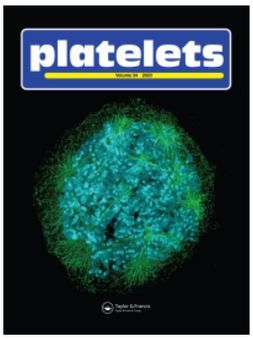

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RESEARCH ARTICLE

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The phenotype of cryopreserved platelets influences the formation of platelet-leukocyte aggregates in an *in vitro* model

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Abstract

Cryopreservation significantly alters the phenotype of platelets; generating distinct subpopulations, which may influence the formation of platelet leukocyte aggregates (PLA). PLAs are immunomodulatory and have been associated with transfusion-associated adverse events. As such, the aim of this study was to examine the effect of cryopreservation on the ability of platelets to form PLAs, using a monocyte-like cell line (THP-1). Platelets were tested pre-freeze, post-thaw and following stimulation with TRAP-6 or A23187, both alone and following co-culture with THP-1 cells for 1 and 24 hours (n = 6). Platelet subpopulations and platelet-THP-1 cell aggregates were analyzed using multi-color imaging flow cytometry using Apotracker Green (ApoT), CD42b, CD62P, CD61, and CD45. Cryopreservation resulted in the generation of activated (ApoT-/CD42b+/CD62P+), procoagulant (ApoT+/CD42b+/CD62P+) and a novel (ApoT+/CD42b+/CD62P-) platelet subpopulation. Co-incubation of cryopreserved platelets with THP-1 cells increased PLA formation compared to pre-freeze but not TRAP-6 or A23187 stimulated platelets. P-selectin on the surface membrane was correlated with increased PLA formation. Our findings demonstrate that cryopreservation increases the interaction between platelets and THP-1 cells, largely due to an increase in procoagulant platelets. Further investigation is required to determine the immunological consequences of this interaction.

Plain Language Summary

What do we know?

- Cryopreserved platelets are an alternative to overcome issues with the short shelf-life of room-temperature stored platelets
- After thawing, cryopreserved platelets exhibit changes in cell structure and receptor abundance
- Activated platelets can attach to leukocytes, forming platelet-leukocyte aggregates and altering their immune function
- Platelet-leukocyte aggregates can increase inflammation, which is associated with adverse events after transfusion, which can negatively affect patient outcomes

What did we discover?

- Cryopreservation results in a heterogenous mix of platelet subpopulations
- Cryopreserved platelets display increased adherence to a monocyte-like cell line (THP-1 cells). Platelet-THP-1 aggregate formation was linked to expression of CD62P on the surface of the platelets
- The increase in cryopreserved platelet-THP-1 cell aggregates was largely due to an increase in procoagulant platelets

What is the impact?

- Our data demonstrate that cryopreservation increases platelet interaction with a monocyte-like cell line
- This may mediate immune responses and/or circulation time of transfused platelets

Keywords

Cryopreservation, immunology, leukocytes, platelets, THP-1

History

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Introduction

Platelet transfusion is commonly used to promote hemostasis in patients presenting with acute bleeding. Currently, platelet components are stored at room-temperature (2024°C), limiting the shelf-life to 5–7 days due to the risk of bacterial proliferation and a gradual reduction in hemostatic effectiveness.[1,2] Consequently, maintaining inventories in remote medical centers is difficult. This has prompted the evaluation of alternative

storage methodologies, including platelet cryopreservation.[3–5] Cryopreservation extends component shelf-life to at least two years and enhances hemostatic parameters *in vitro*, addressing the primary limitations of room-temperature storage.[6,7] The effect of cryopreservation on the hemostatic characteristics of platelets is well documented, and includes the increased exposure of activation makers (phosphatidylserine, P-selectin), decreased surface abundance of selected glycoproteins (GPVI, GPIbα) and an enhanced capacity to generate thrombin.[3–8] However, less information is available regarding the impact of cryopreservation on the immune characteristics of platelets.[9–11]

Platelets can interact with the immune system directly through receptors on the surface membrane and the release of soluble factors.[12,13] Typically, platelet immune function is beneficial, however, dysregulation can contribute to a range of pro-inflammatory disorders and transfusion-associated adverse reactions.[13,14] The cause of adverse transfusion events is multifactorial.[14] However, a strong link has been established between severe adverse events such as Transfusion Related Acute Lung Injury (TRALI), storage-induced platelet activation, and the formation of platelet-leukocyte aggregates (PLAs).[12,13]

The formation of PLAs is a multistep process, first requiring platelet activation and degranulation, exposing P-selectin on the surface membrane.[15,16] P-selectin can then bind to leukocyte PSGL-1, which triggers leukocyte activation and the expression of $\alpha_M\beta_2$ (Mac-1) receptor on the surface membrane of leukocytes.[15,17] Activated Mac-1 can bind platelet GPIbα stabilizing the PLAs.[15] Further, the expression of platelet surface receptors such as CD40L, GPVI, GPIIb/IIIa, or phosphatidylserine can enhance adhesion or provide co-stimulatory signals to adherent leukocytes.[15,17] PLA formation can modulate downstream immune function, promoting pro- or anti-inflammatory signaling, and platelet clearance by phagocytosis.[15,18,19] Notably, the likelihood of PLA formation and functional consequences can be influenced by the phenotype of the adherent platelet.[20]

Once thought to be largely homogenous, distinct subpopulations of platelets have been described based on morphology, phenotype, and functional characteristics.[20–22] The current literature describes resting, aggregatory, procoagulant and apoptotic platelet subpopulations.[20,22–25] Platelet subpopulations were originally investigated to understand their distinct roles in hemostasis and thrombus formation.[26] However, recent work has established links between platelet subpopulations and the formation of PLAs associated with inflammatory disorders.[19] Recent examinations have highlighted that cryopreservation-induced platelet activation does not occur uniformly, and that the majority of cryopreserved platelets are phenotypically aligned with a procoagulant subpopulation.[22] The selective formation of this subpopulation may impact the ability of platelets to bind to leukocytes and form PLAs.

The examination of PLAs has been made more accessible through imaging flow cytometry, which combines the high throughput benefits of multi-color flow cytometry with the imaging capabilities of microscopy.[21] THP-1 cells are commonly used to model plateletleukocyte interactions *in vitro*, including previous work which has examined coculture with cryopreserved platelets and the effect on phagocytosis and pro-inflammatory signaling.[10,27] In this study, imaging flow cytometry was used to examine the interaction of cryopreserved platelets with monocyte-like cells (THP-1 cell line) *in vitro*.

Methods

Platelet collection and processing

Ethics approval was obtained from the Australian Red Cross Lifeblood Ethics Committee prior to commencement of this study (Johnson 10 052 019). All blood donations were obtained from voluntary non-remunerated donors. Pooled platelet concentrates were manufactured by combining the buffy coats from four whole blood donations. Following centrifugation, the platelets and remaining plasma were diluted with platelet additive solution (PASE, SSP+; Macopharma, Tourcoing, France) to achieve a final supernatant composition of 30% plasma/70% PAS-E and leukoreduced using AutoStop BC filters and stored in ELX 1300 mL PVC bags (Haemonetics, Boston, USA). Platelet components were stored at room temperature (20–24°C) on a platelet agitator (Helmer, Noblesville, USA) until they were frozen. Samples were removed on day 2 (10–15 mL), to establish baseline (pre-freeze, PF) characteristics.

Platelet cryopreservation and thawing

Platelet components were cryopreserved within 24 hours of sampling at day 2–3 post-collection by addition of 27% (wt/vol) DMSO in 0.9% saline (Sypharma, Dandenong, Australia) to achieve a final concentration of 5–6%. The platelet concentrate was then transferred to a PVC bag (Macopharma) and centrifuged at 1350 *g* for 10 minutes. Following centrifugation, the majority of the DMSO containing supernatant was removed and the pelleted platelets gently resuspended in the residual supernatant (~25 mL) before being frozen at −80°C.

Platelets were thawed in a 37°C water bath for approximately 4 minutes. Once thawed, the components were transferred to a platelet agitator for 20 minutes then reconstituted in approximately 280 mL resuspension solution. A solution of 30% plasma/70% SPP+ was used to reconstitute the platelets to minimize differences between the PF and post-thaw (PT) supernatant, as detailed previously.[9] Thawed components were sampled (10–15 mL) immediately after resuspension.

Cell culture

THP-1 cells (TIB-202, passage 6, American Type Culture Collection; ATCC) were cultured in Complete Media consisting of RPMI 1640 (Thermo Fisher Scientific, Waltham, USA) without phenol red, containing 10% fetal bovine serum (FBS) and 100 U/mL penicillin/streptomycin (both from Thermo Fisher Scientific). A new vial of THP-1 cells (frozen at passage 9) was thawed for each replicate and cultured at a concentration between $2\text{-}8 \times 10^5$ cells/mL in an incubator (37°C, 5% CO_2, Thermo Fisher Scientific). The media was refreshed, and THP-1 cell viability was assessed by cell counter (Countess 2, Thermo Fisher Scientific) every 2–3 days until expanded sufficiently for testing. THP-1 cells were then concentrated by centrifugation at 125 *g* for 5 minutes before resuspension at a concentration of 1×10^6 cells/mL in fresh Complete Media containing acid citrate dextrose solution (ACD, 10 mM trisodium citrate, 11 mM glucose, 8 mM citric acid). THP-1 cells were tested prior to co-incubation with platelet concentrates to ensure a consistent phenotype (Figure S1).

Platelet co-incubation with THP-1 cells

Resting PF platelets were activated by 10 μM TRAP-6 or 5 μM calcium ionophore A23187 (both from Sigma-Aldrich, St. Louis, USA) for 20 minutes at 37°C.[9,28] Platelet stimulation using TRAP-6

or A23187 has previously been used to generate subpopulations of aggregatory and procoagulant platelets respectively.[9,20,22,28,29] In this study, TRAP-6 and A23187 stimulated platelets were used to assist the classification of cryopreserved platelets into subpopulations. THP-1 cells were analyzed as a platelet free control or co-incubated with 5% (v/v) of PF, PT, TRAP-6 stimulated or A23187 stimulated platelet concentrates in 12 well culture plates (Corning, New York, USA) in an incubator (37°C/5% CO_2). Separate wells were co-incubated for either 1 or 24 hours. Following co-incubation, the supernatant was transferred by pipette to 5 mL tubes. Any adherent THP-1 cells were removed by incubation with 1 × TrypLE Express (no phenol red, Thermo Fisher Scientific) for 7 minutes in an incubator before it was combined with the corresponding sample tube containing non-adherent THP-1 cells.

Staining of platelet concentrates and platelet-THP-1 cell co-culture for analysis by imaging flow cytometry

To assess the platelet phenotype, independent of co-culture, approximately 2×10^6 platelets were diluted in 0.1 µm filtered Tyrode's buffer and stained with Apotracker (ApoT)Green (Biolegend, San Diego, USA), CD42b-PE-Dazzle 594 (HIP-1; Biolegend), CD62P-PE (AC1.2; BD Bioscience, Franklin Lakes, USA), CD61-APC (Y2/51; Dako, Glostrup, Denmark) for 15 minutes. ApoT is a calcium independent stain similar to AnnexinV, which is used to detect phosphatidylserine exposure.[30] The presence of the calcium chelator ACD in the co-culture made staining with Annexin-V impractical.

To examine the phenotype of platelets adhered to THP-1 cells, the co-culture was stained with ApoT-Green, CD42b-PE-Dazzle 594 (HIP-1), CD62P-PE (AC1.2), CD45-APC-H7 (2D1, BD Bioscience) and CD61-APC (Y2/51, Dako). THP-1 cell phenotype were assessed by staining with CD11b-FITC (ICRF44), CD11a/CD18-APC (m24), CD45-APC-H7 (2D1, BD Bioscience), CD162-PE (KPL-1) and CD61-PE-Dazzle 594 (VI-PL2, Biolegend). Samples were incubated for 20 minutes in the dark prior to fixation with 1% paraformaldehyde for 20 minutes at 4°C. Samples were concentrated by centrifugation at 125 *g* for 5 minutes after which the supernatant was removed and the pellet resuspended in 50 µL sterile filtered PBS (Sigma-Aldrich).

Platelets and THP-1 cell/platelet co-cultures were analyzed using an Amnis Imagestream^X Mark II multi-spectral imaging flow cytometer (IS^x, EMD Millipore, Seattle, USA) equipped with one charge coupled device (CCD) camera and two excitation (488 nm: 100 mW, 642 nm: 150 mW) lasers and a side scatter (785 nm: 2 mW) laser. Laser voltages were set to maximize fluorescent signal while avoiding oversaturation. Data were acquired using INSPIRE acquisition software based on area, aspect ratio and CD61 or CD45 positivity to identify platelets and THP-1 cells, respectively. Data collection gates were set by using the relevant unstained and fluorescence minus one (FMO) controls. Single stained controls were employed to generate compensation matrices. Data were collected at a low flow rate with the camera set to 60× magnification with 10 000 platelet or THP-1 cell events collected.

All imaging flow cytometry data was analyzed in IDEAS v6.2 (Luminex Corporation, Austin, USA). Fluorescence minus one (FMO) controls were employed to refine gates and exclude false positive events. The thresholds for intensity masks were set with reference to unstained, positive and FMO controls. Individual platelets and THP-1 cells were identified based on area and aspect ratio in conjunction with the cell specific markers (Figure 1A,B). Masks and the feature finder were then used to identify platelet subpopulations and platelet-THP-1 cell aggregates. A custom platelet mask based on CD61-APC fluorescence data was combined with the Spot Count feature to enumerate the number of platelets bound to each THP-1 cell (Figure 1C,D). Coincidental events (platelet and THP-1 cells in the same field but not attached), platelets occluded from view by other cells and extracellular vesicle-THP-1 cell aggregates were excluded from the data analysis. Platelet subpopulations were classified using CD42b-PE-Dazzle 594, CD62P-PE and ApoT-Green fluorescence (Figure 1E). Intensity masks were sequentially applied in order to classify platelets based on positive or negative antibody/dye binding. Boolean logic was applied to classify each platelet into a subpopulation based on current literature.[20,22–25]

Supernatant collection and analysis

Co-culture supernatant was isolated by three consecutive centrifugation steps at 125 *g* for 5 minutes, 1600 *g* for 20 minutes and 12 000 *g* for 5 minutes to remove most THP-1 cells, platelets and EVs, respectively. The supernatant was frozen at −80°C for later analysis. The concentration of soluble factors was measured using the following commercially available enzyme-linked immunosorbent assay (ELISA) kits: IL-1β, IL-6, IL-8, IL-10 and TNF-α (R&D Systems Minneapolis, MN, USA). All samples were tested in duplicate and measured against a standard curve, as per the manufacturer's instructions.

Statistical analysis

Statistical analysis was conducted using GraphPad Prism 9.4.1 (GraphPad Software, Inc.). Repeated measures one-way analysis of variance (ANOVA) was used to identify differences between groups. Post hoc Bonferroni's multiple comparisons tests were performed to determine differences between the specific groups. Pearson r-test was used to determine the correlation between data points. A p-value of less than 0.05 was considered to be statistically significant.

Results

Platelet activation and cryopreservation significantly altered the abundance of receptors involved in leukocyte adhesion on the surface membrane of platelets (Figure 2). The majority of PF and TRAP-6 platelets were positive for GPIbα (Figure 2A). In comparison, cryopreservation and stimulation with A23187 reduced the proportion of platelets positive for CD42b binding by approximately 10% and 15% respectively (Figure 2A). Only 10% of PF platelets exhibited CD62P binding, compared to approximately 50% of PT and 80–90% of TRAP-6 and A23187 stimulated platelets (Figure 2B). Further, PF and TRAP-6 platelets displayed negligible ApoT staining compared to PT and A23187, where 60–80% of the platelets had externalized phosphatidylserine (Figure 2C).

Platelet activation can occur through several pathways, generating phenotypically distinct subpopulations (Figure 3).[22,26,29] In this study, we defined platelet subpopulations based on morphological and surface phenotype characteristics as described in previous literature.[20,22,26,29,31] Platelets were classified as resting (ApoT-/CD42b+/CD62P-), activated (ApoT-/CD42b+/CD62P+) or procoagulant (ApoT+/CD42b+/CD62P+). In line with previous publications, TRAP-6 and A23187 stimulation were used to generate reference populations of activated and procoagulant platelets, respectively.[22,26,29,31]

The majority of PF platelets exhibited a resting phenotype, with a small proportion (~10%) of activated platelets (Figure 3A,B). In comparison, cryopreservation resulted in the generation of three platelet subpopulations (Figure 3A,C). As

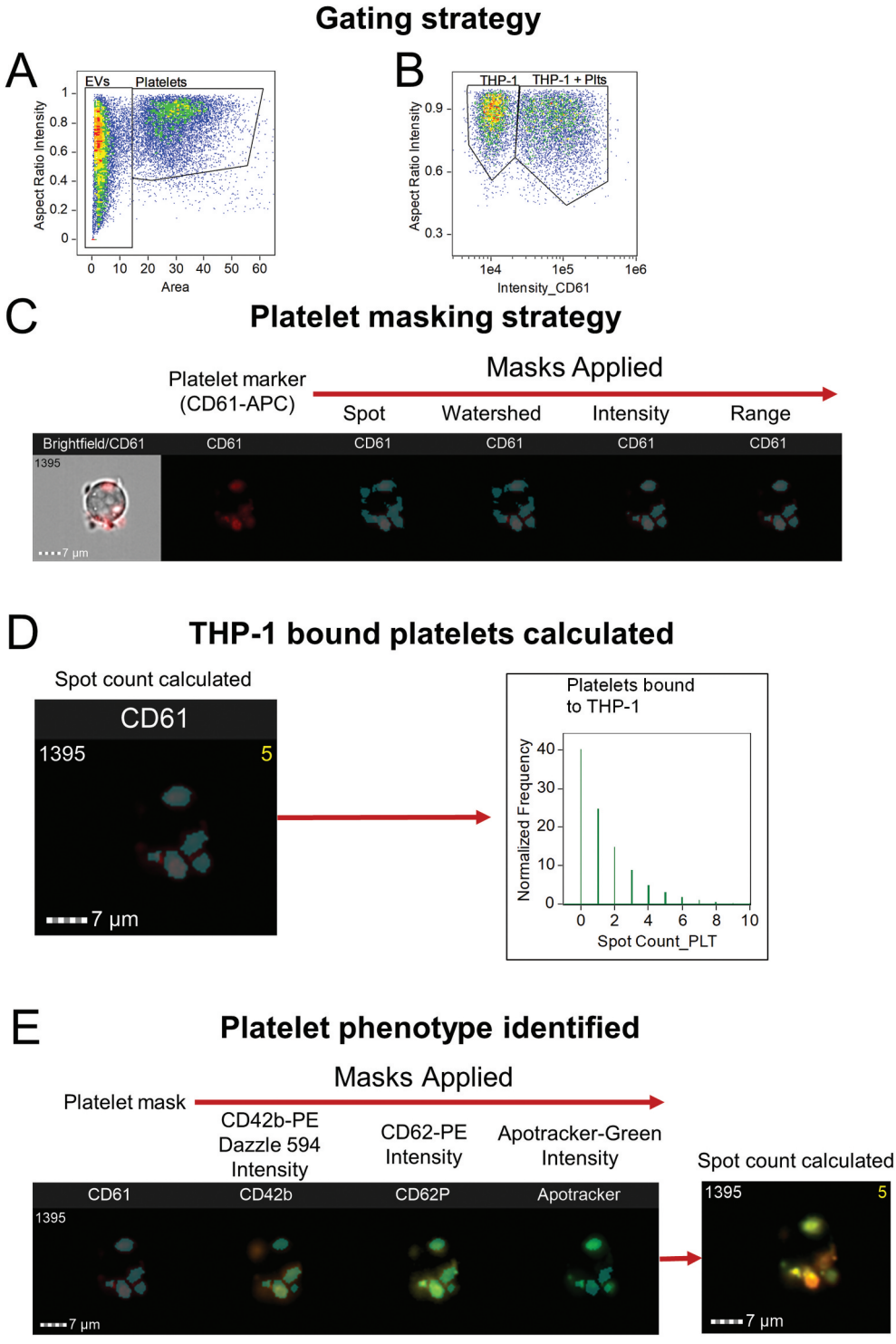

Figure 1. Gating and analysis strategy for enumerating and phenotyping platelet-THP-1 cell aggregates using imaging flow cytometry and IDEAS v6.2 analysis software. Gating strategies are shown for analysis of (A) platelets and (B) platelet-THP-1 cell co-culture. (C) A fluorescence mask based on CD61-APC was created to enumerate the number of platelets bound to THP-1 cells. The platelet mask was created by sequentially applying Spot, Watershed, Intensity and Range masks (blue highlighted area) to reduce the fluorescence background and allow the analysis of individual platelets. (D) The platelet mask was combined with the Spot Count feature to enumerate the number of platelets bound to each THP-1 cell. (E) The phenotype of bound platelets was analyzed based on the staining pattern of CD42b-PE Dazzle 594, CD62P-PE and Apotracker-Green fluorescence images with Intensity masks. The number of platelets counted in an image is indicated by the yellow number in the top right-hand corner of the image. EVs = extracellular vesicles.

expected, PT components contained activated and procoagulant platelets, as well as a population of platelets with a phenotype that did not align with the major subpopulations (ApoT+/CD42b+/CD62P-). Stimulation with either TRAP-6 or A23187 resulted in most platelets exhibiting an activated or procoagulant phenotype, respectively (Figure 3A,D,E).

Platelet binding to THP-1 cells was defined by single color staining, assessing the pattern of CD61, CD42b, CD62P and phosphatidylserine (ApoT) on THP-1 cells. As expected, THP-1 cells were negative for these markers in the absence of platelets (Figure 4A), and suspension of platelets alone in Complete Media did not affect the platelet phenotype (Figure S2). Platelet-THP-1

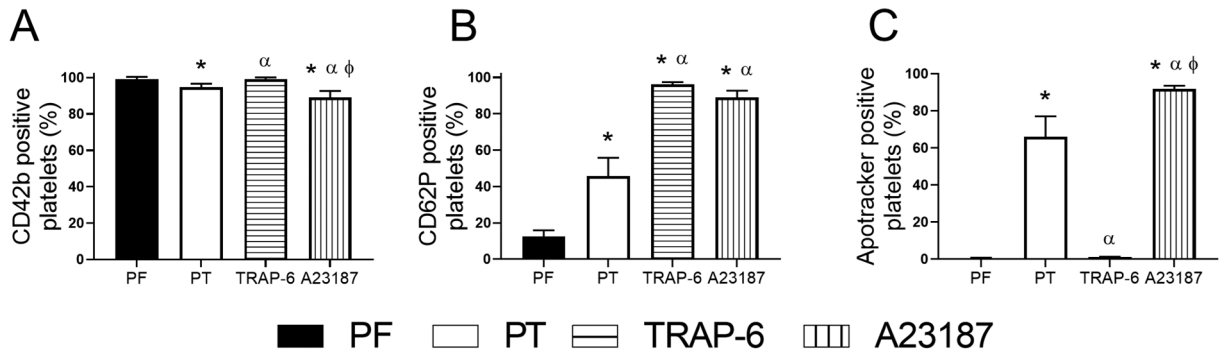

Figure 2. Cryopreservation and activation differentially alters the abundance of leukocyte adhesion receptors on the surface membrane of platelets. Platelets were stained with Apotracker-Green, CD62P-PE, CD42b-PE-Dazzle 594 and CD61-APC and analyzed by imaging flow cytometry at 60x magnification. Platelet events were gated based on area, aspect ratio and CD61-APC fluorescence parameters. Pre-freeze (PF, ■), post-thaw (PT, □), TRAP-6 stimulated (▤) or A23187 stimulated (▥) platelets were identified using the CD61-APC mask, and the proportions of (A) CD42b-PE-Dazzle 594, (B) CD62P-PE and (C) Apotracker-Green positive cells were determined. Data represent the mean + standard deviation (error bars, $n = 6$ in each group) of 10 000 gated platelet events. Significance was determined by one-way ANOVA. * = $p < .05$ compared to PF. α = $p < .05$ compared to PT. Φ = $p < .05$ compared to TRAP-6.

cell aggregates were observed in all treatment groups following co-culture (Figure 4B–E). However, the proportion of platelets binding to THP-1 cells was significantly higher following thawing (PT), and stimulation by TRAP-6 and A23187, compared to PF (Figures 4B–E and 5). Assessment of single markers demonstrated a similar pattern of staining for CD61, CD42b and CD62P, suggesting that the majority of the bound platelets expressed these three markers. Specifically, 40% of THP-1 cells stained positive for CD61, CD42b or CD62P following co-incubation with PF platelets compared to 60% in PT and 80% of TRAP-6 and A23187 stimulated samples (Figure 5A–C). The ApoT staining profile showed a different pattern between the treatment groups, with THP-1 cells demonstrating negligible staining following incubation with PF and TRAP-6 stimulated platelets. In contrast, ApoT+ platelet binding was significantly elevated in THP-1 cells incubated with PT (~40%) and A23187 (~90%) platelets (Figure 5D). In general, the staining pattern of the THP-1 cells mirrored the phenotype of the platelets prior to co-culture.

Characterization of the phenotype of the platelets bound to the THP-1 cells was extended to assess the triple marker staining pattern to allow classification of the specific subpopulations bound (Figure 6). Most PF platelet-THP-1 cell aggregates exhibited an activated phenotype with a smaller proportion of resting platelets bound (Figure 6A,B). In contrast, most PT platelets adhered to THP-1 cells exhibited an activated or procoagulant phenotype (Figure 6A,C). In comparison, following TRAP-6 or A21287 stimulation, most adherent platelets exhibited an activated or procoagulant phenotype, respectively (Figure 6A,D,E).

The density of platelets bound to THP-1 cells was categorized for each treatment group. A classification of low, medium or high binding density was indicative of 1–2, 3–5 or 6–10 platelets bound to THP-1 cells, respectively (Figure 7A,B). The majority of THP-1 cells co-incubated with PF platelets displayed low platelet binding (40%, Figure 7A), with very few THP-1 cells having>2 platelets bound. A similar proportion of low density binding was observed in the PT platelets, although 15 and 5% of the THP-1 cells had medium and high density of platelets bound, respectively. In contrast, approximately 60% of THP-1 cells incubated with TRAP-6 or A23187 treated platelets displayed medium or high density adherence. The presence of CD62P on the platelet surface was associated with the density of platelets adhered to the THP-1 cells. Specifically, low CD62P positivity was correlated with low numbers of platelet adherence to THP-1 cells (Figure 7C; $r = -0.86$). In contrast, a higher proportion of CD62P positive platelets was positively associated with a medium ($r = 0.93$) and high ($r = 0.88$) density of platelets adhered per THP-1 cell (Figure 7D,E).

Upon PLA formation, platelets are capable of altering the soluble factor release and surface phenotype of leukocytes.[10,18] Analysis of the supernatant from the platelet-THP-1 cell co-culture revealed that the concentration of IL-1β, IL-6, IL-8, IL-10 and TNF-α were all below the limit of detection of the assays following 1 or 24 hours of incubation (data not shown). Similarly, the percentage of THP-1 cells binding CD162, CD11a, LFA-1 and CD45 did not vary between treatment groups (Figure S3), indicating their activation status did not change as a result of platelet binding.

Discussion

Platelet cryopreservation is currently under clinical evaluation as it offers potential improvements in shelf-life and hemostatic function compared to conventional storage.[3,5] However, cryopreservation also alters the surface abundance of a range of receptors associated with immune function and leukocyte adhesion,[9,10] which may influence the risk of adverse events. In this study, we demonstrate that cryopreservation-induced changes in receptor abundance increases the occurrence of platelet adhesion to monocyte-like cells (THP-1 cells) compared to PF components, *in vitro*. Further, we demonstrate that cryopreserved platelets are phenotypically heterogenous, and only CD62P positive platelets have the capacity to bind to THP-1 cells.

Platelet subpopulations can be generated by various physiological and non-physiological stimuli.[22,29] In line with previous publications, TRAP-6 or A23187 stimulation predominantly produced subpopulations of activated and procoagulant phenotype platelets, respectively.[22,29,32] Compared to agonist stimulation, cryopreservation induced platelet activation resulted in a heterogenous mix of activated and procoagulant platelets along with a novel subpopulation. The mechanism by which cryopreservation induces platelet activation is still under investigation; however, preliminary work suggests that increased cytosolic calcium concentrations may be important.[33] The question remains as to why cryopreservation does not activate platelets uniformly. Notably, recent work in washed platelets has suggested that individual platelet characteristics, such as the number of mitochondria, may influence whether a platelet progresses from an activated to a procoagulant phenotype.[23]

A

	Platelets classified by subpopulation							
	Dye / Fluorescent Antibody (%)							
	Apotracker-				Apotracker+			
Treatment	CD42b+ CD62P-	CD42b+ CD62P+	CD42b- CD62P-	CD42b- CD62P+	CD42b+ CD62P-	CD42b+ CD62P+	CD42b- CD62P-	CD42b- CD62P+
Pre-freeze	**84 ± 6**	11 ± 3	0 ± 1	0 ± 0	0 ± 0	1 ± 1	0 ± 0	0 ± 0
Post-thaw	7 ± 2*	**34 ± 16***	1 ± 1	0 ± 0	**44 ± 11***	**20 ± 2***	5 ± 4*	0 ± 1
TRAP-6	4 ± 1*α	**95 ± 2*α**	0 ± 0	0 ± 0	0 ± 0	1 ± 0	0 ± 0	0 ± 0
A23187	0 ± 0*α	3 ± 1*αΦ	2 ± 0	1 ± 1	7 ± 3*αΦ	**79 ± 6*αΦ**	3 ± 2	5 ± 2*αΦ
Platelet Subpopulation	**Resting**	**Activated**	**NC**	**NC**	**Novel**	**Procoagulant**	**NC**	**NC**

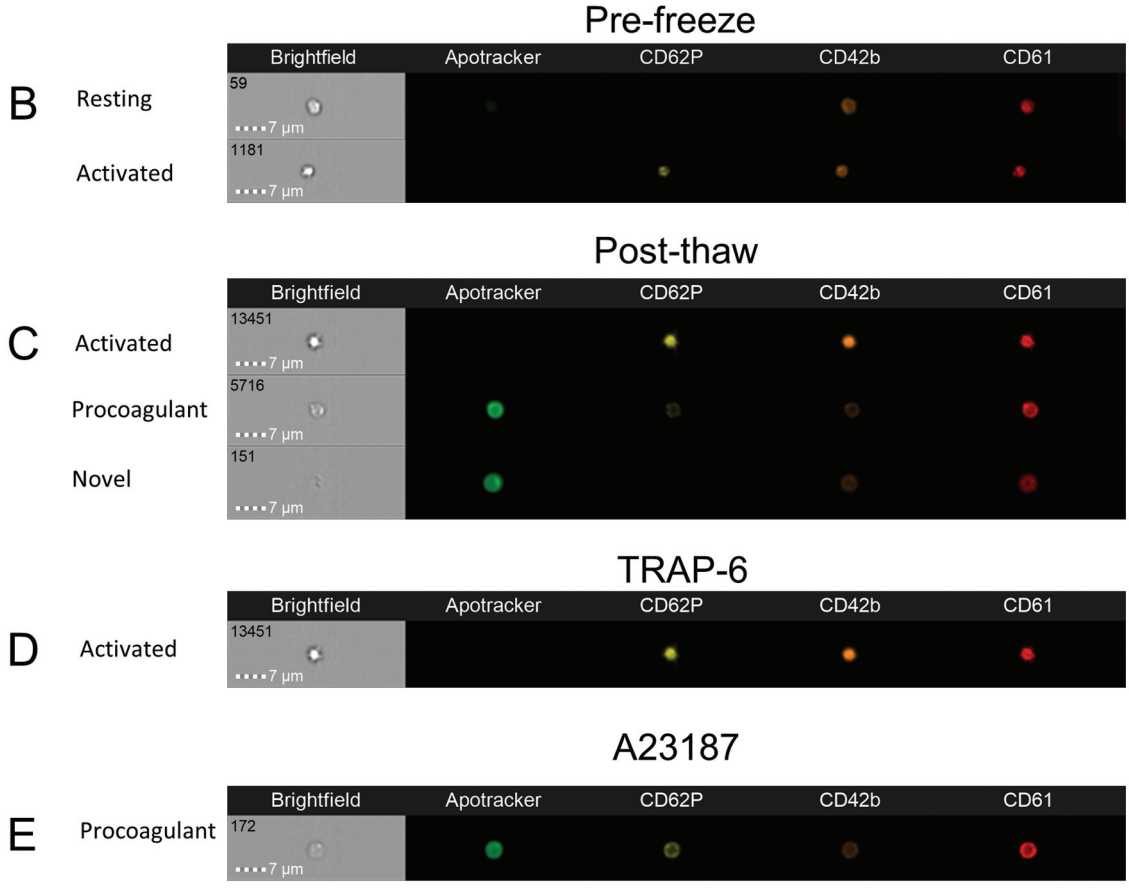

Figure 3. Cryopreservation and activation promote the formation of different subpopulations. Platelets were stained with Apotracker-Green, CD62P-PE, CD42b-PE-Dazzle 594 and CD61-APC and analyzed by imaging flow cytometry at 60× magnification. (A) The staining pattern of the three markers was used to classify platelets into the indicated subpopulations, where the proportions of each phenotype within that treatment group are displayed as a percentage of the total platelet population, with the greatest proportion presented in bold text. Representative brightfield and fluorescence images are shown for each of the major subpopulations in (B) pre-freeze, (C) post-thaw, (D) TRAP-6 stimulated or (E) A23187 stimulated platelets. Data represent the mean ± standard deviation (n = 6 in each group) of 10 000 gated platelet events. Significance was determined by one-way ANOVA comparing each treatment within the platelet subpopulations. * = $p < .05$ compared to PF. α = $p < .05$ compared to PT. Φ = $p < .05$ compared to TRAP-6. NC = no classification.

Previous work has highlighted that platelet activation, which leads to P-selectin exposure, increases the likelihood of PLA formation,[16,18,19,21] a finding reflected in this study. Cryopreservation caused an increase in CD62P positive platelets, consequently, it is unsurprising that an increase in platelet-THP-1 cell aggregates was evident compared to PF samples. Interestingly, the number of THP-1 cells bound by the "activated" subpopulation was comparable between PF and PT samples. As such, the increase in platelet-THP-1 cell aggregates post-thaw was primarily attributable to an increase in procoagulant platelets. However, PT platelets formed platelet-THP-1 cell aggregates at a significantly lower rate than TRAP-6 or A23187 samples. A potential explanation of this finding is that approximately half of PT platelets were CD62P negative.

Approximately half of the cryopreserved platelets exhibited a phenotype (ApoT+/CD42b+/CD62P-) that does not match current literature classifications. In this study, we observed that this novel subpopulation showed phenotypical similarities to procoagulant platelets, including a balloon-like morphology and ApoT binding on the surface membrane. However, this population did not form platelet-THP-1 cell aggregates. The clinical significance

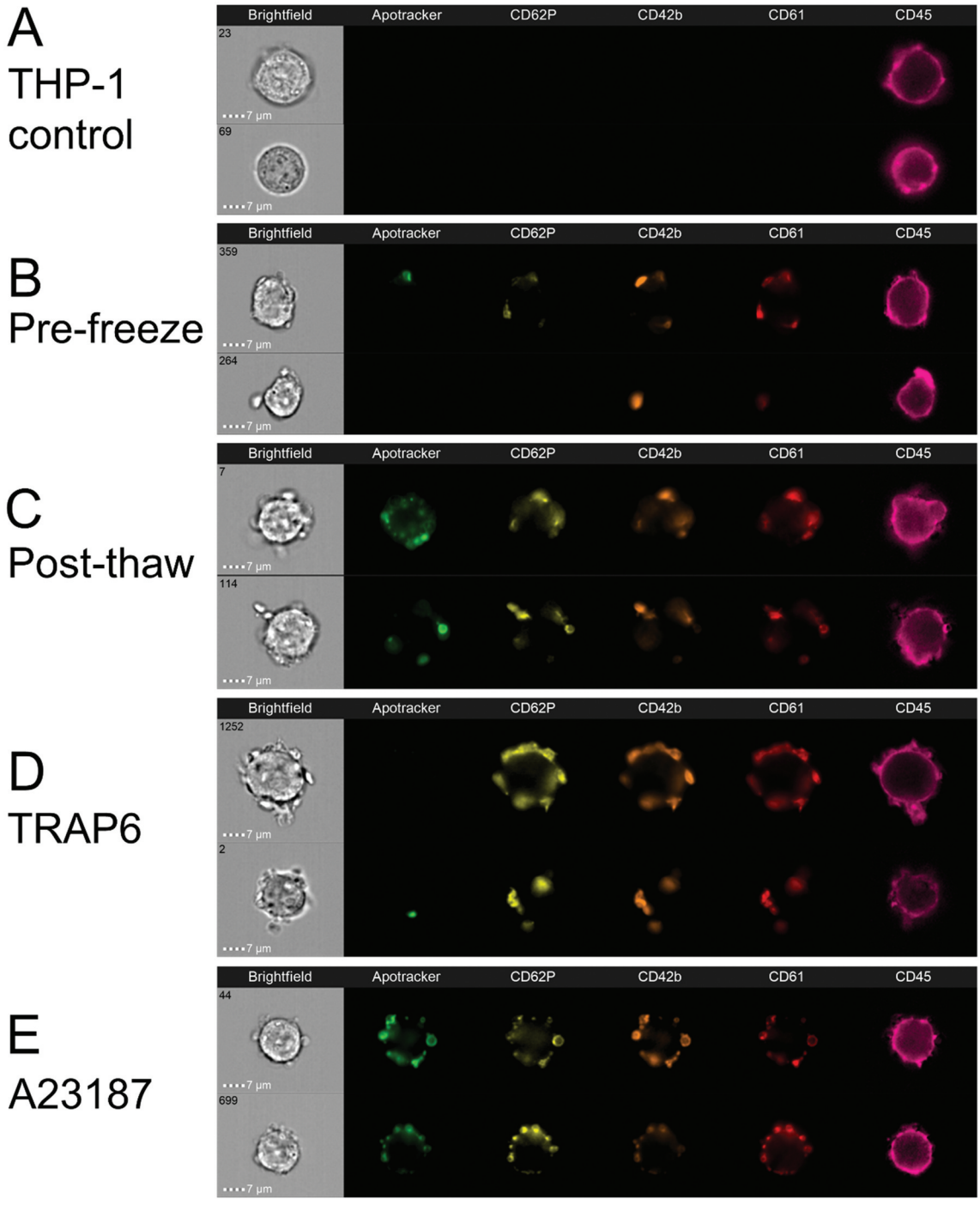

Figure 4. Visualization and quantitation of platelet-THP-1 cell aggregates using imaging flow cytometry. THP-1 cells were examined (A) alone or following co-incubation with (B) pre-freeze, (C) post-thaw, (D) TRAP-6 stimulated or (E) A23187 stimulated platelets for 1 hour. The platelet-THP-1 cell co-culture was stained with Apotracker-Green, CD62P-PE, CD42b-PE Dazzle 594, CD61-APC and CD45-APC-H7 and analyzed by imaging flow cytometry at 60x magnification. Representative brightfield and fluorescence images are shown for each group.

of this subpopulation is currently unclear. But it would be of interest to examine if this subpopulation is present in circulation under physiological or pathological conditions, as well as being generated in platelet components stored under different blood bank conditions, including during cold-storage or after pathogen inactivation.

It also remains to be determined how this novel phenotype arises. Preliminary work from our lab suggests a potential link between procoagulant platelets, the novel subpopulation and extracellular vesicle release, which are all increased post-thaw.[22,34] The novel subpopulation has been shown to exhibit decreased internal complexity, suggesting that degranulation has occurred, exposing P-selectin, which is subsequently lost from the surface membrane.[22] Notably, the concentration of P-selectin in the supernatant is significantly increased post-thaw, which supports this finding.[33,35] The mechanism by which P-selectin is removed from the surface membrane is immunologically relevant, as evidence suggests only the dimeric membrane-bound isoform is pro-inflammatory.[36] The dimeric form of P-selectin is found on the surface of extracellular vesicles, whereas proteolytic cleavage or release of soluble P-selectin generally occurs as a monomer.[37] Although the concentration of extracellular vesicles increases

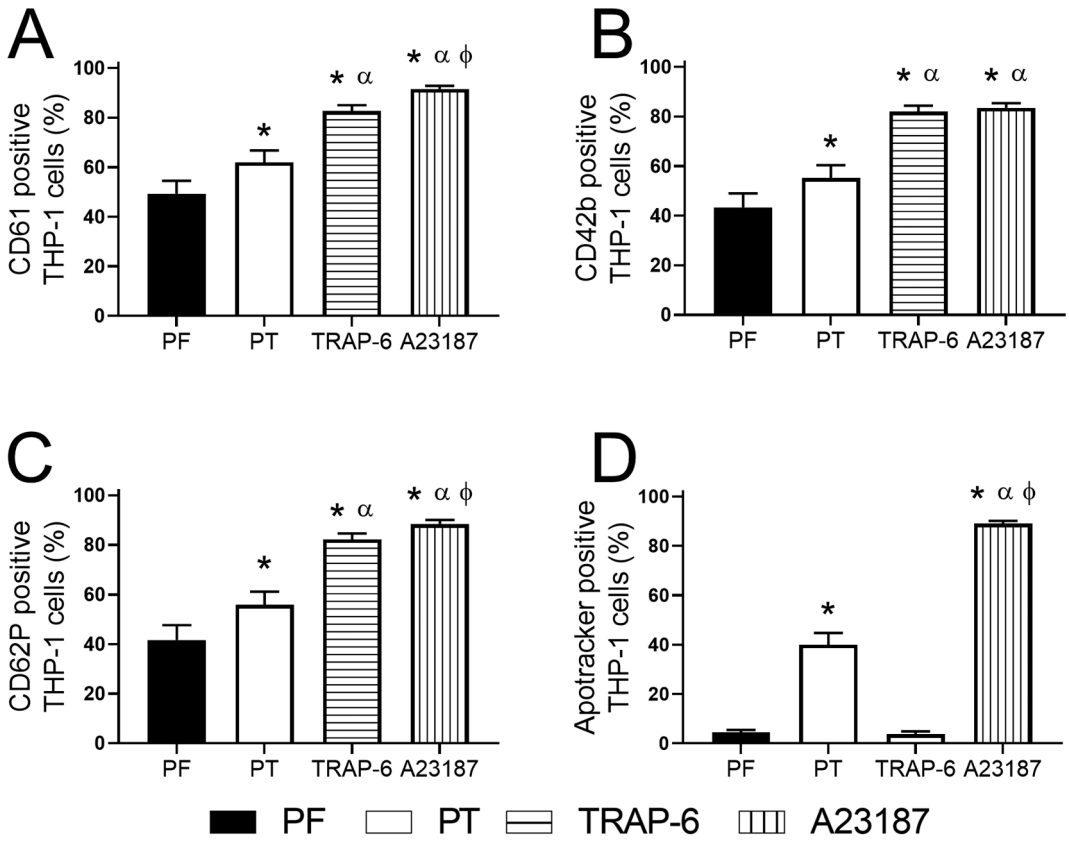

Figure 5. Platelet cryopreservation and activation increases the abundance of platelet-THP-1 aggregates. THP-1 cells were co-cultured with pre-freeze (PF,▬), post-thaw (PT,▭), TRAP-6 stimulated (▭) and A23187 stimulated (▭) platelets for 1 hour. The platelet-THP-1 cell co-culture was stained with Apotracker-Green, CD62P-PE, CD42b-PE Dazzle 594, CD61-APC and CD45-APC-H7 and analyzed by imaging flow cytometry at 60x magnification. The percentage of THP-1 cells positive for (A) CD61-APC, (B) CD42b-PE Dazzle 594, (C) CD62P-PE and (D) Apotracker-Green was measured for each treatment. Data represent the mean + standard deviation (error bars, n = 6 in each group) of 10 000 gated THP-1 cells events per sample. Significance was determined by one-way ANOVA comparing each treatment. * = $p < .05$ compared to PF. α = $p < .05$ compared to PT. Φ = $p < .05$ compared to TRAP-6.

significantly post-thaw,[6] they are not CD62P positive.[34] While further investigation is required, the available information suggests that cleavage of P-selectin from the surface of procoagulant platelets may result in the CD62P negative subpopulation.

The formation of PLAs occurs primarily through P-selectin and PSGL-1,[12,15,16,27] platelets are capable of leukocyte adherence through a range of other receptors, including ICAM-2 and GPVI.[15,17] Notably, ICAM-2 and GPVI are reduced following cryopreservation.[9,22,33] This may help to explain how a proportion of resting platelets (CD62P negative) were observed to generate platelet-THP-1 cell aggregates in the PF group, while the CD62P negative PT platelets did not bind to the THP-1 cells. Alternatively, P-selectin can be shed from the platelet surface membrane shortly after binding to leukocyte PSGL-1,[38] which may account for the observed reduction in CD62P binding.

The post-transfusion recovery of cryopreserved platelets is approximately half that of freshly collected components.[39] Interestingly, the survival time (~7 days) of the cryopreserved platelets that remain in the circulation is lower than freshly isolated platelets, but better than conventionally stored components.[8,39] Platelet clearance from the circulation is mediated through phagocytosis by leukocytes or ingestion by hepatocytes, depending on the surface phenotype. Non-antibody-mediated platelet clearance is typically facilitated by hepatocytes/macrophages in the liver and leukocytes in the circulation.[15,18,40–42] Platelet ingestion/phagocytosis in the liver occurs when GPIbα is clustered and becomes deglycosylated exposing β-N-acetylglucosamine (β-GlcNac).[40–42] Recent work has suggested that cryopreservation does not increase GPIbα clustering[43] and β-GlcNac exposure is decreased PT compared to fresh platelet components.[10] Alternatively, platelets that are dual positive for P-selectin and phosphatidylserine can be phagocytosed by circulating leukocytes.[9,10,15,18,44] P-selectin facilitates platelet-leukocyte adhesion while phosphatidylserine provides a secondary signal, triggering internalization and phagocytosis.[10,15,18,44] In this study, we observed that both activated and procoagulant platelets form aggregates with THP-1 cells. However, only procoagulant platelets exhibited phosphatidylserine exposure, which is required for phagocytosis.[15,18,44] Further, the blockade of phosphatidylserine, using annexin-V has been shown to reduce phagocytosis of cryopreserved platelets by macrophage-like THP-1 cells, *in vitro*.[10] Therefore, subpopulations may influence cryopreserved platelet survival post-transfusion.

The classification of platelets into their subpopulations is complicated by similarities in morphology and phenotype.[20,22] Due to technical limitations, a marker for the activated conformation of GPIIb/IIIa (PAC-1) could not be included in the imaging flow cytometry panel. Consequently, aggregatory platelets could not be definitively identified. Instead, platelets with a comparable morphology and phenotype to the aggregatory subpopulation (ApoT-/CD42b+/CD62P+) were classified as "activated platelets." Likewise, a marker for platelet apoptosis was not included, as recent work suggests that this subpopulation is not present in cryopreserved platelet components.[22]

A

	%THP-1 cells bound by platelet subpopulations							
	Dye / Fluorescent Antibody (%)							
	Apotracker-				Apotracker+			
Treatment	CD42b+ CD62P-	CD42b+ CD62P+	CD42b- CD62P-	CD42b- CD62P+	CD42b+ CD62P-	CD42b+ CD62P+	CD42b- CD62P-	CD42b- CD62P+
Pre-freeze	**24 ± 5**	**43 ± 7**	2 ± 1	1 ± 1	0 ± 0	4 ± 1	0 ± 0	2 ± 1
Post-thaw	2 ± 2*	**44 ± 8***	1 ± 1	2 ± 1	2 ± 1	**42 ± 5***	3 ± 1	4 ± 3
TRAP-6	4 ± 1*	**83 ± 2*α**	0 ± 0	3 ± 0	0 ± 0	18 ± 6*α	0 ± 0	0 ± 0
A23187	0 ± 0*	1 ± 0*αΦ	0 ± 0	0 ± 0	0 ± 0	**89 ± 1***αΦ	0 ± 0	2 ± 1
Platelet Subpopulation	**Resting**	**Activated**	**NC**	**NC**	**Novel**	**Procoagulant**	**NC**	**NC**

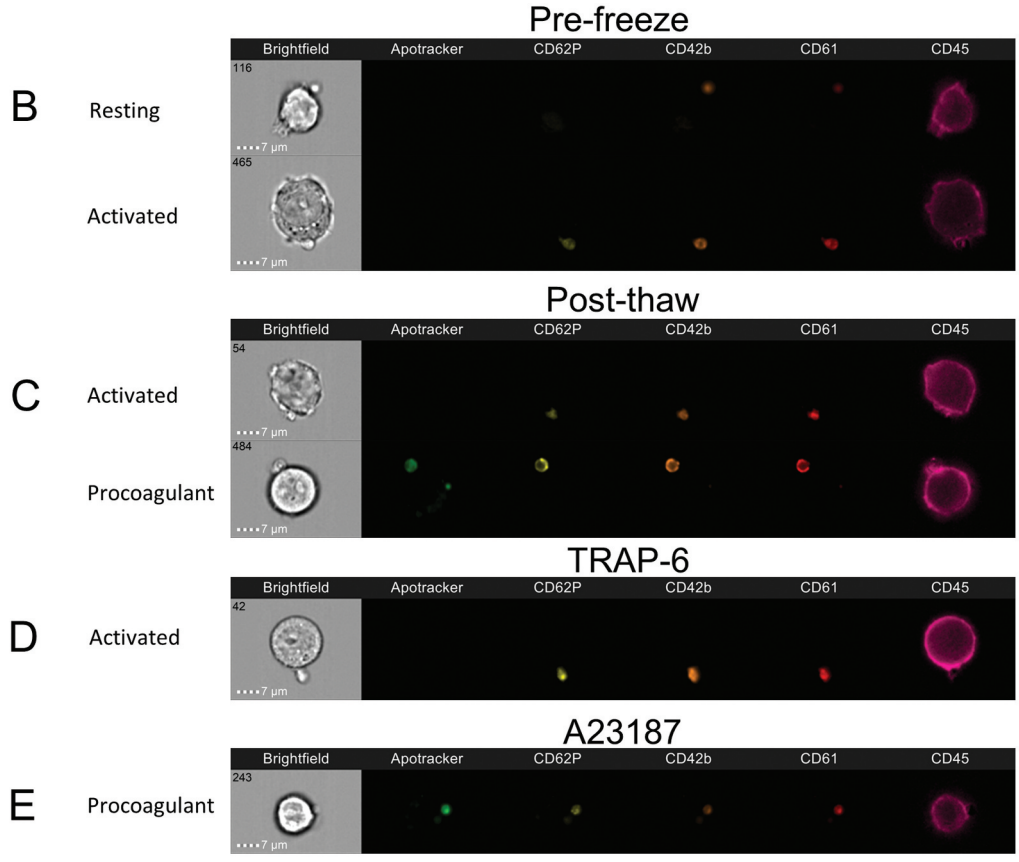

Figure 6. Cryopreservation increases the formation of procoagulant platelet-THP-1 cell aggregates. THP-1 cells were incubated with pre-freeze, post-thaw, TRAP-6 or A21387 platelets for 1 hour. The platelet-THP-1 cell co-culture was stained with Apotracker-Green, CD62P-PE, CD42b-PE Dazzle 594, CD61-APC and CD45-APC-H7 and analyzed by imaging flow cytometry at 60x magnification. (A) The staining pattern of the triple platelet markers was used to classify platelets into the indicated subpopulations. The proportion of THP-1 cells in each treatment group bound by platelets of a certain subpopulation are presented, with the major subpopulations highlighted in bold. The number of platelets bound to THP-1 cells was calculated post-collection using the mask and feature function. Representative brightfield and fluorescence images of (B) pre-freeze, (C) post-thaw, (D) TRAP-6 stimulated or (E) A21387 stimulated platelets bound to THP-1 cells are shown. Data represent the mean ± standard deviation (error bars, n = 6 in each group) of 10 000 gated THP-1 events per sample. Significance was determined by one-way ANOVA comparing the mean of each treatment within each platelet subpopulation. * = $p < .05$ compared to PF. α = $p < .05$ compared to PT. Φ = $p < .05$ compared to TRAP-6. NC = no classification.

While many novel findings are presented, further work is required to fully understand the potential clinical consequences. In this study, the thawed platelets were resuspended in 30% plasma/70% PAS-E to match the pre-freeze composition. However, it is likely that the thawed platelets will be reconstituted in 100% plasma in the clinical setting.[3] While the ratio of plasma can influence the concentration of soluble factors, our prior work has reported a negligible impact on the activation status (CD62P, phosphatidylserine externalization) of post-thaw platelets,[45] which is what appears to be mediating PLA formation. In addition, this study utilized THP-1 cells, which are commonly used to model platelet-leukocyte interaction *in vitro*,[10,27,46] however, these results may not be representative of platelet-leukocyte interactions following co-culture with primary monocytes or neutrophils or leukocytes *in vivo*. Further investigation of plateletneutrophil interactions would be informative due to their dual roles in platelet clearance and adverse events including TRALI.[15]

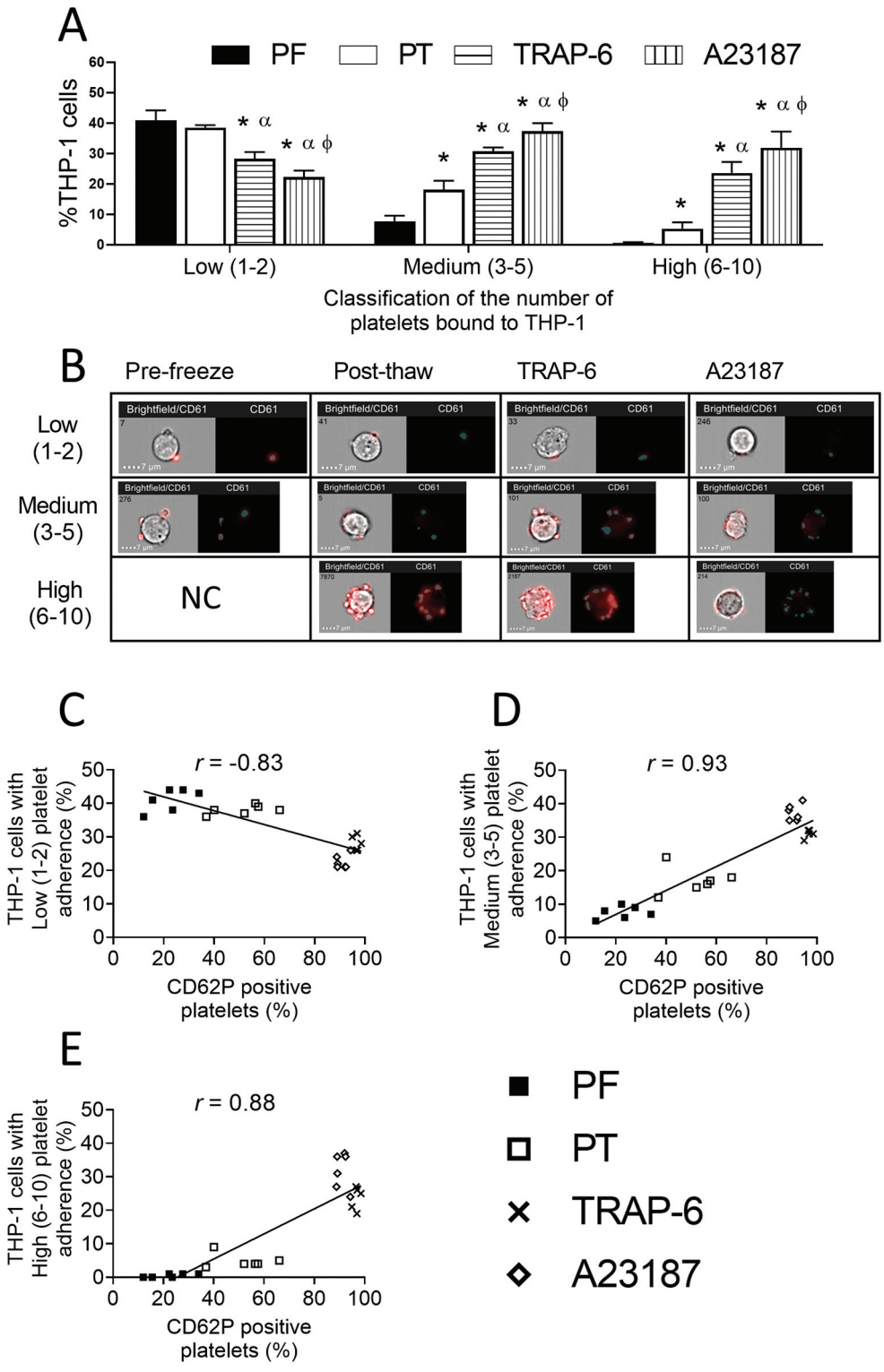

Figure 7. Platelet cryopreservation and activation increases the density of platelets present within the platelet-THP-1 cell aggregates. THP-1 cells were incubated with pre-freeze (PF,■), post-thaw (PT,□), TRAP-6 (▭) or A23187 (▥) platelets for 1 hour. The platelet-THP-1 cell co-culture was stained with Apotracker-Green, CD62P-PE, CD42b-PE Dazzle 594, CD61-APC and CD45-APC-H7 and analyzed by imaging flow cytometry at 60x magnification. (A) THP-1 cells were classified into low (1–2), medium (3–5) and high (6–10) categories based on the number of bound platelets, shown in brackets, and the proportion of platelets in each category was calculated. (B) Representative images of brightfield/CD61-APC composites next to CD61-APC fluorescence images with platelet masks applied (blue dots). The correlation between the percentage of platelets positive for CD62P-PE for PF (■), PT (□), TRAP-6 (x) or A23187 (◇) and the proportion of THP1 cells exhibiting (C) low, (D) medium and (E) high numbers of platelets bound was calculated. Data represent the mean ± standard deviation (error bars, n = 6 in each group) of 10 000 gated THP-1 events per sample or individual data points from each replicate. Significance was determined by one-way ANOVA comparing the mean of each treatment within each platelet subpopulation. * = $p < .05$ compared to PF. α = $p < .05$ compared to PT. Φ = $p < .05$ compared to TRAP-6. NC = no classification. Pearson r-test was used to determine correlation between data points. Linear regression was used to apply a line of best fit.

Despite the differences in the number of platelet-THP-1 aggregates between samples, we observed no measurable difference in THP-1 cell surface phenotype or soluble factor release. Consequently, it is difficult to determine the potential immunomodulatory effects of the observed increases in cryopreserved platelet-THP-1 cell aggregates. However, our findings align with the current literature, which propose that platelets alone, are insufficient to induce the more severe adverse transfusion events such as TRALI.[12,47] Research suggests that TRALI requires the pre-priming of patient leukocytes, caused by an underlying co-morbidity or traumatic injury, which are then further activated by the transfusion of platelet components containing immunomodulatory soluble factors.[12,47] However, cryopreserved platelet components contain lower concentrations of pro-inflammatory cytokines, but more extracellular vesicles than fresh components.[6,9,34] It is important to note that transfusion of cryopreserved platelets is reported to be safe and effective in the context of controlled trials and clinical use.[3–5,48]

Cryopreserved platelets offer advantages over conventionally stored platelets, enhancing hemostatic function *in vitro* and extending the shelf-life, enabling supply to remote medical settings.[6,7] While further work is required, our findings contribute to the body of pre-clinical data being assembled to aid in the interpretation of clinical data as more becomes available.

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Disclosure statement

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Data availability statement

The datasets generated during and/or analyzed during the current study are available from the corresponding author on reasonable request.

Supplementary material

Supplemental data for this article can be accessed online at https://doi.org/10.1080/09537104.2023.2206916

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