Inhibiting GPIbα Shedding Preserves Post-Transfusion Recovery and Hemostatic Function of Platelets After Prolonged Storage

Wenchun Chen,* Xin Liang,* Anum K. Syed, Paula Jessup, William R. Church, Jerry Ware, Cassandra D. Josephson, Renhao Li

Objective—The platelet storage lesion accelerates platelet clearance after transfusion, but the underlying molecular mechanism remains elusive. Although inhibiting sheddase activity hampers clearance of platelets with storage lesion, the target platelet protein responsible for ectodomain shedding–induced clearance is not definitively identified. Monoclonal antibody 5G6 was developed recently to bind specifically human platelet receptor glycoprotein (GP)Ibα and inhibit its shedding but not shedding of other receptors. Here, the role of GPIbα shedding in platelet clearance after transfusion was addressed.

Approach and Results—Both human leukoreduced apheresis-derived platelets and transgenic mouse platelets expressing human GPIbα were stored at room temperature in the presence and absence of 5G6 Fab fragment. At various time points, aliquots of stored platelets were analyzed and compared. 5G6 Fab inhibited GPIbα shedding in both platelets during storage and preserved higher level of GPIbα on the platelet surface. Compared with age-matched control platelets, 5G6 Fab–stored platelets exhibited similar levels of platelet activation, degranulation, and agonist-induced aggregation. 5G6 Fab–stored human GPIbα platelets exhibited significantly higher post-transfusion recovery and in vivo hemostatic function in recipient mice than control platelets. Consistently, 5G6 Fab–stored, 8-day-old human platelets produced similar improvement in post-transfusion recovery in immunodeficient mice and in ex vivo thrombus formation over collagen under shear flow.

Conclusions—Specific inhibition of GPIbα shedding in the stored platelets improves post-transfusion platelet recovery and hemostatic function, providing clear evidence for GPIbα shedding as a cause of platelet clearance. These results suggest that specific inhibition of GPIbα shedding may be used to optimize platelet storage conditions. (Arterioscler Thromb Vase Biol. 2016;36:1821-1828. DOI: 10.1161/ATVBAHA.116.307639.)

Key Words: blood bank ■ glycoprotein ■ platelet transfusion ■ proteolysis

Platelet transfusion is a therapy to treat or prevent hemorrhage in patients with either thrombocytopenia or dysfunctional platelets. Compared with other blood components, platelet products have the shortest storage life. In the blood bank, platelets can only be stored at room temperature under constant agitation for ≤5 days, mainly because of the risk of bacterial growth and accumulated damage to the platelets.1

Pathogen reduction technologies have been developed to inactivate bacteria and viruses in stored platelets and to minimize the risk of contamination and infection, which could potentially extend the platelet shelf life to 7 days.2 Recently, platelet storage at 4°C was approved by the US Food and Drug Administration,3 which may reduce the risk of contamination as well. However, largely independent of bacterial growth and pathogen inactivation, during storage platelets undergo progressive and deleterious modifications that collectively are termed the platelet storage lesion. The extent of the platelet storage lesion is strongly associated with a decrease in post-transfusion platelet survival and function,1,4 but the underlying molecular mechanism is not completely understood.

A characteristic of platelet storage lesion is ectodomain shedding of platelet surface receptor glycoprotein (GP)Ibα, as accumulation of glycocalicin, the product of GPIbα shedding, during platelet storage is reported in many studies.5–7 As a major part of the GPIb-IX complex, GPIbα is the platelet receptor for von Willebrand factor (vWF) and other ligands present in circulation. ADAM17, a widely expressed metalloprotease, cleaves GPIbα at the Gly464-Val465 peptide bond and releases glycocalicin to the plasma.8,9 Recent reports showed that inhibiting ADAM17 activity using a broad-spectrum...
Nonstandard Abbreviations and Acronyms

<table>
<thead>
<tr>
<th>GP</th>
<th>glycoprotein</th>
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<tr>
<td>hTg</td>
<td>transgenic mouse express human GPIbα</td>
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<tr>
<td>IL4R-IbaTg</td>
<td>transgenic mouse express chimeric IL4Rα/GPIbα</td>
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<tr>
<td>LR-ADP</td>
<td>murine leukoreduced apheresis-derived platelets</td>
</tr>
<tr>
<td>SCID</td>
<td>severe combined immunodeficient</td>
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<td>vWF</td>
<td>von Willebrand factor</td>
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metalloprotease inhibitor GM6001 or p38 mitogen-activated protein kinase inhibitors during storage improved the post-transfusion recovery of stored murine platelets. These studies suggest that shedding of GPIbα may play a role in fast clearance of platelets with the storage lesion. However, ADAM17 and other metalloproteases have broad substrate specificities. They cleave GPIbα, tumor necrosis factor-α, and other protein substrates in platelets. Thus, studies using the inhibitors of ADAM17 activity could not rule out the possibility that shedding of a platelet receptor other than GPIbα mediates platelet clearance. The definitive evidence linking GPIbα shedding to platelet clearance is still lacking.

A monoclonal antibody, designated 5G6, was recently developed to specifically bind to sheddase site of human GPIbα and thus limit its access to sheddases. Similar to the full-length antibody, 5G6 Fab fragment inhibited shedding of only GPIbα, but not other receptors, in platelets without inducing platelet activation. Injection of 5G6 does not cause thrombocytopenia in mice. In this study, we report that 5G6 Fab–mediated inhibition of GPIbα shedding during prolonged storage of both human leukoreduced apheresis-derived platelets (LR-ADP) and human GPIbα (hTg) murine platelets significantly improves post-transfusion recovery of stored platelets and markedly enhances their hemostatic function in vivo. This study demonstrates the cause–effect relation between GPIbα shedding and platelet clearance, providing the foundation for further mechanistic investigation of platelet clearance and future development of better platelet storage conditions.

Materials and Methods

Materials and Methods are available in the online-only Data Supplement.

Results

Two models were used to ascertain the effects of 5G6 Fab on stored platelets. In the first model, aliquots of human LR-ADP were stored with 5G6 Fab, mouse IgG Fab (Ctrl Fab), or saline for ≤8 days under standard blood-banking conditions and analyzed periodically for platelet activity (Figure 1A). The clearance of human LR-ADP was assessed in severe SCID (SCID) mice. In the second model, hTg murine platelets that express only human GPIbα (hTg) murine platelets significantly improve post-transfusion recovery of stored platelets and markedly enhance their hemostatic function in vivo. This study demonstrates the cause–effect relation between GPIbα shedding and platelet clearance, providing the foundation for further mechanistic investigation of platelet clearance and future development of better platelet storage conditions.

Treatment of 5G6 Fab During Prolonged Storage

Periodically during storage, LR-ADP and hTg platelets were evaluated for phosphatidylserine exposure, integrin αIIbβ3 activation, and P-selectin expression, all of which are considered markers of platelet activation. As shown in Figure I in the online-only Data Supplement, 5G6 Fab–treated LR-ADP or hTg platelets displayed the same levels of phosphatidylserine exposure, αIIbβ3 activation, and P-selectin expression as saline- or control Fab–treated platelets, suggesting that 5G6 Fab did not alter the activation and functional state of stored platelets.

To determine whether treatment of 5G6 Fab could modulate the function of stored platelets, we performed agonist-induced platelet aggregation assays. Because LR-ADP contains high concentration of acid-citrate-dextrose formula A, agonists at doses higher than those typically used for washed platelets were used to induce platelet aggregation. Throughout the storage of LR-ADP, 5G6 Fab exhibited little effect on ristocetin–, ADP–, or collagen-mediated aggregation (Figure 2A through 2E). Similarly, after storage, 5G6 Fab–treated hTg murine platelets displayed the same aggregation activity as saline-treated or control Fab–treated ones in response to ADP, collagen, and botrocetin (Figure 2F through 2H). Consistently, αIIbβ3 activation and P-selectin expression of stored hTg platelets were unaltered on collagen stimulation (Figure II in the online-only Data Supplement).

Treatment of 5G6 Fab During Prolonged Storage

Improved the Post-Transfusion Recovery of LR-ADP and hTg Platelets In Vivo

Next, we evaluated post-transfusion recovery of stored LR-ADP in SCID mice. SCID mice are capable of identifying lesions imparted to human platelets by prolonged storage as identified by their increased clearance from circulation. We chose to compare 4-day-old LR-ADP, a model for platelets stored within the 5-day shelf life, and 8-day-old LR-ADP, a model for platelets with prolonged storage. The saline-treated 2-day-old LR-ADP, also included in the study, was considered fresh platelets, and the 20-minute recovery of this platelet...
sample in SCID mice was used as the 100% recovery for comparison for all other LR-ADP samples and time points. Because the mouse platelet count does not change significantly throughout the experiment, human platelets were tracked by its relative abundance in the total population of CD41+ platelets (Figure 3A). As shown in Figure 3B, the recovery and survival of both control Fab– and 5G6 Fab–stored 4-day-old LR-ADP was essentially the same as that of 2-day-old sample, suggesting that treatment of 5G6 Fab did not have a significant impact on clearance of platelets within the 5-day shelf life. Consistent with earlier reports, the recovery of control Fab–stored 8-day-old LR-ADP was markedly lower than that of 2-day-old LR-ADP. In comparison, 5G6 Fab–stored 8-day-old LR-ADP produced a significantly improved post-transfusion recovery than its control Fab–stored counterpart (Figure 3C). Moreover, its recovery was similar to that of 2-day-old LR-ADP.

Although immune-mediated rapid clearance of human platelets was significantly diminished in SCID mice, it is notable that LR-ADP, regardless of its age, survived in SCID mice for <24 hours. This may be because of the fact that the innate immunity remains intact in SCID mice. To address this limitation, post-transfusion recovery and survival of stored hTg murine platelets in wild-type mice were measured. After storage of 16 hours, hTg platelets were labeled with fluorescent dye carboxyfluorescein succinimidyl ester and transfused into wild-type mice. At various time points after transfusion, small...
volume of blood was drawn from recipient mice, and the proportion of infused carboxyfluorescein succinimidyl ester+ platelets in total platelets was quantified by flow cytometry (Figure 4A and 4B). Consistent with earlier studies of wild-type platelets,6,9 control Fab–stored hTg platelets exhibited much lower recovery than fresh platelets, with a significant portion (≈35%) being cleared within an hour of transfusion (Figure 4C). In contrast, 5G6 Fab–stored hTg platelets exhibited the same recovery as fresh hTg platelets (Figure 4C). After the first hour, transfused platelets showed similar survival rates as the endogenous ones, suggesting that 5G6 Fab treatment does not affect post-transfusion survival of the platelets. Together, these findings demonstrate that 5G6 Fab during extended storage improved the in vivo recovery of LR-ADP and hTg platelets.

Treatment of 5G6 Fab During Prolonged Storage Preserved Adhesive Function of LR-ADP

To address whether 5G6 Fab affects the adhesive function of stored platelets, the ex vivo adhesion of LR-ADP in reconstituted whole blood on collagen fibrils was assessed in the perfusion chamber as described.6 After 2 minutes of perfusion, saline-treated, control Fab–treated, and 5G6 Fab–treated 2-day-old LR-ADP adhered to collagen equally well (Figure 5). They all covered ≈20% of the collagen surface area, which was comparable to that covered by fresh whole blood as reported before.21 In accordance with the progression of platelet lesion during the course of prolonged storage, the surface area covered by saline-stored and control Fab–stored LR-ADP steadily decreased with the platelet age. Although storage with 5G6 Fab did not alter the overall trend of decrease in adhesive capacity, it visibly delayed such decrease for several days, as the surface area covered by 5G6 Fab–stored 4-day-old LR-ADP was the same as the 8-day-old LR-ADP. Consequently, 5G6 Fab–stored 8-day-old LR-ADP visibly covered significantly larger surface area than its control Fab–stored counterpart (10.1±2.1% versus 3.5±1.2%; Figure 5). These results indicate that incubation with 5G6 Fab during prolonged storage preserved the adhesive function of LR-ADP.

Treatment of 5G6 Fab During Extended Storage Protect Hemostatic Function of hTg Platelets

To investigate whether storage with 5G6 Fab influences hemostatic function of hTg platelets, a tail-bleeding time assay was performed using IL4R-IbαTg mice. In IL4R-IbαTg platelets, the extracellular domain of GPIbα was replaced with that of interleukin-4 receptor.22 As a result, IL4R-IbαTg platelets do not bind vWF and other GPIbα ligands, and hemostasis in IL4R-IbαTg mice is severely impaired.22,23 IL4R-IbαTg mice are also moderately thrombocytopenic22 (W. Deng et al, unpublished data, 2016). In this study, fresh or stored hTg platelets were infused into IL4R-IbαTg mice (≈1×10^8 platelets per mouse). One hour after infusion, 2 mm of the mouse tail tip was amputated, and the time to cessation of bleeding was recorded. In accordance with previous reports,9,22 IL4R-IbαTg mice could not stop tail bleeding within 20 minutes, but transfusion of fresh hTg platelets stopped tail bleeding in most IL4R-IbαTg mice within 500 seconds (Figure 6). Transfusing control Fab–stored hTg platelets did not protect IL4R-IbαTg mice from hemorrhage.
mice from tail bleeding. In comparison, transfusing 5G6 Fab–stored hTg platelets significantly shortened the tail-bleeding time in IL4R-IbTg mice, approaching those transfused with fresh hTg platelets (Figure 6). These results demonstrate that 5G6 treatment during storage helped to preserve the hemostatic function of stored platelets.

Discussion

Platelets after prolonged storage are cleared rapidly on transfusion, but the underlying molecular mechanism remains elusive. During storage, platelets undergo a variety of morphological and biochemical changes, making it difficult to pinpoint the molecular events that critically accelerates platelet clearance.

Figure 3. Treatment of 5G6 Fab during prolonged storage improved the post-transfusion recovery of leukoreduced apheresis-derived platelets (LR-ADP) in vivo. A, Overview of the study. B, Survival plots of 2-day-old LR-ADP stored with saline (white bar), 4-day-old LR-ADP stored with control (Ctrl) Fab (gray bar), and that with 5G6 Fab (black bar). C, Survival plots of 2-day-old LR-ADP stored with saline (white bar), 8-day-old LR-ADP stored with Ctrl Fab (gray bar), and that with 5G6 Fab (black bar). The relative abundance of human platelets at a time point was normalized with that of 2-day-old LR-ADP at 20 minutes post-transfusion being 100%. Data are shown as mean±SEM (n=12). **P<0.01; *P<0.05; n.s., not significant (t test). APC indicates allophycocyanin; FITC, fluorescein isothiocyanate; and SCID, severe combined immunodeficient.

Figure 4. Treatment of 5G6 Fab during storage improved the post-transfusion recovery of human GPIbα (hTg) platelets in vivo. A, The strategy of post-transfusion recovery study of stored hTg platelets. B, The relative amount of infused platelets was measured by flow cytometry. C, Compared with control (Ctrl) Fab, 5G6 Fab enhances stored hTg platelet post-transfusion recovery. Data are shown as mean±SEM (n=6). **P<0.01; *P<0.05; n.s., not significant (t test). APC indicates allophycocyanin; CFSE, carboxylfluorescein succinimidyl ester; PRP, platelet-rich plasma; and WT, wild type.
Adding metalloprotease or p38 mitogen-activated protein kinase inhibitors that blocked ADAM17 activity improved the recovery of stored murine platelets, establishing a close link between ectodomain shedding and platelet clearance. This is an important finding that narrowed the scope of investigation. However, because many receptors can be cleaved by ADAM17 or related metalloproteases and these receptors often elicit different signals, it is still unclear which platelet receptor, on its shedding, can lead to platelet clearance. Shedding of GPIbα was suggested because GPIbα is one of the most abundant platelet receptors undergoing shedding, and it is implicated in platelet apoptosis and clearance. Moreover, a correlation between GPIbα shedding and the extent of storage lesion has been reported. However, it remains unclear whether this is an epiphenomenon or a cause–effect relationship. To address this question, monoclonal antibody 5G6 was recently developed to bind directly to the shedding cleavage site of human GPIbα. 5G6 does not bind wild-type murine platelets but only hTg platelets. The crystal structure of 5G6 Fab in complex with its GPIbα-derived epitope peptide and related mutagenesis analysis identified several residues in the epitope peptide as required for the tight binding with the antibody. Many of these residues are polar residues and unique to human GPIbα, illustrating nicely why 5G6 does not bind murine GPIbα or any other platelet receptors. Consistent with its binding specificity to human GPIbα, we showed earlier that 5G6 inhibits induced shedding of GPIbα but not that of GPVI or glycoprotein V in fresh platelets. In this study, we verified that treatment of 5G6 Fab during storage inhibited GPIbα shedding without affecting the GPVI expression level and the activities of stored human and hTg murine platelets (Figures 1 and 2). Furthermore, 5G6 Fab significantly improved post-transfusion recovery of stored platelets in mice (Figures 3 and 4). It is noteworthy that post-transfusion recovery of 5G6 Fab–stored outdated platelets is similar to that of fresh platelets. These results, together with those demonstrating the binding and inhibitory specificity of 5G6 Fab, provide the first direct evidence supporting the idea that shedding of GPIbα leads to clearance of stored platelets. It remains to be determined whether shedding of another receptor also leads to platelet clearance. With the caveat that platelet clearance in mice may not reflect entirely that in human, our findings suggest that specific inhibition of GPIbα shedding may be potentially used to improve the recovery of stored platelets.

On transfusion, a portion of outdated platelets was cleared quickly, and the remaining ones were cleared at a rate similar to that of endogenous platelets. It is noteworthy that the effect of 5G6 treatment was primarily manifested in the improved recovery of stored hTg platelets, because the survival rate of 5G6 Fab–stored hTg platelets was similar to that of control Fab–stored ones (Figure 4C). Although the survival of LR-ADP in SCID mice was difficult to assess because the infused platelets were cleared within 24 hours, the beneficial effect of

Figure 5. Treatment of 5G6 Fab during prolonged storage improved ex vivo thrombus formation of leukoreduced apheresis-derived platelets (LR-ADP). LR-ADP samples stored with saline, control (Ctrl) Fab, or 5G6 Fab were dyed at 37°C and mixed with freshly prepared human platelet-poor whole blood. Reconstituted blood was perfused over the collagen surface at a shear rate of 1000/s. Two minutes after perfusion, platelet adhesion was visualized by microscope. The percentage of the surface area covered by fluorescent platelets was calculated. A. The extent of thrombus formation was plotted vs the age of stored LR-ADP. Data are shown as mean±SEM (n=4). *P<0.05 (t test). B. Representative images of thrombi formed on the collagen surface. All the images were taken at the same magnification scale and shown at the same contrast (bar=100 µm).

Figure 6. 5G6 Fab protects platelet hemostatic function after storage. IL4R-IlbTg mice were transfused with PBS, fresh human GPIbα (hTg) platelets, control (Ctrl) Fab–stored hTg platelets, or 5G6 Fab–stored hTg platelets. One hour after transfusion, a 1 mm segment of the mouse tail was cut off. Blood drops were absorbed every 20 s using a filter paper until bleeding ceased. Each symbol represents the bleeding time of one mouse. **P<0.01; *P<0.05; n.s., not significant (2-tailed Fisher test).
5G6 storage was clear for the recovery of 8-day-old LR-ADP (Figure 3C). These results suggest that multiple processes participate in the clearance of platelets, and GPIbα shedding may affect a distinct phase of clearance. That same effects of 5G6 treatment were observed in both human and murine platelets suggests that the 2 species may share a similar molecular mechanistic link between GPIbα shedding and platelet clearance.

An important physiological function of platelets is to form a plug at the injured vessel wall to prevent blood loss. GPIbα is a critical receptor mediating the interaction of platelets with subendothelial matrices lining the vessel wall. Collagen, a major component of the subendothelial matrix that becomes exposed to the blood on vessel injury, binds vWF in the plasma and also serves as a ligand for platelet receptors. At high shear rate (>800/s), the collagen-bound vWF captures platelets to the injury site through GPIbα. Platelets of Bernard–Soulier syndrome patients, which expressed little GPIbα, fail to adhere to collagen under flow conditions. Consistently, GPIbα-dependent thrombus formation is absent in IL4R-Ibctg mice. Furthermore, GPIbα may mediate thrombus formation through binding of matrix proteins other than vWF to collagen and also potentially modulating GPVI-mediated signaling responses. Platelets during storage continuously shed GPIbα from the surface and therefore progressively lose their aggregative and adhesive capacity in response to collagen. In this study, using the coated collagen surface in the flow chamber to mimic the physiological scenario of the injured vessel, we made similar observations that the adhesive capacity of untreated LR-ADP decreased with the age of stored platelets (Figure 5). Incubation with 5G6 Fab during blood bank storage preserved the expression level of GPIbα in the LR-ADP and visibly delayed platelet storage lesion-mediated decrease in adhesive capacity (Figure 5). In agreement with the in vitro and ex vivo findings, in vivo study further confirmed the potential effect of 5G6 Fab on protecting the hemostatic function of stored platelets. The transfusion of 5G6 Fab–stored hTg mice into IL4R-Ibctg mice, which exhibits severe bleeding disorder, significantly shortened the tail-bleeding time, whereas transfusion of control Fab–stored platelets did not ameliorate bleeding (Figure 6). These results confirm the importance of GPIbα in mediating platelet aggregation and adhesion to collagen and suggest that maintaining the GPIbα level during prolonged storage of platelets may help to preserve the hemostatic function of outdated platelets in addition to impeding their clearance.

The molecular mechanism by which GPIbα shedding leads to platelet clearance remains to be elucidated. Recent reports have suggested that exposure of β-galactose and N-acetyl-glucosamine on the to-be-cleared platelet, as a result of deglycosylation, can mediate platelet clearance. Particularly, the change of glycans in the extracellular domain of GPIbα has been implicated. Because GPIbα shedding removes the GPIbα extracellular domain from the platelet surface, the released GPIbα extracellular domain, including the glycans therein, should not mediate clearance of platelets. One possibility is that GPIbα shedding leads to the exposure of a new N-terminal end of GPIbα at residue Val465, which may become a ligand for recognition by a clearing receptor. Nicastrin as a part of the γ-secretase complex can recognize the newly exposed N terminus of a shedding product, but there have been no reports of further cleavage of GPIbα by γ-secretase after the initial shedding. Another possibility is that GPIbα shedding transmits a signal through GPIb-IX into the platelet, leading to presentation of a clear-me sign on the platelet surface. Consistently, a peptidomimetic inhibitor of intracellular GPIb-IX signaling can inhibit lipopolysaccharide-induced thrombocytopenia. GPIbα contains a juxtamembrane mechanosensory domain, and shear-induced unfolding of mechanosensory domain, particularly the juxtamembrane Trigger sequence therein, induces GPIb-IX signaling (W. Deng et al, unpublished data, 2016). Because the shedding cleavage site of GPIbα is located in the mechanosensory domain, it is conceivable that after GPIbα shedding the remaining residues from the mechanosensory domain, which contains the Trigger sequence, become unfolded, thereby transmitting a signal into the platelet that leads to its clearance. Consistent with the idea that GPIbα shedding may induce platelet signaling that leads to its clearance, we observed here that the extent of GPIbα shedding during storage was limited, and its inhibition by 5G6 Fab did not seem to impact significantly the platelet response to agonist stimulation (Figures 1 and 2). Finally, desialylation of GPIbα can induce GPIbα shedding, thus potentially inducing platelet clearance via this shedding-mediated mechanism. Additional studies are needed to distinguish between these possibilities and to elucidate the molecular consequences of GPIbα shedding.

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**Disclosures**

Emory University has filed a patent application for 5G6 and related antibodies for which R. Li is an inventor. The other authors report no conflicts.

**References**

• Antibody 5G6 Fab inhibits specifically GPIbα shedding in platelets during storage.

• Specific inhibition of GPIbα shedding improves post-transfusion recovery and hemostatic function of stored platelets.

• Specific inhibition of GPIbα shedding may be used to optimize platelet storage conditions.
Inhibiting GPIbα Shedding Preserves Post-Transfusion Recovery and Hemostatic Function of Platelets After Prolonged Storage
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Figure S.I. 5G6 Fab does not alter the activation state of stored platelets.

LR-ADP (A) and hTg PRP (B) that had been stored with PBS (grey open circle), Ctrl Fab (grey filled circle) or 5G6 Fab (black open circle) were assessed for phosphoserine (PS) exposure and two activation markers (activated αIIbβ3 and P-selectin) by flow cytometry. PS exposure was detected by the binding of GFP-LactC2, and P-selectin expression by binding of anti-P-selectin mAb. Activation of integrin αIIbβ3 was detected by the binding of mAb PAC-1 (LR-ADP) or JON/A (hTg PRP). Data are shown as mean ± SEM (n=5). Note in some cases the error bar is smaller than the symbol.
Figure S.II. 5G6 Fab does not alter integrin activation and P-selectin exposure in response to ADP and collagen

Flow cytometric analysis of αIIbβ3 activation and P-selectin exposure in stored hTg platelets. The hTg platelets were stored at room temperature with PBS (blank), Ctrl Fab (black) or 5G6 Fab (red) for 16 hours. Stored platelets were then stimulated with indicated concentrations of ADP or collagen for 15 minutes, and integrin αIIbβ3 activation (A) and P-selectin exposure (B) were measured using JON/A antibody and anti-P-selectin antibody respectively. Results are depicted as MFI ± SEM (n=6).
Inhibiting GPIbα shedding preserves post-transfusion recovery and hemostatic function of platelets after prolonged storage

Wenchun Chen,¹ Xin Liang,¹ Anum K. Syed,¹ Paula Jessup,² William R. Church,³ Jerry Ware,⁴ Cassandra D. Josephson,¹,² and Renhao Li¹

Materials and methods

Materials and Methods are available in the online-only Data Supplement.

Materials and animals. Allophycocyanin (APC)-labeled anti-human P-selectin, carboxyfluorescein succinimidyl ester (CFSE) were from Biolegend (San Diego, CA). APC anti-human CD41 (clone HIP8), fluorescein isothiocyanate (FITC) anti-mouse CD41 (clone MWRreg30), and FITC PAC-1 antibody were from BD Pharmaningen (San Diego, CA). Phycoerythrin (PE)-JON/A, FITC anti-mouse P-selectin, and FITC JAQ-1 antibodies were from Emfret Analytics (Eibelstadt, Germany). Mouse IgG Fab fragment was from Jackson ImmunoResearch (West Grove, PA). Green fluorescent protein-lactadherin C2 domain (GFP-LactC2) was produced as described¹. Apyrase and prostaglandin I₂ (PGI₂) were from Sigma-Aldrich (Saint Louis, MO). Botrocetin was purified from Bothrops jararaca venom (Sigma-Aldrich) largely as described² (Deng et al. manuscript submitted). The severe combined immunodeficient (SCID) and C57BL/6 (wild-type, WT) mice were procured from Jackson Laboratory. Transgenic mice expressing only human GPIbα (hTg)³ or chimeric IL4Rα/GPIbα (IL4R-IbαTg)⁴ have been described. Six- to 8-week old mice were used in the study as approved by the IACUC of Emory University. All animals were randomized before the experiment, and investigators were blinded to group allocation during data collection. Units of 2-day-old human LR-ADP in 10% Acid-Citrate-Dextrose formula A (ACD-A) was obtained from Bonfils Blood Center (Denver, CO) via the Blood Bank of Children’s Healthcare of Atlanta. Human whole blood was drawn from healthy volunteers in 0.38% sodium citrate. The informed consent and related protocols were approved by Emory University institutional review boards.

Production of 5G6 Fab fragment. 5G6 hybridoma cells were cultured in hybridoma-serum free media (GIBCO). After cell supernatant was collected, the antibody was purified using Protein G Sepharose 4 Fast Flow (GE LifeSciences, Pittsburgh, PA). 5G6 Fab was prepared as previously described¹. All Fab stock solutions were analyzed for purity by SDS-PAGE, buffer exchanged to sterile phosphate-buffered saline (PBS) and stored at 4°C. Mouse IgG Fab was prepared in the same PBS buffer.
Platelet storage. For human platelets, each aliquot of 30 ml 2-day-old LR-ADP was retrieved from the apheresis bag and sterilely transferred into a PermaLife cell culture bag (OriGen Biomedical, Austin, TX). 5G6 Fab (80 µM stock), control Fab (80 µM) or saline (0.9% NaCl) of the same volume was added to the aliquots at a final antibody concentration of 0.4 µM. Platelets were stored for 8 days at 20-24°C on a flatbed agitator (Helmer Inc., Noblesville, IN) with continuous agitation. For murine platelets, whole blood was collected from hTg mice and pooled 10:1 (v/v) with 3.8% sodium citrate, from which platelet-rich plasma (PRP) was isolated. Prepared PRP was added with 0.02 U/ml apyrase and 0.1 µg/ml PGI₂ and divided into equal fractions. 5G6 Fab, control Fab or saline was added to each fraction to 0.4 µM. Platelets were stored in sterile centrifuge tubes at room temperature for 16 hours under agitation conditions.

Platelet aggregometry. Agonist-induced platelet aggregation was monitored in a dual-channel Chrono-Log aggregometer (Havertown, PA). Platelet-poor plasma (PPP) obtained from Precision BioLogic (Dartmouth, Nova Scotia, Canada) was used to adjust the platelet count in each LR-ADP sample to 2.5×10^8 platelets/ml in the 250-µl sample cuvette. Two cuvettes, one with stir bar and 250 µl sample and another one with 250 µl PPP as reference, were placed in the PRP well and reference well, respectively, and then incubated for 3 min at 37°C. With stirring at 1,200 rpm, the channel was calibrated for 0 to 100 % light transmission by pressing the SET BASELINES pushbutton. Aggregation was initiated by the addition of noted agonists and monitored by the optical density, which was converted into percentage activity over the 5-min period after the addition of agonists. The concentrations of the agonists were chosen based on earlier reports of stored LR-ADP^5^.

Western blot. The supernatant of LR-ADP sample was collected by two centrifugation steps (2,000 g for 10 min, followed by 12,000 g for 5 min), and immunoprecipitated with anti-GPIbα WM23 antibody as described^1,^8. The precipitants were resolved in a NuPAGE Bis-Tris SDS gel in the MOPS running buffer under reducing conditions, transferred to PVDF membranes, and blotted with WM23. Seeblue Plus2 pre-stained molecular weight marker (Invitrogen) was used.

Flow cytometry. Platelets or PRP were incubated with noted antibodies or reagents at RT for 20 min, washed once with Tyrode’s buffer containing apyrase and PGI₂, and analyzed on a Becton-Dickinson FACS Canto II instrument.

Platelet transfusion. Stored human platelets were centrifuged for 20 min at 1,000 g. The platelet-poor plasma supernatant was removed, and the platelet pellet was gently resuspended in the residual plasma to about 4×10^11/µl. A 100-µl aliquot of concentrated platelets was injected into the retro-orbital plexus of SCID
mouse with a 1 ml syringe fitted with a 27-gauge needle. Stored hTg platelets were labeled by 2µg/ml CFSE, concentrated (750 g, 5 min) to 1×10⁶/µl, and about 100 µl injected into a WT mouse. At indicated time points after infusion, ~5 µl of whole blood was collected via facial vein into heparinized capillary tubes (Fisher Scientific, West Chester, PA) and transferred to a microcentrifuge tube to incubate with appropriate antibodies for 30 min. All samples were then treated with 200 µl RBC Fix/Lyse solution (eBioscience, San Diego, CA) for 10 min, centrifuged at 400 g for 5 min, and platelet pellets were washed once and resuspended in 200 µl PBS for flow cytometry analysis. Stored human platelet recovery was calculated as \( \frac{\text{anti-hCD41}^{+}\text{cells}}{\text{anti-hCD41}^{+}\text{cells} + \text{anti-mCD41}^{+}\text{cells}} \) and normalized with that at 20 min post-transfusion as 100%. Stored hTg platelet recovery was calculated as \( \frac{\text{CFSE}^{+}\text{cells}}{\text{anti-mCD41}^{+}\text{cells} + \text{CFSE}^{+}\text{cells}} \).

*Flow chamber studies*. The stored LR-ADP sample was labeled with 1 mM 3,3'-dihexyloxacarbocyanine iodide (Sigma-Aldrich) at 37˚C for 10 min and washed once in modified Tyrode's buffer. Platelet-poor whole blood, obtained by centrifugation of freshly drawn human whole blood, was reconstituted with 2.5×10⁸ labeled platelets/ml immediately before perfusion. In the parallel-plate flow chamber, a silicone gasket with a flow path height of 127 µm was placed between a flat perfusion chamber (Glycotech, Rockville, MD) and a 35-mm tissue-culture dish that had been coated with 200 µg/ml bovine type I collagen (Chrono-log) for 1 hour at RT. Perfusion was carried out at a wall shear rate of 1,000/s for 2 min. Platelet adhesion was visualized using a Nikon eclipse Ti inverted microscope equipped with a high-resolution Cool Snap HQ2 camera (Photometrics) and a 20× objective lens. To quantify the extent of thrombus formation, acquired digital color images were processed by ImageJ (NIH, Bethesda, MD) and the percentage of the surface area covered by fluorescent platelets was calculated.

*Tail bleeding time*. Each IL4R-IbaTg mouse was infused with 10⁸ fresh or stored platelets, or PBS. One hour after infusion, mice were placed on a heat plate and anesthetized by mask inhalation of isoflurane vaporized at concentrations of up to 4%. A 2-mm segment of the tail tip was cut off with a scalpel. Tail bleeding was monitored by gently absorbing the drop of blood with a filter paper in 20-s intervals without interfering with the wound site⁹,¹⁰. When no blood was observed on the paper, bleeding was determined to have ceased. The experiment was manually stopped after 20 min.

*Statistics*. All results are presented as means ± SEM. Data sets were compared using the 2-tailed unpaired Student’s *t* test, apart from the two-tailed Fisher’s test, which was applied to assess tail bleeding time. P<0.05 was considered statistically significant.
References


Platelet storage with 5G6 Fab

Less clearance

Storage without 5G6 Fab (No GPIbα shedding)

GPIbα

Clearance

GPIbα

GPIbβ

Cell membrane

GPIX

Shedding

Signal

Clearance

glycocalicin