-human lambda light chain (ThermoFisher) or FITC-labeled goat anti-human kappa light chain (ThermoFisher) before incubation in the dark for 30 minutes. Median fluorescent intensity (MFI) of the labeled antibodies were recorded by flow cytometry and expressed as the percent increase in MFI obtained with and without addition of PF4. *Liquid Chromatography Electrospray Ionization Quadrupole time-of-flight mass spectrometry (LC-ESI-QTOF MS)*

The basic method used for antibody analysis has been previously described[5,6]. Immunoglobulins (Igs) from patient sera or bead eluates were isolated using camelid-derived nanobodies against the constant domains of human Ig gamma heavy chain, kappa light chain, or lambda light chains (Thermo Fisher Scientific). In summary, 10 µL of camelid nanobody beads were incubated with 20 µL of serum or 50 µL of PF4-Heparin Sepharose or PBS-heparin bead eluate diluted into 200 µL of buffered saline (PBS) and incubated for 45 minutes at

ambient temperature. Subsequently, supernatants were removed, and the beads were washed three times with 500 µL of water. Bound immunoglobulin light and heavy chains were eluted using 100 µL of 5% acetic acid and reduced using 50 µL of 100 mM dithiothreitol (DTT) in 1M ammonium bicarbonate to disassociate immunoglobulins and separate light chain from heavy chain Ig components. An Eksigent Ekspert 200 microLC (Framingham, MA) liquid chromatography system was used to separate immunoglobulin chains prior to ionization and to remove eluted PF4 prior to mass spectrometry. The mobile phases included an aqueous phase A (100% water + 1% formic acid) and an organic phase B (90% acetonitrile + 10% isopropanol + 0.1% formic acid). Five microliters of each camelid nanobody bead eluate was injected per analysis onto a Poroshell 300SB-C3 column (1.0 mm X 75 mm) with a 5 µm particle size placed in a 60 ºC column heater. The gradient used has been described previously[5,6]. The flow rate was 25 µL/minute. A SCIEX TripleTOF 5600 quadrupole time-of-flight (Q-TOF) mass spectrometer using electrospray ionization in positive ion mode was used for analysis. Data analysis was performed using Analyst TF v1.8.1 and PeakView ver. 2.2. Overexpressed immunoglobulins were inferred from the light chain +11 (m/z, mass to charge 2020 to m/z 2200) as described elsewhere[5,6]. The mass spectra of the multiply charged LC ions were deconvoluted to obtain an accurate molecular mass using the Bio Tool Kit ver. 2.2 plug-in software. The retention time of the monoclonal LC in each pre-treatment patient sample was tracked using PeakView. The instrument was calibrated every five samples using an automated calibrant delivery system (CDS). Mass measurement accuracy was estimated to be 15 ppm for the duration of the analysis.

Immunofixation Electrophoresis

Serum IFE was performed using Hydrasys 9IF gels (Sebia, Paris, France) following manufacturer's instructions.

Results

Thrombotic history

The patient's first thrombotic event, a pulmonary embolism, was not preceded by heparin administration. He was treated with enoxaparin (which was complicated by bruising and malaise) and was transitioned to apixaban, which was discontinued after four months. One year later, he developed an NSTEMI and was noted to have incidental portal vein thrombosis. Enoxaparin was started (which was associated with rigor and palpitations) with bridging to warfarin. While on warfarin he developed chest pain which was managed with intravenous heparin and was associated with a significant decrease in platelet counts. The patient was discharged on apixaban. Several months later, he underwent further cardiac evaluation for chest pain and exertional dyspnea and was transitioned back to warfarin with fondaparinux bridging. He went on to develop splenic infarction despite a supratherapeutic INR (3.1), which was managed with argatroban and intravenous immunoglobulin G (IVIg). He was discharged on apixaban, despite which he developed a STEMI with occlusion of the left anterior descending coronary artery that was treated with aspiration thrombectomy. The patient has been on apixaban and clopidogrel since this last thrombotic event.

Data Sharing Statement

Data will be shared upon reasonable request to the corresponding author.

References

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4. Kanack AJ, Singh B, George G, et al. Persistence of Ad26.COV2.S-associated vaccine-induced immune thrombotic thrombocytopenia (VITT) and specific detection of VITT antibodies. *Am J Hematol*. 2022;97(5):519-526.
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