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Platelet-RBC interaction mediated by FasL-FasR induces pro-coagulant activity important for thrombosis

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ABSTRACT

Red blood cells (RBCs) influence rheology, release ADP, ATP and nitric oxide suggesting a role for RBCs in hemostasis and thrombosis. Here we provide evidence for a significant contribution of RBCs to thrombus formation. Anemic mice showed enhanced occlusion times upon injury of the carotid artery. A small population of RBCs was located to platelet thrombi and enhanced platelet activation by a direct cell contact via the FasL-FasR (CD95) pathway known to induce apoptosis. Activation of platelets in the presence of RBCs led to platelet FasL exposure that activated FasR on RBCs responsible for externalization of phosphatidylserine (PS) on the RBC membrane. Inhibition or genetic deletion of either FasL or FasR resulted in reduced PS exposure of RBCs and platelets, decreased thrombin generation and reduced thrombus formation in vitro and protection against arterial thrombosis in vivo. Direct cell contacts of platelets and RBCs via FasL-FasR were shown after ligation of the inferior vena cava (IVC) and in surgical specimens of patients after thrombectomy. In a flow restriction model of the IVC, reduced thrombus formation was observed in FasL-/- mice. Taken together, our data reveal a significant contribution of RBCs to thrombosis by the FasL-FasR pathway.
INTRODUCTION

Platelets play an essential role in hemostasis by the formation of a primary hemostatic plug to avoid excessive blood loss upon vessel injury (1, 2). In contrast, uncontrolled platelet activation upon rupture of an atherosclerotic plaque is responsible for acute vessel occlusion, leading to myocardial infarction or stroke (3, 4).

Injury of the endothelium leads to exposure of extracellular matrix proteins that serve as substrates to initiate the adhesion and activation of circulating platelets by the engagement of specific receptors (2). VWF binds the platelet-specific GPIb-V-IX complex, collagens bind the glycoprotein receptor GPVI and integrin α2β1, and fibronectin engages both integrin α5β1 and laminin α6β1 (5). VWF binding to GPIb is essential for platelet recruitment to the vessel wall and initial adhesion in arterioles and stenotic arteries (6). Engagement of GPVI by collagen leads to platelet activation important for stable platelet adhesion mediated by integrin αIIbβ3 binding to vWF and fibrinogen (7, 8) and release of platelet granules. Central for irreversible platelet activation is the release of soluble agonists such as thromboxane (TxA2) and ADP because they increase platelet activation by binding to G-protein coupled receptors (9, 10).

Primary hemostasis is essential for a first coverage of injured vessels but not sufficient for thrombus growth and stability (5). Platelet activation followed by blood coagulation and thrombin generation is crucial for the formation of stable arterial thrombi. Platelets play an important role in these processes because they provide a pro-coagulant surface by exposing phosphatidylserine (PS) to allow assembly of coagulation complexes on their plasma membrane. So far it is known, that PS is exposed by the protein scramblase, which does only require an increased Ca^{2+} concentration but no energy and simultaneous Ca^{2+} induced inactivation of translocase and flippase. This happens for example during early apoptosis (11). Platelets support fibrin formation and regulate the retraction of fibrin clots (12).

Red Blood Cells (RBCs) have been described to participate in hemostasis by influencing rheology. In flowing blood, platelets are pressed to the vessel wall leading to a higher concentration of platelets. Thus, an increase in the hematocrit is accompanied by improved hemostasis, especially upon anemia (13-16). Already in 1961 Hellem and colleagues comment
platelet adhesiveness as the most important platelet function in hemostasis (13). The authors cite a study from Duke from 1910 who gave direct transfusions to patients with anemia and thrombocytopenia. He claimed that RBCs influence the hemostatic mechanism because bleeding time in patients with severe anemia was prolonged. Livio and colleagues investigated uremic patients who are often anemic (15). They observed that bleeding times in uremic patients are profoundly influenced by anemia. Blajchman et al. were able to confirm clinical data also in experimental animals (17). They found that RBC transfusions shorten bleeding time in thrombocytopenic rabbits. Saniabadi proposed already 1985 to manipulate the hematocrit as useful therapeutic option in the control of thrombotic and bleeding tendencies (18). Today the German guidelines of polytrauma and treatment of seriously injured persons pretend to increase the hematocrit to min. 7-9 g/dl (Chapter 2.16, 2.108) and to induce a massive transfusion therapy with fresh frozen plasma, RBCs and platelets at the ratio of 4:4:1 (Chapter 2.16, 2.110).

Beside rheological effects different mechanisms of RBCs to influence hemostasis are described, e.g. by the supply of ADP, ATP or NO (19-22). Recent data provide evidence that RBCs are able to expose PS on their membrane and contribute to the generation of thrombin (23-25). However, a direct contribution of RBCs to arterial thrombus formation and the impact of RBCs in platelet activation and thrombus formation are not well-defined. The present study thus explored the active role of RBCs and the relevant mechanisms critical for platelet function and thrombus formation in vitro and in vivo.
RESULTS

**RBCs are essential for three-dimensional thrombus formation under flow ex vivo**

To investigate RBC driven mechanisms in thrombus formation, we analyzed platelet adhesion and thrombus formation under flow using a flow chamber system. Platelet-rich plasma (PRP) was supplemented with different concentrations of RBCs (1-4x10^6 RBCs/µl; 4x10^6 RBCs/µl corresponds to a physiological hematocrit of 40%, Figure 1A-B, Figure S1, Movie S1, Movie S2) or white blood cells (WBCs, Figure 1C-D) and perfused over a collagen-coated surface at different wall shear rates (150, 1000 and 1700 s⁻¹). No thrombus formation was observed with PRP alone or PRP supplemented with different concentrations of WBCs (4x10^3, 7x10^3, 75x10^3, >15x10⁴; shear rate 1.000 s⁻¹, Figure 1C-D, Figure S1). Moreover, no alterations in thrombus formation were detected with 2 and 4x10^6 RBCs supplemented with 75x10^3 WBCs compared to RBCs alone (shear rate 1.000 s⁻¹, Figure S1). The formation of aggregates and three-dimensional thrombi increased with rising amounts of RBCs. Interestingly, the impact of RBCs in thrombus formation was shown to be shear rate dependent because already the addition of 1x10^6 RBCs/µl to PRP led to the formation of first thrombi and PRP supplemented with 2x10^6 RBCs/µl resulted in robust thrombus growth under a shear rate of 150 s⁻¹ while no thrombus formation (1x10^6 RBCs/µl) or only aggregate formation (2x10^6 RBCs/µl) was observed under a shear rate of 1000 and 1700 s⁻¹, respectively. The addition of 4x10^6 RBCs/µl to PRP induced three-dimensional thrombus formation as observed with whole blood that served as positive control (Movie S1, S3). In contrast, high platelet concentrations (PRP with 4x10^8 platelets/µL) did not result in robust thrombus formation under flow (Figure S1). Because RBCs play an important role in rheology, we performed fixation of 4x10^6/µL RBCs and perfused them together with PRP through the chamber to preserve rheology but to avoid any responses of RBCs upon blood perfusion. Again, we did not observe three-dimensional thrombus formation and only platelet aggregates were detected (Figure 1E-F). This data suggest that RBCs might have an active role in thrombus formation. Raising the hematocrit to 75% (8x10^6 RBCs/µl) did not further increase platelet adhesion and thrombus formation (Figure S1D-E) and the addition
of old RBCs (> 40 days after blood withdrawal) did not result in altered thrombus formation under flow (Figure S1F-G).

**A small population of RBCs in platelet-rich thrombi is sufficient to support thrombus formation under flow**

To characterize the impact of RBCs in thrombus formation under flow we compared the cellular components of whole blood and thrombi that were formed under flow conditions. Blood cells in thrombi were dissolved in Accutase to isolate individual cells of the thrombus and analyzed by flow cytometry according to cell morphology and cell specific membrane proteins using cell type specific antibodies (Figure 2A). While > 94% of cells in whole blood are of RBC origin we only found less than 2% RBCs in thrombi that were formed under flow conditions using whole blood and a shear rate of 150 and 1000 s⁻¹, respectively. The analysis of thrombi by toluidine blue staining (Figure 2B) and scanning electron microscopy (SEM, Figure 2C) revealed that only few RBCs are incorporated into a thrombus confirming the data from flow cytometry. Analysis of thrombi isolated from C57BL/6 mice that underwent FeCl₃-induced injury of the carotid artery showed that a small amount of RBCs are located within a thrombus also in vivo (Figure 2D). In fact, these RBCs are located in small islands within the thrombus (Figure 2D).

To test if hemoglobin influences thrombus formation under flow, we perfused PRP supplemented with 13 mg/ml hemoglobin through the chamber and analyzed thrombus formation (Figure 2E-F). No thrombus formation was observed. However, control experiments revealed that no hemoglobin is released upon platelet adhesion and thrombus formation under flow (Figure S1).

To investigate the molecular mechanisms of RBCs and platelets important for thrombus formation we prepared ghosts. Ghosts have an intact membrane but do not contain any metabolites such as NO or ADP/ATP. Measurements of ATP levels in ghosts confirmed successful preparation (Figure 2G-H). Flow chamber experiments using PRP and ghosts showed platelet adhesion and thrombus formation comparable to experiments where PRP and fresh RBCs (4x10⁶ RBCs/µl) were used (Figure 2I-J, Movie S3, Movie S4) suggesting that
components of the plasma membrane of RBCs and not metabolites of RBC origin play an important role in platelet thrombus formation. This was further supported by unaltered ATP levels in native plasma compared to the flow through in the perfusion system (Figure S2).

**RBCs support occlusive thrombus formation ex vivo and in vivo**

In a further set of experiments, thrombus formation was investigated in another *ex vivo* model, where occlusion of capillaries was analyzed (Figure 3A-D). In all experiments where PRP was used, full occlusion of the collagen-coated capillaries was significantly delayed compared to whole blood. Full occlusion of the capillaries was achieved within 10.68 ± 0.42 minutes using whole blood compared to 19.16 ± 1.82 minutes using PRP (p=0.0027). Because PRP and whole blood have different total cell concentrations we also performed experiments with PRP and fixed RBCs (4x10^6) to avoid differences in rheology (Figure 3D, unaltered size of fixed RBCs shown in Figure S3A). Again we measured significantly delayed occlusion of the capillary where PRP and fixed RBCs were used compared to PRP and fresh untreated RBCs (4x10^6; 8.86 ± 0.64 minutes using PRP and fixed RBCs compared to 20.14 ± 5.71 minutes using PRP and fixed RBCs, p=0.0486).

To investigate the impact of RBCs in hemostasis and arterial thrombus formation in vivo, we used anemic mice. To achieve anemia 200 µl blood was taken from C57BL/6 mice at two consecutive days to reduce the hematocrit to 21-28% (ca. 10 g/dl, Figure S3B-C). We then transfused PRP alone or PRP (up to 45x10^4/µL) with RBCs (number of RBCs until a hematocrit of ~40% was reached) to anemic mice (total platelet counts in the circulation of control and anemic mice was similar after transfusion) and analyzed tail bleeding times as well as arterial thrombus formation in vivo. The determination of bleeding times by amputating the tail tip of mice resulted in arrest of bleeding after 144.2 ± 11.2 (PRP+RBCs) vs. 515.3 ± 112.6 (PRP) seconds, providing strong evidence that increased hematocrit is important for normal hemostasis (Figure 3E). To investigate if reduced hematocrit affects occlusive arterial thrombus formation the right carotid artery was injured by topical application of 15% ferric chloride and blood flow was monitored by an ultrasonic flow probe. As shown in Figure 3F, the
onset of thrombus formation as detected by the formation of small aggregates was not different between control and anemic mice although a slight but not significant increase was measured in anemic mice (mean time: PRP+RBCs 245 ± 61.8 s; PRP 420 ± 74.5 s, \( p= 0.1207 \)). However, anemic mice showed significantly enhanced occlusion times compared to control mice (mean time: PRP+RBCs 537.5 ± 111.3; PRP 1005 ± 56.1, \( p= 0.009 \), Figure 3F) suggesting that the hematocrit influences platelet adhesion and aggregate formation at the injured vessel wall in vivo. Accordingly, only very few RBCs were present within the thrombus of anemic mice compared to control mice (Figure 3G). To investigate if increased hematocrit affects hemostasis or arterial thrombosis, we increased the hematocrit (>50%, Figure S3D) in mice by intravenous injection of fresh RBCs from donor mice from the same genotype and determined bleeding times in these mice (Figure 3H). Mice with increased hematocrit (HCT+) did not show altered hemostasis compared to control mice. Along these lines arterial thrombosis was not altered in mice with increased hematocrit as shown by comparable occlusion times in mice with increased hematocrit and control mice after FeCl\(_3\)-induced injury of the carotid artery (Figure 3I).

**RBCs facilitate thrombin generation and PS exposure of RBCs is important for arterial thrombus formation on collagen**

RBCs are known to expose PS on their membrane and contribute to the generation of thrombin (23-25). To elucidate whether RBCs provide a pro-coagulant surface we performed flow chamber experiments with PRP and \( 4 \times 10^{6}/ \mu l \) RBCs or ghosts and incubated ghosts with AnnexinV before perfusion to neutralize exposed PS on the surface of cells (26). As a result, thrombus formation was strongly reduced when AnnexinV was added to ghosts before PRP and ghosts were mixed and perfused through the flow chamber (Figure 4A-B). To examine the impact of PS of intact cells, we incubated RBCs with Ionomycin to induce PS exposure. Thrombus formation with PRP and Ionomycin treated RBCs was not different to PRP supplemented with fresh RBCs. In contrast, Ionomycin treated RBCs showed significantly reduced thrombus formation under flow when pre-treated with AnnexinV (Figure 4C). In a flow
cytometric analysis we confirmed binding of AnnexinV to ghosts, RBCs treated with ionomycin and old RBCs (> 40 days after blood withdrawal) but not to fresh RBCs (Figure 4D). A detailed characterization of the different RBC/CD235 positive cells used in this study is provided in Figure S4. Moreover, we measured PS exposure of RBCs and ghosts after isolation of cells by Accutase treatment of thrombi at the end of the flow chamber experiment. As expected, increasing concentration of RBCs in the flow chamber led to a stronger AnnexinV signal in flow cytometry. The analysis of ghosts revealed high PS exposure of these cells that was significantly reduced by pre-treatment of ghosts with AnnexinV before starting the flow chamber run (Figure 4E). In control experiments, we approved that treatment with Accutase leads to almost complete dissolution of cells from the coverslip and we were able to exclude any effect of Accutase treatment on PS exposure of RBCs (Figure S5). Flow cytometric analysis confirmed an increased number of CD235 positive cells when we perfused ghosts through the flow chamber compared to different approaches with fresh RBCs or pre-treatment of ghosts with AnnexinV (Figure 4F). The change of mean fluorescence intensity (MFI) of AnnexinV binding to RBCs demonstrated almost no differences comparing RBCs before perfusion through the chamber and thereafter (RBCs that leave the chamber without being recruited to the thrombus, Figure 4G). This data confirmed that most RBCs within a thrombus expose PS on their surface, while the vast majority of RBCs from the flow through do not expose PS (Figure 4E-G).

Next, we determined thrombin generation in PRP and PRP supplemented with RBCs after stimulation of platelets with either thrombin (Figure 4H-J) or ADP (Figure 4I-J). Thrombin was generated faster, as demonstrated by shorter lag time, and thrombin peak was increased when PRP was supplemented with RBCs compared to PRP alone (Figure 4H-J).

**Cell-cell contact of platelets and RBCs is crucial for RBC-platelet interaction**

To determine which signal or mechanism is responsible for the induction of PS externalization of RBCs we perfused whole blood through the flow chamber and allowed thrombus formation on a collagen matrix. Thrombi were then analyzed by transmission electron microscopy (TEM).
As illustrated in Figure 5A, RBCs are located within the thrombus as already shown in Figure 2. Furthermore, we found RBCs close to platelets and with direct contact to platelets. Flow cytometric analysis of static experiments using PRP incubated with fresh RBCs demonstrated that a small subpopulation of RBCs and platelets interact with each other because these cells were positively labeled for the platelet marker GPIbα and the RBC marker CD235a (Figure 5B). Comparable results were obtained with ADP stimulated platelets (data not shown). To disclose the underlying mechanisms we incubated RBCs with the supernatant of CRP stimulated platelets and measured AnnexinV binding of RBCs. As expected, no increase of AnnexinV binding was observed (Figure 5C). However, experiments using PRP and thus intact platelets revealed a significant increase of PS exposure of RBCs providing further evidence that a direct cell contact between platelets and RBCs is necessary to induce PS exposure of RBCs. Further experiments using aspirin and Clopidogrel, respectively to inhibit thromboxane formation or ADP signaling via the ADP receptor P2Y_{12} demonstrated that neither thromboxane nor P2Y_{12} play a major role in platelet mediated induction of PS exposure of RBCs (Figure S6A). All these data prompted us to look for a receptor mediated interaction of platelets and RBCs. Recently, the platelet receptors CD36 and CXCL16 have been identified to bind to PS exposed at the RBC membrane (27). To analyze, if these receptors influence the induction of PS exposure of RBCs, we performed flow chamber experiments. Figure S6 demonstrates that neither blood from Cd36 knock-out mice nor treatment of Cd36 wild-type blood with inhibitory antibodies against CD36 and CXCL16 influence PS exposure of RBCs (Figure S6B-D).

**Fas Receptor (FasR, CD95) mediated cell-cell contact of platelets and RBCs is essential for PS exposure of RBCs upon thrombus formation**

Fas receptor (FasR, CD95) signaling in RBCs is known to induce an apoptotic signal via activation of caspase3 leading to PS externalization in human RBCs (28). To investigate if this apoptotic mechanism plays a role in initiating PS exposure of RBCs, we initially confirmed FasR expression of RBCs (Figure S6E). Unexpectedly, FasR expression on RBCs was increased in the presence of resting and activated platelets (Figure S6F). The incubation of
PRP with RBCs under static conditions showed significantly reduced AnnexinV binding of RBCs when we blocked the FasR of RBCs by antibody treatment upon platelet stimulation with ADP (100% (PRP+RBCs) vs. 151.6% ± 16.1 (PRP+RBCs+ADP) vs. 107.9% ± 6.0 (PRP+RBCs+ADP+FasR antibody), Figure 6A). To analyze the effects of FasR inhibition and reduced PS exposure of RBCs on platelet activation, we determined AnnexinV binding of platelets. In fact, PS exposure of ADP and CRP stimulated platelets was strongly reduced in the presence of RBCs treated with blocking FasR antibody (Figure 6B). Moreover, AnnexinV binding of activated platelets was significantly enhanced in the presence of RBCs compared to activated platelets alone (Figure 6B) suggesting that RBCs amplify PS exposure of activated platelets. In the presence of RBCs, inhibition of FasR reduced significantly the endogenous thrombin potential (ETP) after stimulation of platelets with thrombin (Figure 6C-D) while the inhibiting FasR antibody has no effect in PRP alone (data not shown). To investigate the impact of FasR on RBCs under more physiological conditions we performed flow chamber experiments and found significantly reduced thrombus formation under flow when we inhibited FasR only on RBCs (Figure 6E-F). Accordingly, AnnexinV binding of RBCs and platelets was significantly reduced as measured by flow cytometry (Figure 6G-H). However, thrombus formation and PS exposure of RBCs was shown to be not depending on caspases because experiments performed in the presence of a pan-caspase inhibitor did not alter surface coverage of thrombi, thrombus volume or AnnexinV binding to RBCs (Figure S6G-H).

To analyze if platelets have an activated phenotype in the presence of RBCs we determined P-selectin exposure (degranulation marker) and activated integrin αIIbβ3 (PAC-1 binding) by flow cytometry (Figure S7A-B). We did not detect any platelet activation in the presence of RBCs. Same results were obtained in the presence of WBCs (Figure S7C-E).

To analyze whether FasR on RBCs has any impact on arterial thrombosis in vivo, we analyzed wild-type mice treated with blocking FasR antibody (20 µg/mouse) in a thrombosis model where injury of mesenteric arterioles was induced by FeCl₃. Platelet adhesion and thrombus formation at sites of vessel injury was induced by topical application of 20% FeCl₃ on exposed mesenteric arterioles. Complete vessel occlusion occurred in control mice within 1420 ± 142.6
sec. with an occlusion rate of 100%. In contrast, we never observed occlusion of mesenteric arterioles in mice treated with the inhibitory FasR antibody suggesting that these mice are protected against arterial thrombosis (Figure 6I-J, Movie S5, Movie S6). In contrast, hemostasis as analyzed by tail bleeding experiments was not altered in mice that received blocking FasR antibody (Figure 6K). Taken together, these results clearly show that interfering with platelet-RBC interaction by blocking FasR might be a promising new therapeutic strategy.

Platelet membrane bound Fas ligand (FasL) serves as ligand for erythrocyte FasR to mediate platelet-induced PS exposure of RBCs

FasR is activated upon engagement by Fas ligand (FasL) (29). To investigate if FasL expressed on platelets is responsible for FasR activation on RBCs, we first analyzed FasL exposure on the platelet membrane (Figure 7A). While platelet activation did not lead to a significant increase of FasL externalization, we detected a significant increase in FasL expression on resting and activated platelets in the presence of RBCs. The presence of WBCs did not alter FasL expression of resting platelets but of activated platelets (Figure S7E). In another set of experiments platelet adhesion on immobilized FasR showed an increase of ADP stimulated adherent platelets that was significantly reduced when platelets were treated with human Decoy receptor 3 (hDcR3) while no differences were observed upon platelet adhesion to collagen (Figure 7B-D). HDcR3 binds to FasL without any signal transduction capabilities and prevents FasR-FasL interactions by competitive binding to membrane-bound FasL(30). Accordingly, AnnexinV binding to RBCs was significantly reduced when ADP stimulated platelets were incubated with RBCs in the presence of hDcR3 (Figure 7E). Under flow conditions platelet adhesion and three-dimensional thrombus formation was significantly reduced in hDcR3 treated whole blood (Figure 7F-G). In addition to a reduced surface coverage, we found significantly reduced thrombus volume when we perfused fluorescently labeled platelets through the chamber and measured integrated fluorescence intensity (IFI) (Figure 7H). Consequently, number of RBCs in thrombi was reduced and AnnexinV binding of
RBCs showed a significant reduction of PS externalization in samples from thrombi derived from hDcR3 treated blood (Figure 7I-J).

**Genetic deletion of FasR or FasL is responsible for altered PS exposure and thrombus formation in vitro and in vivo**

To confirm the inhibitory experiments with FasR antibody and hDcR3, we analyzed FasL and FasR knock-out mice. First, we measured platelet and RBC counts, hematocrit and basal levels of PS exposure at the RBC membrane in knock-out mice and found no significant alterations between different mouse lines (Figure S8A-C).

Given that we analyzed human platelets with FasR antibody and hDcR3, we now analyzed thrombus formation under flow with murine blood and inhibiting FasR antibody to confirm human data (Figure 8A). Significantly decreased formation of three-dimensional thrombi was found when we perfused whole blood supplemented with FasR antibody through the chamber using a shear rate of 1.700 s⁻¹ (Figure 8A-B). In addition, thrombus formation and thrombus volume was significantly reduced when we perfused fluorescently labeled FasL knock-out platelets and WT RBCs or WT platelets and FasR knock-out RBCs through the chamber and measured integrated fluorescence intensity (IFI) (Figure 8C-D). Moreover, we determined the number of RBCs in thrombi using the RBC marker TER-119 (red) and the platelet marker GP9 (green). As shown in Figure 8E and G, the number of RBCs in thrombi was significantly reduced when we perfused whole blood from FasL⁻/- mice or FasR⁻/- through the chamber (Figure 8E and G).

AnnexinV binding of RBCs and platelets in the absence or presence of RBCs were measured by flow cytometry under static conditions. As shown in Figure 9, the incubation of ADP-activated platelets from WT or FasL knock-out mice (FasL⁻⁻) with WT RBCs as well as WT platelets and FasR knock-out RBCs (FasR⁻⁻) led to a significant decrease in PS exposure of RBCs (Figure 9A) and platelets (Figure 9B). Moreover, resting FasL knock-out platelets displayed decreased PS exposure in PRP alone and in the presence of WT RBCs.
Accordingly, AnnexinV binding of RBCs (Figure 9C) and platelets (Figure 9D) isolated from thrombi in the flow chamber was significantly reduced when either FasR knock-out RBCs and WT platelets or FasL knock-out platelets and WT RBCs were perfused through the chamber. Capillary occlusion in the T-TAS flow chamber confirmed significantly enhanced occlusion times when either FasR knock-out RBCs and WT platelets or FasL knock-out platelets and WT RBCs were analyzed (Figure 9E-H).

We next investigated the impact of FasR and FasL in shear-dependent arterial thrombus formation in vivo. Topical application of 20% FeCl$_3$ on exposed mesenteric arterioles induced vessel injury that resulted in prompt beginning of thrombus formation (Figure 10A) and complete vessel occlusion in WT mice while most of FasR knock-out mice showed a delay in beginning of thrombus formation and no occlusion at the end of the observation period of 40 min (Figure 10B). An even more pronounced phenotype was observed with FasL knock-out mice that show no aggregate or thrombus formation at all, thus no occlusion of the injured vessel was observed in these mice (occlusion time > 40 min, Figure 10A-D). Furthermore, thrombus size at different time points was significantly reduced in FasR knock-out mice while no thrombi were observed in FasL knock-out mice compared to WT controls (Figure 10E). To confirm the importance of platelet FasL in thrombus formation in vivo, we analyzed mice with a cell-specific knock out of FasL in platelets using the PF4-Cre$^+$ FasL$^{fl/fl}$ mice. As shown in Figure 10F, beginning of thrombus formation in PF4-Cre$^+$ FasL$^{fl/fl}$ mice was significantly delayed compared to PF4-Cre$^+$ FasL$^{+/+}$ mice that served as controls. Furthermore, 5 of 8 PF4-Cre$^+$ FasL$^{fl/fl}$ mice show no occlusion of the injured vessel at the end of the observation period of 40 min while 2 PF4-Cre$^+$ FasL$^{+/+}$ mice showed a prolonged time to occlusion compared to PF4-Cre$^+$ FasL$^{+/+}$ controls (Figure 10G-H) confirming the results obtained from FasL$^{-/-}$ mice.

To investigate the interaction of platelets and RBCs in another mouse model we analyzed C57BL/6 mice after ligation of the inferior vena cava (IVC). C57BL/6 mice received murine platelets isolated from C57BL/6 or FasL$^{-/-}$ mice and labeled with RhodaminB and RBCs isolated from C57BL/6 or FasR$^{-/-}$ mice labeled with DCF. After ligation of the IVC, interactions of RBCs and platelets were defined as co-localization for at least three consecutive images (204 msec.,
Figure 10I-K). Cell interactions were quantified per animal 30, 60 and 90 min. after ligation (Figure 10J). C57BL/6 mice receiving FasR<sup>-/-</sup> RBCs or FasL<sup>-/-</sup> platelets showed significantly reduced platelet-RBC interactions 90 min after ligation compared to WT mice with RBCs and platelets from C57BL/6 (WT vs. FasR<sup>-/-</sup> RBCs: 10.75 ± 1.548 vs. 4.25 ± 0.25, p=0.003; WT vs. FasL<sup>-/-</sup>: 10.75 ± 1.548 vs.5.25 ± 1.25 , p=0.0163, Figure 10J). In a flow restriction model of the IVC, venous thrombus formation as measured by thrombus weight was not altered in mice treated with FasR blocking antibody, although the incidence of thrombus formation was reduced with 30% compared to 60% in IgG control mice (Figure 10L). However, the number of FasR antibody treated mice that were able to form a thrombus is low (3 of 10 mice); thus, the incidence of thrombus formation (50% of IgG treated mice) rather than the thrombus weight represents differences between IgG and FasR antibody treated mice. In contrast, venous thrombus formation was significantly reduced in FasL<sup>-/-</sup> compared to control mice while the incidence of thrombus formation was slightly enhanced in these mice (WT vs. FasL<sup>-/-</sup>: 15.06 mg ± 3.91 vs. 5.683 ± 1.277, p=0.0446, Figure 10M).

Normal hemostasis upon FasR or FasL deficiency was observed when tail bleeding times of wild-type, FasR<sup>-/-</sup> and FasL<sup>-/-</sup> mice were measured (Figure 10N). In line with these results, platelet-specific FasL knock-out mice were described to show no differences in bleeding times compared to controls as shown by Schleicher and colleagues (31).

**FasL-FasR mediated PS externalization of RBCs is relevant under pathological conditions**

To investigate whether platelet mediated PS externalization on the RBC membrane via FasL-FasR interaction influences arterial thrombosis in humans, we analyzed thrombi that were isolated from patients who underwent surgical thrombectomy (table I). Histological sections from these thrombi were stained with hematoxylin and eosin and characterized for thrombus morphology (Figure 11A). As before we found RBCs in small islands within the thrombus as already observed in mice after injury of the carotid artery (Figure 2D). TEM analysis of thrombi provided strong evidence for a direct contact of RBCs and platelets under pathological
conditions (Figure 11B) as already observed in thrombi that resulted from platelet adhesion and thrombus formation under flow (Figure 5A). Flow cytometric analysis of cell types within the thrombi revealed that 56.74% ± 4.64 RBCs and 42.37% ± 4.62 platelets were localized to the thrombi (Figure 11C). Next, we measured platelet activation and AnnexinV binding of platelets and RBCs isolated from these thrombi and compared data with freshly isolated resting and activated platelets and RBCs from healthy volunteers. P-selectin exposure of platelets in the thrombi was significantly enhanced compared to resting platelets but only moderately increased compared to CRP activated platelets (Figure 11D). However, FasL externalization at the platelet membrane as well as PS exposure of RBCs isolated from patients’ thrombi were strongly enhanced compared to CRP activated platelets in the presence of fresh RBCs: 21.34 ± 2.69 PRP +fresh RBCs +CRP vs. 44.43 ± 6.13 platelets from patients (FasL), 6.95 ± 1.79 PRP +fresh RBCs +CRP vs. 23.36 ± 2.91 platelets from patient (AnnexinV), respectively (Figure 11E-F). Interestingly, no differences were observed in AnnexinV binding of activated platelets (Figure 11G). Flow cytometric analysis of whole blood from patients revealed that P-selectin and PS exposure of platelets was significantly enhanced compared to healthy volunteers (Figure S9) while no difference of FasL exposure was observed. We next compared RBC-rich thrombi and platelet-rich thrombi and found no alterations in FasL externalization (Figure 11H). However, AnnexinV binding of RBCs in platelet-rich thrombi was significantly enhanced compared to PS exposure of RBCs in RBC-rich thrombi (Figure 11I). PS externalization of platelets was significantly higher in RBC-rich thrombi (Figure 11J). Taken together these data provide first evidence that cell-cell contact of platelets and RBCs via FasL-FasR interaction is also relevant under pathological conditions.
DISCUSSION

In this study, we have shown that RBCs are essential for effective thrombus formation in vitro and in vivo, because a reduced number of RBCs results in attenuated hemostasis and prolonged occlusion times in anemic mice. Beside effects of RBCs on rheology conditions, a small population of RBCs is sufficient to support platelet mediated thrombus formation by providing a pro-coagulant surface. Mechanistically, in a direct cell-cell contact between platelets and RBCs PS exposure of RBCs occurs, mediated through FasL induced activation of FasR, followed by thrombin generation and platelet activation (Figure 11K). Inhibition or genetic deletion of FasR prevents three-dimensional thrombus formation on collagen under flow ex vivo, arterial and venous thrombosis and RBC-platelet interaction after IVC ligation in vivo suggesting that FasL-FasR interaction is essential for thrombus formation. Mice with global FasL deficiency as well as platelet specific loss of FasL confirmed the importance of platelet FasL for the induction of PS exposure at the RBC membrane and thrombus formation in vivo. High FasL and PS exposure of platelets and RBCs in arterial thrombi of patients provide first evidence that FasL-FasR mediated cell contact of platelets and RBCs represent a pathophysiological mechanism as well.

RBCs have been linked to thrombus formation since decades. They have been described to play a passive role in hemostasis by physical and chemical effects on the interaction between platelets and the vessel wall (16, 32). In flowing blood, RBCs displace platelets to the vessel wall, to induce contact of platelets with pro-thrombotic extra cellular matrix proteins exposed upon vessel injury. In the FeCl₃ mouse model of arterial thrombosis, RBCs were shown to participate in thrombosis by supporting platelet adhesion to the intact endothelium (33). A reduced hematocrit is accompanied by prolonged bleeding times in humans and experimental animals, providing a patho-physiologically reasoned rationale to reduce the risk of bleeding by normalizing the hematocrit with RBC transfusions (15, 17, 18, 34). In this study, the analysis of thrombus formation with PRP supplemented with fixed RBCs (Figure 3D, Figure S1) suggests that RBCs not only influence rheology but play an active role in supporting thrombus formation. Different mechanisms for RBCs and their role in hemostasis have been described.
RBCs release ADP, ATP and NO and thus might influence hemostasis by platelet activation (19-22). Already in 1961 Gaarder and colleagues found ADP released by RBCs to be relevant for platelets adhesion (20) and platelet aggregation (35). More recent data link RBCs with PS exposure (28) and suggest pro-thrombotic properties of RBCs (23, 24, 36). Here we directly provide evidence for the contribution of RBCs to thrombin generation because the supply of RBCs to PRP significantly increased the height of thrombin peak and shortened the time when thrombin generation was initiated (Figure 4H-J). However, only less is known so far about the mechanisms of PS exposure of RBCs. Red cell ICAM-4 was suggested to be a novel ligand for integrin αIIbβ3 on platelets (37, 38). This was strengthened by inhibitory experiments with decreased thrombin formation (39). Moreover, adhesion of RBCs to immobilized platelets depends on PS exposure of RBCs and the PS receptors CD36 and CXCL16 (27). To date, it is still not clear how RBCs come into a first contact with platelets to induce FasL-FasR signaling. However, decreased incorporation of RBCs into a thrombus was observed when the interaction of ICAM-4 and integrin αIIbβ3 was inhibited (39).

PS exposure of RBCs led to increased AnnexinV binding of platelets (Figure 6B and 6H). The activation of platelets by RBC-platelet interactions was already shown by different groups. In contrast to this study, the authors provide evidence for enhanced integrin activation, P-selectin exposure, thromboxane production and ADP release when RBCs were present in platelet suspensions (22, 40, 41). Here we did not detect increased P-selectin exposure or integrin activation (Figure S7). However, neither a direct mechanism is known to induce PS exposure of RBCs in a growing thrombus nor a direct contribution of RBCs by PS exposure, thrombin generation nor platelet activation to arterial thrombus formation was shown to date. Thus, the role of FasL-FasR interaction mediating platelet-RBC contacts upon adhesion to the injured vessel and arterial and venous thrombus formation is a complete novel element in platelet activation and aggregate formation. Here we show that the death receptor Fas (CD95) on RBCs is essential for thrombus formation while inhibition or genetic deletion of FasR protects mice from arterial thrombosis. FasL is a type-II transmembrane protein that belongs to the tumor necrosis factor (TNF) family and binds to FasR to induce apoptosis (29, 42, 43).
Furthermore, FasL-FasR interactions play an important role in the regulation of the immune system and the progression of cancer (44-47). FasL is expressed by activated T-cells and natural killer cells and induces apoptosis of target cells via FasR (48). Activated platelets expose FasL on their surface and induce apoptosis in Fas-positive tumor cells in vitro (49) and in a mouse model of stroke in vivo (31). This study reveals that platelet FasL is a ligand for FasR on the RBC membrane critical for the cell-cell interaction of RBCs and platelets. Interestingly, the expression of FasL on the platelet membrane (Figure 7A) as well as FasR expression on RBCs (Figure S6) increased already under resting conditions when platelets and RBCs were co-incubated suggesting that the cells influence the expression of receptors of the other cell. hDcR3 is a TNF-R1 and FasR related death receptor and does not transduce apoptotic signals but competes with the death receptors for ligand binding to inhibit apoptosis (30, 50). Noteworthy, hDcR3 mediated reduction of thrombus formation and PS exposure of RBCs within the thrombus is significantly reduced but to a lesser extent than FasR inhibition. The same observation was made with FasL knock-out platelets (Figure 8C-D) suggesting that another ligand on the platelet membrane might be able to activate FasR on RBCs. Interestingly, PS exposure of RBCs was shown to be not depending on caspases (Figure S6G-H) although caspase activation following FasL binding to FasR has been shown to reflect a general mechanism to induce apoptosis in target cells, especially in the immune system (47) and in cancer (46). However, in RBCs there might be another mechanism that translates FasR activation to PS exposure. In acute leukemia, an alternative pathway through TRADD and TRAF initiates a phosphorylation cascade leading to the activation of JNK and phosphorylation of p53 and c-Jun, which induces apoptosis through multiple mechanisms including Bcl-2 family proteins (51). An acid sphingomyelinase is also activated in response to FasR activation leading to ceramide production and cell stress that also might account for PS exposure at the RBC membrane (52).

In the last years, it becomes increasingly evident that different cellular processes are involved in hemostasis and thrombosis (53, 54). In this study we observed that FasR blocking by antibody (Figure 6K) or deficiency of either FasL or FasR does not lead to impaired hemostasis
(Figure 10A) but protects against arterial thrombosis while anemia leads to increased occlusion times and impaired hemostasis after vessel injury (Figure 3E-F). This suggests that the rheology-dependent impact of RBCs in thrombus formation is different to the FasL-FasR interaction between platelets and RBCs.

The analysis of thrombi isolated from patients by thrombectomy suggests that FasL-FasR mediated interaction of platelets and RBCs might be relevant under pathological conditions. Polycythemia patients with high RBC counts as well as patients with inherited RBC abnormalities suffer from thrombotic complications and exhibit cardiac and cerebral thrombosis with abnormal PS exposure on RBCs (55-60). Moreover, a correlation between the size of infarct and the hematocrit was shown in patients with carotid occlusion and completed strokes (61). Our studies did not confirm a relation of increased numbers of RBCs and thrombus formation because perfusion of PRP and 8x10⁶ RBCs/µl did not result in accelerated thrombus formation under flow (Figure S1D-E). Moreover, mice with increased hematocrit did not show altered hemostasis or differences in arterial thrombus formation in vivo (Figure 3H-I). However, we performed analysis under conditions of intermediate shear (flow chamber: 1.000 s⁻¹, carotid artery: 295 - 645 s⁻¹). It is tempting to speculate that pathological high shear rates might affect RBC-platelet interaction as already shown by Turitto and Weiss (16). Under pathological conditions, increased pro-coagulant activity of RBCs was measured in patients with sickle-cell disease and with nephrotic syndrome (56, 62). Interestingly, RBC-rich thrombi contain more inflammatory cells in patients with ST-elevation myocardial infarction with impaired myocardial reperfusion (63). So far the consequences of hypoxia or atherosclerotic vessels with decreased vessel lumen and high shear rates on platelet-RBC interaction are not known but might also be relevant e.g. upon myocardial infarction or stroke.

However, high levels of PS externalization on platelets and RBCs in arterial thrombi of patients might be also due to the apoptotic character of these cells because some patients already suffered from arterial thrombosis for several hours or days (for details see table 1).
Taken together, our results indicate that RBCs are essential for hemostasis and arterial as well as venous thrombosis. FasL-FasR mediated cell-cell contact of platelets and RBCs mediate PS externalization on the RBC membrane critical for platelet activation and adhesion upon thrombus formation. The here identified mechanism of RBC-platelet interaction is summarized in Figure 9K. Thus, interfering with the interaction of FasL-FasR might be an innovative and promising approach for a completely novel antithrombotic strategy.
METHODS

Animals
Specific pathogen-free C57BL/6J mice were obtained from Janvier labs. B6Smn.C3-Fas<sup>plq/J</sup> mice were obtained from The Jackson Laboratory, USA. Gene-targeted mice lacking CD36 and FasR (CD95, C57BL/6J.MRL-FAS<sup>lpr</sup>), respectively and the corresponding wild-type littermates were bred from breeder pairs and genotyped by PCR. Fasl<sup>fl/fl</sup> mice were kindly provided by Dr Martin-Villalba (University of Heidelberg) and crossed to PF4-Cre mice, which were purchased from The Jackson Laboratory (C57BL/6-Tg [Pf4-cre] Q3Rsko/J). PF4-Cre<sup>+</sup> Fasl<sup>fl/fl</sup> mice or PF4-Cre<sup>+</sup> Fasl<sup>fl/fl</sup> littermate controls were analyzed for thrombus formation. Experiments were performed with male and female mice aged 2-4 months.

Murine Platelet Preparation
Platelets were prepared as previously described (64, 65). Blood was collected into trisodium citrate and centrifuged at 250 g for 5 minutes at room temperature. To obtain platelet-rich plasma (PRP), the supernatant was centrifuged at 50 x g for 6 min. PRP was washed twice at 650 x g for 5 min and pellet was resuspended in Tyrode’s buffer [136 mM NaCl, 0.4 mM Na<sub>2</sub>HPO<sub>4</sub>, 2.7 mM KCl, 12 mM NaHCO<sub>3</sub>, 0.1% glucose, 0.35% bovine serum albumin (BSA), pH 7.4] supplemented with prostacyclin (0.5 µM) and apyrase (0.02 U/mL). Before use, platelets were resuspended in the same buffer and incubated at 37°C for 30 min.

Study approval
All animal experiments were conducted according to the Declaration of Helsinki and German law for the welfare of animals. The protocol was reviewed and approved by the local government authority (Heinrich-Heine-University Animal Care Committee and by the State Agency for Nature, Environment and Consumer Protection of North Rhine-Westphalia LANUV, Recklinghausen, NRW, Germany and by the local legislation on protection of animals.
Regierung von Oberbayern, Munich, Germany; Permit Numbers: 84-02.05.20.12.A487; O 86/12; 84-02.05.40.16.073; 84-02.04.2013.A210.

Experiments with human tissues and blood were reviewed and approved by the Ethics Committee of the Heinrich-Heine-University who approved the collection and analysis of the tissue. Subjects provided informed consent prior to their participation in the study (patients’ consent); Permit /Study Number 4669, ID No. 2014042327.

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AUTHOR CONTRIBUTIONS

HAH, HS, JS, SM, MK and ME designed the experiments, analyzed data and wrote the manuscript. MS carried out electron microscopic studies, WL performed RBC deformability experiments, KJ conducted thrombin measurements, LP performed IVC ligation experiments, and CK, IK, SZ, K-JK, MS, NSG, AO performed all other experimental work.

Conflict of interest: The authors declare no conflict of interest.
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Figure 1. RBCs are essential for three-dimensional thrombus formation under flow ex vivo. Human whole blood or PRP supplemented with different concentrations of RBCs was perfused over a collagen coated surface at indicated shear rates. The effects of blood components on thrombus formation as indicated by mean surface coverage, was determined. Data are expressed as arithmetic means. (A) Representative phase contrast images at the end of the perfusion period. Scale bar 100 µm. (B) Quantification of surface coverage with increasing (from left to right) RBC concentrations added to PRP in comparison to whole blood. Data are given for three shear rates of 150 s⁻¹ (black column), 1000 s⁻¹ (grey column) and 1700 s⁻¹ (white column). Blood components as indicated below and shear rates as shown on the left. (C) Effect of WBCs on thrombus formation under flow. Representative images of surface coverage of whole blood and PRP in the absence and presence of WBCs (7x10³ WBCs/µl and
>15x10^4 WBCs/µl, respectively). Scale bar 50 µm. (D) Quantitative analysis of surface coverage per visual field. (E-F) Thrombus formation using PRP and fixed RBCs. Bar graphs depict mean values ± s.e.m. (n=3, all experiments); Student’s t-test (A-F). *** $P < 0.001$. PRP=platelet-rich plasma; RBC=red blood cell.
Figure 2. A small population of RBCs in platelet-rich thrombi is sufficient to support thrombus formation. (A-D) Cellular composition of whole blood and thrombi. (A) Cell types were determined by morphology and cell specific antibodies in flow cytometry. The diagram presents the percentage of RBCs (white), platelets (black), WBCs (light gray) and cells that
could not be identified precisely (other, dark grey) (n=3). (B) Semi-thin section through a middle layer of a thrombus. RBCs, some indicated by black arrowheads, are spread through the thrombus. Scale bar 10 µm. (C) Scanning electron microscopy of thrombi. Collagen fibers indicated by white arrowheads, platelets indicated by black arrows and RBCs indicated by black arrowheads (n=3). Scale bar as indicated. (D) Thrombi from mice after injury of the carotid artery were isolated and stained with hematoxylin-eosin to visualize RBCs in arterial thrombi. Representative images were shown, Overview (upper panel, details lower panel. Scale bars 100 µm (upper panel) and 20 µm (lower panel). (E and F) Effects of hemoglobin on thrombus formation ex vivo. (E) Representative images, scale bar 50 µm. (F) Mean surface coverage for PRP (2x10⁵ platelets/µl), PRP + RBCs (4x10⁵ RBCs/µl) and PRP + hemoglobin (13 mg/ml) at a shear rate of 1000 s⁻¹ (n=3). (G) ATP release of RBCs, platelets and ghosts was induced by L-arginine and detected by HPLC. A total amount of 4x10⁹ RBCs, an equivalent amount of ghosts and 2x10⁸ platelets were used. (n=3). (H) Preparation of ghosts was visualized under the microscope for quality inspection. Scale bar 50 µm. (I and J) Thrombus formation in the presence of RBCs or ghosts and PRP at a shear rate of 1000 s⁻¹. (I) Representative images, scale bar 50 µm. (J) Mean surface coverage per visual field (n=3). Bar graphs depict mean values ± s.e.m. WBC=white blood cell. Student’s t-test (J).
Figure 3. RBCs support occlusive thrombus formation ex vivo and in vivo. (A) Flow-chamber system T-TAS. Whole blood and PRP were loaded into the flow chamber at a shear rate of 600 s$^{-1}$ at 37 °C. Bar graphs show full occlusion of the capillary (n=6). (B) Representative pictures of whole blood and PRP were shown. Blood flow direction was from left to right. (C) Whole blood and PRP were measured until full occlusion of the capillary. When flow pressure
increases to 80 kPa blood perfusion stops and the capillary is almost complete occluded by thrombi. (D) Human PRP and RBCs (4x10^6) were perfused through the flow chamber. Where indicated RBCs were substituted with fixed RBCs and occlusion of the capillary was monitored. (E) Bleeding times of control and anemic mice by amputating the tail tip. The time from the incision to the cessation of bleeding was recorded (no blood flow for 1 minute). (F) Time to beginning of thrombus formation (left) and time to occlusion (right) was measured with an ultrasonic flow probe in mice where the carotid artery was injured by topical application of FeCl₃ (n=5-6). (G) After injury of the carotid artery thrombi were isolated and stained with hematoxylin-eosin to visualize RBCs within the thrombus. Representative images of control mice (upper panel, same control sample but different detail as shown in Figure 2D) and anemic mice (middle and lower panel) were shown. Scale bars 50 µm (left and middle panel) and 20 µm (right panel). (H) Bleeding times of mice that receive RBC injections to increase the hematocrit were analyzed. (I) Time to occlusion in mice with increased hematocrit were determined after injury of the carotid artery with FeCl₃ (n=9-10). HCT+=mice with increased hematocrit. Data are mean ± s.e.m. ** P < 0.01, *** P < 0.001. Student’s t-test (J).
Red Blood Cells and arterial thrombus formation

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Figure 4. PS exposure of RBCs is responsible for RBC-mediated three-dimensional thrombus formation on collagen. (A and B) Thrombus formation on collagen under flow using PRP supplemented with freshly isolated RBCs, ghosts and ghosts incubated with AnnexinV to neutralize PS at 1000 s⁻¹. Representative images (A) with blood components as indicated above. (B) Thrombus formation was quantified by surface coverage per visual field (n=3). Scale bar equals 50 µm. (C) PS exposure of RBCs was induced by treatment with ionomycin. Defective aggregate formation under flow using PRP and RBCs treated with ionomycin and AnnexinV. Mean surface coverage per visual field (left panel) (n=3). Representative image of PRP supplemented with RBCs treated with ionomycin and AnnexinV (right panel). Scale bar 50 µm. (D) AnnexinV binding of fresh RBCs, RBCs treated with ionomycin, RBCs after storage for > 40 days (old RBCs) and ghosts was measured by flow cytometry (n=3). (E) Determination of AnnexinV binding of RBCs and ghosts isolated from thrombi developed in the flow chamber system (n=3). Where indicated ghosts were pre-incubated with AnnexinV. (F) Number of CD235 positive cells in thrombi formed under flow was determined via flow cytometry. (G) AnnexinV binding of RBCs before and after the flow through as determined by change of mean fluorescence intensity (MFI) compared to PS exposure of ghosts (positive control). (H-J) Thrombin- (H) and ADP-induced (I) thrombin generation in PRP and PRP supplemented with RBCs was measured with the fluorogenic-calibrated automated thrombogram assay. (J) Lag time (min, left panel) and peak height (nM thrombin, right panel) were shown. Data are expressed as arithmetic means ± s.e.m. Student’s t-test (A, J) and One-way Anova, Tukeys multiple comparison test (B-F). * P < 0.05, ** P < 0.01, *** P < 0.001. PS = phosphatidylserine.
Figure 5. Cell-cell contact of platelets and RBCs is crucial for RBC-platelet interaction. (A) Transmission electron microscopy of thrombi. Overview (left images) and image details (on the right) are shown. RBCs (black arrowheads) and regions of cell-cell contact (white arrowhead) with platelets are highlighted. Scale bars as indicated (n=3). (B) Representative dot blots from PRP incubated with RBCs using cell specific antibodies. Gate1 (G1) shows platelets. Gate2 (G2) shows aggregates of RBCs and platelets. (C) Annexin binding of RBCs
as determined by flow cytometry. s/n = supernatant (n=7). Student’s t-test (C). Agonist concentrations: CRP (5 µg/ml). Data are mean ± s.e.m. *** P < 0.001.
Figure 6. Cell-cell contact of platelets and RBCs via FasR (CD95). (A) Effects of inhibiting FasR antibody on PS exposure of RBCs in the presence of ADP-stimulated platelets. (n=9). (B) Platelet PS exposure was determined by flow cytometry (n=4-6). (C and D) Thrombin-induced thrombin generation in PRP supplemented with RBCs in the presence and absence of FasR antibody was measured by calibrated automated thrombogram (CAT). (C) Representative thrombin curves and (D) endogenous thrombin potential (ETP) (nM x min) were shown. (E-H) Flow chamber experiments with FasR antibody. (E) Representative images of thrombus formation. Scale bar 50 µm. (F) Mean surface coverage per visual field (n=5). (G and H) Effects of FasR antibody on PS exposure of RBCs (G) and platelets (H) (n=7-8). (I and
J) FeCl₃ induced injury of mesenteric arterioles. Wild-type mice were injected with FasR antibody (anti-FasR, 20 µg/mice) and IgG (20 µg/mice) as control. Thrombus formation was monitored by intravital microscopy. (I) Time to irreversible occlusion (right panel). (J) Representative pictures at indicated time points (n=4-6/group). (K) Tail bleeding times were determined in mice (n=5-8/group). Agonist concentrations: ADP (10 µM) and CRP (5 µg/ml). FasR antibody (10 µg/ml). Data are mean ± s.e.m. One-way Anova, Tukeys multiple comparison test (A-B) and Student’s t-test (D-K). * P < 0.05, ** P < 0.01, *** P < 0.001.
Figure 7. FasL serves as ligand for FasR to mediate platelet-induced activation of RBCs.

(A) Exposure of FasL at the platelet membrane after incubation of platelets with ADP (10 µM) and CRP (5 µg/ml) in the absence and presence of RBCs was determined by flow cytometry.
MFI = Mean fluorescence intensity (n=4-10). (B and C) ADP (10 µM) stimulated platelets were allowed to adhere to immobilized FasR (50 µg/ml). Representative pictures (B) and quantification (C) of adherent platelets in the presence of hDcR3 or control IgG-Fc (10 µg/ml). hDcR3 = human Decoy receptor 3, scale bar 50 µm. (D) No effect of hDcR3 on platelet adhesion on collagen (200 µg/ml) was observed. (n=5). (E) Platelets were activated with ADP (10 µM), pre-treated with hDcR3 (10 µg/ml) and incubated with RBCs. Effects of hDcR3 treatment on PS exposure of RBCs was determined (n=10). (F to I) Aggregate formation of hDcR3 (10 µg/ml) treated platelets on collagen under flow using a shear rate of 1000 s⁻¹. Controls receive IgG-Fc protein. (F) Representative phase contrast (left panel) and fluorescence images with mepacrine labeled platelets (middle panel, overlay right panel) at the end of the perfusion period. Scale bar 50 µm. (G and H) Mean surface coverage and relative platelet deposition after treatment with hDcR3 as measured by integrated fluorescence intensity (IFI) per visual field is shown (n=6). (I) Cells of thrombi were isolated by Accutase treatment and number of RBCs was quantified by flow cytometry (n=6). (J) PS exposure of RBCs treated with hDcR3 or control IgG-Fc was determined by flow cytometry (n = 6). Controls were treated with IgG-Fc protein (n=8). Data are mean ± s.e.m. Student’s t-test (D, G-J) and One-way Anova, Tukeys multiple comparison test (A, C, E). * P < 0.05, ** P < 0.01, *** P < 0.001.
Figure 8. Genetic deletion of FasR and FasL reduces thrombus formation in vitro. (A-B) Flow chamber experiments with whole blood from WT mice treated with antibodies as indicated. (A) Representative images of thrombus formation. (B) Relative platelet deposition (IFI) per visual field (n=4). (C-D) Aggregate formation under flow. Platelets from either WT or FasL−/− mice were incubated with WT RBCs; and WT platelets with FasR−/− RBCs were used. Representative images (C) and thrombus volume (D) were shown (n=4-6). (E-G) Detection of RBCs and platelets in thrombi of WT, FasR−/− or FasL−/− mice. (n=3). (E) Number of RBCs in thrombi under flow conditions. (F) Staining of isolated RBCs (TER-119 antibody, red; upper panel) and of RBCs and platelets in thrombi (RBCs: TER-119 antibody, red; platelets: GP9 antibody, green; lower panel). (G) Representative images of RBCs in thrombi from WT, FasR−/− or FasL−/− mice. Arrows indicate RBCs. Data are mean ± s.e.m. Student’s t-test (B) and two-way Anova, Tukeys multiple comparison test (D-E). * P < 0.05, ** P < 0.01, *** P < 0.001.
Figure 9. Genetic deletion of FasR and FasL reduces PS exposure in vitro. (A-B) Effects of FasL and FasR deficiency on PS exposure of (A) RBCs and (B) platelets in vitro. Platelets from either wild-type (WT) or FasL\(^{-/-}\) mice were incubated with WT RBCs; WT platelets were incubated with FasR\(^{-/-}\) RBCs (n=4). (C-D) Flow cytometry of AnnexinV binding of RBCs (C) and platelets (D) isolated from thrombi (n=5). (E-G) Thrombus formation and capillary occlusion in the flow chamber system T-TAS using PRP and RBCs. Time until full occlusion of the capillary was monitored. PRP from WT mice was incubated with either WT (E) or FasR\(^{-/-}\) RBCs (F) and compared to PRP from FasL\(^{-/-}\) mice and WT RBCs (G). (H) Occlusion times, n=4-6. Shear rate of 1.700 s\(^{-1}\) was used. Data are mean ± s.e.m. Two-way Anova, Tukeys multiple comparison test (A-B) and One-way Anova, Dunnetts multiple comparison test (C-H).
Figure 10. Genetic deletion of FasR and FasL reduces PS exposure and thrombus formation in vivo. (A-E) FeCl₃ induced injury of mesenteric arterioles in WT, FasR⁻/⁻ or FasL⁻/⁻ mice (n=5-9/group). Beginning of thrombus formation (A), time to irreversible occlusion (B), and representative intravital microscopy images (C), percentage of mice with no occlusion (D, (WT vs. FasR⁻/⁻: p=0.0002; WT vs. FasL⁻/⁻: p=0.0011)) and thrombus size (E) are shown. The break-off was set at 40 min after vessel injury when no occlusion occurred. (F-H) FeCl₃ induced injury of mesenteric arterioles in PF4Cre(+) FasL⁻/⁻ mice. PF4Cre(-) FasL⁻/⁻ mice served as controls. (F) Beginning of thrombus formation, (G) time to irreversible occlusion, and percentage of mice with no occlusion (H, p=0.003) are shown. (n=8/group). (I-K) Platelet-RBC interactions after ligation of the inferior vena cava (IVC) were analyzed by intravital epifluorescence microscopy. Mice received murine platelets from C57BL/6 or FasL⁻/⁻ mice, labeled with RhodaminB and murine RBCs from C57BL/6 or FasR⁻/⁻ mice labeled with DCF. (I) Number of platelet-RBC interactions per second (WT vs. FasR⁻/⁻ RBCs: p=0.008; WT vs. FasL⁻/⁻ platelets: p=0.072). (J) Cell interaction was quantified (90 min: WT vs. FasR⁻/⁻ RBCs: p=0.003; WT vs. FasL⁻/⁻ platelets: p=0.0163). (K) Representative images of platelet-RBC interactions in WT mice 60 min. after IVC ligation. Scale bar 10 µm. (n=4). (L-M) Venous thrombus formation was investigated in a flow restriction model of the IVC and thrombus weight was determined: (L) IgG vs. FasR antibody treated mice (p=0.2814); (M) FasL⁻/⁻ compared to WT mice (p=0.0446). (N) Tail bleeding times were determined (n=6/group). Data are mean ± s.e.m. Student’s t-test (F-G, M) and one-way Anova, Dunnett’s multiple comparison test (A-B, I-J, N), Log rank (Mantel-Cox) test (D, H), two-way Anova, Sidak’s multiple comparison test (E). * P < 0.05, ** P < 0.01, *** P < 0.001.
Figure 11. FasL/Fas receptor mediated PS exposure is relevant under pathological conditions. Human arterial thrombi from patients with thrombectomy were analyzed for cell content and cell markers. (A) Representative images from hematoxylin-eosin staining of human thrombi. Overview (top, scale bar 500 µm) and details (middle panel, scale bar 100 µm) are shown. Black arrows mark RBCs. Representative pictures from toluol staining of human thrombi (lower panel, 200x). (B) Representative transmission electron microscopic images of a human thrombus. Overview (left panel), with RBC (red) and platelets (green), and details (right panel). Scale bars as indicated. (C) Quantification of RBCs and platelets and (D) determination of P-selectin exposure in human thrombi (patient) (n = 29). Platelets from healthy donors were activated with CRP and incubated with RBCs (controls, n = 9-11). (E) Expression of FasL on platelets from human thrombi (patient) and healthy donors (n = 24). Determination of PS exposure of RBCs (F) and platelets (G) by flow cytometry. Isolated platelets and RBCs (fresh or older than 40 days after withdrawal) from healthy donors served as controls (n = 27). (H-J) Patients’ samples were divided into platelet-rich (Plts>RBCs) and RBC-rich (RBC>Plts) thrombi and analyzed for FasL and PS exposure by flow cytometry. (H) Determination of FasL exposure in platelet-rich and RBC-rich human arterial thrombi (n =9-13). (I) PS exposure of RBCs was analyzed using AnnexinV (n = 11-14). (J) AnnexinV binding of platelets in platelet-rich and RBC-rich human thrombi (n = 4-6). (K) Tentative schematic illustration of RBCs and their impact on thrombus formation. Ca²⁺ = calcium, PLSCR = phospholipid scramblase, PLCβ = phospholipase Cβ. CRP 5 µg/ml. Data are mean ± s.e.m. Student’s t-test (C, H-J) and one-way Anova, Tukeys multiple comparison test (D-G). * P < 0.05, ** P < 0.01, *** P < 0.001. MFI=mean fluorescence intensity; Plts=platelets.
Table 1. Characteristics of patients who underwent surgical thrombectomy

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