

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

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

Marijke J.E. Kuijpers, Susanne de Witt,\* Reyhan Nergiz-Unal,\* Roger van Kruchten, Suzanne J.A. Korporaal, Peter Verhamme, Maria Febbraio, Marc Tjwa, Peter J. Voshol, Marc F. Hoylaerts, Judith M.E.M. Cosemans, Johan W.M. Heemskerk

**Objective**—Platelets abundantly express the membrane receptor CD36 and store its ligand thrombospondin-1 (TSP1) in the  $\alpha$ -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 phosphatidylserine 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, phosphatidylserine 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  $\leq 25000$  copies per cell.<sup>1,2</sup> CD36 consists of a double membrane-spanning protein with 1 large extracellular domain and 2 short N- and C-terminal cytoplasmic domains.<sup>3–5</sup> Its function has remained unclear for long. Earlier, CD36 was thought to be a platelet collagen receptor,<sup>6,7</sup> but it was shown that platelets from CD36-deficient patients have an unchanged response to collagen.<sup>8,9</sup> Subsequent studies suggested a role for CD36 as receptor for thrombospondin-1 (TSP1).<sup>10,11</sup> It was also found that CD36 can bind oxidized lipids, including oxidized low-density lipoproteins (oxLDLs), particularly at conditions promoting atherogenesis.<sup>12,13</sup> 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 P2Y<sub>12</sub> receptors.<sup>14,15</sup> Interestingly,

this signaling promotes Ca<sup>2+</sup>-dependent exposure of procoagulant phosphatidylserine 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  $\alpha$ -granules ( $\approx 101000$  copies per cell).<sup>2</sup> TSP1 is also found in the blood vessel, likely after deposition secreted by platelets and vascular cells.<sup>16–18</sup> In addition to CD36, several other platelet receptors for TSP1 have been proposed. These include the glycoprotein Ib-V-IX complex<sup>19–21</sup> and glycoprotein CD47.<sup>22</sup> 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.<sup>23</sup>

Lack of platelet  $\alpha$ -granules is associated with a mild-to-moderate bleeding tendency in patients with the congenital gray platelet syndrome.<sup>24</sup> The genetic defect has recently been attributed to mutations in the neurobeachin-like 2 (*NBEAL2*)

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Nonstandard Abbreviations and Acronyms	
oxLDL	oxidized low-density lipoprotein
TSP1	thrombospondin-1

gene.<sup>25,26</sup> In mice, deficiency in *Nbeal2* similarly results in defective  $\alpha$ -granule biogenesis, accompanied by diminished platelet adhesion, phosphatidylserine exposure, and thrombus formation.<sup>27</sup> To date, it has remained unclear which of the proteins stored in platelet  $\alpha$ -granules contribute to these responses.

In the present article, we hypothesized that the abundantly expressed TSP1, secreted from the  $\alpha$ -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.<sup>15</sup> To assess this for the mouse system, we measured the adhesion of platelets from *Cd36<sup>+/+</sup>* and *Cd36<sup>-/-</sup>* 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<sup>-/-</sup>* 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<sup>-/-</sup>* platelets failed to stain (Figure IIA and IIB in the online-only Data Supplement). We determined whether phosphatidylserine-exposing microparticles as possible CD36 ligands<sup>28</sup> 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<sup>-/-</sup>* mice, we measured integrin  $\alpha_{\text{IIb}}\beta_3$  activation,  $\alpha$ -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.<sup>15</sup> Similarly, other authors have described that CD36-deficient mouse platelets in suspension are unchanged in activation properties.<sup>12,29</sup>

Considering that CD36 mediates platelet adhesion to immobilized TSP1, we investigated whether platelet-released TSP1

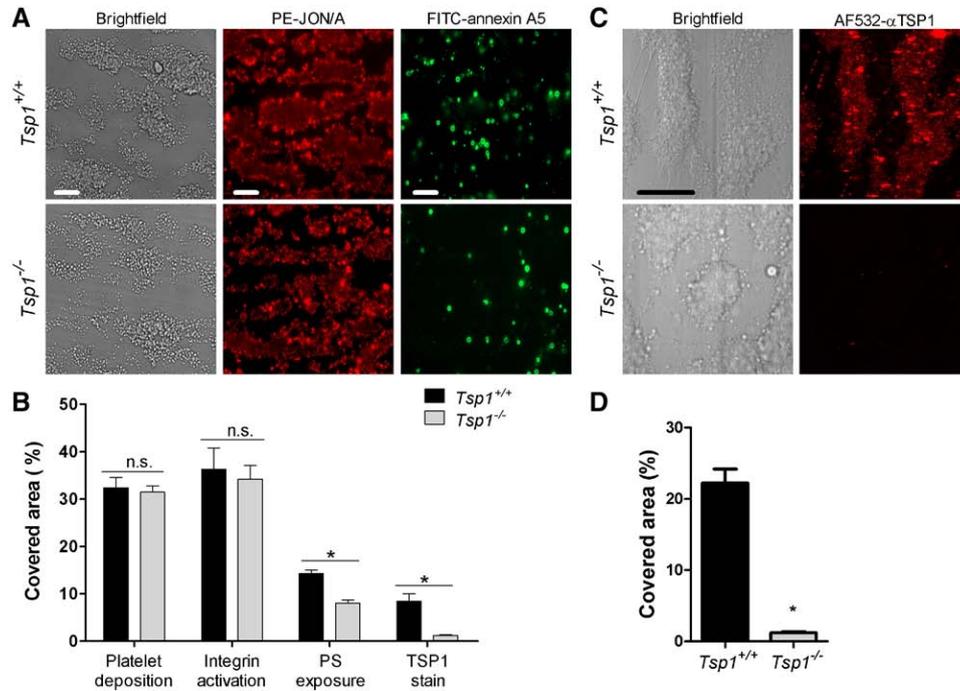
contributes to thrombus formation under flow conditions. Whole blood from *Tsp1<sup>-/-</sup>* or corresponding wild-type mice was perfused over collagen at high wall shear rate. Thrombi formed with *Tsp1<sup>-/-</sup>* and *Tsp1<sup>+/+</sup>* blood differed neither in deposition of platelets (surface area coverage) nor in integrin  $\alpha_{\text{IIb}}\beta_3$  activation (staining with PE-JON/A monoclonal antibody; Figure 1A and 1B). However, thrombi formed with *Tsp1<sup>-/-</sup>* blood were significantly reduced in platelet phosphatidylserine exposure. Immunostaining indicated the presence of released TSP1 in wild-type thrombi, but not in *Tsp1<sup>-/-</sup>* 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<sup>+/+</sup>* thrombi was observed, but not of the *Tsp1<sup>-/-</sup>* thrombi.

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

Staining for integrin  $\alpha_{\text{IIb}}\beta_3$  activation did not show marked differences between *Cd36<sup>-/-</sup>* and *Cd36<sup>+/+</sup>* thrombi formed on collagen alone (Figure 3A and 3B). However,  $\alpha_{\text{IIb}}\beta_3$  activation and phosphatidylserine exposure were significantly increased in *Cd36<sup>+/+</sup>* thrombi ( $P=0.005$  and  $P=0.027$ , respectively), when these were formed on a surface with collagen plus releasate from *Tsp1<sup>+/+</sup>* (but not *Tsp1<sup>-/-</sup>*) platelets (Figure 3A–3D). Notably, the stimulating effect of *Tsp1<sup>+/+</sup>* releasate was absent in *Cd36<sup>-/-</sup>* 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,<sup>30,31</sup> we investigated the stability of thrombi formed with *Cd36<sup>-/-</sup>*, *Tsp1<sup>-/-</sup>*, or wild-type blood. This was done by measuring the rate of dissolution of preformed thrombi during a high shear postperfusion protocol.<sup>30</sup> 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<sup>-/-</sup>* and *Tsp1<sup>-/-</sup>* platelets in comparison with wild type (Figure 4B). Control experiments pointed out that in *Tsp1<sup>-/-</sup>* 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<sup>+/+</sup>* or *Cd36<sup>-/-</sup>* 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.

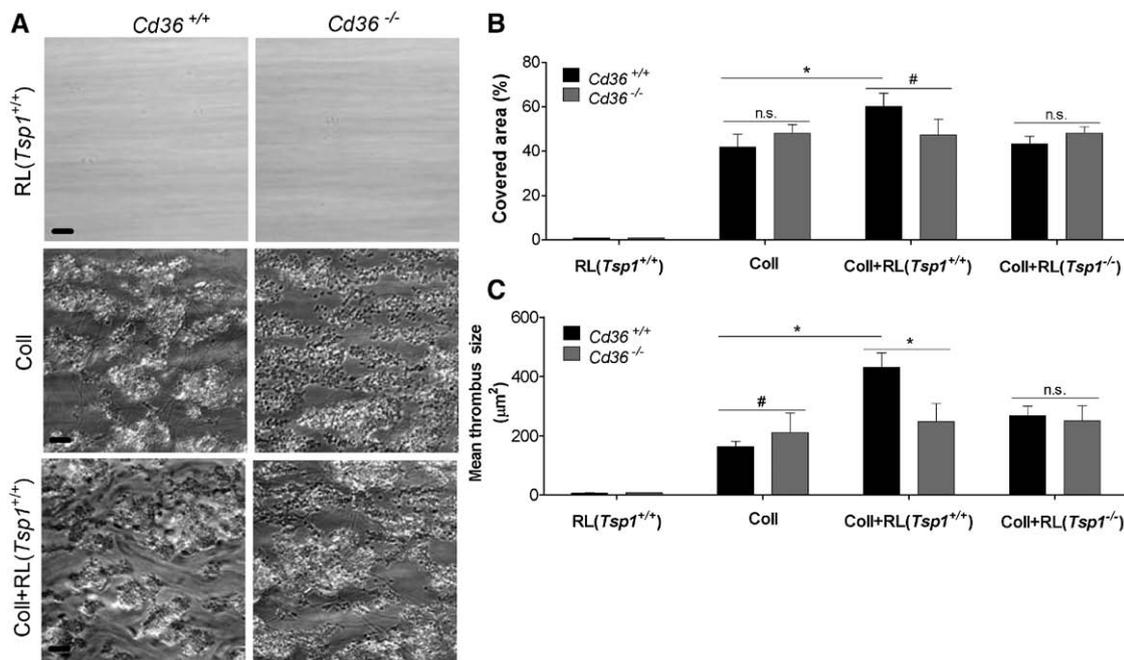
To investigate this under in vivo conditions, we measured the thrombotic process in mesenteric arterioles and venules from



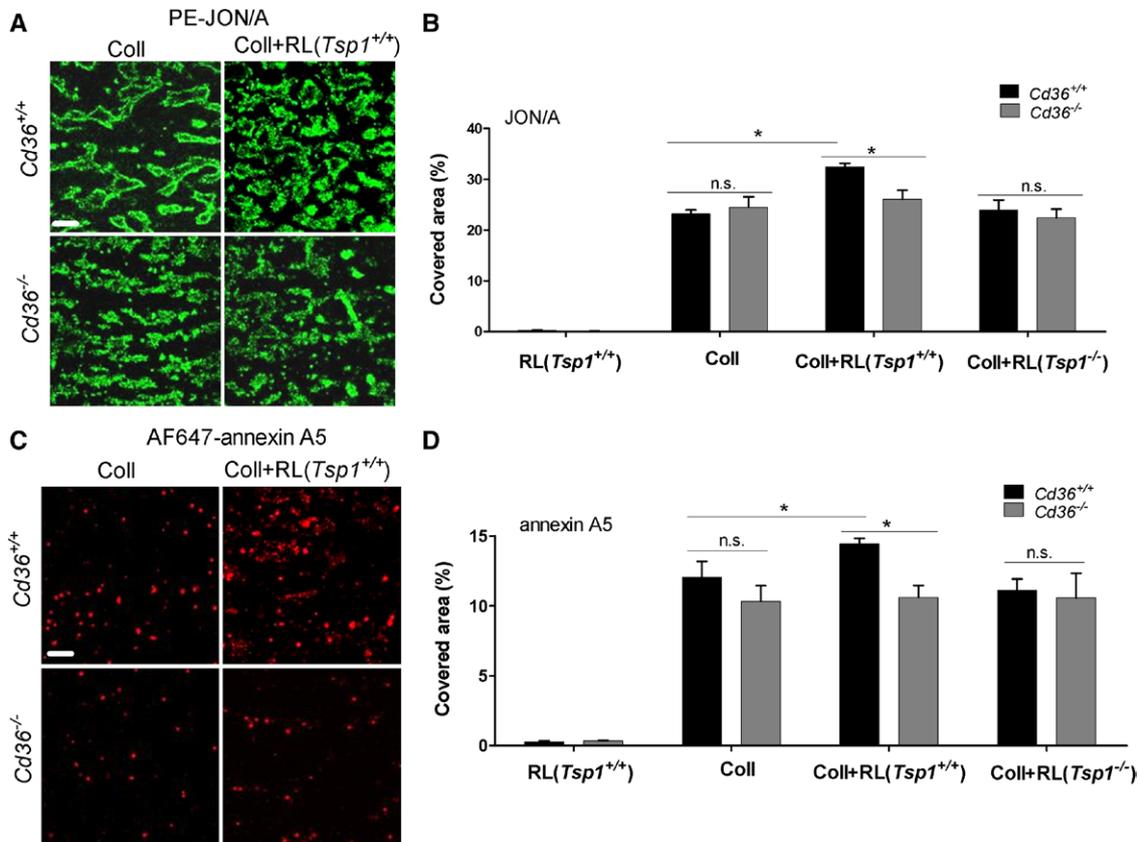
**Figure 1.** Thrombospondin-1 (TSP1) released from platelets during thrombus formation supports phosphatidylserine (PS) exposure. **A** and **B**, Blood from *Tsp1*<sup>+/+</sup> or *Tsp1*<sup>-/-</sup> mice was perfused during 4 minutes over collagen at a wall shear rate of 1000/s. **A**, Representative 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; α<sub>IIb</sub>β<sub>3</sub> 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*<sup>+/+</sup> or *Tsp1*<sup>-/-</sup> 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*<sup>+/+</sup>. FITC indicates fluorescein isothiocyanate; and n.s., not significant.

*Cd36*<sup>+/+</sup> and *Cd36*<sup>-/-</sup> mice, injured with FeCl<sub>3</sub>. In the vessels from *Cd36*<sup>-/-</sup> 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*<sup>-/-</sup> mice (Figure 5B). These results are compatible



**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*<sup>+/+</sup> or *Tsp1*<sup>-/-</sup> platelets, collagen alone (coll), or collagen postincubated with RL, as indicated. Blood from *Cd36*<sup>+/+</sup> or *Cd36*<sup>-/-</sup> 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*<sup>+/+</sup> control.



**Figure 3.** Platelet-derived thrombospondin-1 (TSP1) enhances phosphatidylserine exposure in thrombus formation in the presence of CD36. Blood from *Cd36*<sup>+/+</sup> or *Cd36*<sup>-/-</sup> mice was perfused during 4 minutes at 1000/s over a surface of releasate (RL) from *Tsp1*<sup>+/+</sup> platelets, over collagen alone (coll), or collagen postincubated with RL from *Tsp1*<sup>+/+</sup> or *Tsp1*<sup>-/-</sup> platelets, as indicated. **A** and **C**, Representative fluorescence images (bar, 25  $\mu$ m) of thrombi stained for activated  $\alpha_{IIb}\beta_3$  (PE-JON/A monoclonal antibody) and phosphatidylserine exposure (FITC-annexin A5). **B** and **D**, Quantification of fluorescence area covered by thrombi. Means $\pm$ SEM. (n=6–7); \**P*<0.05 vs *Cd36*<sup>+/+</sup> control.

with our earlier finding that also in mesenteric vessels of *Tsp1*<sup>-/-</sup> mice thrombus formation is delayed and accompanied by embolization.<sup>23</sup> Together, these data indicate that both CD36 and TSP1 play a role in thrombus stabilization in vivo.

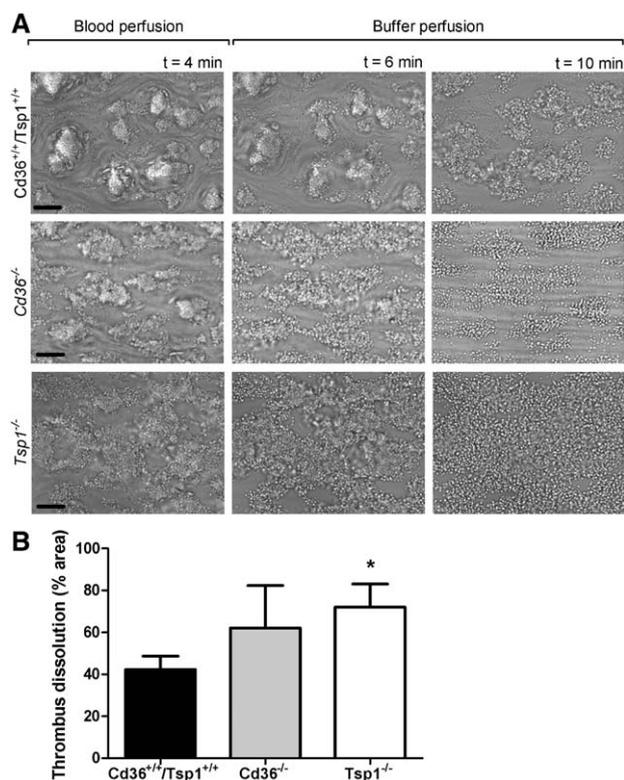
### Discussion

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*<sup>-/-</sup> platelets augments platelet adhesion and phosphatidylserine exposure in a CD36-dependent way, that is, detectable with wild-type but not *Cd36*<sup>-/-</sup> 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 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  $\alpha$ -granules.<sup>2,16,17</sup> 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*<sup>-/-</sup> and *Tsp1*<sup>-/-</sup> platelets are reminiscent—although with a less strong phenotype—of those published for *Nbeal2*<sup>-/-</sup> platelets. These platelets (lacking  $\alpha$ -granules) show impaired adhesion, reduced phosphatidylserine exposure, and diminished thrombus formation accompanied by increased thrombus instability.<sup>27</sup> Hence, TSP1 may be one of the  $\alpha$ -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  $\alpha$ -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.

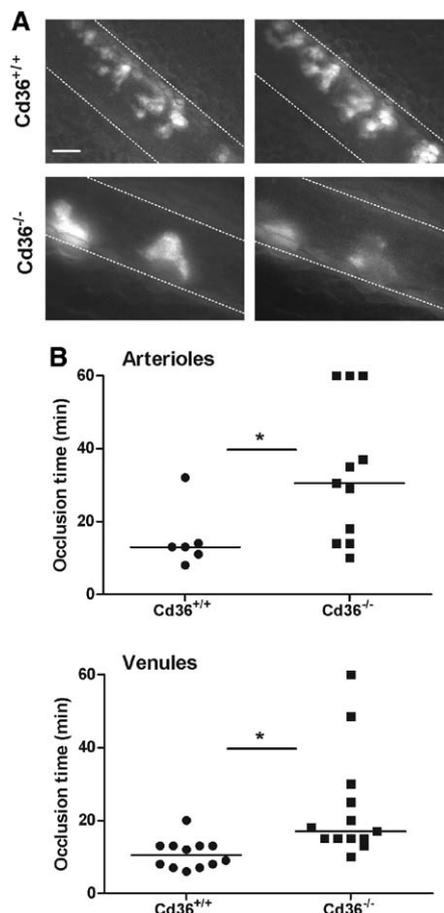
The present results are in agreement with earlier data, showing that immobilized TSP1 activates human platelets via CD36 through a Syk kinase-dependent mechanism, resulting in increased Ca<sup>2+</sup> signaling, activation of  $\alpha_{IIb}\beta_3$ , and exposure of phosphatidylserine.<sup>15</sup> Also, in endothelial cells, a role for TSP1 in signaling via CD36 has been proposed.<sup>32</sup> Others have



**Figure 4.** Platelet CD36 and thrombospondin-1 (TSP1) support thrombus stabilization on collagen. Thrombi were formed by 4 minutes perfusion over collagen at 1000/s, using blood from *Cd36*<sup>-/-</sup>, *Tsp1*<sup>-/-</sup>, or corresponding wild-type mice. Stability of thrombi was evaluated during 6 minutes of postperfusion with modified Tyrode buffer, containing 2 mmol/L CaCl<sub>2</sub> and 1 U/mL heparin. **A**, Representative phase-contrast images taken during postperfusion (bar, 25 μm). **B**, Dissolution of thrombi during postperfusion calculated by subtraction analysis. Representative data of ≥4 experiments. See also Movies in the online-only Data Supplement.

provided evidence that TSP1 can promote platelet activation via the glycoprotein CD47.<sup>22</sup> 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.<sup>23</sup> 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<sup>33</sup> and hyperlipidemia-associated enhanced platelet reactivity.<sup>12</sup> The oxLDL–CD36 pathway is supposed to signal via the mitogen-activated protein kinases JNK,<sup>14</sup> and p38 mitogen-activated protein kinase,<sup>13</sup> and the tyrosine kinase Syk.<sup>15</sup>



**Figure 5.** Platelet CD36 supports formation of stable thrombi in vivo. Thrombus formation was induced in mesenteric vessels from *Cd36*<sup>+/+</sup> and *Cd36*<sup>-/-</sup> mice by FeCl<sub>3</sub> injury. Animals were preinjected with rhodamine 6G to fluorescently label circulating cells. **A**, Representative images showing thrombus instability and embolization in injured arterioles from *Cd36*<sup>-/-</sup> mice. Dotted lines indicate vessel walls; right image is 50 seconds later than left image (bar, 100 μm). See also Movies in the online-only Data Supplement. **B**, Distribution plots of occlusion times in arterioles (**top**) and venules (**bottom**) after injury. Horizontal lines represent median values (\**P*<0.05).

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 α<sub>IIb</sub>β<sub>3</sub> activation and maintenance of platelet–platelet interactions and thereby to stabilization of a formed thrombus.<sup>30,31,34</sup> 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.

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## Significance

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