

RESEARCH ARTICLE

TLT-1-CONTROLS EARLY THROMBUS FORMATION AND STABILITY BY FACILITATING AIIBB3 **OUTSIDE-IN SIGNALING IN MICE.**

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Abstract

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Key points:

- TLT-1 modulates αIIbβ3 fibrinogen-mediated inside-out signaling 1. while stabilizes outside-in signaling.
- 2. Anti-TLT-1 reduced mortality in the pulmonary embolism model demonstrating therapeutic potential for TLT-1.

Platelets regulate inflammation as well as hemostasis. Inflammatory insults often induce hemostatic function through mechanisms that are not always understood. The triggering receptor expressed in myeloid cells (TREM)-like transcript 1 (TLT-1) is an abundantly expressed platelet receptor and its deletion leads to hemorrhage and edema after lipopolysaccharide and TNF- α treatment. To define a role for TLT-1 in immune derived bleeding we used a CXCL-2 mediated local inflammatory reaction in the vessels of the cremaster muscle of treml1 ^{/-} and wild type mice. Our whole mount immunofluorescent staining of the cremaster muscle demonstrated a 50% reduction in clot size and increased extravasation of plasma molecules in *treml1^{-/-} mice* compared to wild type. We demonstrate that the decreased clotting in *treml1*^{-/-} mice is associated with a 2X reduction in integrin β 3 phosphorylation on residue Y773 after platelet activation, which is consistent with treml1--- mice displaying reduced outside-in signaling and smaller thrombi. We further substantiate TLT-1's role in the regulation of immune derived bleeding using the reverse arthus reaction and demonstrate TLT-1's role in thrombosis using the thromboplastin initiated and collagen/epinephrine models of pulmonary embolism. Thus, the data presented here demonstrate that TLT-1 regulates early clot formation though the stabilization of α IIb β 3 outside-in signaling.

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Introduction:-

The relationship between inflammatory and hemostatic systems is dynamic, and although extensively characterized, the complete relationship is not understood(Herter et al., 2014). TREM-Like transcript 1 (TLT-1) is an α -granular platelet receptor that binds fibrinogen and localizes at the platelet surface upon platelet activation(Washington, 2009; Washington et al., 2002; Washington et al., 2004). TLT-1 is abundantly expressed in platelets(Coxon et al., 2017). Based on the works of Simon et. al. (Simon et al., 2014) and those of Rowley et. al(Rowley et al., 2011), TLT-1 is one of the most abundantly expressed proteins in mouse and human platelets (top 3%). These data suggest that it is more abundant than GP1 β (top 7%), P-selectin, and integrin 2a (each are in the top 5%). However, TLT-1's role in hemostasis or inflammation remains undefined.

Invocation of the Shwartzman reaction in *treml1*^{-/-} mice suggests that TLT-1 controls hemorrhage, neutrophil recruitment, and possibly edema during inflammation and points to a role for TLT-1 in the translation of inflammatory response into hemostatic mechanisms(Washington, 2009). The Shwartzman reaction uses lipopolysaccharide and tumor necrosis factor- \Box to induce an immune response(Brozna, 1990; Stetson and Good, 1951; Stetson, 1951b). In the present study, we evaluate the role of TLT-1 in inflammation and determine that TLT-1 mediates early clot formation enabling α IIb β 3 activation by facilitating downstream outside-in signaling. We further demonstrate the relevance of our finding using inflammatory and hemostatic challenges.

Methods:-

Mice

All mice were maintained under specific pathogen–free conditions at the Animal House Facility. Mice were between 8-10 weeks of age and weighed 20-23 g. Animal care was provided in accordance with the NIH *Guidefor the Care and Use of Laboratory Animals* (publication no. 85-23. Revised 1985).

Reverse Arthus Reaction (RAR)

RAR was completed as described in reference(Goerge et al., 2008). For quantification studies of the inflammatory area, edema and hemorrhage were subjected to quantification as described in references(Klopfleisch, 2013; Sylvestre et al., 1996).

Western blotting

Western blotting was completed as in ref(Washington et al., 2002)

Whole mount stain of cremaster muscle

Anesthetized wild type or *treml1*^{-/-} mice were intra-scrotally injected with CXCL-2 (2.5 \Box g/mL). After 3.5 hours incubation, the cremaster muscle was prepared for intravital microscopy as previously described(Zhang et al., 2001).

Pulmonary embolism

Thromboplastin induced pulmonary embolism (PE) was achieved through the I.V injection of 5 μ L thromboplastin [Sigma] and mice were monitored for 30-minutes for breathing cessation after which mortality was recorded and lungs were collected for histology. Collagen/epinephrine PE was induced as in ref(Rowley et al., 2016) using 64 mg/kg of collagen and 5.4 mg/kg of epinephrine and anti-TLT-1 (100ug/100ul) 20 minutes prior to the collagen/epinephrine injection.

Statistics

Paired, two-tailed Student's *t* test analysis available in Prism, version 7.01 (Graph-Pad Software) was applied to evaluate statistical differences for 2 groups. P < 0.05 was considered statistically significant.

Results and discussion:-

To gain insights into TLT-1 function we injected CXCL2 in to the scrotum of wild type (*WT*) and *treml1^{-/-}* mice to visualize the inflammatory changes in the vasculature. CXCL2 attracts neutrophils and leads to clotting in the vasculature. We evaluated the cremaster muscle at 3, 4, and 5 hours after injection and found 3 - 4 hours to be the most insightful. WT mice exhibited more and larger clots with an average area that was approximately twice that of those in the *treml1^{-/-}* mouse (11.68±0.99 μ M² vs. 5.59±0.80 μ M²; figure1a). These results suggested that TLT-1 is responsible for the preliminary stages of clot formation after inflammatory challenge. We also stained for complement factors H (155kD) and I (88kD) to evaluate plasma leakage and found that CXCL2 caused a striking

mobilization of plasma factor H and I from vasculature into the cremaster muscle of $treml1^{-/-}$ mice, which was not observed in WT mice (figure 1b). These results support the concept that TLT-1 regulates edema as well.

To determine the basis for the small size of TLT-1^{-/-} thrombi and inhibition of clot retraction, we examined the kinetics of α IIb β 3 surface expression after low dose thrombin activation. Quantification of mean fluorescence intensity demonstrated that, *WT* platelets exhibited a time dependent increase of α IIb β 3 and TLT-1 surface expression. Although not significant, α IIb seemed to decrease on *treml1*^{-/-} platelets (figure1c-e). These results suggested that TLT-1 continuously participates in agonist-induced signaling that leads into α IIb β 3 stabilization on the surface. *Treml1*^{-/-} mice demonstrate reduced clot retraction compared to WT (figure 1f) and collectively our results suggest that TLT-1 affects α IIb β 3 function. We hypothesized that TLT-1 stabilizes clot formation by facilitating outside-in signaling of α IIb β 3. To test this hypothesis, we examined β 3^{Y773} phosphorylation (p β 3^{Y773}) over time in WT and *treml1*^{-/-} platelets. p β 3^{Y773} is an indicator of early outside-in signaling(Unsworth et al., 2017; Xi et al., 2006). Consistent with published data(Unsworth et al., 2017) we found by 5 minutes, p β 3^{Y773} increased in *WT* mice. *Treml1*^{-/-} platelets demonstrated ~2X less p β 3^{Y773} at 5 minutes and less over the 20-minute period (*p*=0.03; figure 1g). Addition of an anti-TLT-1 monoclonal antibody(Manfredi et al., 2018) yielded a significant decrease in p β 3^{Y773} accumulation and supports the idea that TLT-1 stabilizes α IIb β 3 on the platelet surface by mediating integrin outside in signaling.



Figure 1

TLT-1 controls later stages of platelet activation and function by facilitating aIIbβ3 outside-in signaling. (a) Upper panel - Representative Z-stack images of whole mount cremaster muscle showing clot formation in wild type and treml1^{-/-} mice (red: CD41-platelets; green: GR1-neutrophils; yellow: anti TLT-1; blue: DAPI; n=4 mice per group). The red scale bar = 50 μ M. Lower panel - Quantified thrombus area in wild type and treml1^{-/-} mice. Images were processed using a Nikon Confocal Microscope and analyzed using NIS element viewer 4.20 magnification 40X, (****p<0.001). (b) Confocal analysis of CXCL-2 treated whole mount cremaster muscle showing plasma leakage

in treml1^{-/-} mice compared to wild type mice (green: TLT-1, red: complement factor H, yellow: complement factor I, blue: DAPI). The red scale bar = 100 μ M (c) Time course of aIIb (CD41) and TLT-1 expression in wild type and treml1^{-/-} platelets. These are representative results of more than 5 experiments. The red scale bar = 10 μ M (d) Quantification of MFI (mean fluorescence intensity) of CD41 (comparing the kinetics of surface expression of CD41 between wild type and treml1^{-/-} mice, ***p≤0.0001). (e) Quantification of MFI of surface expression of TLT-1 in wild type platelets, ***p≤0.0001 student t-test. In d and e n>95 individual platelets measured (f) Clot retraction using 3x 10⁸ cells/ml and 0.2 units of thrombin comparing WT and treml1^{-/-} mice. (g) Left panel - representative western blot of protein lysates of platelets from wild type and treml1^{-/-} mice activated with 0.025 units thrombin and probed with anti-phospho $\beta 3^{1773}$ (Novus Biologicals) at different time points, anti TLT-1 from ref(Washington et al., 2004), anti-rabbit (Jackson Immunoresearch) and anti- β actin (Sigma) controls demonstrate differences in phosphorylation at different time points. Right panel- quantification of western blots (n=3)

To extend the applicability of TLT-1's mechanism beyond LPS mediated inflammation seen in the Shwarztman reaction, we used the Reverse Passive Arthus reaction which elicits a localized, dermal, immune response using immune complexes(Stetson, 1951a). Evaluations of the skin lesions show that wild type mice exhibited mild petechia surrounding the injection area (figure 2a). Blinded histological grading of the lesions (scale 1 - 4) revealed that the *treml1*^{-/-} mice have a significant increase in bleeding and associated edema when compared with their wild type counterparts (score= 2.4 ± 0.23 vs 1.3 ± 0.21). Thus TLT-1 has a broad impact in translating the immune challenge into hemostatic response.



Figure 2

TLT-1 deficiency associates with increase survival in a pulmonary embolism model but cause poor hemostasis on inflammation. (a) Top-macroscopic evaluation of wild type and treml1^{-/-} mice subjected to the RPAR and sacrificed at 4 hours, Bottom -hematoxylin and eosin (H&E) staining of RPAR lesions, blue line equals 100µM. Right panel - blinded histological quantification of hemorrhage from wild type and treml1^{-/-} mice (n=3 per group, **p \leq 0.001, student t-test). (b) Left panel - mortality associated with I.V. injection of either 0.9% saline or 5µl/kg thromboplastin and monitored over a 30-min observation period. Treml1^{-/-} mice were found to be protected from thrombosis compared to the littermate heterozygous mice. Middle panel - H&E histologic analysis of the lungs after thromboplastin treatment. Right panel - quantification of the clots in the H&E analysis demonstrated a significant decrease in clot formation in the treml1^{-/-} mice compared to treml1^{-/-} mice (treml1^{+/-} n=6, treml1^{-/-} n=9, control

n=3; p=0.009). (c, e) Kaplan-Meier curves demonstrating the mortality associated with I.V. injection of 64 mg/kg of collagen and 5.4 mg/kg of epinephrine after 17 minutes. (e) mice were given anti-TLT-1 (100ug/100ul) 20 minutes prior to the collagen/epinephrine injection. Animals surviving the 17 minutes were considered survivors (wild type n=11, treml1^{-/-}, n=10; p=0.0025). (d and f) Mice from d and f were perfused with Evans' blue (500ul) after onset of respiratory arrest or those mice that survived the study; lungs were then excised, the treml1^{-/-} mice showed almost complete perfusion (blue) while in the wild type the perfusion was impaired (pink) compared to wild type (wild type n=4, wild type + antibody- n=4; p=0.006).

To investigate if TLT-1 responded to hemostatic triggers, we elicited pulmonary embolism (PE) using two different experimental approaches, both of which cause widespread formation of micothrombi in the lungs(Renné et al., 2005). First, we used an I.V. injection of thromboplastin, which mimics tissue factor exposure by activating coagulation through the conversion of pro-thrombin to thrombin(Zacharski and Rosenstein, 1979). Thromboplastin administration caused a significant increase in mortality in *treml1*^{+/-}heterozygous littermates compared to *treml1*^{-/-} mice (65% vs 100%; figure 2b). Histological analysis revealed a reduction of clot formation in *treml1*^{-/-} mice compared to *treml1*^{+/-} mice. Moreover, quantification compared to *treml1*^{-/-} mice after thromboplastin treatment demonstrated a 40% decrease in clot formation compared to *treml1*^{-/-} mice (p<0.009). Second, we injected I.V. collagen/epinephrine which causes direct platelet activation(Mustonen and Lassila, 1996) (Renné et al., 2005). Administration of collagen/epinephrine caused a significant increase in mortality in *WT* compared to *treml1*^{-/-} mice (p=0.025; figure 2e). The infusion of Evans' blue after onset of respiratory arrest demonstrated almost complete blockage of perfusion on *WT* compared to *treml1*^{-/-} mice (figure 2d, f). To our knowledge, this is the first in vivo demonstration after hemostatic challenges as well as immune challenges.

Here, we demonstrate that TLT-1 contributes to α IIb β 3 inside-out signaling and mediates the phosphorylation of the β 3 subunit, resulting in enhanced outside-in directed clot stabilization and vessel occlusion. It is possible that TLT-1 may play a receptor scaffolding role, recruiting key signaling molecules to the platelet surface. Like α IIb β 3, TLT-1 is a fibrinogen receptor(Washington, 2009). Where α IIb β 3 must convert to its active form, there is no known TLT-1 activation sequence and consequently TLT-1 should bind fibrinogen upon surface expression. Therefore, TLT-1 could sequestrate fibrinogen on the platelet surface and/or abet fibrin formation. Fibrin(ogen) sequestration would be important in forming a hierarchical thrombus core(Stalker et al., 2013), controlling fibrin deposition, and regulation of clot size(Owaynat et al., 2015).

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Author Contributions:-

JMO, FR are responsible for the cremaster studies; FR, SO are responsible for the RAR studies; JMO, LR, MKB, MTR are responsible for the PE studies; JMO, BM, NL are responsible for confocal images and analysis; JMO, NL, AVW are responsible for the western blots; and JMO and AVW are responsible for the writing of the manuscript and overall direction of the project

Disclosures:-

Nothing to disclose

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