Glycoprotein Ibα clustering in platelet storage and function

Eelo Gitz

Title: Glycoprotein Ibα clustering in platelet storage and function

Author: Eelo Gitz, 2013

ISBN: 978-90-889-1644-1

Printed by: proefschriftmaken.nl / printyourthesis.com

Published by: Uitgeverij BOXPress, 's-Hertogenbosch

Cover: Illustration by ©iStockphoto.com/nihatdursun, layout by Eelo Gitz
'Symbolic representation of glycoprotein Ibα clustering in lipid rafts'

Glycoprotein Ibα clustering in platelet storage and function

De rol van glycoproteïne Ibα clustervorming bij het bewaren en de functie van bloedplaatjes

(met een samenvatting in het Nederlands)

Proefschrift

ter verkrijging van de graad van doctor aan de Universiteit Utrecht op gezag van de rector magnificus, prof.dr. G.J. van der Zwaan, ingevolge het besluit van het college voor promoties in het openbaar te verdedigen op dinsdag 25 juni 2013 des middags te 2.30 uur

door

Eelo Gitz

geboren op 27 juli 1984 te Ermelo

Promotoren:	**Prof.dr. J.W.N. Akkerman**
	Prof.dr. H.C. Gerritsen
Co-promotor:	**Dr. R.T. Urbanus**

The studies described in this thesis were supported by a grant from the Landsteiner Foundation of Blood Transfusion Research (LSBR grant no. 0807).

Financial support by the Dutch Heart Foundation for the publication of this thesis is gratefully acknowledged.

Additional financial support for the publication of this thesis was generously provided by BiozymTC, Abbott Diagnostics, Boehringer Ingelheim, Tebu-Bio and BD Biosciences.

Contents

CHAPTER 1

General introduction

1

The role of platelets in hemostasis and thrombosis

Hemostasis describes the process that prevents blood loss from a damaged vessel. Platelets are anucleated, discoid-shaped cells that play an essential role in the formation of a hemostatic plug. They are produced by megakaryocytes in the bone marrow and circulate in the bloodstream at a concentration of 150-400 x 10^9/L. Platelets screen the integrity of the vascular system and, if not consumed in hemostatic events, are cleared by macrophages in liver and spleen after approximately 10 days of circulation.[1] Vascular injury leads to exposure of the subendothelial extracellular matrix (ECM), which presents adhesive proteins to which platelets interact. Initial adhesion to the damaged arterial vessel wall is regulated through the interaction of the glycoprotein (GP) Ib-V-IX complex with von Willebrand factor (VWF) immobilized on subendothelial-exposed collagen fibers. Transiently rolling platelets are then able to interact with collagens via the immunoglobulin-like receptor GPVI, which stimulates platelet activation. Rapid cytoskeletal remodeling subsequently induces a change in cell shape from discoid to spheric, leading to platelet spreading on the adhesive surface (Figure 1).

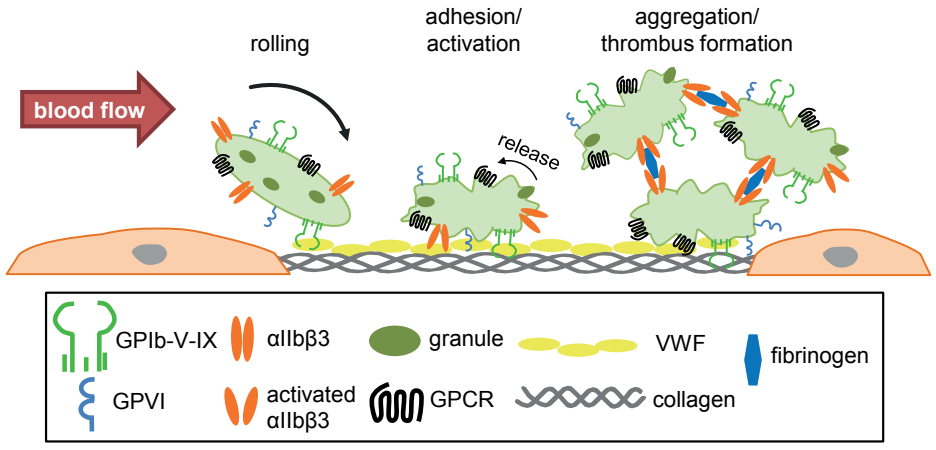


Figure 1. Mechanism of platelet adhesion and thrombus formation at sites of vascular injury. Initial platelet rolling and adhesion to the ECM is mediated predominantly by GPIbα–VWF interactions. Subsequent interaction with the collagen stimulates platelet spreading, integrin activation, and granule secretion. Together with the production and release of TxA_2, secreted soluble agonists activate GPCRs, leading to further αIIbβ3 activation and the formation of a stable thrombus.

Stimulated platelets release compounds that further enhance their activation. These compounds include adhesive proteins, like fibrinogen and VWF secreted from α-granules, and the substances adenosine diphosphate (ADP) and serotonin from dense granules. Together with the production and release of thromboxane A_2 (TxA_2), these soluble agonists activate G protein-coupled receptors (GPCRs), leading to further activation and recruitment of additional platelets into the growing thrombus. In a final step, inside-out signaling triggers activation of integrin adhesion receptors, which stabilizes platelet deposition and induces aggregation. Activation of integrin αIIbβ3 is considered essential as it contributes to platelet adhesion and regulates thrombus growth by bridging platelets through fibrinogen.[2]

Activated platelets also support the coagulation system by providing a negatively charged surface on which coagulation factor complexes assemble.[3] The resulting generation of a fibrin network enhances thrombus stability, which ultimately leads to the formation of a hemostatic plug that is crucial to limit blood loss after vascular injury. A disturbed balance in one of these processes leads to hemorrhage or occlusion of vessels, which can result in severe tissue damage. These life-threatening complications include stroke and myocardial infarction and are among the leading causes of death worldwide. Understanding the details of platelet function in hemostasis is therefore fundamental to develop therapeutic strategies to treat these diseases.

Platelet transfusion

Platelets play a pivotal role in maintaining vascular integrity. Patients with decreased platelet counts (thrombocytopenia) therefore require platelet transfusions to prevent bleeding and other related problems. Thrombocytopenia can be the result of various diseases, including sepsis, auto-immune diseases, leukemia and other malignancies that induce bone marrow failure. Since the 1970s, platelet transfusions are routinely applied as prophylaxis and for the treatment of bleeding in thrombocytopenic patients undergoing invasive surgery or chemotherapy. This resulted in a marked decrease in mortality rate due to bleeding (<1%),[4] but profoundly increased the demand for platelet concentrates.[5] Prophylactic platelet transfusions are prescribed when platelet counts fall below a certain threshold level. The critical level of circulating platelets to maintain hemostasis is 5 x 10^9/L. [6;7] Several studies have documented that there is very little relationship between platelet counts and bleeding risk at counts above this critical level.[8;9] There is, however, concern about the accuracy of platelet counts at these low levels. The current consensus therefore is that patients should receive a platelet transfusion when the platelet count is <10 x 10^9/L, unless there are additional risk factors for bleeding, such as sepsis, or other abnormalities of hemostasis.[10-12] A recent study has additionally demonstrated that more frequent low dose platelet transfusions (1.1 x 10^{11}/m^2 of body surface area) is more cost-effective than normal dose transfusion (2.2 x 10^{11}/m^2 of body surface area). Although the number of transfusions increased, the costs were lower due to a decreased number of total transfused platelets.[9] The effectiveness of platelet transfusion is assessed by determining the platelet recovery (corrected count increment; CCI): post-transfusion increment divided by the number of platelets transfused corrected for blood volume) and platelet survival (days to next transfusion). A platelet transfusion is considered successful when the CCI at 1 hour (h) after transfusion is >7.5 and after 20 h >2.5.[13]

Preparation of platelet concentrates

Platelet concentrates used for transfusion are prepared by isolating platelets either from a whole blood donation or by platelet apheresis. The two main methods of preparing platelet concentrates from whole blood are the platelet-rich plasma (PRP) and the buffy-coat method. The PRP method is the prevalent approach in the United States[14] and first separates two fractions, a red cell concentrate and a mix of the platelets and plasma. A second centrifugation step separates the plasma from the concentrated platelets. For the buffy-coat method, the predominant technique used in Europe,[14] whole blood is separated in one step into plasma, the buffy-coat and a red blood cell fraction. Buffy-coats from 4-8 ABO-matched donors are subsequently pooled and centrifugated to isolate the platelet fraction. Both the PRP and the buffy-coat method include a leukocyte reduction step to decrease the risk of recipient alloimmunization. Platelet apheresis is a procedure for obtaining platelet concentrates from

a single donor. A programmed machine automatically withdraws whole blood and extracts platelets by centrifugation, while the other blood components are returned to the donor. Transfusion of apheresis platelet concentrates from matched donors is performed when patients are refractory due to the presence of human leukocyte antigen (HLA) and/or platelet-specific antigen antibodies.

There is much debate about which source of platelet concentrates is superior, mainly in terms of platelet quality, efficacy of transfusion and safety issues.[15] Human transfusion studies are difficult to perform and compare for several reasons, one being that thrombocytopenic patients often have extremely variable responses to platelet transfusion. Overall, whole blood donations are preferred over apheresis due to its superior cost-effectiveness and reduced risk for the donor. Apheresis platelets may be applied when a specific clinical condition such as neonatal immune thrombocytyopenia or platelet refractoriness due to alloantibodies requires transfusion of matched platelet concentrates. There appears to be no significant difference in quality or transfusion efficacy between buffy-coat and apheresis platelet concentrates, but platelet concentrates prepared by the PRP method are considered inferior.[16;17] Lower bacterial infections rates and increased platelet yield currently argue in favor of the use of the buffy-coat method over PRP-prepared platelet concentrates.[18]

Quality control

The quality of platelet concentrates can be evaluated in vivo by measuring recovery and survival of fresh or stored radiolabeled platelets in healthy individuals. The complexity and high costs involved in these studies stimulated development of in vitro tests to assess platelet viability. Platelets become activated during preparation and storage. Platelet activation and reactivity to agonists can be analyzed by measuring the surface expression of hemostasis markers with flow cytometry. An alternative approach is to measure the platelets' ability to aggregate in response to agonists using light transmission aggregometry. However, it is unclear whether these in vitro tests correlate with in vivo survival and hemostatic function of platelets after transfusion.[19] Other parameters assessed during platelet storage include pH, lactate production and swirling. Accumulation of lactate due to anaerobic metabolism of glucose during storage of platelet concentrates leads to a drop in pH.[20] A pH range between 6.7 and 7.5 correlates with adequate in vivo platelet survival.[21;22] When pH falls below 6.0, platelet undergo an irreversible shape change from discoid to sphere,[23] which can be detected by the "swirling" phenomenon. Swirling indicates the presence of discoid platelets and is determined by examining a platelet concentrate against a light source while gently squeezing the container bag.

Storage conditions

Platelet concentrates are currently stored at room temperature (22-24^{o}C) under gentle agitation for a maximum of seven days. These conditions are far from ideal, as room temperature storage promotes bacterial growth and induces a decline in platelet viability and function, known as the platelet storage lesion. It is estimated that bacteria contaminate one out of 5000 platelet units, resulting in sepsis in more than 10% of contaminated platelet transfusions.[24] Recent developments in bacteria testing and pathogen inactivation have reduced the risk of transfusion-mediated sepsis, which could increase platelet shelf life. These techniques however do not prevent functional deterioration of platelets induced by prolonged storage at room temperature and may even reduce recovery and survival of transfused platelets.[24;25]

An alternative approach is storage of platelet concentrates at low temperature (0-4^{o}C). Cold

storage suppresses metabolic activity[26] and reduces the risk of bacterial growth,[27] which potentially prolongs platelet shelf life. Studies generally report that platelet activation markers increase faster in room temperature-stored platelets than in cooled platelets.[28-31] In addition, platelets stored at low temperature perform better in aggregation assays as their room temperature-stored counterparts.[28;29;32] However, cold storage has been precluded from introduction in transfusion medicine, as transfused platelets stored at low temperature are rapidly cleared from the circulation.[33] Apparently, the mechanisms that control clearance of cold-stored platelets are independent from the hemostatic functions of platelets.[34] Platelet cooling leads to a rapid irreversible disc-to-sphere shape change.[35] Preservation of the discoid shape by addition of the actin polymerization inhibitor cytochalasin D did not improve platelet survival,[36] indicating that other mechanisms are responsible. Another result of platelet cooling is transition of the platelet membrane from a liquid-crystalline to a gel phase. This coincides with aggregation of membrane domains enriched in cholesterol and sphingomyelin, known as lipid rafts.[37;38] Although stabilization of the platelet membrane with the sugar trehalose prevented these cold-induced changes, it was not established whether it prevented platelet clearance in vivo.[39]

In 2003, Hoffmeister et al. discovered a major role for GPIbα, a member of the GPIb-V-IX complex, in the clearance mechanism of cooled platelets.[34] Cold storage induced a redistribution of GPIbα from linear arrays into 'clusters' on the surface of murine platelets. This change was recognized by the carbohydrate-binding αMβ2 integrin present on macrophages, resulting in platelet phagocytosis and clearance from the circulation. Subsequent work published by the same group showed that exposed N-acetyl-D-glucosamine (GlcNAc) residues present on N-linked GPIbα glycans caused αMβ2 recognition.[40] GlcNAc residues are normally covered by galactose, which in turn are capped by terminal sialic acid. Platelet cooling triggered a loss of both sugar residues, as determined by an increase in binding of succinylated wheat germ agglutinin (sWGA), a lectin that specifically recognizes GlcNAc. Restoration of galactosylation by uridine diphosphate–galactose inhibited phagocytosis in vitro and clearly improved survival of two hour-cooled platelets in mice. Although this appeared to provide a simple approach to improve platelet circulation time, galactosylation had no effect on the survival of human platelets stored for more than 48 h at 0°C.[41] Long-term storage of platelets at this temperature increased exposure of galactose residues on GPIbα, which appeared docking sites for the Ashwell-Morell receptor on hepatocytes, initiating platelet destruction.[42] Inhibition of neuraminidase activity during cold storage by addition of N-Acetyl-2,3-dehydro-2-deoxyneuraminic acid (DANA) prevented the increase in GPIbα-bearing galactose residues and significantly improved platelet survival.[43] Whether prevention of deglycosylation by DANA enables cold storage of platelets, without interfering with hemostatic function and maintaining adequate platelet recovery and survival in humans remained to be established.

Apoptosis

Apoptosis in nucleate cells

Apoptosis, the process of programmed cell death, plays a vital role during cell termination, homeostasis, development and lymphocyte interactions. It has enormous implications in pathology, since cancer and neurodegenerative, autoimmune, and cardiovascular diseases all have dysregulated apoptosis as a hallmark. The process of apoptosis can be viewed as a cascade of events initiating from the accumulation of a death signal(s), release of apoptotic

factors from the mitochondria and activation of initiator/effector caspases, ultimately leading to cell death.[44] In nucleate cells, the initiation phase consists of two pathways; the extrinsic and intrinsic pathway (Figure 2). An extrinsic apoptotic signal is mediated by binding of an extracellular ligand to a transmembrane death receptor, which activates the initiator caspase-8. Important death receptors are the Fas receptor and tumor necrosis factor receptor-1 (TNFR1). Although the exact process of ligand binding and receptor oligomerization is unclear, it might require receptor internalization as well as clustering.[45] Once activated, either caspase-9 or caspase-8 cleaves executioner caspase-3, which represents the execution level of the caspase cascade. This gives rise to the morphological hallmarks of apoptosis such as DNA fragmentation, exposure of phosphatidylserine (PS) on the cell surface, membrane blebbing and cell shrinkage and ultimately leads to cell removal by phagocytes.

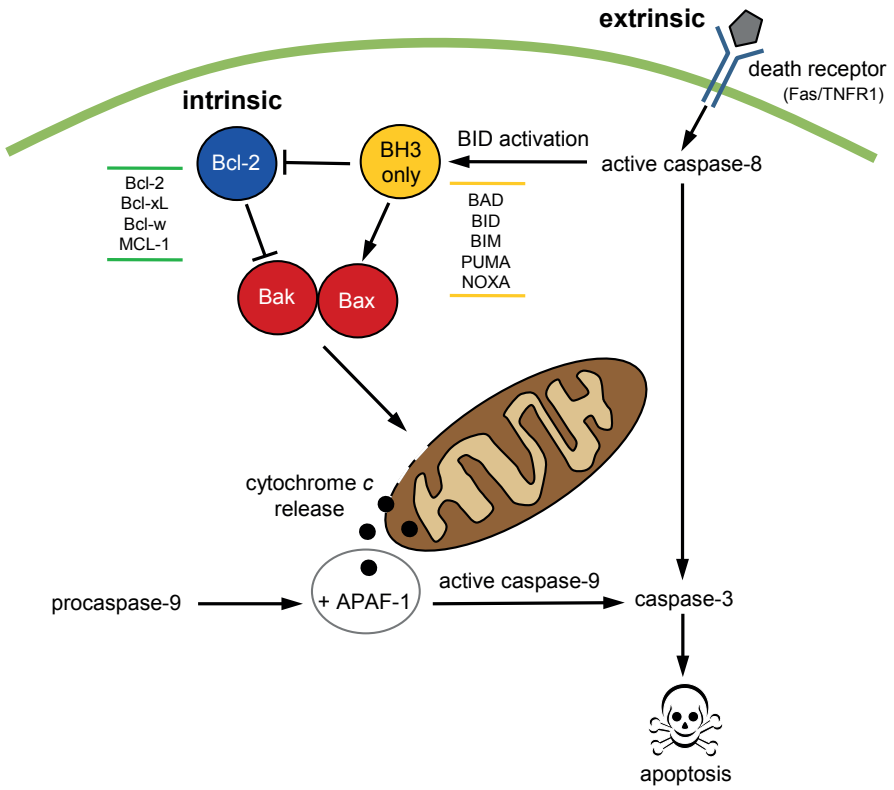


Figure 2. The extrinsic and intrinsic apoptosis pathways. Ligand binding to death receptors initiates the extrinsic pathway. Receptor activation results in cleavage and activation of caspase-8, which can directly activate caspase-3. Alternatively, it can trigger the intrinsic pathway through Bid activation. The intrinsic apoptosis pathway is regulated by the Bcl-2 family of pro-survival and pro-apoptotic proteins. Stress signals activate the BH3-only proteins, leading to inhibition of pro-survival Bcl-2 proteins. Activation of Bak and Bax results in permeabilization of the mitochondria. Cytochrome *c* leaks from the mitochondrial intermembrane space and forms a complex with APAF-1, resulting in caspase activation.

The intrinsic pathway is initiated from within the cell and involves changes in mitochondrial integrity. This is usually in response to cellular signals resulting from DNA damage, a defective cell cycle, detachment from the extracellular matrix, hypoxia, loss of cell survival factors, or other types of severe cell stress.[46] The intrinsic pathway is initiated by active transcriptional upregulation of specific members of the pro-apoptotic Bcl-2 (B-cell lymphoma 2) protein family involved in the promotion of apoptosis, such as the BH3-only proteins Bad, Bim, PUMA and NOXA. These in turn activate the multi-domain pro-apoptotic proteins Bax or Bak which move to the mitochondrial membrane. Here, these proteins disrupt the function of the anti-apoptotic Bcl-2 and Bcl-xL proteins resulting in permeabilization of the mitochondrial membrane.[47;48] This process is facilitated by the pro-apoptotic protein Bad, which dimerizes with Bcl-xL, thereby mediating the release of Bax from Bcl-2 and Bcl-xL proteins. Cytochrome *c* leaks from the intermembrane space of the mitochondria into the cytosol where it associates with the adaptor apoptotic protease activating factor-1 (APAF1). This large multi-protein structure known as the apoptosome then recruits and activates caspase-9, which in turn activates the downstream effector caspase-3, leading to apoptosis.[48] Apoptotic changes in the nucleus include DNA fragmentation, chromatin condensation, and nucleate break-up.[49;50] Cross-talk between the intrinsic and extrinsic pathway can occur via caspase-8 cleavage and activation of the pro-apoptotic BH3-only protein BID. This process relates signaling steps controlled by Bcl-2/Bcl-xL and Bak/Bax to death receptor activation.[51;52]

The role of 14-3-3 proteins in apoptosis

The 14-3-3 family of adaptor proteins consists of abundant 28–33 kDa acidic polypeptides found in all eukaryotic organisms. They belong to a family of proteins with seven isoforms designated with Greek letters δ, ε, τ, γ, η, and ζ.[53-55] 14-3-3 proteins self-assemble into homo- and hetero-dimers, with some family members, such as δ and γ, preferring to homodimerize and other family members, such as ε, preferring to heterodimerize. An important feature of the 14-3-3 proteins is their ability to interact with a diverse array of cellular proteins including transcription factors, biosynthetic enzymes, cytoskeletal proteins, signaling molecules, apoptosis factors and tumor suppressors. This interaction is commonly mediated through a phosphorylated serine or threonine motif.[56;57] The effects of 14-3-3 binding include changes in protein complex formation, enzymatic activity, and subcellular localization. By mediating these effects in a diverse array of proteins, 14-3-3 controls cell cycle progression, tumor suppression, mitogenic signal transduction, metabolism, and apoptosis.

Regulating the balance between survival and apoptotic signaling is a key aspect of cell fate decisions and 14-3-3 proteins contribute to this process in several ways. 14-3-3 regulates many signaling molecules that mediate the transmission of survival and death signals to the mitochondrial death machinery. Importantly, 14-3-3 plays a critical role in suppressing apoptosis by controlling Bad. The interaction of 14-3-3 with Bad was first discovered by a yeast-2-hybrid study, using Bad as bait.[58] In the pro-survival state, Bad is phosphorylated and retained in the cytosol. In order to become pro-apoptotic, Bad requires dephosphorylation and localization into mitochondria where it heterodimerizes with Bcl-2/xL, releasing Bak from the Bcl-2/xL complex to induce apoptosis. In survival mode, 14-3-3 binding leads to sequestration of Bad in the cytoplasm, away from Bcl-2/xL in the mitochondria. This inhibits Bad-induced release of Bak from Bcl-xL and neutralizes the ability of Bax to trigger apoptosis.

Apoptosis in platelets

The mechanisms of apoptosis have been extensively investigated in nucleated cells. However, several studies over the past decade have shown that the nucleus itself is not required for apoptosis, as apoptotic signaling molecules can induce typical apoptotic events in anucleate cytoplasts[59;60] and platelets.[61-66] In recent years, several studies have been performed to investigate the occurrence of apoptosis in platelets. In 1997, Martin et al. were the first to describe that alterations in Bcl-2/Bax protein levels in platelets induced by the calcium ionophore ionomycin lead to a process that resembles apoptosis.[61] Originally, the morphological changes of the platelet, which included platelet shrinkage, cytoplasm condensation, plasma membrane blebbing and extension of filopodia, were described as platelet activation, and only since 1997 did some investigators begin to consider these change as apoptotic.[61;67] Pereira et al. showed that platelet apoptosis was associated with platelet aging using a suppressed thrombopoiesis dog model,[64] while other studies demonstrated the occurrence of platelet apoptosis in mice with thrombocytopenia induced by injection of Tumor Necrosis Factor (TNF)[68] or anti-platelet antibodies.[69] Other studies reported apoptosis in platelets induced by platelet agonists TxA_2 mimetic U46619, collagen and thrombin.[67;70;71]

As the morphological changes of apoptosis resemble those of platelet activation, it has been questioned whether platelets are able to become apoptotic. Zhang et al. demonstrated that platelet apoptosis can be distinguished from platelet activation with the use of ABT-737, a potent inhibitor of Bcl-2 and Bcl-xL.[72] ABT-737 did not lead to platelet aggregation, only induced a modest α-granule release, and was not accompanied by activation of αIIbβ3. ABT-737 did induce cytochrome *c* release, associated with a dramatic increase in caspase-3 activity. These data show that inhibition of Bcl-2 proteins activates the classical markers of apoptosis in platelets. A major finding in platelet apoptosis was reported in 2007, where researchers revealed that the intrinsic pathway for apoptosis regulates platelet lifespan.[66] Genetic mutation or pharmacological inhibition of Bcl-xL caused apoptosis in vitro and reductions in platelet lifespan in vivo, while deletion of pro-apoptotic Bak nearly doubled platelet survival. How this apoptotic program is controlled remains unclear. One possibility is that Bcl-xL degrades during platelet ageing, with levels eventually reaching a threshold below which Bak becomes activated.[66] Alternatively, there may be an active signal that initiates apoptosis.

In addition to DNA fragmentation, membrane blebbing and cell shrinkage, other features of apoptosis include exposure of phosphatidylserine (PS) to the cell surface, and the permeabilization of mitochondrial membranes. For nucleate cells, two general mechanisms have been described to be responsible for the permeabilization of the membranes. One mechanism is mediated by members of the Bcl-2 family that act directly on the outer membrane of the mitochondrion. The other mechanism is defined by the opening of the mitochondria permeability transition pore (MPTP) in the inner mitochondrial membrane, allowing molecules to pass through. This leads to depolarization of mitochondrial inner membrane potential ($\Delta\Psi_m$), and swelling of the mitochondrial matrix following permeabilization of the outer mitochondrial membrane and release of proteins normally confined to the intermembrane space, including cytochrome *c*.[73-75] A recent study showed that, as in nucleated cells, mitochondria play a crucial role in the control of apoptosis in platelets and that the formation of MPTP is a key mechanism in mitochondrial control of platelet apoptosis.[76] Inhibition of MPTP formation by cyclosporin A completely prevented loss of $\Delta\Psi_m$, caspase-3 activation, and, to a lesser extent, the inhibition of PS exposure on the platelet surface (~25%). This might be explained by another recent study on PS exposure

by apoptotic platelets.[77] Herein the authors observed that two distinct pathways can regulate PS exposure of platelets in vitro. One pathway is a calcium-dependent, caspase independent pathway induced by platelet agonists. The second pathway is described by a Bak/Bax-caspase-mediated pathway independent of platelet activation.

Several signaling molecules of the extrinsic pathway of apoptosis in nucleate cells have been found in platelets. However, contradicting results were obtained when these mediators were analyzed on mRNA and protein levels. Platelets appear to express mRNA for death ligand TRAIL, death receptors TNFR1, DR3, DR4 and DR5, and adapter proteins TRADD and RIP, which are all associated with the extrinsic pathway of apoptosis.[63] In contrast, several mRNA and immunoblot assays were unable to detect the Fas receptor and Fas ligand,[63;78;79] and anti-Fas antibodies had no effect on platelets.[67] Contradicting results were also obtained for agonistic TRAIL receptors DR4 and DR5, as one study suggested the presence in platelets,[63] while others did not identify these proteins at the platelet surface either before or after storage of platelet concentrates for up to 11 days.

Storage of platelets and apoptosis

Platelet concentrates stored for transfusion medicine are kept for a maximum of seven days due to a decline in function and viability, possibly initiated by apoptosis.[61-63;67;80] Studies investigating the effects of platelet storage showed that apoptosis was provoked during storage of washed platelets at 37°C in a culture medium or plasma in capped tubes.[62] Platelets stored at 37°C had increased levels of pro-apoptotic caspase-3 and caspase-9 activity, and decreased levels of Bcl-xL.[78;81] Storage of platelet concentrates under standard blood bank conditions however was also associated with signs of apoptosis.[63;79] Platelets stored for seven days had increased caspase-3 activation[80] and depolarization of mitochondrial inner membrane potential,[82] strongly indicating the initiation of apoptotic death. Although hemostatic properties are better preserved, platelet storage at 0-4°C has been shown to induce apoptotic events, including activation of pro-apoptotic Bad and Bax, a fall in $\Delta\Psi_m$, cytochrome *c* release, caspase activation and PS exposure.[28;41;83] Recent work identified a role for GPIbα in cold-induced apoptosis. Under resting conditions, the adaptor protein 14-3-3ζ associates with phosphorylated Bad, thereby preventing initiation of apoptosis. Cold storage triggered the binding of 14-3-3ζ to the cytoplasmic tail of GPIbα, releasing 14-3-3ζ from Bad, inducing its dephosphorylation and activation of the apoptotic machinery.[83]

Glycoprotein Ibα

Structure

Under the high shear conditions present in arteries and arterioles, initial platelet adhesion requires the binding of VWF immobilized on the vessel wall to the platelet receptor complex GPIb-V-IX.[84] The complex consists of the subunits GPIbα, GPIbβ, GPV and GPIX, with a stoichiometry of 2:4:1:2.[85] Each subunit is a type I transmembrane protein and requires stabilization by covalent and noncovalent interactions. Disulfide bonds link GPIbα to two GPIbβ subunits, forming the GPIb complex, which in turn interacts noncovalently with GPIX and GPV. A single platelet contains approximately 25,000 copies of GPIbα, the subunit that interacts with VWF.[86] The ectodomain (residues 1-485) of GPIbα consists of an N-terminal flank, seven leucine-rich repeats, a C-terminal flank, a sulphated region and a macroglycopeptide domain (Figure 3).The ectodomain is heavily glycosylated and contains O- and N-linked glycans. The glycan core consists of GlcNAc residues, covered by galactose

residues which in turn are covered by terminal sialic acid. Residues 486-514 form the transmembrane domain and the cytoplasmic tail consists of 96 amino acid residues (residues 515-610),[87-89] which contain binding sites for multiple intracellular proteins, including filamin A[90] and 14-3-3ζ.[91] These proteins regulate GPIbα anchorage to the cytoskeleton.

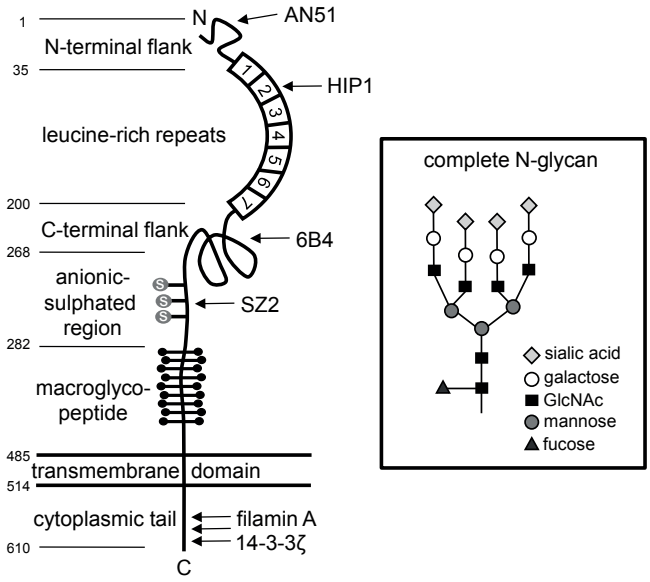


Figure 3. Schematic illustration of GPIbα. Binding sites of the monoclonal antibodies AN51, HIP1, 6B4 and SZ2 are indicated. The insert represents a typical, complete N-glycan.

Interaction with VWF

One of the major functions of GPIbα is slowing down platelets at sites of vascular injury by interacting with VWF. The unique biomechanical properties of both molecules allow the interaction to strengthen upon increasing hemodynamic drag.[92] VWF is a multimeric plasma protein that binds specifically to sites on subendothelial-exposed collagen and is stretched by the shear stress of flowing blood, which leads to the exposure of its A1 domain. This allows platelet binding through the leucine-rich repeat domain of GPIbα (Figure 4).[93] Incubation of VWF with the antibiotic ristocetin mimics shear-induced exposure of the A1 domain, which allows investigation of the GPIbα-VWF interaction in suspension in vitro.[94] Another important regulatory mechanism of the GPIbα-VWF interaction is the conformation of GPIbα. Single molecule measurements demonstrated recently that the GPIbα-A1 complex changes its conformation in a so-called 'flex bond' upon increasing shear force, which strengthens the receptor-ligand interaction.[92] In addition to conformational changes, the receptor function of GPIbα is regulated by intracellular signaling events. A critical step in GPIbα signaling is 14-3-3ζ binding to its cytoplasmic tail.[95;96] This adaptor molecule is expressed as a homodimer and contains two ligand binding sites that recognize phosphorylated serine or threonine motifs.[56;57] The intracellular domain of GPIbα contains multiple 14-3-3ζ binding sites, including residues 551-564,[96] 580-590[97] and 605-610.[98] The GPIbβ subunit of the GPIb complex contains a low-affinity 14-3-3ζ binding site at Ser-166.[95] The RYSGHpSL[610] sequence

with constitutively phosphorylated Ser-609 is the primary site required for high-affinity 14-3-3ζ binding to GPIbα. Genetic deletion or incubation with inhibiting peptides severely impairs GPIbα dependent platelet binding to VWF, which emphasizes the importance of 14-3-3ζ interaction with this site.[95] Interaction of 14-3-3ζ with residues 557-559, a region which also contains the binding site for filamin A, also affects VWF binding.[96] In resting platelets, GPIbα is anchored to the membrane skeleton via Filamin A. Activation by VWF triggers GPIbα dissociation from the membrane skeleton and recruitment to the actin cytoskeleton.[99] Yuan et al. have proposed that phosphorylation-dependent binding of 14-3-3ζ to Ser-559 competes with Filamin A interaction and subsequently links GPIbα to the cytoskeleton.[96] The multiple binding sites may also allow 14-3-3ζ dimers/multimers to crosslink GPIbα molecules, which has been reported to facilitate clustering of the acetylcholine receptor in muscle cells.[100]

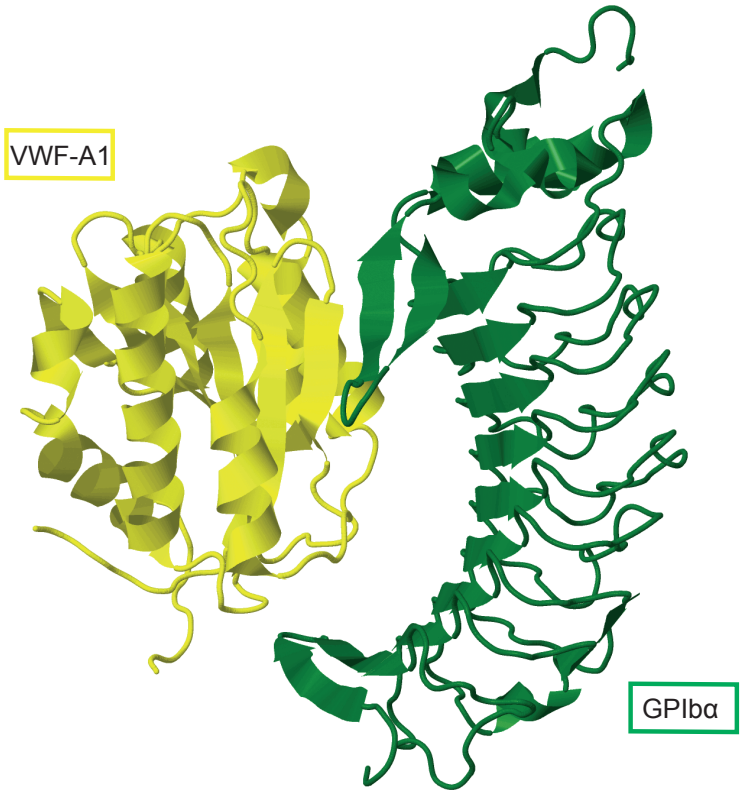


Figure 4. Crystal structure of GPIbα in complex with VWF A1 domain. Stereo view of a ribbon representation of the GPIbα-A1 complex by Huizinga et al.[93]

Several studies have reported on GPIbα signaling events that lead to integrin activation and integrin-dependent stable platelet adhesion and aggregation, which have been extensively reviewed.[101] VWF activation has been shown to induce GPIbα association with members of the Src family of protein kinase (c-Src and Lyn) and phosphoinositide 3-kinase (PI3K),[102] which are important for transmitting early activation signals leading to calcium elevation.[103] There

has been evidence that GPIbα associates with the immunoreceptor tyrosine-based activation motif (ITAM) receptors FcRγ[104] and FcγRIIa[105] upon activation. The ITAM domains interact with Syk, leading to activation of phospholipase Cγ2 (PLCγ2) and calcium mobilization, and may enhance GPIbα-mediated signaling events leading to α-granule release.[106] Other contributors of VWF-induced GPIbα signaling include the mitogen-activated protein kinases (MAPK).[107] GPIbα-VWF association was shown to activate P38MAPK,[108] which releases arachidonic acid from membrane phospholipids by stimulation of cytosolic phospholipase A_2 ($cPLA_2$). Liberated AA is converted to TxA_2 by cyclooxygenase-1 (COX-1) and Tx synthase. Binding of TxA_2 to its platelet receptor TPα subsequently leads to activation of αIIbβ3 and platelet aggregation.[109] As platelets are exposed to increasing hemodynamic drag during VWF binding, activation of P38MAPK by shear[110] may also contribute to GPIbα signaling events (Figure 5).

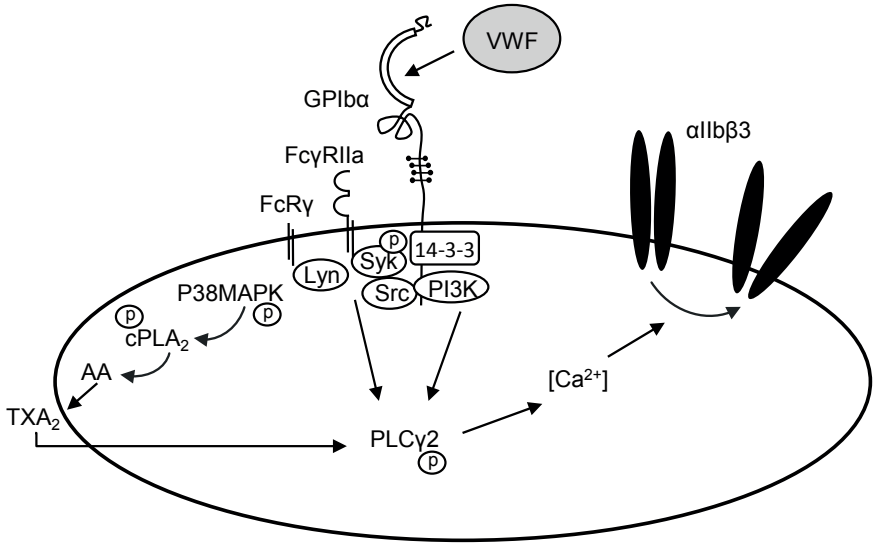


Figure 5. Signaling pathways activated by GPIbα. Binding of VWF induces [14-3-3ζ-GPIbα] complex formation, activation of Src tyrosine kinases and production of TxA_2. Activation of PLCγ2 results in calcium (Ca^{2+}) mobilization and activation of αIIbβ3.

Other functions

In addition to VWF, ligands for GPIbα include P-selectin,[111] αMβ2,[112] α-thrombin,[113] coagulation factors VII,[114] XI[115] and XII,[116] high-molecular-weight kininogen,[117] and β_2GPI.[118] The receptor thus appears to be important in platelet adhesion to activated endothelial cells, in leukocyte recruitment to sites of vascular injury, and in promoting the procoagulant activity of platelets. Another important function is regulating normal platelet morphology and cytoskeletal architecture.[119] This is illustrated by the Bernard-Soulier Syndrome (BSS), a rare hereditary disorder characterized by the lack of a functional GPIb-V-IX complex. Patients suffering from BSS have abnormally large platelets, low platelet counts and an increased bleeding tendency. Cranmer et al. have recently demonstrated that the GPIbα-filamin A interaction is important for membrane stability.[120] The absence or mutation of the intracellular domain of GPIbα may account for the membrane deformability observed in BSS

platelets.[121] The thrombocytopenia is thought to be the result of ineffective platelet production and increased platelet clearance.[122] In cancer, GPIbα is involved in the regulation of tumor metastasis, with some reports showing inhibition and others activation of this process.[123;124]

Presence in lipid rafts

The plasma membrane of a cell consists of a complex mixture of lipids and proteins, which are organized in liquid-disordered and liquid-ordered domains. The ordered domains are described as lipid rafts, of which the term 'raft' refers to distinct structures floating within a sea of fluid, randomly moving lipid. Currently, lipid rafts are defined as 'small' (10–200 nm) highly dynamic, heterogeneous, sterol- and sphingolipid-enriched domains that compartmentalize cellular processes.[125] Small rafts are known to coalesce into larger platforms through protein-protein and protein-lipid interactions, which facilitate transmembrane signal transduction.[126] Lipid rafts are viewed as platforms that can physically concentrate or separate receptors, adaptor proteins and effector enzymes, which regulate signaling events. A wide variety of cellular processes depend on lipid rafts, including T- and B-cell activation,[127;128] focal adhesions and cell migration,[129] the life cycle of the influenza and HIV viruses[130;131] and hormone signaling.[132] The composition of lipid rafts is highly heterogeneous, but enrichment in cholesterol and sphingolipids is considered a general characteristic.[125] Other molecules that preferentially localize to lipid rafts are glycosylphosphatidylinositol (GPI)-linked proteins, dually acylated or palmitoylated proteins, arachidonic acid and gangliosides.[133-137] Gangliosides are glycosphingolipids with different carbohydrate chains that extend out from the cell surface and are involved in cell-cell-recognition, adhesion and signal transduction.[138] The most widely used marker for lipid rafts is monosialotetrahexosylganglioside (GM1).[139]

The importance of lipid rafts in platelets is well established. Platelet signaling events depending on lipid rafts include calcium mobilization,[140] membrane remodeling upon activation,[141] anchorage of surface receptors to the membrane skeleton,[142] and colocalization of receptors that enhance platelet function.[143-145] In 2002, it was discovered that lipid rafts play a crucial role in the interaction of GPIbα with VWF.[145] In resting platelets, approximately 10%–15% of GPIbα molecules were present in rafts, which increased about threefold upon VWF ligation. Disruption of lipid rafts by cholesterol depletion markedly impaired GPIbα-dependent platelet interaction with VWF, which illustrates the importance of these membrane domains in platelet adhesion. Raft localization facilitates GPIbα association with important signaling molecules, including the tyrosine kinases Lyn and Src,[146] , the Fc receptor γ chain (FcRγ)[147] and the low-affinity IgG receptor FcγRIIa.[105] Association with these molecules could amplify GPIbα signaling events elicited by VWF ligation. The local density of GPIbα receptors may also be increased by raft localization, leading to improved ligand binding properties. Studies with Chinese Hamster Ovary (CHO) cells in which GPIbα was artificially dimerized have indeed suggested that receptor clustering increases the overall strength of the VWF-GPIbα interaction.[148;149]

Studies investigating GPIbα localization to lipid rafts have used the conventional method of sucrose density centrifugation.[142;145;146;150] However, preparation of detergent-resistant membrane fractions has a number of clear disadvantages. A major drawback is that the organization of the membrane structure is completely disturbed, which possibly alters protein interactions,[151] and makes it impossible to draw conclusions on spatial organization.

Protein colocalization

Information on the physical distance between molecules is important to understand their relationship and function. A non-invasive technique that allows the study of intact cells is microscopy. A major limitation of conventional (fluorescence) light microscopy is the spatial resolution. The maximal distance at which two objects can be visualized as separate entities using light microscopy is 200 nm.[152] Many fundamental cellular processes occur below this level, which has driven the need for improving the microscopy techniques. Electron microscopy (EM) has improved the spatial resolution 1000-fold, but this technique is costly, time-consuming, and requires the use of (chemically) fixed specimens.

Förster Resonance Energy Transfer

Information on the spatial distribution of molecules can be obtained in an indirect fashion, using Förster Resonance Energy Transfer (FRET). When a (donor) fluorophore is excited at its excitation wavelength, it undergoes a transition from the ground state S_0 to an excited state S_1 or S_2 (Figure 6A). The donor fluorophore can return to the S_0 state by emission of a photon, direct transfer to the ground state without emission of a photon or by transferring energy to an acceptor fluorophore. FRET occurs only when the two fluorophores have sufficiently large spectral overlap, a favorable dipole–dipole orientation, and a large enough quantum yield. The quantum yield is the ratio of the number of photons emitted to the number of photons absorbed. Importantly, the efficiency of FRET strongly depends on the distance between the donor and acceptor fluorophore (inversely proportional to the sixth power). As a consequence, FRET will only occur within the range of 1-10 nm.[153]

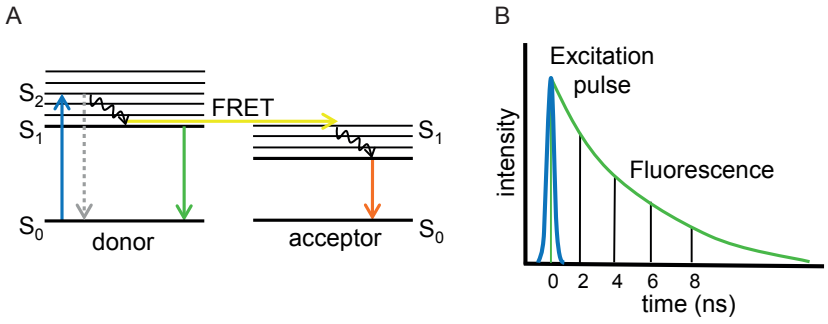


Figure 6. Principles of FRET and FLIM measurements. (**A**) Jablonski diagram for FRET. Excitation of a donor fluorophore results in a transition from the ground state S_0 to the excited state S_1 or S_2 (blue arrow). Return to the S_0 state can be achieved by direct non-radiative decay to the ground state (grey arrow), by emission of a photon (green arrow), or by transferring energy to a nearby acceptor fluorophore (yellow arrow). The acceptor can in its turn return to the S_0 state by emission of a photon. (**B**) Measurements of fluorescence lifetime. A short pulse of light excites the donor fluorophore (blue peak). Emitted light is recorded in four consecutive time gates of two nanoseconds each to accurately fit the decay curve (green).

Fluorescence Lifetime Imaging Microscopy

A popular and relatively easy measurement of FRET is determining the increase in acceptor emission and reduction in donor emission. A major disadvantage of this ratiometric method is that it strongly depends on the concentration of the fluorophores, which is difficult to control or determine. Furthermore, corrections are needed for donor fluorophore emission bleed-through in the acceptor emission channel. FRET can also be determined by Fluorescence

Lifetime Imaging Microscopy (FLIM). The fluorescence lifetime (τ) is defined as the average time that a molecule remains in the excited state before returning to the ground state. FLIM is independent of changes in probe concentration, excitation intensity and only requires determination of the donor fluorescence lifetime. The efficiency of FRET can be calculated by

$$FRET\ Efficiency = \frac{\tau_D - \tau_{D/A}}{\tau_D} \times 100\%$$

where τ is the donor fluorophore lifetime in nanoseconds in the absence (τ_D) and presence ($\tau_{D/A}$) of the acceptor fluorophore.
To determine the fluorescence decay, the donor is excited with a picosecond pulsed laser. Next, the donor emission is fitted using a monoexponential decay curve, described as

$$I(t) = I_0 e^{-t/\tau}$$

where I_0 is the intensity of the donor at t=0 after excitation.

For an accurate fit, the donor emission should be recorded at several points throughout the decay curve (Figure 6B). This can be achieved by recording the emitted light in consecutive time gates of a few nanoseconds. Data collected from four consecutive time gates is sufficient to determine reliable lifetime values without significant loss of sensitivity.[154] Although FLIM measurements require advanced technology, it allows accurate determination and visualization of protein-protein interactions on the intact cell membrane.

Outline of this thesis

The importance of GPIbα in arterial thrombus formation is well recognized. Although several papers have reported that GPIbα is involved in cold storage-induced platelet apoptosis and early clearance in vivo, the exact molecular mechanisms controlling these events remain to be elucidated. Progress in understanding of this pathway may not only potentiate the development of new drugs to prevent arterial thrombosis, but could also allow platelet storage at low temperature.

This thesis aims to provide molecular insights of GPIbα regulation in both platelet storage and function. Chapter 2 and 3 focus on storage of platelets for transfusion medicine and describes the pathway that leads to cold-induced apoptosis. In **chapter 2**, a novel role for AA is reported in mediating the transport of 14-3-3ζ to the cytoplasmic tail of GPIbα, resulting in activation of the apoptotic machinery of cold-stored platelets. By using a FRET/FLIM approach, we describe in **chapter 3** that cold storage induces deglycosylation of GPIbα, resulting in its 14-3-3ζ-mediated clustering in lipid rafts. Interference with these cold-induced signaling events markedly improved the survival times of transfused cold-stored platelets, without affecting hemostatic functions.

In **chapter 4**, we report on GPIbα clustering under physiological conditions and analyzed the effects on platelet interaction with VWF under conditions of elevated shear. Platelet exposure to high shear induced activation of P38MAPK, resulting in the liberation of AA from membrane phospholipids. Liberated AA triggered 14-3-3ζ-induced clustering of GPIbα. **Chapter 5** describes how autoantibody binding to GPIbα induces platelet activation in a patient with immune thrombocytopenia (ITP). FRET/FLIM analysis revealed that autoantibody binding triggered GPIbα translocation to lipid rafts, where it colocalized with FcγRIIa resulting in platelet activation. **Chapter 6** summarizes the results of this thesis and evaluates the findings in relation to current views on the regulatory mechanisms of GPIbα function.

References

1. Leeksma CH, Cohen JA. Determination of the life of human blood platelets using labelled diisopropylfluorophosphanate. *Nature*. 1955;175(4456):552-553.
2. Gruner S, Prostredna M, Schulte V et al. Multiple integrin-ligand interactions synergize in shear-resistant platelet adhesion at sites of arterial injury in vivo. *Blood*. 2003;102(12):4021-4027.
3. Heemskerk JW, Bevers EM, Lindhout T. Platelet activation and blood coagulation. *Thromb Haemost*. 2002;88(2):186-193.
4. Slichter SJ. Controversies in platelet transfusion therapy. *Annu Rev Med*. 1980;31509-540.
5. Sullivan MT, McCullough J, Schreiber GB, Wallace EL. Blood collection and transfusion in the United States in 1997. *Transfusion*. 2002;42(10):1253-1260.
6. Gaydos LA, Freireich EJ, Mantel N. The quantitative relation between platelet count and hemorrhage in patients with acute leukemia. *N Engl J Med*. 1962;266905-909.
7. Slichter SJ, Harker LA. Thrombocytopenia: mechanisms and management of defects in platelet production. *Clin Haematol*. 1978;7(3):523-539.
8. Tinmouth A, Tannock IF, Crump M et al. Low-dose prophylactic platelet transfusions in recipients of an autologous peripheral blood progenitor cell transplant and patients with acute leukemia: a randomized controlled trial with a sequential Bayesian design. *Transfusion*. 2004;44(12):1711-1719.
9. Slichter SJ, Kaufman RM, Assmann SF et al. Dose of prophylactic platelet transfusions and prevention of hemorrhage. *N Engl J Med*. 2010;362(7):600-613.
10. Guidelines for the use of platelet transfusions. *Br J Haematol*. 2003;122(1):10-23.
11. Schiffer CA, Anderson KC, Bennett CL et al. Platelet transfusion for patients with cancer: clinical practice guidelines of the American Society of Clinical Oncology. *J Clin Oncol*. 2001;19(5):1519-1538.
12. Slichter SJ. Evidence-based platelet transfusion guidelines. *Hematology Am Soc Hematol Educ Program*. 2007172-178.
13. Novotny VM, van DR, Witvliet MD, Claas FH, Brand A. Occurrence of allogeneic HLA and non-HLA antibodies after transfusion of prestorage filtered platelets and red blood cells: a prospective study. *Blood*. 1995;85(7):1736-1741.
14. Slichter SJ. Platelet transfusion therapy. *Hematol Oncol Clin North Am*. 2007;21(4):697-729, vii.
15. Schrezenmeier H, Seifried E. Buffy-coat-derived pooled platelet concentrates and apheresis platelet concentrates: which product type should be preferred? *Vox Sang*. 2010;99(1):1-15.
16. Arnold DM, Heddle NM, Kulczycky M, Carruthers J, Sigouin C, Blajchman MA. In vivo recovery and survival of apheresis and whole blood-derived platelets: a paired comparison in healthy volunteers. *Transfusion*. 2006;46(2):257-264.
17. Vassallo RR, Murphy S. A critical comparison of platelet preparation methods. *Curr Opin Hematol*. 2006;13(5):323-330.
18. Devine DV, Serrano K. Preparation of blood products for transfusion: is there a best method? *Biologicals*. 2012;40(3):187-190.
19. Rinder HM, Smith BR. In vitro evaluation of stored platelets: is there hope for predicting posttransfusion platelet survival and function? *Transfusion*. 2003;43(1):2-6.
20. Rock G, Figueredo A. Metabolic changes during platelet storage. *Transfusion*. 1976;16(6):571-579.
21. Murphy S, Sayar SN, Gardner FH. Storage of platelet concentrates at 22 degrees C. *Blood*. 1970;35(4):549-557.
22. Murphy S, Gardner FH. Platelet storage at 22 degrees C: role of gas transport across plastic containers in maintenance of viability. *Blood*. 1975;46(2):209-218.
23. Moroff G, Friedman A, Robkin-Kline L. Factors influencing changes in pH during storage of platelet concentrates at 20-24 degree C. *Vox Sang*. 1982;42(1):33-45.
24. Eder AF, Kennedy JM, Dy BA et al. Bacterial screening of apheresis platelets and the residual risk of septic transfusion reactions: the American Red Cross experience (2004-2006). *Transfusion*. 2007;47(7):1134-1142.
25. Webert KE, Cserti CM, Hannon J et al. Proceedings of a Consensus Conference: pathogen inactivation-making decisions about new technologies. *Transfus Med Rev*. 2008;22(1):1-34.
26. Slichter SJ. In vitro measurements of platelet concentrates stored at 4 and 22 degree C: correlation with posttransfusion platelet viability and function. *Vox Sang*. 1981;40 Suppl 172-86.

27. Currie LM, Harper JR, Allan H, Connor J. Inhibition of cytokine accumulation and bacterial growth during storage of platelet concentrates at 4 degrees C with retention of in vitro functional activity. *Transfusion*. 1997;37(1):18-24.
28. Babic AM, Josefsson EC, Bergmeier W et al. In vitro function and phagocytosis of galactosylated platelet concentrates after long-term refrigeration. *Transfusion*. 2007;47(3):442-451.
29. Hornsey VS, Drummond O, McMillan L et al. Cold storage of pooled, buffy-coat-derived, leucoreduced platelets in plasma. *Vox Sang*. 2008;95(1):26-32.
30. Snyder EL, Hezzey A, Katz AJ, Bock J. Occurrence of the release reaction during preparation and storage of platelet concentrates. *Vox Sang*. 1981;41(3):172-177.
31. Perrotta PL, Perrotta CL, Snyder EL. Apoptotic activity in stored human platelets. *Transfusion*. 2003;43(4):526-535.
32. Choi JW, Pai SH. Influence of storage temperature on the responsiveness of human platelets to agonists. *Ann Clin Lab Sci*. 2003;33(1):79-85.
33. Murphy S, Gardner FH. Effect of storage temperature on maintenance of platelet viability--deleterious effect of refrigerated storage. *N Engl J Med*. 1969;280(20):1094-1098.
34. Hoffmeister KM, Felbinger TW, Falet H et al. The clearance mechanism of chilled blood platelets. *Cell*. 2003;112(1):87-97.
35. Zucker MB, Borrelli J. Reversible alterations in platelet morphology produced by anticoagulants and by cold. *Blood*. 1954;9(6):602-608.
36. Winokur R, Hartwig JH. Mechanism of shape change in chilled human platelets. *Blood*. 1995;85(7):1796-1804.
37. Tablin F, Wolkers WF, Walker NJ et al. Membrane reorganization during chilling: implications for long-term stabilization of platelets. *Cryobiology*. 2001;43(2):114-123.
38. Gousset K, Wolkers WF, Tsvetkova NM et al. Evidence for a physiological role for membrane rafts in human platelets. *J Cell Physiol*. 2002;190(1):117-128.
39. Crowe JH, Tablin F, Wolkers WF, Gousset K, Tsvetkova NM, Ricker J. Stabilization of membranes in human platelets freeze-dried with trehalose. *Chem Phys Lipids*. 2003;122(1-2):41-52.
40. Hoffmeister KM, Josefsson EC, Isaac NA, Clausen H, Hartwig JH, Stossel TP. Glycosylation restores survival of chilled blood platelets. *Science*. 2003;301(5639):1531-1534.
41. Wandall HH, Hoffmeister KM, Sorensen AL et al. Galactosylation does not prevent the rapid clearance of long-term, 4 degrees C-stored platelets. *Blood*. 2008;111(6):3249-3256.
42. Rumjantseva V, Grewal PK, Wandall HH et al. Dual roles for hepatic lectin receptors in the clearance of chilled platelets. *Nat Med*. 2009;15(11):1273-1280.
43. Jansen AJ, Josefsson EC, Rumjantseva V et al. Desialylation accelerates platelet clearance after refrigeration and initiates GPIbalpha metalloproteinase-mediated cleavage in mice. *Blood*. 2012;119(5):1263-1273.
44. Coultas L, Strasser A. The role of the Bcl-2 protein family in cancer. *Semin Cancer Biol*. 2003;13(2):115-123.
45. Lee KH, Feig C, Tchikov V et al. The role of receptor internalization in CD95 signaling. *EMBO J*. 2006;25(5):1009-1023.
46. Thornberry NA, Lazebnik Y. Caspases: enemies within. *Science*. 1998;281(5381):1312-1316.
47. Reed JC, Jurgensmeier JM, Matsuyama S. Bcl-2 family proteins and mitochondria. *Biochim Biophys Acta*. 1998;1366(1-2):127-137.
48. Henry-Mowatt J, Dive C, Martinou JC, James D. Role of mitochondrial membrane permeabilization in apoptosis and cancer. *Oncogene*. 2004;23(16):2850-2860.
49. Roy C, Brown DL, Little JE et al. The topoisomerase II inhibitor teniposide (VM-26) induces apoptosis in unstimulated mature murine lymphocytes. *Exp Cell Res*. 1992;200(2):416-424.
50. Wyllie AH, Kerr JF, Currie AR. Cell death: the significance of apoptosis. *Int Rev Cytol*. 1980;68251-306.
51. Riedl SJ, Salvesen GS. The apoptosome: signalling platform of cell death. *Nat Rev Mol Cell Biol*. 2007;8(5):405-413.
52. Riedl SJ, Shi Y. Molecular mechanisms of caspase regulation during apoptosis. *Nat Rev Mol Cell Biol*. 2004;5(11):897-907.
53. Dougherty MK, Morrison DK. Unlocking the code of 14-3-3. *J Cell Sci*. 2004;117(Pt 10):1875-1884.
54. Mackintosh C. Dynamic interactions between 14-3-3 proteins and phosphoproteins regulate diverse cellular processes. *Biochem J*. 2004;381(Pt 2):329-342.

55. Muslin AJ, Xing H. 14-3-3 proteins: regulation of subcellular localization by molecular interference. *Cell Signal*. 2000;12(11-12):703-709.

56. Muslin AJ, Tanner JW, Allen PM, Shaw AS. Interaction of 14-3-3 with signaling proteins is mediated by the recognition of phosphoserine. *Cell*. 1996;84(6):889-897.

57. Yaffe MB, Rittinger K, Volinia S et al. The structural basis for 14-3-3:phosphopeptide binding specificity. *Cell*. 1997;91(7):961-971.

58. Zha J, Harada H, Yang E, Jockel J, Korsmeyer SJ. Serine phosphorylation of death agonist BAD in response to survival factor results in binding to 14-3-3 not BCL-X(L). *Cell*. 1996;87(4):619-628.

59. Schulze-Osthoff K, Walczak H, Droge W, Krammer PH. Cell nucleus and DNA fragmentation are not required for apoptosis. *J Cell Biol*. 1994;127(1):15-20.

60. Jacobson MD, Burne JF, Raff MC. Programmed cell death and Bcl-2 protection in the absence of a nucleus. *EMBO J*. 1994;13(8):1899-1910.

61. Vanags DM, Orrenius S, guilar-Santelises M. Alterations in Bcl-2/Bax protein levels in platelets form part of an ionomycin-induced process that resembles apoptosis. *Br J Haematol*. 1997;99(4):824-831.

62. Brown SB, Clarke MC, Magowan L, Sanderson H, Savill J. Constitutive death of platelets leading to scavenger receptor-mediated phagocytosis. A caspase-independent cell clearance program. *J Biol Chem*. 2000;275(8):5987-5996.

63. Li J, Xia Y, Bertino AM, Coburn JP, Kuter DJ. The mechanism of apoptosis in human platelets during storage. *Transfusion*. 2000;40(11):1320-1329.

64. Pereira J, Soto M, Palomo I et al. Platelet aging in vivo is associated with activation of apoptotic pathways: studies in a model of suppressed thrombopoiesis in dogs. *Thromb Haemost*. 2002;87(5):905-909.

65. Seghatchian J, Krailadsiri P. Platelet storage lesion and apoptosis: are they related? *Transfus Apher Sci*. 2001;24(1):103-105.

66. Mason KD, Carpinelli MR, Fletcher JI et al. Programmed anuclear cell death delimits platelet life span. *Cell*. 2007;128(6):1173-1186.

67. Wolf BB, Goldstein JC, Stennicke HR et al. Calpain functions in a caspase-independent manner to promote apoptosis-like events during platelet activation. *Blood*. 1999;94(5):1683-1692.

68. Piguet PF, Vesin C, Da KC. Activation of platelet caspases by TNF and its consequences for kinetics. *Cytokine*. 2002;18(4):222-230.

69. Piguet PF, Vesin C. Modulation of platelet caspases and life-span by anti-platelet antibodies in mice. *Eur J Haematol*. 2002;68(5):253-261.

70. Shcherbina A, Remold-O'Donnell E. Role of caspase in a subset of human platelet activation responses. *Blood*. 1999;93(12):4222-4231.

71. Leytin V, Allen DJ, Mykhaylov S, Lyubimov E, Freedman J. Thrombin-triggered platelet apoptosis. *J Thromb Haemost*. 2006;4(12):2656-2663.

72. Zhang H, Nimmer PM, Tahir SK et al. Bcl-2 family proteins are essential for platelet survival. *Cell Death Differ*. 2007;14(5):943-951.

73. Bernardi P, Scorrano L, Colonna R, Petronilli V, Di LF. Mitochondria and cell death. Mechanistic aspects and methodological issues. *Eur J Biochem*. 1999;264(3):687-701.

74. Crompton M. The mitochondrial permeability transition pore and its role in cell death. *Biochem J*. 1999;341 (Pt 2)233-249.

75. Kroemer G, Galluzzi L, Brenner C. Mitochondrial membrane permeabilization in cell death. *Physiol Rev*. 2007;87(1):99-163.

76. Leytin V, Allen DJ, Mutlu A, Gyulkhandanyan AV, Mykhaylov S, Freedman J. Mitochondrial control of platelet apoptosis: effect of cyclosporin A, an inhibitor of the mitochondrial permeability transition pore. *Lab Invest*. 2009;89(4):374-384.

77. Schoenwaelder SM, Yuan Y, Josefsson EC et al. Two distinct pathways regulate platelet phosphatidylserine exposure and procoagulant function. *Blood*. 2009

78. Kuter DJ. Apoptosis in platelets during ex vivo storage. *Vox Sang*. 2002;83 Suppl 1311-313.

79. Plenchette S, Moutet M, Benguella M et al. Early increase in DcR2 expression and late activation of caspases in the platelet storage lesion. *Leukemia*. 2001;15(10):1572-1581.

80. Perrotta PL, Perrotta CL, Snyder EL. Apoptotic activity in stored human platelets. *Transfusion*. 2003;43(4):526-535.

81. Bertino AM, Qi XQ, Li J, Xia Y, Kuter DJ. Apoptotic markers are increased in platelets stored at 37 degrees C. *Transfusion*. 2003;43(7):857-866.

82. Albanyan AM, Harrison P, Murphy MF. Markers of platelet activation and apoptosis during storage of apheresis- and buffy coat-derived platelet concentrates for 7 days. *Transfusion*. 2009;49(1):108-117.

83. van der Wal DE, Du VX, Lo KS, Rasmussen JT, Verhoef S, Akkerman JW. Platelet apoptosis by cold-induced glycoprotein Ibalpha clustering. *J Thromb Haemost*. 2010;8(11):2554-2562.

84. Savage B, Saldivar E, Ruggeri ZM. Initiation of platelet adhesion by arrest onto fibrinogen or translocation on von Willebrand factor. *Cell*. 1996;84(2):289-297.

85. Luo SZ, Mo X, fshar-Kharghan V, Srinivasan S, Lopez JA, Li R. Glycoprotein Ibalpha forms disulfide bonds with 2 glycoprotein Ibbeta subunits in the resting platelet. *Blood*. 2007;109(2):603-609.

86. Du X, Beutler L, Ruan C, Castaldi PA, Berndt MC. Glycoprotein Ib and glycoprotein IX are fully complexed in the intact platelet membrane. *Blood*. 1987;69(5):1524-1527.

87. Dong JF, Li CQ, Lopez JA. Tyrosine sulfation of the glycoprotein Ib-IX complex: identification of sulfated residues and effect on ligand binding. *Biochemistry*. 1994;33(46):13946-13953.

88. Lopez JA, Chung DW, Fujikawa K, Hagen FS, Papayannopoulou T, Roth GJ. Cloning of the alpha chain of human platelet glycoprotein Ib: a transmembrane protein with homology to leucine-rich alpha 2-glycoprotein. *Proc Natl Acad Sci U S A*. 1987;84(16):5615-5619.

89. Korrel SA, Clemetson KJ, Van HH, Kamerling JP, Sixma JJ, Vliegenthart JF. Structural studies on the O-linked carbohydrate chains of human platelet glycocalicin. *Eur J Biochem*. 1984;140(3):571-576.

90. Andrews RK, Fox JE. Identification of a region in the cytoplasmic domain of the platelet membrane glycoprotein Ib-IX complex that binds to purified actin-binding protein. *J Biol Chem*. 1992;267(26):18605-18611.

91. Du X, Harris SJ, Tetaz TJ, Ginsberg MH, Berndt MC. Association of a phospholipase A2 (14-3-3 protein) with the platelet glycoprotein Ib-IX complex. *J Biol Chem*. 1994;269(28):18287-18290.

92. Kim J, Zhang CZ, Zhang X, Springer TA. A mechanically stabilized receptor-ligand flex-bond important in the vasculature. *Nature*. 2010;466(7309):992-995.

93. Huizinga EG, Tsuji S, Romijn RA et al. Structures of glycoprotein Ibalpha and its complex with von Willebrand factor A1 domain. *Science*. 2002;297(5584):1176-1179.

94. Dong JF, Berndt MC, Schade A, McIntire LV, Andrews RK, Lopez JA. Ristocetin-dependent, but not botrocetin-dependent, binding of von Willebrand factor to the platelet glycoprotein Ib-IX-V complex correlates with shear-dependent interactions. *Blood*. 2001;97(1):162-168.

95. Dai K, Bodnar R, Berndt MC, Du X. A critical role for 14-3-3zeta protein in regulating the VWF binding function of platelet glycoprotein Ib-IX and its therapeutic implications. *Blood*. 2005;106(6):1975-1981.

96. Yuan Y, Zhang W, Yan R et al. Identification of a novel 14-3-3zeta binding site within the cytoplasmic domain of platelet glycoprotein Ibalpha that plays a key role in regulating the von Willebrand factor binding function of glycoprotein Ib-IX. *Circ Res*. 2009;105(12):1177-1185.

97. Mangin P, David T, Lavaud V et al. Identification of a novel 14-3-3zeta binding site within the cytoplasmic tail of platelet glycoprotein Ibalpha. *Blood*. 2004;104(2):420-427.

98. Du X, Fox JE, Pei S. Identification of a binding sequence for the 14-3-3 protein within the cytoplasmic domain of the adhesion receptor, platelet glycoprotein Ib alpha. *J Biol Chem*. 1996;271(13):7362-7367.

99. van der Wal DE, Verhoef S, Schutgens RE, Peters M, Wu Y, Akkerman JW. Role of glycoprotein Ibalpha mobility in platelet function. *Thromb Haemost*. 2010;103(5):1033-1043.

100. Lee CW, Han J, Bamburg JR, Han L, Lynn R, Zheng JQ. Regulation of acetylcholine receptor clustering by ADF/cofilin-directed vesicular trafficking. *Nat Neurosci*. 2009;12(7):848-856.

101. Du X. Signaling and regulation of the platelet glycoprotein Ib-IX-V complex. *Curr Opin Hematol*. 2007;14(3):262-269.

102. Wu Y, Asazuma N, Satoh K et al. Interaction between von Willebrand factor and glycoprotein Ib activates Src kinase in human platelets: role of phosphoinositide 3-kinase. *Blood*. 2003;101(9):3469-3476.

103. Mazzucato M, Pradella P, Cozzi MR, De ML, Ruggeri ZM. Sequential cytoplasmic calcium signals in a 2-stage platelet activation process induced by the glycoprotein Ibalpha mechanoreceptor. *Blood*. 2002;100(8):2793-2800.

104. Wu Y, Suzuki-Inoue K, Satoh K et al. Role of Fc receptor gamma-chain in platelet glycoprotein Ib-mediated signaling. *Blood*. 2001;97(12):3836-3845.

105. Sullam PM, Hyun WC, Szollosi J, Dong J, Foss WM, Lopez JA. Physical proximity and functional interplay of the glycoprotein Ib-IX-V complex and the Fc receptor FcgammaRIIA on the platelet plasma membrane. *J Biol Chem*. 1998;273(9):5331-5336.

106. Canobbio I, Bertoni A, Lova P et al. Platelet activation by von Willebrand factor requires coordinated signaling through thromboxane A2 and Fc gamma IIA receptor. *J Biol Chem*. 2001;276(28):26022-26029.

107. Li Z, Zhang G, Feil R, Han J, Du X. Sequential activation of p38 and ERK pathways by cGMP-dependent protein kinase leading to activation of the platelet integrin alphaIIb beta3. *Blood*. 2006;107(3):965-972.

108. Canobbio I, Reineri S, Sinigaglia F, Balduini C, Torti M. A role for p38 MAP kinase in platelet activation by von Willebrand factor. *Thromb Haemost*. 2004;91(1):102-110.

109. Garcia A, Quinton TM, Dorsam RT, Kunapuli SP. Src family kinase-mediated and Erk-mediated thromboxane A2 generation are essential for VWF/GPIb-induced fibrinogen receptor activation in human platelets. *Blood*. 2005;106(10):3410-3414.

110. Sumpio BE, Yun S, Cordova AC et al. MAPKs (ERK1/2, p38) and AKT can be phosphorylated by shear stress independently of platelet endothelial cell adhesion molecule-1 (CD31) in vascular endothelial cells. *J Biol Chem*. 2005;280(12):11185-11191.

111. Romo GM, Dong JF, Schade AJ et al. The glycoprotein Ib-IX-V complex is a platelet counterreceptor for P-selectin. *J Exp Med*. 1999;190(6):803-814.

112. Simon DI, Chen Z, Xu H et al. Platelet glycoprotein ibalpha is a counterreceptor for the leukocyte integrin Mac-1 (CD11b/CD18). *J Exp Med*. 2000;192(2):193-204.

113. Celikel R, McClintock RA, Roberts JR et al. Modulation of alpha-thrombin function by distinct interactions with platelet glycoprotein Ibalpha. *Science*. 2003;301(5630):218-221.

114. Weeterings C, de Groot PG, Adelmeijer J, Lisman T. The glycoprotein Ib-IX-V complex contributes to tissue factor-independent thrombin generation by recombinant factor VIIa on the activated platelet surface. *Blood*. 2008;112(8):3227-3233.

115. Baglia FA, Shrimpton CN, Emsley J et al. Factor XI interacts with the leucine-rich repeats of glycoprotein Ibalpha on the activated platelet. *J Biol Chem*. 2004;279(47):49323-49329.

116. Bradford HN, Pixley RA, Colman RW. Human factor XII binding to the glycoprotein Ib-IX-V complex inhibits thrombin-induced platelet aggregation. *J Biol Chem*. 2000;275(30):22756-22763.

117. Joseph K, Nakazawa Y, Bahou WF, Ghebrehiwet B, Kaplan AP. Platelet glycoprotein Ib: a zinc-dependent binding protein for the heavy chain of high-molecular-weight kininogen. *Mol Med*. 1999;5(8):555-563.

118. Pennings MT, Derksen RH, van LM et al. Platelet adhesion to dimeric beta-glycoprotein I under conditions of flow is mediated by at least two receptors: glycoprotein Ibalpha and apolipoprotein E receptor 2'. *J Thromb Haemost*. 2007;5(2):369-377.

119. Kanaji T, Russell S, Ware J. Amelioration of the macrothrombocytopenia associated with the murine Bernard-Soulier syndrome. *Blood*. 2002;100(6):2102-2107.

120. Cranmer SL, Ashworth KJ, Yao Y et al. High shear-dependent loss of membrane integrity and defective platelet adhesion following disruption of the GPIbalpha-filamin interaction. *Blood*. 2011;117(9):2718-2727.

121. White JG, Burris SM, Hasegawa D, Johnson M. Micropipette aspiration of human blood platelets: a defect in Bernard-Soulier's syndrome. *Blood*. 1984;63(5):1249-1252.

122. Tomer A, Scharf RE, McMillan R, Ruggeri ZM, Harker LA. Bernard-Soulier syndrome: quantitative characterization of megakaryocytes and platelets by flow cytometric and platelet kinetic measurements. *Eur J Haematol*. 1994;52(4):193-200.

123. Erpenbeck L, Nieswandt B, Schon M, Pozgajova M, Schon MP. Inhibition of platelet GPIb alpha and promotion of melanoma metastasis. *J Invest Dermatol*. 2010;130(2):576-586.

124. Jain S, Zuka M, Liu J et al. Platelet glycoprotein Ib alpha supports experimental lung metastasis. *Proc Natl Acad Sci U S A*. 2007;104(21):9024-9028.

125. Pike LJ. Rafts defined: a report on the Keystone Symposium on Lipid Rafts and Cell Function. *J Lipid Res*. 2006;47(7):1597-1598.

126. Lingwood D, Ries J, Schwille P, Simons K. Plasma membranes are poised for activation of raft phase coalescence at physiological temperature. *Proc Natl Acad Sci U S A*. 2008;105(29):10005-10010.

127. Gaus K, Chklovskaia E, Fazekas de St GB, Jessup W, Harder T. Condensation of the plasma membrane at the site of T lymphocyte activation. *J Cell Biol*. 2005;171(1):121-131.

128. Gupta N, DeFranco AL. Lipid rafts and B cell signaling. *Semin Cell Dev Biol*. 2007;18(5):616-626.

129. Gaus K, Le LS, Balasubramanian N, Schwartz MA. Integrin-mediated adhesion regulates membrane order. *J Cell Biol*. 2006;174(5):725-734.

130. Carrasco M, Amorim MJ, Digard P. Lipid raft-dependent targeting of the influenza A virus nucleoprotein to the apical plasma membrane. *Traffic*. 2004;5(12):979-992.

131. Nguyen DH, Hildreth JE. Evidence for budding of human immunodeficiency virus type 1 selectively from glycolipid-enriched membrane lipid rafts. *J Virol*. 2000;74(7):3264-3272.

132. Marquez DC, Chen HW, Curran EM, Welshons WV, Pietras RJ. Estrogen receptors in membrane lipid rafts and signal transduction in breast cancer. *Mol Cell Endocrinol*. 2006;246(1-2):91-100.

133. Brown DA, Rose JK. Sorting of GPI-anchored proteins to glycolipid-enriched membrane subdomains during transport to the apical cell surface. *Cell*. 1992;68(3):533-544.

134. Shaul PW, Smart EJ, Robinson LJ et al. Acylation targets emdothelial nitric-oxide synthase to plasmalemmal caveolae. *J Biol Chem*. 1996;271(11):6518-6522.

135. Shogomori H, Hammond AT, Ostermeyer-Fay AG et al. Palmitoylation and intracellular domain interactions both contribute to raft targeting of linker for activation of T cells. *J Biol Chem*. 2005;280(19):18931-18942.

136. Pike LJ, Han X, Chung KN, Gross RW. Lipid rafts are enriched in arachidonic acid and plasmenylethanolamine and their composition is independent of caveolin-1 expression: a quantitative electrospray ionization/mass spectrometric analysis. *Biochemistry*. 2002;41(6):2075-2088.

137. Prinetti A, Chigorno V, Tettamanti G, Sonnino S. Sphingolipid-enriched membrane domains from rat cerebellar granule cells differentiated in culture. A compositional study. *J Biol Chem*. 2000;275(16):11658-11665.

138. Sonnino S, Prinetti A. Lipids and membrane lateral organization. *Front Physiol*. 2010;1153.

139. Simons K, Ikonen E. Functional rafts in cell membranes. *Nature*. 1997;387(6633):569-572.

140. Dionisio N, Galan C, Jardin I, Salido GM, Rosado JA. Lipid rafts are essential for the regulation of SOCE by plasma membrane resident STIM1 in human platelets. *Biochim Biophys Acta*. 2011;1813(3):431-437.

141. Larive RM, Baisamy L, Urbach S, Coopman P, Bettache N. Cell membrane extensions, generated by mechanical constraint, are associated with a sustained lipid raft patching and an increased cell signaling. *Biochim Biophys Acta*. 2010;1798(3):389-400.

142. Munday AD, Gaus K, Lopez JA. The platelet glycoprotein Ib-IX-V complex anchors lipid rafts to the membrane skeleton: implications for activation-dependent cytoskeletal translocation of signaling molecules. *J Thromb Haemost*. 2010;8(1):163-172.

143. Lee FA, van LM, Relou IA et al. Lipid rafts facilitate the interaction of PECAM-1 with the glycoprotein VI-FcR gamma-chain complex in human platelets. *J Biol Chem*. 2006;281(51):39330-39338.

144. Locke D, Chen H, Liu Y, Liu C, Kahn ML. Lipid rafts orchestrate signaling by the platelet receptor glycoprotein VI. *J Biol Chem*. 2002;277(21):18801-18809.

145. Shrimpton CN, Borthakur G, Larrucea S, Cruz MA, Dong JF, Lopez JA. Localization of the adhesion receptor glycoprotein Ib-IX-V complex to lipid rafts is required for platelet adhesion and activation. *J Exp Med*. 2002;196(8):1057-1066.

146. Jin W, Inoue O, Tamura N et al. A role for glycosphingolipid-enriched microdomains in platelet glycoprotein Ib-mediated platelet activation. *J Thromb Haemost*. 2007;5(5):1034-1040.

147. Falati S, Edmead CE, Poole AW. Glycoprotein Ib-V-IX, a receptor for von Willebrand factor, couples physically and functionally to the Fc receptor gamma-chain, Fyn, and Lyn to activate human platelets. *Blood*. 1999;94(5):1648-1656.

148. Kasirer-Friede A, Ware J, Leng L, Marchese P, Ruggeri ZM, Shattil SJ. Lateral clustering of platelet GP Ib-IX complexes leads to up-regulation of the adhesive function of integrin alpha IIbbeta 3. *J Biol Chem*. 2002;277(14):11949-11956.

149. Arya M, Lopez JA, Romo GM et al. Glycoprotein Ib-IX-mediated activation of integrin alpha(IIb)beta(3): effects of receptor clustering and von Willebrand factor adhesion. *J Thromb Haemost*. 2003;1(6):1150-1157.

150. Geng H, Xu G, Ran Y, Lopez JA, Peng Y. Platelet glycoprotein Ib beta/IX mediates glycoprotein Ib alpha localization to membrane lipid domain critical for von Willebrand factor interaction at high shear. *J Biol Chem*. 2011;286(24):21315-21323.

151. van RJ, Achame EM, Janssen H, Calafat J, Jalink K. PIP2 signaling in lipid domains: a critical re-evaluation. *EMBO J*. 2005;24(9):1664-1673.

152. Schermelleh L, Heintzmann R, Leonhardt H. A guide to super-resolution fluorescence microscopy. *J Cell Biol*. 2010;190(2):165-175.

153. Jares-Erijman EA, Jovin TM. FRET imaging. *Nat Biotechnol*. 2003;21(11):1387-1395.

154. Gerritsen HC, Asselbergs MA, Agronskaia AV, Van Sark WG. Fluorescence lifetime imaging in scanning microscopes: acquisition speed, photon economy and lifetime resolution. *J Microsc*. 2002;206(Pt 3):218-224.

Chapter 2

Arachidonic acid depletion extends survival of cold-stored platelets by interfering with the [glycoprotein Ibα – 14-3-3ζ] association

Eelo Gitz[1*]
Dianne E. van der Wal[1*]
Vivian X. Du[1]
Kimberly S.L. Lo[1]
Cornelis A. Koekman[1]
Sabine Versteeg[2]
Jan-Willem N. Akkerman[1]

[1]Department of Clinical Chemistry and Haematology, University Medical Center Utrecht, Utrecht, the Netherlands
[2]Central Laboratory Animal Research Facility, Utrecht University, Utrecht, the Netherlands

*Both authors contributed equally to this manuscript

Adapted from Haematologica. 2012; 97(10): 1514-1522

Abstract

Cold storage of platelets reduces bacterial growth and better preserves their hemostatic properties than current procedures. However, 0ºC storage induces [14-3-3ζ-glycoprotein Ibα] association, 14-3-3ζ release from phospho-Bad, Bad activation and apoptosis. We investigated whether arachidonic acid, which also binds 14-3-3ζ, contributes to cold-induced apoptosis. Cold storage activated P38-mitogen-activated protein kinase and released arachidonic acid, which accumulated due to cold inactivation of cyclooxygenase-1/thromboxane synthase. Accumulated arachidonic acid released 14-3-3ζ from phospho-Bad and decreased the mitochondrial membrane potential, which are steps in apoptosis induction. Arachidonic acid addition did the same and depletion made platelets resistant against cold-induced apoptosis. Incubation with biotin-arachidonic acid revealed formation of a [arachidonic acid-14-3-3ζ-glycoprotein Ibα] complex. Indomethacin promoted complex formation by accumulating arachidonic acid and released 14-3-3ζ from cyclooxygenase-1. Arachidonic acid depletion prevented the cold-induced reduction of platelet survival in mice. We conclude that cold storage induced apoptosis through a [arachidonic acid-14-3-3ζ-glycoprotein Ibα] complex, which released 14-3-3ζ from Bad in an arachidonic acid-dependent manner. Although arachidonic acid depletion reduced agonist-induced thromboxane A_2 formation and aggregation, arachidonic acid repletion restored these functions, opening ways to reduce apoptosis during storage without compromising hemostatic functions post-transfusion.

Introduction

Current protocols for storage of platelet concentrates recommend a temperature of 22-24°C and a maximum of 7 days.[1] Problems of the relative high temperature are the growth of bacteria which occasionally contaminate platelet concentrates and the gradual loss of hemostatic functions. Improvements are sought in cooling to 0-4°C, but this introduces new problems as it induces redistribution of the von Willebrand factor (VWF) receptor, glycoprotein (GP) Ibα. The GPIbα change starts apoptosis,[2] platelet destruction by macrophages[3] and generation of Thromboxane A_2 (TxA_2) upon rewarming.[4]

We described previously that the cold-induced change makes GPIbα a "sink" for the adapter protein 14-3-3ζ.[2] Formation of the [14-3-3ζ-GPIbα] complex is accompanied with dissociation of [14-3-3ζ-phospho-Bad], inducing Bad activation, a fall in mitochondrial membrane potential ($\Delta\Psi_m$) and caspase-9 activation. These reactions drive the surface exposure of phosphatidylserine (PS), which together with GPIbα and surface-expressed P-selectin mediate binding to macrophages and platelet destruction. Cold storage followed by rewarming starts TxA_2 formation, which initiates a second wave of apoptosis induction, as do most platelet activating agents.[5]

At physiological temperature, VWF binds to GPIbα and activates cytosolic phospholipase A_2 ($cPLA_2$), arachidonic acid (AA) release from membrane phospholipids and TxA_2 formation.[6] $cPLA_2$ is activated through phosphorylation of Ser^{505} by P38-mitogen-activated protein kinase (P38MAPK).[7;8] The major part of released AA is metabolized by cyclooxygenase-1 (COX-1) to endoperoxides and further converted by thromboxane synthase to TxA_2. Released AA is also a substrate for 12-lipoxygenase, which generates hydro (pero)xy-eicosatetraenoic acid[9] and possibly for cytochrome P450 monooxygenase, which catalyzes formation of 14,15-epoxyeicosatrienoic acid.[10] COX-1 is the target of aspirin, which acetylates Ser^{530} blocking access of AA to the active site.[11]

The caspase-9 induction followed by PS surface exposure and binding/phagocytosis by macrophages, are major responses to the relative small effect that association with GPIbα might have on 14-3-3ζ localizations. We therefore searched for other pathways that might contribute to cold-induced apoptosis, acting either in parallel or in synergy with the pathway initiated by GPIbα. A candidate is the release of AA. In U937 phagocytic cells, interference with reacylation/deacylation of membrane phospholipids induces accumulation of free-AA and apoptosis.[12] Neurons stimulated with AA respond with depolarization of the inner mitochondrial membrane and caspase-3 activation.[13] In tumour cells, overexpression of COX-2 to increase AA removal blocks apoptosis. The reduction in cell death correlates inversely with the cellular level of free-AA. Conversely, COX-2 inhibition restores the apoptotic response.[13] In the present report, we describe a novel role for AA in apoptosis induction during cold storage of platelets.

Materials and Methods

Materials

The materials used in this study are described in more detail in the Online Supplementary Materials and Methods.

Platelet isolation and incubations

Human platelets were isolated[14] while maximally preventing their activation using free-

flow blood collection and discarding the first 2 mL of blood and all collections that showed micro-aggregates as determined by particle sizing. Procedures were approved by the Medical Ethical Committee of our hospital; the laboratory is certified for ISO-9001:2008. Platelets were resuspended in Hepes-Tyrode ($2x10^{11}$ cells/L, pH 7.2) and incubated without stirring for 10 minutes at room temperature (RT; defined as fresh platelets) and 4 hours at 0°C followed by 1 hour at 37°C to mimic cold storage and post-transfusion conditions,4 unless stated otherwise. Inhibitors used were for TPα SQ30741 (25 μM), for P38MAPK SB203580 (10 μM), for COX-1 indomethacin (30 μM), for lipoxygenase ETI (25 μM) and for cytochrome P450 monooxygenase SK&F96365 (30 μM), added 15 minutes before the 0°C-incubation. To deplete platelets of AA, FAF-BSA was present (75 g/L in Hepes-Tyrode, pH 7.2) during the 4 hours 0ºC incubation with a concurrent incubation with normal albumin as a control, as applied earlier to platelets.[15-17] Then, platelets were washed in the presence of PGI2 and left at RT for 30 minutes to restore responsiveness.

For isolation of murine platelets, eight weeks old strain-, and sex-matched C57Bl/6 wild type mice from Harlan (Boxmeer, the Netherlands) were used. The experimental protocols were approved by the local ethics committees for animal experiments. Mice were anesthetized with isoflurane and blood was collected in 0.1 volume 130 mM trisodium citrate by cardiac puncture and centrifuged (420*g*, 3 minutes, 22ºC, no brake). The pellet, together with one third of the red blood cell fraction was collected and again centrifuged (960*g*, 1 minute, 22ºC). Platelets were collected and resuspended in Hepes-Tyrode (pH 6.5), washed in 0.1 volume ACD and PGI_2 (2700*g*, 2 minutes, 22ºC) and resuspended in Hepes-Tyrode (pH 7.2) to a final concentration of $2x10^{11}$ platelets/L.

Western blots and immunoprecipitations

Platelet suspensions were added to lysis buffer. Proteins were separated by SDS-PAGE. After blocking with Odyssey Blocking buffer, membranes were incubated with primary antibodies (1 μg/mL) and protein bands visualized with an Odyssey Imaging system (LI-COR Biosciences, Lincoln, NE). Quantification was performed with Image-J software (NIH, Bethesda, MD). For immunoprecipitations, 450 μL washed platelets ($5x10^{11}$ platelets/L) was lysed, (15 minutes, 0°C), centrifuged (10 000*g*, 10 minutes, 4ºC) to remove cell debris and mixed with 55 μL (10% vol/vol) protein G beads together with antibody (1 μg/mL, 30 minutes, 4ºC, rotating). Data were expressed as percentage of fresh platelets. Control studies by FACS analysis with inhibitors of ADAM17 (TAPI-2, GM6001) confirmed that GPIbα ectodomain shedding was absent (not shown).

Flow cytometric analysis

For determination of the mitochondrial membrane potential $\Delta\Psi_m$, 100 μL platelet suspension was incubated with JC-1 (0.5 μM, 30 minutes, 37°C) and 10 000 platelets were measured on a FACS Calibur (BD Biosciences, San Jose, CA). In viable cells, the high $\Delta\Psi_m$ promotes a directional uptake of JC-1 into the matrix where JC-1 forms J-aggregates (λ_{ex} 490 nm, λ_{em} 570-610 nm). In apoptotic cells, the low $\Delta\Psi_m$ preserves the monomeric form (λ_{ex} 490 nm, λ_{em} 535 nm).[18] Changes in $\Delta\Psi_m$ were calculated from the ratio of platelets in lower- over upper-right quartiles and expressed as ratio of treated over fresh platelets.

P38MAPK activity assays

P38MAPK phosphorylation was measured as described.[14] The catalytic activity was measured in immunoprecipitates, resuspended in 15 μL kinase buffer containing 50 μM ATP and 2 μg

Activating Transcription Factor-2 (ATF-2)-fusion protein. After incubation (30 minutes, 30 °C), 15 µL sample buffer with DDT was added and phospho-ATF-2 (Thr^{171}) was measured on western blots.

Measurement of free AA, AA conversion to TxA_2 and COX-1 activity

To estimate free-AA formation at 0°C, platelet suspensions in the presence of SQ30741 (25 µM) were incubated for indicated periods. Samples were then transferred to 37°C and incubated for exactly 60 minutes. Suspensions were put on ice to halt COX-1 activity, centrifuged (15 000xg, 30 seconds, 22°C) and supernatants were collected for TxA_2 analysis. Data were expressed as AA equivalents formed/min/10^{11} platelets during the 60 minutes incubation period. To measure the activity of COX-1/Tx-synthase at different temperatures, platelets in Hepes-Tyrode preincubated with SB203580 (30 minutes, 37°C) to block release of endogenous AA were incubated at indicated temperatures. Then, exogenous AA (50 µM final concentration) was added and 60 minutes later TxA_2 formation was measured. The catalytic activity of COX-1 was measured in platelet lysates by luminal luminescence in a Spectramax-L (MDS Analytic Technology, Sunnyvale, CA) according to Hohlfeld et al.[19]

Platelet survival in vivo

Before transfusion, platelets were isolated from donor mice, resuspended in Tyrode pH 6.5 and labeled with CMFDA (2.5 µM, 1 hour 22°C). After washing in the presence of PGI_2, platelets were resuspended in Tyrode pH 7.2 and incubated for 4 hours at RT (control), in buffer containing either 75 g/L FAF-BSA or normal BSA at 0°C and in Tyrode buffer containing 1 µM AA at 0ºC. Then, platelets were washed in the presence of PGI_2 and resuspended to a concentration of 10^{12} cells/L. After 30 minutes incubation at 22°C to inactivate PGI_2, $1x10^8$ CMFDA-labeled platelets were injected into the tail vein of syngeneic recipient mice. For recovery and survival determinations, blood samples were collected at 2 minutes and 2, 24, 48, and 72 hours after injection in small vacuum EDTA tubes by mandibular puncture. Then, 1 µL whole blood was diluted in Tyrode (1/250 by volume), analyzed by FACS, and percentage of CMFDA-positive platelets was determined in a total of 50 000 platelets per sample. Initial recovery was determined 2 minutes after injection and survival times were calculated as described.[20] Data are means ± SEM of three mice in each group.

Statistical analysis

Data are means ± SEM (n=3). Statistical analysis was performed using GraphPad InStat (San Diego, CA) software. Differences were considered statistically significant when the p-value was less than 0.05 (between fresh and treated samples (*) and between incubations ($\underline{*}$)). Blots are representative examples of three experiments.

Results

Changes in mitochondrial membrane potential induced by AA

We and others reported previously that cold storage starts platelet apoptosis through activation of the pro-apoptotic Bcl-2 protein Bad, depolarization of the mitochondrial membrane ($\Delta\Psi_m$ change), and caspase-9 mediated exposure of surface PS.[2;21] Figure 1A confirms these observations for the $\Delta\Psi$ change and shows a 7 fold increase after 4 hours incubation at 0°C. Cold/rewarming initiates formation of TxA_2.[4] To investigate whether AA metabolites contributed to apoptosis induction, platelets were incubated with the COX-1 inhibitor indomethacin, but this treatment did not inhibit $\Delta\Psi_m$ change. Instead, indomethacin induced a significant increase in this apoptosis parameter. Subsequent rewarming (1 hour, 37°C)

further raised the $\Delta\Psi_m$ change This response was inhibited by indomethacin and therefore caused by TxA_2, a known platelet apoptosis inducer.[5] To identify steps that contributed to apoptosis induction at 0°C, incubations were performed with metabolic inhibitors (Figure 1B). P38MAPK is an upstream regulator of $cPLA_2$ and AA release.[8] Blockade of P38MAPK by SB203580 completely suppressed the cold-induced $\Delta\Psi_m$ change and in addition abolished the effect of indomethacin. Inhibition of the TxA_2 receptor, TPα, (SQ30741) had no effect in agreement with the low TxA_2 formation found at 0°C.[4] Apart from being a precursor of TxA_2 formation through COX-1/Tx-synthase, AA is converted to hydro (pero)xy-eicosatetraenoic acids by 12-lipoxygenase and, possibly, to 14,15-epoxyeicosatrienoic acids by cytochrome P450 monooxygenase.[9;10] Blockade of the respective pathways with ETI and SK&F96365 (SK&F) in combination with indomethacin did not further raise $\Delta\Psi_m$ change. Apparently, AA triggers apoptosis without being metabolized and indomethacin enhances this response by blocking a residual AA conversion to TxA_2 precursors.

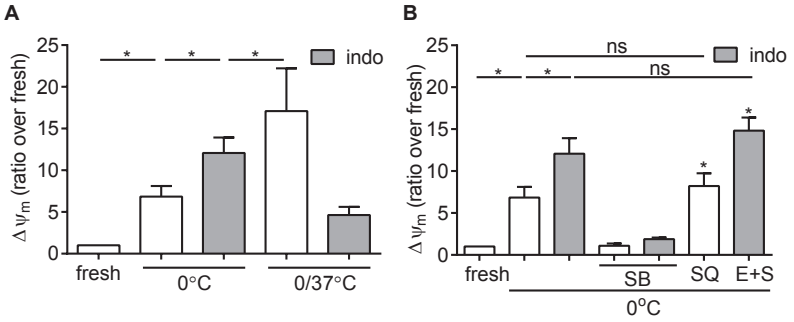


Figure 1. Role of AA in cold-induced platelet apoptosis. Apoptosis induction in platelets incubated at 0°C (4 hours) and 0/37°C (4 hours/1 hour) was analyzed by measuring the change in mitochondrial membrane potential ($\Delta\Psi_m$) and compared with fresh platelets. (**A**) Platelets in the absence (open bars) and presence of indomethacin (indo, 30 μM f.c., grey bars). (**B**) Platelets incubated at 0°C (4 hours) without and with indomethacin, the P38MAPK-blocker SB203580 (SB; 10 μM), the TPα-blocker SQ30741 (SQ; 25 μM) and the combined (E+S) lipoxygenase- (ETI; 25 μM) and cytochrome P450 blockers (SK&F96365, 30 μM). Data are means ± SEM (n=3) with significant difference $P<0.05$ compared with fresh platelets and between treatments (*).

These findings suggest that 0°C incubation activates upstream regulators of AA release and inactivates downstream regulators of AA degradation inducing AA accumulation and $\Delta\Psi_m$ change. Analysis of P38MAPK phosphorylation confirmed that the enzyme was activated upon cooling to 0°C and inhibited by SB203580; subsequent rewarming suppressed enzyme phosphorylation (Figure 2A). To understand the temperature dependence of P38MAPK activation, platelets were incubated at different temperatures and the catalytic activity was inferred from the phosphorylation of ATF-2. After 4 hours incubation, optimal activation was found at 10ºC. Conversely, the capacity of COX-1 to convert added AA to TxA_2 and the catalytic activity of COX-1 were low at 0°C and increased at higher temperature (Figure 2B). A time course at 0ºC showed rapid P38MAPK activation after 10 minutes which was accompanied by accumulation of free-AA (Figure 2C). To confirm that free-AA has apoptotic properties, $\Delta\Psi_m$ was measured in fresh platelets incubated with exogenous AA. There was a dose-dependent increase in $\Delta\Psi_m$ change, both at 0 and 37°C (Figure 2D). Apoptosis induction by endogenous AA was measured in cold-stored platelets with normal and lowered AA content (AA-depleted platelets, in short). These platelets were prepared by prior incubation at 0°C in normal BSA and FAF-BSA containing buffer respectively. AA-depleted platelets

showed a 60% lower $\Delta\Psi_m$ change than controls (Figure 2E). As expected, FAF-BSA incubation lowered TRAP-induced TxA_2 formation and aggregation (supplementary Figure S1A-C). Subsequent repletion of AA stores restored these functions. Importantly, recovery of these functions was not accompanied by apoptosis induction (supplementary Figure S1D).

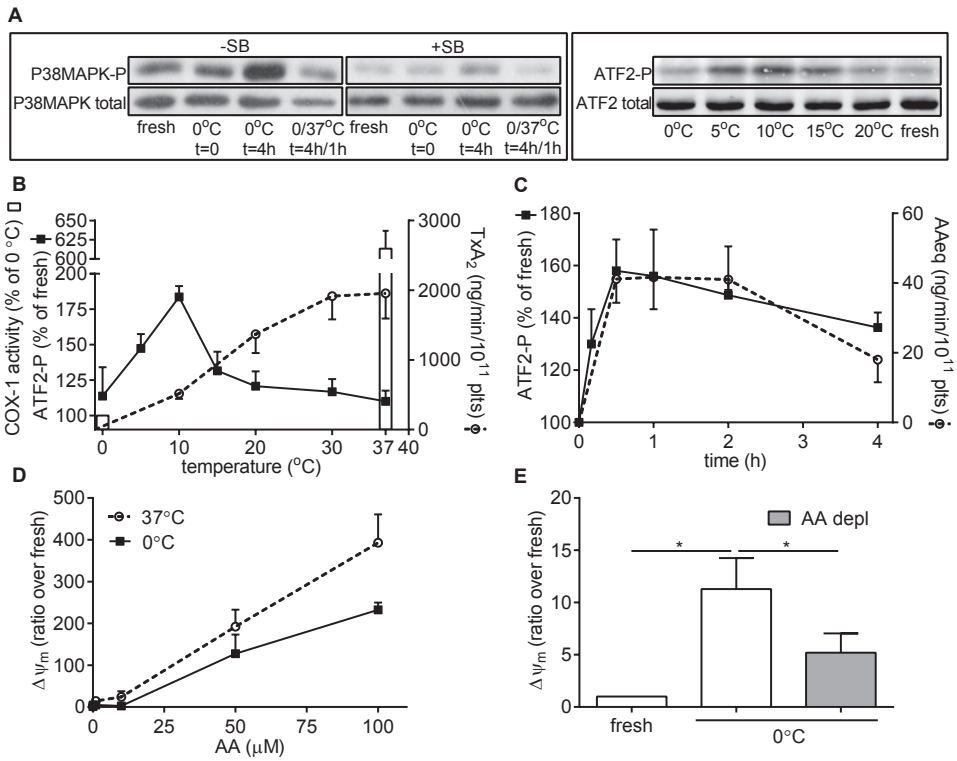


Figure 2. Free AA triggers platelet apoptosis. (**A**, left panel) Cold storage activates P38MAPK. Platelets without and with the P38MAPK inhibitor SB203580 (SB) were incubated for 10 minutes at RT (fresh), at 0°C (0 and 4 hours) and 0/37°C (4 hours/1 hour), and phosphorylated P38MAPK was measured. (**A**, right panel) P38MAPK catalytic activity shown by the phosphorylation of ATF-2 after 4 hours incubation at indicated temperatures. (**B**) Temperature dependence of P38MAPK activity (catalytic assay, ■ - ■), COX-1 activity (bars) and the COX-1/Tx-synthase reaction (O - - O). COX-1 activity measured as relative luminescence units[19] showed a 6 fold difference between platelets stored at 0°C or 37°C. Bars show % activity in platelet lysates at 37ºC and 0ºC (set at 100%). For measurement of the COX-1/Tx-synthase reaction, platelets with blocked P38MAPK (SB203580) to prevent release of endogenous AA, were incubated for 60 minutes with 50 μM exogenous AA at indicated temperatures and TxA_2 was measured. (**C**) Time course of P38MAPK catalytic activity (■ - ■), and accumulation of free-AA (O - - O) during incubation at 0°C. Platelets with blocked TPα-receptor to prevent TxA_2-signaling were incubated for indicated times at 0°C. Then, samples were collected for P38MAPK measurement and for a standard incubation for 60 minutes at 37°C to allow conversion of free-AA to TxA_2. (**D**) Exogenous AA triggers apoptosis. The change in mitochondrial membrane potential ($\Delta\Psi_m$) was measured in platelets incubated with 100 nM-100 μM AA for 10 minutes at 0°C (■ - ■), and 37°C (O - - O). AA concentrations up to 100 μM did not compromise cell integrity. E) AA depletion decreases apoptosis. Platelets were incubated (4 hours, 0°C) in buffer with normal BSA (open bars) and with FAF-BSA to lower platelet AA content (grey bar), and the $\Delta\Psi_m$ change was measured.

At a physiological temperature, AA release contributes to aggregation and secretion through TxA_2 formation and extracellular feed-back signaling. To investigate whether at 37°C, AA preserves apoptotic properties, normal and AA-depleted platelets were stimulated with the Ca^{2+}-ionophore A23187, the PAR-1 activator TRAP and the TxA_2-mimetic U46619. In all

conditions, stimulation led to $\Delta\Psi_m$ change (supplementary Figure S2A). In AA-depleted platelets, the $\Delta\Psi_m$ change was much smaller (A23187) or virtually absent (TRAP, U46619). Thus, apoptosis induction by activators of platelet aggregation occurs mainly through AA. Blockade of the TPα receptor reduced the $\Delta\Psi_m$ change by TRAP (supplementary Figure S2B). Apoptosis induction by U46619 was virtually absent, confirming complete blockade of TPα. AA depletion further reduced the TRAP-induced $\Delta\Psi_m$ change, but the ionophore response remained unchanged.

Regulation of [14-3-3ζ-Bad] association by AA

A key step in apoptosis induction is the release of 14-3-3ζ adapter protein from [14-3-3ζ-phospho-Bad] complex, inducing dephosphorylation of phospho-Bad, Bad activation and further signaling to pro-apoptotic Bax and Bak. We showed earlier that the $\Delta\Psi_m$ change in cold-stored platelets is accompanied by a fall in [14-3-3ζ-Bad].[2] To investigate whether AA contributes to this process, platelets with blocked TPα were incubated at 0°C (4 hours) and subsequently incubated at 37°C (1 hour) and the complex was measured in immunoprecipitates of Bad. Cold/rewarming induced a fall in [14-3-3ζ-Bad] (Figure 3A). At 0°C, AA addition decreased the complex (Figure 3B) and AA depletion prevented the decrease (Figure 3C). The 0/37ºC-induced dephosphorylation of total Ser reported earlier[2] was confirmed for phospho-Ser^{112} and also induced by added AA (Figure 3D). These data show that AA induces dissociation of [14-3-3ζ-Bad] complex and Bad dephosphorylation.

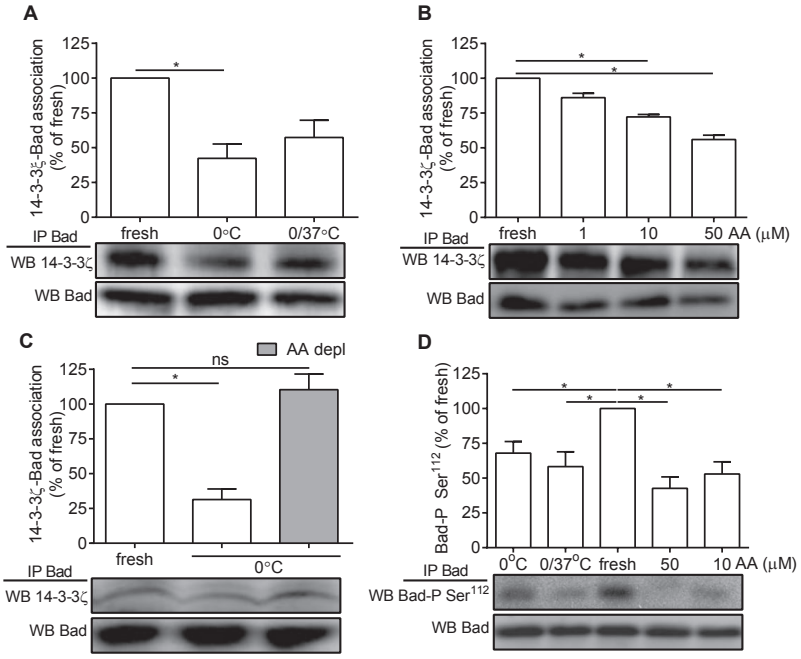


Figure 3. AA dissociates the [14-3-3ζ-Bad] complex. Measurement of [14-3-3ζ-Bad] in (**A**) fresh, 0°C- and 0/37°C-treated platelets, (**B**) fresh platelets and platelets incubated with 1, 10 and 50 μM AA for 10 minutes at 0°C, and (**C**) fresh and 0°C-treated platelets incubated with normal BSA (open bar) and FAF-BSA (AA depl, grey bar). (The shape of the spots is disturbed by the high albumin content of the medium). (**D**) Bad-Ser^{112} dephosphorylation in cold-rewarmed platelets and platelets treated with AA.

Regulation of [14-3-3ζ-COX-1] association by AA

14-3-3 proteins are phospho-Ser/phospho-Thr binding proteins through specific interaction with Arg-X-X-X-Ser-X-Pro and Arg-Ser-X-Ser-X-Pro sequences.[22] COX-1 contains two potential 14-3-3ζ binding sites (Arg[60]-Thr-Gly-Tyr-Ser-Gly-Pro[66] and Arg[149]-Ile-Leu-Pro-Ser-Val-Pro[155]; UniProt: KB-P23219). Immunoprecipitates of 14-3-3ζ of fresh platelets with blocked TPα confirmed the association of 14-3-3ζ with COX-1 (supplementary Figure S3A). Cold storage induced a 40% fall and rewarming induced re-association. AA addition (0°C) induced a dose-dependent decrease in [14-3-3ζ-COX-1], and AA depletion prevented dissociation (supplementary Figure S3B,C). These findings show that cold storage induced a fall in [14-3-3ζ-COX-1] through formation of free-AA, in parallel with the fall in [14-3-3ζ-Bad]. Rewarming restored the complex, to levels above those in fresh platelets, probably as a result of P38MAPK deactivation and AA removal by TxA_2 formation. The 14-3-3 bindings site Arg[149]-Ile-Leu-Pro-Ser-Val-Pro[155] of COX-1 is located in the catalytic site of COX-1 (aa 120-385). Indomethacin binds COX-1 at Tyr[355], forming a salt bridge between the carboxylate site of the drug and Arg[120], preventing binding of AA at Tyr[385].[23] Therefore, addition of indomethacin to cold-stored platelets should displace 14-3-3ζ from COX-1. In fresh platelets, indomethacin induced a 30% fall in [14-3-3ζ-COX-1]. Incubation with AA (0ºC) lowered the complex and again indomethacin induced a further decrease (supplementary Figure S3D).

Cold-induced platelet apoptosis by AA-mediated [14-3-3ζ-GPIbα] association

Earlier studies in our laboratory showed that cold storage increases 14-3-3ζ association with GPIbα and decreases 14-3-3ζ association with Bad enabling Bad dephosphorylation and apoptosis induction.[2] To clarify the relation between the GPIbα pathway and the AA pathway, platelets were pretreated with osge to remove GPIbα ectodomain and with SB203580 to arrest P38MAPK-mediated production of free-AA. The separate treatments (Figure 4A) and the combination (not shown) induced complete blockade of cold-induced $\Delta\Psi_m$ change. Apparently, GPIbα and free-AA are both essential components in apoptosis induction. Cold-induced P38MAPK activation and production of AA equivalents were insensitive to osge treatment and therefore independent of GPIbα (Figure 4B and not shown).

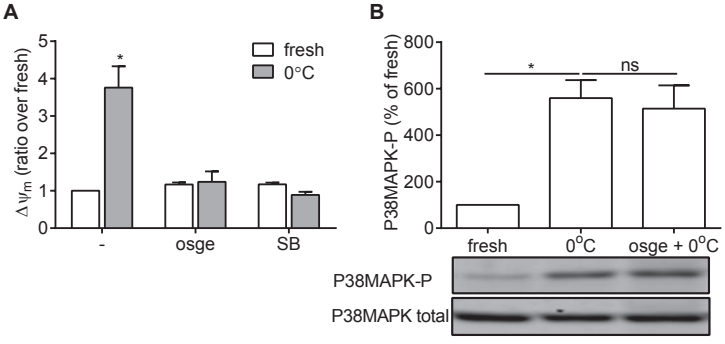


Figure 4. Contribution of GPIbα and arachidonic acid signaling in apoptosis induction. (**A**) Both GPIbα and free-AA are essential for cold-induced apoptosis. Fresh and cold-treated platelets (4 hours, 0ºC) were incubated without and with osge (to remove GPIbα ectodomain) and SB203580 (to block P38MAPK), and the $\Delta\Psi_m$ change was measured. (**B**) Cold-induced P38MAPK activation does not depend on GPIbα. Phosphorylated P38MAPK was measured in fresh and cold-stored platelets (4 hours, 0ºC) without and with osge treatment.

To clarify the contribution of GPIbα and AA in more detail, platelets were treated with 1, 10 and 50 μM biotin-labeled AA (biotin-AA). This treatment induced a dose-dependent association of 14-3-3ζ (Figure 5A). The fall in [14-3-3ζ-Bad] and [14-3-3ζ-COX-1] found earlier might therefore reflect transfer of 14-3-3ζ to AA. Interestingly, also GPIbα bound biotin-AA (Figure 5B) as did COX-1 (Figure 5C). In contrast, there was no binding of Bad to biotin-AA (not shown). Together with 14-3-3ζ binding to GPIbα at 0°C[2] and confirmed in AA-treated platelets (Figure 5D), this data suggest formation of a [AA-14-3-3ζ-GPIbα] complex upon cold storage.

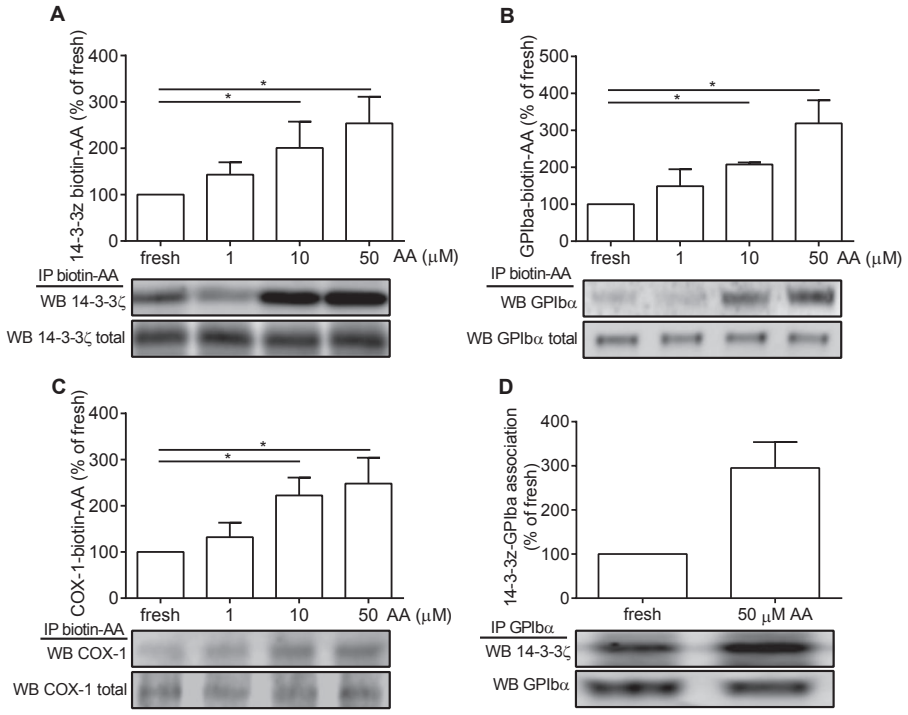


Figure 5. Free arachidonic acid binds 14-3-3ζ, GPIbα and COX-1. (**A-C**) Formation of a [AA-14-3-3ζ-GPIbα] complex. Platelets were incubated with 1, 10 and 50 μM biotin-AA (10 minutes, 0ºC). Lysates from the same incubations were incubated with streptavidin-beads, centrifuged and analyzed for association with (**A**) 14-3-3ζ, (**B**) GPIbα and (**C**) COX-1. (**D**) AA-induced [14-3-3ζ-GPIbα] association. Platelets were incubated with 50 μM AA (10 minutes, 0ºC) and 14-3-3ζ, was measured in IPs of GPIbα.

AA depletion enhances in vivo survival of cold-stored platelets

Apoptosis induction by added AA was also observed in murine platelets and AA depletion inhibited the cold-induced $\Delta\Psi_m$ change (supplementary Figure S4A,B). The question whether AA depletion would improve the survival of cold-stored platelets was addressed by incubating murine platelets for 4 hours at RT (control) and for 4 hours at 0°C either in buffer with fatty acid free BSA (FAF-BSA plts), normal BSA (BSA plts) and in buffer containing 1 μM AA. Then, platelets were washed with protection by PGI_2 and injected into recipient mice (Figure 6A). Recoveries at 2 minutes after injection were ~80% for RT-stored controls and cold-treated FAF-BSA platelets (Figure 6B). For cold-treated BSA platelets and AA-treated

platelets recoveries were ~71% and ~56% respectively. The survival of FAF-BSA platelets (~88 hours) was better than of RT-platelets (~79 hours) whereas BSA-platelets (~70 hours) and AA-treated platelets (~60 hours) showed much shorter survival. Thus, AA depletion prevents the cold-induced reduction of platelet survival.

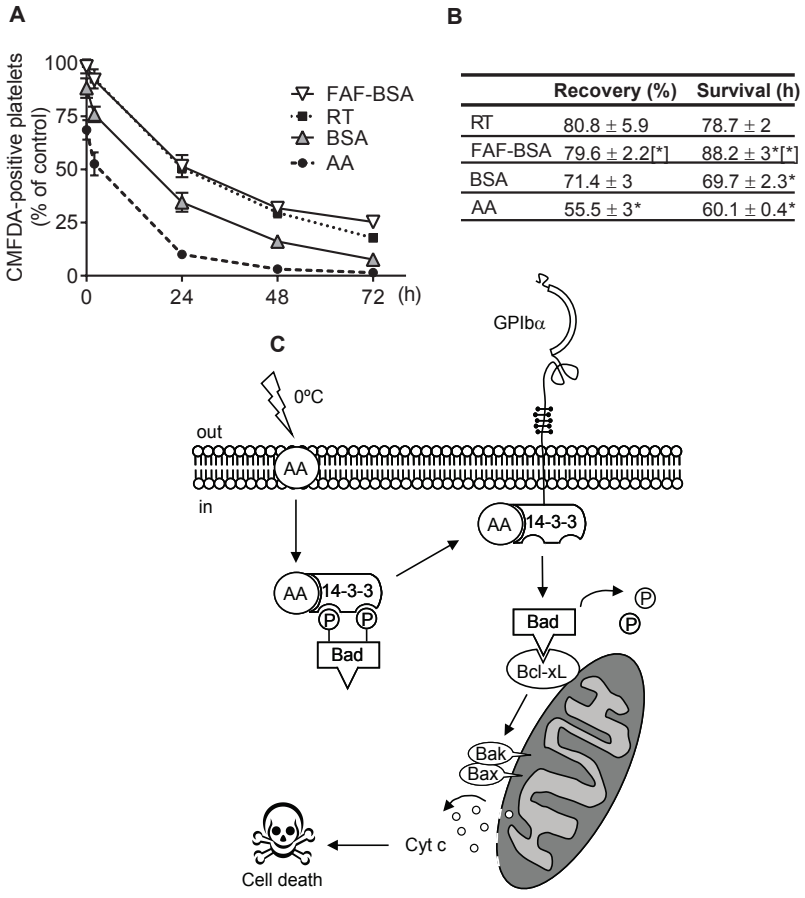


Figure 6. Platelet survival in C57Bl/6 mice. (**A,B**) Mice platelets labeled with CMFDA were incubated for 4 hours at RT (control) and for 4 hours at 0°C either in buffer with fatty acid free BSA (FAF-BSA plts), normal BSA (BSA plts) and in buffer containing 1 μM AA. Then, platelets were washed with protection by PGI_2 and injected into recipient mice. Blood was collected after 2 minutes, 2, 24, 48 and 72 hours after injection. (**A**) Recovery of RT platelets at 2 minutes (80.8 ± 5.9) was set at 100%; other data points are presented relative to this value. (**B**) Recoveries and survival times of CMFDA-labeled platelets (means ± SEM of three mice in each group). Data statistically compared to either RT platelets (*) or BSA platelets ([*]). (**C**) Schematic representation of apoptosis induction in cold-stored platelets. Cold releases free-AA from membrane phospholipids initiating (i) 14-3-3ζ translocation from phospho-Bad to GPIbα, (ii) dephosphorylation/activation of pro-apoptotic Bad, (iii) Displacement of Bak and Bax from pro-survival Bcl-xL by Bad, (iv) Bak/Bax-induced permeabilization of the mitochondrial membrane, (v) Cytochrome C (Cyt c) release and (vi) apoptosis.

Discussion

The main findings of this study are: (i) cold storage releases AA from membrane phospholipids, which accumulates since COX-1/Tx-synthase have little activity at low temperature, (ii) released AA induces formation of a [AA-14-3-3ζ-GPIbα] complex, inducing [14-3-3ζ-Bad]

dissociation, Bad dephosphorylation and $\Delta\Psi_m$ change, (iii) indomethacin increases AA accumulation enhancing apoptosis and releases 14-3-3ζ from COX-1, (iv) AA depletion reduces apoptosis in vitro and improves the survival of cold-stored platelets in vivo (Figure 6C).

Cold storage activates P38MAPK, an upstream activator of $cPLA_2$ and AA release, and inhibits COX-1/Tx-synthase, suppressing AA metabolism to TxA_2. P38MAPK is a member of the stress-activated protein kinase family and especially sensitive to thermal stress.[24] Chilling induces P38MAPK phosphorylation/activation when the temperature falls below 10ºC. At this temperature, the platelet plasma membrane undergoes a phase transition which might be a trigger for P38MAPK phosphorylation.[25] P38MAPK phosphorylation by cold has been found in other cell types, including hepatocytes,[26] endothelial cells,[26] brain,[27] and in plants where it is the result of phosphatase PP2A inhibition.[28] P38MAPK is an upstream regulator of $cPLA_2$ and mediates TxA_2 formation in platelets stimulated with collagen,[7] LPS,[29] VWF[30] and low density lipoprotein at 37°C.[31] The Ca^{2+} increase in cold-stored platelets[32] together with the P38MAPK-induced phosphorylation of $cPLA_2$-Ser^{505}/Ser^{727} translocates the enzyme to the plasma membrane,[33;34] inducing AA release. Induction of $\Delta\Psi_m$ change, but not P38MAPK activation, depends on GPIbα. Chilling triggers desialylation of the receptor, exposing galactose and β-*N*-acetyl-*D*-glucosamine residues which become recognition sites for receptors on macrophages and hepatocytes.[35] Apparently, cold storage also makes GPIbα a participant in apoptosis induction.

Released AA induces a [AA-14-3-3ζ-GPIbα] complex by trapping 14-3-3ζ from [14-3-3ζ-Bad]. Dissociation of [14-3-3ζ-Bad] removes the constraint that prevents Bad dephosphorylation and activation.[36] The role of AA as a Bad activator is supported by the $\Delta\Psi_m$ change upon AA addition and its reduction in AA-depleted platelets. These data support earlier correlations between free-AA and cell death and formation of labeled 14-3-3 in [^{3}H] AA-neurons.[37] The introduction of biotin-AA confirmed that platelet 14-3-3ζ binds AA. An unexpected result was that also GPIbα binds biotin-AA. Apparently, cold-induced apoptosis by a GPIbα change[2] and by accumulated free-AA (present study) act together in activating Bad. Earlier, Li et al. showed that at 37°C, VWF induces a $\Delta\Psi_m$ change, caspase-3 activation and PS exposure in platelets and that a mutated 14-3-3-ζ binding site in GPIbα site abolished apoptosis induction in CHO cells.[38] Thus, Bad activation in cold-stored platelets might have properties in common with VWF-stimulated platelets. Its importance in apoptosis induction is illustrated by the prolonged platelet lifespan in $Bad^{-/-}$ mice.[39]

At 37°C, thrombin and TxA_2-analogue (U46619) affect the downstream target of Bad, the pro-apoptotic Bax, which translocates to the mitochondria and induces a $\Delta\Psi_m$ change.[5;40] Our studies show that free-AA might mediate apoptosis induction by these agents. Without blockade of TPα receptor signaling, the $\Delta\Psi_m$ change by TRAP and TxA_2-analog was lowered by AA depletion, illustrating the contribution of TxA_2 formation and TPα signaling to $\Delta\Psi_m$. With TPα receptor blockade, a slight TRAP induction remained present, which completely disappeared in AA-depleted cells. The difference reflects the contribution of free-AA in TRAP-induced apoptosis. The Ca^{2+}-ionophore A23187 triggers entry of extracellular Ca^{2+} and is a potent apoptosis inducer independent of receptor activation. Without TPα blockade, $\Delta\Psi_m$ change was suppressed by AA depletion, but with TPα blockade, a mechanism for apoptosis induction independent of free-AA and TxA_2 signaling became apparent. Studies by Arachiche et al. confirm that a high cytosolic Ca^{2+} concentration is an independent inducer

of platelet apoptosis.[41]

The COX-1 inhibitor indomethacin increased the $\Delta\Psi_m$ change at 0ºC in the absence of P38MAPK blocker but not with SB203580 present. Thus, at low temperature indomethacin facilitates the accumulation of free-AA. Cold storage induced formation of [14-3-3ζ-COX-1]. Complex formation was optimal in fresh platelets, fell after cold storage and restored to pre-treatment values and more after incubation at 37ºC. These changes parallel variations in [14-3-3ζ-Bad].[42] The capacity to bind 14-3-3ζ might be important, since indomethacin releases 14-3-3ζ from COX-1. Binding of 14-3-3 proteins depends on the phosphorylation status of specific Ser-residues on target molecules. Therefore, factors that control COX-1 Ser-phosphorylation might contribute to 14-3-3ζ translocation. Apart from COX-1, 14-3-3 proteins are known to bind pro-apoptotic Bax,[43] members of the GPIb-V-IX complex,[2;44] GTPase-activating protein Rap1GAP2,[45] Phosphoinositide 3-kinase,[46] c-Raf-1 and insulin receptor substrate-1.[47] Trapping of 14-3-3ζ by accumulated AA might therefore affect many steps that control platelet functions.

Apoptosis induction by added AA was also observed in mouse platelets and AA depletion inhibited the cold-induced $\Delta\Psi_m$ change. In vivo experiments showed that AA depletion improved survival of cold-stored platelets bringing it in the range of RT-stored platelets. PS exposure and platelet binding/phagocytosis by macrophages are downstream steps in cold-induced Bad activation.[2] The improved survival of AA-depleted platelets might therefore result from decreased Bad activation. A problem of AA depletion is that it also impairs TRAP-induced TxA_2 formation and aggregation, which would jeopardize hemostatic functions post-transfusion. The finding that this treatment is fully reversible is important, since it opens ways to suppress apoptotic and hemostatic functions during storage with an expected normalization in the recipient. Equally important is the fact that during the recovery phase, AA addition does not start apoptosis indicating that AA-depleted platelets give priority to restoration of AA stores in the plasma membrane.

The combination of cold and AA-depletion might significantly improve preservation conditions for platelet transfusion. Apart from suppressing bacterial growth, cold storage will decrease platelet energy metabolism extending the time the suspension medium supports metabolic ATP regeneration. Cold also increases the chance that artificial media can fully replace plasma and plasma-buffer combinations, lowering risks for virus infections and transfusion reactions.[48] Furthermore, cold storage better preserves the platelets' capacity to aggregate and secrete granule contents upon later stimulation at 37°C.[49;50] AA-depletion will reduce platelet activation during preparation of platelet concentrates since mechanical disturbances can activate this metabolic pathway.[51] Since this process is reversible, AA addition prior to transfusion, or when transfused platelets bind plasma AA-albumin in the circulation, will fully recover hemostatic functions. Under in vitro conditions, normal platelets incorporate exogenous AA at a rate of 0.7 μmol/min/10^{11} platelets, reaching saturation after 90 minutes (37°C).[52] The uptake might be faster in AA-depleted platelets, but the kinetics of this process in vitro and especially in circulating blood and its implications for the arrest of bleeding remain subjects for further studies.

Acknowledgements

This study was supported by a grant from the Landsteiner Foundation of Blood transfusion Research (LSBR grant nr. 0510). Prof. Dr. J.W.N. Akkerman is supported by the Netherlands Thrombosis Foundation. The authors thank Dr. R.T. Urbanus at the UMCU for discussions.

References

1. Shrivastava M. The platelet storage lesion. *Transfus Apher Sci*. 2009;41(2):105-113.
2. van der Wal DE, DU VX, Lo KS, Rasmussen JT, Verhoef S, Akkerman JW. Platelet apoptosis by cold-induced glycoprotein Ibalpha clustering. *J Thromb Haemost*. 2010;8(11):2554-2562.
3. Hoffmeister KM, Felbinger TW, Falet H et al. The clearance mechanism of chilled blood platelets. *Cell*. 2003;112(1):87-97.
4. van der Wal DE, Verhoef S, Schutgens RE, Peters M, Wu Y, Akkerman JW. Role of glycoprotein Ibalpha mobility in platelet function. *Thromb Haemost*. 2010;103(5):1033-1043.
5. Tonon G, Luo X, Greco NJ, Chen W, Shi Y, Jamieson GA. Weak platelet agonists and U46619 induce apoptosis-like events in platelets, in the absence of phosphatidylserine exposure. *Thromb Res*. 2002;107(6):345-350.
6. Garcia A, Quinton TM, Dorsam RT, Kunapuli SP. Src family kinase-mediated and Erk-mediated thromboxane A2 generation are essential for VWF/GPIb-induced fibrinogen receptor activation in human platelets. *Blood*. 2005;106(10):3410-3414.
7. Saklatvala J, Rawlinson L, Waller RJ et al. Role for p38 mitogen-activated protein kinase in platelet aggregation caused by collagen or a thromboxane analogue. *J Biol Chem*. 1996;271(12):6586-6589.
8. Kramer RM, Roberts EF, Um SL et al. p38 mitogen-activated protein kinase phosphorylates cytosolic phospholipase A2 (cPLA2) in thrombin-stimulated platelets. Evidence that proline-directed phosphorylation is not required for mobilization of arachidonic acid by cPLA2. *J Biol Chem*. 1996;271(44):27723-27729.
9. Maskrey BH, Bermudez-Fajardo A, Morgan AH et al. Activated platelets and monocytes generate four hydroxyphosphatidylethanolamines via lipoxygenase. *J Biol Chem*. 2007;282(28):20151-20163.
10. Zhu Y, Schieber EB, McGiff JC, Balazy M. Identification of arachidonate P-450 metabolites in human platelet phospholipids. *Hypertension*. 1995;25(4 Pt 2):854-859.
11. Picot D, Loll PJ, Garavito RM. The X-ray crystal structure of the membrane protein prostaglandin H2 synthase-1. *Nature*. 1994;367(6460):243-249.
12. Perez R, Matabosch X, Llebaria A, Balboa MA, Balsinde J. Blockade of arachidonic acid incorporation into phospholipids induces apoptosis in U937 promonocytic cells. *J Lipid Res*. 2006;47(3):484-491.
13. Cao Y, Pearman AT, Zimmerman GA, McIntyre TM, Prescott SM. Intracellular unesterified arachidonic acid signals apoptosis. *Proc Natl Acad Sci U S A*. 2000;97(21):11280-11285.
14. Korporaal SJ, van Eck M, Adelmeijer J et al. Platelet activation by oxidized low density lipoprotein is mediated by CD36 and scavenger receptor-A. *Arterioscler Thromb Vasc Biol*. 2007;27(11):2476-2483.
15. Yoshida N, Aoki N. Release of arachidonic acid from human platelets. A key role for the potentiation of platelet aggregability in normal subjects as well as in those with nephrotic syndrome. *Blood*. 1978;52(5):969-977.
16. Ramesha CS, Taylor LA. Measurement of arachidonic acid release from human polymorphonuclear neutrophils and platelets: comparison between gas chromatographic and radiometric assays. *Anal Biochem*. 1991;192(1):173-180.
17. Surya II, Gorter G, Akkerman JW. Arachidonate transfer between platelets and lipoproteins. *Thromb Haemost*. 1992;68(6):719-726.
18. Smiley ST, Reers M, Mottola-Hartshorn C et al. Intracellular heterogeneity in mitochondrial membrane potentials revealed by a J-aggregate-forming lipophilic cation JC-1. *Proc Natl Acad Sci U S A*. 1991;88(9):3671-3675.
19. Hohlfeld T, Zimmermann N, Weber AA et al. Pyrazolinone analgesics prevent the antiplatelet effect of aspirin and preserve human platelet thromboxane synthesis. *J Thromb Haemost*. 2008;6(1):166-173.
20. Baker GR, Sullam PM, Levin J. A simple, fluorescent method to internally label platelets suitable for physiological measurements. *Am J Hematol*. 1997;56(1):17-25.
21. Liu Q, Xu L, Jiao SX, Wang TX, Song Y, Wen ZK. Trehalose inhibited the phagocytosis of refrigerated platelets in vitro via preventing apoptosis. *Transfusion*. 2009;49(10):2158-2166.
22. van Hemert MJ, Steensma HY, van Heusden GP. 14-3-3 proteins: key regulators of cell division, signalling and apoptosis. *Bioessays*. 2001;23(10):936-946.
23. Mancini JA, Riendeau D, Falgueyret JP, Vickers PJ, O'Neill GP. Arginine 120 of prostaglandin G/H synthase-1 is required for the inhibition by nonsteroidal anti-inflammatory drugs containing a carboxylic acid moiety. *J Biol Chem*. 1995;270(49):29372-29377.
24. Cowan KJ, Storey KB. Mitogen-activated protein kinases: new signaling pathways functioning in cellular responses to environmental stress. *J Exp Biol*. 2003;206(Pt 7):1107-1115.

25. Gousset K, Tsvetkova NM, Crowe JH, Tablin F. Important role of raft aggregation in the signaling events of cold-induced platelet activation. *Biochim Biophys Acta*. 2004;1660(1-2):7-15.

26. Laszlo V, Rauen U. Evidence for the involvement of ERK in cold-induced injury [abstract]. Cryobiology 2010; Abstract Annual Meeting Society for Cryobiology:

27. Zheng G, Chen Y, Zhang X et al. Acute cold exposure and rewarming enhanced spatial memory and activated the MAPK cascades in the rat brain. *Brain Res*. 2008;1239171-180.

28. Monroy AF, Sangwan V, Dhindsa RS. Low temperature signal transduction during cold acclimation: protein phosphatase 2A as an early target for cold-inactivation. *The Plant Journal*. 1998;13(5):653-660.

29. Brooks AC, Menzies-Gow NJ, Wheeler-Jones C, Bailey SR, Cunningham FM, Elliott J. Endotoxin-induced activation of equine platelets: evidence for direct activation of p38 MAPK pathways and vasoactive mediator production. *Inflamm Res*. 2007;56(4):154-161.

30. Canobbio I, Reineri S, Sinigaglia F, Balduini C, Torti M. A role for p38 MAP kinase in platelet activation by von Willebrand factor. *Thromb Haemost*. 2004;91(1):102-110.

31. Hackeng CM, Relou IA, Pladet MW, Gorter G, van Rijn HJ, Akkerman JW. Early platelet activation by low density lipoprotein via p38MAP kinase. *Thromb Haemost*. 1999;82(6):1749-1756.

32. Hoffmeister KM, Falet H, Toker A, Barkalow KL, Stossel TP, Hartwig JH. Mechanisms of cold-induced platelet actin assembly. *J Biol Chem*. 2001;276(27):24751-24759.

33. Borsch-Haubold AG, Bartoli F, Asselin J et al. Identification of the phosphorylation sites of cytosolic phospholipase A2 in agonist-stimulated human platelets and HeLa cells. *J Biol Chem*. 1998;273(8):4449-4458.

34. Das S, Rafter JD, Kim KP, Gygi SP, Cho W. Mechanism of group IVA cytosolic phospholipase A(2) activation by phosphorylation. *J Biol Chem*. 2003;278(42):41431-41442.

35. Rumjantseva V, Grewal PK, Wandall HH et al. Dual roles for hepatic lectin receptors in the clearance of chilled platelets. *Nat Med*. 2009;15(11):1273-1280.

36. Xing H, Zhang S, Weinheimer C, Kovacs A, Muslin AJ. 14-3-3 proteins block apoptosis and differentially regulate MAPK cascades. *EMBO J*. 2000;19(3):349-358.

37. Brock TG. Arachidonic acid binds 14-3-3zeta, releases 14-3-3zeta from phosphorylated BAD and induces aggregation of 14-3-3zeta. *Neurochem Res*. 2008;33(5):801-807.

38. Li S, Wang Z, Liao Y et al. The glycoprotein Ibalpha-von Willebrand factor interaction induces platelet apoptosis. *J Thromb Haemost*. 2010;8(2):341-350.

39. Kelly PN, White MJ, Goschnick MW et al. Individual and overlapping roles of BH3-only proteins Bim and Bad in apoptosis of lymphocytes and platelets and in suppression of thymic lymphoma development. *Cell Death Differ*. 2010;17(10):1655-1664.

40. Lopez JJ, Salido GM, Pariente JA, Rosado JA. Thrombin induces activation and translocation of Bid, Bax and Bak to the mitochondria in human platelets. *J Thromb Haemost*. 2008;6(10):1780-1788.

41. Arachiche A, Kerbiriou-Nabias D, Garcin I, Letellier T, chary-Prigent J. Rapid procoagulant phosphatidylserine exposure relies on high cytosolic calcium rather than on mitochondrial depolarization. *Arterioscler Thromb Vasc Biol*. 2009;29(11):1883-1889.

42. Masters SC, Yang H, Datta SR, Greenberg ME, Fu H. 14-3-3 inhibits Bad-induced cell death through interaction with serine-136. *Mol Pharmacol*. 2001;60(6):1325-1331.

43. Nomura M, Shimizu S, Sugiyama T et al. 14-3-3 Interacts directly with and negatively regulates pro-apoptotic Bax. *J Biol Chem*. 2003;278(3):2058-2065.

44. Mangin PH, Receveur N, Wurtz V, David T, Gachet C, Lanza F. Identification of five novel 14-3-3 isoforms interacting with the GPIb-IX complex in platelets. *J Thromb Haemost*. 2009;7(9):1550-1555.

45. Hoffmeister M, Riha P, Neumuller O, Danielewski O, Schultess J, Smolenski AP. Cyclic nucleotide-dependent protein kinases inhibit binding of 14-3-3 to the GTPase-activating protein Rap1GAP2 in platelets. *J Biol Chem*. 2008;283(4):2297-2306.

46. Munday AD, Berndt MC, Mitchell CA. Phosphoinositide 3-kinase forms a complex with platelet membrane glycoprotein Ib-IX-V complex and 14-3-3zeta. *Blood*. 2000;96(2):577-584.

47. Yaffe MB, Rittinger K, Volinia S et al. The structural basis for 14-3-3:phosphopeptide binding specificity. *Cell*. 1997;91(7):961-971.

48. Stroncek DF, Rebulla P. Platelet transfusions. *Lancet*. 2007;370(9585):427-438.

49. Choi JW, Pai SH. Influence of storage temperature on the responsiveness of human platelets to agonists. *Ann Clin Lab Sci*. 2003;33(1):79-85.

50. Babic AM, Josefsson EC, Bergmeier W et al. In vitro function and phagocytosis of galactosylated platelet concentrates after long-term refrigeration. *Transfusion*. 2007;47(3):442-451.

51. Stevens DE, Joist JH, Sutera SP. Role of platelet-prostaglandin synthesis in shear-induced platelet alterations. *Blood*. 1980;56(5):753-758.

52. Bakken AM, Farstad M. The activities of acyl-CoA:1-acyl-lysophospholipid acyltransferase(s) in human platelets. *Biochem J*. 1992;288(Pt 3):763-770.

Supplementary Materials and Methods

Materials

We used the following products (with sources): hematin (Alfa Aesar, Ward Hill, MA), AA (Bio/Data Corporation, Horsham, PA), Biotin-AA, COX activity assay buffer (Cayman chemical, Ann Arbor, MI), BSA fraction V (BSA) and fatty acid free-BSA (FAF-BSA), 5,5',6,6'-Tetrachloro-1,1',3,3'-tetraethyl-imidacarbocyanine iodide (JC-1), indomethacin, luminol (Sigma-Aldrich, St Louis, MO), BSA for blotting (MP Biomedicals, Solon, OH), Cell tracker green, Molecular Probes, Invitrogen, Carlsbad, CA), 5-chloromethyl fluorescein diacetate (CMFDA), lipoxygenase-inhibitor 5, 8, 11-eicosatriynoic acid (ETI, Cayman chemicals, Ann Arbor, MI), Odyssey blocking buffer (LI-COR Biosciences, Lincoln, NE), prostacyclin (PGI_2, Cayman Chemical, Ann Arbor, MI), P38MAPK substrate ATF-2 fusion protein, kinase buffer (Cell Signaling Technology, Danvers, MA), P38MAPK-inhibitor SB203580 and cytochrome P450 monooxygenase inhibitor SK&F96365 (Alexis Biochemicals/Enzo Lifesciences BVBA, Zandhoven, Belgium), *O*-sialoglycoprotein endopeptidase (osge, Cederlane Laboratories, Hornby, Ontario, Canada), TxA_2 Enzyme Immuno Assay (EIA) kit (Assay Designs, Ann Arbor, MI). Tx receptor (TPα) antagonist SQ30741 was a kind gift from Bristol-Meyers-Squibb (Maarssen, The Netherlands).

Antibodies used for western blotting were directed against GPIbα (clone SZ2, Beckman Coulter, Marseille, France), COX-1 (Abcam, Cambridge, UK), phospho ATF-2 (Thr 171), total Bad (Zymed, Invitrogen, Carlsbad, CA), Bad Ser 136, Phospho-P38MAPK (Thr180/Tyr182), total P38MAPK, secondary horseradish peroxidase-labeled anti-rabbit antibodies (Cell Signaling Technology, Danvers, MA), Bad (Enzo life science,Farmingdale, NY) 14-3-3ζ (C-16, Santa Cruz Biotechnology, Santa Cruz, CA). Secondary antibodies were: Alexa-680 (Molecular Probes, Invitrogen, Carlsbad, CA) and IRDYe 800CW (LI-COR Biosciences, Lincoln, NE). Antibodies for immunoprecipitation (IP) were against GPIbα (AK2, Santa Cruz Biotechnology), total Bad (Cell signaling Technology, Danvers, MA) and 14-3-3ζ (V-16, Santa Cruz Biotechnology).

For studies shown in the Supplement, we used the following products (with sources): calcium ionophore A23187 (Calbiochem, Darmstadt, Germany), fibrinogen (Enzyme Research Laboratories, South Bend, IN), thrombin receptor (PAR1)-activating peptide (TRAP, SFLLRN, Bachem, Switzerland), TxA_2-mimetic U46619 (Cayman Chemical, Ann Arbor, MI). The P_2Y_{12} blocker AR-C69931MX was a kind gift from Astra Zenica, Loughborough, UK.

Hemostatic properties after AA depletion/repletion

AA-depleted platelets were prepared by incubation with FAF-BSA using BSA as control. AA-repleted platelets were prepared by subsequent incubation with AA. In non-stirred suspensions, TxA_2 was measured upon stimulation with 5 μM TRAP (10 minutes, 37°C). Aggregation induced by 20 μM TRAP was measured in an optical aggregometer (Chronolog Corporation, Haverford, PA) at 37°C with stirring at 900 rpm in the presence of 100 nM AR-C69931MX and 100 μg/mL fibrinogen.

SUPPLEMENTARY FIGURES

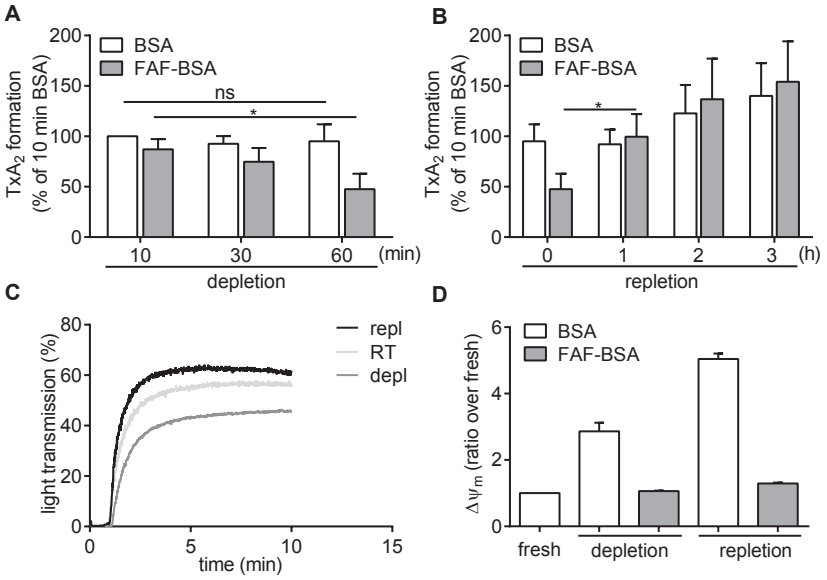


Figure S1. Reversible modulation of TxA_2 formation by AA de-/repletion. (**A**) Depletion of platelet AA. Platelets were incubated in buffer with BSA (open bars) and FAF-BSA (grey bars) for indicated times at 0ºC. Samples were washed in the presence of PGI_2, placed at 37ºC for 30 minutes to restore responsiveness and incubated with 5 µM TRAP (10 minutes, 37ºC) to induce TxA_2 formation. (**B**) Repletion of platelet AA. Platelets incubated with FAF-BSA for 1 hour at 0ºC were washed and incubated in buffer containing 1 µM AA for indicated times. Following a second wash step, TRAP-induced TxA_2 formation was measured. (**C**) Reversible modulation of aggregation. RT-stored platelets (RT), AA-depleted platelets (60 minutes with FAF-BSA, 0ºC) and AA-repleted platelets (AA-depleted platelets incubated for 1 hour with 1 µM AA, 37ºC) were stimulated with 20 µM TRAP in the presence of the P2Y12 blocker AR-C69931MX and aggregation was measured at 37ºC. Mean aggregations were 57.3 ± 2.1, 42.3 ± 3.1 and 60.0 ± 2.8 % respectively. (**D**) AA-repletion does not initiate apoptosis. The change in mitochondrial membrane potential ($\Delta\Psi_m$) was measured in platelets incubated under the conditions of (C). Data are means ± SEM (n=3) with significant difference P<0.05(*).

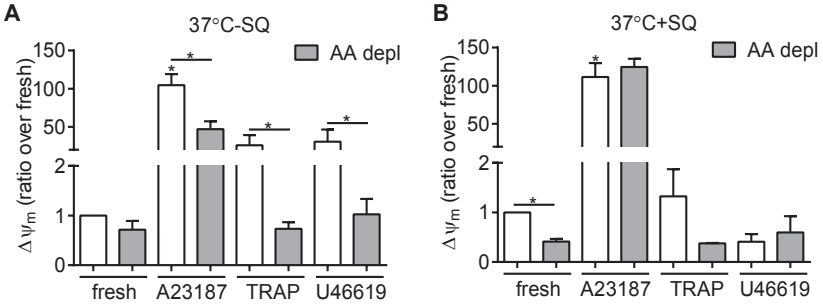


Figure S2. Free AA contributes to agonist-induced platelet apoptosis. Effect of AA depletion on apoptosis induction at 37°C in the absence (**A**) and presence (**B**) of the TPα-blocker SQ30741. Platelets were incubated at 0°C for 4 hours with normal BSA (open bars) and FAF-BSA (grey bars), washed and apoptosis induction was analyzed by measuring the change in mitochondrial membrane potential, $\Delta\Psi_m$. Platelets were stimulated without stirring with Ca^{2+}-ionophore A23187 (3 µM, with 2 mM extracellular Ca^{2+}), TRAP (20 µM) and the TxA_2-analog U46619 (10 µM).

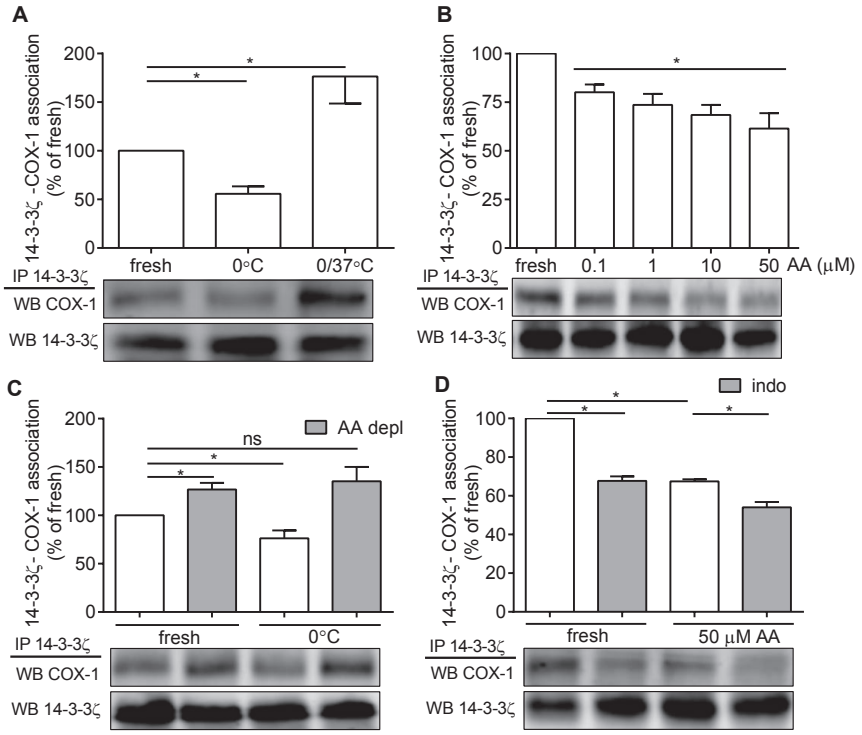


Figure S3. AA dissociates the [14-3-3ζ-COX-1] complex. Measurement of [14-3-3ζ - COX-1] association. (**A**) Fresh, 0°C- and 0/37°C-treated platelets. (**B**) Fresh platelets and platelets incubated with 100 nM, and 1, 10 and 50 μM AA for 10 minutes at 0°C. (**C**) Fresh and 0°C-treated platelets incubated with normal BSA (open bars) and FAF-BSA (AA depl, grey bars). (**D**) Effect of indomethacin. Fresh platelets and platelets incubated with 50 μM AA (10 minutes, 0°C) in absence and presence of indomethacin.

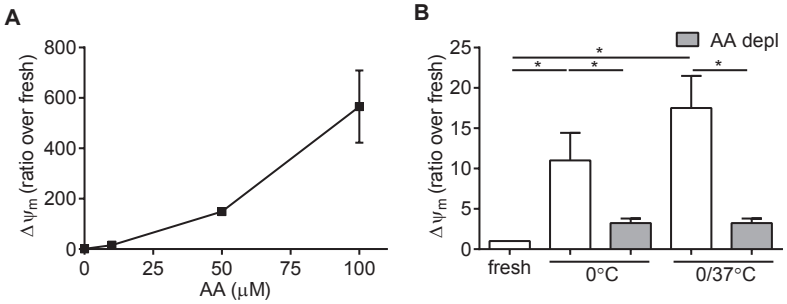


Figure S4. AA triggers apoptosis in murine platelets. (**A**) AA addition induces $\Delta\Psi_m$ -change in murine platelets. The $\Delta\Psi_m$ -change in fresh platelets and platelets incubated with 10, 50 and 100 μM AA for 10 minutes at 0°C. (**B**) AA depletion lowers cold-induced apoptosis induction. The $\Delta\Psi_m$ -change in fresh platelets 0/37°C-treated platelets incubated with FAF-BSA to lower platelet-AA (AA depl, grey bars) and normal BSA (open bars). Data are means ± SEM (n=3) with significant difference $p<0.05$ compared with fresh platelets (*) and between treatments.

CHAPTER 3

Improved platelet survival after cold storage by prevention of glycoprotein Ibα clustering in lipid rafts

Eelo Gitz[1]
Cornelis A. Koekman[1]
Dave J. van den Heuvel[2]
Hans Deckmyn[3]
Jan-Willem N. Akkerman[1]
Hans C. Gerritsen[2]
Rolf T. Urbanus[1]

[1]Department of Clinical Chemistry and Haematology, University Medical Center Utrecht, Utrecht, the Netherlands
[2]Department of Molecular Biophysics, Utrecht University, Utrecht, the Netherlands
[3]Laboratory for Thrombosis Research, KU Leuven, Kortrijk, Belgium

Adapted from Haematologica. 2012; 97(12): 1873-1881

Abstract

Room temperature storage of platelets for transfusion increases the risk of microbial infection and decreases platelet functionality, leading to out-date discard rates of up to 20%. Cold storage may be a better alternative, but this treatment leads to rapid platelet clearance after transfusion, initiated by changes in glycoprotein Ibα, the receptor for von Willebrand factor. We examined the change in glycoprotein Ibα distribution using Förster Resonance Energy Transfer by time-gated Fluorescence Lifetime Imaging Microscopy. Cold storage induced deglycosylation of glycoprotein Ibα ectodomain, exposing *N*-acetyl-D-glucosamine residues, which sequestered with GM1 gangliosides in lipid rafts. Raft-associated glycoprotein Ibα formed clusters upon binding of 14-3-3ζ adaptor proteins to its cytoplasmic tail, a process accompanied by mitochondrial injury and phosphatidyl serine exposure. Cold storage left glycoprotein Ibα surface expression unchanged and although glycoprotein V decreased, the fall did not affect glycoprotein Ibα clustering. Prevention of glycoprotein Ibα clustering by blockade of deglycosylation and 14-3-3ζ translocation raised the survival of cold-stored platelets above levels of room temperature platelets without compromising hemostatic functions. We conclude that glycoprotein Ibα translocates to lipid rafts upon cold-induced deglycosylation and forms clusters by associating with 14-3-3ζ. Interference with these steps provides a means to enable cold storage of platelet concentrates in the near future.

Introduction

Platelet concentrates are currently stored at 22-24°C and a maximum of 5-7 days. The major drawbacks of room temperature (RT) storage are the growth of bacteria, which contaminate one in 2,000 platelet units[1] and the platelet viability and function decline known as platelet storage lesion.[2] Lowering the temperature to 0-4°C may be a better alternative, but this approach introduces new problems as it affects Glycoprotein (GP) Ibα. GPIbα is the major subunit of the receptor for von Willebrand factor (VWF), which traps platelets at sites of vessel damage enabling firm attachment by collagen receptors GPVI and integrin α2β1. The GPIbα change precludes introduction of cold storage in transfusion medicine since it induces apoptosis, starts hemostasis responses upon rewarming,[3] and promotes platelet clearance from the circulation.[4]

There is little insight in the GPIbα change inflicted by cold storage. The ectodomain is highly glycosylated with O- and N-linked carbohydrates, all of which covered by sialic acid. Chilling causes deglycosylation, leading to changes in galactose and *N*-acetyl-D-glucosamine (GlcNAc) exposure. The damaged GPIbα molecules re-arrange and become targets for the lectin binding domain of αMβ2 on liver macrophages and for Ashwell-Morell receptors on hepatocytes, thereby initiating platelet destruction.[4;5] Cold storage affects the cytosolic domain, which is released from the membrane skeleton and becomes a target for the adaptor protein 14-3-3ζ. [14-3-3ζ-GPIbα] association releases phospho-Bad from the [14-3-3ζ - Bad] complex, initiating a fall in mitochondrial membrane potential and caspase-mediated phosphatidylserine (PS) expression.[3]

Chilling reduces the binding affinity of AN51 antibody directed against N-terminal aa 1-35, reflecting a change in a single GPIbα molecule or steric hindrance when GPIbα molecules form clusters. Added GlcNAc preserves normal AN51 binding and inhibits PS expression, suggesting that interference with the ectodomain affects signaling through the cytosolic tail.[6] Possibly, the ectodomain change is caused by receptor clustering, since GPIbα release from the membrane skeleton[7] and dimerizing GPIbα constructs in CHO cells[8] enhance VWF signaling.

Formation of a [14-3-3ζ-GPIbα] complex requires release of arachidonic acid (AA) from membrane phospholipids, which transfers 14-3-3ζ to the cytoplasmic tail of GPIbα.[9] AA is released by cytosolic phospholipase A_2 ($cPLA_2$) upon cold-activation of the stress kinase P38-mitogen-activated protein kinase (P38MAPK).[10] Cooling of platelet suspensions reveal the first signs of P38MAPK activation and [14-3-3ζ-GPIbα] association at 10ºC. This is the phase transition temperature at which membrane phospholipids shift from the liquid-crystalline phase into the gel phase and nanoscale cholesterol-rich domains coalesce to microscale signaling platforms known as lipid rafts.[11-13] The latter property is of specific interest for GPIbα, since at physiological temperature GPIbα depends on raft association to become a signaling receptor for VWF.[14]

In the present study, we investigated the effect of cold storage on GPIbα distribution using Förster Resonance Energy Transfer (FRET), measured by time-gated Fluorescence Lifetime Imaging Microscopy (FLIM). This technique allows determination of the co-localization of two fluorescent molecules within 1-10 nm. We used Alexa Fluor conjugated Fab-fragments of 6B4 antibody against GPIbα[15] to measure [GPIbα-GPIbα] associations and Cholera toxin subunit B (CTB) against the lipid raft marker GM1 ganglioside to assess [GPIbα-GM1] associations.

Materials and Methods

Materials, platelet isolation and incubations, and molecular techniques
A detailed description can be found in the Supplementary Materials and Methods.

Analysis of GPIbα distribution by FRET/FLIM
A detailed description of GPIbα distribution analyzed by FRET/FLIM can be found in the Supplementary Materials and Methods. In short, 6B4-Fab fragments were conjugated to Alexa Fluor-488 or -594 (6B4-488 and 6B4-594, respectively), of which the labeling efficiency on average was 2.5 dye per Fab. Fixed platelet samples were labeled with the Fabs (1 μg/mL) or CTB-594 (5 μg/mL) and clustering of GPIbα and translocation to lipid rafts was determined by FRET using FLIM. The analyzed surface area was a quadrant of 50x50 μm and contained approximately 50 platelets. The fluorescence lifetimes of the donor fluorophore (6B4-488) were determined the absence and presence of acceptor fluorophore (6B4-594 or CTB-594) and subsequently used to calculate the FRET efficiency, defined as

$$FRET\ Efficiency = \frac{\tau_D - \tau_{D/A}}{\tau_D} \times 100\%$$

where τ is the lifetime in nanoseconds in the absence (τ_D) and presence ($\tau_{D/A}$) of the acceptor. To determine variation in FRET efficiency, the lifetimes of three randomly chosen quadrants were quantified and analyzed for statistical significance.

Platelet survival in vivo
Analysis of platelet survival in mice was performed as described previously.[9]

Statistics
Data are means ± SEM (*n*=4), or as indicated. Statistical analysis was performed using GraphPad Prism 5 (San Diego, CA) software. Statistical differences between RT platelets and other incubations were analyzed by Mann-Whitney test. *P*-values less than 0.05 (* or $\underline{*}$) and between incubations (|–*–|) were considered significant.

Results

Cold storage induces clustering of GPIbα

To understand the change in GPIbα ectodomain that introduces macrophage recognition and apoptosis induction, platelets were incubated at 0ºC, fixed and incubated with antibody 6B4 Fab fragments directed against aa 200-268. To enable FRET-FLIM measurements, the 6B4-Fab fragment was conjugated to Alexa Fluor-488 (6B4-488; donor) and -594 (6B4-594; acceptor). Conjugation did not alter the binding capacity to GPIbα (supplementary Figure S2A). Platelets were incubated with 6B4-488, 6B4-594 or a combination to label 50% of GPIbα with donor and 50% with acceptor. Figure 1A shows that dual labeling leads to a uniform distribution of both probes. The average lifetime of 6B4-488 was determined of RT- and cold-stored platelets (4h at 0ºC) the absence and presence of acceptor 6B4-594 (Figure 1B,C). RT platelets showed little reduction in lifetime, resulting in a FRET efficiency of 1.8 ± 0.8%. Cold storage, significantly increased FRET efficiency to 8.8 ± 0.8% indicating that the average distance of GPIbα molecules decreased to <10 nm (Figure 1C). Thus, cold storage induces clustering of GPIbα.

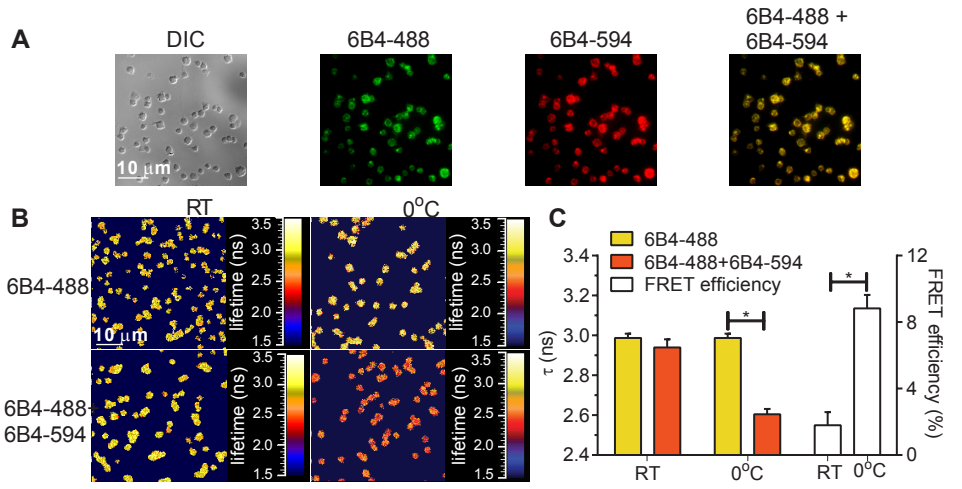


Figure 1. FRET/FLIM analysis of GPIbα distribution. Platelets were kept at RT or 0ºC for 4h, fixed with 2% paraformaldehyde and attached to glass slides by cytospin centrifugation. (**A**) Platelets shown by differential interference contrast (DIC) and fluorescence microscopy. GPIbα was immunostained with 1 µg/mL 6B4-488 (donor) and 1 µg/mL 6B4-594 (acceptor) to obtain 50% GPIbα labeled with 6B4-488 and 50% with 6B4-594. (**B**) False color images of the donor probe fluorescence lifetimes of RT- and cold-stored platelets in nanoseconds (ns) in the absence (top panels) and presence (bottom panels) of acceptor probe. (**C**) Quantification of fluorescence lifetime values of donor probe in the absence (yellow bars) and presence of acceptor probe (orange bars) and corresponding mean FRET efficiencies (open bars) ± SEM (*n*=6).

To determine how fast cold incubation affected the distance between GPIbα molecules, platelets were incubated at 0ºC. The FRET efficiency increased significantly after 1h and rose further at longer incubation reaching 14.9 ± 0.4% after 48h (Figure 2A). To mimic post-transfusion conditions, cold-stored platelets (24h, 0°C) were rewarmed to 37°C. This treatment did not affect the FRET efficiency, indicating that GPIbα clustering was irreversible (Figure 2B). The FRET efficiency increase was accompanied by an increase in [14-3-3ζ-GPIbα]

association (Figure 2C) and depolarization of the mitochondrial membrane (Figure 2D). Cold storage was not accompanied by secretion of the α-granule marker P-selectin or activation of integrin αIIbβ3 (supplementary Figure S2B,C), but subsequent rewarming to 37 ºC increased P-selectin expression about 3-fold. Notably, neither cold storage nor rewarming increased the amount of surface-bound VWF (supplementary Figure S2D). Together, these results demonstrate that cold storage induces a time-dependent redistribution of GPIbα, leading to irreversible clustering in the absence of VWF binding.

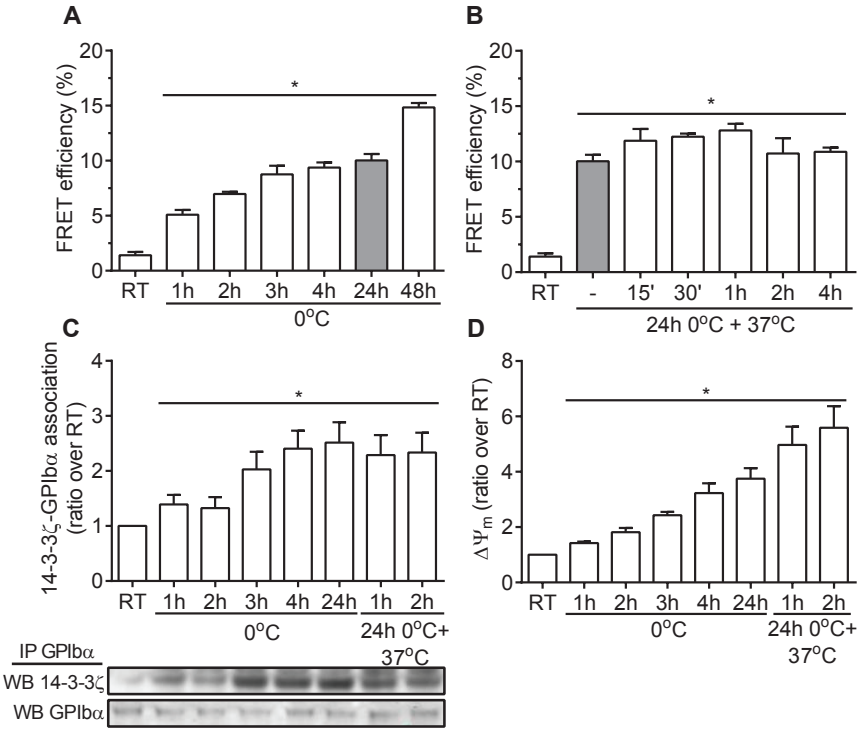


Figure 2. Cold storage induces irreversible clustering of GPIbα and downstream signaling events. (**A**) Platelets were incubated for indicated times at 0°C and GPIbα distribution was analyzed by FRET/FLIM. (**B**) Rewarming of platelets to 37°C after 24h cold storage did not affect the FRET efficiency, indicating that cold-induced GPIbα clustering is irreversible. (**C**) Formation of [14-3-3ζ-GPIbα] complex in RT platelets and during cold storage/ rewarming. (**D**) Cold storage leads to a collapse of the mitochondrial membrane potential ($\Delta\Psi_m$), indicated by the cationic dye JC-1. (C,D) Data are expressed as the ratio of treated platelets over RT platelets.

Clustering of GPIbα is initiated by cold-induced deglycosylation and initiates apoptosis events

One of the factors that might cause the change in GPIbα distribution is the loss of sugar residues during cold incubation.[4;16] GPIbα is a heavily glycosylated receptor, containing O- and N-linked glycans. The core consists of GlcNAc residues, covered by galactose residues which in turn are covered by terminal sialic acid. Earlier work indicated that short-term cold storage leads to increased GlcNAc exposure and long-term cold storage to increased galactose exposure.[4] The neuraminidase inhibitor DANA inhibited galactose exposure.[17]

Using lectins that specifically bind sialic acid, galactose and GlcNAc, we examined whether cold storage leads to changes in glycan structure. Figure 3A shows that during cold storage both sialic acid and galactose were released simultaneously, leading to GlcNAc exposure. Pre-incubation with DANA fully inhibited the loss of sugars. Previous studies showed that the cold-induced GPIbα change revealed by AN51 binding and the [14-3-3ζ-GPIbα] association initiating apoptosis were blocked by addition of GlcNAc in a final concentration (100 mM) that prevented destruction of cold-stored platelets by macrophages.[3;16] In contrast, the same concentration of glucose had no effect.[3] We investigated the effect of these treatments on GPIbα-GPIbα association (Figure 3B). DANA and GlcNAc completely abolished the cold-induced FRET efficiency increase, but glucose had no effect. These findings indicate that the exposure of GlcNAc residues on GPIbα ectodomain drives GPIbα clustering, with which excess GlcNAc interferes. DANA and GlcNAc, but not glucose, also inhibited the change in mitochondrial membrane potential ($\Delta\Psi_m$) and PS exposure, which accompany cold storage (Figure 3C). A titration experiment learned that optimal inhibition by GlcNAc was already induced at 50 μM, highlighting the specificity of GlcNAc interference (Figure 3D). Together, these data suggest that GPIbα-GPIbα association initiates signaling to mitochondrial injury and PS exposure.

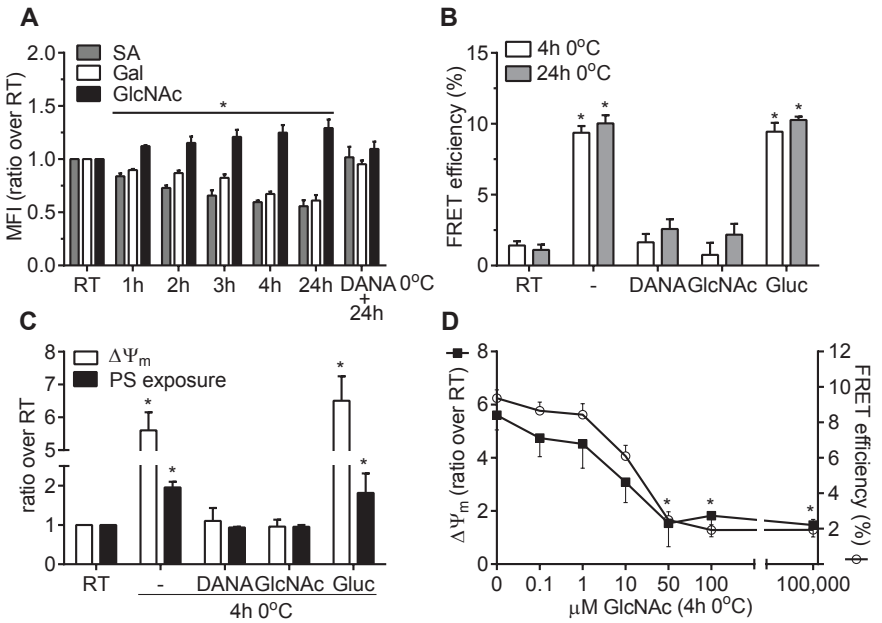


Figure 3. Deglycosylation triggers cold-induced GPIbα clustering leading to apoptosis events. (**A**) FACS analysis of sialic acid (SA), galactose (Gal) and GlcNAc exposure, as detected with SNA, RCA-1 and sWGA FITC-labeled lectins, respectively. Lectin binding to RT and cold-incubated platelets is shown at indicated times, in the absence and presence of neuraminidase inhibitor DANA (200 μM). Data is presented as the ratio of mean fluorescence intensity (MFI) of treated platelets over RT platelets. (**B**) GPIbα distribution measured by FRET/FLIM of platelets stored for 4 (open bars) or 24h (grey bars) at 0°C pre-incubated without or with DANA (200 μM), GlcNAc (100 mM), or glucose (Gluc; 100 mM). (**C**) The mitochondrial membrane potential ($\Delta\Psi_m$; open bars) and PS exposure (black bars) in conditions of Figure 3B. (**D**) GlcNAc dose-dependently inhibits GPIbα redistribution and mitochondrial depolarization. Cold-stored platelets (4h) were preincubated with indicated concentrations of GlcNAc and $\Delta\Psi_m$ (black squares) and GPIbα clustering (open circles) were measured. C,D) Data are expressed as the ratio of treated platelets over RT platelets.

Cold-induced deglycosylation triggers GPIbα to associate with lipid rafts

At physiological temperature, VWF binding triggers GPIbα association with membrane patches enriched in cholesterol and sphingomyelin known as lipid rafts.[14;18;19] To investigate whether cold-induced GPIbα clustering involved raft association, the FRET-FLIM technique was used to determine co-localization of GPIbα, labeled with 6B4-488 (donor), and the raft marker GM1 ganglioside (GM1), labeled with Cholera Toxin Subunit B conjugated to Alexa Fluor-594 (CTB-594; acceptor). Figure 4A shows the presence of GM1 on the platelet surface, which overlaps with staining of GPIbα. FRET-FLIM analysis of [GPIbα-GM1] and [GPIbα-GPIbα] associations revealed that RT platelets have little GPIbα co-localized with GM1 (Figure 4B). Cold storage raised the FRET efficiency of [GPIbα-GM1] association to 9.8 ± 1.9%, which is in the range found for [GPIbα-GPIbα] association (9.4 ± 2.2%). Interestingly, DANA or GlcNAc (50 μM), which blocked clustering of GPIbα, also prevented GPIbα association with lipid rafts. Addition of exogenous GM1 partially decreased the two types of interaction. Addition of GM3, which can interact with GlcNAc-exposing receptors,[20] completely abolished both [GPIbα-GM1] and [GPIbα-GPIbα] association.

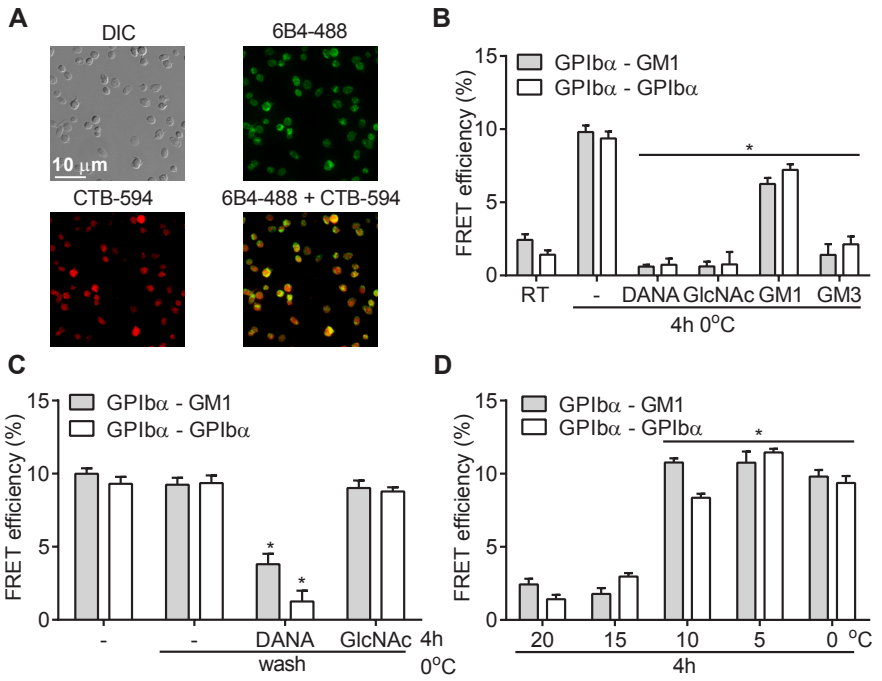


Figure 4. Cold-induced deglycosylation triggers GPIbα to cluster in lipid rafts. (**A**) Platelets shown by DIC and immunostaining of GPIbα with 6B4-488 (donor probe, 1 μg/mL), labeling of raft-specific GM1 Ganglioside with CTB-594 (acceptor probe; 2 μg/mL) and dual staining of donor and acceptor probe. (**B-C-D**) FRET/FLIM analysis of [GPIbα-GM1] (grey bars) and [GPIbα-GPIbα] associations (open bars) of platelets stored for 4h at 0°C. (**B**) Cold-incubation induces [GPIbα-GM1] association. Cold-induced redistribution of GPIbα is blocked by pre-incubation with 200 μM DANA, 50 μM GlcNAc or GM3, and partially by GM1 (50 μM). Data statistically compared to 0°C platelets. (**C**) The effect of removal of DANA or GlcNAc after 4h cold storage on GPIbα redistribution. Platelets were stored at 0°C for 4h in the absence and presence of DANA and GlcNAc. Both agents were subsequently removed by centrifugation (wash; at 0°C) with protection of PGI_2 and again GPIbα distribution was measured. Data statistically compared to 0°C platelets. (**D**) Cooling of platelets triggers GPIbα redistribution. A temperature fall to 10°C and below triggers GPIbα clustering. Data statistically compared to RT platelets.

To assess the applicability of inhibitors of GPIbα clustering to transfusion medicine, platelets were stored (4h, 0°C) in the presence of DANA and GlcNAc and additions were removed by centrifugation (Figure 4C). As expected, removal of DANA left protection against GPIbα clustering intact but GlcNAc removal started a delayed GPIbα-GPIbα association to the range found in GlcNAc-free suspensions. This property would release inhibition of cold-induced GPIbα clustering when transfused platelets enter the circulation. Chilling of platelets is accompanied by raft redistribution below the phase transition temperature of the plasma membrane (about 10 °C).[12;13;21] Analysis of GPIbα associations with GPIbα and GM1 at different temperatures confirmed that both types of association started when the temperature fell to 10°C, suggesting a close dependence on raft redistribution (Figure 4D). Together, these findings show that under different conditions associations between [GPIbα-GM1] and [GPIbα-GPIbα] go hand in hand.

A second cause for cold-induced GPIbα clustering might be shedding of constituents of the $[GPIb\alpha]_2$-$[GPIb\beta]_4$-[GPV]-$[GPIX]_2$ complex, thereby removing steric hindrance for GPIbα to form clusters. During cold storage and subsequent rewarming, there was little change in GPIbα ectodomain content, as demonstrated by flow cytometry, but GPV ectodomain expression fell by 50 % during chilling and another 50% during rewarming (supplementary Figure S3A). To account for possible affinity changes of the antibodies at lower temperature, platelets were stored at 0°C and GPIbα and GPV were measured in immunoprecipitates of pellet and supernatant. Supplementary Figure S3B confirms that surface expression of GPV decreased during cold storage, while expression of GPIbα remained unchanged. Shedding of GPV is regulated by ADAM17 and inhibited by the broad-spectrum matrix metalloproteinase inhibitor GM6001.[22] As expected, the inhibitor prevented GPV loss during cold storage (supplementary Figure S3C), but had no inhibitory effect on [GPIbα-GM1] and [GPIbα-GPIbα] associations (supplementary Figure S3D).

Binding of 14-3-3ζ to the cytoplasmic tail of GPIbα regulates clustering in lipid rafts

We demonstrated recently that cold storage induces the release of AA from the plasma membrane upon activation of $cPLA_2$ by the stress kinase P38MAPK.[9] The liberated AA then acts as a carrier for 14-3-3ζ transfer from multiple 14-3-3ζ-associated proteins to GPIbα. To investigate the contribution of 14-3-3ζ transfer to the GPIbα raft- and self-association, platelet AA content was lowered by incubation with fatty acid-free albumin (AA depletion, in short), as described in detail in an earlier report.[9] This treatment left [GPIbα-GM1] complex formation undisturbed but completely blocked formation of [GPIbα-GPIbα] complexes (Figure 5A). Conversely, when normal platelets were incubated with exogenous AA, [GPIbα-GM1] association remained unchanged but formation of a [GPIbα-GPIbα] complex increased significantly. To confirm that AA affected GPIbα clustering by regulating 14-3-3ζ association, [14-3-3ζ-GPIbα] complex was measured in normal and AA-depleted platelets. Cold incubation induced a 2.5 fold increase in [14-3-3ζ-GPIbα] complex confirming earlier observations.[9] Lowering of endogenous AA completely abolished this increase. The same effect was seen in platelets treated with DANA (Figure 5B). Together these findings suggest that GPIbα must undergo translocation to lipid rafts before 14-3-3ζ can bind and induce GPIbα clusters.

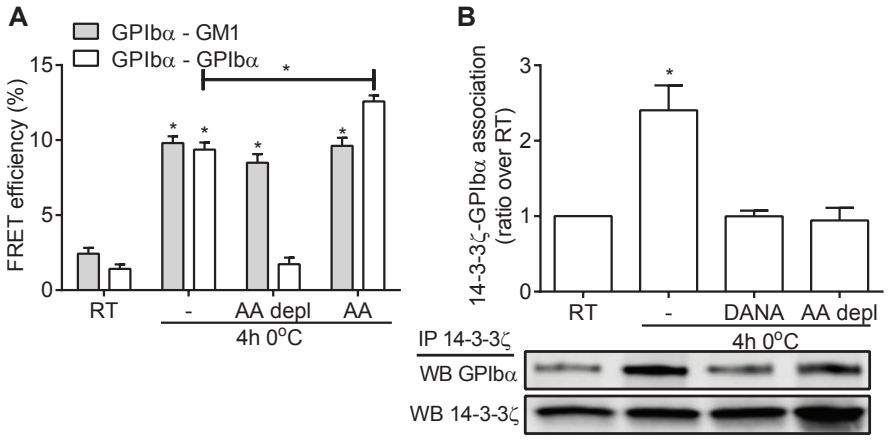


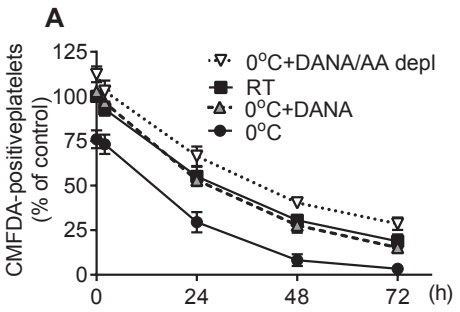
Figure 5. Arachidonic acid transfers 14-3-3ζ to the cytoplasmic tail of GPIbα, leading to clustering in lipid rafts. (**A**) GPIbα co-localization with GM1 (grey bars) and clustering (open bars) of cold-stored platelets after AA depletion and addition. AA content of cold-incubated platelets was lowered by incubation with fatty acid free-BSA (AA depl), leading to a decrease in [GPIbα-GPIbα]. Addition of 10 μM exogenous AA increased cold-induced [GPIbα-GPIbα]. (**B**) The effect of DANA and AA depletion on cold-induced [14-3-3ζ-GPIbα] complex formation. Immunoprecipitations show that addition of 200 μM DANA or AA depletion inhibits 14-3-3ζ binding to GPIbα. Data are expressed as the ratio of treated platelets over RT platelets.

Inhibition of GPIbα clustering improves the survival of cold-stored platelets without loss of hemostatic function

To clarify how the association of cold-damaged GPIbα and [AA-14-3-3-ζ] affected the survival of platelets, we incubated murine platelets for 4h under conditions that interfere with the change in GPIbα and the release of AA. To this end, CMFDA-labeled platelets were stored for 4h in the absence of cold (RT controls), at 0°C to induce GPIbα damage and AA release, at 0°C with GPIbα protection (DANA) and AA release and at 0°C with GPIbα protection and impaired AA release (AA depleted platelets). Recovery and survival of RT platelets were ~83% and 80h, respectively (Figure 6A,B). Cold storage decreased these values with about 25% and 15%. Both parameters improved upon addition of DANA. Importantly, a combination of DANA and AA depletion raised recovery and survival above levels observed with RT platelets. Thus, optimal survival of cold-stored platelets requires both arrest of extracellular GPIbα deglycosylation and intracellular 14-3-3-ζ translocation.

It is unlikely that a neuraminidase inhibitor such as DANA will interfere with the signaling properties of GPIbα and other receptors but depletion of AA stores will compromise thromboxane A_2 production and might disturb hemostatic properties. We addressed this possibility by analyzing surface P-selectin expression after ex vivo stimulation with PAR-4 agonist peptide. P-selectin expression in CMFDA-labeled platelets analysed immediately after cold storage (4h, 0°C) was similar to that observed in RT platelets (Figure 6C; t=0). Treatment with DANA had no effect either. A second analysis of platelet reactivity 24h after transfusion showed that P-selectin expression was preserved without and with DANA. Analysis of αIIbβ3 activation showed similar results (Figure 6D). In contrast, the combination of DANA treatment and AA depletion induced a significant fall in P-selectin expression and αIIbβ3 activation immediately after cold storage. Since DANA alone had no effect, this fall

was due to the reduced AA stores. Interestingly, both responses had normalized following 24h circulation. These data suggest that the recovery of AA stores after prior depletion observed in vitro[9] also occurs in vivo. Thus, inhibition of GPIbα clustering prevents the cold-induced fall in platelet survival without compromising hemostatic functions.



B

	Recovery (%)	Survival (h)
RT	82.5 ± 3.4	81.3 ± 2.4
0°C	62.7 ± 3.4*	64.9 ± 2.9*
0°C+DANA	84.8 ± 1.9[*]	76.7 ± 1.3[*]
0°C+DANA/ AA depl	92.7 ± 3*[*]	89.3 ± 1.9*[*]

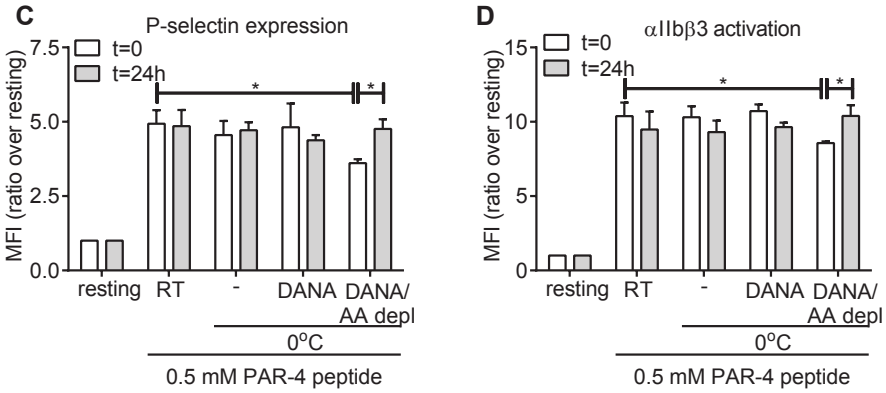


Figure 6. Inhibition of GPIbα redistribution improves the survival of cold-stored platelets without affecting their hemostatic function. (**A**) Platelet survival in mice after cold storage. Mouse platelets labeled with CMFDA were kept at RT or at 0°C (4h) in the absence or presence of 200 μM DANA or depleted of AA in combination with DANA treatment. Then, platelets were washed with protection by PGI_2 and injected into recipient mice. Blood was collected after 2 minutes, 2, 24, 48 and 72h after injection. Recovery of RT platelets at 2 min was set at 100%; other data are expressed as a percentage of this value. (**B**) Recoveries and survival times of CMFDA-labeled platelets (means ± SEM of four mice in each group). Data statistically compared to either RT platelets (*) or 0°C platelets ([*]). (C,D) Flow cytometric analysis of (**C**) surface P-selectin expression and (**D**) activation of integrin αIIbβ3 upon ex vivo platelet stimulation with 0.5 mM PAR-4 agonist peptide. Analysis was performed immediately after cold storage (t=0) and 24h post-transfusion (t=24h). Data refer to CMFDA labeled platelets and are expressed as a ratio of MFI of stimulated over resting platelets.

Discussion

The better preservation of platelet functions and the lower bacterial growth at 0°C make cold storage an attractive alternative for current procedures for platelet preservation at RT.[23-25] Unfortunately, the cold-inflicted changes in GPIbα collectively defined as GPIbα clustering withstands rapid introduction in transfusion medicine. The present results describe the molecular mechanism of cold-induced GPIbα clustering. First, sialic acid and galactose are removed exposing GlcNAc residues on GPIbα ectodomain. Second, GlcNAc residues associate with the raft constituents GM1/3. Third, the adaptor protein 14-3-3ζ binds to the

GPIbα cytoplasmic tail inducing a mechanism that lowers the average distance between GPIbα molecules to less than 10 nm. This subsequently leads to mitochondrial injury and PS exposure. They are also factors controlling platelet survival in vivo since inhibition of sugar loss (DANA) and inhibition of 14-3-3ζ translocation (AA depletion) improves recovery and survival of cold-stored platelets.

Recent findings show that cold storage triggers surface up-regulation of neuraminidase-1 and β-galactosidase, which co-localize in granule-like structures under resting conditions.[17] Neuraminidase inhibition (DANA) blocks both release of sialic acid and galactose and the GPIbα-GPIbα association revealed by FRET/FLIM, indicating that sugar loss is a first step in GPIbα clustering. The removal of sialic acid and galactose induced by cold go hand in hand illustrating that loss of sialic acid residues make galactose residues accessible to β-galactosidase. Conversely, neuraminidase blockade prevents β-galactosidase to reach its substrate.

Loss of sialic acid/galactose exposes GlcNAc residues that associate with ganglioside GM1/3-rich areas in lipid rafts, as determined by FRET/FLIM analysis of GPIbα and GM1. This reaction is accompanied by GPIbα-GPIbα associations, as detected with the same technique. Addition of exogenous GM1, GM3 or GlcNAc inhibits GPIbα-GM1/3 associations, which is in agreement with direct binding of GPIbα-bound GlcNAc to raft-bound GM1/3. Importantly, interference with GPIbα-GM1/3 associations also blocks GPIbα-GPIbα associations. This implies that GPIbα clustering is a direct consequence of its association with specific domains in lipid rafts. Gangliosides are glycosphingolipids with different carbohydrate chains that extend out from the cell surface and are involved in cell-cell-recognition, adhesion and signal transduction.[26] Both GM1 and GM3 concentrate in lipid rafts where they can coincide and form clusters.[27] The carbohydrate-carbohydrate interaction between GlcNAc and GM3 seems quite specific as GM1, which differs from GM3 in that it has an extra galactose and N-acetyl-galactosamine residue, only partially blocked GPIbα clustering and GM3 induced full inhibition. Earlier work showed that cold lowers the binding of an antibody directed against the GPIbα *N*-terminal flank, a change that could be prevented by GlcNAc.[6] This antibody binds to GPIbα aa 1-35 and the affinity change induced by cold appears to parallel the association of GPIbα residues 200-268 covered by 6B4-Fab fragments bound to the FRET/FLIM labels.

Conventional sucrose density fractionation showed earlier that 10-15% of total GPIba is located in rafts in resting platelets, which increases three-fold upon stimulation with VWF.[14;19] GPIbα translocation to rafts is an important step in VWF signaling since cholesterol depletion inhibits the major functions of the receptor complex, including ristocetin-induced platelet aggregation and adhesion to VWF under conditions of flow. The FRET/FLIM technique for GPIbα-GM1/3 interaction shows a 3-4 % FRET efficiency at RT and a 4-fold increase during cold incubation, also indicating that in resting platelets only a minor part of GPIbα is bound to rafts and that this fraction increases upon stimulation. This shift occurs in the absence of VWF and represents a type of ligand-independent raft association. It might also explain why cold storage increases binding of VWF.[28]

A final step in cold-induced GPIbα clustering is the binding of 14-3-3ζ adaptor protein to the cytosolic tail. This reaction is restricted to raft-bound GPIbα since blockade of raft association with DANA inhibits both GPIbα-GPIbα and 14-3-3ζ-GPIbα associations. Both associations are lower in AA-depleted platelets than in controls. Cold storage activates

the stress kinase P38MAPK, which together with c-Phospholipase A_2 releases AA from membrane phospholipids. Lipid rafts are enriched in AA and might be the source of the released AA.[29] Released AA then accumulates due to poor COX-1 activity at low temperature.[9] AA binds directly to 14-3-3ζ, induces 14-3-3ζ multimerization and releases the protein from binding partners.[30] One of these binding partners is ADF/cofilin, a protein that destabilizes actin filaments when dephosphorylated upon release from 14-3-3ζ.[31] In muscle cells, 14-3-3ζ association regulates clustering of the acetylcholine receptor.[32] A similar process may contribute to clustering of GPIbα. The cytoplasmic tail of GPIbα has multiple binding sites for 14-3-3ζ.[33-35] Domain aa 551-564 contains the binding site for Filamin A (aa ser559),[36] which anchors GPIbα to the membrane skeleton under resting conditions. The functional activity of 14-3-3ζ depends on its dimerization[37] and it has been indicated that a single 14-3-3ζ dimer can disrupt this interaction by competitive binding, thereby releasing GPIbα.[35] In a next step, 14-3-3ζ dimer/multimer might bind two or more GPIbα molecules, creating a 'crosslink platform' that facilitates clustering.

AA-depletion disturbs GPIbα-GPIbα interaction, but GPIbα-GM1/3 interaction remains intact. This interesting discrepancy separates GPIbα binding to rafts (AA-independent) from [GPIbα-GPIbα] association (AA-dependent). Cooling of platelets leads to the coalescence of small rafts into larger patches, which then become signaling platforms.[11-13] This is particularly evident when the temperature decreases below ~10°C and membrane phospholipids change from the liquid-crystalline phase into the gel phase. We showed earlier that the P38MAPK mediated AA-release starts below this temperature and the start of GPIbα-GPIbα associations below 10°C might well result from the AA-mediated attachment of 14-3-3ζ to GPIbα cytosolic tail. However, also the GPIbα binding to GM1/3 starts when the temperature falls below 10°C. Since this reaction is AA-independent, a direct effect of the phase transition on GPIbα binding to rafts is evident. Together, these data imply that the phase transition of the plasma membrane contributes to GPIbα clustering at two levels: first, by stimulating GPIbα binding to rafts and second, by inducing GPIbα binding to [AA-14-3-3ζ].

One of the protein complexes that is disturbed by the cold-induced AA accumulation is [14-3-3ζ-phospho-Bad], which upon release of 14-3-3ζ is dephosphorylated and becomes an activator of pro-apoptotic Bax, mitochondrial damage, caspase-9 and PS exposure.[9] These reactions are inhibited completely by arrest of GPIbα deglycosylation (DANA), raft association (GlcNAc) and 14-3-3ζ binding (AA depletion) and therefore critically depend on the clustering of GPIbα molecules. Since this type of apoptosis induction occurs in the absence of VWF, it represents a form of ligand-independent signaling inflicted by cold.

To investigate whether interference with GPIbα clustering changed the survival of cold-stored platelets, murine platelets were stored at RT (controls) and 0°C in the presence of DANA without and with prior depletion of AA. DANA treatment improved platelet recovery by 35% and the combination with AA-depletion by almost 50%, thereby surpassing the recovery observed with platelets stored at RT. The effect of these treatments on platelet survival was smaller albeit significant and again demonstrated that the combined treatments completely prevented the platelet disturbance inflicted by cold. Treatment with DANA alone left P-selectin expression and αIIbβ3 activation induced by PAR-4 agonist peptide undisturbed. In contrast, both responses were reduced following preparation of AA-depleted platelets, probably reflecting a shortage of thromboxane A_2 production, which is known to support these functions. Interestingly, both defects had disappeared 24h after transfusion,

raising the possibility that AA-depleted platelets accumulate extracellular AA and restore their responsiveness in the circulation.

In conclusion, inhibition of GPIbα clustering by DANA and AA depletion provides a simple means to prevent the damage that compromises the recovery and survival of cold-stored platelets.

Acknowledgements
This study was supported by a grant from the Landsteiner Foundation of Blood transfusion Research (LSBR grant no. 0807). Prof. Dr. J.W.N. Akkerman is supported by the Netherlands Thrombosis Foundation. Dr. R.T. Urbanus is a research fellow of the Dutch Heart Foundation (grant no. 2010T068). The authors thank Dr Erik Hofman for helpful discussions.

References

1. Brecher ME, Hay SN, Rothenberg SJ. Evaluation of a new generation of plastic culture bottles with an automated microbial detection system for nine common contaminating organisms found in PLT components. *Transfusion*. 2004;44(3):359-363.
2. Sorensen AL, Hoffmeister KM, Wandall HH. Glycans and glycosylation of platelets: current concepts and implications for transfusion. *Curr Opin Hematol*. 2008;15(6):606-611.
3. van der Wal DE, DU VX, Lo KS, Rasmussen JT, Verhoef S, Akkerman JW. Platelet apoptosis by cold-induced glycoprotein Ibalpha clustering. *J Thromb Haemost*. 2010;8(11):2554-2562.
4. Rumjantseva V, Grewal PK, Wandall HH et al. Dual roles for hepatic lectin receptors in the clearance of chilled platelets. *Nat Med*. 2009;15(11):1273-1280.
5. Hoffmeister KM, Felbinger TW, Falet H et al. The clearance mechanism of chilled blood platelets. *Cell*. 2003;112(1):87-97.
6. van der Wal DE, Verhoef S, Schutgens RE, Peters M, Wu Y, Akkerman JW. Role of glycoprotein Ibalpha mobility in platelet function. *Thromb Haemost*. 2010;103(5):1033-1043.
7. Englund GD, Bodnar RJ, Li Z, Ruggeri ZM, Du X. Regulation of von Willebrand factor binding to the platelet glycoprotein Ib-IX by a membrane skeleton-dependent inside-out signal. *J Biol Chem*. 2001;276(20):16952-16959.
8. Kasirer-Friede A, Ware J, Leng L, Marchese P, Ruggeri ZM, Shattil SJ. Lateral clustering of platelet GP Ib-IX complexes leads to up-regulation of the adhesive function of integrin alpha IIbbeta 3. *J Biol Chem*. 2002;277(14):11949-11956.
9. van der Wal DE, Gitz E, DU VX et al. Arachidonic acid depletion extends survival of cold-stored platelets by interfering with the [glycoprotein Ibalpha - 14-3-3zeta] association. *Haematologica*. 2012;97(10):1514-1522.
10. Kramer RM, Roberts EF, Um SL et al. p38 mitogen-activated protein kinase phosphorylates cytosolic phospholipase A2 (cPLA2) in thrombin-stimulated platelets. Evidence that proline-directed phosphorylation is not required for mobilization of arachidonic acid by cPLA2. *J Biol Chem*. 1996;271(44):27723-27729.
11. Gousset K, Wolkers WF, Tsvetkova NM et al. Evidence for a physiological role for membrane rafts in human platelets. *J Cell Physiol*. 2002;190(1):117-128.
12. Gousset K, Tsvetkova NM, Crowe JH, Tablin F. Important role of raft aggregation in the signaling events of cold-induced platelet activation. *Biochim Biophys Acta*. 2004;1660(1-2):7-15.
13. Bali R, Savino L, Ramirez DA et al. Macroscopic domain formation during cooling in the platelet plasma membrane: an issue of low cholesterol content. *Biochim Biophys Acta*. 2009;1788(6):1229-1237.
14. Shrimpton CN, Borthakur G, Larrucea S, Cruz MA, Dong JF, Lopez JA. Localization of the adhesion receptor glycoprotein Ib-IX-V complex to lipid rafts is required for platelet adhesion and activation. *J Exp Med*. 2002;196(8):1057-1066.
15. Fontayne A, Vanhoorelbeke K, Pareyn I et al. Rational humanization of the powerful antithrombotic anti-GPIbalpha antibody: 6B4. *Thromb Haemost*. 2006;96(5):671-684.
16. Hoffmeister KM, Josefsson EC, Isaac NA, Clausen H, Hartwig JH, Stossel TP. Glycosylation restores survival of chilled blood platelets. *Science*. 2003;301(5639):1531-1534.
17. Jansen AJ, Josefsson EC, Rumjantseva V et al. Desialylation accelerates platelet clearance after refrigeration and initiates GPIbalpha metalloproteinase-mediated cleavage in mice. *Blood*. 2012;119(5):1263-1273.
18. Munday AD, Gaus K, Lopez JA. The platelet glycoprotein Ib-IX-V complex anchors lipid rafts to the membrane skeleton: implications for activation-dependent cytoskeletal translocation of signaling molecules. *J Thromb Haemost*. 2010;8(1):163-172.
19. Geng H, Xu G, Ran Y, Lopez JA, Peng Y. Platelet glycoprotein Ib beta/IX mediates glycoprotein Ib alpha localization to membrane lipid domain critical for von Willebrand factor interaction at high shear. *J Biol Chem*. 2011;286(24):21315-21323.
20. Guan F, Handa K, Hakomori SI. Regulation of epidermal growth factor receptor through interaction of ganglioside GM3 with GlcNAc of N-linked glycan of the receptor: demonstration in ldlD cells. *Neurochem Res*. 2011;36(9):1645-1653.
21. Tablin F, Oliver AE, Walker NJ, Crowe LM, Crowe JH. Membrane phase transition of intact human platelets: correlation with cold-induced activation. *J Cell Physiol*. 1996;168(2):305-313.
22. Rabie T, Strehl A, Ludwig A, Nieswandt B. Evidence for a role of ADAM17 (TACE) in the regulation of platelet glycoprotein V. *J Biol Chem*. 2005;280(15):14462-14468.

23. Slichter SJ. In vitro measurements of platelet concentrates stored at 4 and 22 degree C: correlation with posttransfusion platelet viability and function. *Vox Sang*. 1981;40 Suppl 172-86.

24. Babic AM, Josefsson EC, Bergmeier W et al. In vitro function and phagocytosis of galactosylated platelet concentrates after long-term refrigeration. *Transfusion*. 2007;47(3):442-451.

25. Currie LM, Harper JR, Allan H, Connor J. Inhibition of cytokine accumulation and bacterial growth during storage of platelet concentrates at 4 degrees C with retention of in vitro functional activity. *Transfusion*. 1997;37(1):18-24.

26. Sonnino S, Prinetti A. Gangliosides as regulators of cell membrane organization and functions. *Adv Exp Med Biol*. 2010;688165-184.

27. Fujita A, Cheng J, Hirakawa M, Furukawa K, Kusunoki S, Fujimoto T. Gangliosides GM1 and GM3 in the living cell membrane form clusters susceptible to cholesterol depletion and chilling. *Mol Biol Cell*. 2007;18(6):2112-2122.

28. Sorensen AL, Rumjantseva V, Nayeb-Hashemi S et al. Role of sialic acid for platelet life span: exposure of beta-galactose results in the rapid clearance of platelets from the circulation by asialoglycoprotein receptor-expressing liver macrophages and hepatocytes. *Blood*. 2009;114(8):1645-1654.

29. Pike LJ, Han X, Chung KN, Gross RW. Lipid rafts are enriched in arachidonic acid and plasmenylethanolamine and their composition is independent of caveolin-1 expression: a quantitative electrospray ionization/mass spectrometric analysis. *Biochemistry*. 2002;41(6):2075-2088.

30. Brock TG. Arachidonic acid binds 14-3-3zeta, releases 14-3-3zeta from phosphorylated BAD and induces aggregation of 14-3-3zeta. *Neurochem Res*. 2008;33(5):801-807.

31. Gohla A, Bokoch GM. 14-3-3 regulates actin dynamics by stabilizing phosphorylated cofilin. *Curr Biol*. 2002;12(19):1704-1710.

32. Lee CW, Han J, Bamburg JR, Han L, Lynn R, Zheng JQ. Regulation of acetylcholine receptor clustering by ADF/cofilin-directed vesicular trafficking. *Nat Neurosci*. 2009;12(7):848-856.

33. Du X, Fox JE, Pei S. Identification of a binding sequence for the 14-3-3 protein within the cytoplasmic domain of the adhesion receptor, platelet glycoprotein Ib alpha. *J Biol Chem*. 1996;271(13):7362-7367.

34. Mangin P, David T, Lavaud V et al. Identification of a novel 14-3-3zeta binding site within the cytoplasmic tail of platelet glycoprotein Ibalpha. *Blood*. 2004;104(2):420-427.

35. Yuan Y, Zhang W, Yan R et al. Identification of a novel 14-3-3zeta binding site within the cytoplasmic domain of platelet glycoprotein Ibalpha that plays a key role in regulating the von Willebrand factor binding function of glycoprotein Ib-IX. *Circ Res*. 2009;105(12):1177-1185.

36. Williamson D, Pikovski I, Cranmer SL et al. Interaction between platelet glycoprotein Ibalpha and filamin-1 is essential for glycoprotein Ib/IX receptor anchorage at high shear. *J Biol Chem*. 2002;277(3):2151-2159.

37. Woodcock JM, Murphy J, Stomski FC, Berndt MC, Lopez AF. The dimeric versus monomeric status of 14-3-3zeta is controlled by phosphorylation of Ser58 at the dimer interface. *J Biol Chem*. 2003;278(38):36323-36327.

Supplementary Materials and Methods

Materials and antibodies

The following products were used (with sources): AA (Bio/Data Corporation, Horsham, PA), GlcNAc, fatty acid free-BSA, N-Acetyl-2,3-dehydro-2-deoxyneuraminic acid (DANA), *D*-Glucose and mowiol 4-88 (Sigma-Aldrich, St Louis, MO), Cell tracker green 5-chloromethyl fluorescein diacetate (CMFDA) (Molecular Probes, Invitrogen, Carlsbad, CA), monosialoganglioside GM1 and GM3 (GenWay Biotech Inc, San Diego, CA), GM6001 (Millipore, Billerica, MA), Odyssey blocking buffer (LI-COR Biosciences, Lincoln, NE) and prostacyclin (PGI_2; Cayman Chemical, Ann Arbor, MI). Protease-activating receptor-4 (PAR-4) agonist peptide AYPGKV was synthesized at the Netherlands Cancer Institute (Amsterdam, the Netherlands). Antibodies and lectins used for flow cytometry were directed against GPIbα (clone HIP1; BD Pharmingen (San Diego, CA), GPV (clone CLB-SW16; Monosan, Uden, the Netherlands) and goat anti-mouse $F(ab)_2$ conjugated to Alexa Fluor 488 (Invitrogen, Carlsbad, CA), against PS (fluorescein-conjugated Lactadherin; Haematologic Technologies Inc. Essex Junction, VT), sialic acid (fluorescein-conjugated sambucus nigra, SNA), galactose (fluorescein-conjugated ricinus communis I, RCA-I), and GlcNAc (fluorescein-conjugated succinylated wheat germ agglutinin, s-WGA; Vector Laboratories, Burlingame, CA). To assess murine platelet activation by flow cytometry we obtained PE-labeled anti-active CD41/CD61 (active form of integrin αIIbβ3; clone JON/A), PE-labeled anti-CD62P (P-selectin; clone Wug.E9), and the corresponding PE-labeled negative control (Emfret Analytics GmbH & Co, Eibelstadt, Germany). The cationic dye 5,5′,6,6′-Tetrachloro-1,1′,3,3′-tetraethylimidacarbocyanine iodide (JC-1) was from Sigma-Aldrich, St Louis, MO. Antibodies for immunoprecipitation were against GPIbα (clone AK2; Santa Cruz Biotechnology, Santa Cruz, CA) and for western blotting against GPIbα (clone SZ2; Beckman Coulter, Brea, CA), GPV (clone CLB-SW16; Monosan, Uden, the Netherlands) and 14-3-3ζ (C-16, Santa Cruz Biotechnology, Santa Cruz, CA). Secondary antibodies were Alexa Fluor 680-conjugated goat-α-rabbit and goat-α-mouse (Invitrogen, Carlsbad, CA). The antibody used for FRET analysis of GPIbα co-localization was the recombinant 6B4-Fab fragment directed against aa 200-268 of GPIbα.[1] Other tools for FRET analysis were Alexa Fluor-488 and -594 protein labeling kits and Cholera toxin subunit B conjugated to Alexa Fluor-594 (CTB-594; Invitrogen). O-sialoglycoprotein endopeptidase (osge) was from Cedarlane Laboratories (Burlington, ON, Canada). Polyclonal sheep-anti-VWF was from Abcam (Cambridge, UK), antibodies against P-selectin and activated αIIbβ3 (PAC-1) were from BD Pharmingen (San Diego, CA).

Platelet isolation and incubations

Human platelets were isolated[2] with free-flow blood collection. The first 2 mL of blood and all collections that showed micro-aggregates were discarded. Procedures were approved by the Medical Ethical Committee of our hospital; the laboratory is certified for ISO-9001:2008. Platelets were resuspended in Hepes-Tyrode's ($2x10^{11}$ cells/L, pH 7.3) and kept at room temperature (10 minutes, defined as RT platelets), 4h at 0°C (or indicated) or at 0°C followed by rewarming to 37°C. In a few experiments, D-Glucose (100 mM) or GlcNAc (1 µM to 100 mM) was added prior to platelet storage. To lower AA content in the platelet membrane, fatty acid free-BSA was present (75 g/L in Hepes-Tyrode, pH 7.3) during the 4h incubation at 0ºC, as applied earlier.[3-6] All incubations were without stirring, unless specifically indicated.

For isolation of murine platelets, eight weeks old strain-, and sex-matched C57BL/6 wild type mice from Harlan (Boxmeer, the Netherlands) were used. The experimental protocols were approved by the local ethics committees for animal experiments. Mice were anesthetized with isoflurane and blood was collected in 0.1 volume 130 mM trisodium citrate by cardiac puncture and centrifuged (420*g*, 3 minutes, 22ºC, no brake). The pellet, together with one third of the red blood cell fraction was collected and again centrifuged (960*g*, 1 minute, 22ºC). Platelets were collected and resuspended in Hepes-Tyrode (pH 6.5), washed in 0.1 volume ACD and PGI_2 (2,700*g*, 2 minutes, 22ºC) and resuspended in Hepes-Tyrode (pH 7.3) to a final concentration of $2x10^{11}$ platelets/L.

Ex vivo murine platelet activation

To investigate the hemostatic functions of stored mouse platelets after transfusion, we determined activation of integrin αIIbβ3 and P-selectin expression upon stimulation with 0.5 mM PAR-4 agonist peptide. Platelet activation was analyzed immediately after storage in the aforementioned conditions and 24h after transfusion. Whole blood (25µL) was diluted (1:20 (v/v) with Hepes-Tyrode buffer (pH 7.3), recalcified (1 mM $CaCl_2$), and subsequently incubated with 5 µL PE-conjugated anti-active integrin αIIbβ3 (5 µL, JON/A) or PE-conjugated anti-P-selectin (5 µL, Wug.E9) for 15 minutes at 20°C. Samples were fixed with 1% formaldehyde in phosphate-buffered saline (PBS) (20°C), and CMFDA-positive platelets were analyzed by flow cytometry.

Flow cytometric analysis

Characterization of platelets by FACS was based on FSC and SSC-scatter (FACS-Calibur; BD Biosciences, San Jose, CA). Appropriate antibodies or lectins were added and incubated for 15 minutes at 37°C. A total of 10,000 platelets were analyzed for surface expression of GPIbα, PS, sialic acid, galactose, GlcNAc, VWF, P-selectin, activation of integrin αIIbβ3, and GPV. For determination of the mitochondrial membrane-potential $\Delta\Psi_m$, platelet suspensions were incubated with the JC-1 dye (0.5 µM, 30 minutes, 37°C),[7] which changes emission from ~590 to ~525 nm upon depolarization. Changes in $\Delta\Psi_m$ were expressed as the ratio of platelets in lower- over upper-right quartiles.[8;9]

Immunoprecipitations and western blots

Platelet suspensions were added to lysis buffer.[2] For immunoprecipitations, 450 µL washed platelets ($5x10^{11}$ platelets/L) was lysed, (15 minutes, 0°C), centrifuged (10,000*g*, 10 minutes, 4ºC) to remove cell debris and mixed with 55 µL (10% vol/vol) protein G beads together with antibody (1 µg/mL, 30 minutes, 4ºC, rotating).[7] Proteins were separated by SDS-PAGE and Western Blotted. After blocking with Odyssey Blocking buffer, membranes were incubated with primary antibodies (1 µg/mL) and protein bands visualized with an Odyssey Imaging system (LI-COR Biosciences, Lincoln, NE). Quantification was performed with Image-J software (NIH, Bethesda, MD). Possible lane-to-lane loading variation was corrected by normalization to the immunoprecipitated protein.

Analysis of GPIbα distribution by FRET/FLIM

The 6B4-Fab fragment was labeled with Alexa Fluor-488 or -594, which was performed as recommended by the manufacturer. In short, 300 µg of Fab-fragment was incubated with 40 µg of DMSO dissolved amine-reactive Alexa-Fluor dye for 1h at RT in the dark. Labeled Fab fragment was separated from non-reacting dye using 0.5 mL Zeba desalt spin columns (Thermo Scientific, Waltham, MA). Labeling efficiency was determined with a Nanodrop

spectrophotometer (Nanodrop Technologies, Wilmington, Delaware), and was on average 2.5 Alexa-Fluor label per Fab fragment. Final concentration of the fluorescent 6B4-Fab fragments used for cell labeling varied between 1 and 1.5 µg/mL.

Platelets were prepared for immunohistochemistry and FRET/FLIM analysis by fixation with 2% paraformaldehyde for 30 minutes at room temperature. Platelets were subsequently fixed to glass slides by cytospin centrifugation (Shandon Cytospin 3, Astmoor, UK) and dried for 10 minutes. After three wash steps in PBS, cells were blocked with 1% BSA in PBS (30 minutes) and incubated with 6B4-488, 6B4-594 (both 1 µg/mL) or CTB-594 (2 µg/mL) for 1h at 37°C. Cells were washed again, embedded in mowiol and stored at -20°C until further use. Wide field microscopy was performed using an Axio Observer Z1 microscope equipped with an AxioCam MRm CCD camera using a Zeiss 100x/1.3 EC PlanNeoFluar oil immersion lens (Carl Zeiss MicroImaging GmbH, Gottingen, Germany).

Clustering of GPIbα and translocation to lipid rafts was determined by FRET using FLIM, as previously described[10] with minor adaptations. Compared with conventional FRET approaches this technique has the advantage that it is insensitive to variations in the concentration and emission intensity of fluorophores (for review see Wallrabe et al 2005).[11] A Nikon PCM 2000 confocal scanning laser microscope (CSLM) was equipped with a fluorescence lifetime imaging module (LiMo Nikon Instruments, Badhoevedorp, The Netherlands),[12] which captures four images representing the total fluorescent intensity in four consecutive time gates of approximately 2 nanoseconds each (supplementary Figure S1A; donor and B; donor + acceptor). The analyzed surface area was a quadrant of 50x50 µm and contained approximately 50 platelets. The four-gate intensity decays recorded for each pixel were fitted with a monoexponential decay using the LiMo software to generate lifetime images. An intensity threshold was set to exclude pixels with a photon count too low to accurately fit an exponential decay. The fluorescence lifetimes were plotted in a histogram that was fitted with a Gaussian function (using GraphPad Prism 5; San Diego, CA) to determine the average lifetime (supplementary Figure S1C). To determine variation in FRET efficiency, the lifetimes of three randomly chosen quadrants were quantified and a Student's t-test was performed to determine the statistical significances (supplementary Figure S1D). The lifetime values were subsequently used to calculate the FRET efficiency, defined as

$$FRET\ Efficiency = \frac{\tau_D - \tau_{D/A}}{\tau_D} \times 100\%$$

where τ is the lifetime in nanoseconds in the absence (τ_D) and presence ($\tau_{D/A}$) of the acceptor.

6B4-Fab binding to platelets

Alexa Fluor-488 and -594 conjugated 6B4-Fab (6B4-488 and 6B4-594, respectively) binding to platelets was tested in an ELISA system where the Fab fragment was added, in a 1:2 (vol:vol) dilution series into wells precoated with platelets. Unbound Fab fragment was removed and binding was quantified using a Spectramax M2e microplate reader (Molecular Devices, Sunnyvale, CA). Respective excitation and emission wavelengths for analysis of 6B4-488 binding were 494 and 520 nm, and for 6B4-594 binding 590 and 619 nm.

SUPPLEMENTARY FIGURES

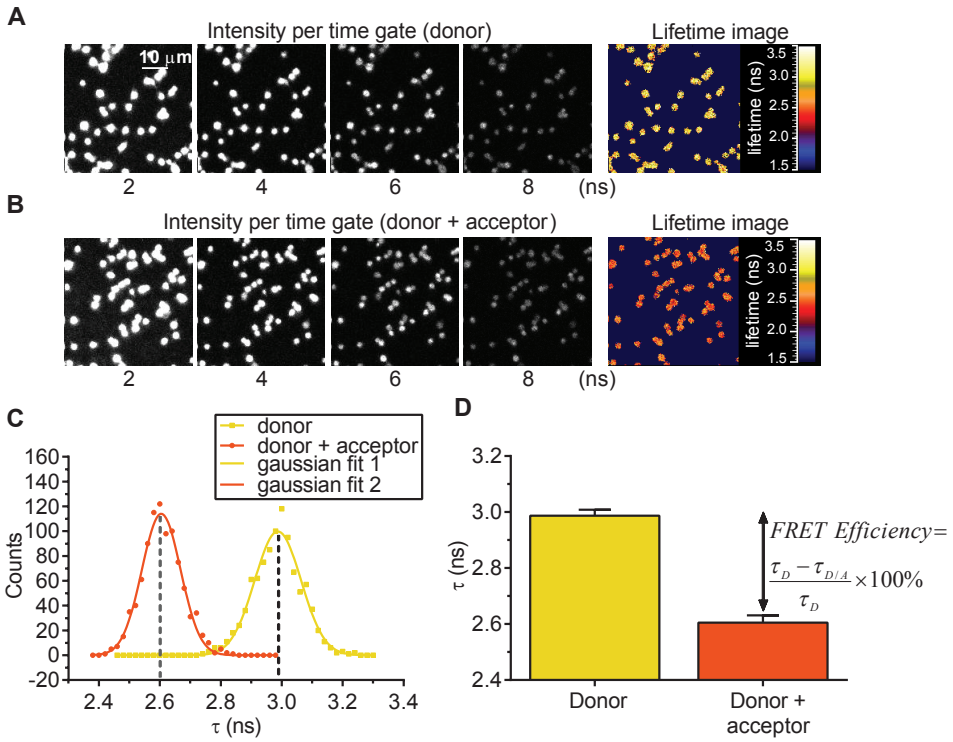


Figure S1. Analysis of FRET/FLIM data. Intensity images from the four consecutive 2 nanoseconds (ns) time gates and the lifetime image of platelets stored for 4h at 0°C labeled with 6B4-488 (donor) in the absence (**A**) and presence (**B**) of acceptor probe 6B4-594. (**C**) Determination of the average τ_{donor} value by Gaussian fitting. (**D**) The average donor lifetime τ ± SEM (n=6) in the absence or presence of acceptor for >50 platelets per measurement and the formula for calculating FRET efficiency.

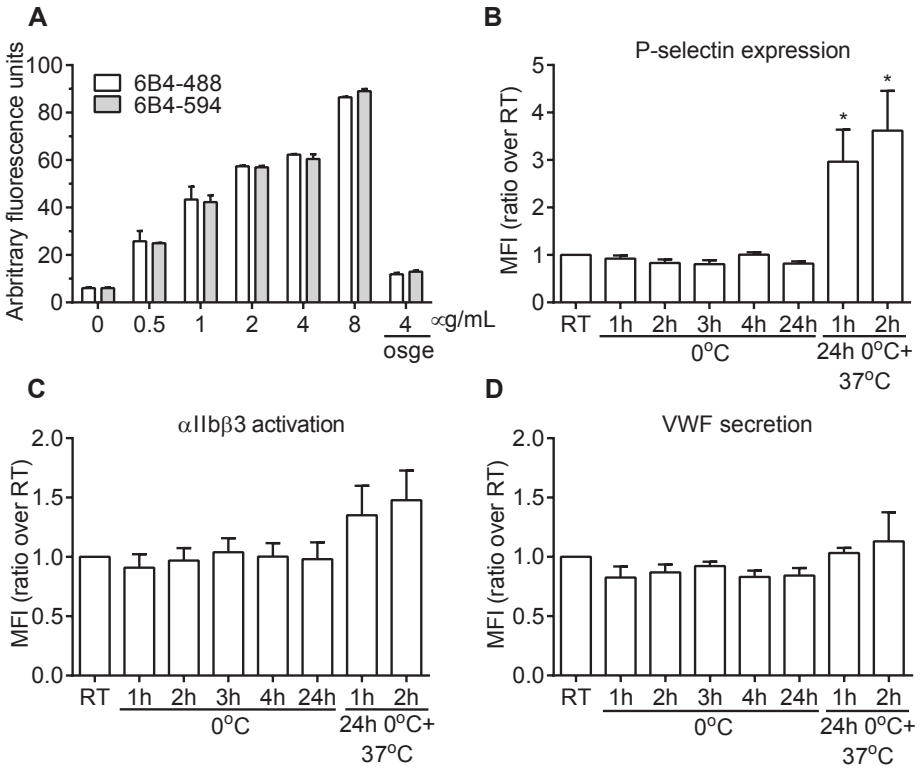


Figure S2. Binding of 6B4-Fab fragments to platelets and activation during cold storage. (**A**) Platelets were incubated with indicated concentrations of Alexa Fluor-488 and -594 conjugated 6B4-Fabs (6B4-488 and 6B4-594, respectively). Unbound 6B4-Fab fragments were removed and the fluorescence intensities were determined. To demonstrate specific binding to GPIbα, platelets were preincubated with 80 µg/mL O-sialoglycoprotein endopeptidase (osge; 30 minutes, 37°C), which selectively cleaves the *N*-terminal part of GPIbα, and again analyzed for 6B4-Fab binding. (**B-D**) Flow cytometric analysis of (**B**) P-selectin expression, (**C**) activation of αIIbβ3 and (**D**) surface-bound VWF during cold storage of platelets and subsequent rewarming. (B-D) Data statistically compared to RT platelets.

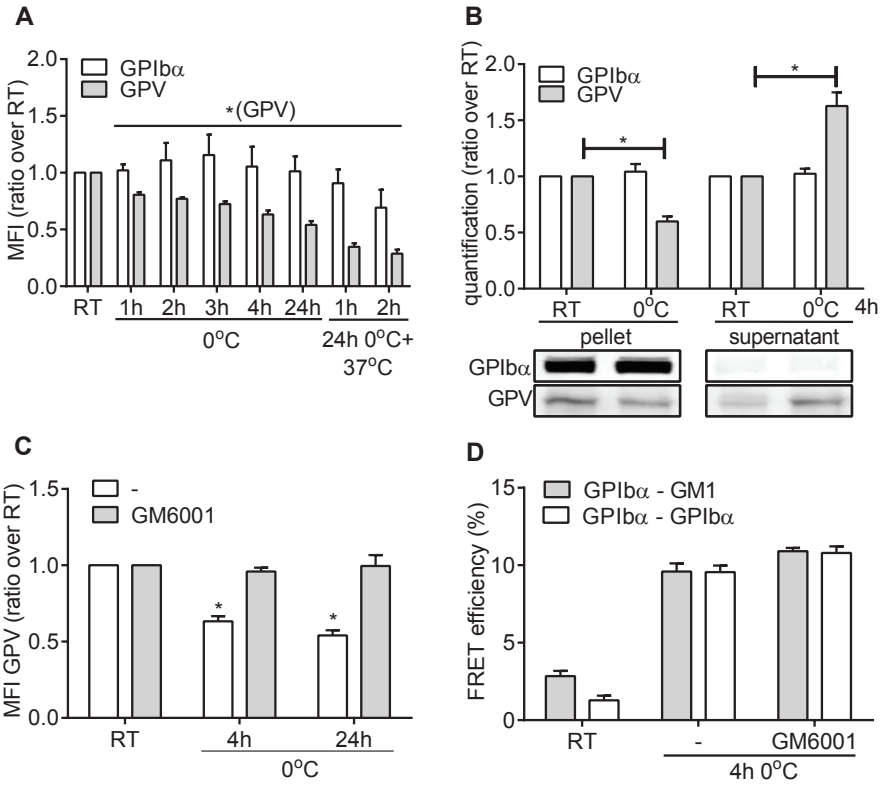


Figure S3. Surface expression of GPIbα and GPV during cold storage of platelets. (**A**) Flow cytometric analysis of surface expression of GPIbα (open bars) and GPV (grey bars) during cold storage and rewarming of platelets at indicated times. (**B**) Assessment of surface expression and shedding of GPIbα and GPV by immunoprecipitation. Platelets were kept at RT or stored for 4h at 0°C, subsequently fixed and collected by centrifugation. In pellet and supernatant the presence of GPIbα and GPV was measured. (**C**) Shedding of GPV is prevented by the broad-spectrum matrix metalloproteinase inhibitor GM6001. Platelets were stored for 4 and 24h at 0°C in the presence of DMSO (control; open bars) or 100 μM GM6001 (grey bars). (**D**) GPV shedding does not change [GPIbα-GM1] (grey bars) and [GPIbα-GPIbα] (open bars) associations, analyzed by FRET-FLIM. Pre-incubation with 100 μM GM6001 does not prevent the cold-induced redistribution of GPIbα. (A-C) Data are expressed as the ratio of treated platelets over RT platelets.

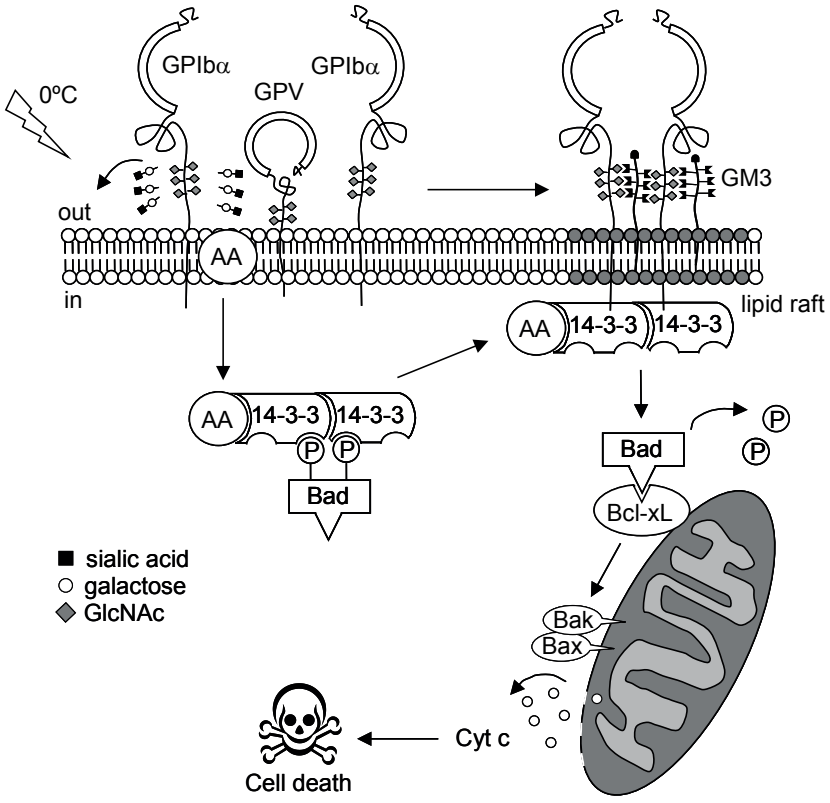


Figure S4. Schematic representation of cold-induced GPIbα clustering in lipid rafts. (1) Cold triggers the removal of sialic acid and galactose from glycans on GPIbα leading to exposure of GlcNAc residues. (2) The GlcNAc residues associate with raft component GM3, mediating GPIbα sequestration in lipid rafts, and surface expression of GPV is reduced. (3) In parallel, AA is released from membrane phospholipids and transfers 14-3-3ζ to the GPIbα cytoplasmic tail inducing clustering. (4) Association of 14-3-3ζ to GPIbα lowers 14-3-3ζ bound to pro-apoptotic Bad, resulting in dephosphorylation/activation. (5) Bad displaces Bak and Bax from pro-survival Bcl-xL, thereby activating permeabilization of the mitochondrial membrane, Cytochrome C (Cyt c) release and apoptosis.

Supplementary References

1. Fontayne A, Vanhoorelbeke K, Pareyn I et al. Rational humanization of the powerful antithrombotic anti-GPIbalpha antibody: 6B4. *Thromb Haemost*. 2006;96(5):671-684.
2. Korporaal SJ, Van EM, Adelmeijer J et al. Platelet activation by oxidized low density lipoprotein is mediated by CD36 and scavenger receptor-A. *Arterioscler Thromb Vasc Biol*. 2007;27(11):2476-2483.
3. Yoshida N, Aoki N. Release of arachidonic acid from human platelets. A key role for the potentiation of platelet aggregability in normal subjects as well as in those with nephrotic syndrome. *Blood*. 1978;52(5):969-977.
4. Ramesha CS, Taylor LA. Measurement of arachidonic acid release from human polymorphonuclear neutrophils and platelets: comparison between gas chromatographic and radiometric assays. *Anal Biochem*. 1991;192(1):173-180.
5. Surya II, Gorter G, Akkerman JW. Arachidonate transfer between platelets and lipoproteins. *Thromb Haemost*. 1992;68(6):719-726.
6. van der Wal DE, Gitz E, DU VX et al. Arachidonic acid depletion extends survival of cold-stored platelets by interfering with the [glycoprotein Ibalpha - 14-3-3zeta] association. *Haematologica*. 2012;97(10):1514-1522.
7. van der Wal DE, DU VX, Lo KS, Rasmussen JT, Verhoef S, Akkerman JW. Platelet apoptosis by cold-induced glycoprotein Ibalpha clustering. *J Thromb Haemost*. 2010;8(11):2554-2562.
8. Leytin V, Allen DJ, Mykhaylov S et al. Pathologic high shear stress induces apoptosis events in human platelets. *Biochem Biophys Res Commun*. 2004;320(2):303-310.
9. Albanyan AM, Harrison P, Murphy MF. Markers of platelet activation and apoptosis during storage of apheresis- and buffy coat-derived platelet concentrates for 7 days. *Transfusion*. 2009;49(1):108-117.
10. Hofman EG, Ruonala MO, Bader AN et al. EGF induces coalescence of different lipid rafts. *J Cell Sci*. 2008;121(Pt 15):2519-2528.
11. Wallrabe H, Periasamy A. Imaging protein molecules using FRET and FLIM microscopy. *Curr Opin Biotechnol*. 2005;16(1):19-27.
12. de Grauw CJ, Gerritsen HC. Multiple time-gate module for fluorescence lifetime imaging. *Applied Spectroscopy*. 2001;55(6):670-678.

CHAPTER 4

Platelet interaction with von Willebrand factor is enhanced by shear-induced clustering of glycoprotein Ibα

Eelo Gitz[1]
Charlotte D. Koopman[1]
Alèkos Giannas[1]
Cornelis A. Koekman[1]
Dave J. van den Heuvel[2]
Hans Deckmyn[3]
Jan-Willem N. Akkerman[1]
Hans C. Gerritsen[2]
Rolf T. Urbanus[1]

[1]Department of Clinical Chemistry and Haematology, University Medical Center Utrecht, Utrecht, the Netherlands
[2]Department of Molecular Biophysics, Utrecht University, Utrecht, the Netherlands
[3]Laboratory for Thrombosis Research, KU Leuven, Kortrijk, Belgium

Submitted for publication

Abstract

Initial platelet arrest at the exposed arterial vessel wall is mediated through glycoprotein Ibα binding to the A1 domain of von Willebrand factor. This interaction occurs at sites of elevated shear force, and strengthens upon increasing hydrodynamic drag. The increased interaction requires shear-dependent exposure of the von Willebrand factor A1 domain, but the contribution of glycoprotein Ibα remains ill defined. We have previously found that glycoprotein Ibα forms clusters upon platelet cooling and hypothesized that such a property enhances the interaction with von Willebrand factor under physiological conditions. We analyzed the distribution of glycoprotein Ibα with Förster resonance energy transfer using time-gated fluorescence lifetime imaging microscopy. Perfusion at a shear rate of 1,600 s^{-1} induced glycoprotein Ibα clusters on platelets adhered to von Willebrand factor, while clustering did not require von Willebrand factor contact at 10,000 s^{-1}. Shear-induced clustering was reversible, not accompanied by granule release or αIIbβ3 activation and improved glycoprotein Ibα-dependent platelet interaction with von Willebrand factor. Clustering required glycoprotein Ibα translocation to lipid rafts and critically depended on arachidonic acid-mediated binding of 14-3-3ζ to its cytoplasmic tail. This newly identified mechanism emphasizes the ability of platelets to respond to mechanical force and provides new insights in how changes in hemodynamics influence arterial thrombus formation.

INTRODUCTION

Platelet adhesion to subendothelial matrices in the damaged vessel wall is the prime event in the arrest of bleeding. Recruitment of platelets to sites of vascular injury is hampered by the rapid flow of blood in arteries and arterioles. In these vessels, the interaction between von Willebrand factor (VWF), a multimeric plasma glycoprotein, and the platelet glycoprotein (GP) Ib-IX-V receptor complex is critical for initial platelet adhesion.[1] This interaction requires unfolding of the VWF A1 domain and allows platelets to decelerate until they firmly attach in a process assisted by platelet integrins.[1;2] Defects in both the GPIb-IX-V complex (Bernard Soulier Syndrome) and VWF (von Willebrand disease; VWD) result in a bleeding diathesis, which underscores the importance of this interaction in hemostasis.[3;4]

The GPIb-IX-V complex consists of four transmembrane subunits; GPIbα, GPIbβ, GPIX and GPV that are expressed in a 2:4:2:1 stoichiometry.[5] Each platelet contains approximately 25,000 copies of GPIbα, the subunit that binds to the VWF A1 domain.[6] The extracellular domain (residues 1-485) of GPIbα consists of an N-terminal flank, seven leucine-rich repeats, a C-terminal flank, a sulphated region and a highly glycosylated macroglycopeptide domain. Residues 486-514 form the transmembrane domain and the cytoplasmic tail consists of 96 amino acid residues (residues 515-610),[7-9] which contain binding sites for multiple intracellular proteins, including filamin A[10] and the adaptor protein 14-3-3ζ.[11] The region that interacts with the VWF A1 domain resides within the concave face of the leucine-rich repeat domain of GPIbα.[12;13] Despite the fundamental importance in initiating platelet adhesion, the molecular mechanism regulating the VWF-GPIbα interaction remains incompletely understood.

Binding of VWF to GPIbα requires the dynamic conditions of flowing blood. The unique biomechanical properties of VWF and GPIbα allow the interaction to strengthen upon increasing hemodynamic drag.[14] An explanation for this counterintuitive finding is that VWF needs to change its conformation to allow GPIbα access to the A1 domain. Elevated shear force and immobilization on a surface trigger this conformational change in vivo, a process mimicked by the antibiotic ristocetin in vitro.[15] The interaction between VWF and GPIbα is also regulated through changes in GPIbα. Its adhesive properties depend on translocation to cholesterol-rich membrane domains known as lipid rafts[16;17], which may increase the local density of GPIbα receptors and stimulate their signaling properties. Indeed, studies with Chinese Hamster Ovary (CHO) cells in which GPIbα was artificially dimerized have suggested that receptor clustering increases the overall strength of the VWF-GPIbα interaction.[18;19]

In an effort to optimize the storage conditions of platelet concentrates used for transfusion, we have recently demonstrated that GPIbα clusters in lipid rafts when platelets are kept at low temperature.[20] Analysis of Förster resonance energy transfer (FRET) by fluorescence lifetime imaging microscopy (FLIM) revealed that cooling of platelets triggers [GPIbα-GPIbα] associations in lipid rafts within a range of 1-10 nm. In the present study, we assessed whether clustering of GPIbα occurs under physiological conditions, investigated its influence on VWF interaction and identified the responsible molecular mechanism.

Materials and Methods

Patient
Citrated blood (10.9 mM f.c.) was obtained from a patient with von Willebrand disease (VWD) type 3. Permission was obtained from the local medical ethics committee. The patient had no detectable plasma VWF (<0.1%), 1% plasma factor VIII, <1% factor VIII activity, no ristocetin-induced platelet aggregation, and normal platelet count and volume.[21]

Materials, antibodies, platelet preparation and incubations
A detailed description can be found in the Supplementary Materials and Methods.

Platelet adhesion and rolling under flow conditions
A parallel plate perfusion chamber[22] was used to investigate platelet adhesion and rolling. Further details are found in the Supplementary Materials and Methods.

Exposure to shear force
Platelets were exposed to shear force by perfusion through a microcapillary (inner diameter 760 μm, blocked with 4% BSA). Washed platelets were resuspended in HT buffer ($2.5x10^{11}$ cells/L, pH 7.3) supplemented with 4% human albumin. Platelet suspensions were prewarmed to 37°C for 5 minutes and perfused through the microcapillary at indicated shear rates for 5 seconds. The length of the microcapillaries was matched with the shear rate, which means that the platelet suspensions have similar shear exposure times at different shear rates. Indicated shear rates are the maximal shear rates to which platelets are exposed near the wall of the microcapillary. The wall shear rate (γ_w) inside a microcapillary is described as

$$wall\ shear\ rate\ (\gamma_w) = \frac{4Q}{\pi r^3}$$

Where Q is the volumetric flow rate and r is the inner radius of the microcapillary.

Agglutination
Platelet agglutination was measured in a Chrono-log Lumi-Aggregometer (model 700, Chrono-log Corporation, Haverton, PA) with Aggrolink 8.0 software. Washed platelets in HT buffer were pre-incubated with prostacyclin (PGI_2) analog iloprost and dRGDW (5 minutes, 37°C) and stimulated with VWF (10 μg/mL) and ristocetin (0.3 mg/mL) with stirring (900 r.p.m.). Data are expressed as percentage of maximal agglutination, with light transmission through HT buffer set at 100%.

Flow cytometric analysis, immunoprecipitations and western blots
A detailed description can be found in the Supplementary Materials and Methods.

Analysis of GPIbα distribution by FRET/FLIM
GPIbα distribution was analyzed by FRET/FLIM as described.[20] In short, 6B4-Fab fragments conjugated to either Alexa Fluor-488 or Alexa Fluor-594 (6B4-488 and 6B4-594 respectively) were incubated with fixed platelet samples under conditions in which each Fab labeled ~50% of total receptor number. GPIbα translocation to lipid rafts was determined by labeling GPIbα with 6B4-488 and monosialo-tetrahexosylganglioside (GM1) with Cholera toxin subunit B conjugated to Alexa Fluor-594 (CTB-594; 5 μg/mL). The fluorescence lifetimes of the donor fluorophore (6B4-488) were determined in the absence and presence of acceptor fluorophore

(6B4-594 or CTB-594) and used to calculate the FRET efficiency, defined as

$$FRET\ Efficiency = \frac{\tau_D - \tau_{D/A}}{\tau_D} \times 100\%$$

where τ is the donor fluorophore lifetime in nanoseconds in the absence (τ_D) and presence ($\tau_{D/A}$) of the acceptor fluorophore. To determine variation in FRET efficiency, the lifetimes of three randomly chosen quadrants were quantified.

Statistical analysis

Data are means ± SEM. Statistical analysis was based on GraphPad Prism 5 (San Diego, CA). Differences between control platelets and incubations were analyzed by Mann-Whitney test. *P*-values less than 0.05 (* or *) and between incubations (|–*–|) were considered significant.

RESULTS

Platelet adhesion to VWF under conditions of flow triggers GPIbα clustering

Platelet adhesion at shear rates above 1,000 s^{-1} depends on the interaction between surface-bound VWF and GPIbα.[1] We analyzed the effect of this interaction on the spatial distribution of GPIbα on the platelet plasma membrane with FRET/FLIM. Whereas GPIbα molecules were dispersed in resting platelets (Figure 1 A-B), indicated by a FRET efficiency of 0.9 ± 0.2%, GPIbα clustered upon adhesion to VWF (FRET efficiency 10.3 ± 0.9%). Clustering was not caused by close contact between adjacent platelets, as FRET efficiency did not differ between single platelets and platelets that adhered as small aggregates (Supplementary Figure S1). As platelet-VWF interaction is influenced by flow conditions, we analyzed GPIbα distribution of platelets adhered to VWF at different shear rates. Adhesion to VWF at 300 s^{-1} left GPIbα dispersed, but perfusion at 750 s^{-1} and higher induced clustering (Figure 1C). To investigate whether changes in GPIbα distribution were specific for adhesion to VWF, platelets were perfused over collagen. Adhesion to collagen at a low shear rate (300 s^{-1}) in the presence or absence of VWF resulted in FRET efficiencies similar to that observed in resting platelets (Figure 1D). Perfusion over collagen at 1,600 s^{-1} in the absence of VWF had little effect on GPIbα distribution. In contrast, addition of VWF prior to perfusion at 1,600 s^{-1} increased FRET efficiency to 8.3 ± 0.6%, indicating that clustering of GPIbα requires the presence of VWF.

Exposure to high shear leads to reversible VWF-independent GPIbα clustering

The change in GPIbα distribution measured on surface-attached platelets might be the result of shear, of rolling/attachment or both. To understand the contribution of shear, we perfused platelets in VWF-free buffer through a microcapillary tube at different shear rates in the absence of an adhesive surface. FRET/FLIM analysis showed that a shear rate of 300 s^{-1} left GPIbα dispersed. Exposure to 1,600 s^{-1} had a minor effect on GPIbα distribution, whereas a shear rate of 10,000 s^{-1} increased FRET efficiency to 9.1 ± 0.6% (Figure 2A). Platelet α-granules also contain VWF,[23] which might be released during platelet isolation and therefore influence GPIbα clustering. To address this possibility, experiments were repeated with platelets from a patient with VWD type 3 who had undetectable levels of VWF. FRET efficiency of VWF-deficient platelets increased to similar levels as control platelets, indicating

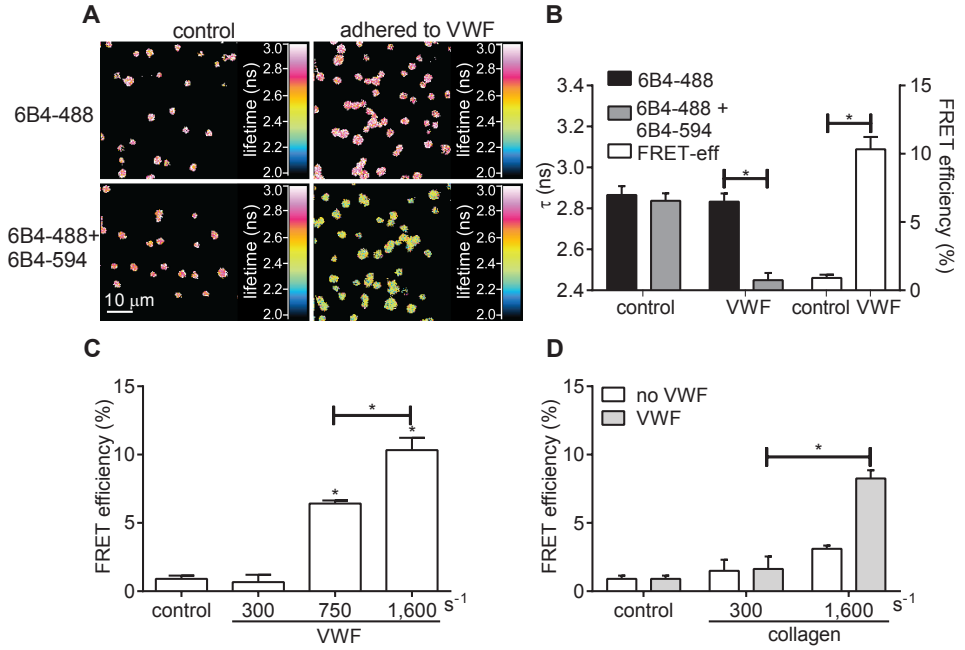


Figure 1. Platelet adhesion to VWF induces clustering of GPIbα. (**A**) Freshly isolated resting platelets (control; left panels) or platelets adhered to VWF after whole blood perfusion (1 minute, 37°C) at a shear rate of 1,600 s^{-1} (right panels) were analyzed for GPIbα distribution by FRET/FLIM. Platelets were fixed with 2% paraformaldehyde and stained with 1 μg/mL 6B4-488 (donor) in the absence (top panels) and presence of 1 μg/mL 6B4-594 (acceptor; bottom panels). The fluorescence lifetimes in nanoseconds (ns) are shown in false color images. (**B**) Quantification of fluorescence lifetime values of donor probe in the absence and presence of acceptor probe of platelets treated under the conditions of (A). Corresponding FRET efficiencies are calculated as described in Materials and Methods. (**C,D**) FRET/FLIM analysis of platelets adhered to VWF after whole blood perfusion (**C**) or to collagen after reconstituted blood perfusion (**D**) at indicated shear rates (1 minute, 37°C). Perfusion of reconstituted blood over collagen was performed in the absence (open bars) and presence (grey bars) of 10 μg/mL VWF (n=4).

that GPIbα clusters independent of the presence of VWF (Figure 2B). GPIbα clustering was reversible, as exposure to shear followed by incubations under static conditions resulted in a gradual decline to the range found in resting platelets (Figure 2C). Platelet exposure to a shear rate of 10,000 s^{-1} did not result in P-selectin expression, αIIbβ3 activation, or VWF binding (Figure 2D). Conversely, clustering was not induced by stimulation with thrombin receptor activating peptide (TRAP) or cross-linked collagen related peptide (CRP) under static conditions (Figure 2E). Transient GPIbα clustering did not affect the ability of platelets to respond to agonists, because stimulation with TRAP or CRP before and after exposure to shear resulted in similar levels of P-selectin expression and αIIbβ3 activation (Figure 2F).

Platelet interaction with VWF is stimulated by clustered GPIbα

To clarify whether changes in GPIbα distribution contributed to platelet responsiveness to VWF, agglutination was measured in platelets with shear-induced clustered GPIbα. VWF and a suboptimal concentration of ristocetin were used and aggregation was prevented by pre-incubation with iloprost, a stable analog of prostacyclin, and dRGDW. Neither agent affected

shear-induced GPIbα clustering (data not shown). Maximal agglutination of platelets with pre-clustered GPIbα was four-fold higher than with controls (Figure 3 A-B).

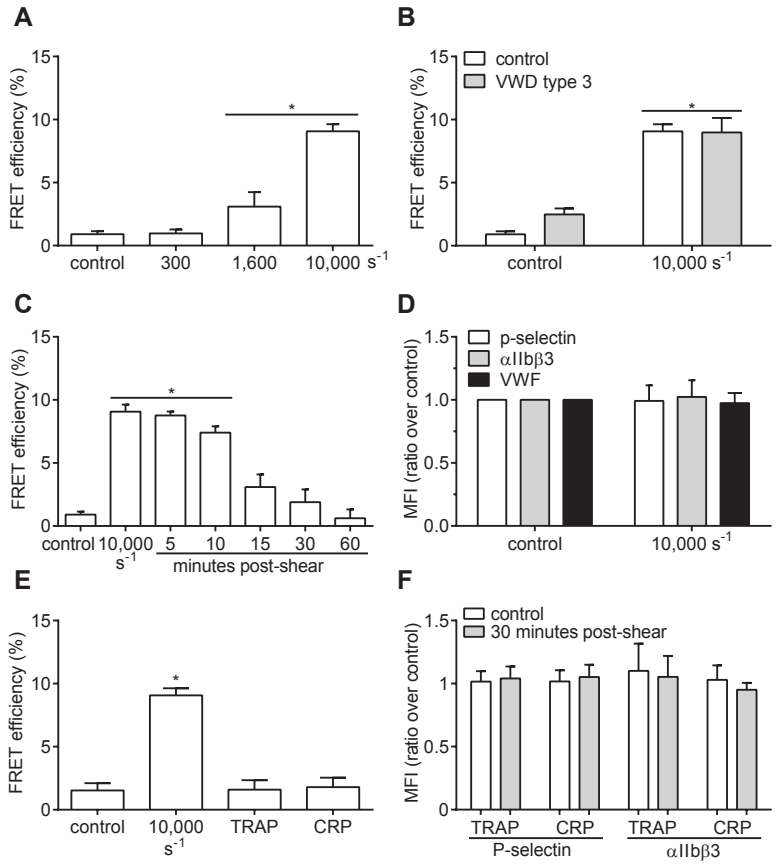


Figure 2. High shear force induces reversible GPIbα clustering in the absence of VWF. (**A**) Platelets resuspended in HT buffer (pH 7.3) supplemented with 4% albumin were exposed to indicated shear rates by perfusion (37°C) through a microcapillary tube for 5 seconds and analyzed for GPIbα distribution by FRET/FLIM. (**B**) Shear-induced GPIbα clustering occurs in the absence of VWF. Platelets from healthy donors and from a VWD type 3 patient were exposed to a shear rate of 10,000 s^{-1} and analyzed for FRET/FLIM. (**C**) Shear-induced GPIbα clustering is reversible. Platelets exposed to 10,000 s^{-1} were monitored for clustering at indicated time intervals post-shear, which after 10 minutes reduced to control levels. (**D**) Platelets were analyzed by FACS for expression of P-selectin, αIIbβ3 activation and VWF binding after exposure to shear. (**E**) FRET/FLIM analysis of platelets activated by TRAP-6 (20 μM) or CRP (1 μg/mL) for 5 minutes at 37°C in the absence of shear. (**F**) The effects of reversible GPIbα clustering on platelet responsiveness were analyzed by stimulating platelets with TRAP or CRP at 30 minutes post-shear. FACS analysis revealed that agonist-induced surface expression of P-selectin or αIIbβ3 activation was similar as control platelets. Data are presented as the ratio of mean fluorescence intensity (MFI) of treated platelets over control platelets (n=4).

VWF enables rolling of platelets over the damaged vessel wall until they firmly attach in an integrin-dependent manner. The effect of GPIbα clustering was measured by platelet perfusion over a VWF-coated surface in the presence of dRGDW to block αIIbβ3-mediated

attachment. Induction of GPIbα clustering prior to perfusion reduced the rolling velocity by 40% (Figure 3 C-D). These data show that GPIbα clustering facilitates platelet-VWF interaction.

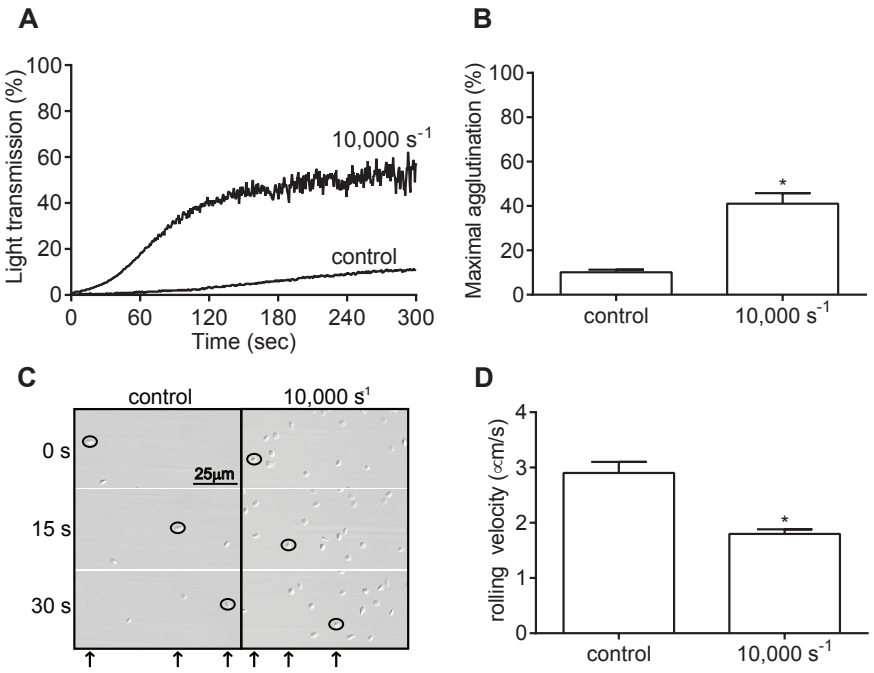


Figure 3. GPIbα clustering improves platelet interaction with VWF. Resting platelets (control) or platelets exposed to 10,000 s^{-1} were pre-incubated with dRGDW and iloprost for 5 minutes at 37°C. (**A,B**) Platelets were stimulated with suboptimal concentrations of ristocetin (0.3 mg/mL) and VWF (10 µg/mL) and agglutination (**A**) was measured at 37°C while stirring. (**B**) Quantification of mean maximal agglutination (n=3). (**C,D**) Analysis of platelet rolling velocity over VWF at a shear rate of 1,600 s^{-1}. (**C**) Single platelets (black circles) were tracked over distance in time from which the rolling velocity was determined. Black arrows indicate the location of a single platelet at 0, 15 and 30 s. (**D**) Quantification of rolling velocity in µm/s. Rolling velocity is significantly reduced after pre-exposure to a shear rate of 10,000 s^{-1} (n=5).

GPIbα translocates to lipid rafts and forms clusters through 14-3-3ζ binding

Platelet binding to VWF depends on reallocation of GPIbα in membrane domains enriched in sphingomyelin and cholesterol, known as lipid rafts.[16;17] To understand the role of raft allocation in GPIbα clustering, GPIbα was labeled with 6B4-488 (donor) and the raft marker GM1 with CTB conjugated to Alexa Fluor-594 (CTB-594; acceptor). The surface of resting platelets showed little co-localization but adhesion to VWF or exposure to high shear (10,000 s^{-1}) induced GPIbα-GPIbα as well as GPIbα-GM1 associations, suggesting that clustering and raft translocation go hand in hand (Figure 4A). Disruption of lipid rafts by cholesterol depletion with methyl-β-cyclodextrin (mβCD) effectively abrogated GPIbα clustering. The time-dependent decay of shear-induced GPIbα clusters closely followed raft translocation, again suggesting a tight interrelationship (Figure 4B).

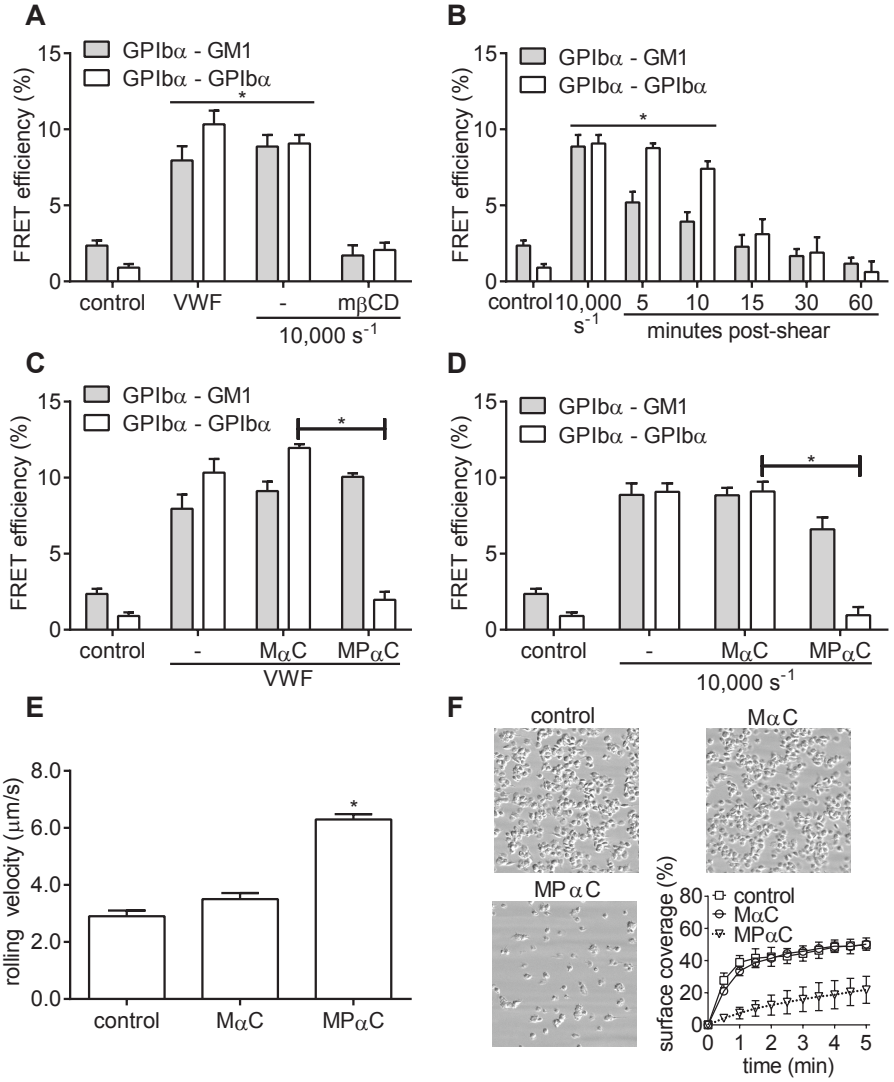


Figure 4. Clustering of GPIbα requires translocation to lipid rafts and 14-3-3ζ binding to its cytoplasmic tail. GPIbα translocation to lipid rafts was measured by labeling GPIbα with 6B4-488 (donor, 1 μg/mL) and raft-specific GM1 ganglioside with CTB-594 (acceptor; 5 μg/mL). (**A-D**) FRET/FLIM analysis of [GPIbα-GM1] and [GPIbα-GPIbα] associations. (**A**) GPIbα distribution of platelets adhered to VWF by whole blood perfusion at 1,600 s^{-1} (VWF) or washed platelets perfused through a microcapillary at 10,000 s^{-1}. Disruption of lipid rafts by cholesterol depletion with mβCD (10 mM, 30 minutes at 37°C) prior to perfusion prevented changes in GPIbα distribution. (**B**) GPIbα translocation to lipid rafts induced by shear force is reversible after 10 minutes. (**C,D**) GPIbα clustering requires 14-3-3ζ binding to its cytoplasmic tail. Binding of 14-3-3ζ to GPIbα was prevented by pre-incubation with the cell-permeable peptide MPαC (100 μM, 5 minutes at 37°C). FRET/FLIM analysis of platelets bound to VWF by perfusion (**C**) or exposed to shear (**D**) in the presence of control peptide MαC or MPαC. (E,F) Platelet interaction with VWF is impaired by prevention of 14-3-3ζ binding to GPIbα. Analysis of platelet rolling velocity over VWF at 1,600 s^{-1} (**E**) and quantification of platelet surface coverage during whole blood perfusion (**F**) in the presence of MαC or MPαC. Snapshots were made after 5 minutes perfusion (n=4).

Binding of the adaptor protein 14-3-3ζ to the cytoplasmic tail of GPIbα is essential for platelet interaction with VWF.[24;25] To assess the role of 14-3-3ζ in GPIbα clustering, we pre-incubated platelets with MPαC. This membrane permeable peptide represents the critical 14-3-3ζ binding site on GPIbα, which includes the constitutively phosphorylated Ser-609 residue on its cytoplasmic tail.[24;26;27] As expected, the peptide prevented VWF-induced 14-3-3ζ binding to GPIbα (Supplementary Figure S2). Figure 4C shows that pre-incubation with MPαC had little effect on lipid raft translocation induced by platelet adhesion to VWF, but completely abrogated clustering of GPIbα. Control peptide MαC, which lacks phosphorylation at Ser-609, had no effect on GPIbα redistribution. FRET/FLIM analysis of platelets exposed to a shear rate of 10,000 s^{-1} provided similar results (Figure 4D), demonstrating that 14-3-3ζ binding to the cytoplasmic tail of GPIbα is essential for its clustering. To elucidate the importance of 14-3-3ζ-induced clustering, we determined the rolling velocity of platelets perfused over VWF in the presence of MPαC (Figure 4E). While control peptide MαC had no effect, rolling velocity of platelets pre-incubated with MPαC increased more than twofold. Moreover, inhibition of 14-3-3ζ-induced GPIbα clustering impaired stable adhesion to VWF during whole blood perfusion (Figure 4F).

Arachidonic acid mediates 14-3-3ζ-induced GPIbα clustering

Platelet interaction with VWF or incubation at low temperature activates the stress kinase P38-mitogen-activated protein kinase (P38MAPK), which liberates arachidonic acid (AA) from membrane phospholipids through cytosolic phospholipase A_2 ($cPLA_2$).[20;28,29] Incubations with inhibitors at 37°C indicated that P38MAPK-mediated AA release might support GPIbα-GPIbα interactions during exposure to shear. The P38MAPK inhibitor SB203580 and the $cPLA_2$ inhibitor $AACOCF_3$ inhibited the rise in FRET efficiency induced by high shear. The low FRET efficiency observed under static conditions increased 12-fold upon addition of AA. The intracellular accumulation of free AA might therefore contribute to GPIbα clustering (Figure 5A). The FRET efficiency of shear-treated platelets decreased upon subsequent incubations under static conditions (Figure 2C). In platelets, liberated AA is metabolized by cyclooxygenase-1 and lipoxygenase to thromboxane A_2 (TxA_2) and other eicosanoids, which could account for the reversibility of shear-induced GPIbα clustering. Indeed, accumulation of AA by inhibition of these enzymes with indomethacin and 5, 8, 11-eicosatriynoic acid (ETI) prevented GPIbα clusters from dispersing after exposure to high shear (Figure 5B). Control studies confirmed that SB203580 blocked shear-induced P38MAPK phosphorylation/activation whereas the other treatments left the enzyme undisturbed (Figure 5C). Treatments that inhibited AA release prevented shear-induced 14-3-3ζ-GPIbα association and blockade of AA degradation and the separate addition of AA preserved the complex (Figure 5D). These data indicate that liberated AA binds 14-3-3ζ and facilitates its translocation to the GPIbα cytosolic tail.[20;28]

The inhibitors of AA release and degradation affected platelet rolling velocity over a VWF surface. Inhibition of AA release induced by shear increased the rolling velocity, whereas treatments that preserved accumulation of AA resulted in reduced velocities (Figure 5E). These data indicate that the AA-mediated transfer of 14-3-3ζ to the GPIbα cytoplasmic tail induces GPIbα clustering which supports platelet interaction with VWF at high shear.

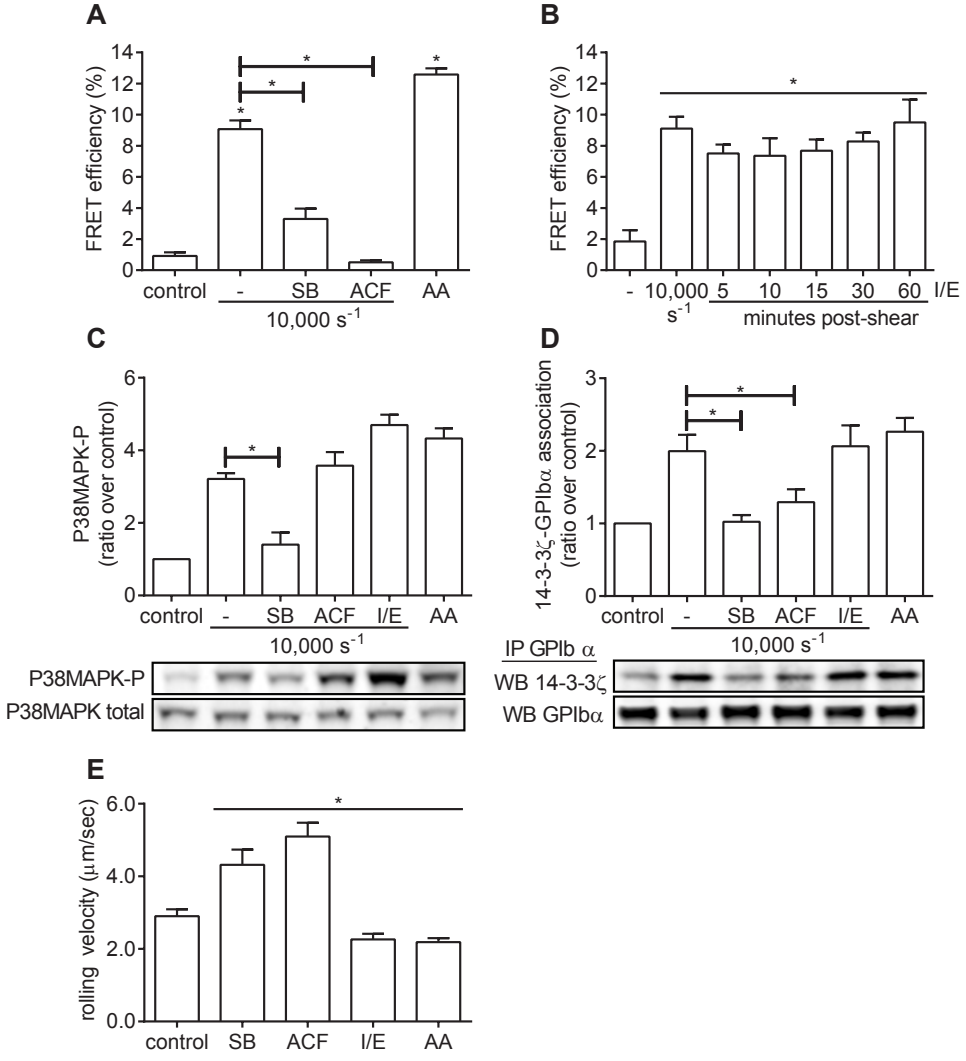


Figure 5. Binding of 14-3-3ζ to GPIbα is regulated by AA. (**A**) Clustering of GPIbα is prevented by inhibitors of upstream regulators of intracellular AA release. Pre-incubation (10 minutes at 37°C) with P38MAPK38 inhibitor SB203580 (10 μM) or $cPLA_2$ inhibitor $AACOCF_3$ (ACF; 20 μM) abolished shear-induced clustering of GPIbα. Platelet incubation with 10 μM AA (5 minutes at 37°C) in the presence of TPα receptor antagonist SQ30741 (25 μM) induced GPIbα clustering. (**B**) Shear-induced clustering of GPIbα is maintained in the presence of inhibitors of AA metabolism. Inhibitors were against cyclooxygenase-1 (indomethacin; 30 μM) and lipoxygenase (ETI; 25 μM), abbreviated as I/E. (**C**,**D**) Analysis of shear-induced phosphorylation of P38MAPK (**C**) and [14-3-3ζ-GPIbα] complex formation (**D**) under conditions described for A and B. (**E**) Platelet rolling velocity increases in the presence of SB203580 and AACOCF3 and is reduced in the presence of AA metabolism inhibitors (I/E) or exogenous AA (n=4).

DISCUSSION

Our results demonstrate that platelet exposure to high shear leads to clustering of GPIbα and enhances the interaction with VWF. Shear-induced clustering is reversible and not associated

with granule release or activation of αIIbβ3. Clustering requires lipid raft translocation and critically depends on AA-mediated 14-3-3ζ binding to the cytoplasmic tail of GPIbα (Figure 6).

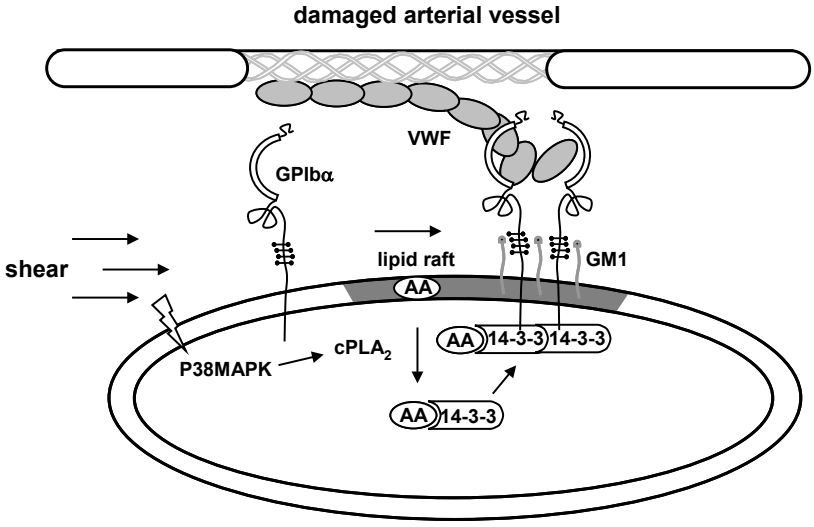


Figure 6. Schematic representation of initial platelet adhesion to VWF at the damaged arterial vessel wall. Shear triggers GPIbα translocation to lipid rafts and activates P38MAPK. This stress kinase subsequently activates $cPLA_2$, which liberates AA from membrane phospholipids. GPIbα clusters upon AA-mediated 14-3-3ζ binding to its cytoplasmic tail, leading to enhanced interaction with VWF.

Previous studies reported a role for receptor clustering in GPIbα-VWF interaction.[18;19] Experiments with CHO cells showed that intracellular dimerization of a modified GPIbα construct increases the overall bond strength with VWF. We found that GPIbα formed clusters in platelets adhered to VWF after perfusion at a shear rate of 750 s^{-1} or higher. At these shear rates, platelet adhesion strongly depends on the interaction between GPIbα and VWF, because only this interaction is sufficiently fast and strong to withstand the associated hemodynamic drag.[1] Our results indicate that GPIbα clustering contributes to GPIbα-mediated platelet adhesion to VWF, as inhibition of clustering strongly attenuated both platelet rolling velocity and adhesion.

Initial adhesion to VWF increases the hemodynamic drag on platelets substantially and results in the formation of membrane tethers that are pulled from the cell surface.[30;31] Upon adhesion to VWF, clustering was observed at a shear rate of 750 s^{-1} or higher. In the absence of an adhesive surface, GPIbα clustering required exposure to 10,000 s^{-1}, a shear rate found in stenotic arteries. By definition, only those platelets nearest to the vessel wall are subjected to this shear rate. Therefore, shear exposure is probably limited to part of the platelet population perfused through a microcapillary. Nevertheless, this short exposure to shear stress induced clustering of GPIbα, illustrating the high sensitivity of platelets for mechanical stress. The combined force of shear exposure and tensile stress exerted on GPIbα when bound to VWF apparently cooperate in facilitating GPIbα clustering. Shear-induced clustering did not coincide with platelet activation, which is in line with earlier reports of VWF-dependent adhesion[32] or aggregation of discoid platelets[33] at high shear rates.

The effects of clustering on the interaction with VWF probably reflect avidity modulation, where an increased local density of GPIbα molecules increases the number of ligand-receptor bonds. Based on crystal structure studies, it is less likely that GPIbα clustering facilitates binding of two GPIbα molecules to a single A1 domain.[12;34] Under the influence of elevated hemodynamic drag, VWF-bound GPIbα can subsequently undergo a conformational change that further strengthens the interaction.[14]

Disruption of lipid rafts by cholesterol depletion strongly impairs platelet adhesion to VWF under conditions of flow, indicating that GPIbα localization to these regions is essential for its function.[16] Lipid rafts are viewed as platforms that can physically concentrate receptors, adaptor proteins and effector enzymes, which lead to amplification of signaling events. We observed little GPIbα localization in rafts on the surface of resting platelets, which increased about threefold upon ligation to immobilized VWF or exposure to high shear in solution. Disruption of rafts prevented GPIbα from clustering. Although translocation was essential for this process, clustering depended critically on the interaction between 14-3-3ζ and GPIbα. The importance of the [14-3-3ζ-GPIbα] association for the interaction of platelets with VWF is well established,[24;25] but the exact mode of action remains poorly defined. It has been suggested that this association participates in αIIbβ3 integrin activation in GPIb-IX-expressing CHO cells.[35;36] We show that inhibition of 14-3-3ζ binding to GPIbα impaired adhesion to VWF and increased rolling velocity. The presence of iloprost and dRGDW excluded involvement of αIIbβ3 integrin in platelet rolling on VWF. Together, these data indicate that 14-3-3ζ association with GPIbα directly improves platelet interaction with VWF by allowing receptor clustering. The dimeric nature of 14-3-3ζ supports this finding.[37] Indeed, a similar mechanism has been described in muscle cells, where clustering of the acetylcholine receptor depends on 14-3-3ζ.[38]

P38MAPK is a kinase that is responsive to stress stimuli, including alterations in thermal[28] and shear conditions.[39] We found that platelet exposure to shear in solution leads to P38MAPK phosphorylation. Phosphorylated P38MAPK subsequently activates $cPLA_2$ to release AA from membrane phospholipids.[40] Our study reveals a central role for AA in GPIbα clustering. Inhibition of AA release during exposure to shear prevented the transfer of 14-3-3ζ to the cytoplasmic tail of GPIbα. Moreover, addition of AA enhanced clustering and inhibition of AA metabolism resulted in irreversible clustering. The findings that AA binding to 14-3-3ζ induces 14-3-3ζ multimerization,[41] and that AA-bound 14-3-3ζ directly associates with GPIbα.[28] both support the concept that the adaptor protein provides a platform for GPIbα clustering. In addition, lipid rafts are enriched in AA,[42] which may explain the dependence of GPIbα cluster formation on these membrane domains. Rolling experiments established the importance of AA-mediated GPIbα clustering, as inhibitors of AA release reduced platelet interaction with VWF, while its accumulation enhanced this initial step in adhesion.

Aspirin, a widely used antithombotic drug, also interferes with AA conversion by inhibiting COX-1 activity. Our studies suggest that the use of aspirin may prolong the presence of GPIbα clusters, which contradicts with the antithrombotic effects of aspirin use. However, the inhibitory effects of aspirin are attributed to inhibition of TxA_2-enhanced platelet activation,[43] which is important for more advanced steps in thrombus formation. Interestingly, several studies have demonstrated that GPIbα-dependent platelet adhesion actually increases upon aspirin intake.[44;45] These unexplained findings may be the result of aspirin-enhanced GPIbα clustering.

In conclusion, we define a central role for GPIbα clustering in platelet interaction with VWF under conditions of flow. Clustering of GPIbα requires translocation to lipid rafts and AA-mediated 14-3-3ζ binding to its cytoplasmic tail. These findings illustrate the mechanosensitive properties of platelets and give a new perspective on the molecular mechanism of arterial thrombus formation.

REFERENCES

1. Savage B, Saldivar E, Ruggeri ZM. Initiation of platelet adhesion by arrest onto fibrinogen or translocation on von Willebrand factor. *Cell*. 1996;84(2):289-297.
2. Schneider SW, Nuschele S, Wixforth A et al. Shear-induced unfolding triggers adhesion of von Willebrand factor fibers. *Proc Natl Acad Sci U S A*. 2007;104(19):7899-7903.
3. Li C, Martin SE, Roth GJ. The genetic defect in two well-studied cases of Bernard-Soulier syndrome: a point mutation in the fifth leucine-rich repeat of platelet glycoprotein Ib alpha. *Blood*. 1995;86(10):3805-3814.
4. Weiss HJ, Rogers J, Brand H. Defective ristocetin-induced platelet aggregation in von Willebrand's disease and its correction by factor VIII. *J Clin Invest*. 1973;52(11):2697-2707.
5. Luo SZ, Mo X, fshar-Kharghan V, Srinivasan S, Lopez JA, Li R. Glycoprotein Ibalpha forms disulfide bonds with 2 glycoprotein Ibbeta subunits in the resting platelet. *Blood*. 2007;109(2):603-609.
6. Du X, Beutler L, Ruan C, Castaldi PA, Berndt MC. Glycoprotein Ib and glycoprotein IX are fully complexed in the intact platelet membrane. *Blood*. 1987;69(5):1524-1527.
7. Dong JF, Li CQ, Lopez JA. Tyrosine sulfation of the glycoprotein Ib-IX complex: identification of sulfated residues and effect on ligand binding. *Biochemistry*. 1994;33(46):13946-13953.
8. Lopez JA, Chung DW, Fujikawa K, Hagen FS, Papayannopoulou T, Roth GJ. Cloning of the alpha chain of human platelet glycoprotein Ib: a transmembrane protein with homology to leucine-rich alpha 2-glycoprotein. *Proc Natl Acad Sci U S A*. 1987;84(16):5615-5619.
9. Korrel SA, Clemetson KJ, Van HH, Kamerling JP, Sixma JJ, Vliegenthart JF. Structural studies on the O-linked carbohydrate chains of human platelet glycocalicin. *Eur J Biochem*. 1984;140(3):571-576.
10. Andrews RK, Fox JE. Identification of a region in the cytoplasmic domain of the platelet membrane glycoprotein Ib-IX complex that binds to purified actin-binding protein. *J Biol Chem*. 1992;267(26):18605-18611.
11. Du X, Harris SJ, Tetaz TJ, Ginsberg MH, Berndt MC. Association of a phospholipase A2 (14-3-3 protein) with the platelet glycoprotein Ib-IX complex. *J Biol Chem*. 1994;269(28):18287-18290.
12. Huizinga EG, Tsuji S, Romijn RA et al. Structures of glycoprotein Ibalpha and its complex with von Willebrand factor A1 domain. *Science*. 2002;297(5584):1176-1179.
13. Dumas JJ, Kumar R, McDonagh T et al. Crystal structure of the wild-type von Willebrand factor A1-glycoprotein Ibalpha complex reveals conformation differences with a complex bearing von Willebrand disease mutations. *J Biol Chem*. 2004;279(22):23327-23334.
14. Kim J, Zhang CZ, Zhang X, Springer TA. A mechanically stabilized receptor-ligand flex-bond important in the vasculature. *Nature*. 2010;466(7309):992-995.
15. Dong JF, Berndt MC, Schade A, McIntire LV, Andrews RK, Lopez JA. Ristocetin-dependent, but not botrocetin-dependent, binding of von Willebrand factor to the platelet glycoprotein Ib-IX-V complex correlates with shear-dependent interactions. *Blood*. 2001;97(1):162-168.
16. Shrimpton CN, Borthakur G, Larrucea S, Cruz MA, Dong JF, Lopez JA. Localization of the adhesion receptor glycoprotein Ib-IX-V complex to lipid rafts is required for platelet adhesion and activation. *J Exp Med*. 2002;196(8):1057-1066.
17. Geng H, Xu G, Ran Y, Lopez JA, Peng Y. Platelet glycoprotein Ib beta/IX mediates glycoprotein Ib alpha localization to membrane lipid domain critical for von Willebrand factor interaction at high shear. *J Biol Chem*. 2011;286(24):21315-21323.
18. Kasirer-Friede A, Ware J, Leng L, Marchese P, Ruggeri ZM, Shattil SJ. Lateral clustering of platelet GP Ib-IX complexes leads to up-regulation of the adhesive function of integrin alpha IIbbeta 3. *J Biol Chem*. 2002;277(14):11949-11956.
19. Arya M, Lopez JA, Romo GM et al. Glycoprotein Ib-IX-mediated activation of integrin alpha(IIb)beta(3): effects of receptor clustering and von Willebrand factor adhesion. *J Thromb Haemost*. 2003;1(6):1150-1157.
20. Gitz E, Koekman CA, van den Heuvel DJ et al. Improved platelet survival after cold storage by prevention of glycoprotein Ibalpha clustering in lipid rafts. *Haematologica*. 2012;97(12):1873-1881.
21. Nichols WL, Hultin MB, James AH et al. von Willebrand disease (VWD): evidence-based diagnosis and management guidelines, the National Heart, Lung, and Blood Institute (NHLBI) Expert Panel report (USA). *Haemophilia*. 2008;14(2):171-232.
22. Sakariassen KS, Aarts PA, de Groot PG, Houdijk WP, Sixma JJ. A perfusion chamber developed to investigate platelet interaction in flowing blood with human vessel wall cells, their extracellular matrix, and purified components. *J Lab Clin Med*. 1983;102(4):522-535.

23. Wencel-Drake JD, Painter RG, Zimmerman TS, Ginsberg MH. Ultrastructural localization of human platelet thrombospondin, fibrinogen, fibronectin, and von Willebrand factor in frozen thin section. *Blood*. 1985;65(4):929-938.

24. Dai K, Bodnar R, Berndt MC, Du X. A critical role for 14-3-3zeta protein in regulating the VWF binding function of platelet glycoprotein Ib-IX and its therapeutic implications. *Blood*. 2005;106(6):1975-1981.

25. Yuan Y, Zhang W, Yan R et al. Identification of a novel 14-3-3zeta binding site within the cytoplasmic domain of platelet glycoprotein Ibalpha that plays a key role in regulating the von Willebrand factor binding function of glycoprotein Ib-IX. *Circ Res*. 2009;105(12):1177-1185.

26. Bodnar RJ, Gu M, Li Z, Englund GD, Du X. The cytoplasmic domain of the platelet glycoprotein Ibalpha is phosphorylated at serine 609. *J Biol Chem*. 1999;274(47):33474-33479.

27. Du X, Fox JE, Pei S. Identification of a binding sequence for the 14-3-3 protein within the cytoplasmic domain of the adhesion receptor, platelet glycoprotein Ib alpha. *J Biol Chem*. 1996;271(13):7362-7367.

28. van der Wal DE, Gitz E, Du VX et al. Arachidonic acid depletion extends survival of cold-stored platelets by interfering with the [glycoprotein Ibalpha - 14-3-3zeta] association. *Haematologica*. 2012;97(10):1514-1522.

29. Kramer RM, Roberts EF, Um SL et al. p38 mitogen-activated protein kinase phosphorylates cytosolic phospholipase A2 (cPLA2) in thrombin-stimulated platelets. Evidence that proline-directed phosphorylation is not required for mobilization of arachidonic acid by cPLA2. *J Biol Chem*. 1996;271(44):27723-27729.

30. Dopheide SM, Maxwell MJ, Jackson SP. Shear-dependent tether formation during platelet translocation on von Willebrand factor. *Blood*. 2002;99(1):159-167.

31. Reininger AJ, Heijnen HF, Schumann H, Specht HM, Schramm W, Ruggeri ZM. Mechanism of platelet adhesion to von Willebrand factor and microparticle formation under high shear stress. *Blood*. 2006;107(9):3537-3545.

32. Ruggeri ZM, Orje JN, Habermann R, Federici AB, Reininger AJ. Activation-independent platelet adhesion and aggregation under elevated shear stress. *Blood*. 2006;108(6):1903-1910.

33. Nesbitt WS, Westein E, Tovar-Lopez FJ et al. A shear gradient-dependent platelet aggregation mechanism drives thrombus formation. *Nat Med*. 2009;15(6):665-673.

34. Uff S, Clemetson JM, Harrison T, Clemetson KJ, Emsley J. Crystal structure of the platelet glycoprotein Ib(alpha) N-terminal domain reveals an unmasking mechanism for receptor activation. *J Biol Chem*. 2002;277(38):35657-35663.

35. Gu M, Xi X, Englund GD, Berndt MC, Du X. Analysis of the roles of 14-3-3 in the platelet glycoprotein Ib-IX-mediated activation of integrin alpha(IIb)beta(3) using a reconstituted mammalian cell expression model. *J Cell Biol*. 1999;147(5):1085-1096.

36. Mangin P, David T, Lavaud V et al. Identification of a novel 14-3-3zeta binding site within the cytoplasmic tail of platelet glycoprotein Ibalpha. *Blood*. 2004;104(2):420-427.

37. Fu H, Subramanian RR, Masters SC. 14-3-3 proteins: structure, function, and regulation. *Annu Rev Pharmacol Toxicol*. 2000;40617-647.

38. Lee CW, Han J, Bamburg JR, Han L, Lynn R, Zheng JQ. Regulation of acetylcholine receptor clustering by ADF/cofilin-directed vesicular trafficking. *Nat Neurosci*. 2009;12(7):848-856.

39. Sumpio BE, Yun S, Cordova AC et al. MAPKs (ERK1/2, p38) and AKT can be phosphorylated by shear stress independently of platelet endothelial cell adhesion molecule-1 (CD31) in vascular endothelial cells. *J Biol Chem*. 2005;280(12):11185-11191.

40. Canobbio I, Reineri S, Sinigaglia F, Balduini C, Torti M. A role for p38 MAP kinase in platelet activation by von Willebrand factor. *Thromb Haemost*. 2004;91(1):102-110.

41. Brock TG. Arachidonic acid binds 14-3-3zeta, releases 14-3-3zeta from phosphorylated BAD and induces aggregation of 14-3-3zeta. *Neurochem Res*. 2008;33(5):801-807.

42. Pike LJ, Han X, Chung KN, Gross RW. Lipid rafts are enriched in arachidonic acid and plasmenylethanolamine and their composition is independent of caveolin-1 expression: a quantitative electrospray ionization/mass spectrometric analysis. *Biochemistry*. 2002;41(6):2075-2088.

43. Patrono C, Garcia Rodriguez LA, Landolfi R, Baigent C. Low-dose aspirin for the prevention of atherothrombosis. *N Engl J Med*. 2005;353(22):2373-2383.

44. Turner NA, Moake JL, Kamat SG et al. Comparative real-time effects on platelet adhesion and aggregation under flowing conditions of in vivo aspirin, heparin, and monoclonal antibody fragment against glycoprotein IIb-IIIa. *Circulation*. 1995;91(5):1354-1362.

45. Grabowski EF. Platelet aggregation in flowing blood at a site of injury to an endothelial cell monolayer: quantitation and real-time imaging with the TAB monoclonal antibody. *Blood*. 1990;75(2):390-398.

SUPPLEMENTARY MATERIALS AND METHODS

Materials and antibodies

We used the following products (with sources): cytosolic phospholipase A_2 ($cPLA_2$) inhibitor $AACOCF_3$ (Santa Cruz Biotechnology, Santa Cruz, CA), arachidonic acid (AA; Bio/Data Corporation, Horsham, PA), bovine serum albumin (BSA) fraction V, methyl-β-cyclodextrin (mβCD), indomethacin (Sigma-Aldrich, St. Louis, MO), lipoxygenase-inhibitor 5, 8, 11-eicosatriynoic acid (ETI) and prostacyclin (PGI_2) (Cayman Chemical, Ann Arbor, MI), human serum albumin fraction V (MP biomedicals, Santa Ana, CA), horm collagen (Nycomed, Linz, Austria), PGI_2 analog iloprost (Bayer AG, Leverkusen, Germany), Odyssey blocking buffer (LI-COR Biosciences, Lincoln, NE), ristocetin (Biopool Us Inc, Jamestown, NY), P38-mitogen-activated protein kinase (P38MAPK) inhibitor SB203580 (Alexis Biochemicals/ Enzo Lifesciences BVBA, Zandhoven, Belgium) and thrombin receptor activating peptide (TRAP-6, SFLLRN; Bachem, Bubdendorf, Switzerland). Thromboxane A_2 receptor (TPα) antagonist SQ30741 was kindly provided by Bristol-Meyers-Squibb (Maarssen, The Netherlands). Cross-linked collagen related peptide (CRP) was a generous gift from Dr. Richard Farndale (Cambridge University, UK). Human VWF was purified as described.[1] The synthetic peptide dRGDW and membrane-permeable myristoylated peptides MPαC (N-myristoyl-SIRYSGHpSL) and MαC (N-myristoyl-SIRYSGHSL) were produced by the NKI (Amsterdam, the Netherlands). The latter two mimic the cytoplasmic binding site for 14-3-3ζ on GPIbα surrounding the Ser^{609} residue. Phosphorylation of Ser^{609} is critical for 14-3-3ζ association.[2]

The antibody used for FRET analysis of GPIbα distribution was the recombinant 6B4-Fab fragment directed against aa 200-268 of GPIbα.[3] Other tools for FRET analysis were Alexa Fluor-488 and -594 protein labeling kits and Cholera toxin subunit B conjugated to Alexa Fluor-594 (CTB-594; Invitrogen, Carlsbad, CA), which binds to the raft marker monosialo-tetrahexosylganglioside (GM1). Antibodies used for flow cytometry were directed against VWF (Abcam, Cambridge, UK), P-selectin and activated αIIbβ3 (PAC-1) (BD Pharmingen, San Diego, CA). Antibodies for immunoprecipitation were against GPIbα (clone HIP1; eBioscience, San Diego, CA) and for western blotting against GPIbα (clone SZ2; Beckman Coulter, Brea, CA), 14-3-3ζ (C-16, Santa Cruz Biotechnology), Phospho-P38MAPK (Thr180/Tyr182) and total P38MAPK (Cell Signaling Technology, Danvers, MA). Secondary antibodies were Alexa Fluor 680-conjugated goat-α-rabbit and goat-α-mouse (Invitrogen), and IRDye 800CW-conjugated goat-α-rabbit IgG (LI-COR Biosciences, Lincoln, NE).

Platelet preparation and incubations

Human platelets were isolated with free-flow blood collection as described.[4] Procedures were approved by the local Medical Ethics Committee. Platelets were resuspended in Hepes-Tyrode's (HT) buffer ($2.5x10^{11}$ cells/L, pH 7.3) and allowed to recover for 30 minutes at room temperature prior to incubations (defined as control platelets). Reconstituted blood was prepared[5] by resuspending washed platelets in HT buffer ($3.3x10^{11}$ cells/L, pH 7.3) supplemented with 4% human albumin. Packed red blood cells were mixed with platelets in a 40:60 (v/v) ratio to obtain reconstituted blood with a platelet count of $2x10^{11}$/L and a hematocrit of 0.4 (L/L). In some experiments, VWF was added to a final concentration of 10 μg/mL.

Inhibitors used were against platelet activation (iloprost; 1 μM), integrin αIIbβ3 occupancy (dRGDW, 200 μM), TPα (SQ30741; 25 μM) and activation of P38MAPK (SB203580; 10 μM), $cPLA_2$ ($AACOCF_3$, 20 μM), cyclooxygenase-1 (indomethacin; 30 μM) and lipoxygenase (ETI; 25 μM), each preincubated for 10 minutes at 37°C. Membrane was depleted of cholesterol by incubating platelets with mβCD (10 mM) for 30 minutes at 37°C. In some experiments, platelets were incubated with control peptide MαC or MPαC (100 μM) for 5 minutes at 37°C. All incubations were without stirring unless indicated otherwise.

Platelet adhesion and rolling under flow conditions

A parallel plate perfusion chamber[6] was used to investigate platelet adhesion and rolling. For adhesion experiments, coverslips were coated with VWF (10 μg/mL) or horm collagen (0.1 mg/mL) and blocked with 4% BSA. Whole blood or reconstituted blood was perfused at defined shear rates (37°C) and adhesion was visualized using an Axio Observer Z1 microscope equipped with an AxioCam MRm CCD camera with a Zeiss EC Plan-NeoFluar 40x/0.75 DICII lens (Carl Zeiss MicroImaging GmbH, Gottingen, Germany). Surface coverage was analyzed using ImageJ software (NIH, Bethesda, MD). For determination of rolling velocity, washed platelets were resuspended in HT buffer ($2.5x10^{11}$ cells/L, pH 7.3) supplemented with 4% human albumin and preincubated with iloprost and dRGDW. Platelets were perfused at 1,600 s^{-1} over a VWF coated coverslip with half of the surface blocked with 4% BSA (upstream). Platelet rolling was analyzed in the middle of the perfusion chamber at the interface of surface immobilized BSA and VWF where flow is laminar. Rolling velocity was determined by tracking 40 platelets per experiment over a distance of 200 μm in time using Axiovision v4.8 software (Carl Zeiss MicroImaging GmbH).

Flow cytometric analysis

Characterization of platelets by FACS was based on FSC and SSC-scatter (FACS-Calibur; BD Biosciences, San Jose, CA). Appropriate antibodies were added and incubated for 15 minutes at 37°C. A total of 10,000 platelets was analyzed for surface-expressed VWF, P-selectin, and activated αIIbβ3.

Immunoprecipitations and western blots

For immunoprecipitations, 900 μL washed platelets ($5x10^{11}$ platelets/L) were lysed with 100 μL of 10x RIPA lysis buffer (15 minutes, 0°C) and mixed with 110 μL (10% vol/vol) protein G beads (GE Healthcare, Little Chalfont, UK) together with antibody (2 μg/mL, o/n, 4ºC, rotating). For lysate analysis, platelet suspensions were lysed, centrifuged (10,000 g, 1 minute) and dissolved in sample buffer under reducing conditions. Proteins were separated by SDS-PAGE and western blotted. After blocking with Odyssey Blocking buffer, membranes were incubated with primary antibodies (1 μg/mL) and protein bands visualized with an Odyssey Imaging system (LI-COR Biosciences). Quantification was performed with ImageJ software. Variations in lane loading were adjusted by normalization to the immunoprecipitated protein.

SUPPLEMENTARY FIGURES

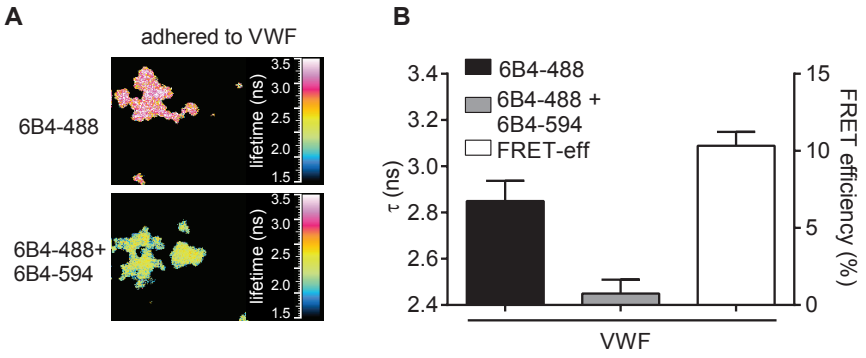


Figure S1. Levels of GPIbα clustering are similar in platelets attached to VWF as small aggregates. (**A**) Platelets adhered to VWF after whole blood perfusion (1 minute, 37°C) at a shear rate of 1600 s^{-1} were analyzed for GPIbα distribution by FRET/FLIM. Platelets were fixed with 2% paraformaldehyde and stained with 1 µg/mL 6B4-488 (donor) in the absence (top panels) and presence of 1 µg/mL 6B4-594 (acceptor; bottom panels). The fluorescence lifetimes in nanoseconds (ns) are shown in false color images. (**B**) Quantification of fluorescence lifetime values of donor probe in the absence and presence of acceptor probe of platelets treated under the conditions of (A). Corresponding FRET efficiencies are calculated as described in Materials & Methods. Data are means ± SEM (n=3).

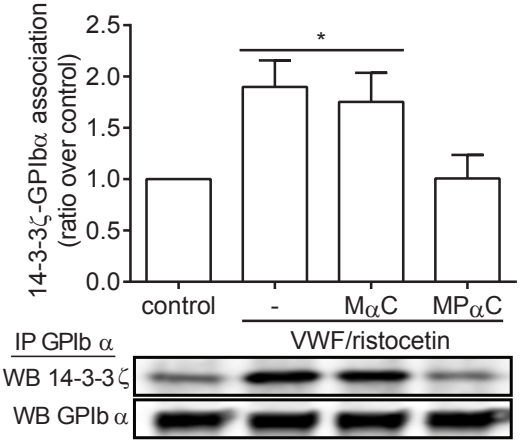


Figure S2. Prevention of [14-3-3ζ-GPIbα] complex formation by MPαC. Washed platelets were pre-incubated in the absence and presence of control peptide MαC and MPαC (100 µM) for 5 minutes at 37°C and subsequently stimulated with ristocetin (0.3 mg/mL) and VWF (10 µg/mL) at 37°C while stirring. After 2 minutes incubation, platelets were lysed and analyzed for [14-3-3ζ-GPIbα] complex formation.

CHAPTER 5

Patient autoantibodies induce platelet destruction signals via raft-associated glycoprotein Ibα and FcγRIIa in immune thrombocytopenia

Rolf T. Urbanus[1]
Dianne E. van der Wal[1]
Cornelis A. Koekman[1]
Albert Huisman[1]
Dave J. van den Heuvel[2]
Hans C. Gerritsen[2]
Hans Deckmyn[3]
Jan-Willem N. Akkerman[1]
Roger E.G. Schutgens[4]
Eelo Gitz[1]

[1]Department of Clinical Chemistry and Haematology, University Medical Center Utrecht, Utrecht, the Netherlands
[2]Department of Molecular Biophysics, Utrecht University, Utrecht, the Netherlands
[3]Laboratory for Thrombosis Research, KU Leuven, Kortrijk, Belgium
[4]Haematology/Van Creveldkliniek, University Medical Center Utrecht, Utrecht, the Netherlands

Accepted for publication in *Haematologica. 2013*

Abstract

The primary targets of autoantibodies in immune thrombocytopenia (ITP) are the αIIbβ3 integrin (70-80%) and the glycoprotein (GP) Ib-V-IX complex (20-40%). Thrombocytopenia in patients with anti-αIIbβ3 antibodies is alleviated by intravenous immunoglobulin G (IVIG), which blocks Fcγ receptor-mediated platelet clearance by macrophages. Additional destruction pathways may regulate thrombocytopenia in patients with anti-GPIbα antibodies, which are often resistant to IVIG. Here we report on the generation of platelet clearance signals induced by anti-GPIbα antibodies in an ITP patient. Patient antibodies induced GPIbα clustering and surface expression of both P-selectin and phosphatidylserine. These clearance signals were generated by autoantibody-induced GPIbα translocation to lipid rafts, where the receptor clustered and associated with the low-affinity Fc-receptor FcγRIIa. Blocking of GPIbα translocation to lipid rafts or FcγRIIa neutralization prevented generation of destruction signals.

Introduction

Immune thrombocytopenia (ITP) is an acquired immune-mediated disorder characterized by thrombocytopenia in the absence of an underlying cause.[1;2] The pathophysiology of ITP is multifactorial and includes the development of autoantibodies that trigger abnormal thrombopoiesis, enhanced platelet destruction, complement activation and T-cell-mediated effects.[3-5] Platelet autoantibodies are detected in about 50% of patients[4] and generally target the fibrinogen receptor αIIbβ3 or the receptor for von Willebrand factor (VWF), the glycoprotein (GP) Ib-V-IX complex. Anti-αIIbβ3 antibodies (70-80% of cases) are thought to induce thrombocytopenia through increased platelet clearance by Fcγ receptor-bearing macrophages. Autoantibodies against GPIb-V-IX (20-40% of cases) often induce a more severe fall in platelet count[6;7] that is less responsive to standard therapies, such as intravenous immunoglobulin G (IVIG).[8;9] Thrombocytopenia induced by GPIb-V-IX autoantibodies has been characterized in little detail. Some monoclonal antibodies against GPIbα are known to induce platelet activation,[10] which may lead to accelerated platelet destruction in ITP patients[11-13] with autoantibodies against this receptor. Here we report how an autoantibody against GPIbα, obtained from a patient with ITP, induces recognition signals for macrophages through interplay between glycoprotein Ibα and the low affinity IgG receptor FcγRIIa in lipid rafts.

Materials and Methods

Patient characteristics

The patient is a 70 year old Caucasian woman. She had a history of nephrectomy and parathyroidectomy; both surgeries were without bleeding complications. In 2007, routine laboratory investigations revealed a thrombocytopenia ($20x10^9$/L; normal $150\text{-}450x10^9$/L). Recently, she suffered from increased spontaneous skin hematomas and melena. Her platelet count was low ($20x10^9$/L) and disturbed by aggregates. Hemoglobin level, leukocytes and differential were normal. There was no detectable monoclonal protein and immunoglobulin levels were normal. IVIG (30 g per day for 5 days) and platelet transfusions failed to increase platelet count to normal levels. Gastro- and colonoscopy did not reveal a clear bleeding focus. (Supplementary Table S1)

Materials, antibodies and platelet incubations

A detailed description is found in the Supplementary Materials and Methods.

Flow cytometric analysis

Characterization of platelets by Fluorescence-Activated Cell Sorting (FACS) was based on forward- and side-scatter (FSC/SSC) (FACS-Calibur; BD Biosciences, San Jose, CA). Platelets were incubated with appropriate antibodies (15 minutes, 37 °C) and 10,000 platelets were analyzed for surface expression of P-selectin, phosphatidylserine (PS), fibrinogen and binding of human IgGs. Results were expressed as ratio of patient data/control data.

Plasma depletion from IgGs

Patient plasma and plasma pooled from 200 healthy donors was depleted from IgG by passage through a Protein G-sepharose column. The effluent was collected and stored at -80 ºC before experiments. Bound IgG was eluted and dialyzed thrice against a large excess of 10 mM Hepes, 154 mM NaCl, pH 7.4 for 6 hours. IgG was stored at -80 ºC until used.

Antibody titer determination by ELISA
Antibody titer of GPIbα antibodies was determined by ELISA. Wells were coated with 10 μg/mL of fully sulphated recombinant GPIbα (residues 1-298),[14] blocked with 4% BSA and washed. Patient plasma was prediluted (1:10) and added in a 1:2 (vol:vol) dilution series. Bound antibody was detected with HRP-conjugated goat-anti-human IgG and quantified at 450 nm using a Spectramax M2e microplate reader (Molecular Devices, Sunnyvale, CA).

Analysis of GPIbα distribution by Förster Resonance Energy Transfer using Fluorescence Lifetime Imaging Microscopy (FRET/FLIM)
GPIbα translocation to rafts, GPIbα clustering and GPIbα association with FcγRIIa were determined by FRET/FLIM and analyzed as described in the Supplementary Materials and Methods.

Statistical analysis
Data are means ± SEM with number of experiments (n), as indicated. Statistical analysis was performed using GraphPad Prism 5 (San Diego, CA) software. Differences between control platelets and incubations were analyzed by Mann-Whitney test. *P*-values <0.05 (*) were considered significant.

Results & Discussion

Platelet activation by patient autoantibodies
Citrated blood from an ITP patient showed a mixture of single platelets and small aggregates. Inhibition of platelet activation with prostacyclin (PGI_2) during blood collection reduced the number of aggregates, but the platelet count remained low (Figure 1A). FACS analysis with gating for single platelets showed increased levels of surface-expressed fibrinogen, P-selectin and PS compared with controls (Figure 1B). Surface-exposed P-selectin and PS are clearance signals, triggering platelet binding to macrophages, followed by their destruction.[15] As expected, matured monocytic THP-1 cells phagocytosed 21-fold more patient platelets than control platelets. The activation observed in blood was caused by a plasma constituent, since normal platelets incubated in patient plasma were also activated and showed a strong increase in surface-bound fibrinogen and P-selectin. IgG depletion prevented the increase in P-selectin expression, which was restored upon repletion of IgG (Figure 1C).

Autoantibody binding to GPIbα leads to FcγRIIa-mediated platelet activation
The observed platelet activation suggested the presence of anti-GPIbα autoantibodies, as some monoclonal anti-GPIbα antibodies are known to induce platelet aggregation.[10] Indeed, removal of GPIbα ectodomain with o-sialoglycoprotein endopeptidase (osge) or addition of excess soluble recombinant GPIbα reduced P-selectin expression on normal platelets incubated in patient plasma (Figure 2A). Binding of VWF to GPIbα triggers its translocation to cholesterol-rich domains known as lipid rafts.[16] Subsequent platelet activation by GPIbα involves signaling through immunoreceptor tyrosine-based activation motif-containing receptors, such as the Fc receptor γ chain (FcRγ)[17] or the low-affinity IgG receptor FcγRIIa.[18] To investigate whether a similar mechanism might function in autoantibody-induced platelet activation, suspensions of normal platelets in patient plasma were incubated with GM3 ganglioside, which inhibits GPIbα translocation to lipid rafts,[19] or with a neutralizing anti-FcγRIIa antibody (clone AT10).[20] Both treatments strongly suppressed P-selectin expression induced by patient plasma (Figure 2A). Antibody titer determination with immobilized recombinant GPIbα confirmed the presence of GPIbα autoantibodies in patient plasma

(Figure 2B). Direct analysis of IgG binding in suspensions of normal platelets in patient plasma confirmed the expected interference by recombinant GPIbα, whereas GM3 and anti-FcγRIIa antibody had no effect (Figure 2C). These data demonstrate that antibody binding is primarily regulated through GPIbα.

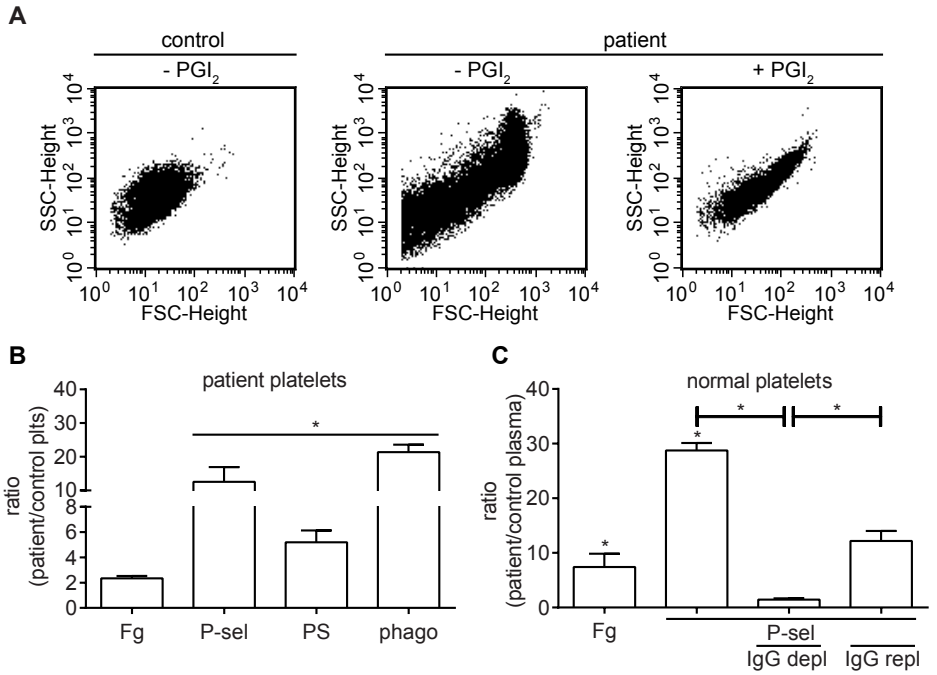


Figure 1. Platelet activation by patient autoantibodies. (A) Platelet aggregation during blood collection. Blood was collected in citrate supplemented with PGI_2 (10 ng/mL) and FSC/SSC-scatter was analyzed by FACS in isolated control platelets (left panel) or patient platelets without and with PGI_2 (middle and right panel). (B) Patient platelets are activated during blood collection. FACS analysis of activated αIIbβ3 (bound fibrinogen; Fg), surface P-selectin (P-sel) and PS exposure. CFMDA-labeled platelets were incubated with matured monocytic THP-1 cells and phagocytosis (phago) was determined. (C) Normal platelets were incubated (2 hours, 22 °C) with autologous (control plasma) or patient plasma and analyzed for bound fibrinogen and P-selectin expression. Depletion of IgGs from patient plasma (IgG depl) reduced surface P-selectin to control levels, while repletion (IgG repl) increased its expression. Results are expressed as ratio of normal platelets in patient plasma over control plasma. Data are means ± SEM (n=4).

FRET measurement using FLIM is a sensitive technique to analyze protein colocalization on the intact platelet membrane.19 Analysis of normal platelets revealed that little GPIbα was present in lipid rafts (Figure 2D). The receptor was dispersed over the surface and formed little associations with FcγRIIa. Incubation in patient plasma induced GPIbα translocation to rafts, the formation of GPIbα clusters and GPIbα association with FcγRIIa. Addition of GM3 not only induced the expected blockade of GPIbα translocation to rafts but also prevented formation of GPIbα clusters and GPIbα association with FcγRIIa. Collectively, these findings indicate that autoantibodies against GPIbα trigger surface expression of GPIbα clusters, P-selectin and PS, which are all 'eat-me' signals for macrophages.15;21 Crucial is the association with lipid rafts, both for formation of GPIbα clusters and activation of FcγRIIa, whose ligand-binding properties are enhanced by localization to rafts.22 The result is a more

severe drop in platelet count compared to patients with anti-αIIbβ3 antibodies.6;7 The fact that αIIbβ3 does not translocate to rafts upon ligand binding may explain the inability of autoantibodies against this receptor to efficiently activate FcγRIIa to generate additional destruction signals.[16] Future studies should reveal whether generation of destruction signals is similar in other ITP patients with autoantibodies against GPIbα. Confirmation of this mechanism in a larger population may open ways to explore the prevention of autoantibody-induced GPIbα association with FcγRIIa in lipid rafts as a possible therapy in ITP.

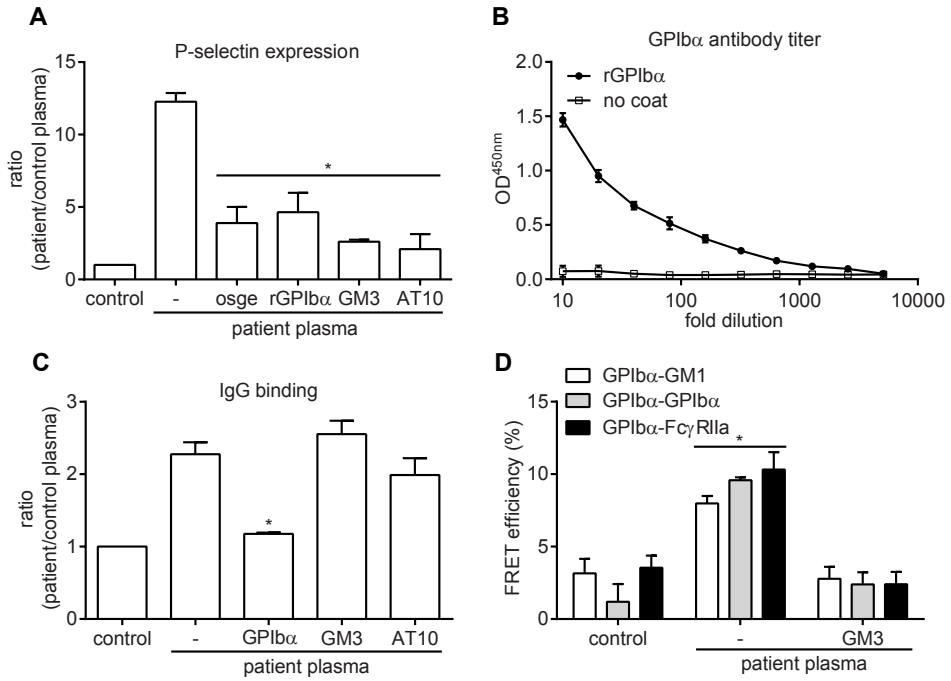


Figure 2. Autoantibody binding to GPIbα leads to FcγRIIa-mediated platelet activation. (A) Normal platelets were incubated with autologous (control plasma) or patient plasma (2 hours, 22ºC) and surface P-selectin expression was measured following removal of extracellular GPIbα (osge; 80 μg/mL), addition of recombinant GPIbα to patient plasma (rGPIbα; 50 μg/mL), GM3 (50 μM) to prevent GPIbα translocation to lipid rafts or a neutralizing anti-FcγRIIa antibody (clone AT10; 50 μg/mL). (B) Determination of GPIbα antibody titer in patient plasma by ELISA. Patient plasma was prediluted (1:10) and added to wells coated with or without recombinant GPIbα to determine IgG binding. (C) Determination of IgG binding to platelets by FACS analysis under conditions as described for (A). (D) FRET/FLIM analysis of GPIbα translocation to lipid rafts, clustering and co-localization with FcγRIIa of normal platelets incubated with patient plasma. Platelets were fixed with 2% paraformaldehyde and stained with 6B4-488, 6B4-594 (1 μg/mL), CTB-594 (5 μg/mL) or CD32-488 (1 μg/mL). The fluorescence lifetimes of the donor fluorophore (6B4-488 or AT10-488) were determined in the absence and presence of acceptor fluorophore (6B4-594 or CTB-594) and subsequently used to calculate the FRET efficiency. Normal platelets incubated in autologous plasma have dispersed GPIbα receptors that do not co-localize with GM1, a marker for lipid rafts, or FcγRIIa. Platelet incubation in patient plasma triggers GPIbα translocation to rafts, leading to its clustering and association with FcγRIIa, which is prevented by addition of GM3. Data are means ± SEM (n=3).

Acknowledgements
This study was supported by a grant from the Landsteiner Foundation of Blood transfusion Research (LSBR grant no. 0807). Prof. Dr. J.W.N. Akkerman is supported by the Netherlands Thrombosis Foundation. Dr. R.T. Urbanus is a research fellow of the Dutch Heart Foundation (grant no. 2010T068).

References

1. Provan D, Stasi R, Newland AC et al. International consensus report on the investigation and management of primary immune thrombocytopenia. *Blood*. 2010;115(2):168-186.
2. Cines DB, Bussel JB, Liebman HA, Luning Prak ET. The ITP syndrome: pathogenic and clinical diversity. *Blood*. 2009;113(26):6511-6521.
3. Olsson B, Andersson PO, Jernas M et al. T-cell-mediated cytotoxicity toward platelets in chronic idiopathic thrombocytopenic purpura. *Nat Med*. 2003;9(9):1123-1124.
4. Stasi R, Evangelista ML, Stipa E, Buccisano F, Venditti A, Amadori S. Idiopathic thrombocytopenic purpura: current concepts in pathophysiology and management. *Thromb Haemost*. 2008;99(1):4-13.
5. Peerschke EI, Andemariam B, Yin W, Bussel JB. Complement activation on platelets correlates with a decrease in circulating immature platelets in patients with immune thrombocytopenic purpura. *Br J Haematol*. 2010;148(4):638-645.
6. Nomura S, Yanabu M, Soga T et al. Analysis of idiopathic thrombocytopenic purpura patients with antiglycoprotein IIb/IIIa or Ib autoantibodies. *Acta Haematol*. 1991;86(1):25-30.
7. Hou M, Stockelberg D, Kutti J, Wadenvik H. Antibodies against platelet GPIb/IX, GPIIb/IIIa, and other platelet antigens in chronic idiopathic thrombocytopenic purpura. *Eur J Haematol*. 1995;55(5):307-314.
8. Webster ML, Sayeh E, Crow M et al. Relative efficacy of intravenous immunoglobulin G in ameliorating thrombocytopenia induced by antiplatelet GPIIbIIIa versus GPIbalpha antibodies. *Blood*. 2006;108(3):943-946.
9. Go RS, Johnston KL, Bruden KC. The association between platelet autoantibody specificity and response to intravenous immunoglobulin G in the treatment of patients with immune thrombocytopenia. *Haematologica*. 2007;92(2):283-284.
10. Cauwenberghs N, Schlammadinger A, Vauterin S et al. Fc-receptor dependent platelet aggregation induced by monoclonal antibodies against platelet glycoprotein Ib or von Willebrand factor. *Thromb Haemost*. 2001;85(4):679-685.
11. Yanabu M, Nomura S, Fukuroi T et al. Synergistic action in platelet activation induced by an antiplatelet autoantibody in ITP. *Br J Haematol*. 1991;78(1):87-93.
12. Olsson A, Andersson PO, Tengborn L, Wadenvik H. Serum from patients with chronic idiopathic thrombocytopenic purpura frequently affect the platelet function. *Thromb Res*. 2002;107(3-4):135-139.
13. Jy W, Horstman LL, Arce M, Ahn YS. Clinical significance of platelet microparticles in autoimmune thrombocytopenias. *J Lab Clin Med*. 1992;119(4):334-345.
14. Huizinga EG, Tsuji S, Romijn RA et al. Structures of glycoprotein Ibalpha and its complex with von Willebrand factor A1 domain. *Science*. 2002;297(5584):1176-1179.
15. Maugeri N, Rovere-Querini P, Evangelista V et al. Neutrophils phagocytose activated platelets in vivo: a phosphatidylserine, P-selectin, and {beta}2 integrin-dependent cell clearance program. *Blood*. 2009;113(21):5254-5265.
16. Shrimpton CN, Borthakur G, Larrucea S, Cruz MA, Dong JF, Lopez JA. Localization of the adhesion receptor glycoprotein Ib-IX-V complex to lipid rafts is required for platelet adhesion and activation. *J Exp Med*. 2002;196(8):1057-1066.
17. Falati S, Edmead CE, Poole AW. Glycoprotein Ib-V-IX, a receptor for von Willebrand factor, couples physically and functionally to the Fc receptor gamma-chain, Fyn, and Lyn to activate human platelets. *Blood*. 1999;94(5):1648-1656.
18. Sullam PM, Hyun WC, Szollosi J, Dong J, Foss WM, Lopez JA. Physical proximity and functional interplay of the glycoprotein Ib-IX-V complex and the Fc receptor FcgammaRIIA on the platelet plasma membrane. *J Biol Chem*. 1998;273(9):5331-5336.
19. Gitz E, Koekman CA, van den Heuvel DJ et al. Improved platelet survival after cold storage by prevention of glycoprotein Ibalpha clustering in lipid rafts. *Haematologica*. 2012;97(12):1873-1881.
20. Pollreisz A, Assinger A, Hacker S et al. Intravenous immunoglobulins induce CD32-mediated platelet aggregation in vitro. *Br J Dermatol*. 2008;159(3):578-584.
21. Hoffmeister KM, Felbinger TW, Falet H et al. The clearance mechanism of chilled blood platelets. *Cell*. 2003;112(1):87-97.
22. Bournazos S, Hart SP, Chamberlain LH, Glennie MJ, Dransfield I. Association of FcgammaRIIa (CD32a) with lipid rafts regulates ligand binding activity. *J Immunol*. 2009;182(12):8026-8036.

Supplementary Materials and Methods

Materials and antibodies

We used the following products (with sources): 5-chloromethyl-fluorescein-diacetate (Cell tracker green\CFMDA, Molecular Probes/Invitrogen, Carlsbad, CA), 96-well microtiter plate (Nunc Ni-chelate; Nalge Nunc International, Rochester NY), anti-FcγRIIa neutralizing antibody (clone AT10; Sanbio, Uden, The Netherlands), bovine serum albumin (BSA) fraction V and vitamin D_3 (Sigma-Aldrich, St. Louis, MO), monosialoganglioside GM3 (GenWay Biotech Inc, San Diego, CA), EDTA-K2 (BD, San Diego, CA), fibrinogen (Enzyme Research Laboratories, South Bend, IN), *O*-sialoglycoprotein-endopeptidase (osge, Cederlane Laboratories, Hornby, Canada), phorbol-12-myristate-13-acetate (PMA, MP Biochemicals, Illkirch, France), platelet-derived human TGF-β1 (R&D systems, Minneapolis, MN), prostacyclin (PGI_2, Cayman Chemical, Ann Arbor, MI), Protein G-sepharose (GE Healthcare, Uppsala, Sweden), recombinant GPIbα (high sulphated),[1] sodium-heparin (Greiner bio-one, Frickenhausen, Germany), and THP-1 monocytic cells (ATCC/LGC Standards, Wesel, Germany).

For FACS analysis we used antibodies against human IgG (Fab-specific; Sigma-Aldrich), P-selectin and FITC-labeled fibrinogen (BD, San Diego, CA). Surface exposure of phosphatidylserine (PS) was deduced from FITC-labeled lactadherin binding (Haematologic Technologies Inc. Essex Junction, VT). For ELISA we used HRP-conjugated goat-anti-human IgG (Surmodics, Eden Prairie, MN). The agents used for FRET analysis were the recombinant Alexa Fluor-488 and -594-conjugated 6B4-Fab fragments (6B4-488 and 6B4-594, respectively) directed against aa 200-268 of GPIbα,[2;3] Alexa Fluor-488-conjugated anti-FcγRIIa (AT10-488; Santa Cruz, biotechnology, Santa Cruz, CA), and Alexa Fluor-594-conjugated Cholera toxin subunit B directed against ganglioside GM1 (CTB-594; Invitrogen, Carlsbad, CA).

Blood collection, platelet isolation and incubations

Procedures were approved by the Medical Ethical Committee of our hospital. Platelets from healthy, medication-free volunteers were isolated[4] and resuspended in Hepes-Tyrode (HT) buffer (pH 7.3). Whole blood and isolated platelets were analyzed using the Cell-Dyn Sapphire hematology analyzer (Abbott Diagnostics, Santa Clara, CA, USA). The patient platelets aggregated spontaneously during blood collection in vacuum tubes containing citrate, coated EDTA-K2 or sodium-heparin, preventing investigations. To reduce aggregation to a minimum, patient blood was collected by free-flow; the first 2 ml was discarded and the remainder collected in 13 mM citrate (f.c.), supplemented with 10 ng/mL PGI_2 to induce transient platelet inhibition. Patient platelets were subsequently isolated as described.[4] Plasma was isolated by centrifugation (10 minutes, 2,000*g*, 22 °C). For plasma reconstitution experiments, platelet-rich plasma from healthy subjects was mixed with 0.1 volume of ACD (2.5% trisodium citrate, 1.5% citric acid and 2% *D*-Glucose), centrifuged (15 minutes, 330*g*, no brake, 22 °C) and platelets were incubated in autologous or patient plasma for 2 hours, 22 °C. Then, platelets were washed (15 minutes, 330*g*, no brake, 22 °C, in the presence of 10 ng/mL PGI_2), resuspended in HT buffer (pH 7.3) ($2x10^{11}$ cells/L) and kept at 22°C for 30 minutes to regain responsiveness. In some experiments, platelets were incubated (30 minutes at 22 °C) with osge (80 μg/mL) prior to plasma incubation to remove GPIbα ectodomain, with recombinant GPIbα to saturate GPIbα binding properties in patient antibody (50 μg/mL), with a neutralizing antibody against FcγRIIa (50 μg/mL) and with GM3 (50 μM) to

interfere with GPIbα translocation to lipid rafts.[3] Phagocytosis of platelets by monocytic THP-1 cells was performed as described before.[5]

Flow cytometric analysis
Characterization of platelets by Fluorescence-Activated Cell Sorting (FACS) was based on forward- and side-scatter (FSC/SSC) (FACS-Calibur; BD Biosciences, San Jose, CA). Platelets were incubated with appropriate antibodies (15 minutes, 37 °C) and 10,000 platelets were analyzed for surface expression of P-selectin, phosphatidylserine (PS), fibrinogen and binding of human IgGs. Results were expressed as ratio of patient data/control data.

Plasma depletion from IgGs
Patient plasma and plasma pooled from 200 healthy donors was depleted from IgG by passage through a Protein G-sepharose column. The effluent was collected and stored at -80 ºC before experiments. Bound IgG was eluted and dialyzed thrice against a large excess of 10 mM Hepes, 154 mM NaCl, pH 7.4 for 6 hours. IgG was stored at -80ºC until used for repletion studies.

Antibody titer determination by ELISA
Antibody titer of GPIbα antibodies was determined by ELISA. Wells were coated with 10 µg/mL of fully sulphated recombinant GPIbα (residues 1-298),[1] blocked with 4% BSA and washed. Patient plasma was prediluted (1:10) and added in a 1:2 (vol:vol) dilution series. Bound antibody was detected with HRP-conjugated goat-anti-human IgG and quantified at 450 nm using a Spectramax M2e microplate reader (Molecular Devices, Sunnyvale, CA).

Platelet phagocytosis
Platelets were resuspended in HT buffer (pH 6.5) to a concentration of $4x10^{11}$ cells/L, labeled with CFMDA (20 µM, 1 hour, 22 °C), centrifuged (330g, 10 minutes, 22°C) in the presence of 10 ng/mL PGI_2 and resuspended in HT buffer (pH 7.3). Functionality was recovered by 30 minutes incubation at 22°C. For measurement of phagocytosis, monocytic THP-1 cells were cultured in a 96-wells plate ($5x10^4$ cells/well) and stimulated with 50 nM vitamin D_3 and 1 ng/mL TGF-β1 (12 hours, 37°C) and 250 nM PMA (2 hours, 37°C). $CaCl_2$ and $MgCl_2$ (1mM each, f.c.) were added and $5x10^5$ platelets were incubated with the maturated THP-1 cells (90 minutes, 37°C). Unbound platelets were removed and phagocytosis was measured on a Fluorstar Galaxy (BMG LABTECH GmbH, Offenburg, Germany). Results were expressed as ratio of patient data/control data.

Analysis of GPIbα distribution by Förster Resonance Energy Transfer using Fluorescence Lifetime Imaging Microscopy (FRET/FLIM)
GPIbα distribution by FRET/FLIM was analyzed as described before.[3] In short, 6B4-Fab fragments conjugated to either Alexa Fluor-488 or Alexa Fluor-594 (6B4-488 and 6B4-594 respectively) were incubated with fixed platelet samples under conditions in which each Fab labeled ~50% of total receptor number. GPIbα translocation to lipid rafts was determined by labeling GPIbα with 6B4-488 and the lipid rafts marker GM1 with CTB-594 (5 µg/mL). GPIbα colocalization with FcγRIIa was analyzed by labeling FcγRIIa with AT10-488 (1 µg/mL) and GPIbα with 6B4-594. The fluorescence lifetimes of the donor fluorophore (6B4-488 or AT10-488) were determined in the absence and presence of acceptor fluorophore (6B4-594 or CTB-594) and subsequently used to calculate the FRET efficiency, defined as

$$FRET\ Efficiency = \frac{\tau_D - \tau_{D/A}}{\tau_D} \times 100\%$$

where τ is the donor fluorophore lifetime in nanoseconds in the absence (τ_D) and presence ($\tau_{D/A}$) of the acceptor fluorophore.

Statistical analysis

Data are means ± SEM with number of experiments (n), as indicated. Statistical analysis was performed using GraphPad Prism 5 (San Diego, CA) software. Differences between control platelets and incubations were analyzed by Mann-Whitney test. *P*-values <0.05 (*) were considered significant.

Supplementary Table

Supplementary Table S1: Hematology parameters. Coagulation assays were performed on a Sta-rack coagulation analyzer (Diagnostics Stago, Asnieres, France).

	Reference	Patient
Platelet count (10^9/L), citrate	150-450	3-29
Platelet count (10^9/L), heparin	150-450	3-20
Platelet count (10^9/L), EDTA	150-450	17
Mean platelet volume (fL)	7.0-9.5	14.8-17.7
Factor VIII:C (%)	60-150	163
VWF:Ag (%)	60-130	120-147
VWF:Ristocetin cofactor activity (%)	40-150	145-173
VWF multimer pattern	normal	normal
VWF protease (%)	62-134	97
Red Blood Cells (10^{12}/L)	3.7-5.0	4.1-4.5
White Blood Cells (10^9/L)	4 - 10	5.1-9.6
Hemoglobin (mM)	7.4-9.6	7.5-8.5
Hematocrit (fraction)	0.36-0.46	0.37-0.42
Prothrombin time (s)	11.5-14.5	12.8- 13.7
Activated partial thromboplastin time (s)	29-39	34-40
Thrombin time (s)	13.9-19.9	15.7
Bleeding time (Surgicut method, (min))	2-8.3	>30

Supplementary References

1. Huizinga EG, Tsuji S, Romijn RA, Schiphorst ME, de Groot PG, Sixma JJ, et al. Structures of glycoprotein Ibalpha and its complex with von Willebrand factor A1 domain. Science. 2002;297(5584):1176-9.
2. Fontayne A, Vanhoorelbeke K, Pareyn I, Van R, I, Meiring M, Lamprecht S, et al. Rational humanization of the powerful antithrombotic anti-GPIbalpha antibody: 6B4. Thromb Haemost. 2006;96(5):671-84.
3. Gitz E, Koekman CA, van den Heuvel DJ, Deckmyn H, Akkerman JW, Gerritsen HC, et al. Improved platelet survival after cold storage by prevention of glycoprotein Ibalpha clustering in lipid rafts. Haematologica. 2012;97(12):1873-81.
4. Korporaal SJ, Van EM, Adelmeijer J, Ijsseldijk M, Out R, Lisman T, et al. Platelet activation by oxidized low density lipoprotein is mediated by CD36 and scavenger receptor-A. Arterioscler Thromb Vasc Biol. 2007;27(11):2476-83.
5. van der Wal DE, Du VX, Lo KS, Rasmussen JT, Verhoef S, Akkerman JW. Platelet apoptosis by cold-induced glycoprotein Ibalpha clustering. J Thromb Haemost. 2010;8(11):2554-62.

CHAPTER 6

General discussion

Introduction

Although the list of biological processes in which platelets play a role is growing, their primary function is to form a temporary hemostatic plug to prevent excessive blood loss from injured vessels. Their essential contribution to hemostasis emphasizes the importance of maintaining sufficient peripheral platelet counts. An excess of platelets could lead to thrombotic complications, whereas low platelet counts result in a bleeding diathesis. Platelet counts are regulated by a balance between production, consumption in hemostatic events and clearance, mediated by the liver and spleen. Several disorders may affect this delicate balance, including sepsis, leukemia, hereditary and auto-immune diseases. Decreased platelets counts are also observed in patients undergoing chemotherapy, which has led to a significant increase in the demand for platelet transfusions since its introduction for the treatment of cancer. The increased need has challenged blood banks to optimize the storage conditions of platelet concentrates. Platelets are currently kept at room temperature, which limits their shelf life to seven days due to increased risk of bacterial infection. Cold storage may be a better alternative, but this introduces changes in glycoprotein (GP) Ibα, leading to platelet apoptosis and early clearance in vivo.

The mechanisms driving thrombus formation consist of a complex interplay between ligands, receptors, intracellular molecules and the local environment. Under high shear conditions present in arteries and arterioles, initial thrombus formation strongly depends on the interaction between the platelet receptor GPIbα and collagen-bound von Willebrand factor (VWF). Defects in this interaction are associated with an increased bleeding risk, whereas enhanced GPIbα-VWF interactions may lead to thrombotic events, including ischemic stroke and myocardial infarction. GPIbα also becomes a target for the development of autoantibodies in some patients with immune thrombocytopenia (ITP), resulting in increased platelet destruction. The underlying molecular mechanisms are however poorly understood. Unraveling the regulatory pathways of GPIbα is therefore crucial to develop therapeutic treatment strategies.

This thesis aims to increase our knowledge on GPIbα in relation to platelet storage and function. We have identified that clustering is an essential component of GPIbα regulation and studied its consequences in cold storage of platelets, platelet interaction with VWF and autoantibody binding in a patient with ITP. This chapter will reflect on these findings in a broader perspective, compare the results in relation to published literature and elaborate on future directions of GPIbα-related research.

Storage of platelet concentrates: time to chill?

Normal hemostatic activity and circulation times are essential properties of platelets that are used in transfusion medicine. Current protocols require storage of platelets at room temperature, which induces a decline in platelet function and viability known as the platelet storage lesion.[1] Efforts to improve the quality of platelet concentrates focus on storage at low temperature, which slows down metabolic activity[2] and reduces the risk of microbial infection.[3] Although cold storage better preserves hemostatic function,[4-6] chilled platelets are rapidly removed from the circulation after transfusion.[7] Cold storage causes deglycosylation of GPIbα, leading to changes in galactose and N-acetyl-D-glucosamine (GlcNAc) exposure. The damaged GPIbα molecules redistribute and become targets for the lectin binding domain of αMb2 on liver macrophages and for Ashwell-Morell receptors on hepatocytes,

thereby initiating platelet destruction.[8;9] Previous work has demonstrated that cold incubation induces apoptosis and the generation of the platelet activator thromboxane A_2 (TxA_2) upon subsequent rewarming to 37°C.[10] A consequence of apoptosis is increased surface exposure of phosphatidylserine (PS), whereas platelet activation upregulates surface expression of both PS and P-selectin. These are 'eat-me' signals for phagocytes[11] and potentially contribute to the accelerated clearance of cold-stored platelets. Chapter 2 and 3 describe the regulatory mechanism underlying cold-induced platelet apoptosis.

A novel function of arachidonic acid in cold-induced apoptosis

We have identified that cold-induced liberation of arachidonic acid (AA) facilitates initiation of the platelet apoptotic machinery (**chapter 2**). In a resting platelet at physiological temperature, this fatty acid is present in the phospholipid membrane. Platelet stimulation triggers activation of P38-mitogen-activated protein kinase (P38MAPK), resulting in the liberation of AA by cytosolic phospholipase A_2 ($cPLA_2$). The major part of released AA is metabolized by cyclooxygenase-1 (COX-1) to endoperoxides and further converted by thromboxane synthase to TxA_2. COX-1 is the target of aspirin, which blocks the access of AA to the active site of this protein by acetylation of Ser-530.[12] Liberated AA is also a substrate for 12-lipoxygenase, which converts it into hydroperoxy-eicosatetraenoic acid.[13] Based on the laws of thermodynamics, it is expected that lowering the temperature decreases the activity of these enzymes.[14] In order to convert substrate into product, enzymes must collide with and bind to the substrate at its active site. An increase in temperature enhances the number of collisions of the enzyme and substrate per unit time. Thus within limits, the rate of the reaction increases. We have provided direct and indirect evidence for activation of P38MAPK and $cPLA_2$ during cold storage of platelets. A potential explanation for this observation is that the platelet membrane undergoes a transition from a liquid-crystalline to a gel phase during cooling.[15] Studies with hibernating ground squirrels showed that the activity of membrane-associated oxidative enzymes is influenced by a conformational change induced by a temperature-dependent phase transition of membrane lipids.[16] In addition, the phase transition potentially decreases the physical distance between enzymes and substrates. A similar mechanism may regulate the cold-induced activity of P38MAPK, which together with a rise in intracellular Ca^{2+} activates $cPLA_2$, resulting in AA liberation from membrane phospholipids.

We have shown that liberated AA binds to and transfers 14-3-3ζ to the cytoplasmic tail of GPIbα. This leads to dissociation of [14-3-3ζ-Bad] complexes, Bad dephosphorylation and activation of the apoptotic machinery. The exact mechanism of how AA binding to 14-3-3ζ stimulates [14-3-3ζ-GPIbα] formation remains to be established. This 14-3-3 isoform was originally thought to have PLA activity,[17] but subsequent studies have found that 14-3-3 proteins do not have this property.[18] The authors have demonstrated that 14-3-3ζ binds AA, which is in agreement with our results. A recent study has identified [14-3-3ζ-AA] associations and demonstrated that elevated concentrations of AA induce 14-3-3ζ multimerization.[19] Together with a possible conformational change induced by AA binding, this property may enhance 14-3-3ζ interaction with the cytoplasmic tail of GPIbα.

Cold-induced GPIbα clustering and apoptosis

Previous investigations indicated that cold storage triggers a change in GPIbα surface distribution.[8;9] Electron microscopy images of the edge of the murine platelet surface indicated that GPIbα redistributes from linear arrays to cluster-like structures. A disadvantage

of this approach is that GPIbα is labeled with whole immunoglobulins (Ig), which are capable of binding two GPIbα molecules. The receptor was visualized by binding of secondary IgGs coupled to 10 nm gold particles, which limits determination of receptor colocalization to >10 nm. The authors suggested that clustering of GPIbα, which is connected to the actin cytoskeleton by Filamin A, is a result of rearrangement of actin filaments induced by cooling.[8;20] The clusters of deglycosylated GPIbα are recognized by αMβ2 receptors on macrophages and Ashwell-Morell receptor on hepatocytes, which leads to phagocytosis of cold-stored platelets. Although prevention of deglycosylation improved platelet circulation time in vivo, the authors did not address whether this affected GPIbα clustering or underlying platelet signaling events.

Chapter 3 describes the molecular mechanism by which GPIbα forms clusters upon platelet cooling and provides evidence for the involvement of GPIbα in cold-induced apoptosis. We investigated the cold-induced change in surface distribution of GPIbα by Förster Resonance Energy Transfer (FRET) using Fluorescence Lifetime Imaging Microscopy (FLIM). The use of Fab fragments to label GPIbα ruled out binding to two receptors. FRET/FLIM allows determination of protein colocalization within the range of 1-10 nm on the surface of multiple cells in one measurement. We have demonstrated that cold storage triggers GPIbα molecules to colocalize within this range. This technique does not discriminate between the presence of many clusters of small size or few clusters of larger size. GPIbα clustering may thus occur between several GPIb-V-IX complexes or within a single complex, as the complex has been described to consist of two GPIbα molecules.[21] Given that the size of a Fab is estimated to be 7 nm,[22] the actual distance of two molecules when FRET occurs may be slightly greater than 10 nm. Clustering in this context is therefore defined as the average distance of GPIbα molecules on the platelet surface being less than 15 nm.

Cold-induced clustering required deglycosylation of GPIbα ectodomain. Exposed N-acetyl-D-glucosamine (GlcNAc) residues on GPIbα associated with gangliosides in lipid rafts through carbohydrate-carbohydrate interactions. Glycosylation is a form of post-translational modification that enhances the functional diversity of proteins and influences their biological activity. The glycan structure of a protein is regulated by glycan-transferring and -hydrolyzing enzymes that are found in the endoplasmic reticulum, lysosome, cytosol or bound to the plasma membrane.[23] Analysis of surface-exposed glycans revealed a loss of sialic acid and galactose during platelet cooling. These GlcNAc-covering sugars are hydrolyzed by the enzymes neuraminidase-1 and β-galactosidase. Cold storage apparently activates these glycosidases, leading to increased GlcNAc exposure. While lowering the temperature usually decreases enzymatic activity, it may induce enzyme relocation, thereby increasing the probability of enzyme-substrate contact. Indeed, recent findings show that cold storage triggers surface upregulation of surface neuraminidase-1 and β-galactosidase, which colocalize in granule-like structures under resting conditions.[24] As we did not find increased P-selectin expression during cold storage, it is unlikely that these enzymes localize to α-granules. The identity of these granule-like structures and the exact trigger of enzyme relocation remain subjects for further studies.

An important observation was that cold-induced GPIbα clustering could be inhibited by the neuraminidase inhibitor N-Acetyl-2,3-dehydro-2-deoxyneuraminic acid (DANA). This sialic acid analog prevented exposure of GlcNAc residues, thereby blocking GPIbα association with gangliosides in lipid rafts. As a consequence, GPIbα failed to form clusters. DANA also

prevented loss of galactose residues, suggesting that the inhibitor blocked β-galactosidase from reaching its substrate. The addition of DANA prevented cold-induced apoptosis and markedly improved survival times of cold-stored platelets in mice, demonstrating the importance of GPIbα clustering in these processes. Protection by DANA could therefore be considered as a simple means to allow cold storage without inducing apoptosis and early clearance in vivo. However, platelet cooling also leads to the release and accumulation of AA, which regulates the final step of cold-induced clustering by promoting 14-3-3ζ binding to the cytoplasmic tail of GPIbα. Although clustering is abrogated when GPIbα translocation to lipid rafts is blocked by DANA, accumulated AA will be converted into TxA_2 upon platelet rewarming (chapter 2). Generation of this platelet agonist will drive surface expression of P-selectin and PS, which enhance phagocytosis.[11;25] Prevention of both GPIbα translocation to rafts (DANA) and AA accumulation (AA depletion) indeed improved platelet recovery and survival even further than after incubation with DANA alone. Importantly, both treatments did not affect the hemostatic properties, and this approach has therefore the potential to enable cold storage of platelet concentrates in the near future.

Clustering of GPIbα under (patho)physiological conditions

Dimerization or oligomerization, defined here as clustering, has been described to regulate the function of a number of receptors. Examples of platelet receptors are αIIbβ3 integrin,[26;27] which forms clusters upon ligand binding, the collagen receptor GPVI, which dimerizes upon receptor occupation[28;29] and the C-type lectin receptor (CLEC-2), which crosslinks upon activation.[30] Clustering induced by ligand binding has also been described as a feature of GPIbα. Several studies have indicated that a mutant GPIbα dimer binds VWF more effectively as compared to the monomeric form.[31;32] Clustering of GPIbα has also been reported as a result of thrombin binding[33] and by interaction with multiple echicetin molecules, a GPIbα-binding snake C-type lectin, bound to multivalent IgM antibodies.[34]

We have now established that GPIbα forms clusters upon adhesion to VWF under conditions of flow as found in arteries (**chapter 4**). Although crosslinking of receptors may have inhibitory or stimulatory effects on their function,[28;35] we have demonstrated that clustering significantly improves platelet interaction with VWF. It has been suggested that GPIbα clustering induces signaling events that lead to activation of αIIbβ3,[32] which potentially explains enhanced platelet interaction with VWF. Although we cannot rule out that sustained adhesion to VWF may result in GPIbα-mediated activation of αIIbβ3, we provide evidence that clustering directly enhances its VWF-binding capacity. Platelet agglutination or rolling experiments where VWF binding to αIIbβ3 was blocked revealed that platelet interaction with this multimeric protein was significantly improved when GPIbα was in a clustered state. How clusters of GPIbα exactly improve the interaction with VWF remains to be determined, but it is probably the result of increased avidity. Adjacent GPIbα receptors bind multiple A1 domains and may synergize in increasing the overall bond strength. The crystal structure of the GPIbα-VWF complex shows that the A1 domain of VWF covers the complete concave face of the leucine-rich repeat domain of GPIbα, which makes binding of two GPIbα molecules to a single A1 domain an unlikely event.[36;37]

The multimeric nature of VWF may explain clustering of GPIbα, as the multiple A1 domains may act as a rake, thereby bringing GPIbα molecules together. However, we have demonstrated that GPIbα clusters upon exposure to high shear force, in a ligand-independent fashion. High shear force induced its translocation to lipid rafts, where GPIbα clustered

by AA-mediated binding of 14-3-3ζ to its cytoplasmic tail. There are other receptors that cluster in a ligand-independent manner. Oligomerization of the erythropoietin receptor is primarily mediated by their transmembrane domains and does not require ligand binding.[38] In apoptosis regulation, the death receptor FasR aggregates in the absence of its ligand, resulting in activation of the caspase cascade.[39] In muscle cells, the acetylcholine receptor clusters ligand-independently through spontaneous tyrosine kinase activity.[40] Interestingly, clustering of this receptor involves 14-3-3ζ binding,[41] another property that it shares with GPIbα cluster formation.

Triggers of GPIbα clustering: similarities and differences

Clustering of GPIbα can be the result of cold storage or shear-induced binding to VWF. Both forms of clustering require GPIbα translocation to lipid rafts and both critically depend on AA-mediated [14-3-3ζ-GPIbα] associations. However, our results indicate that there are some differences in the steps that lead to cluster formation. Cold-induced translocation to rafts is the result of GPIbα deglycosylation. The exposed GlcNAc sugars on GPIbα form carbohydrate-carbohydrate interactions with raft-associated ganglioside GM3, which is blocked by both compounds. Platelet exposure to high shear does not result in a loss of sialic acid or galactose, which are the sugars that cover GlcNAc residues. Nevertheless, GPIbα translocation to lipid rafts may still be the result of sugar interactions. One possibility is that GPIbα constitutively bears a few free GlcNAc residues that associate with gangliosides. Although lectin binding suggests the presence of some GlcNAc residues on the surface of resting platelets, analysis of GPIbα-specific sugar exposure by immunoprecipitation failed to confirm the presence of incomplete glycans. Alternatively, raft translocation may be regulated by carbohydrate-carbohydrate interactions between ganglioside-bearing sugar residues and sialic acid residues present on GPIbα. The fact that exogenous GM1, which differs from GM3 in that it has an extra galactose and N-acetyl-galactosamine residue, also leads to reduction in cold-induced GPIbα raft translocation supports this theory. Although carbohydrate-carbohydrate interactions may facilitate GPIbα interaction with raft-associated gangliosides, the exact trigger of shear-induced raft translocation remains to be elucidated. Earlier investigations suggested that increased palmitoylation may trigger raft translocation,[42] but the authors later found that removal of the palmitoylation sites within the GPIb-V-IX complex had no effect on its localization to lipid rafts.[43] The same study revealed that GPIbβ and GPIX are important for raft association of GPIbα, but the mechanism initiating translocation to these membrane domains was not characterized. In light of our current observations, it could be possible that shear force temporarily affects the angle of GPIbα receptors in relation to the cell membrane, which may allow improved interaction of the GPIbα sugar residues with raft gangliosides.

After translocation to lipid rafts, GPIbα clusters by the formation of a [14-3-3ζ-GPIbα] complex. This step strongly depends on AA liberation from membrane phospholipids, which regulates the transfer of 14-3-3ζ to the cytoplasmic tail of GPIbα. Both during cold storage and exposure to shear force, AA release is the result of $cPLA_2$ activation by P38MAPK, a kinase that is responsive to alterations in thermal and shear conditions.[44;45] At physiological temperature, released AA is converted by COX-1 and Tx synthase into the platelet agonist TxA_2. Cold storage abolishes this conversion as both enzymes show little activity at 0°C. Shear-induced release of AA occurs at physiological temperature, which would allow conversion of this fatty acid into TxA_2. The fact that we did not observe platelet activation after shear exposure, argues against formation of TxA_2. The quantity of liberated AA could

be insufficient to initiate platelet activation and may preferentially associate with 14-3-3ζ. In addition, both AA and 14-3-3 proteins have been found to specifically localize to lipid rafts,[46;47] which may increase their chance to interact and allows the complex to bind and cluster raft-associated GPIbα.

Cold storage results in irreversible clustering, whereas shear exposure induces GPIbα clusters that disperse after 10 minutes of static incubation. Inhibition of both COX-1 and lipooxygenase (LOX) prevented GPIbα from dispersing post-shear. This suggests that in the absence of inhibitors these enzymes can remove AA from the [14-3-3ζ-AA] complex, resulting in 14-3-3ζ dissociation from GPIbα. Rewarming of cold-stored platelets also allows conversion of AA into TxA_2, thereby potentially releasing 14-3-3ζ from GPIbα, followed by dispersal of the receptor. The fact that GPIbα remains in a clustered state after rewarming could be a result of its cold-induced deglycosylation. The interaction between the exposed GlcNAc residues on GPIbα and raft-associated GM3 may prevent the receptor from leaving lipid rafts, thereby maintaining clustering. Alternatively, the quantity of released AA during cold incubation may be sufficient to maintain irreversible GPIbα clusters.

Shear-induced clustering of GPIbα significantly improved its interaction with VWF. Clustering induced by cold started apoptosis and resulted in early platelet clearance upon transfusion. We have not explored whether shear-induced GPIbα clustering initiates similar processes. Exposure to high shear has been reported to induce apoptotic events in platelets, but this was observed after 90 seconds of constant shear force application.[48] The time during which the receptor is clustered as a consequence of passing through a narrow artery may be insufficient to release 14-3-3ζ from pro-apoptotic Bad and start the apoptotic machinery. Binding of clustered GPIbα to VWF potentially makes clustering irreversible, as the positions of the receptors are fixed by their interaction with the VWF A1 domains. Sustained platelet adhesion to this protein may therefore initiate apoptosis. Indeed, recent data confirmed that GPIbα-dependent platelet interaction with VWF elicited upregulation of pro-apoptotic Bak and Bax, depolarization of the mitochondrial inner membrane potential and increased surface expression of PS.[49] The role of apoptotic events in thrombus formation has not been extensively studied, but it may serve as a clearance mechanism for platelets that finished participation in this process of wound healing.

Aspirin, a widely used antithombotic drug that interferes with COX-1 activity, has been described to induce apoptosis in platelets.[50] Although our studies indicate that irreversible clustering requires both COX-1 and LOX inhibition, it may be possible that the use of aspirin prolongs the presence of GPIbα clusters that activate the apoptotic pathway. Clustering will also enhance platelet interaction with VWF, which contradicts with the antithrombotic effects of aspirin use. However, the inhibitory effects of aspirin are attributed to inhibition of TxA_2-enhanced platelet activation,[51] which is important for more advanced steps in thrombus formation. Interestingly, several studies have demonstrated that GPIbα-dependent platelet adhesion actually increases upon aspirin intake.[52;53] These unexplained findings may be the result of aspirin-enhanced GPIbα clustering.

Platelet destruction by anti-GPIbα autoantibodies

Immune thrombocytopenia (ITP) is an acquired autoimmune disease characterized by autoantibody-induced platelet destruction. Most patients with ITP have a mild bleeding diathesis, but more severe bleeding complications, like intracranial hemorrhage, have also

been reported.[54-56] The primary targets of autoantibodies in ITP are the αIIbβ3 integrin (70-80%) and the glycoprotein (GP) Ib-V-IX complex (20-40%).[55;57;58] Platelet clearance induced by anti-αIIbβ3 autoantibodies is believed to be caused by the interaction between the Fc portion of platelet-associated antibodies and Fc receptors on macrophages. Blockage of macrophage Fc receptors by intravenous immunoglobulin G (IVIG) often restores platelet counts in these patients. In contrast, autoantibodies against GPIb-V-IX are associated with resistance to IVIG therapy,[59;60] and can result in a severe fall in platelet count.[61;62] It has been reported that anti-GPIb antibodies or the plasma of some patients with ITP cause platelet activation,[63-66] which may accelerate platelet destruction. In **chapter 5**, we describe that patient autoantibody binding to GPIbα leads to its clustering in lipid rafts, resulting in platelet activation through the low-affinity Fc receptor FcγRIIa.

It has previously been reported that GPIbα colocalizes with FcγRIIa upon activation.[67] Other platelet receptors that form heterotypic associations include the insulin and insulin-like growth factor-1 receptor,[68] the collagen receptor GPVI, which forms a complex with the Fc receptor γ chain (FcRγ),[69-71] and platelet endothelial cell adhesion molecule (PECAM-1), which associates with and inactivates the [GPVI-FcRγ] complex.[72] Chapter 5 reports that the association of GPIbα with FcγRIIa in lipid rafts induced by autoantibody binding initiates surface expression of P-selectin and PS. Together with the formation of GPIbα clusters, these 'eat-me' signals stimulate phagocytosis by liver and spleen macrophages,[8;11] and potentially explains why IVIG is ineffective in patients with these antibodies. The ligand-binding properties of FcγRIIa are markedly enhanced by localization to rafts, which may explain why anti-GPIbα autoantibodies efficiently activate this receptor. The finding that blockage of GPIbα translocation to rafts by the addition of GM3 inhibits FcγRIIa-induced P-selectin expression supports this notion. Preliminary data revealed similar inhibition by addition of GlcNAc or sialic acid, which illustrates the importance of carbohydrate-carbohydrate interactions for raft translocation. Prevention of autoantibody-induced raft translocation and activation of FcγRIIa by these sugars could be considered as a novel therapy in the treatment of ITP patients with antibodies against GPIbα.

Clinical implications and future directions

The observations described in the first part of this thesis have created a detailed understanding of the involvement of GPIbα clustering in cold-induced platelet apoptosis and early clearance in vivo. We have demonstrated that prevention of both GPIbα translocation to rafts and 14-3-3ζ binding to its cytoplasmic tail is important to improve platelet recovery and survival of cold-stored platelets. Prevention of GPIbα deglycosylation by DANA, or another sialic acid analog, may be a relatively simple and effective way to abolish cold-induced raft translocation. Inhibition of AA-mediated [14-3-3ζ-GPIbα] complex formation is not only important to abrogate clustering, but also for preventing platelet activation upon rewarming to 37°C post-transfusion. We have depleted AA from membrane phospholipids during cold storage using fatty acid-free albumin. Importantly, AA depletion did not affect the hemostatic functions after transfusion, indicating that platelets reincorporate extracellular AA into their phospholipid membrane. A disadvantage of this approach is that an additional washing step is required to remove excess albumin prior to transfusion. This potentially increases the risk of mechanical platelet activation during handling and may preclude this method from being implemented in transfusion medicine. An alternative approach is to prevent upstream regulators of AA liberation. The most interesting candidate for pharmacological inhibition is

P38MAPK, which is activated by cold storage and initiates AA release by stimulating $cPLA_2$ activity. The most extensively studied P38MAPK inhibitor is SB203580, which appears to induce reversible inhibition.[73] Future studies should reveal whether the combined use of a sialic acid analog and a reversible P38MAPK inhibitor allows widespread use of cold storage without affecting platelet survival times and function after transfusion.

Once implemented, the quality of cold-stored platelet concentrates can be analyzed by the presence of GPIbα clusters. The FRET/FLIM measurements performed in our studies, however, are laborious and require highly specialized equipment. Previous investigations from our laboratory showed that cold storage reduces the binding affinity of AN51 antibody directed against N-terminal aa 1-35 of GPIbα.[10;25] Comparison of both techniques revealed that the change in AN51 binding correlates with data obtained by FRET/FLIM. This method does not require the use of advanced technology, and may provide a simple and inexpensive screening method for the quality of platelet concentrates.

The second part of this thesis provides new insights into the role of GPIbα clustering in platelet adhesion to VWF. GPIbα is crucial for platelet adhesion under conditions of arterial shear force. Several studies have demonstrated that the development of ischemic stroke critically depends on GPIbα-VWF interactions.[74;75] In humans, allelic variants of GPIbα that cause enhanced interaction with VWF were associated with an increased risk of ischemic stroke.[76] Pharmacological intervention of this interaction is therefore currently explored to treat thrombotic disorders.[75;77] As complete blockade of GPIbα interaction with VWF may introduce bleeding complications, studies should carefully evaluate the inhibiting potential of the compounds of interest. Targeting GPIbα clustering could be interesting for the prevention of thrombotic events. Our experiments suggest that clustering is especially important for platelet adhesion under pathologic shear conditions. Inhibition of GPIbα clustering may therefore leave normal hemostasis unaffected, while occlusion of high shear vessels is prevented. The protective potential and methods of inhibiting GPIbα clustering remain subjects of further studies.

Another interesting question for future investigation is whether the molecular mechanism of autoantibody-induced platelet activation described in chapter 5, is similar in other ITP patients with anti-GPIbα autoantibodies. The fact that addition of GM3, but also GlcNAc or sialic acid, prevents generation of platelet destruction signals may open ways to treat these patients. Oseltamivir, a sialic acid analog, is currently used as antiviral drug to slow the spread of the influenza virus. An ITP patient who suffered from the flu was recently treated with this sialic acid analog.[78] Without intake of any other agent or blood product, her platelet count progressively increased during treatment. Future studies should investigate whether sialic acid analogs can be deployed for the treatment of ITP.

References

1. Sorensen AL, Hoffmeister KM, Wandall HH. Glycans and glycosylation of platelets: current concepts and implications for transfusion. *Curr Opin Hematol*. 2008;15(6):606-611.
2. Slichter SJ. In vitro measurements of platelet concentrates stored at 4 and 22 degree C: correlation with posttransfusion platelet viability and function. *Vox Sang*. 1981;40 Suppl 172-86.
3. Currie LM, Harper JR, Allan H, Connor J. Inhibition of cytokine accumulation and bacterial growth during storage of platelet concentrates at 4 degrees C with retention of in vitro functional activity. *Transfusion*. 1997;37(1):18-24.
4. Babic AM, Josefsson EC, Bergmeier W et al. In vitro function and phagocytosis of galactosylated platelet concentrates after long-term refrigeration. *Transfusion*. 2007;47(3):442-451.
5. Hornsey VS, Drummond O, McMillan L et al. Cold storage of pooled, buffy-coat-derived, leucoreduced platelets in plasma. *Vox Sang*. 2008;95(1):26-32.
6. Choi JW, Pai SH. Influence of storage temperature on the responsiveness of human platelets to agonists. *Ann Clin Lab Sci*. 2003;33(1):79-85.
7. Murphy S, Gardner FH. Effect of storage temperature on maintenance of platelet viability--deleterious effect of refrigerated storage. *N Engl J Med*. 1969;280(20):1094-1098.
8. Hoffmeister KM, Felbinger TW, Falet H et al. The clearance mechanism of chilled blood platelets. *Cell*. 2003;112(1):87-97.
9. Rumjantseva V, Grewal PK, Wandall HH et al. Dual roles for hepatic lectin receptors in the clearance of chilled platelets. *Nat Med*. 2009;15(11):1273-1280.
10. van der Wal DE, Du VX, Lo KS, Rasmussen JT, Verhoef S, Akkerman JW. Platelet apoptosis by cold-induced glycoprotein Ibalpha clustering. *J Thromb Haemost*. 2010;8(11):2554-2562.
11. Maugeri N, Rovere-Querini P, Evangelista V et al. Neutrophils phagocytose activated platelets in vivo: a phosphatidylserine, P-selectin, and {beta}2 integrin-dependent cell clearance program. *Blood*. 2009;113(21):5254-5265.
12. Picot D, Loll PJ, Garavito RM. The X-ray crystal structure of the membrane protein prostaglandin H2 synthase-1. *Nature*. 1994;367(6460):243-249.
13. Maskrey BH, Bermudez-Fajardo A, Morgan AH et al. Activated platelets and monocytes generate four hydroxyphosphatidylethanolamines via lipoxygenase. *J Biol Chem*. 2007;282(28):20151-20163.
14. Laidler KJ, Peterman BF. Temperature effects in enzyme kinetics. *Methods Enzymol*. 1979;63234-257.
15. Gousset K, Wolkers WF, Tsvetkova NM et al. Evidence for a physiological role for membrane rafts in human platelets. *J Cell Physiol*. 2002;190(1):117-128.
16. Raison JK, Lyons JM. Hibernation: alteration of mitochondrial membranes as a requisite for metabolism at low temperature. *Proc Natl Acad Sci U S A*. 1971;68(9):2092-2094.
17. Zupan LA, Steffens DL, Berry CA, Landt M, Gross RW. Cloning and expression of a human 14-3-3 protein mediating phospholipolysis. Identification of an arachidonoyl-enzyme intermediate during catalysis. *J Biol Chem*. 1992;267(13):8707-8710.
18. Robinson K, Jones D, Patel Y et al. Mechanism of inhibition of protein kinase C by 14-3-3 isoforms. 14-3-3 isoforms do not have phospholipase A2 activity. *Biochem J*. 1994;299 (Pt 3)853-861.
19. Brock TG. Arachidonic acid binds 14-3-3zeta, releases 14-3-3zeta from phosphorylated BAD and induces aggregation of 14-3-3zeta. *Neurochem Res*. 2008;33(5):801-807.
20. Hoffmeister KM, Falet H, Toker A, Barkalow KL, Stossel TP, Hartwig JH. Mechanisms of cold-induced platelet actin assembly. *J Biol Chem*. 2001;276(27):24751-24759.
21. Luo SZ, Mo X, fshar-Kharghan V, Srinivasan S, Lopez JA, Li R. Glycoprotein Ibalpha forms disulfide bonds with 2 glycoprotein Ibbeta subunits in the resting platelet. *Blood*. 2007;109(2):603-609.
22. Green NM. Electron microscopy of the immunoglobulins. *Adv Immunol*. 1969;111-30.
23. Monti E, Bonten E, D'Azzo A et al. Sialidases in vertebrates: a family of enzymes tailored for several cell functions. *Adv Carbohydr Chem Biochem*. 2010;64403-479.
24. Jansen AJ, Josefsson EC, Rumjantseva V et al. Desialylation accelerates platelet clearance after refrigeration and initiates GPIbalpha metalloproteinase-mediated cleavage in mice. *Blood*. 2012;119(5):1263-1273.
25. van der Wal DE, Verhoef S, Schutgens RE, Peters M, Wu Y, Akkerman JW. Role of glycoprotein Ibalpha mobility in platelet function. *Thromb Haemost*. 2010;103(5):1033-1043.

26. Fox JE, Shattil SJ, Kinlough-Rathbone RL, Richardson M, Packham MA, Sanan DA. The platelet cytoskeleton stabilizes the interaction between alphaIIbbeta3 and its ligand and induces selective movements of ligand-occupied integrin. *J Biol Chem*. 1996;271(12):7004-7011.

27. Shattil SJ, Newman PJ. Integrins: dynamic scaffolds for adhesion and signaling in platelets. *Blood*. 2004;104(6):1606-1615.

28. Jung SM, Moroi M, Soejima K et al. Constitutive dimerization of glycoprotein VI (GPVI) in resting platelets is essential for binding to collagen and activation in flowing blood. *J Biol Chem*. 2012;287(35):30000-30013.

29. Loyau S, Dumont B, Ollivier V et al. Platelet glycoprotein VI dimerization, an active process inducing receptor competence, is an indicator of platelet reactivity. *Arterioscler Thromb Vasc Biol*. 2012;32(3):778-785.

30. Hughes CE, Pollitt AY, Mori J et al. CLEC-2 activates Syk through dimerization. *Blood*. 2010;115(14):2947-2955.

31. Kasirer-Friede A, Ware J, Leng L, Marchese P, Ruggeri ZM, Shattil SJ. Lateral clustering of platelet GP Ib-IX complexes leads to up-regulation of the adhesive function of integrin alpha IIbbeta 3. *J Biol Chem*. 2002;277(14):11949-11956.

32. Arya M, Lopez JA, Romo GM et al. Glycoprotein Ib-IX-mediated activation of integrin alpha(IIb)beta(3): effects of receptor clustering and von Willebrand factor adhesion. *J Thromb Haemost*. 2003;1(6):1150-1157.

33. Celikel R, McClintock RA, Roberts JR et al. Modulation of alpha-thrombin function by distinct interactions with platelet glycoprotein Ibalpha. *Science*. 2003;301(5630):218-221.

34. Navdaev A, Dormann D, Clemetson JM, Clemetson KJ. Echicetin, a GPIb-binding snake C-type lectin from Echis carinatus, also contains a binding site for IgMkappa responsible for platelet agglutination in plasma and inducing signal transduction. *Blood*. 2001;97(8):2333-2341.

35. Ramarli D, Fox DA, Reinherz EL. Selective inhibition of interleukin 2 gene function following thymocyte antigen/major histocompatibility complex receptor crosslinking: possible thymic selection mechanism. *Proc Natl Acad Sci U S A*. 1987;84(23):8598-8602.

36. Huizinga EG, Tsuji S, Romijn RA et al. Structures of glycoprotein Ibalpha and its complex with von Willebrand factor A1 domain. *Science*. 2002;297(5584):1176-1179.

37. Uff S, Clemetson JM, Harrison T, Clemetson KJ, Emsley J. Crystal structure of the platelet glycoprotein Ib(alpha) N-terminal domain reveals an unmasking mechanism for receptor activation. *J Biol Chem*. 2002;277(38):35657-35663.

38. Constantinescu SN, Keren T, Socolovsky M, Nam H, Henis YI, Lodish HF. Ligand-independent oligomerization of cell-surface erythropoietin receptor is mediated by the transmembrane domain. *Proc Natl Acad Sci U S A*. 2001;98(8):4379-4384.

39. Beltinger C, Fulda S, Kammertoens T, Meyer E, Uckert W, Debatin KM. Herpes simplex virus thymidine kinase/ganciclovir-induced apoptosis involves ligand-independent death receptor aggregation and activation of caspases. *Proc Natl Acad Sci U S A*. 1999;96(15):8699-8704.

40. Sander A, Hesser BA, Witzemann V. MuSK induces in vivo acetylcholine receptor clusters in a ligand-independent manner. *J Cell Biol*. 2001;155(7):1287-1296.

41. Lee CW, Han J, Bamburg JR, Han L, Lynn R, Zheng JQ. Regulation of acetylcholine receptor clustering by ADF/cofilin-directed vesicular trafficking. *Nat Neurosci*. 2009;12(7):848-856.

42. Shrimpton CN, Borthakur G, Larrucea S, Cruz MA, Dong JF, Lopez JA. Localization of the adhesion receptor glycoprotein Ib-IX-V complex to lipid rafts is required for platelet adhesion and activation. *J Exp Med*. 2002;196(8):1057-1066.

43. Geng H, Xu G, Ran Y, Lopez JA, Peng Y. Platelet glycoprotein Ib beta/IX mediates glycoprotein Ib alpha localization to membrane lipid domain critical for von Willebrand factor interaction at high shear. *J Biol Chem*. 2011;286(24):21315-21323.

44. Cowan KJ, Storey KB. Mitogen-activated protein kinases: new signaling pathways functioning in cellular responses to environmental stress. *J Exp Biol*. 2003;206(Pt 7):1107-1115.

45. Sumpio BE, Yun S, Cordova AC et al. MAPKs (ERK1/2, p38) and AKT can be phosphorylated by shear stress independently of platelet endothelial cell adhesion molecule-1 (CD31) in vascular endothelial cells. *J Biol Chem*. 2005;280(12):11185-11191.

46. Pike LJ, Han X, Chung KN, Gross RW. Lipid rafts are enriched in arachidonic acid and plasmenylethanolamine and their composition is independent of caveolin-1 expression: a quantitative electrospray ionization/mass spectrometric analysis. *Biochemistry*. 2002;41(6):2075-2088.

47. Assossou O, Besson F, Rouault JP et al. Subcellular localization of 14-3-3 proteins in Toxoplasma gondii tachyzoites and evidence for a lipid raft-associated form. *FEMS Microbiol Lett*. 2003;224(2):161-168.

48. Leytin V, Allen DJ, Mykhaylov S et al. Pathologic high shear stress induces apoptosis events in human platelets. *Biochem Biophys Res Commun*. 2004;320(2):303-310.

49. Li S, Wang Z, Liao Y et al. The glycoprotein Ibalpha-von Willebrand factor interaction induces platelet apoptosis. *J Thromb Haemost*. 2010;8(2):341-350.

50. Zhao L, Zhang W, Chen M, Zhang J, Zhang M, Dai K. Aspirin Induces platelet apoptosis. *Platelets*. 2013

51. Patrono C, Garcia Rodriguez LA, Landolfi R, Baigent C. Low-dose aspirin for the prevention of atherothrombosis. *N Engl J Med*. 2005;353(22):2373-2383.

52. Turner NA, Moake JL, Kamat SG et al. Comparative real-time effects on platelet adhesion and aggregation under flowing conditions of in vivo aspirin, heparin, and monoclonal antibody fragment against glycoprotein IIb-IIIa. *Circulation*. 1995;91(5):1354-1362.

53. Grabowski EF. Platelet aggregation in flowing blood at a site of injury to an endothelial cell monolayer: quantitation and real-time imaging with the TAB monoclonal antibody. *Blood*. 1990;75(2):390-398.

54. Harrington WJ, Minnich V, Hollingsworth JW, Moore CV. Demonstration of a thrombocytopenic factor in the blood of patients with thrombocytopenic purpura. 1951. *J Lab Clin Med*. 1990;115(5):636-645.

55. McMillan R. Autoantibodies and autoantigens in chronic immune thrombocytopenic purpura. *Semin Hematol*. 2000;37(3):239-248.

56. McMillan R. The pathogenesis of chronic immune (idiopathic) thrombocytopenic purpura. *Semin Hematol*. 2000;37(1 Suppl 1):5-9.

57. McMillan R. Antiplatelet antibodies in chronic adult immune thrombocytopenic purpura: assays and epitopes. *J Pediatr Hematol Oncol*. 2003;25 Suppl 1S57-S61.

58. Beardsley DS, Ertem M. Platelet autoantibodies in immune thrombocytopenic purpura. *Transfus Sci*. 1998;19(3):237-244.

59. Webster ML, Sayeh E, Crow M et al. Relative efficacy of intravenous immunoglobulin G in ameliorating thrombocytopenia induced by antiplatelet GPIIbIIIa versus GPIbalpha antibodies. *Blood*. 2006;108(3):943-946.

60. Go RS, Johnston KL, Bruden KC. The association between platelet autoantibody specificity and response to intravenous immunoglobulin G in the treatment of patients with immune thrombocytopenia. *Haematologica*. 2007;92(2):283-284.

61. Nomura S, Yanabu M, Soga T et al. Analysis of idiopathic thrombocytopenic purpura patients with antiglycoprotein IIb/IIIa or Ib autoantibodies. *Acta Haematol*. 1991;86(1):25-30.

62. Hou M, Stockelberg D, Kutti J, Wadenvik H. Antibodies against platelet GPIb/IX, GPIIb/IIIa, and other platelet antigens in chronic idiopathic thrombocytopenic purpura. *Eur J Haematol*. 1995;55(5):307-314.

63. Cauwenberghs N, Schlammadinger A, Vauterin S et al. Fc-receptor dependent platelet aggregation induced by monoclonal antibodies against platelet glycoprotein Ib or von Willebrand factor. *Thromb Haemost*. 2001;85(4):679-685.

64. Yanabu M, Nomura S, Fukuroi T et al. Synergistic action in platelet activation induced by an antiplatelet autoantibody in ITP. *Br J Haematol*. 1991;78(1):87-93.

65. Olsson A, Andersson PO, Tengborn L, Wadenvik H. Serum from patients with chronic idiopathic thrombocytopenic purpura frequently affect the platelet function. *Thromb Res*. 2002;107(3-4):135-139.

66. Jy W, Horstman LL, Arce M, Ahn YS. Clinical significance of platelet microparticles in autoimmune thrombocytopenias. *J Lab Clin Med*. 1992;119(4):334-345.

67. Sullam PM, Hyun WC, Szollosi J, Dong J, Foss WM, Lopez JA. Physical proximity and functional interplay of the glycoprotein Ib-IX-V complex and the Fc receptor FcgammaRIIA on the platelet plasma membrane. *J Biol Chem*. 1998;273(9):5331-5336.

68. Hunter RW, Hers I. Insulin/IGF-1 hybrid receptor expression on human platelets: consequences for the effect of insulin on platelet function. *J Thromb Haemost*. 2009;7(12):2123-2130.

69. Tsuji M, Ezumi Y, Arai M, Takayama H. A novel association of Fc receptor gamma-chain with glycoprotein VI and their co-expression as a collagen receptor in human platelets. *J Biol Chem*. 1997;272(38):23528-23531.

70. Gibbins JM, Okuma M, Farndale R, Barnes M, Watson SP. Glycoprotein VI is the collagen receptor in platelets which underlies tyrosine phosphorylation of the Fc receptor gamma-chain. *FEBS Lett*. 1997;413(2):255-259.

71. Poole A, Gibbins JM, Turner M et al. The Fc receptor gamma-chain and the tyrosine kinase Syk are essential for activation of mouse platelets by collagen. *EMBO J*. 1997;16(9):2333-2341.

72. Lee FA, van LM, Relou IA et al. Lipid rafts facilitate the interaction of PECAM-1 with the glycoprotein VI-FcR gamma-chain complex in human platelets. *J Biol Chem*. 2006;281(51):39330-39338.

73. Kumar S, Jiang MS, Adams JL, Lee JC. Pyridinylimidazole compound SB 203580 inhibits the activity but not the activation of p38 mitogen-activated protein kinase. *Biochem Biophys Res Commun*. 1999;263(3):825-831.

74. De Meyer SF, Schwarz T, Deckmyn H et al. Binding of von Willebrand factor to collagen and glycoprotein Ibalpha, but not to glycoprotein IIb/IIIa, contributes to ischemic stroke in mice--brief report. *Arterioscler Thromb Vasc Biol*. 2010;30(10):1949-1951.

75. Pham M, Helluy X, Kleinschnitz C et al. Sustained reperfusion after blockade of glycoprotein-receptor-Ib in focal cerebral ischemia: an MRI study at 17.6 Tesla. *PLoS One*. 2011;6(4):e18386.

76. Maguire JM, Thakkinstian A, Sturm J et al. Polymorphisms in platelet glycoprotein 1balpha and factor VII and risk of ischemic stroke: a meta-analysis. *Stroke*. 2008;39(6):1710-1716.

77. Fontayne A, Meiring M, Lamprecht S et al. The humanized anti-glycoprotein Ib monoclonal antibody h6B4-Fab is a potent and safe antithrombotic in a high shear arterial thrombosis model in baboons. *Thromb Haemost*. 2008;100(4):670-677.

78. Alioglu B, Tasar A, Ozen C, Selver B, Dallar Y. An experience of oseltamivir phosphate (tamiflu) in a pediatric patient with chronic idiopathic thrombocytopenic purpura: a case report. *Pathophysiol Haemost Thromb*. 2010;37(2-4):55-58.

CHAPTER 7

Nederlandse samenvatting

Inleiding

Hemostase

Hemostase beschrijft het proces dat bloedverlies tegengaat bij beschadiging van een bloedvat, waarbij bloedplaatjes een essentiële rol spelen. Deze kleine, schijfvormige cellen worden geproduceerd door megakaryocyten in het beenmerg en zijn na rode bloedcellen de meest voorkomende cellen in het bloed. In samenwerking met een groot aantal eiwitten in het bloed (stollingsfactoren) vormen bloedplaatjes een prop die de beschadigde vaatwand afdicht om bloedverlies tegen te gaan. Als de cellen aan de binnenkant van de vaatwand (endotheel) beschadigd raken worden structuren blootgelegd waar bloedplaatjes aan kunnen binden, zoals collageenvezels.

Vooral in slagaderen waar bloed onder hoge snelheid door het vat stroomt, is een speciaal mechanisme nodig om voorbij vliegende bloedplaatjes tot stilstand te brengen. Hierbij is de interactie tussen het in het bloed aanwezige kleefeiwit von Willebrand factor (VWF) en de receptor glycoproteïne Ibα (GPIbα), een eiwit aanwezig op het oppervlak van bloedplaatjes, van cruciaal belang (Hoofdstuk 1; Figuur 1). Het mechanisme is te vergelijken met het landen van een straaljager op een vliegdekschip. Om tot stilstand te komen moet de piloot met behulp van een haak, bevestigd aan de onderkant van het vliegtuig, een kabel grijpen die over de landingsbaan is gespannen. GPIbα fungeert als de haak onder het vliegtuig (het bloedplaatje), terwijl VWF de rol van de kabel vervult om het bloedplaatje te vangen. Het verschil met het vliegtuig is dat bloedplaatjes niet 1 haak hebben, maar 25.000 stuks.

Na het hechten aan de beschadigde vaatwand veranderen bloedplaatjes van vorm en verklonteren ze om zo het gat perfect af te dichten. Om stevigheid aan de prop te geven wordt het bloedstollingsysteem lokaal geactiveerd. In een complexe reactie waar een veelvoud van stollingseiwitten bij betrokken zijn wordt uiteindelijk fibrine gevormd, wat als cement fungeert om de gevormde prop te stabiliseren. Nadat de vaatwand hersteld is wordt de prop weer afgebroken door een proces genaamd fibrinolyse. Het falen van een van deze processen kan leiden tot bloedingen, terwijl bij een te hoge activiteit bloedproppen kunnen ontstaan die een vat geheel afsluiten (trombose). Bekende voorbeelden hiervan zijn het hart- en herseninfarct, welke tot de belangrijkste doodsoorzaken in de westerse wereld behoren.

Het bewaren van bloedplaatjes voor transfusie

Een gezond persoon heeft tussen de 150 en 450 miljard bloedplaatjes per liter bloed. De levensduur van een bloedplaatje is ongeveer 10 dagen, waarna ze opgeruimd worden in de lever en milt. Een tekort aan bloedplaatjes (trombocytopenie) kan leiden tot levensbedreigende bloedingen. Patiënten die hier aan lijden krijgen dan ook vaak een transfusie van bloedplaatjes toegediend om het aantal op peil te houden. Trombocytopenie kan door verschillende ziekten veroorzaakt worden, waaronder infecties, bepaalde vormen van kanker en auto-immuunziekten. Omdat chemotherapie vooral ingrijpt op snel delende cellen, leidt deze behandeling ook vaak tot een tekort aan bloedplaatjes. De introductie van bloedplaatjestransfusies in de jaren zeventig heeft geresulteerd in een significante afname van sterfte als gevolg van bloedingen (<1%), maar heeft de vraag naar deze cellen sterk doen toenemen.

De toegenomen vraag heeft geleid tot het zoeken naar de optimale bewaarcondities van bloedplaatjesconcentraten. Bloedplaatjes worden momenteel bij kamertemperatuur bewaard en constant in beweging gehouden om verklonteren te voorkomen. Bewaren bij

kamertemperatuur heeft echter nadelige gevolgen voor de functie van bloedplaatjes en verhoogt de kans op bacteriële besmetting aanzienlijk. Dit kan leiden tot gevaarlijke infecties na transfusie, waardoor bloedbanken deze cellen niet langer dan vijf tot zeven dagen bewaren. Een alternatief is om bloedplaatjes bij lage temperatuur te bewaren, waardoor zowel de functies beter behouden zouden blijven als de kans op bacteriegroei sterk afneemt. Dit is vooralsnog niet mogelijk, omdat koud bewaren bloedplaatjes dusdanig beschadigt dat ze na transfusie te snel uit de circulatie verwijderd worden. De beschadigingen die optreden bij het koud bewaren worden geïnitieerd door veranderingen aan GPIbα, de bloedplaatjesreceptor voor VWF.

Bevindingen

Bloedplaatjes de koelkast in

In **hoofdstuk 2** en **3** van dit proefschrift staat het mechanisme beschreven hoe het koud bewaren van bloedplaatjes leidt tot versnelde verwijdering uit de circulatie na transfusie. Onder normale omstandigheden is GPIbα bedekt met verscheidene suikergroepen. Op het moment dat bloedplaatjes gekoeld worden naar 0-4^{o}C worden enzymen geactiveerd die deze suikergroepen er afknippen. Als gevolg hiervan verplaatsen de GPIbα-receptoren naar een specifiek deel van het celoppervlak, de zogenaamde lipid rafts. Dit zijn als het ware ‘eilandjes’ in het celmembraan bestaande uit specifieke lipiden (vetten) en eiwitten die er de voorkeur aan geven in elkaars omgeving te gaan zitten. Tegelijkertijd induceert koud bewaren veranderingen aan de binnenkant van het bloedplaatje. Binnen in de cel komt arachidonzuur vrij, een vetzuur dat bij fysiologische temperatuur door een enzym snel wordt omgezet in een vorm die belangrijk is voor het vormen van een bloedprop. Dit enzym staat bekend als cyclo-oxygenase (COX) en wordt geremd door aspirine, een medicijn dat onder andere gebruikt wordt om trombose te voorkomen. Bij lage temperatuur is COX inactief waardoor arachidonzuur niet wordt omgezet en in staat is om zich te binden aan het adaptereiwit 14-3-3ζ. Dit heeft tot gevolg dat 14-3-3ζ aan het gedeelte van GPIbα bindt dat zich aan de binnenkant van de cel bevindt (de intracellulaire staart). Omdat de GPIbα-receptoren zich verzameld hebben in lipid rafts, zorgt het binden van 14-3-3ζ ervoor dat ze nog dichter in elkaars omgeving gaan zitten (Hoofstuk 4; Supplementair Figuur S4). Dit laatste is in dit proefschrift gedefinieerd als clustervorming van GPIbα.

De verplaatsing naar lipid rafts en het clusteren van GPIbα is aangetoond met behulp van een geavanceerde microscopietechniek, genaamd Förster resonance energy transfer (FRET). Deze techniek is gebaseerd op het gebruik van antistoffen gericht tegen een bepaald eiwit (bijvoorbeeld GPIbα) waar een fluorescente kleurstof aan hangt die oplicht als deze wordt bestraald met licht van een bepaalde golflengte. Dit is het beste te vergelijken met ‘glow in the dark’ producten die licht geven nadat ze via een lichtbron aangestraald zijn. Gewone fluorescentiemicroscopie is niet toereikend om de nabijheid van twee dezelfde of verschillende eiwitten te bepalen op hele kleine schaal. Om dit toch mogelijk te maken werd gebruik gemaakt van een methode die gebaseerd is op de overdracht van fluorescente energie van de ene fluorescente kleurstof (donor) naar de andere (acceptor). Het gevolg van deze energieoverdracht (FRET) is dat de tijd waarin de donor fluoresceert afneemt. Aangezien de energieoverdracht sterk afhankelijk is van de afstand tussen de twee kleurstoffen, kan aan de hand van de levensduur van de donorfluorescentie bepaald worden of twee eiwitten op een afstand van enkele nanometers van elkaar verwijderd zijn. Door middel van fluorescence lifetime imaging microscopy (FLIM) kan die levensduur voor het gehele oppervlak van een

cel zichtbaar gemaakt worden en zo de afstand van twee eiwitten worden bepaald (Hoofdstuk 1; Figuur 6).

De gevolgen van het clusteren van GPIbα geïnduceerd door het koud bewaren van bloedplaatjes zijn tweeledig. Nadat 14-3-3ζ koppelt aan de intracellulaire staart van GPIbα wordt een proces op gang gebracht wat leidt tot geprogrammeerde celdood (apoptose). Dit is een mechanisme van cellen om zichzelf op te kunnen ruimen wanneer ze te beschadigd of niet meer nodig zijn. Bij de ontwikkeling van een embryo zorgt dit mechanisme er bijvoorbeeld voor dat de 'zwemvliezen', die bij mensen in aanleg aanwezig zijn, verdwijnen om zo vingers te vormen. Een gevolg van apoptose is dat fosfatidylserine, wat normaal aan de binnen in de cel zit, aan het oppervlak van het bloedplaatje verschijnt. Zowel geclusterd GPIbα als fosfatidylserine zijn 'eet me op'-signalen voor cellen die gespecialiseerd zijn in het verwijderen van andere cellen uit de circulatie. Deze cellen (macrofagen) zijn dan ook verantwoordelijk voor de snelle verwijdering van koud bewaarde bloedplaatjes uit de circulatie, waardoor ze niet voldoende tijd krijgen om te functioneren. Het remmen van de twee belangrijkste stappen in GPIbα clustervorming geïnduceerd door kou, namelijk de verplaatsing van GPIbα naar lipid rafts en het vrijkomen van arachidonzuur, verbetert de circulatietijd van koud bewaarde bloedplaatjes aanzienlijk. Ook blijft het vermogen van bloedplaatjes om bloedingen te stelpen onaangetast en zou dit in de nabije toekomst gebruikt kunnen worden om het koud bewaren van bloedplaatjes mogelijk te maken.

Clustervorming van GPIbα verbetert bloedplaatjesadhesie aan VWF

Het binden van bloedplaatjes aan de beschadigde vaatwand is vooral in slagaderen een uitdaging vanwege de hoge stroomsnelheid van het bloed. Omdat rode bloedcellen vele malen groter zijn zorgen deze cellen ervoor dat bloedplaatjes naar de wand van het bloedvat geduwd worden. Hierdoor zijn ze beter in staat om schade aan de vaatwand te ontdekken. De interactie tussen GPIbα en VWF stelt bloedplaatjes vervolgens in staat om op deze plek af te remmen en te binden. Door schade aan de bekleding van de vaatwand worden onderliggende collageenvezels blootgelegd. Het in het bloed aanwezige VWF bindt aan deze vezels en ontvouwt zich zodat bloedplaatjes via GPIbα in staat zijn hier aan te binden. Het bijzondere is dat de GPIbα-VWF binding sterker wordt naarmate de trekkrachten hierop toenemen. Uit eerdere bevindingen bleek dat GPIbα clusters vormt bij het koud bewaren van bloedplaatjes en in **hoofdstuk 4** staat beschreven of een vergelijkbaar mechanisme de bloedplaatjesadhesie aan VWF bevordert onder (patho)fysiologische omstandigheden.

De hoge stroomsnelheid van bloed in slagaderen zorgt voor een bepaalde wrijvingskracht die het hoogst is bij de wand van een bloedvat. Dit wordt schuifspanning (shear stress) genoemd en wordt naast de stroomsnelheid bepaald door de diameter van het bloedvat en de stroperigheid (viscositeit) van het bloed. Op het moment dat bloedplaatjes aan de beschadigde vaatwand binden, worden ze blootgesteld aan een hoge schuifspanning. In hoofdstuk 4 werden de effecten van deze schuifspanning op bloedplaatjes onderzocht door gebruik te maken van een perfusiesysteem waar onder een definieerde snelheid vers bloed doorheen stroomt. Op de perfusiekamer werd een met VWF bedekt dekglaasje geplaatst, om zo een beschadigd bloedvat na te bootsen. De adhesie van bloedplaatjes aan dit kleefeiwit werd met behulp van videomicroscopie gevisualiseerd. Het binden van bloedplaatjes aan VWF onder een schuifspanning aanwezig in slagaderen, resulteerde in clustervorming van GPIbα. Het verhogen van de schuifspanning naar een niveau die normaal gesproken alleen in vernauwde bloedvaten voor komt, bleek ook in afwezigheid van VWF de receptor te doen

clusteren. Clustervorming geïnduceerd door deze hoge schuifspanning was omkeerbaar, ging niet gepaard met bloedplaatjesactivatie en verbeterde de adhesie aan VWF.

Ook bij blootstelling aan hoge schuifspanning is zowel de verplaatsing van GPIbα naar lipid rafts als het binden van 14-3-3ζ van essentieel belang bij het vormen van clusters (Hoofdstuk 4; Figuur 6). Het verhogen van de lokale concentratie van GPIbα op het bloedplaatjesoppervlak zorgt er waarschijnlijk voor dat bloedplaatjes beter in staat zijn VWF te 'vangen' en de trekkrachten te weerstaan. Deze bevindingen benadrukken dat bloedplaatjes gevoelig zijn voor mechanische krachten en kunnen van belang zijn bij de zoektocht naar medicijnen die trombose tegengaan.

Een patiënt met hyperactieve bloedplaatjes

Immuuntrombocytopenie (ITP) is een auto-immuunziekte waarbij de patiënt antistoffen aanmaakt tegen eiwitten die aanwezig zijn op het oppervlak van bloedplaatjes. De met antistof bedekte bloedplaatjes worden herkend als niet lichaamseigen en verwijderd uit de circulatie. Het resulterende tekort aan bloedplaatjes leidt in de meeste gevallen tot een milde bloedingsneiging, maar er zijn ook gevallen van ernstige bloedingscomplicaties bekend, zoals hersenbloedingen. Bij 70-80% van de patiënten met autoantistoffen zijn deze gericht tegen de integrine αIIbβ3, de bloedplaatjesreceptor voor zowel VWF als fibrinogeen. In 20-40% van de gevallen zijn de autoantistoffen tegen het GPIb-V-IX complex gericht, waarbij patiënten vaak een ernstigere trombocytopenie hebben. Wanneer behandeling noodzakelijk is vindt dit in eerste instantie plaats met corticosteroïden en/of intraveneuze immunoglobulinen (IVIG). Corticosteroïden onderdrukken het afweersysteem van de patiënt, wat de afbraak van bloedplaatjes tegengaat. Een IVIG preparaat bestaat uit een hoge concentratie antistoffen gezuiverd uit ten minste 1000 bloeddonoren. Hoewel de werking van IVIG nog onduidelijk is, leidt het waarschijnlijk tot verzadiging van receptoren op macrofagen die antistoffen binden. Daardoor zijn deze cellen niet in staat zijn om de met antistof bedekte bloedplaatjes te verwijderen. Patiënten met antistoffen tegen het GPIb-V-IX complex lijken minder goed op deze behandeling te reageren.

In **hoofdstuk 5** staat onderzoek beschreven naar het mechanisme van bloedplaatjestekort bij een patiënte met ITP. De patiënte van 70 jaar oud had een zeer laag bloedplaatjesaantal en haar bloedplaatjes verklonterden spontaan tijdens bloedafname. Het toevoegen van een specifieke bloedplaatjesremmer vlak voor de afname maakte onderzoek naar deze cellen mogelijk. Haar bloedplaatjes verkeerden in een staat van verhoogde activiteit, wat werd afgeleid uit de toegenomen aanwezigheid van P-selectine en fosfatidylserine op het celoppervlak. Het opnemen van bloedplaatjes van gezonde donoren in het plasma van de patiënte resulteerde in dezelfde mate van verhoogde activiteit, wat bevestigde dat een component uit het plasma hiervoor verantwoordelijk was.

Het matig reageren op behandeling met IVIG duidde op de aanwezigheid van antistoffen tegen GPIbα, wat werd bevestigd. Door onderzoek naar het mechanisme werd vastgesteld dat het binden van autoantistoffen aan GPIbα leidt tot de verplaatsing van de receptor naar lipid rafts. Op deze 'eilandjes' in het celmembraan bevindt zich een andere receptor die in staat is om antistoffen te binden, genaamd FcγRIIa. De met antistof bedekte GPIbα clustert en vormt een complex met FcγRIIa in lipid rafts, wat een signaalroute aanzet die leidt tot verhoogde bloedplaatjesactiviteit. De gevormde GPIbα clusters en de verhoogde activiteit zorgen ervoor dat deze bloedplaatjes 'opgegeten' worden door macrofagen, waardoor ze te snel uit de circulatie verdwijnen. Het remmen van de autoantistof-geïnduceerde verplaatsing van GPIbα

naar lipid rafts beperkte de vorming van clusters en verhoogde bloedplaatjesactiviteit en zou wellicht in de toekomst gebruikt kunnen worden als behandeling voor ITP.

Conclusies en aanbevelingen

De resultaten beschreven in het eerste deel van dit proefschrift dragen bij aan het optimaliseren van de bewaarcondities van bloedplaatjesconcentraten voor transfusie. Het remmen van zowel de verplaatsing van GPIbα naar lipid rafts als het vrijkomen van arachidonzuur, maakt het koud bewaren van bloedplaatjes mogelijk zonder dat ze na transfusie snel uit de circulatie verwijderd worden. Hoewel de methoden waarop deze twee processen geremd worden wellicht nog geoptimaliseerd dienen te worden, lijkt de stap naar implementatie realistisch. Daarnaast zou het analyseren van GPIbα clustervorming wellicht ingezet kunnen worden om de kwaliteit van (koud bewaarde) bloedplaatjesconcentraten te controleren. In het tweede deel van het proefschrift werd duidelijk dat clustervorming van GPIbα een belangrijke rol speelt bij het binden van bloedplaatjes aan VWF onder hoge stroomsnelheid van het bloed. Momenteel wordt het remmen van de GPIbα-VWF interactie onderzocht om in te zetten als behandeling tegen trombose. Omdat het volledig platleggen van deze interactie tot bloedingen zou kunnen leiden, is het remmen van GPIbα clustervorming wellicht een interessant aangrijpingspunt om als antitromboticum verder te onderzoeken. Aangezien clustervorming van GPIbα vooral essentieel lijkt te zijn voor adhesie onder pathologische omstandigheden, is het mogelijk dat het remmen ervan trombose zou kunnen voorkomen terwijl hemostase kan blijven plaatsvinden. Bij de studie naar een patiënte met ITP werd aangetoond dat autoantistoffen gericht tegen GPIbα de receptor doet verplaatsen naar lipid rafts, waar deze clusters vormt en bloedplaatjes activeert door een complex te vormen met de receptor FcγRIIa. Het remmen van de verplaatsing naar lipid rafts belemmerde het genereren van ‘eet me op’-signalen en zou in de toekomst wellicht ingezet kunnen worden om ITP-patienten met autoantistoffen tegen GPIbα te behandelen.

CHAPTER 8

Abbreviations

488	Alexa Fluor 488
594	Alexa Fluor 594
A23187	Ca^{2+}-ionophore
aa	amino acid
AA	arachidonic acid
AACOCF$_3$	cytosolic phospholipase A_2 inhibitor
ADP	adenosine diphosphate
APAF1	apoptotic protease activating factor-1
AR-C69931MX	P_2Y_{12} blocker
ATF-2	activating transcription factor 2
Bad	Bcl-2-associated death promoter
Bak	Bcl-2 homologous antagonist/killer
Bax	Bcl-2–associated X protein
Bcl-2	B-cell lymphoma 2
Bcl-w	Bcl-2-like protein 2
Bcl-xL	B-cell lymphoma-extra large
Bid	BH3 interacting domain death agonist
BSA	bovine serum albumin
BSS	Bernard-Soulier Syndrome
cAMP	cyclic adenosine monophosphate
CCI	corrected count increment
CHO	Chinese hamster ovary
CLEC-2	C-type lectin receptor-2
CMFDA	5-chloromethyl fluorescein diacetate
COX-1	cyclooxygenase-1
cPLA$_2$	cytosolic phospholipase A_2
CRP	cross-linked collagen related peptide
CTB	cholera toxin subunit B
Cyt c	Cytochrome c
DANA	N-Acetyl-2,3-dehydro-2-deoxyneuraminic acid
DIC	differential interference contrast
DR	death domain-containing receptor
dRGDW	αIIbβ3 inhibitor
ECM	extracellular matrix
EDTA	ethylenediaminetetraacetic acid
EM	electron microscopy

ETI	5, 8, 11-eicosatriynoic acid
Fab	fragment antigen-binding
FAF	fatty acid free
Fas	tumor necrosis factor receptor superfamily, member 6
FcRγ	Fc receptor γ chain
Fg	fibrinogen
FLIM	Fluorescence Lifetime Imaging Microscopy
FRET	Förster Resonance Energy Transfer
Gal	galactose
GlcNAc	N-acetyl-D-glucosamine
Gluc	glucose
GM1	monosialotetrahexosylganglioside
GM3	monosialodihexosylganglioside
GM6001	broad-spectrum matrix metalloproteinase inhibitor
GP	glycoprotein
GPCR	G protein-coupled receptor
GPI	glycosylphosphatidylinositol
HLA	human leukocyte antigen
HT	Hepes-Tyrode's
I	fluorescence intensity
Ig	immunoglobulin
indo	indomethacin
IP	immunoprecipitation
ITAM	immunoreceptor tyrosine-based activation motif
ITP	immune thrombocytopenia
JC-1	5,5′,6,6′-tetrachloro-1,1′,3,3′-tetraethyl-imidacarbocyanine iodide
LiMo	lifetime module
LOX	lipooxygenase
LPS	lipopolysaccharide
MAPK	mitogen-activated protein kinases
Mcl-1	induced myeloid leukemia cell differentiation protein
MFI	mean fluorescence intensity
MPTP	mitochondria permeability transition pore
MPαC	N-myristoyl-SIRYSGHpSL; [14-3-3ζ-GPIbα] complex inhibitor
MαC	N-myristoyl-SIRYSGHSL; control peptide
mβCD	methyl-β-cyclodextrin

nm	nanometer
NOXA	phorbol-12-myristate-13-acetate-induced protein 1
ns	nanosecond
osge	o-sialoglycoprotein endopeptidase
PAR	protease-activated receptor
PGI_2	prostacyclin
PI3K	phosphoinositide 3-kinase
PLCγ2	phospholipase Cγ2
PRP	platelet-rich plasma
PS	phosphatidylserine
P-sel	P-selectin
PUMA	p53 upregulated modulator of apoptosis
RCA-1	ricinus communis I; galactose binding lectin
RIP	receptor-interacting protein
RT	room temperature
SA	sialic acid
SB203580	P38MAPK inhibitor
SEM	standard error of the mean
SK&F96365	P450 monooxygenase inhibitor
SNA	sambucus nigra; sialic acid binding lectin
SQ30741	thromboxane receptor inhibitor
sWGA	succinylated wheat germ agglutinin; GlcNAc binding lectin
τ	fluorescence intensity
TGF	transforming growth factor
THP-1	human acute monocytic leukemia cell line
TNF	tumor necrosis factor
TPα	thromboxane A_2 receptor α isoform
TRADD	tumor necrosis factor receptor type 1-associated DEATH domain
TRAIL	tumor necrosis factor-related apoptosis-inducing ligand
TRAP	thrombin receptor activating peptide
Tx	thromboxane
U46619	thromboxane A_2 mimetic
VWD	von Willebrand disease
VWF	von Willebrand factor
WB	Western blot
ΔΨm	mitochondrial inner membrane potential

8

Chapter 9

Dankwoord

Eindelijk, het dankwoord! Na het intensief bestuderen van het gehele proefschrift bent u vast toe aan iets luchtigere tekst. Het zit er dan nu echt bijna op. In 2007 kwam ik voor het eerst op de afdeling Laboratorium Klinische Chemie & Haematologie voor een onderzoeksstage. Tijdens het afronden van mijn studie was de keuze snel gemaakt en heb ik de mogelijkheid om op dezelfde afdeling te promoveren met beide handen aangegrepen. Hoewel het leven als onderzoeker in opleiding soms wat stress en frustraties kende (zucht.. weer een artikel afgewezen..), was het vooral een ontzettend leuke en leerzame tijd die werkelijk voorbij gevlogen is. Aangezien het een bijna onmogelijke opgave is om niemand te vergeten, wil ik bij deze graag iedereen bedanken die op wat voor manier dan ook bijgedragen heeft aan de totstandkoming van dit proefschrift. Een aantal mensen wil ik in het bijzonder bedanken.

Allereerst mijn promotor en begeleider van het eerste uur, **Jan-Willem Akkerman**. Toen ik als student bij je langs kwam voor een stage werd ik al snel gemotiveerd door je enthousiasme over wetenschap. Ik hoefde dan ook niet lang na te denken toen je me vroeg om na mijn studie als promovendus terug te komen. Tijdens werkoverleg was ik altijd onder de indruk van de manier waarop je via (bijna beroemde) illustraties complexe materie kon vereenvoudigen (of soms nog complexer kon maken). Ik wil je enorm bedanken voor je kritische en zeer nauwkeurige nakijkwerk van artikelen, de pittige discussies die we konden voeren en de (beruchte) kerstdiners, waarbij je samen met Rietje, je groepje zo gastvrij ontving.

Mijn co-promotor en begeleider van het tweede uur, **Rolf Urbanus**. Nadat Jan-Willem met pensioen ging, nam jij het stokje over. Ik bewonder de manier waarop je van begin af aan moeiteloos mee kon denken met het onderzoek en Jan-Willem soms kon overtuigen (knappe prestatie!) van het nut van bepaalde experimenten. Niet voor niets wordt je met regelmaat de ‘wandelende encyclopedie’ van de afdeling genoemd. Enorm bedankt voor al je hulp bij het schrijven van dit proefschrift, waarbij ik vooral geleerd heb hoe een boodschap kort en bondig overgebracht kan worden. Het voelt als een eer om zowel Jan-Willem zijn laatste, als jouw eerste promovendus te zijn.

Flip de Groot, ik wil je hartelijk bedanken dat ik op deze afdeling als student, analist en promovendus onderzoek heb mogen doen. Het mede mogelijk maken van het bezoeken van congressen heeft ertoe geleid dat ik nu zicht heb op een baan als postdoc in Engeland. Ik beschouw het als een eer dat jij de promotieplechtigheid voorzit.

Hans Gerritsen, mijn tweede promotor. Ik wil je ontzettend bedanken voor het beschikbaar stellen van je lab voor de vele metingen die ik heb verricht. De FRET/FLIM-experimenten vormen een essentieel onderdeel van bijna alle hoofdstukken, wat ook blijkt uit de titel die dit proefschrift draagt. Deze proeven had ik nooit kunnen doen zonder hulp van **Dave van den Heuvel**. Dave (of Mr. FRET zoals je hier wel eens wordt genoemd), bedankt voor het meedenken met de proeven, het voorbereiden van de metingen en het uitleggen van de natuurkundige principes van optica, FRET en FLIM, zodat zelfs ik er iets van begreep.

Ik wil **Hans Deckmyn** in het bijzonder bedanken voor het beschikbaar stellen van de antistoffen die de FRET-metingen mede mogelijk hebben gemaakt. **Bernard Nieswandt**, thank you for your kind invitation to join the symposium in Würzburg in 2012 and for providing the Fabs against murine GPIbα, which will be of use in the near future. **Steve Watson**, I would like to thank you for your interest in the research described in this thesis and for giving me the opportunity to come over to your lab in Birmingham.

Graag wil ik al mijn lotgenoten bedanken voor de gezellige tijd die we als AIO’s met elkaar hadden. **Thijs**, wat hebben wij een lol gehad tijdens de NVTH-cursus in Noordwijkerhout

en Koudekerke, de ISTH in Kyoto (sake en karaoke is een gouden combinatie) en het mini-oktoberfest in Wiesbaden! Ja, het leven van een AIO is zwaar… Succes met je toekomstige baan als klinisch chemicus. **Claudia**, ongeveer tegelijk met Thijs begonnen we aan onze promotie. Je ondernemende en competitieve karakter heeft ervoor gezorgd dat we met het lab allerlei leuke activiteiten hadden. De beteuterde gezichten van de mannen die weer eens een adwedstijd van je verloren spraken boekdelen! **Agon**, de man die meer over Nederlandse geschiedenis weet dan menig Nederlander. Indrukwekkend hoe snel je de taal eigen hebt gemaakt en als trotse eigenaar van een bakfiets ben je hier helemaal op je plek! **Marije**, **Peter-Paul** en **Esther**, altijd leuk om reacties uit te lokken door in jullie nabijheid te spreken over hoe artsen altijd typisch van die 'artsen' zijn. **Vivian**, knap om te zien hoe je het promoveren succesvol combineert met het moederschap. **Bert**, daar valt veel over te zeggen, maar één ding is zeker: Je bracht leven in de AIO-brouwerij! **Steven**, toch knap hoe snel je een plekje in de AIO-kamer wist te bemachtigen, terwijl er in eerste instantie geen plek voor was. Hoe kreeg je dat toch voor elkaar? **Sander** en **Susan**, alsof cellen nog niet klein genoeg zijn bestuderen jullie viesukels, zoals Sander dat zo mooi kan uitspreken. Jullie discussies zijn fantastisch: Sander, pas op wanneer Susan je bij je voornaam begint te noemen! **Marco**, als Leidse/Utrechtse AIO ben je volgens mij de ideale kandidaat om het nieuwe bestuurslid van de NVTH te worden. **Jessica**, net gestart als AIO. Veel succes gewenst, je hebt aan Rolf een goede begeleider! Alle AIO's van de **hemato-oncologie**: heel veel succes met jullie promoties! **Jonas**, jou kom ik waarschijnlijk nog wel eens tegen op een festival, verkleed als Baywatch lifeguard of iets dergelijks.

De oud-AIO's/postdocs: **Anja**, oud-stagebegeleidster en nu postdoc bij Harvard, goed bezig! Bedankt voor je hilarische discussies over wip-haist ratio (of was het nu andersom?). **Dianne**, het was een uitdaging om jouw onderzoekslijn op te volgen. Je chaotisch georganiseerde gedachtegang gaf altijd een leuke draai aan werkbesprekingen, terwijl je briljante dansvaardigheden feestjes tot een hoger niveau wisten te brengen! **Maarten P**, jouw promotie was één van de eerste die ik mocht meemaken (hier kom ik nog op terug), waarbij de fietstocht naar de feestlocatie met **Eszter** maar net goed ging :). Het werd me snel duidelijk op wat voor een gezellige afdeling ik terecht was gekomen! **Coen**, onderzoeker met een creatieve (en soms onnavolgbare) geest. Ik ken niemand die na het nuttigen van de nodige biertjes nog zo serieus over wetenschap kan praten. **Suzanne**, jouw zeer nauwkeurige uitleg en pipetteerschema's :) hebben ervoor gezorgd dat de in vivo proeven een succes waren! **Attie**, naast het promoveren ook gewoon nog even een master epidemiologie afronden, petje af! My old lab- and AIO-room neighbour **Valentina**, I do miss the need to put tape on the bench/desk to indicate which part is mine! **Judith**, oud-student en huidige AIO in Duitsland, mede dankzij jou gastvrijheid heb ik nu zicht op een baan in Engeland, dus hartelijk dank daarvoor!

Arnold, je bent een (lab)held! Het runnen van 10 blotjes en tegelijkertijd een paar vergaderingen bijwonen is voor jou kinderspel. Als coauteur op alle artikelen is het duidelijk dat je wetenschappelijke en praktische input zeer belangrijk is geweest en voelt het vertrouwd dat jij mijn paranimf bent. Ontzettend bedankt voor al je hulp! Als laatste der team Akkerman moeten we deze periode nog eens waardig afsluiten (met bier en koekmanworst zou ik zeggen!). **Arjan**, the man in black, koning van de ELISA-robot en microscopen. Onder andere dankzij jouw hulp heb ik mooie filmpjes van rollende bloedplaatjes kunnen maken! **Annet**, kletskous pur sang. Bedankt voor al je gezellige praatjes (en het toespreken van studenten :p)! **Silvie**, bedankt voor al je praktische hulp, maar ook voor het bieden van

onderdak na mijn allereerste Drum & Bass feest! **Sandra**, Lab III werd een stuk rustiger toen je wegging. Gezellig dat je er weer bent! **Brigitte**, bedankt voor je interesse en succes met al je viscometerproeven! Alle regelaars en donoren van de **minidonordienst**, bedankt voor de liters bloed die de experimenten beschreven in dit proefschrift mogelijk hebben gemaakt!

Raymond, je enthousiaste, vrolijke en positieve instelling werkt aanstekelijk. Leuk om te zien hoe je twee onderzoeksvelden bij elkaar brengt! Bedankt voor je hulp en volgens het jaarplan word je dit jaar officieel benoemd tot hoogleraar, dus alvast gefeliciteerd! **Mark**, jouw tactloze opmerkingen zijn legendarisch. Bedankt voor alle zinloze en zinvolle discussies, vooral die over shear stress natuurlijk! **Harry**, altijd geïnteresseerd en bomvol leuke (en soms onuitvoerbare) ideeën. **Richard**, ik ben benieuwd of ik ooit nog een langverwachte presentatie van je mag bijwonen! **Carin**, **Sonja** en vooral **Joukje**, hartelijk dank voor al jullie hulp bij het indienen van artikelen en zoveel andere zaken! **Albert** en **Roger**, jullie hulp bij het patiëntenstuk werd zeer gewaardeerd! **Tuna**, ik mis je scherpe opmerkingen en interessante kijk op de wetenschap tijdens onze wekelijkse werkbespreking

Met veel plezier heb ik tijdens mijn promotietraject drie studenten mogen begeleiden. **Sjoerd**, student uit het oosten van 't land (was niet te horen hoor :p). Toen ik één van de vrouwelijke studenten het woord 'knooien' hoorde gebruiken wist ik dat je bij haar in de smaak viel! Gaan we nog eens motor rijden? **Alèki**, alles veeeejs? (ik kan het nog steeds niet uitspreken) Bedankt voor het bijbrengen van de lokale Utregse straattaal en natuurlijk al die perfusieproeven die je hebt gedaan (hoewel je door de komst van nieuwe vrouwelijke studenten nog wel een afgeleid was). **Lotte**, toen je weg was realiseerde ik me des te meer hoeveel werk je hebt verzet. Ontzettend bedankt voor het perfectioneren en veelvuldig uitvoeren van de experimenten met rollende bloedplaatjes. Ik heb bij een aantal presentaties zeer dankbaar gebruik gemaakt van de mooie filmpjes die je hebt gemaakt!

Mede dankzij mijn indirecte collega's heb ik het hier zo naar mijn zin gehad. **Maarten E**, door jouw wijze en pakkende spreuken vermaakte ik me prima in het CKL. **Cor** (sleutel :P), zelfs jouw flauwe grapjes ben ik gaan waarderen. **Tineke** en **Berris**, jullie strenge (maar rechtvaardige) aanwijzingen hebben me netjes doen leren kweken. **Arno**, altijd leuk om motorverhalen uit te wisselen en ik hoop dat je me nog eens de fijne kneepjes van het whiskyproeven bijbrengt! **Michel**, als ik weer eens een computer liet crashen had jij altijd weer een oplossing, bedankt! **Maaike**, hoewel niemand zin heeft om SOP's te schrijven, weet jij er op een of andere manier toch een positieve draai aan te geven (en dat is knap!). **Rosmina**, bedankt voor al je hulp en veel geluk gewenst met je terugkeer naar Indonesië. Collega's van de **speciële hematologie**, bedankt dat ik jullie altijd weer kon lastig vallen met de vele metingen op de Cell-Dyn! Collega's van de **AMI**, zonder het gebruik van jullie cytospin-apparaat had ik nooit al die FRET-proeven kunnen doen, dus veel dank daarvoor!

Na een week hard werken is het fijn om bij je vrienden terecht te kunnen voor de nodige ontspanning! **Joost**, maatje en partner in crime tijdens vele leuke activiteiten (festivallen, snowboarden, noem maar op). Fijn dat je me deze dag wil ondersteunen als paranimf (die pinguïnpakken staan ons vast goed)! **Tim**, non-stop maatje sinds de kleuterklas. Wat hebben wij samen al een hoop meegemaakt! Ondertussen ben je vader van een schat van een meid en wat staat die rol je goed! **Erik**, grapjas en echte sfeermaker! Bijzonder hoe jij altijd klaar staat om vrienden uit de brand te helpen! Bedankt alle **vriendjes** en **vriendinnetjes** voor de leuke dingen die we met elkaar ondernemen, maar ook voor jullie luisterend oor als ik weer eens een poging doe om uit te leggen wat ik nou precies onderzoek :) Op de squashbaan kon

ik mijn opgebouwde energie (en soms frustratie en agressie) kwijt na een dag op het lab. **Squashmaatjes**, bedankt voor de vele pogingen die ik heb mogen doen om jullie van de baan te slaan!

Lieve **pa** en **ma**, zonder jullie had ik hier nu niet gestaan. Bedankt voor al jullie steun en liefde. Wat is het toch fijn om zo warm ontvangen te worden wanneer we bij jullie langskomen. Pa, bedankt dat je me van jongs af aan hebt betrokken bij al je leuke en sportieve hobby's. Dokter worden heb je me nooit aangeraden, maar als doctor ben ik toch nog een (klein) beetje in je voetsporen getreden. Mama, ongelooflijk hoe sterk jij bent en wat ben ik dankbaar dat jij als trotse moeder me deze dag zal bijstaan. Ik ben zeker zo trots op jou als jij op mij! **Caroline**, wat ben ik blij met jou als zus! Hoewel we qua karakter van elkaar verschillen, zijn we toch de grootste maatjes! Niet alleen delen we dezelfde vriendengroep, maar vertonen we stiekem allebei meer Gitzentrekjes dan we zelf denken/willen :P En wat een mooie zoon hebben jij en **Bart** gekregen. Supertrots ben ik op mijn grote, kleine neefje **Robin** en ik beschouw het als een grote eer dat hij mijn naam draagt. Ik ben benieuwd wat jij gaat doen als je later groot bent! **Robert**, mijn broer op afstand, tijd om die eens kleiner te maken. Mijn schoonfamilie, **John**, **Els** en **Paul**, bedankt dat ik zo hartelijk ben opgenomen in jullie gezin en dat ik met jullie dochter mag trouwen!

Het allermooiste wat mijn tijd op de afdeling me heeft gebracht, mijn liefste **Jerney**. Schat, wat ben ik toch blij dat wij elkaar gevonden hebben! Wie mag ik hier dankbaar voor zijn? De dames van de speciële hematologie, omdat ze jou destijds hebben aangenomen? Jan-Willem, omdat ik bij hem stage mocht lopen? Maarten P, omdat hij zo vriendelijk was ons allebei uit te nodigen voor zijn promotiefeest, waar we elkaar voor het eerst spraken? Of Coen, omdat hij me (ongevraagd?) meenam om bij jou thuis plaatjes te draaien? Jazeker, maar bovenal ben ik jou dankbaar. Wat zorg je toch goed voor me en wat hebben we het fijn samen! Ik ben zo benieuwd naar wat de toekomst ons zal brengen, maar samen met jou weet ik zeker dat het goed is!

En nog zal ik misschien mensen vergeten zijn, iedereen bedankt!

CHAPTER 10

List of publications & curriculum vitae

Journal articles

Urbanus RT, van der Wal DE, Koekman CA, Huisman A, van den Heuvel DJ, Gerritsen HC, Deckmyn H, Akkerman JW, Schutgens RE, Gitz E. Patient autoantibodies induce platelet destruction signals via raft-associated glycoprotein Ibα and FcγRIIa in immune thrombocytopenia. Accepted for publication in *Haematologica*. 2013

Gerrits AJ, Gitz E, Koekman CA, Visseren FL, van Haeften TW, Akkerman JW. Induction of insulin resistance by the adipokines resistin, leptin, plasminogen activator inhibitor-1 and retinol binding protein 4 in human megakaryocytes. *Haematologica*. 2012;97(8):1149-1157

Gitz E, Koekman CA, van den Heuvel DJ, Deckmyn H, Akkerman JW, Gerritsen HC, Urbanus RT. Improved platelet survival after cold storage by prevention of glycoprotein Ibα clustering in lipid rafts. *Haematologica*. 2012;97(12):1873-1881

Gitz E*, van der Wal DE*, Du VX, Lo KS, Koekman CA, Versteeg S, Akkerman JW. Arachidonic acid depletion extends survival of cold-stored platelets by interfering with the [glycoprotein Ibα-14-3-3ζ] association. *Haematologica*. 2012;97(10):1514-1522. *Both authors contributed equally to this study

Zipperle AM, Coyer JA, Reise K, Gitz E, Stam WT, Olsen JL. Clonal architecture in an intertidal bed of the dwarf eelgrass Zostera noltii in the Northern Wadden Sea: persistence through extreme physical perturbation and the importance of a seed bank. *Marine biology*. 2009;156(10): 2139-2148

Abstracts

Gitz E, van der Wal DE, Koekman CA, Huisman A, van den Heuvel DJ, Gerritsen HC, Deckmyn H, Akkerman JW, Schutgens RE, Urbanus RT. Autoantibody binding to glycoprotein Ibα induces FcγRIIa-mediated platelet activation in a patient with immune thrombocytopenia. Selected for oral presentation at the *Symposium of the Netherlands Society on Thrombosis and Haemostasis*, Koudekerke, the Netherlands, 2013

Gitz E, van der Wal DE, Koekman CA, Huisman A, van den Heuvel DJ, Gerritsen HC, Deckmyn H, Akkerman JW, Schutgens RE, Urbanus RT. Autoantibody binding to glycoprotein Ibα induces FcγRIIa-mediated platelet activation in a patient with immune thrombocytopenia. Selected for oral presentation at the *24th Congress of the International Society on Thrombosis and Haemostasis*, Amsterdam, the Netherlands, 2013

Gitz E, Koopman CD, Koekman CA, van den Heuvel DJ, Deckmyn H, Akkerman JW, Gerritsen HC, Urbanus RT. Platelet interaction with von Willebrand factor is enhanced by shear-induced clustering of glycoprotein Ibα. Selected for oral presentation at the *24th Congress of the International Society on Thrombosis and Haemostasis*, Amsterdam, the Netherlands, 2013

Gitz E, van den Heuvel DJ, Deckmyn H, Gerritsen HC, Akkerman JW, Urbanus RT. Shear-induced binding of platelets to von Willebrand factor is enhanced by clustering of Glycoprotein Ibα. Oral presentation at the *17th International Vascular Biology Meeting*, Wiesbaden, Germany, 2012

Gitz E, van den Heuvel DJ, Deckmyn H, Gerritsen HC, Akkerman JW, Urbanus RT. Shear-induced binding of platelets to von Willebrand factor is enhanced by clustering of Glycoprotein Ibα. Oral presentation at the *symposium of the Netherlands Society on Thrombosis and Haemostasis*, Koudekerke, the Netherlands, 2012

Gitz E, van den Heuvel DJ, Deckmyn H, Gerritsen HC, Akkerman JW. Glycoprotein Ibα is a mechanoreceptor which clusters upon cold storage and hydrodynamic force. Poster presentation at the *23rd Congress of the International Society on Thrombosis and Haemostasis*, Kyoto, Japan, 2011

Gitz E, van den Heuvel DJ, Deckmyn H, Gerritsen HC, Akkerman JW. Glycoprotein Ibα is a mechanoreceptor which clusters upon cold storage and hydrodynamic force. Oral presentation at the *Symposium of the Netherlands Society on Thrombosis and Haemostasis*, Koudekerke, the Netherlands, 2011

Gitz E, van den Heuvel DJ, Deckmyn H, Gerritsen HC, Akkerman JW. Glycoprotein Ibα is a mechanoreceptor which clusters upon cold storage and hydrodynamic force. Oral presentation at the *4th Sanquin Spring Seminars*, Amsterdam, the Netherlands, 2011

Gitz E, van der Wal DE, van den Heuvel DJ, Bader A, Akkerman JW. Detection of platelet glycoprotein Ibα clusters by fluorescence lifetime imaging. Oral presentation at the *Joint symposium of the Netherlands Society on Thrombosis and Hemostasis/British Society for Haemostasis & Thrombosis*, Noordwijkerhout, the Netherlands, 2010

Curriculum vitae

Nederlands

De schrijver van dit proefschrift werd geboren op 27 juli 1984 te Ermelo. Na het behalen van het havodiploma aan het Christelijk College Groevenbeek te Ermelo, is hij in 2002 begonnen met de studie Biotechnologie aan het van Hall Instituut te Leeuwarden. Gedurende deze studie liep hij stage bij de Rijksuniversiteit Groningen binnen het vakgebied mariene biologie en bij het Universitair Medisch Centrum Groningen, waar onderzoek naar stamcellen werd verricht. In 2006 werd de studie succesvol afgerond en doorliep hij het premasterprogramma van de studie Biological Sciences aan de Rijksuniversiteit Groningen. In 2007 begon hij met de master Biomolecular Sciences aan de Universiteit Utrecht. Als onderdeel van deze studie begon hij in hetzelfde jaar aan een onderzoeksstage bij de afdeling Klinische Chemie & Haematologie in het Universitair Medisch Centrum Utrecht onder begeleiding van prof.dr. J.W.N. Akkerman. Na het afronden van de master in 2009 begon hij als onderzoeker in opleiding bij dezelfde afdeling, onder begeleiding van prof.dr. J.W.N. Akkerman, prof.dr. H.C. Gerritsen en dr. R.T. Urbanus. De resultaten hiervan staan beschreven in dit proefschrift. In de toekomst zal hij zijn wetenschappelijke carrière voortzetten.

English

The author of this thesis was born on the 27th of July, 1984 in Ermelo, the Netherlands. In 2002, he finished high school at the Christelijk College Groevenbeek in Ermelo, and started with the study biotechnology at the van Hall Instituut in Leeuwarden. During this study, he completed an internship within the field of marine biology at the Rijksuniversiteit Groningen and within the field of biomedical sciences at the University Medical Center Groningen. After finishing this study in 2006, he started with the premaster programme of the study Biological Sciences at the Rijksuniversiteit Groningen. After completing this progamme in 2007, he moved to Utrecht and started with the master Biomolecular Sciences at Utrecht University. His major research project was carried out at the Department of Clinical Chemistry and Haematology at the University Medical Center Utrecht under supervision of prof.dr. J.W.N. Akkerman. After obtaining his master degree in 2009, he started with a PhD project at the same department under supervision of prof.dr. J.W.N. Akkerman, prof.dr. H.C. Gerritsen and dr. R.T. Urbanus. The results of this project are described in this thesis. In the future, he will continue his scientific career.