

RESEARCH ARTICLE

CD36-fibrin interaction propagates FXI-dependent thrombin generation of human platelets

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Abstract

Thrombin converts fibrinogen to fibrin and activates blood and vascular cells in thrombo-inflammatory diseases. Platelets are amplifiers of thrombin formation when activated by leukocyte- and vascular cell-derived thrombin. CD36 on platelets acts as sensitizer for molecules with damage-associated molecular patterns, thereby increasing platelet reactivity. Here, we investigated the role of CD36 in thrombin-generation on human platelets, including selected patients with advanced chronic kidney disease (CKD). Platelets deficient in CD36 or blocked by anti-CD36 antibody FA6.152 showed impaired thrombin generation triggered by thrombin in calibrated automated thrombography. Using platelets with congenital function defects, blocking antibodies, pharmacological inhibitors, and factor-depleted plasma, CD36-sensitive thrombin generation was dependent on FXI, fibrin, and platelet signaling via GPIIb/IIIa and SFKs. CD36-deficiency or blocking suppressed thrombin-induced platelet α IIb β 3 activation, granule exocytosis, binding of adhesion proteins and FV, FVIII, FIX, FX, but not anionic phospholipid exposure determined by flow cytometry. CD36 ligated specifically soluble fibrin, which recruited distinct coagulation factors via thiols. Selected patients with CKD showed elevated soluble fibrin plasma levels and enhanced thrombin-induced thrombin generation, which was normalized by CD36 blocking. Thus, CD36 is an important amplifier of platelet-dependent thrombin

Abbreviations: CAT, calibrated automated thrombography; CKD, chronic kidney disease; DAMP, damage-associated molecular patterns; DFP, defibrinated plasma; etp, endogenous thrombin potential; LDL, low density lipoprotein; PAR, proteinase activated receptor; PFP, platelet-free plasma; PRP, platelet-rich plasma; SFK, src family kinase; TF, tissue factor; TSP-1, thrombospondin-1; vWF, von Willebrand factor.

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generation when exposure of anionic phospholipids is limited. This pathway might contribute to hypercoagulability in CKD.

KEYWORDS

adhesion molecules, coagulation, factor XI, inflammation, scavenger receptors

1 | INTRODUCTION

Platelets are key players in primary hemostasis, but they are also important drivers of thrombin generation in coagulation and thrombo-inflammatory diseases. Vascular cells upon vessel wall injury or monocytes under inflammatory conditions initiate the conversion of prothrombin (factor II (FII)) to thrombin (FIIa) by prevalent assembly of the prothrombinase complex in a tissue factor (TF)/FVIIa-dependent manner.¹ On the contrary, the contact pathway of thrombin generation is initiated by activation of FXII to FXIIa at negatively charged surfaces, for example, collagen, DNA, RNA, polyphosphates, misfolded protein aggregates, leading to activation of FXI to FXIa, which converts FIX to FIXa with subsequent assembly of the tenase and prothrombinase complexes.^{2,3} Thrombin is crucial for the conversion of fibrinogen to fibrin and is one of the most potent platelet agonists. Platelets activated by thrombin via GPIb α ⁴ of the GPIb-V-IX complex and 7-transmembrane G protein-coupled proteinase activated receptors (PARs) exhibit a pro-coagulant surface, which enables the amplification of thrombin generation. Platelet-dependent thrombin generation is propagated by thrombin-mediated activation of FXI to FXIa, which enables increased assembly of the tenase and prothrombinase complexes on the platelet surface.⁵ This FXI-dependent positive feedback loop of thrombin generation has been recently shown to be significantly involved in distal platelet activation and microaggregate formation downstream of thrombus formation under flow in vitro and ex vivo.⁶ Several studies strengthened a major role of FXI in arterial and venous thrombosis.^{7,8} Furthermore, we identified an important role of the FXI-dependent thrombin amplification loop on thrombin-stimulated platelets in vascular inflammation and in arterial hypertension.⁹ However, how platelets regulate FXI-dependent thrombin generation is incompletely understood.

CD36 [glycoprotein (GP) IV], a member of the class B scavenger receptor family, is constitutively expressed on the platelet surface with up to 25 000 copies per platelet.^{10,11} The response to injury protein thrombospondin-1 (TSP-1) represents an important ligand of CD36.¹² CD36-mediated binding of soluble TSP-1 to platelets has been shown to promote platelet aggregation in response to collagen in vitro.¹³ CD36 is partially involved in platelet adhesion to TSP-1 at high shear¹⁴ and supports TSP-1-dependent thrombus stabilization on collagen.¹⁵ CD36 represents also a pattern recognition receptor for molecules with damage-associated

molecular patterns (DAMPs) such as oxidized phospholipids/LDL,^{16,17} amyloids,¹⁸ and MRP-14.¹⁹ Binding of oxidized LDL to CD36 mediates activation of platelets in vitro.^{20,21} CD36 induces a hyperreactive platelet phenotype in the presence of subthreshold concentrations of the physiological platelet agonists ADP and collagen, which is implicated in pro-thrombotic states in hyperlipidemic mice.^{22,23} Thus, platelet CD36 acts as a sensitizer for physiological ligands and DAMPs, and therefore, increases platelet reactivity contributing to a pro-thrombotic phenotype under conditions of metabolic and inflammatory stress. However, the role of CD36 in platelet-dependent thrombin amplification, which is an important process in thrombo-inflammation, is not known so far. Advanced chronic kidney disease (CKD) with uremia and particularly with end stage renal disease (ESRD) represents a thrombo-inflammatory disease, which is characterized by activated and procoagulant platelets.²⁴

In the present study, we investigated whether CD36 contributes to FXI-dependent thrombin formation on human platelets, including selected patients with advanced CKD.

2 | MATERIALS AND METHODS

2.1 | Preparation of human platelets and plasma from healthy donors and patients

Venous blood was collected in trisodium-citrate as anticoagulant (final concentration: 10.6 mmol/L) from healthy donors and volunteers with type II CD36-deficiency, GPIb α -deficiency (Bernard-Soulier syndrome (BSS)), δ -storage pool deficiency (δ -SPD), and from two patients with CKD stage 5, who had not taken any medication affecting platelet function for at least 11 days before the experiment. Platelet CD36-deficiency was caused by the known missense variant C478T in exon 4 of *CD36* as previously published²⁵ and flow cytometric analysis showed absence of platelet CD36 as the mean fluorescence intensity of FITC-conjugated anti-CD36 antibody FA6-125 (MFI: 1) was the same as detected for FITC-conjugated IgG1 isotype control (MFI: 1); all other major receptors were normally expressed on the platelet surface as assessed by flow cytometry; platelet count and size were within the normal range (EDTA-whole blood: 275 000 platelets/mL; MPV: 8,8 fl). BSS was caused by a homozygous frameshift variant in *GPIBA*, rs763978422, GPIb α < 1% as assessed

by flow cytometry; all other major receptors were normally expressed on the platelet surface in relation to the increased mean platelet volume (MPV) as assessed by flow cytometry, defective ristocetin-induced platelet agglutination but normal platelet aggregation in response to other physiological platelet agonists, citrate-whole blood: 21 000 platelets/ μL , MPV > 13 fl). δ -SPD was caused by severe reduction of platelet dense bodies demonstrated by 30% residual mepacrine-uptake *ex vivo* determined by flow cytometry and residual 0.35 nmol ATP release per 10^8 platelets but normal receptor surface expression as assessed by flow cytometry and platelet aggregation induced by high concentration of equine collagen type I (10 $\mu\text{g}/\text{mL}$) determined by lumi-aggregometry, EDTA-whole blood: 230 000 platelets/ μL , MPV: 8,2 fl). Routine coagulation parameters, plasma coagulation factor levels, and TF-induced thrombin generation in platelet-free plasma from volunteers and patients were within normal ranges. All participants gave written informed consent according to our institutional guidelines and the Declaration of Helsinki. Studies with human platelets from probands and patients with CKD-5 were approved by the local institutional ethics committees (Mainz (837.302.12(8403-F), (837.460.15 (10228)), Würzburg (67/92 and 114/04), Vienna (237/2004)).

Platelet-rich plasma (PRP) and platelet-poor plasma (PPP) were prepared by centrifugation of citrated whole blood as described.²⁶ Platelet-free plasma (PFP) was prepared by centrifugation of PPP at 30 000 *g* for 10 minutes at room temperature (RT). Defibrinated plasma (DFP, <0.35 *g/L* fibrinogen) was prepared by incubation of PPP with the snake venom batroxobin (1 U/*mL*; Loxo, Dossenheim, Germany) for 30 minutes at 37°C followed by a frozen cycle at -20°C and subsequent centrifugation at 2000 *g* for 10 minutes at RT. Human platelets were washed as described.²⁷ The platelet pellet was finally diluted in control or patient plasma or coagulation factor-depleted plasma or HEPES buffer (145 mM NaCl, 5 mM KCl, 10 mM HEPES, 10 mM Glucose, 1 mM MgCl_2).

2.2 | Preparation of soluble fibrin and FITC-coupled proteins

Highly purified human fibrinogen, depleted of vWF, fibronectin, plasminogen (Enzyme Research Laboratories, South Bend, IN, USA) was cleaved by the snake venom batroxobin in the presence of the fibrin polymerization inhibiting peptide GPRP (Gly-Pro-Arg-Pro; Bachem, Heidelberg, Germany) as described.²⁸ TSP-1 was purified from thrombin-stimulated human platelets.¹⁴ FITC-coupling of human coagulation factors (factor V, IX, X (Loxo), recombinant human factor VIII (Bayer, Leverkusen, Germany), and of TSP-1 was performed as described.²⁶

2.3 | Thrombin generation measurements

Platelet-dependent thrombin formation was assessed with calibrated automated thrombography (CAT) according to Hemker et al.²⁹ and Jurk et al.²⁶ Platelets in PRP and washed platelets were adjusted to $150 \times 10^3/\mu\text{L}$ with PFP or factor VII, XI, XII, vWF-depleted plasma (Hematologic Technologies, Essex Junction, VT, USA), in the absence or presence of human plasma-derived factor XI (30 nM; kindly provided by P. Wenzel, University Medical Center Mainz, Germany) or recombinant human factor VIII, with a final volume of 80 μL . Platelet function blocking antibodies, IgG isotype controls, and peptides (Table S1) or platelet and coagulation inhibitors (Table S2) were pre-incubated with platelets 10 minutes at RT prior to addition of 20 μL of α -thrombin (2 nM (0.1 U/*mL*), bovine, Sigma-Aldrich, Munich, Germany), recombinant human TF (0.15-1 pM; Innovin Siemens Healthcare, Marburg, Germany), TRAP-6 peptide SFLLRN (20 μM ; Bachem, Heidelberg, Germany), PAR-4 activating peptide AYPGKF-NH₂ (200 μM ; Bachem, Heidelberg, Germany) or convulxin (50 ng/*mL*; Enzo Life Sciences, Lörrach, Germany) as triggers. When blocking IgG antibodies were used, platelets were pre-incubated with anti-CD32 antibody (Table S1) for 10 minutes at RT to prevent potential Fc γ RIIA-mediated platelet activation. In some experiments batroxobin (1 U/*mL*) was added instead of triggers to induce fibrinogen cleavage to fibrin.

Thrombin was quantified in citrated platelet-free plasma supplemented with beads when fibrin- or BSA-coated beads were adjusted to 14 000 beads/ μL with PFP or DFP, respectively, with a final volume of 80 μL . In control experiments, washed platelets from healthy controls were adjusted to 14 000 platelets/ μL with PFP and a final volume of 80 μL , and GPRP (2.5 mM) and thrombin (2 nM) were added. Thrombin generation was started by adding 20 μL of pre-warmed (37°C) FluCa-solution containing HEPES-BSA-buffer, pH 7.35 and CaCl_2 (17 mM; Thromboscope, Maastricht, The Netherlands). Thrombin-mediated cleavage of the fluorogenic substrate Z-Gly-Gly-Arg-AMC (Fluo-substrate, Thromboscope) was monitored at 37°C for 60 minutes in 20 seconds intervals using a Fluoroskan Ascent fluorescence reader (excitation 390 nm, emission 460 nm wavelengths, Thermo Labsystems, Franklin, MA).

Quantification of thrombin formation in whole blood was performed with the calibrated automated thrombography according to Ninivaggi et al.³⁰ with some modifications. Briefly, 10 μL of the rhodamine-based thrombin substrate P₂Rho (300 μM ; Life Technologies, Darmstadt, Germany) and 5 μL α -thrombin (4 nM/0.2 U/*mL*) were added to 30 μL of citrated whole blood. Thrombin generation was started by adding 15 μL of HEPES-buffer containing 0.5% of BSA and 67 mM CaCl_2 . Filter paper discs (5 mm diameter and 180 μm thickness) were wetted with 5 μL of the blood mixture and covered

with 40 μL mineral oil. Thrombin-mediated cleavage of the rhodamine-based substrate P2Rho was monitored at 37°C for 60 minutes in 20 seconds intervals with a Fluoroskan Ascent fluorescence reader (excitation 485 nm, emission 538 nm wavelengths). The Thrombinoscope, Synapse BV software program was used for calculation of thrombin generation parameters.

2.4 | Flow cytometry analysis of platelets

Formaldehyde-fixed platelets in diluted PRP were incubated with monoclonal anti- $\alpha\text{IIb}\beta\text{3}$ -FITC, anti-GPIIb α -FITC, anti-GPIX-FITC, anti-integrin- α1 -FITC, anti-GPVI-eFluor660, anti-CD36-FITC antibodies, and mouse IgG1 as described.³¹ Absolute numbers of antigen binding sites per platelet were calculated by flow cytometry (FACS Canto II flow cytometer with FACS Diva software, Becton Dickinson Biosciences, Heidelberg, Germany) using Quantum Simply Cellular anti-mouse IgG (Bangs-Laboratories, Fishers, IN, USA) according to the manufacturer's instructions. Agonist-induced platelet integrin $\alpha\text{IIb}\beta\text{3}$ activation, P-selectin (CD62P) and CD63-surface expression, binding of fibrinogen, vWF, TSP-1, coagulation factors, annexin-V, and lactadherin were analyzed by flow cytometry as described³² with some modifications. Diluted PRP (1:10 with Tyrode's buffer) was treated with α -thrombin (1 nM/0.05 U/mL) or Ca^{2+} -ionophore A23187 (10 μM , Sigma Aldrich) for 15 minutes at RT in the presence of 1.7 mM CaCl_2 and 3.75 mM GPRP to prevent fibrin polymerization. Stimulated platelets were stained with PAC-1-FITC (5 $\mu\text{g}/\text{mL}$; Becton Dickinson, Heidelberg, Germany), anti-CD62P-FITC (5 $\mu\text{g}/\text{mL}$; Beckman Coulter, Brea, CA, USA), anti-CD63-FITC (5 $\mu\text{g}/\text{mL}$; Beckman Coulter, Brea, CA, USA), anti-fibrinogen-FITC (5 $\mu\text{g}/\text{mL}$; DAKO/Agilent, Santa Clara, CA, USA), anti-vWF-FITC (5 $\mu\text{g}/\text{mL}$; Bio-Rad, Feldkirchen, Germany), anti-TSP-1-PE (2.5 $\mu\text{g}/\text{mL}$; Beckman Coulter), anti-GPVI-eFluor660 (5 $\mu\text{g}/\text{mL}$), anti-FV-FITC, anti-FVIII-FITC, anti-FIX-FITC, anti-FX-FITC, anti-FXI-FITC (all 5 $\mu\text{g}/\text{mL}$; Affinity Biologicals, Ancaster, Canada) antibodies, and corresponding IgG isotope controls and annexin-V-FITC (eBioscience, Frankfurt, Germany), lactadherin-FITC (Hematologic Technologies; experiments without addition of CaCl_2) at saturating concentration for 15 minutes at RT and analyzed by flow cytometry.

2.5 | Flow cytometry analysis of CD36- and fibrin-coated beads

Polystyrene beads (3.0 μm ; Sigma-Aldrich, Munich, Germany) were coated with human albumin (50 $\mu\text{g}/\text{mL}$;

Sigma-Aldrich, Munich, Germany), fibrinogen/soluble fibrin (50 $\mu\text{g}/\text{mL}$) in TBS-buffer, pH 7.4, including 2 mM CaCl_2 , or with recombinant human CD36 (300 mg/mL; abcam, Cambridge, UK) in Tyrode's buffer for 60 minutes at RT. After washing and centrifugation at 10 000 g for 10 minutes at RT coated beads were resuspended in corresponding buffer. For analysis of plasma adhesion protein and coagulation factor binding to CD36-coated beads, CD36- or BSA-coated beads were incubated with diluted platelet-free plasma or defibrinated plasma 1:5 with Tyrode's buffer) in the presence of GPRP (17.5 mM), CaCl_2 (3.4 mM) and in the absence or presence of α -thrombin (0.025 U/mL) for 30 minutes at RT. After washing and centrifugation at 10 000 g for 10 minutes at RT, beads were resuspended in Tyrode's buffer and stained with anti-TSP-1-PE, anti-vWF-FITC, anti-fibrinogen-FITC, anti-FV-FITC, anti-FVIII-FITC, anti-FIX-FITC, anti-FX-FITC, anti-FXI-FITC, and corresponding IgG isotype controls at saturating concentration, respectively. 10 000 beads per sample were analyzed by flow cytometry. Data were calculated as mean fluorescence intensity of CD36-coated beads corrected by mean fluorescence intensity of corresponding BSA- and IgG-coated beads. For analysis of purified adhesion protein binding to CD36-coated beads, CD36- or BSA-coated beads were incubated with Alexa488-conjugated fibrinogen (saturating concentration of 50 $\mu\text{g}/\text{mL}$ in the absence or presence of 1 U/mL batroxobin plus 1.25 mM GPRP), FITC-conjugated human TSP-1 or human recombinant vWF (Baxter BioScience, Vienna, Austria) for 15 minutes at RT. After washing and centrifugation at 10 000 g for 10 minutes at RT beads, which bound vWF were stained with anti-vWF-FITC and washed. 10 000 beads per sample were analyzed by flow cytometry. Data were calculated as mean fluorescence intensity of CD36-coated beads corrected by mean fluorescence intensity of corresponding BSA-coated beads. For analysis of coagulation factor binding to fibrin-coated beads, fibrin-coated beads were incubated with FITC-conjugated coagulation factors at saturating concentration²⁶ in the absence or presence of the thiol-blocker DTNB (2.5 mM; Sigma-Aldrich) for 30 minutes at RT. Binding of coagulation factors to beads was analyzed by flow cytometry measuring 10 000 beads for each sample.

2.6 | Light transmission aggregometry

Platelet aggregation was assessed in native PRP as described³¹ using a four channel light transmission aggregometer (APACT 4S Plus, DiaSys, Flacht, Germany). ADP (10 μM ; Hart Biologicals, Hartlepool, UK), equine collagen type I (2.0 $\mu\text{g}/\text{mL}$; Chrono-Par), TRAP-6 (10 μM ; Bachem), arachidonic acid (0.5 mg/mL; Sigma-Aldrich), and epinephrine (2.0 μM ; Sigma-Aldrich), respectively, were used as platelet agonists. Ristocetin (0.9 mg/mL; American Biochemical & Pharmaceuticals,

London, UK) was used to induce VWF-mediated platelet agglutination and subsequent platelet aggregation.

2.7 | Western blot analysis

Washed platelets were resuspended in HEPES buffer and pretreated with anti-FcγRIIIa antibody IV.3 (10 μg/mL) and anti-CD36 antibody FA6.152 or IgG control followed by incubation at 37°C for 60 seconds in the presence of GPRP (2.5 mM) and in the absence or presence of fibrinogen (500 μg/mL) and thrombin (1 nM). Platelet proteins were separated by SDS-PAGE and transferred to a polyvinylidene-difluoride membrane. Monoclonal mouse anti-Syk (clone 4D10, Cell Signaling, Danvers, MA, USA), rabbit-anti-phospho-Syk-Tyr352 (Cell Signaling, MA, USA), rabbit-anti-phospho-Src family-Tyr416 (detection of Src^{Y416} and potentially other SFKs, that is, Lyn, Fyn, Lck, Yes, Hck, when phosphorylated at equivalent sites) and anti-β-actin antibody (Cell Signaling) were used as primary antibodies and polyclonal goat anti-mouse or goat anti-rabbit IgG conjugated with horseradish peroxidase were used as secondary antibodies (1:1000; Bio-Rad), ECL was used for western blot imaging as described.²⁷

2.8 | Human soluble GPVI enzyme-linked immunosorbent assay

Soluble GPVI was quantified in the supernatant of flow cytometry platelet samples as described above in the presence of the factor Xa inhibitor rivaroxaban (1 μg/mL; Cayman Chemical) after stopping platelet activation with EGTA (10 mM) and with the direct thrombin inhibitor PPACK (40 μM; Calbiochem/Merck Millipore, Burlington, MA, USA) and subsequent centrifugation at 30 000 *g* for 5 minutes at RT according to the manufacturer's instructions (RayBiotech, Peachtree Corners, GA, USA).

2.9 | Quantification of plasma soluble fibrin monomer complexes

Soluble fibrin monomer complexes were determined in PFP using an enzyme-linked immunosorbent assay (Biomatik, Ontario, Canada) according to the manufacturer's instructions.

2.10 | Statistical analysis

Data are expressed as mean ± SD. For statistical analysis GraphPad Prism software (version 6.07 for Windows, GraphPad Software, La Jolla California USA) was used. Normality of distribution (Shapiro-Wilk test) and equal

variance (*F* test, Brown-Forsythe test) were tested. In case of normal distribution and equal variance parametric tests were performed for comparisons between two groups by two-tailed Student's *t* test, for comparisons between >2 groups by one-way or two-way analysis of variance (ANOVA) followed by Tukey's multiple comparisons test. For comparisons of each of a number of treatment groups with a single control group, one-way ANOVA followed by Dunnett post hoc test was performed. For comparison of nonparametric data sets the Mann-Whitney *U* test (comparison of 2 groups) or the Kruskal-Wallis test (comparison of >2 groups) followed by Dunn post hoc test for multiple comparisons was used. *P* < .05 was considered statistically significant.

3 | RESULTS

3.1 | FXI-dependent thrombin generation of human platelets is amplified by CD36

To mimic thrombin generation of human platelets in vitro the calibrated automated thrombography system was used. Here, we identified a significant role of platelet CD36 in thrombin formation induced by 2 nM (0.1 U/mL) α-thrombin, which was dependent on FXI (Figure S1A), but independent on FXII (Figure S1B,C) and TF-FVII complex (Figure S1D,E). PRP from a donor with platelet CD36-deficiency showed significantly decreased thrombin peak and etp, but unaltered lag time compared to PRP from healthy controls (Figure 1A). Pre-incubation of PRP or whole blood with the anti-CD36 antibody FA6.152, which blocks binding of TSP-1 and oxLDL to CD36, resulted in similarly reduced thrombin peak and etp but unaltered lag time compared to IgG isotype control-treated samples (Figure 1B,C). In contrast, another anti-CD36 antibody 185-1G2, blocking oxLDL binding to CD36, did not show any effect on platelet-dependent thrombin generation (data not shown), indicating differential epitope specificity of CD36-blocking antibodies. When recombinant TF was used as trigger, thrombin generation in PRP was less sensitive to CD36 blocking, even at a low TF concentration of 0.25 pM (Figure S2A). TF-triggered thrombin generation showed partial sensitivity to FXI demonstrated with a 40% decreased thrombin peak in FXI-depleted plasma without further effect by CD36 blocking (Figure S2B). These data indicate that CD36-sensitive thrombin formation depends on FXI.

3.2 | GPIIbα is crucially involved in CD36-sensitive thrombin generation

To obtain evidence that platelet activation is important in CD36-dependent thrombin formation, we examined

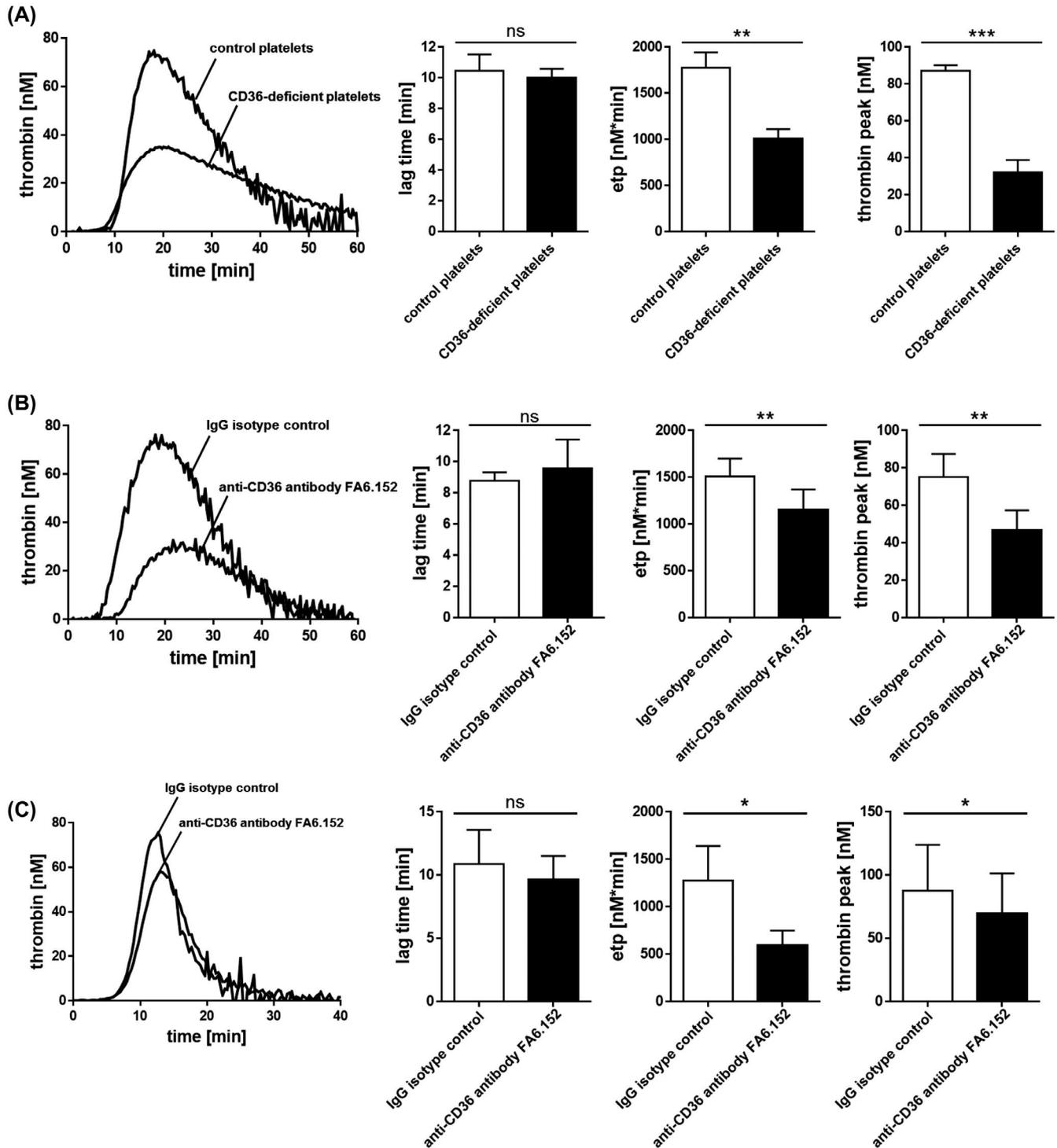


FIGURE 1 Role of CD36 in thrombin generation on human platelets induced by thrombin. A, Thrombin generation in PRP from controls in the presence of IgG isotype control (5 μ g/mL) and anti-CD36 antibody FA6.152 (5 μ g/mL) triggered by 2 nM α -thrombin. B, Thrombin generation in PRP from a proband with CD36-deficiency triggered by 2 nM α -thrombin. C, Thrombin generation in citrated-whole blood from controls in the presence of IgG isotype control (5 μ g/mL) and anti-CD36 antibody FA6.152 (5 μ g/mL) triggered by 2 nM α -thrombin. Means \pm SD (n = 3-5). * P < .05, ** P < .01, *** P < .001, n.s. not significant

receptor-mediated signaling. The thrombin peak was significantly diminished in PRP from a patient with Bernard Soulier syndrome (BSS), expressing less than 1% of GPIIb α on the platelet surface, and when GPIIb α was blocked by the anti-GPIIb α antibody SZ2 (blocking the binding of

vWF, TSP-1, thrombin, and FXI to GPIIb α). Interestingly, no additional inhibition was observed in the presence of the anti-CD36 antibody FA6.152, even when each effector alone showed submaximal inhibition (Figure 2A). The anti-GPIIb α antibody VM16d, blocking the binding of

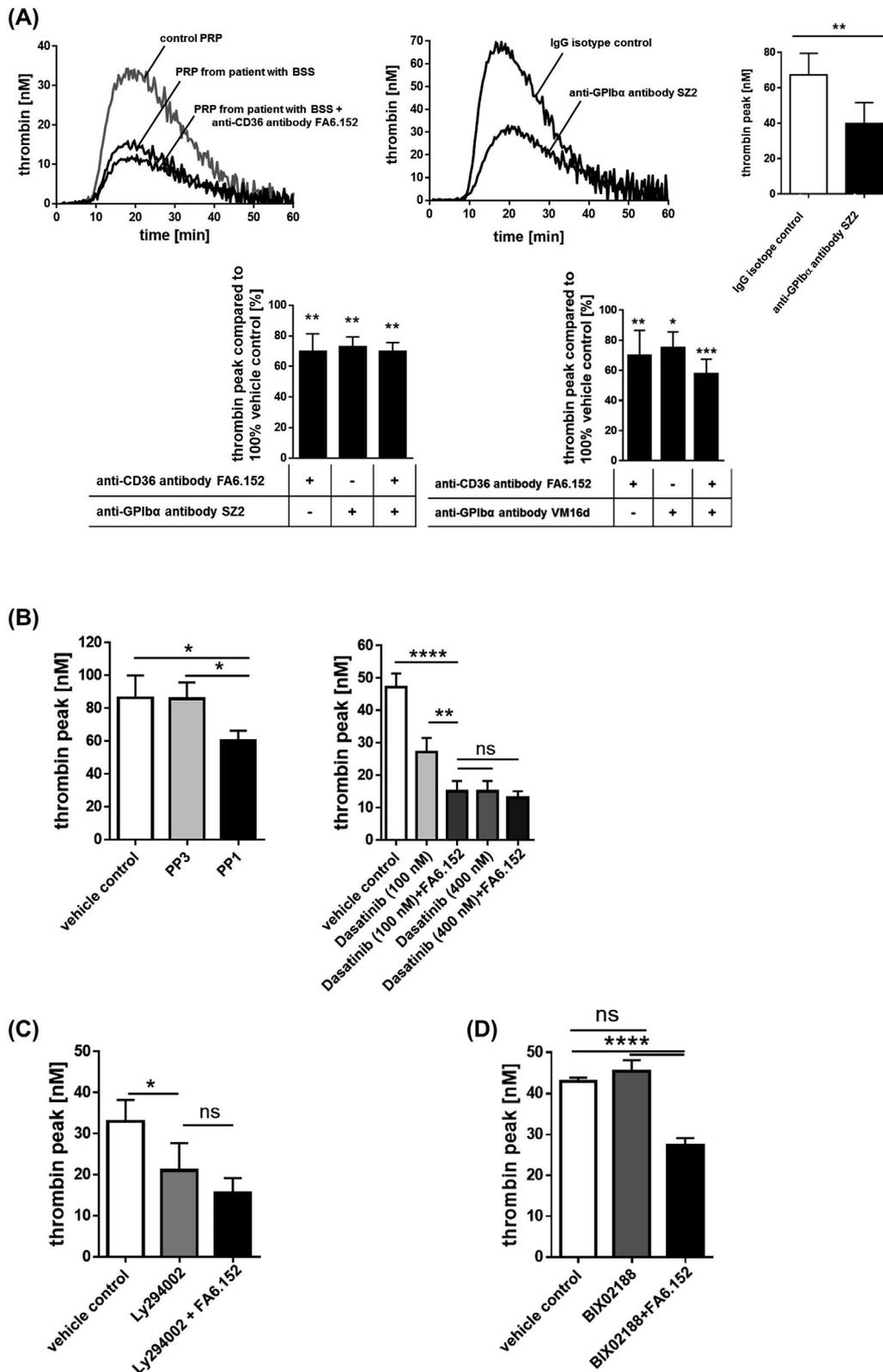


FIGURE 2 Involvement of platelet GPIIb/IIIa and SFK-signaling in CD36-dependent thrombin formation. A, Effect of GPIIb/IIIa-deficiency (patient with Bernard Soulier syndrome (BSS)), of anti-GPIIb/IIIa antibody SZ2 (10 μ g/mL; 5 μ g/mL), of anti-GPIIb/IIIa antibody VM16d (5 μ g/mL), and anti-CD36 antibody FA6.152 (5 μ g/mL) on thrombin generation in PRP triggered by 2 nM thrombin. B, Effect of Src family kinase inhibitors PP1 and dasatinib on thrombin-triggered thrombin generation in PRP in the absence or presence of anti-CD36 antibody FA6.152 (5 μ g/mL). PP1 (20 μ M), PP3 (negative control 20 μ M). C, Effect of PI3K inhibitor Ly294002 (25 μ M) on thrombin-triggered thrombin generation in PRP in the absence or presence of anti-CD36 antibody FA6.152 (5 μ g/mL). D, Effect of ERK5 inhibitor BIX02188 (10 μ M) on thrombin-triggered thrombin generation in PRP in the absence or presence of anti-CD36 antibody FA6.152 (5 μ g/mL). Means \pm SD (n = 3-4). * P < .05, ** P < .01, *** P < .001, **** P < .0001, n.s. not significant vs. 100% control or as indicated

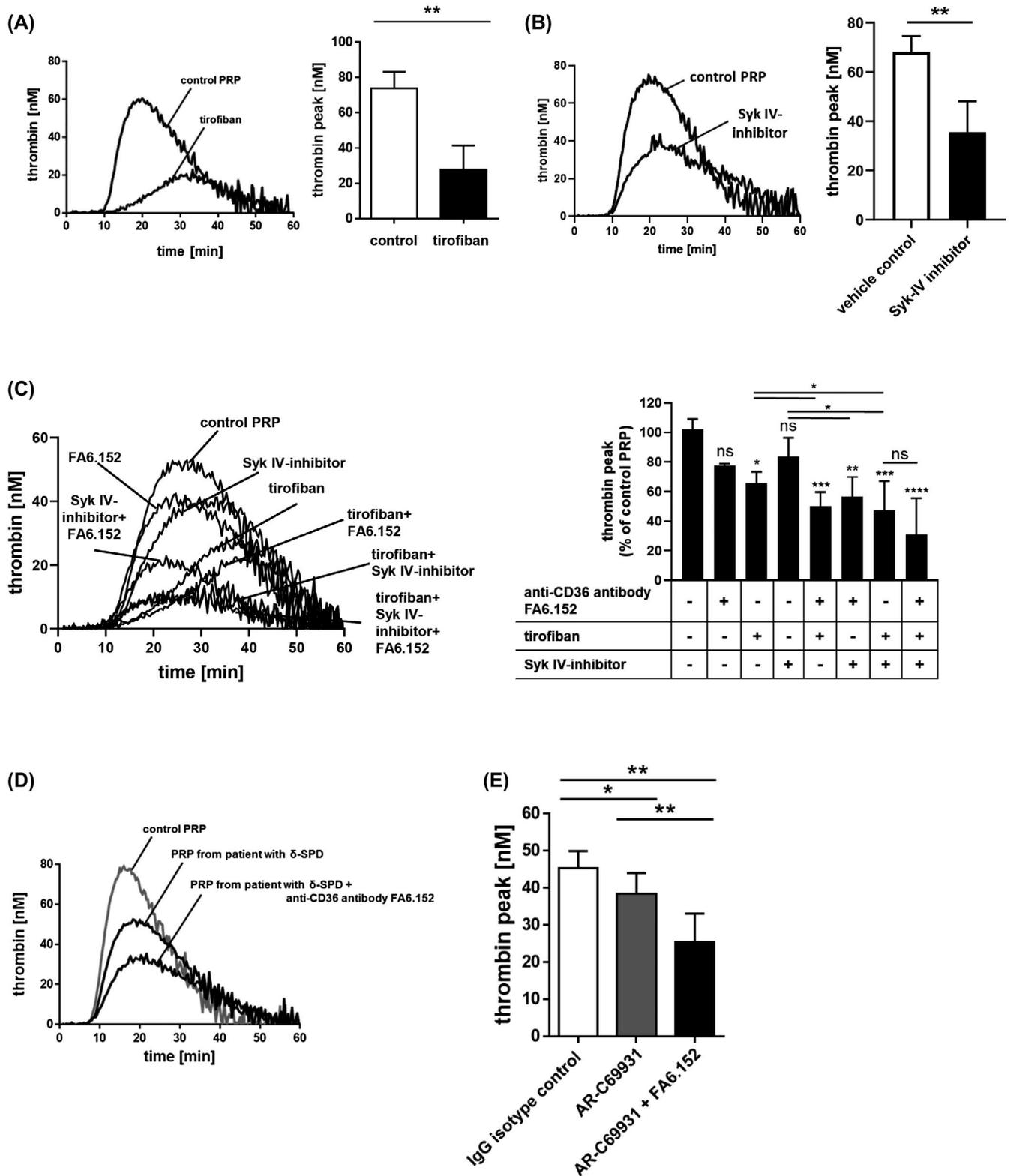


FIGURE 3 Partial role of integrin α Ib β 3 and δ -granule cargo in CD36-dependent thrombin formation. A, Effect of tirofiban (5 μ M) on thrombin generation in PRP triggered by 2 nM thrombin. B, Effect of Syk IV-inhibitor (10 μ g/mL) on thrombin (2 nM)-induced thrombin formation. C, Effect of tirofiban (2.5 μ M) +/- Syk IV-inhibitor (1.25 μ M) +/- anti-CD36 antibody FA6.152 (2.5 μ g/mL) on thrombin generation in PRP triggered by 2 nM thrombin. D, Thrombin generation (trigger: 2 nM thrombin) in PRP from a patient with δ -storage pool deficiency in the absence or presence of anti-CD36 antibody FA6.152 (5 μ g/mL) compared to control PRP. E, Thrombin generation in PRP pretreated with the P2Y₁₂-blocker AR-C69931 (5 μ M) in the absence or presence of anti-CD36 antibody FA6.152 (5 μ g/mL) (trigger: 2 nM thrombin) compared to IgG isotype control (5 μ g/mL). Means \pm SD (n = 3-4). **P* < .05, ***P* < .01, ****P* < .001, *****P* < .0001, n.s. not significant vs. 100% control or as indicated

thrombin to GPIIb α , partially reduced the thrombin peak, and the combination with FA6.152 resulted in an additive effect (Figure 2A). Src family kinases (SFKs) are involved in early signaling of several platelet receptors including GPIIb-V-IX. The SFK inhibitors PP1 and dasatinib as well as the PI3K-inhibitor Ly294002 diminished the thrombin peak in PRP up to 70%, which was not further reduced in the presence of FA6.152, indicating the important role of SFKs in signaling of FXI-dependent and CD36-sensitive thrombin generation (Figure 2B,C). Interestingly, inhibition of ERK5 by BIX02188, which has been recently shown to block CD36-mediated platelet activation and caspase-dependent phosphatidylserine-exposure in response to oxLDL,^{33,34} had no effect on thrombin-induced thrombin formation (Figure 2D) and platelet activation (data not shown).

The reversible integrin α IIB β 3 antagonist tirofiban as well as an inhibitor of the tyrosine kinase Syk, known to be involved in α IIB β 3 outside-in signaling, also diminished the thrombin peak significantly (Figure 3A,B). However, pre-incubation of PRP with tirofiban or Syk inhibitor plus anti-CD36 antibody FA6.152 or tirofiban plus Syk inhibitor resulted in additive inhibition of thrombin generation when each effector alone showed submaximal inhibition (Figure 3C). These data demonstrate that CD36 regulates thrombin generation via α IIB β 3- and Syk kinase-dependent pathways and that Syk does not only play a role in FXI-dependent thrombin formation via α IIB β 3/Fc γ RIIA signaling. This finding was also supported by the observation that blocking of Fc γ RIIA by the antibody IV.3 had no significant effect on FXI-dependent thrombin generation in PRP (data not shown). Decreased thrombin generation was observed in PRP from a patient with δ -storage pool deficiency or when PRP from a healthy donor was pre-incubated with the P2Y₁₂ blocker AR-C69931 and additional inhibition was achieved in the presence of the anti-CD36 antibody FA6.152 (Figure 3D,E).

Thrombin receptor/PAR-mediated thrombin generation was less sensitive to CD36. CD36 blocking by FA6.152 resulted only in 30%-40% inhibition of the thrombin peak but in no significant inhibition of the etp induced by the PAR-1 and PAR-4 activating peptides SFLLRN (TRAP-6) and AYPGKF, respectively (Figure S3A,B). The PAR-4 inhibitor BMS-986120 in a saturating concentration reduced up to 40% TRAP-6-induced thrombin generation, which was not further affected by FA6.152. In contrast, the PAR-1 inhibitor vorapaxar only slightly diminished AYPGKF-induced thrombin formation, which was further reduced by CD36 blocking (Figure S3C,D), suggesting that PAR-4 seems to be more relevant than PAR-1. Our observation that the Syk IV inhibitor neither affects PAR-1- nor PAR-4-induced thrombin generation when CD36 was not blocked (Figure S3E,F), indicates that the role of Syk in CD36-dependent thrombin formation cannot be explained by PAR-signaling.

To test the role of CD36 in FcR γ -chain-ITAM-mediated thrombin generation induced by GPVI-ligation, the selective GPVI-agonist convulxin was used as trigger. CD36 receptor blocking resulted in up to 30% reduction of the thrombin peak, whereas the etp was not affected (Figure S4A). Syk-inhibition led to a similar reduction of the thrombin peak compared to FA6.152, which was not further decreased by FA6.152 (Figure S4B). However, under conditions of CD36-sensitive platelet activation induced by thrombin neither platelet GPVI surface expression was affected (Figure S5A), nor GPVI shedding as marker for GPVI activation was detected in the platelet-free supernatant compared to the supernatant of Ca²⁺-ionophore-treated platelets (Figure S5B). Together, these data suggest that CD36 is only slightly involved in GPVI-mediated thrombin generation in a Syk-dependent manner and that CD36 sensitive platelet activation induced by thrombin does not require GPVI activation.

3.3 | CD36 supports platelet integrin α IIB β 3 activation, granule exocytosis, and binding of fibrinogen/fibrin, vWF, and thrombospondin-1 in response to thrombin

To confirm that CD36 also support thrombin-induced platelet activation, conditions similar to the CAT-assay were used for flow cytometry analysis of platelet function. Thrombin induced full activation of integrin α IIB β 3, P-selectin-, and CD63-surface expression as well as the binding of fibrinogen/fibrin, TSP-1, and vWF to the platelet surface (Figure 4A). These platelet activation responses were significantly diminished by CD36 blocking (Figure 4B) or CD36 deficiency (Figure 4C).

3.4 | CD36 promotes binding of distinct coagulation factors to thrombin-stimulated platelets independently of anionic phospholipids

To pinpoint the role of platelet surface anionic phospholipids in CD36-dependent thrombin formation, negatively charged phospholipids such as phosphatidylserine on the activated platelet surface were blocked by annexin-V. The lag time was significantly prolonged of annexin-V-treated PRP compared to the vehicle control, accompanied by a significant decrease of the thrombin peak and the etp (Figure 5A). Simultaneous blockade of anionic phospholipids and CD36 had an additive inhibitory effect leading to almost complete inhibition of thrombin generation (Figure 5A), demonstrating that CD36-dependent thrombin formation does not depend on the exposure of anionic phospholipids. Flow cytometric analysis revealed that

thrombin-induced binding of annexin-V-FITC (Figure 5B) or lactadherin-FITC (Figure 5C) to CD36-blocked or CD36-deficient platelets was unaltered (10%-18% annexin-V or lactadherin-positive platelets), whereas increased binding of the factors FIX(a), FVIII(a), and FX(a), FV(a) to the

platelet surface was significantly reduced (Figure 5D,E). These data indicate that CD36 supports binding of factors of the intrinsic tenase and prothrombinase complex to thrombin-stimulated platelets independently of anionic phospholipids.

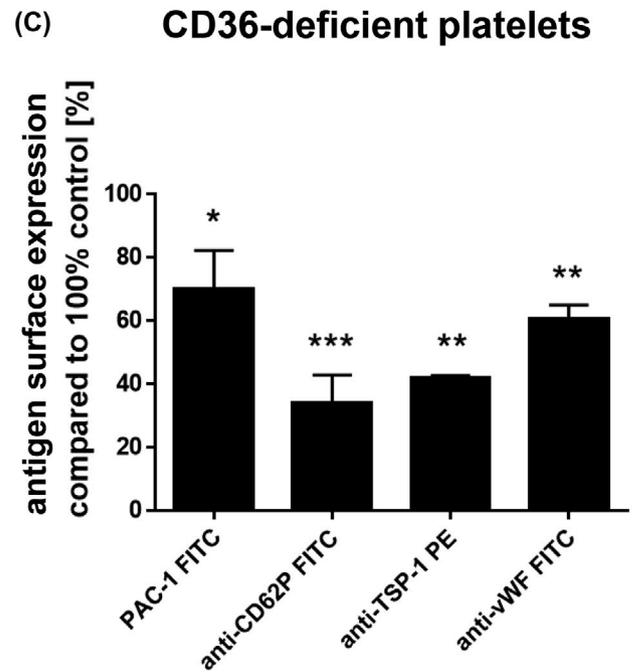
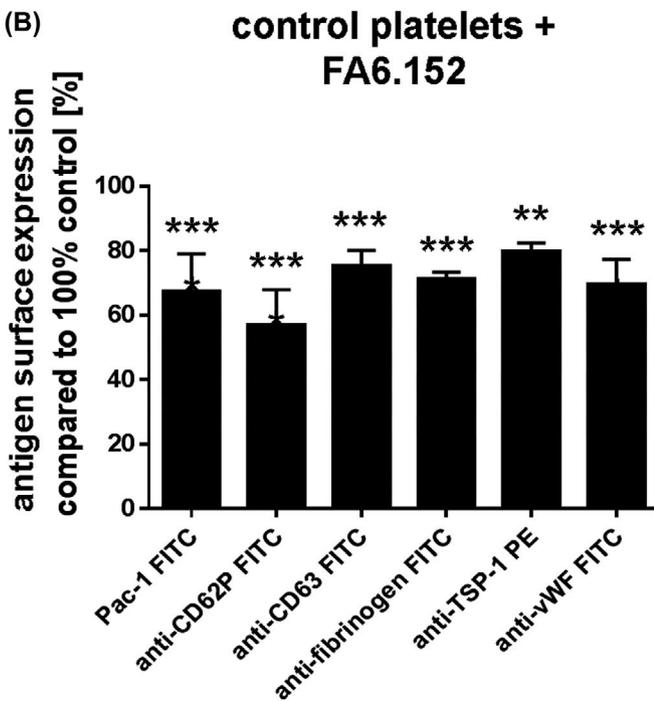
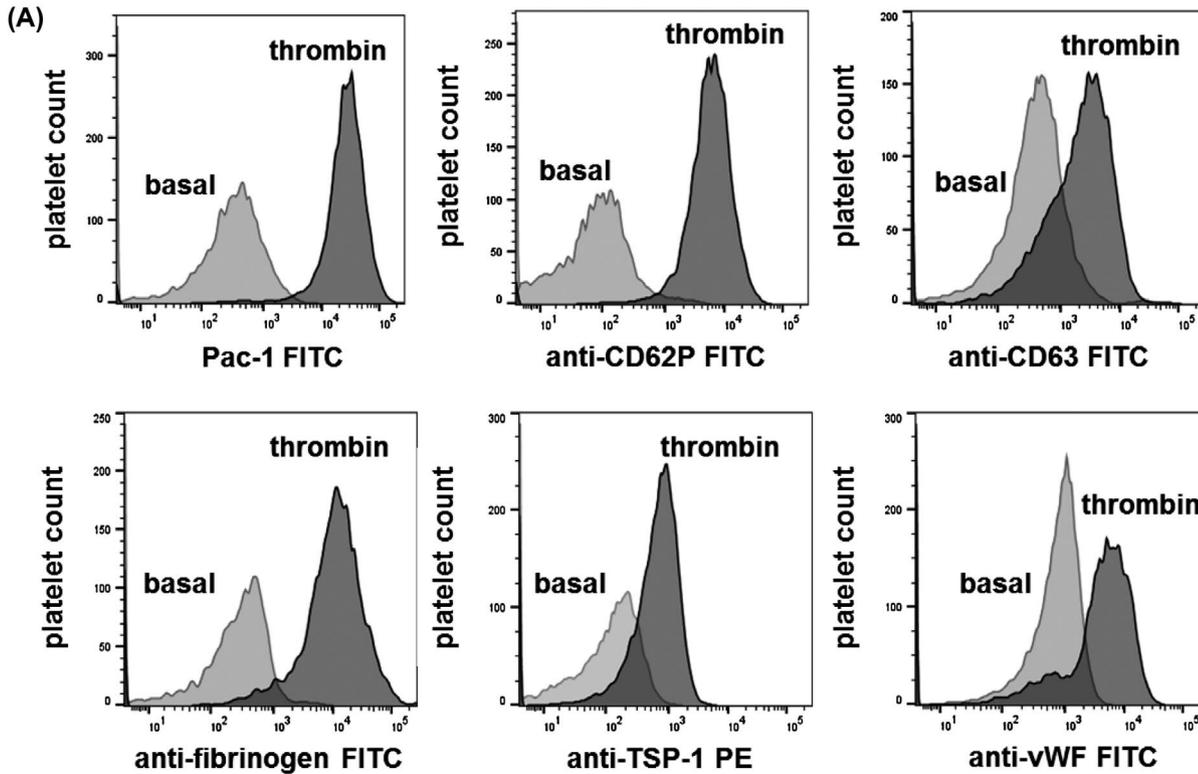


FIGURE 4 Role of CD36 in platelet integrin α IIb β 3 activation, granule secretion, and binding of adhesion proteins. A, Representative flow cytometric histograms of unstimulated (basal) and thrombin (1 nM) stimulated platelets in diluted PRP presenting activated α IIb β 3 integrin (PAC-1 FITC), P-selectin surface expression (anti-CD62P FITC), CD63 surface expression (anti-CD63-FITC), binding of fibrinogen (anti-fibrinogen-FITC), binding of TSP-1 (anti-TSP-1 PE), and vWF (anti-vWF FITC). B, Residual binding in % of antibodies, detecting activated α IIb β 3 integrin (PAC-1 FITC), P-selectin surface expression (anti-CD62P FITC), CD63 surface expression (anti-CD63-FITC), binding of fibrinogen (anti-fibrinogen-FITC), binding of TSP-1 (anti-TSP-1 PE) and vWF (anti-vWF FITC), to thrombin-stimulated platelets in diluted PRP in the presence of anti-CD36 antibody FA6.152 (5 μ g/mL) compared to thrombin-stimulated platelets in diluted PRP as detected by flow cytometry. C, Residual binding in % of PAC-1 FITC, anti-CD62P FITC, anti-TSP-1 PE, and anti-vWF FITC antibodies to thrombin-stimulated platelets in diluted PRP from a proband with CD36-deficiency compared to thrombin-stimulated platelets in diluted PRP from controls as detected by flow cytometry. Normalized 100% reflects mean fluorescence intensity of thrombin-activated platelets treated with 5 μ g/mL IgG1 isotype control for FA6.152. Means \pm SD (n = 3-4). * P < .05, ** P < .01, *** P < .001% vs. 100% control

3.5 | Fibrin mediates CD36-dependent thrombin generation of human platelets

Recently, fibrin was identified as ligand of GPVI, thereby amplifying TF-induced thrombin generation.³⁵ Therefore, washed human platelets in defibrinated plasma were tested for the role of fibrinogen/fibrin in CD36-regulated thrombin generation. The thrombin peak and etp were significantly decreased compared to washed platelets in control plasma and CD36 blocking by FA6.152 did not further reduce thrombin generation. Reconstitution of plasma with highly purified human fibrinogen completely rescued thrombin generation, which was also significantly inhibited by FA6.152 (Figure 6A). In contrast, the addition of purified human TSP-1 or vWF/FVIII to washed platelets in defibrinated plasma did not yield in a significant rescue of the thrombin generation capacity (Figure S6A). Thrombin generation could not be induced when isolated platelets were resuspended in vWF-depleted plasma, as expected due to the additional depletion of FVIII, but addition of FVIII was sufficient to rescue thrombin generation to normal levels (Figure S6B). Blocking the interaction of CD36 with TSP-1 by the anti-TSP-1 antibody A4.1, the TSP-1 peptide CSVTCG or the CD36 peptide P(93-110) also did not significantly affect thrombin generation in PRP in response to thrombin (Figure S6C-E). Thrombin generation induced by the fibrin generating snake venom batroxobin was comparable to that induced by thrombin and was inhibited to a similar extent by FA6.152 (Figure 6B). This process was dependent on platelet activation as iloprost, a stable prostacyclin analog, preventing platelet activation by increasing intracellular cAMP levels, inhibited significantly batroxobin-induced thrombin generation (Figure 6C). The peptide GPRP, which prevents fibrin polymerization did not affect thrombin-induced thrombin generation (Figure 6D).

3.6 | Fibrin mediates activation of SFKs and Syk in human platelets via CD36

To examine whether soluble fibrin induces platelet SFK and Syk signaling in a CD36-dependent manner, which we found

to be important in FXI-dependent thrombin formation, Src family Y416 phosphorylation and Syk activation (Syk Y352 phosphorylation) were analyzed in the presence of GPRP when fibrinogen and/or thrombin was added to washed platelets (Figure 7). Platelet Src Y416 and Syk Y352 phosphorylation was slightly induced by addition of fibrinogen or thrombin, but it was significantly upregulated when soluble fibrin was generated in the presence of fibrinogen plus thrombin. Interestingly, only these enhanced phosphorylations at Src Y416 and at Syk Y352 phosphorylation were inhibited by CD36-blocking to the extent observed for added fibrinogen or thrombin alone. Inhibition of SFKs by PP1 resulted in basal levels of P-Src Y416 in response to thrombin plus fibrinogen and reached comparable levels when CD36 was additionally blocked by FA6.152 (Figure 7A). Using again the SFK inhibitor PP1 similar results were obtained with P-Syk Y352 (Figure 7B). These data indicate a crucial role of SFKs in CD36-mediated activating signaling including Syk via soluble fibrin in human platelets.

3.7 | Fibrin is a ligand of CD36 and mediates binding of distinct coagulation factors to CD36

We analyzed the binding properties of fibrinogen/fibrin, vWF, TSP-1, and FV(a), FVIII(a), FIX(a), FX(a) to polystyrene beads coated with recombinant human CD36 by flow cytometry. Increased binding purified human TSP-1 to CD36-beads and blocking of this interaction by FA6.152, A4.1, CSVTCG, and P93-110 confirmed specific binding of TSP-1 to CD36 in this assay compared to purified vWF (Figure 8A,B). Interestingly, binding of highly purified human fibrinogen converted into fibrin by batroxobin as soluble, non-polymerized form (in the presence of GPRP) was more than 50-fold higher compared to the binding of untreated fibrinogen, which was significantly inhibited by the anti-CD36 antibody FA6.152 but not by 185-1G2 (Figure 8C).

Next, thrombin-treatment of control plasma resulted in clearly increased binding of plasma fibrinogen/fibrin, vWF, TSP-1, and plasma FV(a), FVIII(a), FIX(a), FX(a) to

CD36-coated beads, which was lost in defibrinated plasma (Figure 8D,E). These results demonstrate that fibrin directly or indirectly mediates the binding of distinct coagulation factors to CD36.

The anti-FVIII monoclonal antibody ESH8, directed against the FVIII C2-domain, blocking the binding of FVIII

to soluble fibrin but not to phosphatidylserine-containing vesicles,³⁶ significantly inhibited thrombin-induced thrombin generation in PRP, which was not further affected by CD36 blocking (Figure 8F).

Then, we evaluated whether immobilized fibrin, independent of platelets, mediates thrombin formation in response

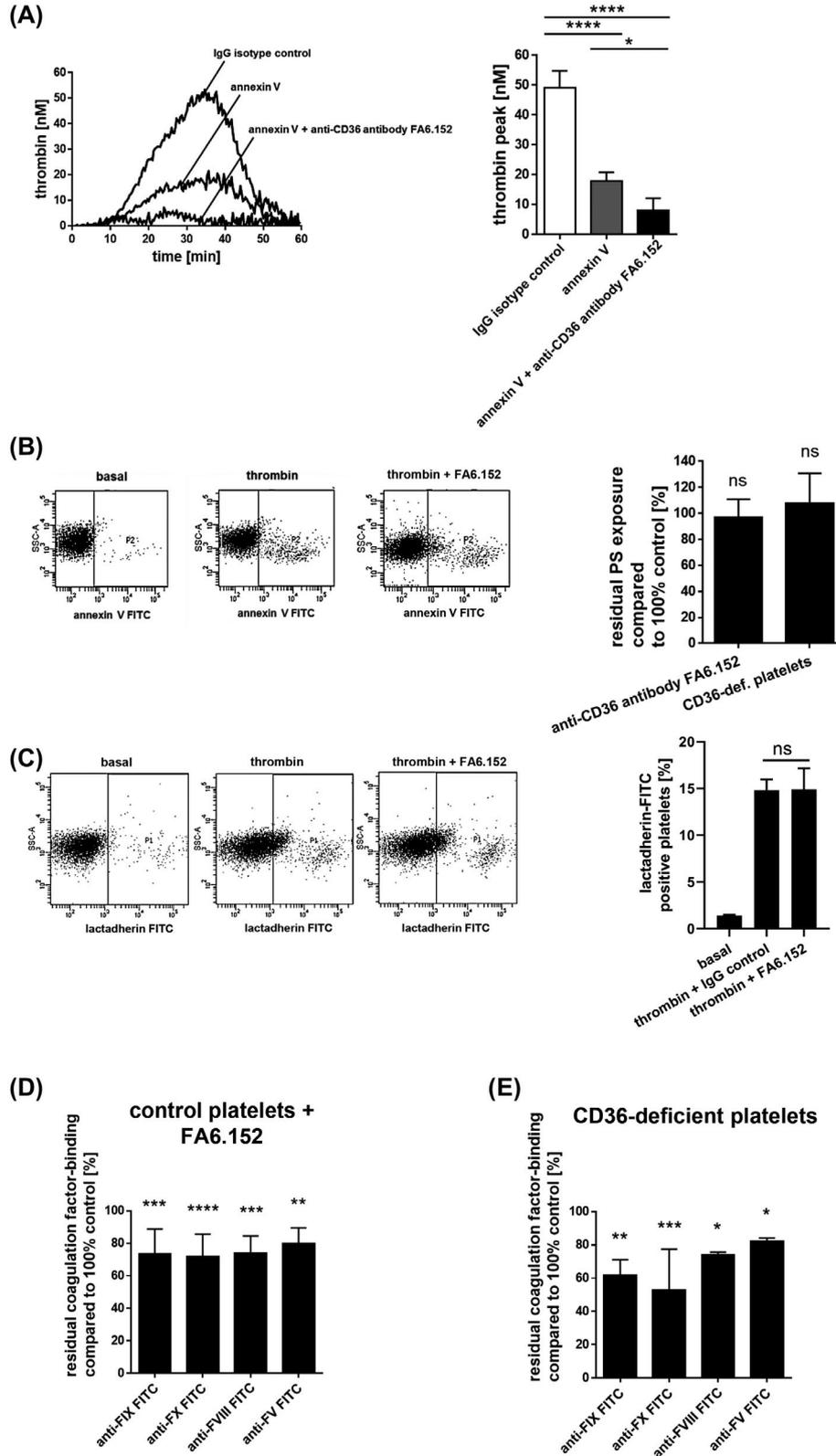


FIGURE 5 Role of platelet phosphatidylserine exposure in CD36-dependent thrombin generation and coagulation factor binding. A, Thrombin generation in annexin V-treated PRP (0.5 $\mu\text{g}/\text{mL}$) in the absence or presence of anti-CD36 antibody FA6.152 (5 $\mu\text{g}/\text{mL}$) compared to IgG isotype control (5 $\mu\text{g}/\text{mL}$); trigger 2 nM thrombin. B, Representative flow cytometric dot plots of unstimulated (basal), thrombin-stimulated platelets in the absence and presence of anti-CD36 antibody FA6.125 (5 $\mu\text{g}/\text{mL}$) in diluted PRP binding annexin-V (P2: annexin-V positive platelets) and residual binding in % of FITC-conjugated annexin V to thrombin-stimulated platelets in diluted PRP from a proband with CD36-deficiency or preincubated with anti-CD36 antibody FA6.152 (5 $\mu\text{g}/\text{mL}$) compared to thrombin-stimulated platelets in diluted PRP from controls as detected by flow cytometry. Normalized 100% reflects percentage of annexin-V positive thrombin-stimulated platelets treated with 5 $\mu\text{g}/\text{mL}$ IgG1 isotype control for FA6.152. C, Representative flow cytometric dot plots and quantification of unstimulated (basal), thrombin-stimulated platelets in the absence and presence of anti-CD36 antibody FA6.125 (5 $\mu\text{g}/\text{mL}$) in diluted PRP binding lactadherin-FITC (P1: lactadherin positive platelets). D, E, Residual binding in % of FITC-conjugated antibodies detecting FIX, FX, FVIII and FV to thrombin-stimulated platelets pre-incubated with anti-CD36 antibody FA6.152 (5 $\mu\text{g}/\text{mL}$) (D) or in diluted PRP from a proband with CD36-deficiency (E) compared to thrombin-stimulated platelets in diluted PRP from controls as detected by flow cytometry. Normalized 100% reflects mean fluorescence intensity of thrombin-activated platelets treated with 5 $\mu\text{g}/\text{mL}$ IgG1 isotype control for FA6.152. Means \pm SD (n = 3-8). * P < .05, ** P < .01, *** P < .001, **** P < .0001, n.s. not significant vs. 100% control or as indicated

to thrombin. Fibrin-coated beads mediated a significantly higher thrombin peak and etp associated with significantly shorter lag time compared to BSA-coated beads (negative control) in defibrinated plasma. The lag time of fibrin-coated beads was in the same range compared to washed platelets in platelet-free plasma (positive control). However, the same concentration of platelets induced a significant higher thrombin peak and etp than fibrin-coated beads (Figure 8G), indicating that immobilized fibrin alone is able to contribute to some extent to thrombin generation.

3.8 | FIX, FVIII, and FV bind to fibrin in a thiol-dependent manner

To test whether distinct coagulation factors ligate directly to fibrin, binding of purified, and FITC-conjugated coagulation factors to fibrin-coated beads was assessed by flow cytometry. We confirmed increased binding of FVIII to fibrin-coated beads as published.³⁶ However, we observed also significantly increased binding of FIX and FV but not of FX to fibrin-coated beads. Blocking of free thiols by the thiol-reactive compound DTNB completely inhibited factor binding to fibrin-coated beads (Table 1). These data demonstrate that not only FVIII but also FIX and FV specifically bind to fibrin in a thiol-dependent manner.

3.9 | CD36 contributes to increased FXI-dependent thrombin generation on platelets from selected patients with advanced chronic kidney disease

To prove the concept that CD36 may be a candidate to trigger a hyperreactive platelet phenotype in chronic inflammatory diseases, we analyzed the in vitro thrombin generation capacity in PRP from two selected patients with advanced CKD (stage 5) before hemodialysis. These patients were without

previous cardiovascular symptomatic events, presented with normal coagulation parameters, normal platelet count, phenotype, and aggregation. However, they showed increased plasma levels of soluble fibrin monomer complex (Table S3) and increased thrombin peak and etp in PRP compared to healthy controls (Table 2). Interestingly, isolated platelets from healthy donors resuspended in patients' platelet-free plasma showed still enhanced thrombin generation, suggesting that distinct factors in the patients' plasma enhance the capacity of FXI-dependent thrombin generation. Using the anti-CD36 antibodies FA6.152 and 185-1G2, which both are known to block the binding of oxidized lipids/proteins to CD36, only FA6.152 but not 185-1G2 diminished the thrombin peak in PRP and in platelet-plasma exchange experiments (Table 2).

4 | DISCUSSION

In this study, we identified CD36 as important mediator to amplify FXI-dependent thrombin generation on human platelets via GPIIb α and SFK signaling (Figure 9). We also identified CD36 as a receptor for fibrin that ligates FV, FVIII, and FIX in a thiol-dependent manner. This pathway does not depend on the limited exposure of anionic phospholipids on thrombin-stimulated platelets (Figure 9), which might be involved in enhanced thrombin generation in CKD.

Using calibrated automated thrombography, we confirmed that 2 nM (0.1 U/mL) of α -thrombin induces FXI-sensitive thrombin generation in PRP to trigger the positive feedback burst of thrombin formation,³⁷ which does not depend on FXII.³⁸ Flaumenhaft and colleagues also validated the physiological relevance of our platelet-dependent thrombin generation assay³⁹ that we used to detect CD36 sensitivity of FXI-dependent thrombin generation on human platelets. We also demonstrated CD36 sensitivity for integrin α IIb β 3 activation and granule release of thrombin-stimulated platelets, suggesting that platelet activation pathways are involved

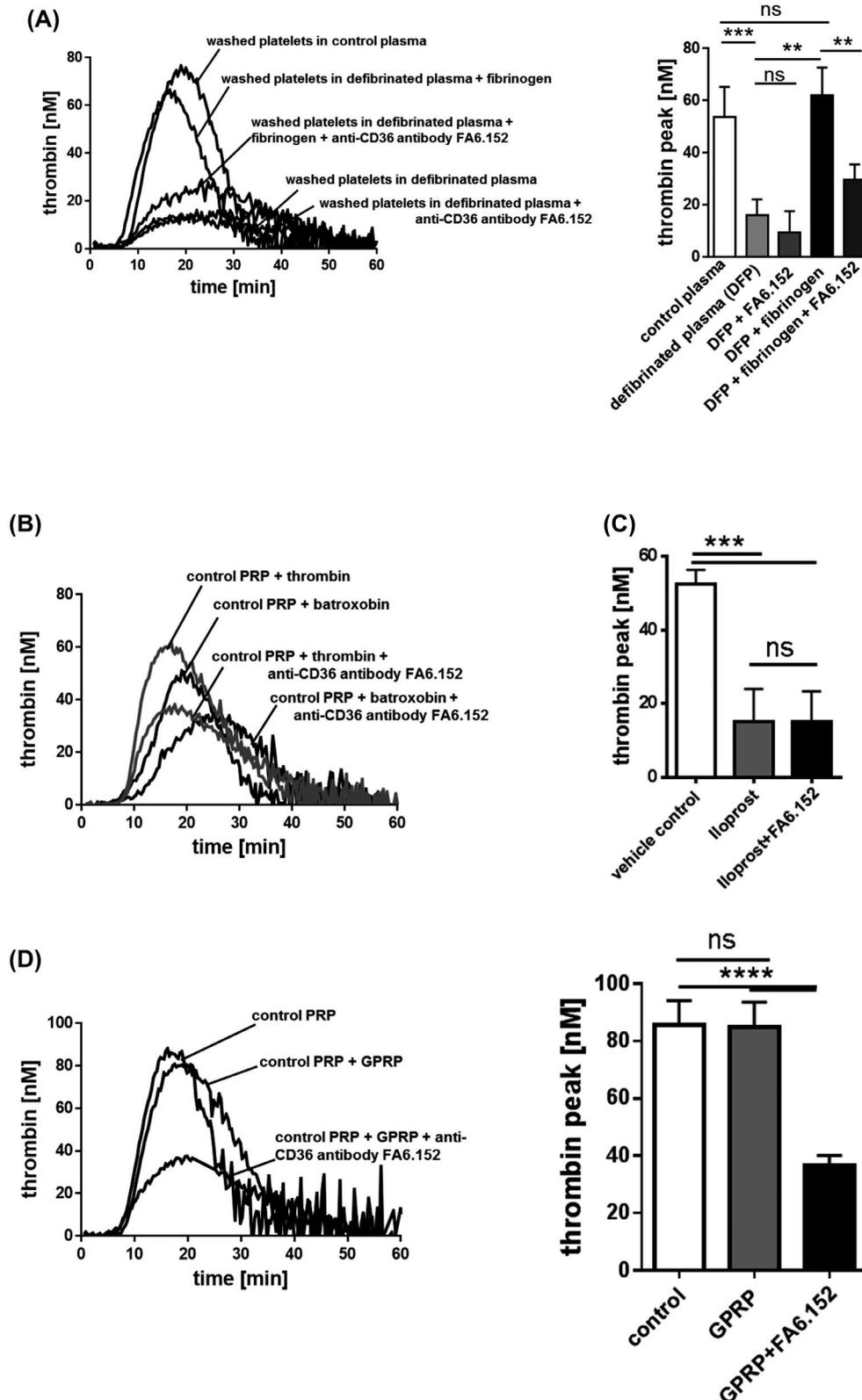


FIGURE 6 Role of fibrinogen/fibrin in platelet-dependent thrombin generation triggered by thrombin. A, Thrombin generation on washed human platelets resuspended in plasma from controls, in defibrinated plasma (DFP), in defibrinated plasma in the presence of anti-CD36 antibody FA6.152 (5 $\mu\text{g}/\text{mL}$) and/or fibrinogen (4 mg/mL); trigger 2 nM thrombin. B, Effect of batroxobin (1 U/mL) on thrombin generation in PRP without additional trigger in the absence or presence of anti-CD36 antibody FA6.152 (5 $\mu\text{g}/\text{mL}$) compared to thrombin generation in control PRP triggered by 2 nM thrombin in the absence or presence of anti-CD36 antibody FA6.152 (5 $\mu\text{g}/\text{mL}$). C, Effect of Iloprost (10 nM) in the absence or presence of anti-CD36 antibody FA6.152 (5 $\mu\text{g}/\text{mL}$) on thrombin peak in PRP triggered by thrombin (2 nM). D, Effect of GPRP (2.5 mM) in the absence or presence of anti-CD36 antibody FA6.152 (5 $\mu\text{g}/\text{mL}$) on thrombin generation in control PRP compared to untreated control PRP. Means \pm SD (n = 5). ** P < .01, *** P < .001, **** P < .0001, n.s. not significant

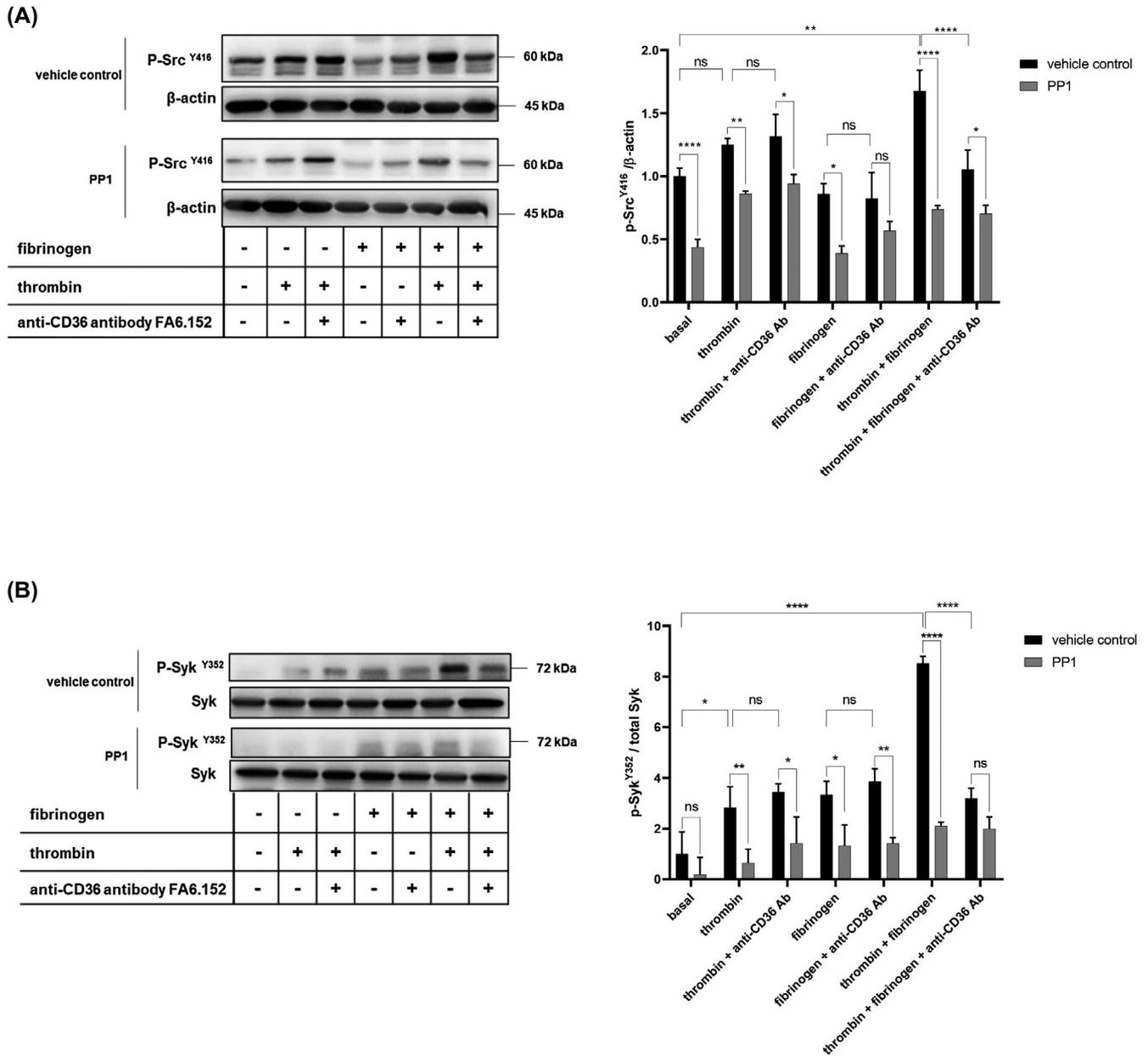


FIGURE 7 Role of SFKs and Syk in CD36-dependent platelet signaling via non-polymerized fibrin. A, Representative western blot and quantification of Src^{Y416} family phosphorylation compared to loading control β-actin and the effect of SFK inhibition by PP1 (20 μM) in washed human platelets plus GPRP (2.5 mM) in the absence or presence of fibrinogen (0.5 mg/mL), thrombin (1 nM), and anti-CD36 antibody FA6.152 (5 μg/mL) incubated for 1 minute at 37°C. B, Representative western blot and quantification of Syk^{Y352} phosphorylation compared to total Syk and the effect of SFK inhibition by PP1 (20 μM) in washed human platelets plus GPRP (2.5 mM) in the absence or presence of fibrinogen (0.5 mg/mL), thrombin (1 nM), and anti-CD36 antibody FA6.152 (5 μg/mL) incubated for 1 minute at 37°C. Means ± SD (n = 3). **P* < .05, ***P* < .01, *****P* < .0001, n.s. not significant

in CD36-dependent thrombin formation. Here, GPIIb/IIIa-mediated pathways were identified to be crucial. Recently, we found that the FXI-dependent thrombin feedback-loop mediated by platelet GPIIb/IIIa triggers angiotensin-II induced vascular inflammation and hypertension in mice as well as spontaneous hypertension in men.⁹ Further work has to show whether FXI-binding to GPIIb/IIIa has impact on CD36-dependent thrombin formation, and whether CD36 modulates the FXI-feedback thrombin loop in arterial hypertension. We

obtained further evidence that a) SFK-mediated downstream signaling via Syk and PI3K is important, b) integrin αIIbβ3 outside-in signaling and platelet released δ-granule cargo such as ADP play a partial role, whereas c) PAR-1 and PAR-4 have a minor role in CD36-dependent thrombin formation induced by thrombin.

However, our data did not support a role of integrin αIIbβ3 outside-in signaling via Syk/FcγRIIA in CD36-sensitive thrombin generation (Figure 9). Very recently, we showed that

activation of Syk mediated via FcR γ -chain-ITAM phosphorylation is very important in GPIb α -mediated platelet activation and that this occurs independently of α IIB β 3 outside-in signaling.²⁷ Thus, GPIb α might contribute to platelet Syk-signaling via the FcR γ -chain pathway in CD36-dependent thrombin generation.

Previously, we demonstrated that isolated human platelets present pronounced surface exposed anionic phospholipids in response to thrombin, when platelet-platelet contact was provoked and that this procoagulant activity depends on thrombin binding to GPIb α .⁴ In our experimental setting and in line with others⁴⁰ thrombin induced only a limited surface

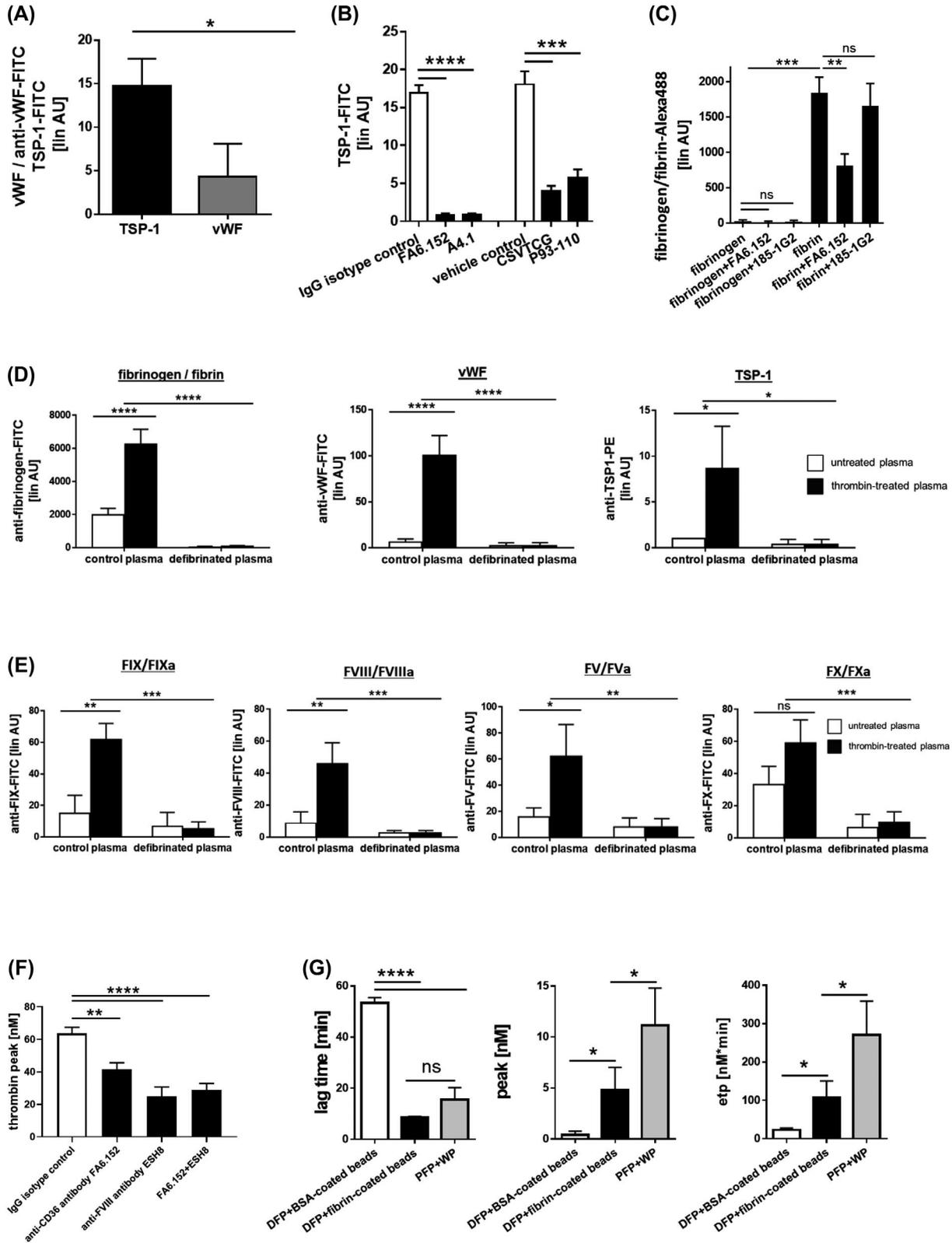


FIGURE 8 Binding of fibrinogen/fibrin, vWF, TSP-1, and FIX, FVIII, FV, FX to CD36-coated beads and thrombin generation in PRP with blocked FVIII-fibrin interaction and of fibrin-coated beads in defibrinated plasma. A, Flow cytometric analysis of human FITC-conjugated TSP-1- and vWF-binding to CD36-coated beads and (B) effect of anti-CD36 antibody FA6.152 (5 $\mu\text{g}/\text{mL}$), anti-TSP-1 antibody A4.1 (5 $\mu\text{g}/\text{mL}$), CSVTCG-, and P93-110-peptide on TSP-1-FITC-binding to CD36-coated beads. vWF was detected by FITC-conjugated anti-vWF antibody. C, Flow cytometric analysis of human fibrinogen-FITC to CD36-coated beads in the absence or presence of batroxobin (1 U/mL plus 1.25 mM GPRP) and anti-CD36 antibody FA6.152 or 185-1G2 (20 $\mu\text{g}/\text{mL}$). D, Binding of fibrinogen/fibrin, vWF, and TSP-1 from control or defibrinated plasma in the absence or presence of thrombin (0.5 nM plus 17.5 mM GPRP). Fibrinogen/fibrin, vWF, and TSP-1 were detected by anti-fibrinogen-FITC, anti-vWF-FITC, and anti-TSP-1-PE antibody, respectively. E Binding of FIX, FVIII, FV, and FX from control or defibrinated plasma in the absence or presence of thrombin (0.5 nM). Plasma coagulation factors were detected by anti-FIX-FITC, anti-FVIII-FITC, anti-FV-FITC, and anti-FX-FITC antibody, respectively. Data are expressed as linear fluorescence intensity in arbitrary units (lin AU) of CD36-coated beads corrected by fluorescence intensity of albumin-coated beads. F, Effect of anti-FVIII antibody ESH8 (10 $\mu\text{g}/\text{mL}$), blocking binding of FVIII to fibrin, on thrombin-induced thrombin generation in PRP. G, Thrombin generation of fibrin-coated beads (14 000/ μL) compared to BSA-coated beads (14 000/ μL) in defibrinated plasma (DFP) compared to washed platelets from healthy donors (14 000/ μL) in PFP plus GPRP (2.5 mM) induced by thrombin (2 nM). Means \pm SD (n = 3-4). * P < .05, ** P < .01, *** P < .001, **** P < .0001, n.s. not significant

exposure of anionic phospholipids (up to 18% of annexin-V or lactadherin positive platelets), when platelets are activated by thrombin without provoked platelet-platelet contact. Our data revealed that CD36-dependent thrombin formation and ligation of FVIII(a), FIX(a), and FX(a) to thrombin-activated platelets are not mediated by exposed anionic phospholipids and require another mechanism. Nevertheless, negatively charged phospholipids are still necessary for binding of vitamin-K-dependent coagulation factors to the platelet surface and for subsequent thrombin generation, and this might also explain why CD36-deficient people or mice do not suffer from major bleeding.

Indeed, our findings showed that fibrin rather than TSP-1 or vWF plays an essential role in FXI- and CD36-dependent thrombin generation. From experiments using GPRP, which prevents polymerization of soluble fibrin, but showed no inhibitory effect on CD36-dependent thrombin generation, we conclude that CD36-dependent thrombin formation occurs also when fibrin is not polymerized. Furthermore, we observed that non-polymerized fibrin-induced Src/SFK and Syk activation in isolated platelets in the presence of thrombin plus fibrinogen plus GPRP, which was inhibited by FA6.152 to basal activation. In contrast, CD36 blocking did not affect Src/SFK and Syk activation induced by soluble fibrinogen or immobilized fibrinogen/fibrin via $\alpha\text{IIb}\beta 3$ outside-in signaling. These results support our data that $\alpha\text{IIb}\beta 3$ outside-in signaling through ligation of fibrinogen/fibrin is not important in CD36-mediated SFK and Syk signaling. Others reported that polymerized fibrin, but not non-polymerized fibrin or fibrinogen via GPVI ligation promotes platelet-dependent thrombin formation and induces GPVI shedding in platelet suspensions.^{35,41} Montague et al demonstrated that under stirring conditions Src or Syk signaling does not seem to be important for platelet GPVI shedding induced by polymerized fibrin.⁴² Based on our results, which did not show significant GPVI shedding under conditions of CD36-sensitive and SFK/Syk-dependent platelet activation, we conclude that GPVI-mediated pathways are not required for CD36-dependent thrombin formation or activation of human platelets. Bead

experiments provided evidence that non-polymerized fibrin is a prominent ligand of CD36. The anti-CD36 antibody FA6.152 but not 185-1G2 blocked this interaction and had an inhibitory effect on thrombin-induced thrombin generation. The data imply epitope(s) specific binding of fibrin to CD36, which is not identical with that of oxidized-modified lipids/proteins. The important role of fibrin in CD36-dependent thrombin generation was further supported by the finding of significantly increased binding of fibrin, but not of fibrinogen, to CD36-coated beads. Although we did not obtain evidence that binding of soluble TSP-1 to CD36 plays an important role in FXI- and CD36-dependent thrombin formation, TSP-1 together with vWF might contribute to this process. It has been previously shown that soluble fibrin acts as ligand for vWF⁴³ but also for TSP-1⁴⁴ and that vWF directly interacts with TSP-1,⁴⁵ suggesting that GPIIb α -bound vWF and TSP-1⁴⁴ could bridge fibrin to CD36 or vice versa in CD36-dependent thrombin generation. The questions whether vWF/TSP-1/fibrin complexes are important in this process and whether the vWF and TSP-1 receptor GPIIb α is involved in binding of such complexes have to be addressed in future studies.

Gilbert et al demonstrated with bead experiments that fibrin acts as ligand for FVIII.³⁶ Our data, also based on bead experiments, revealed that fibrin is not only a ligand for FVIII but also for FIX and FV and that fibrin-bound FVIII but also fibrin-bound FIX and FV triggers CD36-dependent thrombin generation. Interestingly, thrombin generation could be induced by thrombin in defibrinated plasma in the presence of fibrin-coated beads alone to some extent, suggesting that immobilized fibrin in the absence of activated platelets contributes to thrombin-induced thrombin generation by recruitment of coagulation factors. We already published that thiol isomerases on the platelet surface, including protein disulfide isomerase, regulate feedback activation of platelet-dependent thrombin generation via modulation of coagulation factor binding.²⁶ Here, we could show that the interaction between fibrin and distinct coagulation factors was dependent on free thiols as binding of

	Albumin-coated beads	Fibrin-coated beads	Fibrin-coated beads + DTNB (2.5 mM)
FIX-FITC	11.9 ± 0.9	33.2 ± 3.1**	12.2 ± 1.3
FVIII-FITC	12.2 ± 1.2	34.3 ± 5.2**	12.6 ± 3.1
FV-FITC	12.4 ± 1.1	39.1 ± 3.3**	7.9 ± 0.9
FX-FITC	12.1 ± 0.9	15.3 ± 2.5	8.3 ± 0.5

Note: Values are linear fluorescence intensities in arbitrary units. Data are expressed as mean ± standard deviation (SD) from three independent experiments.

***P* < .01 vs. albumin-coated beads.

TABLE 1 Flow cytometric analysis of FIX-FITC, FVIII-FITC, FV-FITC, and FX-FITC binding to fibrin-coated beads

TABLE 2 Platelet-dependent thrombin generation (thrombin peak in nM) in platelet-rich plasma (trigger: 2 nM thrombin) of patients with chronic kidney disease prior dialysis and effect of anti-CD36 blocking antibodies

	Controls	P1	P2
PRP + IgG isotype control (5 µg/mL)	100.00 ± 22.76	188.60 ± 2.45***	181.90 ± 2.83***
PRP + FA6.152 (5 µg/mL)		141.70 ± 9.12##	128.50 ± 11.78##
PRP + 185-1G2 (5 µg/mL)		192.70 ± 6.69 (n.s.)	168.70 ± 6.28 (n.s.)
WP (control) + PFP + IgG isotype control (5 µg/mL)	100.00 ± 17.87	316.90 ± 14.84****	279.00 ± 22.14****
WP (control) + PFP + FA6.152 (5 µg/mL)		242.00 ± 13.15##	219.30 ± 13.16#

Note: Values are % thrombin peak compared to controls (100%) determined in PRP and of washed platelets (WP) from healthy donors resuspended in PFP, respectively. Data are expressed as mean ± standard deviation (SD) from three independent experiments for two patients (P1, P2) and from 5-10 healthy controls.

****P* < .005, *****P* < .001 vs. corresponding controls; #*P* < .05, ##*P* < .01; n.s. (not significant) vs. same patient + IgG isotype control.

FITC-conjugated FVIII, FIX, and FV was completely abolished by the thiol-derivatizing reagent DTNB. However, the precise mechanism by which thiol isomerases might regulate the interaction between coagulation factors and fibrin and whether this process is modulated by colocalization of thiol isomerases, GPIIb/IIIa, αIIbβ3, and CD36 in lipid rafts or in coagulation active cap-like regions on thrombin-activated platelets⁴⁶ needs further investigation.

Thrombin feedback activation of FXI has been shown to be involved in thrombin generation induced by low TF levels.⁴⁷ Our data indicate partial CD36-sensitivity of platelet-dependent thrombin generation triggered by the established low TF concentration of 0.25 pM, which requires FXI. One explanation for the difference in CD36-sensitivity of TF- and thrombin-mediated thrombin formation could be that the CD36-fibrin pathway becomes important for thrombin generation, when the levels of exposed anionic phospholipids are limited as this was observed for thrombin-stimulated platelets. Conversely, TF even at low concentration induces pronounced anionic phospholipid exposure, presenting the dominant mechanism to recruit coagulation factors. Therefore, the CD36-fibrin pathway might be less relevant when platelets come in contact with TF of the injured vessel, but dominates in the center or at the shell of a growing thrombus where platelets get activated by thrombin generated by thrombus-recruited platelets or especially by activated monocytes under inflammatory conditions.

Hyperreactive and procoagulant platelets contribute to a prothrombotic state in chronic inflammatory diseases such as CKD.²⁴ However, the underlying mechanisms are incompletely understood. We observed significantly increased FXI-dependent thrombin generation in PRP from selected patients with advanced CKD prior hemodialysis treatment compared to healthy controls. This altered thrombin generation was nearly normalized by the CD36 blocking antibody FA6.152 but not by 185-1G2. Platelet-plasma exchange experiments indicated that distinct plasma factors contribute to the enhanced CD36-dependent platelet coagulant function in these patients. Here, elevated levels of soluble fibrin monomer complexes detected in the patients' plasma might serve as potential trigger for CD36-mediated platelet hypercoagulability. Large clinical studies are needed to confirm these proof-of-concept data and to evaluate the diagnostic value of platelet CD36 for thrombo-inflammation in CKD. Our data fit with previously published data that platelet CD36 promotes a prothrombotic state in other chronic inflammatory diseases such as atherosclerosis via ligation of oxidized lipoproteins.²² In this study, we identified fibrin as new CD36 ligand and we obtained evidence that this interaction might be important to augment platelet activation and thrombin-induced thrombin generation under physiological and inflammatory conditions of CKD. As enhanced fibrinogen and degraded fibrin have been localized in progressive atherosclerotic lesions and elevated plasma levels of fibrinogen and fibrin degradation

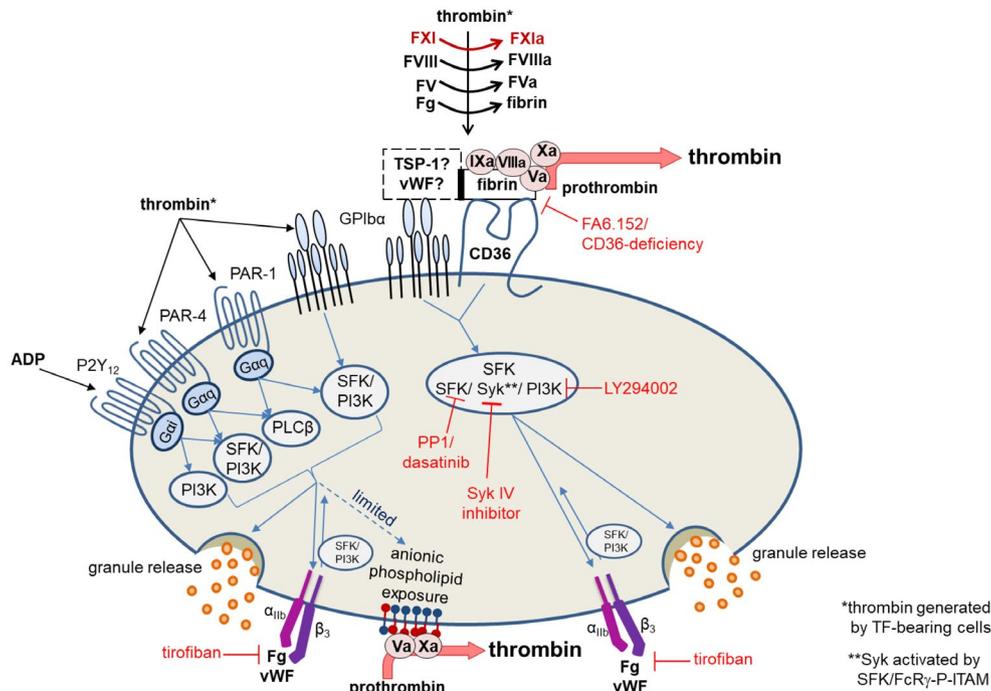


FIGURE 9 Model of the role of CD36 in platelet-dependent thrombin generation induced by thrombin

products have been considered as risk factors for atherothrombosis,⁴⁸ platelet CD36 may be a promising candidate to sense increased plasma fibrin levels in pronounced inflammation. Further work is necessary to elucidate the role of CD36 on monocytes and endothelial cells in thrombin generation and its relation to chronic inflammatory processes.

In conclusion, our data based on studies with both human CD36-deficient platelets and normal platelets with blocked CD36 indicate that CD36 acts as important sensitizer of platelet-dependent thrombin generation via fibrin and represents an attractive target to suppress platelet hyperreactivity under conditions of chronic inflammation.

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CONFLICT OF INTEREST

The authors declare no conflicts of interest.

AUTHOR CONTRIBUTIONS

M. Döhrmann, S. Makhoul, K. Gross, and K. Jurk performed experiments and analyzed data; M. Krause, D. Pillitteri, C. von Auer, J. Lutz, and I. Volf recruited patients with clinical data; K.

Jurk and B. E. Kehrel designed the research; M. Döhrmann, U. Walter, K. Jurk wrote the manuscript, which was finalized by K. Jurk; and all authors corrected and approved the manuscript.

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SUPPORTING INFORMATION

Additional Supporting Information may be found online in the Supporting Information section.

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