Studies on formation and stabilization of pathological thrombi *in vivo*

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Miroslava Požgajová
aus
Nitra, Slowakei

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Mitglieder der Promotionskommission:

Vorsitzender:	Prof. Dr. M. Müller
1. Gutachter:	Prof. Dr. B. Nieswandt
2. Gutachter:	Prof. Dr. M. Schartl
3. Gutachter (graduate program “Target Proteins“):	Prof. Dr. H. Stopper

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Summary

Platelet activation and adhesion resulting in thrombus growth is essential for normal hemostasis, but can lead to irreversible, life-threatening vessel occlusion. In the current study, the contribution of platelet integrins, activation receptors and the contact system of blood coagulation in such pathological conditions was investigated in mice. Firm platelet adhesion to the exposed ECM was analysed in the first part of the presented work. The injury to the common carotid artery in mice deficient in integrin α2 or β1 subunits revealed that multiple integrin-ligand interactions are required for platelet adhesion to the vessel wall, none of which by itself is essential. This process is regulated through GPVI-induced signaling which leads to conformational changes and activation of integrins of β1 and β3 families. Furthermore, the role of the two major collagen receptors in hemostasis (GPVI and α2β1) was assessed by antibody-induced depletion of the activating collagen receptor, GPVI, form the surface of $\alpha2^{-/-}$ platelets. This treatment resulted in dramatically prolonged bleeding times showing severely compromised hemostasis in these mice. In contrast, single deficiency of either receptor does not lead to a bleeding disorder. Similar results were obtained when the production of TxA_2 was inhibited by aspirin in GPVI depleted wild-type mice, but not in control mice, suggesting a central role for G-protein coupled receptors in platelet activation in the absence of functional GPVI. Platelets express receptors that couple to different G-proteins which mediate responses to soluble mediators. Deletion of the G_z-coupled adrenaline receptor, α2A, results in variable bleeding times indicating a moderate hemostatic defect. *In vivo* thrombosis studies revealed impaired stabilization of formed thrombi with enhanced embolus formation. This for the first time demonstrates that adrenaline contributes significantly to thrombus stability.
Besides activated platelets, plasmatic coagulation is known to be an essential co-player in stable plug formation. While the importance of FXII for contact activation initiated coagulation has been known for many years, the protein is considered not to play any role in hemostasis and thrombosis. *In vivo* thrombosis analysis in three distinct arterial beds revealed a severe defect in the formation and stabilization of platelet-rich occlusive thrombi of FXII deficient mice. Although FXII-deficient mice do not suffer from spontaneous bleeding, they are protected against collagen- and epinephrine-induced thromboembolism. These findings establish FXII as a promising new target for antithrombotic therapies with low or no risk of uncontrolled bleeding.

Zusammenfassung

Plättchenaktivierung, -adhäsion und nachfolgende Thrombusbildung ist ein für die Hämostase essentieller Prozess, der jedoch zu irreversiblem lebensbedrohlichen Gefäßverschluss führen kann. In der vorliegenden Arbeit wurde die Rolle von Thrombozyten-Integrinen, aktivierenden Rezeptoren, sowie dem Kontaktsystem der Koagulation unter pathologischen Bedingungen im Maussystem untersucht. Im ersten Teil wurde die Adhäsion von Blutplättchen an exponierte Extrazellulärmatrix analysiert. Im Arteria carotis-Modell konnte an Mäusen mit α2- und β1-knockout gezeigt werden, dass multiple Integrin-Ligand-Interaktionen für die Adhäsion von Blutplättchen an die Gefäßwand verantwortlich sind; dabei ist keine dieser Interaktionen *per se* essentiell. Der Beitrag der zwei Kollagenrezeptoren auf Blutplättchen, GPVI und α2β1 zur Hämostase wurde in α2-knockout Mäusen, deren GPVI durch Antikörperinjektion depletiert wurde, näher untersucht. Diese Mäuse hatten eine stark verlängerte Blutungszeit. Im Gegensatz dazu war die Hämostase aber nicht beeinträchtigt, wenn nur einer der Kollagenrezeptoren ausgeschaltet war. Vergleichbare Ergebnisse wurden erzielt, wenn die Produktion von TXA_2 in GPVI-depletierten Mäusen durch Aspirin gehemmt wurde. Daraus lässt sich ableiten, dass G-Protein-gekoppelten Rezeptoren bei Abwesenheit von GPVI eine entscheidende Rolle bei der Plättchenaktivierung zukommt. Blutplättchen exprimieren verschiedene G-Protein-gekoppelter Rezeptoren, die Aktivierung durch lösliche Agonisten vermitteln. Im Mausmodell führt die Deletion des G_z-gekoppelten Adrenalinrezeptors α2A zu einer variablen Verlängerung der Blutungszeit. *In vivo* Untersuchungen zeigen eine gestörte Thrombusstabilisierung mit vermehrter Embolusbildung. Diese Ergebnisse zeigen, dass Adrenalin-vermittelte Plättchenaktivierung maßgeblich zur Stabilisierung von Thromben beiträgt.

Neben aktivierten Plättchen spielt die plasmatische Gerinnung eine wesentliche Rolle bei der Ausbildung stabiler Thromben. Während die Bedeutung des Gerinnungsfaktors XII im Rahmen der Kontaktaktivierung seit langem bekannt ist, wurde bislang davon ausgegangen, dass ihm für die Prozesse der Hämostase und Thrombose keine Bedeutung zukommt. *In vivo*-Untersuchungen an FXII-knockout Mäusen in drei verschiedenen arteriellen Stromgebieten zeigen jedoch, dass diese Mäuse keine Verschlussthromben ausbilden können. Obwohl FXII-knockout Mäuse keine Blutungsneigung aufweisen, sind sie auch gegen Kollagen- und Epinephrin-induzierte Thromboembolie-Bildung resistent. Diese Ergebnisse weisen FXII als bedeutendes, viel versprechendes Ziel für die antithrombotische Therapie aus.

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A. Introduction

Under physiological conditions blood circulates in a closed system of vessels without any interruptions in flow. Traumatic action on the vessel walls such as cuts destroys the integrity of the vascular wall and allows blood to escape into the surrounding tissue. In order to keep the loss of blood as low as possible, blood platelets in combination with soluble components of plasma form a hemostatic plug that stops bleeding. Hemostasis requires a close interaction between platelets, vessel wall, and plasma coagulation factors. Under pathological conditions, e.g. as observed upon the rupture of an atherosclerotic plaque, platelet adhesion and aggregation on the extracellular matrix may also lead to vessel occlusion and irreversible tissue damage or infarction of vital organs. Our understanding of thrombotic diseases has been improved by the use of a variety of different mouse models. A wide array of stimuli can activate platelets and/or the coagulation system leading to platelet aggregation and intravascular fibrin clot formation. Injury or damage to the endothelium results in formation of pathological, occlusive thrombi. A number of experimental approaches were performed to induce endothelial disruption in mouse thrombosis models using intravital microscopy systems to study formation and stabilization of thrombi under pathological conditions *in vivo* (Figure A.1). It allows us to answer central questions about the contribution of individual receptors in platelet adhesion to the injured vessel wall and the mechanisms of their activation, the role of the two direct collagen receptors GPVI and $\alpha 2\beta 1$ in hemostasis and thrombosis, contribution of G-protein coupled receptors and the coagulation system in thrombus formation and stabilization.

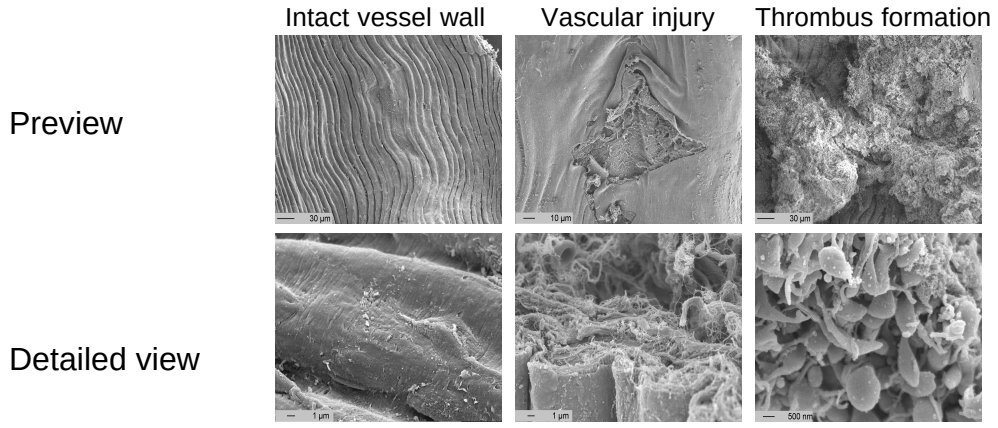


Figure A.1. Scanning electron micrograph of intact and injured blood vessel (Nieswandt et al. 2003)

A.1. Platelets

The mammalian platelet (Figure A.2) is derived from the cytoplasm of megakaryocytes. It is presently not clear how megakaryocyte fragmentation into individual platelets takes place. However, it is likely to be due to shear forces in the circulating blood[1]. In humans, the physiological platelet count in peripheral blood ranges from 200,000 and 400,000 per µL blood. In contrast, in the mouse, the platelet count is about 1 $x10^6$ per µL. Platelets are the smallest corpuscular components of circulating blood. Human platelets have a diameter of 2-4µm and murine platelets of 1-2µm[2]. In contrast to other mammalian cells, platelets do not have a cell nucleus and thus exhibit no or only very limited ability for *de novo* synthesis of proteins. Their cytoplasm consists of mitochondria, glycogen stores, and three different forms of storage granules: dense bodies, α-granules, and lysosomes. Dense bodies contain a series of low-molecular-mass compounds (ADP, ATP, Ca^{2+}, serotonin) that promote aggregation processes. The most abundant granules, α-granules, contain a number of proteins (such as e.g. fibrinogen, P-selectin) that influence different biological functions (adhesion, aggregation, proliferation, inflammation, coagulation). The lysosomal granules contain hydrolytic enzymes. The platelet is surrounded by a typical bilamellar plasma membrane that extends through the multiple channels of the surface connected canalicular system (SCCS), largely increasing the surface area of the platelet. Through the phospholipid bilayer, platelet receptors such as integrins α2β1, and αIIbβ3 or glycoproteins (GPs) GPIb-V-IX, and GPVI are extruded to mediate activatory and inhibitory signals and platelet responses leading to adhesion and aggregation[3].

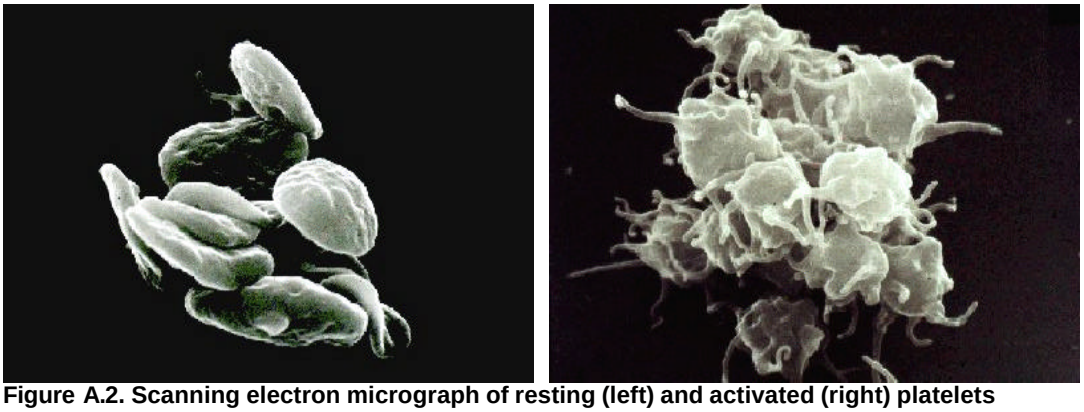


Figure A.2. Scanning electron micrograph of resting (left) and activated (right) platelets

From: www.akh-wien.ac.at/biomed-research/htx/platweb1.htm

A.1.1. Platelets and atherothrombosis

Cardiovascular and cerebrovascular diseases (Figure A.3) continue to be the leading cause of death in the developed world. It is now recognized that blood platelets play a central role in physiologic blood clotting (hemostasis) but also in both the acute and chronic phases of arterial disease. The pathogenesis of atherothrombosis is multifactorial[4], but the main aspect considered of great relevance is the deposition of lipids that are then metabolized abnormally and oxidized in the vascular wall, leading to inflammation and atherosclerotic plaque formation[5]. Under certain conditions, rupture of an advanced atherosclerotic plaque occurs, exposing thrombogenic material to the flowing blood, a process which can lead to acute myocardial infarction or ischemic stroke. In order to prevent thrombotic and/or pro-inflammatory activity of platelets while preserving their hemostatic function, it is essential to characterize receptors and signaling pathways that are responsible for adhesion, activation, aggregation, granule release, and coagulant activity.

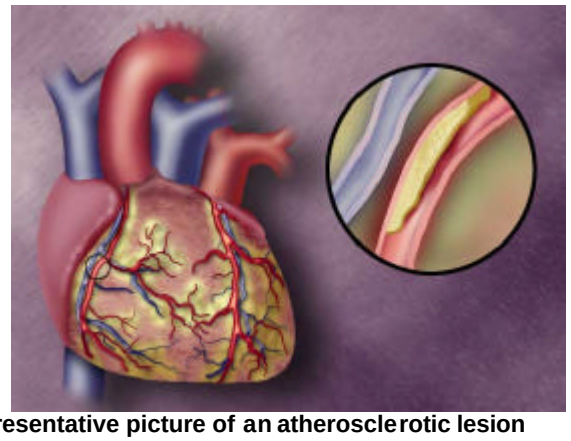


Figure A.3. Representative picture of an atherosclerotic lesion
From: www.medimagery.com/circulatory/atheroscerotic.jpg

A.2. Tethering

Platelets and von Willebrand factor (VWF) both circulate in the blood without any detectable interactions. After vessel injury, the protective endothelial layer is removed and platelets are exposed to numerous adhesive proteins such as collagen, fibronectin and laminin[6]. The first contact between circulating blood platelets and the vessel wall lesions is established by an interaction of the platelet receptor GP Ib-V-IX with collagen-bound VWF. This interaction allows platelets to tether along the matrix at much slower velocities than in the normal flow. Subsequent to this, platelet activation mediated by the interaction of GPVI with collagen on the exposed

subendothelium takes place. This leads to the activation of integrins α2β1 and αIIbβ3, followed by firm platelet adhesion and thrombus growth[7] (Figure A.4).

A.2.1. GPIb-V-IX

GPIb-V-IX is a constitutively active receptor for VWF, expressed at approximately 25,000 copies per platelet. It consists of four subunits: GPIbα, GPIbβ, GPV and GPIX[8]. The membrane GPIb-V-IX complex plays a central role in platelet adhesion and aggregation at sites of vascular injury (Figue A.4). It mediates the initial platelet tethering to the damaged vessel wall under conditions of elevated shear by interacting with collagen-bound VWF. It allows subsequent interactions of other platelet receptors with their ligands[6;9]. In particular, the activated platelet integrin αIIbβ3 then forms stable bonds between tethered platelets through its principal ligand, fibrinogen[6;10]. The GPIbα receptor contains binding sites for P-selectin[11] and Mac-1[12] indicating that the receptor plays an important role in the crosstalk between leukocytes and platelets. Furthermore, GPIbα binds with high affinity to the heparin binding site on α-thrombin[13]. There is considerable evidence that the interaction of thrombin with this site on GPIbα facilitates the response of platelets to thrombin in addition to thrombin responses mediated through members of the PAR family of receptors[14].

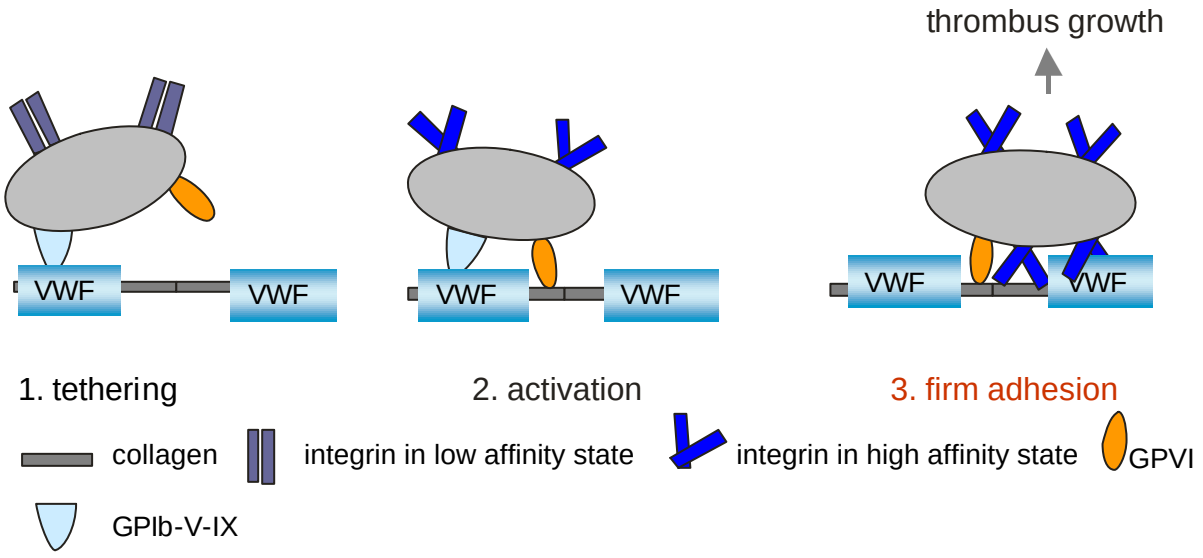


Figure A.4. GPIbα mediated firm adhesion on a collagen surface[15]

A.3. Activation and signal transduction

Many substances can activate platelets. Platelets can form and/or release parts of these agonists themselves, others are formed in the surrounding tissues or in plasma. Each agonist binds its specific receptor(s) on the platelet surface and effects the formation of signals through specific transduction pathways. Collagen represents the most reactive component of the ECM and induces platelet activation through the Ig-like receptor GPVI. Collagen binding to GPVI leads to shape change, integrin activation, release, and procoagulant activity of platelets[16] (Figure A.5).

In vivo, firm adhesion and activation of platelets is facilitated by two collagen receptors, GPVI and integrin $\alpha 2\beta 1$. The interaction of GPVI with collagen generates potent signals to activate not only $\alpha IIb\beta 3$ which binds fibrinogen, fibronectin and VWF, but also $\alpha 2\beta 1$, which contributes to firm adhesion by binding a site on collagen which is distinct from the GPVI binding site[15]. It enhances coagulant activity and release of soluble agonists such as adenosine diphosphate (ADP) and thromboxane A_2 (TxA_2). Downstream of $\alpha 2\beta 1$, phospholipase C (PLC) is activated, the concentration of cytosolic Ca^{2+} increases, and synthesis of cyclic adenosine monophosphate (cAMP) is suppressed.

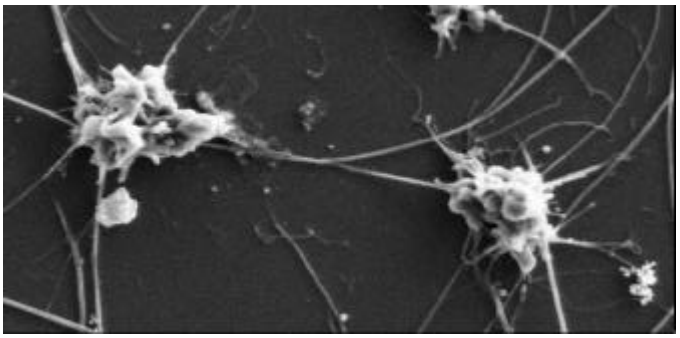


Figure A.5. Platelet adhesion and activation on collagen (Nieswandt et al. 2003, unpublished)

A.3.1. The activating collagen receptor GPVI

GPVI is a member of the immunoglobulin superfamily and serves as a direct collagen receptor. It is a transmembrane glycoprotein (~60 kD) noncovalently associated with the signal transducing Fc receptor (FcR) γ-chain[16]. Crosslinking of GPVI leads to tyrosine phosphorylation of the FcRγ-chain on its immunoreceptor tyrosine-based activation motif (ITAM) by Src kinases Fyn and Lyn. 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 GTP-ases and phosphatidylinositol 3-kinase (PI3-K)[17;18]. Under conditions of elevated shear, platelet adhesion on collagen strictly depends on the interaction of GPIb-V-IX with collagen-bound VWF[6] and that of GPVI with collagen[15]. During this process, ligation of GPVI (and GPIb) leads to platelet activation and the shift of β1 and β3 integrins to a high-affinity state[19]. The importance of these initial processes in arterial thrombus formation has been established through the demonstration that platelet adhesion and thrombus formation on the injured arterial wall are largely inhibited in the absence of functional GPVI or GPIb[20]. Approximately 10% of collagen type I and III consist of repeat glycine, proline and hydroxyproline (GPO) motifs, 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 collagen type III, along with low levels of other extracellular matrix proteins. Collagen-induced platelet activation (Figure A.5) is a complex process that firstly involves platelet tethering at sites of injury by GPIbα-VWF interactions which are essential under conditions of high shear[6]. In a second step, platelets use GPVI to bind to collagen and thereby become activated and upregulate the activity of receptors, including integrins α2β1 and αIIbβ3. High affinity binding of α2β1 to collagen then strengthens firm adhesion and reinforces GPVI-mediated adhesion[15]. Soluble agonists like ADP and TxA_2 that are secreted upon platelet activation are also involved in collagen-induced platelet aggregation ("second wave mediators"). The receptors for these platelet activators couple to several heterotrimeric G proteins.

A.3.2. G-protein coupled receptors

G-protein coupled receptors are comprised of a single polypeptide chain with an extracellular N-terminus and seven transmembrane domains. Binding sites for agonists can involve the N-terminus, the extracellular loops, or a pocket formed by the transmembrane domains. Heterotrimeric G-proteins consist of an α-subunit that binds and hydrolyzes guanine nucleotides, and a tightly, but not covalently associated complex consisting of β- and γ- subunits[21]. In the resting state, the α-

subunit binds GDP. Agonist-induced receptor activation causes the exchange of GDP for GTP. Binding of GTP to the α-subunit induces a conformational change resulting in the dissociation of the α-subunit from the βγ-complex which are now both able to interact with downstream effectors[22;23]. The G-protein definition is based on structural and functional homologies of their α-subunits. They are grouped into four families: $G_{\alpha s}$, $G_{\alpha i}$, $G_{\alpha q}$, and $G_{\alpha 12}$[24]. In platelets, all four family members are present. While $G_{\alpha i}$, $G_{\alpha q}$, $G_{\alpha 12}$ family members are involved in platelet activation, $G_{\alpha s}$ mediates platelet inhibition. Platelet agonists acting through G-protein coupled receptors (Figure A.6, Table A.1) activate phospholipase Cβ via $G_{\alpha q}$, the actin cytoskeleton is reorganized through $G_{12/13}$ and products of G_q-mediated phosphoinositide hydrolysis. The G_i family members are known to suppress cAMP synthesis.

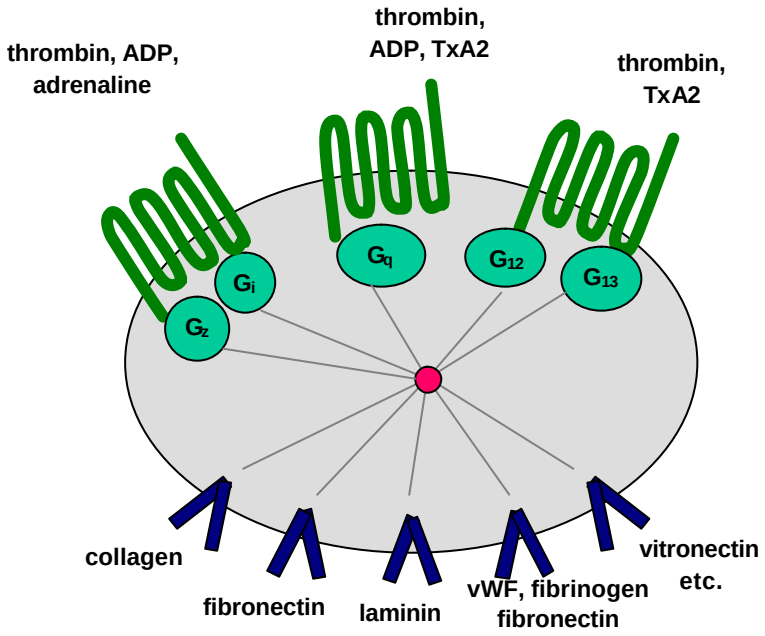


Figure A.6. Receptors coupled to the heterotrimeric G proteins on the platelet surface

A.3.2.1. ADP receptors

ADP is stored in platelet dense granules and released upon platelet activation. When added to platelets *in vitro*, ADP causes TxA_2 formation, protein phosphorylation, an increase in cytosolic Ca^{2+}, shape change, and aggregation[25;26]. Platelets express two ADP-specific purinoreceptors, $P2Y_1$ and $P2Y_{12}$[27]. Optimal platelet response to ADP requires activation of both receptors. $P2Y_1$ couples to G_q to mobilize Ca^{2+}, resulting in shape change and aggregation [28]. In the absence of $P2Y_1$, ADP is still able to inhibit cAMP formation, but the ability to increase cytosolic Ca^{2+} level, shape change, and

aggregation is impaired[29]. $P2Y_{12}$ receptor couples to G_i to inhibit adenylate cyclase. When the $P2Y_{12}$ receptor is inhibited by thienopyridine drugs (e.g., ticlopidine or clopidogrel) or deleted by genetic manipulations, humans[30] and mice[31] suffer from prolonged bleeding time, platelets do not normally aggregate in response to ADP, show impaired aggregation in response to TxA_2 and thrombin, and lack the ability to suppress cAMP formation. A third purinergic receptor on platelets, $P2X_1$, is coupled directly to a nonselective cation channel and is activated by ATP, which causes Ca^{2+} and Na^+ influx[32].

Table A.1. G-protein coupled receptors expressed on human platelets

Agonist	Receptor	G-protein families	Approx. amount of copies per platelet
Thrombin	PAR1	G_q, G_i, G_{12}	2000
	PAR4	G_q, G_{12}	?
ADP	$P2Y_1$	G_q	150
	$P2Y_{12}$	G_i (G_{i2})	600
TxA_2	TPα TPβ	G_q, G_{12}	1000
Adrenaline	α2A-adrenergic	G_i (G_z)	300
Vasopressin	V1	G_q	100
PAF	PAF receptor	G_q	200-2000
SDF-1	CXCR4	G_i	?
PGI_2	IP	G_s	?

From: Woulfe D, et al: Signal transduction during the initiation, extension, and perpetuation of platelet plug formation. In: Platelets, edited by Michelson AD, Elsevier Science, 2002, p. 198.

A.3.2.2. Adrenaline receptor

Adrenaline ($C_9H_{13}NO_3$) is a catecholamine and belongs to the family of biogenic amines. Adrenaline is synthesized in the neurones of the adrenal medulla and stored in chromaffin granules. It is a first messenger hormone which is released when the glucose level in blood is low. Adrenaline also acts as neurotransmitter and is released by nervous stimulation in response to physical or mental stress and binds to a special group of transmembrane proteins - the adrenergic receptors[33]. In platelets, adrenaline binds to the α2A-adrenergic receptor (α2A) that couples to G_z, which belongs to G_i family. This binding suppresses PGI_2-stimulated cAMP levels[34].

Adrenaline alone is unable to promote platelet shape change, aggregation or αIIbβ3 activation[35;36] but it can potentiate different platelet responses and leads to an increase in cytosolic free Ca^{2+} levels[37]. The role of adrenaline/α2A/G_z pathway in human platelet activation and aggregation was assessed after specific inhibition of G_q pathway. In such conditions, co-stimulation of G_{12}/G_{13} and G_z pathways increased intracellular Ca^{2+} levels[38]. The analysis performed with $G\alpha_q^{-/-}$ mice revealed that the concomitant stimulation of G_{12}/G_{13} pathway using U46619 and G_z pathway through adrenaline in these mice was sufficient for irreversible platelet aggregation and αIIbβ3 activation[39], but this is, however, not sufficient for normal hemostasis as the G_q deficient mice display largely prolonged bleeding times[40].

A.3.2.3. Thrombin receptors

Thrombin is a serine protease that exists in an inactive form (prothrombin) in serum but is activated during the coagulation cascade. Thrombin is a potent platelet activator, it increases intracellular Ca^{2+} concentrations, activates phospholipase A_2, and is able to inhibit cAMP formation. Thrombin induces platelet degranulation (which causes cell-surface expression of the adhesion molecules P-selectin and CD40 ligand), aggregation, as well as activation of αIIbβ3[41;42]. Platelet responses to thrombin are to a great extent mediated by protease-activated receptor (PAR) family members[43]. Human platelets express PAR1 and PAR4 receptors, whereas on mouse platelets PAR3 and PAR4 are expressed. Thrombin activates PAR1 and PAR4 on human platelets by binding to the extended N-terminus which causes its cleavage and exposure of new N-terminus that serves as a tethered ligand[41;43]. In mouse platelets, PAR3 primarily promotes cleavage of PAR4 receptor[44].

A.3.2.4. Thromboxane A_2 receptor

Thromboxane A_2 is produced by activated platelets through the sequential conversion of arachidonic acid by phospholipase A_2, cyclooxygenase-1 and thromboxane synthase. The TxA_2 receptor, TP, exists in two splice variants, TPα and TPβ, that differ in their cytoplasmic tail[45]. In human and mouse platelets, the TP receptor couples to G_q and $G_{12/13}$[46]. The stable TxA_2 analog U46619 causes platelet shape change, aggregation, phosphoinositide hydrolysis, phosphorylation, and an increase in cytosolic Ca^{2+}. Inhibition of TxA_2 production by inactivation of

cyclooxygenase-1 is the basis of the antithrombotic action of acetylsalicylic acid (Aspirin).

A.4. Adhesion and aggregation

Firm platelet adhesion to the exposed ECM under high shear conditions follows platelet activation and release of granular stores. In this process activated platelet integrins undergo conformational changes and P-selectin is released from α-granules to reinforce platelet deposition to the site of injury.

Aggregation is defined as the process of cell-to-cell adhesion of two or more platelets. In the first phase of aggregation, platelets are loosely linked to each other by fibrinogen bridges. This process is reversible. During the second phase, platelets release their granule components (secretion). This process is irreversible. Three conditions are decisive for a normal aggregation process: shear forces, Ca^{2+}, and fibrinogen[47]. Platelet aggregation is mediated by the Ca^{2+} dependent binding of the activated αIIbβ3 receptor to fibrinogen. Fibrinogen then functions as a bridge between two platelets, and contributes to platelet recruitment from blood flow, which finally results in the expansion of the thrombus.

A.4.1. Selectins

Selectins are adhesion receptors that mediate the heterotypical interactions of cells. Three selectins have been described: E-selectin, P-selectin, and L-selectin. E-selectin occurs on cytokine-activated endothelial cells and modulates granulocyte adhesion. Resting endothelial cells do not express any E-selectin. L-selectin occurs constitutively on leukocytes and participates in the adhesion of leukocytes to endothelial cells. P-selectin is found in α-granules of platelets and in endothelial cells stored in Weibel-Palade bodies[48]. P-selectin is not expressed on resting cells. However, activation leads to the rapid release and surface expression of P-selectin on both types of cells where it plays a central part in secondary hemostasis and vascular repair processes. P-selectin mediates platelet/endothelial interactions through its specific receptor P-selectin glycoprotein ligand-1 (PSGL-1). PSGL-1 is sulfate-containing sialomucine highly expressed on leukocytes and very low expressed on platelet surface. It mediates calcium dependent rolling and tethering of platelets on activated endothelium *in vivo*[49;50].

A.4.2. Integrins

Integrins represent a class of adhesive and signaling molecules expressed on the platelet surface. They consist of noncovalently associated heterodimers of α and β subunits. Integrins interact with numerous components of the extracellular matrix such as collagen, fibronectin, fibrinogen, laminin, thrombospondin, vitronectin, and VWF. The integrins expressed on platelets are: α2β1, α5β1, α6β1, αIIbβ3 (GPIIbIIIa), and αvβ3. They recognise the arginine-glycine-aspartate (RGD) amino acid sequence that mediates ligand binding in the presence of divalent cations such as Ca^{2+} and Mg^{2+}. The rapid regulation of integrins is based on affinity modulation, i.e. the receptor changes its conformation and is transformed from a low-affinity to a high-affinity functional state. Integrins exhibit both “inside-out” and “outside-in” signaling properties[51].

A.4.2.1. b_1 integrins

Platelets express three β_1 integrins that regulate the primary adhesion of platelets to the vessel wall (α2β1 - collagen receptor, α5β1 - fibronectin receptor, α6β1 - laminin receptor).

- The collagen receptor α2β1, also known as GPIaIIa, plays a role in platelet adhesion to collagen *in vitro*. There are 2000-4000 copies per platelet and its expression is influenced by silent polymorphisms[52]. Collagen interactions with platelets are mediated by the interaction between specific sites on collagen and GPVI which causes activation and conformational changes of α2β1 integrin and enhances its affinity for collagen[15]. Mice with a genetic deletion of either α2 or β1 subunit, which lack the α2β1 receptor, display normal bleeding times and show essentially normal adhesion and aggregation to fibrillar collagen[53]. Defective aggregation of α2β1-null platelets was shown only in the response to pepsin digested collagen. Under low and high shear flow conditions platelet adhesion and thrombus formation on collagen coated surface was only very moderately affected in the absence of α2β1, suggesting that α2β1is not essential for this process[15].
- The fibronectin receptor α5β1 is expressed on the platelet surface and is thought to have a supportive role in adhesion processes at the site of vascular injury. Fibronectins are dimeric glycoproteins that are present in plasma (plasma fibronectin) and in tissue extracellular matrices (cellular fibronectin)[54]. At sites of vascular injury,

platelets get in touch with both cellular and extravasated plasma fibronectin. Studies in mice with a cre/loxP-mediated deletion of plasma fibronectin revealed no major hemostatic defect as shown by normal bleeding times, platelet aggregation, and clot retraction[55]. However, recent studies with these mice in a model of arterial thrombosis demonstrated delayed thrombus formation and reduced thrombus stability, suggesting a role of plasma fibronectin in platelet-platelet interactions[56].

- The laminin receptor α6β1 is expressed on the platelet surface in low affinity state. Upon platelet activation it undergoes conformational changes and is able to bind its ligand laminin. Laminins are highly expressed in the vessel wall and become accessible to the flowing blood at sites of injury. Laminins are a family of structurally related glycoproteins that are tightly assembled with collagen type IV through the action of nidogen[57] in the basement membrane. Therefore, laminins are among the first constituents of the ECM that platelets get in touch with at sites of endothelial denudation and they are closely associated with collagen. Although collagen type IV is a relatively weak platelet agonist compared with collagen types I, III, and VI[58], it is known to activate GPIIbIIIa through the GPVI/FcR$_\gamma$-chain complex[59].

A.4.2.2. b_3 integrins

Platelets express two integrins of the β3 family on their surface (αvβ3 – vitronectin receptor, and αIIbβ3 (GP IIbIIIa) – fibrinogen, fibronectin, and VWF receptor)

- The vitronectin receptor (αvβ3) is expressed in only very low amounts on platelets (~50 receptors per platelet) and its role in platelet function is unclear[60]. Integrin $\alpha_v\beta_3$ makes a decisive contribution to the adhesion and to the migration of endothelial cells as well as smooth muscle cells to the extracellular matrix[61]. When $\alpha_v\beta_3$-positive cells adhere to specific matrix proteins, the receptors are enriched in the region of focal contact sites (clustering).
- The fibrinogen receptor (αIIbβ3) is a component of the platelet plasma membrane, the open canalicular system, and the α-granules. It is the most abundant platelet surface membrane glycoprotein with 50.000 to 80.000 copies per platelet[62]. The main task of αIIbβ3 is the binding of soluble fibrinogen to the activated platelet surface, the first step of platelet aggregation. Activation of platelets causes signaling from inside the cell, which modulates integrin affinity and avidity for ligands[51]. These so called "inside-out" signals are ultimately transmitted to αIIbβ3 to convert it from an

inactive to an active state. This makes the binding of soluble fibrinogen to the platelet surface possible. Fibrinogen possesses two major amino acid sequences (RGD and KQAGDV), which are recognised by specific binding regions within the $\alpha IIb\beta 3$ and mediate ligand binding[63]. Bound ligand induces further changes in the conformation of the receptor. The ligand-induced conformational change of the receptor regulates mechanisms for irreversible fibrinogen binding to $\alpha IIb\beta 3$ and integrin clustering[64] in order to promote outside-in signaling. Outside-in signaling affects cytoskeletal organization and is responsible for full aggregation, granule secretion, and procoagulant activity[65].

A.5. Platelet interactions with the coagulation system

Activated platelets exhibit procoagulant activity, which is characterized by changes in the orientation of phospholipids that allow the association of coagulation factors and the formation of a catalytic prothrombinase complex, which enzymatically converts prothrombin to thrombin[66]. Thrombin is a strong platelet agonist and supports the recruitment of further platelets in the region of the platelet-rich thrombus, it cleaves fibrinogen to fibrin, which is then cross linked (by factor XIII) with covalent bonds, to form a stable clot[67].

A.5.1. Physiology of coagulation

The classical model of blood coagulation involves a series of activation reactions where a precursor protein (zymogen) is converted to an active protease by cleavage of one or more peptide bonds in the precursor molecule. Two pathways lead to the formation of a fibrin clot: the intrinsic and the extrinsic pathway.

The intrinsic pathway requires the clotting factors VIII, IX, X, XI, and XII. Also required are the proteins prekallikrein and high molecular weight kininogen, as well as calcium ions and phospholipids present on the platelet surface. Each of these pathway constituents leads to activation of factor X to factor Xa. Initiation of the intrinsic pathway occurs when prekallikrein, high molecular weight kininogen, factor XI and factor XII are exposed to a negatively charged surface[68], termed “contact phase” (Figure A.7). The activation of factor Xa requires assembly of the tenase complex (Ca^{2+} and factors VIIIa, IXa and X) on the surface of activated platelets. The role of factor VIII in this process is to act as a receptor, in the form of factor VIIIa, for factors IXa and X. The activation of factor VIII to factor VIIIa occurs in the presence of

minute quantities of thrombin. As the concentration of thrombin increases, factor VIIIa is ultimately cleaved by thrombin and inactivated. This dual action of thrombin upon factor VIII, acts to limit the extent of tenase complex formation and thus the extent of coagulation.

The extrinsic pathway is initiated at the site of injury in response to the release/exposure of subendothelial tissue factor[69;70]. Tissue factor is a cofactor in the factor VIIa-catalyzed activation of factor X (Figure A.7). The activation of factor VII occurs through the action of thrombin or factor Xa[71]. Activated factor X (FXa) assembles into prothrombinase through Ca^{2+}-dependent interactions with factor Va to generate picomolar amounts of thrombin. Thrombin as a strong platelet agonist supports platelet recruitment and thereby sustains coagulation. It activates additional factor V and factor VIII to ensure continued prothrombinase activity and intrinsic pathway – the so called “amplifier loop”, which is essential for sustained hemostasis[72;73].

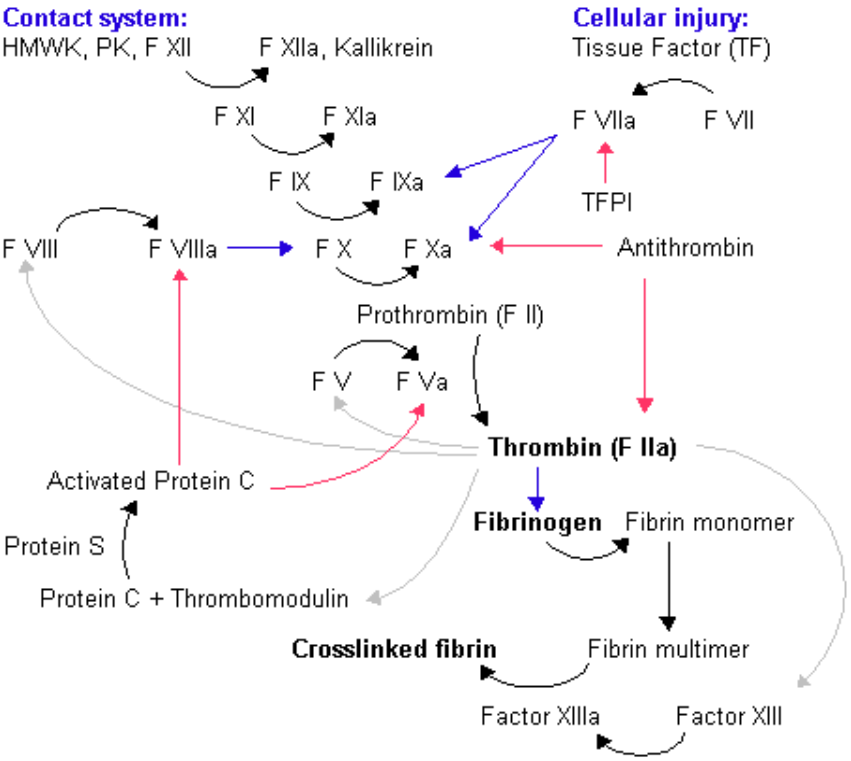


Figure A.7. The coagulation cascade. Legend: HWMK = High molecular weight kininogen, PK = Prekallikrein, TFPI = Tissue factor pathway inhibitor. Black arrow = conversion/activation of factor. Red arrows = action of inhibitors. Blue arrows = reactions catalysed by activated factor. Grey arrow = various functions of thrombin.
From: http://en.wikipedia.org/wiki/Coagulation

The common final step of the coagulation cascade can be divided into three sections: activation of factor X, formation of thrombin from prothrombin, and cleavage of fibrinogen to afford fibrin to form the insoluble, cross-linked clot. The course of the coagulation cascade is regulated by inhibitors (antithrombin, proteins C and S, tissue factor pathway inhibitor [TFPI]) that inhibit the function of specific coagulation factors [74-76].

A.5.2. Plasma coagulation factors

The coagulation factors (Table A.2) whose lack or dysfunction is clearly associated with bleeding risk are: tissue factor, factors V, VII, VIII, IX, X, XIII and prothrombin[77;78]. Coagulation protease zymogens include: factors II (prothrombin), VII, IX, X, XI, XII, and kallikrein. Non-enzymatic protein cofactors include: factors V and VIII, tissue factor, and high-molecular weight kininogen (HMWK).

Table A.2. Plasma coagulation factors

Factor	Molecular Weight	Plasma Concentration (µg/ml)	Required for Hemostasis (% of normal concentration)
Fibrinogen	330,000	3000	30
Prothrombin	72,000	100	40
Factor V	300,000	10	10 - 15
Factor VII	50,000	0.5	5 - 10
Factor VIII	300,000	0.1	10 - 40
Factor IX	56,000	5	10 - 40
Factor X	56,000	10	10 - 15
Factor XI	160,000	5	20 - 30
Factor XIII	320,000	30	1 - 5
Factor XII	76,000	30	0
Prekallikrein	82,000	40	0
HMWK	108,000	100	0

From: http://tollefsen.wustl.edu/projects/coagulation/coagulation.html

A.5.2.1. Tissue factor

Tissue factor (TF) is a non-enzymatic lipoprotein and a member of the class II cytokine receptor family that is constitutively expressed in the subendothelial and, especially, subcutaneous spaces. Exposure of plasma to these tissue surfaces initiates coagulation outside a damaged blood vessel. Endothelial cells also express TF when stimulated by endotoxin, tumor necrosis factor, or interleukin-1, and may be

involved in thrombus formation under pathologic conditions. The physiologic function of rapid induction of TF in response to a range of stimuli is not yet clear. The major function of TF is believed to be related to initiation of the procoagulant pathway[79-82]. TF binds factor VIIa and accelerates factor X activation. Although factor VII is activated by factor Xa, a trace amount of factor VIIa appears to be available in plasma at all times to interact with TF. Factor VIIa also activates factor IX in the presence of TF, providing a connection between "extrinsic" and "intrinsic" pathways.

A.5.2.2. Thrombin

Thrombin (activated Factor II) is a serine protease and is designed to perform the specific cleavage needed to turn fibrinogen into fibrin. Thrombin is a central molecule in the process of blood clotting. It is converted from inactive prothrombin (Factor II) through the prothrombinase complex. This complex is composed of platelet phospholipids, phosphatidylinositol and phosphatidylserine, Ca^{2+}, factors Va and Xa, and prothrombin formed by activated coagulation factors Xa and Va. Thrombin has a number of effects on other coagulation proteins. In addition to fibrinogen, conversion into fibrin, it also activates factor XI and factor VIII to increase its formation through the intrinsic pathway of coagulation. Thrombin activates factor V, which quickly increases its own production from prothrombin. Factor XIII, the coagulation protein that cross-links fibrin molecules to stabilize the fibrin clot, is activated as well. Interestingly, thrombin combines with thrombomodulin present on endothelial cell surfaces forming a complex that converts protein C to protein Ca. The cofactor protein S and protein Ca degrade factors Va and VIIIa, thereby limiting thrombin levels. Thus, thrombin both cleaves fibrinogen to form fibrin and slows down the clotting process. Apart from this, thrombin also initiates fibrinolysis (together with Factor XII). Activation of prothrombin into thrombin is crucial in physiological and pathological coagulation[83]. Thrombin is generated on the membrane of stimulated platelets and in turn leads to increased platelet activation and leukocyte adhesion. It also binds to and leads to the release of G-protein-coupled protease activated receptors (PARs), specifically PAR-1, -3 and -4. Activation of these receptors leads to the activation of numerous signaling cascades that in turn increase release of interleukins, as well as secretion of intercellular adhesion molecule-1 (ICAM-1) and vascular cell adhesion molecule-1 (VCAM-1)[84].

A.5.2.3. Factor XII

Factor XII is ~80 kD single-chain proenzyme that is synthesized in the liver[85]. Factor XII's plasma concentration is approximately 30 µg/mL and its half-life is about 50 hours. The gene for factor XII (Hageman factor) is located on the tip of the long arm of the fifth chromosome (5q33-qter). Hageman factor was first discovered in 1955 when a routine preoperative blood sample of John Hageman was found to have prolonged clotting time in test tubes, even though he had no haemorrhagic symptoms. Factor XII deficiency is usually inherited in an autosomal recessive manner and heterozygous deficiency is relatively common, affecting 1.5 to 3.0 percent of the population[86]. Hageman factor homozygous deficiency is a rare hereditary disorder with a prevalence of about one in a million, although it is a little more common among Asians. This deficiency does not cause excessive hemorrhage, and there is no risk for bleeding. Studies on patients deficient in FXII revealed controversial results in terms of whether or not there is an increase or decrease in thrombosis. Larger studies are required to point out if contact factor abnormalities can predict thrombotic events to any degree of clinical utility[87].

A.5.2.4. Factor XI

Factor XI (FXI) is a serine protease and circulates in its inactive form. It is activated into Factor XIa by Factor XIIa (FXIIa), thrombin, and also autocatalytically. FXI is a member of the "contact pathway". Factor XIa activates factor IX by selectively cleaving Arg-Ala and Arg-Val peptide bonds, and factor IXa, in turn, activates factor X. Deficiency of factor XI causes the rare hemophilia C, which is an autosomal recessive disorder. There is little spontaneous bleeding[88], but surgical procedures may cause excessive blood loss, and prophylaxis is required. High levels of factor XI have been implicated in thrombosis, although it is uncertain what determines these levels and how serious the procoagulant state is[89].

A.6. Studies on thrombus formation and stabilization *in vivo*

Our understanding of how platelets function *in vitro* has advanced enormously over the past three decades. However, defining and measuring *in vivo* platelet function remains a considerable challenge. Experimental animal models can provide very useful insights into the relevant biological activity of platelets in intact organisms, for

better understanding how these anucleate cells participate in hemostasis, thrombosis and vascular biology[90]. Moreover, such models can serve as tool for particular pathologic or biologic processes. For this purpose various genetically modified animals have been generated, from which the most commonly used are mice. Genetic methods that allow targeted manipulations in the mouse genome have opened new ways to study platelet function both *in vitro* and *in vivo*. Despite the unique contribution of the knockout and transgenic technologies, novel approaches in imaging and image analysis are equally important to analyze platelet-dependent thrombotic and inflammatory processes *in vivo*. To examine the process of platelet accumulation, adhesion and subsequent formation and stabilization of three-dimensional thrombi at sites of vascular injury, arterial lesions are induced in different branches of the vascular system and by different methods[91]. Damage to the intact vascular system results in exposure of the ECM which triggers thrombus formation by collagen- and thrombin-dependent mechanisms thereby reflecting naturally occurring vascular lesions.

A.7. Aim of the study

It is commonly accepted that platelets play an important role in hemostasis and thrombosis as well as in atherosclerotic processes. This makes platelet receptors and their intracellular signaling pathways important molecular targets for anti-thrombotic and anti-inflammatory therapy. The aim of this study was to identify the most important aspects of platelet activation, adhesion, aggregation, and procoagulant activity in the process of thrombus formation and stabilization *in vitro* and *in vivo*. The work was focussed on studies of platelet collagen receptors (GPVI and $\alpha 2\beta 1$), the G-protein coupled adrenaline receptor ($\alpha 2A$) and the contact system (FXII).

B. Materials and methods

B.1. Materials

B.1.1. Chemicals

acetylsalicylic acid (Aspisol)	Bayer (Leverkusen, Germany)
ADP	Sigma (Deisenhofen, Germany)
adrenaline	Sigma (Deisenhofen, Germany)
apyrase (grade III)	Sigma (Deisenhofen, Germany)
avertin (2,2,2-tribromoethanol and 2-methyl-2-butanol)	Sigma(Deisenhofen, Germany)
bovine fibronectin	Sigma (Deisenhofen,Germany)
bovine serum albumin (BSA)	Pierce (Rockford, IL, USA)
enhanced chemoluminiscence (ECL) detection substrate	MoBiTec (Göttingen, Germany)
fetal calf serum (FCS)	PAN (Aidenbach, Germany)
fibrilar type I collagen (Horm)	Nycomed (Munich, Germany)
fluorescein-isothiocyanate (FITC)	Molecular Probes (Oregon, USA)
HAT stock (50x)	Roche Diagnostics (Mannheim, Germany)
HEPES	Roth (Karlsruhe, Germany)
high molecular weight heparin	Sigma (Deisenhofen, Germany)
hirudin	Aventis (Frankfurt, Germany)
horseradish peroxidase (HRP)-labeling kit	Zymed (Berlin, Germany)
human FXII	American Diagnostics (Greenwich, USA)
human fibrinogen	Sigma (Deisenhofen, Germany)
immobilized papain	Pierce (Rockford, IL, USA)
immobilized pepsin	Pierce (Rockford, IL, USA)
ketamine	Parke-Davis (Karlsruhe, Germany)
mouse laminin	Sigma (Deisenhofen, Germany)
Nonidet P-40 (NP-40)	Roche Diagnostics (Mannheim)
PD-10 column	Pharmacia (Uppsala, Sweden)
penicillin/ streptomycin	PAN (Aidenbach, Germany)

polyethylene glycol 1500 (PEG 1500)	Roche Diagnostics (Mannheim)
prostacyclin	Calbiochem (Bad Soden, Germany)
protein G sepharose	Pharmacia (Uppsala, Sweden)
R-phycoerythrin (PE)	EUROPA (Cambridge, UK)
RPMI media	PAN (Aidenbach, Germany)
5-carboxyfluorescein diacetate succinimidyl ester	Invitrogen (Karlsruhe, Germany)
thrombin	Roche Diagnostics (Mannheim)
3,3,5,5-tetramethylbenzidine (TMB)	EUROPA (Cambridge, UK)
U46619	Alexis Biochemicals (San Diego, USA)
xylazine	Bayer AG (Leverkusen, Germany)

Human VWF and collagen-related peptide (CRP) were kindly provided by G. Dickneite (Marburg, Germany) and S. P. Watson (Oxford, United Kingdom), respectively. All other chemicals were obtained from Sigma (Deisenhofen, Germany) or Roth (Karlsruhe, Germany)

B.1.2. Monoclonal antibodies (mAbs)

mAbs generated and modified in our laboratory:

antibody	**isotype**	**antigen**	**described in**
JAQ1	IgG2a	GPVI	[16]
LEN1	IgG1	α2	[92]
DOM1	IgG1	GPV	[92]
DOM2	IgG2a	GPV	[92]
JON/A	IgG2b	αIIbβ3	[93]
JON1	IgG2b	αIIbβ3	[92]
EDL1	IgG2a	β3	[92]
ULF1	IgG2a	CD9	[92]
p0p4	IgG2b	GPIbα	[92]
p0p6	IgG2b	GPIX	[92]
21H4	IgG2b	α2 integrin	Unpublished
BRU1	IgG1	P-selectin	Unpublished

Irrelevant control rat IgG, fluorescein isothiocyanate (FITC)-conjugated anti-β1 integrin and α6 were obtained from Pharmingen (Hamburg, Germany).

B.1.3. Polyclonal antibodies (pAbs)/ secondary reagents

rabbit anti-rat IgG (-FITC, -HRP)	DAKO (Hamburg, Germany)
rabbit anti-rat IgG1, IgG2a, IgG2b, IgM, IgA	BD Pharmingen (Hamburg, Germany)

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). Mice deficient in α2 integrin subunit were obtained from Beate Eckes (Cologne, Germany), deficient in α2A were kindly provided by Lutz Hein (Wuerzburg, Germany), deficient in FXII and FXI were obtained from Thomas Renne, (Wuerzburg, Germany), and deficient in $G\alpha_q$-subunit ($G\alpha_q^{-/-}$) as well as the conditional $G\alpha_{13}$-deficient mouse line ($G\alpha_{13}^{-/-}$) were kindly provided by Stefan Offermanns (Heidelberg, Germany). To produce mice carrying the β1-null allele in megakaryocytes, β1(fl/fl) mice were crossed with transgenic mice carrying the Mx-cre transgene (mx-cre$^+$). Deletion of the β1 gene was induced in 4- to 5-week-old (β1(fl/fl)/Mx-cre$^+$) mice by 3 intraperitoneal injections of 250 µg polyinosinic-polycytidylic acid (pI-pC) at 2-day intervals. Littermate β1(fl/fl) control mice were treated similarly. For experiments, mice were used at least 2 weeks after pI-pC injection. The β1(fl/fl) mice were kindly provided by Reinhard Fässler (Martinsried, Germany). To deplete GPVI, C57BL6/J or $\alpha2^{-/-}$ mice were injected with 100 µg JAQ1 intraperitoneally, a treatment which causes *in vivo* GPVI-downregulation. Animals were used for *in vivo* assessment of platelet adhesion/thrombus formation on day 5 after antibody injection.

B.2. Methods

B.2.1. Buffers and Media

All buffers were prepared and diluted using aqua bidest

- Phosphate-buffered saline (PBS), pH 7.14

NaCl	137 mM (0.9 %)
KCl	2.7 mM
KH_2PO_4	1.5 mM
$Na_2HPO_4x2H_2O$	8 mM

- PBS/EDTA

PBS	
EDTA	5 mM

- Tris-buffered saline (TBS), pH 7.3

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

- Storage buffer, pH 7.0

Tris	20 mM
NaCl	0.9 %
BSA	0.5 %
NaN_3	0.09 %

- Tyrode's buffer, pH 7.3

NaCl	137 mM (0.9 %)
KCl	2.7 mM
$NaHCO_3$	12 mM
NaH_2PO_4	0.43 mM
Glucose	0.1 %
Hepes	5 mM
BSA	0.35 %
$CaCl_2$	1 mM
$MgCl_2$	1 mM

- Acid-citrate-dextrose (ACD) buffer, pH 4.5

Trisodium citrate dehydrate	85 mM
Citric acid anhydrous	65 mM
Glucose anhydrous	110 mM

- IP buffer

Tris/HCl (pH 8.0)	15 mM
NaCl	155 mM
EDTA	1 mM
NaN_3	0.005 %

- SDS sample buffer, 2X

β-mercaptoethanol (for red. conditions)	10 %
Tris buffer (1.25 M), pH 6.8	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

ε-amino-n-caproic acid	4 mM
Methanol	20 %

- Digestion buffer (for generation of Fab fragments), pH 7.0

$NaH_2PO_4.H_2O$	20 mM
NaCl	10 mM
Cystein HCl	20 mM

- Digestion buffer (for generation of $F(ab)_2$ fragments), pH 4.5

Sodium acetate	20 mM

- Washing buffer

PBS	
Tween 20	0.1 %

- Coating buffer, pH 9.0

$NaHCO_3$	50 mM

- Coupling buffer, pH 9.0

$NaHCO_3$	160 mM
Na_2CO3	80 mM

- Pen/strep-solution

Penicillin	10.000 U/mL
Streptomycin	10 mg/mL
in 0.9 % NaCl	

- RPMI standard media

RPMI 1640	470 mL
FCS	25 mL
Penicillin/Streptomycin	5 mL

- HAT media

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

- Coomassie Stain

Acetic acid	10 %
Methanol	40 %

Brilliant blue in H_2O	1 g

- Destaining solution

Acetic acid in H_2O	10 %

B.2.2. Production of monoclonal antibodies

B.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 the immunogens.

B.2.2.2. Generation of hybridomas

The rat spleen was removed under aseptic conditions and filtered through a Nitex filter to obtain a single cell suspension. Spleen cells were washed twice in RPMI/pen-strep medium (160 *g*, 5 min, RT), mixed with mouse myeloma cells (Ag14, 10^8 cells / fusion) and washed twice with RPMI/pen-strep by centrifugation at 900 *rpm* for 5 min. Supernatant was removed carefully and 1 mL of polyethylene glycol 1500 (37°C) was added dropwise (over a time period of 2 min). This was followed by slow addition of 10 mL RPMI/pen-strep medium (37°C) over a period of 10 min. Cells were then seeded into 96-well plates and fed with HAT containing medium until screening. Positive hybridomas were left to grow in RPMI/ pen-strep/ 10 % fetal calf serum.

B.2.2.3. Screening of hybridomas

Hybridomas producing 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 100µL of the hybridoma supernatant for 20 min, RT. To prepare this mixture, washed platelets (10^6; prepared as mentioned under B.2.3) were divided into two parts. One part was left untreated and the other was activated by thrombin (0.2 U/mL; 5 min, 37°C), followed by the addition of hirudin to stop thrombin function. Samples were then washed once with 1 mL PBS (2800 *rpm*, 10 min) and stained with

FITC-conjugated rabbit anti-rat Ig for 15 min at RT. Samples were finally analyzed on a FACScalibur (Becton Dickinson, Heidelberg, Germany).

B.2.2.4. Determination of isotype subclass

96-well ELISA plates (BD Falcon, Heidelberg, Germany) were coated with rabbit anti-rat IgG/M (H+L) antibodies (1:1000 in coating buffer) for 2 h at 37°C or overnight at 4°C. After blocking, serial dilutions of purified rat anti-mouse platelet mAbs were added in duplicate wells (1 h, 37°C). Plates were washed and subsequently incubated for 1 h with AP-conjugated isotype specific antibodies (BD Pharmingen; 1:1000, 37°C). After several washing steps, Sigma104 substrate was added to each well and absorbance at 405 nm was recorded on Anthos Reader 2010 (Anthos Labsysteme, Krefeld, Germany).

B.2.2.5. Modification of antibodies – preparation of F(ab) /F (ab)$_2$ fragments

Antibody (whole IgG; 3 mg) was dialyzed overnight against digestion buffer at 4°C. Antibody was then concentrated in a concentrator tube (VivaSpin®, Exclusion size 10 kDa, VivaScience, Hannover) by centrifugation at 4000 *rpm* until volume of 0.5-1.0 mL was reached (approximately 10 min). To generate F(ab) fragments, dialyzed IgG was mixed with digestion buffer and immobilized papain (washed 2x with digestion buffer) in the ratio of 1:1:1 and left to incubate at 37°C for 4-6 h under shaking conditions. To test the efficacy of the digestion, samples (20 µL) were taken at different time points (0, 1, 2, 4, and 6 h), mixed with 20 µL non-reducing sample buffer and separated by SDS-PAGE (12 %). To visualize the protein bands, the gel was then coomassie-stained (overnight), then destained (incubated with destaining solution for at least 4 h or overnight). If the digestion was not complete, antibody was left to incubate for further 2-4 h. Antibody was next dialyzed overnight against PBS at 4°C. To remove remaining Fc parts, F(ab) fragments were incubated overnight with 70 µL protein-G-sepharose 2x washed with PBS. Samples (20 µL) were taken before and after the clearing process, mixed with 20 µL non-reducing sample buffer and were separated by SDS-PAGE (12 %). Finally, 1 % BSA was added to the F(ab) fragments. To generate F (ab)$_2$ fragments, dialyzed IgG was mixed with digestion buffer and immobilized pepsin (washed 2x with digestion buffer) in the ratio of 1:1:1 and left to incubate at 37°C for 48 h under shaking conditions. Samples (20 µL) were

taken at 0, 6, 24, and 48 h, mixed with 20 µL non-reducing sample buffer and separated by SDS-PAGE (12 %). Same procedure as above was then performed.

B.2.2.6. FITC labeling

Affinity purified antibodies were FITC-labeled to a fluorescein/protein ratio of approximately 3:1. Antibody (4 mg) was dialyzed against coupling buffer overnight at 4°C. FITC was dissolved in anhydrous DMSO to a final concentration of 1 mg/mL. 50 µL of this solution was added to the antibody and left to incubate at RT for 8 h. The reaction was then stopped by addition of 100 µL of 1 M NH_4Cl. FITC-labeled antibody was separated from the unreacted FITC by gel filtration on a PD-10 column.

B.2.3. Platelet preparation

B.2.3.1. Platelet washing

Mice were bled under ether anesthesia from the retroorbital plexus. Blood was collected into a tube containg ACD buffer or 20 U/mL heparin in TBS, pH 7.3 (both 300 µL). Blood was centrifuged at 2000 *rpm* for 5 min. Supernatant (plasma with platelets and little bit of erythrocytes) was taken and centrifuged at 800 *rpm* for 6 min at RT to obtain platelet rich plasma (PRP). To wash platelets, PRP was centrifuged at 2800 *rpm* for 5 min in the presence of prostacyclin (PGI_2) (0.1 µg/mL) and the pellet was resuspended in Tyrode's buffer containing PGI_2 (0.1 µg/mL) and apyrase (0.02 U/mL) and left to incubate at 37 °C for 5 min. After a second centrifugation step, platelets were resuspended in the same buffer and incubated at 37°C for 5 min. Platelets were finally centrifuged as above, resuspended in Tyrode's buffer (500µL) containing apyrase (0.02 U/mL) and left to incubate for 30 min at 37°C before analysis.

B.2.3.2. Preparation of platelets for intravital microscopy

Blood from wild-type or mutant mice was drawn from the retro-orbital plexus and collected into a tube containg 20 U/mL heparin in TBS, pH 7.3 (300 µL). Platelet rich plasma was prepared as described above and gently transferred to a fresh polypropylene tube, diluted with 3ml PBS and incubated with 5-carboxyfluorescein diacetate succinimidyl ester (DCF; 5 µg/mL for 2 minutes in the dark), then

centrifuged at 2300 rpm for 10 minutes. Isolated platelets were adjusted to a final concentration of 200 x 10^6 platelets/250 µL of Tyrode`s buffer containing 0.35% BSA and 5mM glucose. Where indicated, platelets were preincubated with 50 µg/mL F´(ab)$_2$ fragments of anti-αIIbβ3 antibody (JON/A) for 5 minutes before infusion. Flow cytometric analysis with FITC-conjugated JON/A confirmed that more than 95% of surface αIIbβ3 integrins were occupied under these conditions.

B.2.4. Platelet counting

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 (Becton Dickinson, Heidelberg, Germany). The diluted blood sample was allowed to settle for 20 minutes in an Improved Neubauer haemocytometer (Superior, Bad Mergentheim, Germany), and platelets were counted under a phase contrast microscope at x 400 magnification.

B.2.5. Immunoblotting

For Western blot analysis, platelets were washed 3x in PBS/EDTA and finally solubilized in 150 µL IP buffer containing 1% NP-40. After 10 min incubation, samples were spun down at 1500 *rpm* for 10 min, supernatant was then transferred to a fresh tube containing 150µL of 2x non-reducing sample buffer and boiled at 95°C for 5 min. Samples were separated by 12 % SDS-PAGE and transferred onto a polyvinylidene difluoride membrane. To prevent non-specific antibody binding, the membrane was incubated in 10 % fat-free milk (dissolved in washing buffer) for 1 h at RT. After that, the membrane was incubated with the required antibody (5 µg/mL) for 1 h at RT. For washing, the membrane was incubated 3x with washing buffer for 10 min at RT. After the washing steps, HRP-labeled secondary reagent was added and left to incubate for 1 h at RT. After several washing steps, proteins were visualized by ECL.

B.2.6. *In vitro* analysis of platelet function

B.2.6.1. Flow cytometry

Platelets (1 x 10^6) were left untreated or activated with the indicated agonists for 10 min at RT, afterwards stained for 15 min with saturating amounts of fluorophore-conjugated antibodies, reaction was stopped by addition of 500 µL PBS, and sample was immediately analyzed on a FACScalibur (Becton Dickinson, Heidelberg, Germany). For a two-color staining, the following settings were used:

Detectors/Amps:

Parameter	Detector	Voltage
P1	FSC	E01
P2	SSC	380
P3	Fl1	650
P4	Fl2	580
P5	Fl3	150

Threshold:

Value	Parameter
253	FSC-H
52	SSC-H
52	Fl1-H
52	Fl2-H
52	Fl3-H

Compensation

Fl1	2.4 % of Fl2
Fl2	7.0 % of Fl1
Fl2	0 % of Fl3
Fl3	0 % of Fl2

B.2.6.2. Aggregometry

To determine platelet aggregation, light transmission was measured using PRP or washed platelets diluted in Tyrode's buffer containing 1 mM CaCl2 (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 over 10 min on an Fibrintimer 4 channel optical aggregometer (APACT Laborgeräte und Analysensysteme, Hamburg, Germany). Before starting the measurements, Tyrode's buffer (for washed

platelets) or plasma (for PRP) was set as 100% aggregation and washed platelet suspension (for washed platelets) or PRP (for PRP) was set as 0% aggregation.

B.2.6.3. Adhesion under flow conditions

Heparinized whole blood (1 vol) was diluted 2:1 in Tyrode`s buffer pH 7.4, containing 0.35% BSA, 5mM glucose, and Ca^{2+}/Mg^{2+} (1 mM each). Coverslips (24 x 60 mm, Roth, Karlsruhe, Germany) were coated with fibrillar (Horm) collagen (0.25 mg/mL, Nycomed, Munich, Germany), dried at 37°C, and blocked for 1 h with 1% BSA. Perfusion studies were performed as follows. Transparent flow chambers with a slit depth of 50 μm, equipped with the coated coverslips and fixed, were connected to a syringe filled with the anticoagulated blood. Perfusion was performed using a pulse-free pump under high shear stress equivalent to a wall shear rate of 1000 s^{-1} (4 min). Where indicated, ADP (5 μM), U46619 (1 μM), or a combination of the two agonists was co-infused to the blood directly before it entered the flow chamber. Thereafter, chambers were rinsed by a 10 min perfusion with Tyrode`s buffer at the same shear stress and phase-contrast images were recorded from at least five different microscope fields (63x objectives). The platelet adhesion results are expressed as the mean count of single platelets per microscopic field. Thrombus formation was evaluated as a percentage of total area covered by thrombi. Image analysis were performed off-line using Metamorph software (Visitron, Munich, Germany)

B.2.6.4. Static adhesion assay

Static adhesion was performed with washed platelets in modified Tyrode`s buffer containing 0.35% BSA and Ca^{2+}/Mg^{2+} (each at 1 mM) on 96-well plates (Nunc, Wiesbaden, Germany). Plates were coated overnight at 4°C with laminin (0.2 μg/well), fibronectin (0.2 μg/well), or VWF (0.4 μg/well) diluted in PBS and then blocked with 5% BSA for 2 hours at 37°C. Resting or CRP-activated (0.2 μg/mL) platelets were allowed to adhere for 60 minutes and adhesion was quantitated colorimetrically using anti GPIbα AP-conjugated mAb. After several washing steps, Sigma104 substrate was added to each well and absorbance at 405 nm was recorded on Anthos Reader 2010 (Anthos Labsysteme, Krefeld, Germany). Where indicated, the experiments were performed in the presence of the function-blocking anti-αIIbβ3 antibody, JON/A (50 μg/mL).

B.2.6.5. Histology

For histologic examination, carotid arteries were perfusion-fixed in situ with 4% paraformaldehyde, pH 7.0. Thereafter, the vessels were excised, fixed in 0.1 M cacodylate-buffered Karnovsky solution (2.5% glutaraldehyde and 1% paraformaldehyde; overnight, RT) and then fixed in 1% osmium tetroxide (2 h) at pH 7.3. The samples were dehydrated in graded ethanols and embedded in the EmBed-812 epoxy resin (all reagents from Science Services, Munich, Germany). After 48 h heat polymerization at 60°C, semithin (0.8 µm) sections were cut with a diamond knife (Diatome, Fort Washington, PA) on a Reichert Ultracut-S ultramicrotome (Leica-Reichert, Leica-Microsysteme, Vienna, Austria) and double stained with aqueous solutions of 1% toluidine blue and basic fuchsin (60°C, 1 minute).

B.2.7. *In vivo* experiments

B.2.7.1. Bleeding time experiments

Mice were anesthetized by intraperitoneal injection of ketamine/xylazine (ketamine 100 mg/kg, Parke-Davis; xylazine 5 mg/kg, Bayer AG), and a 3-mm segment of the tail tip was cut off 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-second intervals, bleeding was determined to have ceased. Otherwise, the experiment was stopped after 20 minutes. In some experiments, mice were injected with 100 mg/kg acetylsalicylic acid diluted in sterile PBS (i.v.) 3 h before the experiments.

B.2.7.2. Collagen and epinephrine-induced pulmonary thromboembolism

Mice were anesthetized and a mixture of collagen (0.8 mg/kg) and adrenaline (60 µg/kg) was injected into the jugular vein. Incisions of surviving mice were sutured, and they were allowed to recover. Platelet counts were determined 2 min after injection by flow cytometry on a FACScalibur. Results are expressed as mean ± S.D or as percent of control.

B.2.7.3. Intravital microscopy of thrombus formation in the carotid artery

Mice were anesthetized and polyethylene catheters (Portex, Hythe, England) were implanted into the right jugular vein. Fluorescently labeled platelets (200 x 10^6/250

µL) of the same genetic background were infused intravenously. The right common carotid artery was dissected free and ligated vigorously near the carotid bifurcation for 30 seconds using a surgical filament to induce vascular injury. Prior to and following vascular injury, the fluorescent platelets were visualized in situ by *in vivo* video microscopy of the right common carotid artery using a Zeiss Axiotech microscope (x 20 water immersion objective; W x 20/0.5; Zeiss, Göttingen, Germany) with a 100-W mercury short arc photo optic lamp (HBO) for epi-illumination. Platelet adhesion was recorded for 5 minutes after the induction of injury and the videotaped images were evaluated using a computer-assisted image analysis program (Visitron, Munich, Germany). The number of adherent platelets was assessed by counting the cells that did not move or detach from the vascular wall for at least 10 seconds. In each mouse, 3 nonoverlapping fields (size, 100 µm x 100 µm) were analyzed for 30 seconds (2.5 - 3.0 minutes after injury) in a slow-motion modus. Clusters of 2 or more platelets were defined as microaggregates. The total number of adherent platelets or microaggregates at t = 3 minutes was calculated by the following formula that reflects concave shape of the vessel wall: vessel diameter (µm) x π (circle constant) x 2 x $\sin^{-1}$ (amplitude of measured area, in µm) x length of measured area (µm) and is presented per mm^2. All experiments performed on animals were approved by the German legislation on protection of animals.

B.2.7.4. In vivo thrombosis model with FeCl3-induced injury

Male and female mice in the age of 4-5 weeks were anesthetized and fluorescently labeled platelets were injected intravenously. The mesentery was exteriorized gently through a midline abdominal incision. Arterioles (35 – 60µm in diameter) were visualized with a Zeiss Axiovert 200 inverted microscope (x10) equipped with a 100-W HBO fluorescent lamp source and a CCD camera (CV-M300) connected to an S-VHS video recorder (AG-7355, Panasonic, Matsushita Electric, Japan). After topical application of one drop of $FeCl_3$ (20%) saturated on a filter paper, arterioles were monitored for 40 min or until complete occlusion occurred (the blood flow stopped for at least 1 min). Platelet adhesion was defined as the number of fluorescently labeled platelets bound to the vessel wall 5 min after injury. A thrombus was defined as a platelet aggregate larger than 10 µm in diameter. Where indicated, human FXII was injected intravenously directly before the experiment.

B.2.7.5. Aorta occlusion model

The abdominal cavity of anesthetized mice was opened with a longitudinal incision, and the abdominal aorta was exposed. An ultrasonic flow probe (Model 0.5PSB274, ALTRON Medical Electronics, Fürstenfeldbruck, Germany) was placed around the aorta, and the blood flow was measured for at least one minute. Endothelial denudation was induced by a single firm compression of the vessel with a forceps upstream of the flow probe. Blood flow was then monitored for 45 minutes or until complete occlusion occurred, the blood flow was equal to zero. After vessel occlusion, the flow probe was observed for another 40 minutes. If the occlusive thrombus was recanalized and blood flow was re-established, this was considered as embolisation. Where indicated, human FXII was injected intravenously directly before the experiment.

B.2.8. Data analysis

The results shown are mean ± SD or SE, respectively. Statistical analyses were performed using Student's *t* test or Wilcoxon rank test with $P < 0.05$ as level of significance.

C. Results

C.1. Multiple integrin ligand interactions are essential for shear-resistant platelet adhesion at sites of arterial injury *in vivo*.

At sites of vascular injury, the subendothelial extracellular matrix (ECM) is exposed to the flowing blood. The ECM comprises different components (including collagens, laminins, fibronectin, and von Willebrand factor) that are responsible for integrin-mediated platelet adhesion. In this process, collagen plays a central role, as it not only supports platelet adhesion but also serves as strong platelet activator[94]. To assess the role of individual platelet integrins in the process of platelet adhesion to the ECM and subsequent thrombus formation, different genetically modified mouse models were analysed.

C.1.1. Platelet adhesion and thrombus growth on the injured arterial wall is unaltered in $a2^{-/-}$ and ß1-null mice

Vascular injury and exposure of the native ECM to blood flow was induced by ligation of the carotid artery with a surgical filament. Parts of the intravital fluorescence microscopy analyses were performed in cooperation with Dr. Sabine Grüner. Platelet attachment and thrombus formation was assessed in mice lacking the integrin $\alpha 2$ or $\beta 1$ subunit as well as mice depleted of the major collagen receptor, GPVI. The $\alpha 2$ deficient platelets lack the collagen specific receptor, integrin $\alpha 2\beta 1$. Cre/loxP-mediated deletion of the integrin $\beta 1$ subunit in platelets causes deletion of $\alpha 2\beta 1$, $\alpha 5\beta 1$ (fibronectin receptor), and $\alpha 6\beta 1$ (laminin receptor). Unexpectedly, *in vivo* fluorescence microscopy revealed that platelet adhesion was indistinguishable between $\alpha 2^{-/-}$, $\beta 1$-null, and wild-type mice. In contrast, platelet adhesion and aggregate formation was virtually abolished in GPVI-deficient mice (Figure C.1 A), the latter confirming previous results[20]. In agreement with these early events, large platelet-rich thrombi were found in the injured arteries of wild-type and $\alpha 2$ or $\beta 1$-deficient mice after 20 minutes, whereas no thrombus had formed in GPVI-deficient mice (Figure C.1 B). This finding demonstrates that $\alpha 2\beta 1$ integrin as well as all other $\beta 1$ integrins are not essential for arterial thrombus formation in mice. However, it does not exclude a supportive role of the integrins in this process. On the other hand,

these results confim that GPVI-collagen interactions are essentially required for this process.

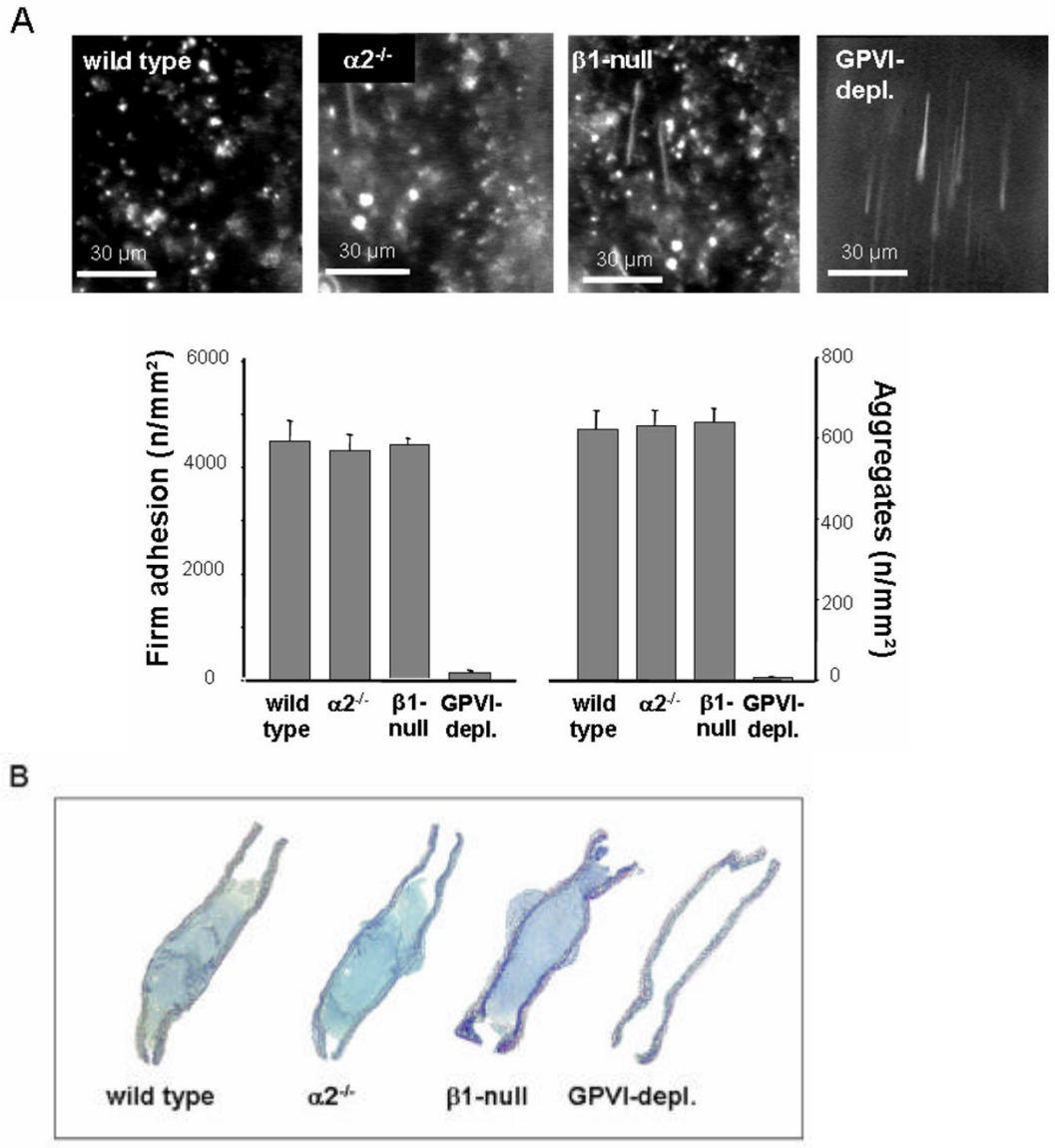


Figure C.1. Platelet adhesion and thrombus formation in a2$^{-/-}$ or b1$^{-/-}$ mice and GPVI-depleted mice. (A) Platelet-vessel wall interactions after vascular injury were investigated in wild-type, α2$^{-/-}$, β1-null, and GPVI-deficient mice by in vivo fluorescence microscopy of the common carotid artery in situ. Lower panel summarizes platelet adhesion and aggregate formation, respectively, of 7 experiments per group. Results are shown as mean ± SEM. (B) Representative histologic sections of carotid arteries 20 minutes after injury demonstrating large platelet-rich thrombi in wild-type, α2$^{-/-}$, β1-null, but not GPVI-deficient mice. Sections stained with toluidine blue/basic fuchsin; original magnification, x 5 [95].

C.1.2. Integrins a5ß1 and a6ß1 are involved in shear-resistant platelet adhesion on the ECM *in vivo*

According to previous results, other receptors can mediate shear-resistant platelet adhesion on the injured arterial wall in the absence of all β1 integrins. The αIIbβ3 receptor could mediate this adhesion, as it binds to VWF and fibronectin, both of

which are present in the ECM. To test this, wild-type mice received fluorescently labeled platelets preincubated with saturating concentrations (50 µg/mL) of $F(ab)_2$ fragments of the blocking anti-αIIbβ3 mAb JON/A[93] prior to carotid injury. Inhibition of the major platelet receptor αIIbβ3 causes 60% reduction in platelet adhesion to the vessel wall in wild-type as well as α2 mutated mice, whereas the adhesion in mice lacking the β1 integrin was nearly completely abolished (Figure C.2). The finding that the aIIbb3 blockade almost completely inhibited platelet adhesion in $\beta1^{-/-}$ but not $\alpha2^{-/-}$ mice demonstrated for the first time that integrins α5β1 and/or α6β1 can mediate shear-resistant platelet adhesion under arterial flow conditions *in vivo*.

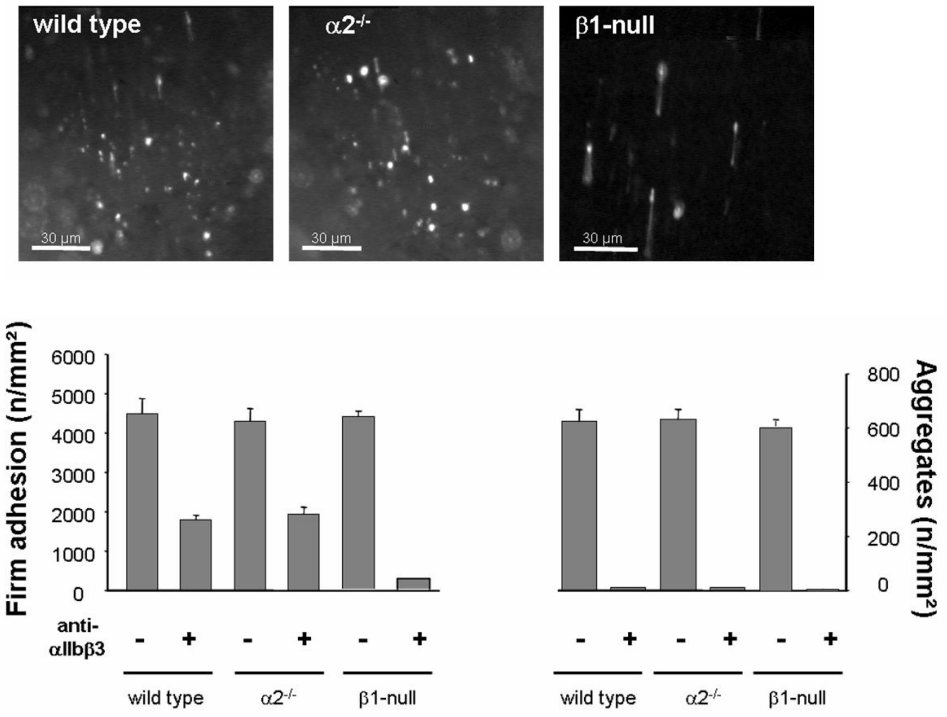


Figure C.2. Inhibition of αIIbβ3 abrogates platelet adhesion and aggregate formation in β1-null mice but not in $\alpha2^{-/-}$ mice. Platelet-vessel wall interactions after vascular injury were investigated in wild-type and $\alpha2^{-/-}$ or β1-null mice in the presence of αIIbβ3 blocking antibody by *in vivo* fluorescence microscopy of the common carotid artery in situ. Fluorescent wild-type and $\alpha2^{-/-}$ or β1-null platelets were preincubated with 50 µg/mL anti-αIIbβ3 (JON/A $F(ab)_2$ fragments) and injected into recipient mice of the same genotype. The lower panel summarizes platelet adhesion and aggregate formation with and without αIIbβ3 inhibition. The results are presented as mean ± SEM of 7 experiments per group[95].

C.1.3. The affinity of platelet a5ß1 and a6ß1 for their ligands is regulated by GPVI

GPVI-mediated integrin activation is an essential prerequisite for platelet adhesion to collagen *in vitro*[15] and platelet adhesion to the subendothelial matrix *in vivo* is virtually abolished in the absence of GPVI[20]. These observations suggested that the affinity of α5β1 and α6β1 for their ligands is also regulated by GPVI-dependent mechanisms.

To test this hypothesis, adhesion of wild-type and mutant platelets was studied on laminin, fibronectin, and, as a control, human VWF (hVWF) under static conditions *in vitro*. Virtually no adhesion of resting platelets on laminin was observed for up to 1 hour. In contrast, when platelets were activated with the GPVI-specific agonist collagen-related peptide (CRP, 0.2 µg/mL), robust adhesion of wild-type and $\alpha 2^{-/-}$ but not β1-null platelets occurred (Figure C.3 A), suggesting that α6β1 is essential to mediate platelet adhesion to laminin. A slightly different picture was observed when adhesion was studied on fibronectin. No adhesion of unstimulated platelets to this ligand was detected for up to 1 hour, whereas strong adhesion of wild-type, $\alpha 2^{-/-}$, and β1-null platelets occurred upon stimulation with CRP. However, when αIIbβ3 was blocked with JON/A (50 µg/mL), adhesion of wild-type and $\alpha 2^{-/-}$ platelets was retained, whereas adhesion of β1-null platelets was almost completely abrogated (Figure 3B). This finding suggests that the two major fibronectin-binding receptors on platelets are αIIbβ3 and α5β1 and that they can independently mediate adhesion to this ligand. Furthermore, these results demonstrate that the affinity of both integrins for fibronectin is up-regulated by GPVI-dependent signaling.

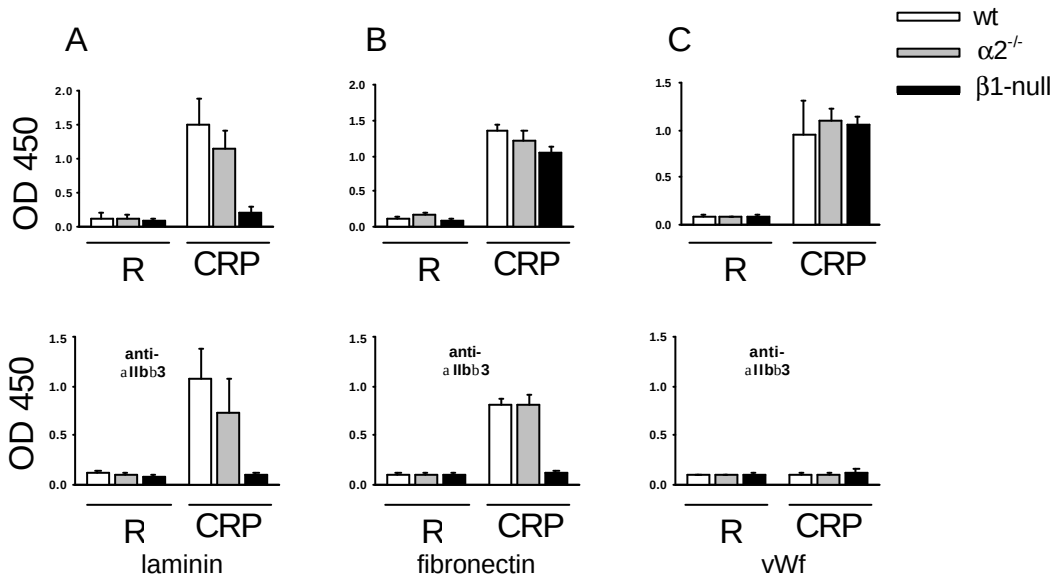


Figure C.3. Platelet adhesion to laminin, fibronectin, and VWF under static conditions. Resting or CRP-activated washed wild-type (□),$\alpha 2^{-/-}$ (▨), or β1-null (■) platelets were allowed to adhere for 60 minutes under static conditions to laminin (A), fibronectin (B), or VWF (C) immobilized to microtiter plates. The experiments were performed in the presence of Mg^{2+}/Ca^{2+} (1 mM each). Where indicated, platelets were preincubated with anti-αIIbβ3 antibody (JON/A, 50 µg/mL). Adherent platelets were quantitated fluorimetrically. The data shown are from a single experiment representative of 6 identical experiments, and are expressed as the mean of quadruplicate reading ± SD for the indicated times.

The normal function of αIIbβ3 in wild-type and mutant platelets was confirmed when adhesion was studied on hVWF. It is important to note that hVWF does not efficiently interact with mouse GPIbα[96]. Therefore, GPIb-dependent αIIbβ3 activation, which has been reported to induce adhesion of human platelets on hVWF[97], does not occur in this system. No adhesion of resting mouse platelets to this ligand was observed for up to 1 hour. However, when platelets were activated with CRP, strong adhesion of wild-type, and α2 or β1 deficient platelets occurred. As expected, this adhesion was completely blocked in the presence of the anti-αIIbβ3 mAb JON/A (50 μg/mL; Figure C.3 C).

Together, these results indicate that platelet attachment and thrombus formation at sites of vascular injury in mice can occur independently of α2β1 or even all β1 integrins on platelets. On the other hand, β1 integrins can mediate shear-resistant platelet adhesion independently of αIIbβ3, demonstrating that β1 and β3 integrins have redundant roles in this process and are regulated by similar mechanisms. At sites of arterial injury, GPVI-collagen interactions are a major trigger of this activation process[20]. However, since GPVI-deficient humans[98] and mice[7] display no major bleeding phenotype, it appears that other agonist receptors/signaling pathways can substitute for GPVI in mediating integrin activation in normal hemostasis. To address this question and better define the function of GPVI and α2β1 in hemostasis we depleted GPVI in $\alpha 2^{-/-}$ mice.

C.2. Anti GPVI treatment compromises hemostasis in the absence of a2ß1 integrin

In agreement with previous reports[7] injection of mice with the anti-GPVI mAb, JAQ1[16] (100μg/mouse), induced depletion of the receptor from the surface of circulating platelets, which was accompanied by a transient thrombocytopenia. However, on day 5 JAQ1-treated wild-type and $\alpha 2^{-/-}$ mice displayed normal platelet counts (Fig. C.4 A), but were GPVI-deficient as shown by analysis with the anti-GPVI mAb JAQ2[99] by flow cytometry (Fig. C.4 B). In contrast, other receptors, including GPIb and αIIbβ3, were not affected by the treatment.

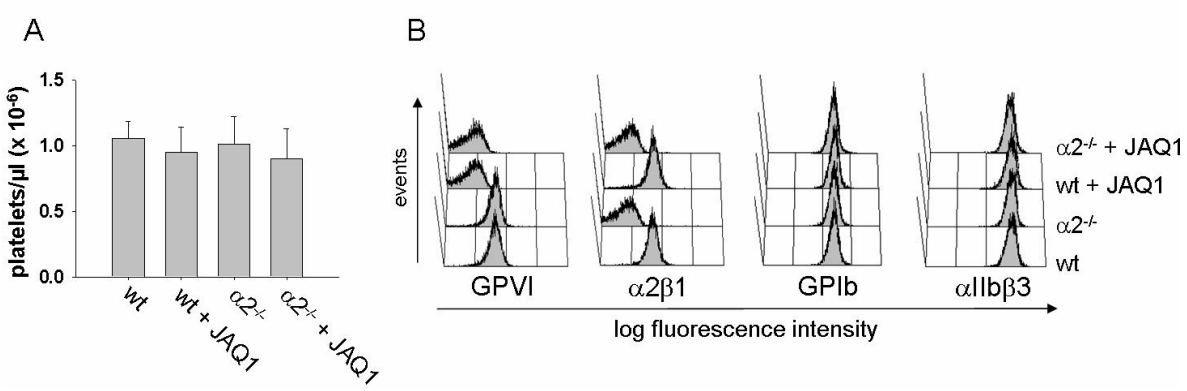


Figure C.4. Platelet count and glycoprotein expression. Wild-type (wt) or α2-deficient ($\alpha2^{-/-}$) mice were injected with 100 µg JAQ1 or irrelevant IgG i.p. On day 5 the mice had normal platelet counts (A) (n=8 per group). (B) Flow cytometric analysis of platelet glycoprotein expression. The results are representative of 8 mice per group.

C.2.1. GPVI depleted wild-type and $a2^{-/-}$ mice do not respond to collagen and display largely prolonged bleeding times

Due to the absence of GPVI, both wild-type and $\alpha2^{-/-}$ platelets did not aggregate in response to high doses of collagen (20 µg/ml), whereas they normally responded to other agonists such as adenosine diphosphate (ADP, 5 µM) or the stable TxA_2 analog U46619 (1 µM) (Fig. C.5 A). To test whether the α2/GPVI double deficiency has any effect under *in vivo* conditions, we determined tail bleeding times as a measure of primary hemostasis. As reported previously[7], JAQ1-treated wild-type mice only displayed slightly increased bleeding times. In marked contrast, however, in $\alpha2^{-/-}$ mice the same treatment induced a severe hemostatic defect, with 20 of 33 mice (60.6%) bleeding longer than 20 min (Fig. C.5 B). Furthermore, the mean bleeding time of the other 13 mice was 2.6 times increased as compared to JAQ1-treated wild-type mice. This finding demonstrated that the presence of either functional GPVI or α2β1 is required for normal primary hemostasis.

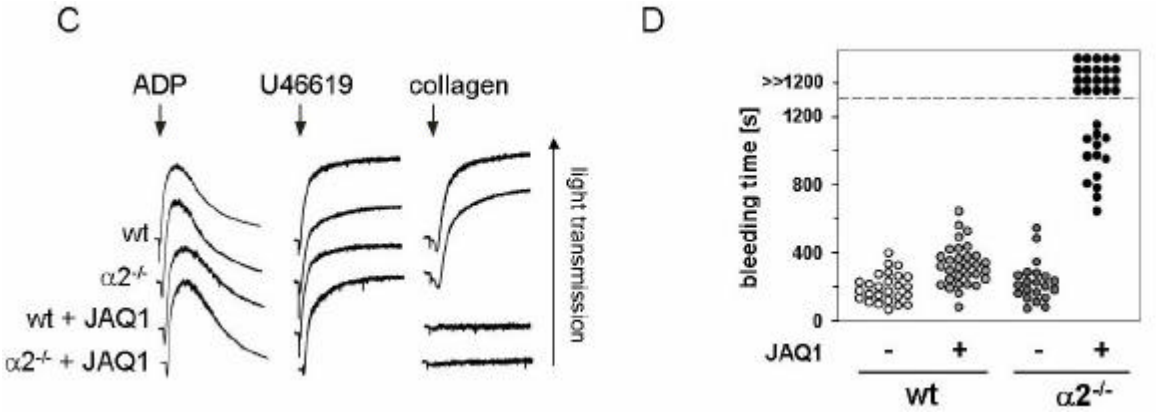


Figure C.5. Anti-GPVI treatment severely compromises hemostasis in $\alpha 2^{-/-}$ mice. (A) Platelet rich plasma from the indicated mice was stimulated with ADP, U46619, or collagen at 37°C under stirring conditions and light transmission was recorded. The results are representative of 8 mice per group. (B) Tail bleeding times (wt: n=26; $\alpha 2^{-/-}$: n=21; JAQ1-treated wt: n=31; JAQ1-treated $\alpha 2^{-/-}$: n=33). The experiment was stopped manually after 20 min. Each symbol represents one individual.

C.2.2. Half levels of a2ß1 are not sufficient to restore hemostatic alterations in the absence of functional GPVI

It is well documented that $\alpha 2\beta 1$ expression levels in platelets vary greatly (up to 10-fold) among the normal population due to several linked polymorphisms within the $\alpha 2$ gene[100]. To test whether reduced $\alpha 2\beta 1$ levels would interfere with the safety of anti-GPVI treatment, we examined $\alpha 2^{+/-}$ mice, which express 50% of normal $\alpha 2\beta 1$ on their platelets[53] (Fig. C.6 A). Five days after injection of 100 µg JAQ1 these mice had normal platelet counts and were GPVI-deficient (not shown). Unexpectedly, the anti-GPVI treatment resulted in a marked bleeding time prolongation in the heterozygous mice that was similar to that observed in the homozygous mutant mice with 8 of 14 animals (57.1%) bleeding longer than 20 min (Fig. C.6 B). Thus, a 50% reduction in the $\alpha 2\beta 1$ level is sufficient to induce a severe hemostatic defect in anti-GPVI treated mice. Although the data obtained in mice cannot be directly extrapolated to the situation in humans, these results strongly suggest that anti-GPVI treatment would severely impair primary hemostasis in humans with low $\alpha 2\beta 1$ levels thereby potentially increasing the risk of uncontrolled bleeding.

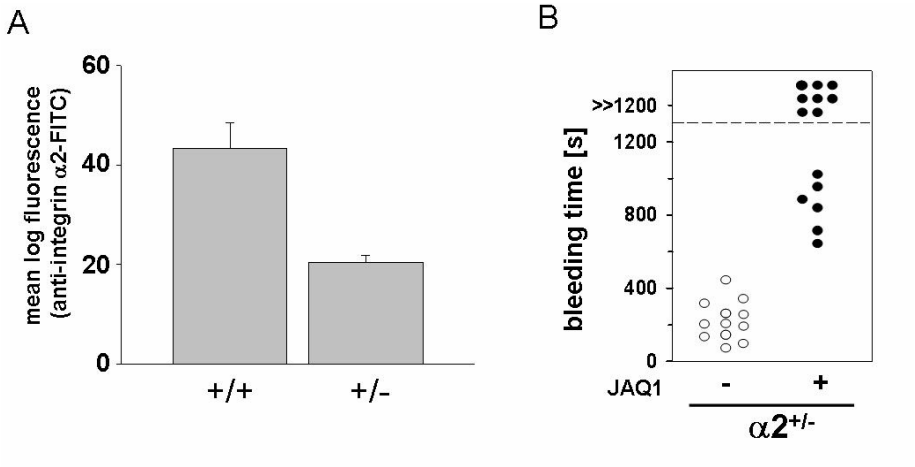


Figure C.6. Defective hemostasis in JAQ1-treated $\alpha 2^{+/-}$ mice. (A) Flow cytometric analysis of integrin α2-levels in wild-type (+/+) and heterozygous α2-knock out (+/-) mice. (B) Integrin $\alpha 2^{+/-}$ mice were injected with 100 µg JAQ1 (n=15) or irrelevant IgG (n=12) i.p. and bleeding times were determined on day 5. The experiment was stopped manually after 20 min. Each symbol represents one individual.

C.2.3. The adhesion of GPVI deficient platelets to collagen can be restored by integrin activation through G-protein coupled receptors signaling pathways

To examine the molecular determinants of hemostasis and in particular the role of α2β1 in the absence of functional GPVI, we assessed platelet adhesion in *ex vivo* whole blood perfusion studies. GPVI-deficient or -blocked platelets fail to adhere to collagen due to defective activation of α2β1 and αIIbβ3[15]. To test whether adhesion of GPVI-deficient platelets can be restored by integrin activation through other signaling pathways, we co-infused ADP, U46619, or a combination of the two agonists into the flowing blood (1000 s^{-1}) directly before entering the flow chamber[15]. In the case of ADP (5 µM), which induces reversible integrin activation at intermediate levels (Fig. C.7 A), both GPVI-depleted wild-type and $\alpha 2^{-/-}$ platelets formed large thrombi on the collagen surface within 2 minutes. These thrombi were, however, very unstable and completely washed out when the flow chamber was rinsed (data not shown). In contrast, co-infusion of U46619 (1 µM), which on its own induces weak but sustained integrin activation[39] (Fig. C.7 A), consistently resulted in firm adhesion of single GPVI-depleted wild-type, but not $\alpha 2^{-/-}$ or $\alpha 2^{+/-}$ platelets (Fig. C.7 B). When strong and sustained integrin activation was induced by the co-infusion of ADP and U46619 in combination[39] (Fig. C.7 A), GPVI-depleted wild-type, $\alpha 2^{-/-}$, and $\alpha 2^{+/-}$ platelets formed large thrombi on the collagen, the size and stability of which was comparable in all groups (Fig. C.7 B).

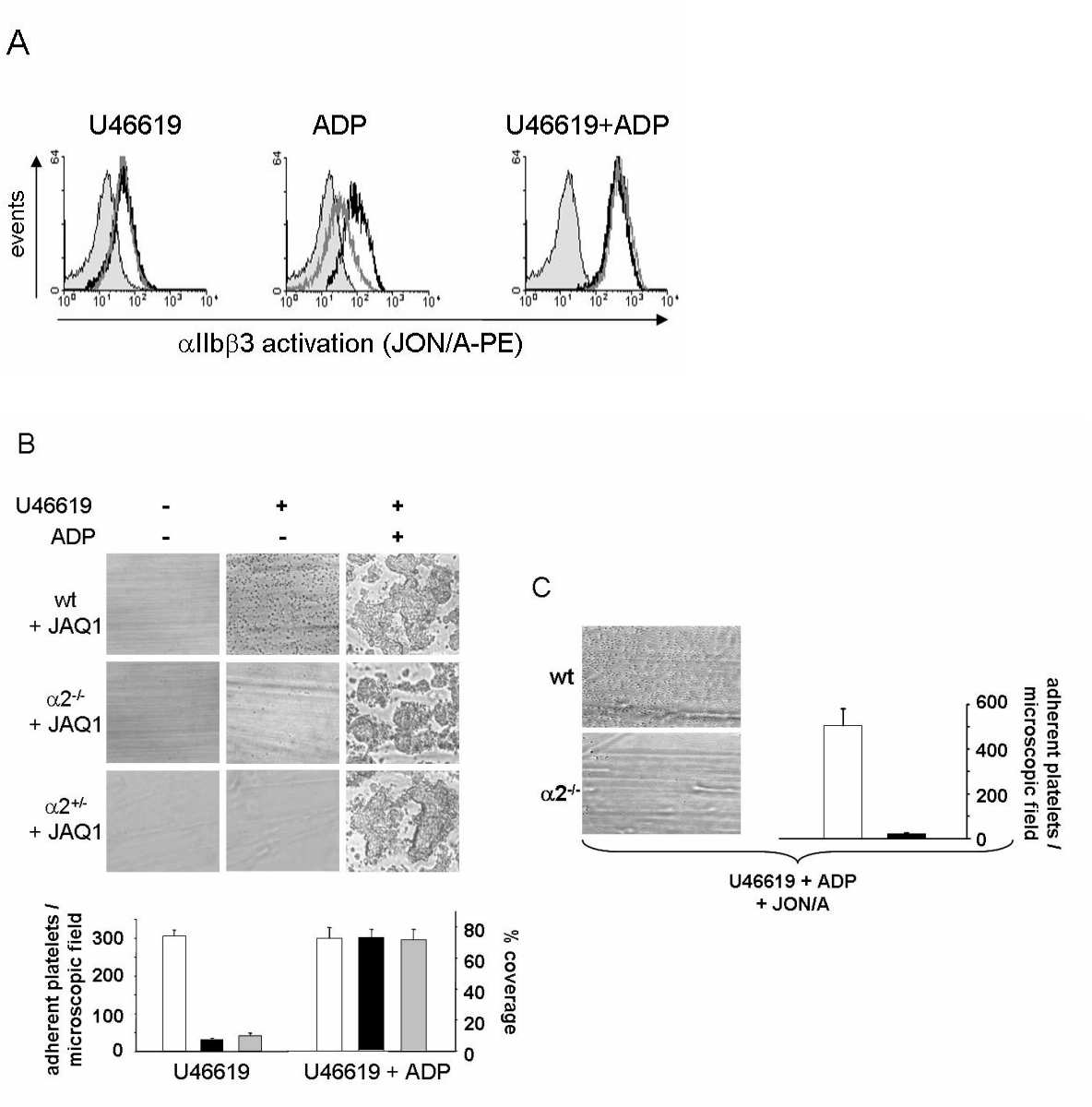


Figure C.7. Adhesion of GPVI-depleted platelets. (A) Integrin activation in platelets in response to different agonists. Washed wild-type platelets were stimulated with ADP, U46619, or a combination of both agonists and αIIbβ3 activation was determined with JON/A-PE after 1 min (black line) and 15 min (gray line). Non-activated platelets are indicated as gray histograms. The data shown are from wild-type platelets, but similar results were obtained with α2-deficient platelets. (B) Whole blood was perfused over a ´Horm type´ collagen-coated cover slip at high shear (1000 s^{-1}). Where indicated, agonists were co-infused to the blood. Upper panel: representative phase contrast images taken at the end of experiment. Lower panel: firmly adherent platelets or surface area covered by thrombi at the end of experiment of JAQ1-treated wt (white bars), $\alpha2^{-/-}$ (black bars), and $\alpha2^{+/-}$ mice (gray bars) (mean ± SD, n = 8). (C) Whole blood from JAQ1-treated wild-type (white bar) or $\alpha2^{-/-}$ (black bar) mice was preincubated with F´(ab)-fragments of the blocking anti-αIIbβ3 antibody, JON/A, and perfused as above together with U46619 and ADP. Representative phase contrast images (left) and numbers of firmly adherent platelets (mean ± SD, n = 8 per group) at the end of experiment are shown.

This α2β1-independent adhesion is mediated by αIIbβ3-vWF interaction as it was completely inhibited in the presence of F(ab) fragments of the anti-αIIbβ3 antibody, JON/A (30 μg/ml, Fig. C.7 C). These findings suggest that α2β1 plays a major role for shear-resistant platelet adhesion on collagen under conditions of weak, but not

strong integrin activation as under the latter conditions αIIbβ3 is sufficient to arrest the cells. This is in line with the well documented requirement for α2β1 for adhesion to monomeric or degraded collagen which is a weak agonist at GPVI and therefore only induces low-level integrin activation[101].

C.2.4. Aspirin-treated GPVI-depleted mice suffer from prolonged bleeding times

On fibrillar collagens, GPVI mediates strong and sustained integrin activation[101], which may explain why α2β1 is not required for hemostasis under normal conditions[53]. In anti-GPVI-treated mice, however, α2β1 is strictly required to arrest bleeding suggesting that the stimuli leading to integrin activation in the absence of functional GPVI must be rather weak, i.e. insufficient to induce αIIbβ3-dependent adhesion. We suspected TxA_2 to play a role in this process as *in vitro* this activation pathway induces α2β1-dependent adhesion of GPVI-deficient platelets to collagen (Fig. C.7 B). To test this hypothesis directly, we inhibited TxA_2 synthesis in control and JAQ1-treated wild-type mice (day 5) with aspirin (100 mg/kg) and determined the tail bleeding times 3 h after aspirin treatment. While aspirin had only a minor effect on bleeding times in control mice, it severely compromised hemostasis in anti-GPVI treated mice with 10 of 18 animals (55.5%) bleeding longer than 20 minutes (Fig. C.8 A). To further substantiate this surprising finding, we used FcRγ-chain-deficient mice, which fail to express GPVI on their platelets[16]. These mice are largely protected from arterial thrombus formation and subsequent neointimal hyperplasia[102] as well as collagen-induced thromboembolism but they display no increased bleeding tendency[7]. Treatment of these mice with aspirin, however, almost completely blocked hemostasis as shown by bleeding times longer than 20 min in 7 of 10 mice (Fig. C.8 A). These results demonstrate that the TxA_2-mediated activation pathway becomes crucial for primary hemostasis in the absence of functional GPVI suggesting that the safety of anti-GPVI treatment might be blunted in patients concomitantly taking aspirin at antithrombotic, analgetic, or antiphlogistic dosage.

In platelets, the TxA_2 receptor couples to the G_q and G_{12}/G_{13} signaling pathways, both of which are crucial for the formation of stable thrombi under flow conditions *in vitro* and *in vivo*[40;103]. To determine which of these signaling pathways is required for U46619-induced adhesion of GPVI-deficient platelets, we injected wild-type, $G\alpha_q$-, and $G\alpha_{13}$-deficient mice with JAQ1 and tested the adhesion of their platelets to

collagen under flow conditions *ex vivo* on day 5. Interestingly, U46619 was unable to induce adhesion of $G\alpha_q^{-/-}$ or $G\alpha_{13}^{-/-}$ platelets, demonstrating that both signaling pathways are essential for this process to occur (Fig. C.8 B).

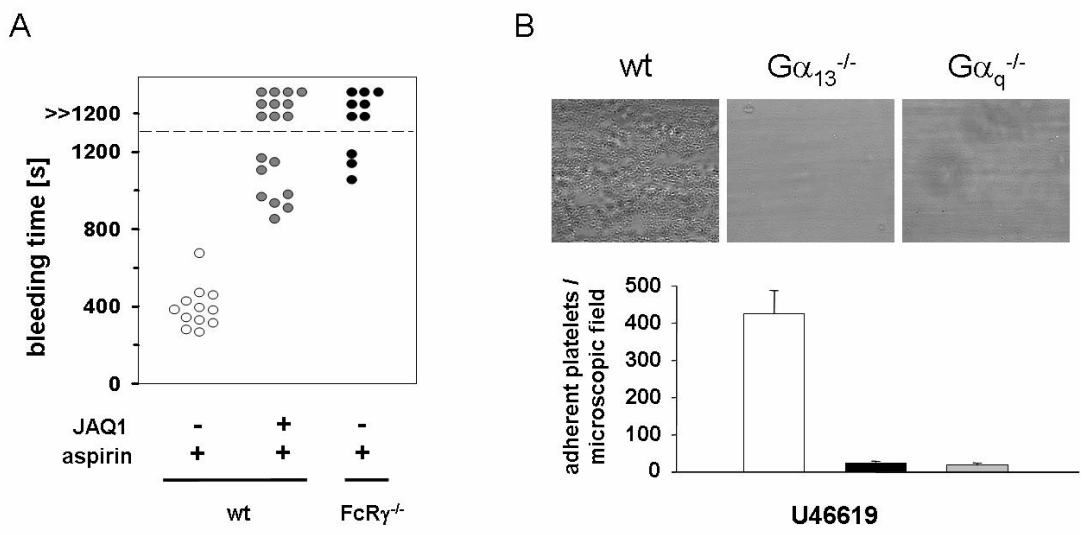


Figure C.8. TxA_2 mediated activation is required for hemostasis in GPVI-deficient mice. (A) Control (n=12), JAQ1-treated (day 5, n=18), or $FcR\gamma^{-/-}$ (n=9) mice received 100 mg/kg acetylsalicylic acid (ASA) and bleeding times were determined after 3 h. The experiment was stopped manually after 20 min. (B) Wild-type (wt, white bar), $G\alpha_{13}^{-/-}$ (black bar), and $G\alpha_q^{-/-}$ (gray bar) were treated with JAQ1. On day 5, whole blood was perfused over a ´Horm type´ collagen-coated coverslip (1000 s^{-1}). Platelets were stimulated by the co-infusion of 1 µM U46619. Upper panel: representative phase contrast images taken at the end of the experiment. Lower panel: firmly adherent platelets (mean ± SD, n = 4 per group).

C.3. Thrombosis models

The above described experiments have demonstrated that multiple integrin-ligand interactions are essential for normal platelet adhesion and aggregation. Additionally, they revealed that GPVI is the major receptor for collagen-induced activation of other platelet receptors, both *in vitro* and *in vivo*. GPVI-depleted platelets fail to firmly adhere to immobilized collagen under conditions of high and low shear, due to defective activation of $\alpha 2\beta 1$ and $\alpha IIb\beta 3$ integrins[15]. On the other hand, mice lacking GPVI show no major bleeding phenotype, but are profoundly protected from arterial thrombosis[7]. However, depletion of GPVI from $\alpha 2^{-/-}$ mice strongly compromises hemostasis. This indicates that the loss of GPVI-signaling in GPVI depleted wild-type mice is compensated by a network of different G-protein-mediated signaling pathways which converge in the activation of $\alpha 2\beta 1$ allowing it to arrest the cells and to reinforce the activation through "outside-in" signals. Receptors expressed on the platelet surface that couple to G-proteins are receptors for soluble agonists, including ADP, thrombin, adrenaline, and TXA_2. These agonists sustain the initial platelet

responses and recruit circulating platelets from the flowing blood into a growing hemostatic plug. Subsequently, the plasma coagulation system contributes to the formation of a fibrin rich clot which is critical for limiting posttraumatic blood loss. However, this process may also occlude diseased vessels inducing infarction in vital organs, which is still the leading cause of death in developed countries. To investigate the role of single platelet receptors and plasma coagulation factors in such processes, suitable *in vivo* arterial thrombosis models had to be established in the laboratory. Arterial lesions can be induced in different branches of the vascular system[91]. For our purposes, the injury was induced on mesenteric arteries and the abdominal aorta.

C.3.1. Intravital fluorescence microscopy model of ferric chloride induced injury on mesenteric arteries

The mesenteric artery model is based on a method previously described by Denis *et al.*[104]. Arterial injury was induced by topical application of ferric chloride ($FeCl_3$) leading to the formation of free radicals and disruption of the protective endothelium. This treatment causes platelets to come into contact with the ECM, which results in their activation and formation of three-dimensional platelet-rich occlusive thrombi. To study single platelet adhesion and subsequent formation of occlusive thrombi, fluorescently labeled platelets obtained from a donor mouse of the same genetic background were intravenously injected. A drop of solubilised $FeCl_3$ was then applied on the exteriorized and visualized arteries using a filter paper tip (Figure C.9).

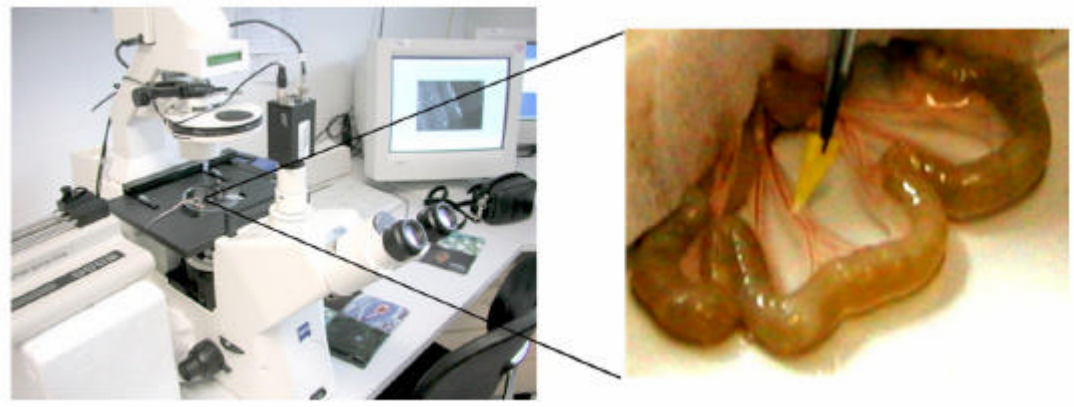


Figure C.9. Topical application of $FeCl_3$ saturated on filter paper tip.

Several approaches were undertaken to define the optimal concentration of $FeCl_3$ (Figure C.10). Experiments performed with 10, 30 and 50% $FeCl_3$ revealed hardly reproducible results, whereas injury induced using 20% $FeCl_3$ consistently resulted in formation of occlusive thrombi usually within 20 – 35 minutes, which corresponds to those described by others[31;56;91].

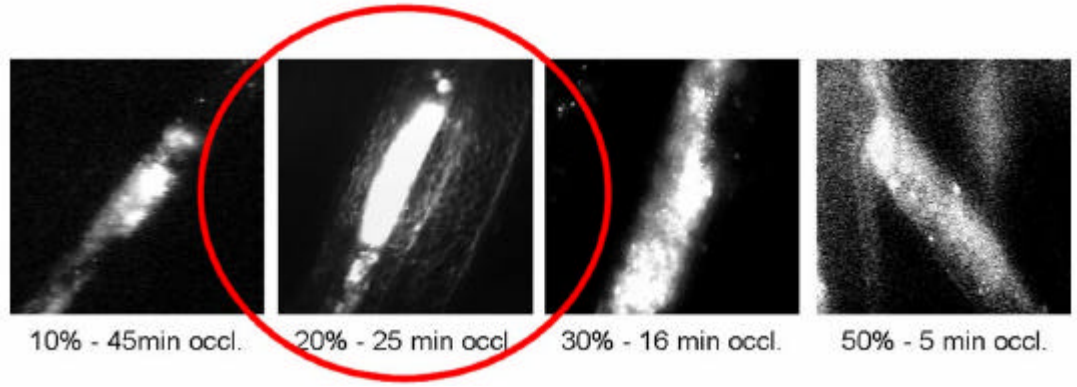


Figure C.10. Representative pictures of formed thrombi after injury induced by different $FeCl_3$ concentrations.

Arterioles were monitored for 40 min or until complete occlusion occurred, i.e. blood flow stopped for more than 1min (Figure C.11). Firm platelet adhesion was determined as the number of platelets that were deposited on the vessel wall until 5 minutes after injury. A thrombus was defined as a platelet aggregate larger than 10µm in diameter. Thrombus stability was characterized as number of embolus events (thrombi in diameter larger than 10µm) that detached during the observation period from the formed thrombi.

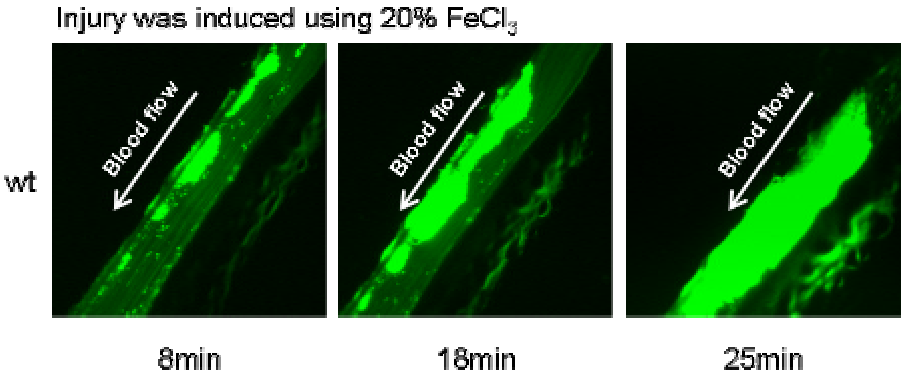


Figure C.11. Formation of occlusive thrombi in wild-type (C57Bl/6) mice.

To exclude artificial effects of $FeCl_3$ on platelets and to test whether thrombus formation in this model depends on activated platelets, the major platelet receptor αIIbβ3 was inhibited with the blocking monoclonal antibody JON/A[93]. Wild-type mice were injected, and donor platelets were preincubated with F(ab)`2 fragments of the antibody. Injury was induced as described above. This treatment still allowed single platelet adhesion, but formation of thrombi or platelet aggregates was completely abolished (Figure C.12).

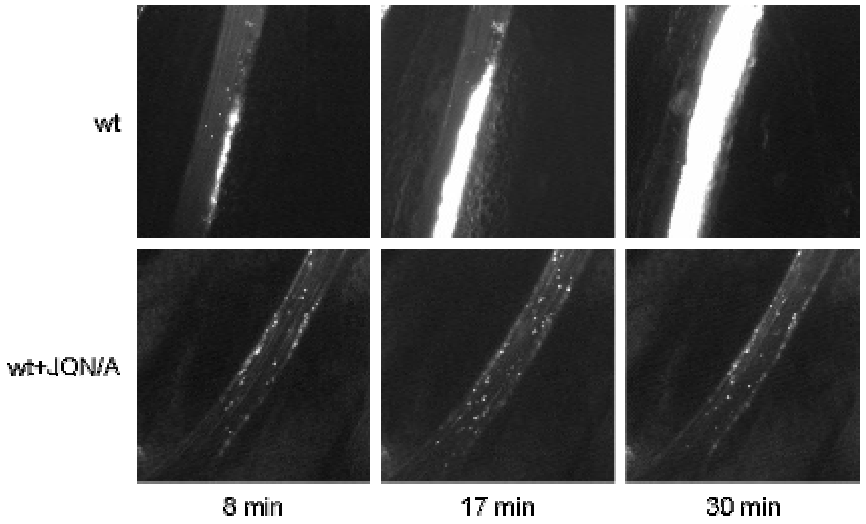


Figure C.12. Inhibition of αIIbβ3 receptor blocks thrombus formation in $FeCl_3$ induced arterial injury.

These results confirmed that thrombus formation in this model depends on αIIbβ3 activation, which leads to recruitment of new platelets from the blood stream and subsequent aggregate formation and thrombus growth. Therefore, this model was used to assess the mechanisms underlying thrombus formation and stabilization.

C.3.2. *In vivo* thrombosis model of injury induced to the abdominal aorta

At present, the exact mechanism underlying thrombotic processes is not well understood. Thus, it was important to establish another *in vivo* thrombosis model, in which a different type of injury is induced in a distinct branch of the vascular system. Thereby, it is possible to not only compare the two models to each other, but also to better study the mechanisms and kinetics of thrombus formation.

In the second model, an ultrasonic flow probe was placed around the abdominal aorta (Figure C.13) which measures blood flow. Endothelial denudation was induced

mechanically by firm compression with a tweezer. This treatment generally leads to the formation of occlusive thrombi in wild-type mice within 10 minutes after injury.

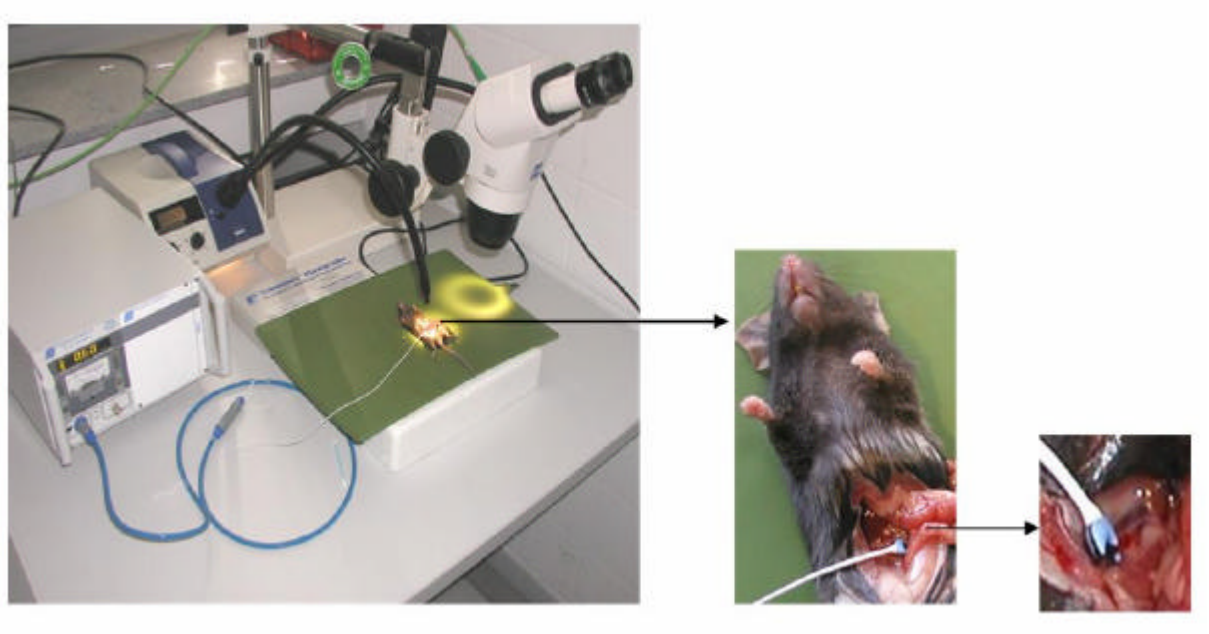


Figure C.13. Ultrasonic flow probe blood flow measurement in the abdominal aorta.

Blood flow is measured before and after injury for 45 minutes or until complete occlusion occurs, defined as a blood flow is 0 mL/min. Re-establishment of blood flow is interpreted as embolisation.

Platelet contribution to vessel occlusion was tested using F(ab)`2 fragments of the monoclonal antibody JON/A, which inhibits αIIbβ3. This treatment inhibited formation of occlusive thrombi, confirming the requirement for activated platelets in this process (Figure C.14.).

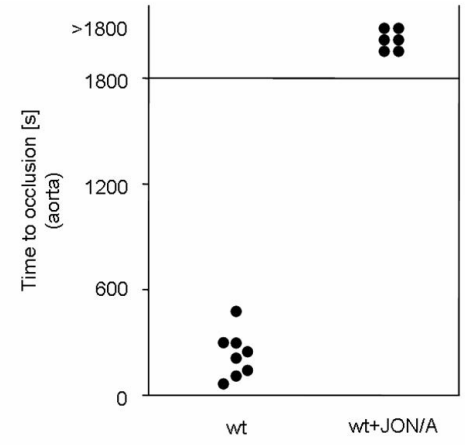


Figure C.14. Inhibition of the αIIbβ3 receptor blocks the formation of occlusive thrombi.

The two above described *in vivo* thrombosis models were further used to investigate the role of the soluble platelet agonist, adrenaline as well as the contribution of plasma coagulation factor XII in the process of thrombus formation and stabilization.

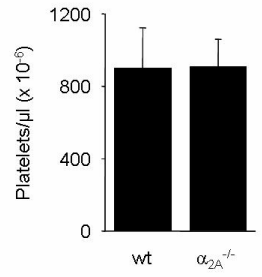
C.4. Reduced thrombus stability in mice lacking the a2A-adrenergic receptor

As shown in C.2.3 the role of GPVI and G-protein coupled receptors in platelet activation is partly redundant. This is also supported by the fact that concomitant stimulation of dimerized GPVI and G_i coupled receptors results in irreversible $\alpha IIb\beta 3$ activation whereas stimulation of either receptor alone does not lead to activation of the fibrinogen receptor[35]. It was previously reported that the G_i coupled ADP receptor, $P2Y_{12}$, plays an important role in thrombus formation and stabilization as its deficiency dramatically affects the thrombotic process *in vivo*[31]. It was therefore important to investigate the role of α2A, which couples to G_z, another G_i family member in the process of thrombus formation and stabilization *in vivo*. The α2A adrenergic receptor is the adrenaline receptor on the platelet surface. Although this receptor has been known for many years and its role in platelet activation *in vitro* is well established, only very little is known about its function *in vivo*. To address this question, mice lacking α2A were analysed.

C.4.1. Platelets from a2A$^{-/-}$ mice respond normally to thrombin-, CRP- or collagen-induced activation

Wild-type and α2A deficient mice had comparable platelet counts as detected by flow cytometry (Figure C.15 A). Deletion of α2A from the platelet surface did not lead to changes of any other tested membrane receptors including GPIb and $\alpha IIb\beta 3$ (Figure C.15 B).

A



B

glycoprotein	wt	$\alpha_{2A}^{-/-}$
GPVI	49,1 ± 2,3	48,6 ± 3,2
integrin αIIbβ3	396,1 ± 83,7	353,4 ± 46,6
GPV	264,6 ± 38,6	269,4 ± 26,4
GPIbα	641,8 ± 128,1	656,0 ± 71,4
GPIX	433,4 ± 60,4	405,6 ± 39,9
integrin α2	38,2 ± 2,3	36,6 ± 1,9
integrin β1	147,0 ± 38,8	138,1 ± 27,3
CD9	1225,4 ± 106,8	1192,5 ± 85,7

Figure C.15. Platelet count and glycoprotein expression in a2A$^{-/-}$ mice. (A) Flow cytometric analysis of platelet count and (B) platelet glycoprotein expression of wild-type and α2A adrenergic knockout mice. Results are expressed as mean ± SD for groups of six mice.

Dose-dependent activation of the αIIbβ3 receptor (Figure C.16 A) and P-selectin expression (not shown) induced by thrombin and collagen related peptide (CRP) was not affected in platelets lacking α2A. Platelet aggregation in response to several collagen concentrations was not influenced by α2A deficiency (Figure C.16 B). Whole blood perfusion studies were performed under high shear flow conditions over a collagen-coated surface. As shown in Figure C.16 C, no difference was detected in the surface coverage of formed thrombi from wild-type and $\alpha 2A^{-/-}$ blood samples.

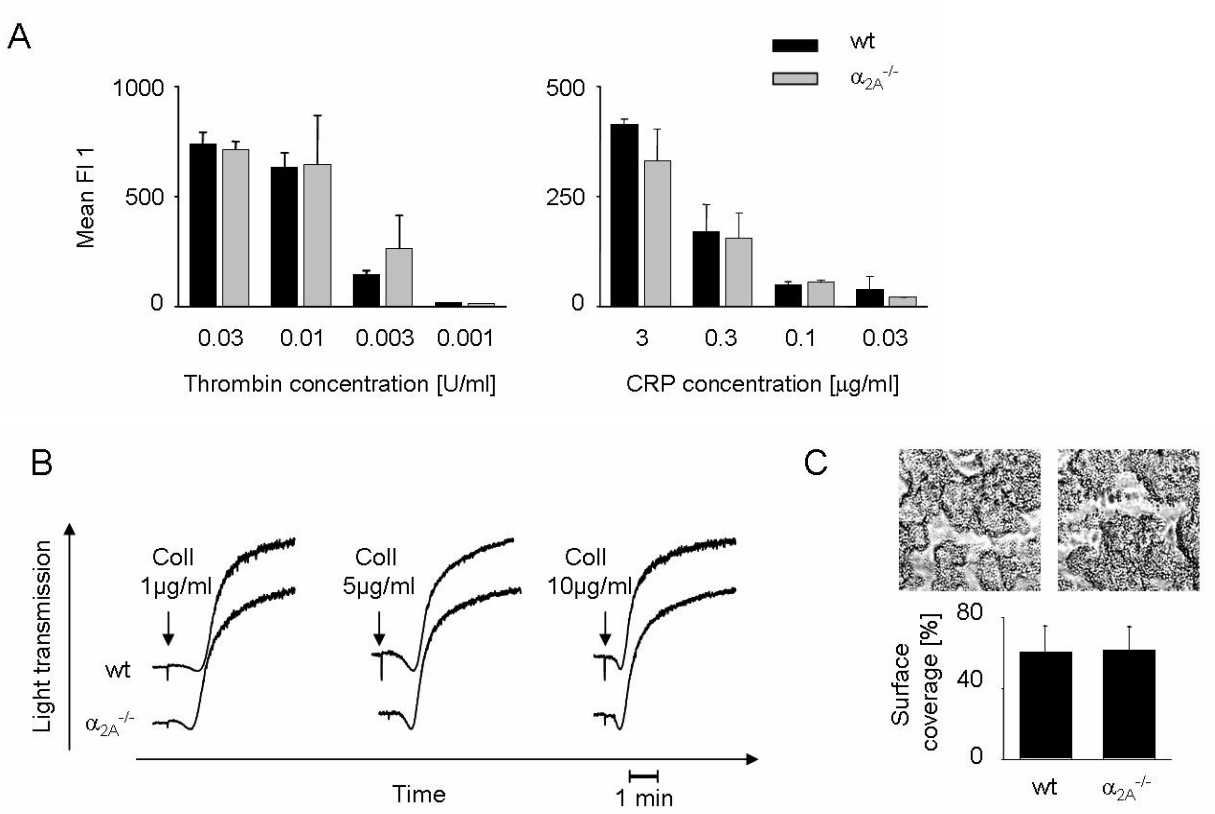


Figure C.16 Unaltered platelet responses to thrombin and collagen in a $2A^{-/-}$ mice.
(A) Diluted whole blood was incubated with different concentrations of thrombin and CRP as indicated. Activation of αIIbβ3 and P-selectin expression (not shown) was analysed by flow cytometry. Results are presented as mean ± SD of 5 mice per group. (B) Heparinized PRP was stimulated with indicated concentrations of fibrilar collagen ("Horm") and light transmission was recorded on a Fibrintimer 4 channel aggregometer. The results shown are representative of 6 individual experiments. (C) Whole blood was perfused over a "Horm type" collagen-coated cover slips at high shear (1000 s^{-1}). Upper panel, phase contrast images taken at the end of the experiment. Lower panel, surface area covered by thrombi at the end of each experiment of wild-type and $\alpha 2A^{-/-}$ mice (mean ± SD, n = 6).

C.4.2. Abnormal responses to adrenaline of platelets obtained from a2A-deficient mice

Aggregometry studies were performed with platelet rich plasma (PRP) obtained from wild-type and $\alpha 2A^{-/-}$ mice. Adenosine diphosphate (ADP, 5µM) induced reversible aggregation of both, α2A as well as wild-type platelets. In combination with adrenaline (10µM), which alone does not induce aggregation, platelets from wild-type

mice responded with full, irreversible aggregation. In contrast, adrenaline failed to enhance reversible aggregation of α2A-deficient platelets (Figure C.17 A). High concentrations of the stable TxA_2 analog U46619 (1µM) induced full aggregation of wild-type and $\alpha2A^{-/-}$ platelets. As expected, neither wild-type nor $\alpha2A^{-/-}$ platelets aggregated at low concentration (0.1µM) of the agonist. Costimulation with adrenaline and low concentration of U46619 caused full aggregation of wild-type, but not $\alpha2A^{-/-}$ platelets did not aggregate (Figure C.17 A), showing that adrenaline is without effect on $\alpha2A^{-/-}$ platelets.

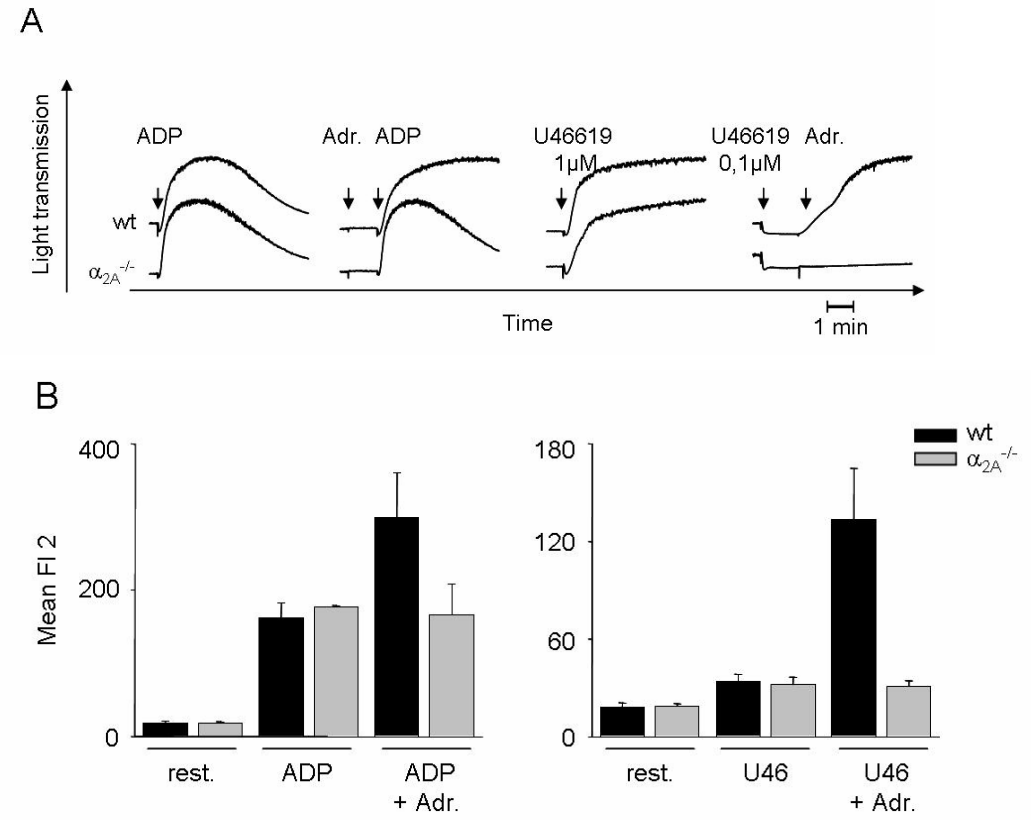


Figure C.17. *In vitro* characterization of α2A-deficient platelets. (A) Heparinized PRP was stimulated with ADP (5µM) and U46619 (1µM or subtreshold concentration 0.1µM) in the presence or absence of 10µM adrenaline and light transmission was recorded on a Fibrintimer 4 channel aggregometer. The results shown are representative of 6 individual experiments. (B) Washed whole blood from wild-type or $\alpha2A^{-/-}$ mice was incubated with 5µM ADP and 1µM U46619 (U46) in the presence or absence of 10µM adrenaline for 15 min at RT. Activation of αIIbβ3 and P-selectin expression was analysed by flow cytometry. Results are presented as mean ± SD of 5 mice per group.

In order to further extend the finding that adrenaline plays a supportive role in platelet activation, flow cytometric studies were performed. In these studies, the activated form of the αIIbβ3 receptor was detected using the specific antibody JON/A-PE[93]. Washed whole blood from wild-type and $\alpha2A^{-/-}$ mice was incubated with ADP (5µM) or U46619 (1µM) in the presence or absence of adrenaline (10µM). Activation of

αIIbβ3 on wild-type platelets was enhanced when adrenaline was added, whereas there was no additional effect of adrenaline on α2A-deficient platelets (Figure C.17 B).

C.4.3. The a2A receptor is involved in primary hemostasis

In tail bleeding time assays, all wild-type mice arrested bleeding within 10 minutes after the tail tip segment was cut. In contrast, $\alpha 2A^{-/-}$ mice displayed very variable bleeding times. In addition, 6 of 31 (19.35%) of the mutant mice were not able to stop bleeding within the 20 minutes observation period (Figure C.18 A).

To analyse platelet function *in vivo*, wild-type and $\alpha 2A^{-/-}$ mice were intravenously injected with collagen/adrenaline (0.8mg/kg / 60µg/kg), which causes lethal pulmonary thromboembolism[7]. All wild-type mice died within 5 minutes after injection, whereas more than 80% of mice lacking α2A survived and were able to completely recover. This suggests that the α2A receptor is the essential receptor for adrenaline responses induced *in vivo*. Mice deficient in FcRγ, which also lack the major collagen receptor, GPVI, on the platelet surface were used as controls[7]. All injected control mice survived the challenge (Figure C.18 B).

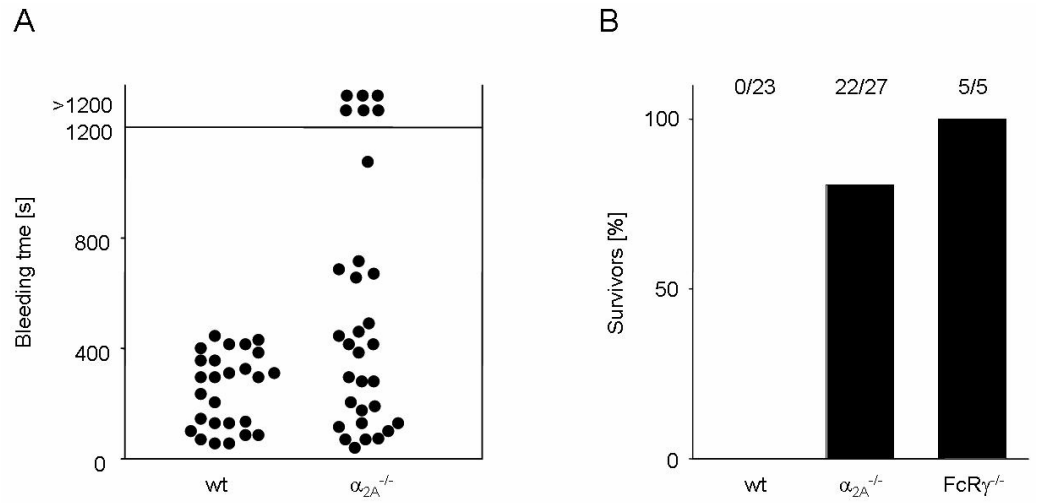


Figure C.18. The a2A adrenergic receptor plays an important role in hemostasis; mice lacking a2A are largely protected from lethal pulmonary thromboembolism. (A) Mice were anesthetized and a 3 mm segment of the tail tip was cut off with a scalpel. Tail bleeding times of wt (n=26) and $\alpha 2A^{-/-}$ (n=31) were monitored by gently absorbing the drop of blood. When no blood was observed on the paper after 15 second intervals, bleeding was determined to have ceased. The experiment was stopped after 20 minutes. Each symbol represents one individual. (B) Thromboembolic mortality was observed following the injection of collagen (0.8 mg/kg) and adrenaline (60µg/kg). All wild-type mice died (23/23), while 81.48% (22/27) of $\alpha 2A^{-/-}$ mice and all of injected $FcR\gamma^{-/-}$ mice survived (5/5).

C.4.4. Reduced thrombus stability in a2A$^{-/-}$ mice

To investigate the relevance of α2A in pathological occlusive thrombus formation *in vivo*, $FeCl_3$ injury was induced on mesenteric arterioles and thrombus formation was assessed by *in vivo* fluorescence microscopy. Mice were anesthetized and injury was induced after intravenous injection of fluorescently labeled syngeneic platelets. Firm platelet adhesion on the denuded vessel wall was determined 5 min after injury. No significant differences were observed between wild-type and α2A$^{-/-}$ mice (Figure C.19 A), suggesting that the α2A adrenergic receptor is not essential for platelet adhesion at high shear conditions *in vivo*.

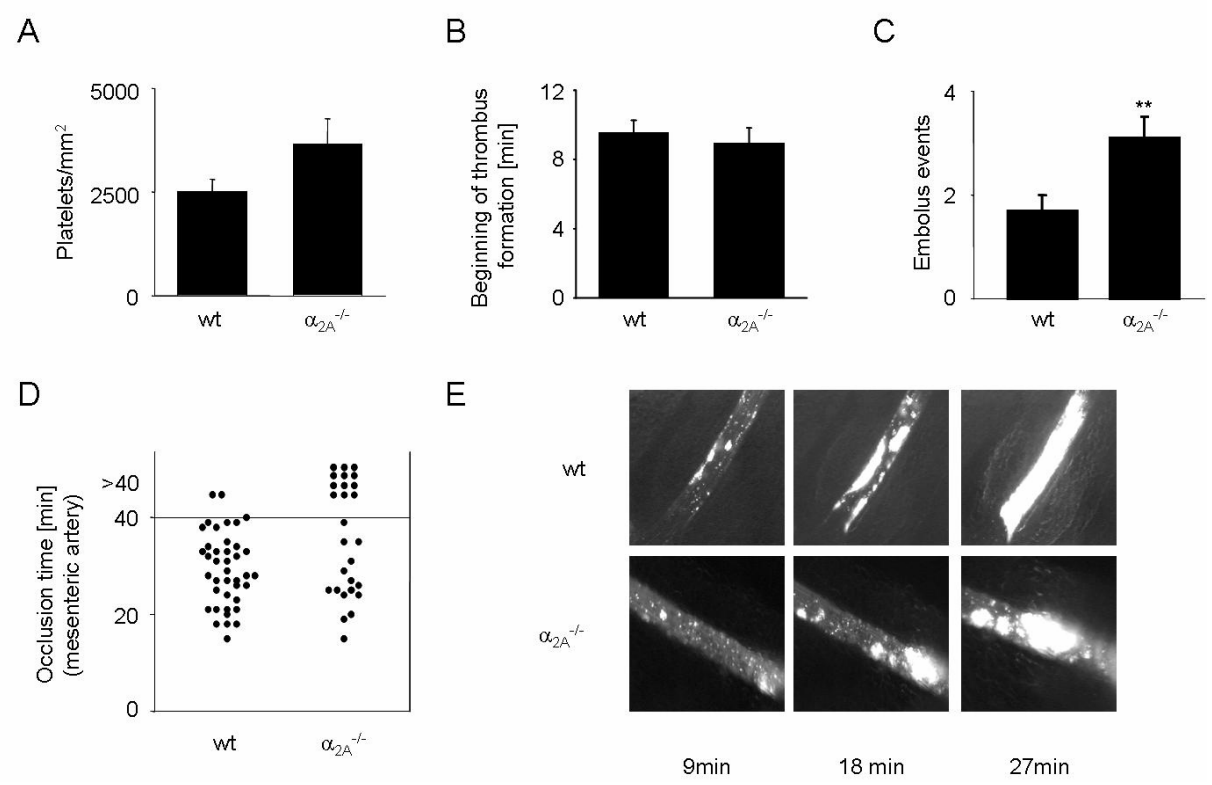


Figure C.19. Enhanced embolus formation in a 2A$^{-/-}$ mice. (A) Single platelet adhesion is detected 5 min after injury in both, wild-type and α2A$^{-/-}$ mice. (B) The first thrombi larger than 10µm were observed 9.5 ± 4.0 minutes after injury in wild-type and 9.0 ± 4.2 min after injury in mutant mice. (C) Quantitative analysis of embolus formation in control (1.7 ± 0.32) and mutant mice (3.1 ± 0.39) are presented as amount of thrombi which detached during the observing period from the viewing field. Results are presented as mean ± SEM. (D) Thrombi formed in wild-type mice occluded the artery in 95% whereas in α2A$^{-/-}$ mice only 55.6% of formed thrombi were able to occlude. Each symbol represents one single monitored arteriole. (E) For better illustration, representative pictures of one experiment are shown, indicated time points represent minutes after $FeCl_3$-induced injury.

On average 9 min after $FeCl_3$ application the first thrombi were observed in both, control and mutant mice (Figure C.19 B). Interestingly, thrombi formed in α2A$^{-/-}$ mice were able to occlude the artery in 55.6% only, whereas 95% of the injured vessels in

wild-type mice occluded (Figure C.19 C). This reduced occlusion rate was due to thrombus instability and increased embolus formation as compared to wild-type mice. The number of embolus events in $\alpha 2A^{-/-}$ mice was twice as high as in wild-type mice (3.1 ± 0.39 vs.1.7 ± 0.32) (Figure C.19 D). These results demonstrate for the first time that the α2A receptor plays a role in thrombus stabilization *in vivo*. Representative pictures of one experiment are shown in Figure C.19 E.

A second *in vivo* injury model was applied to confirm the finding that α2A is involved in the formation of stable thrombi. In this model, thrombosis was induced mechanically in the aorta by firm compression with a forceps and time to occlusion was determined. Blood flow was monitored with an ultrasonic flow probe until complete occlusion occurred. After a transient increase directly after injury, blood flow progressively decreased for several minutes in all animals. Subsequently, in all tested wild-type mice (17/17) this decrease resulted in complete and irreversible occlusion of the vessel within 0.5 to 13.1 min after injury (mean occlusion time 4.0 ± 4.5 min). In contrast, stable thrombus formation was defective in $\alpha 2A^{-/-}$ mice. Occlusive thrombi occurred in 75.86% of all $\alpha 2A^{-/-}$ mice only (22 of 29), and in the remaining 24% (7 of 29) blood flow was re-established between 10 s and 453 s after occlusion (Figure C.20). This result confirmed that the α2A adrenergic receptor plays a role in thrombus formation and stabilization *in vivo*.

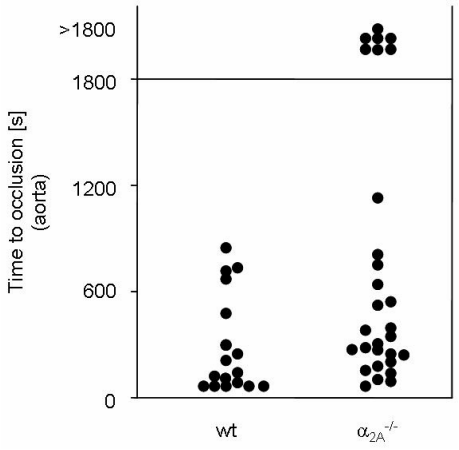


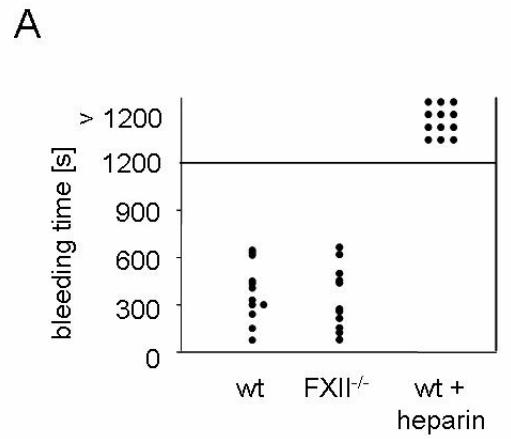
Figure C.20. Compromised thrombus stability in mice lacking the α2A receptor. Thrombosis was induced in the aorta by one firm compression with a forceps. Blood flow was monitored with a perivascular ultrasonic flow probe until complete occlusion. The experiment was stopped after 40 min. Each symbol respresents one individual.

C.5. Defective thrombus formation in mice lacking coagulation factor XII

Injury to a blood vessel triggers activation of blood platelets as well as the plasma coagulation system, leading to formation of a blood clot containing platelets and fibrin. Plasma coagulation proceeds through a series of sequential activations of plasma serine proteases culminating in the generation of thrombin, which converts plasma fibrinogen to fibrin. Thrombin also activates platelets, and activated platelets, in turn, facilitate thrombin generation by exposing procoagulant phosphatidylserine (PS) on the outer surface of their membranes. Induction of fibrin clot formation through intrinsic pathway is initiated when factor XII (FXII, Hageman factor) comes into contact with negatively charged surfaces (contact activation). Although FXII is activated by a variety of polyanions, including constituents of subendothelial matrix (glycosaminoglycans, collagens, sulfatides, and nucleosomes) and non-physiological materials (glass, ellagic acid, kaolin, cilica)[68;105] the mechanism(s) responsible for FXII activation *in vivo* are not known. Induction of fibrin clot formation through contact activation-mediated activation of FXII is the basis of the activated partial thromboplastin time (aPTT) assay. Despite its obvious importance to blood coagulation *in vitro*, the pathophysiologic significance of the FXII-triggered intrinsic pathway of coagulation has been questioned for more than 50 years. Therefore, mice deficient in FXII were used to assess the *in vivo* significance of the intrinsic pathway of coagulation in thrombus formation.

C.5.1. Mice deficient in FXII do not suffer from prolonged bleeding time

Mice homozygous for the FXII null allele ($FXII^{-/-}$) are healthy, fertile, and phenotypically indistinguishable from their wild-type littermates. Similar to humans, $FXII^{-/-}$ mice do not suffer from spontaneous hemorrhage or pathological vascular thrombosis[106]. Moreover, they do not display prolonged bleeding compared to wild-type mice in a tail bleeding time assay (369.5 ± 201.7 and 355.9 ± 176.1 sec, respectively) (Figure C.21 A), despite having prolonged plasma aPTT (71 ± 18 sec) and recalcification times (412 ± 78 sec; wild-type values are 25 ± 4 and 210 ± 31 sec, respectively, Figure C.21 B). Furthermore, the prothrombin time (PT) of $FXII^{-/-}$ mouse plasma (a measure of TF-initiated coagulation) is similar to wild-type plasma (Figure C.21 B). Peripheral blood cell counts in $FXII^{-/-}$ and wild-type mice did not differ (Figure C.19 B).



B

	wt	FXII$^{-/-}$
Platelets	1142 ± 81	1176 ± 141
WBC	7.2 ± 2.3	6.2 ± 1.6
Lymphocytes	6.5 ± 2.9	5.6 ± 2.1
Monocytes	0.05 ± 0.03	0. 04 ± 0.02
Neutrophils	0.65 ± 0.24	0.57 ± 0.21
Erythrocytes	9.3 ± 0.4	9.2 ± 0.7
aPTT [sec]	25 ± 5	71 ± 18
PT [sec]	9.1 ± 1.3	8.9 ± 1.3
recalc. time [sec]	210 ± 31	412 ± 78

Figure C.21. Hemostasis and coagulation in FXII deficient mice. (A) Tail bleeding times of wild-type (n=12) and FXII$^{-/-}$ (n=11) mice. Each symbol represents one individual. (B) Peripheral blood counts in thousands/µl and coagulation parameters for FXII$^{-/-}$ and wild-type mice. The abbreviations are white blood cells (WBC), activated partial thromboplastin time (aPTT) and prothrombin time (PT). Values given are mean values ± SD of 10 mice for each genotype.

C.5.2. FXII contributes to collagen-induced thromboembolism *in vivo*

To determine the consequences of FXII deficiency *in vivo*, we first tested wild-type and FXII$^{-/-}$ mice in a model of lethal pulmonary thromboembolism induced by infusion of a mixture of collagen and adrenaline. All wild-type mice (19/19) died within 5 min, with > 95% reduction in circulating platelet counts within 2 min after challenge (Figure C.22 A, B).

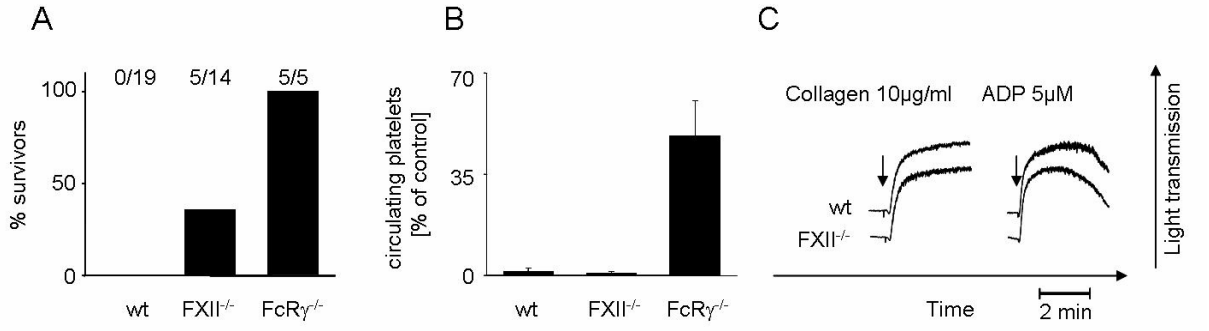


Figure C.22. Collagen induced pulmonary embolism model. (A) Shown is mortality associated with intravenous injection of collagen (0.8 mg/kg) and epinephrine (60µg/kg). All wild-type mice died within 5 min. Animals alive 30 min after challenge were considered survivors. (B) Platelet counts in control (*n* = 19), FXII$^{-/-}$ (*n* = 14) and FcR$\gamma^{-/-}$ (*n* = 5) mice 2 min after infusion of collagen/epinephrine. (C) Heparinized platelet rich plasma from wild-type and FXII$^{-/-}$ mice was stimulated with collagen (10µg/ml) or ADP (5µM) and light transmission was recorded in a standard aggregometer. Shown are representative results for each genotype (6 mice per group).

Consistent with previous reports[7], mice deficient in the Fc receptor (FcR) γ-chain are protected from death in this model, and experience only moderate reductions in platelet count. Five of 14 FXII$^{-/-}$ mice (37.5%) survived this challenge, although their peripheral platelet counts were reduced to a similar degree as in the wild-type

controls. This suggests that the protection conferred by FXII deficiency is not due to a platelet activation defect, but rather a defect in thrombin generation or some other FXII-related activity. Consistent with this premise, *in vitro* studies demonstrate that platelet aggregation in response to collagen and ADP is normal in platelet rich plasma from $FXII^{-/-}$ mice (Figure C.22 C).

C.5.3. Defective arterial thrombus formation in $FXII^{-/-}$ mice

Pathological thrombus formation in arteries is thought to be initiated by fissuring or other disruption of an atherosclerotic plaque, leading to local activation of platelets and plasma coagulation on exposed subendothelial layers of the vessel. The effect of FXII deficiency on thrombus formation in wild-type and $FXII^{-/-}$ mice was studied employing three models of arterial injury. In the first model, oxidative injury was induced in mesenteric arterioles, and thrombus formation was examined by *in vivo* fluorescence microscopy. Wild-type and $FXII^{-/-}$ mice received fluorescently labeled platelets (1×10^8) from donors of the same genotype, and injury was induced by topical application of filter paper saturated with 20% ferric chloride ($FeCl_3$). $FeCl_3$ induces formation of free radicals leading to disruption of the vascular endothelium. Platelets rapidly interacted with the injured vessel wall, and five minutes after injury the number of firmly adherent platelets was similar in FXII and wild-type mice (Figure C.23 A). In wild-type mice additional platelets were recruited into the growing thrombus. Thrombi > 20 µm developed in 100% of wild-type vessels tested (17/17) within 10 min of injury (Figure C.23 B), ultimately leading to complete occlusion in 94.1% (16/17) of vessels within the observation period of 40 min (mean occlusion time: 25.6 ± 8.9 min - Figure C.23 C). In sharp contrast, formation of microaggregates or thrombi did not occur in 50 % (7/14) of vessels in $FXII^{-/-}$ mice (Figure C.23 B). In the remaining vessels (7/14), formed aggregates were unstable and detached from the vessel wall (Figure C.23 D). In no $FXII^{-/-}$ vessel did a thrombus >20 µm in diameter remain attached to the injury site for more than 1 min. Consequently, no vessel occluded within the 40 minutes observation period in $FXII^{-/-}$ mice (Figure C.23 C). To assess the contribution of FXII to thrombus formation more quantitatively, mice heterozygous for the FXII null allele ($FXII^{+/-}$) were analyzed. Such mice have half of the FXII plasma antigen, activity levels of wild-type mice and a normal aPTT (26 ± 5 s vs. 25 ± 5 s for $FXII^{+/-}$ and wild-type mice, respectively[107]). Platelet adhesion occurred normally in $FXII^{+/-}$ animals, and thrombi >20µm developed in 100% of the

vessels (14 out of 14) within 10 minutes after injury, leading to complete occlusion in 11 out of 14 vessels (mean occlusion time: 29.5 ± 7.7 min). In the other three arterioles, stable thrombi occluded >90% of the vessel lumen but did not reach complete occlusion. These results demonstrate that FXII is required for the propagation and stabilization of platelet rich-thrombi in $FeCl_3$-injured arterioles and suggest that FXII-mediated activation of coagulation is involved in this model.

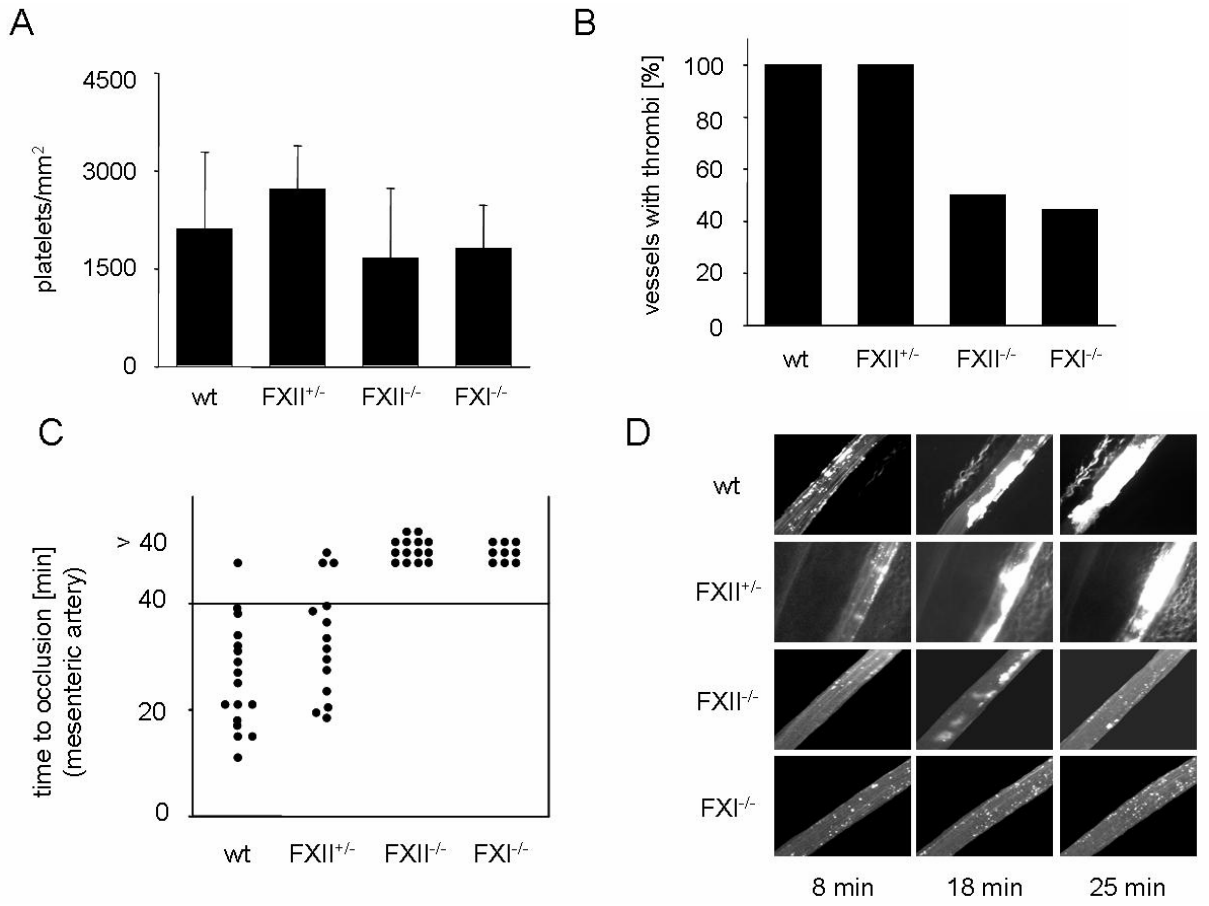


Figure C.23. Defective thrombus formation in $FeCl_3$-injured mesenteric vessels in $FXII^{-/-}$ and factor $XI^{-/-}$ mice. Thrombus formation *in vivo* was monitored on mesenteric arterioles upon injury induced with 20% $FeCl_3$. (A) Shown are the mean number of platelets adherent at five minutes ± SD (129Sv wild-type, n = 14, $FXII^{-/-}$, n=11; $FXI^{-/-}$, n = 9) (B) The number of vessels in which one or more thrombi >20 µm in diameter formed during the observation period (40 min). (C) Time to complete occlusion after injury. Each symbol represents one monitored arteriole. (D) Representative pictures of one experiment.

To examine this possibility further, we analyzed factor XI-deficient mice in the same model. Factor XI is the principal substrate of FXIIa in the intrinsic pathway of the coagulation cascade *in vitro*, and a similar defect in thrombus formation would be expected in FXII and factor XI-deficient mice if this pathway is operating *in vivo*. Similar to $FXII^{-/-}$ mice, normal platelet adhesion at the site of injury was detectable during the first five minutes after injury in factor XI-deficient mice (Figure C.23 A), with formation of thrombi in only 4 of 9 (44.4%) vessels studied. In the remaining

vessels, microaggregates and thrombi were unstable and broke loose from the site of injury (Figure C.23 D). No vessel remained occluded in factor XI-deficient animals within the observation period. This data confirms and extends the previous observation that FXI deficient mice are protected from vascular occlusion in a $FeCl_3$ carotid artery injury model[108].

$FeCl_3$-induced arterial thrombus formation is dependent on platelet activation and thrombin generation, but it is unclear how well this type of injury mimics naturally occurring arterial thrombus formation at the site of rupture of an atherosclerotic plaque. As it is possible that $FeCl_3$-induced oxidative damage produces conditions that artifactually favor FXII-dependent processes, the effect of FXII deficiency was assessed in a second well-established arterial thrombosis model where injury is induced mechanically in the aorta, and blood flow is monitored with an ultrasonic flow probe. After a transient increase directly after injury, blood flow progressively decreased for several minutes in all animals. In all wild-type mice (11/11), this decrease resulted in complete and irreversible occlusion of the vessel within 1.6 to 11.1 min after injury (mean occlusion time 5.3 ± 3.0 min, Figure C.24 A). While a progressive reduction in blood flow was observed during the first minutes after injury in $FXII^{-/-}$ mice, occlusion occurred in only 4 of 10 mice. Occlusive thrombi were all unstable, and rapidly embolized so that blood flow was re-established within 10 to 115 s after occlusion. No vessel in which flow was re-established occluded a second time, and all $FXII^{-/-}$ mice displayed essentially normal flow rates through the injured vessel at the end of the observation period (40 min). Similar results were obtained with factor XI-deficient mice, where 9 of 11 vessels were not occluded at the end of the observation period (Figure C.24 A).

The defect in arterial thrombus formation in $FXII^{-/-}$ mice was confirmed in a third model in which platelet recruitment into thrombi in injured carotid arteries is studied by *in vivo* fluorescence microscopy. Platelets purified from donor mice of the appropriate genotype were fluorescently labeled and injected into recipient mice. Vascular injury was induced by ligation of the carotid artery, a process that consistently causes disruption of the endothelial layer and frequent breaching of the internal elastic lamina, followed by rapid collagen-dependent platelet adhesion and thrombus formation at the site of injury. Large stable thrombi that did not embolize formed rapidly in wild-type animals (thrombus area: 102.821 ± 39.344 μm^2; t = 5min). In contrast, only small and medium-sized aggregates were formed in $FXII^{-/-}$ mice,

which frequently detached from the site of injury (Figure C.24 B, C). Consequently, the thrombus area was dramatically reduced in FXII$^{-/-}$ mice (8.120 ± 13.900 µm^2; t = 5min) although primary platelet adhesion on the vessel wall appeared not to be defective.

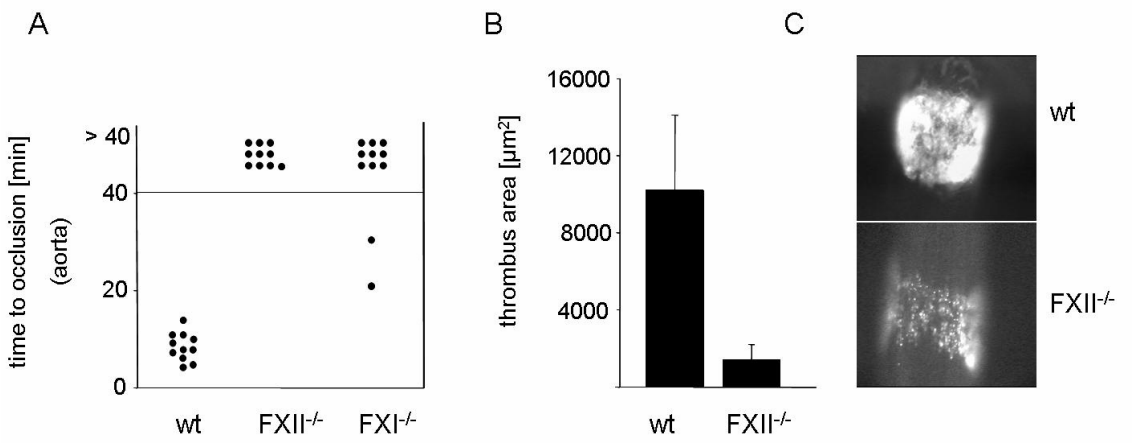


Figure C.24. Defective thrombus formation in the injured aorta and carotid artery in FXII$^{-/-}$ and factor XI$^{-/-}$ mice. (A) Thrombosis was induced in the aorta of wild-type (n=10), FXII$^{-/-}$(n=10), and FXI$^{-/-}$(n=11) mice by one firm compression with a forceps. Blood flow was monitored with a perivascular ultrasonic flow probe until complete occlusion. The experiment was stopped after 40 min. Each symbol represents one individual. (B) Mechanical injury to the carotid artery was induced by ligation with a surgical filament. Five minutes after removal of the filament, thrombus area (µm^2) in wild-type and FXII$^{-/-}$ (n=10 per group) was measured and is expressed as mean ± SD. (C) The photomicrographs show representative images 5 min after injury.

C.5.4. Exogenous human FXII restores arterial thrombus formation in FXII$^{-/-}$ mice

To determine whether the severe defect in thrombus formation in FXII$^{-/-}$ mice results from the absence of plasma FXII, or a secondary effect of chronic FXII deficiency that indirectly alters sensitivity to prothrombotic stimuli, we studied arterial thrombus formation in FXII$^{-/-}$ mice following intravenous administration of human FXII (2 µg/g body weight). This treatment corrects the prolonged aPTT clotting time of FXII-deficient murine plasma to normal (27 ± 6 sec), and fully restores arterial thrombus formation. In $FeCl_3$-injured mesenteric arterioles in wild-type mice or FXII$^{-/-}$ mice treated with human FXII, thrombi >20 µm formed within 10 min after injury, and all vessels were occluded within the observation period (Figure C.25 A and B) with the exception of a single vessel in a wild-type animal. In fact, there was a slight tendency towards faster occlusion in the reconstituted FXII$^{-/-}$ mice compared to untreated wild-type control mice (mean occlusion time: 22.7 ± 8.2 min vs. 25.6 ± 8.9 min). A similar result was obtained with mechanical injury of the aorta (Figure C.25 C), with all vessels completely occluded within 10 min after injury. These results confirmed that the absence of plasma FXII protects FXII$^{-/-}$ mice from arterial thrombus formation in these models.

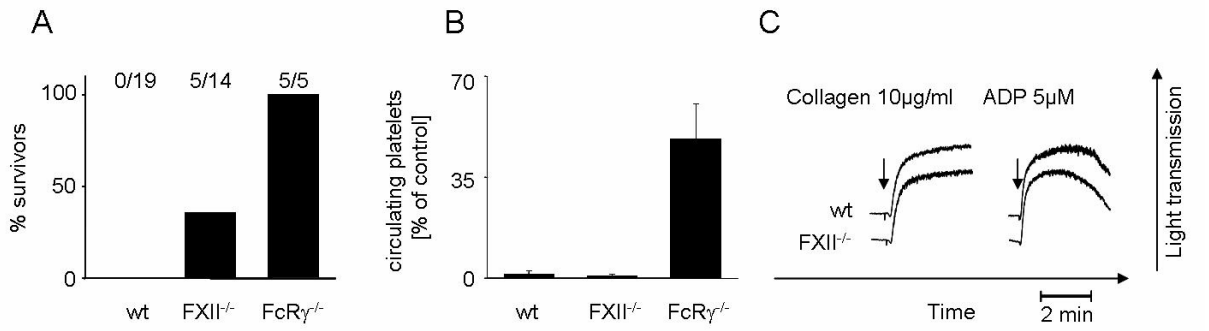


Figure C.25. Thrombus formation in FXII$^{-/-}$ mice reconstituted with exogenous FXII. FXII-deficient animals received human FXII (hFXII, 2mg/kg body weight) and thrombus formation upon $FeCl_3$-induced injury was analysed. (A) The number of vessels in which one or more thrombi >20 µm in diameter formed during the observation period (40 min). (B) Time to complete occlusion after injury. Each symbol represents one monitored arteriole. (C) Representative pictures of one experiment. (D) FXII$^{-/-}$ mice received 2 mg/kg hFXII and thrombosis was induced in the aorta by one firm compression with a forceps. Blood flow was monitored with an perivascular ultrasonic flow probe until complete occlusion. The experiment was stopped after 40 min. Each symbol represents one individual.

D. Discussion

Ischemic cardiovascular or cerebrovascular events such as myocardial infarction or stroke are the leading causes of mortality and morbidity in Western countries. In such pathological processes platelets play a crucial role as they become activated on the exposed extracellular matrix, e.g. upon abrupt disruption of an advanced atherosclerotic lesion which, subsequently, leads to thrombotic vascular occlusion. Therefore, it is essential to study the contribution of single receptors and signaling pathways in platelet activation, adhesion, and aggregation, together with plasma coagulation factors in the formation and stabilization of occlusive thrombi. In the current work, different aspects of the mechanisms underlying thrombus formation and stabilization were studied using the mouse system. Mice with targeted genetic manipulation or depletion of single platelet adhesion and activation receptors or receptor subunits as well as mice lacking coagulation factor XII were analyzed using different *in vitro* and *in vivo* techniques. For this, it was important to establish *in vivo* methods for thrombosis studies that allow to examine platelet adhesion and kinetics of thrombus formation in a living organism. A variety of murine thrombosis models have been described by several investigators using different branches of the vascular system and different types of injury[91]. We used a model of mechanically injured carotid artery to study single platelet adhesion to the exposed subendothelial ECM. To study the mechanisms of formation and stabilization of three dimensional platelet rich occlusive thrombi, models of $FeCl_3$-induced injury on mesenteric arteries and mechanical injury on the abdominal aorta were established, both of which lead to formation of occlusive platelet rich thrombi.

D.1. Thrombosis models

Our modified intravital fluorescence microscopy model of $FeCl_3$-induced injury on mesenteric arterioles is based on a model previously described by Denis *et al.*[104]. In that model, after injection of fluorescently labeled platelets obtained from donor mice, arterioles were exteriorized and a drop of $FeCl_3$ was placed on the vessel wall to induce injury. This *in vivo* fluorescence model allows good and easy accessibility of arterioles, very good visualization of fluorescently labeled platelets, the possibility to observe and record platelet-platelet and platelet-vessel wall interactions as well as formation of occlusive thrombi in real time. A good reproducibility of results and no

need for any special equipment is also a great advantage of this model. Exposure of $FeCl_3$ to the vessel wall causes formation of free radicals leading to denudation of the endothelium and platelet activation, aggregation, and formation of occlusive thrombi. This type of arterial injury is, however, artificial and does not occur under natural conditions. Consequently, it is important to mention that injury induced with chemical compounds such as $FeCl_3$ possibly modifies the chemical properties of the vessel wall. On the other hand, although the exact mechanism by which thrombus formation is triggered in this model is not clear, it has been shown that the morphology of the thrombi is similar to those found in humans[109].

To better understand the kinetics and conditions of vessel occlusion, a model of mechanically induced injury on the abdominal aorta was established. With this method, an injury leading to formation of occlusive thrombi was induced in a big artery which is in contrast to the above described model, where small arterioles were studied. This allows us to study and compare the formation of occlusive thrombi under conditions of high (aorta) and very high (mesenterium) shear forces and upon two distinct types of injury. In the latter model, endothelial denudation is achieved by firm compression with a forceps. This causes exposure of the native ECM and subsequent thrombus formation by collagen- and thrombin-dependent mechanisms. This method might best represent naturally occurring vascular lesions. After abdominal incision, an ultrasonic flow probe is placed around the aorta and blood flow is measured. Mechanical injury is performed upstream of the flow probe and complete, stable occlusion is typically reached within 1-10 minutes after compression with a forceps. This method has good reproducibility, does not require *ex vivo* platelet labeling, but requires special equipment to measure blood flow.

The mechanisms of platelet deposition on the injured arterial wall were studied using a model that had previously been established in our laboratory. In this model, vascular damage was performed by vigorous ligation of the carotid artery with a surgical filament and firm adhesion of fluorescently labeled platelets was analyzed[20].

It is important to mention that in all described injury models thrombus formation occurs in normal healthy arteries while arterial thrombosis in humans occurs in diseased (atherosclerotic) vessels. Despite this limitation, the combination of these models with genetically modified mouse lines and pharmacological tools has largely improved our understanding of hemostasis and thrombosis.

In the first part of the present study, the synergistic action of platelet integrins on *in vivo* platelet adhesion to arterial lesions was studied using mice with targeted deficiency in platelet integrins of the β1 family, with or without specific inhibition of the αIIbβ3 receptor.

D.2. Multiple integrin-ligand interactions synergize in shear-resistant platelet adhesion at sites of arterial injury *in vivo*.

The subendothelial ECM consists mainly of collagen, a principal platelet agonist. Two major collagen receptors are expressed on the platelet surface, the activating receptor GPVI, and integrin α2β1. The contribution of α2β1 and all other β1 family receptors to *in vivo* platelet adhesion and thrombus formation to the injured carotid artery was assessed. Mice deficient in the α2 subunit, lacking the α2β1 integrin only, or β1-null mice which lack all β1 family receptors were analyzed by intravital fluorescence microscopy. Surprisingly, platelet adhesion and thrombus formation were not significantly altered in the mutant mice compared to wild-type controls. This finding demonstrates that α2β1 is not essential for arterial thrombus formation in mice but it does not exclude a supportive role of the integrin in this process. The unexpected finding that platelet adhesion and aggregation on the ECM was not significantly altered in β1-null mice also excludes an essential role of α5β1 and α6β1 in arterial thrombus formation, which is in line with normal tail bleeding times in those mice[15].

In addition to collagen, the ECM contains multiple macromolecules that potentially provide an adhesive substrate for platelet integrins. Among these, VWF, immobilized on fibrillar collagen, is thought to play a role in this process as it is a ligand for the dominant platelet receptor, αIIbβ3[6]. Blocking of αIIbβ3, demonstrates a major role for the receptor in platelet adhesion on the ECM *in vivo* (Figure C.2). This finding is in agreement with *in vitro* studies showing that αIIbβ3-VWF interactions are sufficient to mediate shear-resistant platelet deposition on collagen in whole-blood perfusion experiments in the absence of functional α2β1[6;15]. However, the newly formed aggregates are less stably attached to the collagen substrate as compared with wild-type controls[110] suggesting that αIIbβ3-VWF interactions are unable to fully substitute for the lack of α2β1 in this system. *In vivo*, the situation appears to be different as αIIbβ3 not only binds to VWF but also to other ligands, including fibrinogen deposited

on the surface of the damaged vessel and fibronectin, which is present in the ECM of the vessel wall. This may explain why no defect in adhesion and thrombus formation was detectable in $\alpha 2^{-/-}$ mice. The pivotal role of $\alpha IIb\beta 3$ was revealed in $\beta 1$ deficient mice, where platelet adhesion and thrombus formation was not reduced compared with controls but abrogated on inhibition of $\alpha IIb\beta 3$ (Figure C.2). These results strongly suggest that $\alpha IIb\beta 3$ is the major integrin that supports shear-resistant platelet adhesion on the injured vessel wall *in vivo*, presumably by interacting with multiple ligands in the ECM. The observation that inhibition of $\alpha IIb\beta 3$ abolished platelet adhesion in $\beta 1$-null but not $\alpha 2^{-/-}$ mice provides the first direct evidence that $\alpha 5\beta 1$ and/or $\alpha 6\beta 1$ can contribute to platelet attachment to the damaged vascular wall *in vivo*. It is not clear whether $\alpha 5\beta 1$, $\alpha 6\beta 1$, or both were responsible for the observed adhesion in the absence of functional $\alpha 2\beta 1$ and $\alpha IIb\beta 3$. It appears likely, however, that both integrins are involved in this process as both fibronectin and laminin are highly expressed in the vessel wall and become accessible to the flowing blood at sites of injury. Laminins are assembled with collagen type IV which is known to activate $\alpha IIb\beta 3$ through the GPVI/FcRγ-chain complex[59]. Fibronectins are dimeric glycoproteins that are present in plasma (plasma fibronectin) and in tissue extracellular matrices (cellular fibronectin)[54]. Recent studies in mice with a cre/loxP-mediated deletion of plasma fibronectin in a model of arterial thrombosis demonstrated delayed thrombus formation and reduced thrombus stability[56]. The results obtained with $\beta 1$-null mice extend the role of fibronectins also to the process of platelet adhesion and suggest that both fibronectin-binding integrins on platelets, $\alpha IIb\beta 3$ and $\alpha 5\beta 1$, play a significant role in this process. In the vascular wall, cellular fibronectin is closely associated with collagens including types I and III, both of which are strong platelet agonists on GPVI, suggesting that GPVI-collagen interactions may facilitate platelet adhesion on fibronectin. The adhesion studies (Figure C.3) suggest that $\alpha 5\beta 1$ and $\alpha 6\beta 1$ are in a low-affinity state on resting mouse platelets unable to efficiently mediate adhesion to fibronectin or laminin, respectively, and that ligation of GPVI shifts these integrins to a high-affinity state. This finding is in line with previous studies demonstrating a similar regulation of $\alpha 2\beta 1$ in mouse platelets[15] and indicates that both integrins, together with $\alpha 2\beta 1$, may contribute to shear-resistant platelet adhesion at sites of arterial injury in a GPVI-dependent manner.

GPVI is the major collagen receptor and mediates platelet activation, adhesion, and release of granular contents. The above described data show that GPVI-induced signaling leads to conformational changes of multiple integrins expressed on the platelet surface. These changes mediate a shift of integrins from a low affinity state to a high affinity state leading to firm platelet adhesion to the exposed extracellular matrix. It was previously reported that platelets with either depleted or blocked GPVI show virtually no adhesion to collagen under static or flow conditions even though they express normal levels of the major adhesion molecules α2β1, GPIb-V-IX, and αIIbβ3[15]. Despite this, however, the absence of the GPVI receptor does not lead to a hemostatic disorder in mice[7] as well as in humans. A few patients with low levels of GPVI have been described[98;111;112]. In most cases, the patients display only a mild bleeding phenotype though their platelets exhibit defective aggregation to collagen.
Deficiency of the other major collagen receptor, integrin α2β1 does not affect hemostasis either[53]. To better define the role of the two direct collagen receptors on the platelet surface in hemostasis, mice lacking the α2 integrin subunit and depleted from GPVI were analyzed.

D.3. Anti-GPVI treatment compromises hemostasis in the absence of α2β1 integrin.

The current model of platelet activation and adhesion at sites of vascular injury involves platelet tethering mediated by GPIbα-VWF interactions which are essential under conditions of high shear[6]. This is followed by GPVI binding to collagen which causes activation of integrin α2β1. High affinity binding of α2β1 to collagen increases firm adhesion and reinforces GPVI-mediated adhesion without, however, being essential for these processes. This model of platelet-collagen interactions clearly establishes GPVI as the central collagen receptor on platelets[15]. Treatment of mice with anti-GPVI mAb JAQ1 causes depletion of the receptor[16] for at least 14 days and results in long-term antithrombotic protection against collagen-dependent thromboembolism[7].
Since GPVI-deficient humans[98] and mice[7] display no major bleeding phenotype, it appears that other agonist receptors/signaling pathways can substitute for GPVI in mediating integrin activation in normal hemostasis. The G-protein-coupled receptors for ADP, TxA_2, or thrombin are likely to play major roles in this process. Low levels of

the other main collagen receptor, integrin α2β1, do not seem to affect hemostasis in otherwise hemostatically normal individuals[113]. However, low α2β1 levels may become clinically significant in a setting where the overall platelet-collagen interaction is already partially compromised, as for example in the case of mild type I von Willebrand disease[52]. The data presented here clearly indicate that the loss of GPVI-signaling is compensated by a very sensitive network of different G-protein-mediated signaling pathways which converge in the activation of α2β1 allowing it to arrest the cells and to reinforce activation through "outside-in" signals[114]. Any impairment of this alternative pathway, which may not be of any significance under normal conditions, might lead to an intolerable bleeding risk under anti-GPVI treatment. Although there is only a weak correlation between the bleeding time and bleeding risk[115], it is tempting to speculate that the combination of anti-GPVI agents with other anti-platelet compounds in order to improve the antithrombotic effectiveness of the therapy, as currently discussed for clopidogrel and aspirin[116], would have to be carefully evaluated to avoid uncontrolled bleeding in humans. These observations may have important implications for the development and potential use of anti-GPVI based therapeutics for the prevention of ischemic cardiovascular disease.

It is widely recognized that platelet adhesion to collagen requires prior activation of integrins through inside-out signals generated by GPVI and reinforced by released second-wave mediators ADP and TxA_2 that function though G-protein coupled receptors. Platelet activation induced with these agonists plays an essential role in thrombus stabilization. In platelets, G-proteins of the G_i, G_q and $G_{12/13}$ families are expressed. Among these, a G_i family member, G_z, which couples to the α2A adrenergic receptor, mediates adrenaline responses. Although the α2A receptor has been known for many years, its role in thrombosis and hemostasis is poorly understood. Therefore $\alpha2A^{-/-}$ mice were used to analyze the process of *in vitro* and *in vivo* platelet adhesion and thrombus formation

D.4. The α2A-adrenergic receptor is involved in thrombus stabilization *in vivo*.

The first set of experiments revealed that α2A is not essential for normal megakaryocyte maturation and platelet formation because platelet counts and expression of prominent glycoproteins were not affected in $\alpha2A^{-/-}$ mice. However, $\alpha2A^{-/-}$ mice displayed a mild bleeding tendency, impaired thrombus stabilization, and

enhanced embolus formation, indicating that adrenaline-mediated platelet responses do substantially contribute to stable platelet plug formation. This is in accordance with previous reports form several patients with reduced α2A levels on their platelet surface[117;118]. In these patients, reduced amounts of α2A were accompanied by a bleeding tendency[117], and the platelets showed impaired responses to adrenaline *in vitro*. Comparable data were obtained with $\alpha 2A^{-/-}$ mice, whose platelets remain unresponsive to adrenaline-enhanced αIIbβ3 activation and aggregation in the presence of low concentrations of ADP and U46619 (Fig. C.15.).

Mice deficient in α2A are largely protected against collagen/adrenaline-induced pulmonary thromboembolism (Fig. C.16.), confirming that the contribution of adrenaline to platelet activation *in vivo* is mediated through the α2A receptor. These findings support results previously described by Yang *et al.*[119] showing that platelets obtained from $G_{z\alpha}^{-/-}$ mice are unresponsive to adrenaline and that these animals are protected against collagen/adrenaline-induced lethal thromboembolism. Consistent with this, alterations in the number of α2A receptors on human platelets cause abnormalities in the platelet responses to adrenaline[120-122].

Analysis of $\alpha 2A^{-/-}$ mice in different *in vivo* thrombosis models revealed that they display enhanced embolus formation and thrombus instability. In the model of $FeCl_3$-induced injury on mesenteric arteries, 44.4% of $\alpha 2A^{-/-}$ mice did not occlude due to frequent embolisation. Similarly, in the aorta model, 24% of initially occlusive thrombi recanalized. Our findings demonstrate for the first time that adrenaline-dependent signaling is also required for the formation of stable plugs. Thrombus stability largely depends on the release of second wave mediators, including ADP and TxA_2. Those together with adrenaline share the common property of binding G-protein coupled receptors. These receptors are known to act in concert in order to achieve full platelet activation by cAMP suppression, increase of Ca^{2+} concentration, PLCγ activation, and reorganization of the platelet cytoskeleton[123]. The α2A receptor on the platelet surface inhibits cAMP formation through stimulating one of the G_i family members, G_z[34]. Although adrenaline alone is unable to promote platelet shape change, aggregation, or direct αIIbβ3 activation[35;36], it potentiates different responses and thereby contributes to platelet activation[124;125].

The above described results revealed that α2A is the essential receptor for adrenaline on the platelet surface that regulates adrenaline responses during platelet

activation *in vitro* and *in vivo*. It is important to note that we did not observe any differences in the vascular constriction upon $FeCl_3$-induced injury. This strongly suggests that the observed defect in thrombus stabilization is based on reduced platelet activation caused by suboptimal activation of platelets from $\alpha 2A^{-/-}$ mice, rather than a defect in the vessel wall. Based on our findings, we speculate that adrenaline through α2A contributes to the progression of stable plug formation under pathological conditions. It is known that the density of α2A on the platelet surface can vary in relation to physiological, pathological, and pharmacological factors. Furthermore, it has been reported that age[126], ischemic heart failure[127], and plasmatic catecholamine levels[128;129] can regulate α2A expression. In line with this, Wang *et al.*[130] have found that strenuous exercise increases platelet α2A-adrenergic receptor density and can enhance platelet adhesiveness on fibrinogen-coated surfaces and Ca^{2+} elevation. This α2A/adrenaline mediated platelet responses during strenuous, acute exercise enhance the risk of major ischemic cardiovascular events.

Taken together, the presented results provide strong evidence that signaling initiated by adrenaline through the α2A receptor on the platelet surface contributes to *in vivo* thrombus stabilization under pathological conditions. Although it is difficult to answer the question where the adrenaline comes from, one may speculate that adrenaline that is taken up, concentrated, and stored in platelet dense granules[131-133] is released upon platelet activation and thereby contributes to thrombus formation.

Among the soluble agonists that contribute to the formation of stable occlusive thrombi, thrombin plays a crucial role. Thrombin is generated on the surface of activated platelets which exhibit procoagulant activity characterized by changes in phospholipids orientation[134]. Such negatively charged surfaces initiate, together with plasma coagulation factors, blood clotting. This results in thrombin-induced cleavage of fibrinogen to fibrin. Crosslinking of fibrin fibres, the final step of blood coagulation, leads to stabilization of the formed clot. Blood coagulation is mediated via two pathways: i) intrinsic pathway (contact system), ii) extrinsic pathway. The participation of the so-called "contact system" in formation and stabilization of occlusive thrombi was studied using FXII-deficient mice.

D.5. Defective arterial thrombus formation in FXII-/- mice.

While the importance of FXII for contact activation initiated coagulation has been recognized for more than 50 years[135], the protein is not considered to be an important component of the hemostatic mechanism, as humans lacking FXII do not have a bleeding diathesis. Indeed, for many years FXII has been suspected of having antithrombotic (profibrinolytic), rather than prothrombotic properties, based on reports indicating an association between FXII-deficiency and venous thrombosis[136] and myocardial infarction[137]. However, this premise has been challenged recently by Girolami and coworkers, who demonstrated that in most cases of thrombosis associated with FXII deficiency, other congenital or acquired prothrombotic risk factors are present[138]. Indeed, large clinical studies have not identified a correlation between FXII deficiency and bleeding or thrombosis[139;140]. Current models of hemostasis emphasize the importance of the factor VIIa/TF complex in initiation of thrombin formation. The importance of this pathway is highlighted by the fact that mice lacking factor VII or TF die *in utero* from apparent severe hemorrhage[141]. While it is clear that the TF exposed at a site of vessel injury is well positioned to initiate thrombin generation, it is not known how thrombin generation proceeds on the surface of a growing thrombus once the factor VIIa/TF at the wound site is covered by the clot or neutralized by inhibitors such as tissue factor pathway inhibitor (TFPI)[140]. There is evidence that TF is expressed on procoagulant microparticles that are released at sites of injury or that circulate in blood. These particles can be incorporated into a growing thrombus in a P-selectin-dependent manner[142]. Indeed, mice expressing low levels of TF display defective arterial thrombus formation, although it is not clear whether this is related to a lack of vessel wall or blood cell-derived TF[143]. However, the existence of active TF in blood is still a matter of discussion[144] , and other mechanisms contributing to fibrin formation are possible.

In the present work, three *in vivo* models were used to study platelet recruitment and thrombus formation at sites of arterial injury in FXII-deficient mice. A profound defect in formation and stabilization of platelet rich thrombi was observed. Similar results were obtained in a pulmonary embolism model. The results suggest that a TF-independent pathway may be operative in propagation of pathologic thrombus formation in mice. The protective effect of FXII deficiency in these models is reversed by infusions of human FXII, demonstrating that the absence of plasma FXII is responsible for the observed phenotype (Figure C.25). These results appear to

conflict with the reasonable proposition that the coagulation proteins involved in pathologic thrombus formation are the same as those that are also important for normal hemostasis (cessation of bleeding at a wound site). The presented results support the interesting possibility that hemostasis and thrombosis may be facilitated by different, though probably largely overlapping, mechanisms. Several recent reports support this premise. Mice lacking plasma fibronectin or the secreted growth arrest-specific gene 6 product (Gas6) do not have prolonged bleeding times nor bleed spontaneously, but are unable to form occlusive arterial thrombi or are protected against thromboembolism, respectively[56]. Similarly, mice lacking the platelet collagen binding GPVI/FcRγ complex have a profound defect in injury-induced arterial thrombus formation, but only minor hemostatic abnormalities[20].

FXII-deficient mice, like their human counterparts, have prolonged aPTTs in the absence of a bleeding diathesis. Furthermore, plasma mixing studies demonstrate that murine FXII functions normally in human plasma *in vitro*, while infusion of human plasma into $FXII^{-/-}$ mice results in a phenotype similar to wild-type mice in thrombosis models. Clinical studies have associated elevated plasma FXIIa levels with an increased prevalence of coronary disease and other known plasma cardiovascular risk factors[145], supporting the notion that the FXII-initiated intrinsic pathway might contribute to thrombosis in humans. It remains to be determined if FXII activation is the cause or the consequence of the underlying vascular disease. A particular FXII single nucleotide polymorphism (SNP, 46C>T) has been linked with lower FXIIa and FXII plasma levels and protection from coronary artery diseases in British patients[145]. Because the same SNP has been reported to be a risk factor for ischemic stroke in the Spanish population[146] environmental and/or other genetic factors may influence the effects of FXII plasma levels on thrombotic risk. Indeed, clinical studies have shown that elevated FXIIa levels are associated with increased risk for coronary heart disease. However, in these patients FXIIa activation is linked with other plasma risk factors such as cholesterol, triglyceride or fibrinogen[147]. Moreover, elevated FXIIa levels have been reported as prognostic risk factor for recurrent coronary events[148] supporting our hypothesis that FXII contributes to thrombus formation in humans. Data from heterozygous $FXII^{+/-}$ mice show that 50% of normal FXII plasma levels are sufficient for the formation of large and stable thrombi, though with a slightly reduced rate of complete vessel occlusion (Figure C.21). This suggests that partial FXII deficiency may provide only a limitted protection from the risk of stroke or myocardial

infarction in humans. In contrast, Girolami *et al.* showed that none out of 21 patients with severe (homozygous) FXII deficiency experienced a thrombotic event within a mean observation period of 16 years[149]. But clearly, larger clinical studies will be required to define the significance of severe (homozygous) FXII deficiency for a complex disease such as thrombosis.
Importantly, it seems very unlikely that resistance to thrombus formation in FXII-deficient mice is an artifact of the type of injury inflicted on the vessel, or the vascular bed tested, as observations were consistent across several models and different vascular beds.

Thus, there is no evidence that FXII functions differently in mice and humans. The mechanism through which FXII is recruited into the thrombotic process is not clear. Plaque rupture or fissuring results in exposure of collagen fibrils and other basement membrane components to flowing blood, and it is likely that there is similar exposure in our arterial injury models, which disrupts the vascular endothelium. Early work demonstrated that collagen activates FXII[105], although not all investigators came to the same conclusion[150]. An explanation for the discrepancies may be that procoagulant activities of collagens are highly dependent on the type, available surface area, charge, and method of preparation of the collagen[151]. At sites of vascular injury, platelets come in contact with collagens exposed on endothelial extracellular matrix (ECM). Platelets are initially tethered to the ECM by von Willebrand factor through platelet GPIb[6]. Activation and adhesion then proceeds through interactions between platelet collagen receptors such as GPVI and integrin $\alpha 2\beta 1$ and the ECM[101]. Factor VIIa/TF initiates thrombin formation, which recruits additional platelets into the growing thrombus. While FXII and factor XI may be activated during this early phase, these proteins appear to have little effect on platelet adhesion or recruitment. As the thrombus grows, the exposed ECM and TF are covered, and additional mechanisms are required to maintain spatio-temporal thrombin generation to activate newly recruited platelets and consolidate fibrin formation in the growing thrombus. It is in this propagation phase that $FXII^{-/-}$ and factor XI-deficient mice appear to be defective. In conjunction with the observation that mice expressing low levels of TF also display impaired arterial thrombus formation, our results suggest that factor VIIa/TF (extrinsic pathway) and FXII (intrinsic pathway) are both required for formation of an occlusive thrombus. The

absence of a bleeding diathesis in FXII$^{-/-}$ mice indicates that this mechanism for thrombus growth is not required to seal a hole in a vessel wall (hemostasis). Left unanswered by this model is the long standing question of the physiologic role of FXII, but the findings raise the interesting possibility that formation of a large thrombus may serve non-hemostatic functions, such as inhibition of the spread of invading pathogens or toxins within the blood stream. Therefore the FXII-deficient mice could be an important tool for studying other contact system-linked diseases such as bacterial inflammation and sepsis[152], complement activation[153;154] and kinin-mediated vascular leakage[155].

D.6. Concluding remarks.

The work presented here provides closer insights into *in vitro* and *in vivo* function of platelet integrins, the collagen receptor GPVI, as well as contribution of the G-protein coupled adrenaline receptor α2A, and coagulation FXII, representing the intrinsic coagulation pathway in the process of formation and stabilization of pathological occlusive thrombi.

The major findings are as follows:

- Platelet attachment and thrombus formation at sites of vascular injury in mice can occur independently of all β1 integrins on platelets.
- The α5β1 and/or α6β1 receptor can contribute to platelet attachment to the damaged vascular wall *in vivo*, and this process occurs in a GPVI-dependent manner.
- GPVI-deficiency in mice lacking or expressing low levels of α2β1 results in a severe hemostatic defect
- The combination of anti-GPVI agents with other anti-platelet compounds in order to improve the antithrombotic effectiveness of the therapy, needs to be carefully evaluated in order to avoid uncontrolled bleeding in patients.
- Adrenaline-mediated α2A/G_z activation contributes to normal hemostasis and plays a significant role in pathological thrombus formation and stabilization of the formed thrombi.
- Coagulation FXII plays a central role in thrombus formation *in vivo*, whereas it is dispensable for normal hemostasis. This establishes FXII as a promising new

target for antithrombotic therapies that might be associated with a low risk, or no risk of excessive bleeding.

D.7. Future work.

It is currently not clear by which mechanism FXII is activated *in vivo*. There are unpublished observations suggesting that collagens might be good candidates. Further studies will be required to test this hypothesis. Next candidates might be activated platelets, as they possess negatively charged surfaces. Functional *in vitro* and *in vivo* experiments performed with blocking antibodies directed against FXII or aFXII will extend the data obtained with $FXII^{-/-}$ mice and help to understand the *in vivo* function and regulation of the protein. This will give us an answer if blocking of functional FXII is sufficient to obtain a similar antithrombotic effect as it was observed with FXII knock-out mice. Analysis of FXI/FXII double deficient mice will be required to further clarify the role of the two proteins in thrombosis and hemostasis.

A new intravital fluorescence microscopy model, where the endothelial injury is induced with a high energy laser, will further extend our knowledge about the function of FXII, FXI and individual platelet receptors in normal hemostasis or under thrombotic conditions. With this method, lesions of different type and severity can be produced depending on the intensity and exposure time of the laser beam which may be useful to identify potentially different mechanisms of thrombus formation triggered by the exposure of different layers of the vessel wall.

Another interesting point to study is the synergism of G-protein coupled receptors and the major collagen receptor GPVI. In the next set of experiments, GPVI will be depleted, using mAb JAQ1, in α2A deficient mice, where the G_z pathway is compromised. Functional *in vitro* and *in vivo* analysis comparing single and double deficient mice with control wild-type mice will help us to answer this question. To investigate the co-acting role of G_z, G_q and $G_{12/13}$, all of which are crucial for the formation of stable thrombi under flow conditions *in vitro* and *in vivo*, TxA_2 synthesis will be inhibited by injection of aspirin (100 mg/kg) in $\alpha 2A^{-/-}$ mice. Bleeding time assay will verify the influence of concomitant deficiency to normal hemostasis. Intravital microscopy analysis using different types of arterial injury will improve the knowledge about the role of α2A and other G-protein coupled receptors in thrombotic processes.

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Appendix

Abbreviations

ADP	adenosine diphosphate	HRP	horseradish peroxidase
aPTT	activated partial thromboplastin time	Ig	immunoglobulin
BSA	bovine serum albumin	IP	immunoprecipitation
cAMP	cyclic adenosine monophosphate	ITAM	immunoreceptor tyrosine-based activation motif
CRP	collagen related peptide	mAb	monoclonal antibody
DCF	5-carboxyfluorescein diacetate succinimidyl ester	NP-40	nonidet P-40
DMSO	dimethylsulfoxide	PAR	protease-activated receptor
ECL	enhanced chemiluminiscence	PAGE	polyacrylamide gel electrophorasis
ECM	extracellular matrix	PBS	phosphate buffered saline
EDTA	ethylenediaminetetraacetic acid	PLCγ2	phospholipase Cγ2
ELISA	enzyme linked immunosorbent assay	PS	phosphatidylserine
FcR	Fc receptor	PSGL-1	P-selectin glycoprotein ligand-1
FCS	fetal calf serum	PT	prothrombin time
FITC	fluorescein isothiocyanate	SDS	sodium dodecyl sulfate
FSC/SSC	forward scatter / side scatter	TACE	TNFα converting enzyme
FXI	plasma coagulation factor XI	TF	tissue factor
FXII	plasma coagulation factor XII	TFPI	tissue factor pathway inhibitor
GP	glycoprotein	TMB	3,3,5,5-tetramethyl benzidine
GPO	glycine-proline-hydroxyproline	TP	TxA_2 receptor
HBO	mercury short arc photo optic lamp	TxA_2	thromboxane A_2
HMWK	high molecular weight kininogen	VWf	von Willebrand factor

Curriculum vitae

Name	Miroslava Požgajová (born Prostredná)
Date of Birth	12.09.1977
Place of birth	Nitra, Slovakia
Nationality	Slovak Republic

School education

1983-1995	primary, secondary and high school, Nitra
1995	Slovak high school graduation diploma

University education

1995-2000	Department of human nutrition, Agricultural faculty, Slovak University of Agriculture
2000 (graduation)	Diploma work in human nutrition (Dipl. Ing)

Postgraduate education

2001-2001	Employ at the department of animal nutrition, Research Institute of Animal Production, Slovakia
2002-now	PhD student in the group of Prof. Dr. Nieswandt, Vascular biology at Rudolf-Virchow-Zentrum for Experimental Biomedicine (Bayerische Julius-Maximilians-Universität Würzburg)

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Publications

Articles

Schulte V, Rabie T, **Prostredna M**, Aktas B, Gruner S, Nieswandt B: Targeting of the collagen-binding site on glycoprotein VI is not essential for the *in vivo* depletion of the receptor. *Blood* 2003, 101: 3948-3952

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Požgajová M, Sachs U, Hein L, Nieswandt B: Reduced thrombus stability in mice lacking the α2A-adrenergic receptor. Submitted for publication

Kuijpers MJE, **Požgajová M**, Munnix ICA, Grüner S, Auger JM, Eckes B, Nieswandt B, Heemskerk JWM: Key role of murine integrin α2β1 in thrombus stabilization and embolization. Submitted for publication

Oral presentation:

Prostredna M*, Grüner S*, Aktas B, Moers A, Schulte V, Krieg T, Offermanns S, Eckes B, Nieswandt B: Anti-glycoprotein VI treatment severely compromises hemostasis in mice with reduced α2β1 levels or concomitant aspirin therapy. XVIIIth European Platelet Meeting, October 2003, Giessen, Germany

Požgajová M, Sachs U, Hein L, Nieswandt B: Reduced thrombus stability in mice lacking the α2A-adrenergic receptor. XXth European Platelet Meeting, October 2005, Ede, Netherlands.

Poster presentation:

Požgajová M, Sachs U, Hein L, Nieswandt B: Reduced thrombus stability in mice lacking the α2A-adrenergic receptor. The International Society on Thrombosis and haemostasis XXth Congress, August 2005, Sydney, Australia

Erklärung gemäß §4 Abs. 3 der Promotionsordnung:

Hiermit erkläre ich ehrenwörtlich, dass ich die vorliegende Arbeit selbständig angefertigt und keine anderen als die angegebenen Hilfsmittel und Quellen verwendet habe.

Diese Dissertation hat weder in gleicher noch in ähnlicher Form in einem anderen Prüfungsverfahren vorgelegen.

Ich habe bisher außer den mit dem Zulassungsbesuch urkundlich vorgelegten Graden keine weiteren akademischen Grade erworben oder zu erwerben versucht.

Miroslava Požgajová Würzburg, im November 2005