Refrigeration-induced binding of von Willebrand factor facilitates fast clearance of refrigerated platelets

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Abstract

Objective—Apheresis platelets for transfusion treatment are currently stored at room temperature because after refrigeration platelets are rapidly cleared upon transfusion. In this study, the role of von Willebrand factor (VWF) in the clearance of refrigerated platelets is addressed.

Approach and Results—Human and murine platelets were refrigerated in gas-permeable bags at 4°C for 24 hours. VWF binding, platelet signaling events, and platelet post-transfusion recovery and survival were measured. After refrigeration the binding of plasma VWF to platelets was drastically increased, confirming earlier studies. The binding was blocked by peptide OS1 that bound specifically to platelet glycoprotein (GPI)bα and was absent in VWF⁻/⁻ plasma. Although surface expression of GPIbα was reduced after refrigeration, refrigeration-induced VWF binding under physiological shear induced unfolding of the GPIbα mechanosensory domain on the platelet, as evidenced by increased exposure of a linear epitope therein. Refrigeration and shear treatment also induced small elevation of intracellular Ca²⁺, phosphatidylserine exposure and desialylation of platelets, which were absent in VWF⁻/⁻ platelets or inhibited by OS1. Furthermore, refrigerated VWF⁻/⁻ platelets displayed increased post-transfusion recovery and survival than wild-type ones. Similarly, adding OS1 to transgenic murine platelets expressing only human GPIbα during refrigeration improved their post-transfusion recovery and survival.

Conclusions—Refrigeration-induced binding of VWF to platelets facilitates their rapid clearance by inducing GPIbα-mediated signaling. Our results suggest that inhibition of the VWF-GPIbα interaction may be a potential strategy to enable refrigeration of platelets for transfusion treatment.
Keywords
platelet refrigeration; transfusion; platelet clearance; VWF

Subject code
Basic Sciences; Platelets

Introduction
Platelet transfusion is widely used to treat or prevent hemorrhage in patients. In blood banks, platelets are stored at room temperature (RT) for up to 5 days, during which deleterious changes known collectively as the platelet storage lesion accumulate. The risk of bacterial growth during storage also limits the shelf life of stored platelets\textsuperscript{1,2}. Refrigerating platelets at 4°C is an attractive alternative since refrigeration would inhibit bacterial growth and slow down its metabolism. However, refrigerated platelets are rapidly cleared from the circulation after transfusion\textsuperscript{3}. Therefore, it is important to elucidate the molecular mechanism underlying the rapid clearance in order to enable refrigeration of platelets.

Long-term refrigeration of platelets leads to substantial changes of surface glycans\textsuperscript{4–6}. In particular, the density of exposed, terminal β-galactose is significantly increased after refrigeration, due to surface expression of the lysosomal neuraminidase and subsequent desialylation of the platelet\textsuperscript{5}. The exposed β-galactose is recognized by hepatocyctic Ashwell-Morell receptor (AMR), which mediates clearance of desialylated platelets\textsuperscript{4}. Incubation with 2,3-didehydro-2-deoxy-N-acetylneuraminic acid (DANA), a neuraminidase inhibitor, or asialofetuin, an AMR inhibitor, significantly improves the recovery of refrigerated platelets\textsuperscript{4,5}. However, how refrigeration induces the surface expression of lysosomal neuraminidase and platelet desialylation remains unclear. Curiously, it was noted that clearance of refrigerated murine platelets in which the N-terminal ligand-binding domain (LBD) of platelet glycoprotein (GP)Ib\textsubscript{α} had been cleaved off by O-sialoglycoprotein endopeptidase (OSP) was significantly reduced compared to that of refrigerated uncleaved platelets, suggesting that the LBD may be critical for the rapid clearance of refrigerated platelets\textsuperscript{4}. Increase in binding of von Willebrand factor (VWF) to platelets during refrigeration was noted, albeit its significance was not determined\textsuperscript{4}.

A major subunit of the GPIb-IX receptor complex, GPIb\textsubscript{α} contains the LBD, followed by the macroglycopeptide region, the mechanosensory domain (MSD), the transmembrane domain, and the cytoplasmic domain\textsuperscript{7–9}. It has been implicated in platelet clearance under several circumstances. Antibodies targeting the LBD activate GPIb-IX, induce platelet signaling including platelet desialylation, and lead to rapid platelet clearance\textsuperscript{10,11}. Relatedly, VWF binding to the LBD of GPIb\textsubscript{α} may also play a role in mediating platelet clearance. Under normal conditions, plasma VWF does not bind GPIb\textsubscript{α}. However, mutant VWF from patients with type 2B von Willebrand disease (VWD) exhibits heightened spontaneous binding to GPIb\textsubscript{α} and often leads to accelerated platelet clearance and thrombocytopenia\textsuperscript{12–15}. It was recently reported that binding of type 2B VWF, or botrocetin-
induced binding of wild-type VWF, to platelets under physiological shear exerts a pulling force on GPIbα and induces unfolding of the MSD therein. MSD unfolding thereafter induces GPIb-IX signaling, including the elevation of intracellular Ca^{2+} ([Ca^{2+}]_i), phosphatidylserine (PS) exposure and platelet desialylation, and accelerates platelet clearance. In this study we report that refrigeration-induced VWF binding to platelet GPIbα also results in shear-dependent clearance signaling events, thereby facilitating platelet clearance upon transfusion. Blocking the GPIbα-VWF interaction during refrigeration prevents GPIb-IX signaling and impedes clearance of refrigerated platelets in mice.

Materials and Methods

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

Results

Refrigeration-induced VWF binding to platelets

To test whether VWF binds to the platelet during refrigeration, murine platelets and plasma were obtained from WT and VWF−/− mice of both sexes, and mixed in four groups: WT platelets with WT plasma (WT platelet-rich plasma, PRP), WT platelets with VWF−/− plasma, VWF−/− platelets with WT plasma, and VWF−/− platelets with VWF−/− plasma (VWF−/− PRP). These mixed PRPs were refrigerated in gas-permeable bags at 4°C for 24 hours, then re-warmed at 37°C for 10 minutes before being analyzed for VWF binding by flow cytometry. PRPs freshly prepared from WT and VWF−/− mice were also measured for comparison. Consistent with previous reports, significant VWF binding to the platelet in refrigerated WT PRP was observed, whereas little was observed in fresh WT PRP (Fig. 1A,D). Similar increase in VWF binding was observed for refrigerated human PRP (Fig. 1B). The increase was inhibited by 1 μM OS1, a monomeric 11-residue peptide (CTERMALHNLC) that binds the LBD of human GPIbα with high affinity and inhibits VWF binding (Fig. 1C,E). Furthermore, significant VWF binding was observed in refrigerated VWF−/− platelets mixed with WT plasma, but not in fresh or refrigerated VWF−/− PRP, or refrigerated WT platelets mixed with VWF−/− plasma (Fig. 1B,D). These results suggest that refrigeration induces binding of plasma VWF, not platelet-derived VWF, to the platelet.

Refrigeration induces shear-dependent VWF-mediated platelet signaling

It was reported earlier that refrigeration of platelets results in metalloprotease-mediated ectodomain shedding of GPIbα. Consistently, we found that the level of GPIbα on the platelet surface became significantly reduced after refrigeration (Fig. 2A, B, F). However, similar reduction was observed when 2 mM EDTA, a metalloprotease inhibitor, was included during refrigeration (Fig. 2G). Similar reduction was also observed in refrigerated VWF−/− platelets in the presence or absence of EDTA. These results suggest that in addition to VWF binding, a process that is independent of metalloprotease activity may also mediate refrigeration-induced reduction of GPIbα surface expression.
Plasma VWF does not spontaneously interact with platelet in circulation, but botrocetin can induce VWF binding to GPIbα, leading to platelet activation and agglutination\(^\text{18}\). Recently we reported that binding of botrocetin/VWF induced platelet signaling, such as elevation of \([\text{Ca}^{2+}]_i\) and platelet desialylation, in a shear-dependent manner\(^\text{15}\). To determine whether refrigeration induces platelet signaling under shear via VWF binding, after refrigeration murine PRPs were treated with no shear or a uniform shear of 10 dyn/cm\(^2\) at RT for 5 minutes on a cone-plate viscometer. After the treatment platelets were collected, and signaling events assessed by flow cytometry. In refrigerated WT platelets \([\text{Ca}^{2+}]_i\), PS exposure, and ECL binding were significantly increased compared to fresh ones (Fig. 2C–E, H). It is worth noting that the extent of change in ECL binding appeared to be smaller than what had been reported\(^\text{4}\). Since GPIbα is the most sialylated protein on the platelet, refrigeration-induced reduction in GPIbα level may have reduced the binding level of ECL\(^\text{15}\). In comparison, refrigerated platelets that did not undergo shear treatment did not exhibit platelet signaling. Moreover, refrigeration of VWF\(^{-/-}\) PRP did not lead to increased \([\text{Ca}^{2+}]_i\), PS exposure or ECL binding in platelets, confirming the signaling dependence on VWF binding (Fig. 2I). Overall, these results suggest that refrigeration-mediated VWF binding to GPIbα triggers platelet signaling in a shear-dependent manner.

**Increased post-transfusion recovery in refrigerated VWF\(^{-/-}\) platelets**

To test whether VWF binding-mediated signaling events accelerate the clearance of refrigerated platelets in vivo, platelets in fresh or refrigerated WT and VWF\(^{-/-}\) PRP were fluorescently labeled and infused separately into WT mice at 10\(^8\) platelets per 10 g of body weight, and the recovery and survival of infused platelets were monitored periodically for 4 days (Fig. 3). As expected\(^\text{19}\), the clearance of fresh VWF\(^{-/-}\) platelets were indistinguishable from that of fresh WT ones. Consistent with earlier reports\(^\text{4}\), the initial recovery and survival of refrigerated WT platelets was significantly lower than that of fresh WT ones (Fig. 3, Table 1). In comparison, refrigerated VWF\(^{-/-}\) stored platelets exhibit higher recovery and longer survival than refrigerated WT ones (Table 1), indicating that VWF binding during refrigeration contributes to the rapid clearance of refrigerated platelet. Despite the significant increase, the recovery of refrigerated VWF\(^{-/-}\) platelets were still lower than that of fresh ones, suggesting that an additional process also mediates fast clearance of refrigerated platelets.

**Shear treatment of refrigerated platelets induces MSD unfolding on the platelet**

Mechanical pulling on the LBD of the immobilized GPIb-IX using a bead coated with recombinant A1 domain of VWF induces unfolding of the MSD in GPIb-IX\(^8\). Botrocetin-induced binding of plasma VWF to platelets under physiological shear generates a pulling force on GPIbα and induces unfolding of its MSD, which was identified as a key step in the activation of GPIb-IX signaling and platelet clearance\(^\text{15}\). MSD unfolding on the platelet was detected using monoclonal antibody 5G6 that targets a 10-residue linear epitope in the MSD of human GPIbα\(^\text{15, 16}\). Since 5G6 is a conformation-insensitive monoclonal antibody and it binds the epitope peptide and native GPIb-IX with essentially the same affinity\(^\text{20, 21}\), an increase in 5G6 binding relative to the GPIbα expression level indicates an increased exposure of the 5G6 epitope in GPIbα on the platelet and is considered as an indicator of MSD unfolding\(^\text{15}\). To test whether refrigeration-induced VWF binding leads to MSD
unfolding under shear, PRP obtained from transgenic mice expressing only human GPIbα (hTg) and human donors were refrigerated as described. Afterwards they underwent shear or no shear treatment in the presence of EDTA that precluded metalloprotease-mediated shedding of GPIbα. FITC-labeled SZ2, a conformation-insensitive monoclonal antibody that binds near the LBD of human GPIbα, or FITC-labeled 5G6 was added to the PRP mixture for measurement by flow cytometry. Consistent with observations of WT and VWF−/− PRPs, hTg platelets exhibited reduced surface expression of GPIbα after refrigeration as detected by SZ2 binding (Fig. 4A,B,F). Thus, the ratio of 5G6 and SZ2 binding levels was calculated to account for the reduction in GPIbα expression. While the shear treatment of fresh hTg platelets did not increase the 5G6/SZ2 ratio as expected, same treatment of refrigerated ones significantly increased the 5G6/SZ2 ratio. The increase was inhibited by the OS1 peptide. Similar increase in the 5G6/SZ2 ratio and its inhibition by OS1 were also observed for human platelets (Fig. 5A,B,F). These results suggest that refrigeration-induced VWF binding can lead to MSD unfolding on the platelet under physiological shear stress.

**OS1 peptide inhibits the post-recovery of refrigerated platelets**

Similar to the observations in refrigerated WT murine platelets (Fig. 2), shear treatment of refrigerated hTg and human PRP resulted in GPIb-IX signaling as evidenced by the increased $[Ca^{2+}]_i$, PS exposure and platelet desialylation (Fig. 4, 5). Importantly, addition of OS1 eliminated these signals (Fig. 4G, 5G), indicating that they were dependent on the VWF interaction with GPIbα. To investigate whether this inhibition can be translated into ameliorating the rapid clearance of refrigerated platelets in vivo, hTg PRP was refrigerated in the presence and absence of 1 μM OS1. After the refrigeration, hTg platelets were labeled with CFSE, and infused into WT recipient mice, and their post-transfusion recovery and survival was monitored for 4 days. As shown in Figure 4H and Table 2, OS1-treated refrigerated hTg platelets displayed significantly higher recovery than those without OS1 treatment. The survival of hTg platelets was not altered by refrigeration and the incubation of OS1. Overall, these results suggest that inhibiting VWF binding to GPIbα can block refrigeration-induced GPIb-IX signaling and impede rapid platelet clearance.

**Discussion**

In this study, a novel role of VWF in the rapid clearance of refrigerated platelets is described. We confirmed that refrigeration of PRP resulted in dramatically increased VWF binding to platelets through GPIbα (Fig. 1). Moreover, the ligation of VWF under shear induced GPIb-IX signaling in both human and murine platelets, including Ca$^{2+}$ mobilization, PS exposure and β-galactose exposure (Fig. 2, 4, 5). Shear was required to generate a pulling force on GPIbα and to induce unfolding of the MSD therein, which triggers GPIb-IX signaling and downstream events. Finally, deficiency of VWF (Fig. 2, 3) or inhibition of VWF binding by an exogenous peptide during refrigeration (Fig. 4, 5) diminished these shear-dependent signaling events and improved the post-transfusion recovery of refrigerated platelets. These data provide the first evidence supporting a role of VWF binding in facilitating rapid clearance of refrigerated platelets.
Our findings address a key step in the molecular mechanisms underlying the rapid clearance of refrigerated platelets. Changes of glycans on the refrigerated platelets, particularly the exposure of β-galactose as the result of desialylation, have been observed and demonstrated to mediate rapid uptake of refrigerated platelets by hepatocytes through the Ashwell-Morell receptor. One neuraminidase mediating platelet desialylation is neuraminidase-1 (NEU1), which resides in the platelet lysosome and is trafficked to the plasma membrane after refrigeration. NEU1 is membrane-bound and can desialylate platelet glycoproteins including GPIbα. However, it was not clear how refrigeration induces movement of NEU1 from the lysosome to the plasma membrane. It was reported that the proteolytic removal of the LBD of GPIbα inhibits the fast clearance of refrigerated murine platelets, and it was proposed that the desialylation of N-glycans on the LBD is a critical step. However, murine GPIbα does not contain any NXS/T sequence motif for N-glycosylation. Our results suggest a potential model to account for the importance of LBD in the clearance of refrigerated murine platelets: Refrigeration induces VWF binding to GPIbα on the platelet, which upon shear induces GPIb-IX-mediated signaling in the platelet, including likely the trafficking of NEU1 to the platelet surface and subsequent desialylation of platelet glycoproteins. In this model, the LBD is required for VWF binding and the initiation of GPIb-IX signaling, but not for subsequent platelet desialylation. Thus, whether it has N-glycans is not essential. This model is also consistent with previous studies that activation of GPIb-IX by VWF or anti-GPIbα antibodies leads to increased [Ca2+]i, PS exposure, and platelet desialylation, and that GPIb-IX is associated with the procoagulant activity of platelets. Moreover, refrigeration-induced VWF binding may also enhance the GPIbα-dependent platelet activation under high shear. Here we report the requirement of shear stress for refrigeration-induced platelet signaling (Fig. 2), which fits the observation that GPIb-IX is a mechanoreceptor and requires shear for its signaling function. In our experiments a shear stress of 10 dyn/cm² was utilized. Our results suggest that physiological shear stress (4–30 dyn/cm²) is sufficient to induce GPIb-IX signaling in refrigerated platelets. During refrigeration little shear is present. However, upon entering the circulation refrigerated platelets should experience shear stress that triggers GPIb-IX signaling, leading to their clearance.

It is noteworthy that the extent of refrigeration-induced β-galactose exposure as detected by ECL binding appeared relatively small compared to those of changes in [Ca2+]i and PS exposure (Fig. 2). One possible reason is that the reduction in surface expression of GPIbα, which is a highly siaiylated protein, might contributed to the reduction in the overall amount of glycans on the platelet surface, including the exposed β-galactoses. It is also possible that refrigeration induces fast platelet clearance via a different pathway than desialylation. Consistently, the reduction in clearance by the OS1 peptide was not complete as the initial recovery of OS1-treated refrigerated hTg platelets was still lower than that of fresh ones (Fig. 4, Table 2). A similar difference was observed between refrigerated and fresh VWF−/− platelets (Fig. 3). This suggests that an additional mechanism that does not require VWF contributes to the rapid clearance of refrigerated platelets. Clustering of GPIbα in the platelet membrane after refrigeration has been observed, and it was thought to induce GPIb-IX signaling, apoptosis and platelet clearance. Since VWF is a multimeric protein, refrigeration-induced VWF binding could presumably induce clustering of GPIbα on the
platelet surface. It remains to be tested whether refrigeration induces GPIbα clustering independent of VWF binding via a mechanism that is similar to high shear-induced clustering\textsuperscript{31}. It was suggested that deglycosylation of platelet and GPIbα leads to the exposure of N-acetyl-D-glucosamine, which may induce GPIbα clustering through its interaction with gangliosides GM1 and GM3 in lipid rafts\textsuperscript{32}. In this scenario, it remains to be determined whether deglycosylation of refrigerated platelets can be induced in the absence of VWF.

It remains unclear how refrigeration induces binding of VWF to GPIbα. One possibility is that cold temperature induces a change in VWF that promotes its binding to GPIbα. VWF can undergo an activating conformational change in the absence of shear, as evidenced by various type 2B VWD mutations\textsuperscript{33}. Conversely, cold temperature may induce a change in GPIbα that promotes its binding to VWF. Crystal structures of the LBD in complex with the A1 domain of VWF bearing type 2B and/or platelet-type VWD mutations are similar to that of the wild-type complex but with local differences in the contact interface\textsuperscript{34, 35}. Regardless of the conformation of the refrigerated VWF or GPIbα, we have demonstrated in this study that the OS1 peptide can inhibit refrigeration-induced binding of VWF to GPIbα, inhibit subsequent GPIb-IX signaling, and improve the post-transfusion recovery of refrigerated platelets. These proof-of-concept results suggest that inhibition of the VWF-GPIbα interaction may be utilized in future development of a practical protocol to refrigerate platelets for transfusion purposes.

In addition to the potential contribution from GPIbα clustering, significant reduction of GPIbα surface expression after refrigeration (Fig. 2) may be another factor. GPIbα can be shed by metalloprotease ADAM17 and released from the platelet surface\textsuperscript{36, 37}. Shedding of GPIbα in platelets during storage at RT is well documented. Inhibition of GPIbα shedding by inhibitors of ADAM17 activity or an anti-GPIbα antibody improves the post-transfusion recovery of RT-stored platelets\textsuperscript{38–40}. Little GPIbα shedding was observed after refrigeration of Adam17\textsuperscript{ΔZn/ΔZn} murine platelets that express only an inactive ADAM17, but refrigerated Adam17\textsuperscript{ΔZn/ΔZn} platelets were cleared as rapidly as the WT control\textsuperscript{5}. The apparently disparate effect of inhibition of GPIbα shedding on RT-stored and refrigerated platelets remains to be explained. In this study, we observed refrigeration-induced reduction of GPIbα surface expression in the presence of EDTA, a broad-spectrum inhibitor of metalloproteases including ADAM17 (Fig. 2). The reduction also occurred in refrigerated VWF\textsuperscript{−/−} platelets (Fig. 2). These results suggest that GPIbα expression can be reduced by another mechanism that requires neither metalloproteases nor VWF. Whether such reduction of GPIbα surface expression contributes to rapid clearance of refrigerated platelets remains to be determined.

**Supplementary Material**

Refer to Web version on PubMed Central for supplementary material.
Acknowledgments

WC, CDJ, KMH and RL designed research; WC, SAD and YW performed experiments and analyzed results; JW provided critical reagents; WC and RL wrote the paper; KMH edited manuscript. We thank the Emory Children’s Pediatric Research Center Flow Cytometry Core for technical support.

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Nonstandard Abbreviations and Acronyms

- **GPIbα**: Glycoprotein Ibα
- **VWF**: von Willebrand factor
- **hTg**: transgenic mouse expressing only human GPIbα
- **WT**: wild-type
- **[Ca^{2+}]_i**: intracellular Ca^{2+}
- **ECL**: Erythrina cristagalli lectin
- **PS**: phosphatidylserine
- **CFSE**: carboxyfluorescein succinimidyl ester
- **MSD**: mechanosensory domain
- **LBD**: ligand-binding domain
- **PRP**: platelet-rich plasma

References


Highlights

1. Refrigeration-mediated VWF binding induces shear-dependent unfolding of GPIbα mechanosensory domain and signaling in refrigerated platelets.

2. Inhibition of VWF binding to GPIbα during refrigeration improves recovery and survival of refrigerated platelets.
Figure 1.
Enhanced VWF binding to platelets after refrigeration. (A,B) Representative flow cytometry histograms of VWF binding to murine platelets. Murine WT and VWF<sup>−/−</sup> platelets were mixed with either WT or VWF<sup>−/−</sup> plasma, and then were refrigerated at 4°C for 24 hours. VWF binding was measured by flow cytometry using FITC-labeled anti-VWF antibody. The histogram of the negative control is filled by grey. Histograms denoted by grey dotted line, black dashed line, and solid black line are labeled in each figure following the description in panels D and E. (D) Quantitation of VWF binding to murine platelets. The composition of mixed PRP and storage condition are listed on the left. Each VWF binding was quantitated by mean fluorescence intensity (MFI) of the histogram, subtracted by that of the negative control, and normalized with that of WT murine platelets mixed with WT plasma without refrigeration storage as 1 (n=6). (C, E) Representative flow cytometry histograms and quantitation of VWF binding to human platelets. Human PRP were refrigerated with or without 1 μM OS1, and VWF binding was measured and quantitated as that for murine
platelets (n=3). Statistical analysis was performed using one-way ANOVA. All data are shown as mean ± SD. **, $P < 0.01$; and ***, $P < 0.001$. 
Figure 2.
Physiological shear stress induces VWF-dependent signaling in refrigerated platelets. Fresh or refrigerated murine WT and VWF−/− PRPs were treated with or without a uniform shear of 10 dyn/cm² for 5 min. (A–B, F–G) Representative flow cytometry histograms and quantitation plots of GPIba surface expression level on platelets, which was measured using FITC-labeled antibody Xia.G5. The formats follow those shown in Figure 1. GPIba level was quantitated and normalized with those of fresh WT or VWF−/− being 1 (n=4). (C–E) Representative flow histograms of platelet signaling following refrigeration and shear treatment. [Ca²⁺], PS exposure and β-galactose exposure were determined by Fura2-AM, GFP-LactC2 and Fluorescein-labeled ECL, respectively. (H,I) Quantitation plots of each signaling output under denoted conditions (n=4). Statistical analysis was performed using
two-way ANOVA. All data are shown as mean ± SD. n.s., not significant; *, $P < 0.05$; **, $P < 0.01$; and ***, $P < 0.001$. 
Figure 3.
Clearance traces of platelets following transfusion in mice. CFSE-labeled fresh (black) or refrigerated (black dashed) WT, fresh (grey) or refrigerated (grey dashed) VWF−/− platelets were transfused into recipient WT mice. At indicated times after transfusion, 30 μl blood was drawn from these mice, and the number of transfused platelets was counted. The recovery of WT fresh platelets at 1-hour post transfusion was normalized as 100% (n=6–8). Statistical analysis was performed by student t-test. All data are shown as mean ± SD. *, P < 0.05; ***, P < 0.001.
Figure 4.
Effects of shear and OS1 on MSD unfolding, signaling and post-transfusion recovery of refrigerated hTg platelets. (A–B, F) Representative flow cytometry histograms and quantitation plots of binding of anti-GPIIbα antibodies SZ2 and 5G6 on fresh or refrigerated hTg platelets that had been treated with or without uniform shear. The formats follow those shown in Figure 1. Binding levels of 5G6, SZ2 and their ratio were normalized with that of fresh hTg as 1 (n=4). (C–E, G) Representative flow histograms of platelet signaling following refrigeration and shear treatment. OS1 was added during refrigeration when noted, and [Ca^{2+}]_i, PS exposure and β-galactose exposure were determined (n=4). (H) Clearance traces of hTg platelets that had been refrigerated with or without OS1. The platelets were labeled with CFSE prior to transfusion into WT mice. The measurement follows that described in Figure 3. Staticstical analysis was performed by two-way ANOVA (F and G) and student t-test (H). All data are shown as mean ± SD (n=6). **, P < 0.01.
Figure 5.
Effects of shear and OS1 on MSD unfolding and signaling in refrigerated human platelets. (A–B, F) Representative flow cytometry histograms and quantitation plots of binding of anti-GPIbα antibodies SZ2 and 5G6 on fresh or refrigerated human platelets that had been treated with or without shear and OS1. The formats follow those shown in Figure 4. The measurements were normalized with those of fresh human platelets as 1 (n=3). (C–E, G) Representative flow histograms and quantitation plots of [Ca2+]i, PS exposure and β-galactose exposure showing the effect of OS1 on refrigerated human platelets. Statistical analysis was performed by two-way ANOVA. All data are shown as mean ± SD (n=3). *, P < 0.05; **, P < 0.01; and ***, P < 0.001.
Refrigerated VWF\(^{-/-}\) platelets display improved post-transfusion recovery and survival compared to refrigerated WT platelets.

<table>
<thead>
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<th>Platelets</th>
<th>Recovery (%)</th>
<th>Survival (T(_{1/2}); hour)</th>
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<tr>
<td>Fresh WT platelets</td>
<td>100 ± 6.56</td>
<td>33.82 ± 1.2</td>
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<tr>
<td>Fresh VWF(^{-/-}) platelets</td>
<td>97.5 ± 9.76</td>
<td>35.64 ± 4.2</td>
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<tr>
<td>Refrigerated WT platelets</td>
<td>54.21 ± 12.45*</td>
<td>25.56 ± 6.12*</td>
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<tr>
<td>Refrigerated VWF(^{-/-}) platelets</td>
<td>68.53 ± 12.73**</td>
<td>36.6 ± 8.14</td>
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Fresh or refrigerated WT or VWF\(^{-/-}\) platelets were transfused into WT mice (\(10^9/10\)g). The recovery and half-life time (T\(_{1/2}\)) of the transfused platelets were measured. The recovery of fresh WT platelets was normalized as 100%. Refrigerated WT platelets displayed lower recovery and shorter half-life time compared to refrigerated VWF\(^{-/-}\) platelets (n=6–8).

* \(P < 0.05\);

** \(P < 0.01\) (Student t-test).
### Table 2

Blocking VWF binding with OS1 enhanced the recovery of refrigerated hTg platelet.

<table>
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<tr>
<th>Platelets</th>
<th>Recovery (%)</th>
<th>Survival (T₁/₂; hour)</th>
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<tr>
<td>Fresh hTg platelets</td>
<td>100 ± 11.90</td>
<td>28.21 ± 2.8</td>
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<tr>
<td>Refrigerated hTg platelets</td>
<td>69.0 ± 5.4</td>
<td><strong>29.4 ± 3.92</strong></td>
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<tr>
<td>Refrigerated hTg platelets with OS1</td>
<td>82.96 ± 7.60</td>
<td>29.52 ± 5.25</td>
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The indicated platelets were transfused into WT mice (10⁸/10g), and the recovery and half-life time (T₁/₂) of the transfused platelets were measured. The recovery of fresh hTg platelets was considered as 100%. As indicated, the recovery of refrigerated hTg platelets without OS1 was lower than those with OS1 (n=6).

**P < 0.01 (Student t-test).**