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BRIEF REPORT

Thioredoxin-related transmembrane protein 1 negatively regulates coagulation and phosphatidylserine exposure

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Abstract

Background: Five secreted platelet protein disulfide isomerases (PDIs) and 1 transmembrane PDI regulate platelet function and thrombosis. Thioredoxin-related transmembrane protein 1 (TMX1) was the first member of the PDI family found to negatively regulate platelet aggregation and platelet accumulation *in vivo*. The effect of TMX1 on coagulation is unknown.

Objectives: To determine the effect of TMX1 on coagulation.

Methods: TMX1$^{-/-}$ mice were used to study platelet accumulation and fibrin deposition *in vivo* in the laser-induced thrombosis injury model. Annexin V deposition at the site of vascular injury was studied using conditional TMX1 knockout mice. Annexin V binding to platelets was studied using human platelets, anti-TMX1 antibodies, and TMX1-deficient platelets.

Results: TMX1$^{-/-}$ mice had increased fibrin deposition that was reversed with infusion of recombinant TMX1. Infusion of recombinant TMX1 inhibited platelet accumulation and fibrin deposition in wild-type mice and inhibited fibrin deposition in β_3-null mice. Platelet accumulation is absent in β_3-null mice, suggesting that TMX1 inhibits coagulation independently of platelets. Annexin V binding was increased in activated human platelets incubated with an anti-TMX1 antibody and mouse platelets lacking TMX1. Addition of recombinant TMX1 decreased annexin V binding to platelets. Annexin V binding was increased at the site of vascular injury in Tie2-Cre/TMX1$^{fl/fl}$ mice deficient in endothelial cell TMX1.

Conclusion: TMX1 decreases coagulation at the site of vascular injury and negatively regulates phosphatidylserine exposure on endothelial cells and platelets.

KEYWORDS
annexin V, coagulation, phosphatidylserine, platelets, protein disulfide isomerase, TMX1

Essentials

- Six members of the protein disulfide isomerase family regulate platelet function and thrombosis.
- Thioredoxin-related transmembrane protein 1 (TMX1) inhibits platelet function, but the effect of TMX1 on coagulation is unknown.
- TMX1 is a dual negative regulator of platelets and coagulation.
- TMX1 inhibits annexin V binding to vascular cells.

1 | INTRODUCTION

There are 6 members of the protein disulfide isomerase (PDI) family of enzymes known to regulate hemostasis and thrombosis. PDI, ERp57, ERp72, and ERp46 are secreted prothrombotic vascular thiol isomerases containing CGHC active sites [1–11], each with distinct functions [8,10]. A fifth PDI, ERp5, also with CGHC active sites, can have either prothrombotic [12,13] or antithrombotic properties [14,15]. PDI, ERp57, ERp72, and ERp46 mediate activation of the $\alpha_{IIb}\beta_3$ platelet integrin, platelet aggregation, and platelet accumulation *in vivo* [1–10]. Thioredoxin-related transmembrane protein 1 (TMX1) is a transmembrane member of the PDI family with a single CPAC active site that negatively regulates platelet aggregation by oxidizing sulfhydryl groups in the $\alpha_{IIb}\beta_3$ integrin to disulfide bonds [16].

PDI, ERp57, and ERp72 also mediate fibrin generation *in vivo* independently of platelet deposition, suggesting they have a direct role in coagulation [1,2,6,8]. Coagulation factors regulated by PDI include tissue factor, factor (F)XI, and platelet FV [11]. PDI regulates tissue factor–dependent thrombin generation by mononuclear cells [17] and platelet- and endothelial cell–dependent coagulant activity [18,19]. While PDI on the surface of endothelial cells was reported to be a negative regulator of phosphatidylserine exposure and coagulation [19], *in vivo* PDI supports coagulation [1,2,7]. PDI-catalyzed disulfide exchange supports the binding of coagulation factors to the platelet surface after platelets are activated and phosphatidylserine is exposed [18].

TMX1 is expressed on the surface of platelets and acts extracellularly at the final common pathway of platelet aggregation by inhibiting activation of $\alpha_{IIb}\beta_3$ [16]. Whole-body TMX1-knockout mice ($TMX1^{-/-}$ mice) had increased platelet incorporation into a thrombus in a $FeCl_3$-induced mesenteric arterial injury and shortened tail-bleeding times [16]. The role of TMX1 in coagulation has not been studied. We now found that TMX1 inhibits coagulation *in vivo* and inhibits phosphatidylserine exposure on platelets and endothelial cells.

2 | METHODS

2.1 | Materials

Suppliers for antibodies were polyclonal mouse anti-TMX1 antibody against the full-length TMX1 protein (H00081542-B01P, Novus Biologicals); normal mouse immunoglobulin G (Sigma); anti-CD41 F(ab)2 (BD Bioscience) was conjugated with Alexa 488 (Invitrogen); antifibrin antibody hybridoma (clone 59D8) was from Dr H. Weiler [8]; Dylight 649 anti-CD42c (Thermo Fisher Scientific); and Alexa 488-Annexin V (Yeasen Biotechnology). Proteins were SFLLRN (Sigma, S1820), Convulxin (Enzo), and FITC annexin V (Becton Dickinson).

2.2 | Generation and characterization of TMX1-deficient mice

We previously characterized the generation of TMX1 knockout mice using the knockout first conditional-ready strategy [16]. The germ-line transmitted targeted allele was confirmed by genotyping of TMX1-deficient ($TMX1^{-/-}$, knockout first) mice. Reverse transcription-polymerase chain reaction (PCR) confirmed the absence of TMX1 mRNA in platelets. The mRNA levels of PDI, ERp57, and ERp72 were comparable with wild-type (WT) mice, indicating successful targeting of TMX1. Platelet counts were comparable to those of WT $TMX1^{+/+}$ control mice. Resting platelets from $TMX1^{-/-}$ mice had normal expression of the major platelet surface glycoproteins (GPs; $\alpha_{IIb}\beta_3$, GPVI, or GPIb) and P-selectin and did not have activation of $\alpha_{IIb}\beta_3$. The prothrombin time and partial thromboplastin time were normal in the $TMX1^{-/-}$ mice.

2.2.1 | β_3 integrin-null mice

Previously described β_3 integrin-null mice on a C57BL/6 background with matched C57BL/6 WT control mice were used in some experiments [7,8].

2.3 | Intravital microscopy of laser-induced thrombosis of the cremaster muscle arterioles

Laser-induced injury of the mouse cremaster muscle arteriole was performed as previously described [6–8,20]. Alexa 647–labeled antifibrin antibody and Alexa 488–labeled anti-CD41 F(ab)2 fragments (BD Biosciences) were infused at 0.1 μg/g into the jugular vein followed by infusion of recombinant glutathione S-transferase (GST)-TMX1 protein or GST as a control (150 μg/mouse). In the experiments using β_3-null mice, intravenous administration of the cationic lipophilic fluorochrome 3,3'-dihexylcarbocyanine iodide (DiOC6; 2.5 μL of a 100 μM solution/g of body weight) [8] was used to visualize platelets instead of anti-CD41 antibody, as the mice did not express CD41 antigen on platelet surface. After 5 minutes, arterioles (30-45 μm diameter) were injured using a Laser Ablation system (Intelligent Imaging Innovations [3i]) through a Zeiss microscope (Axio Examiner D1) objective parfocal with the focal plane. The laser power was set to 55% to 65%, and the laser was fired at the vessel wall in 1 to 3 pulses until thrombi were induced. Approximately 10 thrombi were studied in a single mouse. Injuries in which puncture of the vessel occurred or injuries in which no thrombus formed were excluded. The average number of laser pulses/injuries was equal for all conditions. In some experiments, mice were infused with Dylight 649 anti-CD42c against the platelet GPIb beta chain (0.5 μg/g, Thermo Fisher Scientific) to monitor platelet accumulation and Alexa 488-Annexin V (0.5 μg/g, Yeasen) [20]. Data were captured using a charge coupled device

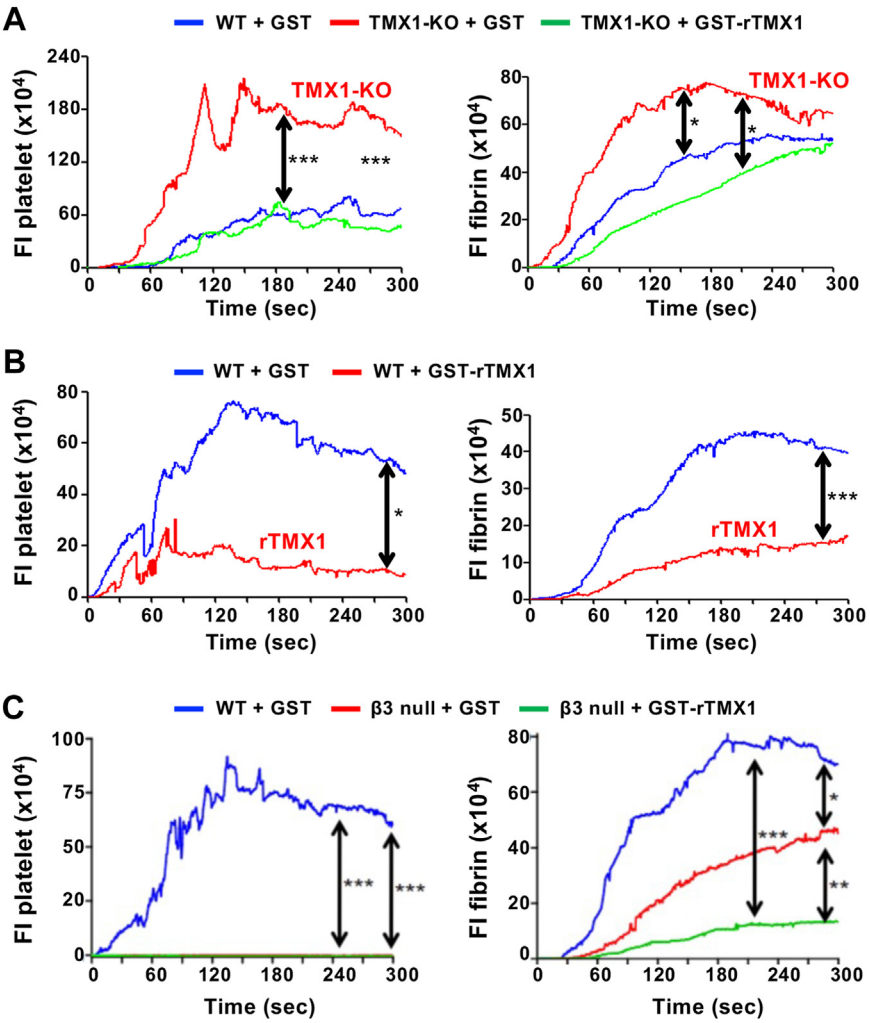

FIGURE 1 Thioredoxin-related transmembrane protein 1 (TMX1) negatively regulates platelet accumulation and fibrin deposition in mice in the laser-induced cremaster arteriole injury model. Cremaster arteriole injury was induced in (A) whole-body TMX1 knockout (KO) mice and wild-type (WT) control mice. (B) WT mice infused with the 150 μg recombinant TMX1 (rTMX1) glutathione S-transferase (GST) fusion protein or GST. (C) WT or $\beta3^{-/-}$ mice. Mice were infused with 150 μg GST or GST-rTMX1. Shown are the integrated results from 30 injuries for each condition. Only significant differences are shown; $^{*}P < .05$, $^{**}P < .01$, $^{***}P < .001$. The areas under the curves over 300 seconds for each lesion are in Supplementary Figure. FI, fluorescence intensity.

camera (Cool SnapTM HQ2) and Slidebook 6.0 image acquisition and analysis software (3i). The median integrated fluorescence intensities over 240 to 300 seconds were collected after vessel wall injury. Image analysis was performed using Slidebook version 6.0 (3i). Data were obtained from 3 to 4 mice for a total of 28 to 30 thrombi per group for each experimental condition, and the area under the curve (AUC) for each thrombus was quantified and analyzed. Mice (8-12 weeks old) were anesthetized using 1% pentobarbital by intraperitoneal injection (100 mg/kg body weight) [3]. All experiments with mice were performed in accordance with Temple University and Soochow University institutional guidelines and approval of the animal care committees.

2.4 | Generation of recombinant human TMX1 protein

The N-terminal extracellular fragment of human TMX1 containing the CPAC active site (27-180) [21] (UniProtKB-Q9H3N1) was expressed as a GST fusion protein in *Escherichia coli* strain ArcticExpress (DE3) and purified on a Glutathione Sepharose High Performance column (GE Healthcare) as previously described [16]. The purified proteins were analyzed on 12% sodium dodecyl sulfate–polyacrylamide gel electrophoresis gels.

2.5 | Flow cytometry

Flow cytometry studies on human platelets were performed using washed platelets suspended into Tyrode's buffer as described [8]. Flow cytometry studies on mouse platelets were performed using platelet-rich plasma prepared and diluted into Tyrode's buffer as described [8]. The platelet-rich plasma was prepared from blood obtained in acid-citrate dextrose solution through the inferior vena cava. All studies on human platelets were performed after approval by the Temple University or the Soochow University Institutional Review Boards.

2.6 | Statistical analysis

Data were analyzed using the statistical software GraphPad Prism 5. For parametric comparison, the values were expressed as the mean ± SEM; 1-way analysis of variance followed by the Tukey's test for

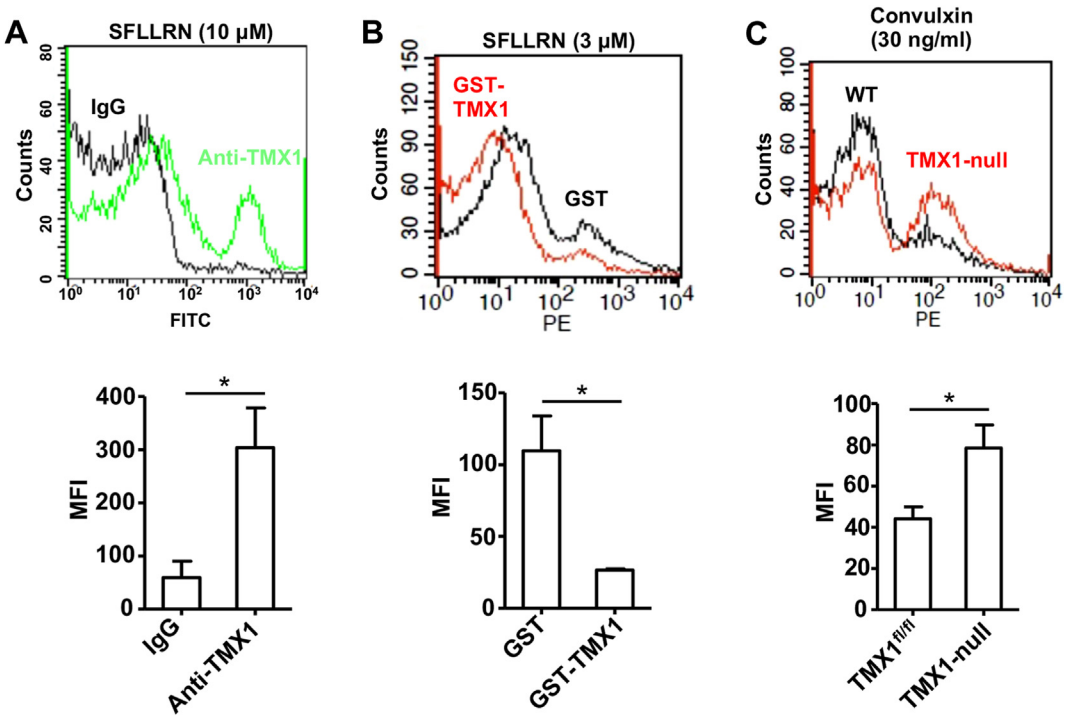

FIGURE 2 Platelet thioredoxin-related transmembrane protein 1 (TMX1) inhibits phosphatidylserine exposure in activated platelets. Human platelets were preincubated with (A) 30 μg/mL normal mouse immunoglobulin (Ig) G or the polyclonal mouse anti-TMX1 antibody, or (B) with 2 μM GST or Glutathione S-transferase-Thioredoxin-related transmembrane protein 1 (GST-TMX1) for 10 minutes at room temperature, and the platelets were activated with (A) 10 μM or (B) 3 μM SFLLRN. (C) Platelets from Pf4-Cre/TMX1$^{fl/fl}$ (TMX1-null) or wild-type (WT, Cre-negative littermate) mice were activated with 30 ng/mL convulxin. Washed human and mouse platelets were adjusted to 10^8/mL using Tyrode's buffer. $CaCl_2$ (1 mM) and the agonist were added, and the samples were incubated for 8 minutes. The samples were diluted (1:6) and incubated with FITC annexin V for 15 minutes, and the binding was measured by flow cytometry. Mean fluorescence intensity (MFI) ± SEM, n = 3, t-test, *P < .05. PE, phycoerythrin.

multiple groups and the 2-tailed Student's t-test for 2 groups were used. For nonparametric comparison between 2 groups, the AUC of the laser injury experiments was analyzed with the Wilcoxon–Mann Whitney test [6]. For the nonparametric comparison between multiple groups, the AUC of median fluorescence intensity over 240 to 300 seconds was analyzed with a Kruskal–Wallis test [8]. A P value less than .05 was considered significant.

3 | RESULTS AND DISCUSSION

3.1 | TMX1 negatively regulates platelet accumulation and fibrin deposition in mice in the laser-induced cremaster arteriole injury model

Previous studies implicated platelets and endothelial cells as the major *in vivo* sources of extracellular thiol isomerases [13,22]. Platelet accumulation and fibrin deposition were increased at the sites of injury in TMX1$^{-/-}$ whole-body knockout mice lacking both vascular sources of TMX1 (Figure 1A). Infusion of GST-tagged recombinant WT TMX1 (GST-rTMX1) reversed the increases in platelet accumulation and fibrin deposition, while GST alone had no effect. These results suggest that TMX1 inhibits these processes. GST-rTMX1 infused into WT mice inhibited platelet accumulation and fibrin formation (Figure 1B).

To determine whether TMX1 can directly inhibit fibrin formation independently of platelet accumulation, we employed β_3-null mice in which platelet accumulation is almost completely absent [8]. Since platelets cannot be detected using anti-CD41 antibody in β_3-null mice, we used DiOC6 to detect platelets (we previously showed that platelet accumulation in WT mice visualized by DiOC6 was comparable to anti-CD41 antibody [8]). As expected, platelet accumulation was absent at the site of injury in β_3-null mice, with a significant reduction of fibrin formation (Figure 1C). The platelet contribution to fibrin deposition is consistent with our previous reports showing that platelets support ~50% to 70% of the fibrin deposition in our laser-induced injury model [6,7]. Infusion of GST-rTMX1 into β_3-null mice further inhibited fibrin formation (Figure 1C). This suggests that TMX1 inhibits coagulation independently of platelets.

3.2 | TMX1 negatively regulates annexin V binding to platelets

TMX1 is expressed on the surface of activated platelets [16], and a subpopulation of platelets express phosphatidylserine on the surface

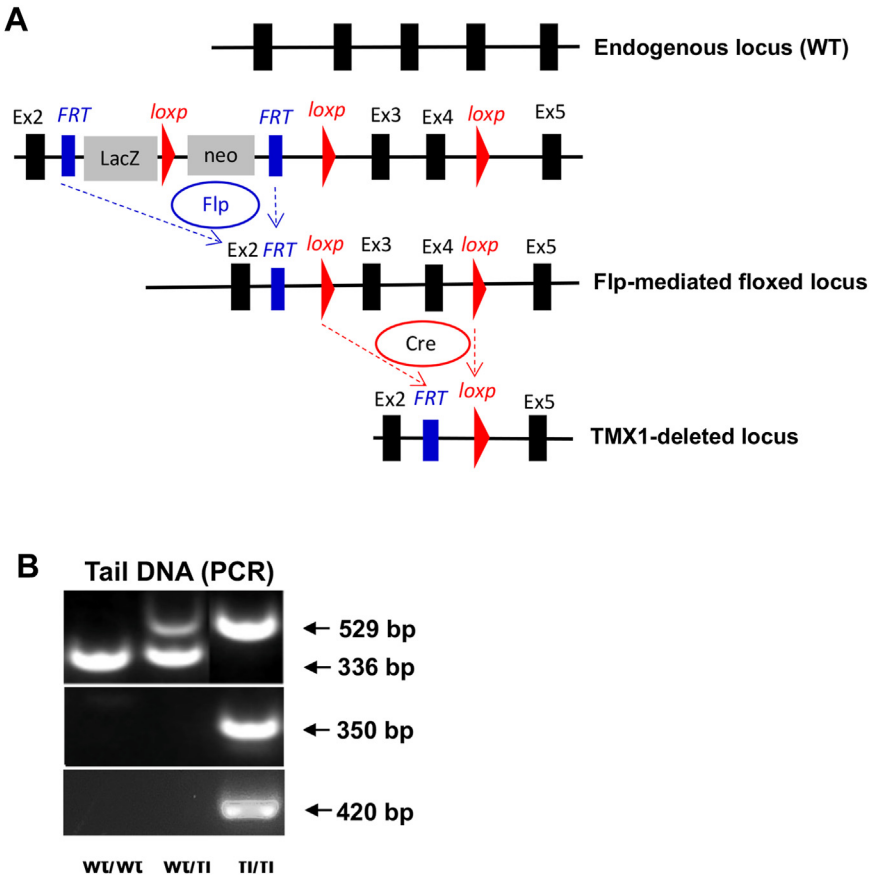

FIGURE 3 Generation and characterization of thioredoxin-related transmembrane protein 1 (TMX1)-deficient mice. (A) Scheme of the targeting strategy. The embryonic stem cells that were generated by EUCOMM (Clone ID: EPD0510_2_E08, MGI Allele ID: 1919986) harbored a modified *TMX1* allele in which the third and fourth exons were flanked by loxP sites, and a phosphoglycerate kinase (PGK)-Neomycin (neo) drug resistance cassette was flanked by flippase recognition target (FRT) sites. The embryonic stem cell clones were injected into murine blastocysts and transferred to pseudopregnant females. The chimeric offspring were mated with wild-type (WT) C57BL/6 mice, and the germ-line transmission of the targeted allele (knockout first) was confirmed by polymerase chain reaction (PCR) analysis of tail DNA. To generate conditional TMX1-floxed (fl) mice, the neo cassette was removed by crossing with mice expressing Flp recombinase; the offspring were confirmed by PCR analysis of tail DNA. (B) Genotyping of TMX1-fl mice. TMX1-fl mice were crossed with *Tie2-Cre* recombinase to delete exons 3 and 4, generating *TMX1*-deleted allele. PCR of genomic DNA yielded a 529-bp product for Flp-excised-fl allele, a 336-bp product for WT allele (wt), a 350-bp product for Tie2-Cre gene, and a 420-bp product for the Pf4-Cre gene (bottom panel). The presence of Pf4-Cre was confirmed by PCR analysis of tail DNA using the following primers: 5'-CCAAGTCCTACTGTTTCTCACTC-3' and 5'-TGCACAGTCAGCAGGTT-3'. The presence of Tie2-Cre was confirmed by PCR analysis of tail DNA using the following primers: 5'-ATTTGCCTGCATTACCGGTC-3' and 5'-ATCAACGTTTTCTTTTCGG-3'.

with physiologic activating agents [23]. Preincubation of platelets with the anti-TMX1 antibody increased binding of FITC annexin V to a subpopulation of human platelets activated with the SFLLRN thrombin receptor activation peptide (Figure 2A). We previously showed that this antibody did not cross-react with other transmembrane (TMX) thiol isomerase proteins, was specific for TMX1 in platelets on immunoblotting, inhibited TMX1 activity, and did not enhance aggregation of TMX1-null platelets [16]. These results suggest that TMX1 on the platelet surface inhibits phosphatidylserine exposure on platelets. Consistent with this conclusion, GST-rTMX1 inhibited annexin V binding to platelets while GST did not inhibit binding (Figure 2B). Additionally, TMX1 deficiency in platelets enhanced annexin V binding (Figure 2C). These data imply that TMX1 is a negative regulator of phosphatidylserine exposure on platelets. TMX1 inhibits integrin $\alpha_{IIb}\beta_3$ activation [16], and $\alpha_{IIb}\beta_3$ has a role in promoting coagulation and phosphatidylserine exposure on platelets [24]. Therefore, TMX1 regulation of $\alpha_{IIb}\beta_3$ may contribute to the inhibition of phosphatidylserine exposure on platelets.

The conditional TMX1 knockout mice were generated by introducing loxp sites flanking coding exons 3 and 4 of the TMX1 gene by gene targeting, using the knockout first conditional-ready strategy (reporter-tagged insertion with conditional potential) [25] (Figure 3A). The mice with Flp-excised allele (TMX1-floxed [fl] mice) were mated with mice expressing either Pf4-Cre or Tie2-Cre to induce the combination of 2 loxp sites. PCR of genomic DNA yielded the expected 529-bp product for Flp-excised-fl allele, a 336-bp product for WT allele, and the expected 350-bp product for the Tie2-Cre gene or 420-bp product for the Pf4-Cre gene (Figure 3B). Tie2-Cre deletes the target protein from endothelial cells and platelets [8].

3.3 | TMX1 inhibits phosphatidylserine exposure at the site of vascular injury

The activated endothelial cell surface is a source of procoagulant phospholipid that supports binding of annexin V and coagulation FXa

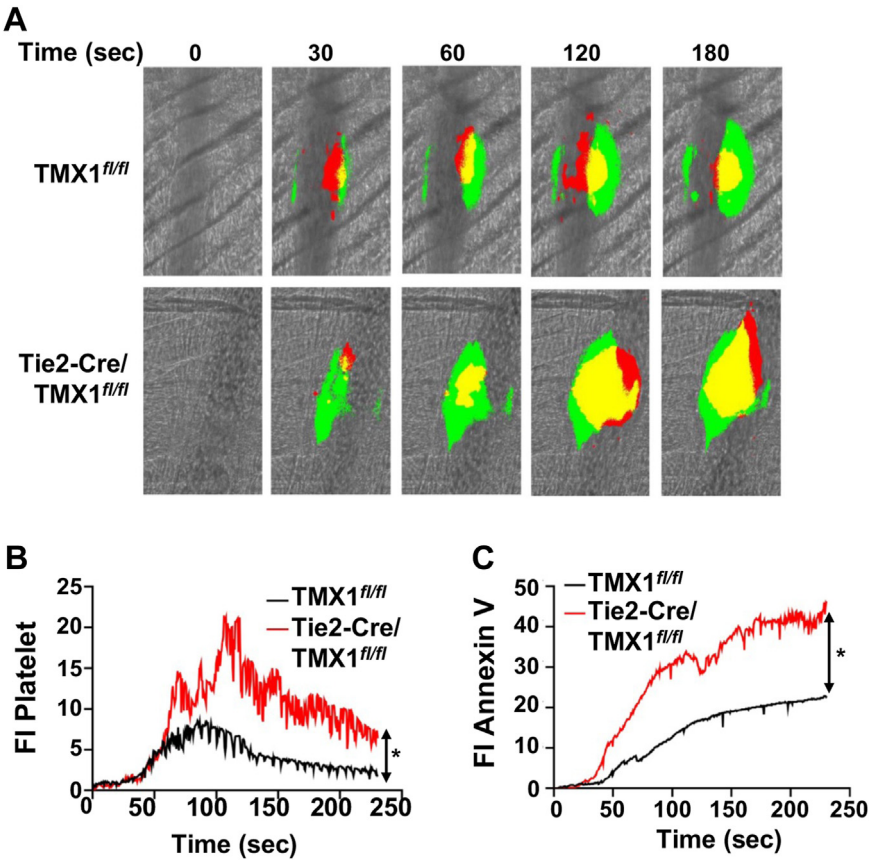

FIGURE 4 Platelet accumulation (red) and annexin V binding (green) at the site of vascular injury. (A–C) Cremaster arteriole injury was induced in Tie2-Cre/thioredoxin-related transmembrane protein 1 floxed/floxed ($TMX1^{fl/fl}$) mice and their Cre-negative $TMX1^{fl/fl}$ littermate control mice. Platelets and annexin V accumulation at the site of injury were detected using Dylight 649 anti-CD42c and Alexa 488-Annexin V. (A) Representative fluorescence images in widefield intravital microscopy for platelet accumulation and annexin V accumulation at the indicated time points after injury. The kinetics of integrated fluorescent intensities (FIs) for (B) platelet accumulation and (C) annexin V over 240 seconds. Shown are the integrated results over 240 seconds from 28 injuries; $^*P < .05$. When the signal decreased, the mice received another injection of Alexa 488-Annexin V (0.25 μg/g). TMX1, thioredoxin-related transmembrane protein 1.

and FVa and thrombosis in laser-induced injury of the cremaster arterials [26–28]. Increased annexin V binding and increased platelet accumulation were demonstrated in Tie2-Cre/$TMX1^{fl/fl}$ conditional knockout mice compared with $TMX1^{fl/fl}$ control mice using widefield microscopy of laser-induced injury of the cremaster arterials (Figure 4). There was nonoverlap of annexin V binding and platelets at the site of injury. Since Tie2-Cre targets endothelial cells [8], the nonoverlap of annexin V with platelets may be from annexin V binding to endothelial cells. This suggests that TMX1 negatively regulates phosphatidylserine exposure on endothelial cells and platelets *in vivo*.

Phosphatidylserine exposure in platelets and endothelial cells supports binding of the vitamin K-dependent coagulation factors as well as the coagulation cofactors FVa and FVIIIa [23]. A previous study using the laser-induced injury model found excellent agreement between the distribution of annexin V and FXa on the surface of endothelial cells *in vivo* [26], and similar colocalization may also apply to FIXa and FVIIIa of the intrinsic Xase. Phosphatidylserine exposure on cell membranes is a thiol-dependent process [29,30] that is critical for coagulation [23]. It is possible that TMX1 inhibits phosphatidylserine exposure in these cells by regulating the thiol-dependent enzymes involved in this process. TMX1 may also inhibit coagulation by other mechanisms in addition to inhibition of phosphatidylserine exposure.

In conclusion, vascular TMX1 is a dual negative regulator of platelets and coagulation. One mechanism by which TMX1 inhibits coagulation appears to be the downregulation of phosphatidylserine exposure in the vascular cells. While monocyte phosphatidylserine exposure can support coagulation, white blood cells are not involved in our laser injury model [7]. Phosphatidylserine exposure on procoagulant platelets is known to support coagulation *in vivo* [23], and platelet TMX1 may inhibit phosphatidylserine exposure at the site of vascular injury through inhibition of $\alpha_{IIb}\beta_3$ activation [24]. Future studies will determine the relative contribution of endothelial cell TMX1 to inhibition of phosphatidylserine exposure and fibrin generation and further define how TMX1 maintains the balance between thrombosis and hemostasis.

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AUTHOR CONTRIBUTIONS

J.Z. designed and performed research and collected and analyzed data. M.C.R. performed research, collected and analyzed data, and helped write the manuscript. L.R. and M.P. supplied reagents and assisted with the *in vivo* hemostasis and thrombosis models and the study

design and interpretation. D.W.E. designed and supervised the research, analyzed the data, and wrote the manuscript.

RELATIONSHIP DISCLOSURE

There are no competing interests to disclose.

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SUPPLEMENTARY MATERIAL

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