TMEM16F ENHANCES PROCOAGULANT ACTIVITY AND THROMBOSIS THROUGH PLATELET MICROPARTICLE GENERATION

by

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A thesis submitted to the Faculty of the University of Delaware in partial fulfillment of the requirements for the degree of Master of Science in Biological Sciences

Fall 2016

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ACKNOWLEDGMENTS

I would like to express my sincerest gratitude to my advisor, Dr. Donna Woulfe for her unwavering support, mentorship and guidance on the completion of this project. Her immense dedication to students and her passion for research is beyond measure! She is the perfect advisor any graduate student could have! I also want to acknowledge her patience and the flexibility she creates in allowing me to develop professionally and to think independently as a researcher. It is because of her superb training and enthusiasm that I have cultivated an insatiable passion for thrombosis research.

I want to thank my committee members – Dr. Terry Papoustakis and Dr. Gary Laverty – for their support, suggestions and insights into my project. Their contributions have helped to shape my scientific skills.

My sincerest gratitude goes to my former and present lab members – Dr. Aasma Khan, Dr. Stephan Lindsey, Fawziya Barnawi, Cara Giordano, Martin Ciskanik, Pernilla Mpasi and Omar Montero. I specially want to acknowledge Aasma for her initial mentoring and help with experiments when I joined the lab and Stephan for his assistance with performing and analyzing experiments on flow cytometry.

I owe a debt of gratitude to Minh Pham, Julie Myers and Dr. Gwen Talham from the animal facility. Minh was exceptional in performing and training me in

mouse retro-orbital blood collection for my project. Julie and Gwen provided phenomenal care of our animals and were always available and willing to go above and beyond to make sure everything regarding our mice was going well. I would like to acknowledge Dr. Mary McLane from the Medical Laboratory Science Department for performing CBC analysis on the mice samples.

I am grateful to Betty Cowgill from our department who has been instrumental in keeping me on track with all administrative-related issues and for her generous support over the years.

Finally, I thank my parents, siblings, relatives and close friends for their unwavering love and dedication. Their continuous immeasurable support has been the cornerstone of my life!

*****I DEDICATE THIS THESIS IN HONOR OF ALL THOSE WHO HAVE LOST THEIR LIVES DUE TO THROMBOSIS*****

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ABSTRACT

Increased procoagulant plasma microparticles are detected in prothrombotic and hypercoagulable states and may contribute significantly to thrombosis, the single greatest cause of death worldwide. Platelet microparticles are strongly procoagulant because of the exposed membrane phosphatidylserine (PS) that interacts with the coagulation cascade enabling rapid, localized and efficient thrombin generation subsequently resulting in fibrin formation and further platelet activation. Recently, TMEM16F, a novel putative 8-transmembrane spanning calcium-activated calcium-permeable channel, emerged as an essential component required for calcium-dependent PS exposure on platelet membranes, procoagulant activity and thrombosis, yet the role of TMEM16F in microparticle formation and subsequent TMEM16F-dependent microparticle generation on thrombosis has been unclear.

To determine whether TMEM16F is required for optimal microparticle formation and PS exposure, platelets from mice lacking (TMEM16F KO) or expressing TMEM16F (WT) were treated with physiological agonists or calcium ionophore and microparticle numbers and PS exposure on platelets were assessed by flow cytometry. TMEM16F KO platelets displayed an impaired ability to generate optimal PS+ and total microparticles and also PS exposure in response to either thrombin and collagen or A23187. Moreover, both CD41+ (platelet- and

megakaryocyte- derived) and total microparticles in healthy circulation were lower in TMEM16F KO mice compared to WT mice, underscoring the physiological requirement for TMEM16F in microparticle formation *in vivo*.

It was demonstrated that TMEM16F-dependent platelet microparticles are important for procoagulant activity and thrombosis, since both platelet-rich plasma (PRP) and platelet-poor plasma (PPP) from TMEM16F KO mice had diminished thrombin generation compared to those isolated from WT mice. Subsequent addition of platelet-derived microparticles to either TMEM16F-deficient PRP or PPP completely restored thrombin generation to the level of that observed in WT PRP. Furthermore, TMEM16F KO mice displayed defective thrombosis *in vivo* using a $FeCl_3$-injury to the carotid artery model and the thrombotic defect was overcome with injection of isolated platelet microparticles, underscoring the requirement of TMEM16F-dependent platelet microparticles for thrombosis. Notably, number of platelets, leukocytes and erythrocytes in addition to platelet aggregation responses remained intact in TMEM16F KO mice, though mean platelet volume was slightly higher compared to WT mice, suggesting that loss of TMEM16F does not interrupt the steady-state levels of platelets and other circulating cells or platelet aggregation pathways, but platelets may be larger on average because of reduced microparticle formation.

Taken together, these data indicate that TMEM16F is required for platelet microparticle formation and that TMEM16F-dependent platelet microparticles subsequently play a role in procoagulant activity that can contribute to thrombosis.

Chapter 1

INTRODUCTION

1.1 Targeting Procoagulant Microparticles for Novel Targeted Antithrombotic Therapies

Thrombosis is a pathologic state characterized by formation of an inappropriate thrombus in the vascular lumen leading to occlusion and subsequent disruption of normal blood flow. Manifestation of such pathological thrombus in an artery (arterial thrombosis) is the primary underlying cause of heart attack and stroke, placing these diseases among the leading cause of death and disability in the United States of America[1-4] and around the world[5-8]. Similarly, thrombus formation in a vein (venous thrombosis) results in high mortality and morbidity and presents as deep vein thrombosis (DVT) and pulmonary embolism (PE)[9-11]. Over the years several efficacious antithrombotic agents have been developed and routinely used in clinical practice[12-15], however these therapeutics carry a significant bleeding risk as they strongly interfere with normal platelet and coagulation responses in hemostasis[16-19]. The goal therefore is to identify and modulate biological targets that selectively bolster thrombosis while leaving hemostasis uncompromised. With this in mind, procoagulant microparticles have emerged as a novel target for thrombosis as they have been shown to be elevated in plasma of patients in prothrombotic and hypercoagulable states[20-22],

and in various diseases that carry a significant thrombotic risk, such as diabetes and cancer[23,24]. Consequently, it is essential to understand the generation of these microparticles and their contribution to coagulation and thrombus formation with the goal of ultimately informing development of drugs to reduce their formation and release, leading to more targeted and safer therapy for thrombosis.

1.2 Introduction to Hemostasis and Thrombosis

Hemostasis is a normal physiological process designed to keep blood circulating in fluid state throughout an intact cardiovascular system and to swiftly form a localized clot upon vessel injury to prevent blood loss. Tight control of hemostasis is paramount[25,26], as impaired hemostasis results in hemorrhage while dysregulated and accelerated hemostasis yield thrombosis – formation of occlusive thrombus inside the blood vessel. Though thrombosis and hemorrhage affect the general population, thrombosis occurs significantly greater in the population at risk of dying.[4,27]. The two major constituents[28,29] of physiological and pathological thrombus are 1) activated platelets, the cellular component and 2) fibrin, the plasma proteinaceous component. Platelets, being the smallest (2-4 μm) of the three blood cells, are anucleated as a result of cytoplasmic fragmentation of megakaryocytes in bone marrow[30,31]. Upon release into bloodstream, platelets circulate for approximately 7-10 days and are discoidal in its quiescent form but acquire a contractile spiky phenotype when fully activated. In hemostasis and thrombosis, platelets not only aggregate resulting in a platelet (or hemostatic) plug, but also provide a negatively

charged membrane surface for accelerated, localized and efficient coagulation[32]. Coagulation is controlled by several serine proteases in plasma known as coagulation factors that are synthesized predominantly by liver. These factors participate in an exquisitely orchestrated enzymatic reaction generating thrombin on the surface of platelets, which further stabilizes the platelet-rich thrombus[33-35]. Importantly, both platelets and coagulation factors are consistently kept quiescent in a healthy intact vasculature enabling blood to remain thrombus-free, but are rapidly activated locally at the site of vessel injury and in other thrombophilic conditions[25,26,36].

1.3 Normal Blood Flow in Intact Vasculature

Ordinarily, blood circulates in a clump-free liquid state within an intact vasculature, mediated by the uninjured endothelium exerting antiplatelet, anticoagulant and fibrinolytic activities[25,37,36.] From these unmarred endothelial cells, prostacyclin (PGI_2) and nitric oxide (NO) are produced and released into circulation preventing platelets from activating and maintaining vessel patency[38]. Additionally, soluble platelet agonist adenosine diphosphate (ADP) is degraded by ecto-ADPase CD39 released from the endothelial cells[39]. Similarly, coagulation is inhibited by healthy endothelium[40] via 1) expression of membrane-bound thrombomodulin which binds residual circulating thrombin subsequently enabling activation of proteins C and S thereby leading to inactivation of FVa and FVIIIa[41] and 2) expression of heparin-like molecules that bind and activate antithrombin III resulting in inactivation of thrombin, FXa, FIXa, FXIa and FXIIa[42]. Furthermore, the healthy endothelium 1) produces

tissue plasminogen activator (t-PA) that degrades unnecessary fibrin dissolving undue clot[43] and 2) acts as a physical barrier between blood and the subendothelial matrix, securely protecting blood from exposure of collagen, von Willebrand Factor (vWF) and tissue factor which are all influential initiators of clot formation[29].

1.4 Formation of Platelet Plug

Upon vascular injury, von Willebrand Factor (vWF) - an immensely large multimeric protein - is released from the damaged endothelium and attaches to the subendothelium. This vWF, later cleaved by ADAMTS-13, acts as a "sticky glue" for nearby circulating platelets to adhere to the exposed subendothelial surface via glycoprotein GPIb-V-IX complex on platelet membranes[44,45]. Adherent platelets, cemented by vWF and in contact with subendothelial collagen are activated weakly by GPIb-V-IX[46,47] and strongly by platelet collagen GPVI receptors[48,49]. Although collagen generally binds to GPVI with low affinity, this interaction and signal transduction are amplified through potentiation by another collagen receptor $\alpha_2\beta_1$[50], GPVI receptor dimerization and vWF bound GPIb-V-IX[51]. Activated platelets transmit complex intracellular signals that lead to many changes[52]. Most visibly, is that activated platelets change their shape through rearrangement of actin and myosin cytoskeletal proteins – platelets essentially convert from a classically discoidal morphology to a swollen phenotype that eventually transform into long pseudopod-like structures[53]. Besides, activated platelets release ADP in addition to other molecules, such as serotonin and calcium from their δ (dense) granules[54-56]. ADP is a

platelet agonist that binds P2Y1 and P2Y12 G-Protein Coupled Receptors (GPCRs) on surfaces of neighboring platelets leading to platelet recruitment and activation[57,58]. Additionally, activated platelets metabolize and release TXA_2 through PLA_2-mediated production of arachidonic acid and its subsequent conversion by cyclooxygenase (COX) -1[59,60]. TXA_2 also recruits and activates nearby platelets through surface-embedded GPCRs[61]. Ultimately, signaling from platelet agonists (vWF, collagen, ADP, TXA_2 and thrombin generated from coagulation cascade) reversibly[61] position $\alpha_{2b}\beta_3$ integrin (GPIIb IIIa) - the most numerous platelet glycoprotein - into its active configuration[63,64]. Once activated, $\alpha_{2b}\beta_3$ binds plasma fibrinogen in the presence of calcium and is the fundamental mechanism for platelets to clump to each other thus forming a platelet plug **(Fig. 1).**

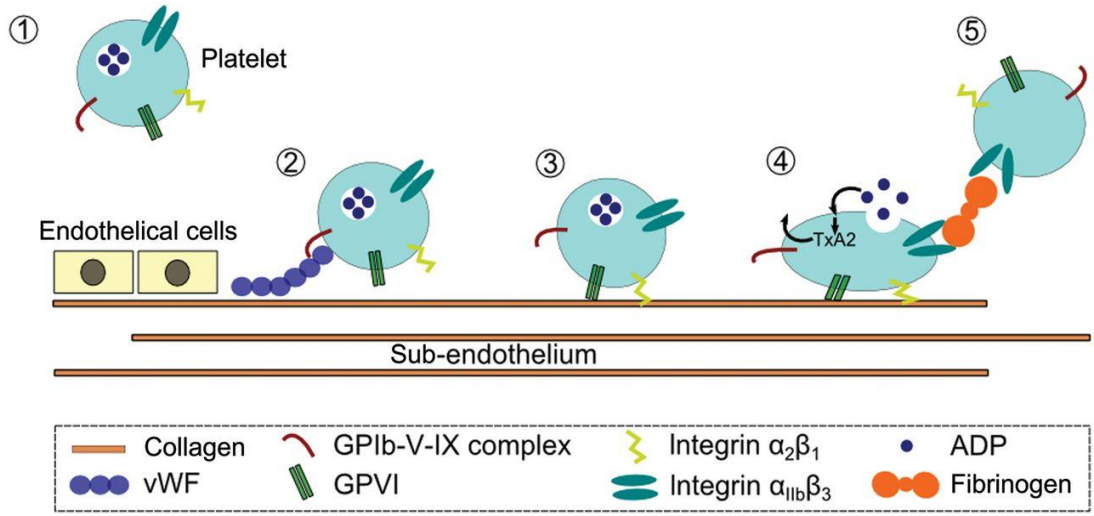


Fig. 1: Steps involved in vascular injury-induced platelet plug formation. (1) In an intact vasculature, platelets are quiescent and circulate in a virtually non-adhesive fashion. (2) Upon vascular injury, vWF released from damaged endothelium binds circulating platelets via GPIb-V-IX complex. (3) vWF-bound platelets interact with exposed collagen in subendothelium and become activated through collagen receptors - GPVI and $\alpha_2\beta_1$. (4) Activated platelets change shape (by spreading initially), secrete soluble agonists ADP and TXA_2 (to activate and recruit neighboring platelets) and expose $\alpha_{2b}\beta_3$ to bind fibrinogen. (5) Finally, platelet plug is maintained through fibrinogen binding among activated platelets. (Image adapted from van der Stoep *et al*.[65]).

1.5 Platelet Procoagulant Activity

Activated platelets do not only aggregate during thrombus formation, but also directly regulate and localize coagulation by externalizing phosphatidylserine (PS) on their membranes[32,66] and releasing some of their PS+ membrane vesicular fragments called microparticles[67-70]. PS, a negatively charged (acidic) membrane phospholipid, binds to vitamin-K dependent γ-carboxylated regions of prothrombin (FII), FX, FIX and FVII in the presence of calcium thus enabling augmented and anchored catalytic activity of these factors[71]. Moreover, FVa and FVIIIa (cofactors of FX and FIX respectively) require and physically interact with PS surfaces, to dramatically enhance tenase and prothrombinase activities **(Fig. 2)**[72,73]. Though trace amounts of thrombin can still be formed in a PS-independent fashion, thrombin generation is expedited maximally and efficiently on PS+ membranes thereby facilitating fibrin deposition on the aggregated platelets in order to strengthen the plug. This platelet procoagulant response is essentially the connection between platelet plug formation and blood coagulation.

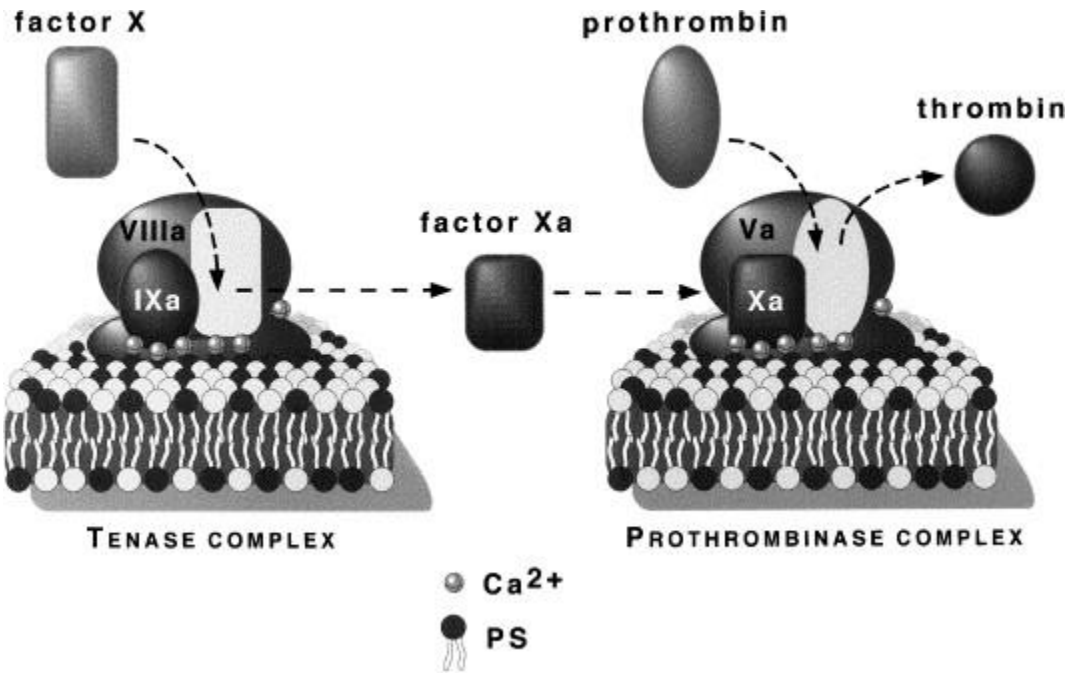


Fig. 2: Platelet procoagulant activity. Tenase complex consisting of FIXa, FVIIIa and calcium is rooted on PS-exposing membrane and allows FX to be activated (FXa). Subsequently, FXa, FVa and calcium form the prothrombinase complex on PS-exposing membrane enabling prothrombin to be rapidly and effectively cleaved with resulting thrombin. (Image adapted from Zwaal *et al*.[74]).

1.5.1 Platelet Microparticles

Principally defined as heterogeneous and small (0.1-1 μm) membrane bound vesicles, circulating microparticles are generated from the plasma membrane of several cell types undergoing activation[75,76], including platelets, and are increasingly shown to be mediators in many cell communication processes such as coagulation and inflammation[68,77-81]. In addition to size, microparticles can be distinguished based on detection of identical cell surface receptors of their parent cells[82]. Notably, a low

quantity of microparticles is normally present in a healthy circulation with over 80% of those microparticles stained positive for CD41 (α_{2b} component of integrin $\alpha_{2b}\beta_3$) suggesting origins from platelets and megakaryocytes[83,84]. However, the number and populations of microparticles can alter dramatically in various pathological conditions[85]. In the context of prothrombotic and hypercoagulable states[20,21], as well as diseases that carry a thrombotic risk[23,24], there is growing body of evidence that procoagulant microparticles are remarkably increased and therefore could have substantial contributions to accelerated coagulation[86-89]. Since activated platelets are central to thrombus formation and are required for coagulation to proceed rapidly and efficiently[32,66], the contributions of microparticles derived from platelets are explored.

The precise cellular mechanisms of platelet microparticle formation remain an enigma, however agonists that elevate and sustain intracellular calcium such as thrombin and collagen, or calcium ionophores, lead to microparticle production **(Fig. 3)**[90-92]. Generally, microparticles are formed upon calcium-dependent PS exposure on the activated platelet outer membrane, and as such these microparticles also have exposed PS on their outer membranes exhibiting strong procoagulant activity[68,21] – even dramatically higher (50- to 100- fold) procoagulant activity than activated platelet membranes[93]. Another important component in microparticle formation is that increased cytosolic calcium in platelets also activates calpain resulting in cleavage of cytoskeleton and budding of plasma membrane[94]. Still other components such as membrane cytoskeleton adhesion[94], membrane PIP2 levels[95], among others[96,97] have a role in microparticle formation.

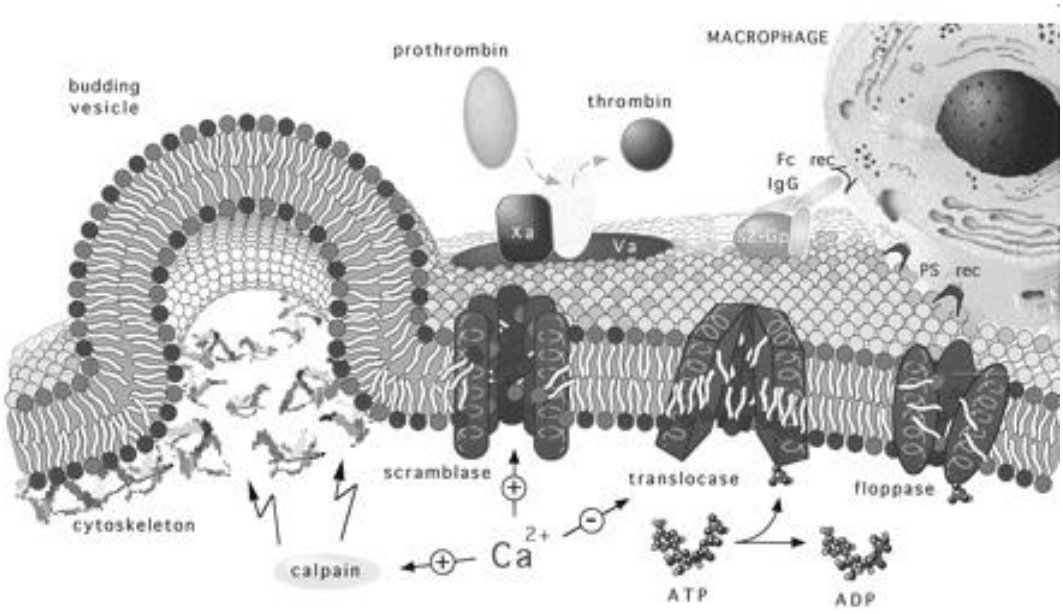


Fig. 3: Micoparticle formation. Upon platelet activation, elevated and sustained intracellular calcium disrupt membrane asymmetry by activating scramblase-mediated PS exposure. Intracellular calcium activates calpain, in addition to scramblase, leading to cytoskeleton rearrangement, membrane vesiculation and ultimately microparticle formation. (Image adapted from Zwaal & Schroit[92]).

1.5.2 Platelet Phosphatidylserine Exposure

Platelets, as with other mammalian cells, have an uneven localization of phospholipids in the two halves of their plasma membranes[98-102]. Negatively-charged aminophospholipids phosphatidylserine (PS) and phosphatidylethanolamine (PE) are cloistered on the cytosolic membrane leaflet by action of ATP-dependent flippase[103,104], while neutral cholinophospholipids phosphatidylcholine (PC) and sphingomyelin are congregated on the exofacial membrane leaflet by ATP-dependent floppase[105,106]. Activation of platelets, especially in a physiological context by dual

agonists thrombin and collagen[107,66], elicits an elevated and lengthened intracellular calcium mobilization[108,109] that switches on a phospholipid scramblase resulting in rapid disruption of membrane phospholipid asymmetry swifty exposing PS on the outer membrane surface. Though there is at least two pathways leading to PS exposure[110,111], it has been demonstrated that agonist-induced PS exposure in platelets occurs rapidly and requires a sustained rise in cytosolic calcium and therefore is most pertinent for hemostasis. Conversely, apoptosis-induced PS exposure happens gradually in a calcium-independent fashion serving as a phagocytic signal for removal of dying cells. Nevertheless, the calcium response elicited by co-activation of thrombin and collagen is not entirely clear. It is proposed that both agonists together evoke a receptor operated calcium entry (ROCE) involving non-selective cation channels and that either agonist alone induces a store-operated calcium entry involving STIM1 sensors on endoplasmic reticulum and Orai1 plasma membrane channels[112-114]. The calcium response elicited by platelet agonists has even extended to the mitochondria, in that the elevated cytosolic calcium triggers increased mitochondrial calcium leading to mitochondrial permeability transition pore with ensuing PS exposure[115,116].

1.6 Blood Coagulation

In the setting of vascular injury, tissue factor from damaged endothelium and unmasked subendothelium (smooth muscle cells, fibroblasts) gets exposed to blood

recruiting and fully activating its circulating cofactor FVIIa **(Fig. 4)**[117,118,29]. TF:FVIIa complex will activate FX (FXa) and subsequent FXa along with FVa released from activated platelets or immediately activated by FXa[119], will cleave two amino acids in prothrombin to generate small amounts of thrombin. TF:VIIa also activates FIX which successively results in FXa and thrombin. It is this initial and minute supply of thrombin that will sustain and amplify its own generation by: 1) activating FVIII (cofactor for IX in the tenase complex) and FV (cofactor for FXa in the prothrombinase complex); both tenase and prothrombinase complexes require a PS surface **(Fig. 2)** for augmented and effective enzymatic activity 2) activating FXI that sequentially activates FIX. A separate yet converging coagulation pathway can be initiated intrinsically by autoactivation of FXII when exposed collagen, platelet contents such as polyposphate (polyP), or other blood cell contents in the vicinity are released, resulting in activation of FXI. Ultimately at the end of the cascade, thrombin is generated on platelet surfaces and will 1) cleave fibrinogen into fibrin that will consolidate the platelet plug 2) activate FXIII to crosslink fibrin monomers resulting in a stable fibrin clot 3) potently activate platelets through PAR-1 and PAR-4 Gq-coupled receptors 4) activate protein C and cofactor Protein S to inactivate FVa and FVIIIa leading to slowing and shutting down of the coagulation system.

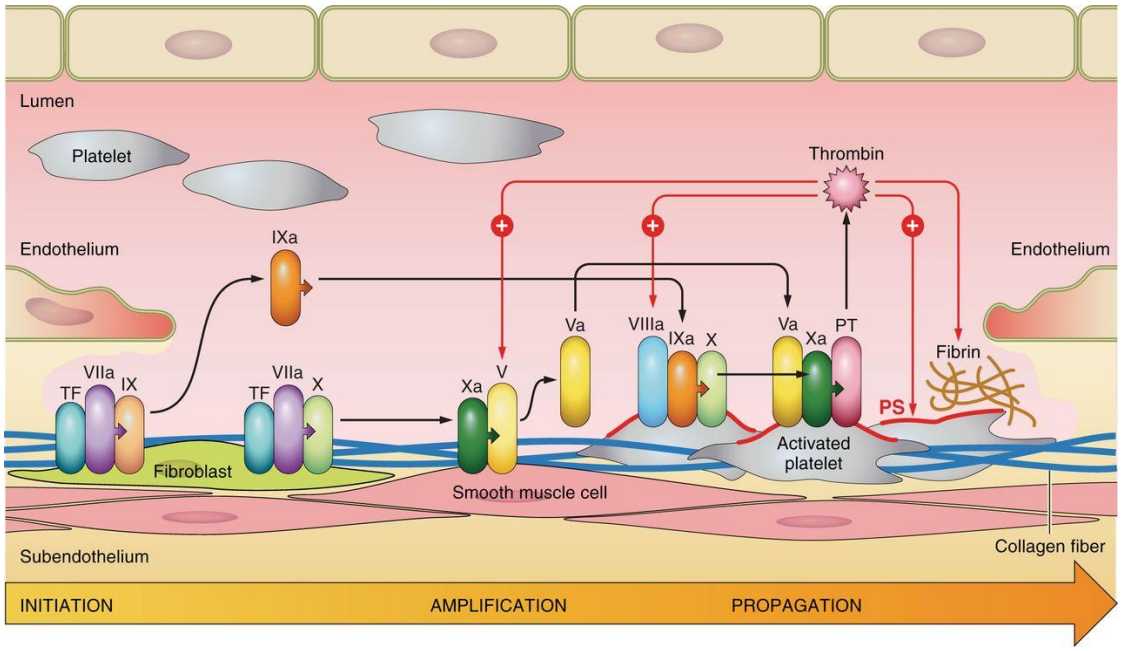


Fig. 4: Blood coagulation. Blood coagulation is initiated *in vivo* at the site of vascular injury when tissue factor comes in contact with FVIIa. The TF:FVIIa complex leads to activation of FIX and FX, culminating in trace amounts of thrombin. This minute thrombin activates FV, FVIII, and FXI leading to subsequent activation of FIX and FX on PS-exposing resulting in elevated thrombin generation. In parallel, FXII is auto activated converging in FXI activation. (Image adapted from Bevers & Williamson[118]).

1.7 Putative Role of TMEM16F in Platelet Procoagulant Activity

Impaired platelet-dependent procoagulant activity is evident in Scott Syndrome, an extremely rare mild bleeding disorder[120] whereby activated platelets[121] as well as other blood cells[122-124] lose their ability to present optimal PS on their exofacial membranes due to absent/mutated scramblase activity[125]. Platelets from patients with Scott Syndrome, though having unaltered structure, levels, adhesion and aggregation profiles fail to support maximum thrombin generation and resulting fibrin,

even with the addition of normal plasma[126,74,121]. Notably, both FVIIIa- and FVa-impaired binding sites have been identified on activated platelet surfaces of Scott Syndrome[127,128]. Curiously, the PS exposure defect in activated Scott platelets has been shown to be associated with reduced platelet microparticle generation[77,126], extending the possible link between scramblase activity and microparticle formation. Furthermore, the impaired platelet vesiculation (microparticle formation) of activated Scott Syndrome is demonstrated to be directly coupled to platelet FV receptor availability, underscoring the importance of microparticles for efficient coagulation[129]. Recently, a novel 106kDa eight-transmembrane protein TMEM16F (also called anoctamin 6) **(Fig. 5)** from the TMEM16 family of calcium channels emerged as indispensable for calcium-mediated PS exposure on plasma membranes[130]. A complementary DNA library constructed from mouse B cells with calcium-ionophore induced PS exposure was expressed in native mouse B cell lines, resulting in selection of an isolated clone that had spontaneous PS exposure. The cDNA associated with this heightened PS exposure encoded a constitutively active TMEM16F variant (D409G). Moreover, elimination of TMEM16F from mouse B cells resulted in reduced calcium-ionophore induced PS exposure. Importantly, these authors performed sequence analyses which confirmed loss-of-function mutations in the TMEM16F gene of a Scott Syndrome patient. Due to G-to-T mutations in splice acceptor site of intron 12 of the Scott Syndrome patient, exon 13 of the TMEM16F gene was missing resulting in premature termination of the protein. Castodi and colleagues[131] conducting sequence analyses on the TMEM16F gene of a different Scott Syndrome patient revealed that

intron 6 had G-to-A mutations interfering with the splice-acceptor site and also that point insertions exist in exon 11 arresting translation of the TMEM16F gene. Interestingly, a canine model presenting defective platelet procoagulant activity also carries a mutation in TMEM16F[132-134]. The human TMEM16 family is widely expressed in various cell types and consists of 10 members, with each member showing the most conservation in the transmembrane domain yet having distinct functions[135,136].

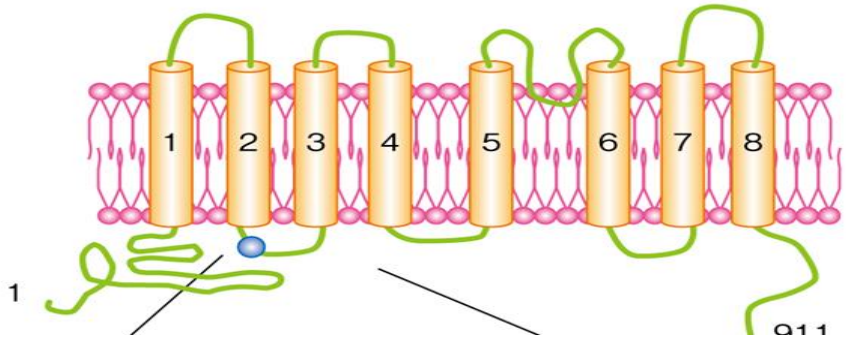


Fig. 5: Schematic representation of TMEM16F. TMEM16F is an eight-transmembrane ion channel. (Image adapted from Suzuki *et al*.[130]).

1.8 Hypothesis and Aims

We hypothesize that the absence of TMEM16F protects animals from thrombosis and is mediated through reduced microparticle generation. We will test this hypothesis in the following 3 aims: Aim I: To determine whether TMEM16F is required for optimal platelet microparticle formation, platelet PS exposure and circulating microparticle formation *in vivo*. Aim II: To determine if TMEM16F

promotes optimal procoagulant activity and thrombosis *in vivo* and whether platelet-derived microparticles restore procoagulant activity and thrombosis due to the loss of TMEM16F. Aim III: To determine if TMEM16F-dependent thrombosis is influenced by the number, morphology, or aggregation of circulating platelets or the levels of other circulating blood cells. Together, the completion of these aims will help us to understand the impact of TMEM16F expression on platelet microparticle formation and procoagulant activity and ultimately shed light on whether it may ultimately provide an attractive target for antithrombotic therapy development to reduce procoagulant microparticles in prothrombotic states.

Chapter 2

METHODS

2.1 Mice

TMEM16F knock out (KO) mice were generated (Ehlen *et al.* 2013) and provided by the laboratory of Andrea Vortkamp (University of Duisburg-Essen) and were backcrossed in C57BL/6 background. All mice used were between 8-12 weeks old. All experiments and care of animals were approved by the Institutional Animal Care and Use Committee (IACUC) at the University of Delaware.

2.2 Platelet-Derived Microparticle Generation and PS Exposure

Blood was obtained from inferior *vena cava* into anticoagulated syringe (150 U/mL heparin) from fully sedated mice (100 mg/kg Pentobarbital) and combined with equal volume of HEPES-Tyrode's Buffer (137 mM NaCl, 20 mM HEPES, 5.6 mM glucose, 1 g/L BSA, 1 mM $MgCl_2$, 2.7 mM KCl, 3.3 mM NaH_2PO_4) and 0.02 U/mL apyrase. PRP extracted from centrifuged blood (800 rpm, 3 min, 25 ^oC, Eppendorf Centrifuge 5810 R) was gel-filtered with 1X Annexin V Binding Buffer on a Sepharose CL-2B and platelets were counted on a Coulter counter (Z1; Beckman-Coulter, Hialeah, FL). $0.5X10^8$ platelets treated (37 ^oC at 15 mins) with combinations

of thrombin (5 U/ml) and either collagen (10 µg/ml) or convulxin (5 µg/ml) and A23187 calcium ionophore (1 µM, 10 µM) were stained in dark with 5 µL Annexin V-PE (25 ^{o}C at 30 mins) and 2 µL of 0.1 µg/µl CD41-FITC (25 ^{o}C at 15 mins). Samples were read upon dilution of 400 µL 1X Annexin V Binding Buffer on FACS Aria.

2.3 *In vivo* Circulating Microparticles Characterization

Blood was collected from inferior *vena cava* of fully sedated (100 mg/kg Pentobarbital) mice and anticoagulated with 150 U/mL heparin. 0.02 U/ml apyrase and equal volume of HEPES-Tyrode's Buffer (137 mM NaCl, 20 mM HEPES, 5.6 mM glucose, 1 g/L BSA, 1 mM $MgCl_2$, 2.7 mM KCl, 3.3 mM NaH_2PO_4) were mixed with blood. Platelet Rich Plasma (PRP) was obtained by initial blood centrifugation (800 rpm, 3 min, 25 ^{o}C, Eppendorf Centrifuge 5810 R), followed by 1 µM PGE1. From second centrifugation (1800 rpm, 10 min, 25 ^{o}C, Eppendorf Centrifuge 5810 R), Platelet Poor Plasma (PPP) was recovered as the supernatant of spun PRP. 100 µl of PPP stained with 2 µl of 0.1 µg/µl CD41 (FITC-conjugated) and 3 µl of 0.04 µg/µl CD62P (PE-conjugated) antibodies for 15 min in dark at 25 ^{o}C. Samples were diluted with 300 µL PBS and read on FACS Aria.

2.4 Thrombin Generation

Blood isolated from retro-orbital sinus of isoflurane (5%) anesthetized mice was collected into Eppendorf tubes already containing 0.11M sodium citrate (9 parts blood: 1 part 0.11M sodium citrate). With an equal volume of HEPES-Tyrode's

Buffer (137 mM NaCl, 20 mM HEPES, 5.6 mM glucose, 1 g/L BSA, 1 mM $MgCl_2$, 2.7 mM KCl, 3.3 mM NaH_2PO_4) added, the diluted blood was spun at (800 rpm, 2 mins, 25 ^oC, Eppendorf Centrifuge 5810 R) to obtain PRP. Platelet concentration was adjusted with Tyrode's Buffer to $1.5X10^8$/ml in a volume of 250 µl. Of this 250 µl volume; 40 µl was used per sample as PRP (2 technical replicates each were done for all biological replicates); remaining volume was spun at 1500 x g for 10 mins to pellet platelets and obtain PPP, 40 µl of PPP per sample was used (2 technical replicates each were done for all biological replicates). Thrombin generation was performed using TECHNOTHROMBIN® TGA Kit (Diapharma, Ohio, USA) according to manufacturer's recommendation. Briefly, a complex containing 50 µl of TGA substrate (fluorogenic thrombin substrate and calcium) and 10 µl of TGA Reagent B (low concentration of phospholipid micelle containing low concentration of tissue factor) was added to 40 µl of both PRP and PPP in a 96-well microplate. Samples were immediately read on a GloMax®-Multi Microplate Multimode Reader (Promega, Wisconsin, USA), where fluorescence was monitored at 365 nM excitation wavelength and 410-460 nM emission wavelength and at 37 ^oC in 1 minute intervals for 1 hour. The fluorescence obtained was adjusted on a standard thrombin calibration curve generated using TECHNOTHROMBIN® thrombin calibrator and the fluorogenic thrombin substrate.

2.5 Platelet-Derived Microparticles Contribution to Thrombin Generation

Blood was obtained from inferior *vena cava* into anticoagulated syringe (150 U/mL heparin) from fully sedated mice (100 mg/kg Pentobarbital) and combined with equal volume of HEPES-Tyrode's Buffer (137 mM NaCl, 20 mM HEPES, 5.6 mM glucose, 1 g/L BSA, 1 mM $MgCl_2$, 2.7 mM KCl, 3.3 mM NaH_2PO_4) and 0.02 U/mL apyrase. PRP extracted from centrifuged blood (800 rpm, 3 min, 25 ^oC, Eppendorf Centrifuge 5810 R) was washed in HEN Buffer (10 mM HEPES, 1 mM EDTA, 150 mM NaCl) and platelet concentration was adjusted to $3X10^8$/ml in 500 µl volume. Adjusted platelet samples were stimulated with 10 µM A23187 for 30 minutes at 37 ^oC and pelleted by centrifugation at 1500 x g for 10 mins. Recovered microparticle supernatant was washed with HEN Buffer, spun at 21000 x g at 1 hour at 4 ^oC to pellet microparticles before resuspending in HEPES-Tyrode's Buffer for a final volume of approximately 150 µl. Resuspended microparticles were added to blood samples and the thrombin generation protocol was followed as described above in 2.4.

2.6 *In vivo* $FeCl_3$-Induced Arterial Thrombosis

Carotid artery of anesthetized (100 mg/kg Pentobarbital) mice was surgically exposed and placed on a Doppler flow probe to measure blood flow. A strip of filter paper soaked in 10% ferric chloride was applied to the carotid artery for 2.5 minutes then rinsed with PBS. Arterial blood flow was monitored for 30 minutes.

2.7 Platelet-Derived Microparticles Contribution to *in vivo* $FeCl_3$-Induced Arterial Thrombosis

Blood was obtained from inferior *vena cava* into anticoagulated syringe (150 U/mL heparin) from fully sedated mice (100 mg/kg Pentobarbital) and combined with equal volume of HEPES-Tyrode's Buffer (137 mM NaCl, 20 mM HEPES, 5.6 mM glucose, 1 g/L BSA, 1 mM $MgCl_2$, 2.7 mM KCl, 3.3 mM NaH_2PO_4) and 0.02 U/mL apyrase. PRP extracted from centrifuged blood (800 rpm, 3 min, 25 ^oC, Eppendorf Centrifuge 5810 R) was washed in HEN Buffer (10 mM HEPES, 1 mM EDTA, 150 mM NaCl) and platelet concentration was adjusted to $3X10^8$/ml in 500 µl volume. Adjusted platelet samples were stimulated with 10 µM A23187 for 30 minutes at 37 ^oC and pelleted by centrifugation at 1500 x g for 10 mins. Recovered microparticle supernatant was washed with HEN Buffer, spun at 21000 x g at 1 hour at 4 ^oC to pellet microparticles before resuspending in normal saline solution for a final volume of approximately 150 µl. Resuspended microparticles were retro-orbitally injected into anesthetized mice and ferric chloride-induced arterial thrombosis protocol was followed as described in 2.6.

2.8 Complete Blood Count (CBC)

Whole blood isolated from retro-orbital sinus of isoflurane (5%) anesthetized mice was collected into EDTA tubes and analyzed by COULTER AC•T diff Hematology Analyzer for CBC.

2.9 Platelet Aggregation

Blood was collected from inferior vena cava of fully sedated (100 mg/kg Pentobarbital) mice and anticoagulated with 150 U/mL heparin. Equal volume of HEPES-Tyrode's Buffer (137 mM NaCl, 20 mM HEPES, 5.6 mM glucose, 1 g/L BSA, 1 mM $MgCl_2$, 2.7 mM KCl, 3.3 mM NaH_2PO_4) was mixed with blood and PRP was obtained by blood centrifugation (800 rpm, 3 min, 25 ^oC, Eppendorf Centrifuge 5810 R). 2.5 µl of each agonist was added to each PRP sample at $2.5X10^8$/ml in 250 µl volume and platelet aggregation was evaluated on a Chronolog Corp. lumi-aggregometer (Havertown, Pennsylvania, USA).

Chapter 3

RESULTS

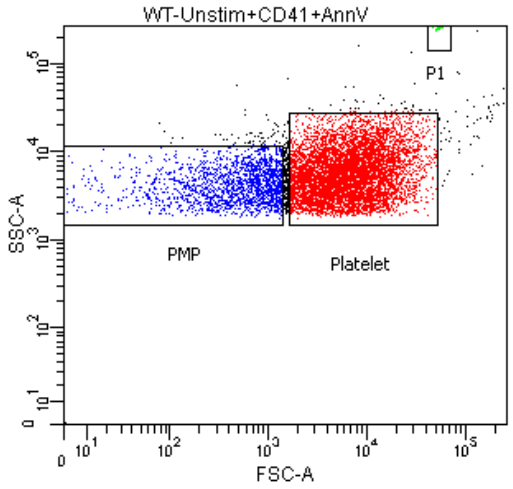
3.1 Platelets Lacking TMEM16F Generate Reduced PS+ and Total Microparticles

TMEM16F, a putative 8-transmembrane-spanning domain protein, was discovered using a screen to identify the calcium-dependent scramblase required for phosphatidylserine exposure in calcium-activated platelets[130]. While previous studies have verified that presence of this protein on platelets is required for platelet PS exposure and also for procoagulant activity[138], it has remained less clear whether the protein is also required for microparticle formation. As detailed in the introduction, high levels of circulating microparticles are found in multiple conditions associated with elevated risk of thrombosis[20-24], so the overall aim of our study was to determine the role of TMEM16F in microparticle generation, and to further determine whether its role in microparticle formation contributed to prothrombotic activity. We sought to do this in 3 specific aims.

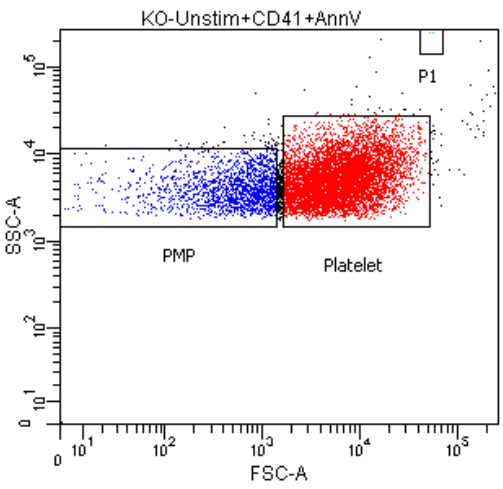
Aim I: To determine whether TMEM16F is required for optimal platelet microparticle generation, platelet PS exposure and microparticle formation *in vivo*. To first explore the role of TMEM16F in platelet microparticle formation, gel-filtered platelets isolated from TMEM16F KO and WT mice were stimulated with either

combinations of thrombin and collagen, thrombin and convulxin or with calcium ionophore A23187. The resulting platelet and microparticle containing samples were stained with PE-conjugated Annexin V (Ann V) to determine whether they expressed PS on their surfaces and characterized by flow cytometry. Microparticles were gated based upon forward and side scatter profiles previously selected to match standards of particles below 1 μm in size **(Fig 6A)**. The results show that platelets from both WT and TMEM16f KO mice generate Annexin V+ microparticles when stimulated with thrombin and collagen, thrombin and convulxin, or calcium ionophore. As other reports show[139,85,140,141], agonist- and ionophore-dependent microparticles (above no treatment background) are predominantly AnnexinV-positive, and so we used AnnexinV-positivity to verify the microparticles were specifically agonist-dependent and membrane-derived. Importantly, platelets from TMEM16f KO mice generated significantly reduced numbers of Ann V+ microparticles **(Fig. 6B)** and total microparticles **(Fig. 6C)** compared to WT platelets when treated with combinations of thrombin and collagen, 1 μM A23187 and 10 μM A23187.

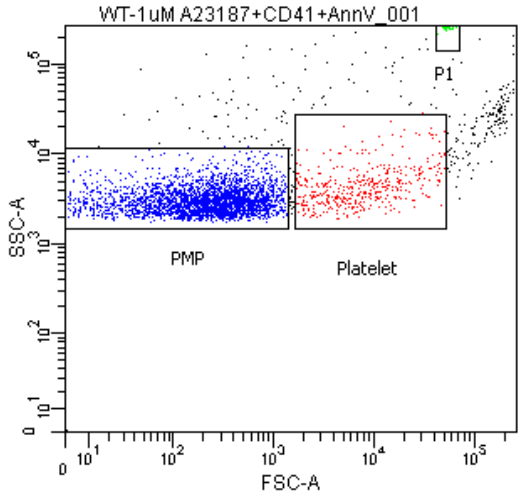
A.



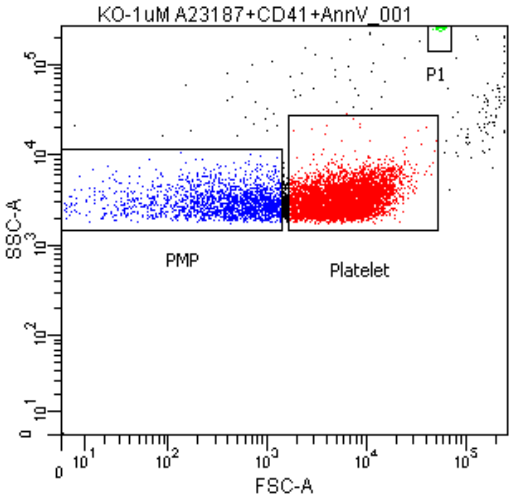
WT – untreated



TMEM16F KO - untreated

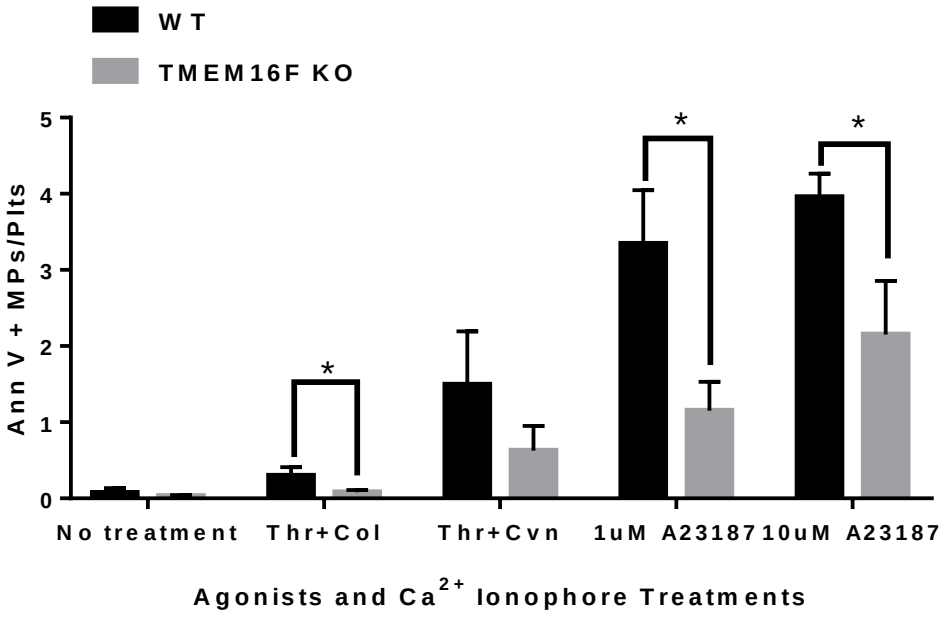


WT – 1 μM A23187



TMEM16F KO – 1 μM A23187

B.


WT
TMEM16F KO
Ann V + MPs/Plts
0
1
2
3
4
5
*
*
*
No treatment
Thr+Col
Thr+Cvn
1uM A23187
10uM A23187
Agonists and Ca2+ Ionophore Treatments

C.

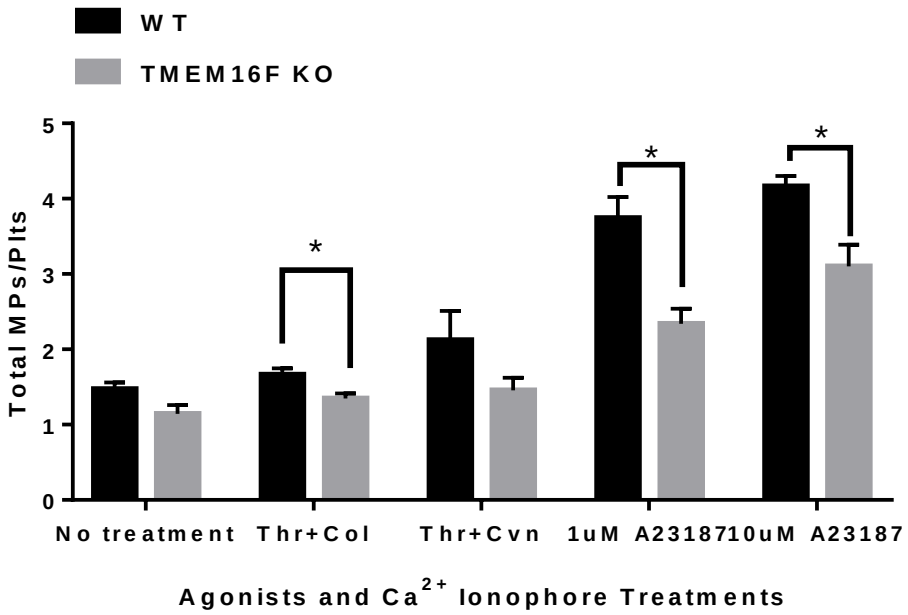

WT
TMEM16F KO
Total MPs/Plts
0
1
2
3
4
5
*
*
*
No treatment
Thr+Col
Thr+Cvn
1uM A23187
10uM A23187
Agonists and Ca2+ Ionophore Treatments

Fig. 6. Microparticles generated from activated platelets. Platelets were exposed to thrombin + collagen (thr+col), thrombin + convulxin (thr+cvn), 1 μM A23187 and 10 μM A23187 and samples were incubated with Annexin-V and evaluated on flow cytometry by gating below 1 μm (A) for detection of (B) Annexin V + and (C) total microparticles. (The averages +/- SEM represent 3-5 independent experiments. *P < 0.05 using a Student's t test).

3.2 Platelets Lacking TMEM16F have Attenuated PS Exposure

To determine whether TMEM16f is required for platelet scramblase activity, we evaluated exposure of PS on the membrane surfaces of platelets isolated from WT vs TMEM16F KO mice after stimulation with either a combination of physiological agonists or calcium ionophore. Activated platelet samples were then stained with Annexin V, evaluated by flow cytometry, and normalized to maximal Annexin V bound with high concentration of ionophore. We found that loss of TMEM16F resulted in less phosphatidylserine exposure on platelets with dual agonist thrombin + collagen or with 1 μM A23187 **(Fig. 7)**. This defect in PS exposure in TMEM16F-lacking platelets, however, was overcome in platelets stimulated with high concentrations of calcium ionophore. This suggests that scramblase activity can be compensated by another scramblase at very high calcium concentrations.

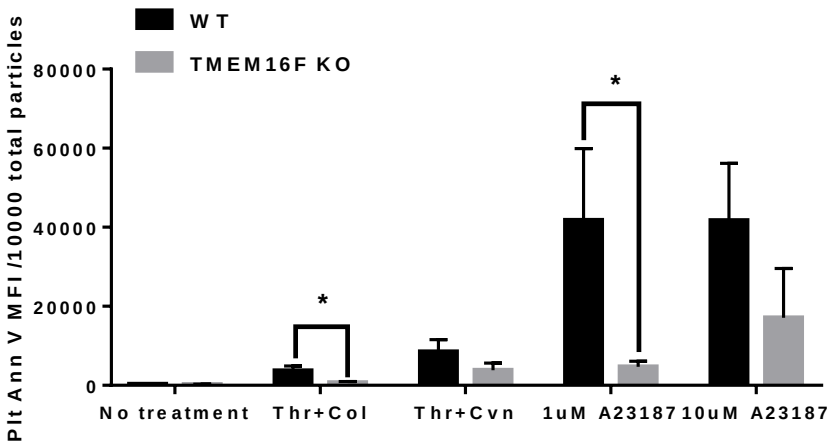


Fig. 7. Mean fluorescence intensity of stimulated platelets. Platelets stimulated with thrombin + collagen (thr+col), thrombin + convulxin (thr+cvn), 1 μM A23187, 10 μM A23187 were stained with Annexin-V and the platelet mean fluorescence intensity (MFI) measured upon counting 10000 total events. (The averages +/- SEM represent 4-7 independent experiments. *P < 0.05 using a Student's t test).

3.3 Loss of TMEM16F Reduces Normal Circulating Microparticles *in vivo*

Next, we turned to the importance of TMEM16F for generating microparticles *in vivo*. To determine whether TMEM16F contributes to steady-state circulating levels of microparticles in healthy animals, and therefore may contribute to both physiological and pathological levels *in vivo*, circulating levels of microparticles were evaluated in WT and TMEM16F KO mice. Normal, healthy plasma from TMEM16F KO and WT mice was isolated, stained with FITC-conjugated anti-CD41 antibody and evaluated on flow cytometry for total and CD41+ microparticles (CD41 is a marker for platelet- and megakaryocyte- derived microparticles). As shown in **Fig. 8**, the loss of TMEM16F reduces the normal CD41+ and total circulating microparticles *in vivo.*

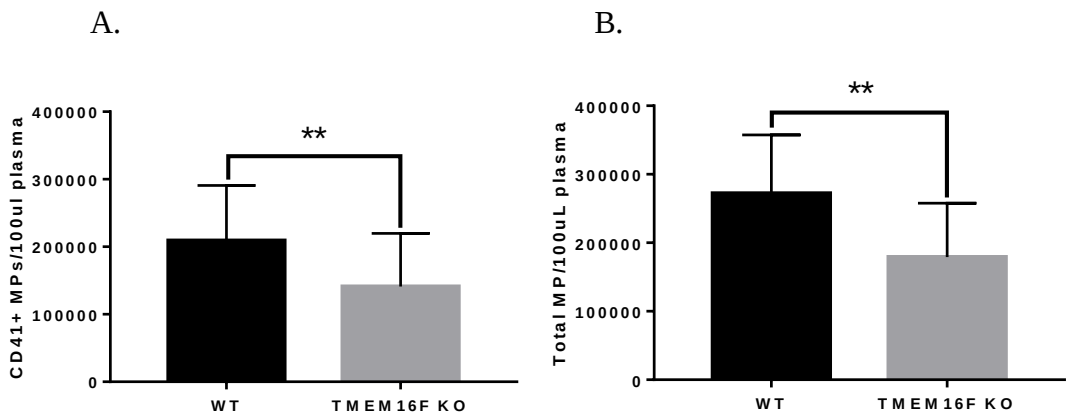
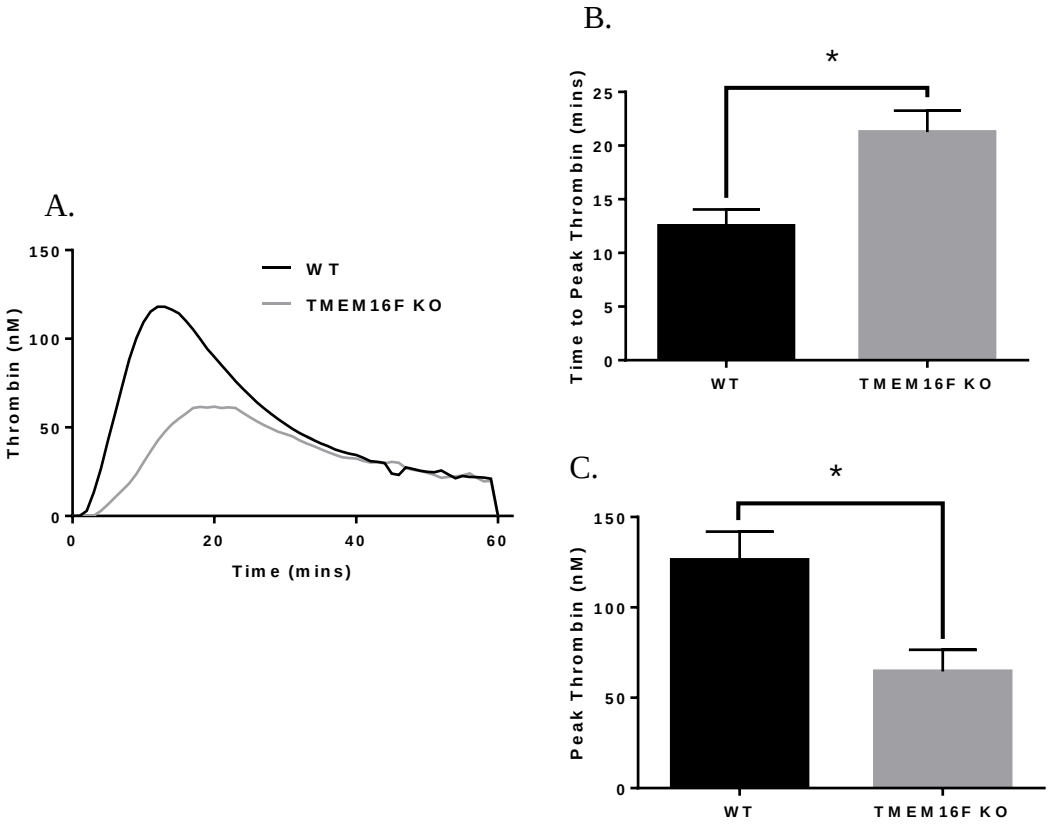


Fig. 8. CD41+ and total normal circulating microparticles *in vivo*. Plasma from both TMEM16F and WT mice was isolated and either remained unstained or stained for with Anti-CD41 and evaluated for (A) CD41+ microparticles and (B) total microparticles on flow cytometry (The averages +/- SEM represent 3 independent experiments. (**$P < 0.005$ using a paired Student's t test).

3.4 Loss of TMEM16F Dramatically Attenuates Thrombin Generation

Blood coagulation proceeds rapidly on exposed PS surfaces, such as platelet microparticles, as the exposed PS interacts with the tenase and prothrombinase complexes leading to maximum thrombin generation[32,20]. Since we found that loss of TMEM16F impairs platelet microparticle generation, platelet PS exposure and normal circulating microparticles *in vivo*, we tested whether procoagulant activity was also reduced with deficiency of TMEM16F. To assess procoagulant activity, PRP and PPP isolated from both TMEM16F KO and WT mice were surveyed for thrombin generation upon tissue factor initiation. Though the PRP of WT and TMEM16FKO had no statistically significant difference in the time taken for the initial burst of thrombin, TMEM16F KO PRP took almost twice as long to generate peak thrombin (TMEM16F KO = 21.25 mins vs WT = 12.5 mins) and could only generate half as

much peak thrombin (TMEM16F KO = 64.575 nM vs. WT = 126.125 nM **(Fig. 9A-C)**. In PPP samples, thrombin generation overall significantly plummeted compared to PRP samples **(Fig. 9)**, however both PRP of TMEM16F KO and WT had vastly different thrombin generation profiles. Similar in trend to the PRP samples, TMEM16F KO PPP had almost doubled the time to generate peak thrombin WT = 20 mins vs TMEM16F KO = 32.25 mins) and had a twice as less peak thrombin formed (WT = 49.3 nM vs TMEM16F KO = 23.8) **(Fig. 9C-D)**.



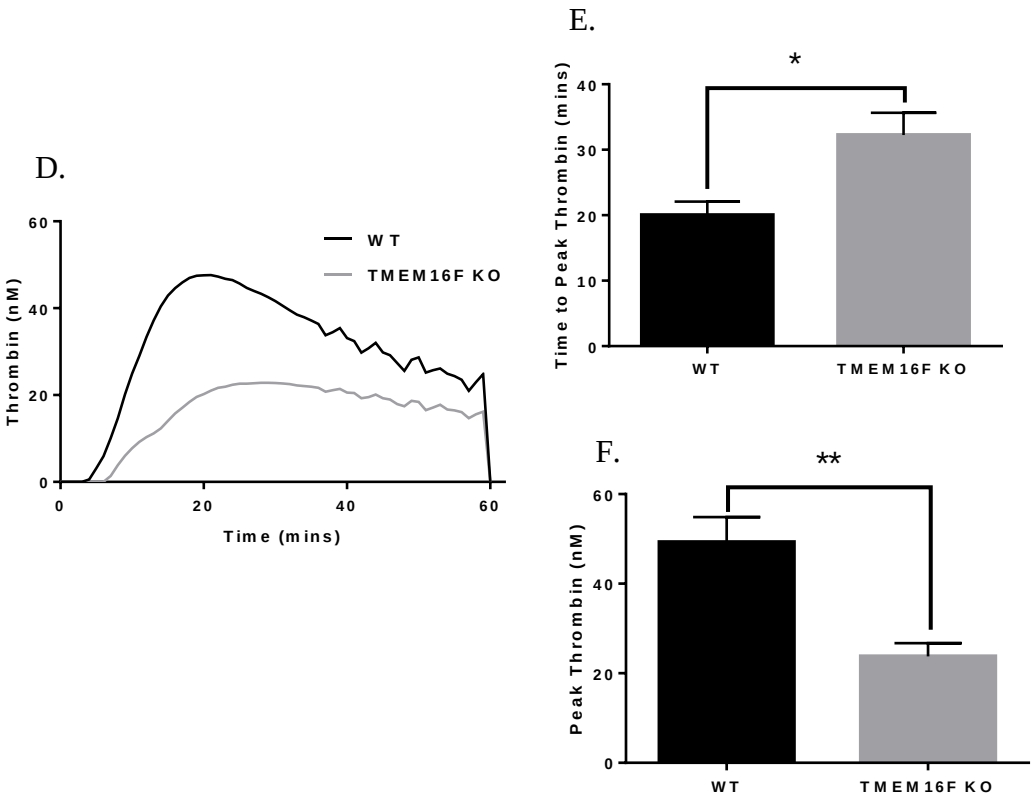
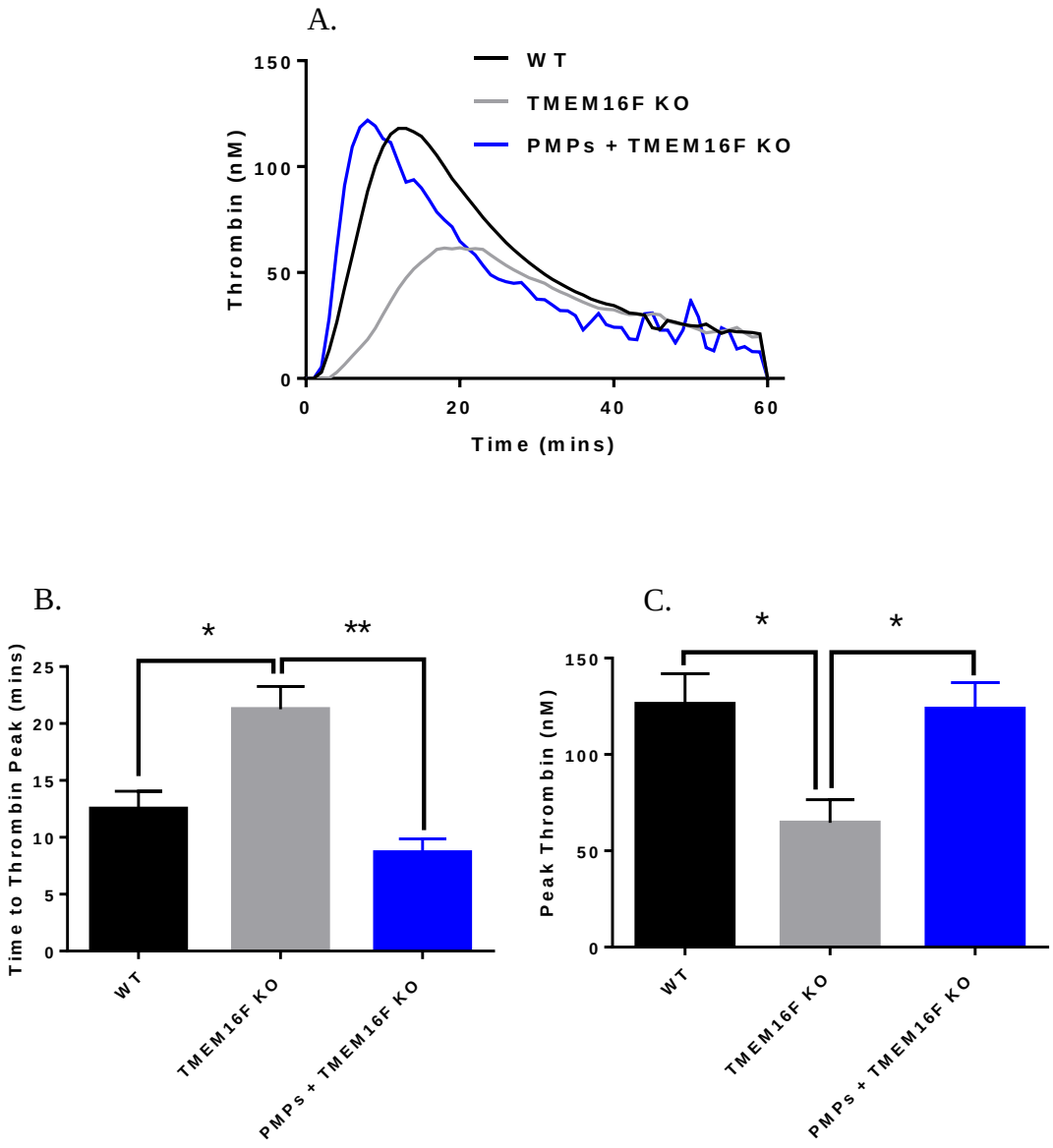


Fig. 9. Thrombin generation in plasma. Upon tissue factor initiation, thrombin generation in PRP and PPP from both WT and TMEM16F KO mice were evaluated for 1 hour at 37^{o}C. (A) Average thrombin generation curve in PRP. (B) Average time to peak thrombin in PRP. (C) Average peak thrombin generated in PRP. (D) Average thrombin generation curve in PPP. (E) Average time to peak thrombin in PPP. (F) Average peak thrombin generated in PPP. (The mean thrombin generated (+/- SEM) from four independent experiments are shown. *P < 0.05 and **P<0.005 using a Student's t test).

3.5 Addition of Platelet Microparticles to Plasma Lacking TMEM16F Completely Restores Thrombin Generation

Platelet microparticles have a 50-100 fold higher procoagulant activity than activated platelets[93], and the exposure of platelet FVa receptor, which is required for tenase activity and efficient coagulation, is directly coupled to platelet microparticle formation[129]. We therefore reasoned that perhaps the reduced microparticles generated from the TMEM16F-deficient platelets were the reason for the attenuated thrombin generation observed with the loss of TMEM16F. Accordingly, we tested the ability of platelet microparticles to recover thrombin generation due to the absence of TMEM16F. Essentially A23187-generated microparticles from WT platelets were added the blood of TMEM16F KO mice and subsequent tissue factor-induced thrombin generation in both PRP and PPP was assessed. As shown in **Fig. 10**, platelet microparticles completely restore the time taken and amount of peak thrombin generated in the both TMEM16F-null PRP and TMEM16F-null PPP to the level of that seen in WT PRP.


A.
WT
TMEM16F KO
PMPs + TMEM16F KO
Thrombin (nM)
150
100
50
0
0
20
40
60
Time (mins)
B.
Time to Thrombin Peak (mins)
25
20
15
10
5
0
*
**
WT
TMEM16F KO
PMPs + TMEM16F KO
C.
Peak Thrombin (nM)
150
100
50
0
*
*
WT
TMEM16F KO
PMPs + TMEM16F KO

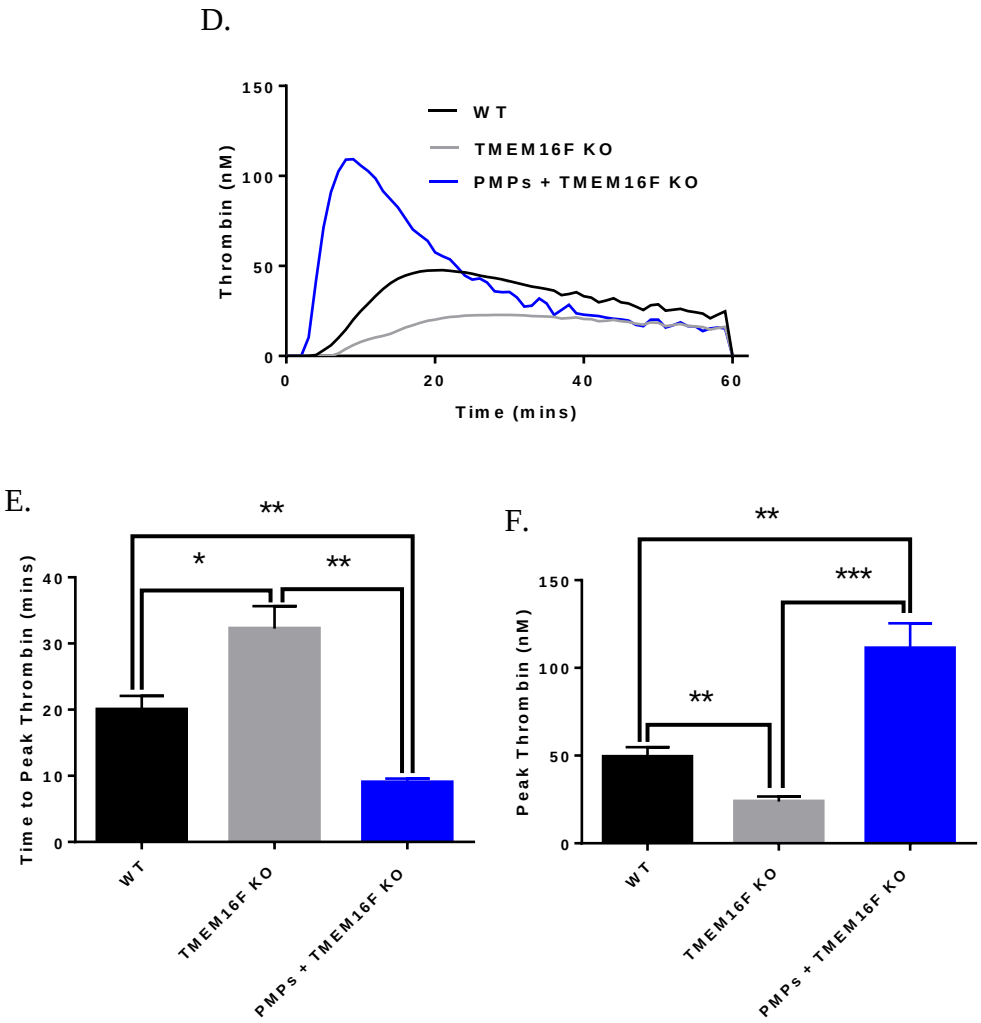
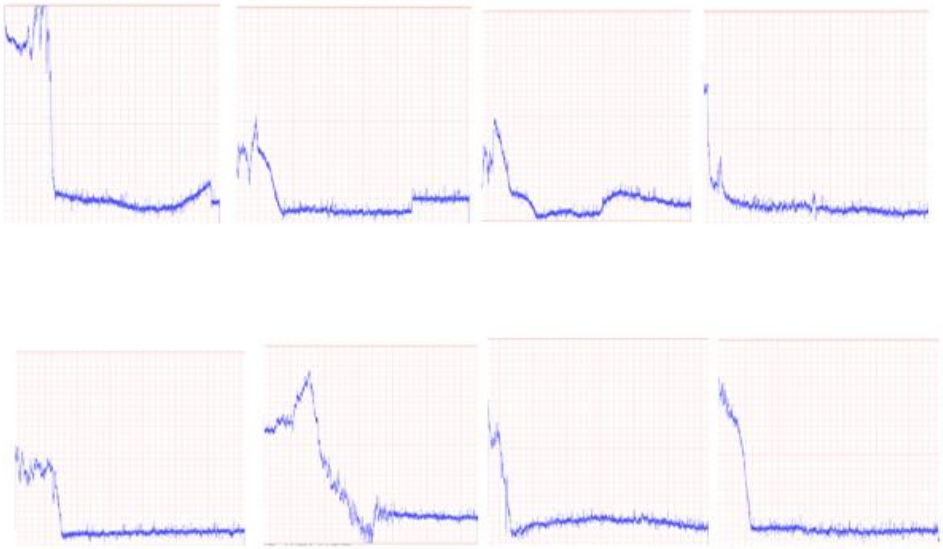


Fig. 10. Platelet microparticles recovery of thrombin generation in TMEM16F plasma. 10 μM A23187-generated WT platelet microparticles were added to TMEM16F KO blood, then both PRP and PPP were evaluated for tissue factor-induced thrombin generation for 1 hour at 37^{o}C. (A) Average thrombin generation curve in PRP. (B) Average time to peak thrombin in PRP. (C) Average peak thrombin generated in PRP. (D) Average thrombin generation curve in PPP. (E) Average time to peak thrombin in PPP. (F) Average peak thrombin generated in PPP. (The mean thrombin generated in MPs + TMEM16F KO plasma (+/- SEM) from three independent experiments are shown as well as the mean thrombin generated in WT and TMEM16F KO plasma (+/- SEM) of four independent experiments. *P < 0.05, **P<0.005 and ***P<0.0005 using a Student's t test).

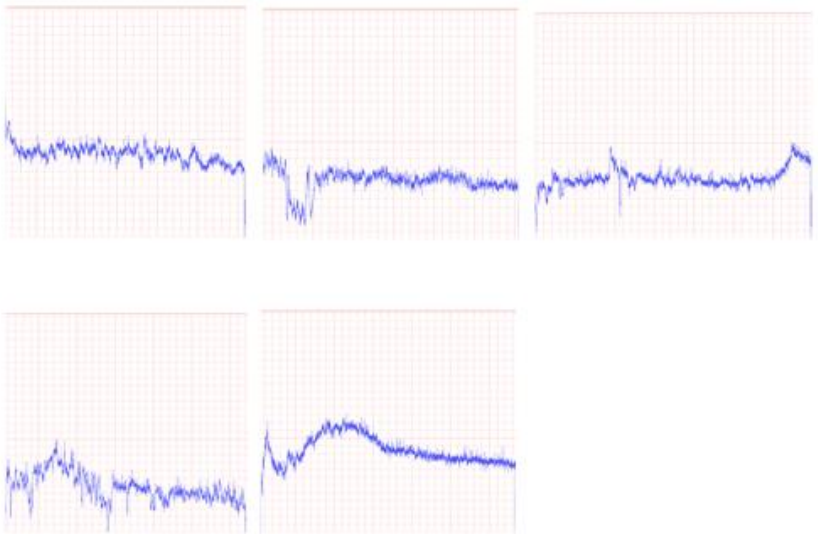
3.6 Mice lacking TMEM16F are Protected from Thrombosis *in vivo* upon $FeCl_3$ Injury to Carotid Artery

Next, we explored the loss of TMEM16F on thrombus formation *in vivo*. Arterial blood flow was monitored in WT and TMEM16F KO mice upon $FeCl_3$ insult to the carotid artery for 30 minutes. Mice were scored as having: stable thrombus (if flow rate remained at 0.00 ml/min after 15 mins), unstable thrombus (if flow rate became 0.00 ml/min then deviated after 15 minutes) or no thrombus (if there were full flow rate after 15 minutes). As shown in **Fig. 11**, all WT mice developed an occlusive thrombus while the TMEM16F KO mice were completely protected from thrombosis.

A. WT Mice



B. TMEM16F KO MICE



C.

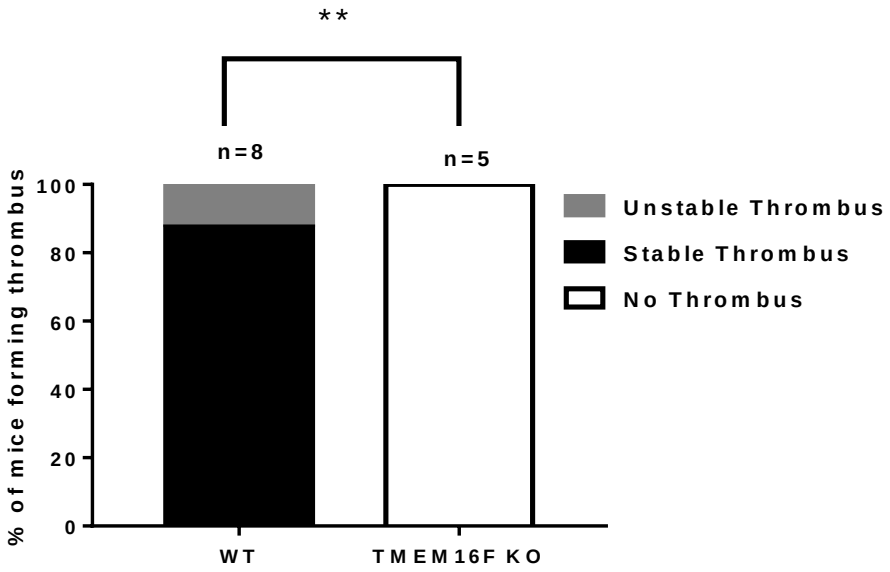
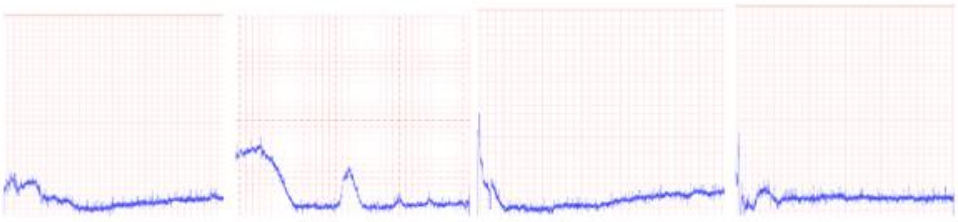


Fig. 11. $FeCl_3$-induced arterial thrombosis. (A and B) Tracings for all mice tested using 2 min and 30 sec exposure to $FeCl_3$ and subsequent monitoring of blood flow rate for 30 minutes. Normal flow rate is approximately 1 ml/min and a flow rate of 0 indicates complete arterial occlusion. (C) Distribution of averages of mice and corresponding thrombotic response. (**P<0.001 using Fischer exact probability test.)

3.7 Reinjection of Platelet Microparticles in Mice Lacking TMEM16F Restores Thrombosis *in vivo* upon $FeCl_3$ Injury to Carotid Artery

Considering that TMEM16F KO mice have defects in thrombosis and procoagulant activity, and that addition of platelet microparticles completely restore procoagulant activity *in vitro*, we examined the introduction of platelet microparticles in TMEM16F KO mice to restore thrombosis *in vivo*. 10 μM A23187 was used to generate microparticles from WT mice platelets and these microparticles were retro-orbitally injected in TMEM16F KO mice and the mice were examined for thrombus formation by $FeCl_3$ injury to the carotid artery. Mice were scored as having: stable thrombus (if flow rate remained at 0.00 ml/min after 15 mins), unstable thrombus (if flow rate became 0.00 ml/min then deviated after 15 minutes) or no thrombus (if there were full flow rate after 15 minutes). We found that the addition of platelet microparticles completely restored thrombosis in the TMEM16F KO mice **(Fig. 12)**.

A. Microparticles added to TMEM16F KO



B.

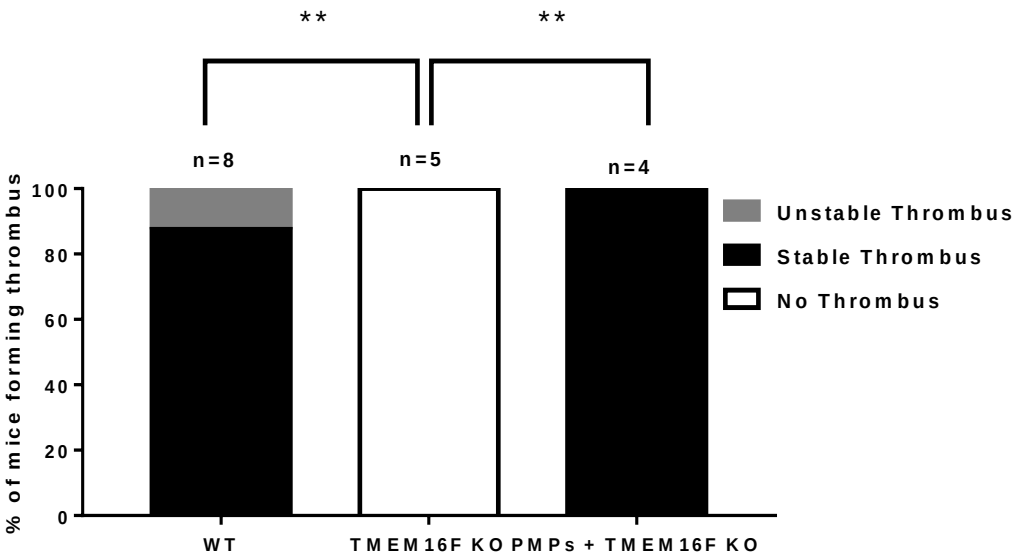


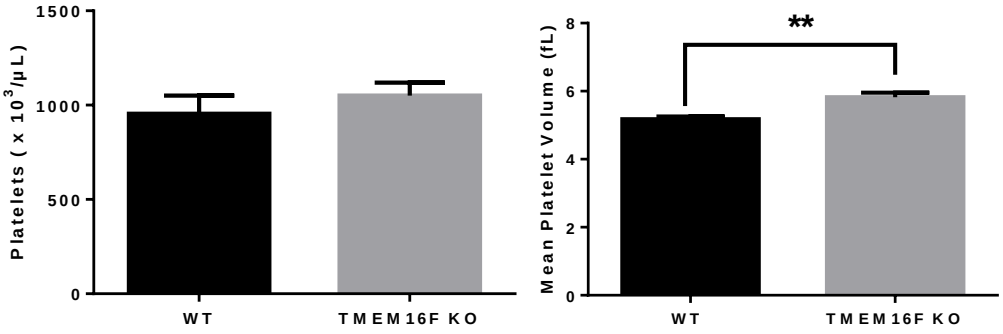
Fig. 12. Platelet microparticle contribution to $FeCl_3$-induced arterial thrombosis. TMEM16F KO mice were reinjected with platelet microparticles as shown (A) Tracings for all mice tested using 2 min and 30 sec exposure to $FeCl_3$ and subsequent monitoring of blood flow rate for 30 minutes. Normal flow rate is approximately 1 ml/min and a flow rate of 0 indicates complete arterial occlusion. (B) Distribution of averages of mice and corresponding thrombotic response. (**P<0.001 using Fischer exact probability test.)

3.8 Loss of TMEM16F has no Effect on Number of Platelets, Leukocytes and Erythrocytes, Distribution of Leukocytes or Indices of Erythrocytes, but Slightly Increases Mean Platelet Volume

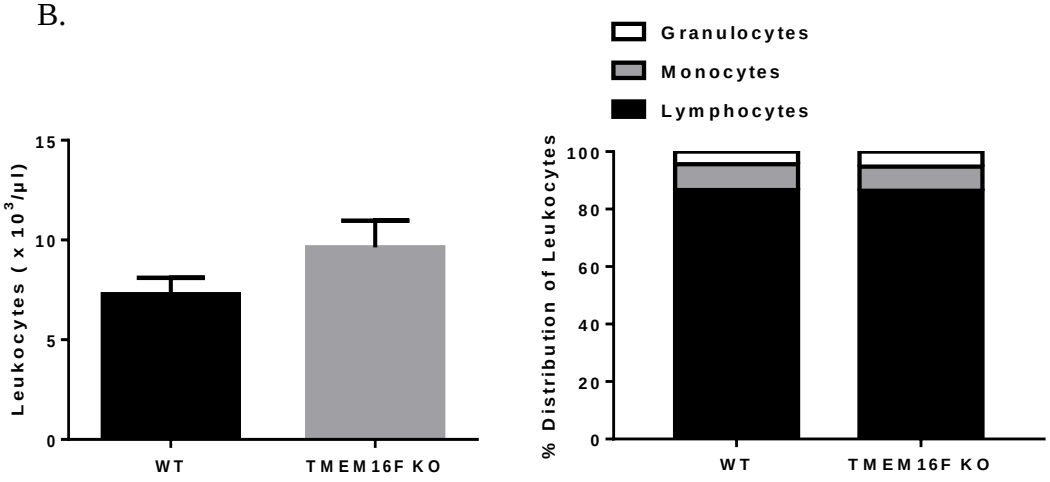
Thrombosis can be multifactorial, and inflammatory components contributed by circulating leukocytes[147,148] have also been implicated in the process. We therefore sought to determine whether any of the steady-state circulating blood cell counts were altered in TMEM16F KO mice. Complete blood counts of WT and TMEM16F KO

mice show that loss of TMEM16F slightly increases mean platelet volume, but not platelet number **(Fig. 13)**. This usually indicates that the platelets themselves are larger in size on average than wildtype platelets. Reasons may be because platelets are shedding fewer microparticles, so remain larger in size. Whether TMEM16F may play any role in platelet elaboration from megakaryocytes and thereby contribute to changes in mean platelet volume is in unknown and remains to be examined.

A.



B.



C.

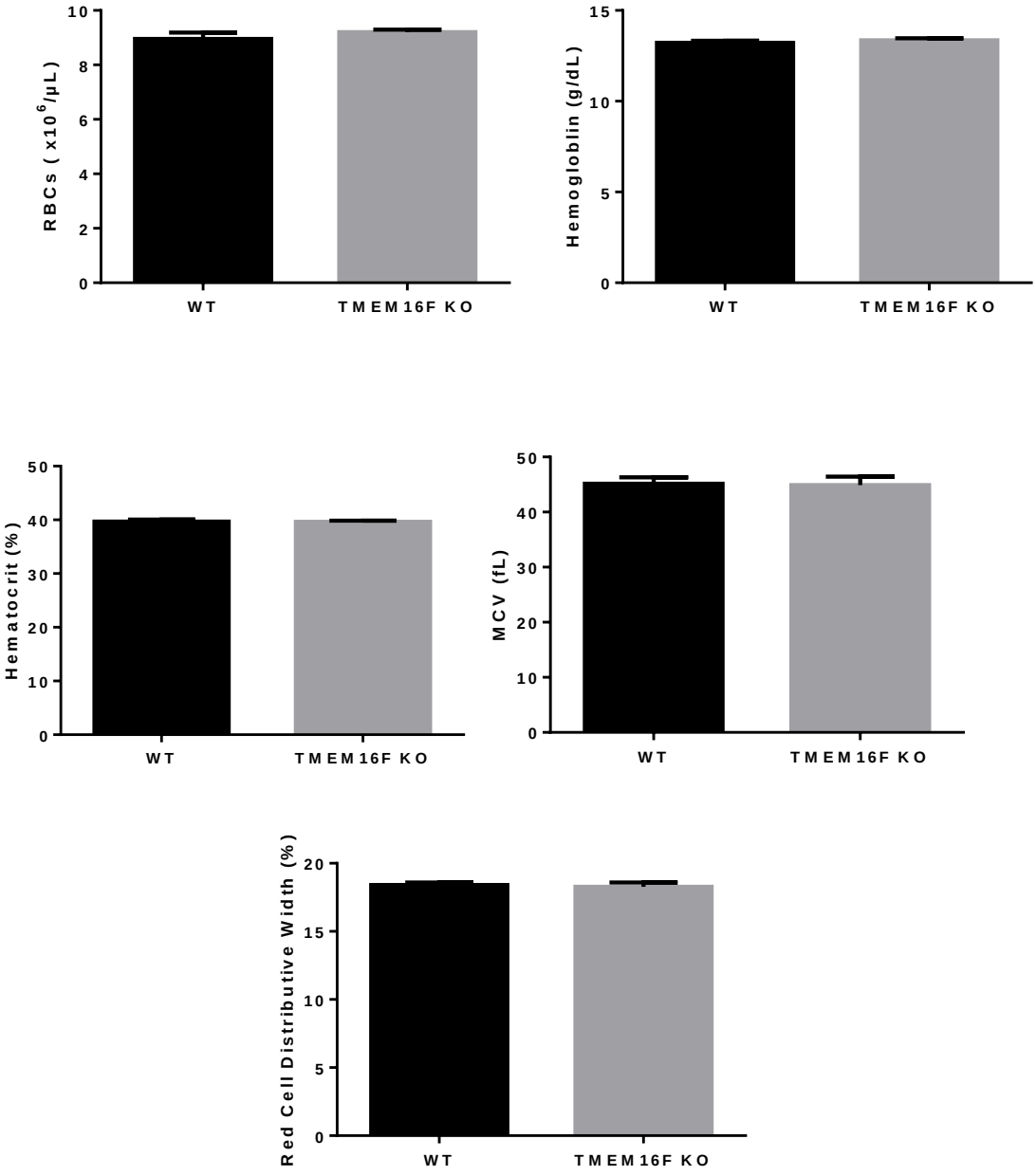
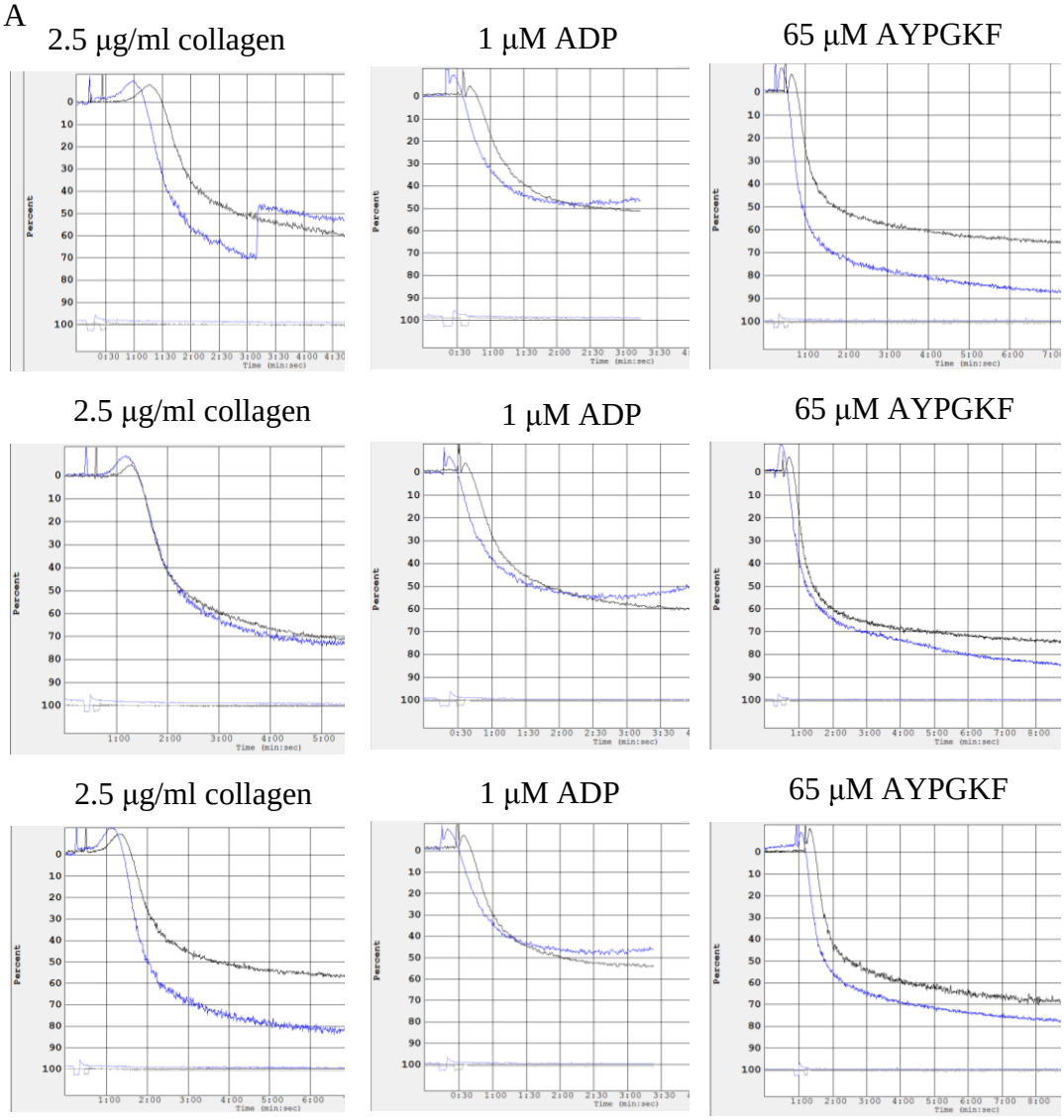


Fig. 13. Complete blood count. Blood from TMEM16F KO and WT mice were evaluated for A) platelet count and mean platelet volume B) leukocyte count and distribution C) erythrocyte count and erythrocyte indices. (Values represented are mean (+/- SEM) of 5-8 independent experiments. *P < 0.05 using a Student's t test).

3.9 Loss of TMEM16F has no Effect on Platelet Aggregation

Considering that platelets lacking TMEM16F have defects in thrombosis *in vivo*, it is important to ascertain whether platelets lacking TMEM16F are still capable of normal aggregation, since a defect in this pathway could also contribute to defects in thrombosis. To determine the role of TMEM16F in platelet aggregation, we compared the aggregation responses of PRP from TMEM16F-deficient and WT mice upon administration of platelet agonists. As expected, TMEM16F-null platelets had normal aggregation responses to all agonists tested (2.5 ug/ml collagen, 1 uM ADP and 65uM AYPGFK) as shown in **Fig 14**. Accordingly, the fact that TMEM16F KO mice exhibit a thrombosis defect and that this defect is corrected in the presence of reconstituted microparticles suggests that the thrombosis defect is attributable to the loss of TMEM16f-dependent microparticle generation.

A



Blue tracing – WT Black tracing – TMEM16F KO

B.

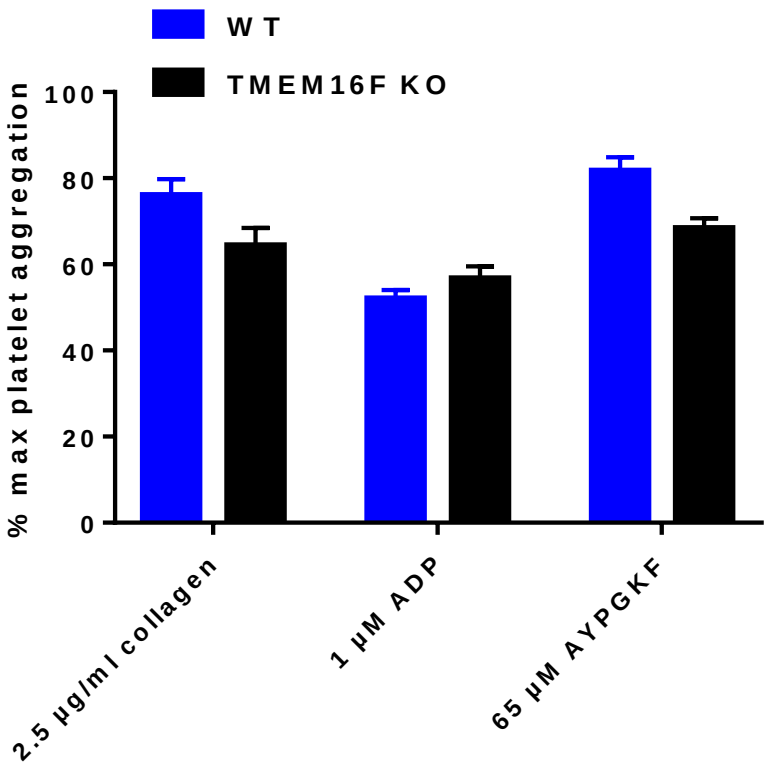


Fig. 14. Platelet aggregation. PRP extracted from TMEM16F KO and WT mice was evaluated for platelet aggregation upon initiation with 2.5 µg/ml collagen, 1 µM ADP or 65 µM AYPGKF in a lumi-aggregometer, and represented by (a) individual tracing of three independent experiments and their (B) averages (+/- SEM).

Chapter 4

DISCUSSION

Procoagulant plasma microparticles are elevated in numerous prothrombotic and hypercoagulable states[20,21,23,24], and could have a dominant role in heightened coagulation and subsequent thrombosis. Microparticles generated from platelets are strongly procoagulant because of their exposed PS membranes which provide additional membranous binding sites for localized enzymatic activities of the tenase (FIXa, FVIIIa and FX) and prothrombinase (FXa, FVa and FII) complexes, resulting in elevated thrombin and fibrin formation[68,21,72,73]. Interestingly, platelet microparticles have been shown to exhibit 50-100 fold higher procoagulant activity compared to activated platelet membranes[93]. The precise mechanisms of platelet microparticle formation are unknown, nevertheless increased intracellular calcium, platelet PS exposure and calpain activation are some known components that contribute to their formation[90,91,92,94].

Reduced platelet-generated microparticles are found in Scott Syndrome[77,126], a rare mild bleeding disorder characterized by impaired platelet procoagulant activity[120]. Due to defective scramblase activity, activated platelets from Scott Syndrome patients have impaired ability to expose PS on the exofacial membrane [125]. Recently, Suzuki and colleagues identified TMEM16F, a novel eight transmembrane protein, to be an

essential component required for calcium-dependent PS exposure on membranes[130]. Importantly, impaired platelet vesiculation (microparticles) of activated Scott Syndrome is demonstrated to be candidly coupled to platelet FV receptor availability[129]. Despite previous studies demonstrating that TMEM16F is required for PS exposure, procoagulant activity and thrombosis[138], the role of TMEM16F in microparticle formation and the subsequent contribution of TMEM16F-dependent microparticles to thrombosis has been unclear. Therefore, we sought to understand how TMEM16F contributes to platelet microparticle formation, procoagulant activity, and thrombosis in this study.

By exposing TMEM16F KO platelets to physiological agonists or calcium ionophore, we have shown that TMEM16F is required for PS+ and total platelet-derived microparticle formation as well as PS exposure on platelet membranes. Previously, Yang *et al.* demonstrated that loss of TMEM16F impairs A23187-induced platelet PS exposure and that TMEM16F forms a calcium activated calcium-permeable ion channel that is required for platelet PS exposure[138]. While data from TMEM16F KO mice originally showed that TMEM16F is required for both scramblase activity and calcium entry, the recently solved crystal structure helped to reveal the calcium-permeable pore[114] and helped to confirm evidence that the protein is likely sufficient for both calcium permeability and scramblase activity. Given this evidence, it is also likely that both calcium channel and scramblase functions are involved in PS+ platelet microparticle formation. The requirement for calcium activity is likely due to the requirement for activation of calcium-dependent calpain for

proteolytic cleavage of the cytoskeleton resulting in vesiculation, while scramblase activity allows PS exposure of the ultimately PS+ extruded membrane surfaces of the microparticles. Our data are consistent with PS exposure on platelet surface during microparticle formation.

As noted in many prior studies[145,146,141,144,90], we observed that platelet-derived microparticle formation elicited by natural agonists occurs with less efficacy than that elicited by calcium ionophore, and in fact requires dual agonist stimulation, such as that elicited by thrombin and collagen to attain consistently significant levels. The reason for this appears to be the requirement of multiple concomitant calcium – activating pathways to attain a threshold level of calcium that is sufficient to stimulate PS exposure on a subset of platelets: this subset of PS+ pro-coagulant platelets were previously often called "COATED" platelets, for collagen and thrombin-activated platelets[142,143]. The snake venom toxin convulxin is often substituted for collagen[141,149,150], as it exhibits a higher affinity interaction with the collagen receptor GPVI than collagen itself, so we have used both agents here. In sum, it is speculated that microparticle formation and by extension platelet PS exposure occurs when several signaling pathways become activated by multiple agonists at the site of platelet activation *in vivo*. Interestingly, Delaney and colleagues have shown that physiological shear significantly increases agonist-dependent microparticle formation and PS exposure from platelets[139].

Our data shows a clear TMEM16F-dependency on the steady-state circulating levels of total and CD41+ (platelet- and megakaryocyte- derived) microparticle

formation *in vivo*, corroborating our *in vitro* platelet microparticle data. These results underscore the physiological requirement of TMEM16F in microparticle formation and suggests that TMEM16F may have a role in pathological microparticle formation as well, such as in prothrombotic conditions. It is worth pointing out that majority of the CD41+ microparticles in healthy normal circulation are most likely derived from megakaryocytes – the precursor cells of platelets, as Flaumenhaft and colleagues[84] demonstrated using various platelet and megakaryocyte specific markers. However, in pathological agonist-driven prothrombotic conditions[151-153,20-24], we would expect that the majority of these circulating microparticles are indeed derived from platelets.

We demonstrated that TMEM16F is required for procoagulant activity *in vitro* and thrombosis *in vivo*, corroborating previously published data[138]. However, for the first time we have shown that addition of platelet-generated microparticles to PRP of TMEM16F KO plasma completely restores thrombin generation to the level of that seen in WT. Further, platelet-derived microparticles added to TMEM16F PPP completely restored thrombin generation to the level seen in WT PRP, suggesting that these microparticles by themselves (without platelets) have significantly higher procoagulant activity compared to activated platelets. These data together suggest the TMEM16F-dependent platelet-derived microparticles significantly elevate coagulation-promoting activity and contribute significantly to thrombosis *in vivo*. Further, these data also suggest that the defect in thrombosis seen in TMEM16F KO mice is attributable to their defect in TMEM16f-dependent procoagulant microparticle generation.

The reconstitution of thrombosis *in vivo* in TMEM16F KO mice with platelet microparticles demonstrates that TMEM16F-dependent microparticles are a major culprit in thrombosis. The fact that TMEM16F KO mice have unaltered platelet count and platelet aggregation responses, but slight increase in mean platelet volume, suggests that platelets may be shedding fewer microparticles and therefore remain larger in size. The increased mean platelet volume observed in TMEM16F KO mice could also suggest that the megakaryocytes are turning over platelets more quickly than normal. The impact of TMEM16F on platelet generation remains to be established.

Overall, our data suggest that TMEM16F is required for platelet microparticle formation and that those microparticles subsequently elevate procoagulant activity that can result in thrombosis. The inhibition of TMEM16F may represent a novel anti-thrombotic therapeutic target by reducing platelet microparticle formation.

Chapter 5

FUTURE DIRECTIONS

We have found that TMEM16F is required for microparticle formation. The precise signaling mechanisms downstream of TMEM16F activation remains to be defined. Notably calpain, a calcium-activated protease, is demonstrated to be required for microparticle formation[91]. Since TMEM16F is a calcium-activated calcium permeable channel[138], we would like to examine the connection between TMEM16F and calpain activity and how loss of TMEM16F impacts calpain activity.

We have demonstrated that TMEM16F-dependent microparticles restores thrombosis *in vivo* due the loss of TMEM16F, highlighting the importance of TMEM16F-dependent platelet microparticles in thrombus formation. We would like to get a more detailed picture of this process by performing a laser injury thrombosis model and using intravital microscopy. Details regarding the incorporation of TMEM16F-dependent microparticles in the thrombus, fibrin deposition, platelet accumulation and thrombin generation in an *in vivo* setting will be evaluated.

The fact that TMEM16F appears to not only regulate normal circulating CD41+ microparticles (platelet- and megakaryocyte- derived microparticles) but also normal circulating total microparticles, leaves unanswered questions about microparticles derived from leukocytes, erythrocytes and endothelial cells. We

therefore would like to characterize all microparticles from TMEM16F KO and WT mice both under normal physiological conditions and in prothrombotic pathological conditions.

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Appendix A

ANIMAL CONSENT FORM

University of Delaware
Institutional Animal Care and Use Committee
Annual Review

(Please complete below using Arial, size 12 Font.)

RECEIVED
JUN 1 2015
IACUC

Title of Protocol: Microparticles and platelet signaling in thrombosis	
AUP Number: 1257-2015-2	← **(4 digits only)**
Principal Investigator: Donna Woulfe	
Common Name: mouse **Genus Species:** mus musculus	

Pain Category: ***(please mark one)***

USDA PAIN CATEGORY: ***(Note change of categories from previous form)***

Category	Description
☐ B	Breeding or holding where NO research is conducted
☐ C	Procedure involving momentary or no pain or distress
X D	Procedure where pain or distress is alleviated by appropriate means (analgesics, tranquilizers, euthanasia etc.)
☐ E	Procedure where pain or distress cannot be alleviated, as this would adversely affect the procedures, results or interpretation

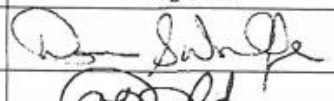
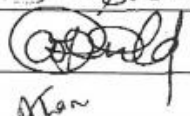
Official Use Only

IACUC Approval Signature: Gwen Talken

Date of Approval: 9/1/15

SIGNATURE(S) OF ALL PERSONS LISTED ON THIS PROTOCOL

I certify that I have read this protocol, accept my responsibility and will perform only the procedures that have been approved by the IACUC.

Name	Signature
1. Donna S. Wolfe	
2. CONROY FIELD	
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