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Thrombospondin-1 promotes haemostasis through modulation of cAMP signaling in blood platelets.

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Key points:

- Thrombospondin-1 (TSP-1)-deficient platelets show increased bleeding times and diminished thrombosis in vivo.
- Platelet-derived TSP-1 corrects haemostasis and is associated with CD36-dependent platelet PGI_2 hyposensitivity and diminished cAMP signaling.

ABSTRACT

Thrombospondin-1 (TSP-1) is released by platelets upon activation and can promote platelet activation, but its role in haemostasis *in vivo* is unclear. We show that TSP-1 is a critical mediator of haemostasis that promotes platelet activation by modulating inhibitory cAMP signaling. Genetic deletion of TSP-1 did not affect platelet activation *in vitro*, but *in vivo* models of haemostasis and thrombosis demonstrated that TSP-1 deficient mice had prolonged bleeding, defective thrombosis and increased sensitivity to the prostacyclin mimetic iloprost. Adoptive transfer of wild type (WT), but not TSP-$1^{-/-}$ platelets, ameliorated the thrombotic phenotype, suggesting a key role for platelet-derived TSP-1. In functional assays, TSP-1-deficient platelets showed an increased sensitivity to cAMP signaling, inhibition of platelet aggregation and arrest under flow by PGI_2. Plasma swap experiments showed that plasma TSP-1 did not correct PGI_2 hypersensitivity in TSP-$1^{-/-}$ platelets. By contrast, incubation of TSP-$1^{-/-}$ platelets with releasates from WT platelets or purified TSP-1, but not releasates from TSP-$1^{-/-}$ platelets, reduced the inhibitory effects of PGI_2. Activation of WT platelets resulted in diminished cAMP accumulation and downstream signaling, which was associated with increased activity of the cAMP hydrolyzing enzyme phosphodiesterase 3A (PDE3A). PDE3A activity and cAMP accumulation were unaffected in platelets from TSP-$1^{-/-}$ mice. Platelets deficient in CD36, a TSP-1 receptor, showed increased sensitivity to PGI_2/cAMP signaling and diminished PDE3A activity, which was unaffected by platelet-derived or purified TSP-1. This suggests that the release of TSP-1 regulates haemostasis *in vivo* through modulation of platelet cAMP signaling at sites of vascular injury.

INTRODUCTION:

The controlled activation of blood platelets at sites of vascular damage is essential for haemostasis. Vascular injury exposes platelets to prothrombotic extracellular matrix (ECM) proteins von Willebrand factor (vWF) and collagen, which stimulate their transition from a quiescent to an activated state. To moderate excessive activation and return platelets to their quiescent state after transient activation, the endothelium releases prostacyclin (PGI_2), which inhibits platelets through a cAMP-dependent signaling cascade[1]. This complex signaling system involves enzymes that generate, propagate and terminate cAMP signaling. PGI_2 activates membrane adenylyl cyclases (ACs) through $G\alpha_s$-coupled receptors (GPCRs) to increase cAMP levels[2]. Elevations in cAMP result in the activation of protein kinase A (PKA) isoforms and the subsequent phosphorylation of protein substrates, which underpin the ability of cAMP signaling to control multiple aspects of platelet function[3]. Increased platelet cAMP is associated with reductions in Ca^{2+} mobilisation, dense granule secretion, integrin $\alpha_{IIb}\beta_3$ activation and aggregation *in vitro*[4] as well as reduced platelet accrual at sites of vascular injury *in vivo*[5].

The marginalisation of platelets during blood flow facilitates their continual exposure to PGI_2 throughout the circulation, ensuring they remain haemostatically inactive. At sites of vascular injury, platelets must overcome the inhibitory effects of PGI_2 to ensure rapid haemostasis. This is achieved through the direct inhibition of cAMP generation by platelet-derived adenosine diphosphate (ADP) binding to Gαi-coupled P2Y12 receptors to inhibit AC activity[6]. Thrombin- and collagen-mediated cAMP hydrolysis may also contribute.[7] The reduction in cAMP synthesis in platelets removes the tonic inhibition of cAMP signaling, thereby promoting platelet activation and haemostasis.

TSP-1 is a homotrimeric multidomain glycoprotein present in the ECM, plasma and platelet α-granules.[8,9,10] The amount of TSP-1 in platelets is estimated to be 0.5µg/10^8 platelets, making it one of the most abundant granule proteins[11]. Under physiological conditions, TSP-1 plasma levels vary between 50-450ng/mL, but its release from platelets can increase plasma concentrations by 10-fold.[10,12,13] TSP-1 binds to platelet GPIV (CD36) with high affinity,[14] but it does not cause platelet activation.[15] Rather, it may have a critical role in the regulation of platelet-endothelium crosstalk. Under flow, TSP-1 facilitates platelet-endothelial cell interactions and supports platelet adhesion in vitro in CD36- and CD47-dependent manners.[16] Interestingly, while TSP-1-deficient platelets aggregate normally,[17] elegant studies using TSP-$1^{-/-}$ and $vWF^{-/-}$ animals show that TSP-1 contributes to vWF-dependent thrombus formation.[18] A second area of TSP-1 biology concerns its ability to influence cyclic nucleotide signaling. Elegant studies Isenberg and colleagues showed that TSP-1 modulated platelet sensitivity to the NO-cGMP inhibitory pathway and increased sensitivity to platelet agonists.[19] Our previous work showed that exogenous TSP-1 could modulate cAMP signaling *in vitro*. Building on these observations, we present evidence that *in vivo* platelet-derived TSP-1 promotes haemostasis and regulates thrombosis by reducing platelet sensitivity to PGI_2.

MATERIALS and METHODS

Reagents

Experimental animals

CD36$^{-/-}$ mice (from Prof. Maria Febbraio, University of Alberta, Canada), TSP-1$^{-/-}$ mice (from The Jackson Laboratory, Bar Harbor, ME), and wild-type (WT) littermates were all on C57BL/6 backgrounds. All procedures were approved by the UK Home Office under the Animals (Scientific Procedures) Act 1986.

Platelet aggregation, flow assays, flow cytometry, intravital microscopy, immunoprecipitation, immunoblotting, PDE activity assay and cAMP measurement.

Detailed protocols are described in the supplemental methods.

Statistics.

Results are expressed as means ± SEM, unless otherwise stated, and statistical analyses were performed on GraphPad Prism 6.0 (La Jolla, CA). Comparisons between groups were performed by an unpaired, non-parametric Mann-Whitney U test. Statistical significance was accepted at $P<0.05$.

RESULTS

TSP-1- deficient mice display haemostatic defects.

We examined the role of platelet-derived TSP-1 in haemostasis and thrombosis using mice deficient in TSP-1 **(Figure 1A)**. In tail bleeding experiments, an indicator of haemostatic capacity, bleeding times for TSP-1$^{-/-}$ mice were significantly increased when compared with WT mice *(257.3±36 s vs 172.5±14 s, P<0.02)* (**Figure 1B**). Arterial thrombosis in carotid arteries of TSP-1-deficient mice induced by $FeCl_3$ injury was delayed, reduced *(mean peak thrombus size at 30 min: WT 5586±1407 vs TSP-1$^{-/-}$ 2580±1030 px, P=0.01)* **(Figure 1C)** and less stable **(Suppl. Video 1)**. Immunoblotting of WT plasma post-injury showed elevated TSP-1 levels in the plasma compared to a non-injured carotid artery **(Figure 1D)**, indicating that platelet activation and thrombosis are associated with TSP-1 secretion. Platelet α-granules are a potential major source of TSP-1 in the vasculature, although leukocytes and endothelial cells could also contribute[20,21]. To examine whether platelet-derived TSP-1 was required for thrombosis, we performed an adoptive transfer of WT or TSP-1$^{-/-}$ platelets into TSP-1$^{-/-}$ recipient mice. The infusion of WT platelets led to a partial correction of $FeCl_3$-induced thrombosis, with peak thrombus size increased *(mean peak thrombus size at 30 min, 2032±327 vs 3102±342 p, P<0.02)*. In contrast, thrombosis in mice that received an infusion of TSP-1$^{-/-}$ platelets was similar to that observed inTSP-1$^{-/-}$ mice that received no infusion (*TSP-1$^{-/-}$ 2032±327 vs TSP-1$^{-/-}$ /TSP-1$^{-/-}$ 1910±220 px, P=0.08)*. **(Figure 1E)**. This data suggests that the absence of platelet-derived TSP-1 accounts for increased bleeding time and diminished thrombosis in TSP-1-deficient mice.

TSP-1 deficiency leads to platelet hypersensitivity to PGI_2.

Thrombosis involves the rapid accrual of platelets at the site of injury, along with activation of coagulation. *In vitro* studies demonstrated that platelet aggregation **(Figure 2A)**, integrin $\alpha_{IIb}\beta_3$ activation (Jon/A binding) **(Figure 2B)** and α-granule secretion [TREM-like transcript 1 expression (TLT-1)] **(Figure 2C)**[22] in response to thrombin or collagen (CRP-XL for flow cytometry) was equivalent in both strains of mice. Consistent with the flow cytometry results, we found that the absence of TSP-1 did not affect dense granule secretion **(Suppl. Figure 1)**. Analysis of TSP-1 deficient platelets revealed that receptor expression levels of GPIb, GPVI, $\alpha_{IIb}\beta_3$, and CD36 **(Suppl. Figure 2)** were comparable to WT platelets. Consistent with previous studies, TSP-1$^{-/-}$ platelet α-granule contents, assessed by immunoblotting of vWF and fibrinogen, were comparable with those in WT **(Suppl. Figure 3).**[17] Similarly, immunostaining showed that vWF release from adherent WT and TSP-1$^{-/-}$ platelets was similar **(Suppl. Figure 4)**. We measured thrombin generation to determine if the observed phenotype was related to defective secondary haemostasis. Tissue factor-induced thrombin generation was similar in WT and TSP-1$^{-/-}$ plasma (AFU; 50825±15216 WT vs 59017±11874 TSP-1$^{-/-}$, P=0.17) **(Suppl. Figure 5)**. Maximal thrombin generation and time to reach maximal generation were also comparable in WT and TSP-1$^{-/-}$ mice (not shown).

We have previously shown that exogenous TSP-1 promotes activation of human platelets by inhibiting cAMP signalling.[15] To explore whether this contributed to our observations *in vivo*, we evaluated the effect of PGI_2 on platelet aggregation in the presence of apyrase to prevent secreted ADP affecting cAMP signaling **(Suppl. Figure 6)**.[23] TSP-1 secretion from α-granules was not impaired by apyrase **(Suppl. Figure 7)**. PGI_2 (0-100 nM) caused a concentration-dependent inhibition of collagen-induced aggregation in both WT and TSP-1$^{-/-}$ mice. However, TSP-1$^{-/-}$ platelets showed increased sensitivity to PGI_2 with a significantly lower EC_{50} (10.4±1 vs 28.7 ±2.4nM, P<0.001) **(Figure 2Ci)**. Pretreatment of platelet-rich plasma with PGI_2 (10 nM) caused a significant reduction in the number of platelets expressing activated $\alpha_{IIb}\beta_3$ integrin and TLT-1 in response to CRP-XL in both strains **(Figure 2D and E)**. Inhibition was greater in TSP-1$^{-/-}$ platelets than WT under the same conditions for integrin activation *(62.5±10 WT* vs *35.5±8.4 TSP-1$^{-/-}$, P=0.05)* and TLT-1 expression (75.5±5 WT vs 50.4±7 TSP-1$^{-/-}$, P=0.018). Similar data were obtained when thrombin was used to

stimulate platelet activation **(Suppl. Figure 8)**. TSP-1$^{-/-}$ platelets are therefore hypersensitive to PGI_2, resulting in altered expression of $\alpha_{IIb}\beta_3$ and TLT-1 following activation.

Regulation of PGI_2 signaling requires the release of TSP-1 from α-granules.

To better understand the relative contribution of platelet-derived and plasma TSP-1 to the platelet response to PGI_2, we performed plasma-swap experiments. PGI_2-induced inhibition of aggregation of WT platelets resuspended in plasma from either WT or TSP-1$^{-/-}$ mice was indistinguishable **(Figure 3A).** However, inhibition by PGI_2 of TSP-1$^{-/-}$ platelets resuspended in TSP-1$^{-/-}$ plasma was greater than WT platelets in WT plasma (61±4 vs 40.3%; P<0.05). To assess the ability of TSP-1$^{-/-}$ platelets to respond to PGI_2 in TSP-1-deficient plasma we performed reciprocal experiments. Inhibition by PGI_2 of TSP-1$^{-/-}$ platelets resuspended in TSP-1$^{-/-}$ plasma was indistinguishable from TSP1$^{-/-}$ platelets that were resuspended in WT plasma (40±2.8 vs 38±5.7%) **(Figure 3A)**.

To substantiate the role of platelet-derived TSP-1, we treated TSP-1$^{-/-}$ platelets with releasates from either WT or TSP-1$^{-/-}$ platelets. WT releasates, containing TSP-1 **(Suppl. Figure 7),** reduced platelet sensitivity to PGI_2 as aggregation increased from 17.3±2.6 to 41±4.5% (P<0.01) **(Figure 3B)**. In contrast, TSP-1$^{-/-}$ releasates did not affect PGI_2-induced platelet inhibition **(Figure 3B),** indicating that the loss of TSP-1 (and not other released factors) was likely responsible for our observations. TSP-1 released from platelets binds rapidly (within 1 min) to platelet surface receptors[24], but importantly treatment of TSP-1$^{-/-}$ platelets with WT releasate neither initiates nor potentiates platelet aggregation in the absence of PGI_2 **(Figure 3C)**. When TSP-1$^{-/-}$ platelets, adhered to collagen, were treated with WT releasate, we found that TSP-1 from the releasate could bind to the surface of TSP-1$^{-/-}$ platelets **(Suppl. Figure 9),** confirming that these platelets retain their ability to bind TSP-1. Finally, incubation of TSP-1$^{-/-}$ platelets with purified human TSP-1 also caused a partial reduction of platelet sensitivity to PGI_2 *(% aggregation; 11.7±2.2 vs 22.3±1.4, P=0.01)* **(Figure 3D)**. Thus, our data suggest that platelet-derived TSP-1 has the potential to alter platelet sensitivity to PGI_2.

TSP-1 regulates cAMP signaling in platelets.

Given the hypersensitivity of TSP-1$^{-/-}$ platelets to PGI_2, we assessed the cAMP signaling pathway. Surprisingly, we found no significant difference in cAMP concentrations between WT and TSP-1$^{-/-}$ platelets following treatment with PGI_2 **(Figure 4A)**. Importantly, human TSP-1 added exogenously to TSP-1$^{-/-}$ platelets still prevents PGI_2-induced cAMP accrual **(Figure 4B)**. Thus, we reasoned that TSP-1 must be released from α-granules to signal in an autocrine and/or paracrine fashion. Hence, platelets were treated with PGI_2 (10 nM), then stimulated with collagen (10 µg/mL) in the presence of apyrase for measurement of cAMP. In WT platelets, PGI_2-induced cAMP formation was significantly reduced after stimulation with collagen (1850±120 vs 1115±295 cAMP fmol/10^{x7}; P<0.04), but activation of TSP-1$^{-/-}$ platelets had no effect on cAMP levels (2048±180 vs 2016±101 cAMP fmol/10^{x7}, P<0.4) **(Figure 4C)**. This data suggests that released TSP-1 may modulate PGI_2/cAMP signaling via increased hydrolysis rather than reduced synthesis of cAMP. To verify this, we measured the activity of PDE3A, an enzyme responsible for cAMP degradation in platelets[25]. The activity of immunoprecipitated PDE3A was not affected by the activation status of platelets (**Suppl. Figure 10**), and it retained sensitivity to milrinone (**Suppl. Figure 11**). Collagen caused a significant increase in PDE3A activity over basal in WT, but this was muted in TSP-1$^{-/-}$ platelets **(Figure 4D)**. Nitric oxide (NO)-induced increases in cGMP may inhibit platelet PDE3A[26], and under some conditions hydrolyse cGMP. We therefore measured cGMP to ensure that it was not affected under our experimental conditions. The NO donor S-nitrosglutathione (GSNO; 10 µM) caused a significant increase in cGMP, neither TSP-1 or PGI_2 alone had any effect. Moreover, TSP-1 did not inhibit basal cGMP concentrations **(Suppl. Figure 12)**, indicating that changes in cGMP were not involved in our observations.

To confirm that modulation of cAMP affects downstream signaling, we measured the phosphorylation of VASP, a target for platelet PKA. Here, we used phosphoflow cytometry to discern subtle changes in phosphorylation in the physiological context of whole blood[27]. We found that PGI_2 increased phosphoVASP^{Ser157} in WT platelets and that phosphorylation was reduced upon stimulation with CRP-XL *(fold change; 5.1±0.3 vs 3.4±0.8, P=0.03)*. In contrast, PGI_2-induced phosphoVASP^{Ser157}was not affected by stimulation with CRP-XL in TSP-1$^{-/-}$ platelets *(Fold change; 4.9±0.3 Versus 4.9±0.08)* (**Figure 4E)**. Critically, immunoblotting showed no differences in key components of the cAMP signalling machinery including PKARI, PKARII, PKAc, PKG and PDE3A in TSP-1$^{-/-}$ and WT platelets **(Suppl. figure 13)**.

TSP-1 modulation of cAMP signalling requires CD36.

We have previously shown that crosstalk exists between cAMP and CD36 signaling pathways.[15] To confirm a role for CD36 in transducing the effects of TSP-1, we used CD36-deficient platelets. We reasoned that PGI_2 hypersensitivity would not be observed in the absence of this key receptor. Platelet aggregation, TLT-1 expression and $\alpha_{IIb}\beta_3$ activation (Jon/A binding) in response to collagen/CRP-XL **(Figure 5A-C)** or thrombin **(Suppl. Figure 14)** in WT and CD36$^{-/-}$ mice were indistinguishable. In contrast, PGI_2-mediated inhibition of platelet aggregation was greater in CD36$^{-/-}$ than WT platelets **(Figure 5A)**. Moreover, PGI_2-mediated reductions of CRP-XL-mediated TLT-1 surface expression and integrin $\alpha_{IIb}\beta_3$ activation were greater in CD36$^{-/-}$ than in WT **(Figure 5B and C)**. Similarly, pretreatment with PGI_2 significantly inhibited thrombin-mediated TLT-1 expression and integrin activation **(Suppl. Figure 14)**.

To demonstrate that observations with CD36$^{-/-}$ platelets were not due to lack of TSP-1, we showed that releasates from CD36$^{-/-}$ platelets, containing TSP-1 **(Suppl. Figure 15),** decrease the sensitivity of TSP-1-deficient platelets to PGI_2 (14±1 vs 26.7±2, P<0.0004) **(Figure 5D)**. The effects of releasates were blocked when TSP-1$^{-/-}$ platelets were treated with the CD36 blocking antibody FA6.152[28] but not IgG control **(Figure 5D).** In contrast, CD36$^{-/-}$ platelet releasates did not affect PGI_2-induced inhibition of aggregation in CD36$^{-/-}$ mice **(Figure 5E)**. Similar data was obtained when purified human TSP-1 was substituted for the releasate **(Figure 5F)**.

We next examined the cAMP signaling pathway in CD36$^{-/-}$ mice. Collagen significantly increased PDE3A activity in WT but not CD36$^{-/-}$ platelets **(Figure 5G)**. Incubation of whole blood from WT mice with PGI_2 increased , which was reduced by stimulation of the blood with CRP-XL. Phosphoflow showed that VASP (Ser157) phosphorylation was elevated in CD36$^{-/-}$ platelets in response to PGI_2 **(Figure 5H),** but like TSP-1$^{-/-}$ platelets, CRP-XL did not affect PGI_2-induced phosphorylation. Together, this data suggests that the modulation of cAMP signaling by TSP-1 requires at least partial involvement of surface CD36.

TSP-1 modulates PGI_2 control of thrombosis *in vitro* and haemostasis *in vivo*

We next examined if altered sensitivity to PGI_2 was evident in the blood of TSP-1$^{-/-}$ mice. Here, whole blood from WT or TSP-1$^{-/-}$ mice was treated with apyrase and perfused over immobilized collagen in the presence or absence of PGI_2. Consistent with previous studies, we found no difference in thrombus size between TSP-1$^{-/-}$ and WT platelets[16]. However, pre-treatment with PGI_2 caused a greater reduction in thrombus size in TSP-1$^{-/-}$ mice versus controls *(WT 25.1±5.8 vs TSP-1$^{-/-}$ 52.6±05.6%, P=0.01)* **(Figure 6A)**. Our *in vivo* studies suggested that thrombi in TSP-1$^{-/-}$ mice were less stable (**Suppl. Video 2**). To test thrombus stability[29], we flowed modified Tyrode's buffer over preformed thrombi. In both strains of mice, thrombi were relatively stable when challenged with buffer alone. However, in PGI_2-supplemented buffer, preformed thrombi in TSP-1$^{-/-}$ blood disintegrated within 4 min while WT thrombi remained stable *(WT 15±4 vs TSP-1$^{-/-}$ 42±12.9%, P=0.05)* **(Figure**

6B). This data highlights the potential importance of TSP-1 in generating and stabilizing thrombi when exposed to PGI_2 at high shear flow.

Having established that the absence of TSP-1 or CD36 renders platelets hypersensitive to PGI_2 *in vitro*, we confirmed these findings by partially recreating the haemostatic response *in vivo* with intravenous injection of a PGI_2 analogue[30]. Iloprost (800 ng/mL) caused a modest but significant increase in the bleeding time of WT mice *(120±3 s vs 264±62 s, P< 0.03)* **(Figure 6C)**. In contrast, it significantly prolonged bleeding times in TSP-$1^{-/-}$ mice *(190±15 s vs 555±160 s, P<0.04),* while the absence of CD36 markedly prolonged bleeding times in $CD36^{-/-}$ mice *(214±21 s vs 524±118 s, P<0.01).* Phosphoflow performed after iloprost injection revealed an increase in intraplatelet VASP (Ser157) phosphorylation **(Suppl. Figure 16)**.

DISCUSSION

The dynamic activation of blood platelets during response to injury requires a rapid suppression of the tonic inhibitory actions of endothelial- derived PGI_2 and NO. In this study, we provide evidence that platelet-derived TSP-1 plays a key role in promoting haemostasis and thrombosis by desensitising platelets to inhibitory cAMP signaling. Our data show that (i) TSP-1-deficient mice have a bleeding phenotype, defective thrombosis and increased sensitivity to the PGI_2mimetic iloprost, (ii) transfusion of WT platelets into TSP-$1^{-/-}$ mice corrects defective thrombus formation and improves clot stability, and (iii) platelet-derived TSP-1, through CD36, reduces platelet cAMP concentration by activating PDE3A.

Bleeding times in TSP-$1^{-/-}$ mice were significantly increased, suggesting a haemostatic defect. Interestingly, some TSP-$1^{-/-}$ mice showed normal bleeding times, and while the reasons for this are unclear, it is likely linked to variations in thrombus stability we observed both *in vitro* and *in vivo*, which can be associated with rebleeding.[31] Given that TSP-1 is expressed by monocytes, endothelium and stromal fibroblasts and that it circulates in the plasma, we explored the possibility that these individual sources play distinct roles in haemostasis,[20] focusing on the importance of platelet-derived TSP-1. While we found that thrombosis was defective in the absence of TSP-1, the use of global rather than tissue-specific TSP-1 knockout mice limited our ability to definitively conclude that platelet-derived TSP-1 is critical. Thus, our observations that (i) thrombotic defects could be ameliorated by the adoptive transfer of WT but not TSP-$1^{-/-}$ platelets, and (ii) TSP-$1^{-/-}$ mice possess normal concentrations of other key adhesive proteins such as vWF and fibrinogen[17] strongly suggest that platelet-derived TSP-1 is largely responsible for thrombus formation and stability *in vivo*. Given that our data, consistent with previous studies, showed TSP-$1^{-/-}$ platelets to aggregate normally, we were initially surprised by our *in vivo* findings[17,19] as they suggest that the haemostatic defect may be unrelated to platelet activation. We first confirmed that TSP-1 deficiency did not affect coagulation in TSP-$1^{-/-}$ mice, which is particularly relevant given that plasma TSP-1 correlates with thrombin generation[32] and potentially increases plasmin generation[33]. Having shown that coagulation was not defective, we turned our attention to the mechanisms of platelet activation *in vivo*. Elegant studies by Sim and colleagues showed that modulation of platelet cAMP delayed thrombus formation *in vivo*[5]. This observation, coupled to our previous work showing that exogenous TSP-1 can reduce platelet sensitivity to PGI_2 *in vitro*, led us to examine the *in vivo* relevance of this pathway. To approach this issue, we used the PGI_2 mimetic iloprost, which is known to modulate platelet function *in vivo*[34]. As expected, we found that iloprost increased bleeding times in both strains but that absence of TSP-1 caused significantly longer bleeding times, suggesting that these animals are hypersensitive to platelet cAMP. Interestingly, our in *vitro* experiments revealed that the phenotype was related to thrombus stability. In agreement with our recent report[35], flow experiments demonstrate that PGI_2 can induce the dissolution of preformed thrombi but that this is more acute in the absence of TSP-1. The significance of this is not yet clear, but it could reveal a new role for cAMP signaling in controlling thrombosis and will require detailed investigation in the future.

The biology of TSP-1 is extremely complex given that is reportedly interacts with ECM proteins, cell receptors, growth factors, cytokines and proteases often simultaneously[36]. It therefore likely that TSP-1 has multiple roles in the haemostatic process, with different pools of TSP-1 having distinct functional roles. Our plasma swap experiments suggest that basal plasma concentrations of TSP-1 do not make a major contribution to platelet regulation by cAMP. This does not preclude plasma TSP-1 regulating other elements of haemostasis, including platelet adhesion and thrombosis[16,18,37]. One key factor is that TSP-1 binds promiscuously to plasma proteins, which could either reduce its bioavailability for platelets, or more likely, induce changes in TSP-1 that direct it to specific functions. A recent study demonstrated that neutrophil-mediated cleavage of TSP-1 promotes platelet adhesion and string formation[38]. Since TSP-$1^{-/-}$ platelets showed increased sensitivity to PGI_2 in aggregation assays,

which were partly reduced by addition of purified human TSP-1 or releasates from WT platelets (containing TSP-1) but not TSP-1$^{-/-}$ platelets, it suggests that platelet TSP-1 could be directed to this specific function. Clearly, data from our cAMP experiments show that platelet activation, and seemingly TSP-1 secretion, is required to dampen cAMP signaling. Given that TSP-1 diminishes cAMP accumulation upon exposure to PGI_2 and this is blocked *in vitro* by the PDE3A inhibitor milrinone, [3915] it is likely that TSP-1 induces the breakdown rather than accumulation of cAMP. To this end, we show that TSP-1 signaling is linked directly to activation of PDE activity in platelets. The physiological relevance of these biochemical assays is strengthened by novel whole blood signaling experiments, demonstrating clearly that PGI_2-cAMP signaling responses, as evidenced by VASP phosphorylation, are maintained in TSP-1$^{-/-}$ mice. These data suggest that released TSP-1 increases PDE3A activity, which accelerates cAMP breakdown, leading to diminished PKA activity and reduced sensitivity to PGI_2. In the absence of TSP-1, platelets are hypersensitive to inhibitory cAMP signaling. We observed that sensitivity to PGI_2 never fully recovers after the addition of either recombinant or platelet-derived TSP-1 for reasons that are unclear. As we omitted ADP signaling from our experiments, we anticipate that a better recovery could be achieved. It is also possible that in the complex conditions found *in vivo* that platelet derived TSP-1 requires processing to be fully effective[36] or that it engages other partner proteins that affect cAMP availability such as multidrug resistance proteins (MRP4) which promotes cAMP efflux from platelets,[40] but these possibilities require further exploration.

Several structurally and functionally distinct receptors for TSP-1 have been identified on platelets, including integrins αvβ3 and αIIbβ3, CD36, and integrin-associated protein (IAP or CD47)[14,41–43]. We have shown that ligation of CD36 by TSP-1 *in vitro* can modulate platelet sensitivity to cAMP signalling[15,19,44,45]. CD36 deficiency (known as the Naka^{a-} phenotype) is highly prevalent in Asian populations (~3%) but less common in the western world.[46–48] The lack of evidence for a haemostatic defect in CD36-deficient individuals suggests that binding of collagen to other receptors (e.g., GPVI) might be sufficient to avoid an overt bleeding diathesis. In this study, we present three pieces of evidence to demonstrate a link between CD36 and platelet-derived TSP-1 modulation of platelet function. First, CD36-deficient platelets, like TSP-1$^{-/-}$ deficient platelets, display a markedly increased sensitivity to PGI_2, which interestingly cannot be rectified by platelet releasates or purified TSP-1. Second, reduced cAMP signaling and increased PDE3A activity in response to platelet activation in the platelets of WT animals is absent in CD36$^{-/-}$ platelets. Finally, the ability of platelet releasates, containing TSP-1, to increase platelet sensitivity to cAMP was blocked by a CD36 blocking antibody. Gosh et al[49] showed that, like TSP-1- deficient mice, CD36$^{-/-}$ mice display delayed thrombus formation in $FeCl_3$-injured arteries. The authors concluded that CD36 ligands are generated following vascular injury and that signals from these ligands contribute to thrombus formation and stability. Our results show that TSP-1, which is generated during vascular damage, is a likely ligand, and that CD36 is required, at least in part, for TSP-1 mediated "disinhibition" of PGI_2 signaling, but given the multivalent nature of TSP-1, it is likely that other receptors may contribute to the modulation of platelet function. The mechanisms underpinning these effects likely involves CD36-mediated tyrosine kinase pathway that are activated by TSP-1 and other ligands[7,15,45,50,51].

Our data suggests that in circulating platelets TSP-1 is localized in alpha-granules, limiting its availability. At sites of vascular injury, activated platelets release TSP-1, which acts to suppress inhibitory cAMP signaling and promote haemostasis. Our data shows that TSP-1 can alter this balance to promote platelet activation, falling in line with previous studies showing TSP-1$^{-/-}$ platelets exhibit higher sensitivity to inhibition by NO[19]. We postulate that control of cAMP and cGMP signaling by TSP-1 represents an important mechanism effective haemostasis. However, this mechanism is potentially subverted by circulating pathological ligands such as oxidised LDL to promote thrombosis[44,45]. The clinical importance of the observations with TSP-1 now needs to be elucidated,

given the increasing interest in the development of therapeutic strategies targeting TSP-1 in advanced primary cancers[52] and the prothrombotic platelets generated by these therapies.[53] Our data and that of others shows that TSP-1 has multifaceted roles in thrombosis and haemostasis, which should be considered in detail when developing new drug interventions.

Authorship Contributions

AA. designed and performed experiments, analysed data and wrote the manuscript. M.B, K.W., B.S., and B.W. performed experiments. M.F. provided essential materials. AWP., designed the research. K.M.N. designed the research, analysed data and wrote the manuscript.

Conflict of Interest

Nothing to declare

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Figure 1: TSP-1 deficiency delays thrombus formation and prolongs bleeding time.
(A) Representative immunoblot of TSP-1 from lysates of WT and TSP-1$^{-/-}$ platelets. **(B)** Animals were counted after cessation of bleeding for 1 minute. Data expressed as scatter plot, every dot represents an individual animal, black line indicates mean. N=7 for WT and N=8 for TSP-1$^{-/-}$ mice, (P<0.02). **(C)** *In vivo* thrombus formation following ferric chloride injury of the carotid artery of WT mice was compared to that of TSP-1$^{-/-}$ (*See supplemental videos*). Platelets were labelled with Dylight488-conjugated rat anti-CD42b antibody. *(i)* Representative images of thrombus accural at indicated times, *(ii)* representative graph of continuous monitoring of real-time thrombus formation (WT=black line and TSP-1$^{-/-}$=grey line), and *(iii)* time to peak thrombus size expressed as mean±SD of N= 8 WT(black bar) and N=8 TSP-1$^{-/-}$mice (grey bar) (*P<0.01). **(D)** Plasma TSP-1 pull-down from non-injured mice and from mice 30 min after ferric chloride injury examined by immunoblotting. Representative of 5 mice. **(E)** Peak thrombus size analysis (20 min post-injury) following transfusion of WT or TSP-1$^{-/-}$ donor platelets into TSP-1$^{-/-}$ recipient mice followed by ferric chloride injury. Data expressed as mean±SD and represents N=5 (*P<0.02).

Figure 2: TSP-1 deficiency has no effect on platelet activation.
(A)(i) Platelet-rich plasma (PRP) from WT (black bars) and TSP-1$^{-/-}$ mice (grey bars) were stimulated with thrombin (0.01-0.1 U/mL) and platelet aggregation was measured under constant stirring (1000 rpm) at 37°C for 4 min. Percentage aggregation is presented as mean±SEM; N=5). **(ii)** PRP from WT and TSP-1$^{-/-}$ mice were stimulated with thrombin (0.001-0.025 U/mL) for 20 min and Jon/A binding was assessed by flow cytometry. Data is expressed as % positive platelets (N=5). (iii) and in (ii) except TLT-1 surface expression (α-granule secretion) was measured. Data expressed as % positive cells (n=5).% **(B)** as in **(A)** except (i) where aggregation was induced by collagen (2.5 or 10 µg/mL), and (ii)/(iii) where CRP-XL 1 and 5µg/ml was used (N=4). **(C)** PRP from WT (solid line) and TSP-1$^{-/-}$ mice (dashed line) (incubated with apyrase (2 U/mL) were treated with PGI_2 (0-100 nM) for 1 min before stimulation with collagen (10 µg/mL), and plat (N=5)elet aggregation was recorded over 3 min. *(i)* Percentage aggregation is presented as mean±SEM (P<0.05; N=5). *(ii)* Representative traces using PGI_2 (5 nM). **(D)** PRP from WT (black) and TSP-1$^{-/-}$ mice (grey) (incubated with apyrase (2 U/mL) were treated with PGI_2 (10 nM) for 1 min before stimulation with CRP-XL (10 µg/mL) for 20 min, and Jon/A binding was measured. Data is presented as % positive platelets, mean±SEM (N=6; *P<0.05, Mann-Whitney U Test 2). **(E)** as in **(D)** except surface expression of TLT-1 was measured by flow cytometry N=6 (*P<0.02).

Figure 3: Platelet-derived, but not plasma-derived, TSP-1 modulates the platelet response to PGI_2.
(A) WT and TSP-1$^{-/-}$ washed platelets were re-suspended in either WT or TSP-1$^{-/-}$ plasma to a concentration of ($2x10^8$ plt/mL) in the presence of apyrase (2U/ml). Platelets, in the presence and absence of PGI_2 (5 nM), were stimulated with collagen (10 µg/mL), and aggregation was measured under constant stirring (1000 rpm) at 37°C for 4 min. *(i)* Representative traces, and *(ii)* percentage aggregation is presented as mean±SEM (N= 5, **P< 0.01 compared to platelets treated with collagen and PGI_2, Mann-Whitney U Test). **(B)**. PRP from TSP-1$^{-/-}$ mice (treated with apyrase) were incubated with releasates from WT platelets or TSP-1$^{-/-}$ platelets (or PPP 1:10 v/v), then stimulated with collagen (10 µg/mL) in the presence or absence of PGI_2 (5 nM). *(i)* Representative traces and *(ii)* percentage aggregation is presented as mean±SEM (N=5, *P< 0.05 compared to platelets treated with collagen and PGI_2, Mann-Whitney U Test. **(C)** as in **(B)** except platelets were stimulated with collagen (2 µg/mL) in the absence of PGI_2. **(D)** PRP from TSP-1$^{-/-}$ mice (treated with apyrase) were incubated with human platelet-derived TSP-1 (h-TSP-1) prior to treatment with PGI_2 (5 nM) for 1min and stimulation with collagen (10 µg/mL) *(i)* representative traces; *(ii)* percentage aggregation is presented by mean±SEM (N= 4, *P< 0.05,Mann-Whitney U Test). Intracellular cAMP levels presented as mean±SEM (N=5, *P< 0.05, Mann-Whitney U Test).

Figure 4: Platelet-derived TSP-1 modulates intracellular cAMP levels by increasing PDE3 activity.
(A) WT (black) and TSP-$1^{-/-}$ (grey) platelets ($2x10^8$ plt/mL) were treated with PGI_2 (0-50 nM) for 1 min and lysed before intracellular cAMP concentrations were measured. Mean ± SEM (N=5). **(B)** TSP-$1^{-/-}$ (black) platelets ($2x10^8$ plt/mL) were treated with PGI_2 (100 nM) for 1 min in the presence or absence of hTSP-1(10 μg/ml) and lysed before intracellular cAMP concentrations were measured. Mean±SEM (N=5,*P< 0.05, Mann-Whitney U Test$_2$). **(C)** WT (black bars) and TSP-$1^{-/-}$ (grey bars) platelets ($2x10^8$ plt/mL) incubated with apyrase were treated with PGI_2 (10 nM) alone for 2 min or stimulated with collagen (10 μg/mL) 1 min after PGI_2 treatment. Reactions were stopped with lysis buffer, and intracellular cAMP levels were measured by ELISA. Intracellular cAMP levels are presented as mean±SEM (N=6, *P<0.05, Mann-Whitney U Test $_2$). **(D)** WT (black bars) and TSP-$1^{-/-}$ (grey bars) platelets ($5x10^8$ plt/mL) incubated with apyrase were stimulated with collagen (20 μg/mL) for 1 min before stopping the reaction with lysis buffer. PDE3A was immunoprecipitated, and enzyme activity was measured. Data is expressed as % activity above basal and presented as mean±SEM (N=6; P<0.05). **(E)** Whole blood from WT (black bars) and TSP-$1^{-/-}$ (grey bars) mice were incubated with apyrase and treated with PGI_2 (10 nM) alone for 2min or stimulated with CRP-XL (10 μg/mL) for 1 min after PGI_2 treatment. Blood was fixed, permeabilised and and incubated with anti-$VASP^{Ser157}$ followed by secondary fluorescent-conjugate (Alexa 647) and analysed by flow cytometry. Data is expressed as mean ± SEM fold increase in phosph-$VASP^{Ser157}$ over basal, N=4 (p<0.03 *p<0.05, Mann-Whitney U Test).

Figure 5: Platelet-derived TSP-1 modulates PDE3 activity in CD36-dependent manner.
(A) PRP from WT and CD36$^{-/-}$ mice (treated with apyrase) were stimulated with collagen (10 μg/mL), in the presence or absence of PGI_2 (5 nM), and aggregation was measured. Mean±SEM (N= 5, **P< 0.01, Mann-Whitney U Test). **(B)** PRP from WT and CD36$^{-/-}$ mice, (treated with apyrase) were treated with PGI_2 (10 nM) for 1 min prior to stimulation with CRP-XL (5 μg/mL) for 20 min, and JonA binding was measured by flow cytometry (N=7; P<0.05). **(C)** As in **(B)** except TLT-1 surface expression was measured (N=7; P<0.05). **(D)** PRP from TSP-$1^{-/-}$ (treated with apyrase) was stimulated with collagen (10 μg/mL) in the presence and absence of PGI_2 (5 nM) for 1 min prior to stimulation with collagen (10 μg/ml). In some cases, platelets were pretreated with CD36-derived releasates and the CD36 receptor blocking antibody FA6.152 (2μg/mL) or CD36-derived releasates and IgG (2μg/mL). Percentage aggregation is presented as mean±SEM (N= 5, **P< 0.01 compared to platelets treated with thrombin and PGI_2, Mann-Whitney U Test). **(E)** PRP from CD36$^{-/-}$ (treated with apyrase) was stimulated with collagen (10 μg/mL) or treated with PGI_2 (5 nM) for 1 min prior to stimulation in the presence or absence of CD36-derived releasates. Platelet aggregation is presented as mean±SEM (N=6). **(F)** As in **(E)** except platelets were treated with hTSP-1 (10μg/mL). **(G)** WT and CD36$^{-/-}$ platelets ($5x10^8$ plt/mL; incubated with apyrase) were stimulated with collagen (10 μg/mL) for 1 min before stopping the reaction with lysis buffer. PDE3A was immunoprecipitated, and its activity was measured. Data is expressed as % activity above basal activity and presented as mean±SEM (N=3; P<0.05). **(H)**. Whole blood from WT and CD36$^{-/-}$ mice (incubated with apyrase) was treated with PGI_2 (50 nM) alone, CRP-XL (10 μg/mL) alone, or PGI_2 followed by CRP-XL. Blood was fixed, permeabilized, and stained for VASP (Ser157) phosphorylation. (i) Representative heat map of fold increase in phosphorylation. (ii) Quantification is presented as fold change in median fluorescence intensity over basal (N=4, *P<0.05, Mann-Whitney U Test).

Figure 6: TSP-$1^{-/-}$ and CD36$^{-/-}$ display prolonged bleeding times following iloprost injection.
(A) Whole blood was incubated alone or with PGI_2 (50 nM) for 2 min, then perfused at arterial shear $1000s^{-1}$ for 2 min over a collagen matrix (50 μg/ml). Images of adherent platelets were taken by fluorescence microscopy. (i) Representative images of arterial flow experiments, (ii) data presented as inhibition of surface coverage (%), mean±SEM (N=5; *P<0.05, Mann-Whitney U Test). **(B)** Whole blood from WT and TSP-$1^{-/-}$ was labelled with DIOC6 and perfused over collagen (50 mg/ml) at a shear rate of 1000^{-s} for 2 min. The thrombi were subjected to a post-flow with Tyrode's BSA buffer with or without PGI_2 (50 nM) at 1000^{-s} for 4 min. *(i)* Representative images at the end of the post-flow. (ii) Data presented as reduction in surface coverage (%); N=5, (P<0.05). **(C)** WT, TSP-$1^{-/-}$ and CD36$^{-/-}$ mice were transfused with iloprost (80 ng/mL) for 2 min or vehicle control, and bleeding time was

examined by tail bleeding assay. Animals were counted after complete cessation of bleeding for 1 minute**.** Data expressed as scatter plot, every dot represents an individual animal, black line indicates mean (N=9; **p<0.01).