Use of Specialized Pro-Resolving Mediators to Alleviate Cold Platelet Storage Lesion

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BACKGROUND: Cold-stored platelets are an attractive option for treatment of actively bleeding patients due to a reduced risk of septic complications and preserved hemostatic function compared to conventional room temperature–stored platelets. However, refrigeration causes increased platelet activation and aggregate formation. Specialized pro-resolving mediators (SPMs), cell signaling mediators biosynthesized from essential fatty acids, have been shown to modulate platelet function and activation. In this study, we sought to determine if SPMs could be used to inhibit cold-stored platelet activation.

METHODS: Platelets were collected from healthy donors (n = 4-7) and treated with SPMs (resolvin E1 [RvE1], maresin 1 [MaR1], and resolvin D2 [RvD2]) or vehicle (VEH; 0.1% EtOH). Platelets were stored without agitation in the cold and assayed on Days 0 and 7 of storage for platelet activation levels using flow cytometry, platelet count, aggregation response using impedance aggregometry, and nucleotide content using mass spectrometry.

RESULTS: Compared to VEH, SPM treatment inhibited GPIb shedding (all compounds), significantly reduced both PS exposure and activation of GPIIb/IIIa receptor (RvD2, MaR1), and preserved aggregation response to TRAP (RvD2, MaR1) after 7 days of storage. Similar to untreated cold-stored platelets, SPM-treated samples did not preserve platelet counts or block the release of P-Selectin. Nucleotide content was unaffected by SPM treatment in cold-stored platelets.

CONCLUSIONS: SPM treatment, particularly Mar1 and RvD2, led to reduced platelet activation and preserved platelet function after 7 days of storage in the cold. Future work is warranted to better elucidate the mechanism of action of SPMs on cold platelet function and activation.

Transfusion of platelets is vital for restoring hemostatic balance after traumatic injury, and the administration of platelet products, along with red blood cells and plasma, for damage control resuscitation correlates with lower rates of mortality.[1–5] Platelets are commonly stored at room temperature but are restricted to no more than 5-7 days of use due to the risk of bacterial contamination. Cold storage of platelets is highly desirable because of its decreased risk of bacterial growth and contamination, preservation of platelet metabolism, and superior hemostatic function over conventional standard-of-care room temperature storage.[6–15] Currently, the U.S. Food & Drug Administration allows the use of 72-hour cold-stored platelets for treatment of actively bleeding patients, and the U.S. Army has obtained a variance to store platelets under refrigeration for up to 14 days.[16] Despite the clinical benefits, there are also several drawbacks associated with cold storage. Of note is the increased risk of visible aggregate formation, or clumping, in the bag that is believed to occur due to activation of platelet GPIIb/IIIa receptor and platelet binding to plasma fibrinogen.[17] Use of a platelet additive solution (PAS) during cold storage to dilute plasma fibrinogen can minimize clumping but

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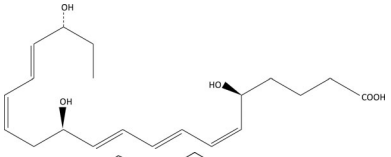
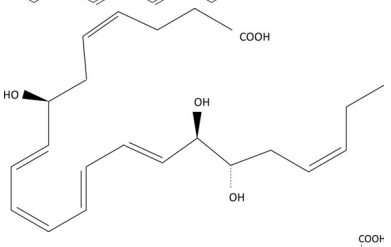
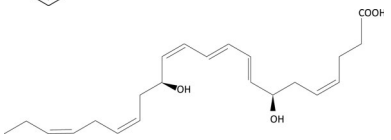
TABLE 1. Properties of SPMs used in study

Compound used	Chemical structure	Molecular formula	Description
Resolvin E1 (RvE1)		$C_{20}H_{30}O_5$	Lipid mediator derived from eicosapentaenoic acid (EPA)
Resolvin D2 (RvD2)		$C_{22}H_{32}O_5$	Lipid mediator derived from eicosapentaenoic acid (EPA)
Maresin 1 (MaR1)		$C_{22}H_{32}O_4$	Lipid mediator derived from docosahexaenoic acid (DHA)

does not inhibit activation of P-Selectin and exposure of phosphatidylserine (PS) during long-term storage.[17]

Specialized pro-resolving mediators (SPMs) are enzymatically derived products of essential fatty acids, such as eicosapentanoic acid (EPA) and docosahexaenoic acid (DHA), that aid in the resolution of acute inflammation.[18] More specifically, SPMs play dual roles acting as both anti-inflammatory and pro-resolving mediators for numerous cell types. SPM activity includes limiting advancement of neutrophil infiltration as well as inducing nonphlogistic apoptosis of macrophages

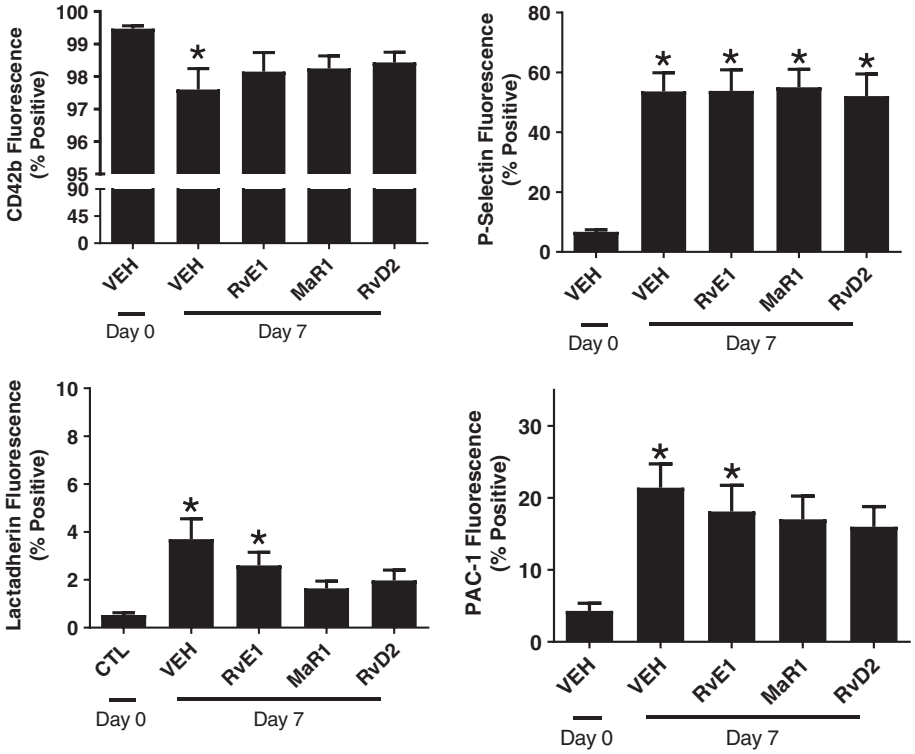


Fig. 1. Platelet activation markers in untreated and SPM-treated cold-stored platelets. Data are means ± SEM for n = 7 donors. * = $p < 0.05$ as compared to Day 0 VEH.

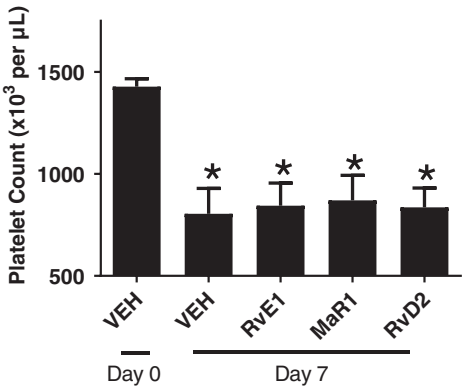


Fig. 2. Platelet count of control and SPM-treated cold-stored platelets. Data are means ± SEM for n = 7 donors. * = p < 0.05 as compared to Day 0 VEH.

during inflammation.[19] Four families of SPMs are known to exist: resolvins, lipoxins, protectins, and maresins.[18] Recent reports have shown that SPMs are capable of differentially regulating platelet function. For example, treatment of platelets with Maresin-1 (MaR1) leads to enhancement of platelet aggregation and spreading and suppression of proinflammatory mediators[20] while resolvin E1 (RvE1) counterregulates platelet P2Y12 signaling and decreases platelet aggregation response to ADP.[21,22] Similar results have been found through the use of resolvin D2 (RvD2).[20]

In this study, we evaluated the use of SPMs in cold-stored platelets. We hypothesized that the presence of SPMs would mitigate cold-stored platelet activation and allow for greater preservation of platelet function. In particular, we evaluated the use of RvD2, MaR1, and RvE1.

MATERIALS AND METHODS

Compounds

RvD2, RvE1, and MaR1 (Table 1) were acquired from Cayman Chemical. SPMs were resuspended in ethanol (EtOH) and stored at −80°C until use according to manufacturer's instructions.

Platelet handling

Apheresis platelets were obtained from healthy volunteers (n = 4 donors) in 100% plasma according to an approved U.S. Army Institute of Surgical Research standard operating procedure. Freshly drawn platelets were aliquotted (10 mL) into 15-mL mini-bags (BSCI). Bags were treated with one of the following compounds for 15 minutes at room temperature: RvD2 (100 nM), RvE1 (100 nM), MaR1 (100 nM), and 0.1% EtOH (vehicle [VEH]). After this incubation period, all bags were placed in the refrigerator (1°C-6°C) for storage. Platelets were assayed on Day 0 (day of collection) and Day 7 of storage unless specified otherwise. Platelet counts were obtained using an ABX Micros60 (Horiba). For hemostatic function analyses, platelets were diluted (to 250×10^3/µL) with ABO-compatible fresh frozen plasma (FFP).

Flow cytometry

To assess platelet activation, 5-µL of apheresis platelets was taken from each sample and diluted in 95 µL of Hank's Buffered Saline Solution (HBSS). Anti-CD42b (BioLegend), anti-CD62P (P-Selectin; BD Biosciences), PAC-1 (BD Biosciences), and lactadherin (PS; Haematologic Technologies), were added to a polystyrene flow tube along with 5 µL of diluted platelet sample. Mixtures were incubated at room temperature for 15 minutes in the dark, diluted with 900 µL HBSS, and analyzed using a FACSCanto II flow cytometer (BD Biosciences). Platelets were gated based on their forward-scatter and side-scatter characteristics as well as their platelet marker positivity. Thirty thousand events were recorded for each sample, and marker-positive gates were set using mouse IgG1 (clones MOPC-21 and MOPC-22; BioLegend) and IgM (clone G155-228; BD Biosciences) isotype controls. Data were reported as percent positivity (%).

Multiplate aggregometry

Platelet aggregation response was assessed using Multiplate impedance aggregometry (Diapharma) as previously described.[12] Briefly, platelets (250×10^3/µL in FFP) were added to $NaCl/CaCl_2$ and incubated for 3 minutes prior to

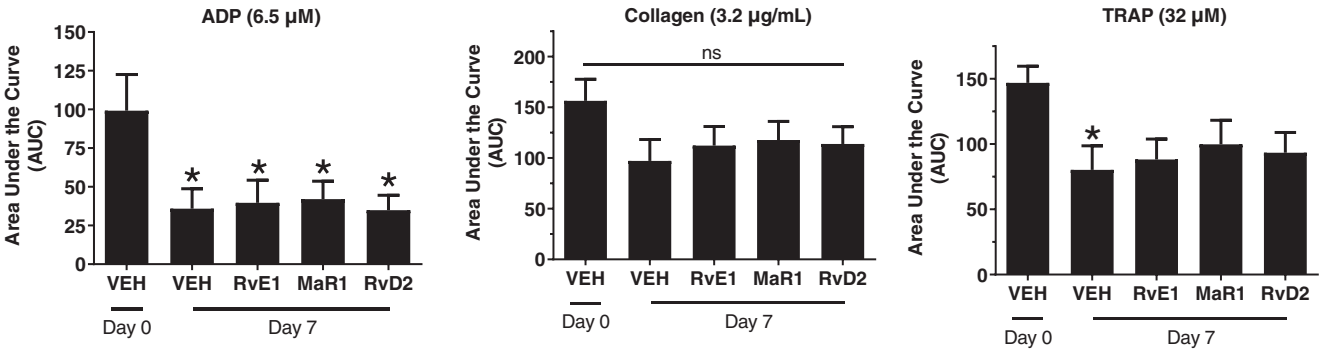


Fig. 3. Aggregation response of SPM-treated cold-stored platelets. Data are means ± SEM for n = 4 donors. * = p < 0.05 as compared to Day 0 VEH.

addition of the following agonists: ADP (6.5 μM; Diapharma), collagen (3.2 μg/mL; Helena Laboratories), and thrombin receptor activating peptide (TRAP, 32 μM; Diapharma). Tests were run for 12 minutes and data were reported as area under the curve (AUC).

Mass spectrometry

Stored samples (Day 7 only) were evaluated for intracellular purine and inositol phosphate levels using liquid chromatography tandem mass spectrometry as previously described.[23] Adenosine and guanosine di- and triphosphate (ADP, ATP, GDP, and GTP), inositol triphosphate (IP3), and inositol monophosphate (IP1) extraction was performed for 100 μL of platelet samples and analyzed using a TSQ Quantiva (Thermo Scientific). Data were reported as $\mu g/10^3$ platelets.

Statistics

Data were reported as means ± SEM. Statistical significance was determined using one-way ANOVA followed by post-hoc Dunnet's test to determine multiple comparisons ($p < .05$).

RESULTS

Resolvins minimize activation of cold-stored platelets

Refrigerated storage of platelets causes cleavage of surface receptor GPIb by metalloprotease ADAM17.[24–26] GPIb receptor expression (Fig. 1) was preserved in all SPM-treated samples compared to Day 0 VEH whereas a significant decrease in GPIb expression was observed in Day 7 VEH ($p = .011$). Increased P-Selectin expression was observed on Day 7 for all samples (VEH and SPMs) with SPMs failing to inhibit activation compared to Day 7 VEH. PS exposure of stored platelets was significantly increased in Day 7 VEH ($p = .0009$, vs. Day 0 VEH) and RvE1 ($p = .043$, vs. Day 0 VEH), but not in MaR1 or RvD2-treated samples. Increased GPIIb/IIIa expression was exhibited in all stored samples (treated and untreated) but only significant for RvE1 ($p = .0161$, vs. Day 0 VEH) and VEH ($p = .0038$, vs. Day 0 VEH).

SPM treatment does not inhibit platelet aggregate formation during storage

Cold storage of platelets leads to an increased occurrence of visible aggregate formation.[27] Platelet counts were determined on Days 0 and 7. After storage, all samples (untreated and treated) showed a significant drop in platelet count (Fig. 2). Along with a decrease in platelet count, we observed an increased incidence of macroaggregates in the bag (data not shown). SPM treatment failed to inhibit aggregate formation as treated samples showed no differences in platelet count from Day 0 VEH control.

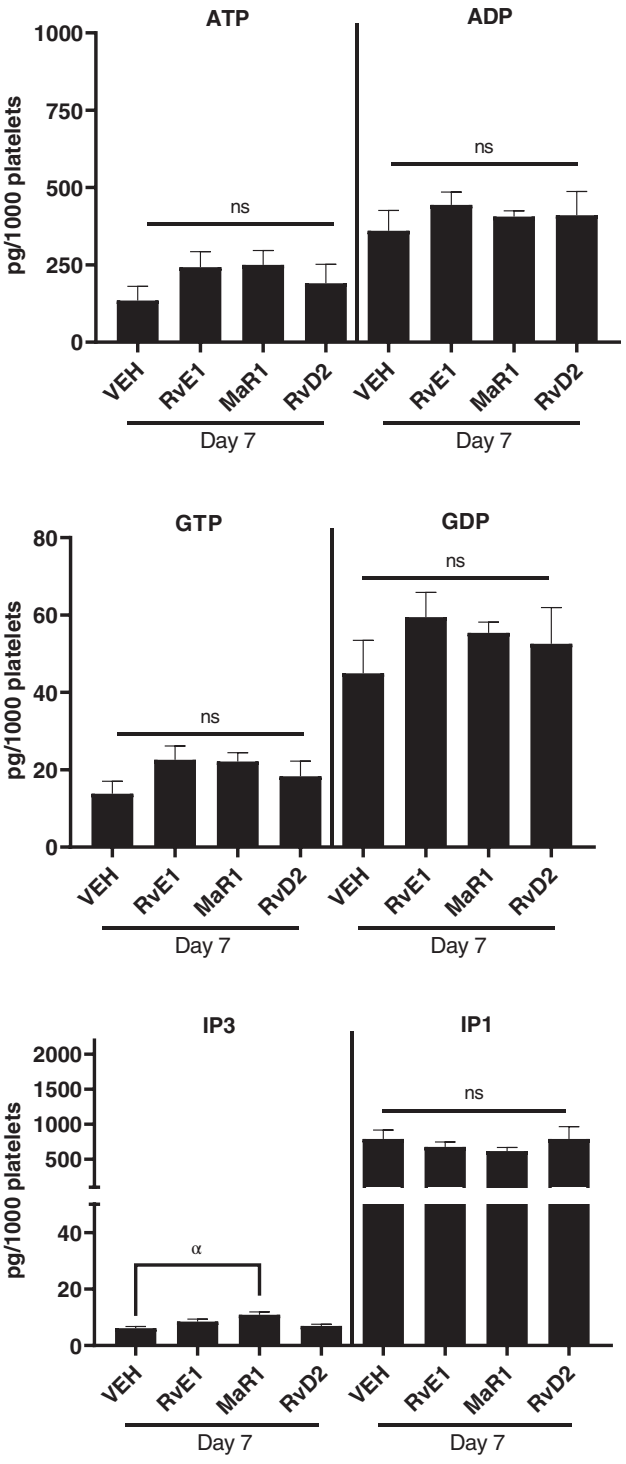


Fig. 4. SPM-treated cold-stored platelet intracellular ATP, ADP, GTP, GDP, IP1, and IP3 levels after 7 days of storage. Data are means ± SEM for n = 4 donors. α = $p < 0.05$ as compared to VEH. Nonsignificant differences are denoted by ns.

SPMs preserve platelet aggregation response

Platelet aggregation, or platelet-platelet binding, at the site of vascular injury is critical for hemostasis.[28] We evaluated

the aggregation response of untreated and SPM-treated platelets after the addition of the agonists ADP, collagen, and TRAP (Fig. 3). All stored samples (treated and untreated) exhibited a significant reduction in ADP aggregation response compared to VEH Day 0. SPM treatment preserved platelet aggregation function to both collagen and TRAP. VEH-treated samples resulted in comparable aggregation function to Day 0 VEH for TRAP only (Day 0: 146.9 ± 12.82%, VEH: 80.25 ± 18.37%, p = .0273).

SPMs preserve nucleotide metabolism in cold-stored platelets

Platelet storage, particularly at room temperature, is known to increase nucleotide metabolism resulting in decreased platelet function.[29] Since SPM treatment led to better preservation of aggregation response and decreased activation compared to VEH, we chose to evaluate levels of intracellular signaling mediators to better understand these differences. We discovered that RVE1, MaR1, and RvD2 treatment caused no significant changes in adenine and guanosine (Fig. 4) di- and triphosphate levels compared to VEH, though MaR1 and RvD2 levels were relatively higher than VEH. IP3 concentration for MaR1-treated samples was significantly higher than that of VEH (MaR1: 10.9 ± 1.0 μg/10^3 platelets, VEH: 6.1 ± 0.6 μg/10^3 platelets, p = .0031). IP1 levels were around 50 times higher than IP3 levels for all samples (VEH and SPM-treated), indicating enhanced metabolic breakdown of IP3.

DISCUSSION

Refrigeration of platelets has gained increased attention as an alternative to conventional room temperature storage due to a decreased risk of bacterial contamination, better preservation of platelet function, and a potentially prolonged shelf life;[6–15] however, cold storage also leads to increased platelet activation.[12,30] In this study, we stored platelets in the cold for 7 days with and without SPMs, compounds that have been proven to reduce inflammation and mitigate platelet function. This work evaluated the utility of three SPMs—RvE1, RvD2, and MaR1—in refrigerated platelet storage. Each of these SPMs have been shown to modulate human platelet function or activation: RvE1 reduces ADP-stimulated P-selectin surface mobilization and blocks platelet actin polymerization[21,22] MaR1 treatment enhances platelet hemostatic function and inhibits the release of inflammatory mediators in thrombin-activated platelets;[20] and RvD2-treated platelets show no difference in platelet aggregation response but dampening of CD62P expression.[20] We discovered that the addition of SPMs (RvE1, RvD2, and MaR1) helped minimize platelet activation and even preserved platelet function during long-term storage.

Platelets play a crucial role in maintaining vascular integrity by initiating hemostasis and promoting repair of vessels to prevent blood loss. The initial capture of platelets during vascular injury is facilitated by platelet receptor GPIb-V-IX and the exposed extracellular matrix proteins, collagen, and von Willebrand Factor.[28,31,32] Dysfunctional GPIb-V-IX, or a lack thereof, can result in unusually large platelets, mild thrombocytopenia, and prolonged bleeding time.[33,34] Metalloproteinase-mediated shedding of GPIbα is a known consequence of the platelet storage lesion, and is associated with accelerated platelet clearance upon transfusion.[26,35,36] Consistent with others,[7,26,37] we observed a significant loss of GPIb expression in Day 7 VEH-treated platelets. Contrastingly, SPM treatment of cold-stored platelets inhibited shedding. This data suggests that long-term cold-stored platelets treated with SPMs may result in better platelet adhesion to damaged endothelium and/or better platelet recovery than untreated cold-stored platelets.

As reported by others, a common disadvantage of storing platelets in the cold is the development of visible aggregates.[27] Aggregate formation is believed to occur after exposure to the cold temperature causes an increase in intracellular calcium, inside-out activation of platelet GPIIb/IIIa receptor, and binding to plasma fibrinogen.[30,38] Inhibiting activation of GPIIb/IIIa should theoretically lessen platelet fibrinogen binding and aggregate formation. In this study, the addition of RvD2 and MaR1 significantly reduced GPIIb/IIIa activation compared to untreated control. Though we did not measure fibrinogen binding, this reduction in GPIIb/IIIa activation did not equate to a significant reduction in aggregate formation as platelet counts still dropped. MaR1 and RvD2 also inhibited platelet PS exposure, though none of the SPMs tested demonstrated inhibition of P-Selectin mobilization. Increased PS exposure is often indicative of platelet apoptosis, dysfunction, and increased clearance in vivo but can also suggest a beneficial procoagulant state as the enhanced surface area allows for binding of coagulation factors.[31,39–44] We postulate that beyond 7 days of storage, SPM-treated (RvD2 and MaR1) platelets may be more protective against apoptosis than untreated cold-stored platelets. Alternatively, SPM-treated platelets could be less procoagulant and therefore less desirable for therapeutic transfusion purposes.

Platelet aggregation response is an intricate process that is largely dependent upon the phosphorylation of various signaling enzymes by ATP and GTP.[45–48] Though no significant differences in intracellular intermediates were observed in our study, ATP and GTP levels of RVE1- and MaR1-treated, cold-stored platelets were higher than VEH suggesting better preservation of nucleotide metabolism in SPM-treated platelets. The higher nucleotide levels also correlated with a preservation of TRAP aggregation response in all SPM-treated platelets. Taken together with previously published data,[20–22] our work further demonstrates the complexity of SPM effects on stored platelet function and activation. Clear differences in the actions SPMs have on cold-stored platelets were observed, and more extensive research is needed to fully understand their potential.

Platelet storage in PAS has gained increasing popularity amongst blood banks due to an enhanced preservation of pH

and reduction of the number of allergic transfusion reactions compared with plasma-stored platelets.[49] Of particular importance for cold storage, utilization of PAS reduces the amount of fibrinogen content available in the bag and significantly reduces the chance of platelet aggregate formation. Though cold storage of platelets in PAS provides better preservation of platelet counts and hemostatic function than storage in plasma[8,17,23,35,50] increased P-Selectin expression and PS exposure remains. Further testing is warranted to determine if the addition of SPMs to a commercial PAS can minimize platelet activation and allow for an extension of refrigerated platelet shelf life (21 days or more).

This study is not without limitations. For instance, we evaluated only resolvin and maresin SPMs (no lipoxins or protectins), and the concentrations (100 nM for each) tested were determined based on previously published works.[20–22] It is highly probable that other categories of SPMs or concentrations could result in significant improvements in functional response and activation markers. Furthermore, we chose to evaluate these samples using mini storage bags. These bags have been utilized by several researchers for similar applications, but are known to exacerbate the platelet storage lesion due to differences in gas exchange.[10,12] Performing these studies in regular sized platelet collection bags may result in improved outcomes. Additionally, this study was only performed out to 7 days of storage. A more pronounced effect of SPMs on platelet function may occur at 10 or 14 days of storage, though platelet count and platelet activation may presumably be impacted. Furthermore, the stability of the SPMs during extended refrigerated storage is currently unknown, and it is possible that the compounds produced transient or transitory effects on platelets that were undetected at our chosen timepoints. Lastly, this study was performed with a small sample size (4-7 donors). While some assays with high donor variability may have presumably benefited from a larger sample size, the number of donors tested was still enough to see significant treatment effects.

Refrigerated platelets are an attractive option for the treatment of actively bleeding patients over conventional room temperature stored platelets due to a reduced risk of septic complications and better hemostatic function, as exhibited in both the in vitro and in vivo settings.[6–15] Targeted therapeutic approaches to extend the shelf life of platelet products without compromising platelet function would expand platelet inventories as well as access to under-served patient populations. The results of this study demonstrate a potential use of SPMs as additives in long-term cold platelet storage. Future work is indicated to determine the benefit of SPMs in refrigerated storage of PAS platelets and whole blood.

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CONFLICT OF INTEREST

The authors have disclosed no conflicts of interest.

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