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THROMBOSIS AND HEMOSTASIS

Off-the-shelf cryopreserved platelets for the detection of HIT and VITT antibodies

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KEY POINTS

- Cryopreservation of healthy donor platelets under controlled cooling rates maintains platelet activatability even after prolonged storage.
- Quantification of TSP1 release from PF4- or heparin-treated cryopreserved platelets is accurate for HIT and VITT diagnosis.

Heparin-induced thrombocytopenia (HIT) is suspected much more often than it is confirmed. Technically simple platelet factor 4 (PF4)-polyanion enzyme-linked immunosorbent assays (ELISAs) are sensitive but nonspecific. In contrast, accurate functional tests such as the serotonin release assay, heparin-induced platelet activation assay, and PF4-dependent P-selectin expression assay require fresh platelets and have complex assay end points, limiting their availability to specialized reference laboratories. To enable broad deployment of functional testing, we sought to extend platelet viability significantly by optimizing storage conditions and developed a simple functional assay end point by measuring the release of a platelet α-granule protein, thrombospondin-1 (TSP1), in an ELISA format. Platelet cryopreservation conditions were optimized by freezing platelets at controlled cooling rates that preserve activatability. Several-month-old cryopreserved platelets were treated with PF4 or heparin and were evaluated for their ability to be activated by HIT and vaccine-induced immune thrombotic thrombocytopenia (VITT) antibodies in the TSP1 release assay (TRA). HIT and spontaneous HIT patient samples induced significantly higher TSP1 release using both PF4-treated (PF4-TRA) and heparin-treated cryopreserved platelets relative to samples from patients suspected of HIT who lacked platelet-activating antibodies. This latter group included several patients that tested strongly positive in PF4-polyanion ELISA but were not platelet-activating. Four VITT patient samples tested in the TRA activated PF4-treated, but not heparin-treated, cryopreserved platelets, consistent with recent data suggesting the requirement for PF4-treated platelets for VITT antibody detection. These findings have the potential to transform the testing paradigm in HIT and VITT, making decentralized, technically simple functional testing available for rapid and accurate in-hospital diagnosis.

Introduction

Heparin-induced thrombocytopenia (HIT) and vaccine-induced immune thrombotic thrombocytopenia (VITT) are prothrombotic syndromes characterized by antibodies to platelet factor 4 (PF4).[1-9] Currently, 2 classes of assays, antigen based and functional (platelet activation-based), are used for diagnosis. PF4-polyanion complex-based enzyme-linked immunosorbent assay (ELISA) tests are simple from a technical standpoint, but are highly nonspecific.[10,11] More accurate functional assays such as the serotonin-release assay (SRA),[12] PF4-dependent P-selectin expression assay (PEA),[11] and heparin-induced platelet activation assay,[13] require complex techniques and handling of radioactive reagents, flow cytometry, or platelet aggregometry. Heparin-treated and PF4-treated platelets are used in HIT functional testing,[11,12] and recent studies suggest that only PF4-treated platelets consistently support the detection of VITT antibodies.[14-16] Functional assays require platelets to be freshly obtained (often from pedigreed "reactive" donors) and have limited shelf lives. As a result of these factors, functional assays are generally available only in the context of specialized reference laboratories. Thus, diagnosis relies much more heavily on ELISA vs functional tests, especially in the early period when HIT is suspected. The increased reliance on HIT ELISA testing coupled with its modest positive predictive value has caused an "epidemic of overdiagnosis"[17] and excessive use of alternative anticoagulants, which are costly[18] and have a worse bleeding risk profile relative to heparin.[10,19] Thus, HIT is a challenging clinicopathologic diagnosis, and delays in definitive testing cause significant morbidity and mortality.

Recent studies suggest that the use of PF4-treated platelets in functional testing is equivalent to heparin-treated platelets for HIT diagnosis[11] and superior for detecting VITT antibodies.[5,14,20] Newer functional assays such as the PEA[11] and PF4-induced platelet activation[21] test, although accurate, have similar limitations as previous functional assays, including the requirement for fresh donor blood and complex technologies such as flow cytometry. Thus, a critical need exists to develop a self-contained functional in vitro diagnostic assay (IVD) that includes activatable platelets (to avoid the need for fresh donor platelets) coupled with a technically simple assay end point. Such a test will enable accurate HIT and VITT functional testing in the near-patient, in-hospital environment. In this study, we demonstrate that platelets cryopreserved under controlled freezing rates maintain their ability to be activatable after long periods of storage. Furthermore, we show that platelet activation of long-term cryopreserved platelets can be assessed with a technically simple ELISA end point by measuring the release of the abundant platelet α-granule protein thrombospondin-1 (TSP1).[22] We demonstrate that PF4-treated cryopreserved platelets support the accurate detection of pathogenic, platelet-activating HIT and VITT antibodies, whereas heparin-treated cryopreserved platelets can be used for the accurate detection of platelet-activating HIT antibodies.

Materials and methods

Patient samples

Thirty-four and 29 serum samples from patients suspected of having HIT were evaluated in the PF4-TSP1 release assay (TRA) and heparin-TRA, respectively. These were sample remnants available in adequate volumes after HIT diagnostic testing (PF4-polyanion ELISA and SRA) had concluded. Ten samples (6 with PEA-positive results and 4 with PEA-negative results) overlapped between the two cohorts. Four VITT samples available in adequate volumes, all from patients with Ad26.COV2.S-associated VITT, also were included in the study. These studies were approved by the institutional review board of Mayo Clinic.

Platelet donors

Healthy, nonpedigreed blood group O individuals made a routine whole blood donation (Community Blood Center, Appleton, WI) and consented to the use of the blood product for research studies. Platelet-rich plasma (PRP) units were obtained by differential centrifugation and were shipped to the research facility after all Food and Drug Administration-mandated infectious disease testing was complete and results were noted to be negative. As noted below, platelets were isolated from 2-day-old PRPs, were cryopreserved, and tested in the TRA.

Platelet cryopreservation

Anticoagulant citrate dextrose-A (117 mM sodium citrate; 136 mM dextrose, pH 5.9) and prostaglandin E-1 (50 μg/mL stock; Millipore Sigma, St. Louis, MO) were added at 1:10 (volume-to-volume) and 1:1000 (volume-to-volume) to 2-day-old PRP units obtained from whole blood donations. The PRP was centrifuged at 100*g* for 15 minutes. Supernatant was collected and centrifuged again at 1000*g* for 15 minutes to pellet platelets. Supernatant was discarded, and platelets were resuspended at a concentration of 1 × 10^6/μL and incubated at 37°C for 2 hours in a buffer containing the cryoprotectant trehalose (9.5 mM HEPES; 100 mM NaCl; 4.8 mM KCl; 5.0 mM glucose; 12 mM $NaHCO_3$; 50 mM trehalose, pH 6.8). Bovine serum albumin (Millipore Sigma, St. Louis, MO) was added to a final concentration of 4% (weight-to-volume ratio). This platelet solution then was aliquoted in 500 μL volume per tube (or 1 mL in preliminary studies presented in Figure 1A-B). Platelets were cryopreserved in a Thermo Scientific CryoMed 7450 controlled rate freezer at a rate of 4°C/min to −80°C, and were stored at −80°C. For some studies, platelets were shipped on dry ice and stored in a −80°C freezer on receipt for various periods before testing.

PF4-TRA and heparin-TRA

Initial testing was performed with apheresates from therapeutic plasma exchange procedures from 3 patients with confirmed HIT. TSP1, a highly expressed protein in platelet α-granules released on HIT antibody-mediated platelet activation, was measured by ELISA. Cryopreserved platelets were used immediately after thawing. Briefly, cryopreserved platelets were thawed at 37°C for 3 minutes and were centrifuged at 1000*g* for 5 minutes (15 minutes in preliminary studies presented in Figure 1A-B). Supernatants were discarded, and platelets were resuspended in a modified Ringer's citrate dextrose buffer containing trehalose (108 mM NaCl; 3.8 mM KCl; 1.7 mM $NaHCO_3$; 22.9 mM sodium citrate; 27.8 mM Glucose; 50 mM trehalose, pH 6.5) and centrifuged at 1000*g* for 5 minutes (15 minutes in preliminary studies presented in Figure 1A-B). Supernatants were discarded. Platelets were resuspended in 80 μL (or 175 μL in preliminary studies) of a phosphate-buffered saline-based activation buffer (155 mM NaCl; 2.97 mM Na_2HPO4; 1.06 mM KH_2PO4; 1% [weight-to-volume ratio] bovine serum albumin; 50 mM trehalose; pH 7.4), with the addition of 150 μg/mL PF4 (Protein Foundry, Wauwatosa, WI) and incubated at room temperature for 20 minutes. Alternatively, unfractionated heparin (Millipore Sigma, St. Louis, MO) was added to the activation buffer at concentrations of 1.56 U/mL or 125 U/mL. Twenty microliters of patient serum was added to 80 μL of PF4- or heparin-treated platelets per reaction (attaining a final PF4 concentration of 120 μg/mL and a final heparin concentration of 1.25 U/mL or 100 U/mL), incubated for 30 minutes at room temperature, and supernatants were collected after centrifugation at 1000*g* for 5 minutes (15 minutes in preliminary studies presented in Figure 1A-B). TSP1 was quantified in platelet supernatants using a Thrombospondin-1 DuoSet ELISA kit (R&D Systems, Minneapolis, MN) using manufacturer instructions. TSP1 release was expressed as concentration of TSP1 in the releasate or as fold increase over TSP1 release from platelets induced by healthy donor serum. In some studies, cryopreserved platelets were thawed, washed, treated with PF4 (150 μg/mL), and incubated with an isotype control or monoclonal antibody IV.3 (4 μg/mL), an anti-FcγRIIa blocking antibody, before being incubated with a healthy donor control, HIT, or VITT sample. In other studies, cryopreserved platelets were thawed, washed, and stimulated with thrombin receptor-activating peptide (TRAP; 25 μg/mL) or vehicle control, and TSP1 release was quantified by ELISA.

Additional laboratory testing

The PEA was performed as previously described with minor modifications.[11,23] Briefly, prostaglandin E1 (50 ng/mL) was added

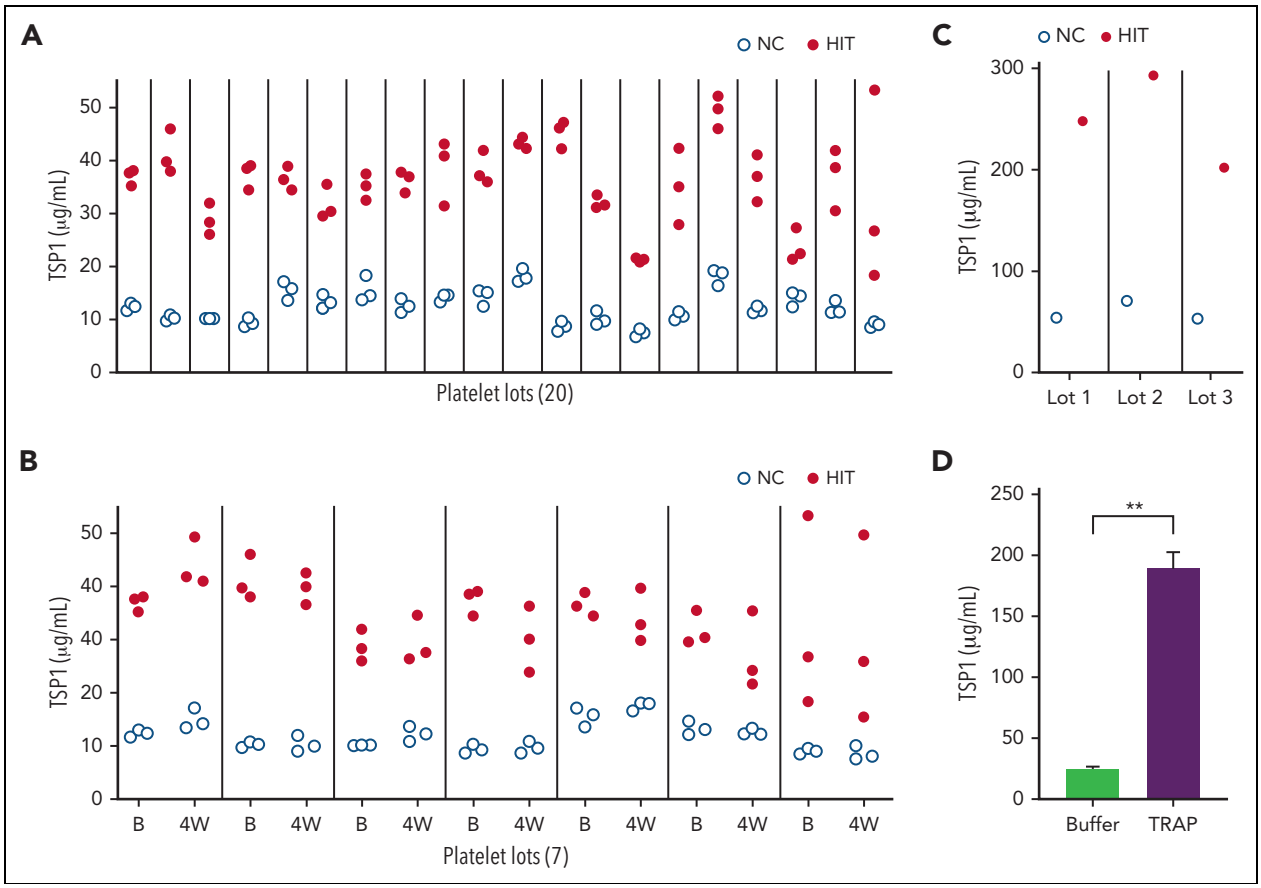

Figure 1. HIT antibodies activate PF4-treated cryopreserved platelets, including 1-year-old platelets, and TSP1 release correlates with serotonin release. (A) TRAs were performed with 3 platelet-activating HIT (closed circles) and 3 NC (open circles) samples using 20 lots of cryopreserved platelets. Each data point represents the mean of technical triplicates. (B) TSP1 release from 7 of the 20 cryopreserved platelet lots stored for >4 weeks at −80°C (4W) were compared with activation of platelets stored for ≤1 week at −80°C (baseline [B]). Four-week-old platelets ranged in storage age from 29 to 36 days, whereas baseline platelets ranged in storage age from 1 to 7 days. Each data point represents the mean of technical triplicates. (C) PF4-dependent platelet activation induced by a HIT (closed circle) and NC sample (open circle) with 3 cryopreserved platelet lots stored at −80°C for 12 months. Each data point represents the mean of technical triplicates. (D) Platelet activation induced by TRAP. Data are from technical triplicates with mean ± SD displayed. Groups are compared using two-tailed, unpaired *t* test with Welch's correction. $^{**}P < .005$. Studies were performed at Retham Technologies (Wauwatosa, WI) on cryopreserved platelets that were not subject to shipping. NC, normal control.

to citrated whole blood obtained from healthy volunteers and centrifuged at 200*g* for 15 minutes to obtain PRP. PRP then was centrifuged at 1000*g* for 15 minutes to pellet platelets. The platelet pellet was resuspended in phosphate-buffered isotonic saline (pH 7.4)-1% bovine serum albumin. Platelets (1×10^6) were treated for 20 minutes at room temperature with PF4 (37.5 µg/mL). Ten microliters of patient sample was added to 40 µL PF4 treated platelets, and the mixture was incubated for 1 hour at room temperature, giving a final PF4 concentration of 30 µg/mL. After the addition of labeled anti-P-selectin (monoclonal antibody HB-299; ATCC, Manassas, VA) and anti-glycoprotein IIIa (monoclonal antibody HB-242; ATCC, Manassas, VA) antibodies, platelet events were gated by glycoprotein IIIa positivity in flow cytometry, and P-selectin expression (median fluorescence intensity [MFI]) was recorded. In addition to a normal sample calibrator, patient samples with known positive and negative findings were included in each run. Maximum P-selectin expression (100%) was measured by treating platelets with TRAP (25 µg/mL). Results were expressed as the percentage of maximum P-selectin expression corrected for background signal obtained with a healthy donor (normal) calibrator sample as follows: PEA percent activation = [(patient sample MFI-calibrator serum MFI)/(TRAP MFI-calibrator serum MFI)] × 100.

The SRA was performed in various Clinical Laboratory Improvement Amendments–approved reference laboratories, as determined by the treating physician. All patient samples were tested in the Lifecodes PF4 immunoglobulin G ELISA (Immucor, Waukesha, WI), per diagnostic kit manufacturer instructions.

Statistical analysis

TSP1 release was correlated with SRA and PEA results using linear regression analysis. An ordinary 1-way analysis of variance was used to compare TSP1 release in HIT and VITT groups in the PF4-TRA and heparin-TRA. TSP1 release induced by platelet-activating vs nonactivating samples in ELISA optical density (OD)-matched groups of HIT samples were compared using a 2-tailed, unpaired Student *t* test.

Results

We initially evaluated the ability of refrigerated (4°C) platelets to support the detection of HIT antibodies using PF4-treated platelets. Compared with 2-day-old "fresh" platelets stored at ambient temperature, 1-week-old refrigerated platelets demonstrated unacceptably high platelet activation, as

evidenced by elevated P-selectin (CD62P) expression upon incubation with healthy donor sera (supplemental Figure 1, available on the *Blood* website). We then explored the possibility of using cryopreserved platelets to detect platelet-activating HIT antibodies. After optimization, platelets were cryopreserved at a controlled rate, thawed, and treated with PF4 as described in "Materials and methods," and then incubated with platelet-activating HIT or healthy donor samples to determine their ability to elicit platelet activation. As shown in Figure 1A, 3 HIT samples stimulated higher TSP1 release relative to 3 healthy donor (normal) control samples when the TRA was performed using 20 platelet lots, each drawn from a different donor, despite some variability in the amount of TSP1 released between lots. To assess the longer-term viability of cryopreserved platelets for detecting HIT antibodies, the TRA was repeated on a subset of platelet lots (n = 7) stored at −80°C for at least 4 weeks. Figure 1B shows that platelets preserved for 4 weeks remained capable of activation and degranulation. Of note, the reactivity of platelets was highly reproducible within each platelet lot (baseline ["B"] vs 4 weeks ["4W"]; Figure 1B). Three platelet lots were stored at −80°C for 12 months and retested in the TRA to assess their viability after extended storage periods. As seen in Figure 1C, all 3 12-month-old platelet lots supported platelet activation, as demonstrated by an elevated release of TSP1 upon incubation with HIT samples relative to control samples. During the process of method optimization for cryopreservation, the protocol was changed slightly (as noted in "Materials and methods") with platelets being frozen in smaller volumes (500 μL per tube vs 1 mL in preliminary studies presented in Figure 1A-B) and steps after thaw using shorter centrifugation times (5 minutes vs 15 minutes in preliminary studies presented in Figure 1A-B). This accounted for the difference in TSP1 concentrations noted in platelet releasates (Figure 1A-B vs Figure 1C). To further validate its utility as a marker of platelet activation, the release of TSP1, an abundant platelet α-granule protein,[22] was confirmed to be highly correlated with serotonin release in a cohort of five patients with confirmed HIT and one healthy control using standard noncryopreserved platelets (linear regression, R^2 = 0.961; supplemental Figure 2). Furthermore, cryopreserved platelets were highly responsive to protease-activated receptor 1-mediated platelet activation (Figure 1D), suggesting a general preservation of platelet viability conferring the ability to activate in response to multiple agonists.

To define the diagnostic accuracy of the TRA, a cohort of 16 platelet-activating sera (with positive PEA results) and 18 nonactivating (with negative PEA results) HIT-suspected patient samples were selected for evaluation in the PF4-treated cryopreserved platelet-coupled TRA (PF4-TRA). PEA and SRA results were concordant among all 34 patient samples as shown in Figure 2A and included samples from 2 patients with spontaneous HIT. The PEA-negative cohort was selected specifically to include several cases commonly encountered in clinical practice with positive results in PF4-polyanion ELISA, but negative results in functional testing (Figure 2B, closed triangles). All 16 PEA-positive samples stimulated significantly higher TSP1 release from cryopreserved platelets relative to the PEA-negative group (Figure 2B). The mean and 95% confidence interval (CI) for TSP1 release (fold increase over release induced by normal serum) were 2.67-fold and 2.44- to 2.90-fold, respectively, in the PEA-positive group compared with an average of 0.73-fold TSP1 release (95% CI, 0.62-fold to 0.84-fold) in the PEA-negative group. Notably, no overlap was found between the PEA-positive and PEA-negative groups in the PF4-TRA, although 11 of the 18 patients in the PEA-negative cohort showed positive results in the ELISA (Figure 2B, closed triangles). Similar to the PEA-positive HIT samples, all 4 VITT samples stimulated an average 2.20-fold increase in TSP1 release (95% CI, 1.24-fold to 3.15-fold; Figure 2B). In a subanalysis, TSP1 release induced by PEA-positive and PEA-negative samples matched for strong ELISA positivity (1.3-2.3 OD) from within this cohort were compared (Figure 2C). The platelet-activating group (with positive PEA results) induced significantly higher TSP1 release (average fold increase, 2.51; 95% CI, 2.34-2.69) compared with the PEA-negative subgroup (average fold increase, 0.76; 95% CI, 0.55-0.96; Figure 2C), despite highly overlapping ELISA ODs. TSP1 release was highly correlated with both PEA (Figure 2D; R^2 = 0.795) and SRA (Figure 2E; R^2 = 0.868) results associated with the patient samples.

To evaluate the diagnostic utility of heparin-treated platelets (the "technology" used in the SRA) for the detection of HIT and VITT antibodies, the TRA was performed on unfractionated heparin-treated cryopreserved platelets (heparin-TRA) as described in "Materials and methods." This cohort of 29 patients included 1 SRA-PEA discordant sample that showed negative test results in the PEA (0%) and ELISA (<0.075 OD), but was weakly positive (21%) in the SRA and was deemed HIT-negative based on clinical review (Figure 3A). PEA-positive samples stimulated an average 2.81-fold increase in TSP1 release relative to release induced by healthy donor serum (95% CI, 2.50-3.12). In comparison, the 19 nonactivating (PEA-negative) samples stimulated a 1.43-fold increase in TSP1 release from cryopreserved platelet (95% CI, 1.03-1.83; Figure 3B). Of note, TSP1 release was elevated significantly in 2 samples (samples P1 and P2) from the PEA-negative group, despite both patients showing negative results in the ELISA with ODs of 0.102 and 0.089, respectively (Figure 3B). Attenuation of platelet degranulation in the presence of high concentrations of heparin is a commonly used technique for confirming the presence of HIT antibodies. High heparin concentrations (100 U/mL) significantly inhibited induction of platelet activation by the PEA-positive cohort, confirming the presence of HIT antibodies in that group (Figure 3C). In contrast, platelet activation induced by P1 and P2 were resistant to inhibition by high heparin concentrations, pointing to non-HIT antibody-related factors as the cause of platelet activation (Figure 3C). As expected, VITT samples did not activate cryopreserved platelets in the presence of high concentrations of heparin (Figure 3C). Furthermore, P1 and P2 sera also stimulated significant TSP1 release in the PF4-TRA and when the TRA was performed without the addition of PF4 or heparin, confirming that platelet activation induced by these samples was not mediated by HIT antibodies (supplemental Figure 3). None of the VITT samples stimulated TSP1 release in the heparin-TRA (mean change of 0.81-fold relative to normal serum; 95% CI, 0.681-0.938; Figure 3B). Interestingly, 2 of the 4 VITT patient samples showed positive results in the TRA using platelets untreated with either PF4 or heparin (supplemental Figure 4). TSP1 release induced by patients suspected of having HIT in the presence of low concentrations of heparin (excluding the 2 heparin uninhibitable samples, P1 and P2) was correlated highly with both PEA (Figure 3D; R^2 = 0.814) and SRA (Figure 3E; R^2 = 0.832) results. Heparin-TRA and PF4-TRA results for 10 overlapping samples from the 2 cohorts tested in both

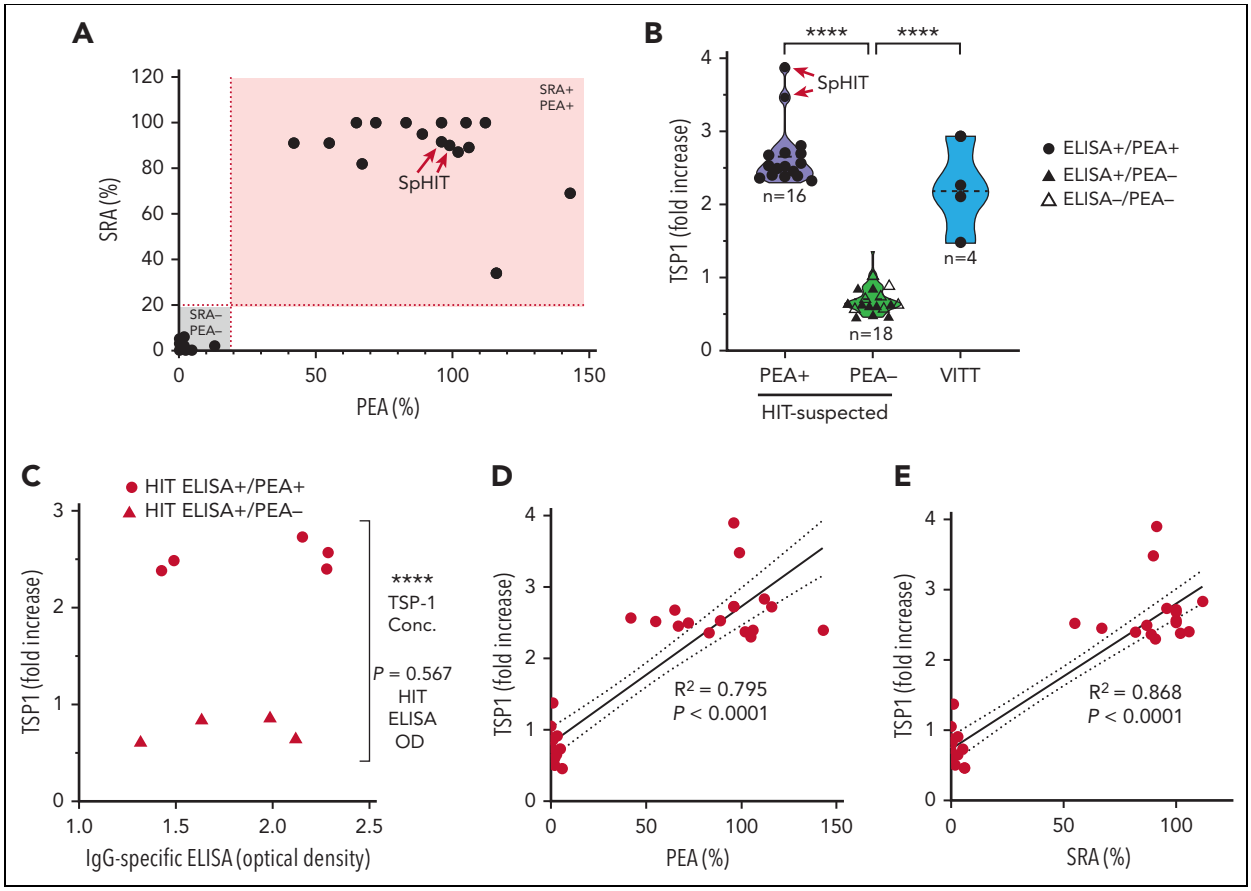

Figure 2. The PF4-TRA is highly sensitive and specific for the detection of platelet-activating HIT and VITT antibodies. (A) PEA and SRA results for the cohort of HIT-suspected patient samples tested in the PF4-TRA are shown on the x-axis and y-axis, respectively. Red dotted lines represent positive cutoff values (19% P-selectin expression for the PEA and 20% serotonin release for the SRA). (B) PF4-TRA results for 34 HIT-suspected patient samples and 4 VITT patient samples are shown. ELISA-positive/PEA-positive samples, ELISA-positive/PEA-negative samples, and ELISA-negative/PEA negative samples are indicated by closed circles, closed triangles, and open triangles, respectively. Each data point represents the mean of technical duplicates. An ordinary 1-way analysis of variance was used for comparisons, and means are shown as dotted lines. (C) TRA results for OD-matched HIT ELISA-positive/PEA-positive patient samples (closed circles) vs HIT ELISA-positive/PEA-negative patient samples (closed triangles) are shown. Each data point represents the mean of technical duplicates. A 2-tailed, unpaired Student *t* test was used to compare OD values and TSP1 fold changes. (D) PEA and (E) SRA results were correlated with TSP1 release for the HIT-suspected patient samples tested in the PF4-TRA. Linear regression analysis (black solid line) with 95% CI (dashed lines) are presented. The fold increase in TSP1 released from platelets incubated with the HIT/VITT cohort samples relative to release seen with a normal serum control is depicted on the y-axis for (B), (C), (D), and (E). ****$P < .0001$. IgG, immunoglobulin G; SpHIT, spontaneous HIT.

assays were highly correlated (Figure 4A), and activation of cryopreserved platelets by both HIT and VITT antibodies was mediated through the platelet immunoglobulin G receptor FcγRIIa (Figure 4B).

The ability of cryopreserved platelets to maintain activatability after several hours to days of transport is an essential consideration for its inclusion in a viable IVD assay. Studies presented in Figures 2-4 were conducted on cryopreserved platelets shipped on dry ice for approximately 30 hours (shipped from Retham Technologies, Wauwatosa, WI, to Mayo Clinic, Rochester, MN). To simulate more extended transport, cryopreserved platelets were held on dry ice in standard shipping containers for 72 hours and tested. Results demonstrated continued activatability of platelets even after this prolonged hold on dry ice (supplemental Figure 5).

Discussion

The requirement for fresh platelets in functional HIT and VITT assays and the complexity of test end points are major barriers to the decentralization of accurate HIT testing. The current testing paradigm, where PF4-polyanion ELISA-positive samples are sent out for functional confirmation, causes delays in diagnosis and results in excessive use of nonheparin anticoagulants.[17] This study assessed the feasibility of using long-term stored off-the-shelf cryopreserved platelets from non-pedigreed donors coupled to a novel ELISA-based TSP1 end point. Results demonstrated that platelets from all donors tested were activatable by HIT or VITT antibodies, including 12-month-old platelets. The study also provides proof-of-concept that measurement of TSP1, an abundant α-granule protein,[22] in a technically simple ELISA format offers a convenient method for assessing platelet activation. The large size of TSP1 (~155 kD) also lends itself well to development of sandwich ELISAs using antibodies that do not overlap in epitope specificity. Like functional assays such as the SRA,[24] interdonor variability in reactivity to HIT antibodies was noted in the TRA. We believe that this challenge can be mitigated using a pool of platelets from multiple donors in testing as we have done in a recently launched HIT functional assay, the PEA.[11,25] The process envisioned involves pooling 2-day-old PRPs from 5 to 10

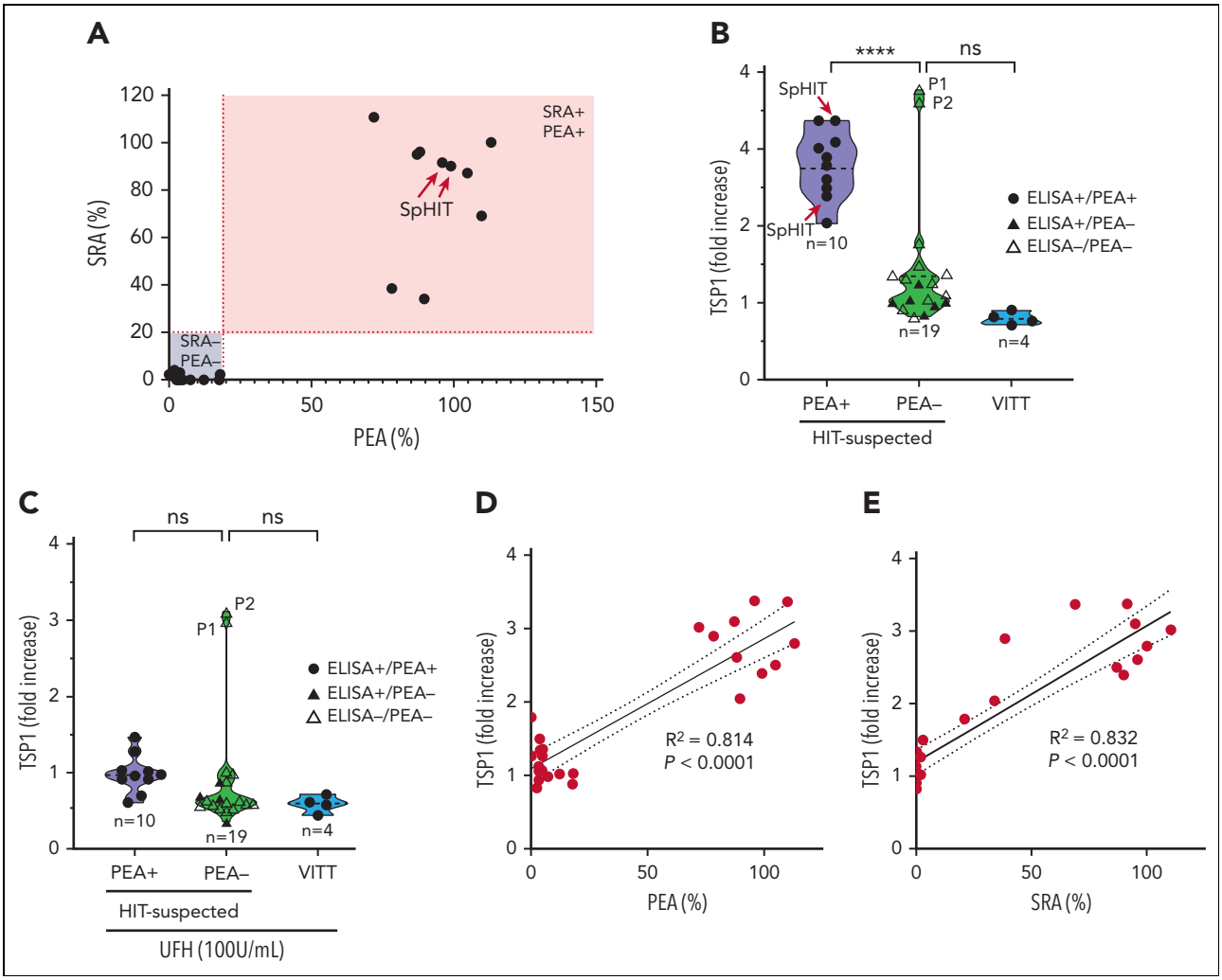

Figure 3. The heparin-TRA is highly sensitive and specific for detection of platelet-activating HIT, but not VITT antibodies. (A) PEA and SRA results for the cohort of HIT-suspected patient samples tested in the heparin-TRA are shown on the x-axis and y-axis, respectively. Red dotted lines represent positive cutoff values (19% P-selectin expression for the PEA and 20% serotonin release for the SRA). (B) Heparin-TRA results are displayed for 29 HIT-suspected patient samples and 4 VITT patients. ELISA-positive/PEA-positive samples, ELISA-positive/PEA-negative samples, and ELISA-negative/PEA-negative samples are indicated by closed circles, closed triangles, and open triangles, respectively. Each data point represents the mean of technical duplicates. Ordinary 1-way analysis of variance was used for comparisons, and means are shown as dashed lines. (C) Results from the TRA performed in the presence of high concentrations of UFH (100 U/mL) are presented. ELISA-positive/PEA-positive samples, ELISA-positive/PEA-negative samples, and ELISA-negative/PEA-negative samples are indicated by closed circles, closed triangles, and open triangles, respectively. Each data point represents the mean of technical duplicates. Means are shown as dashed lines. (D) PEA and (E) SRA results were correlated with TSP1 release in the heparin-TRA for HIT-suspected patient samples (excluding the heparin-noninhibitable samples, P1 and P2). Linear regression analysis (black solid line) with 95% CI (dashed lines) are presented. The fold increase in TSP1 released from platelets incubated with the HIT/VITT cohort samples relative to release seen with a normal serum control is depicted on the y-axis for (B), (C), (D), and (E). ****$P < .0001$. ns, not significant; SpHIT, spontaneous HIT; UFH, unfractionated heparin.

platelet donors and cryopreserving platelets isolated from this mixture using the methods described. Alternatively, platelets isolated from PRPs obtained from reactive individual platelet donors can be cryopreserved and used in testing. A positive cutoff of ≥2-fold and ≥1.5-fold over TSP1 release induced by a negative control serum that is inhibitable by high concentrations of heparin could be used to differentiate HIT- and VITT-positive samples from HIT- and VITT-negative samples, respectively. The data further demonstrated that platelets retain activatability after several days of storage on dry ice to simulate shipping conditions, an essential consideration for decentralization of functional testing. Platelets used in most of the experiments of this study were stored for ~2 to 4 months before being run in the PF4-TRA and heparin-TRA. Furthermore, after shipment of platelets on dry ice, platelets were stored for varying times (at −80°C) before being used in TRA studies (range, 9-78 days). Thus, the results obtained herein suggest robust preservation of activatability of these platelets with both cryotransport and long-term cryostorage.

Using a heterogeneous group of HIT samples, including from patients with strongly ELISA-positive results with nonactivating antibodies and from patients with spontaneous HIT, cryopreserved platelets coupled to a TSP1 ELISA end point demonstrated high sensitivity and specificity for the detection of platelet-activating HIT antibodies using both PF4- and heparin-treated cryopreserved platelets. The use of a high heparin concentration step for HIT antibody confirmation, as is performed routinely in SRA and PEA testing, is critical, as highlighted by the 2 noninhibited PEA-negative samples (P1

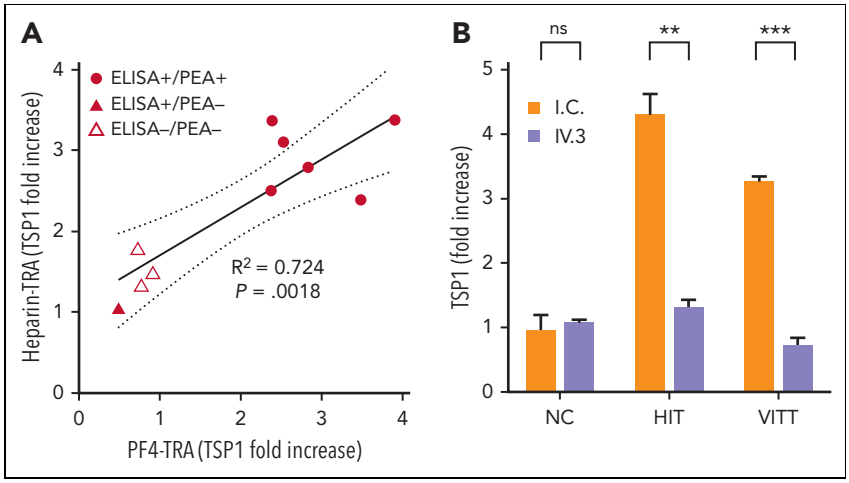

Figure 4. TSP1 release in the PF4-TRA and heparin-TRA were well correlated, and cryopreserved platelets support FcγRIIa-mediated platelet activation. (A) Ten HIT-suspected patient samples with sufficient volumes to test in both the PF4-TRA and heparin-TRA stimulated similar levels of TSP1 release in both the PF4-TRA and heparin-TRA ($R^2 = 0.724$; $P = .0018$). Data are from technical duplicates, and correlation was determined by linear regression analysis (black solid line) with 95% CI (dashed lines) displayed. (B) TSP1 release induced by HIT and VITT samples from cryopreserved platelets incubated with monoclonal antibody IV.3 or IC in the PF4-TRA are shown. Data are from technical duplicates with mean ± SD displayed. $^{**}P < .005$; $^{***}P < .001$. IC, isotype control; ns, not significant.

and P2). One possible mechanism of platelet activation in these cases is HLA antibody mediated, which, unlike HIT antibody-mediated platelet activation, is expected to be resistant to inhibition by high concentrations of heparin. Recent data suggest that VITT antibodies are detected consistently using PF4-treated, as opposed to heparin-treated, platelets.[5,14-16,20] Heparin and VITT antibodies compete for binding to the heparin-binding site on PF4.[26] As a result, functional assays that use heparin-treated or heparin-treated plus PF4-treated platelets show significantly lower diagnostic sensitivity for VITT antibodies than tests that use PF4-treated platelets.[16] Thus, our finding that VITT patient samples do not activate heparin-treated cryopreserved platelets was not surprising.

Based on these data, we believe that patients suspected of HIT may be evaluated by either the cryopreserved platelet PF4-TRA (analogous to the PEA that is used for HIT evaluation), or heparin-TRA (analogous to the SRA). The study further suggested that use of the high-concentration heparin inhibition step is critical for HIT confirmation, similar to its use for HIT confirmation in both the PEA and SRA. This condition also may be important to differentiate TSP1 released as a result of platelet activation vs high background TSP1 levels that may be found in some medical conditions.[27] In the former setting, a decrease in released TSP1 is expected if platelet activation is mediated by HIT or VITT antibodies, whereas no decrease is expected in the setting of high baseline TSP1. In addition, sera are used at a 1:5 dilution in the assay, minimizing the serum contribution of TSP1 in the releasate. Patients suspected of having VITT are not likely to induce platelet activation in the heparin-TRA and should be tested only in the PF4-TRA, also with a heparin inhibition step for confirmation of VITT antibodies. Although some VITT antibodies can activate platelets not treated with PF4,[5] this technique risks missing some VITT antibodies, as seen in our study (supplemental Figure 4).

In our hands, the time required to perform both steps of the TRA, platelet activation and TSP1 ELISA testing, is similar to that of the PEA (6 hours), but offers a significantly simpler technical end point (ELISA vs flow cytometry) and the convenience of not having to obtain fresh platelets. Thus, phlebotomists and highly trained technical personnel are not required for assay performance. A limitation of the study is the lack of 4Ts scores in all patients, although scores available in approximately half the patients tested in the study suggested a higher likelihood of low and intermediate scores in those without activating HIT antibodies (supplemental Figure 6), consistent with expectations.[28] To address this issue, patient samples were tested in 2 different gold standard functional assays, SRA and PEA, each with high sensitivity and specificity to minimize the risk of misclassification of the reference standard against which the TRA was assessed. While the study provides strong proof-of-concept results for use of long-term stored cryopreserved platelets in HIT and VITT diagnosis, given the limited number of samples evaluated in this study exclusively by laboratories with a high level of expertise in platelet testing, additional, more extensive multicenter studies to conclusively evaluate the diagnostic performance of the heparin-TRA and PF4-TRA are required.

In summary, the technological breakthrough in platelet storage described herein has the potential to transform the diagnostic testing paradigm in HIT and VITT. The deployment of self-contained IVD assays such as the cryopreserved platelet-coupled TRA that do not require fresh platelets or access to complex end point-compatible devices such as radioactive counters, aggregometers, and flow cytometers have the potential to make in-hospital functional testing available for the rapid and accurate diagnosis of HIT and VITT.

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Authorship

Contribution: A.J.K., C.G.J., and B.S. performed the TSP1 release assay and flow cytometry studies; N.P.S. helped perform VITT sample testing; R.R.L., N.M.H., R.K.P., D.C., G.G., M.Y.A.-I., G.D.W., and K.G. provided helpful advice and clinical and laboratory correlates; A.P. conceived the experimental plan and directed the laboratory studies; A.J.K., C.G.J., and A.P. wrote the first draft; and all authors provided input and approved the final version.

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Technologies) and reports equity ownership and employment in Retham Technologies. R.K.P. reports honoraria for advisory board participation from CSL Behring, Genentech, Bayer Healthcare AG, HEMA Biologics, Instrumentation Laboratory, and Merck. G.D.W. receives honoraria for advisory board participation from Diagnostica Stago. A.P. reports pending or issued patents (Mayo Clinic, Retham Technologies, and Versiti BloodCenter of Wisconsin), equity ownership in and serving as an officer of Retham Technologies, and member of the advisory board of Veralox Therapeutics. The remaining authors declare no competing financial interests.

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Footnotes

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Data will be shared upon reasonable request to corresponding author (padmanabhan.anand@mayo.edu).

The online version of this article contains a data supplement.

There is a *Blood* Commentary on this article in this issue.

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