In Vitro and *In Vivo* Studies on the Activating Platelet Collagen Receptor Glycoprotein VI in Mice

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Summary

Damage to the integrity of the vessel wall results in exposure of the subendothelial extracellular matrix (ECM) to the flowing blood. Thus, platelets come in contact with collagen, the major component of the ECM which is considered as the most thrombogenic substrate therein. Upon adhesion to collagen platelets get activated and a thrombus is formed to limit blood loss and finally re-establish the normal blood circulation. However, formation of occlusive thrombi, e.g. as observed upon the rupture of an atherosclerotic plaque, can result in life-threatening ischemic syndromes such as myocardial infarction or stroke.

The work summarized here focused on the characterization of the murine platelet collagen receptor glycoprotein (GP) VI and was performed to evaluate its potential as an antithrombotic target. The first mAb against (mouse) GPVI, JAQ1, was generated and used to demonstrate that GPVI requires the FcRγ-chain for its expression and function and that this receptor is the central molecule in collagen-induced platelet activation. Blocking the major collagen binding site on GPVI with JAQ1 revealed the presence of a second activatory epitope within collagen. Additionally, the collagen receptor integrin $\alpha_2\beta_1$ was found to be required for activation *via* this second pathway but not to be essential for collagen-induced activation of normal platelets.

In studies with mice expressing reduced levels of the GPVI-FcRγ-complex, differential responses to GPVI ligands were observed. Most importantly, the striking difference between platelet responses to collagen and the GPVI specific synthetic collagen related peptide (CRP) confirmed the supportive role of other collagen receptor(s) on platelets. Irrespective of yet undefined additional receptors, studies with mice deficient in GPVI (FcRγ-chain) or $\alpha_2\beta_1$ showed that GPVI, but not $\alpha_2\beta_1$ is essential for platelet-collagen interaction. Based on these results, the model of platelet attachment to collagen was revised establishing GPVI as the initial activating receptor which upregulates the activity of integrins, thus enabling firm attachment of platelets to the ECM.

While the mAb JAQ1 had only limited inhibitory effects on collagen-induced activation *in vitro*, its *in vivo* application to mice resulted in completely abolished platelet responses to collagen and the GPVI specific agonists CRP and convulxin. This effect was found to be due to antibody-induced irreversible down-regulation of GPVI on circulating platelets for at least two weeks. Further studies revealed that GPVI depletion occurs independently of the targeted epitope on the receptor and does not require the divalent form of IgG as it was also induced by mAbs (JAQ2, JAQ3) or the respective Fab fragments directed against epitopes distinct from the major collagen binding site. The internalization of GPVI *in vivo* resulted in a long-term protection of the mice from lethal collagen-dependent thromboembolism whereas it had only moderate effects on the bleeding time, probably because the treatment did not affect other activation pathways. These results establish GPVI as a potential pharmacological target for the prevention of ischemic cardio-vascular diseases and may open the way for a completely new generation of antithrombotics.

Zusammenfassung

Wird ein Blutgefäß verletzt, kommt die extrazelluläre Matrix (EZM) in Kontakt mit dem Blutstrom. Kollagen, der thrombogenste Bestandteil der EZM, aktiviert daraufhin die Thrombozyten und dient als Adhäsionssubstrat für den anschließenden Thrombusaufbau. Dieser Prozess ist essentiell für die Blutstillung (Hämostase), kann aber auch, etwa nach dem Aufbrechen atherosklerotischer Plaques, zu lebensbedrohlichen ischämischen Zwischenfällen wie Herzinfarkt oder Schlaganfall führen.

In der vorliegenden Arbeit wurde untersucht, ob der thrombozytäre Kollagenrezeptor Glykoprotein (GP) VI eine geeignete Zielstruktur für neue Antithrombotika darstellt. Dazu wurden monoklonale Antikörper (mAk) gegen murines GPVI hergestellt (JAQ1, 2 und 3) und deren *in vitro* und *in vivo* Effekte im Maussystem untersucht. Es wurde erstmals gezeigt, dass die Expression und Funktion von GPVI auf Thrombozyten von der Assoziation mit der signaltransduzierenden Fc Rezeptor γ-Kette abhängt. Obwohl GPVI als zentraler Kollagenrezeptor auf Thrombozyten identifiziert wurde, hat die Blockade der Hauptbindestelle für Kollagen mit JAQ1 die Aktivierung nicht vollständig inhibiert, was erstmals die Existenz zweier unabhängiger aktivierender Motive im Kollagen zeigte. Der Kollagenrezeptor Integrin $\alpha_2\beta_1$ ist essentiell für eine Aktivierung durch diesen alternativen Signalweg, nicht jedoch für die Kollagen-induzierte Aktivierung normaler Thrombozyten. Die Präsenz anderer Kollagenrezeptoren neben GPVI wurde in Untersuchungen an Thrombozyten mit reduzierten Expressionsraten des GPVI-FcRγ-Komplexes bestätigt. Unabhängig davon wurde jedoch anhand von GPVI-, FcRγ- und $\alpha_2\beta_1$-defizienten Mäusen belegt, dass GPVI, nicht aber wie zuvor angenommen $\alpha_2\beta_1$ der zentrale Kollagenrezeptor auf Thrombozyten ist. Diese Ergebnisse wurden in einem veränderten Modell der Thrombozyten-Kollagen Interaktion zusammengefasst, in dem GPVI als der initiale Rezeptor zur Integrinaktivierung und somit zur festen Adhäsion der Thrombozyten an die EZM etabliert wird.

Im Gegensatz zu den *in vitro* Resultaten mit JAQ1 waren Thrombozyten von anti-GPVI-behandelten Mäusen weder durch Kollagen noch durch andere GPVI Liganden aktivierbar. Es zeigte sich, dass die Antikörper *in vivo* die Internalisierung sowie den proteolytischen Abbau des Rezeptors induzierten. Dieser Effekt war unabhängig von der Bindungsstelle auf GPVI und konnte auch mit monovalenten anti-GPVI Fab Fragmenten erzielt werden. Während der mindestens zweiwöchigen GPVI-Defizienz der zirkulierenden Thrombozyten waren die JAQ1-behandelten Mäuse vor Kollagen-induzierter Thromboembolie geschützt. Darüber hinaus hatte die GPVI-Depletion nur geringe Effekte auf die Blutungszeit, wahrscheinlich weil diese Behandlung keine anderen Aktivierungswege beeinflusste. Diese Ergebnisse zeigen, dass GPVI ein viel versprechendes pharmakologisches Zielprotein für die prophylaktische Therapie kardiovaskulärer Krankheiten ist, das als Grundlage zur Entwicklung neuer Antithrombotika dienen kann.

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A Introduction

Platelets are discoid, anucleated cells that originate from the cytoplasm of bone marrow megakaryocytes. Circulating in the blood, platelets survey the integrity of the vascular system. As a response to vascular injury, platelet adhesion to exposed tissues and to one another results in the formation of a platelet plug, which, in combination with the coagulation system, allows the rapid re-establishment of normal blood flow in the disrupted vasculature. However, neither platelets nor other components of the hemostatic process can distinguish between traumatic wounds and other lesions that may develop in the vessel wall. Formation of occlusive thrombi, e.g. as observed upon the rupture of an atherosclerotic plaque, then becomes a life-threatening disease mechanism that may cause death or serious pathological complications, such as ischemic syndromes of the heart and brain. Knowledge of the mechanisms of platelet function has enabled the development of potent and selective antithrombotic drugs, and first-generation antiplatelet compounds that specifically block activating receptors or adhesion receptors have already proven beneficial in the clinic.

A.1 Platelet production, structure and function

Megakaryocyte maturation involves nuclear duplication without cell division, resulting in giant cells. Cytoplasmic organelles are organized into domains representing nascent platelets, demarcated by a network of invaginated plasma membranes. Within the bone marrow, megakaryocytes are localized next to the sinusoidal walls, which facilitates the exit of large segments of cytoplasm into the circulation. The fragmentation of megakaryocyte cytoplasm into individual platelets then results from the shear forces of circulating blood, perhaps largely in the pulmonary circulation[1].

Human platelets are about 3 µm in diameter and 1 µm thick, hence they are the smallest cells of the hematopoietic system. The platelet count in humans normally ranges from 200,000 to 400,000 platelets/µl, the lifespan of individual platelets thereby being approximately ten days. The platelet is surrounded by a typical bilamellar plasma membrane that extends through the multiple channels of the surface connected canalicular system, greatly increasing the surface area of the platelet. Through the phospholipid bilayer, intrinsic platelet glycoproteins (GPs) such as GPIa/IIa, GPIb-V-IX, GPIIb/IIIa and GPVI are extruding which serve as platelet receptors mediating activatory and inhibitory signals and platelet responses such as adhesion and aggregation[2]. Close contact with elements of the intraplatelet contractile system have been demonstrated for the intracytoplasmic tails of several GPs, e.g. GPIbα and GPIIb/IIIa[3].

The dense tubular system is the platelet equivalent of the smooth endoplasmatic reticulum in other cells; it is the site where calcium is sequestered and enzymes involved in prostaglandin synthesis are localized.

Platelets are capable of only limited protein synthesis. There are numerous organelles dispersed in the cytoplasm, including (few) mitochondriae, glycogen particles, lysosomes, and peroxisomes. α-granules and dense granules are platelet-specific storage granules. α-granules contain mainly proteins important for coagulation and platelet aggregation such as fibrinogen, fibronectin, thrombospondin, plasminogen activator inhibitor I, and von Willebrand factor (vWf), as well as platelet-specific proteins like platelet factor 4, β-thromboglobulin, and platelet-derived growth factor. Dense granules are rich in calcium, inorganic phosphates, and activating substances like serotonin and adenosine diphosphate (ADP)[4].

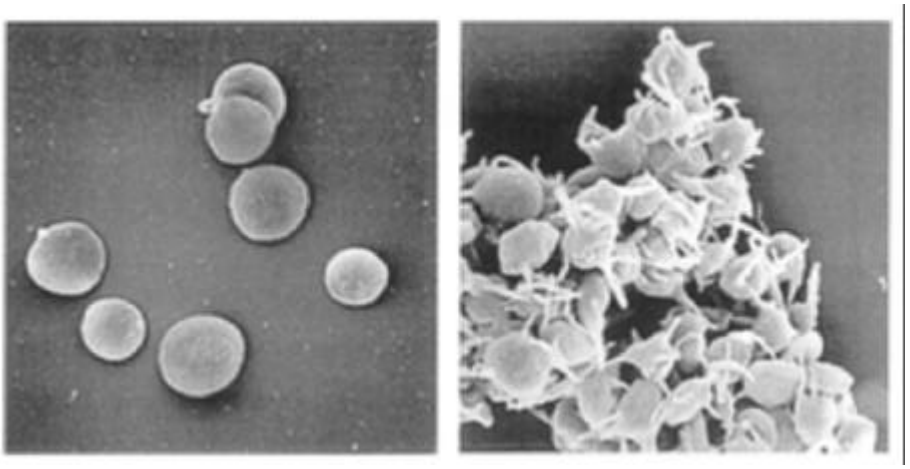


Figure A.1. Scanning electron micrograph of resting and activated platelets. Platelets from wild type mice were incubated with (right) or without (left) adenosine diphosphate (ADP), fixed and examined under a scanning electron microscope (from collaborative studies with C. Gachet, INSERM, Strasbourg, France).

At sites of vascular injury, the thromboresistant endothelium is disrupted and prothrombotic subendothelial vessel wall constituents (e.g. collagen) are exposed to the flowing blood, thereby inducing hemostatic processes to re-establish the normal blood circulation. Platelet activation is one of the initial events, upon which they change their discoid shape into a spherical form and extend long, spiky pseudopods as well as bulky surface protrusions (Fig. A.1). The organelles are contracted towards the platelet center and are enclosed by a tight fitting-ring of newly reassembled microtubules and microfilaments. Finally, degranulation results in release of granule contents and diffusion of internal granular membrane proteins such as P-selectin into the plasma membrane. While dense granule contents are easily released, α-granule release requires higher agonist concentrations. In addition to release of granule contents, platelets also produce and secrete pharmacologically active substances

like thromboxane A_2 (TxA_2) and platelet activating factor (PAF) during their activation and aggregation, establishing a positive feedback system that is known as “second(ary) wave“ of platelet aggregation. Furthermore, activated platelets can provide a procoagulant surface by exposure of negatively charged phosphatidylserine (PS) on their outer membrane, which potently stimulates prothrombinase activity and thereby blood coagulation[5].

A.2 The role of platelets in hemostasis

Platelet aggregation is a key mechanism for normal hemostasis limiting blood loss after vascular injury[6;7] which is often initiated by abrupt disrupture of an atherosclerotic plaque[8;9]. Damage to the integrity of the vessel wall results in exposure of the subendothelial extracellular matrix (ECM) to the flowing blood followed by platelet activation and thrombus formation. Although several of the macromolecular components of the subendothelial layer such as laminin, fibronectin, and vWf all provide a suitable substrate for platelet adhesion, fibrillar collagen is considered the most thrombogenic constituent of the ECM, as it not only supports platelet adhesion but is also a strong platelet activator[10;11]. The interaction between platelets and collagen is complex and involves the synergistic action of integrins, Ig-like receptors, G-protein-coupled receptors, and leucine-rich receptors[12;13]. Besides GPIb-IX-V, which indirectly interacts with collagen *via* vWf[14], several collagen receptors have been identified on platelets, including integrin $\alpha_2\beta_1$[15], and the nonintegrin GPVI[16;17]. The previously accepted model for platelet attachment to the ECM will be described in detail in part A.4.

A.3 Platelet surface receptors

A.3.1 Integrins

Integrins are non-covalently linked heterodimers of α and β subunits. They are trans-membrane proteins that are constitutively expressed in a low affinity state on platelets and require activation in order to bind their ligand. Integrins exhibit both “outside-in” and “inside-out” signaling properties.

3.1.1 b_1-integrins

Platelets express three different integrins of the β_1 subfamily: $\alpha_2\beta_1$ (collagen receptor), $\alpha_5\beta_1$ (fibronectin receptor), and $\alpha_6\beta_1$ (laminin receptor). While the roles of $\alpha_5\beta_1$ and $\alpha_6\beta_1$ on platelets are still poorly defined, the collagen receptor $\alpha_2\beta_1$, also termed GPIa/IIa, has been

intensively studied. The first indication that it might play a fundamental role in hemostasis came from platelet pathobiology. Nieuwenhuis et al.[18] described a patient with a pronounced bleeding disorder whose platelets showed selective nonresponsiveness to collagen, markedly impaired adhesion to collagenous substrates, and a selective deficiency of the α_2 integrin subunit (GPIa) resulting in the absence of platelet surface $\alpha_2\beta_1$ integrin. Subsequent studies confirmed the role of $\alpha_2\beta_1$ as a collagen receptor on platelets[19] but also gave rise to conflicting results on its importance for platelet-collagen interactions. So far, only very few patients with impaired $\alpha_2\beta_1$ function have been described and it is not clear whether some or all of them suffered from additional defects that might contribute to their phenotype. Recently, the group of R. Fässler (MPI for Biochemistry, Martinsried, Germany) generated conditional knock out mice with a deletion of the β_1 gene in the hematopoietic system of adult mice. Those mice produce platelets which lack all three β_1 integrins, $\alpha_2\beta_1$, $\alpha_5\beta_1$, and $\alpha_6\beta_1$. In collaborative studies, these mice were used to examine the role of $\alpha_2\beta_1$ in platelet function.

3.1.2 Integrin $a_{IIb}b_3$ (fibrinogen receptor, GPIIb/IIIa)

Integrin $\alpha_{IIb}\beta_3$ is exclusively expressed on cells of the megakaryocytic lineage with about 40,000-80,000 copies/cell expressed on resting platelets. Additional receptors can be recruited from intracellular compartments and are exposed on the platelet surface upon platelet activation. Receptor activation is believed to induce conformational changes in the N-terminal domains of the α and β subunits where the ligand binding sites for fibrinogen, vWf, fibronectin, and vitronectin are located, resulting in a high-affinity state of the receptor.

In addition to the adhesive functions of $\alpha_{IIb}\beta_3$, this integrin serves as a bidirectional mediator for biochemical and mechanical information flow across the platelet plasma membrane. For example, intracellular signals modulate the ligand-binding function of the receptor (“inside-out”), and signals generated by ligation and clustering of $\alpha_{IIb}\beta_3$ regulate the extent of platelet aggregation and spreading (“outside-in”)[20].

$\alpha_{IIb}\beta_3$ is the most intensively studied platelet glycoprotein and has become an important antithrombotic target. Inhibition of $\alpha_{IIb}\beta_3$ blocks the final common pathway of platelet aggregation, irrespective of the stimulus that activates the cell. However, as platelet aggregation is also a key mechanism of normal hemostasis, such treatment includes a potential bleeding risk, which accounts for the need of new, safer, antithrombotic therapies.

Integrins and other adhesion receptors

Receptor	Ligand/ Agonist	Molecular Weight	Distribution	Other Names
GPIIb/IIIa ($\alpha_{IIb}\beta_3$)	fibrinogen, vWf, fibronectin, vitronectin	125α-22β/95	Mg	CD 41/61
GPIa/IIa ($\alpha_2\beta_1$)	collagen	153/130	broad expression	CD 49b/29
GPIc/IIa ($\alpha_5\beta_1$)	fibronectin	160/130	broad expression	CD 49e/29
GPIc'/IIa ($\alpha_6\beta_1$)	laminin	160/130	broad expression	CD 49f/29
$\alpha_V\beta_3$	vitronectin/ thrombospondin	195/95	Mg, EC	CD 51/61
GPIb/IX	vWf, thrombin, P-selectin, Mac-1	143α-22β/22	Mg	CD 42a-c
GPV	collagen?	89	Mg	CD 42d
GPIV	thrombospondin, collagen?	88	Mg, Ma, B	CD 36
CD9		24	Mg, Mo, pB, EC, F	P24
P-selectin	PSGL-1	140	Mg, EC	CD 62P

Table 1. Prominent platelet glycoproteins (GPs)

Abbreviations: B = B-cell; pB = pre B-cell; EC = endothelial cell; F = fibroblast; Ma = macrophage; Mg = megakaryocyte; Mo = monocyte; PSGL-1 = P-selectin glycoprotein ligand-1

A.3.2 The GPIb-V-IX complex (von Willebrand factor receptor)

GPIb-V-IX is a constitutively active receptor for von Willebrand factor (vWf), expressing ~25,000 copies per platelet and causing immediate platelet attachment to exposed perivascular vWf[21]. Binding sites for vWf and various other ligands have been identified on the N-terminal region of the GPIbα subunit[22]. The initial tethering of platelets to vWf *via* GPIb-V-IX is intrinsically short-lived, therefore it only mediates platelet rolling and does not result in firm adhesive contacts[23]. However, it is crucial under conditions of high shear as found in arterioles or larger arteries with obstructed lumina (e.g. in atherosclerotic vessels) because it allows subsequent interactions of other platelet receptors with their ligands[14;23]. In particular the activated platelet integrin $\alpha_{IIb}\beta_3$ then forms stable bonds between tethered platelets and the substrate[14;24].

Furthermore, GPIbα contains binding sites for P-selectin[25] and Mac-1[26] indicating that the receptor might play an important role in the crosstalk between leukocytes and platelets.

A.3.3 G-protein-coupled activating receptors

Platelets can be activated by a variety of physiological agonists, e.g. thrombin, TxA_2, collagen, ADP, PAF, serotonin, and epinephrine that exert their effects through the interaction with specific receptors on the plasma membrane. Most of these agonist receptors are coupled to activatory as well as inhibitory guanine-nucleotide binding proteins (G-proteins)[27;28]. Prominent members of this group are PAR1, PAR3, and PAR4 (thrombin receptors)[29], $P2Y_1$ and $P2Y_{12}$ (ADP receptors)[30;31], and TPα (thromboxane A_2 receptor)[32]. Irrespective of the agonist, intracellular signaling events lead to activation of second messenger-generating enzymes like adenylyl cyclase, phospholipase C and phospholipase A_2. The final common pathway of platelet activation is the transformation of integrins like $\alpha_{IIb}\beta_3$ from the low-affinity to the high-affinity state which is a prerequisite for stable platelet adhesion and aggregation.

Activating receptors

Receptor	Stimulus	G-protein
PAR-1/3	thrombin	$G\alpha_i$, $G\alpha_q$, $G\alpha_{12/13}$
PAR-4		$G\alpha_i$, $G\alpha_q$, $G\alpha_{12/13}$
$P2Y_1$	ADP	$G\alpha_q$
$P2Y_{12}$		$G\alpha_i$
TPα	Thromboxane A_2	$G\alpha_q$, $G\alpha_{12/13}$
α_{2A}	epinephrine	$G\alpha_i$
5-HT_{2a}	serotonin	$G\alpha_q$
GPVI	collagen	FcRγ-chain

Table 2. Activating platelet receptors

A.3.4 The activating collagen receptor GPVI

Among the activating receptors on platelets, the collagen receptor GPVI is unique as it is not coupled to G-proteins but signals through a mechanism similar to that used by immuno-receptors. GPVI was initially described in 1982 by Clemetson and co-workers who identified a 60-65 kD platelet glycoprotein by 2-D gel electrophoresis[33]. Seven years later, the first indication that GPVI may be an important collagen receptor came from studies on a patient

whose platelets lacked GPVI. A mild bleeding time prolongation and unresponsiveness of platelets to collagen was observed whereas activation by other stimuli was normal[34]. A small number of patients with low levels of GPVI displayed similar phenotypes confirming the important role of GPVI in collagen-induced platelet activation[16;17]. Later, additional studies using synthetic collagen-related peptides (CRP, see A.3.4.1) provided evidence that GPVI recognizes the quaternary structure of the collagen molecule[35].

The recent cloning of human and mouse GPVI demonstrated this receptor as a type I transmembrane glycoprotein (~60 kD) belonging to the immunoglobulin (Ig) superfamily[36;37]. GPVI is noncovalently associated with the signal transducing FcRγ-chain[38;39]. Cross-linking of GPVI leads to tyrosine phosphorylation of the FcRγ-chain on its immunoreceptor tyrosine-based activation motif (ITAM) by the Src kinases Fyn and Lyn[40-42]. This leads to binding and subsequent activation of the tandem SH2-domain-containing tyrosine kinase, Syk, which initiates a downstream signaling cascade that culminates in activation of a number of effector enzymes including PLCγ2, small G-proteins and PI3-kinase (Fig. A.2). The adapters LAT and SLP-76 play critical roles in this signaling cascade. Several reports have shown that GPVI signaling cascade takes place in cholesterol-rich membrane domains known as rafts, which are enriched in essential signaling molecules[43].

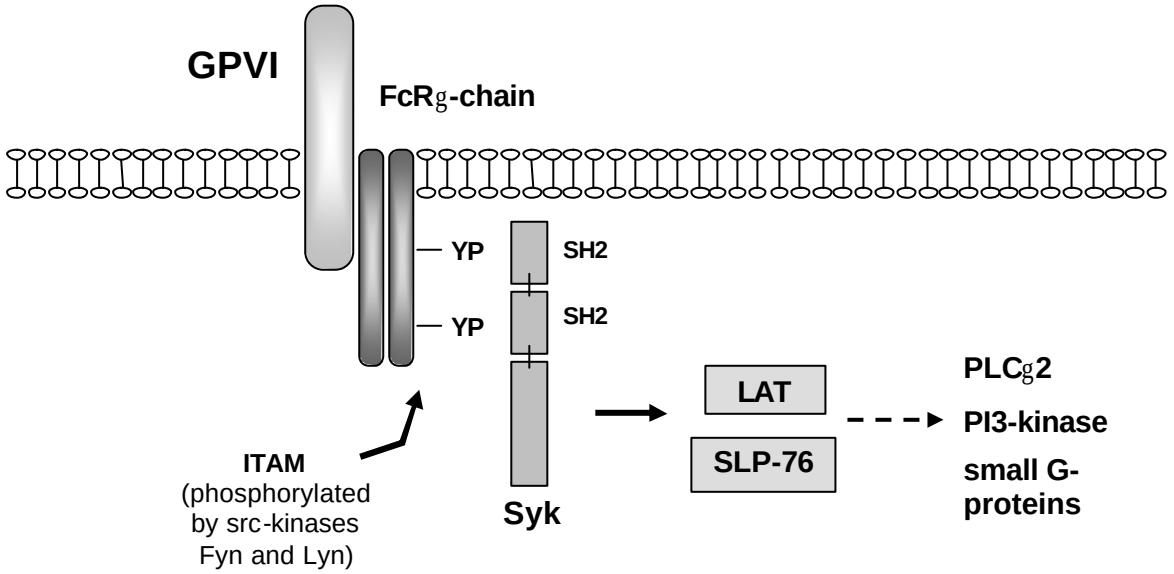


Figure A.2. Collagen activates platelets through GPVI by an ITAM-based signaling pathway
Cross-linking of the GPVI-FcRγ-chain complex leads to tyrosine phosphorylation of the ITAM, enabling recruitment of the tyrosine kinase Syk via its tandem SH2 domains. This is followed by autophosphorylation and subsequent activation of Syk, leading to the phosphorylation / activation of PLCγ2, PI3-kinase and small G-proteins.

The early studies on the GPVI-deficient patients provided compelling evidence for a key role of the glycoprotein in platelet activation by collagen but this was not initially recognized because of the multiplicity of other candidates for this role, most notably $\alpha_2\beta_1$. Despite its pivotal role in platelet activation by collagen, only very limited information has been available on the involvement of GPVI in the adhesion process. One reason for this might be that no GPVI knock out has been described so far. But, as shown in the present study, platelets from FcRγ-chain-deficient mice fail to express GPVI and do not aggregate in response to collagen. Together with mAb-induced depletion of GPVI from circulating platelets (see C.4.2), two *in vivo* systems are available to further investigate the role of GPVI.

3.4.1 Physiological and non-physiological GPVI ligands

Several tools have been developed to investigate the function of GPVI, including different types and preparations of collagen, receptor-specific synthetic collagens, snake toxins, and antibodies.

Collagens consist of repeat GXY motifs where G is glycine and X and Y are frequently proline (amino acid code = P) and hydroxyproline (amino acid code = O). The sequence GPO makes up approximately 10% of collagens I and III. The GXY repeat sequence forms monomeric chains cross-linking of which results in fibrillar collagen, the predominant structure that platelets come into contact with in the ECM. The most commonly used preparation of collagen for platelet studies, 'Horm' collagen, is a suspension of fibrils made up of equine collagen type I and a small amount of type III, along with low levels of other extracellular matrix proteins. For several studies, especially under flow conditions, monomeric (pepsin-digested) collagen is used which can have important experimental implications[44] because it interacts selectively with the integrin $\alpha_2\beta_1$ in suspension and requires cross-linking or immobilization to a surface at a sufficient density to stimulate platelet activation via GPVI. This is probably due to the relatively low frequency of the GPO motif in monomeric collagen.

A number of synthetic collagens have been generated, one of which consists of a repeat GPO motif and was termed collagen related peptide (CRP). Significantly, CRP is unable to support adhesion under the same conditions under which collagen does, but can induce platelet activation in the presence of $\alpha_2\beta_1$-blocking antibodies[45]. CRP was therefore the first selective agonist for the major collagen signaling receptor in the platelet[34;38;46].

Additionally, snake venom peptides that mediate their actions through GPVI are used as powerful tools to study this collagen receptor. The multimeric snake toxin convulxin was identified in the venom of the tropical rattlesnake *Crotalus durissus terrificus* and it induces platelet activation by clustering the receptor[47;48].

Within the last few years, several antibodies to GPVI have been raised. GPVI-specific antibodies are powerful platelet agonists when cross-linked by secondary antibodies, but on their own several of them can serve as receptor antagonists. So far, the mAb JAQ1, described in the present study, is the only one to be directed against the major collagen binding site of the receptor.

A.4 Previous model of platelet attachment to the extracellular matrix (ECM)

Platelet thrombus formation at sites of vascular injury is a complex process that can be separated into two steps: platelet adhesion to the ECM, and platelet cohesion to adherent platelets, also termed aggregation. To form stable bonds with components of the ECM, e.g. collagen, circulating platelets must slow down and attach to the reactive substrate, thereby resisting the force of flowing blood, which would tend to move platelets away from the vessel wall. At least four platelet receptors, $\alpha_{IIb}\beta_3$, $\alpha_2\beta_1$, GPVI, and GPIb-V-IX play a role in this event[14;49;50].

A previously accepted model of platelet adhesion to a collagen matrix[51-53] suggested the following sequence of events: First, the GPIbα-vWf interaction mediates initial tethering of platelets to the ECM, thereby reducing the velocity of the cells. Second, platelets firmly adhere to collagen via $\alpha_2\beta_1$ which enables the third step, a low affinity interaction of GPVI with collagen finally resulting in a variety of platelet responses like release of granule contents and upregulation/activation of surface receptors. Additional platelet agonists like second wave mediators ADP and TxA_2 as well as the coagulation protease thrombin then act synergistically to promote further platelet activation and aggregation, resulting in a growing platelet thrombus (Fig. A.3)[54].

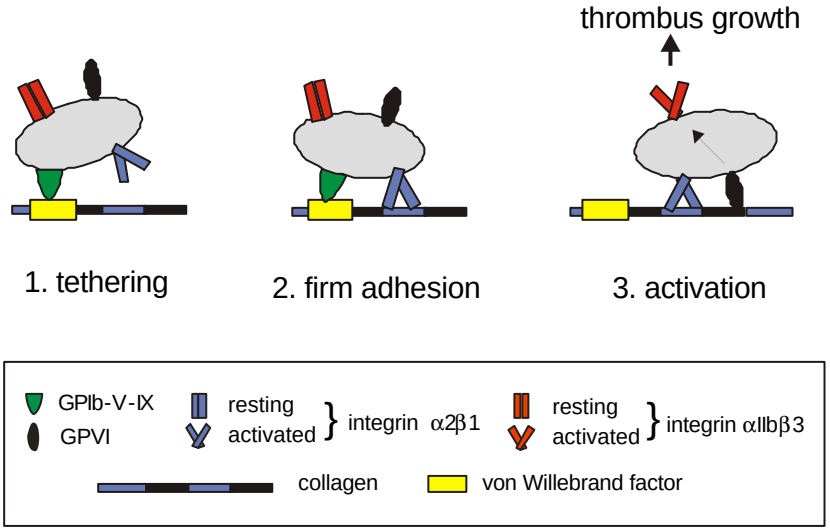


Figure A.3. Previous model of platelet adhesion to collagen

This model implies that $\alpha_2\beta_1$ would be constitutively active on platelets. That this is not the case and that the adhesion events to collagen occur in a different sequence will be demonstrated in the present work (for discussion of the revised model, see D.5).

A.5 Platelets and disease

A.5.1 Thrombosis

Arterial thrombosis frequently causes acute and irreversible damage or infarction of target organs, most notably heart and brain, leading to death or permanent disability. The pathophysiology differs in some basic respects from that of venous thrombosis. Venous thrombi tend to form in the absence of underlying vascular damage. They generally result from the combined effects of reduced blood flow (stasis) and activated coagulation proteins. Venous thrombi are composed predominantly of red blood cells enmeshed in fibrin, hence the term “red thrombi“. In contrast, arterial thrombi tend to form under conditions of elevated wall shear stress at sites of vascular injury and disturbed blood flow. They develop most commonly in atherosclerotic vessels. Arterial thrombi (“white thrombi“) are composed predominantly of platelets and relatively little fibrin or red blood cells. Therefore, it is presently thought that the platelet is the central player in the development of arterial, but not venous, thrombosis.

A.5.2 Antiplatelet therapy

The major clinical indication for antiplatelet therapy has been the prevention of arterial thrombosis. Aspirin, the prototype antiplatelet agent which irreversibly inactivates cyclooxygenase 1 (COX1) and therefore blocks platelet TxA_2 synthesis, has been in clinical use for almost half a century. However, clinical trials have exposed the limitations of aspirin, and there has been considerable effort dedicated to the development of more effective antiplatelet agents. Some of these newer agents are rationally based on the interruption of specific mechanisms in the sequence of platelet activation, including TxA_2 receptor antagonists, and ADP receptor blockers like ticlopidine and clopidogrel[55]. The clinical efficacy of these agents may be limited by their actions, which are restricted to single, specific platelet receptors of weak agonists involved in the secondary wave of platelet activation/aggregation. Antithrombin therapy with hirudin, heparin, or synthetic peptides, on the other hand, also interferes with coagulation, therefore having drastic effects on normal hemostasis. To

improve antiplatelet therapy, specific agents targeting activating platelet receptors stimulated by strong agonists like collagen are needed.

Compared to agents specifically blocking activating platelet receptors, inhibitors of initial platelet adhesion or platelet aggregation should improve clinical efficacy. Platelet aggregation induced by any stimulus is abolished if binding of fibrinogen (or vWf) to $\alpha_{IIb}\beta_3$ is blocked. Two general strategies have been used to target this interaction: monoclonal antibodies to $\alpha_{IIb}\beta_3$ (abciximab), and RGD analogs that compete with ligand binding to the receptor (disintegrins)[56;57]. Increased bleeding complications, as observed in clinical trials, however, represent the major disadvantage of $\alpha_{IIb}\beta_3$ inhibitors, particularly when treatment is combined with invasive procedures.

Agents that block the interaction of vWf with its principal platelet membrane receptor, GPIbα, are thought to have antithrombotic efficacy due to different effects: 1) inhibition of initial adhesion of circulating platelets to the injured vasculature, and 2) prevention of platelet release of activatory substances downstream of platelet activation. However, in contrast to $\alpha_{IIb}\beta_3$ blockers, the development of antagonists of the GPIb-vWf interaction has only just begun.

So far, the activating collagen receptor GPVI has not been in the focus of antiplatelet treatment, which was mainly due to the insufficient knowledge about its exact role in platelet physiology. In the light of the progress in understanding the role of GPVI, partly reported in the present work, this platelet glycoprotein turns out to be a promising target for new antithrombotic strategies.

A.6 Mouse platelets and *in vivo* models

Monoclonal antibodies (mAbs) are routinely used to study human platelet glycoprotein receptors *in vitro*. Blocking and activating mAbs against most of the prominent platelet receptors have been applied to different methods of platelet analysis, including aggregometry, flow cytometry, biochemical and cell biological methods to identify the mechanisms of platelet activation and aggregation and to define potential antithrombotic targets. However, antibodies can not easily be used to influence platelet function in humans whereas the mouse system appears to be the ideal model for such studies, as transgenic and knock out strains with defined defects in platelet function (integrins, G-protein coupled receptors, signaling molecules, etc.) have been established (Table 3).

Protein	Status	Platelet phenotype	Reference
Membrane GP			
β_3 -/-	Impaired viability	no aggregation	Hodivala-Dilke et al.[58]
β_3 (Y747, 759F)	viable	reversible aggregation	Law et al.[59]
β_1 -/-	EL	-	Fässler et al.[60]
platelet β_1 -/- (cre/loxP)	viable, no bleeding	delayed collagen activation	Nieswandt et al.[61]
α_2 -/-	viable, no bleeding	delayed collagen activation	Holtkötter et al.[62]
GPV -/-	viable	hyperreactive to thrombin	Ramakrishnan et al.[63]
P-selectin -/-	viable	decreased rolling	Subramaniam et al.[64]
GPIbα -/-	viable, bleeding	giant platelets	Ware et al.[65]
Signaling			
SLP-76 -/-	impaired viability	defective collagen activation	Clements et al.[66]
LAT -/-	viable	defective collagen activation	Pasquet et al.[67]
FcRγ -/-	viable	no response to collagen	Poole et al.[68]
Gαq -/-	viable	defective aggregation	Offermanns et al.[28]
Src -/-	viable	none	Soriano et al.[69]
Fyn -/-	viable	none	Appleby et al.[70]
Lyn -/-	viable	none	Hibbs et al.[71]
VASP -/-	viable	faster response to collagen, increased fibrinogen binding	Aszodi et al.[72]
cGK I -/-	impaired viability	hyperresponsive	Massberg et al.[73]
Agonist receptor			
Thrombin (PAR1) -/-	viable	none	Connolly et al.[74]
Thrombin (PAR3) -/-	viable	diminished thrombin response	Kahn et al.[75]
Thromboxane A2 R -/-	viable	bleeding	Thomas et al.[76]
P2Y1 -/-	viable	impaired ADP response	Leon et al.[30] Fabre et al.[77]
Glycoprotein ligand			
Fibrinogen -/-	viable	bleeding	Suh et al.[78]
Thrombospondin 1 -/-	viable	none	Lawler et al.[79]
Thrombospondin 2 -/-	viable	bleeding	Kyriakides et al.[80]
vWf -/-	viable	bleeding	Denis et al.[81]
Vitronectin -/-	viable	increased response to thrombin, prothrombotic	Fay et al.[82]

Table 3. **List of knock out mice with defects in platelet-expressed proteins.** (GP=Glycoprotein; EL=embryonic lethal)

Our understanding of the hemostatic system has already been improved significantly by the availability of mouse models of human disease and by the “knock out“ of genes that have not yet been identified as abnormal or missing in humans. Human and mouse platelets appear to share many of the same receptor systems and functional capacities. Platelet counts in mice on average are four times those of humans and platelets are only approximately one-half the volume of human platelets. Reduced responses of mouse platelets towards weak agonists

like epinephrine, serotonin, PAF, or ADP may reflect minor differences in signal transduction pathways. However, since mouse platelets are inhibited by many of the same physiological and pharmacological agents as human platelets, the mechanisms responsible for platelet activation in mice and humans seem to be very similar[83].

A.7 Aim of the study

The aim of the present study was to investigate the function of the platelet collagen receptor GPVI and to evaluate its potential role as an antithrombotic target. These studies were performed in the mouse system. Therefore, monoclonal antibodies against mouse GPVI (JAQ1, 2, and 3) were generated and characterized. The mAbs were used to study platelet activation by various agonists as well as platelet adhesion under static and flow conditions. Furthermore, the effects of *in vivo* application of the mAb were tested.

B Material and Methods

B.1 Material

B.1.1 Chemicals

acrylamide stock solution (30%)	Roth (Karlsruhe, Germany)
AEC substrate	EUROPA (Cambridge, UK)
alkaline phosphatase (AP)-labeling kit	Roche Diagnostics (Mannheim, Germany)
annexin-V (oregon green^{488} labeled)	Nexins Research (Hoeven, The Netherlands)
aprotinin	Roche Diagnostics (Mannheim, Germany)
apyrase (grade III)	Sigma (Deisenhofen, Germany)
bovine serum albumine (BSA)	Pierce (Rockford, IL, USA)
calcein acetoxymethyl ester	Molecular Probes (Leiden, The Netherlands)
dithioerythritol (DTE)	Sigma (Deisenhofen, Germany)
enhanced chemoluminiscence (ECL) detection substrate	MoBiTec (Göttingen, Germany)
EZ-Link sulfo-NHS-LC-biotin	Pierce (Rockford, IL, USA)
fetal calf serum (FCS)	Sigma (Deisenhofen, Germany)
fibrillar type I collagen (Horm)	Nycomed (Munich, Germany)
fluorescein-isothiocyanate (FITC)	Sigma (Deisenhofen, Germany)
HAT stock (50x)	Roche Diagnostics (Mannheim, Germany)
hematoxylin	Sigma (Deisenhofen, Germany)
high molecular weight heparin	Sigma (Deisenhofen, Germany)
hirudine	Hoechst (Frankfurt, Germany)
horseradish peroxidase (HRP)-labeling kit	Roche Diagnostics (Mannheim, Germany)
human fibrinogen	Sigma (Deisenhofen, Germany)
immobilized papain	Pierce (Rockford, IL, USA)
immobilized pepsin	Pierce (Rockford, IL, USA)
indomethacin	Calbiochem (Bad Soden, Germany)
ketamine (Imalgene 1000®)	Mérial (Lyon, France)
leupeptin	Roche Diagnostics (Mannheim, Germany)
monomeric collagen type I	Sigma (Deisenhofen, Germany)
N-ethyl maleimide (NEM)	Sigma (Deisenhofen, Germany)
2-[N-morpholino]ethanesulfonic acid (MES)	Sigma (Deisenhofen, Germany)
Nonidet P-40 (NP-40)	Roche Diagnostics (Mannheim, Germany)
PD-10 column	Pharmacia (Uppsala, Sweden)

penicillin/streptomycin	Sigma (Deisenhofen, Germany)
pepstatin	Roche Diagnostics (Mannheim, Germany)
D-Phe-Pro-Arg chloromethyl ketone (PPACK)	Calbiochem (Bad Soden, Germany)
pHix	Sigma (Deisenhofen, Germany)
phorbol 12-myristate 13-acetate (PMA)	Sigma (Deisenhofen, Germany)
Polyethylene glycol 1500 (PEG 1500)	Roche Diagnostics (Mannheim, Germany)
prostacyclin	Calbiochem (Bad Soden, Germany)
protein A sepharose	Pharmacia (Uppsala, Sweden)
protein G sepharose	Pharmacia (Uppsala, Sweden)
R-phycoerythrin (PE)	EUROPA (Cambridge, UK)
RPMI media	Sigma (Deisenhofen, Germany)
succinimidyl 4-(N-maleimidomethyl) cyclohexane-1-carboxylate (SMCC)	Pierce (Rockford, IL, USA)
thrombin	Roche Diagnostics (Mannheim, Germany)
3,3,5,5-tetramethylbenzidine (TMB)	EUROPA (Cambridge, UK)
Unopette™ kits	Becton Dickinson (Heidelberg, Germany)
xylazine (Rompun®)	Bayer (Leverkusen, Germany)

Human vWf was kindly provided by G. Dickneite (Marburg, Germany). Convulxin was obtained from the venom of the tropical rattlesnake *Crotalus durissus terrificus* and was kindly donated by M. Leduc and C. Bon (Institute Pasteur, Paris, France). The collagen related peptides, CRP, were kindly provided by S. P. Watson (Oxford University, UK).
All other chemicals and reagents were purchased from Sigma (Deisenhofen, Germany) or Roth (Karlsruhe, Germany).

B.1.2 Monoclonal antibodies

rat anti-mouse control mAb (IgG1)	BD Pharmingen (Hamburg, Germany)
mouse anti-phosphotyrosine (4G10)	Biomol (Hamburg, Germany)
rabbit anti-LAT (2E9)	Biomol (Hamburg, Germany)
hamster anti-β_1 integrin (Ha31/8)	BD Pharmingen (Hamburg, Germany)
rat anti-β_1 integrin (9EG7)	BD Pharmingen (Hamburg, Germany)
hamster anti-α_2 integrin (Ha1/29)	BD Pharmingen (Hamburg, Germany)
rat anti-α_5 integrin (5H10-27)	BD Pharmingen (Hamburg, Germany)
rat anti-α_6 integrin (GoH3)	BD Pharmingen (Hamburg, Germany)

The following antibodies were generated and modified in our laboratories:

Antibody	**Isotype**	**Antigen**	**described in**
JON/A	IgG2b	GPIIb/IIIa	Bergmeier et al.[84]
JON1	IgG2b	GPIIb/IIIa	Nieswandt et al.[85]
DOM1	IgG1	GPV	Nieswandt et al.[85]
DOM2	IgG2a	GPV	Nieswandt et al.[85]
EDL1	IgG2a	GPIIb/IIIa	Nieswandt et al.[85]
ULF1	IgG2a	CD9	Nieswandt et al.[85]
p0p1	IgG2a	GPIb-IX	Bergmeier et al.[86]
96A7	IgG1	GPIIIa	Nieswandt et al.[85]

Rat anti-mouse P-selectin mAb RB40.34 was provided by D. Vestweber (Münster, Germany), rabbit anti-Syk and anti-PLCγ2 were from M. Tomlinson (DNAX, Palo Alto, USA), and the blocking anti-GPV antibody was from F. Lanza (Strasbourg, France).

B.1.3 Polyclonal antibodies / secondary reagents

rabbit anti-human fibrinogen (-FITC)	DAKO (Hamburg, Germany)
rabbit anti-human vWf	DAKO (Hamburg, Germany)
rabbit anti-FcRγ-chain	Biomol (Hamburg, Germany)
rabbit anti-rat-Ig (-FITC, -HRP)	DAKO (Hamburg, Germany)
goat anti-rat-IgG/M (H+L)	Dianova (Hamburg, Germany)
rabbit anti-rat IgG1, IgG2a, IgG2b, IgM, IgA	BD Pharmingen (Hamburg, Germany)
rabbit anti-hamster IgG (-FITC)	BD Pharmingen (Hamburg, Germany)
rabbit anti-mouse IgG	Dianova (Hamburg, Germany)
goat anti-rabbit IgG (-HRP)	Dianova (Hamburg, Germany)
rabbit anti-FITC-HRP	DAKO (Hamburg, Germany)
goat serum, control	Sigma (Deisenhofen, Germany)
streptavidin-HRP	Dianova (Hamburg, Germany)
streptavidin-FITC	DAKO (Hamburg, Germany)

Sheep anti-SLP-76 polyclonal antiserum was provided by G. Koretzky (University of Pennsylvania, USA).

B.1.4 Animals

Specific-pathogen-free mice (NMRI, C57Bl/6) and rats (Wistar) 6 to 10 weeks of age were obtained from Charles River (Sulzfeld, Germany) and C57Bl/6 mice deficient in the FcRγ-chain were from Taconics (Germantown, USA). FcRγ heterozygous mice were generated by crossing FcRγ-deficient mice with mice from the appropriate wild-type strain. Mice lacking β_1 integrin on their platelets were generated by crossing mice carrying a floxed β_1 gene with mice expressing *cre* recombinase under control of an Mx promoter[87]. Two weeks after injection of polyinosinic-polycytidylic acid, Mx-cre-mediated deletion was almost complete in bone marrow and platelets. Studies using β_1-null mice were performed in collaboration with the group of R. Fässler (MPI for Biochemistry, Martinsried, Germany).

B.1.5 Cell lines

The mouse myeloma cell line *Ag8.653* was kindly provided by D. Männel (Regensburg, Germany).

B.2 Methods

B.2.1 Buffers and Media

All buffers were prepared and diluted in $aqua_{bidest}$.

- *Phosphate-buffered saline (PBS), pH 7.3*

NaCl	137 mM (0.9%)
KCl	2.7 mM
KH_2PO_4	1.5 mM
Na_2HPO_4 x 2 H_2O	8.0 mM

- *PBS/T (PVDF membrane washing buffer)*

0.1 % tween-20 in PBS

- *Tris-buffered saline (TBS), pH 7.3*

NaCl	137 mM (0.9%)
Tris/HCl	20 mM

- *Tyrode's buffer (modified), pH 7.3*

NaCl	137 mM (0.9%)
KCl	2.9 mM
$NaHCO_3$	12 mM
NaH_2PO_4	0.34 mM
Glucose	5.5 mM
Hepes	5 mM
BSA	0.35%

- *IP buffer*

Tris/HCl	40 mM
NaCl	0.3 M
EDTA	1 mM
NaN_3	0.05%

- *Lysis buffer, 2X, pH 7.3 (for tyrosine phosphorylation)*

Tris	20 mM
NaCl	300 mM
EDTA	10 mM
NP-40	2% (v/v)
PMSF	1 mM
Na_3PO_4	2 mM
Aprotinin	10 µg/ml
Leupeptin	10 µg/ml
Pepstatin A	1 µg/ml

- *SDS-sample buffer, 2X*

β-mercaptoethanol (when reducing)	10%
Tris buffer (1.25 M)	10%
Glycerin	20%
SDS	4%
Bromophenolblue	0.02%

- *Laemmli buffer*

Tris	40 mM
Glycin	0.95 M
SDS	0.5%

- *Blotting buffer A*

Tris, pH 10.4	0.3 M
Methanol	20%

- *Blotting buffer B*

Tris, pH 10.4	25 mM
Methanol	20%

- *Blotting buffer C*

e-Amino-n-caproic acid, pH 7.6	4 mM
Methanol	20%

- *Dialysis buffer, pH 7.0*

NaH_2PO_4	50 mM
EDTA	1 mM

- *Exchange Buffer, pH 6.0*

MES	50 mM
EDTA	2 mM

- *C Reaction Buffer, pH 9.0*

$NaHCO_3$	100 mM

- *Storage Buffer, pH 8.2*

Tris-HCl	10 mM
NaCl	150 mM
pHix	1 drop

- *Pen/Strep-solution*

Penicillin	10,000 U/ml
Streptomycin	10 mg/ml
in 0.9% NaCl	

- *RPMI standard media*

RPMI 1640	460 ml
FCS	25 ml
Pen/Strep	5 ml

- *HAT media*

RPMI 1640	435 ml
Pen/Strep	5 ml
FCS	50 ml
HAT stock (50x)	10 ml

B.2.2 Production of monoclonal antibodies

2.2.1 Immunization

Female Wistar rats, 6-8 weeks of age, were immunized either with 0.5 x 10^9 washed mouse platelets or purified antigens obtained by immunoprecipitation from 0.5 x 10^9 washed mouse platelets.
Rats were immunized repeatedly (5 x in intervals of 17 days) intraperitoneally and intramuscularly with platelets.

2.2.2 Generation of hybridomas

The rat spleen was removed aseptically and filtered through a sterile Nitex filter to generate a single cell suspension. After two washing steps in RPMI/Pen-Strep media (160 x *g* for 5 min), rat spleen cells were combined with mouse myeloma cells (Ag8.653, 10^8 cells per fusion) and the cells were washed once in RPMI/Pen-Strep media. Supernatant was removed as complete as possible prior to dropwise addition of 1 ml polyethylene glycol 1500 within 2 min. Subsequently, 10 ml of RPMI/Pen-Strep media was added within 5 min. After another washing step, pelleted cells were resuspended in HAT media and seeded into 96-well plates. Cells were fed with HAT media until screening and positive hybridoma were then grown in RPMI/Pen-Strep/10% fetal calf serum.

2.2.3 Screening

Hybridomas secreting mAbs directed against platelet receptors were identified by flow cytometry. A 1:1 mixture of resting and thrombin-activated platelets (10^6) was incubated with 100 µl hybridoma supernatant for 30 min at RT, washed with 1 ml PBS (1200 x *g*, 10 min) and stained with FITC-labeled rabbit anti-rat Ig for 15 min at RT. The samples were analyzed on a FACScan. Platelets were gated by FSC/SSC-characteristics. Positive hybridomas were subcloned twice prior to large scale production. MAbs were produced and purified according to standard methods. Isotype subclasses were determined by an ELISA method where 96-well plates (BD Falcon, Heidelberg, Germany) were coated with rabbit anti-rat IgG/M (H+L) antibodies diluted 1/1000 in coating buffer for 2 h at 37°C or over night at 4°C. After blocking, serial dilutions of purified rat anti-mouse platelet glycoprotein antibodies were added to duplicate wells (1 h, 37°C). Plates were washed and subsequently incubated for 1 h with AP-conjugated isotype-specific antibodies (1/1000, 37°C). After extensive washing, Sigma104 substrate was added to each well and the absorbance at 405 nm was recorded on Anthos Reader 2010 (Anthos Labsysteme, Krefeld, Germany).

B.2.3 Modification of antibodies

2.3.1 F(ab)$_2$ / Fab fragments

F(ab)$_2$ and Fab fragments of mAbs (10 mg/ml) were generated by incubation with immobilized pepsin (24-48 h) or papain (4-8 h), respectively, according to the instructions of the manufacturer. After digest, the preparations were applied to an immobilized protein A column followed by an immobilized protein G column to remove Fc fragments and any undigested IgG. The purity of F(ab)$_2$ and Fab fragments was checked by SDS-PAGE and silver staining (Bio-Rad) of the gel.

2.3.2 Fluorescent derivatives

- FITC

Affinity-purified antibodies were fluoresceinated to a fluorescein/protein ratio of approximately 3:1 by standard methods. FITC was dissolved in anhydrous dimethylsulfoxide (DMSO) at a concentration of 1 mg/ml. 50 µl of this solution was added in aliquots of 5 µl to 1 mg mAb in C reaction buffer and the sample was incubated for 8 h at 4°C in the dark. Reaction was stopped by addition of 50 µl 1M NH_4Cl. FITC-labeled antibodies were separated from unreacted FITC by gel filtration over a PD-10 column.

- PE

Purified mAbs were labeled with PE (MW: 240 kD) at a ratio of 1:2.

(A) Preparation of SMCC-activated PE

PE (20 mg/ml) was extensively dialyzed against PBS followed by dialysis buffer. 11 µl SMCC (10 mg/ml in DMSO) was added per mg of PE while mixing and rotated at RT for 60 min in the dark. PE-SMCC-conjugates were separated from free compounds on a PD-10 column pre-equilibrated with exchange buffer.

(B) Reduction of IgG

Purified mAb (1.5 mg in PBS) was reduced by addition of 20 mM DTE while vortexing. After 30 min incubation at RT, the reduced mAb was exchanged over a PD-10 column into exchange buffer and the protein concentration was determined colorimetrically.

(C) Covalent conjugation

Pooled antibody was added to PE-SMCC and incubated for 60 min at RT, followed by addition of 50 µg NEM (30 min, RT) to block free sulfhydryls. The product was exchanged over a PD-10 column into storage buffer.

The purity of fluorophore conjugates was tested by flow cytometry and they were diluted in storage buffer and kept at 4°C.

2.3.3 Labeling with AP and HRP

AP- and HRP-labeling of purified mAbs was performed with labeling kits according to the instructions of the manufacturer (*AP*: Roche Diagnostics, Mannheim; *HRP*: Zytomed, Berlin, both Germany).

B.2.4 Platelet preparation and counting

Mice were bled under ether anesthesia from the retroorbital plexus. Blood was collected in a tube containing 10% (v/v) 0.1 M sodium citrate or 10 U/ml heparin and platelet rich plasma (prp) was obtained by centrifugation at 300 x *g* for 10 min at RT. To prevent desensitization of platelet ADP receptors during storage, 0.02 U/ml of the ADP scavenger apyrase (adenosine 5'-triphosphate diphosphohydrolase) was added to prp for 30 min at 37°C[88].

For determination of platelet counts, blood (20 µl) was obtained from the retroorbital plexus of anesthetized mice using siliconized microcapillaries and immediately diluted 1:100 in Unopette kits. The diluted blood sample was allowed to settle for 20 min in an Improved Neubauer haemocytometer (Superior, Bad Mergentheim, Germany), and platelets were counted under a phase contrast microscope.

B.2.5 Platelet washing

Prp was centrifuged at 1200 x *g* for 5 min at RT in the presence of prostacyclin (0.1 µg/ml), and the pellet was then resuspended in Tyrode's buffer containing prostacyclin (0.1 µg/ml) and apyrase (0.02 U/ml) and let rest for 5 min at 37°C. After two washing steps with Tyrode's/prostacyclin/apyrase including 5 min resting, pelleted platelets were finally resuspended in Tyrode´s buffer at a density of 10^9 platelets/ml and were incubated for 30 min at 37°C in the presence of 0.02 U/ml of apyrase[88]. Platelet preparations were only used when surface-expressed P-selectin or fibrinogen was detectable on less than 0.2% of the cells. The final dilution prior to experiments was done in Tyrode's buffer containing 1 mM $CaCl_2$ (and 1 mM $MgCl_2$ if required). When used for Western blot analysis, freshly isolated platelets were washed twice in PBS/EDTA (5 mM) and then resuspended in IP buffer at a density of 10^8 platelets/ml.

B.2.6 Flow cytometry

2.6.1 Fluorophore-labeled Abs

Labeling of mAbs with fluorophores FITC and PE was performed as described above. To determine the optimal staining concentration of the conjugates, a titration of antibody on platelets was performed for each reagent. To minimize unspecific staining, fluorophore-labeled antibodies were only used in flow cytometry when binding to platelets could be completely blocked by a preincubation of cells with the respective unlabeled antibody.

2.6.2 Staining of glycoprotein receptors ex vivo

For analysis of platelets from control, genetically modified, or antibody-treated mice, 50 µl of diluted whole blood (1/20 in TBS/heparin, 10 U/ml) were stained with 10 µl of the indicated fluorophore-labeled Abs for 15 min at RT. Reaction was stopped by addition of 500 µl of PBS and samples were immediately analyzed.

2.6.3 Staining of glycoprotein receptors on resting and activated platelets

- 25 µl of washed platelets (4 x 10^4/µl) were incubated with saturating amounts of directly labeled mAbs (FITC, PE) and samples were incubated for 15 min at RT. After staining was stopped by addition of 500 µl of PBS, samples were immediately analyzed. Platelets were gated by FSC/SSC characteristics and fluorescence. When indicated, platelets were pretreated with 50 µg/ml of unlabeled antibody for 30 min at RT.

- Activation markers on the surface of activated platelets
  Platelet activation by ADP, CRP, and thrombin was determined flow cytometrically by staining of platelets for surface-expressed P-selectin, fibrinogen, vWf, or integrin $\alpha_{IIb}\beta_3$ (activated form).

Flow cytometric analysis was performed on a FACScan or FACScalibur (Becton Dickinson, Heidelberg, Germany) and data were analyzed using WinMDI software (versions 2.5-2.8) from J. Trotter (La Jolla, CA, USA).

B.2.7 Immunoprecipitation and Western blot analysis

2.7.1 Biotinylation of platelet surface glycoproteins

After two washing steps with PBS/EDTA (5 mM), pelleted platelets were resuspended in PBS at a concentration of ~ 10^8 platelets/ml and were incubated with EZ-Link sulfo-NHS-LC-biotin solution (50 µl/ml of 3 mg/ml stock, freshly prepared in PBS) for 15 min at RT on a rotation wheel. Biotinylation was stopped by addition of 50 µl/ml 1 M NH_4Cl and platelets were washed twice with PBS (each 1200 x *g* for 5 min).

2.7.2 Solubilization of platelet surface proteins

Washed (biotinylated) platelets were solubilized in 1 ml of IP buffer (1% NP-40) for 30 min at 4°C. Subsequently, cell debris was removed by centrifugation (20,000 x *g*, 10 min) and the lysate was rotated with 25 µl of protein G-sepharose for 8 h at 4°C (preclearing).

2.7.3 Detection of protein tyrosine phosphorylation

Experiments were performed in the presence of EGTA (100 µM) and indomethacin (10 µM) to prevent secondary events and facilitate protein isolation. Stimulation of washed platelets was carried out using 150 or 500 µl of platelet suspension, containing 0.7-1 x 10^8 cells/ml. Platelets were incubated with the indicated agonists with stirring at 37°C. Reactions were stopped at indicated time points by addition of 1 volume of 2X ice-cold lysis buffer, and platelets were lysed for 30 min at 4°C. Western blot analysis and, where indicated, immuno-precipitation were then carried out as described below.

2.7.4 Immunoprecipitation

10 µg mAb or 200 µl hybridoma supernatant was added together with 25 µl of protein G-sepharose to the precleared lysate of 10^8 platelets and precipitation took place overnight by rotation at 4°C. After repeated washing in lysis buffer (1200 x *g*, 5 min, 4°C), the protein G-sepharose pellet was dissolved in 25 µl of reducing or nonreducing SDS-sample buffer and heated for 5 min at 95°C.

2.7.5 SDS polyacrylamide gel electrophoresis (SDS-PAGE)

Samples were separated along with a molecular weight marker (Bio-Rad) on a Multigel-Long apparatus (Biometra, 200 V, 35 mA) using 12% or 9%–15% gradient SDS-polyacrylamide gels prepared with Rotiphorese™ stock solutions (Roth, Karlsruhe, Germany).

2.7.6 Detection of biotinylated proteins

Biotinylated proteins separated by SDS-PAGE were transferred onto a polyvinylidene difluoride (PVDF) membrane (immobilon P™, Millipore) using a semi-dry technique (Biometra). A blotting sandwich was prepared as follows: Whatman™ paper was soaked in blotting buffers A and B (3 sheets each) and placed on the blotting apparatus, followed by the methanol-activated PVDF membrane, the gel and another three sheets of paper soaked in blotting buffer C. Blotting was performed for 45 min with 15 V and 0.8 mA/cm^2. After 1 h blocking with 10% BSA or 10% milk powder, the membrane was incubated for 1 h at 37°C with streptavidin-HRP (1 µg/ml), followed by extensive washing with three changes of PBS/T. Biotinylated proteins were visualized by ECL.

2.7.7 Western blot analysis

Whole cell extract or immunoprecipitated platelet proteins were separated by SDS-polyacrylamide gel electrophoresis and transferred onto a PVDF membrane (see 2.7.6). The membrane was first incubated with 5 µg/ml FITC-labeled mAb followed by rabbit anti-FITC-HRP (1 µg/ml). Alternatively, the membrane was first incubated with hybridoma supernatant followed by rabbit anti-rat-HRP. Proteins were visualized by ECL and membranes were exposed to Hyperfilm (Amersham Biosciences, Freiburg, Germany). Densitometric analysis of results was performed using Quantity One (version 4) densitometry software (Bio-Rad).

B.2.8 Aggregometry

To determine platelet aggregation, light transmission was measured using prp or washed platelets diluted in Tyrode's buffer containing 1 mM $CaCl_2$ (200 µl with 0.5 x 10^6 platelets/µl). Platelets were incubated under stirring conditions (1000 s^{-1}) with the indicated agonists at 37°C. Transmission was recorded on a Fibrintimer 4 channel aggregometer (APACT Laborgeräte und Analysensysteme, Hamburg, Germany) over ten minutes and was expressed as percent transmission relative to diluted plasma or Tyrode's buffer.

B.2.9 Immunohistochemistry

Acetone-fixed cryosections (6 µm) were blocked (5% normal goat serum, 0.5% BSA, in PBS) for 30 min at RT. Primary mAbs were added at a final concentration of 2 µg/ml. After 90 min, the sections were washed three times with PBS und subsequently incubated with the adequate HRP-labeled secondary antibodies at a final concentration of 2 µg/ml for 60 min at RT. The 3-amino-9-ethyl-carbazole (AEC) substrate was added after three washing steps, and the sections were then counterstained with hematoxylin.

B.2.10 Platelet adhesion

2.10.1 Static conditions

Fibrillar or monomeric collagen (0.2 µg/well) was immobilized on F96-MaxiSorp plates (Nunc, Wiesbaden, Germany) at 4°C over night. The plates were then saturated with 5% BSA in PBS for 3 h at 37°C or over night at 4°C. Washed platelets in Tyrode´s buffer (10^6/well) were incubated in the wells for up to 45 min. The plates were washed three times with Tyrode's and then incubated with HRP-labeled anti-GPIb-IX (p0p1) for 30 min at RT, and 50 µl 3,3,5,5-tetramethylbenzidine (TMB) was added to each well after three washing steps. The reaction was stopped by addition of 1 N H_2SO_4 after 10 min. Absorbance at 450 nm was recorded on Anthos Reader 2010 (Anthos Labsysteme, Krefeld, Germany).

2.10.2 Low and high shear conditions

Rectangular coverslips (24 x 60 mm) were coated with 150 µl fibrillar or non-fibrillar collagen (0.25 mg/ml in 0.5 M acetic acid) for 1h at RT and blocked with 1% BSA for 1h at RT. Mouse blood (1 vol) was collected into 0.5 vol of Tyrode's buffer containing 120 µM PPACK and 15 U/ml heparin and was incubated with 2.5 µM calcein acetoxymethyl ester for 30-60 min at 30°C. Flow chambers with a slit depth of 50 µm, equipped with the collagen-coated

coverslips, were rinsed with Tyrode's buffer (2 mM $CaCl_2$ and 1 U/ml heparin), and connected to a syringe filled with the anticoagulated blood. Perfusion was carried out at RT using a pulse-free pump under low shear stress conditions (10 min, flow rate of 1.12 ml/h, equivalent to a wall shear rate of 150 s^{-1}) or at high shear stress (4 min, flow rate of 7.53 ml/h, equivalent to a wall shear rate of 1000 s^{-1}). During perfusion, microscopic phase-contrast images were real-time recorded. Thereafter, the chambers were rinsed by a 10 min perfusion with Tyrode's buffer (2 mM $CaCl_2$ and 1 U/ml heparin) at the same shear stress. Both phase-contrast and fluorescence images were recorded from at least five different microscopic fields (63x or 80x objectives), using a fluorescence digital imaging system equipped with two intensified, charge-coupled device cameras[5]. Image analysis was performed off-line using Quanticell 700/900 software (Visitech, Sunderland, UK). The platelet adhesion results are expressed as the mean percentage of total area covered by platelets or calcein fluorescence.

B.2.11 In vivo experiments

2.11.1 Bleeding time experiments

Mice were anesthetized and 3 mm of the tail tip was amputated with a scalpel. Tail bleeding was monitored by gently absorbing the bead of blood with a filter paper without contacting the wound site. When no blood was observed on the paper after 15 s intervals, bleeding was determined to have ceased. When necessary, bleeding was manually stopped at the 10 min time point to prevent death.

2.11.2 Thromboembolism induced by collagen/epinephrine

Monoclonal antibodies (in 200 µl PBS) were injected intraperitoneally. At the indicated days after this treatment, mice were anesthetized by intraperitoneal injection of 150 µl of a mixture of 0.08% xylazine base and 1.6% ketamine (Imalgene® 1000). Anesthetized mice received a mixture of collagen (0.8 mg/kg) and epinephrine (60 µg/kg) injected into the jugular vein[89]. Necroscopy and histological studies were performed on lungs fixed in 4% formaldehyde and paraffin sections were stained with hematoxylin/eosin.

B.2.12 Data analysis

Results are shown as mean ± S.D. Statistical analyses were performed using Student's *t* test with $P < 0.05$ taken as the level of significance.

C Results

C.1 Expression and function of GPVI

C.1.1 Characterization of the murine collagen receptor GPVI with the mAb JAQ1

A monoclonal antibody (mAb) against mouse GPVI (JAQ1, rat IgG2a) was generated from rats immunized with mouse platelets. JAQ1 blocked platelet aggregation induced by collagen (up to 5 µg/ml) in platelet rich plasma (prp) and on washed platelets, whereas it had no effect on aggregation induced by PMA (prp) or α-thrombin (washed platelets) (Fig. 1). Incubation of prp with varying concentrations (3-30 µg/ml) of JAQ1 under stirring (1000 rpm, 37°C) or static conditions did not induce any signs of platelet activation (flow cytometric analysis of surface expression of P-selectin or fibrinogen, not shown). Consequently, no aggregation was induced by the mAb itself.

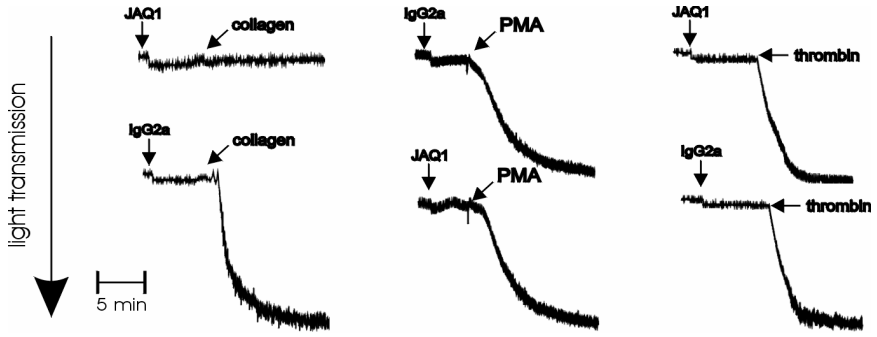


Figure 1. Platelet aggregation by collagen, PMA, and α-thrombin in the presence of JAQ1
Heparinized prp was incubated with JAQ1 (20 µg/ml) or irrelevant rat IgG2a (20 µg/ml) before addition of collagen (5 µg/ml) or PMA (50 ng/ml). Thrombin-induced aggregation (0.1 U/ml) was performed with washed platelets.

JAQ1 bound to mouse platelets (Fig. 2a) and precipitated a single chain protein of an apparent molecular weight of approximately 60 kD under nonreducing conditions (Fig. 2b, *nonred.*) which slightly shifted to approximately 65 kD under reducing conditions (Fig. 2b, *red.*), demonstrating that the apparent molecular weight of mouse GPVI is very similar to its human homolog[33;36]. In Western blot analysis JAQ1 recognized GPVI only under nonreducing conditions (Fig. 2c), indicating that the antibody binds to a conformational epitope on the receptor. On human platelets, polyclonal antibodies against GPVI have been

reported to induce platelet aggregation when used as IgG or F(ab)$_2$ fragments[36;90] while monovalent Fab fragments had no activatory effect, suggesting that clustering of GPVI is required for signal transduction. To induce clustering of GPVI on mouse platelets, surface-bound JAQ1 was cross-linked by the addition of polyclonal rabbit anti-rat IgG antibodies (10 µg/ml). As shown in Fig. 2d, such treatment rapidly resulted in irreversible platelet aggregation, whereas addition of irrelevant rat IgG2a followed by polyclonal rabbit anti-rat IgG antibodies (10 µg/ml) had no effect.

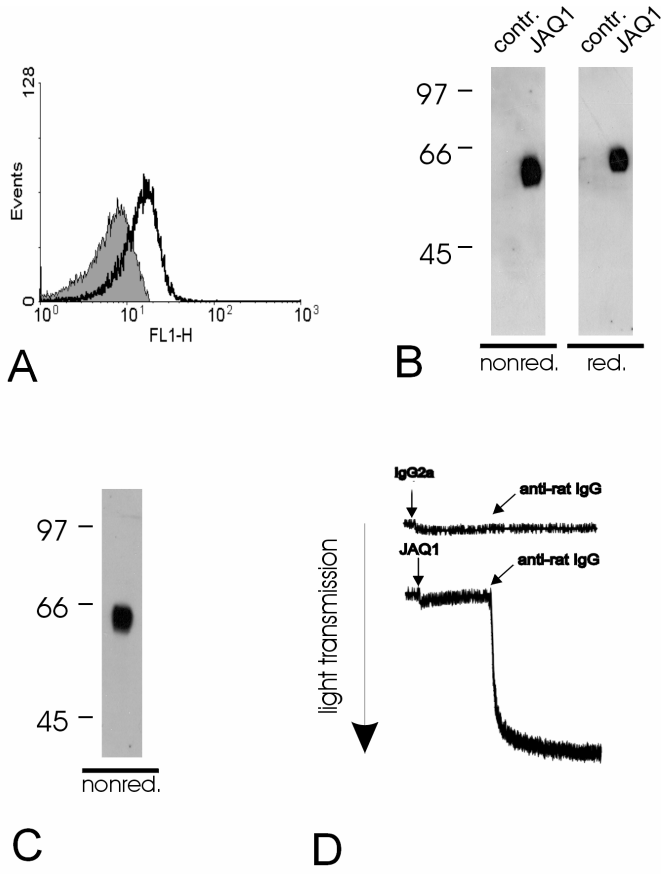


Figure 2. JAQ1 binds to mouse GPVI

A, flow cytometric detection of GPVI on mouse platelets. Platelets were incubated with JAQ1 (10 µg/ml, *solid line*) or irrelevant rat IgG2a (10 µg/ml, *shaded area*) for 30 min at RT. Bound JAQ1 was detected with rabbit anti-rat IgG^{FITC}. ***B***, immunoprecipitation of GPVI from surface-biotinylated mouse platelets. NP-40 lysates were incubated with 10 µg/ml nonimmune rat IgG2a (*contr.*) or JAQ1, followed by protein G-Sepharose. Proteins were separated on a 9%-15% gradient SDS-PAGE gel under reducing (*red.*) or nonreducing (*nonred.*) conditions, transferred onto a PVDF membrane, and detected by streptavidin-HRP and ECL. ***C***, platelet proteins were separated by SDS-PAGE and immunoblotted with JAQ1^{FITC}. Bound JAQ1 was detected by HRP-labeled rabbit anti-FITC. ***D***, heparinized prp was incubated with JAQ1 (20 µg/ml) or irrelevant rat IgG2a (20 µg/ml) followed by addition of polyclonal rabbit anti-rat IgG antibodies (10 µg/ml).

Together, these results demonstrated that GPVI is a major collagen receptor on mouse platelets, and suggested that clustering of the receptor mediates activation.

C.1.2 GPVI is undetectable on platelets and megakaryocytes from FcRγ-chain-deficient mice

On human platelets, GPVI has been described to be physically and functionally associated with the FcRγ-chain[35;38]. Furthermore, platelets from FcRγ-chain-deficient mice do not aggregate in response to collagen[68]. Based on these findings, JAQ1 was expected not to induce platelet aggregation upon cross-linking on platelets from FcRγ-chain-deficient mice. Indeed, addition of increasing concentrations of JAQ1 (3-30 µg/ml) followed by polyclonal rabbit anti-rat IgG antibodies (10 µg/ml) had no effect on FcRγ-chain-deficient platelets, whereas normal aggregation was observed with platelets from C57Bl/6 control mice (Fig. 3). This finding again demonstrated that the antigen recognized by JAQ1 is mouse GPVI and further suggested that activation through mouse GPVI requires its association with the FcRγ-chain.

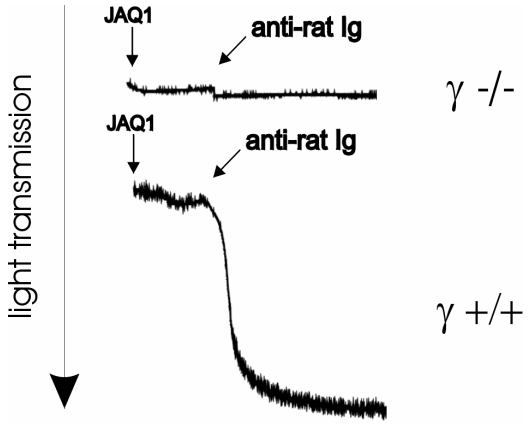


Figure 3. JAQ1 has no effect on platelets from FcRγ-chain-deficient mice
Heparinized prp from FcRγ-chain-deficient (γ-/-) and control (γ+/+) mice was incubated with JAQ1 (20 µg/ml) followed by addition of polyclonal rabbit anti-rat IgG antibodies (10 µg/ml).

Therefore, the expression of GPVI in platelets and megakaryocytes from C57Bl/6 mice in comparison to FcRγ-chain-deficient mice was assessed. JAQ1 did not bind to FcRγ-chain-deficient platelets as determined by flow cytometry (Fig. 4a). Immunohistochemical examination of splenic megakaryocytes from FcRγ-chain-deficient mice demonstrated no specific binding of JAQ1, whereas megakaryocytes from C57Bl/6 control mice were clearly stained (Fig. 4b).

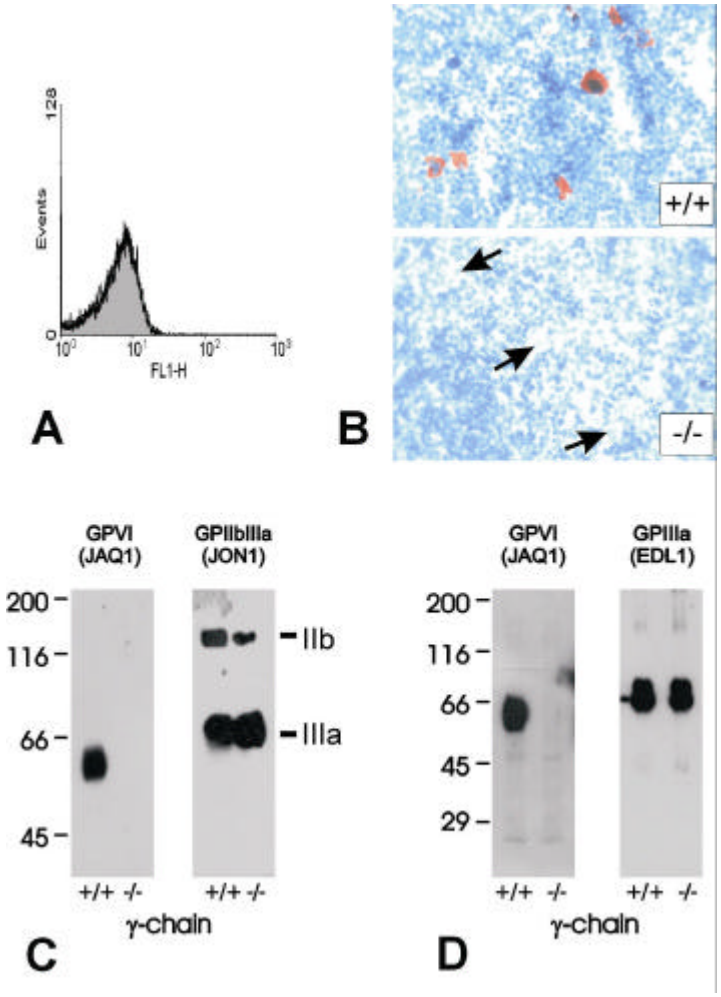


Figure 4. Mouse GPVI is not expressed in the absence of the FcRg-chain

A, flow cytometric analysis of FcRγ-chain-deficient platelets stained with JAQ1. Platelets were incubated with JAQ1 (10 µg/ml, *solid line*) or irrelevant rat IgG2a (10 µg/ml, *shaded area*) for 30 min at RT, followed by rabbit anti-rat IgG^{FITC}. ***B***, immunohistochemical detection of GPVI on splenic megakaryocytes from wild type (+/+) or FcRγ-chain-deficient (-/-) mice on acetone-fixed frozen sections. Unstained megakaryocytes are indicated by arrows. ***C***, immunoprecipitation of GPVI and GPIIb/IIIa from surface-biotinylated control and FcRγ-chain-deficient platelets. NP-40 lysates were incubated with 10 µg/ml JAQ1 or JON1 (anti-GPIIb/IIIa), followed by protein G-Sepharose. Proteins were separated on a 9%-15% gradient SDS-PAGE gel under reducing conditions, transferred onto a PVDF membrane and detected by streptavidin-HRP and ECL. ***D***, whole platelet proteins were separated by SDS-PAGE and immunoblotted with FITC-labeled JAQ1 or EDL1 (anti-GPIIIa). Bound mAb was detected by HRP-labeled rabbit anti-FITC.

Moreover, in the absence of the FcRγ-chain, GPVI-specific bands were detectable neither in JAQ1 immunoprecipitates from surface-biotinylated platelets nor in a Western blot analysis of whole platelet lysate. These data strongly suggest that GPVI is not expressed *in vivo* in the absence of the FcRγ-chain, although the possibility cannot definitively be ruled out that the protein is expressed but fails to undergo the folding necessary for JAQ1 to bind. In contrast to GPVI, similar amounts of GPIIb/IIIa were immunoprecipitated from control and FcRγ-chain-

deficient platelets and GPIIIa was detected in both platelet preparations by Western blot analysis (Fig. 4c,d). Furthermore, flow cytometric studies demonstrated that the expression of GPIIb/IIIa, the collagen receptor integrin $\alpha_2\beta_1$, and the GPIb-IX-V complex was undistinguishable between platelets from wild-type and FcRγ-chain-deficient mice (see App.1).

C.2 Collagen contains two distinct epitopes for activation of mouse platelets

C.2.1 The inhibitory effect of JAQ1 is overcome by high concentrations of collagen

A shown above, JAQ1 inhibits platelet aggregation in response to low concentrations of collagen (5 µg/ml). To further characterize this inhibition and the effect of JAQ1 on platelet activation, aggregation studies were performed and protein tyrosine phosphorylation was determined. Surprisingly, the inhibitory effect of JAQ1 against collagen could be overcome by increasing the concentration of the agonist. Thus, in the presence of intact JAQ1 (10 µg/ml) or monovalent Fab fragments of the mAb (10 µg/ml), collagen at a concentration of 10 µg/ml stimulated partial aggregation which reached almost maximal levels at 20 µg/ml (Fig. 5a,b). Flow cytometric preincubation studies demonstrated that bound $JAQ1^{FITC}$ was not displaced by a 50-fold excess of unlabeled JAQ1 for at least 30 min (not shown), indicating that JAQ1 binding to GPVI is essentially irreversible over the time course of this study. Therefore, collagen must be able to bind either to a second site on GPVI or to a second surface receptor to mediate activation.

In order to help distinguish between these two possibilities, the effect of JAQ1 on collagen-induced tyrosine phosphorylation of key proteins in the GPVI signaling cascade was investigated. In the absence of cross-linking, JAQ1, but not control IgG2a (not shown), stimulated a low level of tyrosine phosphorylation of the adapters LAT and SLP-76, and PLCγ2 (Fig. 5c), despite the fact that it inhibits aggregation to low concentrations of collagen. JAQ1 alone also stimulated tyrosine phosphorylation of FcRγ-chain and Syk as shown later (see Fig. 8). JAQ1-Fab fragments did not induce detectable tyrosine phosphorylation of any of these proteins (not shown), suggesting that antibody-mediated dimerization of GPVI is required to induce this subliminal signaling.

Despite this low level of stimulation, JAQ1 inhibited the collagen-induced tyrosine phosphorylation of LAT, SLP-76 and PLCγ2 (Fig. 5c). Because of the weak stimulatory effect of the antibody itself, this inhibition was more clearly seen at higher concentrations of collagen. Nevertheless, higher concentrations of collagen (10 and 30 µg/ml) were able to stimulate significant tyrosine phosphorylation of SLP-76 and PLCγ2 in the presence of JAQ1, consistent with the restoration of aggregation to collagen under these conditions (Fig. 5a,b).

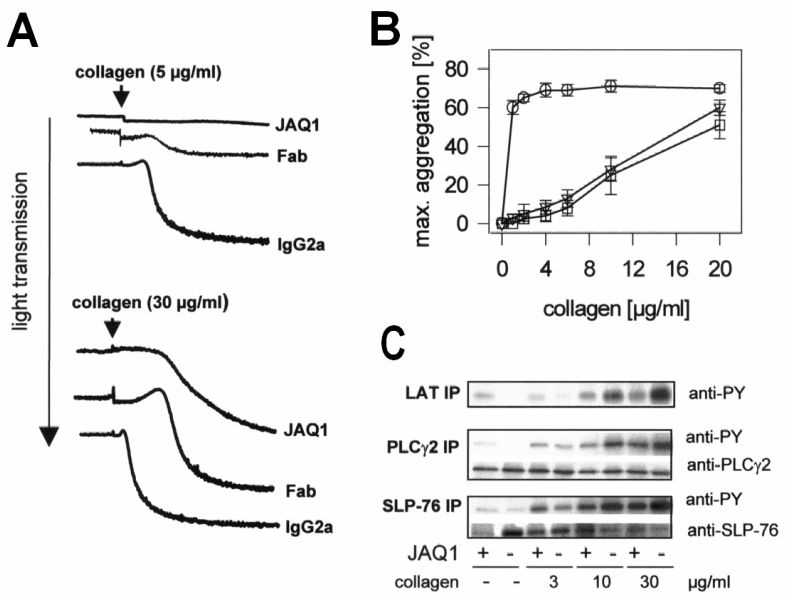


Figure 5. The inhibitory effect of JAQ1 is overcome by high concentrations of collagen
A and ***B***, heparinized prp was incubated under stirring conditions in the presence of irrelevant rat IgG2a (10 µg/ml, *circles*), Fab fragments of JAQ1 (10 µg/ml, *triangles*), or JAQ1-IgG (10 µg/ml, *squares*) for 5min before addition of the indicated concentrations of collagen. Results are given as mean ± S.D. (n = 3-6). ***C***, washed platelets were incubated for 5 min with stirring in the presence or absence of JAQ1 (10 µg/ml) followed by stimulation with varying concentrations of collagen. After 2 min, samples were dissolved in reducing SDS buffer following immunoprecipitation (*IP*) for LAT, PLCγ2, and SLP-76. After separation by 12.5% SDS-PAGE and transfer to PVDF membranes, the proteins were incubated with the anti-phosphotyrosine antibody 4G10 (*anti-PY*) and detected by anti-mouse IgG-HRP and ECL. The membranes were partly stripped and reprobed for the immunoprecipitated proteins. The results are representative of four experiments.

The relative increase in tyrosine phosphorylation of PLCγ2 and SLP-76 induced by high concentrations of collagen was similar in the presence or absence of JAQ1. In contrast, the increase of LAT phosphorylation was much weaker in response to higher concentrations of collagen in the presence of JAQ1 (Fig. 5c).

C.2.2 JAQ1 completely inhibits platelet activation to CRP

The presented findings raise two possible explanations. First, there is a second epitope on the collagen molecule that is capable of binding to GPVI in the presence of JAQ1 or, second, the adhesion molecule is binding to an activatory receptor distinct from GPVI. In the latter case, it is possible that binding is mediated through the same epitope in the collagen molecule. In order to investigate this, the effect of JAQ1 on responses to the collagen related peptide (CRP) was investigated.

Intact JAQ1 as well as Fab fragments (both 10 µg/ml) completely blocked platelet aggregation in response to CRP at concentrations up to 100 µg/ml (Fig. 6a,b). This was confirmed when CRP-induced tyrosine phosphorylation in whole cell lysates was reduced to basal in the presence of JAQ1 (Fig. 6c). These findings suggested that JAQ1 occupies the major collagen binding site on GPVI as this is supposed to be the only target structure for platelet activation by CRP.

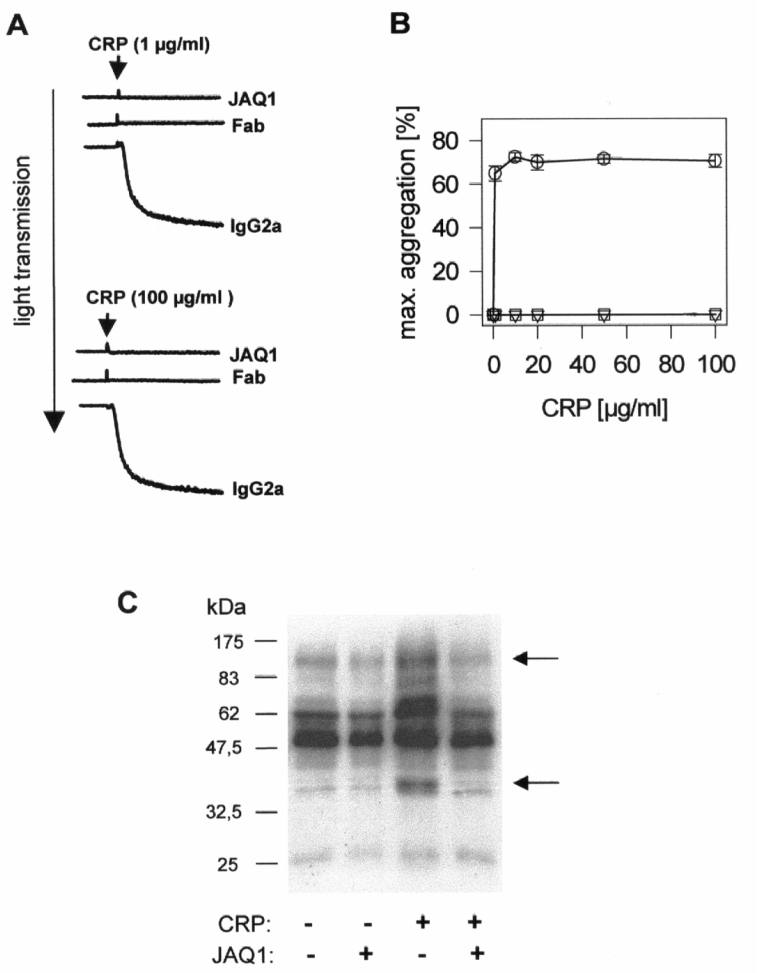


Figure 6. JAQ1 completely inhibits platelet aggregation and decreases protein tyrosine phosphorylation induced by CRP

A and ***B***, heparinized prp was incubated with stirring in the presence of irrelevant rat IgG2a (10 µg/ml, *circles*), Fab fragments of JAQ1 (10 µg/ml, *triangles*), or JAQ1-IgG (10 µg/ml, *squares*) for 5 min before addition of CRP (1-100 µg/ml). Results are shown as mean ± S.D. (n = 3-6). ***C***, washed platelets were incubated for 5 min under stirring conditions in the absence or presence of JAQ1 (10 µg/ml) followed by stimulation with 100 µg/ml CRP. After 2 min, samples were dissolved in nonreducing SDS buffer. After separation by 12.5% SDS-PAGE and transfer to PVDF membranes, blots were incubated with the anti-phosphotyrosine antibody 4G10, and proteins were detected by anti-mouse IgG-HRP and ECL. *Arrows* indicate the positions of 145 and 36 kD proteins, which have previously been shown to represent PLCγ2 and LAT, respectively. Results are representative of two experiments.

C.2.3 Clustering of GPVI by JAQ1-cross-linking stimulates aggregation and protein phosphorylation

Cross-linking of JAQ1 by addition of anti-rat IgG antibodies (10 µg/ml) stimulates platelet aggregation (Fig. 2). In view of the ability of JAQ1 alone to stimulate a minimal increase in protein tyrosine phosphorylation, and also the lack of tyrosine phosphorylation of LAT in response to high concentrations of collagen in the presence of JAQ1, the effect of cross-linking JAQ1 on tyrosine phosphorylation was examined next. The question was whether cross-linking of JAQ1 induces a similar pattern of tyrosine phosphorylation to that seen in response to activation by the GPVI specific agonist convulxin, and specifically whether LAT is phosphorylated.

Cross-linking of surface-bound JAQ1 on platelets stimulated a pattern and time course of tyrosine phosphorylation in whole cell lysates similar to those detected in platelets activated by the GPVI selective agonist, convulxin (5 µg/ml), albeit with a lower intensity of phosphorylation (Fig. 7a). As quantitated by densitometry, phosphorylation in response to convulxin was maintained for all proteins up to 90 sec, with the exception of a band of 25 kD (nonreducing conditions) which comigrates with FcRγ-chain. In contrast, tyrosine phosphorylation in response to cross-linking of JAQ1 declined more rapidly, most notably for protein bands of 25 and 36 kD (Fig. 7b,c and not shown). Immunoprecipitation studies confirmed that cross-linking of JAQ1 stimulated tyrosine phosphorylation of the same pattern of proteins as seen with convulxin, including namely FcRγ-chain, Syk, SLP-76, LAT, and PLCγ2 (Fig. 8). Tyrosine phosphorylation of all of these proteins was less than that seen in response to convulxin, and was particularly weak for the FcRγ-chain, which may partly be explained by the more rapid decline in tyrosine phosphorylation in response to cross-linking of JAQ1. Aggregation and protein phosphorylation in response to cross-linking of JAQ1 were completely inhibited in the presence of the Src family kinase inhibitor, PP1 (not shown), as previously reported for activation by CRP[41]. The finding that cross-linking of GPVI by JAQ1 stimulates a similar pattern of protein tyrosine phosphorylation and aggregation to that seen in response to activation by convulxin indicates that both agonists use comparable mechanisms to activate platelets via the collagen receptor GPVI. The question, whether these observations will help to elucidate the collagen-induced activation processes is to be discussed later in the text.

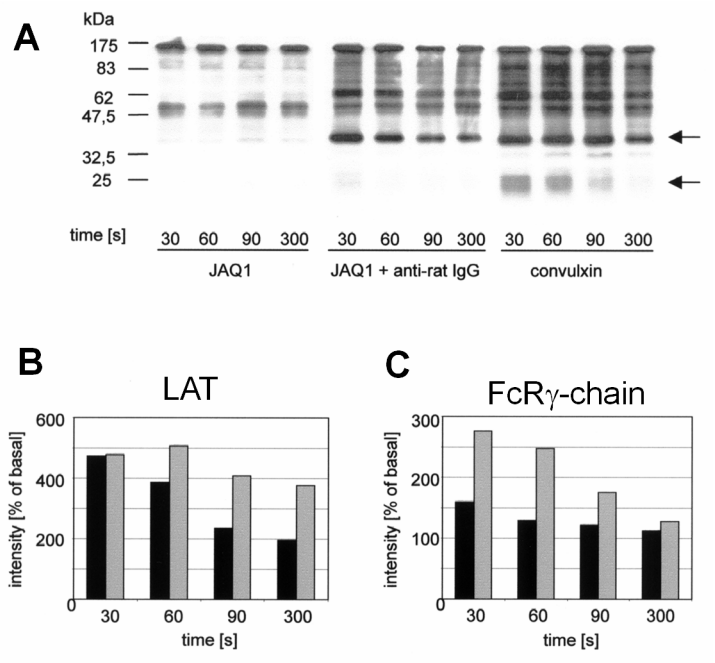
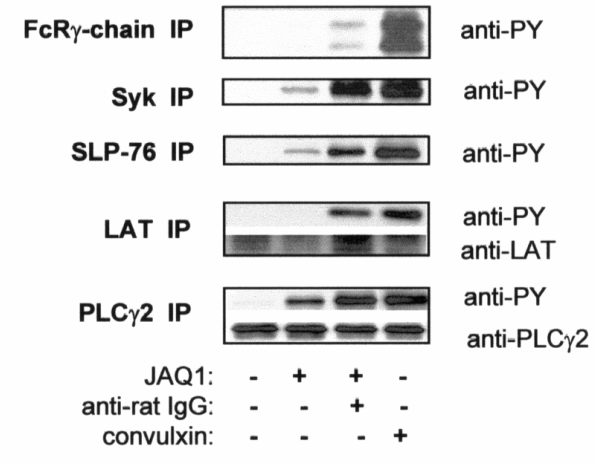


Figure 7. Cross-linking of GPVI by JAQ1 mimics platelet activation by convulxin

Washed platelets were stimulated under stirring conditions with JAQ1 (10 µg/ml), JAQ1 and polyclonal rabbit anti-rat IgG (both 10 µg/ml), or convulxin (2 µg/ml). ***A***, for the time-course assay aliquots were removed at the indicated times and dissolved in reducing SDS buffer. Protein-tyrosine phosphorylation was determined as described in the legend to figure 6. *Arrows* indicate the migration of 36- and 25-kD proteins which have previously been identified as LAT and the FcRγ-chain, respectively. Densitometric quantitation of tyrosine phosphorylation was performed with bands corresponding to LAT (***B***) and the FcRγ-chain (***C***). *Black bars*, JAQ1 cross-linked; *gray bars*, convulxin.

Figure 8. Cross-linked JAQ1 and convulxin induce tyrosine phosphorylation of FcRg-chain, Syk, SLP-76, LAT, and PLCg2

Washed platelets were incubated as described in the legend to Fig. 7. Samples were lysed after 2 min by addition of an equal volume of 2X ice-cold NP-40 buffer and were immunoprecipitated for FcRγ-chain, Syk, SLP-76, LAT, and PLCγ2. After separation by nonreducing 12.5% SDS-PAGE and transfer to PVDF membranes, the proteins were incubated with the anti-phosphotyrosine antibody 4G10 (*anti-PY*) and were detected by anti-mouse IgG-HRP and ECL. The membranes were stripped and reprobed for the immunoprecipitated proteins. Results are from a representative experiment ($n = 3$).



C.3 Differential effects of reduced GPVI levels on platelet activation by GPVI ligands

These experiments were partly performed in collaboration with the group of S. P. Watson (University of Oxford, UK), and mice transgenic in FcRγ-chain (FcRγ-Tg/KO) were kindly provided by T. Saito (Chiba University, Japan) and will be described elsewhere (Arase et al., manuscript submitted).

C.3.1 Modulation of GPVI-FcRγ expression in murine platelets

So far, the significance of GPVI expression levels for activation processes has only been investigated using transfected cell lines[36]. In these systems, it has been shown that collagen responses in the absence of $\alpha_2\beta_1$ can be mediated by GPVI-FcRγ in a receptor density-dependent manner[91]. According to the fact that expression of GPVI on murine platelets strictly depends on the presence of the FcRγ-chain (see C.1.2), we generated mice with reduced GPVI levels by crossing FcRγ-chain-deficient with wild type mice of the same strain. Indeed, platelets from these FcRγ heterozygous (+/-) mice were found to have 50% of the normal levels of the FcRγ-chain and a comparable decrease in GPVI levels as measured by flow cytometry and Western blotting (Fig. 9). Platelets from FcRγ-Tg/KO mice expressed 20% of the wild-type level of FcRγ-chain, and GPVI level was also 20% of controls (Fig. 9a). Flow cytometric analysis showed that the surface expression level of the collagen receptor integrin $\alpha_2\beta_1$, as well as of several other glycoproteins, was unaffected by a decrease in the levels of the GPVI-FcRγ-complex (see App.1).

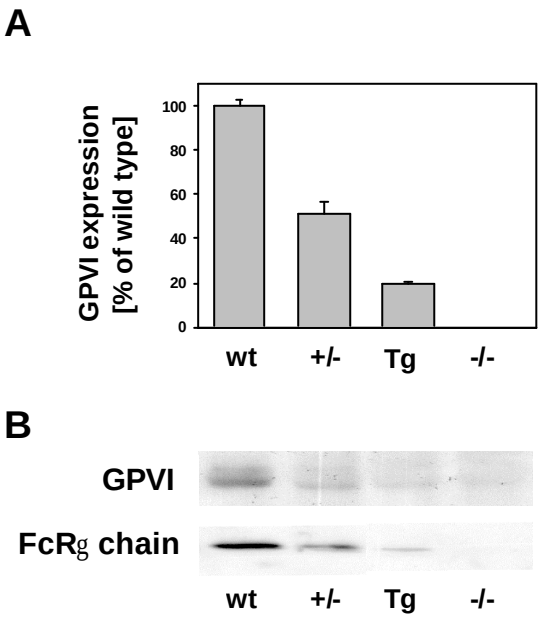


Figure 9. Different levels of GPVI expression in platelets from FcRg heterozygous and transgenic mice

A, flow cytometric analysis of GPVI expression levels with JAQ1^{PE} on platelets from wild type (*wt*), FcRγ heterozygous (+/-), FcRγ-Tg/KO (*Tg*), and FcRγ-deficient (-/-) mice. Results are shown as mean ± S.D. (*n* = 6). ***B***, platelet proteins were separated by SDS-PAGE, transferred to PVDF membranes and immunoblotted with HRP-labeled JAQ1 (anti-GPVI) or anti-FcRγ and anti-rabbit-HRP.

C.3.2 Influence of the level of GPVI-FcRg expression on responses to collagen

To examine the effect of reduced GPVI-FcRγ levels on collagen-induced activation, platelet aggregation and tyrosine phosphorylation of various intracellular signaling proteins was studied.

The dose-response curve for collagen-induced aggregation (0.1-10 µg/ml) was right shifted by approximately 2- and 5-fold in platelets expressing levels of 50% and 20% of GPVI-FcRγ complex, respectively, relative to wild type controls (Fig. 10a). In all cases, high concentrations of collagen induced a maximal aggregation response. However, there was a clear delay in onset of aggregation in response to low concentrations of collagen in the platelets from FcRγ (+/-) and FcRγ-Tg/KO mice. This is illustrated in Fig. 10b for a concentration of 2µg/ml collagen, where a 50% reduction in the level of GPVI-FcRγ led to a significant increase in the time taken for the aggregation trace to fall below the basal level after the initial shape change response, namely from 59 ± 15 to 103 ± 30 s (n=10 per group; $P < 0.05$). It was further tested whether the dose-response curve for tyrosine phosphorylation was also right shifted following a decrease in GPVI-FcRγ levels. Due to the varying delay in activation, tyrosine phosphorylation was measured at 150 s, at which time the aggregation response had reached a plateau in all groups of platelets. There was no detectable reduction in tyrosine phosphorylation in platelets expressing 50% and 20% levels of GPVI as compared with that in platelets expressing control (100%) levels (Fig. 11), whereas phosphorylation was blocked in FcRγ-chain deficient platelets (not shown).

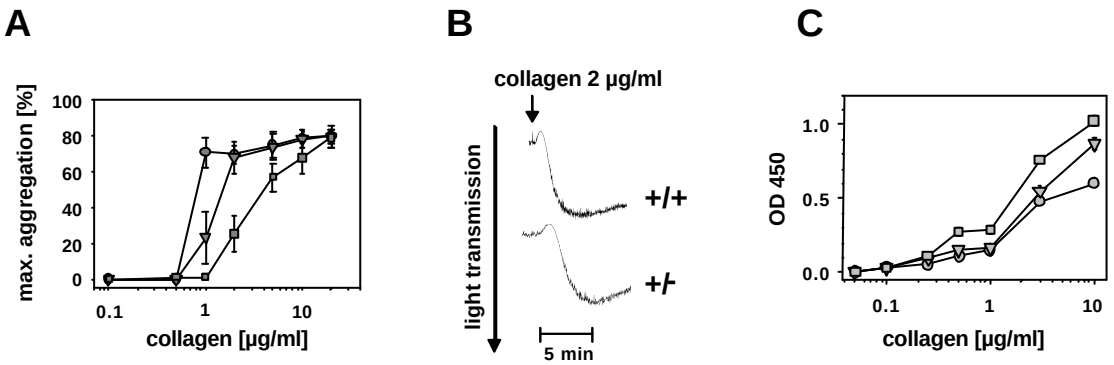


Figure 10. Influence of the level of GPVI-FcRg expression on platelet responses to collagen
A, heparinized prp from wild type (*circles*; +/+), FcRγ heterozygous (*triangles*; +/-), or FcRγ-Tg/KO (*squares*) mice was incubated with stirring in the presence of different concentrations of collagen. Dose-responses to collagen are shown as mean ± S.D. (*n* = 6-10). ***B***, aggregation traces for the indicated concentration of collagen. ***C***, washed platelets from wild-type (*circles*), FcRγ heterozygous (*triangles*), or FcRγ-Tg/KO (*squares*) mice were incubated for 60 min in microtiter plates coated with the indicated collagen concentrations, and adherent platelets were quantified fluorimetrically. The data shown are representative of three identical experiments and are expressed as mean± S.D of triplicate readings.

The effect of reduced GPVI-FcRγ levels on platelet adhesion to collagen was investigated under static conditions. Adhesion to immobilized collagen was dose-related over the concentration range of 0.05-10 µg of collagen per well. Platelets with 50% and 20% of control levels of GPVI-FcRγ adhered to collagen to the same extent as control platelets at all concentrations when measured at 15 min (not shown) and 60 min (Fig. 10c). FcRγ-chain-deficient platelets did virtually not adhere to the collagen-coated surface (not shown), indicating a crucial role for GPVI-FcRγ in this process. Platelet adhesion to collagen-coated surfaces will be addressed in more detail later in this study (C.6.3 and C.6.4.).

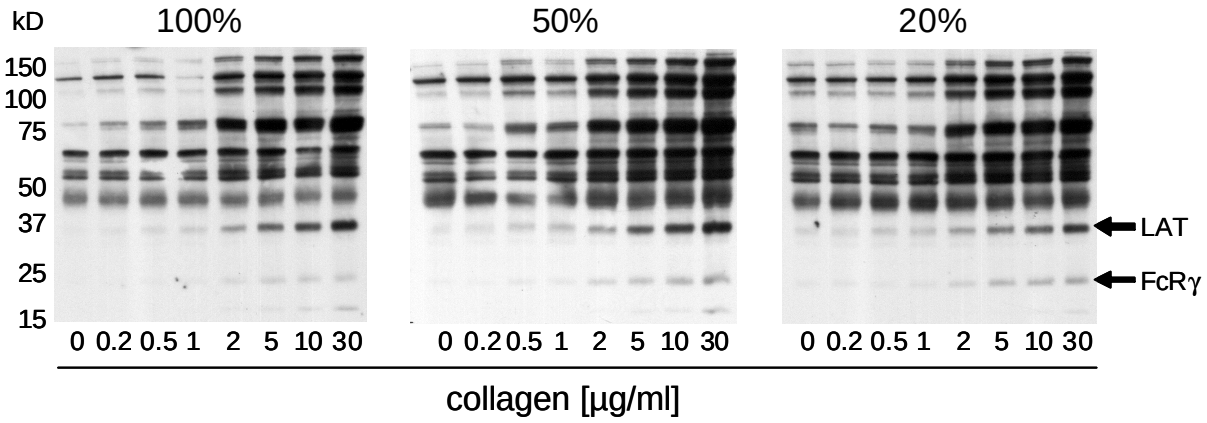


Figure 11. Effect of a decrease in GPVI-FcRγ levels on tyrosine phosphorylation in response to collagen

Washed platelets from mice expressing 100, 50, and 20% of control levels of GPVI were incubated at 37°C and stimulated with the indicated concentrations of collagen for 150 s. Aliquots were lysed after 60 s by addition of NP-40 buffer and dissolved in reducing SDS buffer. After separation by 4-12% SDS-PAGE and transfer to PVDF membranes, blots were incubated with the anti-phosphotyrosine antibody 4G10, and proteins were detected by anti-mouse IgG-HRP and ECL. Results are representative of four experiments.

C.3.3 Effect of blocking antibodies to other collagen receptors on activation of platelets expressing a reduced level of GPVI

It has been proposed that the interaction of collagen with GPVI is supported by other receptors including integrin $\alpha_2\beta_1$[52;51] and GPV[82]. Blocking antibodies to $\alpha_2\beta_1$ and GPV were used to investigate the importance of these receptors in platelets expressing a reduced level of GPVI. The $\alpha_2\beta_1$-blocking antibody delayed the onset of aggregation in response to collagen in wild-type platelets, and shifted the curve to the right by approximately 2-fold (Fig. 12a). In the presence of the blocking antibody, the onset of aggregation was further delayed in platelets from FcRγ (+/-) mice (not shown), and the curve was shifted a further 2-fold to the right (Fig. 12a). The $\alpha_2\beta_1$-blocking antibody induced an even greater delay in

aggregation of platelets from FcRγTg/KO mice (not shown), and again shifted the dose-response curve 2-fold further to the right (Fig. 12a). A blocking antibody to GPV (DOM1) did not cause an obvious delay in the onset of aggregation and caused a shift of less than 2-fold in the dose-response curve for aggregation in platelets from wild type, FcRγ (+/-) and FcRγ-Tg/KO mice (Fig. 12a). The combination of antibodies to $\alpha_2\beta_1$ and GPV resulted in a similar delay in the onset of aggregation and shift in the dose-response curve to collagen to those seen with the $\alpha_2\beta_1$-blocking antibody alone (not shown).

Blocking the CRP binding site with JAQ1, it was then investigated whether platelet aggregation shows a greater dependency on the levels of GPVI under these conditions. As shown in Fig. 12b, JAQ1 induced a similar shift to the right in the dose-response curve to collagen in platelets expressing 100%, 50%, and 20% levels of GPVI-FcRγ.

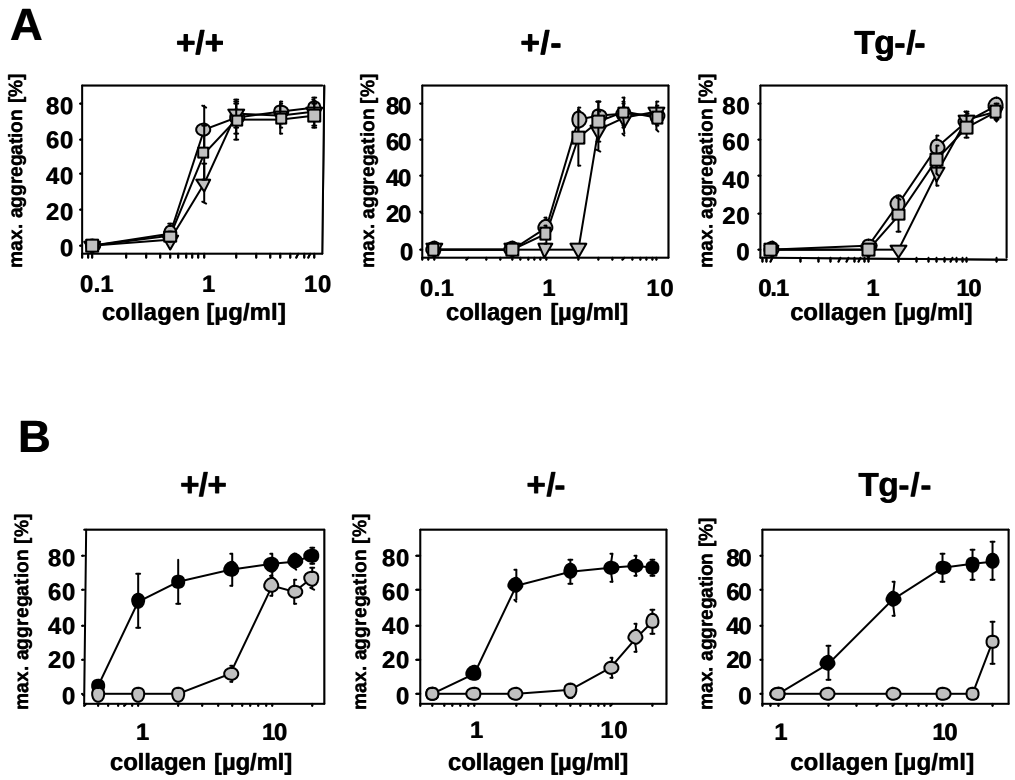


Figure 12. Effects of blocking antibodies against integrin a_2b_1, GPV and GPVI on aggregation in platelets expressing a reduced level of GPVI

A, heparinized prp from wild type (+/+), FcRγ heterozygous (+/-), or FcRγ-Tg/KO (*Tg -/-*) mice was incubated under stirring conditions with control IgG (*circles*), anti-GPV (*squares*) or anti-α_2 (*triangles*) monoclonal antibodies (all 20 µg/ml) for 5 min before addition of collagen (0.1-10 µg/ml). ***B***, heparinized prp from wild type (+/+), FcRγ heterozygous (+/-), or FcRγ-Tg/KO (*Tg-/-*) mice was incubated with (*gray symbols*) or without (*black symbols*) JAQ1 (20 µg/ml) for 5 min before addition of collagen (0.5-20 µg/ml). Results are shown as mean ± S.D. (*n* = 6-10).

Together, these results demonstrated that the contribution of $\alpha_2\beta_1$ and GPV to the collagen-induced aggregation is rather supportive than essential which is maintained even at low expression levels of GPVI.

C.3.4 Effect of a reduction in GPVI-FcRγ levels on platelet responses to GPVI-specific ligands

The effects of a reduction in the level of the GPVI-FcRγ complex on responses to three GPVI specific ligands were investigated. The dose-response curves for aggregation to the snake toxin convulxin and cross-linked mAb JAQ1 were shifted approximately 2- and 5-fold to the right in platelets expressing 50% and 20%, respectively, of the endogenous level of GPVI-FcRγ (Fig. 13a,b). In marked contrast, the dose-response curve for aggregation to the synthetic GPVI ligand CRP was shifted 20-fold to the right in the FcRγ (+/-) platelets and abolished in cells expressing 20% of the endogenous level of GPVI-FcRγ (Fig. 13c).

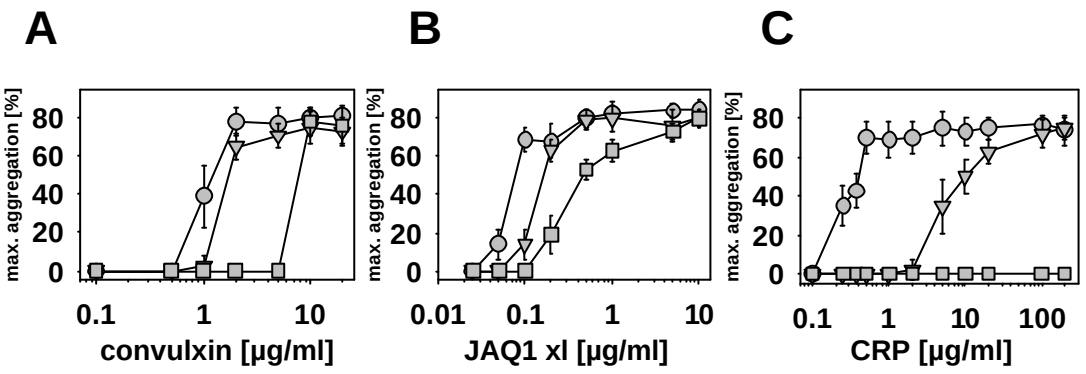


Figure 13. Effect of a decrease in GPVI-FcRγ-levels on aggregation in response to GPVI-specific ligands

Heparinized prp from wild type (*circles*), FcRγ heterozygous (*triangles*), or FcRγ-Tg/KO (*squares*) mice was stimulated under stirring conditions with different concentrations of convulxin (***A***), cross-linked JAQ1 (***B***), or CRP (***C***) and aggregation was recorded. Results are shown as mean ± S.D. (*n* = 6-10).

Consistent with these observations, the dose-response curve for tyrosine phosphorylation to convulxin was right-shifted in platelets expressing 50% of the endogenous level of GPVI-FcRγ, and right-shifted further in platelets with 20% of the control level (Fig. 14a). High concentrations of CRP stimulated protein tyrosine phosphorylation in the FcRγ (+/-) platelets (not shown), but had no effect in platelets expressing 20% of the control level of the GPVI-FcRγ (Fig. 14b), altogether indicating, that platelet activation by CRP is based on different

mechanisms than that used by other GPVI specific ligands and, most importantly, than that induced by collagen.

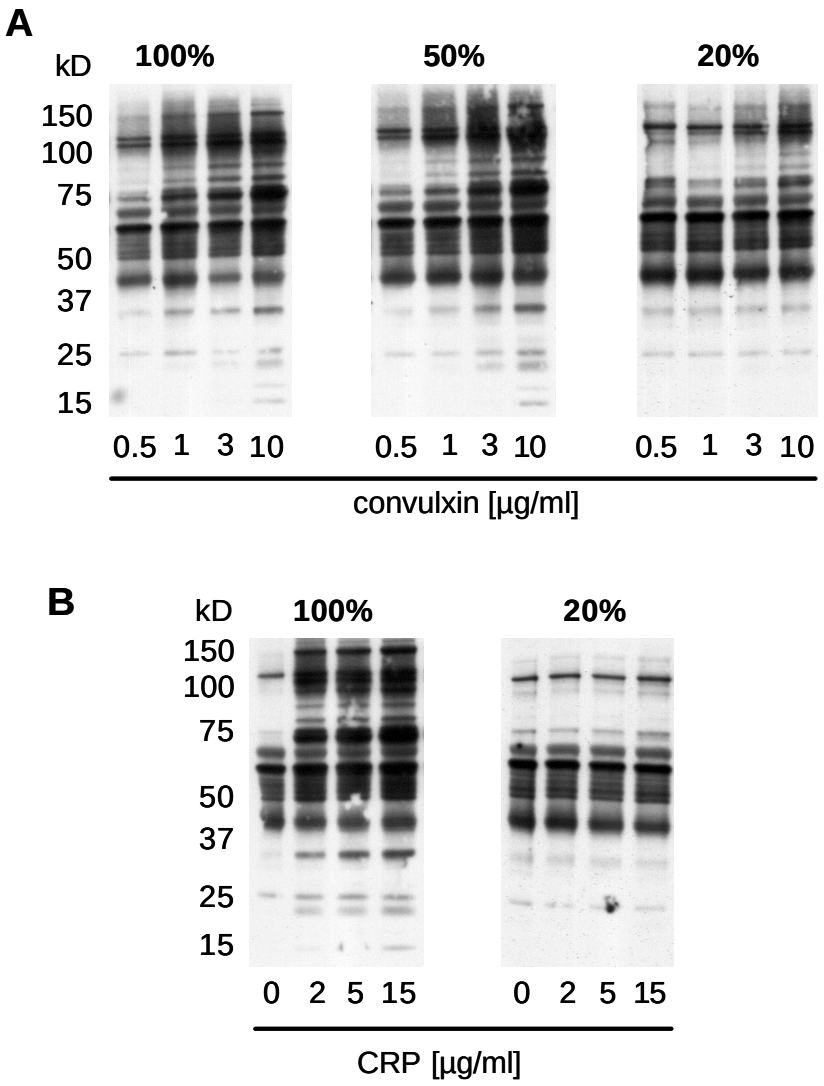
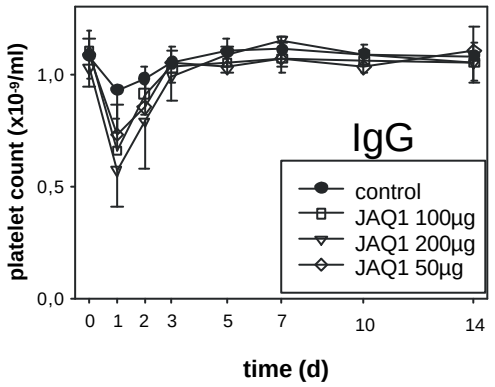


Figure 14. Effect of a decrease in GPVI-FcRg-levels on protein tyrosine phosphorylation in response to convulxin and CRP

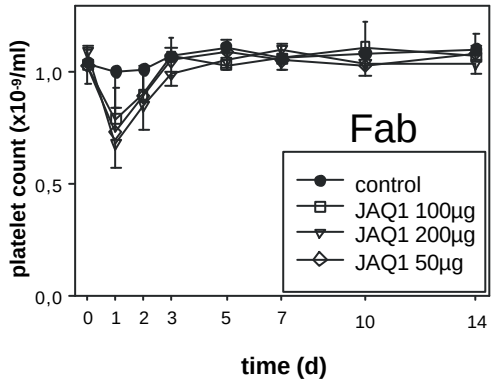
Washed platelets from mice expressing 100, 50, or 20% of GPVI-FcRγ level were stimulated with the indicated concentrations of convulxin (***A***) or CRP (***B***) for 150 s and reactions were stopped by the addition of reducing sample buffer. After separation by 4-12% SDS-PAGE and transfer to PVDF membranes, blots were incubated with the anti-phosphotyrosine antibody 4G10, and proteins were detected by anti-mouse IgG-HRP and ECL. Basal phosphorylation for the convulxin samples (from the same experiment) are shown in *B*. Results are representative of three experiments.

C.4 In vivo depletion of platelet GPVI in mice

In the next part of this work, the effects of JAQ1 were investigated *in vivo*. Therefore, NMRI mice were injected intraperitoneally with a bolus of 50 µg, 100 µg, or 200 µg JAQ1-IgG, JAQ1-Fab, or 100 µg irrelevant rat IgG and platelets were monitored for two weeks. Treatment with either amount of JAQ1 caused mild and transient thrombocytopenia with a maximum drop of platelet counts of approximately 34 ± 7.4% on day 1 and a return to normal after 72 h where they remained for at least 11 more days (Fig. 15a). The transient drop of platelet counts was not Fc-dependent as Fab fragments of JAQ1 had similar effects (Fig. 15b). JAQ1-treated mice did not show any signs of anaphylactic reactions as known to be induced by anti-GPIIb/IIIa mAbs[93] and did not develop spontaneous bleeding for at least three weeks. JAQ1 was immunohistochemically detectable on splenic and bone marrow-derived megakaryocytes 3h after antibody injection, demonstrating that the mAb reached these cells in both organs (not shown).



A



B

Figure 15. JAQ1 induces transient thrombocytopenia

Mice received purified IgG (***A***) or Fab fragments (***B***) of JAQ1 or irrelevant control Ab intraperitoneally in 200 µl sterile PBS. Platelet counts were determined at the indicated times using an improved Neubauer hemocytometer. Results are expressed as the mean platelet count ± S.D. for groups of each six mice.

C.4.1 JAQ1 treatment abolished platelet responses to collagen and collagen related peptides (CRP) ex vivo for at least two weeks

The effect of JAQ1 (100 µg) on circulating platelets was then studied *ex vivo* at different time points after antibody injection. Surface expression of various receptors as well as platelet activation was analyzed by flow cytometry. The basal expression of the major surface receptors GPIb-IX-V, CD9, and integrins $\alpha_{IIb}\beta_3$ and $\alpha_2\beta_1$ was unchanged as compared to control platelets at 3, 7, and 14 days after antibody injection (see App.1). At no time after antibody injection did circulating platelets show any signs of activation, as indicated by the lack of surface bound fibrinogen and surface expressed P-selectin (not shown). On day 3, 7, and 14, platelets from JAQ1-treated mice were resistant towards activation with the strong GPVI-specific platelet agonist CRP whereas ADP induced normal activation of these platelets, as shown by fibrinogen binding (Fig. 16a). Furthermore, platelets from JAQ1-treated mice were completely resistant to activation with collagen at concentrations of up to 50 µg/ml *ex vivo* and this profound inhibitory effect also lasted for at least 14 days upon a single injection of 100 µg JAQ1 (Fig. 16b). In contrast to collagen, ADP and PMA induced normal aggregation of these platelets, indicating that JAQ1 specifically blocked GPVI-dependent platelet activation pathways whereas other functions were not affected (Fig. 16b). These findings stand in contrast to *in vitro* effects of JAQ1, where saturating concentrations of the antibody (20 µg/ml) display only a limited inhibitory effect on collagen-induced platelet aggregation (see Fig. 5)

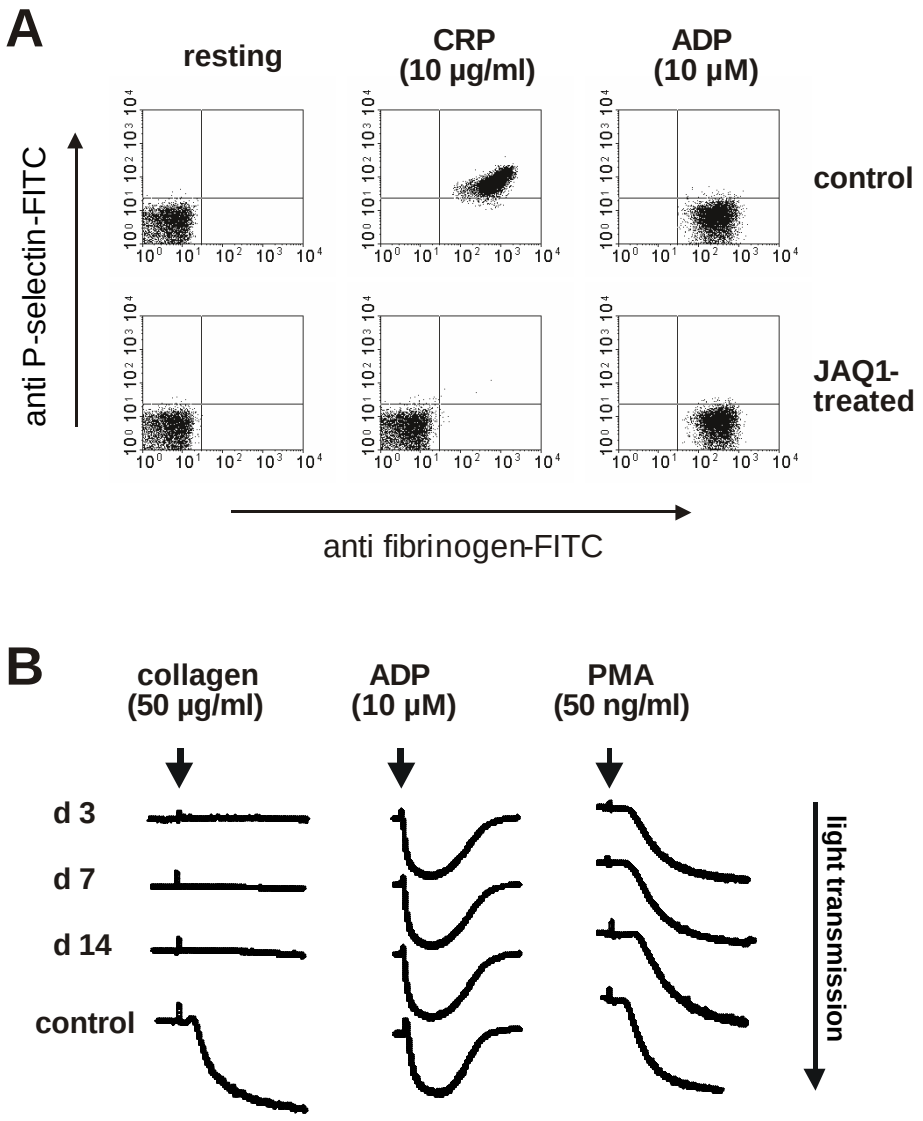


Figure 16. Platelets from JAQ1-treated mice do not respond to CRP and collagen

A, flow cytometric analysis of platelets from JAQ1-treated or control mice 3 d after Ab injection. Diluted whole blood was stimulated with 10 µM ADP or 10 µg/ml CRP for 2 min and subsequently incubated with anti-fibrinogen^{FITC} and anti-P-selectin^{PE} Abs for 10 min at RT and analyzed directly. Platelets were gated by FSC/SSC characteristics. The data shown are representative of six mice per group. Similar results were obtained on days 7 and 14 after Ab injection. ***B***, heparinized prp from the indicated mice was stimulated with collagen (50 µg/ml), ADP (10 µM) or PMA (50 ng/ml) and aggregation was recorded.

C.4.2 JAQ1 induces the loss of GPVI on circulating platelets

The observed discrepancy between the only limited inhibitory effect of JAQ1 on collagen-induced aggregation *in vitro* and the complete inhibition *ex vivo* was surprising and suggested that mechanisms other than pure blockage of an epitope on GPVI must be involved. Therefore, platelets from JAQ1-treated mice were tested for the presence of GPVI in a Western blot analysis of whole cell lysates. As shown in Fig. 17a, GPVI was not detectable in platelets from JAQ1-treated mice for at least 14 days upon a single injection of JAQ1 (100 µg), whereas GPIIIa was present in normal amounts at any time point. In contrast, in all mice tested, new platelets expressing functional GPVI were detectable after 28 days. The absence of GPVI on platelets from JAQ1-treated mice was confirmed by studies with the GPVI-specific agonist convulxin, which showed a lack of aggregation for platelets from JAQ1-treated mice on day 3, 7, and 14 (not shown). Additionally, flow cytometric analysis demonstrated that FITC-labeled convulxin did not bind to platelets from JAQ1-treated mice (Fig. 17b). Together, these results strongly suggested that GPVI had been irreversibly inactivated and removed from these platelets *in vivo*.

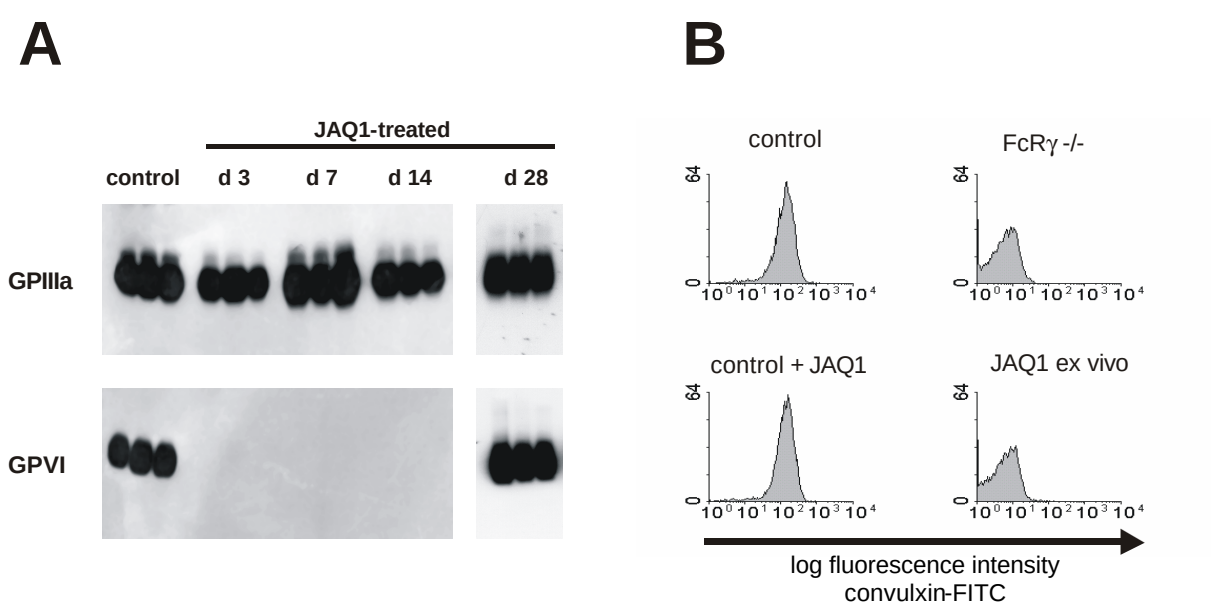
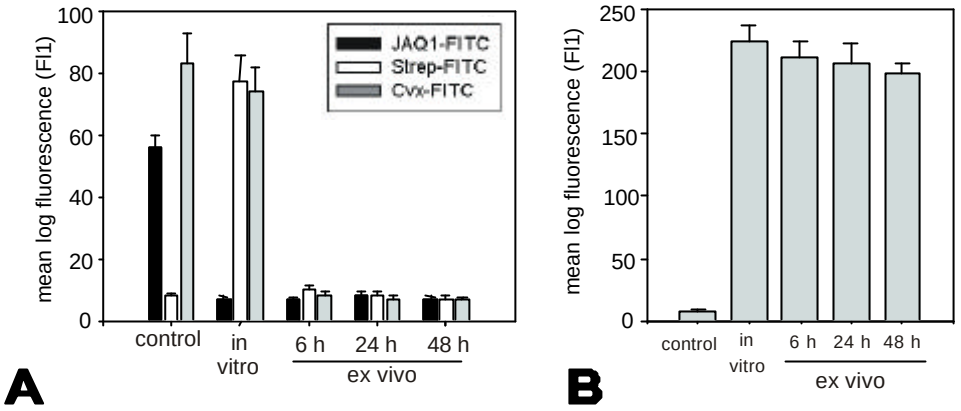


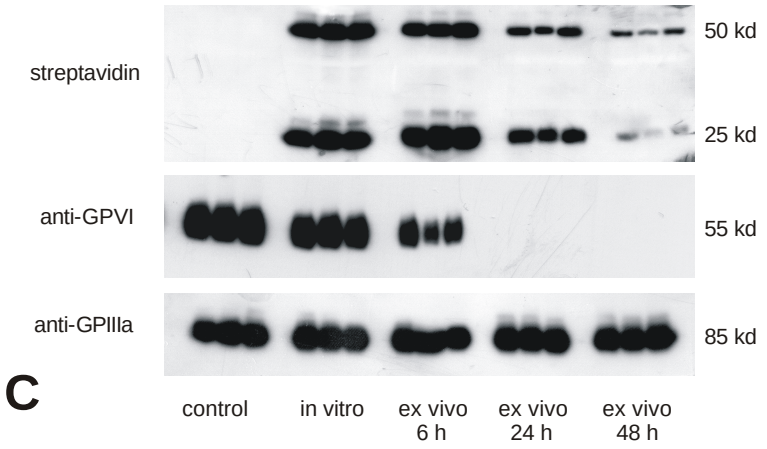
Figure 17. GPVI is not detectable in platelets from JAQ1-treated mice for at least two weeks
A, whole platelet proteins were separated by SDS-PAGE under nonreducing conditions and immunoblotted with FITC-labeled JAQ1 (anti-GPVI) or EDL1 (anti-GPIIIa). Bound mAb was detected by HRP-labeled rabbit anti-FITC and ECL. ***B***, washed platelets from the indicated mice were incubated with convulxin^{FITC} (5 µg/ml) for 15 min at RT and then analyzed flow cytometrically. The data shown are from one experiment representative of six.

C.4.3 JAQ1-induced GPVI loss occurs rapidly in vivo and is Fc-independent

To examine the mechanisms underlying the loss of GPVI, mice were injected with biotinylated JAQ1 and the amount of surface-bound mAb was determined by flow cytometry (streptavidin^{FITC}) *ex vivo* at early time points after injection. Interestingly, as soon as 6 h after injection only very low levels of surface-bound JAQ1 were detectable and the signals further decreased to control level after 24 and 48 h while JAQ1^{FITC} and Cvx^{FITC} bound to the platelets at no time point (Fig. 18a). These data suggested that the JAQ1-GPVI complex had been cleared from the surface of those platelets within 6 h. In contrast, platelets from mice injected with a biotinylated mAb against GPV (DOM1) constantly yielded positive staining with streptavidin^{FITC} (Fig. 18b). In the next step, whole cell lysates from platelets of JAQ1-treated mice were tested for the presence of GPVI and the biotinylated mAb (JAQ1). As shown in Fig.18c, JAQ1 was detectable in platelets 6h after injection whereas signals markedly decreased at 24 h and even more at 48 h. GPVI was only detectable after 6 h but no more after 24 h, strongly suggesting that the JAQ1-GPVI complex had become internalized and was degraded within two days. In contrast to its *in vivo* effects, JAQ1 did not induce any detectable down-regulation of surface GPVI within 6h incubation at 37°C in whole blood (Fig. 18a), indicating that a second signal may be required to induce this effect and that this signal is absent *in vitro*.

To determine whether the Fc part of JAQ1 or the divalent form of the antibody is required for internalization/degradation of GPVI, mice received 100 µg Fab fragments of the mAb and the platelets were tested for the presence of GPVI after 48 h. As shown in Fig. 18d, the Fab fragments, like the intact IgG, induced the complete loss of GPVI from circulating platelets and the cells were completely resistant towards activation with CRP, collagen, or convulxin, whereas ADP-induced aggregation was not affected.





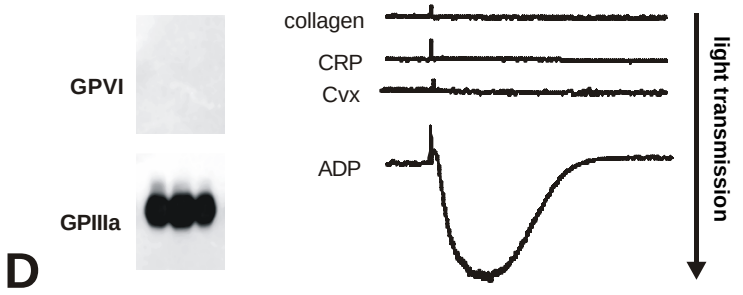


Figure 18. JAQ1 induces internalization of GPVI *in vivo* Fc-independently

Mice were injected with 100 µg biotinylated JAQ1 or DOM1 (anti-GPV) and platelets were analyzed at the indicated time points. ***A***, flow cytometric analysis. Diluted control blood was incubated with biotinylated rat IgG2a (10 µg/ml; *control*) or JAQ1 (10 µg/ml; *in vitro*) for 6 h at 37°C. Subsequently, these samples and samples from JAQ1-treated mice (*ex vivo*) were incubated with FITC-labeled JAQ1 (5 µg/ml), streptavidin (*Strep*, 5 µg/ml), or convulxin (*Cvx*, 5 µg/ml) for 15 min at RT and analyzed directly. Platelets were gated by FSC/SSC characteristics and Fl2 intensity (anti-mouse GPIIb/IIIa^{PE}). ***B***, detection of surface-bound biotinylated DOM1 (anti-GPV) *ex vivo*. The staining was performed as described for biotinylated JAQ1. Results in panels ***A*** and ***B*** are expressed as mean log Fl1 ± S.D. (n = 6). ***C***, *upper*: whole platelet proteins were separated by SDS-PAGE under reducing conditions and biotinylated JAQ1 was detected with HRP-labeled streptavidin/ECL. For detection of GPVI (*middle*) and GPIIIa (*lower*), the proteins were separated under nonreducing conditions and immunoblotted with FITC-labeled JAQ1 (anti-GPVI) or EDL1 (anti-GPIIIa) followed by HRP-labeled rabbit anti-FITC and ECL. ***D***, mice were injected with 100 µg JAQ1-Fab and platelets were analyzed in a Western blot for the presence of GPVI and GPIIIa after 48 h. These platelets did not aggregate in response to collagen (50 µg/ml), CRP (30 µg/ml) or convulxin (*Cvx*, 10 µg/ml) whereas ADP (10 µM) induced normal aggregation.

C.4.4 Targeting of the collagen binding site is not essential for in vivo depletion of GPVI

4.4.1 Generation of additional anti-GPVI mAbs recognizing distinct epitopes on the receptor

To determine whether the JAQ1-induced *in vivo* depletion of GPVI is dependent on targeting of the ligand binding site on the receptor, new mAbs against different epitopes on GPVI were generated and their *in vitro* and *in vivo* activity was tested. Both antibodies (JAQ2, JAQ3, rat IgG2a) precipitated a single chain protein of an apparent molecular weight of approximately 60 kD (Fig. 19a, IP). The identity of the precipitated protein with GPVI was verified by immunoblotting with JAQ1 (not shown). JAQ2 and JAQ3 also recognized GPVI in Western blot analysis under nonreducing conditions (Fig. 19a, WB). In flow cytometric analysis, JAQ2 and JAQ3 did not bind to platelets from FcRγ-chain-deficient mice (not shown) which have been shown to lack GPVI (C.1.2). Similar to JAQ1, JAQ2 and JAQ3 alone did not induce platelet aggregation whereas strong and irreversible aggregation occurred upon cross-linking of the bound mAbs by anti-rat Ig antibodies (Fig. 19b), confirming that multivalent clustering of GPVI is required to induce platelet activation[94]. Flow cytometric preincubation studies demonstrated that JAQ1, JAQ2, and JAQ3 do not block each others binding suggesting that they recognize different epitopes on GPVI (Fig. 19c).

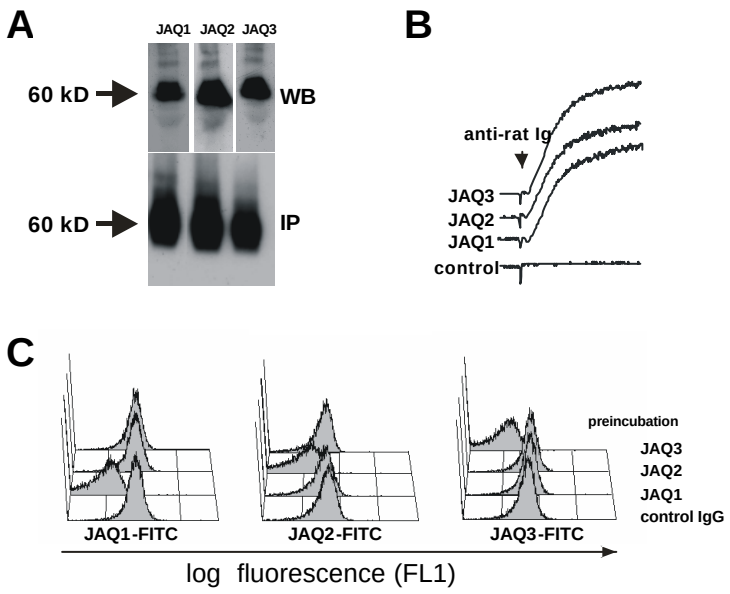


Figure 19. JAQ 1, JAQ2 and JAQ3 bind to different epitopes on mouse GPVI
A, whole platelet proteins were separated by SDS-PAGE under nonreducing conditions and immunoblotted with the indicated antibodies. Bound mAb was detected by HRP-labeled rabbit anti-rat Ig and ECL (*WB*). Surface biotinylated platelets were lysed and immuno-precipitation was performed with the indicated antibodies. Proteins were detected with streptavidin-HRP and ECL (*IP*). ***B***, heparinized prp was incubated with JAQ1, JAQ2, JAQ3, or irrelevant IgG2a (all 20 µg/ml) followed by addition of polyclonal rabbit anti-rat Ig antibody (10 µg/ml) and aggregation was recorded. ***C***, platelets were pre-incubated with the indicated antibodies (20 µg/ml, 30 min at RT) and washed; binding of FITC-labeled JAQ1, JAQ2, or JAQ3 was detected by flow cytometry.

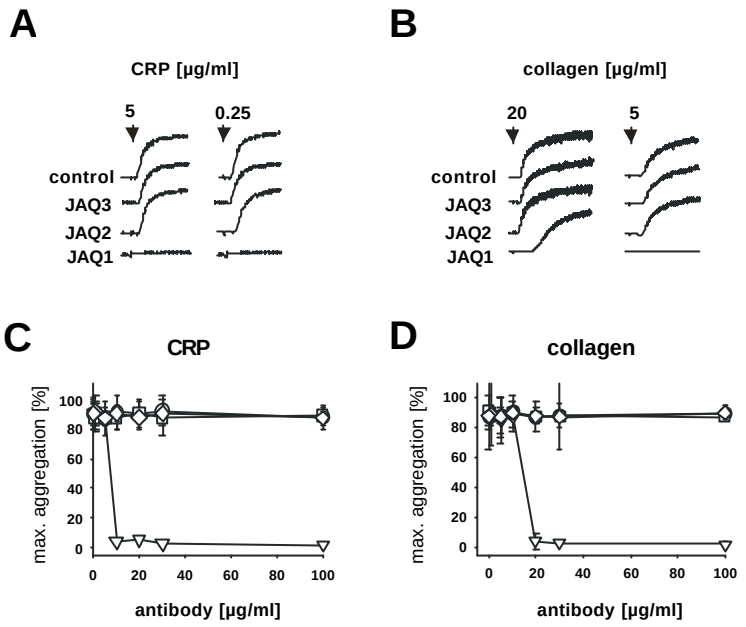


Figure 20. JAQ2 and JAQ3 do not inhibit platelet aggregation induced by CRP and collagen

A, B, heparinized prp was stimulated with CRP or collagen and aggregation was recorded. The results shown are representative of six experiments. ***C, D***, heparinized prp was incubated with different concentrations of control IgG2a (*circles*), JAQ1 (*triangles*), JAQ2 (*squares*), or JAQ3 (*diamonds*) for 5 min before the addition of CRP (0.25 µg/ml) or collagen (5 µg/ml) and aggregation was recorded. Results are expressed as mean ± S.D. of six mice per group.

4.4.2 MAb-induced in vivo depletion of GPVI is independent of the binding epitope

To test the effects of JAQ2 and JAQ3 on the *in vivo* expression of GPVI, mice received 100 µg of the respective antibodies and platelets were monitored for 10 days. As controls, mice received 100 µg non-specific rat Ig or DOM2, a rat IgG2a directed against mouse GPV. Similar to JAQ1 (Fig. 15a), injection of JAQ2, JAQ3, and DOM2 caused a mild and transient effect on platelet counts with a maximum drop of ~40% on day 1 and a return to almost normal after 48-72 h (Fig. 21a). Circulating platelets in JAQ-treated mice were not activated at any time point after injection as shown by flow cytometric analysis of P-selectin expression and integrin activation (Fig. 21b). GPVI expression on the platelet surface was analyzed by flow cytometry at different time points after antibody injection using $JAQ1^{FITC}$. Unexpectedly, as soon as 6 h after antibody injection GPVI was undetectable on platelets from JAQ2- or JAQ3-treated mice and this remained unchanged for 10 days (Fig. 21c). In contrast, GPVI levels on circulating platelets were not altered significantly at any time point in DOM2-treated mice. These findings suggested that GPVI had been specifically depleted by JAQ2 and JAQ3 *in vivo*. This was confirmed when separate groups of mice received the antibodies (100 µg/mouse) and platelets were tested on day 5 for the presence of GPVI in Western blot analysis. As shown in Fig. 21d, GPVI was undetectable in platelets from mice treated with JAQ1, JAQ2, or JAQ3 whereas normal amounts of GPIIIa were found in all platelets. The expression of other receptors including GPIb-IX, GPV, and $\alpha_2\beta_1$ was not altered in any group of mice as confirmed by flow cytometry (see App.1 and not shown). In agreement with the absence of GPVI, these platelets were completely resistant to collagen- and CRP-induced aggregation but reacted normally to ADP (Fig. 22).

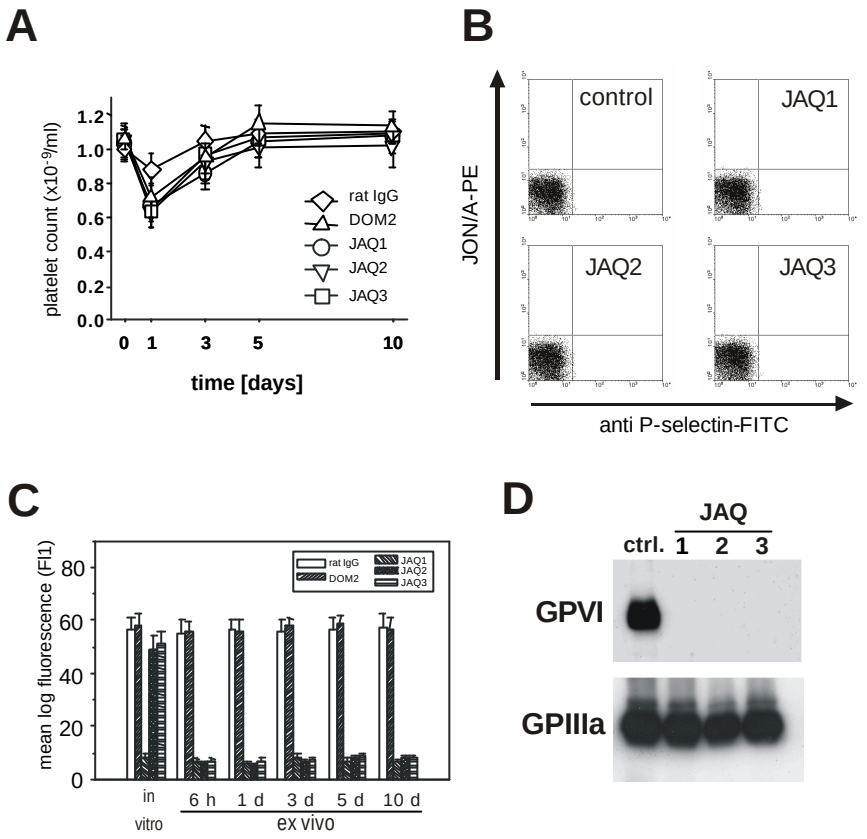


Figure 21. JAQ1, JAQ2 and JAQ3 deplete GPVI *in vivo*

A, platelet counts from antibody-treated mice were monitored for 10 days after injection of the indicated antibodies (all 100 µg). Results are expressed as mean ± S.D. of nine mice per group. ***B***, flow cytometric analysis of P-selectin expression and GPIIb/IIIa activation on platelets from JAQ1-, JAQ2-, JAQ3-treated mice or control mice on day 3 after injection. The results shown are representative of six mice per group. Similar results were obtained at all other time points tested. ***C***, diluted blood collected at the indicated time points after antibody injection was incubated with $JAQ1^{FITC}$ (5 µg/ml) for 15 min at RT and analyzed directly. Results are presented as mean ± S.D. of nine mice per group. ***D***, whole platelet proteins were separated by SDS-PAGE under nonreducing conditions and immunoblotted with HRP-labeled JAQ1 (anti-GPVI) or EDL1 (anti-GPIIIa) followed by ECL.

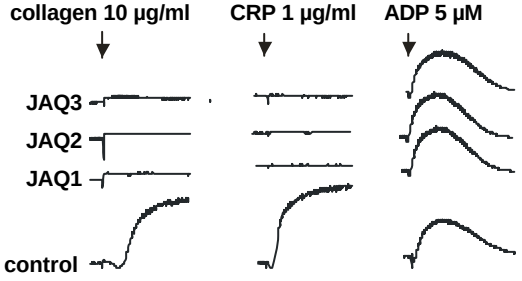


Figure 22. *Ex vivo* analysis of GPVI-depleted platelets

Heparinized prp from mice on day 5 after treatment with irrelevant IgG2a or the indicated anti-GPVI mAb were stimulated with collagen, CRP, or ADP and aggregation was recorded. The results shown are representative of six mice per group.

4.4.3 Dimerization of GPVI is not required for depletion by JAQ2 and JAQ3

For JAQ1 it was shown that neither the Fc part nor the divalent form of the antibody plays a role in depletion of the receptor. To address this question for the new mAbs, monovalent Fab fragments of JAQ2 and JAQ3 were produced and tested *in vivo*. After injection of Fab fragments (100 µg/mouse) platelet counts and GPVI expression were monitored for 10 days. JAQ2- or JAQ3-Fab induced a similar transient decrease in platelet counts as the intact IgG (Fig. 23a). Strikingly, Fab fragments of both JAQ2 and JAQ3 induced the depletion of GPVI (Fig. 23b). However, compared to intact IgG the duration of Fab-induced GPVI depletion was significantly reduced as very low levels of GPVI were detectable in platelets already after 72 h (Fig. 23c,d; see Fig. 18d for 48 h result of JAQ1-Fab) and this increased to almost normal on day 10. Consequently, during the first 3 days, platelets from these mice were resistant to activation with CRP (Fig. 23d).

In summary, these results demonstrated, that mAb-induced depletion of GPVI from circulating platelets occurs irrespective of the targeted epitope and independent of dimerization of the receptor.

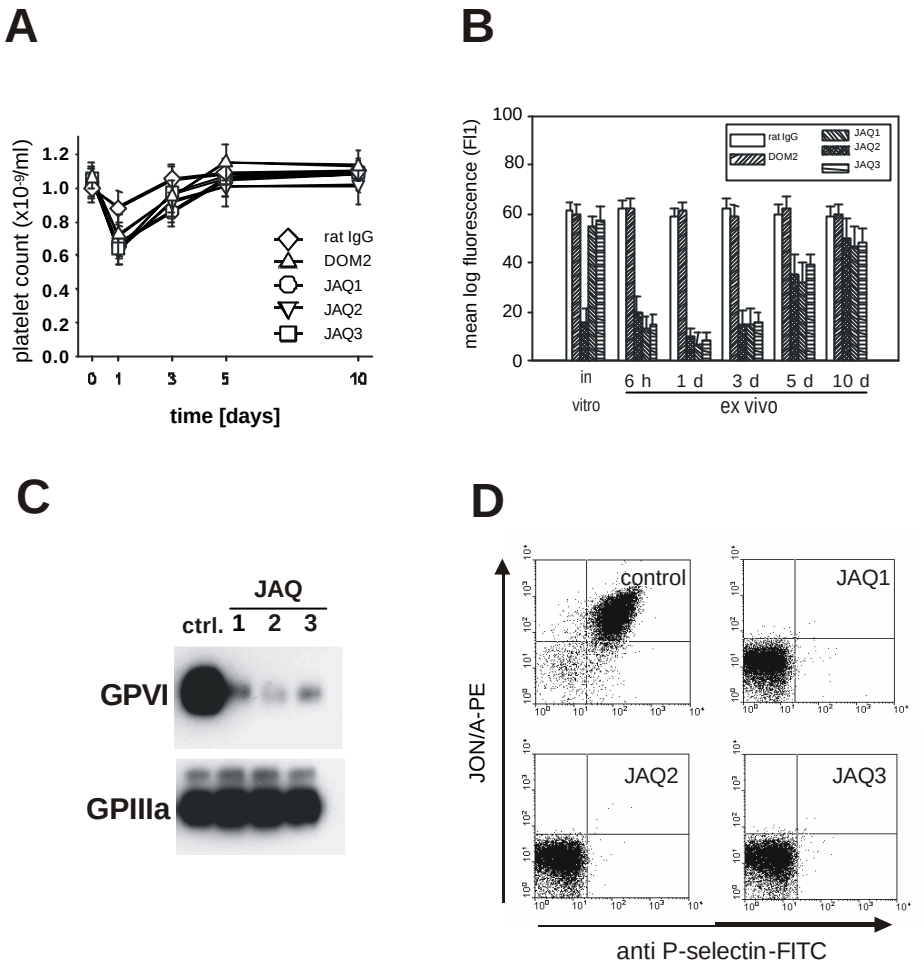


Figure 23. *In vivo* depletion of GPVI by monovalent Fab fragments of JAQ1, JAQ2, and JAQ3

A, platelet counts were monitored for 10 days after injection of the indicated Fab fragments or control IgG (all 100 µg). Results are expressed as mean ± S.D. (n = 6). ***B***, flow cytometric analysis of GPVI expression on platelets from mice treated with JAQ1-, JAQ2-, JAQ3 Fab fragments or control IgG. Diluted blood collected at the indicated time points was incubated with $JAQ1^{FITC}$ (5 µg/ml) for 15 min at RT and analyzed directly. Results are presented as mean ± S.D. (n = 6). ***C***, western blot analysis of GPVI expression on day 3 after injection of anti-GPVI Fab fragments. Whole platelet proteins were separated by SDS-PAGE under nonreducing conditions and immunoblotted with HRP-labeled JAQ1 (anti-GPVI) or EDL1 (anti-GPIIIa) followed by ECL. ***D***, platelets from Fab fragment-treated or control mice were stimulated with CRP (5 µg/ml) and analyzed for P-selectin expression and GPIIb/IIIa activation by flow cytometry. The results shown are representative of six mice per group.

C.5 Long-term antithrombotic protection after JAQ1 treatment

C.5.1 Moderately increased bleeding times in GPVI-depleted mice

To examine the effects of GPVI depletion on normal hemostasis, tail bleeding times were determined on day 7 after a bolus injection of JAQ1 (100 µg). GPVI-depleted mice show significantly increased bleeding times compared to control mice (330 ± 103 vs. 158 ± 89 s, respectively), but consistently lower than in mice pre-treated with 100 µg blocking $F(ab)_2$ fragments against GPIIb/IIIa[85] (Fig. 24).

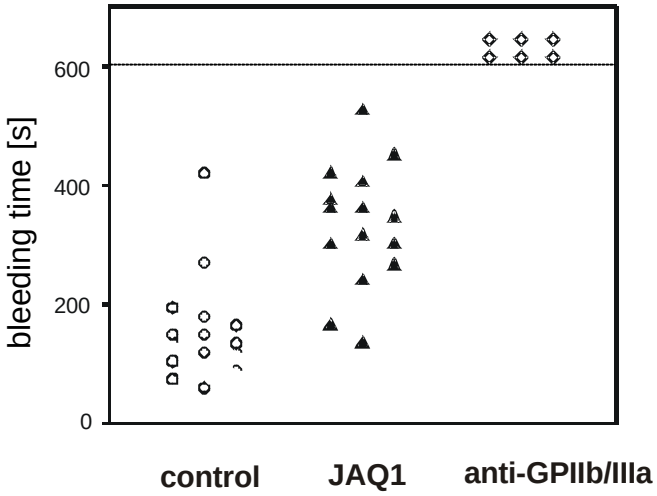


Figure 24. Bleeding time of JAQ1-treated mice
Bleeding times were determined in mice 7 d after injection of 100 µg nonimmune IgG2a or JAQ1 (n = 15 per group). As a control, mice received 100 µg $F(ab)_2$ fragments of JON/A (anti-GPIIb/IIIa) 24 h before the experiment (n = 6). Each point represents one individual.

C.5.2 Anti-GPVI treatment induces long-term antithrombotic protection

In the next step, the protective effect of JAQ1 treatment in an *in vivo* thrombosis model was investigated. In this model, the infusion of a mixture of collagen (0.8 mg/kg body weight) and epinephrine (60 µg/kg body weight) induces lethal pulmonary thromboembolism[89]. Among control mice pre-treated with irrelevant rat IgG2a, 95% (19 of 20) died within 5 min from widespread pulmonary thrombosis and cardiac arrest. In contrast, all mice pre-treated with JAQ1 (100 µg) survived, irrespective of whether they had received the mAb 3, 7, or 14 days before challenge (n=8 per group) (Fig. 25a). While the platelet counts in JAQ1 pre-treated mice had not been influenced significantly by the infusion of collagen/epinephrine, there was a sharp decrease detectable in control mice (n=8) which was determined 3 min after induction of thromboembolism in a separate group (Fig. 25b).

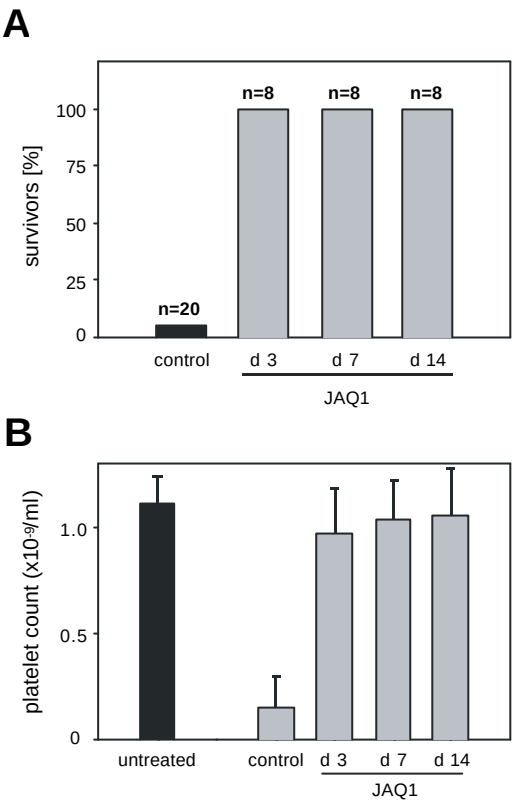


Figure 25. JAQ1 induces long-term protection from intravascular thrombosis

Thromboembolism in response to a bolus injection of a mixture of collagen (0.8 mg/kg body weight) and epinephrine (60 µg/kg body weight). ***A***, mortality in control mice and mice treated with 100 µg JAQ1 at the indicated times before challenge. ***B***, platelet counts in control and JAQ1-treated mice 3min after infusion of collagen/epinephrine (n = 8 per group).

For histological examination, control and JAQ1 pre-treated (3, 7, and 14 days) mice received the same treatment in parallel experiments but the lungs were removed after 3 min. While the vast majority of large and small vessels were obstructed by platelet rich thrombi in the lungs of control mice, there were only very few thrombi detectable in the lungs of mice which received JAQ1 (Fig. 26).

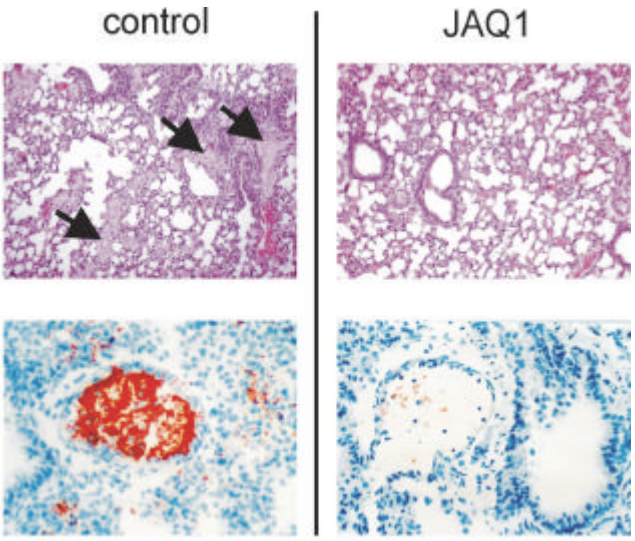


Figure 26. Immunohistochemical analysis of lungs from control and GPVI-depleted mice after challenge with collagen/epinephrine

Top: representative histology of the lungs (original magnification: X100); obstructed vessels are indicated by *arrowheads*. *Bottom*: immunohistochemical detection of platelets in the thrombi (original magnification: X400). Acetone-fixed frozen sections were reacted with a platelet-specific antibody (anti-GPIb-IX) and counterstained with hematoxylin. The red HRP reaction product shows high density of platelets in the thrombus.

C.6 GPVI but not integrin a_2b_1 is essential for platelet interactions with collagen

The current model for platelet-collagen interactions proposed a crucial role for integrin $\alpha_2\beta_1$ in platelet adhesion to and activation by collagen. To investigate the roles of the collagen receptors integrin $\alpha_2\beta_1$ and GPVI in more detail and to understand how $\alpha_2\beta_1$ becomes activated *in vivo*, mice lacking β_1 integrins on their platelets were used in combination with the GPVI blocking antibody JAQ1 for studies of platelet activation and adhesion. Experiments involving β_1-null mice were performed in collaboration with the group of R. Fässler (Martinsried, Germany). These mice display normal platelet counts and no significant bleeding phenotype[61]. As expected, β_1-null platelets also lack α_2, α_5, and α_6, while the surface expression of β_3 as well as other major platelet receptors, including GPVI, CD9, and all subunits of the GPIb-V-IX complex was unchanged (see App.1).

C.6.1 Collagen-induced aggregation of b_1-null platelets is delayed, but not reduced

$\alpha_2\beta_1$ integrin was reported to play a major role during collagen-induced platelet aggregation *in vitro*[18;95]. To directly test this, the aggregation of normal and β_1-null platelets was induced using fibrillar type I collagen. While no difference was observed in the dose-response characteristic and in the maximum aggregation of control and mutant platelets, β_1-null platelets displayed a significant delay in the onset of aggregation that became more evident when lower concentrations of collagen were used (Fig. 27a). A blocking mAb against the α_2 integrin revealed a similarly delayed onset of platelet aggregation (not shown). The delay in aggregation was reflected in the time course of tyrosine phosphorylation observed upon collagen-induced activation, whereas the pattern of phosphorylated proteins was comparable between normal and β_1-null platelets (Fig. 27b). Aggregation of β_1-null platelets induced by other agonists, such as CRP, convulxin, thrombin, and ADP, was normal compared to control (not shown), indicating that the loss of β_1 integrin is not associated with a general delay or other defects in platelet aggregation.

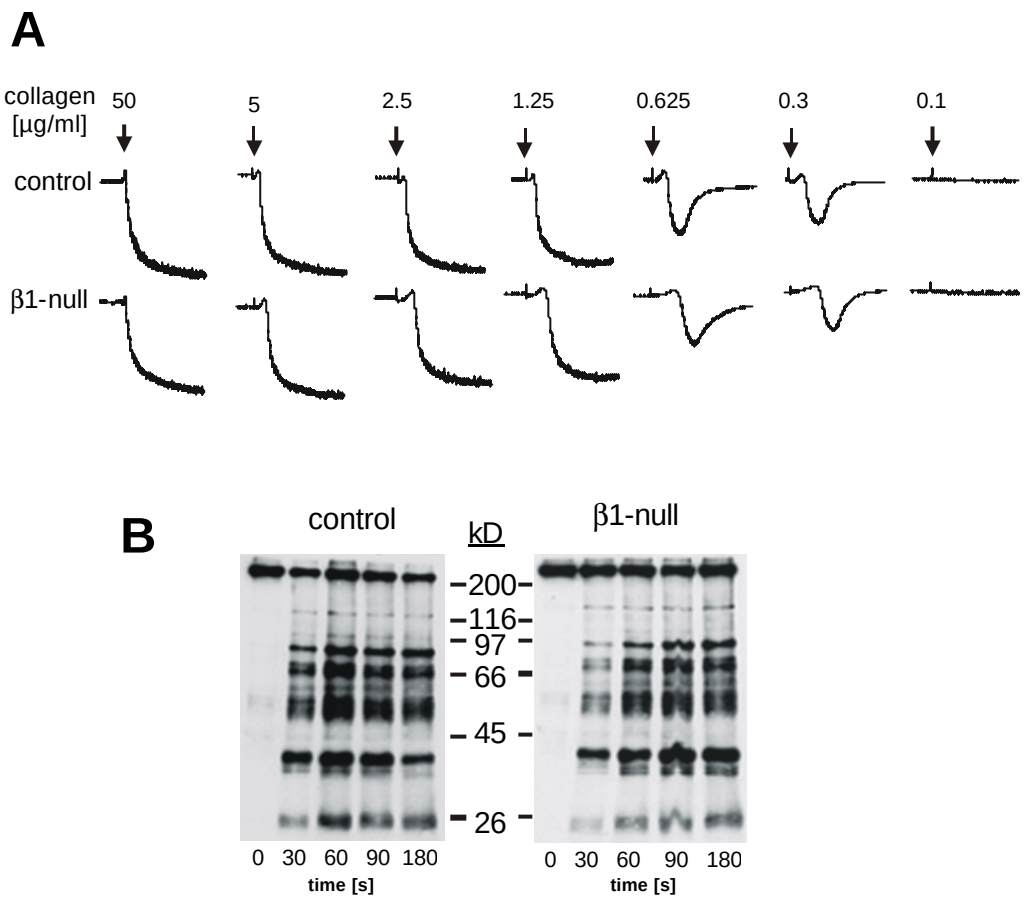


Figure 27. Aggregation of b_1-null platelets in response to fibrillar collagen
A, heparinized prp from control and β_1-null mice was stimulated with the indicated concentrations of fibrillar collagen and aggregation was recorded. ***B***, washed platelets were stimulated with fibrillar collagen (5 µg/ml), and samples were lysed at the indicated time points. Protein tyrosine phosphorylation was detected by immunoblotting with the anti-phosphotyrosine antibody 4G10.

These data indicate that the signaling machinery leading to platelet activation is not altered in β_1-null platelets, that GPVI-mediated platelet activation is not affected by the absence of β_1 integrins, and that $\alpha_2\beta_1$ integrin facilitates fibrillar collagen-induced platelet activation, but is not essential.

C.6.2 GPVI mediates platelet activation by two different pathways only one of which involves a_2b_1

As shown earlier, JAQ1 inhibits platelet aggregation in response to low, but not high collagen concentrations (Fig. 5b). To test whether $\alpha_2\beta_1$ integrin is involved in the activation process upon high collagen concentrations, normal and β_1-null platelets were incubated with 20 µg/ml

JAQ1 Fab fragments and subsequently stimulated with 5 µg/ml or 50 µg/ml of fibrillar collagen. Interestingly, under these conditions, even 50 µg/ml of collagen did not induce aggregation of β_1-null platelets (Fig. 28a) suggesting that an $\alpha_2\beta_1$-dependent pathway mediates this activation. This was confirmed in further studies on wild type platelets where aggregation in response to 50 µg/ml of collagen was completely abolished in the presence of JAQ1 and a blocking antibody against α_2 integrin (not shown). On the other hand, GPVI-depleted and FcRγ-chain-deficient platelets failed to aggregate in response to high concentrations of fibrillar collagen (Fig. 28a and ref[68]) demonstrating that GPVI is strictly required for the $\alpha_2\beta_1$-dependent pathway.

Cells secrete monomeric procollagen which is converted into collagen by proteolytic cleavage followed by assembling into cross-striated fibrils that occur in the extracellular matrix of connective tissue[96]. Soluble collagen is normally not found in tissues but frequently used for *in vitro* analysis of platelets[44;97;98]. It has been reported that platelet activation by soluble collagen is greatly dependent on $\alpha_2\beta_1$ integrin[97]. Indeed, soluble collagen induced irreversible aggregation of normal, but not of β_1-null platelets (Fig. 28b). The aggregation response, however, was also abolished with normal platelets after preincubation with JAQ1 as well as with GPVI-depleted and FcRγ-chain-deficient platelets demonstrating a critical role of GPVI in this activation process. These results were further confirmed by tyrosine phosphorylation studies showing that phosphorylation induced by soluble collagen was abolished in β_1-null (Fig. 28c) as well as GPVI-depleted and FcRγ-chain-deficient platelets (not shown).

In summary, these data show that GPVI is the central collagen receptor for platelet activation that only requires cooperation with $\alpha_2\beta_1$ when signaling through the CRP binding site is insufficient or blocked (by JAQ1).

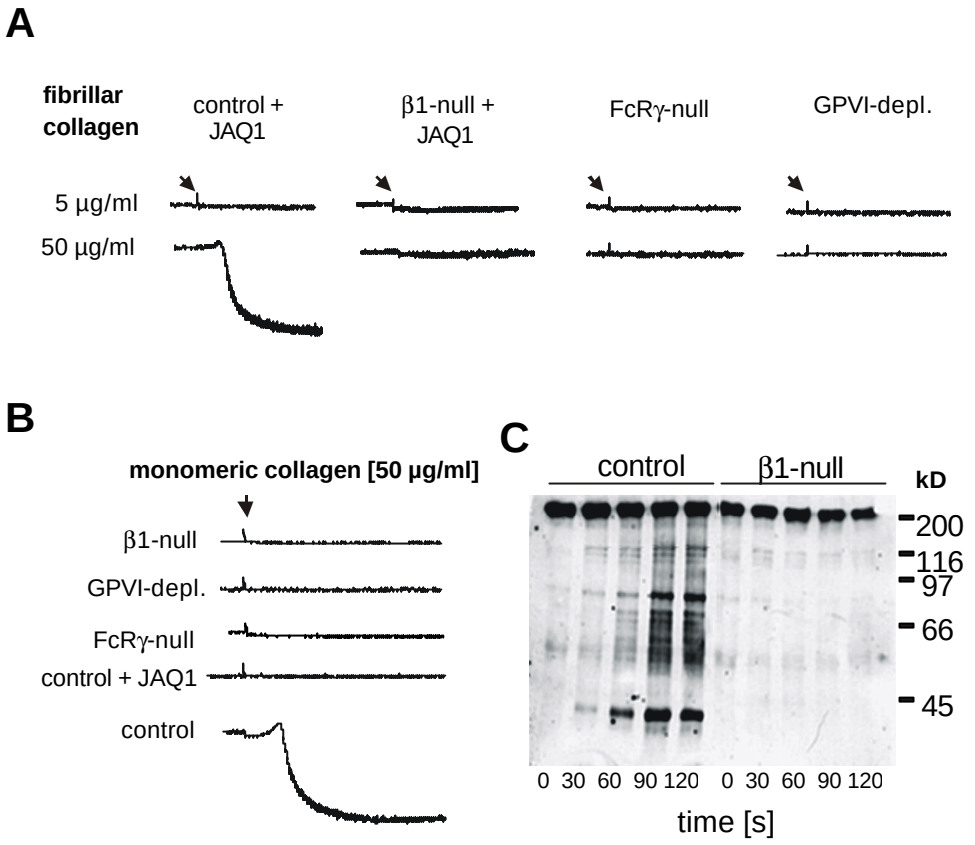


Figure 28. GPVI mediates two activation pathways, one of which involves $\alpha_2\beta_1$
A, heparinized prp from control and β_1-null mice was preincubated with 20 µg/ml JAQ1 Fab fragments and then stimulated with fibrillar collagen (5 or 50 µg/ml). As a control, prp from GPVI-depleted and FcRγ-chain-deficient mice was stimulated with collagen in the absence of JAQ1. ***B***, prp from the indicated mice was stimulated with soluble collagen (50 µg/ml). Where indicated, the prp was pretreated with JAQ1 Fab fragments (20 µg/ml) for 5 min. ***C***, washed platelets from control and β_1-null mice were stimulated with soluble collagen, and samples were lysed at different time points. Protein tyrosine phosphorylation was detected by immunoblotting with the anti-phosphotyrosine mAb 4G10.

C.6.3 Adhesion to fibrillar collagen is GPVI, but not $\alpha_2\beta_1$-dependent

A current model suggests that *in vivo*, platelets adhere firmly to collagen through GPIb-V-IX (via vWf) and $\alpha_2\beta_1$ integrin which allows subsequent collagen-GPVI interactions leading to platelet activation[51-53]. To test this model, normal and β_1-null platelets were isolated and allowed to adhere to fibrillar collagen under static conditions. Integrin $\alpha_2\beta_1$ binding depends on the presence of Mg^{2+}/Ca^{2+}(ref[99]) and therefore the adhesion assays were performed in the presence or absence of these divalent cations. Both normal and β_1-null platelets adhered to

fibrillar collagen in a time-dependent manner in the presence or absence of Mg^{2+}/Ca^{2+} (Fig. 29). When the CRP binding site on GPVI was blocked with JAQ1 Fab fragments (20 µg/ml), normal platelets adhered to fibrillar collagen in the presence, but not in the absence of Mg^{2+}/Ca^{2+}, suggesting that the attachment involves $\alpha_2\beta_1$ integrin. This was confirmed with β_1-null platelets in the presence of JAQ1 where adhesion was abrogated with and without Mg^{2+}/Ca^{2+}, as also observed for FcRγ-chain-deficient platelets, confirming the strict requirement for GPVI in this process (Fig. 29).

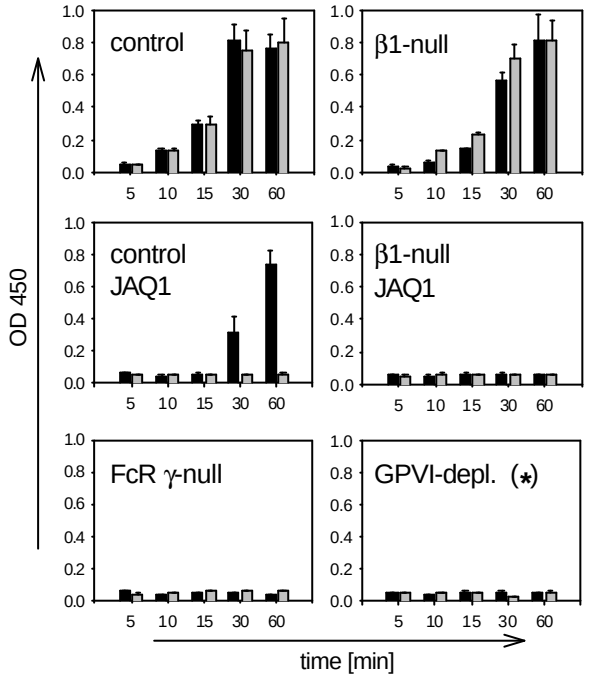


Figure 29. Platelet adhesion to fibrillar collagen under static conditions
Washed platelets from the indicated mice were allowed to adhere under static conditions to fibrillar collagen immobilized in microtiter plates. The experiments were performed in the presence (*black*) or absence (*gray*) of Mg^{2+}/Ca^{2+} (1 mM each). Where indicated, platelets were preincubated with JAQ1 Fab fragments (20 µg/ml). Adherent platelets were quantitated fluorimetrically. The data shown are from a single experiment, representative of four identical experiments and expressed as the mean of triplicate readings ± S.D. for the indicated times. (*) To exclude any residual GPVI activity, the platelets were used in the presence of (10 µg/ml) JAQ1.

To test whether this defect was based on impaired integrin activation, adhesion was analyzed in the presence of Mn^{2+} cations that are known to activate β_1 and β_3 integrins directly[100]. As shown in Fig. 30, FcRγ-chain-deficient platelets strongly adhered to fibrillar collagen in the presence of Mn^{2+} and comparable results were obtained with GPVI-depleted platelets (not shown). This adhesion was nearly abolished when α_2 integrin was blocked with an antibody whereas blockage of $\alpha_{IIb}\beta_3$ had no significant effect. A different picture was found when the collagen substrate had previously been exposed to vWf. Under these conditions, blockage of either $\alpha_2\beta_1$ or $\alpha_{IIb}\beta_3$ only had minor effects, whereas concurrent blockage of both receptors abolished adhesion suggesting that $\alpha_{IIb}\beta_3$ mediated adhesion *via* vWf. When the collagen substrate had been pre-exposed to plasma, even the blockage of both $\alpha_2\beta_1$ and $\alpha_{IIb}\beta_3$ only had partial inhibitory effects, indicating that additional platelet receptors may interact with adhesive plasma proteins immobilized on collagen under these experimental

conditions. Flow cytometric studies with control, GPVI-depleted, and FcRγ-chain-deficient platelets demonstrated that collagen activates integrins β_1 and $\alpha_{IIb}\beta_3$ in a GPVI dependent manner (not shown, see ref[61]).

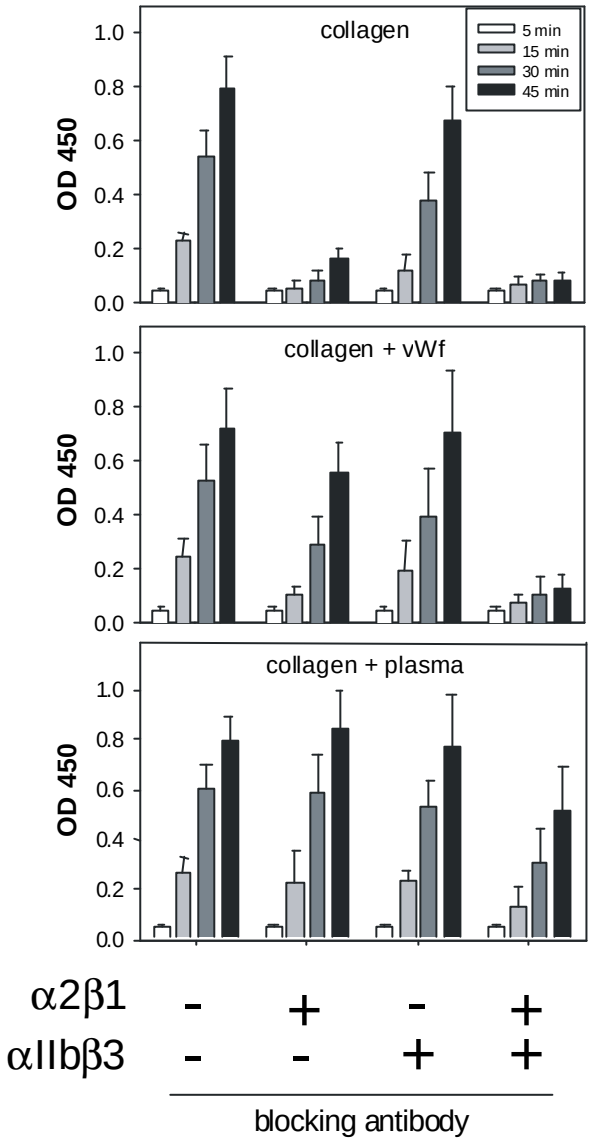


Figure 30. GPVI-mediated integrin activation is a prerequisite for platelet adhesion to collagen
Washed FcRγ-chain-deficient platelets were allowed to adhere to fibrillar collagen immobilized in microtiter plates in the presence of Mn^{2+} with or without blocking antibodies against $\alpha_2\beta_1$ or $\alpha_{IIb}\beta_3$. Where indicated, the collagen substrate had been pre-exposed to vWF (20 µg/ml) or plasma for 30 min. Adherent platelets were quantitated fluorimetrically. The data shown are from a single experiment, representative of three identical experiments, and are expressed as the mean of triplicate readings ± S.D. for the indicated times.

Static adhesion assays were also performed on immobilized soluble collagen. This process has been reported to be entirely $\alpha_2\beta_1$-dependent[98]. Normal platelets adhered to soluble collagen in the presence, but not in the absence of Mg^{2+}/Ca^{2+} cations suggesting that the attachment is mediated by $\alpha_2\beta_1$ integrin (Fig. 31). This was confirmed by the abolished adhesion of β_1-null platelets. However, normal platelets pre-treated with JAQ1 as well as GPVI-depleted and FcRγ-chain-deficient platelets also failed to adhere to soluble collagen (Fig. 31) demonstrating that this interaction depends on both $\alpha_2\beta_1$ integrin and GPVI.

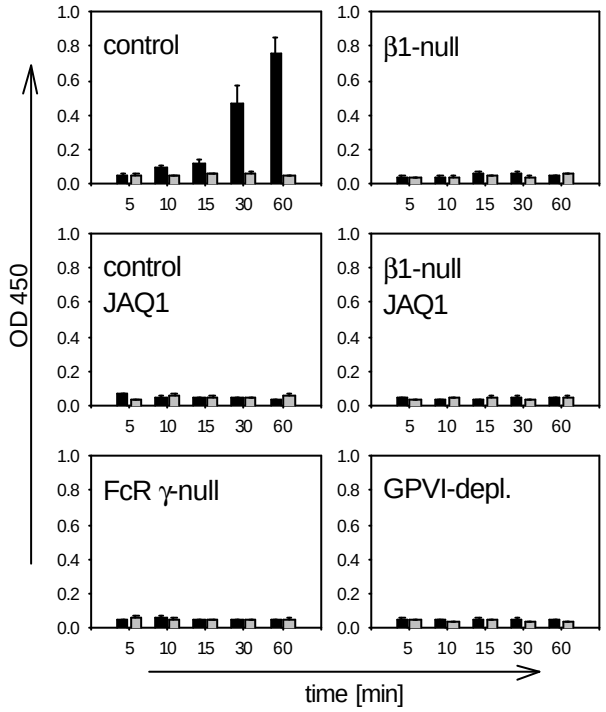


Figure 31. Platelet adhesion to soluble collagen under static conditions
Washed platelets from the indicated mice were allowed to adhere under static conditions to soluble collagen immobilized in microtiter plates. The experiments were performed as described for Fig. 29.

C.6.4 Adhesion to fibrillar collagen under low and high shear conditions is GPVI-, but not a_2b_1-dependent

To further define the role of the two collagen receptors in adhesion under more physiological conditions, whole blood perfusion studies were performed under conditions of low and high shear stress (150 and 1000 s^{-1}, respectively). At low shear, wild type and β_1-null platelets formed large aggregates on fibrillar collagen within 5 min, indicating that integrin $\alpha_2\beta_1$ is not essential for adhesion under these conditions. Blocking the CRP binding site on GPVI with 20 µg/ml JAQ1-Fab drastically reduced the adhesion of normal platelets and completely blocked the formation of aggregates (Fig. 32). Also, β_1-null platelets did not adhere in the presence of JAQ1 (not shown). These data demonstrate that adhesion and subsequent thrombus formation is predominantly dependent on the CRP binding epitope on GPVI, and to

very minor part facilitated by $\alpha_2\beta_1$. Also, this minor $\alpha_2\beta_1$-dependent adhesion required GPVI, since adhesion of GPVI-depleted and FcRγ-chain-deficient platelets was virtually abolished (Fig. 32).

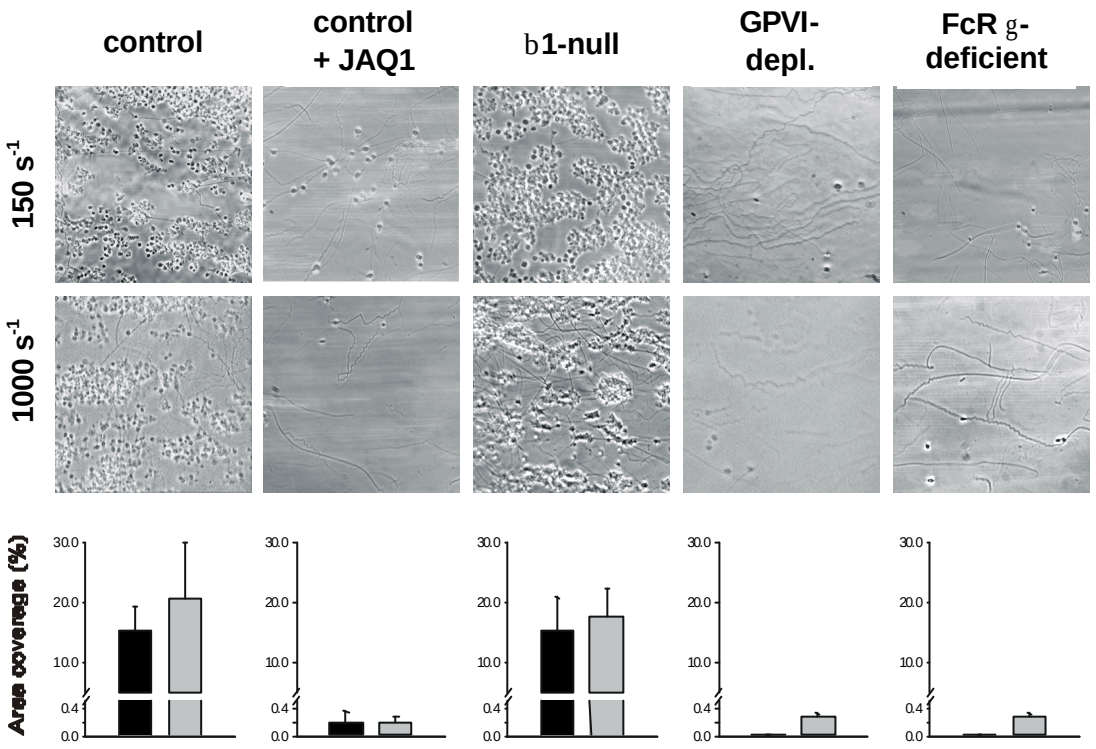


Figure 32. GPVI, but not a2b1, is essential for platelet adhesion to collagen under flow
Whole blood from the indicated mice was loaded with calcein and perfused at wall shear rates of 150 s^{-1} (10 min) or 1000 s^{-1} (4 min) over a collagen-coated surface. Blood from control mice was assessed with (20 µg/ml) or without JAQ1-Fab. *Upper panels*: representative phase-contrast microscopic images after perfusion (original magnification: X630). *Lower panels*: surface coverage of calcein fluorescence after perfusion at low (*black bars*) and high (*gray bars*) shear (mean ± S.E.M., *n* = 3-6). Similar results were obtained when analyzing surface area coverage of platelets from phase-contrast images.

At high shear stress (1000 s^{-1}), platelet adhesion greatly depends on interactions between GPIbα and vWf[14]. Under these conditions, adhesion and thrombus formation occurred significantly faster than under low shear conditions (< 2 min) and no difference was found between normal and β_1-null platelets at the end of the perfusion period (Fig. 32). However, aggregates formed by β_1-null platelets were less stable and detached more frequently from the immobilized surface during flow. In contrast, preincubation of platelets with JAQ1-Fab almost completely abrogated adhesion and formation of aggregates. Comparable results were found with GPVI-depleted and FcRγ-chain-deficient platelets confirming the critical role of GPVI in the adhesion process at high shear.

Adhesion under low and high shear flow conditions was also tested on soluble collagen. Similar to the results obtained in the static assay, normal platelets adhered under both

conditions in the absence but not in the presence of JAQ1Fab, whereas no adhesion occurred with β_1-null, FcRγ-chain-deficient, or GPVI depleted platelets, confirming the critical role of both $\alpha_2\beta_1$ and GPVI for adhesion to soluble (monomeric) collagen and aggregate formation under flow conditions (not shown).

C.6.5 a_2b_1 facilitates procoagulant response induced by GPVI

The role of GPVI and integrin $\alpha_2\beta_1$ for collagen-dependent procoagulant activity of platelets was studied under high shear flow conditions. Therefore, whole blood from wild type and from mice lacking either collagen receptor on their platelets was perfused over a collagen coated surface and exposure of phosphatidylserine (PS) was detected by binding of fluorescent annexin-V. As expected, only few GPVI-deficient platelets attached to collagen and these showed no signs of procoagulant activity (Fig. 33b,e). Strikingly, platelets lacking β_1 integrins were less annexin-V positive than wild type platelets (Fig. 33d vs. f), although both exert virtually the same surface area coverage of adherent platelets (Fig. 33a vs. c). These findings demonstrated, that $\alpha_2\beta_1$ integrin potentiates the GPVI-dependent PS-exposure, i.e., procoagulant activity.

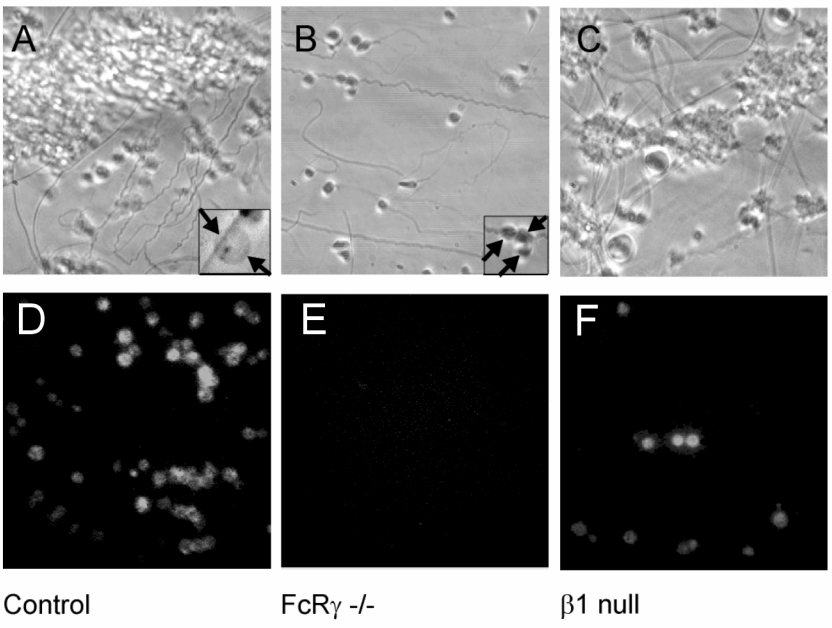


Figure 33. Platelets lacking GPVI or integrin a_2b_1 differ in collagen-mediated aggregate formation and procoagulant activity under flow.

A*-*C, phase contrast microscope images after perfusion of whole blood from the indicated mice at wall shear rate of 1000 s^{-1} over a collagen-coated surface (4 min). ***D*-*F***, fluorescence images after staining with OG^{488}-labeled annexin-V, detecting PS-exposing platelets (original magnification: X800). Inserts are magnifications of platelets at a collagen fiber. Images shown are representative of 4-9 independent experiments.

D Discussion

D.1 GPVI expression depends on its association with the FcRg-chain

The first mAb against mouse GPVI (JAQ1) was characterized and its *in vitro* effects on platelet activation were described. Using JAQ1 in combination with FcRγ-chain deficient mice it was demonstrated that the correct expression and function of mouse GPVI is strictly dependent on the presence of the FcRγ-chain. This observation, combined with reports that human GPVI is also physically and functionally associated with the FcRγ-chain[38;101], indicates that the mechanisms leading to collagen-induced platelet activation are similar in mice and humans.

So far, JAQ1 was the first mAb directed against mouse GPVI and therefore the only divalent anti-GPVI reagent described. This may explain why JAQ1, in contrast to polyclonal antibodies against human GPVI, did not induce platelet activation / aggregation by itself but only upon cross-linking (Fig. 3). Thus, like in the human system[36;90], receptor clustering seems to be required for signaling through mouse GPVI. The finding that blockage of GPVI with JAQ1 specifically inhibited collagen-induced platelet activation, strongly suggested that GPVI is the principal collagen receptor for platelet activation in mice.

D.2 Collagen contains two distinct epitopes for activation of murine platelets

Further studies revealed that the blocking effect of JAQ1 can be overcome by increasing the concentrations of collagen (Fig. 5), whereas aggregation was completely blocked in response to the selective agonist CRP (up to 100 µg/ml, Fig. 6b), indicating that JAQ1 recognizes the “CRP binding site” on GPVI or at least sterically occupies it by binding in close vicinity. This strongly suggests that there is a second activating sequence within collagen which is distinct from the GPO motif represented by CRP. It appears that the binding site for this novel epitope within collagen is located on either GPVI or on a novel receptor which also signals through the FcRγ-chain. The latter hypothesis, however, seems unlikely since both, GPVI-depleted and FcRγ-chain-deficient platelets, are unresponsive to collagen, strongly relating the dependency for collagen activation rather to GPVI than (only) to the FcRγ-chain.

Consistent with the aggregation response, tyrosine phosphorylation was also restored with higher concentrations of collagen in the presence of JAQ1. However, under these conditions the phosphorylation of LAT was very limited in contrast to a substantial signal for SLP-76 and

PLCγ2. This raises the possibility that phosphorylation of LAT is mainly induced *via* the GPVI-FcRγ complex whereas signal transduction leading to phosphorylation of SLP-76 and PLCγ2 also occurs upon stimulation of another receptor(s). In this context, it is important to note that tyrosine phosphorylation of PLCγ2 and platelet activation in response to CRP are heavily reduced but not abolished in LAT-deficient platelets[67]. Further studies will be required to clarify whether receptors other than GPVI transduce activation signals in platelets in response to collagen.

D.3 Effects of reduced expression levels of GPVI-FcRg complex on platelet activation by GPVI ligands

The knowledge about an additional activating epitope within collagen may help to understand how the platelets get activated by collagen fibers. However, the underlying mechanisms are still poorly defined. To address the question whether the expression level of GPVI affects collagen-induced platelet activation, mice expressing reduced amounts of the GPVI-FcRγ complex were used for studies on aggregation and tyrosine phosphorylation.

It was demonstrated that the responsiveness of platelets to collagen, convulxin, and cross-linked JAQ1 decreased in parallel with the expression level of GPVI-FcRγ complex by 2- and 5-fold, respectively (Figs. 10a and 13a,b). For activation by collagen, the presence of blocking antibodies against $\alpha_2\beta_1$ and GPV did not affect this proportional sensitivity to GPVI levels (Fig. 12a). In contrast to the above mentioned agonists, the platelet response to CRP was shifted 20-fold to the right in platelets expressing 50% of the GPVI-FcRγ complexes and abolished in platelets expressing only 20% of the complexes (Fig. 13c).

These results indicate that the platelet responses to GPVI ligands are determined by a combination of receptor affinity and avidity. In terms of CRP this means that the “apparent affinity” of this multimeric ligand for the platelet surface is determined through its multiple interaction sites (i.e. GPO motifs) with GPVI. Upon reduction of GPVI levels, the weak affinity of CRP for the single receptor molecule then accounts for the strongly impaired or absent reaction. In contrast, the high affinity of the GPVI specific ligands convulxin and JAQ1 enables activatory signals mediated even when GPVI amounts are reduced to 20%. On the other hand, platelet responses to collagen are likely to be maintained despite low levels of GPVI because of the ability of collagen to bind to additional receptors. However, since blocking of integrin $\alpha_2\beta_1$ did not abolish the activation, the presence of further receptors for platelet-collagen interaction is suggested.

The present findings may also clarify, why platelets from “GPVI deficient” patients are unresponsive to CRP but are partially activated by collagen. This observation by Kehrel and

co-workers was originally explained by the hypothesis that in the absence of GPVI collagen activates platelets (mainly) *via* $\alpha_2\beta_1$[35;102]. Considering the present results, a residual level of GPVI expression on the patient's platelets may have been sufficient to support collagen- but not CRP-induced responses.

Finally, it should be emphasized that while a reduction in the level of GPVI produces only a small shift in the dose-response curve to collagen, it also induces a significant delay in platelet activation. This is likely to have considerable implications *in vivo*, thereby explaining the mildly increased bleeding tendencies experienced by individuals with a low level of GPVI[16;34].

D.4 Glycoprotein VI but not a_2b_1 integrin is essential for platelet interaction with collagen

To study the role of $\alpha_2\beta_1$ for platelet-collagen interactions, mice lacking β_1 integrins on their platelets were used. β_1-null platelets respond normally to ADP, thrombin, and CRP[61]. Strikingly, these platelets can be efficiently activated with collagen *in vitro*, although the onset of activation is delayed (Fig. 27a,b). Together, these results demonstrate that the signaling machinery leading to platelet activation is not altered in β_1-null platelets, that GPVI-mediated platelet activation is not affected by the absence of β_1 integrins, and that $\alpha_2\beta_1$ integrin facilitates fibrillar collagen-induced platelet activation, but is not essential.

Additionally, β_1-null platelets adhere to fibrillar collagen under static (Fig. 29) as well as low and high shear conditions (Fig. 32), although exerting a reduced procoagulant activity (Fig. 33). These findings are in sharp contrast to observations made in patients with low or no expression of $\alpha_2\beta_1$ on platelets. They suffered from severe bleedings and their platelets failed to respond to collagen *in vitro*[18;95]. Possible explanations for the discrepancy could be species-specific differences or, more likely, that the few patients reported so far had additional defects on their platelets. Mice with β_1-deficient platelets clearly demonstrate that $\alpha_2\beta_1$ integrin is dispensable for hemostasis under normal conditions *in vivo*. It is possible, however, that $\alpha_2\beta_1$-mediated adhesion of platelets to collagen is crucial if additional components such as vWf or fibronectin are absent.

It was found, however, that $\alpha_2\beta_1$ integrin was essential for adhesion to soluble collagen under static and flow conditions (Fig. 31 and not shown). These results confirm the role of $\alpha_2\beta_1$ as a collagen receptor on platelets and may at least partly explain the conflicting data on the determinants of the platelet-collagen interaction reported in the literature (reviewed in[103]). Numerous studies of platelet adhesion under both static and flow conditions were performed

with type I collagen solubilized by pepsin. Such soluble collagen has served as a powerful tool to characterize the recognition sites for individual collagen receptors within the molecule. Although collagens are degraded under certain pathological circumstances, they are still predominantly present in fibrillar form in normal vessels. Thus, the presented data suggested that platelet interactions with fibrillar collagen *in vivo* must be different and require more detailed investigation.

Since the described results strongly suggested that $\alpha_2\beta_1$ plays a supportive rather than an essential role in platelet interactions with native, fibrillar collagen, GPVI which binds collagens as a low affinity and signal transducing receptor, was analyzed. Interference with GPVI function revealed an essential role of this receptor in adhesion of normal as well as β_1-null platelets to collagen. A function blocking antibody to GPVI (JAQ1) or the absence of the receptor on platelets efficiently inhibited platelet adhesion and aggregation to collagen under flow conditions (Fig. 32). Under static conditions, GPVI was also essential for adhesion to fibrillar (Fig. 29) as well as soluble collagen (Fig. 31) suggesting that GPVI-collagen interactions are a prerequisite for integrin-mediated adhesion.

Despite blocking the major collagen binding site on GPVI, the mAb JAQ1 is unable to inhibit activation of platelets by high concentrations of fibrillar collagen (Fig. 5b) strongly suggesting the presence of a second, GPVI-independent collagen receptor. It was speculated that $\alpha_2\beta_1$ could be this receptor since it is present on resting platelets in a low affinity binding state requiring high levels of collagens for interaction. Indeed, β_1-null platelets fail to respond to high concentrations of fibrillar collagen in the presence of JAQ1 (Fig. 28a). Interestingly, however, while this response is independent of the CRP binding site blocked by JAQ1, it is still dependent on the presence of GPVI as high collagen concentrations are unable to aggregate GPVI-depleted as well as FcRγ-chain-deficient platelets (Fig. 28a), despite normal expression of $\alpha_2\beta_1$. A possible explanation for this finding could be that, in addition to the CRP binding site, GPVI has a second, low affinity binding site for fibrillar collagen mediating activation of $\alpha_2\beta_1$ integrin which then contributes to signaling. Alternatively, binding of the multivalent fibrillar collagen to $\alpha_2\beta_1$ could coaggregate $\alpha_2\beta_1$ and GPVI within lipid rafts. It has been shown recently that receptor aggregates in lipid rafts can signal in a ligand-independent manner[104;105]. In contrast, soluble monomeric collagen may not be able to mediate clustering of its receptors in lipid rafts due to the lack of highly repetitive recognition sites within the molecule.

Resting platelets express integrins in a low affinity binding state to avoid interactions with fibrinogen or plasma fibronectin[106;107]. When platelets become activated, their integrins shift to a high affinity state and bind their ligands. In the present study, multiple lines of evidence are provided that stimulation of GPVI can shift $\alpha_2\beta_1$ and $\alpha_{IIb}\beta_3$ integrins from a low to high affinity state, thereby confirming and extending previous observations[108;109]. First, GPVI- and

FcRγ-chain-deficient platelets failed to adhere to collagen (Fig. 29 and 31) despite normal expression of $\alpha_2\beta_1$ and $\alpha_{IIb}\beta_3$ which are in a low affinity state as later demonstrated by FACS analysis[61]. Second, $\alpha_2\beta_1$ / $\alpha_{IIb}\beta_3$ integrin-mediated binding of these platelets to collagen could be induced by Mn^{2+} (Fig. 30), which is known to activate β_1 and β_3 integrins directly[100]. These findings, together with the observation that collagen induced the activation of both integrins in a GPVI-dependent manner[61] demonstrate that GPVI triggers integrin-mediated platelet-collagen interactions.

D.5 A new model of platelet-collagen interactions

Altogether, these findings were combined in a revised model of platelet-collagen interactions shown in Fig. 34 and discussed below.

A previous model incorporated the idea that $\alpha_2\beta_1$ integrin is essential for shear-resistant thrombus growth by mediating firm platelet adhesion to collagen. This first binding event would then allow a second step involving low affinity interactions of GPVI with collagen leading to platelet activation and thrombus formation[51-53]. However, the analysis of β_1-null platelets clearly demonstrates that they can adhere to collagen under low as well as high shear stress (Fig. 32). The number and size of platelet aggregates did not differ significantly between normal and β_1-null platelets, although further studies revealed that they display a looser structure and detach more frequently during the shear flow[110]. In contrast, virtually no adhesion was observed in the absence of GPVI. These findings establish a new sequence of events in the initial phase of hemostasis and thrombosis (Fig. 34). First, platelets tether at sites of injury by GPIbα-vWf interactions which are essential under conditions of high shear[14]. In a second step, the platelets use GPVI to bind to collagen and thereby become activated and upregulate the activity of integrins, including $\alpha_2\beta_1$ and $\alpha_{IIb}\beta_3$. High affinity binding of $\alpha_2\beta_1$ to collagen then strengthens firm adhesion and reinforces GPVI-mediated adhesion without, however, being essential for these processes. This revised model of platelet-collagen interactions clearly establishes GPVI as the central collagen receptor on platelets

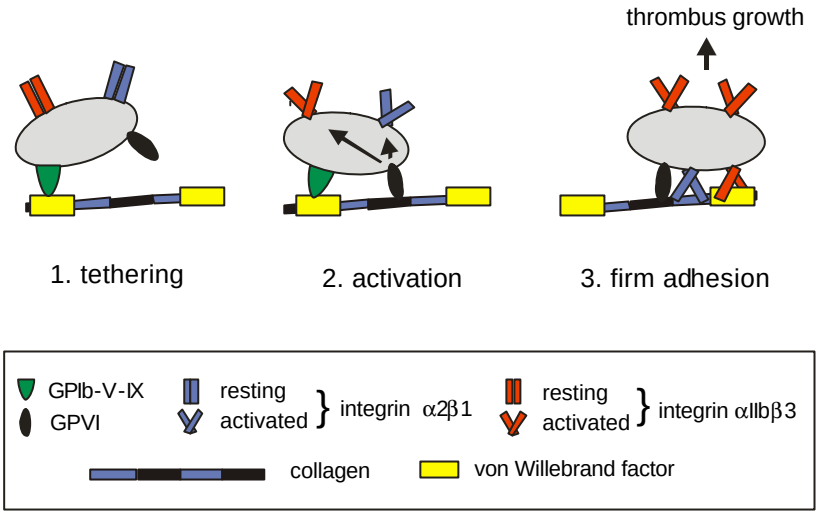


Figure 34. Model for platelet adhesion to collagen

1. Initial tethering to the reactive surface, mediated predominantly by GPIbα-vWF interactions, is important at high shear rates (> 500 s^{-1}), but may be dispensable at lower shear rates[14]. **2**. GPVI-collagen interactions lead to cellular activation followed by shifting of integrins to high affinity state. This step is proposed to be a strict pre-requisite for adhesion. **3**. Firm adhesion of platelets to collagen through activated $\alpha_2\beta_1$ (directly) and $\alpha_{IIb}\beta_3$ (indirectly *via* vWF or other ligands). Integrin $\alpha_2\beta_1$-collagen interactions are not essential for this process. This scheme only includes interactions examined in the current study (with the exception of GPIbα-vWF) and does not exclude the involvement of additional receptor-ligand interactions.

D.6 Long-term antithrombotic protection by GPVI depletion in mice

D.6.1 JAQ1 treated mice are protected from collagen-induced thromboembolism

The potential role of the receptor as a target for antithrombotic strategies was tested in an *in vivo* mouse model. The results of these studies confirmed the proposed critical role of GPVI in collagen-induced activation of platelets *in vivo.* It was demonstrated that treatment of mice with the GPVI-specific mAb, JAQ1, results in profound long-term antithrombotic protection against collagen-dependent thromboembolism. This indicates that anti-GPVI agents might be effective in preventing arterial thrombosis induced by atherosclerotic plaque rupture, where platelets are thought to become activated mainly by the subendothelium under conditions of high shear stress[8;111;112]. Among the matrix proteins which support platelet adhesion and subsequent activation, collagen has a critical role, at least in normal hemostasis as patients with defects in collagen receptors display mild bleeding disorders[18;34;113]. Although the role of GPIb, $\alpha_{IIb}\beta_3$ and their respective ligands vWf and fibrinogen in thrombosis are well documented[114], the finding that vWf and fibrinogen double knockout mice are still able to

form occlusive thrombi suggests that collagen and its platelet receptors might also have a critical role in thrombosis[115].

The profound inhibitory effect of JAQ1 *in vivo* was unexpected since it was based on clearing of GPVI from circulating platelets and no such specific depletion of a platelet receptor has been described to date. The complete loss of functional GPVI on circulating platelets in JAQ1-treated mice was confirmed by different approaches. First, the protein was not detectable in a Western blot analysis of platelet lysates for at least two weeks (Fig. 17a) which exceeds the normal life-span of platelets[116]. Second, the GPVI-specific snake toxin convulxin, which activates platelets in the presence of JAQ1 suggesting that it binds to a different epitope than the antibody, did not bind to platelets from JAQ1-treated mice indicating the absence of GPVI from the platelet membrane. Additionally, 2D-gel electrophoresis (performed in collaboration with C. Gachet, INSERM, France) demonstrated that a ~60 kD protein with an isoelectric point of ~5.6 (which is similar to that described for human GPVI[33]) is absent in the lysate of platelets from JAQ1-treated mice[117] and the same protein is absent in platelets from FcRγ-chain-deficient mice which are shown to lack GPVI (C.1.2). Most importantly, the functional platelet responses to collagen were completely abolished by JAQ1 *in vivo* (Fig. 16b), whereas the antibody has only limited inhibitory effects *in vitro* (Fig. 5a,b). These results demonstrate that JAQ1 induced the clearing of GPVI from the surface of circulating platelets *in vivo*. This finding is also supported by the observation that GPVI as well as biotinylated JAQ1 was detectable in the lysates, but not on the surface, of platelets 6 h after injection (Fig. 18a-c). Furthermore, the decreasing signals for both GPVI and JAQ1 after 24 and 48 h, respectively, (Fig. 18c) strongly suggest that the internalized complex was degraded in the intracellular compartments.

GPVI is closely related to immunoreceptors, some of which may become internalized when stimulated appropriately[118;119]. In the case of JAQ1-GPVI it was difficult to define what the appropriate stimulus is, but it seems clear that the observed phenomenon is not dependent on the antibodies Fc part or dimerization / clustering of the receptor as Fab fragments produced the same effect (Fig. 18d). *In vitro*, JAQ1 did not induce the down-regulation of GPVI from the platelet membrane (Fig. 18a-c) suggesting that a second signal may be required for the induction of this process that is provided only *in vivo*, possibly by other cells. This assumption may be supported by the observation that JAQ1 and Fab fragments of the mAb induced transient thrombocytopenia. The reason for this is not clear, but it might be due to weak activation of $\alpha_{IIb}\beta_3$ leading to formation of loose aggregates and their temporary sequestration to the spleen where the actual loss of GPVI may occur. Currently, very little is known about the cellular regulation of GPVI but the results with JAQ1 indicate that occupancy of the receptor induces a signal that finally results in its down-regulation. Studies with two additional anti-GPVI mAbs, JAQ2 and JAQ3, demonstrate that the antibody-induced

depletion of GPVI occurs independently of the exact binding epitope and confirmed that the divalent form of the antibody is not required to induce the receptor down-regulation.

Irrespective of the underlying mechanism, platelets from JAQ1-treated mice were completely unresponsive towards activation with high concentrations of CRP or collagen whereas they respond normally to ADP or PMA. This strongly suggests that JAQ1 selectively induced a transient GPVI deficiency in mice while other membrane glycoproteins, including GPIb-IX-V, CD9, and integrins $\alpha_2\beta_1$ and $\alpha_{IIb}\beta_3$ were not affected in their surface expression and/or function. JAQ1-treated mice display prolonged bleeding times (Fig. 24) which confirms the important role of GPVI in normal hemostasis and correlates well with the bleeding diathesis in GPVI deficient patients[16;34]. Very interestingly, one GPVI deficient patient developed highly specific antibodies against the absent receptor[90] which may be difficult to explain. Based on the results presented here, however, one can speculate that this patient may suffer from an acquired GPVI deficiency, based on autoantibody-induced clearing of GPVI from her circulating platelets.

$\alpha_{IIb}\beta_3$ antagonists[120] are currently considered the most powerful inhibitors of platelet participation in thrombosis[57], as they inhibit the final common pathway of platelet aggregation, irrespective of the agonist that stimulates the cells. It has been suggested that this more or less complete inhibition of platelet function may come with a potential safety risk as platelet aggregation is also required for normal hemostasis[56]. Importantly, JAQ1 induced significantly shorter bleeding times than blocking antibodies against $\alpha_{IIb}\beta_3$ in mice, indicating that GPVI-depleted platelets still contributed significantly to normal hemostasis *in vivo*. Although there is no clear correlation between the bleeding time and bleeding risk[121], it is tempting to speculate on the grounds of these results that anti-GPVI therapy might be associated with a relatively low risk of clinical hemorrhage.

Recent evidence suggests that GPVI is exclusively expressed in platelets and mature megakaryocytes[37;122] and this was confirmed by immunohistochemical studies with JAQ1 (not shown). Therefore, the effects of anti-GPVI agents should be restricted to platelets and, very importantly, megakaryocytes. JAQ1 was detectable on megakaryocytes in spleen and bone marrow 3 h after antibody injection (not shown), suggesting that the next generation of platelets was also affected by the mAb. This assumption may be confirmed by the fact that GPVI was not detectable in platelets for at least two weeks, although the normal life-span of mouse platelets is only approximately 4-5 days[116]. Bearing in mind that the injected IgGs circulate in the blood system for about one week before they get cleared, indicates that GPVI molecules on the surface of megakaryocytes are targeted by JAQ1 and receptor down-regulation is induced resulting in formation of GPVI-negative platelets.

Preliminary results show that a second injection of JAQ1 two weeks after the first injection has no influence on platelet counts, but prolongs the absence of GPVI on circulating platelets

(not shown). This indicates that the second dose of the mAb affects newly differentiated megakaryocytes, but has no effect on circulating (GPVI-depleted) platelets. Thus, JAQ1 can be used to induce a GPVI knock out-like phenotype in mice for several weeks, allowing studies on platelet function in the absence of this critical activating receptor *in vitro* and *in vivo*.

Taken together, the results indicate that GPVI might become an interesting target for long-term prophylaxis of ischemic cardiovascular diseases and provide the first evidence that it is possible to specifically deplete an activating (glycoprotein) receptor from circulating platelets *in vivo*.

D.7 Concluding remarks

The work summarized here provides information on the murine collagen receptor GPVI obtained by *in vitro* and *in vivo* studies using the first monoclonal antibodies against GPVI (JAQ-antibodies). The major results are:

- The murine GPVI shows major homologies to its human counterpart with regard to receptor expression and signal transduction.
- Absence of the collagen receptor integrin $\alpha_2\beta_1$ does not abrogate platelet adhesion/ thrombus formation indicating rather a supporting than an essential role for this integrin in platelet-collagen interactions.
- A revised model for the initial steps of platelet-collagen interactions was established proposing that GPVI-mediated integrin activation is essential for platelet attachment to a collagenous surface.
- Irreversible antibody-induced depletion of GPVI from circulating platelets occurs independently of the targeted epitope on the receptor and results in long-term protection from collagen-dependent intravascular thrombosis with only moderate effects on bleeding time.

D.7.1 Recent progress

7.1.1 Analysis of α_2-null mice

Meanwhile, the group of B. Eckes (University of Cologne, Germany) generated mice deficient in α_2 integrin which lack the collagen receptor $\alpha_2\beta_1$ but no other β_1 integrins. Studies in aggregometry in combination with adhesion assays under static and flow conditions revealed normal activation of α_2-deficient platelets induced by fibrillar collagen whereas responses to soluble collagen were abolished. Additionally, these mice were shown to have normal bleeding times. In summary, this indicates that the integrin $\alpha_2\beta_1$ is dispensable for platelet adhesion to immobilized fibrillar collagen type I under stasis and flow[62] confirming the previous findings with β_1-null mice[61]. Recent *in vivo* experiments with α_2-deficient mice in a model of arterial thrombosis showed that thrombus formation in the injured carotid artery is not affected by the absence of $\alpha_2\beta_1$. When integrin $\alpha_{IIb}\beta_3$ was blocked, α_2-deficient platelets still attach to the ECM, although to a lesser extent than without blockage, indicating that additional receptors on platelets are able to maintain shear-resistant platelet adhesion under physiological conditions. The observation that adhesion of β_1-null platelets is virtually abolished in the presence of $\alpha_{IIb}\beta_3$ blocking antibody suggested, that β_1 integrins other than $\alpha_2\beta_1$ are involved in platelet adhesion to the ECM *in vivo* and that they are sufficient for this process in the absence of functional $\alpha_2\beta_1$ and $\alpha_{IIb}\beta_3$. Adhesion assays provided evidence *in vitro*, that the platelet receptors for fibronectin and laminin, integrins $\alpha_5\beta_1$ and $\alpha_6\beta_1$, respectively, can mediate stable platelet attachment to their respective ligands in a GPVI-dependent manner (Grüner et al, submitted). Although the exact contribution of each single receptor was not clarified, it appears likely, that $\alpha_5\beta_1$ and $\alpha_6\beta_1$ are involved in platelet attachment to the ECM as both, fibronectin and laminin, are highly expressed in the vessel wall and become accessible to the flowing blood at sites of injury[123;124].

7.1.2 A crucial role for GPVI in platelet recruitment to the injured arterial wall in vivo

Concerning the role of GPVI in arterial thrombosis, recent studies in collaboration with the group of M. Gawaz (Munich, Germany) have shown that this collagen receptor plays a major role in platelet recruitment to the injured arterial wall *in vivo*[125]. It was demonstrated by intravital fluorescence microscopy of the mouse carotid artery that inhibition or absence of GPVI abolishes platelet-vessel wall interactions after endothelial denudation. Additionally, platelet tethering to the subendothelial matrix was markedly reduced, and stable arrest as well as aggregate formation at sites of injury were virtually abolished under these conditions. These results were confirmed using GPVI-depleted mice in different models of arterial injury,

altogether revealing an unexpected role of GPVI in the initiation of platelet attachment at sites of vascular injury *in vivo*. These platelet-collagen interactions *via* GPVI were thereby identified as the major determinants of arterial thrombus formation, again reflecting the importance of GPVI as a target for the development of antithrombotics.

D.7.2 Ongoing projects

The observed antibody-induced depletion of GPVI from circulating platelets is a unique process which needs further investigations to reveal the underlying mechanisms. The required signals are still unknown as well as detailed knowledge about the process of receptor down-regulation. Therefore, *in vivo* studies using gold-labeled (5 nm) JAQ-antibodies may provide information about the intracellular "degrading pathway" of the internalized receptor including definitive evidence that internalization but not shedding is responsible for GPVI depletion *in vivo*. Furthermore, studies of signal transduction may reveal the communication / cross-talk between the collagen receptors involved in the activation of integrins after collagen binding. As GPVI and $\alpha_2\beta_1$ belong to two important receptor families in cellular activity, immunoglobulin receptors and integrins, respectively, a similar cross-talk may be instrumental in the functioning of other cellular types.
Finally, the generation of mice deficient in more than one (collagen) receptor will contribute to a better understanding of the processes involved in platelet-collagen interaction.

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Appendix

App.1. Surface expression of platelet receptors in wild-type and genetically modified mice

	wild type	FcRγ (+/-)	FcRγ (-/-)	GPVI-depleted			β1-null
				day 3	day 7	day 14	
GPVI	43.5 ± 10.7	28.3 ± 10.8	6.6 ± 2.3	6.7 ± 1.1	7.8 ± 2.3	7.4 ± 1.3	45.2 ± 12.9
GPIIa (b_1)	128.3 ± 13.2	130.2 ± 14.1	130.3 ± 12.2	133.5 ± 12.7	129.4 ± 13.2	124.5 ± 11.6	7.3 ± 2.1
GPIa (a_2)	39.1 ± 9.5	43.8 ± 8.3	41.8 ± 7.3	40.3 ± 6.5	35.2 ± 7.8	36.7 ± 6.2	8.4 ± 3.3
GPIc (a_5)	27.2 ± 5.1	n.d.	n.d.	n.d.	n.d.	n.d.	6.6 ± 1.4
GPIc' (a_6)	87.3 ± 10.2	n.d.	n.d.	n.d.	n.d.	n.d.	5.9 ± 1.7
GPIIb/IIIa	347.8 ± 17.5	358. ± 21.4	355.6 ± 25.8	356.5 ± 22.7	350.5 ± 23.4	348.1 ± 19.6	359.5 ±21.4
GPIIIa (b_3)	169.7 ± 10.2	n.d.	n.d.	175.7 ± 13.5	173.9 ± 15.1	182.3 ± 14.7	176.1 ± 16.2
GPIb-IX	295.8 ± 21.2	279.7 ± 22.8	280.3 ± 30.4	275.4 ± 18.0	269.5 ± 15.9	293.4 ± 22.0	287.7 ± 26.1
GPV	143.4 ± 15.9	150.4 ± 15.3	149.5 ± 15.5	163.3 ± 14.1	169.1 ± 15.3	158.1 ± 10.5	149.5 ± 14.3
CD9	545.9 ± 41.1	541.7 ± 49.6	551.3 ± 46.6	554.3 ± 14.6	549.5 ± 19.6	557.0 ± 13.0	537.7 ± 51.8

Diluted whole blood from the indicated mice was incubated with FITC-labeled Abs at saturating concentrations for 15 min at RT and platelets were analyzed directly. Results are expressed as mean log fluorescence ± S.D. (n=6-10); n.d. = not determined.

Abbreviations

A	ampere
ADP	adenosine diphosphate
AEC	3-amino-9-ethyl-carbazole
AP	alkaline phosphatase
BSA	bovine serum albumine
CRP	collagen-related peptide
cvx	convulxin
DMSO	dimethylsulfoxide
DTE	dithioerythritol
ECL	enhanced chemiluminiscence
ECM	extracellular matrix
EDTA	ethylenediaminetetraacetic acid
ELISA	enzyme linked immunosorbent assay
FcR	Fc receptor
FCS	fetal calf serum
FITC	fluorescein isothiocyanate
FSC/SSC	forward scatter/side scatter
GP	glycoprotein
GPO	glycine-proline-hydroxyproline
HRP	horseradish peroxidase
Ig	immunoglobulin
IP	immunoprecipitation
ITAM	immunoreceptor tyrosine-based activation motif
kD	kilo dalton
LAT	linker for activation of T cells
M	molar (mol/l)
mAb	monoclonal antibody
MES	2-(N-Morpholino)ethane-sulfonic acid
min	minute
NEM	N-ethyl maleimide
NP-40	nonidet P-40
PAF	platelet activating factor
PAGE	polyacrylamide gel electrophoresis
PBS	phosphate buffered saline
PE	R-phycoerythrin
PLCγ2	phospholipase Cγ2
PMA	phorbol 12-myristate 13-acetate
PMSF	phenylmethylsulfonyl fluoride
PPACK	D-Phe-Pro-Arg chloromethyl ketone
prp	platelet-rich plasma
PS	phosphatidylserine
PVDF	polyvinylidene difluoride
RT	room temperature
s	second
SDS	sodium dodecyl sulfate
SMCC	succinimidyl 4-(N-maleimidomethyl) cyclohexane-1-carboxylate
TBS	Tris-buffered saline
TMB	3,3,5,5-tetramethyl benzidine
TxA2	thromboxane A_2
vWf	von Willebrand factor

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Studies in collaboration with other groups are indicated.

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Publications

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Poster

Schulte V, Snell D, Bergmeier W, Zirngibl H, Watson SP, Gessner JE, and Nieswandt B. Expression and function of the mouse collagen receptor glycoprotein VI is strictly dependent on its association with the FcRγ-chain. Joint Annual Meeting of Immunology. Nov 29-Dec 1, 2000, Düsseldorf, Germany.

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