Characterization of Long-term Cold Storage Effects on Platelet Hemostatic Function and GPIbα Glycan Composition

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ABSTRACT
Platelet transfusion is an important, life saving therapy for treatment of thrombocytopenia and active bleeding related complications. To be clinically effective, transfused platelets must have sufficient ability to mediate hemostatic regulatory function and circulate. Current practices for storing platelet concentrates (PCs) for transfusion therapy are suboptimal. PCs are standardly stored at room temperature (22-24 °C) as cold exposure (4-6 °C) irreversibly compromises platelets, making them susceptible to rapid clearance from circulation. Room temperature (RT) storage increases risks of bacterial contamination, thus PCs are restricted to a shelf life of 5 days leading to PC shortages and waste. Cold storage of platelets is an increasingly desirable alternative as risks of bacterial contamination are reduced and PC shelf life extended with cold storage. This study re-evaluates the consequences of long-term (5 day) RT and cold storage on platelet functionality to better inform development of current platelet storage standards. Specifically platelet hemostatic function and platelet GPIbα receptor glycan exposure, as it relates to cold-induced platelet clearance, was evaluated by flow cytometric analysis and aggregometry at select time points. The results of this study demonstrate cold storage better preserves platelet hemostatic functionality than RT storage, thus supporting cold storage as a desirable condition to optimize for PC storage. Furthermore results from this study support a recently proposed, novel, long-term storage platelet clearance mechanism and provide insight into the GPIbα receptor glycan exposure dynamics of long-term RT and cold stored platelets.
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INTRODUCTION

Platelets

Platelets, or thrombocytes, are small blood cells with big roles in hemostasis. While platelets themselves are anucleate, they are derived from nucleated myeloid cells known as megakaryocytes. Despite being small anucleate cellular fragments, platelets are metabolically active cells containing functional organelles including endoplasmic reticulum, Golgi and mitochondria. Critical to platelet functionality are platelets’ wide array of surface receptors, adhesion molecules, and granules. The primary function of platelets is to bind to damaged blood vessels and aggregate with other platelets, fibrin, and blood cells to form thrombi/clots to plug wound sites. Platelets initiate such aggregation only in states of relative activation as opposed to baseline states of inactivation.

Platelet hemostatic function

Successful platelet plug formation requires a series of events to overcome baseline resistance to platelet activation. This is a non-trivial task as there are physiological regulations in place to prevent unwarranted platelet activation. The endothelial cell monolayer lining blood vessel walls acts as a physical barrier separating platelets from agonists embedded in the sub-endothelial vessel wall, notably collagen (Figure 1A). In addition to serving as a physical barrier, endothelial cells release molecules that inhibit platelet activation, such as PGI₂ and NO (Brass, 2003; Gryglewski et al., 1988; Ignarro, 1989; Murata et al., 1997)(Figure 1A). PGI₂ and NO have a net effect of depressing the high cytosolic levels of Ca²⁺ necessary to promote platelet activation by raising cyclic adenosine monophosphate (cAMP) levels (Brass, 2003; Moncada et al., 1976; Riddell and Owen, 1999). Given these barriers, platelet activation ideally occurs only in the event of substantial injury that penetrates through the endothelial cell monolayer into the vessel lumen.

Platelet activation and plug formation can be thought of as developed over three broad stages: initiation, extension, and perpetuation. We can consider initiation as being the recruitment and activation of the first platelet responders to the site of vascular injury. In the initiation stage, these first responders for the primary
platelet monolayer in direct contact with the wound (Figure 1B). In the extension stage additional platelets are recruited and platelet activation is amplified through the local accumulation of agonists created by the first responding platelets (Figure 1C). Perpetuation refers to the events that ultimately stabilize the platelet plug, critically the formation of an extensive fibrin network throughout the clot (Figure 1D)

**Figure 1: Stages in platelet activation and plug formation.** (A) Prior to vascular injury, endothelial cells restrain platelets from becoming activated by (1) physically barring platelets access to underlying collagen lining the subendothelial vessel wall and (2) secreting inhibitory factors NO and PGI2. (B) Initiation begins at sites of vascular injury; damaged endothelial cells and exposed subendothelial collagen matrices. Binding of platelets to collagen bound vWF mediates platelet rolling. Rolling platelets slow and bind collagen, leading to platelet activation. (C) Extension refers to the recruitment of more platelets to platelet plug formation. Primary monolayer of activated platelets creates a local density of agonists that include ADP, thrombin and TBXA2, activating platelets in the vicinity. (D) Perpetuation refers to the stabilization of the blood clot mediated by formation of a fibrin network around the platelet plug and recruitment of lymphocytes and adhesion to endothelial cells via platelet P-selectin.
Initiation starts at the wound site where damaged endothelial cells in the vessel wall expose subendothelial collagen. Free von Willibrand factor (vWF) in plasma, can bind this exposed collagen (Jaffe et al., 1974; Sakariassen et al., 1979). It is with these collagen bound vWF that platelets, circulating under high shear conditions, interact via their platelet glycoprotein (GP)Ib-IX-V receptor complexes (Clemetson, 2012; Houdijk et al., 1985; Yun et al., 2016). These interactions slow the platelet so it may subsequently bind exposed collagen directly via its GPIa/IIa and GPVI receptors, successfully halting and anchoring the platelet to the damaged vessel wall (Clemetson et al., 1999; Clemetson and Clemetson, 2001; Farndale et al., 2004; Nieuwenhuis et al., 1985). Binding of these collagen receptors has the downstream effect of activating phospholipase C in the platelet (Clemetson and Clemetson, 2001). Activated phospholipase C mobilizes accumulation of Ca\(^{2+}\) from the dense tubular system into the cytosol (G. White, 1972; Ghoshal and Bhattacharyya, 2014). Increase in intracellular Ca\(^{2+}\) over a certain threshold is the critical event that induces platelet activation. High intracellular concentrations of Ca\(^{2+}\) promote activation of several kinases that mediate platelet activation. Several key events are characteristic of this primary platelet activation (1) platelet morphology change, including formation of filopodia extensions to increase platelet surface area, and formation of platelet derived microparticles (PDMP) (2) platelet granule secretion, most critically alpha and dense granule secretion (3) presentation of a procoagulant surface, notably presentation of negatively charged phospholipids including phosphatidylinerse to the outer membrane leaf and (4) activation of G proteins mediating downstream amplification signals and activation of phospholipase A2 (Fox, 1993; Jurk and Kehrel, 2005; Kramer et al., 1993; Lemons et al., 2000; Rendu and Brohard-Bohn, 2001; Sangkuhl et al., 2011).

These primary activation responses of the initiation stage are what enable the following extension stage events. The presentation of the procoagulant surface results in the recruitment of certain coagulation factors to the surface of the activated platelet, enabling catabolism of prothrombin into active thrombin (coagulation factor II), a protease essential in platelet activation amplification (Coughlin, 2005) (Figure 2). Adenosine diphosphate (ADP), adenosine
triphosphate (ATP), and serotonin are among the contents released from platelet dense granules as a result of primary activation (Yun et al., 2016)(Figure 2). Activated phospholipase A2 mediates the release of Arachidonic Acid (AA) through the cleavage of fatty acids, especially phosphatidylcholine and phosphatidylamine. AA is further processed to produce thromboxane A2 (TBXA2) (Parise et al., 1984; Sangkuhl et al., 2011; Stassen et al., 2004) (Figure 2). These agonists — thrombin, ADP, ATP and TBXA2 — are among many others that further amplify platelet activation via their respective platelet G protein coupled receptors (GPCRs), effectively supporting positive feedback loops within a platelet and activating other platelets local to the site of injury (Figure 2). To be more specific, thrombin, through its proteolytic activity cleaves protease-activated receptors (PAR) on the platelet surface, inducing GPCR mediated platelet activation (Brass, 2003). PAR1 mediates human platelet activation at low thrombin concentration, while PAR4 requires high concentrations of thrombin to induce platelet activation (Covic et al., 2000). ADP and TBXA2 act upon platelet P2Y₁ and P2Y₁₂, and TBXA2 GPCRs respectively, promoting platelet activation.

The varieties of platelet GPCRs differ in their respective agonist antigens, potency, and preference for intracellular effector pathways. Some, such as the thrombin receptors (PAR1 & PAR4), TBXA2 receptor and P2Y₁ receptor cause phosphoinositide hydrolysis resulting in a rise in cytosolic Ca²⁺, which we know promotes platelet activation (Brass, 2003; Offermanns et al., 1997). Others such as the ATP and ADP P2Y₁₂ receptor lead to inhibition of adenylyl cyclase and activation of PI3-kinase, lowering concentrations of cAMP (Brass, 2003; Yang et al., 2002).

We lastly have the perpetuation stage where all the earlier platelet activation signals ultimately converge upon a common pathway, the upregulation of functional integrin adhesion receptors. The most important of these is the platelet GPIIbIIIa receptor, which in its activated conformation binds fibrin (Du et al., 1991). Fibrin is a product of fibrinogen catabolism by thrombin. GPIIbIIIa effectively mediates formation of fibrin crosslinks between receptors of different platelets, forming a fibrin network that holds the platelet plug together.
At this stage binding between platelets and other cell types further promotes plug stabilization. Binding between platelets and leukocyte/endothelial cells are primarily mediated by P-selectin (CD62p) adhesion receptors. While P-selectin is not expressed on the surface of resting platelets, upon platelet activation P-selectin is mobilized from alpha-granules to the platelet surface. Surface expressed P-selectin on an activated platelet is thus able to crosslink platelets to leukocytes and endothelial cells, enlargening the clot and promoting better adhesion to the endothelium.

Figure 2: Common platelet activation pathways.

**Platelet storage practices and complications**

Much like whole blood transfusion, the practice of transfusing platelet concentrates (PC) isolated from whole blood is an important and life saving therapy. PCs became widely available in the 1970s with the advent of better methods of their storage and realization of their use in curbing a major cause of death, thrombocytopenia as a result of then-new chemotherapeutic agents (Gardner and Cohen, 1960; Hersh et al., 1965; Jackson et al., 1959). Administration of platelet concentrates derived from healthy donors is to this day one of the only strategies for treating patients with active bleeding and
incidences of thrombocytopenia as a result of hereditary diseases, hematological malignancies and chemotherapy treatments. The largest patient groups that use PCs are those with hematological malignancies (~67%), patients receiving cardiac surgery (~10%) and patients receiving intensive care (~8%) (Cameron et al., 2007; Estcourt, 2014).

Despite the importance of PCs, current practices for storing them are unfortunately less than optimal. Since the 1960’s a standard has been set for platelets to be stored at 22-24 °C. This practice has been instilled primarily due to the fact that platelets, unlike any other blood components, are compromised when exposed to cold temperatures under 15 °C. Cold exposure induces irreversible, biochemical changes that result in platelets becoming vulnerable to rapid clearance from circulation (Hoffmeister et al., 2003a). Storing platelets at room temperature however comes with the risk of bacterial and viral contamination. Studies conducted in the US have found the risk of bacterial contamination associated with platelet transfusion to be one in every 2000-3000 platelet units (Dykstra et al., 1998; Goodnough et al., 2003; Hillyer et al., 2003; Jacobs et al., 2001). Although rare, these contamination events elevate risks of causing sepsis in platelet transfusion recipients. Studies in the last decade have suggested incidences of clinical sepsis in about 1 of 20,000 platelet transfusions and related fatality in about 1 per 60,000 platelet transfusions (Blajchman, 2002; Brecher and Hay, 2003; Hillyer et al., 2003; Ness et al., 2001). In comparison the fatality risks from bacterially contaminated red blood cells are much lower, about 1 per 500,000 transfusions (Blajchman, 2002). These significant differences in bacterial contamination risks between platelet and red blood cell components can be largely attributed to the restriction on platelet storage to be held at room temperature.

To curb risks of bacterial contamination, regulations are in place limiting the storage shelf life of PCs to five days maximum in the US and seven in Europe. PCs are also limited to having short storage shelf lives as they quickly lose hemostatic functionality when stored at room temperature, a phenomena collectively known as ‘platelet storage lesions’ (Bode, 1990). As a result of these short
storage shelf lives, according to a US department of health report, ~13% of all apheresis units (PCs derived from extracorporeal, machine-automated methods) and ~35% of all whole blood derived units (PCs derived from manual cell fractionation of whole blood), totaling to ~50% of all PCs, expire and are discarded before they can ever be used (Whitaker et al., 2011). This leads to PCs constantly being in short supply.

Additionally demand for PCs, unlike other blood components, is currently on the rise worldwide. In the UK there has been a considerable increase in the demand for PCs; approximately 24% increase between 2002 and 2012 (Bolton-Maggs et al., 2012; Taylor et al., 2009). In the US the number of PCs transfused increased by 32% between 2004 and 2011 (Whitaker et al., 2011; Whitaker and Hinkins, 2011). Similar patterns have been seen across Europe and Australia (Estcourt, 2014; Farmer et al., 2013). Such rising demands have been attributed to a combination of factors including an increasing and ageing population and importantly, an increase in the prevalence of hematological malignancies and improved survival rates for such malignancies (Estcourt, 2014).

**Platelet cold storage**

In the 1970’s, Murphy et al.’s and Slichter et al.’s demonstration of the adverse effects of cold temperatures on platelet viability motivated standards for room temperature PC storage still held today (Murphy and Gardner, 1969; Slichter and Harker, 1976). The discovery that discoid platelets underwent extensive morphological changes when exposed to temperatures below 15 °C followed (Zucker, 1954). Later demonstrations that discoid shape predicts good platelet viability (Slichter and Harker, 1976) led to the conclusion that cold-induced shape change was responsible for compromised platelet viability after cold exposure. This theory was accepted for many years until recently with new evidence to suggest discoid shape is not a definite indicator of platelet viability (Berger et al., 1998; Hoffmeister et al., 2003a; Hoffmeister et al., 2003b; Michelson et al., 1996).
A new theory attributing compromised platelet viability to cold-induced reduced circulatory survival of platelets has taken center stage. In 2003 Hoffmeister et al. demonstrated that reduced circulatory survival of short-term (≤2 hours) cold-stored platelets in mice was due to irreversible clustering of glycoprotein Ib alpha (GPIbα), a major platelet adhesion receptor that binds vWF (Hoffmeister et al., 2003a). They elegantly demonstrated that the resultant GPIbα clusters became recognition targets for Kupffer cells via their integrin receptor αMβ2 (complement receptor type 3 (CR3) / Mac-1), leading to platelet phagocytosis and clearance (Hoffmeister et al., 2003a). Hoffmeister et al. was able to further demonstrate that cold-induced changes in glycan composition also mediate platelet clearance. Sialic acid and galactose residues cover β-N-acetylgalactosamine (NAG) residues on most cell surface glycans including those of platelets (Korrel et al., 1988; Tsuji and Osawa, 1987; Tsuji et al., 1983).

Experiments conducted by Hoffmeister et al. indicate αMβ2 recognition of GPIbα clusters on chilled platelets is assisted via lectin-mediated interaction with exposed NAG residues on N-linked oligosaccharides on GPIbα (Hoffmeister et al., 2003b). It seems when platelets are refrigerated, sialic acid residues are removed from the GPIbα ligand-binding domain leading to subsequent exposure and removal of underlying galactose residues and finally exposure of NAG residues underneath (Hoffmeister, 2011). Other studies have followed to support Hoffmeister et al.'s results (Badlou et al., 2006; Josefsson et al., 2005).

More recently a preliminary investigation has revealed the possibility of a second, independent, cold-induced platelet clearance mechanism. Results of this investigation suggest Hoffmeister et al.'s proposed platelet clearance mechanism is primarily relevant to short-term cold storage but that a second, independent mechanism is applied for long-term (>48 hours) cold stored platelets (Rumjantseva et al., 2009). This second proposed mechanism is similarly a lectin-mediated process linked to platelet glycan exposure, with the distinction being the identity of the target glycan and phagocyte. Rumjantseva et al. propose that increased GPIbα galactose exposure with long-term cold storage mediates Ashwell-Morell asialoglycoprotein receptor (Asgr) recognition and hepatocyte mediated platelet phagocytosis. In other words, instead of NAG, galactose is the
target glycan in this second mechanism and the effector cell is a hepatocyte instead of a Kupffer cell.

Figure 3: Proposed mechanism for short-term cold storage induced platelet clearance. Platelet von Willebrand factor vWF factor receptor complexes, particularly GPIbα receptor, are coated in N-linked glycans. Cold exposure induces GPIbα clustering. Short-term (<2 hours) cold exposure induces desialylation and deglycosylation mediated by surface expressed Neuraminidase and Galactosidase, exposing underlying β-N-acetylgalactosamine residues. Clustering of GPIbα and exposure of β-N-acetylgalactosamine initiates platelet phagocytosis via Kupffer cell αMβ2 integrin receptors.

Though the mechanism behind cold-induced desialylation and degalactosylation is not yet fully understood, some studies suggest platelet derived-sialidases and galactosidases, expressed to the cell surface by cold-induced mechanisms, are responsible for the desialylation and degalactosylation respectively, evident in cold stored platelets (Jansen et al., 2012).

While platelet ability to survive in circulation is the primary concern that motivates platelets for transfusion to being stored at room temperature, platelet functionality and reactivity are another matter. At the discovery of adverse effects of cold exposure on platelets in the 1970's however, this distinction was not clear. Until Hoffmeister et al.'s discoveries, a dogma was held that assumed
cold-stored platelets do not circulate because they do not function. This old dogma is being challenged with more recent demonstrations that a platelet’s ability to survive in circulation is entirely separate from its ability to function in hemostasis. However, there is still some controversy surrounding the impact of cold exposure on platelet activation states and reactivity.

Some investigators have reported that refrigeration does not lead to platelet activation. Specifically, studies have demonstrated that cold exposure retards the release of platelet α-granule contents, a platelet activation biomarker, such as β-thromboglobulin (Perez-Ceballos et al., 2004; Sandgren et al., 2006; Snyder et al., 1982). Inversely, some investigators have reported that refrigeration does stimulate platelet activation or relative activation-like states. Investigators have reported increased surface expression of P-selectin following refrigeration, an indication of α-granule release given that P-selectin is a content of such granules (Babic et al., 2007; Leytin et al., 2004).

More investigators now, however, are finding that cold stored platelets show improved reactivity to platelet agonists such as ADP, epinephrine and collagen as compared to room temperature stored platelets (Babic et al., 2007; Becker et al., 1973; Choi and Pai, 2003; Kattlove et al., 1972; Lindenblatt et al., 2005).

If in fact cold stored platelets have superior functionality and hemostatic reactivity than room temperature stored platelets, the disadvantage to cold storing platelets would be the consequence of rapid clearance from circulation.

Some investigators have attempted to tip the scales and develop methods of preventing cold stored platelets from being rapidly cleared from circulation. Hoffmeister et al. demonstrated that galactosylation of exposed NAG residues on platelet glycoproteins, primarily GPIbα, prevented the phagocytosis of chilled murine platelets (Hoffmeister et al., 2003b). Galactosylation of human platelets also prevented platelet phagocytosis after cold storage in an in vitro phagocytic model using a human monocytic leukemia derived cell line (THP-1) (Hoffmeister et al., 2003b). Unfortunately, a following study failed to prevent the accelerated
clearance of refrigerated human platelets by the same galactosylation treatment (Wandall et al., 2008). The discrepancy in these results was likely attributed to the difference in cold exposure time: in the earlier murine study, platelets were exposed to cold for only 2 hours (short-term) whereas in the following human study platelets were exposed to cold for 48 hours (long-term) (Wandall et al., 2008). Preliminary studies, motivated by this discrepancy, have now proposed the existence of a second mechanism that independently mediates clearance of long-term as opposed to short-term platelets (Rumjantseva et al., 2009). However, this new proposed mechanism has yet to be affirmed by other investigators and developed. Additionally, a means of protecting platelets from long-term cold storage induced clearance is yet to be found.

**Characterizing platelet long-term cold storage effects**

My project is motivated by the pressing need to re-evaluate our current platelet storage standards. Particularly I hope to re-evaluate the viability of platelet cold storage by investigating the long-term (5 days) cold storage effects on platelets in conditions more applicable to current standards for PC storage. Many previous investigations have largely investigated the effects of short-term cold storage on platelets. A recently proposed long-term cold storage induced platelet clearance mechanism needs further development and evaluation. Furthermore, many previous investigations that largely inform our understanding of how cold stored platelets are cleared from circulation, have examined platelets in variable media conditions. For example, Hoffmeister et al. examined platelets in artificial buffer suspensions, otherwise known as washed platelets. More relevant however would be to investigate cold storage effects on platelet concentrates derived by similar methods to standard blood transfusion practices and stored in standard blood transfusion media. I thus aim to elucidate the long-term cold storage effects on platelets stored in concentrate, and treated with acid citrate dextrose. To consider the platelet activation status and hemostatic functionality, I will be monitoring platelet activation biomarkers and platelet *in vitro* aggregation ability. To consider the mechanism of cold storage induced platelet clearance I will be examining the relative glycan compositions of platelet GPIbα surface receptors, and Neuraminidase 1 (NEU1) expression.
MATERIAL AND METHODS

Reagents
HEPES Saline (HS) 10mM HEPES, 0.15M NaCl, pH7.4, Components from Sigma; HS-BSA-Azide: HS + 0.35% BSA and 0.05% NaN3, Components from Sigma; HT-BSA: 137mM NaCl, 2.8mM KCl, 1mM MgCl2, 12mM NaHCO3, 0.4mM Na2HPO4, 0.35% bovine serum albumin, 10mM HEPES, 5.5mM glucose, Components from Sigma; Formaldehyde, Ultrapure 04018-1 Polysciences; ADP, ChronoLog 101312; TRAP, Bachem #H-2936.0025; Arachidonic acid, BioData 101297; Collagen, ChronoLog 385; LTA cuvettes, ChronoLog 312; LTA stir bars, Chronolog 311; Eptifibatide, Millenium/Schering Plough SCH 60936; Lactose, S25375, Fisher Science; N-acetyl-D-glucosamine, A13047, Components from Sigma; Triton X-100, 9002-93-1, Sigma-Aldrich; CaCl2, 10043-52-4, Components from Sigma; GPRP, Bachem H-2935.0100; Convulxin, Centerchem #11902-50UG-B; Counting beads, Spherotech RFP-50-5.

Instruments

Antibodies
IgG1-PE, BD Pharmingen 55749; CD42b-PC5, BD Pharmingen 551141; PAC1-FITC, BD Biosciences 340507; AK4 P-CD62P-PE, BD Pharmingen 555524; CD61-PE, Miltenyl 130-081-501; ECL-Fl, Vector Laboratories FL-1141; sWGA-Fl, Vector Laboratories FL-1021S; CD41a-PerCP-Cy5.5, BD custom research conjugate 340981; Neu1 Antibody (F-8) PE, Santa Cruz Biotechnology sc-155824 PE; Annexin V-FITC, BD Biosciences 556419; CD41a-PE, BD Biosciences 555467.

Platelet concentrate preparation
Single-donor platelet concentrates prepared by apheresis are currently the most popular method of preparing platelet concentrates for storage and transfusion. Automated apheresis instruments utilize differential centrifugation to separate platelet rich plasma (PRP) concentrates from residual red cell and plasma components (McLeod et al., 1997). Apheresis platelets are standardly treated
with citric acid, trisodium citrate and dextrose (ACD-A), a mild anticoagulant cocktail. Apheresis product concentrations range between 1000 $\times 10^3$ - 1500 $\times 10^3$ platelets per μL by US standards. To best approximate these platelet storage standards, PCs of $\sim$1000 $\times 10^3$ platelets per μL were procured from single-donor ACD-A treated whole blood (WB) by manual cell fractionation methods. Venous blood was obtained from volunteers by butterfly syringe venipuncture into 10% volume ACD-A vacutainer tubes. Approval for blood drawing was obtained from the Institutional Review Board of Boston Children’s Hospital, and informed consent was approved according to the Declaration of Helsinki. Blood was drawn from healthy volunteers who were free from antiplatelet agents and non-steroidal anti-inflammatory drugs 10 days prior to the donation. The same phlebotomist performed blood draws. WB was "soft spun" at 110g for 12 minutes to procure PRP. PRP (supernatant) was gently aspirated, pooled and aliquoted into polypropylene microcentrifuge tubes. PRP was "hard spun" at 200g for 15 minutes to procure a soft platelet pellet and fractionated platelet poor plasma (PPP). Top 1/3 of supernatant (PPP$_1$) was aspirated, pooled and aliquoted into polypropylene microcentrifuge tubes, as was the next 1/3 of the supernatant (PPP$_2$). Platelet pellets in the remaining 1/3 plasma were gently resuspended on a rotating mixer to procure concentrated PRP (cPRP). Complete blood cell counts of PPP$_1$, PPP$_2$ and cPRP fractions were obtained on a Sysmex XN-1000 Hematology Analyzer. A total of three, separate, single donor PC samples were produced (n=3).

**Platelet concentrate storage**

Matched samples of cPRP were stored at room temperature (RT: 22 °C) and cold temperature (C: 4 °C). To best approximate platelet storage standards, samples were stored in 1.5mL polypropylene tubes in 1 mL aliquots with gentle agitation on rotating mixers. Samples were rewarmed for 15 minutes at 37 °C before in vitro analysis. In vitro analysis was performed on matched samples 2, 24, 72, and 120 hours after storage.
**Light transmission platelet aggregometry**

Light transmission aggregation (LTA) was performed in a Chronolog-Log model 700 aggregometer. cPRP samples, diluted with PPP to \(400 \times 10^3\) platelets/μL, were assessed by LTA 2, 24, 72, and 120 hours after storage with 1/10th volume ADP (20μM), TRAP (20μM), Arachidonic acid (500μg/mL) and Collagen (2μg/mL). A total of three trials were conducted, each trial testing samples from different single donors \((n=3)\).

**Flow cytometric analysis**

Platelets stored for 2, 24, 72 and 120 hours at RT and cold temperature conditions were assessed for expression of certain platelet activation biomarkers (surface P-selectin, GPIbα, activated GPIIbIIIa, platelet derived microparticle (PDMP) expression, and procoagulant platelet surfaces) and cold-induced platelet clearance biomarkers (GPIbα galactose exposure, GPIbα NAG exposure, and platelet Neuraminidase 1 expression) by flow cytometric technique using appropriate fluorescently labeled antibodies. Before antibody labeling of platelets were performed, cPRP smaples were rewarmed for 15 minutes at 37 °C and appropriately diluted with HT-BSA. Samples were fixed with 1% formaldehyde in HEPES saline buffer prior to analysis on a FACSCalibur flow cytometer. A total of three trials were conducted for each assay, each trial testing samples from different single donors \((n=3)\).

**Platelet surface P-selectin, GPIbα, and activated GPIIbIIIa**

Platelets surface expression of P-selectin, GPIbα and activated GPIIbIIIa was assessed by flow cytometric technique. Analysis was performed using the following three-color cocktail of fluorescently labeled antibodies: PE-conjugated anti-CD62P (directed against P-selectin), FITC-conjugated PAC1 (directed against the activated conformation of GPIIbIIIa) and PE-Cy5-conjugated anti-CD42b (directed against GPIbα). Appropriate isotype controls were also performed using the following fluorescently labeled antibody cocktail: Eptifibatide (1.25μg/mL), PE-conjugated anti-mouse IgG1, FITC-conjugated PAC1, and PE-Cy5-conjugated anti-CD42b. Diluted cPRP \((25 \times 10^3 \text{ platelets/μL})\) was simultaneously treated with vehicle (HS), ADP (20μM) or TRAP (20μM) and
labeled with appropriate antibody cocktail for 15 minutes at ambient
temperature. Platelets were gated based on forward scatter, side scatter and
platelet identifier GPIbα expression.

Platelet derived microparticles (PDMPs) and procoagulant platelet surface
Platelet production of Annexin V positive PDMP and presentation of Annexin V
positive procoagulant platelet surface was assessed by flow cytometric
technique. Analysis was performed using the following three-color cocktail of
fluorescently labeled antibodies: FITC-conjugated Annexin V (directed against
aminophospholipids), PE-conjugated anti-CD41a and PE-Cy5-conjugated anti-
CD42b. cPRP (62.5E3 platelets/μL) was treated with fibrinogen inhibiting Gly-
Pro-Arg-Pro (GPRP) before being reacted with vehicle (HS), CaCl₂ (4.7mM) or
Agonist cocktail (ADP (20μM), TRAP (20μM), convulxin (0.2ng/mL), CaCl₂
(4.7mM)) for 15 minutes at 37 °C. This treatment was immediately followed by
antibody labeling with the fluorescent antibody cocktail described earlier for 15
minutes at ambient temperature. Annexin V negative and positive platelets and
PDMP were gated based on forward scatter, platelet identifier GPIbα and CD41a
expression, and Annexin V positivity.

Platelet GPIbα galactose exposure
Surface receptor galactose exposure of platelets was assessed by flow cytometric
technique. Analysis was performed using the following two-color cocktail of
fluorescently labeled antibodies: PE-conjugated anti-CD61, and FITC-conjugated
Erythrina cristagalli (ECL) (lectin directed against galactose). Isotype controls
were also performed using an appropriate inhibiting sugar, Lactose. Diluted
cPRP (10 × 10^3 platelets/μL) was labeled with the antibody cocktail for 15
minutes at ambient temperature. Platelets were gated based on forward scatter,
side scatter and platelet identifier CD61 expression.

Platelet GPIbα β-N-acetyl-glucosamine exposure
Surface receptor β-N-acetyl-glucosamine (NAG) exposure was assessed by flow
cytometric technique. Analysis was performed using the following two-color
cocktail of fluorescently labeled antibodies: PE-conjugated anti-CD61, and FITC-
conjugated succinylated wheat germ agglutinin (sWGA) (lectin directed against NAG). Isotype controls were also performed using an appropriate inhibiting sugar, free NAG in suspension. Diluted cPRP (10 × 10^3 platelets/μL) was labeled with the antibody cocktail for 30 minutes at ambient temperature. Platelets were gated based on forward scatter, side scatter and platelet identifier CD61 expression.

**Platelet surface and total Neuraminidase 1 (NEU1) expression**
Surface and total expression of Neuraminidase 1 (NEU1) was assessed by flow cytometric technique. Analysis was performed using the following two-color cocktail of fluorescently labeled antibodies: PE-conjugated anti-NEU1, and PerCP-Cy5.5-conjugated anti-CD41a. Appropriate isotype controls were also performed using the following fluorescently labeled antibody cocktail: PE-conjugated anti-mouse IgG1 and PerCP-Cy5.5-conjugated anti-CD41a. Diluted cPRP (62.5 × 10^3 platelets/μL) was simultaneously treated with vehicle (HS) or 1% Triton (cell permeablizer) and labeled with appropriate antibody cocktail for 1 hour at ambient temperature. Platelets were gated based on forward scatter, side scatter and platelet identifier CD41a expression.

**Data processing and analysis**
Data was processed, graphed and statically analyzed (Two-way-ANOVA) with the use of Graphpad Prism 5.0. Titration curves produced in the development of flow cytometric glycan exposure detection assays and Neuraminidase 1 expression detection assays were fitted using Graphpad Prism 5.0 non-linear regression analysis. Specifically a one-site, specific binding, non-linear fit was utilized with the constraint of Kd being shared across all data sets for a given antibody/lectin.

**RESULTS**

**Development of flow cytometric glycan exposure detection assays**
Recent studies have demonstrated cold-induced platelet clearance is attributed to rapid phagocytosis by Kupffer cells and hepatocytes (Hoffmeister et al., 2003a; Rumjantseva et al., 2009). Crucially, there is evidence to suggest that lectin-
mediated interaction of exposed N-acetyl-glucosamine (NAG) and galactose residues on clustered platelet GPIbα receptors mediate recognition and clearance of cold-stored platelets by Kupffer cells and hepatocytes respectively (Hoffmeister et al., 2003a; Rumjantseva et al., 2009) (Figure 3). Though a clear mechanism behind cold-induced platelet galactose and NAG exposure is still lacking, current understanding suggests platelet refrigeration induces removal of sialic acid residues from the GPIbα ligand-binding domain leading to subsequent exposure and removal of underlying galactose residues and finally exposure of NAG residues underneath (Hoffmeister, 2011). Monitoring relative galactose and NAG exposure over a long-term platelet storage time course may provide more insight into the kinetics of these glycan exposure mechanisms that may inform the larger platelet clearance mechanism. To this effect, and to determine the reproducibility of other studies findings in conditions that better approximate standard platelet storage practices, I am interested in characterizing the differences in galactose and NAG exposure of platelets stored long-term in room temperature (RT; 22 °C) and cold (4 °C) temperature conditions. I thus necessarily developed flow cytometric assays for detecting platelet surface beta-galactose and NAG exposure.

In developing the two glycan exposure detection assays the following considerations were made: (1) deciding effective platelet concentrations for use in assay (2) deciding a platelet identifying/gating antibody (3) optimization of galactose/NAG specific lectin working concentrations (4) deciding antibody staining incubation-times and (5) optimization of inhibiting sugar for development of an effective isotype.

Platelet working concentrations a few fold lower than concentrations used in previous GPIbα monitoring assays were adopted for these assays under the rational that our residues of interest are components located on GPIbα.

While anti-GPIb is a standard platelet-gating antibody, anti-CD61 was opted for use, as there was concern regarding the possibility that anti-GPIb might sterically inhibit our lectins from accessing glycans on GPIbα. Appropriate
effective concentrations of anti-CD61 for use as a platelet-gating antibody were adopted from assays previously developed utilizing this antibody as a platelet identifier.

*Riccinus communis* -I (RCA-I) and *Erythrina cristagalli* (ECL) lectins specifically bind beta-galactose residues and have been used for the purpose of monitoring beta-galactose exposure on platelets in past studies (Hoffmeister et al., 2003a; Hoffmeister et al., 2003b; Korrel et al., 1988; Tsuji and Osawa, 1987). Succinylated wheat germ agglutinin (sWGA) specifically binds NAG and has been used for monitoring NAG exposure on platelets in past studies (Hoffmeister et al., 2003a). Fluorescein conjugated ECL (ECL-FL) and Fluorescein conjugated sWGA (sWGA-FL) were thus chosen as the galactose and NAG detecting lectin for development of respective lectin exposure detection assays.

In developing the isotypes for these assays, it was recognized that an ideal isotype would be an FL conjugated lectin that binds non-specifically to the platelet cell surface. Such a lectin however was not at our disposal. Thus an alternative approach, to produce an isotype cocktail that effectively inhibits the ECL/sWGA lectin from binding their targets on the platelet surface specifically, was chosen. A cocktail saturated with free inhibiting sugar (lactose and NAG for the galactose and NAG detection assays respectively) would effectively bind the lectins, inhibiting their specific binding to exposed residues on the platelet surface.

Optimal working concentrations and incubation-times for these lectins and inhibiting sugars were determined through titration assays. A preliminary, small-scale titration assay of ECL-FL, with and without ADP stimulation was performed. There was no observable plateau in the titration curve obtained from this assay, indicating a higher range of concentrations and/or incubation-times were necessary to achieve saturation (data not shown). The assay demonstrated that ECL-FL mean fluorescence intensity (MFI) was significantly larger for stimulated samples indicating that moving forward, stimulated samples may
serve better for testing the upper range capabilities of a given ECL-FL concentration (data not shown).

Titration curves produced in succeeding ECL-FL titration assays demonstrated, of the range of concentrations tested, none quite reached saturating concentrations, though there was an observable, ECL-FL concentration dependent decrease in slope indicative of approaching plateau (Figure 4A). However, a clear incubation-time dependent plateau was achieved indicating that by around 15 minutes incubation-time, ECL-FL binding was equilibrated and stable (Figure 4B). While an effective concentration that achieves saturation would have been optimal, the high concentrations required would have been economically unreasonable. It was thus decided that a lower concentration that still demonstrates effective ECL-FL binding equilibration would be best for our purposes. The effective concentration of 30μg/mL ECL-FL, incubated for 15 minutes was thus decided to be optimal.

A range of lactose concentrations was tested against effective, 30μg/mL ECL-FL concentrations in the development of the isotype. A plateau was nearly reached in the resulting titration curve, indicating that near saturating concentrations of lactose was attained by around 10mg/mL effective concentration (Figure 4C). It was thus decided that a more generous concentration of 14mg/mL would be a sufficient amount of lactose for an effective isotype.

An sWGA-FL versus NAG (inhibiting sugar) titration matrix was run to determine optimal sWGA-FL concentrations and optimal NAG concentrations for isotype simultaneously. A plateau was nearly achieved in the 0mM NAG titration curve indicating that saturating concentrations of sWGA were almost reached among the higher concentrations tested (Figure 5). We found 10μg/mL sWGA-FL to reasonably achieve near saturating concentrations (Figure 5). Inclusion of 45.2mM NAG significantly reduced sWGA-FL signals, indicating free NAG is effectively inhibiting sWGA-FL at these concentrations (Figure 5).
Figure 4: ECL-FL titration assays for galactose exposure assay development. (A & B) PRP was procured by WB centrifugation cell fractionation at 110g for 12 minutes at 22 °C. Platelets were stained with fluorescent antibody cocktails composed of CD61-PE and ECL-FL, and simultaneously treated with ADP (20μM). A range of incubation-times (5, 10, 15, 20, 25 and 30 minutes) and ECL-FL concentrations (0.5, 5.0, 10.0, 20.0, 30.0, 40.0, 50.0 and 100μg/mL) were assessed. Data were fit, using Graphpad Prism 5.0 computation, to one-site, specific binding, non-linear regressions with constraints on Kd. (C) PRP was procured by WB centrifugation cell fractionation at 110g for 12 minutes at 22 °C. Platelets were stained for 15 minutes with fluorescent antibody cocktails composed of CD61-PE, ECL-FL (30μg/mL effective), and varying lactose concentrations (0.45, 0.9, 1.8, 3.6, 7.2, and 14.4 mg/mL). Data were fit, using Graphpad Prism 5.0 computation, to one-site, specific binding, non-linear regressions with constraints on Kd.
It was thus decided that the effective concentration 10μg/mL sWGA-FL, incubated for 30 minutes was optimal for NAG exposure detection and inhibiting sugar concentrations ≥45.2mM NAG was optimal for use in the assay isotype.

![Graph showing sWGA-FL (MFI) vs sWGA-FL (μg/mL)](image)

**Figure 5: NAG exposure assay development titration assay.** PRP was procured by WB centrifugation cell fractionation at 110g for 12 minutes at 22 °C. Platelets were simultaneously treated with ADP (20μM) and stained for 30 minutes with fluorescent antibody cocktails composed of CD61-PE, varying sWGA-FL concentrations (0.2, 2 and 20μg/mL) and varying NAG concentrations (0, 0.452, 4.52 and 45.2mM). Data were fit, using Graphpad Prism 5.0 computation, to one-site, specific binding, non-linear regressions with constraints on Kd.

**Development of flow cytometric platelet Neuraminidase 1 expression detection assay**

Recent studies have demonstrated that cold-induced platelet surface desialylation, which contributes to macrophage mediated platelet clearance, may be attributed to cold-induced translocation of intracellular Neuraminidase 1 (NEU1) from lysosomes to the platelet surface (Jansen et al., 2012). Critically, immunoblotting and enzymatic activity assays of previous studies suggest active NEU1 localizes at the platelet surface upon refrigeration induced release, while NEU1 found in the extracellular media are inactive (Jansen et al., 2012). Monitoring possible translocation of NEU1 over a long-term platelet storage time course may provide more insight into the kinetics of this cold-induced enzymatic expression, which may inform the larger platelet clearance mechanism. To this
effect, and to determine the reproducibility of other studies findings regarding this phenomenon in conditions that better approximate standard platelet storage practices, I am interested in characterizing the total (intracellular and extracellular) and surface expression of NEU1 over long-term platelet storage in room versus cold temperature conditions. I thus necessarily developed a flow cytometric assay for measuring relative total and surface expression of NEU1.

In developing this NEU1 expression monitoring assay the following considerations were made: (1) optimization of platelet effective concentration (2) deciding a platelet identifying/gating antibody (3) optimization of NEU1 specific antibody working concentrations (4) deciding an effective isotype antibody and (5) optimization of cell permeant for effective entry of antibody into platelets for total NEU1 assessment. Platelet working concentrations a few fold higher than concentrations used in previous GPIbα monitoring assays were adopted for these assays. While anti-GPIb is a standard platelet-gating antibody, anti-CD41a was opted for use, as there was concern regarding the possibility that anti-GPIb might sterically inhibit our anti-NEU-1 from accessing GPIbα localized surface NEU-1. Appropriate effective concentrations of anti-CD41a for use as a platelet-gating antibody were adopted from assays previously developed utilizing anti-CD41a as a platelet identifier.

An anti-NEU1 antibody that has been previously used for confocal microscopy detection of NEU1 on platelet surfaces was opted for use in this assay (Jansen et al., 2012). A nonspecifically binding antibody, mouse IgG, was opted for use as the isotype antibody as per recommendation of the anti-NEU1 provider. Optimal working concentrations for these antibodies were determined by a titration assay.

Observable plateau in titration curves was hard to achieve for the anti-NEU1 (Figure 6). Given economical constraints we compromised with a lower anti-NEU1 concentration that would give a relative measure of NEU1 expression rather than an absolute. Despite little indication that saturating concentrations of NEU1 were achieved, the significantly higher MFI signals achieved from anti-NEU1 as compared to the nonspecific mIgG antibody was an encouraging
indication that NEU1 was binding specifically. 1% Triton by volume was used to permeate cells for detection of total NEU-1. This concentration of triton seemed reasonable given the significant increase in MFI for permeated cells as compared to untreated cells (Figure 6). To conclude the effective concentration of 10μg/mL NEU-1, incubated for 1 hour was chosen for the assay and a matching concentration of mlgG for the isotype.

![Graph](image)

**Figure 6: NEU1 expression assay development titration assay.** PRP was procured by WB centrifugation cell fractionation at 110g for 12 minutes at 22 °C. Platelets were either stained for an hour with test fluorescent antibody cocktails composed of CD41a-PerCP-Cy5.5 and varying anti-NEU1-PE concentrations (0, 3, 5 and 10μg/mL) or isotype fluorescent antibody cocktails composed of CD41a-PerCP-Cy5.5 and varying mlgG concentrations (0, 3, 5 and 10μg/mL). To detect total NEU1 levels, platelets were stained with appropriate antibody cocktail and simultaneously treated with 1% Triton. Data were fit, using Graphpad Prism 5.0 computation, to one-site, specific binding, non-linear regressions with constraints on Kd.

**GPIIbα galactose and NAG exposure increases in RT and cold stored platelets**
Platelet surface galactose and NAG exposure of long-term, RT and cold stored platelets was monitored at select time points (2, 24, 72 and 120 hours). Relative levels of galactose and NAG exposed on the platelet surface did not change significantly over the duration of storage (Figure 7C & D). Average galactose and
NAG exposure on the surface of cold stored platelets were observably lower than that of RT stored platelets across most time points (Figure 7C & D). However, of greater interest are the levels of exposed galactose and NAG on the platelet surface GPIbα receptors specifically, given galactose and NAG exposure on GPIbα, not the general platelet surface, has been implicated to mediate cold-induced platelet clearance (Figure 3, 7A). GPIbα levels on the platelet surface were found to significantly (p<0.1) decrease with longer storage time for both temperature conditions (Figure 7B). As levels of GPIbα on the platelet surface proved to decline over the storage period, it was crucial to normalize total platelet surface galactose and NAG exposure levels to GPIbα levels for characterization of exposed galactose and NAG levels specifically on GPIbα. Normalized levels of exposed galactose and NAG had observable, but statistically insignificant, increasing average levels over the duration of storage in both temperature conditions (Figure 7E & F). Crucially, the normalized levels of exposed galactose and NAG were very similar across RT and cold stored platelets at every measured time point (Figure 7E & F). Taken together, these results suggest desialylation and levels of galactose and NAG exposure on GPIbα increased comparably between RT and cold stored platelets over the duration of long-term storage.

**Total and surface expression of Neuraminidase 1 is static in RT and cold stored platelets**

Platelet surface and total (surface and intracellular) NEU1 expression of long-term, RT and cold stored platelets was monitored at select time points (2, 24, 72 and 120 hours). The percent of platelets expressing surface or total NEU1 was found to not significantly change past 2 hours of storage, nor significantly differ between cold and RT storage at any measured time point (Figure 8A). The average levels of NEU1 expression on a given NEU1 positive platelet was also found to not significantly change past 2 hours of storage, nor significantly differ between cold and RT storage (Figure 8B). These results together suggest past 2 hours of storage, NEU1 production and translocation rates are static and similar in both RT and cold stored platelets.
Figure 7: Galactose and NAG exposure on platelet surface over RT and cold long-term storage. Concentrated PRP was procured from ACD-A treated WB by centrifugation cell fractionation methods and stored with gentle agitation in either RT (22 °C) or cold (4 °C) temperature conditions. Platelet surface GPIbα, exposed galactose and exposed NAG levels were monitored 2, 24, 72 and 120 hours after storage in respective temperature conditions. Platelets were rewarmed (37 °C) for 15 minutes before analysis. Red lines indicate RT-stored platelets while blue lines indicate cold-stored platelets. Values are reported as mean ± SEM, n=3. (A) Schematic of the GPIbα with notable sugar glycans; sialic acid, galactose and NAG. Sialic acid cleavage is mediated by NEU1 while galactose cleavage is mediated by Galactosidase, together leading to exposed NAG. This phenomenon is implicated as being cold-induced and mediating cold-induced platelet clearance (B) GPIbα is significantly cleaved from the platelet surface over time in both RT and cold stored platelets (two-way-ANOVA, p = 0.0335). (C) Total platelet surface galactose exposure does not change significantly over time in both RT and cold conditions, with no significant difference between RT and cold stored platelets (D) Total platelet surface NAG exposure does not change significantly over time with no significant difference between RT and cold stored platelets (E) Galactose exposure levels normalized to GPIbα have observable increase over time with no significant difference between RT and cold stored platelets. (F) NAG exposure levels normalized to GPIbα levels have observable increase over time with no significant difference between RT and cold stored platelets.
Surface A-GPIIbIIIa and surface P-selectin expression of RT and cold stored platelets

Platelet activation induces a conformational change in GPIIbIIIa, transforming the receptor from its unactivated form to its fibrin-adhesive-activated form (A-GPIIbIIIa). Surface A-GPIIbIIIa presentation on the platelet surface is thus a biomarker for platelet activation. Upon activation, platelets also release an adhesion receptor, P-selectin, from their alpha-granules which are then presented on the cell surface. Surface P-selectin expression can thus, also serve as a platelet activation biomarker.

In an effort to characterize the activation status and hemostatic functionality of long-term RT and cold stored platelets over the duration of their storage, surface expression of platelet activation biomarkers, P-selectin and A-GPIIbIIIa were monitored at select time points (2, 24, 72 and 120 hours) with and without agonist stimulation. Specifically both the percent of surface A-GPIIbIIIa/P-selectin positive platelets (% gated) and mean levels of surface A-GPIIbIIIa/P-
selectin per A-GPIIbIIIa/P-selectin positive platelet (MFI) were monitored. Monitoring surface A-GPIIbIIIa/P-selectin of platelets without agonist treatment (HS treated) gives insight into the baseline activation status of the platelet; lower percent surface A-GPIIbIIIa/P-selectin positive platelets indicate lower baseline activation and vise versa. The relative change between baseline levels of surface A-GPIIbIIIa/P-selectin and agonist-stimulated levels of surface A-GPIIbIIIa/P-selectin give insight into relative platelet reactivity; a larger percent positive surface A-GPIIbIIIa/P-selectin fold increase between baseline and agonist stimulated platelets indicates a more reactive platelet and vise versa.

**Surface A-GPIIbIIIa of baseline platelets**

Percentage of surface A-GPIIbIIIa positive platelets, among HS treated (baseline) platelets, did not statistically change over the course of either RT or cold storage (Figure 9A). Nor was the percentage of surface A-GPIIbIIIa positive platelets, throughout the duration of storage, significantly different between RT and cold stored baseline platelets (Figure 9A). Relatedly, average surface A-GPIIbIIIa levels of baseline platelets did not statistically change over the duration of storage in either RT or cold storage conditions. Nor were the absolute surface A-GPIIbIIIa levels, throughout the duration of storage, statistically different between RT and cold stored baseline platelets (Figure 9B).

Together, these findings suggest the baseline activation status of the platelets remain largely unchanged over the duration of long-term storage in either RT or cold conditions. More significantly, these findings suggest the baseline activation statuses of platelets are not significantly different between RT and cold stored platelets.

**Surface A-GPIIbIIIa of agonist stimulated platelets**

Though not statistically significant, the average surface GPIIbIIIa positive platelet percent fold change between baseline and agonist (ADP, TRAP) stimulated RT stored platelets had an observable decline over the duration of storage (Figure 9C). There is a similar, storage-time-dependent, decline in the surface A-GPIIbIIIa level fold increase between baseline and TRAP stimulated RT platelets.
(Figure 9D). These results suggest RT-stored platelets reactivity toward agonist decreases over the duration of storage.

Cold stored platelets on the other hand don’t seem to exhibit as clear a storage-time-dependent trend and have a fairly static average surface A-GPIIbIIIa positive percent fold change over the duration of storage (Figure 9C). Interestingly, cold-stored platelets seem to exhibit an ADP-stimulated fold increase in surface A-GPIIbIIIa levels that increases with longer storage time, while TRAP stimulated fold increase in surface A-GPIIbIIIa level is largely static over the duration of storage (Figure 9D).

These results demonstrate cold stored platelets generally maintain hemostatic function over the duration of storage. Additionally these results suggest, of the cold-stored platelets that do react to agonist, the relative level of their activation is stronger or equally strong over the duration of storage.

It is also worth noting that the average surface A-GPIIbIIIa positive percent fold change is observably lower in cold-stored platelets compared to RT-stored platelets in the shorter durations of storage (2, 24 and 72 hours) but not the 120 hour long storage (Figure 9C).

Collectively, these results suggest cold-stored platelets better maintain hemostatic function over the duration of storage as compared to RT-stored platelets, which decline in hemostatic function over the duration of storage. Interestingly however RT-stored platelets demonstrate more robust reactivity to agonist in the earlier stages of storage than cold-stored platelets.
Surface P-selectin of baseline platelets

Percentage of surface P-selectin positive platelets among baseline RT and cold-stored platelets increased significantly (p<0.0001) over the duration of storage (Figure 10A). The average surface P-selectin level of both RT and cold stored baseline platelets also significantly increased with storage time (p=0.0245) (Figure 10B). The percentage of surface P-selectin positive platelets was not statistically different between RT and cold stored baseline platelets. Nor was the
mean level of surface P-selectin statically different between RT and cold stored baseline platelets.

Though not statistically significant, worth noting is the observably higher percent of surface P-selectin positive platelets and absolute surface P-selectin levels among baseline RT-stored platelets as compared to baseline cold-stored platelets at the 120 hour storage mark (Figure 10B).

Taken together, these results suggest both RT and cold stored platelets have storage-time-dependent increasing baseline levels of activation. In other words, both RT and cold stored platelets seem to become more pre-activated at similar rates with longer storage time. Larger baseline activation levels of RT as compared to cold stored platelets by 120 hours of storage might suggest RT platelets are pre-activated over the duration of storage at a faster rate than cold platelets.

**Surface P-selectin of agonist stimulated platelets**

Surface P-selectin positive percent fold change between baseline and ADP-stimulated platelets of both RT and cold stored platelets are insignificant at every measured time point (Figure 10C). Similarly, ADP-stimulated surface P-selectin level fold increase from baseline is insignificant in both RT and cold stored platelets at every measured time point (Figure 10D). In other words, platelets of both temperature conditions appear relatively unresponsive to ADP activation in the context of surface P-selectin expression.

While ADP stimulation has little impact, TRAP stimulation, at most but not all measured time points, does produce an observable percent fold change increase in surface P-selectin positive platelets in both RT and cold stored baseline platelets (Figure 10C). The exception is by 120 hours of storage where TRAP stimulated increase in surface P-selectin positive platelets is small in both RT and cold stored platelets (Figure 10C). Similarly, TRAP stimulation also increases absolute surface P-selectin levels of baseline platelets at most but not all measured time points, with the exception being by 120 hours of storage again.
By 120 hours of storage TRAP-stimulated surface P-selectin level fold increase is very weak in RT stored platelets but robust in cold stored platelets (Figure 10D).

There is a significant, storage-time-dependent decrease (p<0.0001) in TRAP stimulated surface P-selectin positive percent fold increase in both RT and cold stored platelets (Figure 10C). However it is worth noting the observable difference in the rate of this decline between cold and RT stored platelets. The rate of decline in TRAP stimulated surface P-selectin positive percent fold increase in cold stored platelets appears to be slower than for RT stored platelets. This trend is especially apparent if we note that cold stored platelets show lower percent fold increase in surface P-selectin positive platelets at all but the last, 120-hour storage time point, where percent fold increase in surface P-selectin is comparable between cold and RT stored platelets.

There is also a significant, storage-time-dependent decrease (p<0.1) in TRAP stimulated surface P-selectin level fold increase in both cold and RT stored platelets (Figure 10D). There is also an observable difference in the rate of this decline in TRAP stimulated surface P-selectin level fold increase between cold and RT stored platelets. The rate of decline in TRAP stimulated surface P-selectin level fold increase in cold stored platelets appears to be slower than for RT stored platelets. This trend is especially apparent if we note that cold stored platelets demonstrate lower TRAP stimulated surface P-selectin level fold increase platelets at all but the last, 120 hour storage time point where surface P-selectin fold increase is greater for cold than RT stored platelets.

These results collectively suggest there is a storage-time-dependent decrease in platelet TRAP stimulated activation in both RT and cold stored platelets. There is also the suggestion that cold stored platelets are generally less reactive than RT platelets to agonist stimulation, but are more resistant to change in hemostatic functionality than RT stored platelets, leading cold stored platelets to having, by the end of the storage time course, similar or better hemostatic function than RT platelets.
Figure 10: Baseline and agonist stimulated P-selectin expression of platelets under RT and cold long-term storage. Concentrated PRP was procured from ACD-A treated WB by centrifugation cell fractionation methods and stored with gentle agitation in either RT (22 °C) or cold (4 °C) temperature conditions. Expression P-selectin of platelets treated with HS buffer (control) ADP (20μM) and TRAP (20μM) were monitored 2, 24, 72 and 120 hours after storage in respective conditions. Platelets were rewarmed (37 °C) for 15 minutes before analysis. Values are reported as mean ± SEM, n=3. (A) Percent of surface P-selectin positive platelets. Percent of surface P-selectin positive platelets among baseline RT and cold stored platelets increased significantly over the duration of storage (two-way-ANOVA, p<0.0001) (B) Average levels of surface P-selectin presentation on baseline RT and cold stored platelets. Average surface P-selectin level of both RT and cold stored baseline platelets significantly increased with storage time (two-way-ANOVA, p=0.0245) (C) ADP and TRAP stimulated percent fold increase in P-selectin positive platelets from baseline levels. Dotted line indicates 1:1 fold change between baseline and agonist-stimulated P-selectin positive platelet percent. TRAP stimulated surface P-selectin positive percent fold increase in both RT and cold stored platelets declined over storage duration (two-way-ANOVA, p<0.0001) (D) ADP and TRAP stimulated fold increase in levels of P-selectin presentation from baseline levels. Dotted line indicates 1:1 fold change between baseline and agonist-stimulated P-selectin presentation level. TRAP stimulated surface P-selectin level fold increase in both cold and RT stored platelets declined over storage duration (two-way-ANOVA, p=0.0382).
**Annexin V positive PDMP and Annexin V positive procoagulant surface production**

Platelet cell membranes have an asymmetric distribution of different types of phospholipids. For a resting platelet, uncharged phospholipids such as phosphatidylcholine and sphingomyelin mainly comprise the outer leaflet of the membrane bilayer whereas the inner leaflet contains negatively charged aminophospholipids phosphatidylserine (PS) and phosphatidylethanolamine (PE). During platelet activation, this distribution becomes disrupted by translocase activity and aminophospholipids are flipped to the extracellular membrane leaflet, becoming exposed on the cell surface. Like activated platelets, platelet derived microparticles (PDMPs), which are produced by activated platelets, similarly present these aminophospholipids on their surface. Thus, surface aminophospholipid expression and PDMP production can be biomarkers for platelet activation.

Annexin V is a protein that binds with high affinity and specificity to exposed aminophospholipids in the presence of Ca$^{2+}$ ions. We are thus able to use Annexin V and forward scatter as parameters to differentiate between aminophospholipid expressing platelets and PDMPs by flow cytometric analysis.

In an effort to better elucidate the activation status and reactivity of platelets stored long-term in RT and cold temperature conditions, production of Annexin V positive PDMPs and platelets were monitored.

Results indicate that there was no significant difference in levels of Annexin V positive PDMP between cold stored baseline and cold stored agonist-stimulated platelets at any time points (Figure 11A). There was also no clear storage time dependent trend in the levels of Annexin V positive PDMP produced by baseline and agonist stimulated cold-stored platelets (Figure 11A). RT-stored platelets however had observable increases in average Annexin V positive PDMP concentrations from baseline with agonist stimulation (Figure 11A). RT-stored platelets also exhibited an observable storage time dependent decline in agonist-stimulated Annexin V positive PDMP production (Figure 11A).
Together these results suggest RT-stored platelets respond to agonist stimulation and produce Annexin V positive PDMP whereas cold-stored platelets have high baseline levels of Annexin V positive PDMP and do not respond to agonist stimulation to produce more Annexin V positive PDMP. On the other hand, while RT-stored platelets are reactive to agonist throughout the course of storage, their reactivity observably decreases over the duration of storage.

Monitoring of Annexin V positive platelets revealed that with agonist-stimulation, both RT and cold-stored baseline platelets have observable increases in percent Annexin V positive platelets. Results indicate the agonist stimulated increase in percent Annexin V positive platelets of baseline RT platelets significantly decreases over the duration of storage (p<0.01) (Figure 11B). Cold-stored platelets however do not have a clear storage time dependent trend in agonist stimulated percent increase in Annexin V positive platelets (Figure 11B). Together these results might suggest cold-stored platelets are less subject to change in reactivity while RT-stored platelets decline in reactivity with longer storage.

Also worth noting is the difference in cold and RT stored baseline percent Annexin V positive platelets. Baseline cold stored platelets maintain a fairly high and static percent Annexin V positive platelet values throughout the duration of storage. RT stored platelets however exhibit an increase in percent Annexin V positive platelets over the duration of storage. These results suggest cold stored platelets have a stable, relatively high level of pre-activation through the duration of storage while RT stored platelets have a gradually increasing level of pre-activation through the duration of storage.
Cold stored platelets better preserve aggregate formation ability as compared to RT stored platelets

Light transmission aggregometry (LTA) is a long-standing reference method for assessing platelet viability. LTA assesses platelet in vitro agonist induced activation and aggregate formation ability. PRP is stirred in a cuvette placed between a light source and a photocell in the LTA instrument. A PRP solution with freely suspended platelets is homogenous and cloudy and impedes light transmission through solution. Upon the addition of a platelet activating agonist, platelets aggregate allowing for the larger transmission of light through plasma between aggregates. Thus we can determine the percentage of maximal aggregate formation by the relative light transmission detected (high light transmission % = higher % aggregates).
In an effort to elucidate how RT and cold-storage might influence the reactivity, specifically the in vitro aggregate formation ability, of platelets, LTA was performed at select time points (2, 24, 72 and 120 hours).

Results of these LTA assays demonstrate that RT-stored platelets on average exhibit an observable decrease in aggregate formation in response to agonist (ADP and TRAP especially) with longer periods of storage (Figure 12A, B, C). Cold-stored platelets on average however seem to retain or even improve ability to form aggregates in response to agonists over the duration of storage (Figure 12A, B, C). Statistically however only aggregate formation in response to ADP was significantly different (p<0.01) between RT and cold stored platelets. Cold stored platelets had higher % aggregate formation (p<0.01) than RT stored platelets in response to ADP with longer storage (Figure 12A).

Collectively these LTA results suggest cold-stored platelets better retain platelet hemostatic function, specifically ability to form agonist induced (especially ADP) aggregates, as compared to RT-stored platelets.
Figure 12: Agonist induced aggregate formation ability of RT and cold-stored platelets over time. Concentrated PRP was procured from ACD-A treated WB by centrifugation cell fractionation methods and stored with gentle agitation in either RT (22 °C) or cold (4 °C) temperature conditions. ADP (20μM), Arachidonic acid (500μg/mL) and TRAP (20μM) induced platelet aggregate formation ability was assessed 2, 24, 72 and 120 hours after storage in respective temperature conditions by LTA. Platelets were rewarmed (37 °C) for 15 minutes before analysis. Red lines indicate RT-stored platelets while blue lines indicate cold-stored platelets. Values are reported as mean ± SEM, n=3. (A) ADP induced % platelet aggregate formation. Aggregate formation in response to ADP was significantly different between RT and cold stored platelets (two-way-ANOVA, p=0.0079) (B) AA induced % platelet aggregate formation. (C) TRAP induced % platelet aggregate formation.
DISCUSSION

Platelet hemostatic function is better preserved in cold rather than RT conditions over long-term storage. The rate at which hemostatic function was lost over the duration of long-term storage was observably faster for RT stored platelets as compared to cold stored platelets. This conclusion is based on the demonstration that percent fold increase between baseline and agonist stimulated platelets in activation biomarker positive platelet levels and average activation biomarker levels across several different biomarkers declined more rapidly amongst RT stored platelets as compared to cold stored platelets. Specifically, the activation biomarkers, surface A-GPIIbIIIa and P-selectin demonstrated these trends. The following evidence demonstrated in this study supports this conclusion, (1) percent fold increase in both surface A-GPIIbIIIa positive platelets and average surface A-GPIIbIIIa levels between baseline and agonist stimulated (ADP, TRAP) platelets, was static or increased over the duration of cold storage, but declined over the duration of RT storage, and (2) percent fold increase in both surface P-selectin positive platelets and average surface P-selectin levels between baseline and agonist stimulated (TRAP) platelets, declined at a slower rate over the duration of cold storage as compared to RT storage. Additionally cold stored platelets better maintained agonist stimulated, particularly ADP stimulated, in vitro aggregate formation ability over the duration of storage as compared to RT stored platelets. These results challenge the old dogma that attribute poor viability of cold stored platelets to compromised platelet hemostatic functionality and corroborate the growing consensus that cold storage better preserves platelet hemostatic function than RT storage. Many previous studies have presented similar findings, especially regarding cold stored platelets having better aggregation ability than RT stored platelets in response to ADP stimulation (Babic et al., 2007; Becker et al., 1973; Kattlove et al., 1972; Leytin et al., 2004; Reddoch et al., 2014).

Effectively these results demonstrate the advantage of storing platelets in cold temperature over RT and motivate re-evaluation of current RT PC storage practice.
Interestingly, in this study RT stored platelets, especially at earlier time-points of storage, exhibited more robust activation responses in the form of activation biomarker (surface A-GPIIbIIIa, P-selectin, Annexin V PDMP, Annexin V platelets) presentation with agonist stimulation than cold stored platelets. The following evidence supports this finding (1) percent fold increase in surface A-GPIIbIIIa positive platelets between baseline and agonist stimulated (ADP, TRAP) RT stored platelets was greater than that of cold stored platelets at earlier storage time points (2, 24, 72 hours), (2) percent fold increase in surface P-selectin positive platelets between baseline and agonist stimulated (TRAP) RT stored platelets was greater than that of cold stored platelets at earlier time points (2, 24, 72 hours), (3) degree of agonist stimulated (ADP/TRAP/convulxin) Annexin V positive PDMP production was higher for RT stored platelets as compared to cold stored platelets and (4) degree of increase in percent Annexin V positive platelets between baseline and agonist stimulated (ADP/TRAP/convulxin) RT stored platelets was higher as compared to cold stored platelets at earlier time points (2, 24, 72 hours). These results suggest the mechanisms that dictate hemostatic function impairment induced by RT versus cold temperature storage is kinetically different. We might speculate that hemostatic function impairment in cold stored platelets is initially (within the first two hour of storage) rapid but quickly stabilizes to a relatively moderate level of hemostatic ability whereas hemostatic function impairment in RT stored platelets is gradual and continual throughout the duration of storage. Ultimately, these kinetic differences in cold-induced and RT-induced platelet storage lesions might suggest mechanisms underlying these phenomena are inherently different. RT-induced platelet storage lesions have been attributed to an aging process inducing progressive loss in energy generating machinery. In contrast, cold-induced platelet storage lesions have been speculated as being attributed to biophysical changes such as membrane rafting, GPIba clustering and cytoskeletal rearrangement (G. White, 1972; Gitz et al., 2012; Gousset et al., 2004; Hoffmeister et al., 2003a; Rumjantseva et al., 2009; White and Rao, 1998). RT and cold induced platelet storage lesion mechanisms have yet to be fully characterized or understood.
However, results of this study also suggest by 120 hours of storage, RT-stored platelets have comparable or worse hemostatic function than cold stored platelets. Specific evidence demonstrative of this distinction include (1) percent fold increase in average surface A-GPIIbIIIa levels between baseline and agonist stimulated (ADP, TRAP) RT stored platelets was lower as compared to cold stored platelets by 120 hours of storage and (2) percent increase in average surface P-selectin levels between baseline and agonist stimulated (TRAP) RT stored platelets was lower compared to cold stored platelets by 120 hours of storage. The demonstration that RT stored platelets begin with better but end with worse hemostatic function than cold stored platelets through the duration of storage speaks to the high rate with which RT stored platelets lose hemostatic function over time as compared to cold stored platelets. We can further speculate if the rate of decline in hemostatic function we observe for RT stored platelets was maintained with longer storage time, the disparity in hemostatic functionality between cold and RT stored platelets would increase with longer storage periods. In fact, Babic et al. demonstrates that beyond 14 days of RT-storage, PC platelets lose ability to aggregate while PC platelets stored for the same time in cold temperature retain this ability (Babic et al., 2007). Hence these results further demonstrate the advantage of storing platelets in cold temperature as opposed to RT.

While cold storage may better maintain platelet hemostatic function, the problem still stands that cold stored platelets are susceptible to clearance in vivo upon transfusion. The proposed mechanisms behind cold-induced platelet clearance are lectin and glycan mediated phagocytic processes carried out by Kupffer cells and hepatocytes. In an effort to provide more insight into the nature of long-term cold stored platelet clearance mechanisms particularly, galactose and NAG exposure levels on surface GPIbα were monitored at select time points over the duration of long-term (5 days) storage. The results of this time course reveal increased binding of ECL and sWGA (normalized to surface GPIbα levels) to cold stored platelets over duration of storage, suggesting GPIbα galactose and NAG exposure increases with longer storage duration. This is in line with results of previous studies demonstrating galactose exposure levels of cold stored
platelets increases over a 48-hour storage period (Rumjantseva et al., 2009). These results are also in agreement with the long-term cold storage induced platelet clearance mechanism posed by Rumjantseva et al.; increased galactose exposure over long-term cold storage mediates hepatocytic phagocytosis. Unexpectedly however, RT stored platelets demonstrated having similar increases in ECL and sWGA binding (normalized to surface GPIbα levels) to cold stored platelets, suggesting RT and cold stored platelets have comparable, increasing levels of GPIbα galactose and NAG exposure over the duration of storage. Previous studies have demonstrated relative levels of platelet surface galactose and NAG differ between short-term RT and cold stored platelets (≤2 hours) (Hoffmeister et al., 2003b) but none to my knowledge have thus far compared galactose/NAG exposure levels of long-term (≥2 hours) RT and cold stored platelets. The results of this study suggest unlike short-term RT and cold stored platelets, relative levels of GPIbα galactose and NAG exposure are not significantly different between long-term RT and cold stored platelets. If we make the reasonable assumption that RT stored platelets of this study do not rapidly clear from circulation as cold stored platelets do (a distinction well characterized between RT and cold stored platelets in previous studies), these results suggest glycan exposure alone cannot predict platelet clearance susceptibility. Other investigations have demonstrated the extent of galactose exposure on the surface of refrigerated platelets is less than that of ST3GalIV+/− platelets, yet ST3GalIV+/− platelets unlike refrigerated platelets were not susceptible to rapid clearance and retained normal circulation lifetime (Ellies et al., 2002; Grewal et al., 2008). Do these results implicate glycan exposure is not critical to cold-induced platelet clearance? Not necessarily. These results may suggest it is the interactive effect of glycan exposure and other cold-induced mechanisms that determine platelet clearance susceptibility of cold exposed platelets. For example, GPIbα clustering, mediated by cold-induced cytoskeletal and membrane phase change, (Hoffmeister et al., 2001) could be responsible for enhancing lectin avidity, which may crucially determine platelet clearance fate. Hence the nature of glycan re-composition may in fact be similar between long-term RT and cold stored platelets, and cold-induced platelet clearance is
determined by the interactive effect of multiple cold-induced mechanisms including glycan exposure.

The results of this study suggest NEU1 expression/production does not change significantly over the duration of long-term storage nor differ significantly between RT and cold stored platelets at any measured time point. Previous studies have demonstrated surface expression of NEU1 becomes detectable with platelet cold storage, suggesting NEU1 is responsible for cold-induced desialylation of GPIbα receptors (Jansen et al., 2012). Although not explicitly applied to the cold-stored platelet, separate studies have also demonstrated that platelet activation via GPIbα induces surface presentation of NEU1, leading to increased desialylation of platelets and acute thrombocytopenia (Li et al., 2015). Reversal of these NEU1 mediated effects with the application of dehydro-2-deoxy-N-acetylneuraminic acid (DANA), a neuraminidase inhibitor, were also further demonstrated (Li et al., 2015). Results of our study do not explicitly discount or support this theory. We might speculate cold-induced surface expression of NEU1 happens very rapidly, on the order of hours, but past this initial translocation, NEU1 surface expression level and production remains largely static for the remaining duration of storage. A static level of NEU1 surface expression does not compromise the possibility that NEU1 continues actively desialylating platelet glycans, mediating the increasing exposure of galactose apparent over the duration of long-term storage.

In future I hope to expand the sample size of this study to strengthen my statistical analyses, as there was often significant within-group variance that overwhelmed between-group variance. Future studies would additionally benefit with the inclusion of extra time points for experimental analysis, especially earlier in the course of storage to simultaneously characterize short-term storage effects (0 hours ‘fresh’ platelet reference especially). Future studies would also benefit with the use of apheresis PC products instead of manually procured cPRP used in this study. Apheresis PCs would enable procurement of larger PC volumes from a single donor, which would more practically enable analysis at more storage time points, eliminate a significant amount of platelet
pre-activation and handling attributed to the manual cell fractionation processes, and better approximate platelet storage standards. Future in vivo studies that complement this in vitro study would also be highly beneficial. For example, monitoring platelet clearance rates of RT and cold stored platelets in in vivo mice models in conjunction with this study would better inform the true platelet clearance susceptibility status with different durations and temperature conditions of storage. Monitoring bleeding rates of mice models transfused with platelets stored in RT and cold conditions for different durations could also inform the validity of in vitro results of this study.

Concluding remarks

Overall, this study demonstrates storing platelets in cold temperature conditions long-term better maintains platelet hemostatic responsiveness and function as compared to storing in RT conditions. Such findings demonstrate the promise of refrigerating platelets for use in transfusion therapy and encourage the pursuit for a means of protecting cold stored platelets from in vivo clearance for optimal application in transfusion therapy. Additionally, this study demonstrates platelet GPIbα galactose and NAG exposure is comparable between long-term RT and cold stored platelets. This is contrary to previous findings demonstrating greater GPIbα galactose and NAG exposure among short-term cold stored as compared to RT stored platelets. These distinctions in glycan exposure between short and long-term stored platelets is in line with the existence of a second, cold-induced, glycan-integrin mediated platelet clearance mechanism that is independent of a short-term cold stored platelet clearance mechanism (Rumjantseva et al., 2009; Wandall et al., 2008).
REFERENCES


