

# Dual roles for hepatic lectin receptors in the clearance of chilled platelets

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## ABSTRACT.

Platelets for transfusion are stored at room temperature because refrigerated platelets are cleared rapidly from circulation. Room temperature storage causes major inconveniences for blood banks and medical risks for recipients. To address this clinically important problem a macrophage-dependent clearance mechanism was defined for platelets refrigerated < 4 h in buffer. Short-term platelet cooling (< 4 h) leads to exposure of  $\beta$ GlcNAc residues on GPIIb $\alpha$ . Exposed  $\beta$ GlcNAc are recognized by lectin domains on hepatic macrophages  $\alpha_M\beta_2$ , which leads to increased clearance. Capping of exposed  $\beta$ GlcNAc by galactosylation using endogenous galactosyl transferases prevented clearance of short-term cooled platelets. In a follow up clinical study, galactosylation did not improve the circulation of human platelets stored in plasma at 4°C for > 48 h (**Paper 1**). To investigate this different outcome we developed a mouse model for studies of storage of platelet-rich plasma (PRP) at 4°C for 48 h. *In vitro* characterization of the 4°C stored mouse platelets showed similar changes as measured on human platelets after 48 h of refrigeration. Importantly, using the mouse model we demonstrated that galactosylation has no effect on the survival of > 48 h long-term refrigerated mouse platelets (**Paper 1**).

Using the mouse platelet storage model we dissected the clearance mechanism of platelets stored for > 48 h in plasma at 4°C, showing that 48 h refrigerated platelets are removed by an additional macrophage-independent mechanism, i.e. by the hepatic galactose-dependent lectin the Ashwell-Morell Receptor (AMR) (**Paper 2**). This conclusion was based on the following evidence: (a) macrophage depletion in mice dramatically improved the circulation of 4 h chilled platelets, but not clearance of 48 h refrigerated platelets; (b) streptavidin-POD staining revealed the localization of long-term refrigerated biotinylated platelets in hepatocytes; (c) 48 h refrigeration tremendously increased the binding of the  $\beta$ galactose-recognizing lectin RCA-I to platelets; (d) KO mice lacking *Asgpr-1* or *Asgpr-2* subunits of the AMR supported 48 h refrigerated platelet circulation; (e) co-injection of asialofetuin, a competitive inhibitor of the AMR, restored the survival of 48 h refrigerated platelets but not of isolated platelets stored for <4 h; (f) ingestion of fluorescently labeled long-term refrigerated platelets in a hepatocyte culture was prevented by asialofetuin. Circulation of 48 h platelets was markedly improved by removal of the N-terminal domain of GPIIb $\alpha$  using O-sialopeptidase (**Paper 2**).

Platelets from mice lacking the sialyltransferase ST3GalIV were used to investigate the increased clearance of platelets with decreased sialylation. We demonstrated that platelets with increased galactose exposure due to the lack of ST3GalIV expression are also removed by a macrophage-independent mechanism, through AMRs on hepatocytes (**Paper 3**).

In conclusion, our results defined a new hepatic-based clearance mechanism for desialylated platelets, representing the first example of blood cell removal by a non-myeloid cell.

**Keywords:** platelet storage, Asialoglycoprotein receptor, GPIIb $\alpha$ ,  $\alpha_M\beta_2$ , transfusion, clearance

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## **LIST OF PUBLICATIONS.**

### **I. Galactosylation does not prevent the rapid clearance of long-term, 4°C-stored platelets**

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### **III. 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**

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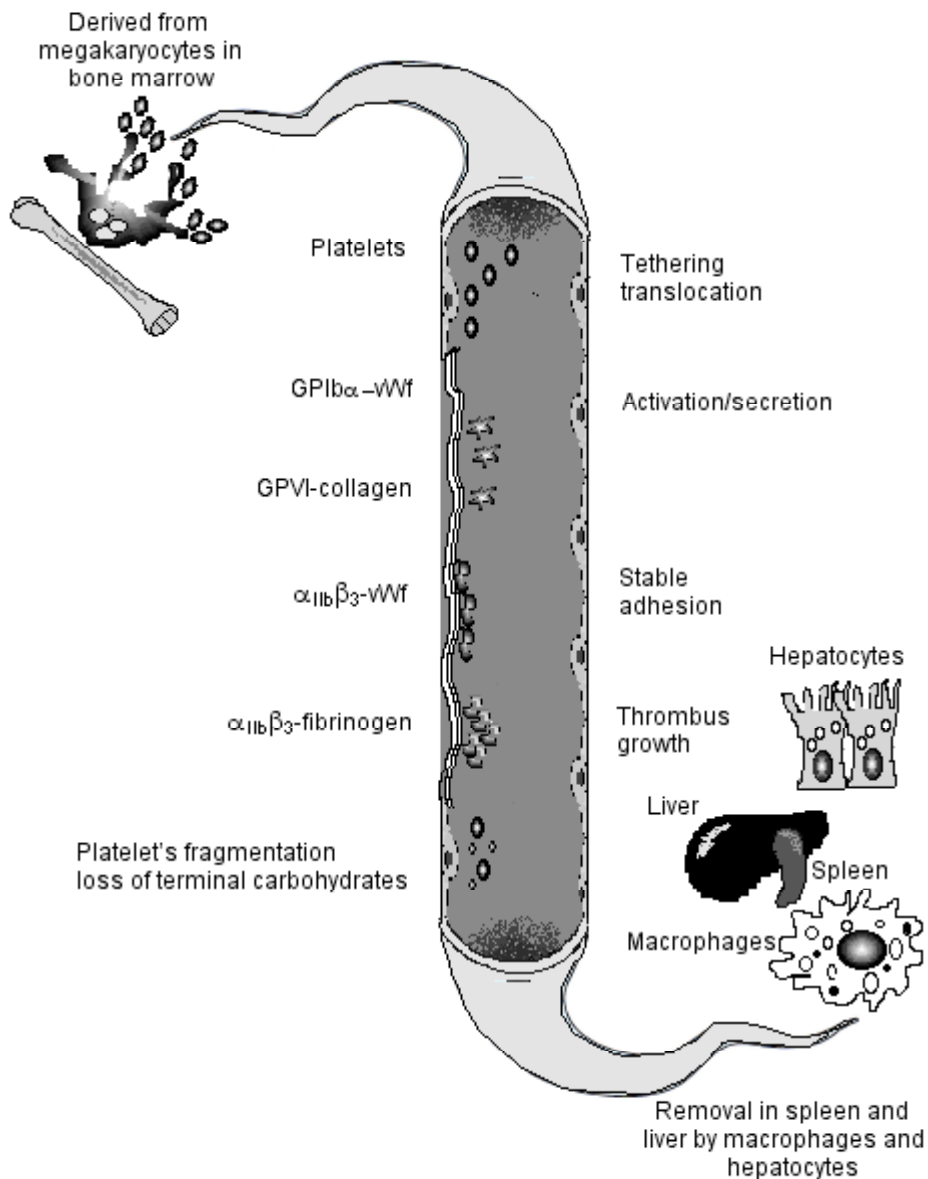
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## ABBREVIATIONS.

<b>Ab</b>	antibody
<b><math>\alpha_{IIb}\beta_3</math></b>	(GPIIb-IIIa, CD41/CD61)
<b><math>\alpha_M\beta_2</math></b>	(Mac-1, CR3, CD11b/CD18)
<b>Asgpr</b>	Asialoglycoprotein receptor
<b>AMR</b>	Ashwell-Morell receptor
<b><math>\beta</math>-Gal</b>	$\beta$ galactose
<b><math>\beta</math>-GlcNAc</b>	$\beta$ -D-GlcNAc-1-Me, methylated $\beta$ -N-acetylglucosamine
<b>CMTMR</b>	5-(and-6)-(((4-chloromethyl)benzoyl)amino)tetramethylrhodamine
<b>CMFDA</b>	5-chloromethylfluorescein diacetate
<b>CRP</b>	collagen related peptide
<b>C-T</b>	carboxyl-terminal
<b>DMS</b>	demarcation membrane system
<b>EM</b>	electron microscopy
<b>FcR</b>	Fc receptor
<b>FITC</b>	fluorescein isothiocyanate
<b>GPIb<math>\alpha</math></b>	glycoprotein Ib $\alpha$
<b>GT</b>	glycosyltransferase
<b>h</b>	hours
<b>HepG2</b>	hepatocellular carcinoma, human
<b>IP</b>	immunoprecipitation
<b>ICAM</b>	intercellular adhesion molecule
<b>ITAM</b>	immunoreceptor tyrosine-based activation motif
<b>KO</b>	knock out
<b>LRR</b>	leucine rich repeat
<b>Man</b>	mannose
<b>mAb</b>	monoclonal antibody
<b>Min</b>	minute
<b>MGL</b>	macrophage galactose lectin
<b>N-T</b>	amino-terminal
<b>OCS</b>	open canalicular system
<b>POD</b>	peroxidase
<b>PAR</b>	protease activated receptor
<b>PE</b>	phycoerythrin
<b>PRP</b>	platelet rich plasma
<b>PS</b>	phosphatidyl serine
<b>RCA-I</b>	ricinus communis agglutinin
<b>RT</b>	room-temperature
<b>SR</b>	scavenger receptor
<b>s-WGA</b>	succinylated wheat germ agglutinin
<b>THP-1 cells</b>	human monocytic cell line
<b>TRAP</b>	thrombin receptor activating peptide
<b>WT</b>	wild-type
<b>vWf</b>	von Willebrand factor
<b>vWfR</b>	von Willebrand factor receptor, (GPIb $_{\alpha,\beta}$ /IX) $_2$ V

## INTRODUCTION TO THE PLATELET LIFE CYCLE.



**Fig. 1 Platelet lifespan.** Platelets derive from precursor bone marrow megakaryocytes and are released as anuclear discs into the bloodstream. Upon injury or inflammation, platelet rolling is initiated by binding of  $\text{GPIb}\alpha$  (the  $\alpha$ -chain of the von Willebrand factor receptor complex) to von Willebrand factor (vWf) exposed on subendothelium. Further platelet adhesion and activation is mediated through  $\text{GPVI}$  binding to collagen, mediating platelet shape change, activation and granule release. Firm platelet adhesion is substantiated by  $\alpha_{\text{IIb}}\beta_3$ -integrin binding to vWf and fibrinogen, inducing further platelet activation and thrombus formation. During their lifespan platelets fragment, lose terminal glycan residues such as sialic acids, and are eventually removed by macrophages and hepatocytes in the liver and spleen.



Platelets, anuclear discoid cell fragments that measure 2-4  $\mu\text{m}$  in diameter, are released from megakaryocytes and circulate in blood as small discs with a half-life of 8-10 days in humans and 4-5 days in mice. Unequivocally, platelets are crucial for hemostasis and coagulation. Emerging literature increasingly suggests that platelets not only regulate hemostatic events but also play a substantial role in inflammation, metastasis and tumor growth. The following chapters will briefly address platelet birth, life and death (Fig. 1).

### ***Platelet birth.***

Megakaryocytes differentiate from hematopoietic stem cell precursor cells in the bone marrow [1-3]. The primary signal for megakaryocyte production and differentiation is thrombopoietin (TPO). TPO is sufficient but not necessary [4] for inducing differentiation of progenitor cells in the bone marrow towards a final megakaryocyte phenotype. Other molecular signals for megakaryocyte differentiation include GM-CSF, IL-3, IL-6, IL-11, chemokines (SDF-1; FGF-4) [5], and erythropoietin [6]. During their differentiation, megakaryocytes undergo endomitosis and become polyploid through repeated cycles of DNA replication without cell division [7]. The megakaryocyte cytoplasm continues to expand and the DNA complement can increase up to 64N.

After the process of endomitosis is completed, the megakaryocyte begins a maturation stage in which the cytoplasm rapidly fills with platelet-specific proteins, organelles and membrane systems that will be ultimately subdivided and packaged into platelets. During the stage of maturation the megakaryocyte acquires its distinct ultrastructural features, including the development of a demarcation membrane system, the assembly of a dense tubular system, and the formation of granules [8]. However, the mechanisms by which megakaryocytes form and release platelets remain poorly understood. Interestingly, there is a remarkable contrast between the relative paucity of megakaryocytes in normal bone marrow and the relative abundance of platelets in peripheral blood. This contrast implies that platelet assembly and release processes are highly dynamic and efficient.

The prevalent model for thrombopoiesis is the proplatelet model [2, 9], which receives support mostly from megakaryocyte differentiation cultures [10, 11]. This model proposes that megakaryocytes extend plump pseudopodia that give rise to long ( $>100\text{-}\mu\text{m}$ ) branched proplatelet processes that appear "beaded" by virtue of intermediate swellings [10-12]. It is unclear whether proplatelets detach from megakaryocytes in a bulk and fragment further into platelets [6], or whether barbell-shaped platelet pairs detach exclusively from proplatelet ends [12]. The second model is the platelet territory model which proposes that "platelet-vesicles" form internally from demarcation membranes and are released as mature platelets into the blood stream [13, 14]. However, recently platelet production was observed *in vivo*, confirming the concept of proplatelet formation *in vivo* [15]. The investigators observed that megakaryocytes extended dynamic proplatelet-like protrusions into microvessels which appeared to be sheared from their trans-endothelial stems by flowing blood, resulting in the appearance of proplatelets in peripheral blood [15]. However, this observation remains purely descriptive and lacks insight into the mechanism of platelet formation.

## ***Platelet life.***

Platelet dysfunction or low blood count predisposes to hemorrhage, while platelet hyper-activation increases the risk of thrombosis. Once platelets are activated, they respond rapidly and in a sequential fashion to chemical and physical agents by changing shape, binding, spreading, secreting granules and aggregating to promote wound closure and healing [16, 17]. The following section will briefly address the complex process of granule release and the function of major platelet receptors (Fig. 1).

### **Platelet receptors.**

Platelet adhesion, activation and aggregation are mediated by specific surface receptors that include integrins, such as the fibrinogen receptor  $\alpha$ IIb $\beta$ <sub>3</sub>; the leucine-rich von Willebrand factor receptor, (GPIb <sub>$\alpha,\beta$</sub> /IX)<sub>2</sub>V; and the immunoglobulin-family receptors such as GPVI and P-selectin. The major glycoproteins in circulating platelets are the Willebrand factor receptor (vWfR) and the fibrinogen receptor  $\alpha$ <sub>IIb</sub> $\beta$ <sub>3</sub> (GPIIb-IIIa or CD41/CD61) which interact with both soluble and tethered ligands to activate platelets[18].

#### ***The $\alpha$ <sub>IIb</sub> $\beta$ <sub>3</sub> integrin.***

Integrins are non-covalent heterodimers composed of  $\alpha$ - and  $\beta$ -subunits that mediate cell-cell and cell-matrix interactions.  $\alpha$ <sub>IIb</sub> $\beta$ <sub>3</sub> integrin is a calcium-dependent receptor for fibrinogen, and it is essential for platelet aggregation.  $\alpha$ <sub>IIb</sub> $\beta$ <sub>3</sub> integrin expression is restricted to platelets and megakaryocytes.  $\alpha$ <sub>IIb</sub> $\beta$ <sub>3</sub> has a very low affinity for fibrinogen on resting platelets [19]; however, after platelet activation,  $\alpha$ <sub>IIb</sub> $\beta$ <sub>3</sub> receives signals from inside the cell to rapidly change its conformation to bind fibrinogen with high affinity. Its absence or deficiency leads to Glanzmann thrombocytopenia, a rare hematopoietic disorder associated with moderate to severe bleeding tendency and normal platelet morphology [18].

#### ***G-coupled receptors.***

Thrombin is a potent activator of platelets *in vivo*. When added to platelets *in vitro*, it causes phosphoinositide hydrolysis, which leads to an increase in intracellular Ca<sup>2+</sup> concentration, shape change, granule release, and aggregation. Thrombin also suppresses cAMP synthesis in platelets by inhibiting adenylate cyclase [20]. All of these effects require that thrombin be proteolytically active. The PAR class of receptors has a distinctive mechanism of activation involving specific cleavage of the N-terminal extracellular domain. This exposes a new N-terminus, which by refolding acts as a ligand to the receptor. The first human thrombin receptor to be identified was PAR-1 [21]. Subsequently, other PAR receptors, PAR-2[22], PAR-3 [23] and PAR-4 [24] have been identified. Mouse platelets express only PAR-3 and PAR-4 while human platelets express PAR- 1 and PAR-4, although PAR-1 appears to be the primary thrombin receptor on human platelets at low thrombin concentrations. The differential role of these receptors remains to be elucidated.

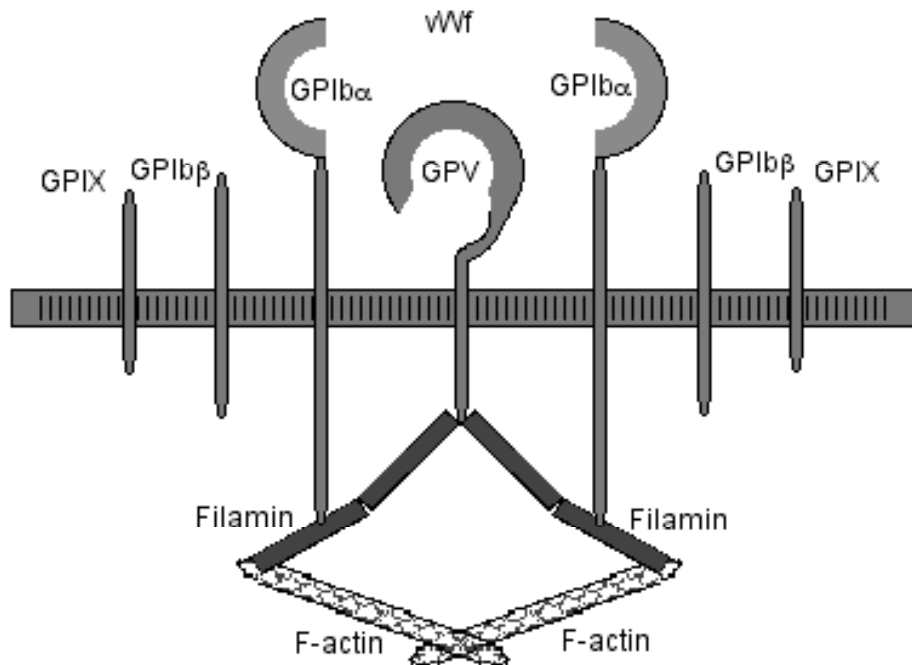
#### ***P-selectin and PSGL-1.***

Selectins are cell adhesion molecules expressed on platelets, endothelial cells and lymphocytes. They belong to the C-type lectin family. There are three types of selectins: P-,

E- and L-selectins. Platelets and endothelial cells express P-selectin on the membrane of their  $\alpha$ -granules and in Weibel-Palade bodies, respectively. P-selectin moves to the membrane surface during platelet and endothelial cell activation and plays an essential role in the initial recruitment of white blood cells to the site of injury during inflammation [25]. A well-characterized ligand for P-selectin is PSGL-1, which is a mucin-type glycoprotein expressed on all white blood cells as well as on platelets [25, 26]. The affinity between the lectin-like domain of P-selectin and carbohydrate residues and sulfated tyrosines present on PSGL-1 mediates the adhesion and rolling of white blood cells along the blood vessel wall and allows white blood cells to leave the blood vessel and enter the site of inflammation [27].

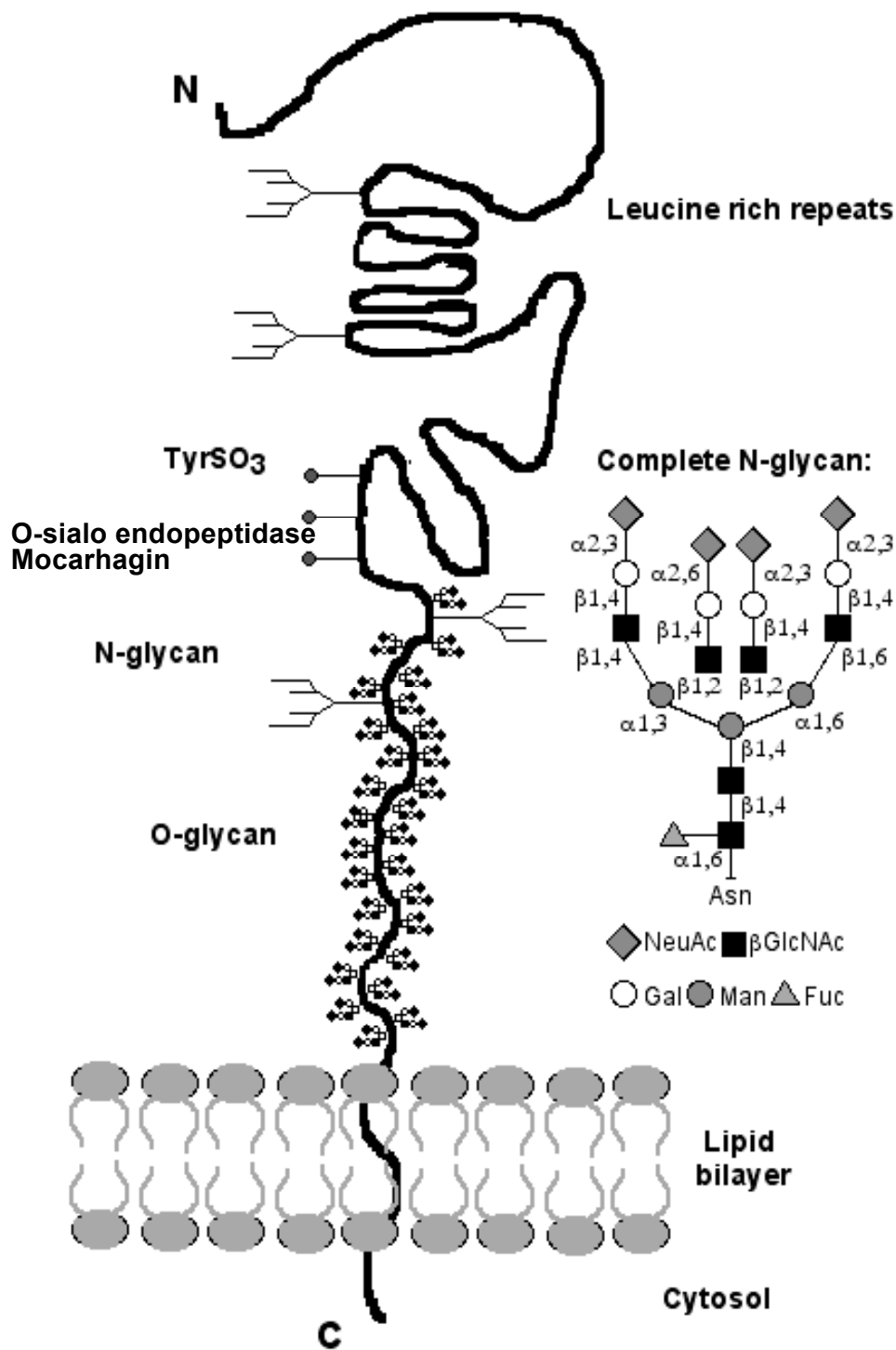
### The von Willebrand factor receptor, (GPIb $_{\alpha,\beta}$ /IX) $_2$ V.

The von Willebrand factor receptor, (GPIb $_{\alpha,\beta}$ /IX) $_2$ V, is a complex of 4 polypeptides: GPIb $_{\alpha}$ , GPIb $_{\beta}$ , GPIX and GPV [28-30] (Fig. 2). It belongs to the leucine-rich family of proteins and consists of four distinct transmembrane subunits: GPIb $_{\alpha}$ , connected to GPIb $_{\beta}$  by a disulfide bond, and the non-covalently associated GPIX and GPV. This receptor complex is present at



**Fig. 2 Von Willebrand factor receptor (vWfR) complex.** The vWfR complex consists of four subunits GPIb $_{\alpha}$ , GPIb $_{\beta}$ , GPIX and GPV. Filamin binds to the cytoplasmic tail of the GPIb $_{\alpha}$  subunit and links the vWfR complex to actin cytoskeleton (F-actin). vWf binds to GPIb $_{\alpha}$  under high shear stress and triggers activation of multiple signaling proteins.

~25,000-30,000 copies per platelet surface. In resting platelets, this highly glycosylated von Willebrand factor receptor (vWfR) complex is linked to underlying actin filaments by filamin A molecules [31]. The vWfR complex mediates activation and platelet adhesion to collagen-bound vWf under high shear rates. Absence or deficiency of GPIb $_{\alpha}$ , GPIb $_{\beta}$  or GPIX is responsible for Bernard-Soulier Syndrome, a bleeding diathesis characterized by severe thrombocytopenia with abnormally large platelets and impaired platelet adhesion [32].



**Fig. 3**  
**Schematic diagram of the human von Willebrand factor receptor subunit GPIb $\alpha$**   
 typical, complete N-glycan identified on GPIb $\alpha$  is shown (insert).

The  $\alpha$ -subunit, GPIb $\alpha$ , is the best-described subunit of the vWfR. It is a type I membrane-spanning subunit containing an external N-terminal, ligand-binding domain which includes leucine rich loops, an anionic peptide sequence with three tyrosine residues, a sialomucin core with multiple O-glycans, a transmembrane domain and a cytoplasmic tail (Fig. 3) [32].

The extracellular N-terminal domain of GPIb $\alpha$  contains the binding site for vWf and interacts with various other ligands such as thrombin, platelet P-selectin, and neutrophil/macrophage  $\beta$ 2 integrin  $\alpha_M\beta_2$  (Mac-1, CD11b/CD18) [32] [33], an integrin activated leukocytes use to adhere firmly to platelets. After vessel wall injury, platelet GPIb-IX-V mediates the initial adhesion contact to vWf bound to collagen within the exposed vascular subendothelium under arterial shear rates, allowing platelets to roll at much slower velocities.

### ***The collagen receptor GPVI.***

GPVI is the major signaling receptor for collagen on the platelets[34, 35] and it is coupled to a disulfide-linked Fc receptor (FcR)  $\gamma$ -chain homodimer in the membrane of human platelets [36]. Each FcR-chain contains one copy of the immunoreceptor tyrosine-based activation motif (ITAM) that undergoes tyrosine phosphorylation by Src family kinases upon crosslinking of GPVI. This leads to binding and activation of the tyrosine kinase Syk, and initiates downstream signaling events. PLC $\gamma$ 2 is recognized as a central target for this signaling cascade [37].

### ***Platelet secretion.***

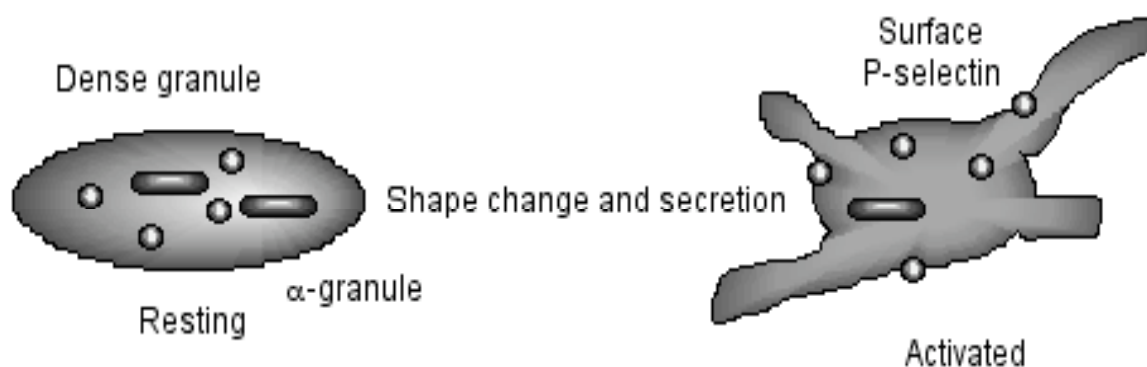
Platelets contain three major types of granules:  $\alpha$ -granules, dense granules and lysosomes. Platelet secretion or exocytosis releases molecules at the site of injury to activate other cells or to facilitate cellular adhesion [38-40]. Platelets secrete molecules from intracellular granules. These molecules play central roles in hemostasis, thrombosis, and vascular remodeling [41-44].

$\alpha$ -granules contain adhesive proteins such as vWf, fibrinogen, thrombospondin, and glycoprotein receptors such as the vWfR or the fibrinogen receptor  $\alpha$ IIb $\beta$ <sub>3</sub> embedded in their membranes [45, 46]. Additionally,  $\alpha$ -granules contain several growth factors, such as insulin-like growth factor 1, platelet-derived growth factor TGF $\beta$ , platelet factor 4 (a heparin-binding chemokine) and the adhesion molecule P-selectin [47-49]. Emerging literature documents that  $\alpha$ -granules are packed differentially and that their molecular composition may come from different sources [50]. For example, vWf is primarily produced by megakaryocytes, while fibrinogen is believed to be endocytocytized from its surroundings during differentiation followed by packaging into  $\alpha$ -granules [51, 52]. Granule secretion has been proposed to be regulated differentially [44], implying a highly regulated system of granule release.

Dense granules are smaller than  $\alpha$ -granules, serve as pools for intracellular calcium, and contain molecules such as adenine nucleotides (ADP and ATP), serotonin, histamin, and granulophysin [16, 17, 53]. Surprisingly, recent reports indicated that dense granules also contain proteins such as P-selectin, which were originally attributed to  $\alpha$ -granules only [54]. This indicates that our current understanding of granule formation and status is only preliminary (Fig. 4).

## Platelet death.

Until recently, the only well-established mechanisms affecting platelet survival were antibody-mediated platelet clearance [55, 56], consumption of platelets by coagulation reactions, and loss due to massive bleeding. Clearance of senile platelets is believed to occur primarily in the spleen and liver and be mediated by macrophages. However, the signals regulating platelet clearance mechanisms are poorly understood. Platelets manipulated *in vitro* to express high levels of phosphatidylserine (PS) on their surfaces are rapidly ingested by macrophages *in vitro* [57]. Apoptotic mechanisms associated with senescent platelets lead to loss of mitochondrial membrane potential and subsequent distribution of phospholipids



**Fig. 4**

**Secretion.** Upon activation, platelets undergo shape change and release dense and  $\alpha$ -granule contents.

<p><math>\alpha</math>-granule contents:</p> <ul style="list-style-type: none"> <li>-Adhesion molecules (P-selectin, vWf, fibrinogen, <math>\alpha</math>IIb<math>\beta</math><sub>3</sub>)</li> <li>-Chemokines</li> <li>-Coagulation and fibrinolytic factors</li> <li>-Growth and angiogenesis factors</li> <li>-Immunologic molecules</li> </ul>	<p>Dense granule contents:</p> <ul style="list-style-type: none"> <li>-Ions (<math>\text{Ca}^{2+}</math>, <math>\text{Mg}^{2+}</math>)</li> <li>-Nucleotides (ATP, GTP, ADP, GDP)</li> <li>-Coagulation and fibrinolytic factors</li> <li>-Membrane proteins (CD63)</li> <li>-Transmitters: Serotonin</li> </ul>
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with increased phosphatidylserine exposure. However, it is unclear if those events are related to *in vivo* survival [58, 59]. In recent years, it has become increasingly apparent that the production of platelets and their subsequent life span in circulation are regulated, at least in part, by apoptotic mechanisms. The review by Kile *et al.* examines the role of the intrinsic apoptosis pathway, regulated by the Bcl-2 family of proteins in platelet biology [60].

Platelet sizes are heterogeneous in blood. It has been postulated that size may be related to platelet age. *In vivo* data show that when platelet fragments are shed by megakaryocytes they certainly exceed normal platelet dimensions [15]. It is therefore likely that younger platelets are larger and are homeostatically more active because of their increased granule content [61-63] [8]. While platelets circulate, their average platelet size and density declines [64], which presumably reflects the loss of membrane, granules and RNA [65, 66]. It is therefore possible

that platelets are continuously “active/secretive” while circulating, shedding their plasma membrane components and granule contents to continuously promote vascular integrity and prevent bleeding, as consistently observed during thrombocytopenia. Such a process could contribute to the “aging/dying” process of circulating platelets. Whether such shedding plays a role in clearance is unknown, although conditions that lead to micro-vesiculation also lead to the activation of platelet calpain and promote the up-regulation of PS to the cell surface [8]. However, activation *per se* or platelet shape-change does not alter platelet survival [67-69].

Two additional phenomena were proposed to be associated with platelet aging: 1) sialic acid loss from terminal glycans [70] and 2) accumulation of IgG on platelet surfaces following the removal of sialic acid [71, 72]. However, the extent to which the two events contribute to platelet clearance has not been demonstrated.

## **PLATELET STORAGE.**

The invention of platelet transfusions has allowed tremendous advancements in hematopoietic progenitor cell transplantations and chemotherapy, procedures often accompanied by severe thrombocytopenia and bleeding. However, therapeutic advances led to a disproportional demand for platelet transfusions [73] which perpetuated the demands on transfusion agencies. Improved platelet storage has permitted millions of transfusions per year, yet current blood banking practices for platelets are far from ideal. In 1969, Murphy and Gardner demonstrated that transfused human platelets stored at 4°C are rapidly cleared from circulation [74, 75]. They and others showed that platelets stored at room temperature (22°C) show significantly better recovery and survival, with a typical life span of 7-9 days versus only 2-4 days for refrigerated platelets [74, 75] [76, 77]. This odd phenomenon of chilled platelet clearance has had profound consequences for blood banking. For decades, all platelet products have been stored at room temperature, and because of the attendant risk of bacterial growth, platelet storage has been limited to five days.

Refrigerated storage of platelet products would theoretically reduce the risk of bacterial growth [78, 79], permitting extended storage of platelets and bolstering product inventories. Competing technologies are now under development and might ultimately lessen the impact of these potential benefits [80]. For example, platelet bacterial testing may lead to the safe extension of room temperature storage of platelets [81]. Pathogen reduction technology could virtually eliminate not only bacteria, but viral and protozoal pathogens as well [82, 83]. Furthermore, improvements in platelet storage—such as second-generation storage containers with better O<sub>2</sub>/CO<sub>2</sub> exchange and bigger platelet storage volumes—have stabilized pH values and resulted in better platelet viability [76, 84-86]. Storage of platelets in synthetic media (platelet additive solutions) may further reduce platelet metabolic rate and inhibit platelet activation during storage [87-89]. Although the usage of platelet additive solutions may be successful, this assessment is mostly based on *in vitro* studies. Only limited clinical evaluations have been published that either study the effectiveness of platelets in initially developed platelet additive solutions, or were specifically done in combination with pathogen reduction technologies. More clinical studies are needed to substantiate *in vitro* results before platelet additive solutions may safely replace plasma and allow extended storage with maintenance of quality [88, 90].

Does platelet refrigeration offer any unique advantages? It is possible, although yet unproven, that 4°C storage could yield platelet products with clinically superior hemostatic function relative to their counterparts after extended storage at room temperature (i.e. > 5 days).

### ***Platelet function: cold versus warm platelets.***

To be clinically effective, transfused platelets must (1) circulate and (2) function in clotting (i.e. prevent or stop bleeding). Currently, the gold standard test to evaluate platelet products is *in vivo* survival and count increment of transfused radiolabeled platelets [91, 92]. It is assumed that if a platelet product circulates normally, it should function appropriately. However, both parameters fail to assess the functional quality of transfused platelets. Furthermore, efficacy of a transfused platelet product is clearly patient-dependent, complicating the assessment of platelet functionality [93]. Unequivocally, clinical experience shows that platelet transfusions work. Improvements in treatment have rendered fatal hemorrhage considerably less common today, although thrombocytopenic patients receiving prophylactic transfusions in randomized platelet “trigger” trials have had clinically significant bleeding complications (WHO Grades 2-4) at rates of 17-21.5% irrespective of study arm [94, 95]. It seems therefore reasonable to question if higher functioning platelet products could reduce current bleeding rates.

Platelets are metabolically active during room temperature storage, a factor that continuously diminishes platelet function. Metabolic products such as lactate accumulate during room temperature storage and precipitate a fall in pH [96]. pH levels below 6.0-6.2 have been associated with severely diminished platelet viability [92, 97-100]. In contrast, there is minimal lactate accumulation and no reduction in pH levels at 4°C [101, 102]. Platelets undoubtedly become activated during room temperature storage [103-107]. Key mediators of thrombosis stored in the  $\alpha$ -granules of resting platelets, such as  $\beta$ -thromboglobulin and platelet factor 4, accumulate in the platelet storage medium over time [108]. Moreover, platelet surface P-selectin expression increases during room temperature storage [109], indicating that platelets continuously release their granule content, i.e. become activated.

Whether cooling itself causes platelet activation is controversial. In many ways, platelets stored at 4°C seem to be less activated than platelets stored at room temperature. For example, 4°C platelet storage does not lead to  $\beta$ -thromboglobulin release into the storage medium [108]. On the other hand, platelet chilling induces rapid, irreversible disc-to-sphere shape change [110]. Also, P-selectin exposure indicates  $\alpha$ -granule content release [111-114]. P-selectin exposure had initially been suggested to accelerate platelet clearance [103, 105, 113]; however, despite initial enthusiasm for the use of P-selectin as a marker of platelet quality in transfusion settings, P-selectin levels do not predict platelet survival after transfusion [67, 68, 105, 106]. Studies using platelets lacking P-selectin [68] or platelets activated with thrombin [67, 115] revealed that P-selectin exposure or loss of platelet discoid shape had no effect on platelet clearance. Conversely, spherical platelets characteristic in mice lacking  $\beta$ 1-tubulin circulate normally [69, 112, 116]. Uncapping and fragmentation of actin filaments is one prerequisite for conversion of platelet discs to spheres following cooling [117]. However, preservation of discoid shape using actin-remodeling inhibitors did not diminish refrigerated platelet clearance [69, 112, 116]. Taken together, these studies show that preservation of platelet discoid shape does not predict platelet survival.



Phosphatidyl serine exposure and loss of the vWfR complex  $\alpha$ -chain (GPIb $\alpha$ ) following storage occur independent of the storage temperature [105, 111, 113, 118-121]. Unfortunately, it appears that as of today none of the measured surface markers reliably predicts platelet survival.

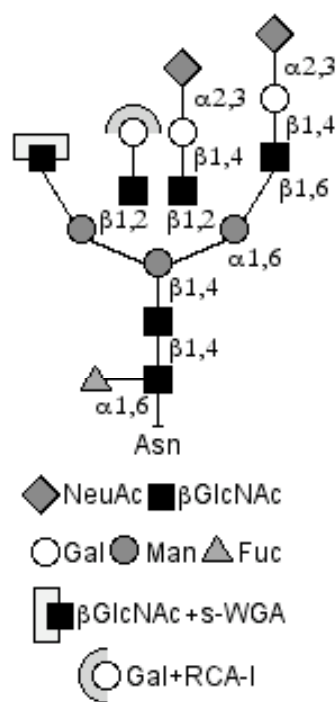
It is assumed that both the retention of platelet discoid shape (as measured photometrically by the extent of shape change) and hypotonic shock response (indicating metabolic efficacy) report on platelet viability [96, 122-124]. Not surprisingly, platelets stored at room temperature perform better in both tests because platelets rapidly lose their “discoid shape” when chilled, implying that refrigerated platelets are not metabolically efficient and viable [75, 110, 124]. However, most investigators agree that 4°C stored platelet products show better pH stability [101, 102], a reduced rate of glycolysis, and a better response when stimulated by ADP, epinephrine or collagen than do room temperature stored platelets [75, 125]. These results imply that refrigerated platelets function better.

### ***In vivo studies of refrigerated human platelets.***

One key concept that has emerged from recent refrigerated platelet studies is that a platelet’s ability to survive normally in circulation may be entirely separable from its ability to function in blood clotting [112]. This concept challenges the “old” conclusion that refrigerated platelets are removed from the circulation because they do not function.

A small number of human studies performed in the early 1970s suggest that platelets stored at 4°C have better *in vivo* function than room temperature platelets despite having poor survival in the circulation. Becker, Aster and colleagues [75] tested the effects of platelets refrigerated for  $\leq 72$  h in 68 thrombocytopenic patients. The patients, with pre-transfusion bleeding times of  $>15$  minutes, were given fresh platelet concentrates, room temperature platelet concentrates that had been stored for 24, 48 or 72 hours, or 4°C platelet concentrates that had been stored for equivalent periods. Bleeding times were measured one hour after transfusion. Platelets refrigerated for 48 or 72 hours corrected the bleeding time in 63% of cases, while room temperature platelets stored for an equivalent period corrected the bleeding time in only 24% of patients. In similar studies performed in aspirin-treated volunteers, bleeding times were corrected almost to baseline within four hours after transfusion with 4°C platelets. In contrast, almost no effect was seen at this early timepoint in subjects receiving platelets stored at room temperature for 72 hours. At 24 hours post-transfusion, substantial correction of the bleeding time was seen in the recipients of room temperature platelets [75] [77]. Another study showed that refrigerated ( $\leq 72$ hrs) platelets transfused into 41 leukemia patients were clinically effective (i.e. stopped bleeding) and were considered “safe” in over 100 transfused patients [126]. Similar observations were made by Handin and Valeri [127], who found that room temperature platelets corrected the bleeding time of aspirin treated volunteers 24 hours post-transfusion but had no effect immediately post-transfusion. Together, this evidence suggests that loss of *in vitro* platelet function may be reversible upon transfusion [127]. Based on these studies, the suggestion was made that platelets specifically intended to treat acute/active bleeding (i.e. trauma or surgical patients) should be stored at 4°C, while platelets used for bleeding prophylaxis should be stored at room temperature [124, 128].

However, this approach never gained acceptance, as studies performed subsequently failed to confirm that transfused room temperature platelets showed a significant delay in hemostatic function. A study by Slichter and Harker showed that room temperature platelets consistently corrected the bleeding times of aplastic thrombocytopenic patients within 2 1/2 hours. In contrast, refrigerated (< 72 hours) platelets corrected the bleeding time in 6/8 cases shortly post-transfusion, but the effect was not sustained beyond 2 1/2 hours [76] [129]. Filip and Aster repeated the bleeding time experiments done previously in their laboratory [129] by administering either room temperature or 4°C platelets to 41 thrombocytopenic patients. They reported that platelets stored at room temperature for 24 hours produced the greatest



**Fig. 5 Detection of exposed glycan residues using lectins.** Sequential loss of terminal sialic acid or galactose residues exposes underlying galactose or  $\beta$ GlcNAc, respectively. Galactose or  $\beta$ GlcNAc residues are detectable with RCA-I or s-WGA lectins, respectively.

attachment of oligosaccharids through N- or O-linkage. Except for cytosolic O-GlcNAc glycosylation [130], glycosylation is confined to the endoplasmic reticulum and the Golgi apparatus. During modification, individual carbohydrate residues are added sequentially by glycosyltransferases. The synthesis of glycoconjugates is generally completed by addition of sialic acid or fucose which cap the structures and prevent further chain elongation (Fig. 5) [131]. Glycoconjugates without these terminal structures (also known as “immature” glycans) expose underlying incomplete carbohydrates such as  $\beta$ Galactose ( $\beta$ Gal) and  $\beta$ GlcNAc residues. Immature glycans are produced and presented on cell surfaces when their biosynthesis is affected, for instance in cancer cells. In contrast, the surface of normal mammalian cells is decorated with completed mature glycans. Glycans play essential roles in

bleeding time correction one hour post-transfusion, while platelets stored at 4°C for 72 hours yielded the least bleeding time correction, although the difference was not statistically significant. The investigators attributed the relative ineffectiveness of room temperature platelets in their earlier study to inadequate (low) storage volume [76].

The sole reason platelets are currently stored at room temperature is that chilled platelets do not circulate for an acceptable period of time in the recipient. It appears that the mechanisms dictating platelet removal from circulation, including for refrigerated platelets, are independent of platelet function and remain poorly understood.

## CLEARANCE MECHANISMS FOR REFRIGERATED PLATELETS: DUAL ROLES FOR HEPATIC LECTINS.

### Platelet glycobiology.

Glycosylation is a posttranslational modification of proteins and lipids by

cell-cell and cell-matrix interactions under physiological and pathophysiological conditions such as leukocyte trafficking and cancer metastasis [132].

One of the major glycoproteins on the platelet surface is GPIb $\alpha$ , which contains 60% of the total platelet glycan content, typically with up to four N-linked core-fucosylated glycans (two located within the N-terminus [133]) and O-linked glycans within the mucin rich region [134]. The glycans' structures and their localization are indicated in Fig. 3. Normally, glycans on GPIb $\alpha$  are fully sialylated when completely assembled. However, upon refrigeration, loss of sialic acid exposes subterminal glycans such as  $\beta$ Gal or  $\beta$ GlcNAc. Such residues are detectable by their binding to specific lectins, for example *Ricinus communis* agglutinin (RCA) I and succinylated wheat germ agglutinin (s-WGA) (Fig 5). GPIb $\alpha$  N-terminal region and its N-linked carbohydrates have been implicated in the clearance of refrigerated platelets [135], [136]. However, it needs to be determined if GPIb $\alpha$  and its carbohydrate play a role in clearance of aging platelets *in vivo*.

### **Lectin receptors.**

Lectins are sugar-binding proteins, highly specific for their sugar moieties. They typically play a role in biological recognition of cells and glycoproteins. Lectins have different biological functions ranging from regulation of cell adhesion to glycoprotein synthesis and the control of protein levels in the blood. In general it is believed that desialylated serum glycoproteins have drastically reduced survivals [137], a phenomenon originally demonstrated for desialylated ceruloplasmin [138, 139]. Resialylation significantly prolonged the survival of ceruloplasmin, pointing to the importance of sialic acid in preventing removal by  $\beta$ Gal recognizing receptors (Asgpr) [140]. The C-type lectin family consists of two galactose-recognizing receptors: the hepatic Ashwell-Morell receptor [141, 142] [143] and the macrophage scavenger receptor [144-146]. Both receptors show overlapping specificity and may function in parallel with each other [147, 148]. Unexpectedly, recent studies showed that Ashwell-Morell receptors not only recognize and clear desialylated proteins, but also mediate the clearance of sialic acid-deficient platelets [136, 149, 150]. Earlier, we demonstrated another unexpected macrophage-lectin based platelet clearance mechanism. We showed that the  $\alpha_M\beta_2$  integrin removes refrigerated platelets from the circulation by recognizing exposed  $\beta$ GlcNAc residues. Two major functions are attributed to  $\alpha_M\beta_2$  (or CR3, CD11b/CD18, MAC-1). First, it mediates adhesion and migration of leukocytes into inflammatory sites in tissues via binding to the intercellular adhesion molecule (ICAM-1) expressed on stimulated endothelium [151]. Second,  $\alpha_M\beta_2$  serves as a phagocytic receptor for the iC3b fragment of complement [152].  $\alpha_M\beta_2$  also contains a cation-independent sugar-binding lectin-site [153, 154] which either recognizes microbial surface polysaccharides or binds to GPI-linked signaling partners, thereby promoting phagocytosis of microbes [155]. That  $\alpha_M\beta_2$  and the Ashwell-Morell receptor mediate platelet clearance mechanisms is surprising and unexpected due to the size differences between platelets and proteins or bacteria. Whether the Ashwell-Morell receptor on hepatocytes or the  $\alpha_M\beta_2$  integrin are also involved in removal of senile platelets remains to be established. In the following paragraphs we will elucidate the fine interplay of two lectin receptors in the removal of sialic acid deficient platelets from the circulation.

## Role of the macrophage $\alpha_M\beta_2$ -integrin in refrigerated platelet clearance.

Earlier, we reported that the macrophage carbohydrate-binding  $\alpha_M\beta_2$  recognizes clustered GPIb $\alpha$  subunits of the vWfR complex following short-term refrigeration (2 h, 0°C), which results in the phagocytosis and clearance of platelets *in vivo* in mice and *in vitro* by human THP-1 macrophages [33, 112, 135, 156]. Experiments using  $\alpha_M\beta_2$ -deficient mice [68] markedly improved the survival of refrigerated platelets. Removal of GPIb $\alpha$ 's ligand binding-domain using the *O*-sialoglycoprotein endopeptidase restored the circulation of refrigerated mouse wild-type platelets, which indicates that the external domain of GPIb $\alpha$  initiates clearance [112]. Subsequent work narrowed carbohydrate recognition by  $\alpha_M\beta_2$  to exposed  $\beta$ GlcNAc ( $\beta$ -N-acetylglucosamine) residues on N-linked GPIb $\alpha$  glycans (Fig. 3) [33, 135]. To remedy the poor circulation of refrigerated platelets a “capping strategy adding galactose residues to exposed  $\beta$ -GlcNAc residues” (galactosylation) was attempted. Surprisingly, both human and mouse platelets have functional platelet galactosyltransferase(s) on their surface. These enzyme(s) respond to the simple addition of UDP-galactose by transferring galactose onto exposed  $\beta$ GlcNAc residues of human or mouse GPIb $\alpha$  [135]. Galactosylation markedly improved the survival of mouse platelets refrigerated for 2 hours [135]. Galactosylation therefore provided a simple approach to improve refrigerated platelet circulation.

While depriving the  $\alpha_M\beta_2$  lectin-domain of its  $\beta$ GlcNAc ligand on refrigerated platelets, galactosylation theoretically provides a new ligand for Asgprs. Hence, it was surprising that refrigerated mouse platelet circulation could be improved by galactosylation. It was postulated that the number of exposed  $\beta$ GlcNAc residues on GPIb $\alpha$  was small, such that even after clustering and galactosylation, the galactose density was insufficient to engage galactose-recognizing lectins [135].

## PAPER I.

A clinical trial was performed using apheresis platelets refrigerated for 48 h. This phase-I clinical trial administered autologous, radiolabeled, galactosylated apheresis platelets refrigerated for 48 h into human volunteers. The results clearly showed that the galactosylation procedure did not extend their circulation time [119]. For logistical reasons, the early studies used isolated platelets and had not stored mouse platelets for clearance studies longer than hours. In contrast, platelets for transfusion were stored for days in platelet rich plasma. Consequently, the question arises if the mouse and human models differ and if prolonged platelet refrigeration led to a new clearance mechanism. We therefore developed a mini-bag methodology (Fig. 6) to support the storage of mouse platelet-rich plasma (PRP) at 4°C for up to 48 h. When mouse platelet-rich plasma was stored in mini-bags, a gradual loss of recoveries and survivals with longer storage times at 4°C was observed [119]. *In vitro* characterization of the 4°C stored mouse platelets demonstrated stable pH, but some increase in P-selectin exposure ( $18.1 \pm 2.8$  compared to  $5.9 \pm 1.4$  % binding for fresh platelets) and moderate increase in annexin V binding ( $5.9 \pm 1.4$  compared with  $2.3 \pm 0.6$  % binding for fresh platelets). Importantly, using a mouse transfusion system we subsequently found that just as with human platelets stored in plasma, galactosylation has no effect on the survival of > 48 h long-term refrigerated mouse platelets [119]. Furthermore, galactosylated mouse platelets stored for 48 hrs in plasma did not show improved circulation time when transfused into

$\alpha_M\beta_2$  integrin deficient mice [119]. These results suggested that different mechanisms are involved in the clearance of platelets refrigerated for days.

## **PAPER II.**

### **The role of the hepatic Asialoglycoprotein (Ashwell-Morell) receptor in refrigerated platelet clearance.**

Using the mouse transfusion model, we have dissected the clearance mechanism for platelets refrigerated for > 48 h in plasma. We have found that as with short-term cooling, biotinylated or radiolabeled platelets refrigerated for 48 h in plasma (designated throughout the following text as long-term refrigerated platelets) are removed from the recipients' circulation by the liver [136, 157] but, unexpectedly by hepatocytes [136] and not by Kupffer cells (macrophages), as we previously reported for short-term cooled isolated platelets [112]. The *in situ* finding that hepatocytes can ingest long-term refrigerated platelets was confirmed using cultured hepatocyte cell lines (HepG2 cells) and human platelet concentrates refrigerated for up to 10 days. Further evidence that long-term refrigerated platelets are ingested by hepatocytes and not by macrophages was obtained by purging recipient mice of mature hepatic and splenic macrophages by injecting toxic clodronate-encapsulated liposomes [158] prior to transfusions of fresh room temperature platelets or short- or long-term refrigerated and/or galactosylated platelets. As expected, removal of macrophages greatly improved recovery and survival of short-term cooled platelets, which confirmed our earlier results. In contrast, the recovery and survival of long-term refrigerated platelets were not affected by macrophage depletion, thus showing that removal of long-term refrigerated platelets is macrophage-independent and rather mediated by hepatocytes [136]. Interestingly, these studies also revealed that macrophages rapidly remove a large fraction of transfused fresh room temperature [136]. This result possibly reflects the detection of damage inflicted during isolation, consistent with a loss of ~30-40% platelet recovery that observed following the transfusion of fresh platelets into healthy volunteers [119].

### **The Asialoglycoprotein (Ashwell-Morell) receptor.**

How does the hepatic galactose lectin or the Ashwell-Morell receptor recognize long-term refrigerated platelets? We reported that short-term cooled platelets have exposed/clustered  $\beta$ GlcNAc residues that are recognized and phagocytosed by the  $\alpha_M\beta_2$  hepatic macrophage receptors [112, 135]. In contrast, platelets refrigerated for long periods have severely increased galactose exposure, as evidenced by the galactose-binding lectins (RCA-I and ECA), which suggests a ligand for the hepatic Asialoglycoprotein receptor. Consistent with this hypothesis, experiments using mice lacking *Asgr-1* or *Asgr-2* subunits of the Ashwell-Morell receptor showed a significantly improved recovery and circulation of long-term refrigerated platelets. Similarly, co-injection of asialofetuin, a competitive inhibitor of the Ashwell-Morell receptor, restored the recovery and circulation of long-term refrigerated platelets, but not of short-term refrigerated platelets. Conversely, asialofetuin inhibited phagocytosis of human platelets refrigerated for up to 10 days by HepG2 cells *in vitro* [136]. Not surprisingly, long-term refrigerated and galactosylated platelets are cleared slightly faster independent of macrophage depletion [136]. Our studies, combined with a recent report by Grewal *et al.*, point out the importance of a hepatic-based platelet removal system that uses the Ashwell-Morell receptor to recognize defectively sialylated proteins and remove platelets

which express desialylated glycans on their surface [149, 150] [136]. Whether hepatocytes also remove senile/desialylated platelets remains to be established.

### **Desialylated glycans reside on GPIb $\alpha$ following long-term refrigeration.**

We investigated whether GPIb $\alpha$  also plays a role in the removal of long-term refrigerated platelets, as we previously reported for short-term cooled platelets. Two of the putative N-linked glycans are localized within the 45 kDa of GPIb $\alpha$  extracellular domain (Fig. 3) [133]. Removal of the N-terminal 282 residues of GPIb $\alpha$  from human platelets using the snake venom protease mocarhagin [159] or *O*-sialoglycoprotein endopeptidase eliminates two putative N-glycan residues, as well as the vWf-binding region of GPIb $\alpha$  [159]. Removal of GPIb $\alpha$ 's 45 kDa domain from mouse or human platelets significantly restored the circulation of long-term refrigerated wild-type mouse platelets and prevented human platelet ingestion by HepG2 cells *in vitro*, which indicates that most exposed  $\beta$ Gal and  $\beta$ GlcNAc residues reside within the external domain of GPIb $\alpha$  initiating clearance [136]. It is tempting to speculate that most of the exposed  $\beta$ Gal residues reside within N-linked glycans on GPIb $\alpha$ , as shown previously for  $\beta$ GlcNAc [135]. However, it remains to be determined whether N-linked glycans are solely involved in this interaction.

Does GPIb $\alpha$  presentation influence platelet clearance? Platelets from ST3GalIV<sup>-/-</sup> mice have exposed galactose on surface glycoproteins as they lack  $\alpha$ 2-3-sialyltransferase activity [143, 150]. In long-term refrigerated and rewarmed platelets, the mechanism of galactose exposure remains to be determined. Release, or activation, of sialidase activity during storage could cleave terminal sialic acid residues, thus revealing underlying galactose residues. We observed increased clustering of GPIb $\alpha$  with prolonged refrigeration [136]. Hence, clustering of GPIb $\alpha$  subunits in the platelet with cooling might amplify the galactose signal and enhance lectin avidity and binding, as reported earlier for  $\beta$ GlcNAc [135]. However, the functional relationship between GPIb $\alpha$  clustering and platelet clearance by hepatocyte Ashwell-Morell remains to be established, particularly if proteolytic removal of GPIb $\alpha$ 's N-Terminal 45 kDa portion alters clustering.

Are glycoproteins other than GPIb $\alpha$  glycans involved in the clearance of cooled platelets? vWf binding increases with storage [136]. It appears that preferentially desialylated vWf binds to long-term refrigerated platelets, which indicates that desialylation of vWf molecules may promote binding to GPIb $\alpha$ . In support of the notion that desialylated vWf binds better to platelets, we found that despite a 50% reduction of vWf concentration in ST3Gal-IV<sup>-/-</sup> plasma, binding of deficiently sialylated vWf to the vWfR on WT platelets is enhanced *in vitro* and *in vivo* [149]. Proteolytic removal of the GPIb $\alpha$  N-terminal region deprives GPIb $\alpha$  of its vWf-binding domain and binds less vWf. Whether vWf-glycans contribute to recognition of platelets by Ashwell-Morell receptor remains to be determined.

## **PAPER III.**

### **Models for sialic acid deficient platelet uptake by hepatic Asialoglycoprotein receptor (Asgpr).**

A number of specific sialyltransferase genes have been targeted in mice, and mice deficient in several of these enzymes exhibit defects in the hematopoietic cell lineage [160-162]. In particular, the ST3Gal-IV enzyme that transfers sialic acid in a  $\alpha$ 2,3 linkage to glycans with terminal Gal $\beta$ 1-4GlcNAc, Gal $\beta$ 1-3GlcNAc and Gal $\beta$ 1-3GalNAc sequences [163, 164] operates as a dominant modifier of hemostasis by concealing asialoglycoprotein ligands on platelets and vWf [160].

The impact of deficient sialylation on platelet clearance following transfusion was tested using the ST3Gal-IV knock out mouse model with autosomal recessive thrombocytopenia. Platelets from ST3GalIV<sup>-/-</sup> mice lack  $\alpha$ 2-3-sialyltransferase (ST3GalIV) activity [160], increasing exposure of penultimate  $\beta$ Gal on vWf and platelets and decreases plasma levels of these to 50% and 30% of normal values, respectively [160]. We have therefore used the ST3GalIV<sup>-/-</sup> mice as a model to identify if ST3GalIV deficiency leads to similar platelet clearance mechanisms as found for long-term refrigerated platelets.

In mice deficient in the ST3Gal-IV sialyltransferase gene we showed that thrombocytopenia is caused by the recognition of terminal galactose residues exposed on the platelet surface in the absence of sialylation. This results in accelerated clearance of transfused ST3Gal-IV<sup>-/-</sup> platelets by liver macrophages and hepatocytes [160], a mechanism that was recently shown to induce thrombocytopenia during *S. pneumoniae* sepsis [150]. We also identified platelet GPIb $\alpha$  as a major counter receptor on ST3Gal-IV<sup>-/-</sup> platelets for asialoglycoprotein receptors, as shown for long-term refrigerated platelets. Moreover, we reported that deficient sialylation increases vWf-mediated platelet activation and suggests that increased binding of deficiently sialylated vWf to platelets contributes to the accelerated clearance of ST3Gal-IV<sup>-/-</sup> platelets. Taken together the data generated using ST3GalIV deficient mice and long-term refrigerated platelets establish the importance of sialylation for platelet clearance by hepatic lectins and vWf function.

The Sections “Platelet storage” and “Clearance mechanisms for refrigerated platelets: Dual roles for hepatic lectins” will be published as a review article in *Transfusion and Apheresis Science*, December 2009.

## **METHODS.**

### ***Platelet preparation.***

#### **Platelet isolation.**

Human and mouse platelets were obtained from whole blood, separated from plasma and other cell components, washed, and resuspended at a concentration of 10<sup>9</sup>/mL in 140 mM NaCl, 3 mM KCl, 0.5 mM MgCl<sub>2</sub>, 5 mM NaHCO<sub>3</sub>, 10 mM glucose, and 10 mM Hepes, pH 7.4 [112].

#### **Platelet staining methods.**

Human and mouse platelets were labeled with 1.8  $\mu$ M CellTracker<sup>TM</sup> Orange CMTMR, or 2.5  $\mu$ M CellTracker Green CMFDA [165] for 20 min at 37 °C. Unincorporated dye was

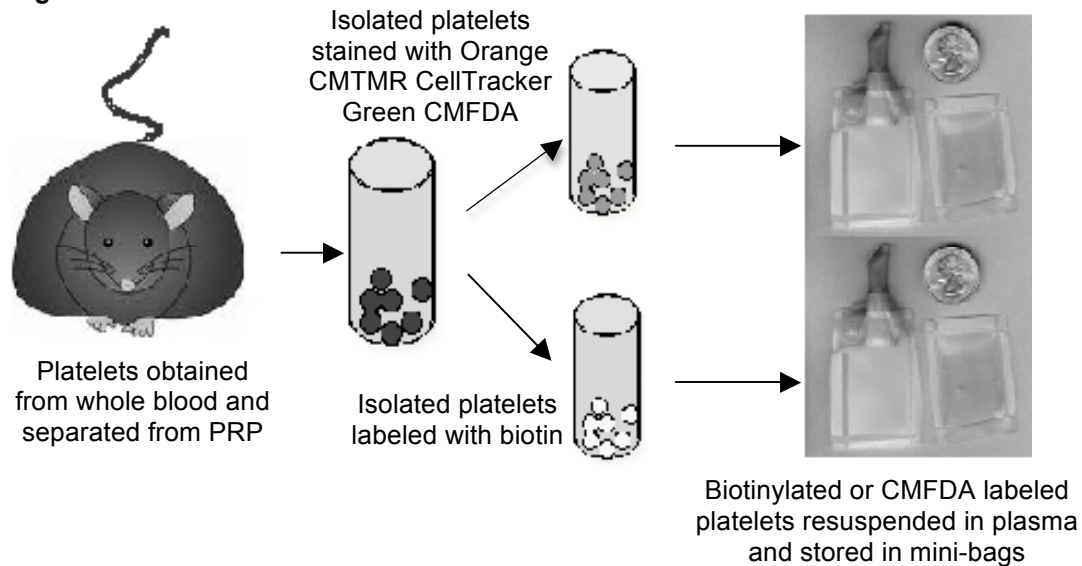
removed by centrifugation [112]. To study the fate of refrigerated platelets, isolated mouse platelets were biotinylated by incubation with  $1 \mu\text{g ml}^{-1}$  Biotin-NHS for 1 h at RT. Unbound biotin was removed by centrifugation. Biotinylated or CMFDA labeled platelets were then resuspended in plasma and stored in mini-bags, as described below, before injection into mice [136].

### Platelet temperature and storage protocols.

To study the effects of cold on platelet survival and/or function, isolated platelets were incubated for 2 h at ice bath temperatures, a process designated as short-term cooling or as “0° C”, or resuspended in PPP and stored for 48 h at 4 °C (4° C), a process designated as long-term cooling or as “4° C”.

In PAPER I a method for mouse platelet storage was developed. Mouse platelets were injected into mini transfusion bags (0.9 mL/transfusion bag) using a 27.5-gauge needle, sealed with a heat sealer (SEBRA, Tucson, AZ), and placed in the dark for 6, 48, or 72 hours

**Fig. 6**



at 4°C without agitation or for 6 hours at room temperature (22°C) on a horizontal table-top shaker rotating at 60 cycles/minute. Platelet mini-bags (Fig. 6) with a storage volume of 1 mL to 2 mL were made by heat-sealing 20-mm by 30-mm squares from gas-permeable platelet storage bags (Gambro BCT, Lakewood, CO). All platelets stored in the cold were rewarmed for 15 minutes at 37 °C before use [119].

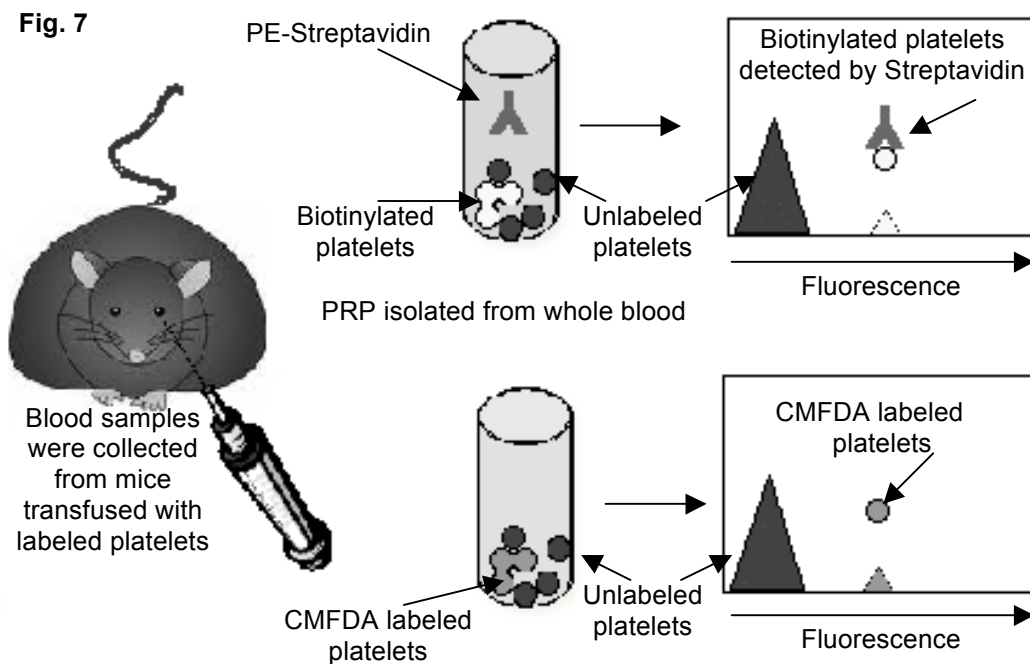
### *In vivo platelet studies.*

#### Mouse platelet recovery, survival studies.

To follow the fate of platelets stored at 0 °C, 4 °C or 22 °C , these platelets were injected into the retro-orbital venous plexus of syngeneic recipient mice. For recovery and survival determinations, blood samples were collected immediately (2 min), and at 5 min, 10 min, 0.5 h, 2 h, 24 h, 48 h, and 72 h. Platelet rich plasma (PRP) was isolated from whole blood and analyzed by flow cytometry (Becton Dickinson Biosciences) and the percentage of CMFDA positive platelets determined [165]. Biotinylated platelets were distinguished from



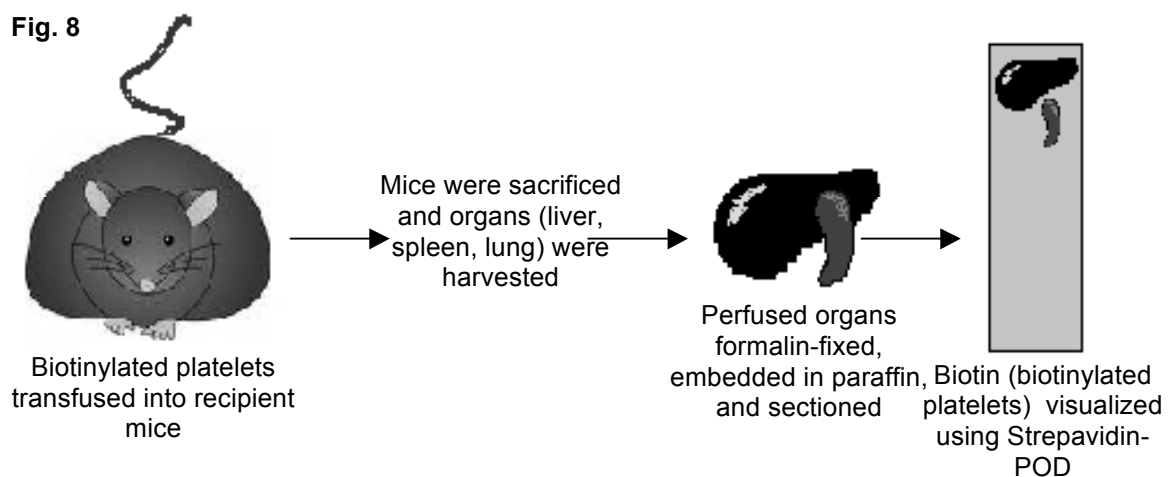
endogenous platelets by addition of Phycoerythrin (PE) conjugated Streptavidin (PE-Streptavidin). PRP was incubated with PE-Streptavidin for 20 min at room temperature and the percentage of biotinylated platelets was determined by flow cytometry (Fig. 7).



## Mouse platelet fate.

### *Immunohistology.*

Mice were infused with  $3 \times 10^9$  of biotinylated platelets. Organs were collected at 5, 15, and 30 min and 24 h and were formalin-fixed, embedded in paraffin, and sectioned every 3  $\mu\text{m}$ .



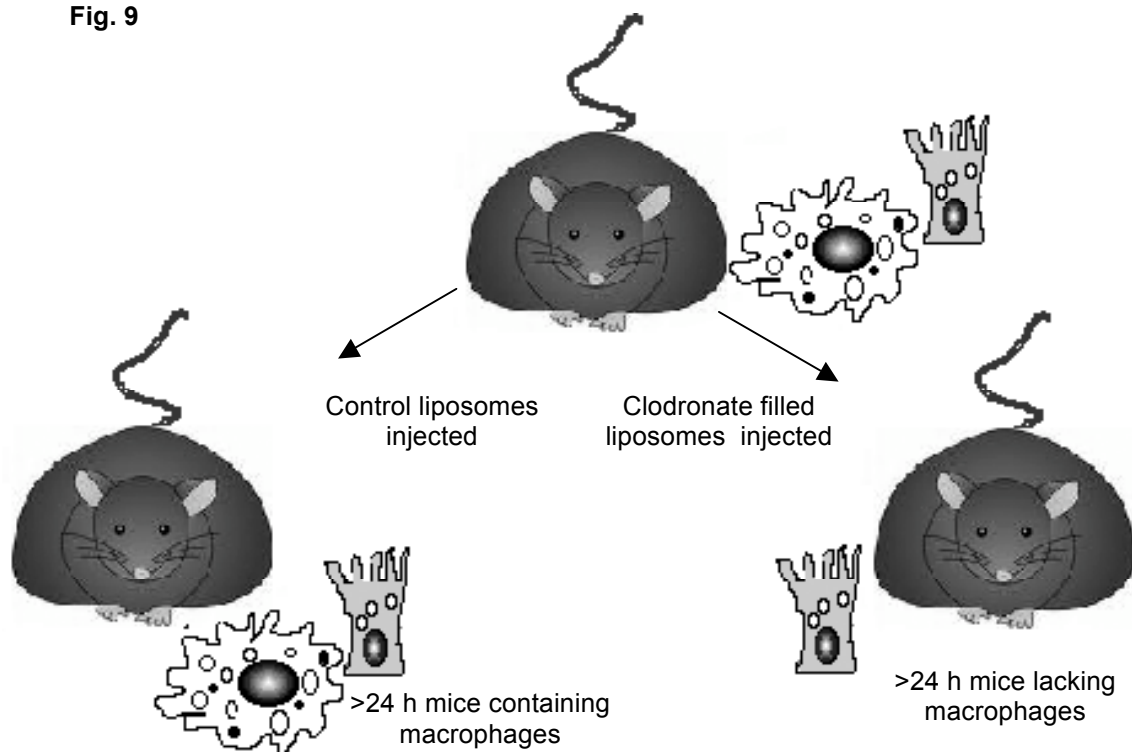
The distribution of biotin (biotinylated platelets) was visualized using Streptavidin-POD conjugate and ImmunoHisto™ Peroxidase Detection Kit (PIERCE). Sections were counterstained with H&E according to the manufacturer's recommendations. Quantitative analysis of staining was done in blinded samples. Ten tissue sections were selected from

mice with similar levels of injected platelets and scored for hepatocytes and macrophages containing biotinylated platelets (Fig. 8).

### ***Radioactive labeled platelet fate.***

To evaluate the fate of platelets, tissues were harvested from recipient mice 1 h after the injection of  $0.4 \times 10^9$   $^{111}\text{In}$ -labeled (NEN Life Science Products) platelets. Platelets were radiolabeled after storage as described before [112]. Organ-weight was measured and radioactivity was determined from  $\gamma$ -count. Results are expressed as % radioactivity per g organ relative to the total radioactivity injected. For recovery and survival determinations of radioactive platelets, blood samples were collected immediately ( $< 2$  min), and at 0.5 h, 1 h and 24 h after platelet infusion and  $\gamma$ -count determined [166].

**Fig. 9**



### ***Macrophage depletion.***

Mice were depleted of phagocytic cells by a single injection of liposomes containing dichloromethylene bisphosphonate (clodronate liposomes) [158, 167]. Mice were injected intravenously with 0.02 ml of clodronate liposomes per 10 g body weight 24 h prior to platelet transfusions. This treatment depletes 99% of Kupffer cells and 95% of splenic macrophages [158, 167]. Staining for macrophages was performed using an antibody to mouse F4/80 (SEROTEC) in tissue sections of clodronate treated and untreated mice. No F4/80 staining was detected following clodronate-liposome treatment. Control liposomes were prepared with PBS in place of clodronate (Fig. 9).

### ***Platelet clearance inhibition studies using asialofetuin and fetuin.***

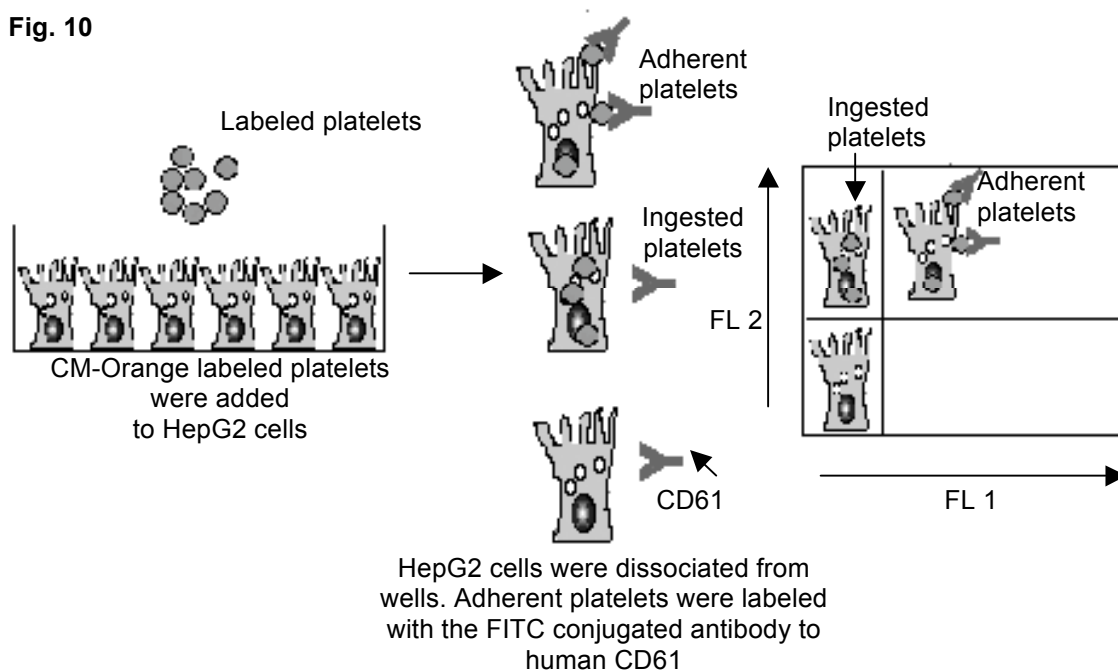
A bolus of 10 mg asialofetuin or fetuin was injected via the retro-orbital venous plexus into WT C57/Bl6 mice 2 min before the platelet transfusion. Following platelet transfusion, 5 mg asialofetuin or fetuin was injected intravenously at time points of 10, 20, 30 and 60 min. The recipient animals were bled at 2, 10, 30, 90 min and the survival of labeled platelets determined by flow cytometry, as described above.

### ***In vitro platelet ingestion studies.***

#### **In vitro HepG2 based platelet ingestion assay.**

We maintained human HepG2 hepatocarcinoma cells in  $\alpha$ MEM (GIBCO Invotrogen), 2% heat-inactivated bovine calf serum (BCS), 3% standard fetal bovine serum (FBS), and 1% of a penicillin and streptomycin solution composed of 10,000 U ml<sup>-1</sup> penicillin G and 10 mg ml<sup>-1</sup> streptomycin sulphate. HepG2 cells were not passaged > 2 times before use. For assays, the HepG2 cells were transferred to 24-well plates (10<sup>6</sup> per well), allowed to adhere for 24 h, and starved for 30 min.

**Fig. 10**



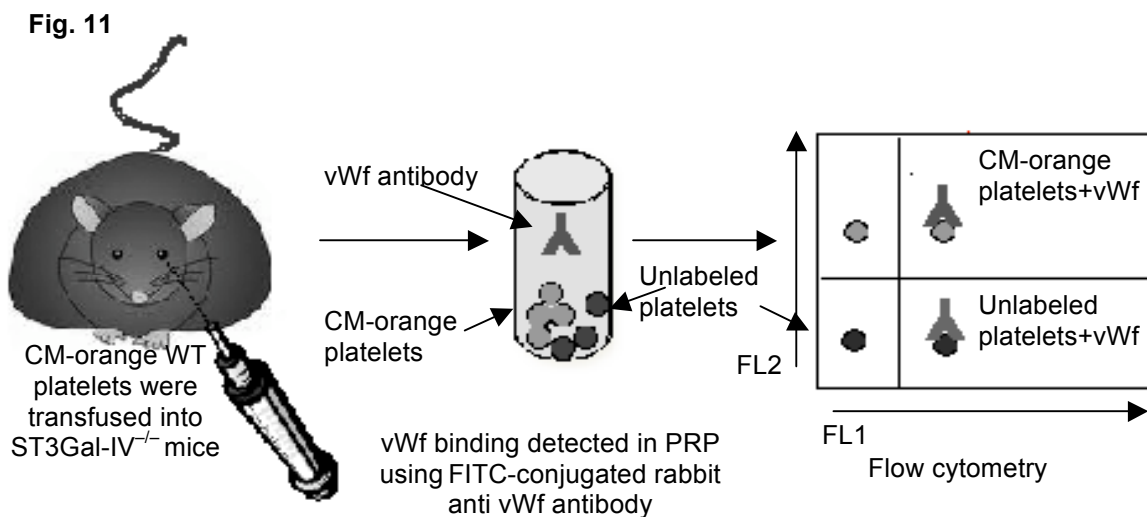
Cytochalasin D (SIGMA) was diluted into  $\alpha$ MEM media at the indicated concentrations and added to the HepG2 cells. DMSO was used as control. To study if the AMR mediated the ingestion of platelets in vitro, asialofetuin (competitive asialoglycoprotein receptor inhibitor) and fetuin (control) were diluted into  $\alpha$ MEM media at 1, 10 or 100  $\mu$ g/ml, respectively, and added to the HepG2 cells.  $1 \times 10^8$  CM-Orange-labelled platelets (fresh 22 °C platelets or platelets cooled at 0 °C or 4 °C) were added per well, with or without cytochalasin D. Hepatocytes and platelets were incubated for 5-30 min at 37 °C with gentle agitation. After the incubation period, the HepG2 monolayers were washed 3 times by removing and changing the buffer. HepG2 cells were dissociated from the wells with 0.05% trypsin, 0.53

mM EDTA in HBSS (GIBCO Invitrogen) at 37 °C for < 10 min. CM-Orange-labeled platelet ingestion was quantified by flow cytometry. HepG2 cells were gated according to their forward and side scatter characteristics. HepG2 with ingested platelets acquired orange fluorescence. Platelets adherent to HepG2 cells were labeled with the FITC conjugated antibody to human CD61 (Fig. 10).

### ***Studies of platelets surface glycoproteins involved in platelet-hepatocyte interaction.***

#### **Removal of the extracellular domain of GPIb $\alpha$ .**

GPIb $\alpha$  was enzymatically cleaved from the surface of human or mouse platelets in platelet buffer containing 1 mM Ca<sup>2+</sup> and 10  $\mu\text{g ml}^{-1}$  of the snake venom metalloprotease mocarhagin [168] or 10  $\mu\text{g ml}^{-1}$  O-sialoglycoprotein endopeptidase, respectively (Fig. 3). After the enzymatic digestion, the platelets were washed by centrifugation, resuspended in platelet poor plasma (PPP), and allowed to rest for 30 min at 37 °C before storage at 4 °C. The extent of GPIb $\alpha$  removal was monitored by flow cytometry using 5  $\mu\text{g ml}^{-1}$  of the FITC-conjugated antibody to human GPIb $\alpha$  (clone SZ2) or 5  $\mu\text{g ml}^{-1}$  of the PE-conjugated antibody to mouse GPIb $\alpha$  (clone Xia.G5).



#### **Measurement of glycan exposure on platelet surfaces.**

Fresh 22 °C platelets or platelets stored in platelet buffer for 2 h at 0 °C, or in plasma for 48 h at 4 °C, were diluted 4-fold with platelet buffer, collected by centrifugation at 830 x g for 5 min, and resuspended in platelet buffer at 1x10<sup>7</sup> ml<sup>-1</sup>. Surface galactose was analyzed by flow cytometry following incubation with FITC-conjugated RCA I or ECA at 0.1 and 10  $\mu\text{g ml}^{-1}$ , respectively. Surface  $\beta\text{GlcNAc}$  was measured by incubation with FITC-conjugated s-WGA at 0.1  $\mu\text{g ml}^{-1}$ . Samples were incubated at RT for 20 minutes and diluted 5-fold with platelet buffer before analysis by flow cytometry (Fig. 5).

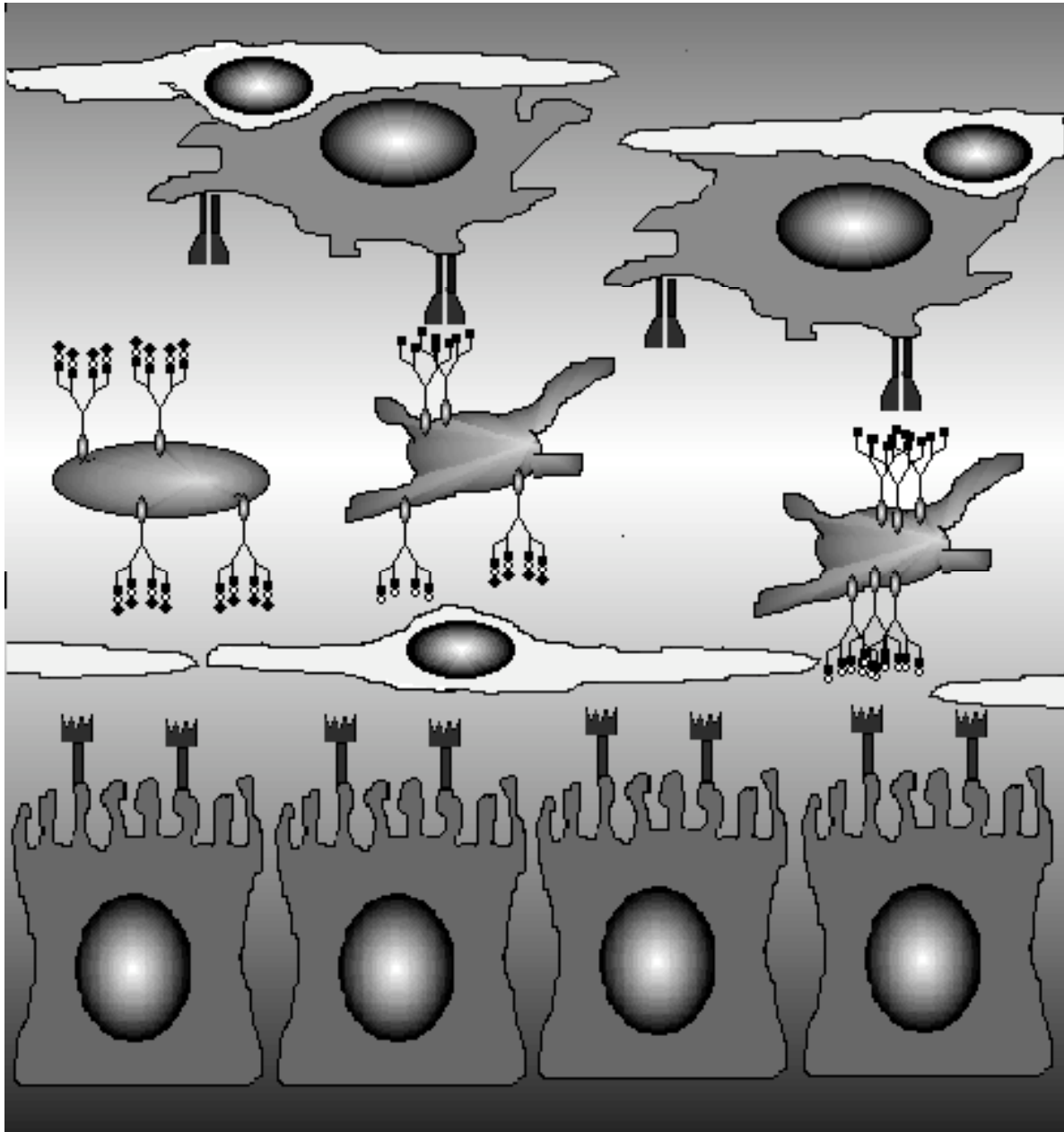
### **Detection of bound vWf to transfused platelets.**

WT platelets were fluorescently labeled by incubation with 1.8  $\mu$ M CM-Orange (Invitrogen) and transfused into WT or ST3Gal-IV<sup>-/-</sup> mice. Blood samples were obtained immediately (1 minute) after transfusion, and vWf binding was measured in PRP by flow cytometry using FITC-conjugated rabbit anti-vWf antibody (Emfret). The percentage of platelets positive for green and orange fluorescence was determined (Fig. 11).

**Additional Materials and Methods used in PAPER I, II and III are published [119, 136, 149].**

## DISCUSSION.

The major focus of this thesis was to determine clearance mechanism for platelets stored at



**Fig. 12 Dual roles of lectin receptors in platelet clearance.** The von Willebrand factor receptor (vWfR) complex, specifically GPIb $\alpha$ , has complete and incomplete N-linked glycans with exposed  $\beta$ GlcNAc and/or galactose residues. Upon short-term refrigeration, vWfRs and exposed  $\beta$ GlcNAc aggregate, initiating recognition and phagocytosis by the macrophage  $\alpha_M\beta_2$  integrin. Extended refrigeration is followed by additional surface changes such as "hyperclustering" of receptors, galactose exposure, and binding of vWf with increased galactose exposure. Increase of galactose density on platelets induces platelet recognition by asialoglycoprotein receptors on hepatocytes (Ashwell-Morell receptors), which recruits clearance mechanisms in addition to macrophage depended platelet clearance through  $\alpha_M\beta_2$  integrins.

4°C for 48 hrs in plasma (long-term refrigerated platelets). Our results revealed an unexpected macrophage-independent clearance mechanism for sialic acid deficient platelets mediated by the Ashwell-Morell receptors on hepatocytes [150]. The mechanisms leading to clearance of short- and long-term refrigerated platelets are schematically depicted in Fig. 12. This observation, however, contradicts previously reported data that galactosylation of exposed  $\beta$ GlcNAc residues on short-term refrigerated platelets improves chilled platelet circulation [135]. The original finding that galactosylation rescued the circulation of short-term cooled platelets was rationalized by the hypothesis that exposed  $\beta$ GlcNAc and  $\beta$ Gal only represent a minority of the exposed glycans on the surface of platelets, while the majority of the glycans are fully sialylated, which prevents the recognition of the platelets by different glycan receptor systems.

The amount of immature carbohydrate exposure on the cell surface is critical in eliciting cell clearance by lectins [169]. Sequential transfusions of neuraminidase-treated rabbit platelets into rabbits have shown that >10% of the total platelet surface sialic acid must be removed to elicit recognition by the Asgpr system. In accordance with this notion, we show that exposure of galactose residues [169] increases with storage and refrigeration, although the mechanism of galactose exposure is unclear. It is possible that platelets release active sialidase that can cleave terminal sialic acid residues, revealing underlying galactose residues. Evidence that supports this mechanism is the increased binding of galactose-specific lectins to long-term refrigerated platelets but not to short-term refrigerated platelets. Glycans associated with GPIb $\alpha$  are the major targets of this activity as judged in platelet lysates and GPIb $\alpha$  immunoprecipitates from long-term refrigerated platelets. Platelets from ST3GalIV<sup>-/-</sup> mice have exposed galactose on surface glycoproteins because they lack  $\alpha$ 2-3-sialyltransferase activity [143, 170]. However, the extent of galactose exposure on refrigerated platelets is less than that present on the surface of ST3GalIV<sup>+/-</sup> platelets that retain their ability to circulate with normal lifetimes when transfused into WT mice [149], a finding that further substantiates the importance of glycan density and/or presentation for lectin recognition. The observed increase in protein clustering [136], and/or alterations in lipid-glycans following long-term refrigeration, may lead to changes in glycan presentation and density, thus amplifying the galactose signal and allowing recognition by Asgprs. Clustering of GPIb $\alpha$  subunits, as we observed in electron micrographs, increases the localized density of galactose or glycan presentation, which enhances lectin avidity and binding [136]. Clustering in the cold could be facilitated by cytoskeletal and membrane phase changes [171, 172]. It is possible that physiologically functional microdomains present at 37°C aggregate into macrodomains in human blood platelets as they chill below their membrane lipid phase transition temperatures [173, 174].

Deficient sialylation of vWf increases vWf binding to platelets *in vivo* and *in vitro* [133, 140, 146]. This suggests that increased binding of deficiently sialylated vWf to platelets may additionally contribute to the accelerated clearance of ST3Gal-IV<sup>-/-</sup> platelets and long-term refrigerated platelets. Evidence for the hypothesis that desialylated vWf may contribute to platelet clearance comes from experiments that remove the 45 kDa GPIb $\alpha$  domain which binds to vWf. Removal of the 45 kDa domain restored the circulation of long-term refrigerated platelets and platelets derived from ST3GalIV<sup>-/-</sup> mice. However, removal of the 45 kDa GPIb $\alpha$  domain does not completely rescue the initial recovery of long-term refrigerated platelets, indicating that other factors influence the initial recovery [136]. Cleavage of the GPIb $\alpha$  by ADAM 17 causes a dramatic loss of platelet recovery following

transfusion [175]. Loss of GPIb $\alpha$  following refrigeration and rewarming could modulate the recoveries of long-term refrigerated platelets. It is therefore possible that desialylation or changes in glycan structure on other highly glycosylated proteins, such as  $\alpha_{IIb}\beta_3$ , play a role in the recognition of both long-term refrigerated platelets and ST3GalIV deficient platelets. It remains to be determined if, during storage, platelets release active sialidases, thus affecting sialylation of plasma proteins, glycolipids or plasma proteins such as vWf, fibrinogen or fibronectin. This would affect platelet clearance.

### **Macrophage versus hepatocyte mediated platelet clearance.**

Macrophages initially remove a large fraction of transfused platelets (~40%) [136], indicating their regulatory role in platelet recoveries. Although macrophages are widely regarded as the key phagocytes in regulating platelet clearance [176-178] [136], we have found that fresh platelets isolated from ST3GalIV deficient mice as well as long-term refrigerated and fresh room temperature platelets are cleared even in the absence of macrophages. Macrophage removal in mice somewhat improved platelet recoveries of ST3GalIV<sup>-/-</sup> platelets and long-term refrigerated platelets. Platelets refrigerated for 48 h have exposed  $\beta$ GlcNAc residues [135]. Covering exposed  $\beta$ GlcNAc residues on long-term refrigerated platelets by galactosylation deprives the macrophage  $\beta$ 2-integrin of its ligand, but promotes recognition by Asgprs. Macrophage depletion should therefore improve non-galactosylated short-term cooled platelet survival but have no effect or decrease galactosylated platelet survival. Consistent with these predicted results, we show that non-galactosylated platelet recoveries and survivals are significantly improved and recoveries of long-term refrigerated and galactosylated are further decreased in platelets of macrophage-depleted mice. These results show that that the recoveries of both short-term and long-term cooled platelets are governed by  $\beta$ 2-integrin lectins on macrophages. However, survivals of transfused platelets (independent of their pretreatment) remain unaffected by macrophage depletion, thus revealing a fine interplay between macrophage and hepatic lectin receptors in the removal of platelets. Taken together, these findings imply that platelet lifespan is defined by hepatocyte Ashwell-Morell receptors and that functional macrophages do not play a substantial role in determining platelet survival, but regulate platelet recoveries following platelet transfusion. Interestingly, macrophage depletion had the biggest impact on fresh room temperature platelet recoveries, which implies that “defining” platelet recovery, or the immediate clearance of a transfused platelet, may involve more than lectin-mediated mechanisms. However, we cannot exclude that a small portion of macrophages may still be present following clodronate treatment.

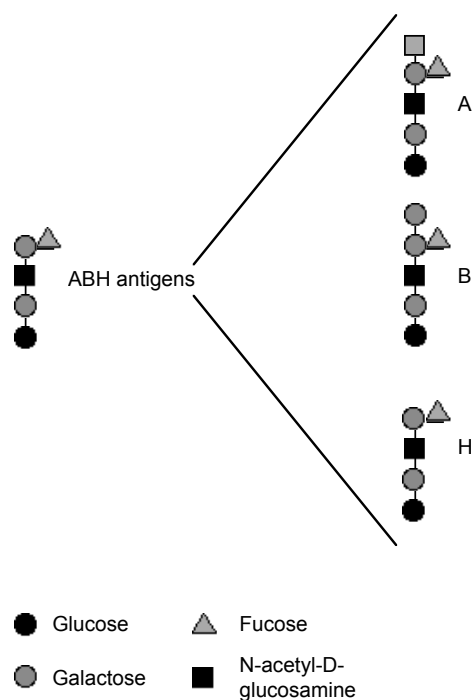
Whether the hepatic-based system also functions to remove senile platelets at the end of their functional life remains to be established. In this case, platelets would lose sialic acid while circulating (aging), possibly due to release of endogenous sialidases. In addition, lifespan is limited by endogenous platelet apoptotic machinery [179] and scavenger receptors. Furthermore, some loss of platelets may also be attributed to platelets that function to ensure vascular integrity, i.e. bind to damaged vessels [180, 181]. How adherent platelet thrombi and debris are removed, if not by macrophages and hepatocytes, remains to be determined.

Multiple hepatic clearance systems contribute to varying extents in clearing soluble epithelial mucins that aberrantly enter circulation. Receptors collaborating in the clearance of human colon carcinoma mucins included the hepatocyte ASGPR (HL-I/II), Kupffer cell Gal-Rs



(MGL-1 and 2) and Man/GalNAc-4-SO<sub>4</sub>-R, endothelial cell Man/GalNAc-4-SO<sub>4</sub>-R, hyaluronic acid receptor for endocytosis, and SRs (SRAI/II, SRD, and SRBI). It appears that secretory mucins entering circulation are cleared before they can cause pathologic events, such as those involving platelet and leukocyte aggregation [182]. Whether or not such "complex" high-capacity clearance mechanisms take place in the clearance of "pathologic platelets or platelets with pathologic glycan exposure" has yet to be determined.

### Glycans in transfusion medicine.



**Fig. 13 Schematic representation of ABH antigens.**

The 1930 Nobel Prize in Medicine was awarded to Karl Landsteiner "for his discovery of human blood groups" as the major cause of blood transfusion reactions. The ABH blood group polymorphisms of humans and other primates is determined by expression of A, B or H (O) antigens of types 1-4, which are terminal neutral glycan sequences found in abundance on glycoproteins and glycolipids (Fig 13).

Platelets, like red blood cells and other tissues [183], express ABH antigens on a variety of membrane glycoproteins and glycosphingolipids [184-186]. Clinically, transfusion of ABH-incompatible platelets can be associated with decreased recovery, [186, 187] shortened survival [188], and an increased incidence and onset of HLA-immune refractoriness[187, 189]. Platelet activation, including platelet refrigeration, causes structural changes in surface glycoproteins, altering the presentation of immature glycans on the platelet surface (unpublished observation).

Incomplete glycans on activated platelets may induce clearance by lectins on phagocytic cells, including antigen-presenting cells, thereby facilitating immune responses leading to alloimmunization following repeated platelet transfusions. On the other hand, "activated platelets" survive normally once transfused [67, 68] indicating that immature glycans may not always be recognized as "pathologic". We did not test if long-term refrigerated platelets bind antibodies or if repeated long-term refrigerated platelet transfusions induce antibody production, a question that should be addressed in the future.

The question remains: Can cooled platelets circulate? Glycans associated with GPIb $\alpha$  and vWf appear to be a major target of sialidase activity. Blocking sialidase activity during refrigeration of long-term stored platelets may be one of approach to prevent sialic acid loss and improve platelet circulation. Once the role and contribution of lectin receptors has been settled, future studies should also investigate if re-glycosylation, i.e. a combination of galactosylation and sialylation of immature platelet surface glycans, can rescue refrigerated platelet survival to accommodate their refrigeration for transfusion. The surprising presence of multiple glycosyltransferases (galactosyltransferases and sialyltransferases) in and on the surface of platelets may have implications for platelet functionality as suggested previously. Specific GPIb $\alpha$  glycans would be altered by specific sialidases and sialyltransferases leading

to lectin-mediated clearance. It is possible that donor sugars and secreted glycosyltransferases regulate platelet function and survival per se. The specific role of neuraminidases and sialyltransferases in platelets needs to be addressed in the future.

## CONCLUDING REMARKS.

In an attempt to address the problem of why platelets stored at 4°C do not circulate, forcing platelets to be stored at room temperature for transfusion, we identified novel and unexpected platelet mechanisms. Earlier work showed that the macrophage  $\alpha_M\beta_2$  integrin recognizes GPIb $\alpha$  associated  $\beta$ GlcNAc moieties following short-term refrigeration (2 h) in the absence of plasma, resulting in phagocytotic clearance of mice platelets *in vivo*. Galactosylation of  $\beta$ GlcNAc residues blocks the recognition of short-term refrigerated platelets by macrophage  $\alpha_M\beta_2$  and allows short-term refrigerated platelets to circulate in mice. However, galactosylation does not prevent the removal of human platelets refrigerated long-term in plasma. The aim of this thesis was to investigate the clearance mechanism of long-term refrigerated platelets in plasma.

### The major results of this thesis are:

We developed a mouse model of platelet storage for 48 hours at 4°C and demonstrated that:

- I) UDP-galactose treatment of mouse platelets also did not prevent their rapid clearance in agreement with the human platelet study. These results suggested that exposed  $\beta$ GlcNAc residues were not the sole factor determining the clearance of long-term 4°C stored platelets in either mice or humans. This was confirmed by the rapid clearance of 48 h, 4°C stored galactosylated mouse platelets in  $\alpha_M\beta_2$  integrin knockout mice.
- II) *In vitro* measured surface changes (P-selectin exposure, annexin V binding, von Willebrand factor binding) are comparable between mouse and human platelets stored in plasma for 48 h at 4°C.
- III) Short-term cooling (2 h, 0°C) leads to  $\beta$ GlcNAc exposure, but not to an increased galactose exposure on mouse and human platelets. In contrast, storage of mouse and human platelets in plasma for  $\geq$  48 h at 4°C leads to increase in galactose exposure.
- IV) Exposed galactose residues on platelets are recognized by galactose lectins, specifically by hepatic Asialoglycoprotein (Ashwell-Morell) receptors. These conclusions are based on the following evidence:
  - a) Macrophage depletion in mice using clodronate encapsulated liposomes significantly improves the circulation of 2 h chilled and transfused platelets but does not prevent the clearance of 48 h refrigerated platelets;
  - b) Immunohistology revealed abundant location of 48h-refrigerated platelets in liver hepatocytes;
  - c) Binding of the  $\beta$ Gal-recognizing lectin RCA-I increases by  $\sim$ 1.5-fold following 48 h-refrigeration;
  - d) Mice lacking Asgr-1 or Asgr-2 subunits of the Asgr1/2 support 48 h-refrigerated platelet circulation times comparable to room temperature stored platelets;
  - e) Co-injection of asialofetuin, a competitive inhibitor of asialoglycoprotein-receptors, restores the survival of 48 h-refrigerated platelets in wild type mice;
  - f) The human hepatic cell line (HepG2 cells) ingest fluorescently labeled long-term refrigerated platelets in culture, and asialofetuin prevents this ingestion;

- g) The glycosylated GPIb $\alpha$  N-terminal domain (the binding site for von Willebrand factor) on 48 h platelets is a major target for Ashwell-Morell receptor;
- h) Circulation of 48 h-refrigerated platelets is markedly improved by removal of GPIb $\alpha$ 's N-terminal domain using *O*-sialoglycoprotein peptidase;
- i) Immunoblotting of long-term refrigerated platelets revealed that increased galactose exposure is associated with GPIb $\alpha$ 's N-terminus and a protein of the same molecular weight as vWf as defined using an antibody specific for vWf.

V) Using mice lacking sialyltransferase ST3GalIV<sup>-/-</sup> we determined that clearance of platelets with increased galactose exposure is also mediated by asialoglycoprotein receptors on hepatocytes and macrophages. These conclusions are based on the following evidence:

- a) Platelets from ST3Gal-IV<sup>-/-</sup> have increased exposure of  $\beta$ Gal residues on their surface, as detected by immunoblotting and flow cytometry using peroxidase or FITC labeled RCA I lectin;
- b) Immunohistology staining revealed ST3Gal-IV<sup>-/-</sup> platelets in both hepatocytes and macrophages;
- c) Macrophage depletion in WT mice improved transfused ST3Gal-IV<sup>-/-</sup> platelet recoveries and survivals;
- d) Co-injection of asialofetuin, a competitive inhibitor of asialoglycoprotein-receptors, restores the survival of ST3Gal-IV<sup>-/-</sup> platelets in WT mice;
- f) HepG2 cells ingest platelets ST3Gal-IV<sup>-/-</sup> in culture, and asialofetuin prevents this ingestion;
- g) The glycosylated N-terminal domain of GPIb $\alpha$ , the binding site for vWf on ST3Gal-IV<sup>-/-</sup>, is a major target for asialoglycoprotein receptors;
- h) The circulation of ST3Gal-IV<sup>-/-</sup> platelets is markedly improved by removal of GPIb $\alpha$ 's N-terminal domain using *O*-sialoglycoprotein peptidase, pointing to the fact that increased galactose exposure on the surface of ST3Gal-IV<sup>-/-</sup> platelet is associated with of GPIb $\alpha$  and vWf;
- i) WT mice have increased binding to vWf with sialic acid deficiency *in vivo* and cleared faster upon transfusion.

Using two mouse platelet models with exposed  $\beta$ Gal residues we have shown that clearance of desialylated platelets is mediated by asialoglycoprotein receptors on hepatocytes and to some extent macrophages for ST3GalIV deficient platelets. These results demonstrate a totally unexpected platelet-carbohydrate and lectin based clearance mechanism. It is likely that additional lectin receptors are involved in platelet clearance. It is important to determine if the demonstrated hepatic platelet clearance systems also contribute to "senescent platelet clearance" and to understand why hepatocytes ingest desialylated platelets.

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