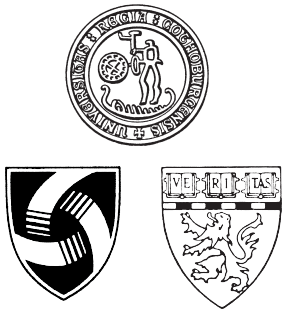
Role of GPIbα clustering and N-linked carbohydrates in the clearance of refrigerated platelets

Emma Josefsson



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The thesis focuses on understanding the mechanisms by which: **1)** the macrophage α_M-subunit recognizes βN-acetylglucosamine (βGlcNAc) residues on the von Willebrand factor receptor complex ($(GPIb_{\alpha,\beta}/IX)_2V$ or vWfR) on refrigerated platelets and **2)** refrigeration changes vWfR to elicit recognition through $\alpha_M\beta_2$. Until recently, the only well-established mechanisms affecting platelet survival were antibody-mediated platelet clearance, consumption of platelets by coagulation reactions, and loss due to massive bleeding. An effort to address a practical problem, how to refrigerate platelets for transfusion, led us to define a previously unsuspected platelet clearance mechanism. We found that (1) macrophages recognize βGlcNAc residues of N-linked glycans on clustered GPIbα subunits following short-term refrigeration (2 h) of platelets in the absence of plasma and (2) phagocytosis and clearance are mediated by the $\alpha_M\beta_2$ integrin receptor of macrophages. Galactosylation of GPIbα blocks ingestion by the macrophage $\alpha_M\beta_2$ and allows short-term refrigerated murine platelets to circulate but does not prevent the removal of platelets stored long-term in plasma.

Work detailed in this thesis demonstrates that the ingestion of short-term refrigerated platelets is dependent on the α_M lectin-domain, not the I-domain which is involved in the recognition of most $\alpha_M\beta_2$ ligands. To address this question, CHO cells were directed to express different α_M/α_x receptor subunit chimeras and the relative contribution of α_M-subdomains to platelet ingestion evaluated in these cells. Critically, the recognition and ingestion of refrigerated platelets by CHO cells occurs only when the α_M-subunits contain the α_M lectin-subdomain. The I- or cation binding subdomains of the α_M-subunit are not required. Soluble recombinant α_M lectin-domain, but not a soluble α_M I-domain, also inhibited the phagocytosis of refrigerated platelets by differentiated macrophages and Sf9 cells expressing solely recombinant α_M lectin-domain constructs bound refrigerated platelets. We conclude, therefore, that refrigeration exposes N-glycan βGlcNAc residues on vWfR which are recognized by the lectin-domain of $\alpha_M\beta_2$ to initiate platelet clearance.

Next, the relationship between vWfR clustering/conformational changes and refrigeration was investigated. Clustering of vWfR is detectable by fluorescent resonance energy transfer (FRET) measured by flow cytometry. Refrigeration of platelets for 24 h markedly increases the FRET efficiency between GPIbα and GPV subunits, whereas the FRET between GPIbα and α_{IIb} is unaltered. We conclude that vWfR aggregation begins immediately following refrigeration but becomes maximal only after extended refrigeration. A panel of monoclonal antibodies (mAbs) that recognize different vWfR subunits was employed to further probe for structural changes. We found that certain epitopes on GPIbα become cryptic as platelets are refrigerated, possibly due to clustering of the vWfR complex, and that the rate of epitope sequestration due to clustering is slowed in the presence of plasma. Changes in binding efficacy of the mAbs are not caused by the loss of GPIbα from the platelet surface as determined by immunoblotting of total GPIbα. Some vWf binding in cold plasma was detected that may influence the binding of mAbs which bind to GPIbα near its vWf binding site. These further changes in vWfR in platelets refrigerated long-term in plasma may be related to the additional phagocytic mechanisms involved in their removal.

Key words: Platelets, GPIbα, vWfR, $\alpha_M\beta_2$, phagocytosis, refrigerated platelets.

List of Publications

This thesis is based on the following papers, which are referred to in the text by their Roman numerals:

I **The Macrophage $\alpha_M\beta_2$ Integrin α_M Lectin Domain Mediates the Phagocytosis of Chilled Platelets**
Emma C. Josefsson, Harry H. Gebhard, Thomas P. Stossel, John H. Hartwig, Karin M. Hoffmeister
J Biol Chem., 2005; 280 (18): 18025-18032

II **Glycosylation Restores Survival of Chilled Blood Platelets**
Karin M. Hoffmeister, Emma C. Josefsson, Natasha A. Isaac, Henrik Clausen, John H. Hartwig, Thomas P. Stossel
Science, 2003; 301: 1531-1534

III **Differential Changes in the Platelet vWf Receptor Following Refrigeration for Short or Long Periods**
Emma C. Josefsson, Viktoria Rumjantseva, Herve Falet, Claes Dahlgren, John H. Hartwig, Karin M. Hoffmeister
Manuscript, 2006

Contents

Abbreviations

Ab	antibody
$\alpha_{IIb}\beta_3$	(GPIIb-IIIa, CD41/CD61)
$\alpha_M\beta_2$	(Mac-1, CR3, CD11b/CD18)
ASGPR	asialoglycoprotein receptor
β-GlcNAc	β-D-GlcNAc-1-Me, methylated β-N-acetylglucosamine
CHO cells	chinese hamster ovary cells
CMFDA	5-chloromethylfluorescein diacetate
CRP	collagen related peptide
C-T	carboxyl-terminal
DMS	demarcation membrane system
EM	electron microscopy
FcR	Fc receptor
FITC	fluorescein isothiocyanate
FRET	fluorescent resonance energy transfer
GPI	glycosylphosphatidylinositol
GPIbα	glycoprotein Ibα
GT	glycosyltransferase
h	hours
IP	immunoprecipitation
ICAM	intercellular adhesion molecule
ITAM	immunorecepor tyrosinebased activation motif
JAM	junctional adhesion molecule
KO	knock out
LB	ligand-binding
LRR	leucine rich repeat
mAb	monoclonal antibody
Min	minute
MGL	macrophage galactose lectin
N-T	amino-terminal
OCS	open canalicular system
PAR	protease activated receptor
PCT	photochemical treatment
PE	phycoerythrin
PRP	platelet rich plasma
PS	phosphatidyl serine
RCA I	ricinus communis agglutinin
RT	room-temperature
sWGA	succinylated wheat germ agglutinin
THP-1 cells	human monocytic cell line
TRAP	thrombin receptor activating peptide
WT	wild-type
vWf	von Willebrand factor
vWfR	von Willebrand factor receptor, $(GPIb_{\alpha,\beta}/IX)_2V$

1. Introduction

1.1 The life of the blood platelet – platelet formation and clearance.

Platelets are specialized subcellular fragments, released from megakaryocytes [1-4], that circulate in blood as thin discs and the tubulin ring maintains the disc shape. The life span of platelets in humans is about seven days [5] and four days in mice [6] and the normal human platelet count is $2.5x10^8$ cells/ml and the murine count is $1x10^9$ cells/ml. Platelets are involved not only in hemostasis but also in a range of other less well understood functions, e.g. in inflammation, pathological thrombosis, antimicrobial host defense, tumor growth and metastasis.

Megakaryocytes arise in bone marrow but can migrate into the blood stream and platelet biogenesis has been suggested also to occur in blood [7] and lung [8-12]. Megakaryocytes come from pluripotent stem cells and undergo multiple DNA replications without cell divisions by the unique process of endomitosis. Upon completion of endomitosis, polyploid megakaryocytes begin a rapid cytoplasmic expansion phase characterized by the development of an elaborate demarcation membrane system (DMS) and the accumulation of cytoplasmic proteins and granules essential for platelet hemostatic function. Three models have been proposed to explain the mechanics of platelet production: 1) cytoplasmic fragmentation via DMS, 2) platelet budding, and 3) proplatelet formation [13]. In the proplatelet model, proplatelet formation requires megakaryocytes to first form long cytoplasmic extensions that appear as platelet-sized beads linked together by thin cytoplasmic strands called proplatelet intermediate structures. Blood platelets are then assembled principally at the ends of proplatelet processes produced. In this model, the DMS functions primarily as a membrane reservoir for the extension of proplatelets [14].

Until recently, the only well-established mechanisms affecting platelet survival were antibody-mediated platelet clearance, consumption of platelets by coagulation reactions and loss due to massive bleeding. The normal clearance of senile platelets occurs primarily in the spleen and liver by macrophages that recognize phagocytic signals expressed on the platelet surface. Not much is known about the platelet clearance mechanism, but one pathway involved in the clearance of damaged platelets is the macrophage scavenger receptor system. For example, platelets manipulated *in vitro* to express high levels of phosphatidylserine (PS) on their surfaces are rapidly ingested by macrophages *in vitro* and cleared from the circulation *in vivo* [15]. Whether PS expression increases as platelets age in the circulation system has not yet been established.

Platelets are a heterogenous collection of sizes in blood, and it has been postulated that size is related to platelet age. In particular, it has been suggested, based on the ability of platelets to vesiculate into microparticles *in vitro*, that size decreases with age as membrane is shed [13]. Whether such shedding plays a role in clearance is unknown, although conditions that lead to microvesiculation also lead to the activation of platelet calpain and promote the up-regulation of PS to the cell surface [13]. Activation *per se* does not diminish platelet survival. Thrombin activated platelets transfused in both primates and mice circulate normally [16,17], eliminating shape change or P-selectin up-regulation, in the clearance of platelets. Conversely, spherical β1 tubulin-lacking platelets circulate normally [18]. We first take a closer look at normal platelet function, activation, and the platelet surface receptors involved.

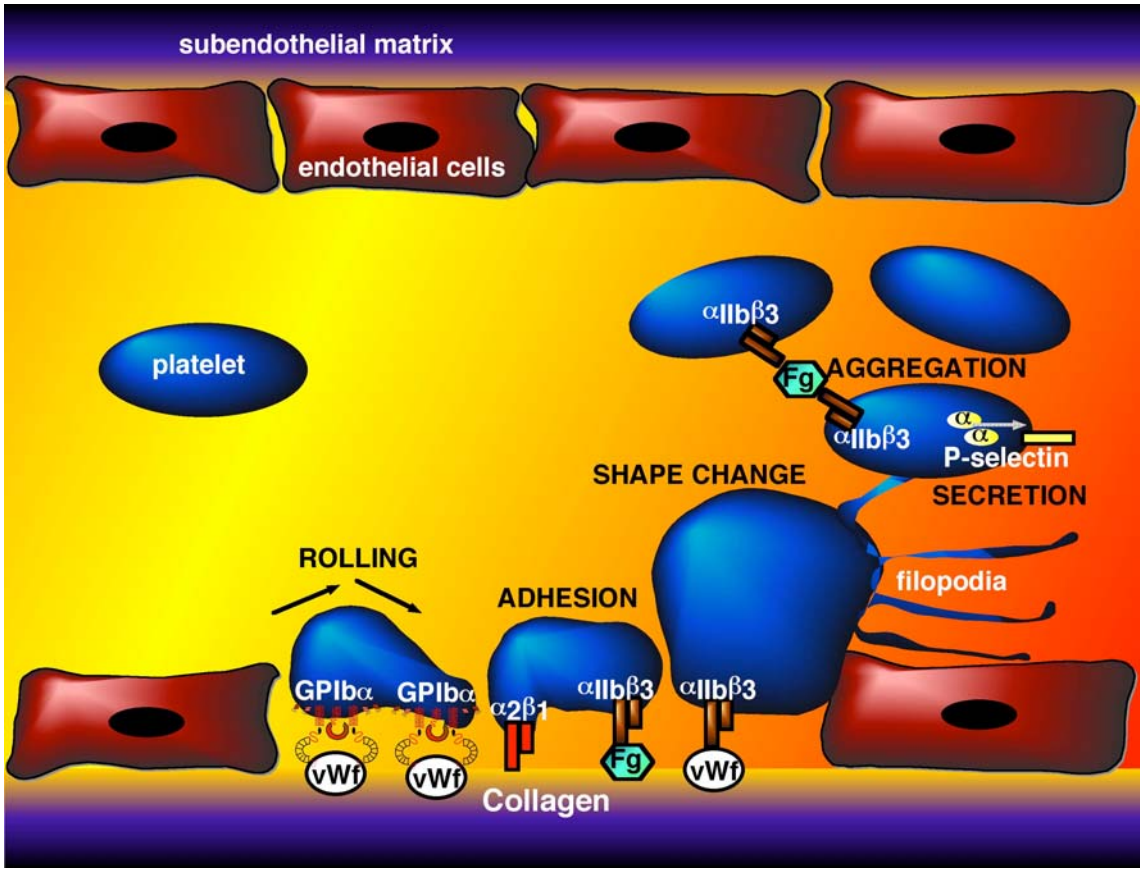

Fig. 1. Platelet activation.
Upon vessel injury, platelets first roll then adhere to the exposed subendothelium. Subsequently, platelets change shape and secrete soluble factors to recruit other platelets and to form a firm platelet aggregate. Platelet rolling is initiated by binding of the GPIbα subunit of the von Willebrand factor receptor complex to von Willebrand factor (vWf) bound to the exposed subendothelium. Firm platelet adhesion is mediated by integrin receptors such as the collagen receptor $\alpha_2\beta_1$ or the fibrinogen (Fg) receptor $\alpha_{IIb}\beta_3$. Fibrinogen acts as a bridge between $\alpha_{IIb}\beta_3$ receptors on activated platelets enabling them to aggregate and form thrombi.

1.2 Platelet activation and granule secretion.

The main function of platelets is hemostasis and their major receptors have a direct role in this process either in activating platelets or as adhesive receptors attaching platelets to damaged vascular walls or with other platelets and leukocytes to form a thrombus. Platelets avidly react, roll, adhere, spread, secrete, and interact with one another to form an aggregate that seals the damaged surface [19]. At sites of vascular injury, circulating von Willebrand factor (vWf) is bound and linearized on subendothelial collagen fibers which exposes the vWf A1 domain that binds the GPIbα subunit of the von Willebrand factor receptor (vWfR) to initiate platelet rolling **(Fig. 1)**. Platelets also bind collagen through GPVI and $\alpha_2\beta_1$ integrin. Ligand binding to vWfR or GPVI initiates inside-out signals that activate the platelet integrin $\alpha_{IIb}\beta_3$, to bind fibrinogen and a RGD motif on linearized vWf to mediate firm adhesion and platelet aggregation [20-22]. Different mechanisms play a role in this complex process. Recruitment of additional platelets is accomplished by the amplification of platelet filopods, the delivery of P-selectin receptors to the platelet surface, and by the release of attractive molecules such as ADP and serotonin during secretion and the production and release of thromboxane. A recent report has shown

that GPVI and vWfR are physically associated on the platelet surface [23] which suggests that the receptors that initiate platelet activation are organized on the surface with a special topology.

Platelets major function is to detect damage, change shape and secrete substances to plug wounds. Platelet counts of 30,000/ml are necessary to prevent spontaneous bleeding. Platelet shape change requires the remodeling of the cytoskeleton, composed of actin and tubulin and their associated proteins, and actin assembly [24,25]. Platelets have an open canalicular system (OCS), a system of internal membranes formed into a network of tubules, which runs throughout the platelets. In the activated platelet, the OCS serves as a channel into which the platelet granules fuse and release their contents and as a source of surface membrane for cell spreading. In the platelet cytoplasm are organelles such as mitochondria, lysosomes, granules and residual packages of endoplasmatic reticulum membrane called the dense membrane system. There are two types of granules: α- and dense granules. α-Granules store matrix adhesive proteins and have glycoprotein receptors embedded in their membranes. P-selectin is stored in their membranes as well as a portion of the major platelet adherence receptors, vWfR and the integrin $\alpha_{IIb}\beta_3$. Matrix adhesive proteins include fibrinogen, fibronectin, thrombospondin, vitronectin, and vWf. Dense granules carry soluble activating agents such as ADP, serotonin, divalent cations, and a small amount of P-selectin [19].

Over 30 years ago, Jamison and Barber [26] proposed that an externally disposed glycosyltransferase (GT) activity mediates platelet adhesion and other functions. Subsequent work ruled out ecto-GT activity in nucleated cells and established Golgi as the primary site of such enzymes, although no further studies examined platelets. The Hoffmeister lab has defined the existence of GT activity in platelets [27] and found that megakaryocytes package and deliver Golgi-associated GTs into platelets and their surfaces using dense granules, that release upon platelet activation [28]. These exciting findings suggest possible new roles of platelet GTs and carbohydrates in platelet function, survival and interaction with immune cells. Platelet surface receptors have key roles in platelet signaling, activation and clearance and are described in detail below.

1.3 Platelet surface receptors. (Table 1.)

Receptors (vWfR, GPVI, G-protein coupled receptors, or ADP receptors) interact with both soluble and tethered ligands to activate platelets. Here, because of the relevance of the vWfR changes in refrigerated platelets, I focus on the vWfR complex, which begins the activation process in flowing blood that leads to platelet rolling, adherence, and $\alpha_{IIb}\beta_3$ integrin-based aggregation **(Fig. 2)**.

1.3.1 The von Willebrand factor receptor (vWfR) complex and GPIbα.

The vWfR receptor is a complex of 4 polypeptides: GPIbα, GPIbβ, GPIX and GPV [29-31], present at ~25,000-30,000 copies per platelet **(Fig. 2)**. In resting platelets, this highly glycosylated $(GPIb_{\alpha,\beta}/IX)_2V$ -complex is linked to underlying actin filaments by filamin A molecules in an interaction that occurs between the cytoplasmic tail of GPIbα [32,33] and repeat 17 in the carboxyl terminus of filamin A [34,35]. GPIbα's extracellular domain, called glycocalacin, when cleaved from the surface in a soluble form, can be divided into 1) the ligand-binding (LB) domain, encompassing the most

amino-terminal (N-T) 45 kDa of the subunit including the N-T flank, leucine rich repeat (LRR) 1-7, the carboxyl-terminal (C-T) flank, and the sulfated region; and 2) the macroglycopeptide, a mucin-like region that separates the LB-domain from the plasma membrane [36].The main function of the macroglycopeptide domain is believed to be to posit the N-T 45 kDa LB domain at a sufficient distance from the plasma membrane to enable it to capture its ligand when bound to a surface [37]. vWf bound to the subendothelial matrix undergoes a conformational change that reveals the normally cryptic A1 domain which contains the binding site for the $(GPIb_{\alpha,\beta}/IX)_2V$-complex [38]. Soluble vWf also binds to the $(GPIb_{\alpha,\beta}/IX)_2V$-complex under the influence of high shear forces [39] by induction of conformational changes in either vWf or GPIbα, or both [40,41]. Controversy exists where the exact binding sites of vWf are located within the LB-domain of GPIbα. Evidence from co-crystal structures of GPIbα and vWf revealed that the N-T LB-domain of GPIbα contains the binding sites for vWf N- and C-T to LRR 2-4 [42-45]. However LRR 2-4 has been identified as crucial under shear conditions [42], and it is possible therefore that different sites in the LB-domain of GPIbα interact with vWf when adhering under static of flow conditions. The LB-binding domain contains binding sites for the leukocyte integrin $\alpha_M\beta_2$ (α_M I-domain) [46], thrombin [47,48], high molecular weight kininogen [49], and coagulation factors XI [50] and XII [51]. vWfR also mediates interaction of unactivated platelets with endothelium by binding to endothelial P-selectin [52]. There is a progressive and reversible down regulation of vWfR from the cell surface following platelet activation and a portion of the receptor becomes inaccessible to antibodies [53-58]. The molecular mechanism of this reversible vWfR redistribution has not been completely established, but rearrangements of the actin cytoskeleton, actin assembly and myosin II activation are necessary [59].

Glycocalacin, released from GPIbα by the proteolytic action of calpain, has both N- and O-glycosidically linked carbohydrate chains [60]. Glycocalacin can be split into a 90 kDa highly O-glycosylated fragment (the macroglycopeptide) and the 45 kDa LB-domain containing 4 potential N-glycosylation sites [36,61], two of which have been shown to be N-glycosylated [62] **(Fig. 4)**. The N-linked carbohydrate chains of GPIbα are of the complex-type and di-, tri-, and tetra- antennary structures [61,63]. A more detailed description of complex N-linked glycans on GPIbα can be found in section 2.2 and in figure 4.

Studies have shown that stable expression of a functional vWfR in the plasma membrane of cells requires co-expression of $GPIb_{\alpha\beta}$ and GPIX, but not GPV [64]. However, recent studies have revealed that GPV influences signaling in two ways. First, it acts as a negative modulator of thrombin induced platelet activation since its cleavage releases a previously cryptic binding site for thrombin on GPIbα [65]. The platelets of GPV null mice generate a more robust hemostatic response than do the platelets of normal mice. This response is characterized by shortened bleeding times [66] and accelerated thrombus growth in response to vascular injury. Both of these may be related to enhanced thrombin-induced platelet activation in these animals rather than enhanced binding of $(GPIb_{\alpha,\beta}/IX)_2V$ to vWf [67]. Second, GPV also plays a role in collagen signaling pathways leading to platelet activation and facilitates GPVI-dependent collagen interactions [68]. Membrane proximal sequences of GPIbβ and GPV directly bind calmodulin, a cytosolic regulatory protein that is dissociated from the $(GPIb_{\alpha,\beta}/IX)_2V$ upon platelet activation [69]. Although the role of $(GPIb_{\alpha,\beta}/IX)_2V$

associated calmodulin is unknown, calmodulin also binds to GPVI, where it regulates GPVI-dependent Ca^{2+} signaling.

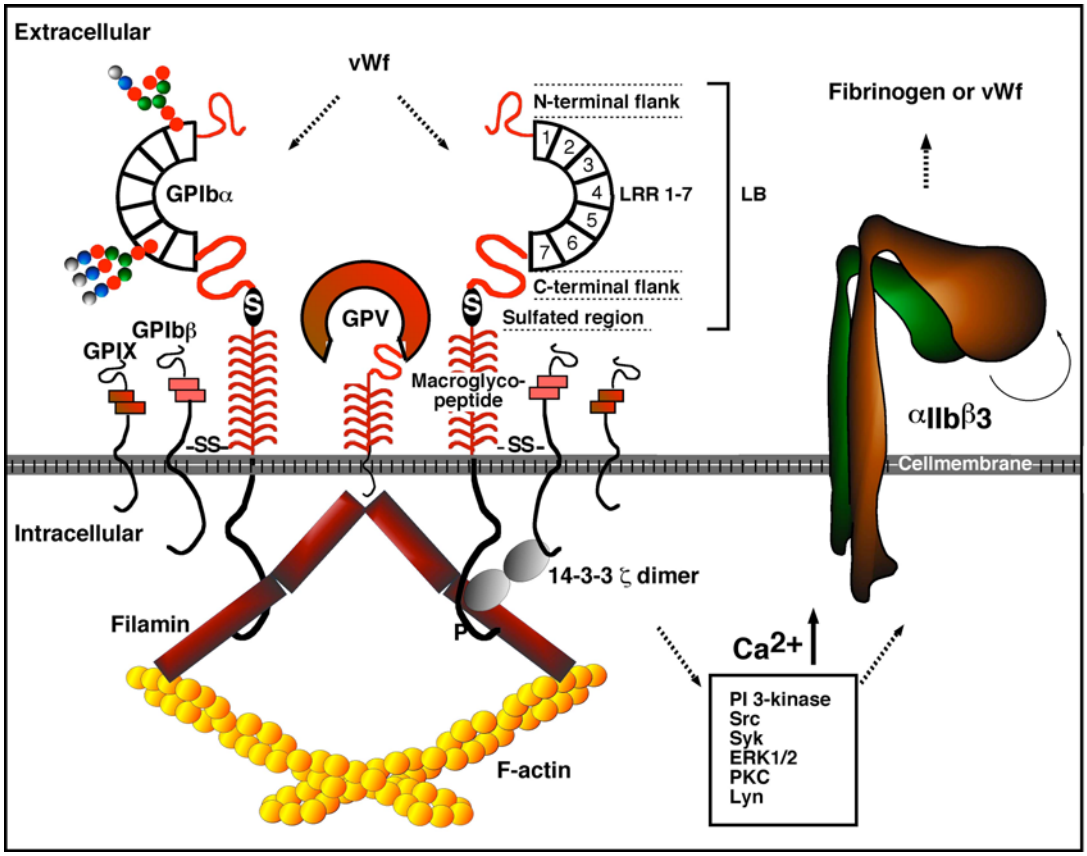

Fig. 2. Platelet receptors: the human von Willebrand factor receptor (vWfR) complex and the $\alpha_{IIb}\beta_3$ integrin.

The vWfR complex consists of four subunits GPIbα, GPIbβ, GPIX and GPV. Filamin binds to the cytoplasmic tail of the GPIbα subunit and links the vWfR complex to the actin cytoskeleton (F-actin). GPIbα's extracellular domain can be divided into 1) the ligand-binding (LB) domain, including the N-terminal flank, leucine rich repeats (LRR 1-7), the C-terminal flank, and the sulfated region; and 2) the C-terminal macroglycopeptide region. N-linked glycosylation sites on GPIbα are indicated in LRR 1 and 6. vWf binds to GPIbα under high shear stress conditions and triggers activation of multiple signaling proteins (PI3-kinase, Src, Syk, ERK1/2, PKC, and Lyn). Thus inside-out signaling eventually results in the binding of talin to the cytoplasmic tail of β_3 and activation the $\alpha_{IIb}\beta_3$ integrin. Fibrinogen binding mediates outside-in signaling and platelet aggregation follows. Dashed arrows indicate the signaling pathway directions.

1.3.2 Integrin $\alpha_{IIb}\beta_3$.

$\alpha_{IIb}\beta_3$ (GPIIb-IIIa, CD41/CD61) **(Fig. 2)** is the major integrin (50,000-80,000 receptors/platelet) on the platelet surface and its expression is restricted to platelets and megakaryocytes. It is activated downstream of the adhesion receptors GPVI and the vWfR, or G-protein coupled receptors, i.e., thrombin (PAR-1 or PAR-4), or ADP receptors ($P2Y_1$ or $P2Y_{12}$) that reinforce $\alpha_{IIb}\beta_3$-dependent platelet aggregation. Inside-out activation of $\alpha_{IIb}\beta_3$ is Ca^{2+}-dependent and involves changes in the conformations of both the ligand-binding extracellular region and the cytoplasmic tails [20-22]. Following ligand binding, outside-in signals and altered interactions with cytoskeletal proteins, such as talin and tyrosine kinases [70,71], control postadhesion events, such as spreading and contraction. The combination of conformational changes and clustering

of integrins is required for full outside-in signaling [72,73]. Additional $\alpha_{IIb}\beta_3$ molecules, which are present in the membrane of the platelet α-granules, can be translocated to the platelet surface after platelet activation [74]. $\alpha_{IIb}\beta_3$ binds fibrinogen or vWf after activation and crosslinks platelets together to form a thrombus.

1.3.3 GPVI and other collagen receptors.

Platelet adhesion to collagen occurs both indirectly, via binding of platelet GPIbα to plasma vWf which binds exposed collagen [75], and directly, via interactions with the platelet integrin $\alpha_2\beta_1$ [76], GPVI [77], and possibly other collagen receptors. GPVI is the major signaling receptor for collagen on the platelet surface [78,79]. It is coupled to a disulfide-linked Fc receptor (FcR) γ-chain homodimer in the membrane via a salt-bridge between charged amino acids within the transmembrane sequences and through specific sequences in the cytosolic tails [80]. Each FcR γ-chain contains one copy of the immunoreceptor tyrosine based activation motif (ITAM) that undergoes tyrosine phosphorylation by Src family kinases upon crosslinking of GPVI, leading to binding and activation of the tyrosine kinase Syk, initiating downstream signaling events. PLCγ2 is recognized as a central target for this signaling cascade [81]. Several lines of evidence suggest that GPVI cross-linking induces signaling, that GPVI functions as a homodimer, and that it is associated with $(GPIb_{\alpha,\beta}/IX)_2V$ on the membrane of resting and activated platelets [23,79,82-86].

1.3.4 Protease activated receptor (PAR) -1 and -4.

Thrombin is a potent activator of platelets *in vivo*. When added to platelets *in vitro*, it causes phosphoinositide hydrolysis, that lead to increases in intracellular Ca^{2+} concentrations, shape change, granule secretion, and aggregation. Thrombin also suppresses cAMP synthesis in platelets by inhibiting adenylate cyclase [87]. All of these effects require thrombin to be proteolytically active. The PAR class of receptors has a distinctive mechanism of activation, involving specific cleavage of the N-T 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 [88], and other PAR receptors, PAR-2 [89], PAR-3 [90] and PAR-4 [91] 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.

1.3.5 ADP receptors.

ADP activates platelets via the G protein-coupled purinergic receptors, $P2Y_1$ and $P2Y_{12}$. $P2Y_1$ coupled to $G\alpha_q$ regulates Ca^{2+} dependent signaling events initiating platelet shape change and a rapid, reversible $\alpha_{IIb}\beta_3$ -dependent platelet aggregation [92,93]. $P2Y_{12}$ is $G\alpha_i$-linked and activates $\alpha_{IIb}\beta_3$ by a mechanism involving inhibition of cAMP production by adenylate cyclase. The current view of the relationship between the two platelet ADP receptors is that $P2Y_1$ initiates aggregation, reinforcing $P2Y_{12}$ [94]. ADP is released from dense granules when platelets are activated by other agonists (including collagen, vWf, or thrombin) and acts on $P2Y_1/P2Y_{12}$ receptors in an autocrine mechanism to promote stable platelet aggregation [95].

Table 1. Major Receptors on the Human Platelet Surface

Class of Receptor		Receptor	Other Names	Number of Receptors/Platelet	Ligand/s
Integrins					
	Adhesion	$\alpha_2\beta_1$	GPIa/IIa,CD49b,VLA-2	~2-4,000	Collagen
		$\alpha_5\beta_1$	GPIc/IIa,CD49e,VLA-5	~4,000	Fibronectin
		$\alpha_6\beta_1$	GPIc/IIa,CD49f, VLA-6	~1,000	Laminin
		$\alpha_L\beta_2$			
	Aggregation	$\alpha_{IIb}\beta_3$	GPIIb-IIIa, CD41/CD61	~50- 80,000	Fibrinogen, vWf
		$\alpha_v\beta_3$	CD51/CD61	~500	Vitronectin, osteo-pontin, vWf, fibrinogen
Leucine-rich receptor		$(GPIb_{\alpha\beta}/IX)_2V$	CD42b,c,a,d	~25-30,000	vWf, thrombin, $\alpha_M\beta_2$
			vWfR		HK, Factor -XI, -XII
G protein-coupled					GPVI, P-selectin
receptors	A) Thrombin	PAR-1		~2,000	Thrombin
	receptors	PAR-4		Low	Thrombin
	B) ADP	$P2Y_1$			ADP
	receptors	$P2Y_{12}$			ADP
	C) Prosta-	TXA_2/PGH_2			Thromboxane
	glandin	PGI_2		~1,000	Prostaglandin I_2
	receptors	PGD_2			
		PGE_2			
	D) Lipid	PAF(R)		~300	PAF
	receptors	LPA(R)			LPA
	E) Chemokine	CXCR1 and R2		~2,000 each	Interleukin-8
	receptors	CXCR4		~2,000	SDF-1
		CCR1 and R3			RANTES
		CCR4		~2,000	MDC
	F) Others	V_{1a} Vasopressin R			Vasopressin
		A_{2a}-Adenosine R			Adenosine
		β_2-Adrenergic R		~700	Epinephrine
		$5\text{–}HT_{2A}$			Serotonin
		Dopamine R	D3, D5		Dopamine
Immunoglobulin		GPVI		1-3,000	Collagen,FcγRIIA,GPIbα
superfamily		FcγRIIA	CD32	~1,000	IgG (Fc), GPVI
receptors		FcεRI			IgE
		PTA-1	CD226		
		JAM-1, -3	F11		β_2 integrins
		ICAM-2			β_2 integrins
		PECAM-1	CD31	1,600-4,600	PECAM-1
		Integrin-assoc. protein	CD47		TSP., SIRPα, $\alpha_{IIb}\beta_3$, $\alpha_2\beta_1$
Selectins		P-selectin, PADGEM	CD62P, GMP-140	~10,000 if activated	PSGL-1, GPIbα
Tetraspanins		CD9	P24	~40,000	Assoc. with integrins
		CD63	GP-53		Assoc. with integrins
		CD82			
		PETA-3	CD151		Assoc. with β_1 integrins
GPI-Anchored Proteins		DAF	CD55		
		CD59			
		CD109			
		PrPC R		~1,800-4,300	

Tyrosine Kinase receptors	CD110	c-mpl		Thrombopoietin
	Tie-1 R			Angiopoietin
	Insulin R			Insulin
	PDGF R			PDGF
ADP- or ATP-driven Ca^{2+}-channel family	$P2X_1$			ADP/ATP
Others	GPIV	GPIIIb, CD36	~25,000	Collagen, TSP
	p65			Collagen
	C1q R	p33		
	C3-Specific binding protein			
	Serotonin Re-Uptake Receptor			Serotonin
	LAMP-1, -2			
	CD40 L	CD154		Interacts with CD40
	Collagen Type -I, -III R			Collagen
	Tight Junction Receptors:			
	Occludin and Zonula Occludens Protein-1			

ADP, Adenosine diphospate; CD, Cluster differentiation; DAF, Decay accelerating factor; GMP, Granule membrane glycoprotein; GP, Glycoprotein; HK, High molecular weight kininogen; LAMP, Lysosomal-associated membrane protein; MDC, Macrophage-derived chemokine; PADGEM, Platelet activation-dependent granule-external membrane protein; PDGF, Platelet-derived growth factor; PECAM-1, Platelet-endothelial cell adhesion molecule-1; PETA, Platelet and endothelial cell tetraspan antigen; PrPC, Prion protein; PSGL-1, P-selectin glycoprotein ligand-1; PTA, Platelet and T cell antigen; SIRPa, Signal-regulatory protein a; SDF-1, Stromal cell-derived factor 1; Tie, Tyrosine kinase with immunoglobulin and epidermal growth factor homology; TSP, Thrombospondin.

Source: Platelet Membrane Proteins and Their Disorders, in Blood: Principles and Practice of Hematology, editors R.I. Handin, S.E. Lux, T.P. Stossel, 1081-1101, 2nd edition, Lippincott Williams and Wilkins, 2002; Platelet receptors, K.J. Clemetson, in Platelets, editor A.D. Michelson, 65-84, 1st edition, Academic Press, 2002; Arthur, Gardiner et al., Thromb Haemost, 2005, 93 (4), 716-23.

1.4 Platelet storage for transfusion - in room temperature or cold?

Thrombocytopenia is a major clinical problem and is in most cases caused by diminished platelet survival time. Many clinical disorders such as atherosclerosis, sepsis and preeclampsia are often accompanied by thrombocytopenia. The maintenance of normal circulating platelet counts is essential for vascular integrity. The only known treatment for acute thrombocytopenia remains platelet transfusion. Platelet storage is complex, because unlike erythrocytes, platelets cannot be refrigerated. Rather, platelets are stored with agitation in plasma at room temperature (RT) in gas permeable bags to allow gas exchange and prevent acidification. Storage at RT is limited to 5 days, because of the increased risk of bacterial growth [96]. The available data indicate that transfusion-associated sepsis develops after 1 in 25,000 platelet transfusions and 1 in 250,000 red blood cell transfusions. One of the most widely used strategies for decreasing bacterial sepsis risk is bacterial detection [97].

It has been known for over 30 years that platelets stored at 4°C have shorter circulation times that 22°C stored platelets, when transfused in human volunteers [98]. When refrigerated murine platelets are injected into mice they also show a dramatically reduced half-life [99]. Storage of platelets at temperatures below 15°C causes shape change in platelets and instead of being discoid, refrigerated platelets change to spiny spheres with irregular projections [100]. The Hartwig/Hoffmeister lab and others have previously shown that short-term platelet refrigeration increases cytosolic calcium [101,102], actin polymerization and shape change [102,103], and induces GPIbα to redistribute from linear arrays (RT) into aggregates on the surface of murine platelets [99]. Crowe *et al.*, have proposed that chilling-induced activation of human blood platelets can be ascribed in part to a thermotropic phase transition of membrane lipids [104]. Low temperature leads to passage of platelet membrane lipids through a phospholipid phase transition between 10 and 20°C [105]. Passage through this transition is correlated with shape changes during chilling [105] but the transition *per se*

is only part of the story; the shape changes seen during the phase transition are completely reversible for up to 24 h in the cold, after which they become irreversible. The same group showed that platelet membranes also undergo lateral phase separation during prolonged storage in the cold [106,107] and that CD36 (GPIV), but not the GPI-anchored protein CD55 or the α_{IIb} integrin, is selectively enriched within detergent resistant membrane domains of cold activated platelets. They have presented evidence that membrane microdomains are maintained intact in the platelets freeze-dried in the presence of the anti-freeze compound, trehalose [108]. Other groups have also tried to circumvent the changes induced to refrigerated platelets by pretreatment of platelets with flavonoids before refrigeration to prevent an increase in cytosolic calcium concentration, actin polymerization and platelet shape change [109], and to metabolically suppress platelets (without glucose and with antimycin A to block energy generation) before storage at 4°C to better preserve platelet *in vitro* function [110].

The discoid shape of the platelets was for long thought to be the best predictor for normal platelet survival time in the circulation. A pharmacological approach used by the Hartwig/Hoffmeister lab to hold refrigerated platelets in a discoid shape using cytochalsin B (actin assembly inhibitor) and EGTA-AM (intracellular calcium chelator) [102], however, did not increase the circulation time of transfused murine platelets [99] nor of baboon platelets [111]. A new effort to address this clinically relevant problem, how platelets are cleared from the circulation, led to the definition of a previously unsuspected platelet clearance mechanism. We found that the macrophage $\alpha_M\beta_2$ recognizes clustered GPIbα subunits of the vWfR complex following short-term refrigeration (2 h) in the absence of plasma, resulting in the phagocytosis and clearance of platelets *in vivo* in mice and *in vitro* by human THP-1 macrophages [99]. Experiments using $\alpha_M\beta_2$ deficient but not vWf, complement or P-selectin deficient, mice [112], improved markedly the survival of refrigerated platelets and the removal of GPIbα's LB-domain by *O*-sialoglycoprotein endopeptidase cleavage restored the circulation of refrigerated platelets [99]. The interaction between platelet GPIbα and macrophage $\alpha_M\beta_2$ is further investigated and discussed in the succeeding publications in this thesis.

2. Short- and long-term platelet refrigeration – implications in platelet clearance.

2.1 Short-term refrigerated platelets are recognized and phagocytized by the macrophage α_M lectin-domain.

We investigated the detailed mechanism mediating the phagocytosis of platelets refrigerated short-term (2 h) by the $\alpha_M\beta_2$ integrin, focusing on which α_M domains were involved [113]. $\alpha_M\beta_2$ (or CR3, CD11b/CD18, MAC-1) **(Fig. 3)** has two main functions. 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 [114,115]. Second, $\alpha_M\beta_2$ serves as a phagocytic receptor for the iC3b fragment of complement [116-118]. The $\alpha_M\beta_2$ receptor shares functional characteristics with other integrins including the bidirectional signaling via conformational changes in the extracellular region that are produced by inside-out signaling [119,120]. The receptor also forms complexes with glycosylphosphatidylinositol

(GPI)-anchored receptors such as FcγRIIIB (CD16b) or uPAR (CD87) providing a transmembrane signaling mechanism for these receptors [119,120]. $\alpha_M\beta_2$, like all integrins, consists of two chains: the α_M- and the β_2 -chain. α_M contains the ligand binding I-domain, a cation-binding region, and a lectin-site. Protein ligands bind to partially overlapping sites contained within the I-domain [121,122] and include ICAM-(1-2), fibrinogen, iC3b, factor X, heparin, junctional adhesion molecule (JAM) 3 [123], and GPIbα [46,124-127]. $\alpha_M\beta_2$ also contains a cation-independent sugar-binding lectin-site, located C-T to its I-domain [128,129], which binds to β-glucans, mannans, and GlcNAc (N-acetyl-D-glucosamine). The lectin-site of α_M recognizes either microbial surface polysaccharides or binds to GPI-linked signaling partners. C3 opsonized microorganisms display iC3b in combination with cell wall polysaccharides, such that both the I-domain and lectin-site of $\alpha_M\beta_2$ become attached to microbial pathogens, stimulating phagocytosis and cytotoxic degranulation [130]. Target cells bearing only iC3b, but not $\alpha_M\beta_2$ binding polysaccharides, do not trigger phagocytosis and/or degranulation, despite avid attachment of the target cells to the I-domain. Particulate, or high molecular weight soluble β-glucans, that are large enough to cross-link the lectin domains of multiple membrane surface $\alpha_M\beta_2$ molecules, stimulate degranulation and the release of inflammatory mediators in the absence of the iC3b-opsonin [131].

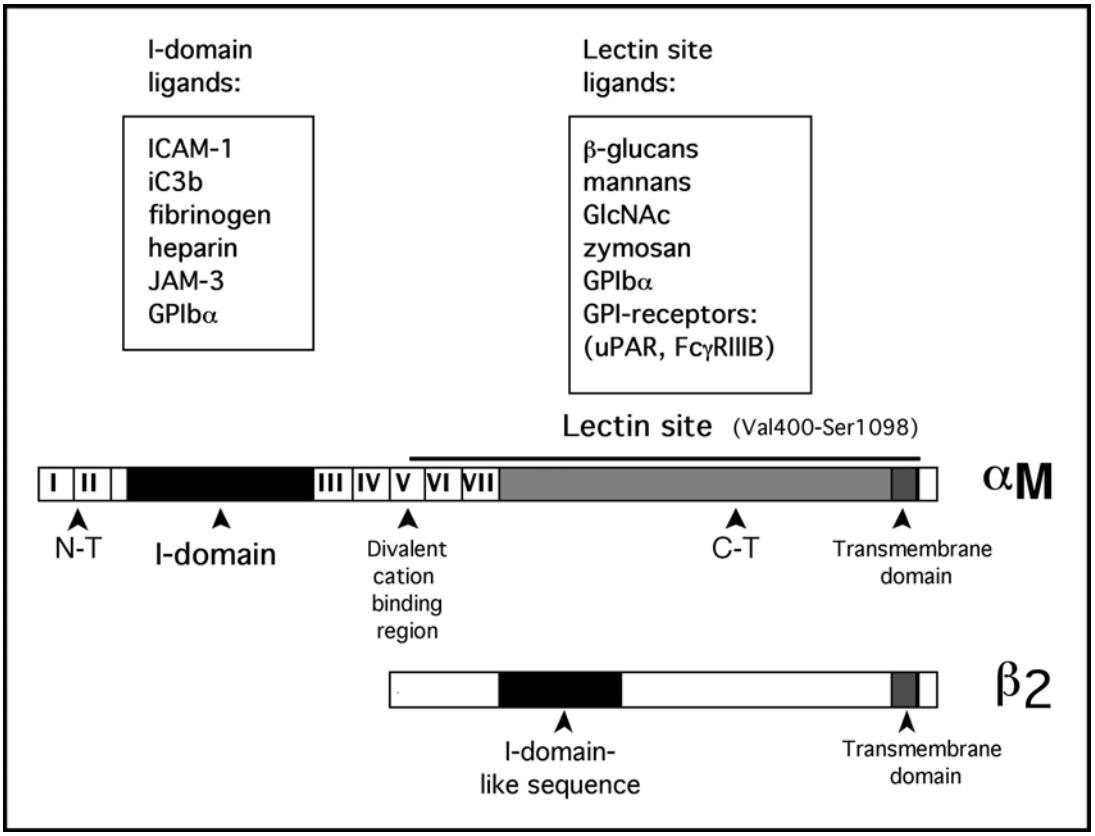

Fig. 3. Structure of the $\alpha_M\beta_2$ (MAC-1) integrin.

The $\alpha_M\beta_2$ receptor is a heterodimer composed of α_M and β_2 subunits. α_M contains multiple ligand binding sites: the ligand-binding I-domain, a divalent cation-binding region, and a lectin site. The drawing illustrates the binding sites of several αM ligands: receptors (GPIbα, ICAM-1, JAM-3, GPI-receptors), soluble protein ligands (iC3b, fibrinogen, heparin), and carbohydrates (β-glucans, mannans, GlcNAc, zymosan).

To dissect the α_M domains involved in the ingestion of human platelets refrigerated short-term in the absence of plasma, they were fed to Chinese hamster ovary (CHO) cells expressing $\alpha_M/\alpha_X\beta_2$- chimeras. Platelet phagocytosis was evaluated by flow cytometry and immunofluorescent microscopy [113]. Ingestion of short-term refrigerated platelets was dependent on the α_M lectin-domain and did not require the I-domain or the presence of divalent cations, showing that exposed carbohydrate residues on refrigerated platelets target the lectin-domain of $\alpha_M\beta_2$. Additional evidences for this conclusion are: 1) a soluble recombinant α_M lectin-domain, but not a soluble α_M I-domain, inhibited the phagocytosis of refrigerated platelets by differentiated macrophages; and 2) Sf9 cells expressing solely recombinant α_M lectin-domain constructs bound refrigerated platelets [113].

2.2 Glycosylation of platelet surface proteins as an approach to protect refrigerated platelets from clearance via $\alpha_M\beta_2$.

Subsequent work narrowed carbohydrate recognition by $\alpha_M\beta_2$ to exposed βGlcNAc residues on N-linked GPIbα glycans [27]. GPIbα N-linked glycans are complex-type branched carbohydrates that are covalently attached to asparagine residues. When completely assembled, they are capped by sialic acid **(Fig. 4)**.

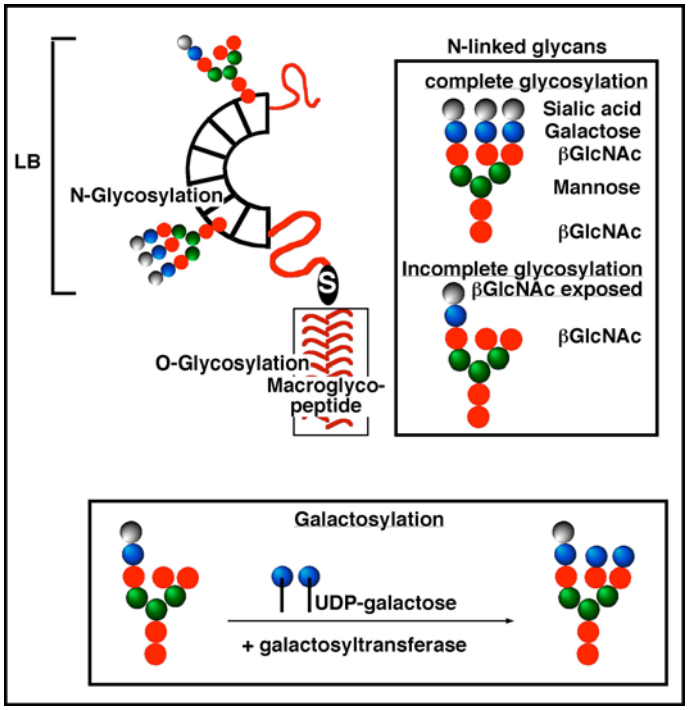

Fig. 4. Location of the N- and O-glycosylation sites on GPIbα.
N-glycosylation sites are located within the ligand binding (LB) (leucine rich repeat 1 and 6) region. The macroglycopeptide is highly O-glycosylated. When mature, N-glycosylated carbohydrate chains are fully covered by sialic acid (complete glycosylation), although platelet GPIbα also contains incomplete N-glycans with exposed β-GlcNAc residues (incomplete glycosylation, right panel). The lower panel summarizes the platelet galactosylation process. A galactosyltransferase enzyme transfers galactose onto exposed β-GlcNAc residues using UDP-galactose as the substrate.

Removal of sialic acid (desialylation) exposes galactose and degalactosylation reveals βGlcNAc. The exposure of individual sugars is detectable by their binding to specific lectins, e.g., Ricinus Communis Agglutinin (RCA I) binds galactose and succinylated Wheat Germ Agglutinin (sWGA) binds βGlcNAc. Resting platelets bind some

sWGA, while refrigerated platelets show increased binding of the same lectin, indicating clustering of immature glycans with exposed βGlcNAc residues. Removal of these residues with the enzyme hexosaminidase converted the cold-dependent ingestion of platelets by THP-1 cells into a temperature independent recognition and ingestion, presumably because removal of βGlcNAc residues exposed mannose residues, which engaged mannose receptors on THP-1 cells [27]. Clustering of βGlcNAc residues attached to GPIbα, evidenced by electron microscopy and by increased sWGA binding to platelets, promoted the phagocytic ingestion of refrigerated platelets [27,99]. We found that human and murine platelets have functional platelet galactosyltransferases and that the simple addition of UDP-galactose was enough to transfer galactose onto the exposed βGlcNAc residues of human or mouse platelet GPIbα [27] **(Fig. 4)**. Platelet galactosylation prevented phagocytosis by macrophage THP-1 cells of short-term (2 h) refrigerated human platelet *in vitro* and the clearance of short-term (2 h) refrigerated murine platelets *in vivo* [27].

2.3 Long-term platelet refrigeration reveals new insights into platelet clearance.

We investigated the *in vitro* function and phagocytosis of galactosylated and non-galactosylated human platelet concentrates prepared under routine blood banking conditions following long-term refrigeration for up to 14 days. We found that platelets in concentrates can be galactosylated in plasma, and that galactosylation is stable following refrigeration for 14 days [132]. Galactosylation prevented phagocytosis of long-term refrigerated platelets by macrophages *in vitro*. Refrigeration with, or without galactosylation, preserved *in vitro* function during extended storage [132]. Using human platelet concentrates, it became clear that there were two important protocol differences between our initial experiments using the murine platelet transfusion model and the human platelet storage conditions for transfusion. For logistical reasons, we worked with isolated platelets and did not store mouse platelets for clearance studies in mice for longer than 2 h. In contrast, a) human platelets for transfusion are stored for days concentrated in plasma, b) accelerated clearance of refrigerated platelets only occurs when human platelet-rich plasma is stored > 8 h in the cold [133]. To directly compare storage conditions, we designed miniature storage containers for mouse platelets resembling those used for human platelet concentrates. Like human platelets, mouse platelets refrigerated in plasma do not clear rapidly unless subjected to long-term storage in the cold, and galactosylation of murine platelets did not prevent clearance although it prevents the clearance of washed platelets refrigerated for 2-4 h [27] (Hoffmeister *et al*., unpublished). Although the clinical relevance of our findings reported here remains to be established in a human clinical setting (a not yet published study lead by Dr. S. Slichter), we were disappointed to find that long-term refrigerated galactosylated murine platelets were cleared with similar rates as non-galactosylated refrigerated platelets. Evidently, different mechanisms account for the clearance of short-term and long-term refrigerated platelets. We therefore begun experiments to understand why and how long-term refrigerated (48 h) platelets were cleared. Like short-term refrigerated platelets, long-term refrigerated platelets are removed in the liver of primates [134] and mice, but are cleared primary by liver *hepatocytes* (Rumjantseva, Hoffmeister *et al.,* unpublished).

Critically, we have acquired evidence that GPIbα plays still a major role in the clearance of long-term refrigerated platelets by transfusing platelets isolated from a

chimeric-mouse model where the extracellular portion of the human GPIbα has been replaced by human IL-4 [135]. These transgenic platelets were refrigerated for 48 h in plasma and their survival after transfusion compared to wild-type (WT) platelets (Hoffmeister *et al.*, unpublished). Although freshly isolated IL-4/GPIbα chimeric platelets are cleared faster than WT platelets, refrigeration was much less effective in accelerating the rate of their clearance. These experiments indicate that the external domain of GPIbα is still a major initiator of cold-induced platelet clearance after long-term refrigeration in plasma. Experiments were, therefore, designed to investigate in more detail, changes that occur in the vWfR after short- (2 h) and long-term (48 h) platelet refrigeration in the absence and presence of plasma [136].

2.4 Differential changes in the platelet vWfR following refrigeration for short or long periods.

In general, most investigators agree that the vWfR remains on the surface of refrigerated platelets, although some investigators have reported vWfR to be slowly lost in the cold due to microvesicle shedding [137]. We further investigated the relationship between vWfR clustering/conformational changes and refrigeration. To study the changes in the vWfR complex occurring following short- and long-term platelet refrigeration, the binding of monoclonal antibodies (mAbs) specific for the vWfR complex subunits GPIbα, GPIX and GPV was analyzed by flow cytometry. We find that certain mAbs can detect changes in the vWfR complex when human and murine platelets are refrigerated, possibly due to conformational changes or aggregation of the vWfR complex [136]. Further, changes in the binding of some anti-human GPIbα mAbs are slowed in plasma, suggesting a retarding effect of plasma on the vWfR rearrangements. Changes in binding efficacy of the mAbs are not related to the loss of GPIbα from the platelet surface as determined by immunoblotting of total GPIbα. Some fibrinogen and vWf binding to platelets refrigerated for 48 h in plasma was detected which may influence the binding of mAbs that bind to GPIbα epitopes near its vWf binding site. Murine and human platelets stored and refrigerated under laboratory and human blood banking conditions respectively do, however, lose significant GPV from their surface, whereas RT stored human platelet concentrates (for 5 days) do not. Murine platelets lacking GPV are hyper-responsive to thrombin activation [65,66], thrombogenesis and embolus formation [67]. It is therefore tempting to speculate that loss of GPV from the platelet surface promotes GPIbα clustering/ rearrangements. Clustering was detectable by fluorescent resonance energy transfer (FRET) in flow cytometry. Refrigeration of platelets for 24 h markedly increased the FRET efficiency between GPIbα and GPV, whereas the FRET between GPIbα and α_{IIb}, a control for a general aggregation of platelet surface glycoproteins, was unaltered [136]. We conclude that vWfR aggregation begins immediately following refrigeration but requires extended refrigeration to become maximal.

3. Discussion

3.1 Cold platelet clearance.

Glycoengineering by platelet galactosylation succeeded in improving the survival of transfused platelets after short-term refrigeration (2 h, no plasma) [27], but was insufficient to accommodate long-term platelet cold storage (48 h, plasma) in mice (Hoffmeister *et al.*, unpublished). Galactosylation substitutes an exposed galactose for

the previously exposed βGlcNAc. While depriving the $\alpha_M\beta_2$ lectin-domain of its βGlcNAc ligand, galactosylation theoretically provides a new ligand for the asialoglycoprotein receptor (ASGPR). Hence, it was surprising that we could improve refrigerated mouse platelet circulation by galactosylation. Galactose exposure can also result from desialylation of mature glycans. We postulated that the number of exposed βGlcNAcs on GPIbα was small, such that even after clustering and galactosylation, the galactose density was insufficient to engage ASGPRs [27]. Now we propose that whereas short-term cold exposure causes clustering of βGlcNAc residues, long-term cold storage in plasma could result in more profound clustering of both exposed β-GlcNAc and galactose residues to an extent that the clearance via ASGPRs takes precedence **(Fig. 5)**.

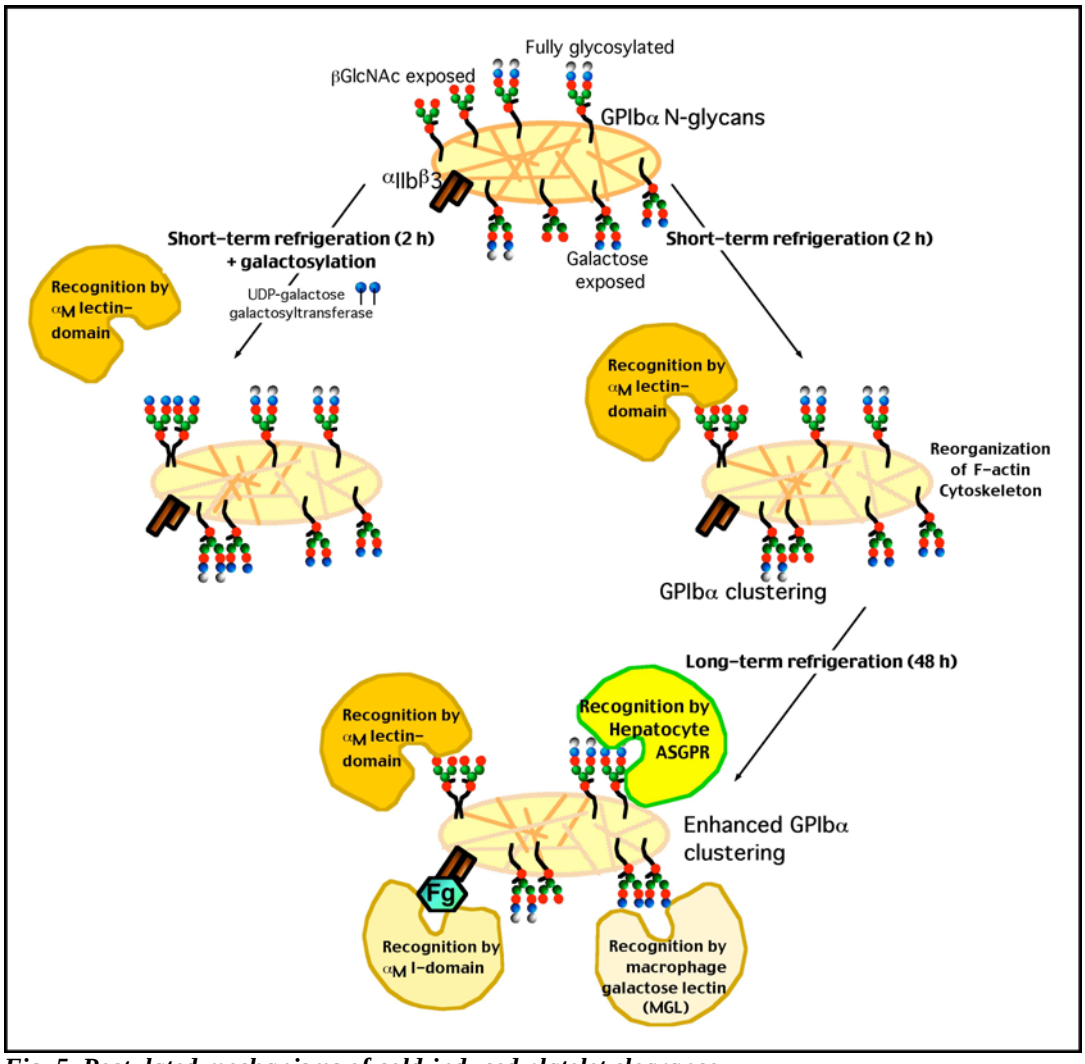

Fig. 5. Postulated mechanisms of cold-induced platelet clearance.
The platelets surface is composed of GPIbα receptors having mature N-linked glycans covered by sialic acid and incomplete carbohydrate chains with exposed βGlcNAc and galactose residues. Short-term refrigeration initiates GPIbα clustering and leads to recognition of exposed β-GlcNAc residues by the macrophage $\alpha_M\beta_2$ integrin lectin-domain. Galactosylation of exposed β-GlcNAc by the addition of galactose prevents $\alpha_M\beta_2$ engagement and platelet phagocytosis. Platelet refrigeration in plasma delays the clustering. However, during extended refrigeration, hyperclustering of GPIbα occurs, which aggregates the exposed galactose and β-GlcNAc residues. Clustered galactose is recognized by hepatocyte asialoglycoprotein receptors (ASGPR) (HL-1/2) and the macrophage galactose lectin (MGL1) receptors. Clustered βGlcNAc is recognized by macrophage/hepatocyte α_M-lectin-domains. Platelet bound fibrinogen (Fg) or platelet GPIbα can also bind to the α_M-I-domains.

Since both galactose-hepatocyte ASGPR and βGlcNAc macrophage/hepatocyte $\alpha_M\beta_2$ interactions could contribute to the clearance of refrigerated platelets, a combination of both galactosylation and sialylation might be required to rescue the circulation of long-term refrigerated platelets. Support for the new hypothesis comes from data showing increased clustering of vWfR after 24 h measured by FRET [136], and data showing that refrigeration for 48 h in plasma increases the binding of the RCA lectin (Hoffmeister *et al.*, unpublished), indicative of galactose exposure. ASGPR-mediated clearance of desialylated proteins takes place in cells carrying this receptor, and we have provocative new data indicating that long-term refrigerated platelets accumulate in murine hepatocytes (Rumjantseva *et al.*, unpublished) as opposed to macrophages. Another potential explanation of the increased clearance of galactosylated and non-galactosylated murine platelets after long-term refrigeration could be increased binding of platelet associated fibrinogen (see manuscript III) [136], which could engage the binding to the α_M I-domain on both macrophages and hepatocytes in a cation-dependent fashion, or the possible involvement of the macrophage galactose lectin (MGL) receptor, or other phagocytic receptors on macrophages and hepatocytes. However, these changes also occur on *in vitro* activated platelets which circulate with normal kinetics.

3.2 Clustering of the vWfR complex.

We have found the redistribution of GPIbα from linear arrays (RT) into aggregates on the surface of 2 h refrigerated murine [99] and human [136] platelets by immunogold electron microscopy. Platelet refrigeration for 24 h promotes a further GPIbα aggregation, which is demonstrated by a 2.8-fold increase in the FRET efficiency between fluorescently labeled $F(ab')_2$ towards GPIbα (VM16d) and GPV (NAM12-6B6) [136]. The FRET could result from both a closer proximity between GPIbα and GPV in the single receptor complex, which seems unlikely, or from the clustering between neighboring vWf receptors. $(GPIb_{\alpha,\beta}/IX)_2V$ clustering has been suggested to promote platelet activation. In CHO cells, chemically inducted oligomerization of GPIb-IX$(FKBP)_2$ increased vWf-binding affinity under flow conditions (assessed by optical tweezers) [138]. Elimination of GPIbα binding sites for the adapter protein 14-3-3ζ and the putative cytoskeletal-linkage protein filamin facilitates receptor lateral mobility and clustering [139,140] suggesting a regulated mechanism for $(GPIb_{\alpha,\beta}/IX)_2V$ clustering. Platelets refrigerated even for short periods have enhanced binding to vWf under shear stress conditions [141] and a decrease in plasma vWf in whole blood refrigerated up to 6 h, has been attributed to increased sequestering of vWf by GPIbα binding [142]. Our finding that prolonged platelet refrigeration, but not short-term platelet refrigeration, induces vWf binding as assessed in flow cytometry, suggests that vWfR clustering may play a role in the vWf binding induced by refrigeration.

Treatment of human platelets with latrunculin A, to depolymerize cytoskeletal actin, induced a clustering of GPIbα on the platelet surface at RT as shown by immune electron microscopy [136] and increased the FRET efficiency by 1.7 fold when compared to fresh RT control platelets. Pretreatment of human platelets with actin polymerization inhibitors also enhances ristocetin induced platelet aggregation and shear-induced platelet aggregation [143] and fibrinogen binding to $\alpha_{IIb}\beta_3$ [144]. Cytoskeletal rearrangements may, therefore, promote GPIbα clustering.

Murine and human platelets stored and refrigerated under laboratory and human blood banking conditions respectively do, however, lose significant GPV from their surface, whereas RT stored human platelet concentrates (for 5 days) do not [136]. Murine platelets lacking GPV are hyper-responsive to thrombin activation [65,66], thrombogenesis and embolus formation [67]. It is therefore tempting to speculate that loss of GPV from the platelet surface promotes GPIbα clustering. The loss of GPV following platelet refrigeration may be mediated by enzymatic or proteolytical cleavage [145-147], however, the exact mechanism remains to be determined.

vWfR clustering following platelet refrigeration could also be triggered by lipid raft aggregation. Others have provided evidence that lipid raft clustering plays a role in platelet activation. Lipid rafts are believed to act as platforms for signal transduction by selectively attracting certain proteins, while excluding others, and recent studies indicate that rafts are important in GPVI receptor signaling [148-150], and in cold activation of platelets [151]. It remains to be determined if changes in the lipid bilayer facilitate GPIbα clustering induced by refrigeration.

3.3 New approaches in platelet transfusion.

Bacterial contamination and growth in platelet products remains an important complication of platelet transfusion. Some investigators have shown that bacterial screening technology is useful for eliminating the transfusion of platelet units that contain high levels of contaminating bacteria, but poorly detect lower bacterial levels, which often become test-positive only upon longer storage [97]. These data suggest that bacterial screening does not prevent all transfusion-transmitted bacterial infections. Pathogen inactivation of blood products is another possible approach to overcome the bacterial contamination issue. New technology of psoralen inactivation of pathogens is under development [152,153]. The technology is based on psoralen-based compounds that intercalate into helical regions of DNA or RNA and on illumination with ultraviolet A light psoralen reacts with pyrimidine bases to form internucleic and intranucleic acid strand cross-links. The photochemical treatment (PCT) inhibits replication of any DNA or RNA. This approach can achieve reduction of a broad range of viruses, bacteria, and protozoa to levels below those likely to transmit infection [152,154]. Extensive toxicology, mutagenicity, carcinogenicity, phototoxicity, and pharmacologic studies established an adequate safety profile for PCT platelets [155,156]. *In vitro* platelet function of PCT platelets was preserved following up to 7 days of storage [157] , although the viability of 5 day-old PCT platelets was worse when compared to control platelets [158]. In the phase 3 SPRINT trial the incidence of grade 2 bleeding was equivalent for PCT and conventional platelets, although post transfusion platelet count increments and days to next transfusion were decreased for PCT compared with conventional platelets [152]. The overall safety profile of PCT platelets was comparable to untreated platelets [159], although it has been reported that mitochondrial DNA in platelets is substantially modified by PCT and that these modifications can be documented by a PCR inhibition system [160]. The long-term effects of such changes still remain to be determined.

4. Concluding Remarks

An attempt to tackle a practical problem, how to refrigerate platelets for transfusion, led us to define a previously unidentified platelet clearance mechanism. The macrophage $\alpha_M\beta_2$ recognizes GPIbα associated βGlcNAc moieties following short-term refrigeration (2 h) in the absence of plasma, resulting in phagocytosis of human platelets *in vitro* and clearance of murine platelets *in vivo* [27,99].

The major findings of this thesis are:
1) The macrophage α_M-subunit lectin-domain recognizes β-GlcNAc carbohydrates on GPIbα on short-term refrigerated platelets and this recognition is sufficient to induce phagocytosis [113]. Human platelets short-term refrigerated in the absence of plasma were fed to CHO cells expressing $\alpha_M/\alpha_X\beta_2$-chimeras and platelet phagocytosis was determined by flow cytometry and immunofluorescence microscopy. Platelet ingestion was dependent on the α_M lectin-domain and did not require the I-domain or the presence of divalent cations.

2) Galactosylation of clustered GPIbα N-glycan βGlcNAc residues blocks ingestion by the macrophage $\alpha_M\beta_2$ and allows short-term refrigerated platelets to circulate in mice [27], but does not prevent the removal of murine platelets refrigerated long-term in plasma.

3) GPIbα clustering is a key event in the changes that mediate platelet clearance. We further investigated the relationship between vWfR clustering/conformational changes following short- and long-term platelet refrigeration. **A) We found that refrigeration of platelets causes certain epitopes on the vWfR to become cryptic.** The binding of some anti-human GPIbα mAbs is reduced during early stages of refrigeration when compared to platelets refrigerated in plasma, suggesting a retarding effect of plasma on the vWfR changes. Changes in binding efficacy of the mAbs are not caused by the loss of GPIbα from the platelet surface as determined by immunoblotting of total GPIbα. **B) Some fibrinogen and vWf binding to platelets refrigerated for 48 h in plasma was detected that could influence the binding of mAbs which bind to GPIbα near its vWf binding site.** Murine and human platelets stored and refrigerated under laboratory and human blood banking conditions respectively do, however, lose significant GPV from their surface, whereas RT stored human platelet concentrates (for 5 days) do not. Murine platelets lacking GPV are hyper-responsive to thrombin activation [65,66], thrombogenesis and embolus formation [67]. It is therefore tempting to speculate that loss of GPV from the platelet surface promotes GPIbα clustering. Clustering was evident by FRET in flow cytometry when human platelets were refrigerated for 24 h as increased FRET efficiency between GPIbα and GPV**. C) We conclude that vWfR aggregation begins immediately following refrigeration but requires extended refrigeration to become maximal [136].**

Our findings demonstrate that prolonged platelet refrigeration induces more profound changes in platelet surface receptors, notably GPIbα and GPV, than observed previously following short-term refrigeration, implying that additional phagocytic mechanisms might operate after long-term platelet refrigeration in plasma.

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