Supporting Roles of Platelet Thrombospondin-1 and CD36 in Thrombus Formation on Collagen

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Objective—Platelets abundantly express the membrane receptor CD36 and store its ligand thrombospondin-1 (TSP1) in the α-granules. We investigated whether released TSP1 can support platelet adhesion and thrombus formation via interaction with CD36.

Approach and Results—Mouse platelets deficient in CD36 showed reduced adhesion to TSP1 and subsequent phosphatidylinerine expression. Deficiency in either CD36 or TSP1 resulted in markedly increased dissolution of thrombi formed on collagen, although thrombus buildup was unchanged. In mesenteric vessels in vivo, deficiency in CD36 prolonged the time to occlusion and enhanced embolization, which was in agreement with earlier observations in TSP1-deficient mice. Thrombi formed using wild-type blood stained positively for secreted TSP1. Releasate from wild-type but not from TSP1-deficient platelets enhanced platelet activation, phosphatidylinerine expression, and thrombus formation on collagen. The enhancement was dependent on CD36 because it was without effect on thrombus formation by CD36-deficient platelets.

Conclusions—These results demonstrate an anchoring role of platelet-released TSP1 via CD36 in platelet adhesion and collagen-dependent thrombus stabilization. Thus, the TSP1–CD36 tandem is another platelet ligand–receptor axis contributing to the maintenance of a stable thrombus. (Arterioscler Thromb Vasc Biol. 2014;34:1187-1192.)

Key Words: blood platelets ■ CD36 ■ mice ■ thrombosis ■ thrombospondin 1

Glycoprotein IV or CD36 forms one of the most abundant glycoproteins on the surface of mouse and human platelets, expressed at ≤25,000 copies per cell.1,2 CD36 consists of a double membrane-spanning protein with 1 large extracellular domain and 2 short N- and C-terminal cytoplasmic domains.3–5 Its function has remained unclear for long. Earlier, CD36 was thought to be a platelet collagen receptor,6,7 but it was shown that platelets from CD36-deficient patients have an unchanged response to collagen.8,9 Subsequent studies suggested a role for CD36 as receptor for thrombospondin-1 (TSP1).10,11 It was also found that CD36 can bind oxidized lipids, including oxidized low-density lipoproteins (oxLDLs), particularly at conditions promoting atherogenesis.12,13 Earlier, we and other have demonstrated that interaction of CD36 with surface-immobilized TSP1 or oxLDL leads to outside-in signaling events in platelets via c-Jun N-terminal kinases (JNK) and spleen tyrosine kinase (Syk), and enforced via autocrine loops of secreted ADP and P2Y13 receptors.14,15 Interestingly, this signaling promotes Ca2+-dependent exposure of procoagulant phosphatidylinerine at the platelet surface.

See accompanying editorial on page 1120

The multidomain matrix glycoprotein TSP1 is one of the most highly expressed proteins in platelet α-granules (≈101,000 copies per cell).3 TSP1 is also found in the blood vessel, likely after deposition secreted by platelets and vascular cells.16–18 In addition to CD36, several other platelet receptors for TSP1 have been proposed. These include the glycoprotein Ib-V-IX complex19–21 and glycoprotein CD47.22 TSP1 can also indirectly influence platelet activity via binding to collagen, fibrinogen, and von Willebrand factor and protect the latter from cleavage by matrix proteinases.23

Lack of platelet α-granules is associated with a mild-to-moderate bleeding tendency in patients with the congenital gray platelet syndrome.24 The genetic defect has recently been attributed to mutations in the neurobeachin-like 2 (NBEAL2)
In mice, deficiency in Nbeal2 similarly results in defective α-granule biogenesis, accompanied by diminished platelet adhesion, phosphatidylserine exposure, and thrombus formation. To date, it has remained unclear which of the proteins stored in platelet α-granules contribute to these responses.

In the present article, we hypothesized that the abundantly expressed TSP1, secreted from the α-granules and interacting with its counter-receptor CD36, starts an additional autocrine feed-forward loop and supports the interactions of platelets in a thrombus. This hypothesis was tested using mice deficient in either CD36 or TSP1 by assessment of platelet adhesion, activation, and thrombus formation.

**Materials and Methods**

Materials and Methods are available in the online-only Supplement.

**Results**

In human platelets, CD36 acts as an adhesive receptor for immobilized TSP1. To assess this for the mouse system, we measured the adhesion of platelets from Cd36+/- and Cd36−/- mice to surfaces coated with purified human TSP1 or a TSP1-containing releasate from activated mouse platelets. With either surface, deficiency in Cd36 resulted in a markedly reduced static adhesion (Figure IA in the online-only Data Supplement). Labeling with fluorescein isothiocyanate-annexin A5 indicated that the lower adhesion of Cd36−/- platelets was accompanied by a lower phosphatidylserine exposure, suggesting reduced platelet activation (Figure IB in the online-only Data Supplement). Immunofluorescence experiments indicated that surface-coated releasate from wild-type platelets stained positively for TSP1, whereas surface-coated releasate from Tsp1−/- platelets failed to stain (Figure IIA and IIB in the online-only Data Supplement). We determined whether phosphatidylserine-exposing microparticles as possible CD36 ligands may contribute to the adhesion of platelets to surface-coated releasates. However, staining of these coatings with fluorescein isothiocyanate-annexin A5 did not result in a detectable fluorescence signal, pointing to the absence of microparticles on the coated surface (Figure III in the online-only Data Supplement). Together, the data indicated that CD36 acts as an adhesive receptor for mouse platelets on released and immobilized TSP1.

To determine the activation tendency of platelets from Cd36−/- mice, we measured integrin αIIbβ3 activation, α-granule secretion, and phosphatidylserine exposure in response to ADP or a glycoprotein VI agonist, convulxin. No difference was observed in any of the responses between the knockout and wild-type platelets (Figure IC–IE in the online-only Data Supplement). These results are in agreement with our previous data that blockage of CD36 does not affect agonist-induced activation of suspended human platelets. Similarly, other authors have described that CD36-deficient mouse platelets in suspension are unchanged in activation properties.

Considering that CD36 mediates platelet adhesion to immobilized TSP1, we investigated whether platelet-released TSP1 contributes to thrombus formation under flow conditions. Whole blood from Tsp1−/- or corresponding wild-type mice was perfused over collagen at high wall shear rate. Thrombi formed with Tsp1−/- and Tsp1+/+ blood differed neither in deposition of platelets (surface area coverage) nor in integrin αIIbβ3 activation (staining with PE-JON/A monoclonal antibody; Figure 1A and 1B). However, thrombi formed with Tsp1−/- blood were significantly reduced in platelet phosphatidylserine exposure. Immunostaining indicated the presence of released TSP1 in wild-type thrombi, but not in Tsp1−/- thrombi. To stimulate activation and secretion, thrombi on collagen were treated with ADP and then immunostained for TSP1 (Figure 1C and 1D). Strong staining of the Tsp1−/- thrombi was observed, but not of the Tsp1−/+ thrombi.

The roles of CD36 and TSP1 in thrombus formation were studied using Cd36−/- blood, which was perfused over collagen surfaces containing releasate from Tsp1−/- or Tsp1+/+ platelets. Control experiments indicated that surfaces with only releasates did not result in adhesion of Cd36−/- or Cd36+/+ platelets (Figure 2A and 2B). However, in combination with collagen, releasate from Tsp1+/+ but not from Tsp1−/- platelets enhanced the process of thrombus formation in perfusions with Cd36−/- blood only (P=0.035). Image analysis indicated that mean thrombus size was largest for the combination of Tsp1+/+ releasate and Cd36−/- blood (P=0.001; Figure 2C). When using Cd36−/- blood, no increase in thrombus size was observed in the presence of releasate from Tsp1−/+ or Tsp1−/- platelets.

Staining for integrin αIIbβ3 activation did not show marked differences between Cd36−/- and Cd36+/+ thrombi formed on collagen alone (Figure 3A and 3B). However, αIIbβ3, phosphatidylserine exposure were significantly increased in Cd36−/- thrombi (P=0.005 and P=0.027, respectively), when these were formed on a surface with collagen plus releasate from Tsp1+/+ (but not Tsp1−/-) platelets (Figure 3A–3D). Notably, the stimulating effect of Tsp1+/+ releasate was absent in Cd36−/- thrombi. Together, this pointed to a platelet-activating effect of immobilized TSP1 via CD36, thus supporting collagen-dependent thrombus formation and procoagulant activity.

Considering that paracrine platelet agents such as ADP, thromboxane, and Gas6 contribute to thrombus stabilization, we investigated the stability of thrombi formed with Cd36−/-, Tsp1−/-, or wild-type blood. This was done by measuring the rate of dissolution of preformed thrombi during a high shear postperfusion protocol. Strikingly, whereas the wild-type thrombi remained stable for a longer period, the thrombi of platelets deficient in either CD36 or TSP1 disintegrated within several minutes (Figure 4A; see Movies in the online-only Data Supplement). Quantification of thrombus dissolution during a 6-minute period indicated a significant increase in this parameter for Cd36−/- and Tsp1−/- platelets in comparison with wild type (Figure 4B).

Control experiments pointed out that in Tsp1−/- blood the blocking anti-mouse CD36 antibody MAB1258 did not have an additional effect (data not shown), suggesting that no other CD36 ligand was involved in thrombus stabilization. Furthermore, pretreatment of Cd36+/+ or Cd36−/- blood with a blocking antibody against CD47 did not change platelet deposition or thrombus stability (data not shown). Together, these findings pointed to a role of TSP1–CD36 interaction in the stabilization of thrombi at high shear flow.

To investigate this under in vivo conditions, we measured the thrombotic process in mesenteric arterioles and venules from
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Cd36+/+ and Cd36−/− mice, injured with FeCl3. In the vessels from Cd36−/− mice, thrombus formation was delayed and more unstable, that is, more embolization, in comparison with wild-type mice (Figure 5A and Movies in the online-only Data Supplement). This resulted in prolonged occlusion times in both the arterioles and venules of Cd36−/− mice (Figure 5B). These results are compatible with a role for CD36 in stabilizing the thrombus.

Figure 1. Thrombospondin-1 (TSP1) released from platelets during thrombus formation supports phosphatidylserine (PS) exposure. A and B, Blood from Tsp1+/+ or Tsp1−/− mice was perfused during 4 minutes over collagen at a wall shear rate of 1000/s. A, Representative brightfield and fluorescence images recorded poststaining (bar=25 μm). B, Quantification of covered area of all platelets (platelet deposition); labeling with PE-JON/A monoclonal antibody (mAb; αIIbβ3 activation); labeling with AF647-annexin A5 (PS exposure); and staining with biotin anti-TSP1 mAb followed by AF532-streptavidin (TSP1 stain). C and D, Tsp1+/+ or Tsp1−/− thrombi formed on collagen in the presence of ADP (10 μmol/L), staining with biotin anti-TSP1 mAb and AF532-streptavidin. C, Representative images (bar=25 μm). D, Quantification of covered area of TSP1 stain. Means±SEM. (n=3–4). *P<0.05 vs Tsp1+/+. FITC indicates fluorescein isothiocyanate; and n.s., not significant.

Figure 2. Platelet-derived thrombospondin-1 (TSP1) supports thrombus formation on collagen in a CD36-dependent way. Coverslips were coated with releasate (RL) from thrombin-stimulated Tsp1+/+ or Tsp1−/− platelets, collagen alone (coll), or collagen postincubated with RL, as indicated. Blood from Cd36+/+ or Cd36−/− mice was perfused during 4 minutes at 1000/s. A, Representative brightfield images of thrombi formed after flow (bar, 25 μm). B, Quantification of platelet surface area coverage. C, Morphometric analysis of images for mean thrombus size. Means±SEM. (n=4–6). *P<0.05, #P<0.1 vs Cd36+/+ control.
with our earlier finding that also in mesenteric vessels of Tsp1−/− mice thrombus formation is delayed and accompanied by embolization.23 Together, these data indicate that both CD36 and TSP1 play a role in thrombus stabilization in vivo.

Discussion

In this study, we hypothesized that platelet-secreted TSP1 by interacting with CD36 provides an additional autocrine feed-forward loop that enforces the interactions of platelets in a growing thrombus. The results of this article agree with a stimulatory effect of the TSP1–CD36 axis in thrombus formation and stabilization. Our data indicate that, similarly to purified human TSP1, the releasate from wild-type but not from Tsp1−/− platelets augments platelet adhesion and phosphatidylserine exposure in a CD36-dependent way, that is, detectable with wild-type but not Cd36−/− platelets. In addition, we find that only the releasate from wild-type platelets enhances collagen-induced thrombus formation, integrin activation, and phosphatidylserine exposure at high shear flow conditions. Furthermore, we find that stable thrombus formation is impaired in mice deficient in TSP1 or CD36 both in vitro and in vivo experiments. In vitro, we could establish that the impairment was not further increased by blockage of CD36.

Jointly, these results indicate that platelet-derived TSP1 and CD36 support adhesion and activation of platelets to phosphatidylserine exposure and thrombus stabilization. These are considered to be relevant findings because TSP1, although also present in the vessel wall, is accumulated within the blood in platelet α-granules.2,16,17 The TSP1 that is secreted from activated platelets thus may deposit on collagen or a growing thrombus and then act in an autocrine way to enforce the thrombus-forming process. Interestingly, the present findings with Cd36−/− and Tsp1−/− platelets are reminiscent—although with a less strong phenotype—of those published for Nbeal2−/− platelets. These platelets (lacking α-granules) show impaired adhesion, reduced phosphatidylserine exposure, and diminished thrombus formation accompanied by increased thrombus instability.27 Hence, TSP1 may be one of the α-granular proteins contributing to full and stable thrombus formation. This idea is supported by the fact that both TSP1 and CD36 are highly expressed in (mouse and human) platelets, thus allowing multiple possible interaction sites. Likely, also other α-granule–derived proteins than TSP1 will play a role in thrombus formation, but we like to note that TSP1—differently from von Willebrand factor, fibrinogen, and Gas6—is mostly stored in the blood in platelets and is present in plasma at only low concentrations.

The present results are in agreement with earlier data, showing that immobilized TSP1 activates human platelets via CD36 through a Syk kinase–dependent mechanism, resulting in increased Ca2+ signaling, activation of αIIbβ3, and exposure of phosphatidylserine.15 Also, in endothelial cells, a role for TSP1 in signaling via CD36 has been proposed.19 Others have...
provided evidence that TSP1 can promote platelet activation via the glycoprotein CD47. However, in our experiments, blocking CD47 did not influence the process of thrombus formation in the presence or absence of CD36. These experiments, however, do not rule out that TSP1 can act by binding to other platelet ligands in the extracellular space, for example, von Willebrand factor and fibrinogen. In the absence of TSP1, also other effects may occur, given the previously identified role of TSP1 in protecting von Willebrand factor from ADAMTS13 (a disintegrin and metalloproteinase with a thrombospondin type 1 motif, member 13) degradation. Similarly, in the absence of CD36, also the interaction with other ligands, such as oxLDL, will be prevented. In other words, although the present results clearly point to an overlap of the functions of CD36 and TSP1 in thrombus formation, they do not impose that these functions are identical.

Various authors have pointed to a role of either CD36 or TSP1, although in a different context and not focusing on the TSP1–CD36 axis. Platelet CD36, by acting as a scavenging receptor of oxLDL, was found to contribute to atherosclerotic lesion development and hyperlipidemia-associated enhanced platelet reactivity. The oxLDL–CD36 pathway is supposed to signal via the mitogen-activated protein kinases JNK, and p38 mitogen-activated protein kinase, and the tyrosine kinase Syk.

In earlier work, we demonstrated that continued inside-out signaling via ADP/ADP receptors and Gas6/Gas6 receptors, as well as PEAR1 (platelet endothelial aggregation receptor 1), contributes to perpetuated αIIbβ3 activation and maintenance of platelet–platelet interactions and thereby to stabilization of a formed thrombus. The present findings extend this concept by revealing the involvement of another autocrine axis in thrombus stabilization, namely the interaction of CD36 with platelet-derived TSP1.

Taken together, our results point to defined roles of murine CD36 and platelet-derived TSP1 in collagen-dependent thrombus formation under high shear flow conditions. Thus, TSP1 binding to platelet CD36 can be considered as another of the multiple receptor–ligand interactions required for the buildup of a stable thrombus.

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Disclosures

None.

References


Significance

Platelet thrombospondin-1 via CD36 supports platelet adhesion plus anchoring and stabilization of collagen-dependent thrombus formation under flow.
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Supplemental Materials and Methods

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Materials and Methods

Materials

H-Phe-Pro-Arg chloromethyl ketone (PPACK) and TSP1 from human platelets were obtained from Calbiochem (La Jolla CA, USA). Bovine serum albumin (BSA), ADP and thrombin and FeCl₃ were from Sigma-Aldrich (St. Luis MO, USA). Low molecular weight heparin (fragmin) was from Pfizer (Capelle a/d IJssel, The Netherlands). Glycoprotein VI agonist, convulxin, was purified to homogeneity from the crude venom of Crotalus durissus terrificus (Latoxan, Valence, France).¹ Fibrillar Horm type I collagen was from Nycomed Pharma (Munich, Germany). Annexin A5 (detecting surface-exposed phosphatidylserine) labeled with fluorescein isothiocyanate (FITC) was from PharmaTarget (Maastricht, the Netherlands). FITC-labeled anti-mouse CD62P mAb (detecting surface exposure of granular P-selectin) and PE-labeled JON/A mAb (detecting the activated conformation of mouse integrin αIIbβ3) were from Emfret Analytics (Würzburg, Germany); biotin-conjugated anti-mouse TSP1 mAb from Abcam (Cambridge, UK); blocking anti-mouse CD36 mAb (MAB1258, clone 63) from Millipore (Temecula, CA, USA); blocking antibody against mouse CD47 (clone miap301) from eBiosciences (San Diego, CA, USA). Annexin A5 labeled with Alexa fluor (AF)647 and streptavidin labeled with AF532 were from Molecular Probes (Eugene OR, USA). Rhodamine 6G was from Invitrogen (Grand Island, NY, USA). Other materials were from sources described before.²

Animals

Experiments were approved by the local Animal Experimental Committees. Mice homozygous in CD36 deficiency (Cd36⁻/⁻) were generated as described, and crossed back >6 times to a C57Bl/6 genetic background.³ Mice deficient in TSP1 (Tsp1⁻/⁻) also of C57Bl/6 background were kindly provided by dr. J. Lawler (Harvard Medical School, Boston MA, USA).⁴ Wild type mice (C57Bl/6) were used from the same sources. Blood counts of platelets and erythrocytes of knockout and wild type mice were in the normal ranges.

Blood collection and platelet preparation

Mice were anesthetized by subcutaneous injection of ketamine and xylazine (0.1 mg/g and 0.02 mg/g body weight), after which blood was obtained by retro-orbital puncture. For flow studies, blood was collected into 40 µmol/L PPACK, 5 U/mL heparin and 40 U/mL fragmin. For platelet preparation, blood was collected into acid citrate dextrose (ACD). Isolated platelets were washed and resuspended in modified Tyrode's Hepes buffer (5 mmol/L Hepes, 136 mmol/L NaCl, 2.7 mmol/L KCl, 0.42 mmol/L NaH₂PO₄, 2 mmol/L MgCl₂, 0.1% glucose and 0.1% BSA, pH 7.45).² Platelet count was determined with a Coulter counter.

To prepare platelet releasates, aliquots of suspended Tsp1⁺⁺ or Tsp1⁻⁻ platelets (1 × 10⁹/mL) were activated with thrombin (20 nmol/L) for 10 minutes, after which PPACK (200 nmol/L) was added to inactivate residual thrombin. Releasates were isolated by centrifuging for 1 minute at 2650 g, and were stored at -80 °C until
use.

Flow cytometry
Washed platelets (1 × 10^8/mL) in modified Tyrode’s buffer with 2 mmol/L CaCl$_2$ were activated with ADP (0.5-5 μmol/L) or convulxin (20-50 ng/mL) without stirring. After 10 minutes, activation of integrin α$_{IIb}$β$_3$, α-granule secretion, and phosphatidylserine exposure were detected with PE-labeled JON/A mAb (150 μg/mL), FITC-labeled anti-CD62P mAb (1:40) and AF647-labeled annexin A5 (1 μg/mL), respectively. Fluorescence was measured with a BD Accuri C6 flow cytometer (San Jose, CA, USA).

Static platelet adhesion and activation
Round glass coverslips were coated with purified TSP1 (3 μL, 100 μg/mL) or releasate from activated platelets (3 μL, ~3 × 10^6 platelets), and blocked with 1% BSA in modified Tyrode’s buffer. Coverslips were mounted in an open chamber and incubated with platelets in buffer containing 2 mM CaCl$_2$ (1.5 × 10^8/mL). Platelet adhesion was determined by phase-contrast microscopy, and exposure of phosphatidylserine by labeling with FITC-annexin A5 and fluorescence microscopy.

Thrombus formation under flow
Rectangular coverslips were coated with fibrillar type I collagen (3 μL, 50 μg/mL), and blocked with 1% BSA in modified Tyrode’s buffer. Where indicated, coated coverslips were post-incubated with releasate from activated platelets (3 μL, ~3 × 10^6 platelets). After mounting into a transparent parallel-plate flow chamber (depth 50 μm, width 3 mm), coverslips were perfused with PPACK/heparin-anticoagulated mouse blood for 4 minutes at a wall shear rate of 1000 s$^{-1}$. Using confocal microscopy, surface expression of activation markers on adhered platelets was measured by staining with PE-labeled JON/A mAb (probing activated integrin α$_{IIb}$β$_3$) and AF647-annexin A5 (probing phosphatidylserine exposure). Using Metamorph software (Molecular Devices, Sunnyvale CA, USA), brightfield and fluorescence images were analyzed for surface area coverage, mean thrombus size, or fluorescence intensity. Where indicated, images were recorded with an EVOS inverted digital fluorescence microscope (AMG, Bothell WA, USA).

Immunostaining of thrombi
Coverslips coated with collagen and/or platelet releasates, before or after thrombus formation, were stained with biotin-conjugated anti-murine TSP1 mAb (4 μg/mL) for 15 minutes. After rinse with modified Tyrode’s buffer, the coverslips were post-stained with AF532-streptavidine (1:200) for 15 minutes. Control stainings were without primary mAb. No fixation or permeabilization was performed. Fluorescence images were recorded after a second rinse using an LSM7 Live confocal microscope (Zeiss, Jena, Germany).

In vivo thrombosis in mesenteric vessels
Mice were anesthetized by sodium pentobarbital (60 mg/kg, i.p.) and thrombus formation in the mesenteric vessels was provoked, as described previously. Briefly, Rhodamine 6G was injected i.v. (3.3 mg/kg) for fluorescent labeling of circulating cells. After opening of the abdomen, mesenteric vessels were exposed on the table of an inverted epifluorescent microscope with 20x objective (Zeiss Observer, Jena, Germany), coupled to Axiovision software (Zeiss). A standardized vascular injury was induced by placement of a filter paper soaked with 5% FeCl$_3$ for 5 minutes. Platelet
deposition, thrombus formation and embolization in arterioles and venules were monitored by microscopic fluorescence imaging in real-time until complete occlusion occurred. Time to occlusion was defined as the arrest of blood flow for at least 1 minute. Per animal, 2 measurements were performed. When occlusion had not occurred for 60 minutes, the experiment was terminated and occlusion time was determined as 60 minutes.

Statistical analysis
Significance of differences was determined with a parametric t-test or a non-parametric Mann-Whitney U test, as appropriate. The statistical package for social sciences was used (SPSS version 17, Chicago IL, USA).

References
Supplemental Material

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Supplemental Figures

Supplemental Figure I. Roles of murine CD36 in platelet adhesion to TSP1 and in agonist-induced platelet activation. A-B, Adhesion of washed platelets from Cd36+/+ or Cd36−/− mice (1.5×10^8/mL) to surfaces coated with purified TSP1 or with releasate from activated wild type platelets. Platelet adhesion and activation were measured by phase-contrast and fluorescence microscopy after 45 minutes. A, quantification of adhered platelets (platelets/mm^2). B, quantification of FITC-annexin A5 positive platelets (% total). C-E, Washed platelets from Cd36+/+ and Cd36−/− mice were stimulated with ADP (0.5-5 µmol/L) or convulxin (20-50 ng/mL) during 10 minutes, and analyzed by flow cytometry. C, Activation of αIIbβ3 detected with PE-labeled JON/A mAb, D, expression of P-Selectin detected with FITC-anti-CD62P mAb, and E, expression of phosphatidylserine detected with FITC-annexin A5. Means ± S.E.M. (n=4-5). *P<0.05 vs Cd36+/+; n.s., not significant.

Supplemental Figure II. Detection of immobilized TSP1 in platelet releasate. Collagen-containing coverslips were post-incubated with releasate (RL) from thrombin-stimulated Tsp1+/+ or Tsp1−/− platelets, as indicated. Surfaces were stained with biotin-conjugated anti-TSP1 antibody and AF532-streptavidin. A, Representative images of differential interference contrast (DIC) and confocal AF532-streptavidin fluorescence (AF532); bars, 25 µm. B, Integrated fluorescence intensity from coated surfaces (arbitrary units). Strep indicates control staining with only AF532-streptavidin; TSP1 indicates staining with anti-TSP1 antibody and AF532-streptavidin. Means ± SEM (n=3).

Supplemental Fig. III: Absence of phosphatidylserine-exposing microparticles in coated releasate from Tsp1+/+ or Tsp1−/− platelets. Coverslips were coated with 3 µL releasate from Tsp1+/+ or Tsp1−/− platelets, as in Suppl. Fig. II. Surfaces then were blocked with 1% BSA, and stained with 0.5 µg/mL FITC-annexin A5 for 5 minutes. After rinse with Tyrode's Hepes buffer, phase contrast and fluorescence images were captured from the coated surface. Image size is 107 x 142 µm.

Supplemental movies of in vitro thrombus formation. Blood from wildtype (Wildtype.avi), Tsp1−/− (TSP1.avi) or Cd36−/− (CD36.avi) mice perfused over collagen during 4 minutes at 1000 s⁻¹. Representative phase-contrast movies of thrombus dissolution during post-perfusion for 6 minutes. Movies were created at 5 images/second (50× real time). Image sizes, 142 x 107 µm.
Supplemental movies of in vivo thrombus formation. Thrombus formation was induced in mesenteric arterioles of $Cd36^{+/+}$ mice (Wildtype in vivo.avi) and $Cd36^{-/-}$ mice (CD36 in vivo.avi) by 5% FeCl$_3$ injury (5 minutes). Circulating cells were fluorescently labeled with Rhodamine 6G. Note difference in stability of the wildtype and CD36-deficient thrombi. Movies were created at 6 images/second (3x real-time).
Supplemental Figure II
Supplemental Figure III

Phasecontrast

BSA  $Tsp1^{+/+}$ releasate  $Tsp1^{-/-}$ releasate

FITC-Annexin A5

$Supplemental Figure III$