Flow Chamber Analyses in Cardiovascular Research: Impact of Platelets and the Intercellular Crosstalk with Endothelial Cells, Leukocytes, and Red Blood Cells

Kim Jürgen Krott[1] Tobias Feige[1] Margitta Elvers[1]

[1] Department of Vascular- and Endovascular Surgery, University Hospital Düsseldorf, Heinrich-Heine-University, Düsseldorf, Germany

Address for correspondence Margitta Elvers, PhD, Department of Vascular- and Endovascular Surgery, University Hospital Düsseldorf, Moorenstraße5, 40225 Düsseldorf, Germany (e-mail: margitta.elvers@med.uni-duesseldorf.de).

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Abstract

Platelets are main drivers of thrombus formation. Besides platelet aggregate formation, platelets interact with different blood cells such as red blood and white blood cells (RBCs, WBCs) and endothelial cells (ECs), to promote thrombus formation and inflammation. In the past, the role of different proteins in platelet adhesion, activation, and aggregate formation has been analyzed using platelets/mice with a genetic loss of a certain protein. These knock-out mouse models have been investigated for changes in experimental arterial thrombosis or hemostasis. In this review, we focused on the Maastricht flow chamber, which is a very elegant tool to analyze thrombus formation under flow using whole blood or different blood cell components of genetically modified mice. Besides, the interaction of platelets with RBCs, WBCs, and ECs under flow conditions has been evaluated with regard to thrombus formation and platelet-mediated inflammation. Importantly, alterations in thrombus formation as emerged in the flow chamber frequently reflect arterial thrombosis in different mouse models. Thus, the results of flow chamber experiments in vitro are excellent indicators for differences in arterial thrombosis in vivo. Taken together, the Maastricht flow chamber can be used to (1) determine the severity of platelet alterations in different knock-out mice; (2) analyze differences in platelet adhesion, aggregation, and activation; (3) investigate collagen and non–collagen-dependent alterations of thrombus formation; and (4) highlight differences in the interaction of platelets with different blood/ECs. Thus, this experimental approach is a useful tool to increase our understanding of signaling mechanisms that drive arterial thrombosis and hemostasis.

Keywords

- arterial thrombus formation
- collagen
- glycoprotein VI
- platelets
- flow chamber

Introduction

Platelets are anuclear cells derived from megakaryocytes in the bone marrow and play a key role in arterial thrombosis and hemostasis. Activation and aggregate formation of platelets is crucial for these processes. Many efforts have been made in the past to analyze the role of different proteins such as platelet receptors, protein kinases or phosphatases, small GTPases or other signaling or scaffold proteins in platelet adhesion, activation, and aggregate formation. Often, the role of a certain protein or signaling pathway in platelets is concluded from the consequences of a genetic loss of this protein using knock-out mouse models that have been analyzed for changes in experimental arterial thrombosis or hemostasis.[1] Many of these studies have been resulted in the concept of collagen-induced arterial thrombus formation.[2–5]

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They rely on the concept that the exposure of subendothelial collagen to flowing blood is the key trigger of shear-dependent thrombus formation. As a prerequisite for thrombus formation, collagen causes platelet adhesion and activation. von Willebrand factor (vWF) binding of collagen is indispensable for the capture of platelets to allow platelet adhesion and activation under high shear conditions. Firm platelet adhesion to vWF–collagen allows platelet activation and requires synergistic action of the vWF receptor glycoprotein (GP)Ib–V–IX, the collagen receptors GPVI, and integrin α2β1.[6–10]

Several collagen receptors are exposed at the platelet membrane. However, GPVI was identified to be the major collagen receptor on the platelet surface that triggers intracellular signals leading to platelet activation, the release of second wave mediators, and integrin activation important for platelet adhesion and aggregation.[11,12] GPVI specifically binds to the repeat GPO motif of collagens (G = glycine, P = proline, O = hydroxyproline) and represents an immunoreceptor tyrosine-based activation motif–coupled receptor with two Ig domains, a mucin-like region and a cytosolic structure coupled to the Fc (fragment crystalized) receptor (FcR) y-chain homodimer in the platelet membrane.[11] Binding of collagen by GPVI exerts downstream signaling and includes the activation of Src family and Syk tyrosine kinases, resulting in the activation of phospholipase C (PLC)y2 and intracellular Ca^{2+} mobilization including the involvement of ORAI1, STIM1, and cyclophilin A.[3,13–15] Furthermore, several isoforms of protein kinase C, phosphoinositide 3-kinases (PI3K), and small GTPases such as RAC1, CDC42, and RHOA become activated.[16–20] Different signaling pathways contribute to GPVI-dependent platelet activation such as phospholipase D,[21] pannexin-1,[22] the proteoglycan biglycan,[23] and the GP reelin.[24,25]

All these signaling pathways contribute to the activation of the platelet fibrinogen receptor, integrin αIIbβ3, the release of granules, and the secretion of thromboxane-A2. The release of second wave mediators and the binding to G-protein-coupled receptors are important for the capture and incorporation of platelets from the blood stream into the growing thrombus.[3–5]

Procoagulant activity in a subpopulation of platelets in the thrombus depends on collagen, the most important agonist for the formation of adherent procoagulant platelets in the presence of physiological concentrations of extracellular calcium.[26] Signaling via GPVI is the major pathway for the formation of procoagulant platelets. This includes the externalization of the Fas ligand (FasL) at the platelet membrane that is important for procoagulant activity of platelets and red blood cells (RBCs).[27]

All these signaling components play a role in collagen-dependent thrombus formation. However, the role of different proteins is still missing and there is limited knowledge about the contribution of platelet interactions with blood and endothelial cells (ECs). Flow chamber experiments appear to be important to understand the alterations in thrombus phenotypes and to predict the consequences of a gene knockout on thrombosis models in vivo.

Analysis of Thrombus Formation under Flow Using Genetically Modified Mouse Strains

Although great efforts have been made in the last years to understand the mechanism of thrombus formation on collagen, there is still limited knowledge about the relative contribution of different proteins. In a recent synthesis approach, a quantitative scale of 1,514 published studies of arterial thrombus formation (in vivo and in vitro), thromboembolism, and tail-bleeding of genetically modified mice was developed to overcome differences in sample size and heterogeneity in approaches or methods to make different studies of mice with single-gene defects comparable. The authors compared 431 mouse genes and found 17 consistently contributing to thrombus formation without affecting hemostasis. Importantly, ranking analysis indicated high correlations between collagen-dependent thrombosis models in vivo and in vitro. The authors could provide evidence that flow chamber analyses where thrombus formation on collagen under flow was studied in vitro predict the consequences of a genetic loss of a certain protein in mice undergoing arterial thrombosis in vivo.[28] This emphasizes the importance of flow chamber experiments in the analysis of thrombus formation in arterial thrombosis and hemostasis.

In the last decade, microfluidic devices (especially the Maastricht flow chamber) have been applied for the analysis of thrombus formation, using whole blood of genetically modified mouse strains. Mostly, collagen-I was used as adhesive surface at arterial wall shear rates of 450 to 1,700/s using anticoagulated blood for flow chamber perfusion. Thrombus formation on collagen-I was analyzed by brightfield microscopy, often combined with fluorescence microscopy where platelets were labeled by specific antibodies or mepacrine. Different approaches where no coagulation (with thrombin inhibitors) or controlled coagulation with citrate-anticoagulated blood that contains collagen-I supplemented with or without tissue factor (TF) can be applied to analyze platelet aggregation and thrombus formation.[1] An overview of different genetically modified mouse strains and the impact of targeted proteins on thrombus formation under flow conditions, arterial thrombosis in vivo, and tail bleeding times is given in ►Table 1.

Platelet Transmembrane Proteins and Their Regulators: Role of GPVI and CLEC-2 in Thrombus Formation under Flow

The role of GPVI as major collagen receptor has been analyzed using the anti-GPVI antibody JAQ1 that depletes GPVI from the platelet membrane when injected into mice. Defective platelet adhesion to collagen under flow and almost no thrombus formation has been observed with whole blood from JAQ1-injected mice that were protected from lethal pulmonary thromboembolism but displayed normal hemostasis.[29] Treatment of whole blood from wild-type mice with JAQ1 reduced thrombus formation on collagen as well but does not avoid thrombus formation using

Table 1 Overview of genetically modified mouse strains analyzed for thrombus formation under flow using the Maastricht flow chamber

Gene	Protein	(Genetic) modification	Background	Thrombus formation under flow (%)	Thrombosis phenotype	Bleeding phenotype	Reference
APP isoform 751 (human)	APP	*B6.Cg-Tg (Thy1-APP) 3Somm/J: APP23*	C57BL/6	↑	↑	↓	94,96
Psen1, APPSwe, tauP301L	Presenilin, APP, tau	*B6;129-Tg (APPSwe, tauP301) 1Lfa Psen1tm1 Mpm/Mmjax* (3xTg-AD)	C57BL/6/SV129	↑	n.d.	n.d.	95
Bgn	Biglycan	$Bgn^{-/0}$	C57BL/6	○	↓	↑	23
Cdc42	Small GTPase CDC42	$Cdc42^{fl/fl}$-*Pf4-Cre*	C57BL/6 × 129SV	○	↑	↑	18
Clec1b	CLEC-2	INU1 antibody[a]	C57BL/6	↓	↓	↑	33
Clec1b	CLEC-2	*Clec1bfl/flGPIbα-Cre*	C57BL/6	↓	↓	n.d.	35
Clec1b	CLEC-2	$Clec\text{-}2^{-/-}$ *chimera*	C57BL/6	↓	↓	○	36
CypA (Ppia)	Cyclophilin A (peptidyl-prolyl cis-trans isomerase A)	*129.Cg-Ppia tm1Lubn/J*	129S6/SvEvTac	↓	↓	○	13
Faslg	Tumor necrosis factor ligand superfamily member 6 (Fas ligand)	$FasL^{-/-}$	C57BL/6	↓	↓	○	27
Fas	Ophn1TNF receptor superfamily member 6)	$FasR^{-/-}$	C57BL/6	↓	↓	○	27
Gp6	GPVI	JAQ1 antibody[a]/GPVI null mice	C57BL/6	↓	↓	○	29,31
Gp6/Clec1b	GPVI/CLEC-2	JAQ1/INU1 antibodies[a]	C57BL/6	n.d.	↓	↑	34
Panx1	Pannexin-1	$Panx1^{fl/fl}$-*Pf4-Cre*	C57BL/6	↓	n.d.	n.d.	22
Plcg2	Phospholipase C-γ2	$Plcg2^{Ali5}$	C3HeB/FeJ	↑	↑	n.d.	32
Pld1	Phospholipase D1	$Pld1^{-/-}$	C57BL/6	↓	↓	○	21
Pld2	Phospholipase D2	$Pld2^{-/-}$	C57BL/6	○	○	○	35
Pld1/Pld2	Phospholipase D1/2	$Pld1^{-/-}/Pld2^{-/-}$	C57BL/6	n.d.	↓	○	35
Sgk1	Serum- and glucocorticoid-inducible kinase 1	$sgk1^{-/-}$	C57BL/6	↓	n.d.	○	36
Smpd1	Acid sphingomyelinase	$Smpd1^{-/-}$	C57BL/6	↓	↓	○	37
Ophn1	Oligophrenin 1	$ophn1^{-/-}$	C57BL/6	↑	↑	↓	39
Rac1	Small GTPase RAC1	$Rac1^{fl/fl}$-*Mx-Cre*	C57BL/6	↓	↓	↑	19
Reln	Reelin	*Reeler, B6C3Fe a/a-Relnrl/J*	C57BL/6	↓	↓	○	24,25
Rhoa	Small GTPase RHO-A	$Rhoa^{fl/fl}$-*Pf4-Cre*	C57BL/6 × 129SV	○	↓	↑	20
Stim1	stromal interaction molecule 1 (STIM1)	$Stim1^{-/-}$	C57BL/6	↓	↓	○	40

Abbreviations: APP, amyloid precursor protein; n.d., not determined; ↑, increased/prolonged; ↓, decreased/shortened; o, unchanged.
Notes: Thrombus formation as percentage of surface coverage for mice with indicated genetic deficiencies in comparison to wild-type mice. Further indicated are published effects of the gene deficiency (same mouse strain) on in vivo arterial thrombosis, pulmonary thromboembolism, and/or (tail) bleeding.
[a]Antibody-mediated deficiency of CLEC2 and/or GPVI.

whole blood from human GPVI knock-in mice (hGP6tg/tg), suggesting that binding of JAQ1 to a structurally conserved epitope in GPVI differently affects receptor function in human and murine platelets.[30] JAQ1-mediated effect on thrombus formation under flow was confirmed by the analysis of GPVI-deficient mouse platelets because thrombus formation on collagen was abolished.[31] An adaption of the microfluidic whole blood assay with the flow chamber revealed an early involvement of GPVI in thrombus formation using the newly synthesized antibody EMF-1.[32] Following GPVI signaling in platelets, the enzymatic activity and activation of PLCγ2 is tightly regulated as shown by the analysis of mutant mice. The gain-of-function mutation in the Plcγ2 gene in $Plcg2^{Ali5/+}$ mice led to virtually unlimited thrombus formation on collagen under flow resulting in a prothrombotic phenotype in vivo.[33] Modulators of GPVI activation have been described to also modulate thrombus formation on collagen under flow conditions. The extracellular matrix protein biglycan (proteoglycan) was recently identified to bind GPVI. Thus, thrombus formation on a mixed collagen/biglycan matrix was significantly enhanced compared to collagen alone suggesting a crucial role of biglycan for GPVI activation. In vivo, the deficiency of biglycan resulted in reduced platelet adhesion to the injured carotid artery and prolonged bleeding times.[23] The secreted GP reelin has been shown to regulate GPVI activation as well.[25] This together with reelin-mediated activation of GPIb modulates thrombus formation under high shear conditions.

Thus, genetic deletion of reelin protects mice against arterial thrombosis by impairing GPIb–GPVI-mediated integrin activation.[24]

The C-type lectin–like receptor 2 (CLEC-2) has been shown to play an important role in thrombus formation under flow conditions. Mice were injected with an anti-CLEC-2 antibody leading to a specific loss of CLEC-2 on circulating platelets. CLEC-2-deficient platelets displayed normal adhesion but reduced aggregate formation following platelet adhesion under flow. Delayed thrombus formation with increased embolization was observed and responsible for protection against arterial thrombosis and increased bleeding times.[34] Results from antibody-induced deletion of CLEC-2 in mice were confirmed using mice with a platelet-specific knockout of CLEC-2 which had reduced aggregate formation on collagen at arterial shear rates.[35] Another study demonstrates that CLEC-2 is important for thrombus stabilization as shown in in vitro and in vivo experiments using mice after transplantation of fetal liver cells from CLEC-2-deficient embryos.[36] Deletion of both, GPVI and CLEC-2, by antibody treatment of mice resulted in delayed thrombus formation and smaller thrombus volume with increased bleeding times but protection against arterial thrombosis in mice.[37]

Impact of Cytosolic Platelet Proteins in Thrombus Formation In Vitro

Different intracellular platelet proteins have been shown to contribute to thrombus formation under flow conditions. Phospholipase D1 (PLD1)-deficient platelets displayed impaired integrin activation and defective GPIb-dependent aggregate formation under high shear conditions. These defects resulted in a reduced thrombosis phenotype while tail bleeding times were not affected by the loss of PLD1.[21] In contrast, genetic deficiency of PLD2 did not affect thrombus formation under flow.[38] Thus, arterial thrombosis, ischemic stroke, and hemostatic function were unaltered in PLD2-deficient mice. However, mice lacking both PLD isoforms (PLD1 and PLD2) showed defective thrombus formation in vivo.[38]

The serum- and glucocorticoid-inducible kinase 1 (SGK1) is expressed in platelets and plays a role in calcium signaling by regulating the expression of ORAI1 in megakaryocytes. Thus, thrombus formation under flow was significantly impaired while tail bleeding times were unaltered in SGK1-deficient mice.[39] The impact of platelet calcium signaling for thrombus formation has also been shown in stromal interaction molecule 1 (STIM1)-deficient mice. STIM1 has been identified as the Ca^{2+} sensor in the endoplasmic reticulum (ER) that activates Ca^{2+} release-activated channels in platelets.[10] Thus, mice with a genetic loss of STIM1 have marked defects in agonist-induced Ca^{2+} responses, impaired activation, and thrombus formation under flow in vitro.[40] This leads to protection against arterial thrombosis, an ischemic brain infarction with only a mild bleeding phenotype. The STIM1 regulator cyclophilin A (CypA) has been identified as important calcium regulator in platelets. CypA deficiency strongly blunted activation-induced calcium mobilization from intracellular stores and calcium influx from the extracellular compartment. This leads to impaired platelet activation including reduced thrombus formation under flow. In a mouse model of arterial thrombosis, mice with genetic loss of CypA were protected against thrombosis while hemostasis was unaltered.[13]

Acid sphingomyelinase (Asm) participates in the regulation of platelet secretion and PS exposure. Thus, Asm-deficient mice display reduced degranulation of alpha and dense granules and impaired PS exposure leading to reduced thrombus formation under high shear conditions. In vivo, Asm-deficient mice are protected against arterial thrombosis while bleeding times were unaffected, suggesting normal hemostasis in these mice.[41]

Consequences of Cytoskeletal Defects in Platelets

Cytoskeletal reorganization of platelets by Rho GTPases is essential for the increase of the platelet surface, platelet adhesion, and thrombus formation in hemostasis and thrombosis.[42] Rac1 was identified to be crucial for the formation of lamellipodia but also plays an important role in the regulation of PLCγ2 activation following GPVI engagement.[19] Consequently, Rac1 deficiency resulted in impaired thrombus formation under flow conditions in vitro and protection against arterial thrombosis in vivo accompanied by elevated bleeding times. Lack of Cdc42 in platelets resulted in elevated platelet secretion, increased aggregation, and enhanced aggregate formation on collagen under flow. Thus, occlusion of ferric chloride-injured arterioles was faster compared to control mice.[18] The activation of Rho GTPases is regulated by GTPase-activating proteins (GAPs) stat stimulate their GTPase activity to terminate Rho signaling. The Rho GAP oligophrenin (OPHN1) was identified as important regulator of platelet cytoskeletal reorganization. Loss of OPHN1 led to abnormal Rho activation with increased platelet cytoskeletal reorganization including platelet adhesion and lamellipodia formation.[43] Enhanced platelet activation in OPHN1 deficient mice resulted in elevated thrombus formation under flow conditions in vitro and promoted arterial thrombosis and hemostasis in vivo suggesting a pro-thrombotic phenotype in these mice. Reelin not only modulates GPIb and GPVI activation of platelets but also amplifies integrin αIIbβ3 outside-in signaling. Thereby reelin promotes platelet adhesion, cytoskeletal reorganization and clot retraction as mediated by the activation of the Rho GTPases Rac1 and RhoA.[25] This together with the modulatory effect on GPVI activation resulted in abolished arterial thrombosis when reelin deficient mice were treated with JAQ1 to deplete GPVI in these mice.

Platelet Procoagulant Defects and Thrombus Formation

Procoagulant activity of platelets is crucial for thrombin generation and stabilization of the growing thrombus.[26] Thus, the formation of procoagulant platelets is important

for thrombosis. Different signaling pathways in platelets are known to contribute to procoagulant activity. In detail, Asm modifies PS exposure of platelets. This together with defective platelet degranulation in Asm-deficient mice led to reduced thrombus formation in vitro and in vivo.[41] Another example is the loss of PLD1 in platelets that not only impairs integrin αIIbβ3 activation but also display reduced coagulant activity leading to impaired thrombus formation under flow conditions and protection against arterial thrombosis.[21] The reduction in thrombus formation has also been observed in SGK1-deficient mice which show reduced procoagulant activity as a consequence of defective calcium mobilization.[39] Signaling via GPVI represents a major pathway for the formation of procoagulant platelets.[26] Thus, it is not surprising that the gain-of-function mutation in PLCγ2 causes platelet hyperreactivity including enhanced PS exposure. This together with enhanced integrin activation and granule secretion amplifies thrombus formation under flow in vitro and results in a prothrombotic phenotype in vivo.[33]

Impact of Different Blood Cells on Thrombus Formation

The adhesion of platelets, the rheological influence of RBCs, the incorporation of these two cell types (as well as white blood cells [WBCs]) during thrombus growth, and hemodynamic aspects have a strong impact on the process of thrombus forming and its shape in relation to the site of origin.[44] This complex shear-dependent process is fundamental to thrombosis and hemostasis. In the past, it became clear that the process of thrombus formation strongly depends on the flow velocity accompanied by incorporation of RBCs in different manners. Lower flow velocity leads to a meshwork of fibrin, platelets, and high amounts of RBCs inside the clot, which is the opposite in thrombi formed under high shear rates.[44] Platelets are essential for initial thrombus formation, because they adhere and aggregate at the site of blood vessel injury. The initial cell adhesion is induced by the interaction of the platelet GP receptor, GPIbα, which interacts with vWF accompanied by the binding with the extracellular matrix molecule collagen (GPIb–vWF–collagen complex).[45] The concomitant activation of integrin $\alpha_{IIb}\beta_3$ leads to fibrinogen binding resulting in cross linkage of platelets to each other.[46] However, platelets are only weakly connected via protein bridges between cross-linked $\alpha_{IIb}\beta_3$ integrins. Thus, the initial thrombus is unstable and has to be stabilized due to the incorporation of fibrin during the phase of the secondary hemostasis.[47] Flow perfusion chambers allow mimicking and investigating these aspects mentioned earlier as well as platelet function, aggregation, and coagulation at the same time. In addition, they allow the use of different matrices, most typically collagen type I/III, vWF, fibrinogen, fibronectin, TF, thrombospondin, and proteins of the extracellular matrix.[48–50] Due to the flow chamber system, a quantity of pathways in platelets leading to calcium signaling, integrin activation, and secretion which contribute to thrombus formation including the feature of the hemodynamic environment were unraveled.[51–53]

Over the last two decades, it became apparent that beside the traditional aspects of platelets and the rheological influence of RBCs on thrombus formation, RBCs play an important, clinically significant role in blood clotting, hemostasis, and thrombosis. The analysis of platelet–RBC interaction revealed that binding of platelet FasL to the FasR exposed at the RBC membrane induced procoagulant activity of both cells important for thrombus formation under flow conditions. Thus, either deletion of FasL or FasR resulted in reduced PS exposure, decreased thrombus formation under flow in vitro, and protection against arterial thrombosis in vivo while tail bleeding times were unaltered in these mice.[27] This points to a direct role of RBCs in thrombin generation, thrombus formation, and stabilization in hemostasis and thrombosis.[27] High FasL and PS exposure of platelets and RBCs in arterial thrombi of patients provides evidence that FasL–FasR-mediated cell contact between platelets and RBCs represents a pathophysiological mechanism as well.[27] In addition, PS-positive RBCs facilitate the formation of the prothrombinase complex, thereby increasing thrombin generation[54] and they can bind to platelets via the receptors CXC chemokine ligand 16 (CXCL16) and CD36.[55] Furthermore, the incorporation of RBCs into a thrombus leads to structural alterations, which affect the stabilization and the character of the thrombus[56] accompanied by fibrinolytic resistance.[57] However, the role of RBCs in hemostasis and the underlying mechanisms of how RBCs contribute to thrombosis is still elusive.

In addition to the plug formation, which transiently stops bleeding, platelets and RBCs provide a surface for the subsequent steps of the coagulation cascade leading to fibrin formation.[27,58] Monocytes and macrophages have long been recognized as the major cells for activation of the coagulation system. Leukocytes (WBCs) form mixed aggregates with platelets influencing the architecture of thrombi. After migration and activation inside thrombi, they secrete different proteases that enhance the vWF-dependent platelet adhesion such as elastase, matrix metalloproteinases (MMPs), and cathepsin G.[59] Especially elastase secreted from neutrophils promote an alternative pathway of fibrinolysis,[59] including two different aspects: (1) direct degradation of fibrin and (2) effects on the plasminogen–plasmin system.[60] Furthermore, leukocyte-released MMPs induce shedding of P-selectin from the surface of activated platelets.[61,62] Common features of neutrophils are neutrophil extracellular traps (NETs) and the observations that the extracellular components are formed by DNA, histones, and granule constituents, which have an impact on procoagulant and prothrombotic effects.[63,64] NETs are known in the context of thrombus growth and can bind to both platelets and vWF under shear.[65] Furthermore, they are also known for RBC recruitment into the growing thrombus.[66] In addition, neutrophils are able to form heterotypic aggregates with annexin V-positive platelets and thereby contribute to the clearance of PS-positive platelets within the growing thrombus.[67,68]

Beyond the aspect that all three types of blood cells (platelets, RBCs, WBCs) are in a resting, low-adhesive state while circulating in blood under physiological conditions, they could interact with each other, with vascular ECs and the subendothelium after cell-specific stimulation in

hemostasis and thrombosis as well as under pathophysiological conditions. ►Fig. 1 summarizes the interaction of different blood cells and their impact in thrombus formation.

Platelet Interaction with Erythroid, Inflammatory, and Endothelial Cells under Flow Conditions

Flow chamber experiments not only allow the analysis of thrombus formation on different matrices but are also a very elegant tool to investigate the interaction of platelets with RBCs, WBCs, and ECs to analyze the impact of these interactions in thrombus formation and inflammation.

Platelet–RBC Conjugates

RBCs are not only passive bystanders in hemostasis; different aspects have been described with regard to their contribution to thrombus formation, especially the interaction with platelets. Goel and Diamond who investigated deep vein thrombosis (DVT) demonstrated tethering, adherence, and strong binding of RBCs to platelets under depressed venous shear rates.[69] Flow chamber experiments under depressed shear rates (25/s) on a surface of collagen-adherent platelets showed approximately 600 events of tethering RBCs (detached within 1 second) and adherent RBCs (detached within 10–50 seconds) in experimental time period of 5 minutes. These events inversely correlated to the shear rate (e.g., for a shear rate of 100/s), and approximately 25 RBCs were counted.[69] Results of Walker et al and Noh et al confirmed the hypothesis that PS on RBCs can act as an adhesion molecule for the binding of platelets and that PS-exposing RBCs contribute to the development of a thrombus.[55,70] In addition, RBCs can bind to platelets via the receptors CXC chemokine ligand 16 (CXCL16) and CD36[55] and RBCs ICAM-4 was suggested to be a novel ligand for integrin αIIbβ3 on platelets.[71] Recent studies of Klatt et al demonstrated that RBCs are essential for thrombus formation in vitro and in vivo, because the reduction of RBCs results in prolonged occlusion times in anemic mice. In addition, a leak of RBCs in flow chamber experiments leads to reduced thrombus growth in a dose-dependent manner. Furthermore, platelet–RBC interactions via FasL–FasR-mediated signaling provide evidence at contribution of RBCs in thrombin generation, thrombus formation and thrombus stabilization in hemostasis and thrombosis.[27] In addition, under pathological conditions, in diabetes mellitus and sickle cell crisis, PS exposure directly contributes to thrombotic events.[72] Besides the known effects of elevated viscosity and the direct binding to platelets via PS exposure, RBCs perform a signaling role in hemostasis via the release of ADP and ATP. Thus, RBCs promote platelet aggregation and degranulation under low pH and in response to mechanical deformation.[73,74] Taken together, RBCs are important binding partners of platelets and an essential component of clot formation.

Platelet–WBC Conjugates

In recent years, it was shown that besides their pivotal role in coagulation and thrombus formation platelets are capable of modulating inflammatory processes.[75,76] The interaction of platelets with circulating leukocytes, either by direct cell–cell interaction or the release of proinflammatory cytokines, is key mechanism of platelet-mediated immune response regulation. The formation of these circulating heterotypic platelet–leukocyte aggregates is promoted by platelet activation,

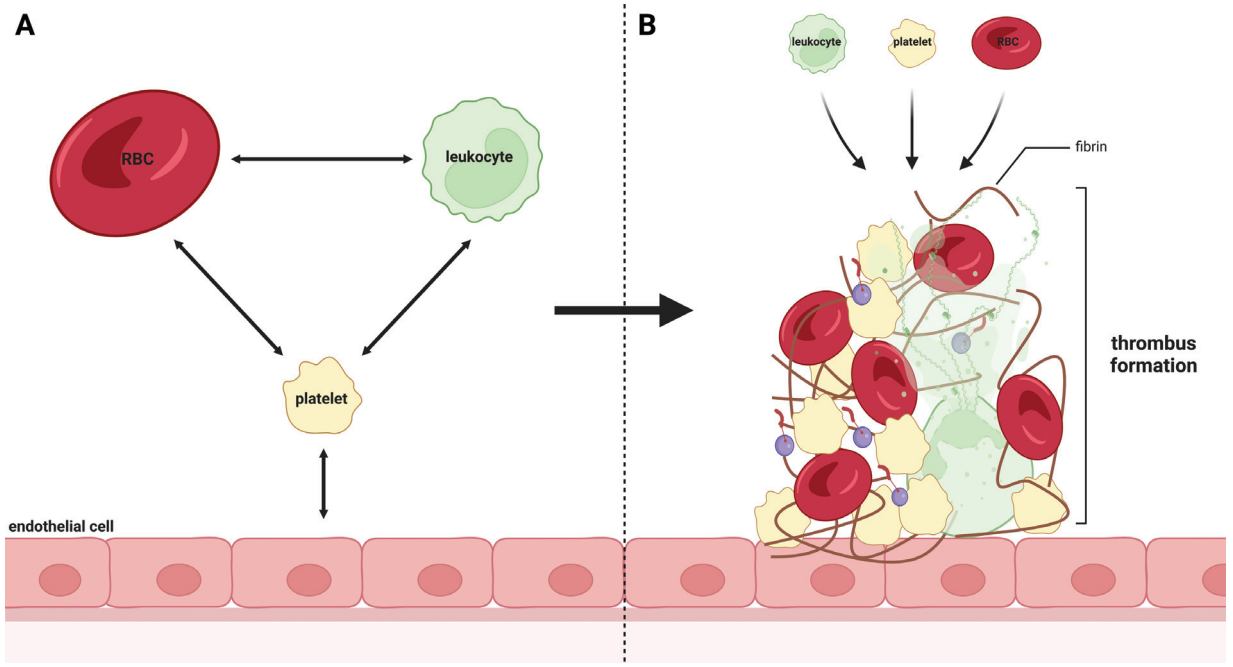

Fig. 1 Overview of platelet interaction partners involved in thrombus formation. (**A**) Scheme of the interaction of platelets with RBCs, leukocytes, and endothelial cells. (**B**) Recruitment of different blood cells during clot formation following vascular injury. RBCs, WBCs, and platelets are incorporated into the growing thrombus by various mechanisms. RBCs, red blood cells. WBCs, white blood cells.

proinflammatory cytokines release, and enhanced shear stress.[77,78] Thereby, aggregate formation of activated platelet and circulating leukocytes is mediated by binding of exposed P-selectin on the platelet surface and the GP ligand-1 (PSGL-1) on leukocytes. PSGL-1 activation results in an upregulation of Mac-1 (CD11b/CD18) on the leukocyte surface that promotes binding to platelet integrin αIIbβ3 via fibrinogen. In addition, binding of platelet GP Ibα to PSGL-1 promotes platelet–leukocyte interactions under thromboinflammatory conditions. Furthermore, platelet–leukocyte interactions are mediated by binding of ICAM-2 to integrin αLβ2, TREM-1L to TREM-1, and the CD40L–CD40 axis. Thereby, these mechanisms of platelet–leukocyte interactions are crucial for leukocyte recruitment in thrombus formation under high arterial shear rates in the context of arterial thrombosis.[79–81] Klier and colleagues provided strong evidence that platelet-mediated leukocyte recruitment under flow is at least partially depending on PLD1 because the recruitment of leukocytes to collagen-adherent *Pld1*$^{-/-}$ platelets was significantly reduced under high shear conditions using the Maastricht flow chamber system.[82] The PLD1-mediated integrin αIIbβ3 activation was causative for reduced adhesion and binding of leukocytes to *Pld1*$^{-/-}$ platelets.

Platelet–EC Conjugates

The direct interaction of platelets with ECs plays an important role in thrombus formation as well as in inflammatory processes, including leukocyte recruitment. Platelet interaction with activated ECs is mediated by different mechanisms including binding of platelet integrin αIIbβ3 via fibrinogen to the endothelial integrin αvβ3 or ICAM-1, binding of P-selectin to PSGL-1, and the interaction of the platelet GPIb–IX–V complex and P-selectin.[83,84] Results using the flow chamber systems revealed the binding of the platelet GPIb–IX–V complex to endothelial vWF, together with P-selectin–PSGL1 interactions as the most important mechanisms for platelet tethering on activated ECs.[85–87] However, for the stable adhesion of platelets on activated ECs under flow condition, the platelet integrin αIIbβ3 plays a pivotal role. Blocking of the integrin αIIbβ3 on the platelet surface, that prevents the binding of both fibrinogen and vWF, results in reduced platelet adhesion and thrombus growth, as already mentioned, and interacts the platelet integrin αIIbβ3 via fibrinogen or vWF with the endothelial integrin αvβ3. Blocking of the endothelial αvβ3 in flow chamber experiments, in turn, also leads to a strongly reduced platelet adhesion on activated ECs. Indicating that both receptors are key mediators for the stable adhesion of platelets on the endothelium.[88,89] Klier et al reported reduced adhesion of platelets under inflammatory conditions in an EC-coated flow chamber system (TNF-α pretreated MHEC5-T cells) at high arterial shear rates (1,700/s) in a PLD1-depending manner. In contrast, platelet adhesion to activated ECs was unaltered under low shear conditions (150/s) indicating that platelet PLD1 is crucial for EC-mediated platelet recruitment under high shear rates.[82] A detailed overview about the different mechanisms involved in cell–cell interaction upon thrombus formation and inflammation is shown in ►Fig. 2.

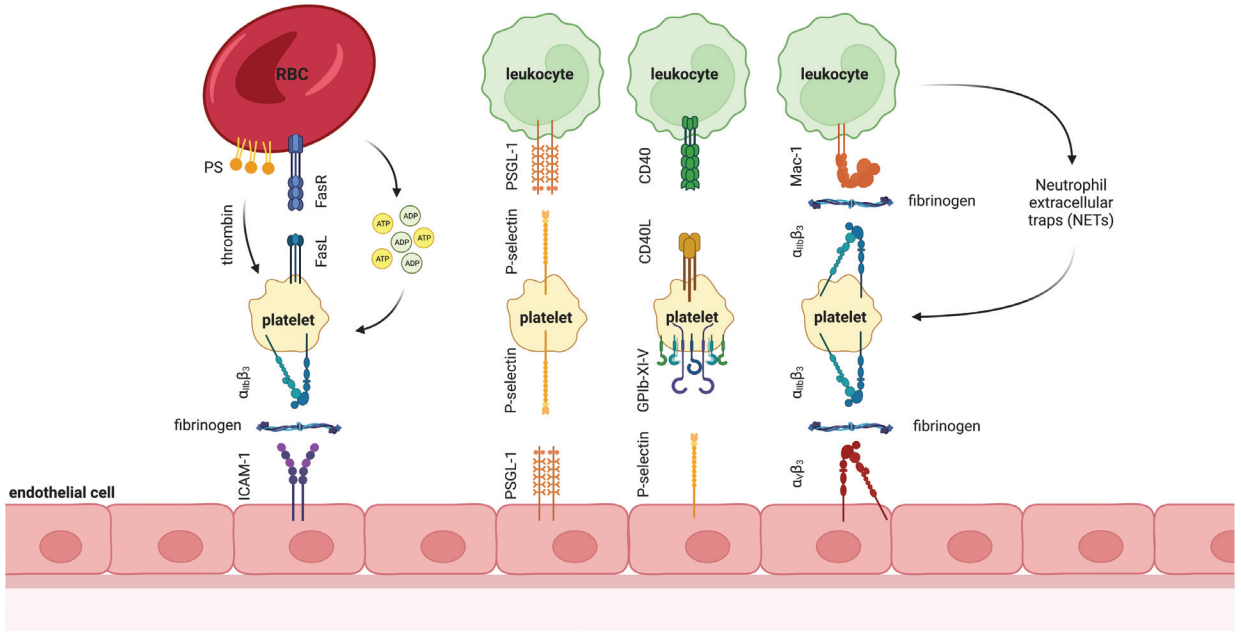

Fig. 2 Platelet–RBC, platelet–WBC, and platelet–endothelial cell interactions during thrombus formation. The endothelium and different shear rates in the vessel influence the interaction of RBCs, WBCs, and platelets. RBCs are able to bind to platelets via a FasL–FasR-driven mechanism thereby influencing platelet activation and aggregation and the release of ATP and ADP. Thrombus formation and stabilization is further supported by the release of extracellular vesicles, thrombin generation and an enhanced procoagulant state of RBCs. Platelet interaction with activated endothelial cells is mediated by different mechanisms as illustrated in the cartoon. Leukocytes bind to adherent platelets in a PSGL-1/P-selectin, CD40L–CD40-, and $\alpha_{IIb}\beta_3$–Mac-1-dependent manner upon thrombus formation and growth. RBCs, red blood cells; PS, phosphatidylserine; ICAM-1, intercellular adhesion molecule 1.

Pathophysiological Thrombus Formation in Experimental Mice

Liver Disease

Cirrhosis and chronic liver diseases are characterized by alterations in hemostasis. Cholestatic liver disease impacts platelet function as analyzed in bile duct ligated (BDL) mice. BDL in mice affects GPVI-induced platelet activation leading to strongly reduced thrombus formation under flow conditions in vitro and bleeding complications as detected by tail bleeding times.[90]

After partial hepatectomy (PHx), platelet activation defects resulted in reduced thrombus formation under flow conditions and impaired hemostasis 1 and 3 days postsurgery compared to sham controls. Platelet activation defects were caused by enhanced plasma levels of nitric oxide, prostacyclin, and bile acids leading to impaired tail bleeding times. Unexpectedly, occlusive thrombosis in the carotid artery of PHx mice was unaltered following ferric chloride-induced injury of the vessel.[91]

In a mouse model of LPS-induced sepsis, PLD1-deficient mice showed reduced organ damage and disseminated intravascular coagulation (DIC) that was probably due to reduced platelet activation, thrombus formation, and procoagulant activity in PLD1-deficient mice.[21,92]

Alzheimer's Disease

Platelets play a pivotal role in Alzheimer's disease (AD).[93] In an experimental mouse model of AD (APP23), platelet activation was enhanced leading to elevated thrombus formation under flow conditions in vitro and accelerated vessel occlusion in vivo suggesting that these mice are at high risk of arterial thrombosis leading to cerebrovascular and cardiovascular complications.[94] Platelets in 3xTg-AD mice, another mouse model of AD, showed an increased ability to form thrombi under shear.[95] GPVI and integrin αIIbβ3 are major players in these processes.[96,97] Cerebral amyloid angiopathy (CAA) is a pathological hallmark of AD and characterized by deposits of amyloid-β (Aβ) 40 in the walls of cerebral vessels.[98] Platelets are able to bind to soluble Aβ40 by GPVI and integrin αIIbβ3 leading to platelet activation, reactive oxygen species generation, and cell membrane scrambling.[99] Consequently, thrombus formation on a mixed collagen-Aβ40 matrix was elevated compared to collagen alone. Furthermore, Aβ40 injection into mice induced increased binding of activated platelets to the injured carotid artery in vivo.[99] Besides, platelets induce the formation of fibrils from soluble Aβ40 suggesting that platelets directly contribute to CAA by supporting the formation of Aβ aggregates indicating a role for platelets in the progression of AD. This was further supported by results from clopidogrel-treated APP23 mice, which showed reduced CAA after a 3-month period of clopidogrel intake.[96]

In conclusion, the analysis of thrombus formation under flow conditions using the Maastricht flow chamber allows the determination of pathophysiological platelet activation and the consequences for thrombus formation under flow conditions in vitro. Thus, defective platelet activation as observed in liver disease is reflected by reduced thrombus formation while elevated platelet activation as detected in experimental AD mice (APP23) is reflected by enhanced thrombus formation under flow conditions. As expected, elevated thrombus formation under flow in vitro was paralleled by enhanced thrombosis in vivo using AD transgenic mice. However, it is not clear to date why reduced thrombus formation under flow using whole blood from mice with liver disease (BDL, PHx) did not result in any alterations in arterial thrombosis in vivo.

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Conflict of Interest

The authors declare that they have no conflict of interest.

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