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CD248 enhances tissue factor procoagulant function, promoting arterial and venous thrombosis in mouse models

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Abstract

Background: CD248 is a pro-inflammatory, transmembrane glycoprotein expressed by vascular smooth muscle cells (VSMC), monocytes/macrophages, and other cells of mesenchymal origin. Its distribution and properties are reminiscent of those of the initiator of coagulation, tissue factor (TF).

Objective: We examined whether CD248 also participates in thrombosis.

Methods: We evaluated the role of CD248 in coagulation using mouse models of vascular injury, and by assessing its functional interaction with the TF-factor VIIa (FVIIa)-factor X (FX) complex.

Results: The time to ferric chloride-induced occlusion of the carotid artery in CD248 knockout (KO) mice was significantly longer than in wild-type (WT) mice. In an inferior vena cava (IVC) stenosis model of thrombosis, lack of CD248 conferred relative resistance to thrombus formation compared to WT mice. Levels of circulating cells and coagulation factors, prothrombin time, activated partial thromboplastin time, and tail bleeding times were similar in both groups.

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AUTHOR CONTRIBUTIONS

PRK conducted and supervised experiments and helped write the manuscript. NSS, JLH, SCM, KG, HL, and VL provided technical support and helped interpret data. WR, AEM, and ELGP helped design studies and write the manuscript. EMC supervised the design and performance of the studies, helped write the manuscript, and bears responsibility for the content.

CONFLICTS OF INTEREST

The authors have no competing interests or conflicts.

SUPPORTING INFORMATION

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

Proximity ligation assays revealed that TF and CD248 are <40 nm apart, suggesting a potential functional relationship. Expression of CD248 by murine and human VSMCs, and by a monocytic cell line, significantly augmented TF-FVIIa-mediated activation of FX, which was not due to differential expression or encryption of TF, altered exposure of phosphatidylserine or differences in tissue factor pathway inhibitor expression. Rather, conformation-specific antibodies showed that CD248 induces allosteric changes in the TF-FVIIa-FX complex that facilitates FX activation by TF-FVIIa.

Conclusion: CD248 is a newly uncovered protein partner and potential therapeutic target in the TF-FVIIa-FX macromolecular complex that modulates coagulation.

Keywords

coagulation; endosialin; enzyme; fibrin; inflammation; perivascular; thrombin

1 | INTRODUCTION

Tissue factor (TF) is a transmembrane glycoprotein, expressed by perivascular cells, stromal/adventitial cells, circulating leukocytes, and microvesicles (MVs).[1] In health, the critical coagulation function of TF is constitutively expressed on the surface of extravascular cells in an encrypted form.[2] In response to injury or cell stimulation, TF is decrypted and thus transformed to an active state by mechanisms that typically require both thiol-disulfide exchange pathways and exposure of negatively charged phospholipids on the cell surface (reviewed in Zelaya et al.[3] and Ansari et al.4 When activated, TF-bound factor VIIa (FVIIa) more efficiently generates factor Xa (FXa) and initiates the coagulation cascade, and thrombin and fibrin clot formation.[3,5–8]

Beyond coagulation, TF also has functions in inflammation, cell proliferation and differentiation, apoptosis, cell motility, and angiogenesis, mediated via direct and indirect interplay with other cellular components. These include, for example, endothelial protein C receptor (EPCR), β1-integrin, protease activated receptors (PARs), and the insulin-like growth factor 1 receptor (IGF-1R).[9–12] Not surprisingly, excess expression of TF has been implicated in the pathogenesis of several disorders, including arterial and venous thrombosis, cardiovascular disease, atherosclerosis, obesity, cancer, and coagulopathies associated with sepsis and pregnancy.[13–16] Defining the mechanisms by which TF is regulated is therefore of major interest, as this may yield therapeutic insights.

CD248, also referred to as endosialin, is a type 1 transmembrane glycoprotein comprising an N-terminal C-type lectin-like domain, a complement control protein domain, three epidermal growth factor (EGF)-like repeats, a mucin-like domain, a single transmembrane segment, and a short cytoplasmic tail.[17–19] In healthy adults, there is low level expression of CD248 in cells of mesenchymal origin, including those surrounding the vasculature (pericytes, vascular smooth muscle cells [VSMCs], stromal/adventitial cells, adipocytes), as well as in monocytes/macrophages.[20–25] During inflammation and cancer, CD248 is upregulated in stromal and perivascular cells, adipocytes, and some tumor cells.[24,26–30] Loss-of-function studies in mice revealed a role for CD248 in promoting cancer, inflammation (e.g., arthritis, atherosclerosis, obesity), and fibrosis.[22,24,25,30–33] Mechanisms

by which CD248 functions remain largely unknown, although its interaction with platelet derived growth factor receptor-alpha (PDGFR-α) on fibroblasts implicates PDGF-triggered signaling pathways.[34]

The role of CD248 in coagulation and thrombosis has not been previously evaluated. However, in view of the overlapping cellular patterns of expression and inflammatory and pathobiological properties of TF and CD248, we considered that CD248 may also participate in parallel with TF to regulate coagulation. In this report, we showed that mice lacking CD248 are protected against injury-induced venous and arterial thrombosis. Using several cell lines, including VSMC and monocytes, we established that CD248 is in close proximity to TF and the TF-FVIIa-FX complex, where it amplifies the procoagulant activity of TF-FVIIa. Conformation-specific antibodies revealed that CD248 induces allosteric changes in the TF-FVIIa-FX complex that amplifies FX activation, and thus are consistent with the increased thrombosis in mice expressing CD248. These findings provide new understanding of the mechanisms by which TF activity is regulated, and thus uncover potential new therapeutic opportunities for thrombotic disorders.

2 | MATERIAL AND METHODS

See Appendix S1 in supporting information for full details.

3 | RESULTS

3.1 | Lack of CD248 conferred protection against arterial and venous thrombosis

To examine the role of CD248 in thrombosis, we compared the response of CD248 wild-type (WT) and knockout (KO) mice using two models; the $FeCl_3$ carotid artery injury model and a modified inferior vena cava (IVC) stenosis model. Under unchallenged conditions, CD248 WT and KO mice are healthy.[24,25,30–33]

Following exposure of the carotid artery of 9- to 10-week-old male mice to $FeCl_3$, blood flow was continuously monitored for a minimum of 40 minutes. There was a significant prolongation in the time to first occlusion (TTO; $P < .01$) of the arteries of the KO mice compared to WT mice (Figure 1A). For all the WT mice, once occluded, doppler-detected blood flow through the carotid arteries remained absent for the entire duration of the experiment, that is, for a minimum of 40 minutes. For the KO mice, in addition to prolongation of the TTO, one of the KO mice had a partial occlusion at 337 seconds, detected by transiently reduced but persistent doppler-monitored blood flow (Figure 1A-symbol &). In another KO mouse, TTO occurred at 422 seconds and this was followed 10 seconds later by full resumption of doppler-monitored normal blood flow, likely reflecting clot embolization (Figure 1A-symbol #). Overall, lack of CD248 conferred protection against thrombosis in this model.

To study the role of CD248 in a venous thrombosis model, the IVC was partially ligated to limit flow by 85% to 90% for 24 and 48 hours. In previous descriptions of this model, either no side branches, or all visible and/or side branches were ligated or cauterized.[35–37] By this approach, the endothelium was reportedly spared from damage. In

contrast, immediately prior to IVC stenosis, we ligated or cauterized all visible anterior, side, and posterior branches. This induced IVC vascular endothelial cell damage, revealed immunohistochemically by staining for VCAM-1 24 hours post-ligation, which was not detectable under quiescent conditions (Figure S1 in supporting information). Moreover, in contrast to the healthy unperturbed IVC, anti-thrombomodulin immunostaining of the post-ligation IVC revealed endothelial gaps that were not observed with healthy IVC (Figure S1).

Based on previous reports and our pilot studies showing that thrombi are larger in male than female rodents, we assessed males and females separately.[38–40] Thrombi from female KO mice weighed significantly less than those from female WT mice following IVC stenosis at 24 and 48 hours (Figure 1B; $P < .001$ and $P = .0035$, respectively). As is often found with this model, no thrombus was detected in some mice, suggesting that either no clot formed or that the clot resolved/lysed by the time of analysis.[35,37,41] This was observed more frequently with KO mice ($n = 0$ for WT mice; $n = 7$ and 4 for KO mice at 24 and 48 h, respectively). In mice with visible clots, KO clot weights were significantly less than those from WT mice ($P = .0124$ and $P = .0430$ at 24 and 48 h, respectively). Hematoxylin and eosin (H&E) staining of clots from WT and KO mice at 24 h did not reveal obvious differences (Figure S2 in supporting information).

The results from the IVC stenosis model in male mice at 24 h (Figure 1C) were similar to females, that is, clot weights were significantly less in KO mice compared to WT mice ($P = .0079$ and $P = .0464$ including and excluding no-clot animals, respectively). However, by 48 h, there was no significant difference in clot weight between male WT and KO mice, whether or not the mice without clots were excluded, although there were still more male KO mice in which no clots were detected.

Under baseline conditions, there were no differences between WT and KO mice in plasma levels of prothrombin, fibrinogen, FVII, factor VIII (FVIII), factor IX (FIX), FX, protein C, or thrombin-antithrombin (TAT) complexes (Table S1 in supporting information), nor were there genotype-dependent differences in hemoglobin, total and differential peripheral blood leukocyte counts, platelets, activated partial thromboplastin time (APTT) or prothrombin time (PT). APTT and PT were also not different between sibling males and females of the same genotype ($n = 4–7$, data not shown). We did not detect significant CD248-dependent differences in tail bleeding times in male or female mice (Figure 1D).

3.2 | CD248 colocalizes with TF in the IVC of mice

CD248 is expressed by VSMC and monocytes/macrophages.[24,25,28,42] Based on our findings with the thrombosis models, we hypothesized that CD248 may modulate TF activity on the surface of these cells, thereby promoting clot formation. We first showed by immunostaining that CD248 is expressed in the walls of the IVC of WT mice, and that it colocalizes with subendothelial TF (Figure 2A). Both CD248 and TF were readily distinguished from the vascular endothelium, the latter detected by thrombomodulin (TM) staining.[24,28,43] In IVC from CD248 KO mice, TM and TF were still readily detected, the quantities of which, measured by intensity of immunostaining, were not affected by the

presence or absence of CD248 (Figure S3 in supporting information). Specificity of each staining was confirmed with corresponding pre-immune isotype-matched antibodies.

3.3 | CD248 in close proximity to tissue factor (TF)

We used the proximity ligation assay (PLA), a highly sensitive and specific technique, to assess whether CD248 and TF are located in close proximity, that is, within 40 nm of each other.[44,45] In cultured primary murine aortic VSMC (mVSMC) from WT and KO mice, fluorescent micrographs from the PLA revealed that CD248 and TF are in close proximity to each other (Figure 2B). The absence of a PLA signal with KO mVSMC and with WT cells using non-specific antibodies (NSIg) in place of the anti-CD248 or the anti-TF antibody, confirmed the specificity of the CD248-TF interaction. The same finding was observed with cultured human aortic vascular smooth muscle cells (hVSMC), that is, CD248 and TF were in close proximity (Figure 2C). siRNA suppression of CD248 to <5% in these cells significantly reduced the PLA signals representing the CD248-TF interaction (% red area/cell 39.3 ± 0.06 vs. 4.1 ± 0.02 for control siRNA oligo- vs. targeted siRNA-treated cells, respectively, n=6 per group, $P < .005$). Notably, reduction in the PLA signal with knockout or knockdown of CD248 was not due to changes in TF expression: mVSMC from KO mice expressed similar amounts of TF by Western blot and by qRT-PCR (Figure 3A,B; Figure S4 in supporting information for full blots), and siRNA knockdown of CD248 also had no effect on TF mRNA levels in hVSMC (Figure 3C).

3.4 | CD248 specifically enhanced TF-FVIIa activation of FX

The close proximity of CD248 to TF and diminished *in vivo* thrombosis in CD248 KO mice suggested that CD248 may affect TF pro-coagulant activity. This was first tested with mVSMC from WT and KO mice. Purified FVIIa was incubated with FX for 30 min on the surface of equal numbers of WT and KO mVSMC, after which FXa generated was quantified. FXa was not generated in the absence of either FX or FVIIa, and CD248 did not affect the amidolytic activity of FVIIa (not shown). However, following incubation with FVIIa and FX, the rate of FXa generated was significantly increased with WT mVSMC compared to CD248 KO mVSMC (Figure 3D,E). We did not detect significant sex-dependent differences in TF-FVIIa triggered FXa generation, using four independently isolated mVSMC preparations for each sex (Figure S5 in supporting information).

To test whether the enhanced FVIIa-mediated FX activation by CD248 requires TF, we co-incubated neutralizing anti-murine TF antibody 1H1[46,47] on mVSMC in the presence of FVIIa and FX. This entirely blocked TF-FVIIa-mediated activation of FX in both WT and KO mVSMC (Figure 4A). The findings supported the notion that TF is essential for FXa generation by FVIIa, and showed that CD248, by itself, was not sufficient to support FX activation by FVIIa.

We validated our preceding findings in the murine system by comparing TF-FVIIa-mediated generation of FXa on the surface of equal numbers of primary hVSMC that express normal levels of CD248 versus undetectable levels, the latter achieved via siRNA knockdown (Figure 3C). In pilot studies, the number of cells and incubation times were adapted to accommodate the difference in TF expression by these cells compared to mVSMC, and

to optimize the sensitivity of the assay (Figure S6 in supporting information). As with mVSMC, CD248 significantly increased the amount of FXa generated by TF-FVIIa (Figure 4B). This was again shown to be TF-dependent, as neutralizing anti-human TF antibodies 5G9 completely suppressed FXa generation.[48,49]

In a third approach to confirm the cofactor activity of CD248 in TF-FVIIa activation of FX, we used a human melanoma cell line that lacks TF (A7 cells) or stably expresses relatively high amounts of human TF (A7/TF cells; Figure S6B).[10,50] These cells were transfected with either an empty plasmid vector or one with the cDNA encoding human CD248. Expression of CD248 had no effect on the amount of TF expressed by the A7/TF cells, as shown by Western immunoblots (Figure 4C, Figure S7 in supporting information for full blots). Incubation of equal numbers of A7 or A7+CD248 cells with FVIIa and FX resulted in generation of undetectable amounts of FXa (Figure 4D), again indicating the requirement for TF in FX activation. With the A7/TF cells, incubation of FVIIa with FX resulted in generation of FXa, with significantly more with A7/TF+CD248 cells (Figure 4D). These findings again confirmed that CD248 enhanced TF-FVIIa-mediated activation of FX.

Because monocytes express CD248 and TF and are believed to play an important role in TF-dependent thrombosis with the IVC stenosis model,[35] we also attempted to measure TF-FVIIa activation of FX on the surface of isolated monocytes from the CD248 WT and KO mice. Even after lipopolysaccharide or tumor necrosis factor-stimulated upregulation of TF mRNA by the murine monocytes, we could not reliably generate measurable amounts of FXa in our system (not shown). We therefore used the human monocyte/macrophage cell line Mono Mac 6 (MM-6), which constitutively expresses both TF and CD248 (Figure S6C).[51] Similar to hVSMC, we measured TF-FVIIa-mediated activation of FX on the surface of equal numbers of MM-6 cells that express normal mRNA levels of CD248 versus ~40% levels, the latter achieved via siRNA knockdown (Figure 4E, Figure S6C). Suppression of CD248 again significantly reduced the amount of FXa generated (Figure 4E). This was also confirmed to be TF-dependent, as neutralizing anti-human TF antibodies 5G9 significantly suppressed FXa generation.[48,49]

3.5 | CD248 did not affect TF decryption

Under quiescent conditions, TF exists in an encrypted form that is decrypted by thiol-disulfide-dependent mechanisms and interactions with cell surface lipids during injury or inflammation.[4,52–54] We considered whether CD248 might decrypt TF, thereby enhancing TF-FVIIa activation of FX. We therefore exposed WT and KO mVSMC to the calcium ionophore A23187 for 30 min prior to measuring TF-FVIIa activation of FX. In different cells, A23187 variably decrypts TF, augmenting TF procoagulant activity. The concentration used did not cause the cells to detach or undergo morphologic changes.[55] A23187 augmented TF-FVIIa-nduced FXa generation on WT and KO cells compared to their corresponding controls (dimethylsulfoxide for A23187; Figure 3D,E). However, in spite of the increases, there remained a significant difference between the FXa generated with WT cells compared to KO cells, indicating that CD248 does not appear to modulate TF decryption to enhance TF-FVIIa-mediated FX activation. These findings were confirmed

with similar studies using A7/TF and A7/TF+CD248 cells, pre- and post-exposure to the calcium ionophore ionomycin 100 μM (Figure S8A,B in supporting information). As with the mVSMC, the calcium ionophore augmented TF-FVIIa-mediated activation of FX in the A7/TF and A7/TF+CD248 cells, but to a similar extent in both.

We directly evaluated whether CD248 modulates the effect of protein disulfide isomerase (PDI) on TF activation, by treating two different TF-expressing cell lines with the PDI inhibitor PACMA31 (Figure S9 in supporting information). PACMA31 suppressed TF-FVIIa-mediated activation of FX by ~50%, in A7/TF and A7/TF+CD248 cells; but the A7/TF+CD248 cells still supported more FXa activity. Exposure of hVSMC to the same concentration of PACMA31 totally suppressed TF-FVIIa-mediated activation of FX with normal and siRNA-reduced levels of CD248 (<2% by qRT-PCR). The findings indicate that (1) CD248 does not affect PDI-mediated activation of TF, and (2) CD248 alone cannot support TF-FVIIa activation of FX.

3.6 | CD248 did not alter the phospholipid surface of cells

We considered the possibility that increased FX activation by TF-FVIIa in CD248-expressing cells might be mediated via increased cell surface exposure of phosphatidylserine. Annexin V has a high affinity for the anionic phospholipid phosphatidylserine. There was no significant difference in annexin V staining of mVSMC from CD248 WT or KO mice, with cells from either sex (Figure S10 in supporting information). Similarly, hVSMC with normal or siRNA-suppressed levels of CD248 did not exhibit differences in annexin V cell surface staining, suggesting that there were no CD248-dependent changes in exposure of phosphatidylserine (Figure S11 in supporting information). We further tested this functionally with A7/TF and A7/TF+CD248 cells that were pretreated for 15 minutes with varying concentrations of lactadherin, which blocks exposed phosphatidylserine through high affinity specific binding.[56] As followed by measurements of TF-FVIIa-dependent activation of FX (Figure 5A), there was a concentration-dependent reduction in FXa generation by lactadherin for cell lines either with or without CD248, showing that absolute inhibition was the same in both to a maximal concentration of 400 nM (Figure 5B). Moreover, although ionomycin increased exposure of phosphatidylserine on the surface of both the A7/TF and A7/TF+CD248 cells, the increase was not significantly affected by the presence of CD248, as measured by annexin V staining (Figure S8C). Overall, in several cell systems, our data indicate that CD248 does not augment phosphatidylserine cell surface exposure as an explanation for the CD248-dependent increase in TF-FVIIa activation of FX.

3.7 | Tissue factor pathway inhibitor expression is not regulated by CD248

Even with TF levels being unchanged in the WT and KO mVSMC, it was possible that a CD248-dependent decrease in tissue factor pathway inhibitor (TFPI) expression by WT mVSMC[57] might explain the relatively higher TF-FVIIa-mediated FXa generation on WT cells. To test this, we first showed by Western blot that TFPI levels in WT and KO mVSMC were similar (Figure 5C, Figure S5 for full blots). We next added neutralizing anti-murine TFPI antibodies[58] to WT and KO mVSMC during the TF-FVIIa-FX reaction (Figure 5D). As expected, this resulted in increased FXa generation on both genotype cells. However,

the amount of FXa generated was still significantly increased with WT cells. Similar results were obtained with hVSMC using anti-human TFPI antibodies[58] (Figure 4B). Thus, the CD248-dependent increase in TF-FVIIa activation of FX was not affected by differential effects on TFPI.

3.8 | CD248 induced allosteric changes in the TF-FX-FVIIa complex

Because CD248 is in close proximity to TF and accelerates TF-FVIIa activation of FX, we used PLA with well-characterized, conformation-dependent anti-FVII/VIIa antibodies to assess whether changes in the spatial alignment of the TF-FVIIa-FX complex could be induced by CD248 to explain its procoagulant activities (Figure 8). The monoclonal antibody 12C7 recognizes an epitope in the FVIIa protease domain.[59,60] Different pairs of antibodies to FVII/VIIa, FX (F21–4.2C[61]), TF, and CD248, and corresponding species/isotype matched controls were used in the PLA with the A7, A7/TF, and A7/TF+CD248 cells. The antibody 3G12 detects the FVIIa light chain, but the epitope is hidden when TF-FVIIa is complexed with FX.[6] The anti-FX antibody recognizes an epitope that is fully available in the TF-FVIIa-TFPI complex (Figure 8A). Images were quantified (Figure S13 in supporting information). Figure 8 provides a diagrammatic representation of the results.

We first confirmed the absence of non-specific signals with this system using A7 cells that lack CD248 and TF. Following incubation of these cells with FX and FVIIa, there was no evidence of a PLA signal using any combination of pairs of antibodies that recognize CD248, FVIIa, FX, and TF (Figure S12 in supporting information).

3.8.1 | PLA confirmed that FX normally blocks 3G12 recognition of its epitope on FVIIa—When A7/TF cells (lacking CD248) were incubated with FX and FVIIa individually (Figure 6Ai–iii), PLA confirmed expected interactions between TF and FX, and TF and FVIIa, the latter detected with either 3G12 or 12C7. No PLA signal was observed with control antibodies (Figure 6Avii–viii). When FX and FVIIa were co-incubated, the TF-FX interaction was detected with the anti-TF antibody:12C7 pairing (Figure 6Aiv, v) but not with anti-TF antibody:3G12 pairing (Figure 6Avi), the latter due to epitope blockade by the presence of FX, which comes in close contact with the FVIIa-Gla-EGF1 domain[62] (Figure 8A).

3.8.2 | PLA revealed close proximity of CD248 to FX that is not dependent on presence of TF—With A7+CD248 cells (lacking TF), PLA revealed that FX and CD248 were in close proximity, whether or not FVIIa was present (Figure 6B i,ii; Figure 8B left, center). In the absence of FX, FVIIa was not in close proximity to CD248 (Figure 6Biii, iv; Figure 8B right). However, when FVIIa and FX were co-incubated, FVIIa was positioned so that a CD248-FVIIa interaction could be detected when the anti-CD248 antibody was paired with 12C7; but not with 3G12 (Figure 6Bv,vi; Figure 8B center). Control antibodies excluded non-specific signals (Figure 6Bvii,viii).

3.8.3 | Co-expression of TF and CD248 positions the protease domain of FVIIa in close proximity to CD248—Using A7/TF+CD248 cells, we confirmed that TF and CD248 were in close proximity, whether incubated with FVIIa, FX, or both (Figure

7i–iii; controls iv–v; Figure 8C left, center). As expected, TF-FX interactions were observed when FX was present, with or without FVIIa (Figure 7vi,vii). As above, CD248 was in close proximity to FX when alone or with FVIIa (Figure 7viii,ix; control x). When only FVIIa was added, CD248 was in close proximity to it when the CD248 antibody was paired with 12C7 (Figure 7xi), but not when paired with 3G12 (Figure 7xii; Figure 8C, right). This was in contrast to the A7+CD248 cells (Figure 6Biii,iv) in which FX was required for detection of a PLA signal with the anti-CD248 antibody paired with 12C7. This indicated that binding of FVIIa to TF already brought the protease domain of FVIIa (detected by 12C7) in close proximity to CD248. The addition of FX did not affect these CD248-FVIIa interactions, that is, there was a PLA signal of anti-CD248 antibodies with 12C7 but not with 3G12 (Figure 7xiii,xiv, respectively).

3.8.4 | CD248 alters the allostery of TF–FVIIa–FX to expose the recognition site on FVIIa for 3G12—Finally, using A7/TF+CD248 cells in the presence of CD248 and FVIIa, with or without FX present (Figure7xv–xviii), the PLA yielded a *positive* signal when the anti-TF antibody was paired not only with 12C7, but also with 3G12 (Figure 8C center). Because 3G12 recognizes an epitope of FVIIa that is normally blocked by the presence of FX, these findings are notable, and indicate a CD248-dependent physical change in the intermolecular interactions and organization of substrate FX interacting with TF-FVIIa.

Because the 3G12 epitope is also hidden in the inhibited TF–FVIIa–FXa–TFPI complex that is stabilized by the tight interaction of FXa with TF--FVIIa, it is reasonable to consider that CD248 interacting with FX/FXa destabilizes the organization in a way to be more favorable for the release of FXa and therefore enhanced product formation on cells (Figure 8C right).

4 | DISCUSSION

TF is a type II transmembrane receptor that is best known as the principal activator of the coagulation cascade. It plays key roles in hemostasis, in thrombosis, and in cell signaling pathways that regulate inflammation and cell proliferation.[2,63] Structure-function studies of the TF–FVIIa–FX complex, facilitated by conformation-dependent antibodies and mutational analyses, have revealed how TF activates the serine protease domain of FVIIa via allosteric interactions, and how TF--FVIIa recognizes and releases its substrates, FX and FIX.[64–66] Pharmacologic or genetic reduction of TF expression in preclinical models not only limits thrombosis, but also protects against the morbidity and mortality associated with numerous disorders,[13,14,16,47,63] highlighting its importance and the relevance of delineating the mechanisms by which it is regulated.

CD248 is constitutively expressed in a pattern that is similar to that of TF, particularly by perivascular VSMC and circulating monocytes. In this report, we show that the membrane-anchored CD248 is physically in close proximity to TF, its substrate FX, and its enzyme partner, FVIIa, where it enhances TF-FVIIa-mediated generation of FXa (Figure 8C). We have therefore identified CD248 as a newly discovered modulator for TF in the initiation of coagulation. The pathophysiologic importance of these observations is evident by our *in vivo* data demonstrating that lack of CD248 confers protection against carotid artery and

venous thrombosis in mouse models of vascular injury, but without significantly altering blood loss following tail clip injury. Although genome-wide analyses have not identified CD248 as a risk factor for vascular disease, we recently reported that CD248 transcript levels in white adipocytes of humans correlate tightly and positively with obesity and insulin resistance, disorders that are major risk factors for venous thrombosis and atherothrombotic disease.[25,67] The relevance of our findings in human disease remains to be proven.

Interestingly, in the non-occlusive IVC model, we observed sex-dependent differences in the CD248-dependent response, that is, 48 h post-stenosis, the protective effect of deleting CD248 was not apparent in male mice, but was retained in female mice. This finding is in line with observational studies in humans that conclude that men are at increased risk of first and recurrent venous thrombosis (reviewed in Roach et al.68). This male-associated heightened risk of thrombosis is also found in mouse models.[39,69] The mechanisms that enhance thrombotic risk in males remain enigmatic and under-explored, although differential expression patterns of inflammatory cytokines and naturally occurring coagulation inhibitors (e.g., protein C, antithrombin), have been implicated.[39,69] We also do not understand why male CD248 KO mice are apparently less protected from thrombosis in the IVC stenosis model. We did not detect sex-dependent differences in TF-FVIIa–mediated activation of FX on isolated mVSMC, nor did we observe differences in cell surface expression of phosphatidylserine on those same cells. One could speculate that it is due to lower baseline CD248 levels in males. We did not test this; however, it seems unlikely, given the profound protection afforded the KO mice in the carotid artery injury model. Alternatively, there may be unidentified sex-dependent modifiers of the coagulant function of CD248 in the circulation or within the vessel wall. Further studies of the role of CD248 and other determinants of sex-dependent differences in thrombotic risk will be important to pursue.

Do the murine macrovascular injury models that we used provide guidance as to whether the VSMC or monocyte TF/CD248 principally drives thrombosis? Although neither model can be relied upon to definitively localize the thrombotic trigger, our findings with the $FeCl_3$ carotid artery injury model strongly implicated vessel wall VSMC-derived TF (and thus, CD248) as a major participant.[70] The IVC stenosis model, as most often performed, implicates circulating TF expressed by monocytes and/or TF-laden microparticles as the source of the thrombogenic trigger.[41] Indeed, circulating CD14-positive monocytes are also a source of TF, upregulated by inflammatory mediators, endotoxins, and histones.[1] Neutrophils and eosinophils[71] also express TF and/or acquire it from blood-borne, soluble sources or microvesicles/microparticles, all of which may participate in thrombogenesis (reviewed in Cimmino and Cirillo72). Although less is known about the expression profile of CD248, it is found on the cell surface of some leukocytes, including monocytes/macrophages,[25] and in a human monocyte cell line, knockdown of CD248 reduced TF-FVIIa–mediated activation of FX on the cell surface. The findings are consistent with the notion that lack of monocyte-expressed CD248 in the KO mice is a reasonable explanation for the reduced thrombosis observed in the IVC stenosis model. Nonetheless, without further study, we cannot exclude a contribution from the VSMC. In our modification of the model, we uniquely disrupted all the IVC branches, including the posterior branches, inducing injury and loss of vascular endothelial integrity, that could expose subendothelial VSMCs to the circulation. Further studies, using conditional cell-specific *CD248* gene

inactivation strategies are under way to help clarify the cellular source(s) of CD248 that impact thrombogenesis in these and other models.

In attempting to delineate the mechanisms by which CD248 augments TF-FVIIa-mediated generation of FXa, we excluded several possibilities. In four separate cell lines, we showed that there are no major CD248-dependent changes in quantitative expression of TF. Nor does it appear that CD248 plays a role in decryption of TF, in altering the integrity of the phospholipid membrane surface, or in modulating the expression of TFPI.

We did, however, demonstrate that CD248 is in close proximity to TF on/in the cell and to FX when added to the cell surface; and the FX-CD248 interaction does not require the presence of TF. We did not perform binding studies; indeed, we do not know whether CD248 directly binds to FX or TF, or whether there are intervening proteins. However, our findings suggest that CD248 can act as a receptor/scaffold for FX, independent of TF. Our PLA studies further revealed that addition of FVIIa and FX to the A7+CD248 cells (lacking TF, but expressing CD248), results in the assembly of a CD248–FVIIa–FX complex. This parallels the known stabilization of TF-FVIIa by FX and similarly of the herpes simplex virus-encoded protein gC in complex with FVIIa.[73] Notably, this multimolecular CD248–FVIIa–FX complex does not, by itself, have the capacity to trigger activation of FX, that is, TF is an absolute requirement for the allosteric activation of FVIIa that is necessary for proteolytic activation of FX.

These data demonstrate that CD248 participates in the TF-FVIIa–FX complex to enhance its pivotal procoagulant function. Insights into the mechanisms were gained with conformation-specific antibodies that recognize FVII/FVIIa (Figure 8). CD248 expressed by A7/TF+CD248 cells triggered exposure of an otherwise hidden epitope on the light chain of FVIIa that is recognized by 3G12, presumably by reorganizing the spatial alignment of FX with the TF-FVIIa complex. This likely resulted from a direct interaction of FX with CD248, thereby positioning FX for more efficient release from TF-FVIIa (Figure 8). Detailed studies will be required to characterize the molecular sites of interaction between CD248 and components of the TF-FVIIa–FX complex, as well as the impact on the kinetics of turnover of macromolecular substrates.

TF is also a receptor and scaffold for multi-protein assemblies that lead to cleavage of PARs that participate in inflammation, angiogenesis, and cell proliferation.[3] CD248 might also modulate these biological effects via interactions of TF and/or its protein partners. The extracellular region of TF binds to the active form of β1-integrin, enhanced by FVIIa–β1–integrin binding.[10,60] In this form, TF lacks pro-coagulant activity but induces PAR2-dependent and -independent signaling that triggers activation/phosphorylation of mitogen-activated protein (MAP) kinases, PI3 kinase, and pro-inflammatory responses with cytokine release and cytoskeletal changes that promote cell migration.[15] TF–FVIIa–FXa dependent on the endothelial protein C receptor also cleaves PAR2 and can alter macrophage polarization in cancer.[74] By altering FX interaction with TF-FVIIa, CD248 may shift the balance from TF–FVIIa–FXa to TF-FVIIa complex signaling. Intriguingly, CD248 has well-established pro-inflammatory, pro-fibrotic, cell migratory, and proliferative properties, mediated in part via activation/phosphorylation of MAP kinases and release of

cytokines.[30,32] It will be interesting and clinically important to determine whether CD248 and TF also cooperate in these important and related biological systems.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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REFERENCES

1. Osterud B, Bjorklid E. Sources of tissue factor. Semin Thromb Hemost. 2006;32:11–23. 10.1055/s-2006-933336 [PubMed: 16479458]
2. Grover SP, Mackman N. Tissue factor: an essential mediator of hemostasis and trigger of thrombosis. Arterioscler Thromb Vasc Biol. 2018;38:709–725. 10.1161/ATVBAHA.117.309846 [PubMed: 29437578]
3. Zelaya H, Rothmeier A, Ruf W. Tissue factor at the crossroad of coagulation and cell signaling. J Thromb Haemost. 2018;26:1941–1952. 10.1111/jth.14246
4. Ansari SA, Pendurthi UR, Rao LVM. Role of cell surface lipids and thiol-disulphide exchange pathways in regulating the encryption and decryption of tissue factor. Thromb Haemost. 2019;119:860–870. 10.1055/s-0039-1681102 [PubMed: 30861549]
5. Hoffman M, Monroe DM 3rd. A cell-based model of hemostasis. Thromb Haemost. 2001;85:958–965. [PubMed: 11434702]
6. Kamikubo Y, Mendolicchio GL, Zampolli A, et al. Selective factor VIII activation by the tissue factor-factor VIIa-factor Xa complex. Blood. 2017;130:1661–1670. 10.1182/blood-2017-02-767079 [PubMed: 28729433]
7. Shobe J, Dickinson CD, Edgington TS, Ruf W. Macromolecular substrate affinity for the tissue factor-factor VIIa complex is independent of scissile bond docking. J Biol Chem. 1999;274:24171–24175. 10.1074/jbc.274.34.24171 [PubMed: 10446191]
8. Gajsiewicz JM, Nuzzio KM, Rienstra CM, Morrissey JH. Tissue factor residues that modulate magnesium-dependent rate enhancements of the tissue factor/factor VIIa complex. Biochemistry. 2015;54:4665–4671. 10.1021/acs.biochem.5b00608 [PubMed: 26169722]
9. Pendurthi UR, Rao LV. Factor VIIa interaction with tissue factor and endothelial cell protein C receptor on cell surfaces. Semin Hematol. 2008;45:S21–S24. [PubMed: 18544419]
10. Dorfleutner A, Hintermann E, Tarui T, Takada Y, Ruf W. Cross-talk of integrin alpha3beta1 and tissue factor in cell migration. Mol Biol Cell. 2004;15:4416–4425. 10.1091/mbc.E03-09-0640 [PubMed: 15254262]
11. Aberg M, Eriksson O, Siegbahn A. Tissue factor noncoagulant signaling: mechanisms and implications for cell migration and apoptosis. Semin Thromb Hemost. 2015;41:691–699. 10.1055/s-0035-1564046 [PubMed: 26418256]

12. Aberg M, Eden D, Siegbahn A. Activation of beta1 integrins and caveolin-1 by TF/FVIIa promotes IGF-1R signaling and cell survival. Apoptosis. 2020;25:519–534. 10.1007/s10495-020-01611-7 [PubMed: 32458278]
13. Pawlinski R, Pedersen B, Erlich J, Mackman N. Role of tissue factor in haemostasis, thrombosis, angiogenesis and inflammation: lessons from low tissue factor mice. Thromb Haemost. 2004;92:444–450. [PubMed: 15351839]
14. Pawlinski R, Pedersen B, Schabbauer G, et al. Role of tissue factor and protease-activated receptors in a mouse model of endotoxemia. Blood. 2004;103:1342–1347. [PubMed: 14576054]
15. Kocaturk B, Versteeg HH. Tissue factor-integrin interactions in cancer and thrombosis: every Jack has his Jill. J Thromb Haemost. 2013;11(suppl 1):285–293. 10.1111/jth.12222 [PubMed: 23809132]
16. Badeanlou L, Furlan-Freguia C, Yang G, Ruf W, Samad F. Tissue factor-protease-activated receptor 2 signaling promotes diet-induced obesity and adipose inflammation. Nat Med. 2011;17:1490–1497. 10.1038/nm.2461 [PubMed: 22019885]
17. Christian S, Ahorn H, Koehler A, et al. Molecular cloning and characterization of endosialin, a C-type lectin-like cell surface receptor of tumor endothelium. J Biol Chem. 2001;276:7408–7414. [PubMed: 11084048]
18. Opavsky R, Haviernik P, Jurkovicova D, et al. Molecular characterization of the mouse tem1/endosialin gene regulated by cell density in vitro and expressed in normal tissues in vivo. J Biol Chem. 2001;276:38795–38807. [PubMed: 11489895]
19. Rettig WJ, Garin-Chesa P, Healey JH, Su SL, Jaffe EA, Old LJ. Identification of endosialin, a cell surface glycoprotein of vascular endothelial cells in human cancer. Proc Natl Acad Sci U S A. 1992;89:10832–10836. [PubMed: 1438285]
20. Naylor AJ, Azzam E, Smith S, et al. The mesenchymal stem cell marker CD248 (endosialin) is a negative regulator of bone formation in mice. Arthritis Rheum. 2012;64:3334–3343. 10.1002/art.34556 [PubMed: 22674221]
21. St Croix B, Rago C, Velculescu V, et al. Genes expressed in human tumor endothelium. Science. 2000;289(5482):1197–1202. 10.1126/science.289.5482.1197 [PubMed: 10947988]
22. Mogler C, Wieland M, Konig C, et al. Hepatic stellate cell-expressed endosialin balances fibrogenesis and hepatocyte proliferation during liver damage. EMBO Mol Med. 2015;7(3):332–338. 10.15252/emmm.201404246 [PubMed: 25680861]
23. Huang HP, Hong CL, Kao CY, et al. Gene targeting and expression analysis of mouse Tem1/endosialin using a lacZ reporter. Gene Expr Patterns. 2011;11(5–6):316–326. 10.1016/j.gep.2011.03.001 [PubMed: 21402174]
24. Hasanov Z, Ruckdeschel T, Konig C, et al. Endosialin promotes atherosclerosis through phenotypic remodeling of vascular smooth muscle cells. Arterioscler Thromb Vasc Biol. 2017;37:495–505. 10.1161/ATVBAHA.116.308455 [PubMed: 28126825]
25. Petrus P, Fernandez TL, Kwon MM, et al. Specific loss of adipocyte CD248 improves metabolic health via reduced white adipose tissue hypoxia, fibrosis and inflammation. EBioMedicine. 2019;44:489–501. 10.1016/j.ebiom.2019.05.057 [PubMed: 31221584]
26. Brady J, Neal J, Sadakar N, Gasque P. Human endosialin (tumor endothelial marker 1) is abundantly expressed in highly malignant and invasive brain tumors. J Neuropathol Exp Neurol. 2004;63:1274–1283. [PubMed: 15624764]
27. MacFadyen JR, Haworth O, Roberston D, et al. Endosialin (TEM1, CD248) is a marker of stromal fibroblasts and is not selectively expressed on tumour endothelium. FEBS Lett. 2005;579:2569–2575. [PubMed: 15862292]
28. Christian S, Winkler R, Helfrich I, et al. Endosialin (Tem1) is a marker of tumor-associated myofibroblasts and tumor vessel-associated mural cells. Am J Pathol. 2008;172(2):486–494. 10.2353/ajpath.2008.070623 [PubMed: 18187565]
29. Simonavicius N, Robertson D, Bax DA, Jones C, Huijbers IJ, Isacke CM. Endosialin (CD248) is a marker of tumor-associated pericytes in high-grade glioma. Mod Pathol. 2008;21(3):308–315. 10.1038/modpathol.3801006 [PubMed: 18192970]
30. Maia M, de Vriese A, Janssens T, et al. CD248 and its cytoplasmic domain: a therapeutic target for arthritis. Arthritis Rheum. 2010;62:3595–3606. 10.1002/art.27701 [PubMed: 20722022]

31. Nanda A, Karim B, Peng Z, et al. Tumor endothelial marker 1 (Tem1) functions in the growth and progression of abdominal tumors. Proc Natl Acad Sci U S A. 2006;103:3351–3356. [PubMed: 16492758]
32. Maia M, DeVriese A, Janssens T, et al. CD248 facilitates tumor growth via its cytoplasmic domain. BMC Cancer. 2011;11(1):11–162. 10.1186/1471-2407-11-162 [PubMed: 21223585]
33. Smith SW, Croft AP, Morris HL, et al. Genetic deletion of the stromal cell marker CD248 (Endosialin) protects against the development of renal fibrosis. Nephron. 2015;131:265–277. 10.1159/000438754 [PubMed: 26633297]
34. Hong YK, Lee YC, Cheng TL, et al. Tumor endothelial marker 1 (TEM1/Endosialin/CD248) enhances wound healing by interacting with platelet-derived growth factor receptors. J Invest Dermatol. 2019;139(2204–14):e7. 10.1016/j.jid.2019.03.1149
35. von Bruhl ML, Stark K, Steinhart A, et al. Monocytes, neutrophils, and platelets cooperate to initiate and propagate venous thrombosis in mice in vivo. J Exp Med. 2012;209:819–835. 10.1084/jem.20112322 [PubMed: 22451716]
36. Brandt M, Schonfelder T, Schwenk M, et al. Deep vein thrombus formation induced by flow reduction in mice is determined by venous side branches. Clin Hemorheol Microcirc. 2014;56:145–152. 10.3233/CH-131680 [PubMed: 23403491]
37. Brill A, Fuchs TA, Chauhan AK, et al. von Willebrand factor-mediated platelet adhesion is critical for deep vein thrombosis in mouse models. Blood. 2011;117:1400–1407. 10.1182/blood-2010-05-287623 [PubMed: 20959603]
38. Bailey AL, Scantlebury DC, Smyth SS. Thrombosis and antithrombotic therapy in women. Arterioscler Thromb Vasc Biol. 2009;29:284–288. 10.1161/ATVBAHA.108.179788 [PubMed: 19221205]
39. Wong JH, Dukes J, Levy RE, et al. Sex differences in thrombosis in mice are mediated by sex-specific growth hormone secretion patterns. J Clin Invest. 2008;118:2969–2978. 10.1172/JCI34957 [PubMed: 18618017]
40. Liu H, Lu Z, Lin B, et al. Inferior vena cava stenosis-induced deep vein thrombosis is influenced by multiple factors in rats. Biomed Pharmacother. 2020;128:110270. 10.1016/j.biopha.2020.110270 [PubMed: 32497864]
41. Diaz JA, Saha P, Cooley B, et al. Choosing a mouse model of venous thrombosis: a consensus assessment of utility and application. J Thromb Haemost. 2019;17:699–707. 10.1111/jth.14413 [PubMed: 30927321]
42. Suresh Babu S, Valdez Y, Xu A, et al. TGFβ-mediated suppression of CD248 in non-cancer cells via canonical Smad-dependent signaling pathways is uncoupled in cancer cells. BMC Cancer. 2014;14:113. [PubMed: 24555435]
43. Bagley RG, Honma N, Weber W, et al. Endosialin/TEM 1/CD248 is a pericyte marker of embryonic and tumor neovascularization. Microvasc Res. 2008;76:180–188. [PubMed: 18761022]
44. Miki Y, Iwabuchi E, Ono K, Sasano H, Ito K. Exploring protein(−) Protein interaction in the study of hormone-dependent cancers. Int J Mol Sci. 2018;19:3173. 10.3390/ijms19103173
45. Soderberg O, Leuchowius KJ, Gullberg M, et al. Characterizing proteins and their interactions in cells and tissues using the in situ proximity ligation assay. Methods. 2008;45:227–232. 10.1016/j.ymeth.2008.06.014 [PubMed: 18620061]
46. Kirchhofer D, Moran P, Bullens S, Peale F, Bunting S. A monoclonal antibody that inhibits mouse tissue factor function. J Thromb Haemost. 2005;3:1098–1099. 10.1111/j.1538-7836.2005.01253.x [PubMed: 15869619]
47. Chantrathammachart P, Mackman N, Sparkenbaugh E, et al. Tissue factor promotes activation of coagulation and inflammation in a mouse model of sickle cell disease. Blood. 2012;120:636–646. 10.1182/blood-2012-04-424143 [PubMed: 22661702]
48. Ruf W, Edgington TS. Two sites in the tissue factor extracellular domain mediate the recognition of the ligand factor VIIa. Proc Natl Acad Sci U S A. 1991;88:8430–8434. [PubMed: 1924302]
49. Versteeg HH, Schaffner F, Kerver M, et al. Inhibition of tissue factor signaling suppresses tumor growth. Blood. 2008;111:190–199. 10.1182/blood-2007-07-101048 [PubMed: 17901245]

50. Sutherland MR, Ruf W, Pryzdial EL. Tissue factor and glycoprotein C on herpes simplex virus type 1 are protease-activated receptor 2 cofactors that enhance infection. Blood. 2012;119:3638–3645. 10.1182/blood-2011-08-376814 [PubMed: 22374699]
51. Tenno T, Botling J, Oberg F, Nilsson K, Siegbahn A. Tissue factor expression in human monocytic cell lines. Thromb Res. 1997;88:215–228. 10.1016/s0049-3848(97)00232-6 [PubMed: 9361374]
52. Rothmeier AS, Marchese P, Langer F, et al. Tissue factor prothrombotic activity is regulated by integrin-arf6 trafficking. Arterioscler Thromb Vasc Biol. 2017;37:1323–1331. 10.1161/ATVBAHA.117.309315 [PubMed: 28495929]
53. Rothmeier AS, Marchese P, Petrich BG, et al. Caspase-1-mediated pathway promotes generation of thromboinflammatory microparticles. J Clin Invest. 2015;125:1471–1484. 10.1172/JCI79329 [PubMed: 25705884]
54. Langer F, Spath B, Fischer C, et al. Rapid activation of monocyte tissue factor by antithymocyte globulin is dependent on complement and protein disulfide isomerase. Blood. 2013;121:2324–2335. 10.1182/blood-2012-10-460493 [PubMed: 23315166]
55. Wolberg AS, Kon RH, Monroe DM, Ezban M, Roberts HR, Hoffman M. Deencryption of cellular tissue factor is independent of its cytoplasmic domain. Biochem Biophys Res Commun. 2000;272:332–336. 10.1006/bbrc.2000.2783 [PubMed: 10833414]
56. Shi J, Gilbert GE. Lactadherin inhibits enzyme complexes of blood coagulation by competing for phospholipid-binding sites. Blood. 2003;101:2628–2636. 10.1182/blood-2002-07-1951 [PubMed: 12517809]
57. Ghrib F, Brisset AC, Dupouy D, et al. The expression of tissue factor and tissue factor pathway inhibitor in aortic smooth muscle cells is up-regulated in synthetic compared to contractile phenotype. Thromb Haemost. 2002;87:1051–1056. [PubMed: 12083485]
58. Wood JP, Petersen HH, Yu B, Wu X, Hilden I, Mast AE. TFPIalpha interacts with FVa and FXa to inhibit prothrombinase during the initiation of coagulation. Blood Adv. 2017;1:2692–2702. 10.1182/bloodadvances.2017011098 [PubMed: 29291252]
59. Dickinson CD, Shobe J, Ruf W. Influence of cofactor binding and active site occupancy on the conformation of the macromolecular substrate exosite of factor VIIa. J Mol Biol. 1998;277:959–971. 10.1006/jmbi.1998.1639 [PubMed: 9545384]
60. Rothmeier AS, Liu E, Chakrabarty S, et al. Identification of the integrin-binding site on coagulation factor VIIa required for pro-angiogenic PAR2 signaling. Blood. 2018;131:674–685. 10.1182/blood-2017-02-768218 [PubMed: 29246902]
61. Muller-Calleja N, Hollerbach A, Ritter S, et al. Tissue factor pathway inhibitor primes monocytes for antiphospholipid antibody-induced thrombosis. Blood. 2019;134:1119–1131. 10.1182/blood.2019001530 [PubMed: 31434703]
62. Ruf W, Shobe J, Rao SM, Dickinson CD, Olson A, Edgington TS. Importance of factor VIIa Gla-domain residue Arg-36 for recognition of the macromolecular substrate factor X Gla-domain. Biochemistry. 1999;38:1957–1966. 10.1021/bi982254r [PubMed: 10026279]
63. Graf C, Ruf W. Tissue factor as a mediator of coagulation and signaling in cancer and chronic inflammation. Thromb Res. 2018;164(suppl 1):S143–S147. 10.1016/j.thromres.2018.01.023 [PubMed: 29703473]
64. Ruf W, Miles DJ, Rehemtulla A, Edgington TS. Tissue factor residues 157–167 are required for efficient proteolytic activation of factor X and factor VII. J Biol Chem. 1992;267:22206–22210. [PubMed: 1429572]
65. Norledge BV, Petrovan RJ, Ruf W, Olson AJ. The tissue factor/factor VIIa/factor Xa complex: a model built by docking and site-directed mutagenesis. Proteins. 2003;53:640–648. 10.1002/prot.10445 [PubMed: 14579355]
66. Baugh RJ, Dickinson CD, Ruf W, Krishnaswamy S. Exosite interactions determine the affinity of factor X for the extrinsic Xase complex. J Biol Chem. 2000;275:28826–28833. 10.1074/jbc.M005266200 [PubMed: 10889208]
67. Morange PE, Alessi MC. Thrombosis in central obesity and metabolic syndrome: mechanisms and epidemiology. Thromb Haemost. 2013;110:669–680. 10.1160/TH13-01-0075 [PubMed: 23765199]

68. Roach RE, Cannegieter SC, Lijfering WM. Differential risks in men and women for first and recurrent venous thrombosis: the role of genes and environment. J Thromb Haemost. 2014;12:1593–1600. 10.1111/jth.12678 [PubMed: 25081183]

69. Alvarado CM, Diaz JA, Hawley AE, Wrobleski SK, Sigler RE, Myers DD Jr. Male mice have increased thrombotic potential: sex differences in a mouse model of venous thrombosis. Thromb Res. 2011;127:478–486. 10.1016/j.thromres.2011.01.004 [PubMed: 21296387]

70. Wang L, Miller C, Swarthout RF, Rao M, Mackman N, Taubman MB. Vascular smooth muscle-derived tissue factor is critical for arterial thrombosis after ferric chloride-induced injury. Blood. 2009;113:705–713. 10.1182/blood-2007-05-090944 [PubMed: 18931346]

71. Maugeri N, Brambilla M, Camera M, et al. Human polymorphonuclear leukocytes produce and express functional tissue factor upon stimulation. J Thromb Haemost. 2006;4:1323–1330. 10.1111/j.1538-7836.2006.01968.x [PubMed: 16706978]

72. Cimmino G, Cirillo P. Tissue factor: newer concepts in thrombosis and its role beyond thrombosis and hemostasis. Cardiovasc Diagn Ther. 2018;8(5):581–593. 10.21037/cdt.2018.10.14 [PubMed: 30498683]

73. Lin BH, Sutherland MR, Rosell FI, Morrissey JH, Pryzdial ELG. Coagulation factor VIIa binds to herpes simplex virus 1-encoded glycoprotein C forming a factor X-enhanced tenase complex ori-ented on membranes. J Thromb Haemost. 2020;18:1370–1380. 10.1111/jth.14790 [PubMed: 32145149]

74. Graf C, Wilgenbus P, Pagel S, et al. Myeloid cell-synthesized coagulation factor X dampens antitumor immunity. Sci Immunol. 2019;4(39):eaaw8405. 10.1126/sciimmunol.aaw8405 [PubMed: 31541031]

Essentials

- CD248 is a pro-inflammatory glycoprotein with a cell distribution like tissue factor (TF).
- The potential role of CD248 in modulating thrombosis was previously unexplored.
- Lack of CD248 confers resistance to thrombosis in arterial and venous injury models in mice.
- CD248 enhances tissue factor (TF)-factor VIIa (FVIIa) activation of factor X (FX) by altering the TF-FVIIa-FX complex

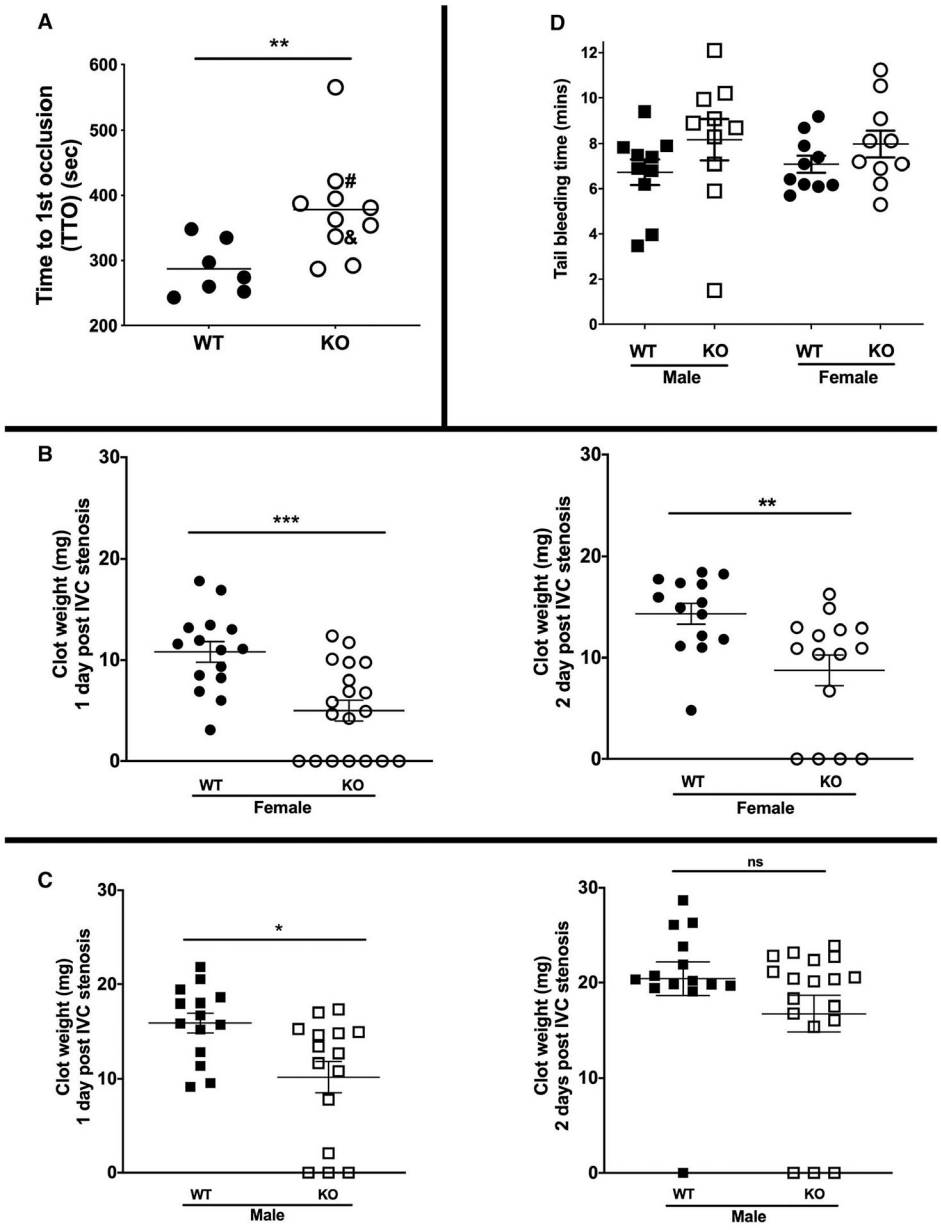

FIGURE 1.

CD248 knockout (KO) mice were protected against macrovascular thrombosis. The thrombotic response of wild-type (WT) and KO mice was assessed using (A) the $FeCl_3$ carotid artery injury model ($n = 7$ WT and 10 KO), and (B, C) the modified inferior vena cava (IVC) stenosis model in females (24 h: $n = 15$ WT and 19 KO; 48 h: $n = 14$ WT and 15 KO) and males (24 h: $n = 14$ WT and 15 KO; 48 h: $n = 14$ WT and 18 KO). In (A), & indicates a mouse in which partial occlusion occurred at 337 s and # indicates a mouse in which transient occlusion occurred at 422 s followed by embolization and resumption of blood flow 10 s later. D, Tail bleeding times were performed in WT and KO mice ($n = 10$ per group per sex). $*P < .05$, $**P < .01$, $***P < .001$

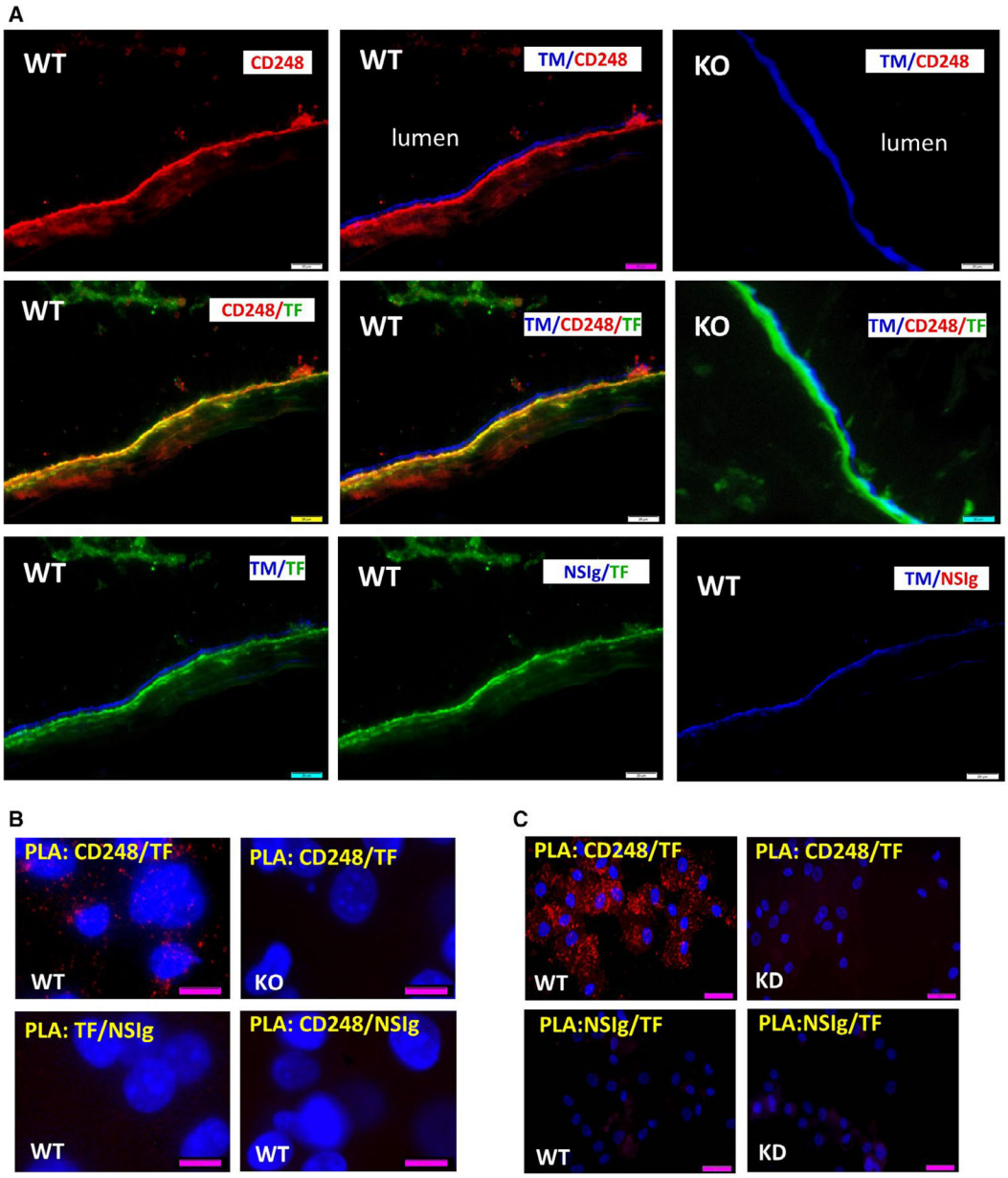

FIGURE 2.

CD248 and tissue factor (TF) are in close proximity to each other. A, Adjacent sections of inferior vena cava (IVC) from 9- to 11-week-old female CD248 wild-type (WT) and knockout (KO) mice 24 h post-IVC stenosis were immunostained with fluorophore-labeled antibodies for thrombomodulin (for endothelial cells; blue), CD248 (red), and TF (green; H-9 antibody) in different combinations. No CD248 was found in the IVC or other tissues from KO mice. Controls with corresponding non-specific antibodies (NSIg) revealed the absence of any signal. Size bar =20 μm. B, C, The proximity ligation assay (PLA) was performed on (B) primary cultured murine vascular smooth muscle cells (mVSMC) from CD248 WT and KO mice (size bar =10 μm) and (C) human VSMC (hVSMC) that have normal expression of CD248 or reduced expression from siRNA knockdown (KD; size bar

=50 μm). The PLA assay was performed using rabbit anti-CD248 antibodies (ProteinTech) and goat anti-TF antibodies, both of which specifically react with the corresponding human and mouse proteins. Pairs of antibodies are indicated in yellow at top of each panel. Pre-immune NSIg matched the concentration and species of the corresponding anti-CD248 or anti-TF antibodies were used. Red dots indicate that CD248 and TF were in close proximity to each other. Results are representative of experiments performed a minimum of five times

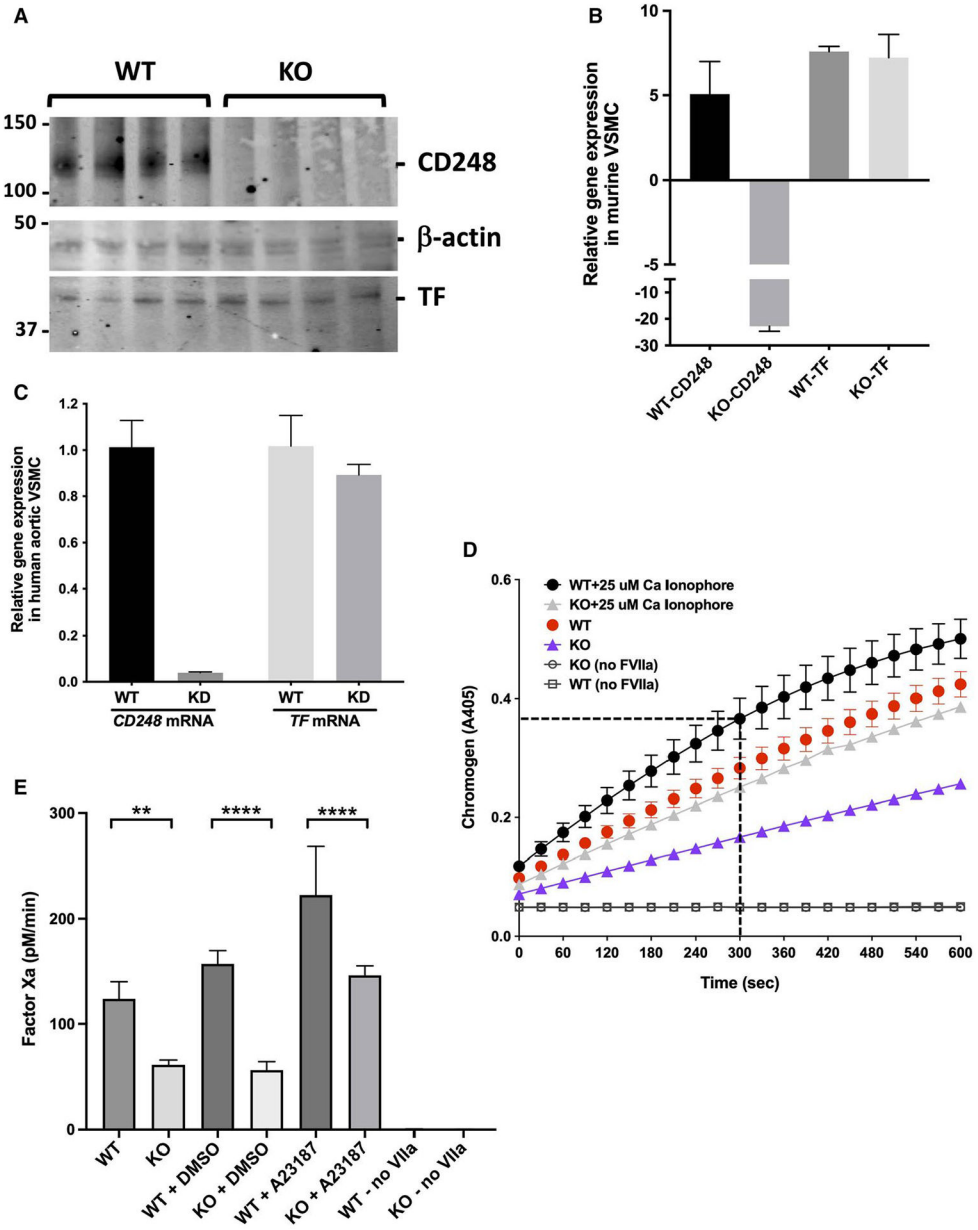

FIGURE 3.

CD248-dependent generation of factor Xa. A, Western blots of cell lysates and (B) qRT-PCR were used to confirm that primary aortic murine vascular smooth muscle cells (mVSMC) isolated from wild-type (WT) and knockout (KO) mice expressed similar amounts of tissue factor (TF, detected with H-9). A, Molecular weight markers (kDa) are shown on the left. B, Results reflect means ± standard deviation (SD), $n = 4$ preparations. Each qRT-PCR measurement was performed in triplicate. C, Human VSMC (hVSMC) were transfected with specific CD248 siRNA oligos (knockdown, KD) or control siRNA oligos (WT) as described in Methods (see Appendix S1). *CD248* gene expression by hVSMC relative to *GAPDH* was suppressed by specific siRNA KD without detectable effects on the gene expression of *TF*. $n = 4$ Independent clones. D, E, mVSMC from WT and KO mice,

10,000 per well, were cultured in 96-well plates and incubated for 30 min with FX and FVIIa as noted in Methods, in the absence/presence of calcium ionophore A23187. A buffer containing dimethylsulfoxide at the same concentration as the diluent for the calcium ionophore was included as a control. D, The FXa thus generated was subsequently measured by dynamic monitoring of cleavage of the specific chromogenic substrate. E, The amount of FXa generated was quantified from the chromogenic assay obtained over 300 seconds (from [D]), using a standard curve with purified FXa. In the absence of FVIIa, no FXa was formed. Experiments were performed in triplicate. Results reflect the means ±SD. $n = 3$ Independent experiments. $^{**}P < .01$, $^{****}P < .0005$

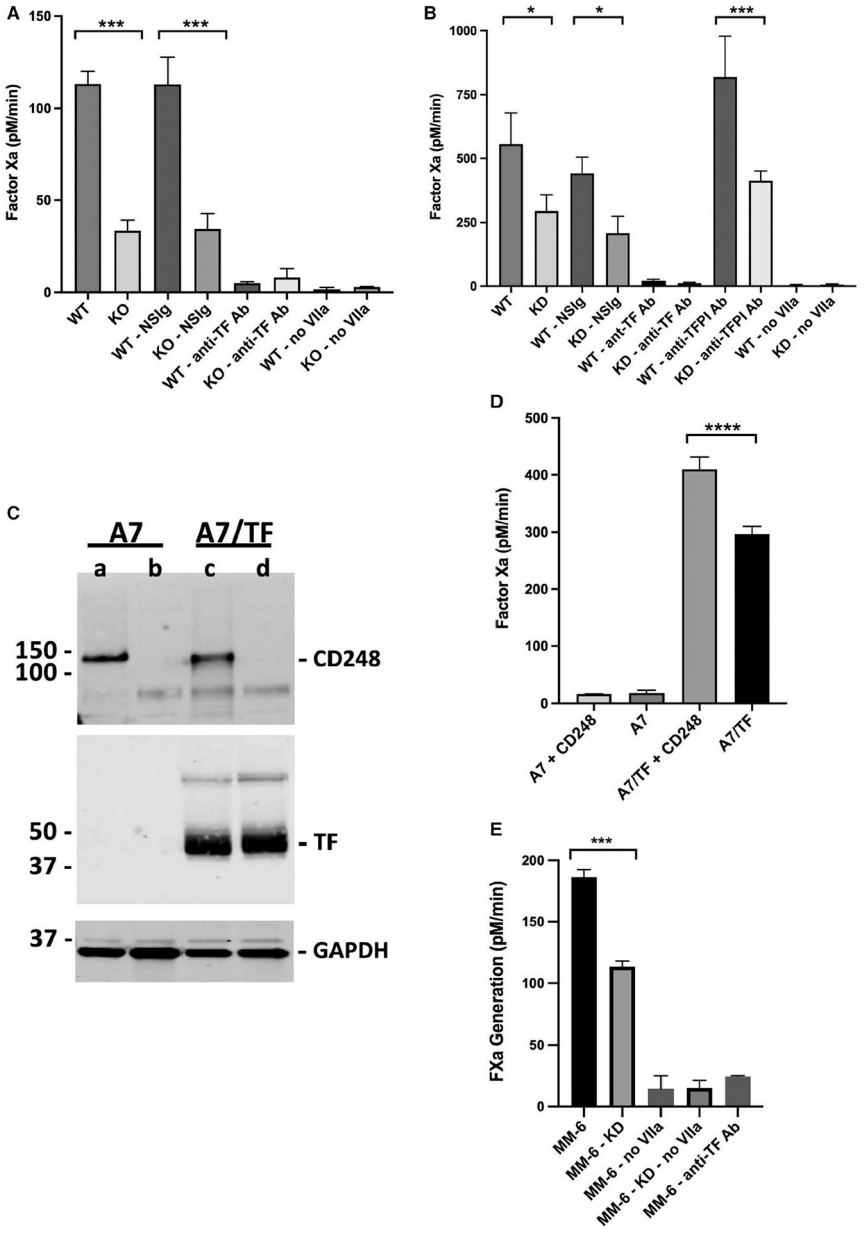

FIGURE 4.

CD248 requires tissue factor (TF) to enhance generation of factor Xa. A, Murine vascular smooth muscle cells (mVSMC) from wild-type (WT) and knockout (KO) mice, 10,000 cells/well, were cultured for 30 min with factor X (FX) and factor VIIa (FVIIa) as noted in Methods in the presence of neutralizing anti-TF antibodies (1H1; 20 μg/ml) or corresponding non-specific antibodies (NSIg). B, Human VSMC (hVSMC) were transfected with control siRNA oligos (WT) or CD248 siRNA oligos (knockdown, KD). Suppressed expression of CD248 by the KD cells was confirmed by qRT-PCR blot (see Figure 3C). The hVSMC were incubated as above with FX and FVIIa (7500 cells per well for 5 min), and FXa generated was quantified. FXa generation was significantly increased in WT vs. KD cells and reduced to almost undetectable levels by the addition of neutralizing anti-TF

antibodies (5G9; 5 μg/ml) to the reaction mixture. NSIg are matched antibodies for 5G9 anti-TF antibodies. The addition of anti-tissue factor pathway inhibitor antibodies resulted in an increase in FXa generated for WT and KD cells, but the significant difference between WT and KD cells was retained. * $P < .05$, *** $P < .001$. Results reflect means ± standard deviation (SD), $n = 4$ experiments. C, A7 and A7/TF cells were transfected with an empty plasmid vector (lanes b, d) or a plasmid vector encoding human CD248 (lanes a, c). Western immunoblots were used to confirm expression of CD248 and that expression of TF by the A7/TF cells was not altered by CD248. Expression of GAPDH confirms equal loading per lane. D, Generation of FXa was quantified on the surface of the A7 and A7/TF cells with/without CD248 expression, upon incubation of FVIIa and FX onto the surface of 10,000 cells per well for 10 min. E, MM-6 cells were transfected with control siRNA oligos or CD248 siRNA oligos (KD), after which FVIIa and FX were incubated with 100,000 cells/well for 5 min to measure FXa generation. In some experiments, the anti-TF antibody (5G9, 20 μg/ml) was added. Non-specific antibodies that matched 5G9 had no effect (not shown). Results reflect the means ±SD. $n = 3$ independent experiments. *** $P < .0005$, **** $P < .0001$

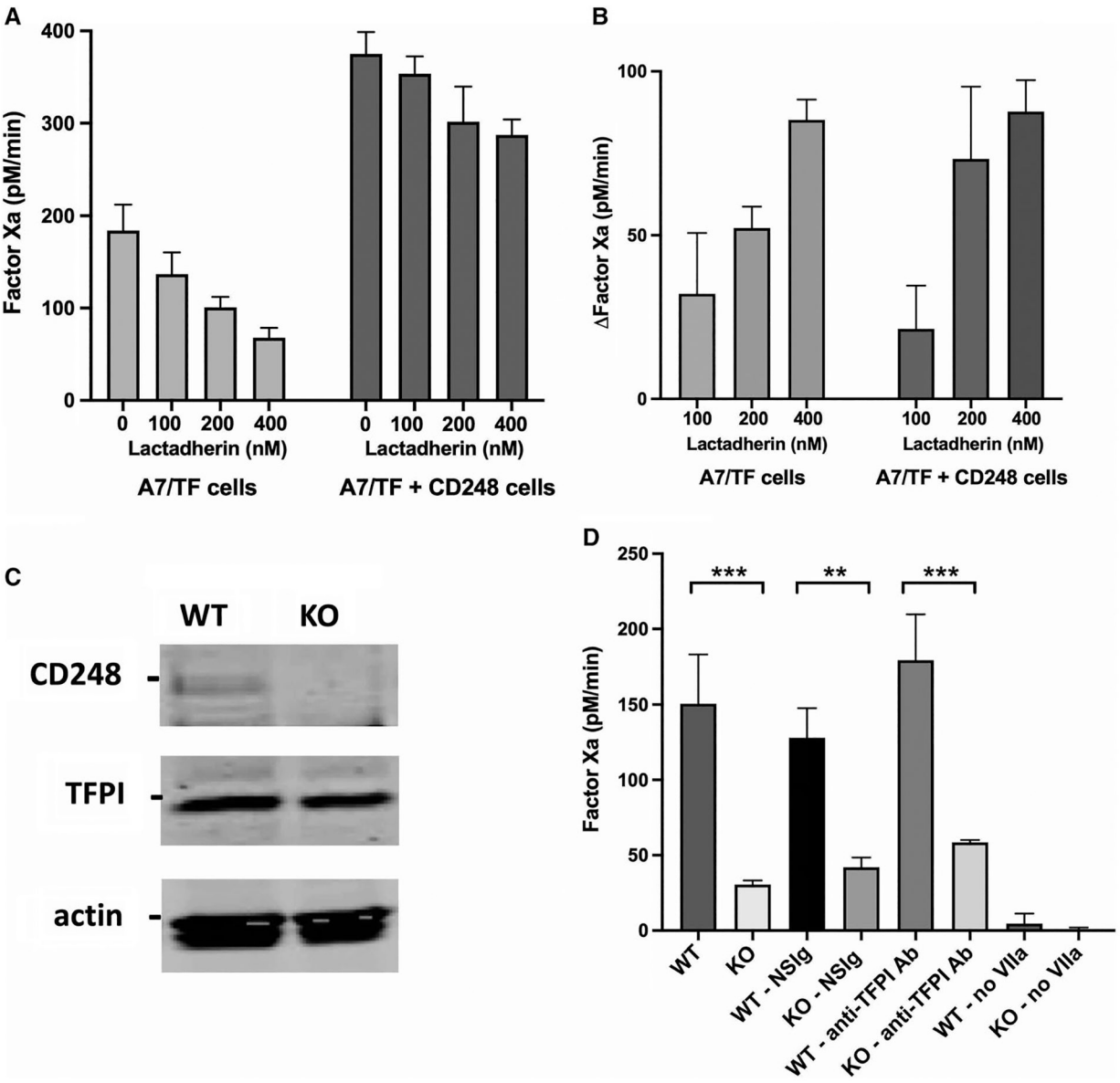

FIGURE 5.

The tissue factor (TF) cofactor activity of CD248 was independent of phosphatidylserine and tissue factor pathway inhibitor (TFPI). A, A7/TF and A7/TF+CD248 cells were preincubated with varying concentrations of lactadherin, after which TF-factor VIIa (FVIIa)–mediated activation of factor X (FX) was quantified (n=3 independent experiments, replicates =3). B, There was no CD248-dependent difference in the absolute lactadherin-mediated suppression of FXa relative to no lactadherin. C, Western blots of lysates of cultured murine vascular smooth muscle cells (mVSMC) from CD248 wild-type (WT) and knockout (KO) mice confirm that the levels of TFPI were not different. D, mVSMC from WT and KO mice were incubated for 30 min (10,000 cells/well) with FVIIa and FX, in the presence or absence of neutralizing anti-TFPI antibodies (5 μg/ml) or corresponding non-specific antibodies (NSIg), after which the generated FXa was quantified. NSIg had no

significant effect on WT and KO cells. The addition of anti-TFPI antibodies resulted in an increase in the amount of FXa generated for WT and KO cells, but the significant difference between WT and KO cells was retained. Results reflect means ± standard deviation, $n = 4$ experiments. $^{**}P < .01$, $^{***}P < .001$

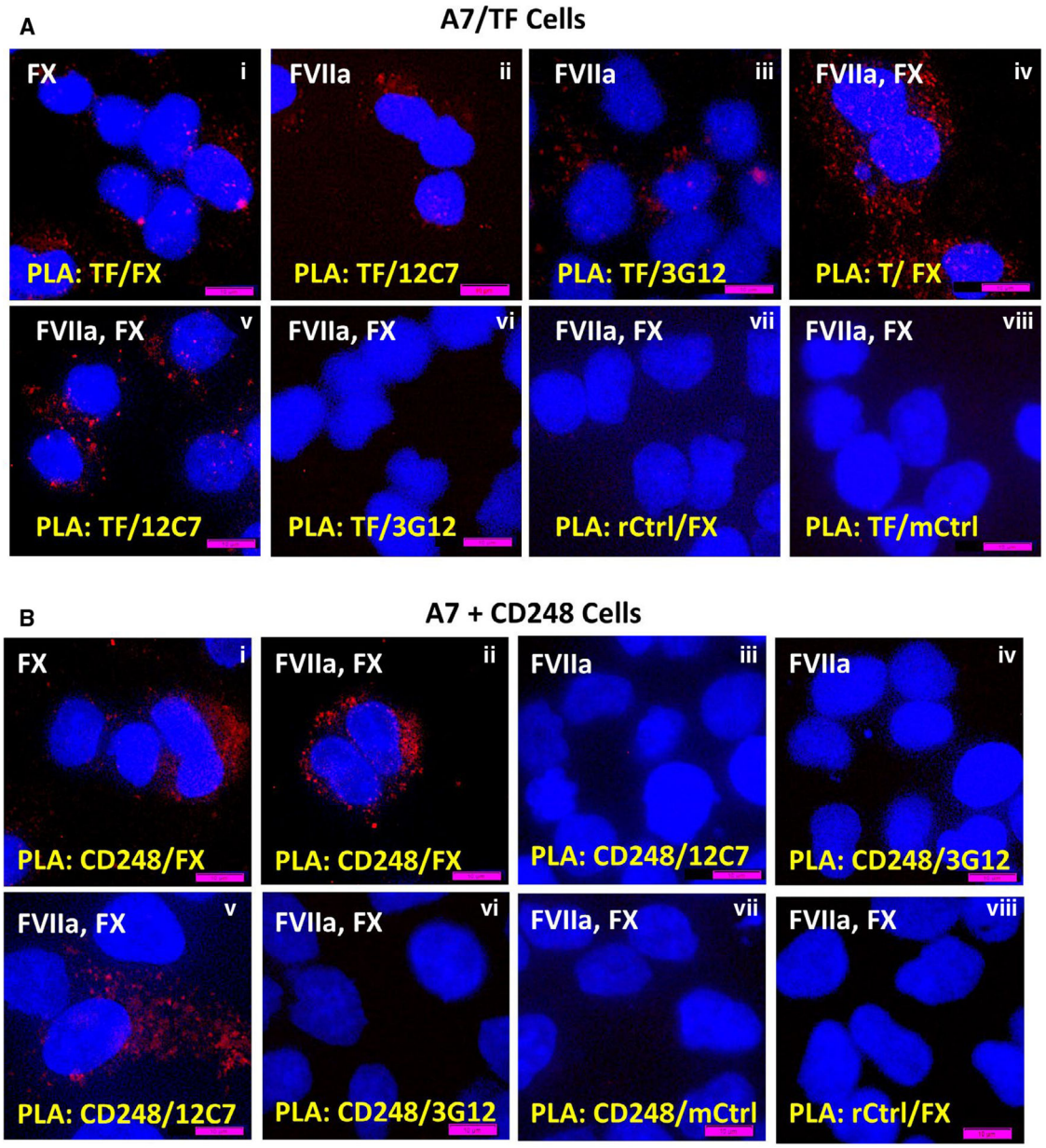

FIGURE 6.

Factor X (FX) is in close proximity to CD248. Proximity ligation assay (PLA) was performed on (A) A7/TF cells and (B) A7+CD248 cells that were preincubated with different combinations of factor X (FX) and factor VIIa (FVIIa), as indicated on each panel in white (upper left corner). PLA was performed with pairs of antibodies as noted in yellow (bottom left corner). Red dots from PLA reflect a close interaction. Anti-FVII/VIIa antibodies were 3G12 and 12C7. mCtrl and rCtrl are non-specific murine monoclonal and rabbit polyclonal antibodies, respectively. Polyclonal rabbit anti-human TF antibody (ab48647) was used in panels A.i-vi, viii. Results are representative of independent experiments performed a minimum of three times. Scale bar =10 μm. See Figure S13 for quantification of PLA signals

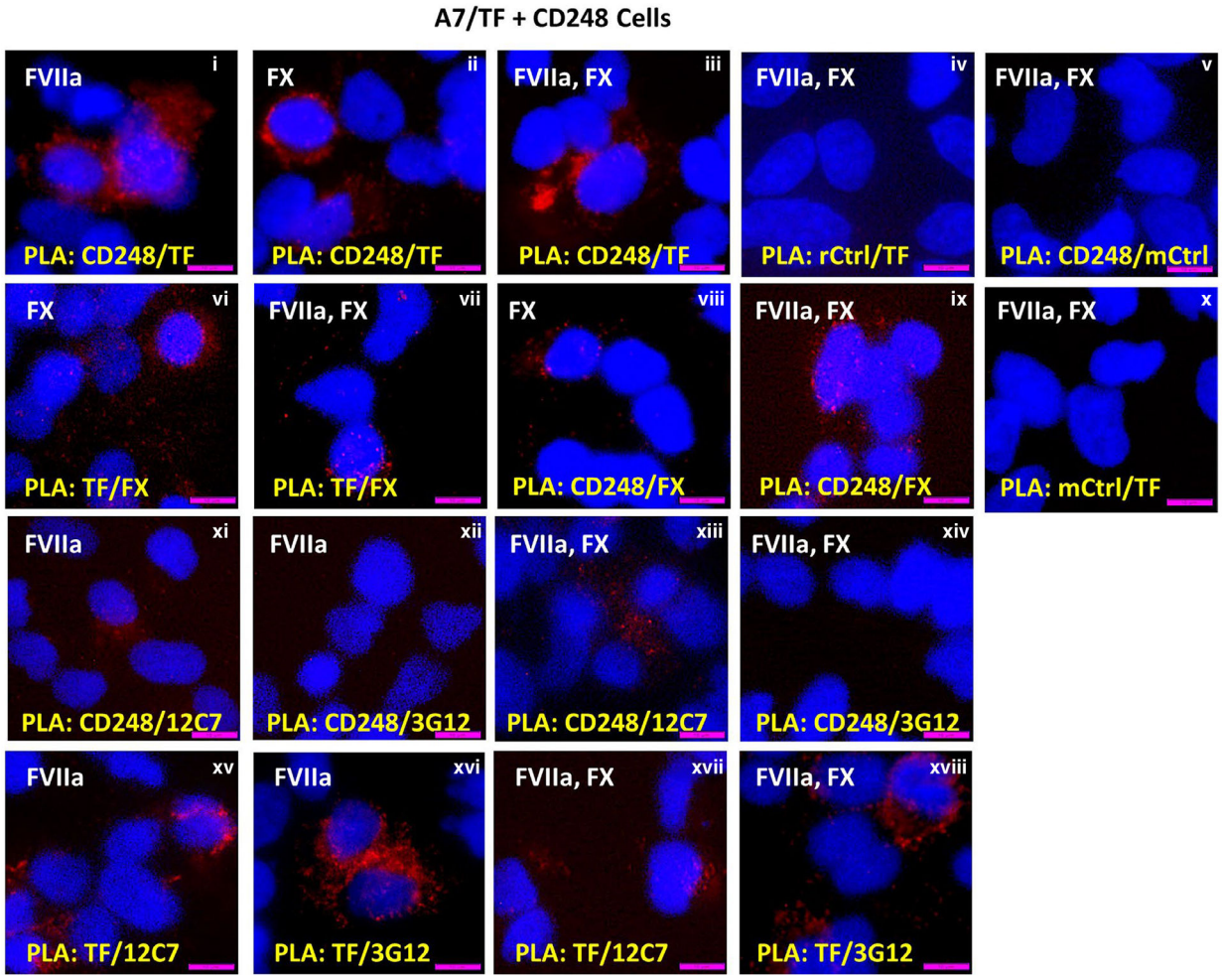

FIGURE 7.

Conformation-dependent anti-factor VII (FVII) antibodies reveal CD248-dependent allosteric changes in tissue factor (TF)-FVIIa–factor X (FX) complex. Proximity ligation assay (PLA) was performed on A7/TF+CD248 cells that were preincubated with different combinations of FX and FVIIa, as indicated on each panel in white (upper left corner). PLA was performed with pairs of antibodies as noted in yellow (bottom left corner). Red dots from PLA reflect close proximity. As in Figure 2B and C, TF and CD248 are in close proximity (panels i--iii). FX is in close proximity to TF and to CD248 in the presence or absence of FVIIa (vi--ix). In the presence or absence of FX, FVIIa is in close proximity to CD248 with anti-FVII/VIIa antibody 3G12 (xi, xiii), but not with 12C7 (xii, xiv). In the presence of CD248, the close TF-FVIIa interaction was observed when the anti-TF antibody was paired with either 3G12 or 12C7 in the absence of FX (xv, xvi) or in the presence of FX (xvii, xviii). Anti-FVII/VIIa antibodies are 3G12 and 12C7. Monoclonal anti-human TF antibody 5G9 was used in panels i-iv. Polyclonal rabbit anti-human TF antibody (ab48647) was used in panels vi-vii, x, xv-xviii. mCtrl and rCtrl are non-specific murine monoclonal and rabbit polyclonal antibodies, respectively. Results are representative of independent experiments performed a minimum of three times. Scale bar =10 μm. See Figure S13 for quantification of PLA signals

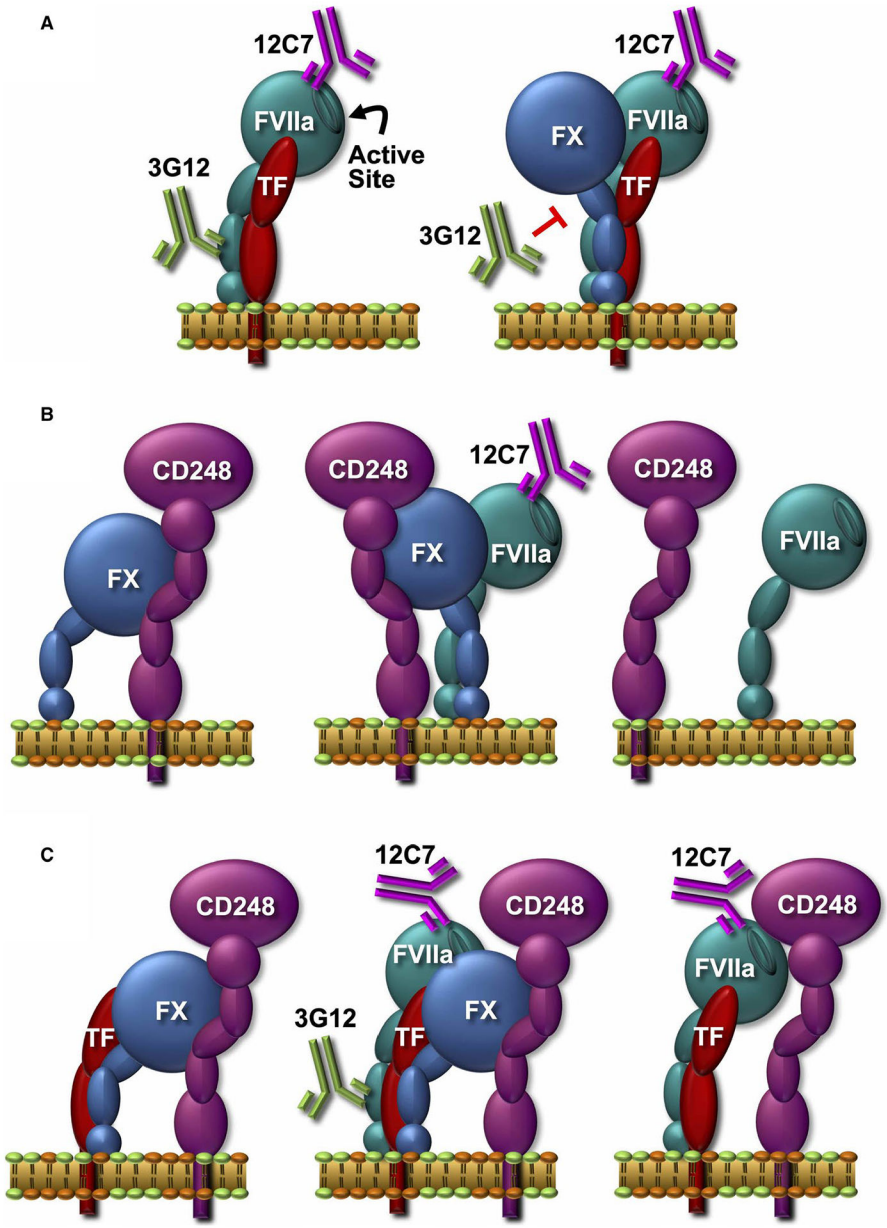

FIGURE 8.

Schematic of interactions between CD248, tissue factor (TF), factor X (FX), and factor VIIa (FVIIa) based on proximity ligation assay (PLA) findings. Schematic representations of the TF–FX-FVIIa–CD248 complex with reference to description of results of PLA studies with conformation specific antibodies. A, On A7/TF cells, the monoclonal antibody 12C7 recognizes a site on the protease domain of FVIIa. The monoclonal antibody 3G12 recognizes an epitope on the light chain of FVIIa, but is blocked when FX is bound to TF (see Figure 6A). B, On A7+CD248 cells, in the absence of TF, FX and CD248 are in close proximity in the presence or absence of FVIIa. However, the CD248–FVIIa–FX complex (center panel) cannot generate FXa. C, Through incompletely defined associations with FX,

CD248 brings the FVIIa active site into more favorable proximity for proteolysis of FX in the presence of TF