Arteriosclerosis, Thrombosis, and Vascular Biology

BASIC SCIENCES

Alterations to Sphingomyelin Metabolism Affect Hemostasis and Thrombosis

Jue Wang, Shiva Keshava, Kaushik Das, Richard Kolesnick, Xian-Cheng Jiang, Usha R. Pendurthi, L. Vijaya Mohan Rao

BACKGROUND: Our recent studies suggest that sphingomyelin levels in the plasma membrane influence TF (tissue factor) procoagulant activity. The current study was performed to investigate how alterations to sphingomyelin metabolic pathway would affect TF procoagulant activity and thereby affect hemostatic and thrombotic processes.

METHODS: Macrophages and endothelial cells were transfected with specific siRNAs or infected with adenoviral vectors to alter sphingomyelin levels in the membrane. TF activity was measured in factor X activation assay. Saphenous vein incision–induced bleeding and the inferior vena cava ligation–induced flow restriction mouse models were used to evaluate hemostasis and thrombosis, respectively.

RESULTS: Overexpression of SMS (sphingomyelin synthase) 1 or SMS2 in human monocyte-derived macrophages suppresses ATP-stimulated TF procoagulant activity, whereas silencing SMS1 or SMS2 increases the basal cell surface TF activity to the same level as of ATP-decrypted TF activity. Consistent with the concept that sphingomyelin metabolism influences TF procoagulant activity, silencing of acid sphingomyelinase or neutral sphingomyelinase 2 or 3 attenuates ATP-induced enhanced TF procoagulant activity in macrophages and endothelial cells. Niemann-Pick disease fibroblasts with a higher concentration of sphingomyelin exhibited lower TF activity compared with wild-type fibroblasts. In vivo studies revealed that LPS+ATP-induced TF activity and thrombin generation were attenuated in ASMase$^{-/-}$ mice, while their levels were increased in SMS2$^{-/-}$ mice. Further studies revealed that acid sphingomyelinase deficiency leads to impaired hemostasis, whereas SMS2 deficiency increases thrombotic risk.

CONCLUSIONS: Overall, our data indicate that alterations in sphingomyelin metabolism would influence TF procoagulant activity and affect hemostatic and thrombotic processes.

GRAPHIC ABSTRACT: A graphic abstract is available for this article.

Key Words: hemostasis ■ metabolism ■ sphingomyelin ■ thrombosis ■ tissue factor

As the primary initiator of the coagulation cascade, TF (tissue factor) is essential for hemostasis,[1,2] but an aberrant expression of TF leads to thrombotic occlusions, the precipitating event in acute myocardial infarction, unstable angina, and ischemic stroke.[3–6] Typically, TF exists in a cryptic, coagulant inactive state in cells.[7–9] Cell injury leads to the transformation of cryptic TF to procoagulant active TF through post-translational modifications of TF, alterations in phospholipid composition, or both.[8–11] However, molecular differences between encrypted and decrypted TF forms and the mechanism of TF transformation from the cryptic state to the procoagulant state are not fully understood; and they are often controversial.[10,12] Recent studies from our laboratory indicate that sphingomyelin levels in the plasma membrane play a critical role in modulating TF activity through encryption and decryption.[13,14]

Sphingomyelin metabolism is altered in many disease settings, including atherosclerosis, diabetes, sepsis, cancer, and viral infections,[15–18] the same disease settings

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Nonstandard Abbreviations and Acronyms

ASMase	acid sphingomyelinase
EV	extracellular vesicle
IVC	inferior vena cava
MDM	monocyte-derived macrophage
nSMase	neutral sphingomyelinase
NPD	Niemann-Pick disease
PBMC	peripheral blood mononuclear cell
SMS	sphingomyelin synthase
TF	tissue factor
TAT	thrombin-antithrombin

that also induce aberrant expression or activation of TF.[3–5,19] SMSs (sphingomyelin synthases) and SMases (sphingomyelinases), including ASMase (acid sphingomyelinase) and nSMase (neutral sphingomyelinase), play a central role in sphingomyelin metabolism.[15,18,20–23] SMSs are shown to play a role in various biological functions, including cell migration, apoptosis, and autophagy, as well as in human disorders, such as cardiovascular diseases and cancer.[24] ASMase plays a crucial role in tissue homeostasis.[25] ASMase deficiency or chronic activation of ASMase leads to various diseases, such as Niemann-Pick disease, depression, atherosclerosis, cancer, and sepsis.[15,25–28] Recent studies showed that SARS-CoV-2 infection activates ASMase, and the ASMase-induced membrane changes play a crucial role in SARS-CoV-2 entry into epithelial cells.[29] nSMase has 3 different forms, nSMase 1, nSMase 2, and nSMase 3. nSMases are shown to play a role in apoptosis, inflammation, embryonic and post-natal development, cancer, neurological pathologies, and cardiovascular pathophysiology.[30–33]

Our earlier studies showed that the high content of sphingomyelin in the plasma membrane plays a crucial role in maintaining TF in an encrypted (coagulant inactive) state in resting cells, and the hydrolysis of sphingomyelin following cell injury removes the inhibitory effect of sphingomyelin on TF activity, thus leading to TF decryption.[14] Our recent in vivo studies suggest that LPS-induced TF procoagulant activity not only requires de novo synthesis of TF but also decryption of newly synthesized TF as the administration of ASMase functional inhibitors suppressed LPS-induced TF procoagulant activity without affecting de novo synthesis of TF.[13] Our more recent studies show that SARS-CoV-2 infection activates ASMase and the activated ASMase is responsible for the enhanced TF procoagulant activity associated with SARS-CoV-2 infection.[34] At present, there is no information on how other enzymes involved in sphingomyelin metabolism influence TF activity and whether genetic deficiency of the enzymes involved in sphingomyelin metabolism affects hemostasis or thrombosis.

Highlights

- Overexpression or inhibition of sphingomyelin by silencing SMS1 (sphingomyelin synthase 1), SMS2, or SMase in different cell types modulated TF (tissue factor) activity on the cell surface and released extracellular vesicles.
- In vivo studies revealed that LPS+ATP-induced TF activity and thrombin generation were attenuated in ASMase$^{-/-}$ mice, while their levels were increased in SMS2$^{-/-}$ mice.
- Studies using the saphenous vein injury bleeding model and inferior vena cava ligation model showed impaired hemostasis and reduced thrombus formation in ASMase$^{-/-}$ mice, whereas enhanced hemostatic activity and thrombus formation in SMS2$^{-/-}$ mice.
- Overall, our data indicate that changes in the SM metabolism influence hemostasis and thrombosis in vitro and in vivo through modulation TF encryption and decryption processes.

The present study was performed to investigate how alterations in sphingomyelin metabolism influence TF procoagulant activity in vitro and in vivo and affect hemostasis and thrombosis in murine models. Alterations of sphingomyelin levels by genetic manipulation of SMS1, SMS2, ASMase, nSMase 2, or nSMase 3 were found to significantly affect TF procoagulant activity without altering TF protein levels in monocytes/macrophages, endothelial cells, and fibroblasts. Fibroblasts from ASMase-deficient Niemann-Pick disease patients exhibited lower levels of TF procoagulant activity compared with fibroblasts from healthy individuals. In vivo studies showed that genetic deficiency of enzymes involved in the sphingomyelin metabolism affects LPS-induced TF procoagulant activity in monocytes and intravascular thrombin generation in a murine model. Studies with ASMase$^{-/-}$ and SMS2$^{-/-}$ mice show that ASMase deficiency induces the bleeding phenotype, whereas SMS2 deficiency promotes thrombosis. Our findings provide clues on new targets for developing novel therapeutics to treat thromboinflammatory disorders associated with infection, cardiovascular diseases, and cancer where sphingolipid metabolism is altered.

MATERIALS AND METHODS

Generation of Human Monocyte-Derived Macrophages

Human peripheral blood mononuclear cells (PBMCs) were isolated by density gradient centrifugation using Ficoll-Paque (GE Healthcare, Pittsburg, PA). PBMCs were cultured for 7 days in an RPMI medium supplemented with a macrophage colony-stimulating factor to differentiate them into monocyte-derived macrophages (MDMs), as described earlier.[14]

Culturing of Endothelial Cells

Primary human umbilical vein endothelial cells (HUVECs) were grown to the confluence at 37 °C and 5% CO_2 in a humidified incubator in EBM-2 basal media supplemented with 2% fetal bovine serum and growth supplements.

Isolation of Murine PBMCs

Murine PBMC were isolated by density gradient centrifugation as described in our earlier publication.[13]

Mice

Wild-type mice (WT, C57BL/6J) were obtained initially from The Jackson Laboratory (Bar Harbor, ME) and then bred in-house. Breeding pairs of heterozygous ASMase$^{-/+}$ mice were provided by Dr Richard Kolesnick, Memorial Sloan Kettering Cancer Center, New York, whereas breeding pairs of homozygous SMS2$^{-/-}$ mice were obtained from Dr Xian-Cheng Jiang, SUNY Downstate Medical Center, New York. ASMase$^{-/-}$ and SMS2$^{-/-}$ mice are in the C57BL/6J genetic background as they were backcrossed with C57BL/6J mice for at least 6 generations. Heterozygous ASMase$^{-/+}$ mice were bred in-house to obtain ASMase knockout mice (ASMase$^{-/-}$) and littermate controls. Four or more (out of ten) littermate controls used in our experiments came from the same cages as of ASMase$^{-/-}$ mice. SMS2$^{-/-}$ mice were generated by homozygous breeding. C57BL/6J WT mice bred in-house in the same room were used as controls for SMS2$^{-/-}$ mice. Where feasible, thrombosis procedure in control groups was performed contemporarily with the respective knockouts.

Generation of SMS1 and SMS2 Adenovirus

SMS1 and SMS2 plasmids in pCDNA3.1 were kindly provided by Dr Joost Holthuis, University of Osnabrueck, Germany. They were excised with *PmeI* and *BamHI* and subcloned into pacAd5 CMVK-NpA shuttle vector (Cell Biolabs). The rest of the procedures to generate adenovirus encoding SMS1 and SMS2 were the same as described earlier.[35]

Inhibition of SMS1, SMS2, ASMase, or nSMases by siRNA

HUVECs or MDMs were transfected with a control transfection reagent (mock transfection), scrambled oligonucleotide (scRNA), or siRNA specific for SMS1, SMS2, ASMase, nSMase 1, nSMase 2, or nSMase 3 (all were used at 100 nM) using Lipofectamine RNAiMax transfection reagent (ThermoFisher, Waltham, MA).

Isolation of Extracellular Vesicles

Extracellular vesicles (EVs) from the conditioned culture media or murine platelet-poor plasma were isolated by centrifugation at 21 000×*g* for 1 hour as described earlier.[13,14]

Measurement of Tissue Factor Activity

Cell surface- and EV-associated TF activity were measured in a factor X activation chromogenic assay as previously described.[36,37]

FACS Analysis

Human MDMs were fixed with 4% paraformaldehyde at room temperature for 1 hour. Fixed cells were blocked with 10% FBS in Tyrode buffer for 1 hour. To label sphingomyelin, non-permeabilized cells were incubated with sphingomyelin-specific binding protein, lysenin (0.5 µg/mL; Peptide Institute, Inc, Osaka, Japan) in 2% bovine serum albumin in PBS for 4 hours. After removing the unbound lysenin and washing cells, the cells were incubated with rabbit polyclonal anti-lysenin antiserum (Peptide Institute, Inc; 200 X dilution) at 4 °C overnight. After washing the cells 3 times with Tyrode buffer, cells were incubated with AF546 conjugated donkey anti-rabbit IgG (2 µg/mL) for 90 minutes. Cells were analyzed using an Attenux flow cytometer (Becton and Dickinson, San Jose, CA). For TF analysis, non-permeabilized fixed MDMs were incubated with mouse anti-human TF monoclonal antibodies (TF9-9C3, 10 µg/mL, a gift from J.H. Morrissey, University of Michigan) followed by AF488 conjugated donkey anti-mouse IgG.

Immunofluorescence Microscopy, Image Acquisition, and Quantification of Fluorescence Signals

Immunofluorescence confocal microscopy, image acquisition, and quantification of fluorescence signals were performed essentially as described in our earlier publications.[38,39]

Animal Studies

All animal studies were conducted following the animal welfare guidelines outlined in the Guide for the Care and Use of Laboratory Animals and the Department of Health and Human Services. All animal procedures were approved by the Institutional Animal Use and Care Committee.

TF Decryption In Vivo

Wild-type, ASMase$^{-/-}$, or SMS2$^{-/-}$ mice were injected intraperitoneally with saline or LPS (*E coli* O111:B4, 5 mg/kg) to induce TF protein expression. Four hours after the LPS challenge, a group of animals was injected intraperitoneally with bz-ATP (50 mg/kg). After 15 minutes, animals were anesthetized, and blood was drawn by cardiac puncture. The whole blood was carefully loaded on a Ficoll gradient and subjected to centrifugation at 400×*g* for 40 minutes to separate the plasma and PBMCs. PBMCs were washed 2 times with Tyrode buffer before they were used for measuring TF activity. The plasma was processed to isolate EVs. TF activity on intact PBMCs and EVs was measured in a factor X activation assay.

Saphenous Vein Incision-Induced Bleeding

Mice were subjected to the saphenous vein incision as described earlier.[40,41] The average time to achieve hemostasis was determined by the number of hemostatic plugs formed during 30-minute observational period. Total blood loss from the incision site during the 30-minute observational period was measured as described in our earlier studies.[40,41]

Venous Thrombosis

Ligature-based inferior vena cava (IVC) stasis model was used to evaluate venous thrombosis as described earlier.[42,43] Briefly,

anesthetized mice were subjected to a ventral midline incision, and the IVC was separated from the aorta. The IVC that was inferior to the renal veins and all side branches were ligated. All the IVC back branches, from the renal veins to the iliac bifurcation, were cauterized. After closing the laparotomy site, mice were returned to the individual cages. After 24 hours, the peritoneum was opened, and the ligated IVC containing the clot was excised. The clot length and weights were measured.

Measurement of Thrombin-Antithrombin Complexes in Murine Plasma

Thrombin-antithrombin complex levels in the mouse plasma were determined using AssayMax mouse thrombin-antithrombin complex ELISA kit (Assaypro LLC, MO 63301) and following the protocol included in the kit.

Data Analysis

Each experiment was repeated at least 3 times or more, and data are expressed as mean±SEM. The normal distribution of the data was analyzed by Shapiro-Wilk or Kolmogorov-Smirnov test. Where a sufficient number of sample sizes or experimental repeats were available for the analysis, most of the data passed the normality test. Statistical significance among multiple groups was analyzed by 1-way ANOVA followed by Dunnett post hoc test. Statistical significance between the 2 groups was calculated using the Mann-Whitney test. $P<0.05$ was considered statistically significant. Prism (vs 9.01; GraphPad) software was used for preparing graphics and calculating statistical significance.

RESULTS

Alterations of Sphingomyelin Levels by Genetic Manipulation of Enzymes Involved in Sphingomyelin Metabolism Influence TF Activity

To investigate the influence of sphingomyelin levels on TF activity, first, we overexpressed SMS1, SMS2, or both in MDMs by infecting the cells with adenovirus (AdV) encoding SMS1 or SMS2. As shown in Figure 1A, SMS1, SMS2, or SMS1 plus SMS2 adenoviruses infection markedly increased sphingomyelin levels on the cell surface of MDMs. The flow cytometric analysis confirmed a significant increase in the sphingomyelin levels in cells infected with SMS1, SMS2, or both AdVs (Figure S1A). Overexpression SMSs did not alter the gross cell morphology or size of MDMs. Overexpression of SMSs did not affect either cell surface or total TF antigen expression in MDMs (Figure 1B; Figure S1B). Increased SMs levels by overexpression of SMSs significantly reduced the ATP-induced enhanced TF activity on the cell surface (TF decryption; Figure 1C) and TF activity associated with EVs (Figure 1D). Next, we transfected MDMs with siRNAs specific for SMS1, SMS2, or both. As expected, the silencing of SMS1, SMS2, or both markedly reduced the sphingomyelin content in MDMs (Figure 1E; Figure S1C). As observed in flow cytometry (Figure 1F) or Western blot analysis (Figure S1D), silencing of SMSs had no detectable effect on TF protein expression. However, the silencing of SMSs significantly enhanced basal cell surface TF activity, reaching a level obtained with ATP-induced decrypted TF (Figure 1G). ATP treatment did not further increase the cell surface TF activity in SMSs silenced MDMs (Figure 1G). SMSs silencing also increased EV-TF activity (Figure 1H).

To assure that the above-observed increase in FX activation in cells where sphingomyelin levels were reduced has resulted from TF-dependent increased procoagulant activity and not from potential changes in PS-dependent procoagulant activity, MDMs were preincubated with annexin V or anti-TF antibodies before they were treated with either bacterial sphingomyelinase (bSMase) or ATP that activates ASMase to lower sphingomyelin levels on the cell surface.[14] As shown in Figure S2, annexin V pretreatment did not affect the enhanced FX activation observed in cells treated with bSMase, whereas anti-TF antibodies fully attenuated FX activation. Similarly, anti-TF antibodies also completely blocked ATP-induced enhanced procoagulant activity on the cell surface. In contrast, consistent with our earlier published data,[14] annexin V inhibited FX activation modestly in ATP-stimulated MDMs. We obtained similar results in measuring EV-TF activity (Figure S2B). Overall, the above data indicate that the enhanced FX activation in cells following the lowering of sphingomyelin levels in the plasma membrane comes from increased TF procoagulant activity that is independent of PS.

To further strengthen our findings that alterations in sphingomyelin levels modulate TF activity, MDMs were transfected with siRNA specific for ASMase, nSMase 1, nSMase 2, or nSMase 3. As shown in Figure 2A, silencing of ASMase, nSMase2, nSMase3, and not nSMase 1 significantly increased sphingomyelin levels in the plasma membrane. Flow cytometric analysis confirmed increased sphingomyelin levels in MDMs transfected with ASMase, nSMase2, or nSMase3 siRNAs (Figure S3A; 30% to 65% increase in mean fluorescence intensity). Analysis of cell surface TF expression by flow cytometry (Figure 2B) or total TF antigen levels by immunoblot analysis (Figure S3B) showed no change in TF expression in MDMs transfected with ASMase, nSMase 1, nSMase 2, or nSMase 3 siRNA. The silencing of ASMase, nSMase2, or nSMase3 significantly reduced the ATP-induced increase in TF procoagulant activity on cell surfaces (Figure 2C) and EV-TF activity (Figure 2D). Silencing of nSMase 1 had no significant effect on ATP-induced TF activity on the cell surface or EVs (Figure 2C and 2D).

To further strengthen the data that alteration in sphingomyelin levels by up- or down-regulation of enzymes involved in sphingomyelin metabolic pathway influence TF activity, we modulated sphingomyelin

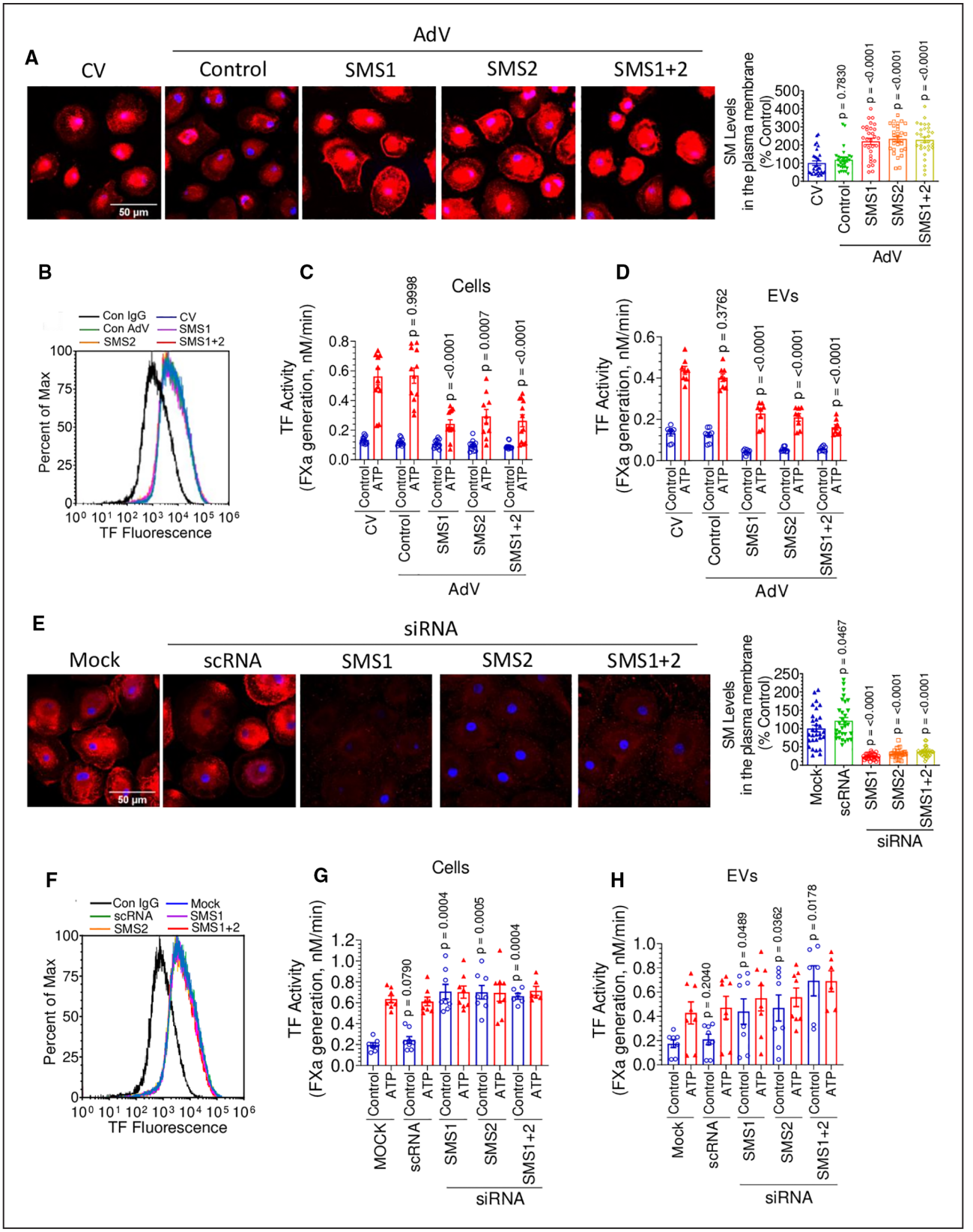

Figure 1. Alterations in sphingomyelin levels by genetic manipulation of SMS1 (sphingomyelin synthase 1) or SMS2 modulate TF (tissue factor) activity.

A, Increased SM levels in human monocyte-derived macrophage (MDMs) infected with adenovirus encoding SMS1 or SMS2. MDMs were treated with a control vehicle (CV) or infected with a control adenovirus (AdV) or AdV encoding SMS1, SMS2, or both (10 pfu/cell). After culturing MDMs for 48 hours, the cells were fixed and stained with an sphingomyelin (SM)-binding protein, lysenin, (*Continued*)

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levels in endothelial cells by overexpressing SMS1 and SMS2 or silencing ASMase or nSMases. Infection of HUVEC with SMS1, SMS2, or SMS1 plus SMS2 AdV significantly increased sphingomyelin levels in HUVECs (Figure S4A). Since HUVECs do not constitutively express TF, they were stimulated with TNFα (10 ng/ml) plus IL1β (10 ng/ml) to induce TF expression. Overexpression of SMS1 or SMS2 significantly decreased TNFα+IL1β-induced TF activity on the endothelial cell surface and EVs (Figure S4B and S4C). As observed in MDMs, silencing ASMase, nSMase 2, or nSMase 3, but not nSMase 1, significantly enhanced sphingomyelin levels on the cell surface (Figure S5A). The silencing of SMases had no detectable effect on TF protein expression in endothelial cells (Figure S5B and S5C). ASMase, nSMase 2, or nSMase 3 silencing gave a modest but statistically significant reduction in cell surface TF activity as well as TF activity associated with EVs (Figure S5D and S5E). nSMase 1 silencing, which did not alter sphingomyelin levels in the plasma membrane, had no significant effect on TF activity (Figure S5D and S5E). Overall, the above data provide strong evidence that alterations in sphingomyelin metabolism influence TF procoagulant activity by modulating its encryption and decryption processes.

Increased Sphingomyelin Levels and Reduced TF Activity in Fibroblasts of Niemann-Pick Disease

Niemann-Pick disease types A (NPD A) and B (NPD B) are rare autosomal recessive lysosomal storage disorders caused by mutations in the ASMase gene.[44] Decreased ASMase activity leads to the accumulation of sphingomyelin in macrophages and other cell types and leads to severe to variable multiorgan disease.[44,45] To determine the effect of ASMase genetic deficiency on TF activity levels, fibroblasts from 3 NPD patients (1 type A and 2 Type B) obtained from Coriell Institute of Medical Research (Hamden, NJ) were cultured, and sphingomyelin levels and TF activity on their cell surfaces were evaluated. As shown in Figure 3A, fibroblasts from NPD patients showed higher sphingomyelin levels than wild-type fibroblasts. No significant differences were found in TF protein expression levels among wild-type and NPD fibroblasts (Figure 3B). Analysis of TF activity on the cell surface and EVs shows that NPD fibroblasts have lower TF activity compared with wild-type fibroblasts, both at basal levels as well as ATP-induced increased TF activity (Figure 3C and 3D). These data suggest that high levels of sphingomyelin in cells suppress TF activity in NPD fibroblasts.

ASMase and SMS2 Deficiency Affect LPS and LPS+ATP-Induced TF Procoagulant Activity in Monocytes In Vivo

To investigate the effect of alterations in sphingomyelin levels on TF activity and coagulation in vivo, first, we evaluated sphingomyelin levels in PBMCs of wild-type (WT), ASMase$^{-/-}$, and SMS2$^{-/-}$ mice by lysenin binding to the cells. As shown in Figure 4A, sphingomyelin levels in PBMCs from ASMase$^{-/-}$ mice were much higher than in WT mice, whereas sphingomyelin levels were significantly lower in PBMCs of SMS2$^{-/-}$ mice. Next, WT, ASMase$^{-/-}$, and SMS2$^{-/-}$ mice were challenged with LPS, and LPS-induced de novo expression of TF antigen in monocytes was analyzed by flow cytometry (Figure 4B) or immunoblot analysis (Figure S6). Analysis of cell surface TF antigen levels by flow cytometry showed that ASMase deficiency

Figure 1 Continued. as described in Methods. Cells were imaged by confocal microscopy, and the fluorescence intensity of SM (red color) in the plasma membrane (30 cells) was quantified using Image J (FIJI). DAPI was used to label nuclei (blue color). The left panel shows representative images, and the right panel shows quantified data by 1-way ANOVA followed by Dunnett post hoc test. The fluorescence intensity measured in MDMs treated with a CV was taken as 100%. **B**, Increased synthesis of SM by overexpression of SMS1 or SMS2 does not alter TF antigen levels on the cell surface. MDMs, infected with a control, SMS1, SMS2, or both AdV as described in **A**, were immunostained with TF mAb. As controls, MDMs treated with a control vehicle were stained with control IgG (Con IgG) or TF mAb (CV). Immunostained cells were subjected to FACS analysis. Please note that histograms of TF immunostained cells of various treatments were completely overlapped. **C** and **D**, SMS1 or SMS2 overexpression attenuates ATP-induced increased TF activity on the cell surface (**C**) and EVs (**D**). MDMs were treated with a control vehicle (CV) or infected with control, SMS1, SMS2, SMS1+SMS2 AdVs as described in **A**. After culturing cells for 48 hours, they were treated with a control vehicle (control) or ATP (200 μM) for 15 minutes. At the end of 15-minute treatment, cell supernatants were collected, and EVs from cell supernatants were isolated by centrifugation. TF activity associated with cells and EVs were determined in a factor X activation assay by adding FVIIa (10 nM) and FX (175 nM) and measuring the rate of factor Xa generation in a chromogenic assay. The data were analyzed by 1-way ANOVA followed by Dunnett post hoc test (n=12 for **C** and n=9 for **D**). *P* were calculated relative to CV|ATP. **E**, Reduced expression of SM (red) in MDMs transfected with SMS1 or SMS2 siRNA. MDMs were transfected with a mock transfection reagent (Mock), scrambled RNA (scRNA), or siRNA specific for SMS1, SMS2, or both. After culturing cells for 48 hours, SM was stained and quantified as described in **A**. Nuclei were stained with DAPI (blue). The left panel shows representative images of SM staining, and the right panel shows quantification of SM staining in the plasma membrane by 1-way ANOVA followed by Dunnett post hoc test (n=30). **F**, Silencing of SMS1, SMS2, or both does not affect cell surface expression of TF in MDMs. Non-permeabilized MDMs, transfected with Mock, scRNA, or siRNA specific for SMS1, SMS2, or both, were immunostained for TF and subjected to FACS analysis. As controls, Mock transfected cells were stained with control IgG (Con IgG). Histograms of TF immunostained cells of various treatments were completely overlapped. **G** and **H**, Silencing of SMS1 or SMS2 led to increased cell surface TF activity (**G**) and EV-associated TF activity (**H**). MDMs were transfected with scRNA, or siRNA specific for SMS1, SMS2, or both. MDMs were treated with a control vehicle or ATP, and TF activity associated with the cell surface and EVs were measured as described in **C** and **D**. The data were analyzed by 1-way ANOVA followed by Dunnett post hoc test (n=7 for **G** and n=8 for **H**). *P* were calculated relative to Mock Control.

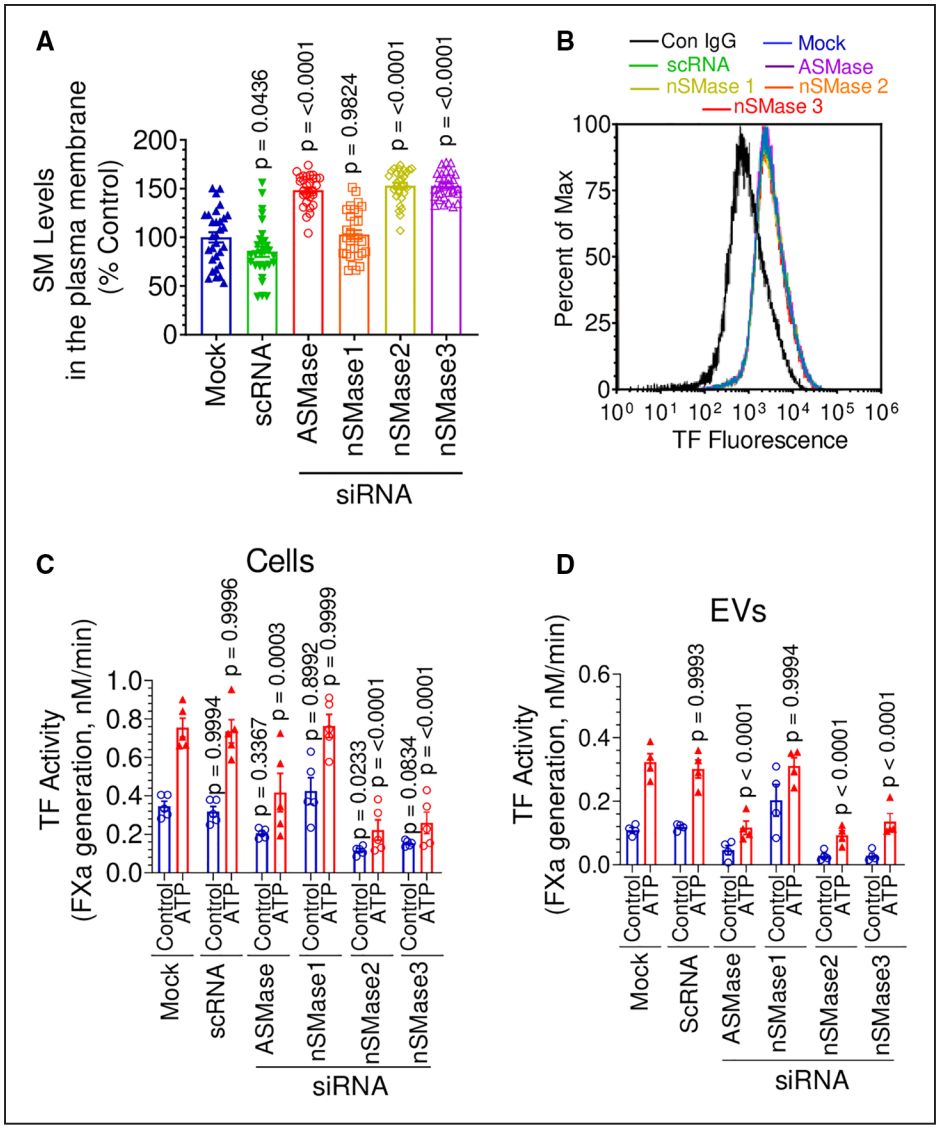

Figure 2. Silencing of ASMase (acid sphingomyelinase), nSMase (neutral sphingomyelinase) 2, or nSMase 3, and not nSMase 1, increases sphingomyelin (SM) levels in the plasma membrane and reduces cell surface TF (tissue factor) activity and TF activity associated with extracellular vesicles (EVs).

A, Silencing of ASMase, nSMase 2, or nSMase 3, and not nSMase 1, increases SM levels in the plasma membrane of monocyte-derived macrophages (MDMs). MDMs were transfected with scrambled RNA (scRNA) or siRNA specific for ASMase, nSMase 1, nSMase 2, or nSMase 3 (100 nM). After culturing cells for 48 hours, cells were fixed and stained for SM using lysenin as described in Figure 1A. The cells were analyzed by confocal microscopy, and the fluorescence intensity of SM staining in the plasma membrane was quantified from 30 cells. The data were analyzed by 1-way ANOVA followed by Dunnett post hoc test. *P* were calculated relative to Mock. **B**, Silencing of SMases had no effect on TF expression. MDMs were transfected with scrambled RNA (scRNA) or siRNA specific for ASMase, nSMase 1, nSMase 2, or nSMase 3. TF expression on the cell surface was analyzed by flow cytometry by immunostaining non-permeabilized cells with TF mAb. As controls, mock-transfected cells were stained with control IgG (Con IgG) or TF mAb (Mock). Histograms of all TF immunostained cells of various treatments were overlapped, therefore individual histograms are not discernible. **C** and **D**, Silencing of ASMase, nSMase 2, or nSMase 3, and not nSMase 1, reduces TF activity at the cell surface and curtails TF$^+$ EVs release. MDMs, transfected with scRNA or siRNA specific for ASMase, nSMase 1, nSMase 2, or nSMase 3, were stimulated with a control vehicle or ATP for 15 minutes. At the end of 15 minutes, cell supernatants were collected and EVs were isolated from the cell supernatants. Cell surface TF activity and TF activity associated with EVs were measured in a factor X activation assay. Cellular TF activity in **C** (n=5) and EV associated TF activity in **D** (n=4) were analyzed by 1-way ANOVA followed by Dunnett post hoc test. *P* were calculated relative to Mock ATP in **C** and **D**. **C**, *P* were also calculated for controls (relative to Mock control).

had no detectable effect on LPS-induced TF expression, whereas SMS2 deficiency appeared to lower TF expression slightly (Figure 4B, ~20% decrease in mean fluorescence intensity). Further analysis of total TF antigen levels by immunoblots and quantification of TF band intensities of immunoblots confirmed that LPS-induced TF protein expression levels in monocytes of SMS2$^{-/-}$ mice were significantly lower than that were found in WT mice (Figure S6). It may be pertinent to point out here that lower expression of TF protein in SMS2$^{-/-}$mice in response to

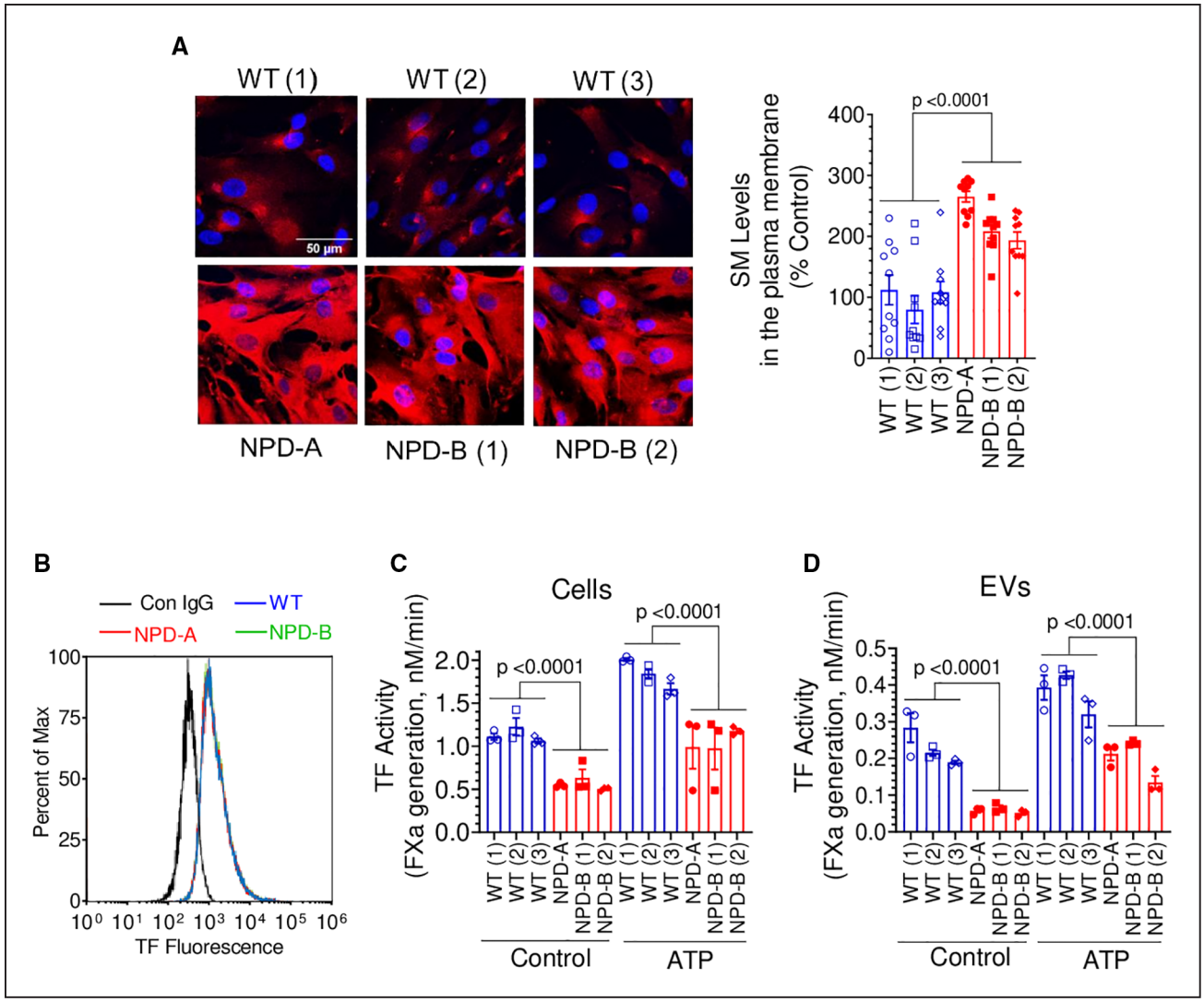

Figure 3. Increased sphingomyelin (SM) levels and reduced TF (tissue factor) activity in fibroblasts from Niemann-Pick disease (NPD) patients.

A, Increased SM levels in fibroblasts from NPD patients. Fibroblasts from 3 healthy individuals (WT) and 3 NPD patients (1 NPD type A and 2 NPD type B) were stained for SM (red) using lysenin. DAPI was used to label nuclei (blue). Representative images from WT and NPD patients' fibroblasts were shown in the left panel. Quantified values of fluorescence intensity in the plasma membrane were shown in the right panel (number of cells analyzed in each group, 10). **B**, Flow cytometry analysis of cell surface tissue factor expression in WT and NPD fibroblasts. Intact, nonpermeabilized WT and NPD fibroblasts were labeled with TF mAb. As a control, WT fibroblasts were also stained with control IgG (Con IgG). The immunostained cells were subjected to FACS analysis. Histograms of TF staining of fibroblasts from WT and NPD patients were overlapped. **C** and **D**, TF activity on the cell surface (**C**) or in EVs (**D**) of WT and NPD fibroblasts. WT and NPD fibroblasts derived from 3 different subjects were treated with a control vehicle (Control) or ATP (200 μM, 15 min). EVs released into the supernatant medium were isolated. TF activity associated with cells and EVs was determined in a factor X activation assay (n=3). For statistical analysis (for all panels), data from 3 cell lines of the same experimental group were combined and statistical significance was determined by nonparametric Mann-Whitney test.

LPS should not be construed as SMS2 regulating TF protein expression as the inhibition or overexpression of SMS2 had no significant effect on TF protein expression in MDMs or endothelial cells. LPS was shown to induce varying levels of TF activity in monocytes in human blood (inter-subject variability/low and high responder phenomenon).[46,47] It is conceivable that the founder mice of SMS2$^{-/-}$ are low responders.

Next, WT, ASMase$^{-/-}$, and SMS2$^{-/-}$ mice were treated with control vehicle, LPS, or LPS, followed by ATP. Measurement of TF activity in PBMCs in ex vivo showed that LPS treatment significantly increased TF activity in all 3 genotypes (Figure 4C). ATP treatment further enhanced LPS-induced TF procoagulant activity in all 3 genotypes (Figure 4C). However, the extent of LPS- or LPS+ATP-induced TF procoagulant activity in ASMase$^{-/-}$ mice was significantly lower than that observed in WT mice. Conversely, LPS- or LPS+ATP-induced TF procoagulant activity was significantly higher in PBMCs of SMS2$^{-/-}$ mice than in WT mice

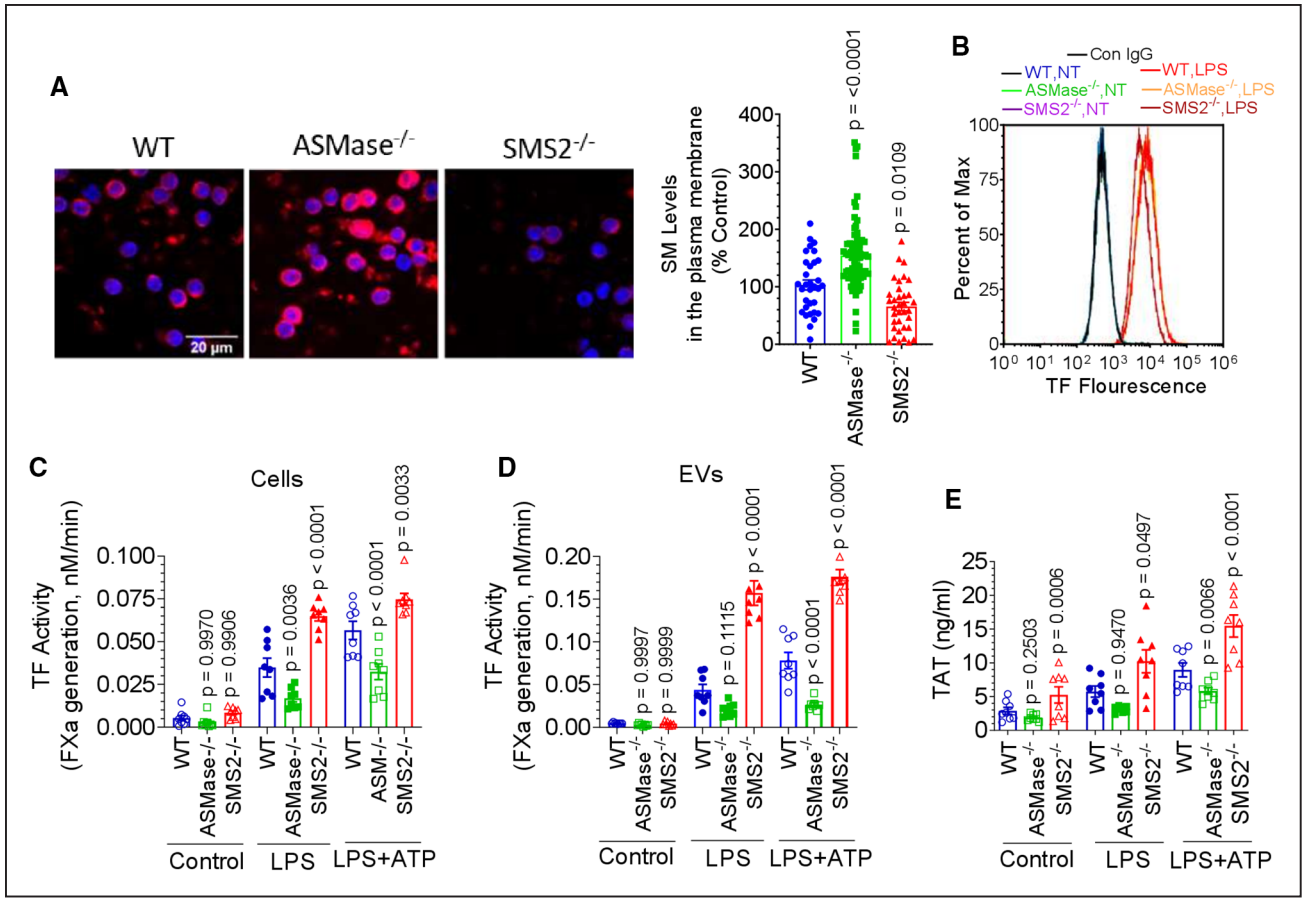

Figure 4. ASMase (acid sphingomyelinase) or SMS2 (sphingomyelin synthase 2) deficiency alters TF (tissue factor) activity in vivo in mononuclear cells by modulating the encryption and decryption of TF.

A, Sphingomyelin (SM) levels in peripheral blood mononuclear cells (PBMCs) isolated from WT, ASMase$^{-/-}$, and SMS2$^{-/-}$ mice. PBMCs were isolated from WT, ASMase$^{-/-}$, or SMS2$^{-/-}$ mice by Ficoll gradient centrifugation. Cells were fixed, and SM was stained by lysenin binding as described in Methods. Immunostained cells were analyzed by fluorescence microscopy, and the fluorescence intensity of SM (red) staining in the plasma membrane was quantified using Image J (visible cells in the image were monocytes; other mononuclear cells were much smaller and stained weakly, and thus not readily visible; n=30 to 80 cells). DAPI was used to label nuclei (blue). The left panel shows representative images, and the right panel shows quantified data. **B**, Cell surface tissue factor expression levels in PBMCs. WT, ASMase$^{-/-}$, and SMS2$^{-/-}$ mice were administered with LPS (5 mg/kg, IP) to induce de novo synthesis of TF or left untreated (NT). After 4 hours of LPS administration, blood was collected, and PMBCs were isolated. Nonpermeabilized PBMCs were fixed, immunostained for TF, and subjected to flow cytometry. As a control, PBMCs from LPS administered WT mice were stained with control IgG (Con IgG). Histograms of TF immunostaining of PBMCs from untreated WT, ASMase$^{-/-}$ and SMS2$^{-/-}$ mice, and control IgG stained PBMCs of LPS-treated WT mice were overlapped (far left histogram). Histograms of TF immunostaining of PBMCs from LPS administered WT and ASMase$^{-/-}$ were also overlapped (far right histogram). Histogram of TF immunostaining of PBMCs from LPS-treated SMS2$^{-/-}$ showed a slight shift to the left compared with histograms of WT and ASMase$^{-/-}$ mice. **C**, TF activity levels in PBMCs of WT, ASMase$^{-/-}$ and SMS2$^{-/-}$ mice (number of mice/group=8). WT, ASMase$^{-/-}$, and SMS2$^{-/-}$ mice were administered with saline (control), LPS (5 mg/kg, 4 hours) or LPS (5 mg/kg, 4 hours) plus ATP (50 mg/kg, 15 minutes, following 4 hours of LPS). At the end of the experimental treatment period, blood was collected, and PBMCs were isolated by Ficoll gradient centrifugation. TF activity associated with PBMCs was measured in a factor X activation assay. **D**, TF activity associated with EVs in plasma (number of mice/group=8). WT, ASMase$^{-/-}$, and SMS2$^{-/-}$ mice were treated with saline, LPS, or LPS+ATP as described above. EVs were isolated from the plasma, and TF activity associated with EVs was measured in a factor X activation assay. **E**, Thrombin generation in the plasma of WT, ASMase$^{-/-}$, and SMS2$^{-/-}$ mice. WT, ASMase$^{-/-}$, and SMS2$^{-/-}$ mice were treated with saline, LPS, or LPS+ATP as described above. Thrombin generation in murine blood was measured as levels of thrombin: antithrombin (TAT) complexes formed in the blood (number of mice/group=8). Statistical analysis was performed using 1-way ANOVA followed by Dunnett post hoc test. *P* were calculated relative to WT in **A**, and WT within the specific treatment group in **C**, **D**, and **E**.

(Figure 4C). Measurement of TF activity associated with EVs isolated from the plasma of control vehicle, LPS, or LPS+ATP treated animals showed similar results, that is, reduced TF activity in EVs isolated from ASMase$^{-/-}$ mice and increased TF activity in EVs isolated from SMS2$^{-/-}$ mice (Figure 4D). Interestingly, quantification of EV numbers by nanoparticle tracking analysis showed a marked reduction in the number of EVs in the plasma of SMS2$^{-/-}$ mice compared with WT and ASMase$^{-/-}$ mice (Figure S7). LPS failed to generate EVs in SMS2$^{-/-}$ mice (Figure S7). Measurement of thrombin-antithrombin levels in plasmas showed that ASMase deficiency substantially reduced LPS- and LPS+ATP-induced thrombin generation, while SMS2 deficiency significantly enhanced

thrombin generation (Figure 4E). Overall, the above data suggest that a genetic deficiency of ASMase or SMS2 that alter sphingomyelin levels in the plasma membrane modulates LPS-induced TF procoagulant activity on monocytes, probably by regulating TF encryption and decryption processes. A genetic deficiency of ASMase or SMS2 also influences thrombin generation in vivo.

Impact of ASMase and SMS2 Deficiencies on Hemostasis and Thrombosis

Since experiments described in the preceding section showed that ASMase and SMS2 deficiencies influence TF activity, we evaluated the impact of ASMase and SMS2 deficiencies on hemostasis and thrombosis using saphenous vein incision-induced bleeding and the IVC ligation-induced thrombus formation, respectively. Our earlier studies showed the importance of TF in hemostasis in the saphenous vein-induced bleeding model, as mice expressing low levels of TF exhibited prolonged bleeding time compared with WT mice.[48] Since using wild-type mice, we found no influence of sex on the saphenous vein incision-induced bleeding (Figure S8), we combined the data from both male and female mice in experimental groups for robust analysis of the data. As shown in Figure 5A, the average time to achieve hemostasis in ASMase$^{-/-}$ mice was significantly prolonged following the saphenous vein incision compared with that observed in WT mice. In the case of SMS2$^{-/-}$ mice, the average time to achieve hemostasis was significantly shorter than that observed in WT mice. Consistent with these data, blood loss from the wound site was significantly higher in ASMase$^{-/-}$ mice and lower in SMS2$^{-/-}$ mice compared with WT mice (Figure 5B).

Next, we used the IVC ligation-induced stasis thrombosis model[42,43] to investigate the effect of ASMase and SMS2 deficiencies on thrombosis. Earlier studies showed that the IVC ligation rapidly induces TF expression in a murine model, and the IVC ligation-induced thrombosis was dependent on TF.[49] It had been suggested that TF decryption might be responsible for enhanced TF procoagulant activity and thrombus formation in this model.[49] Other studies also indicate that, although monocytes, platelets, and neutrophils contribute to the flow restriction-induced venous thrombosis, TF appears to be the central player.[50] Recent studies suggest that TF and platelets, not FXII and neutrophils, are rate-limiting factors in the mouse model of venous thrombosis.[51] Therefore, the IVC ligation-induced stasis model is appropriate to investigate the effect ASMase and SMS2 deficiencies on thrombosis. In the IVC ligation-induced stasis model, ASMase deficiency significantly reduced thrombus formation, whereas SMS2 deficiency led to increased thrombus formation (Figure 6A through 6C). Compared with WT mice, the clot length and weight were significantly lower in ASMase$^{-/-}$ mice, whereas they were significantly higher in SMS2$^{-/-}$ mice (Figure 6B and 6C). Considering sex differences in vascular anatomy and response to inflammation may influence thrombus formation in the IVC ligation model,[52] we analyzed the data separately for male and female mice. We obtained similar results in both groups, that is, ASMase deficiency reduced thrombus formation, whereas SMS2 deficiency increased thrombus formation (Figure S9). In additional studies, we measured TF antigen and activity levels in tissue lysates of the saphenous vein and the IVC excisions. We found no differences in TF antigen and activity levels in these blood vessel lysates among WT, ASMase$^{-/-}$ and SMS2$^{-/-}$ mice (Figures S10 and S11). Overall, the above data strongly indicate that alterations in sphingomyelin levels significantly affect hemostatic and thrombotic processes.

DISCUSSION

Typically, most of the TF expressed on the surface of resting cells exists in an "encrypted" state and possesses very little procoagulant activity.[8–11] Damage to the plasma membrane upon cell injury, pathophysiologic stimulus, apoptosis, or other cell perturbations markedly

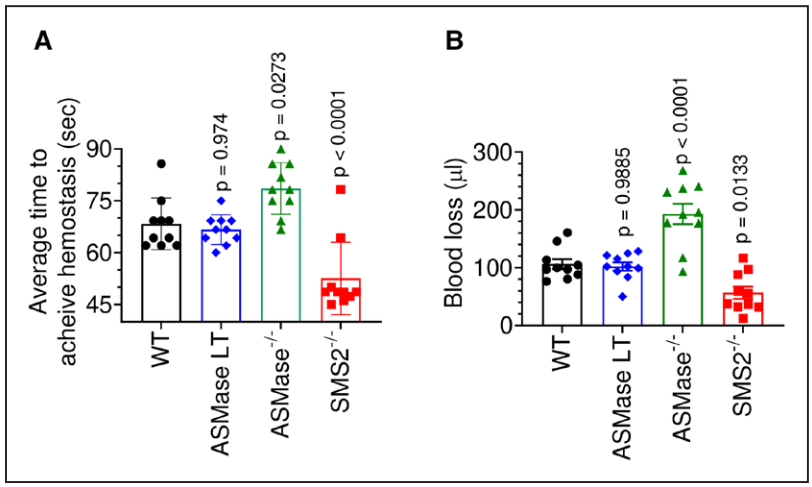

Figure 5. Effect of ASMase (acid sphingomyelinase) or SMS2 (sphingomyelin synthase 2) deficiency on hemostasis.
WT, ASMase littermates (ASMase LT), ASMase$^{-/-}$, and SMS2$^{-/-}$ mice were subjected to the saphenous vein incision. The average time to achieve hemostasis (**A**) and blood loss from the wound site (**B**) were determined as described in Methods (10 mice/group). Statistical analysis was performed by 1-way ANOVA followed by Dunnett post hoc test. *P* were calculated relative to WT. For ASMase$^{-/-}$ group, *P* were calculated relative to ASMase LT.

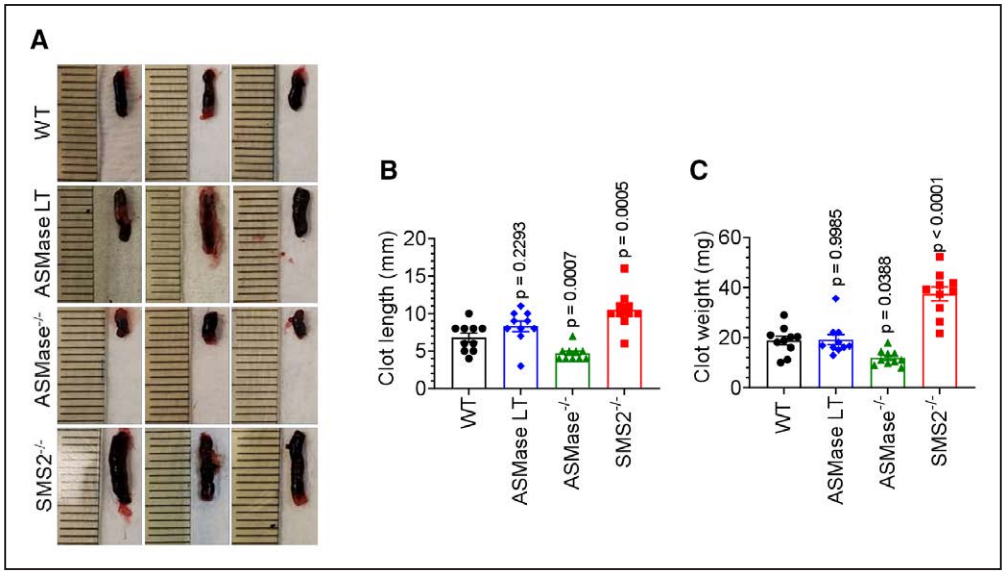

Figure 6. Impact of ASMase (acid sphingomyelinase) or SMS2 (sphingomyelin synthase 2) deficiency on venous thrombosis. ASMase littermates (ASMase LT), wild-type, ASMase$^{-/-}$, and SMS2$^{-/-}$ mice were subjected to inferior vena cava (IVC) ligation-induced stasis thrombosis as described in Methods (number of mice/group=10). After 24 hours of stasis, the clot formed in the ligated IVC was excised. Representative images were shown in **A**, and the clot length (**B**) and weight (**C**) were measured. Statistical analysis was performed by 1-way ANOVA followed by Dunnett post hoc test. *P* were calculated relative to WT. For ASMase$^{-/-}$ group, *P* were calculated relative to ASMase LT.

enhances the cell surface TF activity without increasing TF protein levels.[8–11] Various post-translational mechanisms, such as thiol-disulfide exchange pathways involving protein-disulfide isomerase or thioredoxin system, or changes in phosphatidylserine content in the outer leaflet, are thought to be responsible for regulating the TF procoagulant activity through TF encryption and decryption.[8–11] Our recent studies revealed altogether a novel mechanism, that is, high sphingomyelin content in the outer leaflet of the plasma membrane maintains TF in an encrypted state in resting cells, and the hydrolysis of sphingomyelin by either bSMases or ASMase that is translocated to the outer leaflet upon cell activation leads to TF decryption.[13,14] These data suggest that modifiers that affect sphingomyelin metabolism could be key regulators of TF and hence blood coagulation influencing hemostasis and thrombosis. At present, the pathophysiologic significance of altered sphingomyelin metabolism in hemostasis and thrombosis is unknown. The present study provides convincing evidence that alterations in metabolic enzymes involved in the sphingomyelin metabolism that control sphingomyelin content in the plasma membrane regulate TF procoagulant activity in vitro and in vivo and thrombin generation in vivo. Furthermore, our data show that alterations in sphingomyelin metabolism would influence hemostasis and thrombosis.

Sphingomyelin levels in the plasma membrane are regulated by the enzymes in sphingomyelin metabolic pathways that either drive the synthesis of sphingomyelin or the breakdown of sphingomyelin.[20] SMS isoforms, SMS1 and SMS2, play a primary role in the synthesis of sphingomyelin and increasing the sphingomyelin content in the membrane.[18,20] Sphingomyelinases, such ASMase and nSMases, modulate the sphingomyelin content in the plasma membrane by hydrolyzing the sphingomyelin to ceramide and phosphatidylcholine.[18,20] Our observations that overexpression of SMS1 or SMS2 suppresses TF procoagulant activity and the silencing of SMS1 or SMS2 upregulates TF procoagulant activity indicate that the net content of sphingomyelin in the plasma membrane dictates the status of TF procoagulant activity. Since the overexpression or silencing of SMS isoforms did not alter TF antigen levels on the cell surface, it suggests that the altered TF procoagulant activity in cells with altered sphingomyelin metabolism comes from changes in TF encryption and decryption processes. Here, it may be pertinent to note that our earlier studies showed that lowering sphingomyelin levels in the plasma membrane by the hydrolysis of sphingomyelin by bSMase did not induce PS externalization.[14] Consistent with this finding, the data reported in the current study show that increased TF procoagulant activity observed in cells expressing low levels of sphingomyelin was mostly independent of PS.

Consistent with the observation that sphingomyelin metabolism modulates TF procoagulant activity, silencing SMases, either ASMase or nSMases (nSMase 2 and 3), which increase the net amount of sphingomyelin in the plasma membrane, suppressed TF procoagulant activity. Silencing nSMase1, which is localized in the nucleus and does not translocate to the plasma membrane,[53] failed to affect the sphingomyelin levels on the plasma membrane and therefore did not affect TF procoagulant activity. These data further strengthen the concept that sphingomyelin content in the plasma membrane plays a crucial role in controlling the procoagulant activity of TF.

At present, it is not entirely clear how sphingomyelin metabolism leads to TF decryption. Sphingomyelin levels in the membrane could influence the membrane curvature, lipid raft formation, and phospholipid/protein packing in the membrane.[54,55] Decrease in sphingomyelin levels in the plasma membrane induces upward curvature of the membrane.[56–58] All the above membrane-dependent changes could influence TF activity through modulation factor VIIa and factor X binding to TF or to the membrane. For example, TF associated with lipid raft/caveolae was shown to exhibit low procoagulant activity and disruption of these structures increased TF activity.[60,64,65] Therefore, it is possible that TF decryption induced by sphingomyelin hydrolysis or reduced sphingomyelin synthesis may involve the disruption of lipid rafts. However, it is possible that direct interaction of sphingomyelin with TF could also play a role in regulating TF activity. Molecular modeling studies showed that the TF ectodomain might directly interact with PS and thus modulate TF-FVIIa conformation to be more favorable to FX activation.[52] It is possible that the TF ectodomain may also directly interact with sphingomyelin, and this keeps TF-FVIIa conformation in an unfavorable condition to FX activation. Recent biochemical and molecular dynamic simulation studies by Mallik et al[59] suggest that the OH group at the C3 position in the ceramide moiety of sphingomyelin is responsible for the inhibition of TF activity as the OH contributes to the formation of tight assembly of sphingomyelin in the membrane, restricting the protein-lipid/protein-protein interactions (Figure 7, a schematic representation).

The involvement of sphingomyelin metabolic pathway enzymes and altered sphingomyelin content in various pathologies—such as cancer, neurological, and cardiovascular diseases—is well established.[15,16,31,33] However, little is known about their effects on hemostasis and thrombosis. A cross-sectional study of patients with type B Nieman-Pick disease, a disease caused by ASMase deficiency and the accumulation of sphingomyelin in cells,[27] documented bleeding episodes in 49% of the 59 patients enrolled.[60] A systematic evaluation of morbidity and mortality of a large cohort of type B Nieman-Pick disease revealed that bleeding disorder is one of the major morbidities in these patients.[61] The bleeding disorders noted in NPD-type B patients are thought to be in part to thrombocytopenia present in these patients.[44,60] However, our present studies raise an interesting and novel possibility that impairment in TF decryption in ASMase deficiency could contribute to the bleeding phenotype. Although we did not notice a spontaneous bleeding phenotype in ASMase$^{-/-}$mice, bleeding times were significantly prolonged in ASMase$^{-/-}$mice following the vascular injury induced by the saphenous vein incision. Although not statistically significant, prolonged bleeding times in ASMase$^{-/-}$ mice were also noted in the tail vein incision-induced bleeding model in an earlier study.[62] It may be important to note here that there were no significant differences in hematologic parameters, including platelet counts, mean platelet volume, and platelet aggregation, between ASMase$^{-/-}$ and wild-type mice.[62] In contrast to ASMase$^{-/-}$ mice, the bleeding times of SMS2$^{-/-}$ mice were significantly shorter than WT

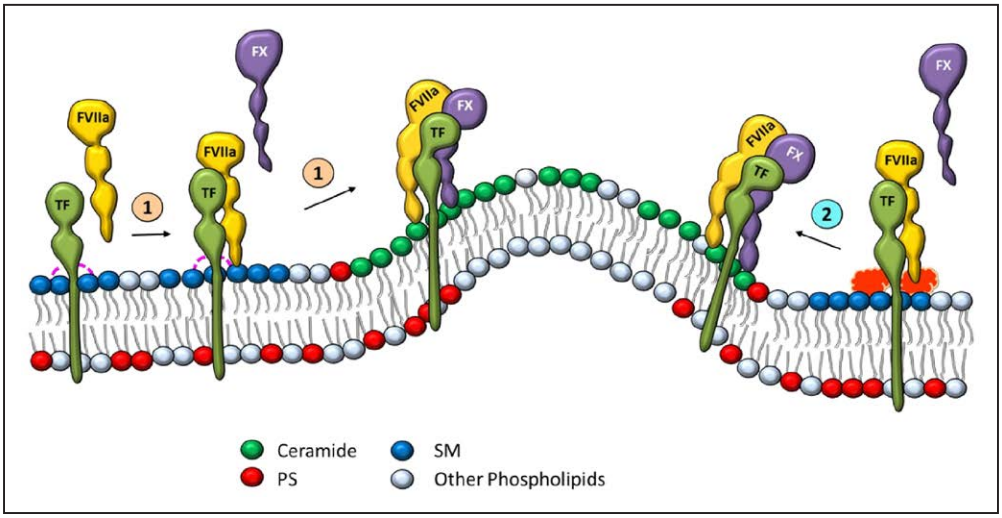

Figure 7. A schematic representation of potential mechanisms by which sphingomyelin metabolism could regulate TF (tissue factor) decryption.
(1) As observed in molecular modeling of TF interaction with phosphatidylserine,[52] TF ectodomain may directly interact with the head groups of sphingomyelin (SM) that keeps TF in a specific conformation that allows FVIIa binding but not TF-FVIIa interaction with FX. Hydrolysis of SM disrupts TF ectodomain interaction with SM, which leads to conformational changes in TF that favor TF-FVIIa interaction with FX. (2) Extensive inter-molecular bonding between SM molecules leads to tight assembly of lipid surface. This tight packing restricts the interaction of Gla domains of FVIIa or FX with the phosphate moiety of lipids.[59] The altered lipid-protein interaction may also destabilize the protease domain and block the substrate contact sites on the membrane. When SM is metabolized to ceramide, where the head group of SM is removed, the tight assembly of the lipids in the membrane is relaxed and the gla domain FVIIa and FX could interact with the lipid surface. Other potential mechanisms, not shown in the figure, include changes in the membrane fluidity and disruption of lipid rafts associated with the hydrolysis of SM.

mice. SMS2 $^{-/-}$ mice, as in ASMase$^{-/-}$ mice, had normal levels of platelets.[63] Therefore, the possibility that potential differences in platelet number could be the reason for observed differences in hemostasis in ASMase$^{-/-}$ and SMS2 $^{-/-}$ mice following the vascular injury is unlikely. However, our current observation with SMS2$^{-/-}$mice differs from the earlier data of Guo et al[64] who reported significantly prolonged bleeding time in SMS2$^{-/-}$ mice. The use of different bleeding model systems in these 2 studies could be a potential reason for the observed differences. The tail-cut bleeding, the model used in the earlier study, causes injury to multiple tissues, including 3 large blood vessels (the central artery and 2 lateral veins). Bleeding in this model is influenced by vasoconstriction/dilation.[65] Mice with platelet abnormalities show severe hemostatic defects.[65] In general, a single bleeding time point, the time to initial cessation of bleeding, is recorded in this model. In contrast, the saphenous vein incision-induced bleeding, the model used in the present study, involves repeated disruption of the clots and the bleeding times were calculated by the number of clots formed over the course of 30 min period. This bleeding model has better sensitivity and lower variability than tail-cut bleeding and is thought to be more suitable to detect subtle defects in the hemostatic system.[66] Since platelets of SMS2$^{-/-}$ mice were found to have impaired platelet aggregation (Figure S12),[64] it is possible that SMS2$^{-/-}$ mice exhibited prolonged bleeding time in the tail-cut bleeding model. It is also possible that saphenous vein-incision caused a greater degree of TF exposure than tail transection, and thus bleeding in this model captures changes in TF activity more readily. Here, it may be important to note that our earlier studies showed that mice expressing low levels of TF exhibit prolonged bleeding time following the saphenous vein incision, indicating that hemostasis in this bleeding model is dependent on TF.[48]

Existing limited data on whether alterations in sphingomyelin metabolism affect thrombosis is ambiguous. Earlier studies showed that platelets secrete ASMase upon thrombin stimulation.[61,62] Munzer et al[62] showed that thrombin-induced ATP release and P-selectin exposure were significantly reduced in platelets of ASMase$^{-/-}$ mice compared with platelets from WT mice. This study also showed that in vivo thrombus formation after $FeCl_3$ injury was significantly blunted in ASMase$^{-/-}$ mice compared with WT mice, indicating ASMase promotes thrombosis. From the above studies, it is difficult to discern whether the observed influence of ASMase on platelet activation and thrombus formation is the direct action of ASMase on platelet degranulation or through its effect on increasing sphingomyelin levels in platelets. Interestingly, a recent study reported similar results with SMS2$^{-/-}$ mice. SMS2$^{-/-}$ mice displayed delayed thrombus formation after $FeCl_3$ injury.[64] Similar results on thrombus formation following $FeCl_3$ injury in ASMase$^{-/-}$ and SMS2$^{-/-}$ mice that have opposing effects on the sphingomyelin metabolism indicate that the altered thrombus formation observed in the above studies might not have been related to alterations in sphingomyelin metabolism. Here, it may be important to point out that the $FeCl_3$ injury-induced thrombus formation is primarily based on collagen-, and not TF-, dependent thrombin generation.[67]

Our data, employing the IVC ligation-induced thrombosis, which is more biologically relevant than $FeCl_3$ injury, provide convincing evidence that altered sphingomyelin metabolism plays a crucial role in venous thrombosis. The observation of reduced thrombus formation in ASMase$^{-/-}$ mice that have elevated levels of sphingomyelin and enhanced thrombus formation in SMS2$^{-/-}$ mice that are documented to have lower sphingomyelin levels indicate that sphingomyelin levels modulate thrombus formation. Here, it may be pertinent to note that the IVC ligation induces TF expression[49] and the thrombus formation induced by the IVC ligation was dependent on TF.[68] Potential differences in TF protein expression in ASMase$^{-/-}$ and SMS2$^{-/-}$ mice could not be a reason for differences in thrombus formation. Therefore, it is likely that decryption of TF in SMS2$^{-/-}$ mice, due to decreased sphingomyelin content in the plasma membrane, could be responsible for enhanced thrombus formation in these mice. In the current study, we used a single model of venous thrombosis. The complete stasis venous thrombosis model system used in the present study is appropriate for mechanistic studies as the model system yields robust and reproducible thrombus formation.[43] However, it is important to include additional model systems that better reflect the pathogenesis of venous thrombosis in future studies to evaluate the translational potential of our findings. The stenotic flow-restricted model and the electrolyte inferior vena cava model could be appropriate models[43] for such future studies.

Sphingomyelin metabolism is altered in many disease settings, including atherosclerosis, diabetes, sepsis, and cancer,[15,16,18,32,69] the same disease settings were also known to induce aberrant expression or activation of TF.[4,5,70] Several pathogenic stimuli, for example, LPS, cytokines, ATP, irradiation, and bacterial and viral infections that promote TF procoagulant activity, were also found to translocate lysosomal ASMase to the outer leaflet or secrete intracellular ASMase to the extracellular space.[16] It will be interesting to examine whether ASMase breakdown of sphingomyelin in the plasma membrane is responsible for prothrombotic TF associated with the above disorders.

Finally, although our present data are consistent with the hypothesis sphingomyelin metabolism influences TF procoagulant activity, and thus affects hemostatic and thrombotic processes, current experimental approaches make it difficult to conclude firmly that changes in TF procoagulant activity associated with sphingomyelin levels in the plasma membrane is solely responsible for the observed defects seen in hemostatic and thrombotic processes in ASMase$^{-/-}$ and SMS2$^{-/-}$ mice. Global alterations in sphingomyelin metabolism may, in addition to affecting TF activity in monocytes and other cell types, also affect platelet function, the function of other membrane-dependent

procoagulant complexes, and the formation of neutrophil extracellular traps.[64,71–73] All the above processes could affect hemostatic and thrombotic processes in vivo. Despite the above limitation, our findings provide valuable insights into how alterations in sphingolipid metabolism, often associated with infection, cardiovascular diseases, and cancer, could affect the pathogenesis of bleeding and thromboinflammatory disorders.

ARTICLE INFORMATION

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Disclosures

None.

Supplemental Material

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Figures S1–S12
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REFERENCES

1. Rapaport SI, Rao LVM. The tissue factor pathway: how it has become a "prima ballerina". *Thromb Haemost.* 1995;74:7–17.
2. Mackman N. Role of tissue factor in hemostasis, thrombosis, and vascular development. *Arterioscler Thromb Vasc Biol.* 2004;24:1015–1022. doi: 10.1161/01.ATV.0000130465.23430.74
3. Osterud B. Tissue factor: a complex biological role. *Thromb Haemost.* 1997;78:755–758.
4. Williams JC, Mackman N. Tissue factor in disease and health. *Front Biosci.* 2012;E4:358–372. doi: 10.2741/383
5. Taubman MB, Fallon JT, Schecter AD, Giesen P, Mendlowitz M, Fyfe BS, Marmur JD, Nemerson Y. Tissue factor in the pathogenesis of atherosclerosis. *Thromb Haemost.* 1997;78:200–204.
6. Chu AJ. Tissue factor, blood coagulation, and beyond: an overview. *Int J Inflam* 2011;2011:367284. doi: 10.4061/2011/367284
7. Bach RR. Tissue factor encryption. *Arterioscler Thromb Vasc Biol.* 2006;26:456–461. doi: 10.1161/01.ATV.0000202656.53964.04
8. Rao LV, Pendurthi UR. Regulation of tissue factor coagulant activity on cell surfaces. *J Thromb Haemost.* 2012;10:2242–2253. doi: 10.1111/jth.12003
9. Chen VM, Hogg PJ. Encryption and decryption of tissue factor. *J Thromb Haemost.* 2013;11:277–284. doi: 10.1111/jth.12228
10. Langer F, Ruf W. Synergies of phosphatidylserine and protein disulfide isomerase in tissue factor activation. *Thromb Haemost.* 2014;111:590–597. doi: 10.1160/TH13-09-0802
11. 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. doi: 10.1055/s-0039-1681102
12. Rao LV, Kothari H, Pendurthi UR. Tissue factor encryption and decryption: facts and controversies. *Thromb Res.* 2012;129:S13–S17. doi: 10.1016/j.thromres.2012.02.021
13. Wang J, Pendurthi UR, Rao LVM. Acid sphingomyelinase plays a critical role in LPS- and cytokine-induced tissue factor procoagulant activity. *Blood.* 2019;134:645–655. doi: 10.1182/blood.2019001400
14. Wang J, Pendurthi UR, Rao LVM. Sphingomyelin encrypts tissue factor: ATP-induced activation of A-SMase leads to tissue factor decryption and microvesicle shedding. *Blood Adv.* 2017;1:849–862. doi: 10.1182/bloodadvances.2016003947
15. Hannun YA, Obeid LM. Sphingolipids and their metabolism in physiology and disease. *Nat Rev Mol Cell Biol.* 2018;19:175–191. doi: 10.1038/nrm.2017.107
16. Kornhuber J, Rhein C, Muller CP, Muhle C. Secretory sphingomyelinase in health and disease. *Biol Chem.* 2015;396:707–736. doi: 10.1515/hsz-2015-0109
17. Kornhuber J, Hoertel N, Gulbins E. The acid sphingomyelinase/ceramide system in COVID-19. *Mol Psychiatry.* 20211; doi: 0.1038/s41380-021-01309-5
18. Bienias K, Fiedorowicz A, Sadowska A, Prokopiuk S, Car H. Regulation of sphingomyelin metabolism. *Pharmacol Rep.* 2016;68:570–581. doi: 10.1016/j.pharep.2015.12.008
19. Versteeg HH, Ruf W. Thiol pathways in the regulation of tissue factor prothrombotic activity. *Curr Opin Hematol.* 2011;18:343–348. doi: 10.1097/MOH.0b013e32834981de
20. Milhas D, Clarke CJ, Hannun YA. Sphingomyelin metabolism at the plasma membrane: implications for bioactive sphingolipids. *FEBS Lett.* 2010;584:1887–1894. doi: 10.1016/j.febslet.2009.10.058
21. Bianco F, Perrotta C, Novellino L, Francolini M, Riganti L, Menna E, Saglietti L, Schuchman EH, Furlan R, Clementi E, et al. Acid sphingomyelinase activity triggers microparticle release from glial cells. *EMBO J.* 2009;28:1043–1054. doi: 10.1038/emboj.2009.45
22. Perrotta C, Clementi E. Biological roles of Acid and neutral sphingomyelinases and their regulation by nitric oxide. *Physiology (Bethesda).* 2010;25:64–71. doi: 10.1152/physiol.00048.2009
23. Hannun YA. Functions of ceramide in coordinating cellular responses to stress. *Science.* 1996;274:1855–1859. doi: 10.1126/science.274.5294.1855
24. Taniguchi M, Okazaki T. The role of sphingomyelin and sphingomyelin synthases in cell death, proliferation and migration-from cell and animal models to human disorders. *Biochim Biophys Acta.* 2014;1841:692–703. doi: 10.1016/j.bbalip.2013.12.003
25. Truman JP, Al Gadban MM, Smith KJ, Hammad SM. Acid sphingomyelinase in macrophage biology. *Cell Mol Life Sci.* 2011;68:3293–3305. doi: 10.1007/s00018-011-0686-6
26. Claus RA, Bunck AC, Bockmeyer CL, Brunkhorst FM, Losche W, Kinscherf R, Deigner HP. Role of increased sphingomyelinase activity in apoptosis and organ failure of patients with severe sepsis. *FASEB J.* 2005;19:1719–1721. doi: 10.1096/fj.04-2842fje
27. Schuchman E, Desnick R. Niemann-Pick disease types A and B: acid sphingomyelinase deficiency. *The Metabolic and Molecular Bases of Inheridted Diseases* 2001. Scriver CR, Beaudet AL, Sly WS, Valle D. NewYork: McHraw-Hill
28. Smith EL, Schuchman EH. The unexpected role of acid sphingomyelinase in cell death and the pathophysiology of common diseases. *FASEB J.* 2008;22:3419–3431. doi: 10.1096/fj.08-108043
29. Carpinteiro A, Gripp B, Hoffmann M, Pohlmann S, Hoertel N, Edwards MJ, Kamler M, Kornhuber J, Becker KA, Gulbins E. Inhibition of acid sphingomyelinase by ambroxol prevents SARS-CoV-2 entry into epithelial cells. *J Biol Chem.* 2021; 296:100701. doi: 10.1016/j.jbc.2021.100701
30. Chatterjee S. Neutral sphingomyelinase: past, present and future. *Chem Phys Lipids.* 1999;102:79–96. doi: 10.1016/s0009-3084(99)00077-8

31. Pavoine C, Pecker F. Sphingomyelinases: their regulation and roles in cardiovascular pathophysiology. *Cardiovasc Res.* 2009;82:175–183. doi: 10.1093/cvr/cvp030
32. Shamseddine AA, Airola MV, Hannun YA. Roles and regulation of neutral sphingomyelinase-2 in cellular and pathological processes. *Adv Biol Regul.* 2015;57:24–41. doi: 10.1016/j.jbior.2014.10.002
33. Horres CR, Hannun YA. The roles of neutral sphingomyelinases in neurological pathologies. *Neurochem Res.* 2012;37:1137–1149. doi: 10.1007/s11064-011-0692-y
34. Wang J, Pendurthi UR, Yi G, Rao LVM. SARS-CoV-2 infection induces the activation of tissue factor-mediated coagulation by activation of acid sphingomyelinase. *Blood.* 2021; 138:344–349. doi: 10.1182/blood.2021010685.
35. Kothari H, Nayak RC, Rao LV, Pendurthi UR. Cystine186-cystine 209 disulfide bond is not essential for the procoagulant activity of tissue factor or for its de-encryption. *Blood.* 2010;115:4273–4283. doi: 10.1182/blood-2009-09-241356
36. Pendurthi UR, Williams JT, Rao LV. Acidic and basic fibroblast growth factors suppress transcriptional activation of tissue factor and other inflammatory genes in endothelial cells. *Arterioscler Thrombosis Vasc Biol.* 1997;17:940–946. doi: 10.1161/01.atv.17.5.940
37. Pendurthi UR, Ghosh S, Mandal SK, Rao LV. Tissue factor activation: is disulfide bond switching a regulatory mechanism?. *Blood.* 2007;110:3900–3908. doi: 10.1182/blood-2007-07-101469
38. Nayak RC, Sen P, Ghosh S, Gopalakrishnan R, Esmon CT, Pendurthi UR, Rao LV. Endothelial cell protein C receptor cellular localization and trafficking: potential functional implications. *Blood.* 2009;114:1974–1986. doi: 10.1182/blood-2009-03-208900
39. Nayak RC, Keshava S, Esmon CT, Pendurthi UR, Rao LV. Rab GTPases regulate endothelial cell protein C receptor-mediated endocytosis and trafficking of factor VIIa. *PLoS One.* 2013;8:e59304. doi: 10.1371/journal.pone.0059304
40. Sundaram J, Pendurthi UR, Esmon CT, Rao LV. Blockade of endothelial cell protein C receptor augments factor VIIa hemostatic effect in hemophilia treatment. *Blood.* 2014;124:3031–3033. doi: 10.1182/blood-2014-09-600254
41. Keshava S, Sundaram J, Rajulapati A, Esmon C, Pendurthi U, Rao LVM. Factor VIIa interaction with EPCR modulates the hemostatic effect of rFVIIa in hemophilia therapy: Mode of its action. *Blood Adv.* 2017;1:1206–1214. doi: 10.1182/bloodadvances.2016004143
42. Wrobleski SK, Farris DM, Diaz JA, Myers DD Jr, Wakefield TW. Mouse complete stasis model of inferior vena cava thrombosis. *J Vis Exp* 2011; 15:2738. doi: 10.3791/2738
43. Diaz JA, Saha P, Cooley B, Palmer OR, Grover SP, Mackman N, Wakefield TW, Henke PK, Smith A, Lal BK. Choosing a mouse model of venous thrombosis. *Arterioscler Thromb Vasc Biol.* 2019;39:311–318. doi: 10.1161/ATVBAHA.118.311818
44. McGovern MM, Avetisyan R, Sanson BJ, Lidove O. Disease manifestations and burden of illness in patients with acid sphingomyelinase deficiency (ASMD). *Orphanet J Rare Dis.* 2017;12:41. doi: 10.1186/s13023-017-0572-x
45. Cox GF, Clarke LA, Giugliani R, McGovern MM. Burden of illness in acid sphingomyelinase deficiency: a retrospective chart review of 100 patients. *JIMD Rep.* 2018;41:119–129. doi: 10.1007/8904_2018_120
46. Osterud B. The high responder phenomenon: enhancement of LPS induced tissue factor activity in monocytes by platelets and granulocytes. *Platlets.* 1995;6:119–125. doi: 10.3109/09537109509013263
47. Osterud B, Bjorklid E. The production and availability of tissue thromboplastin in cellular populations of whole blood exposed to various concentrations of endotoxin. An assay for detection of endotoxin. *Scand J Haematol.* 1982;29:175–184. doi: 10.1111/j.1600-0609.1982.tb00580.x
48. Keshava S, Sundaram J, Rajulapati A, Pendurthi UR, Rao LV. Pharmacological concentrations of rFVIIa restore hemostasis independent of tissue factor in antibody-induced hemophilia mice. *J Thromb Haemost.* 2016; 14:546–550. doi: 10.1111/jth.13244
49. Zhou J, May L, Liao P, Gross PL, Weitz JI. Inferior vena cava ligation rapidly induces tissue factor expression and venous thrombosis in rats. *Arterioscler Thromb Vasc Biol.* 2009;29:863–869. doi: 10.1161/ATVBAHA.109.185678
50. von Bruhl ML, Stark K, Steinhart A„Chandraratne S, Konrad I, Lorenz M, Khandoga A, Tirniceriu A, Coletti R, Köllnberger M„ et al. Monocytes, neutrophils, and platelets cooperate to initiate and propagate venous thrombosis in mice in vivo. *J Exp Med.* 2012;209:819–835. doi: 10.1084/jem.20112322
51. Heestermans M, Salloum-Asfar S, Streef T, Laghmani EH, Salvatori D, Luken BM, Zeerleder SS, Spronk HM, Korporaal SJ, Kirchhofer D, et al. Mouse venous thrombosis upon silencing of anticoagulants depends on tissue factor and platelets, not FXII or neutrophils. *Blood.* 2019;133:2090–2099. doi: 10.1182/blood-2018-06-853762
52. 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. doi: 10.1016/j.thromres.2011.01.004
53. Mizutani Y, Tamiya-Koizumi K, Nakamura N, Kobayashi M, Hirabayashi Y, Yoshida S. Nuclear localization of neutral sphingomyelinase 1: biochemical and immunocytochemical analyses. *J Cell Sci.* 2001;114:3727–3736. doi: 10.1242/jcs.114.20.3727
54. Barenholz Y, Thompson TE. Sphingomyelins in bilayers and biological membranes. *Biochim Biophys Acta.* 1980;604:129–158. doi: 10.1016/0005-2736(80)90572-6
55. Barenholz Y. Sphingomyelin and cholesterol: from membrane biophysics and rafts to potential medical applications. *Subcell Biochem.* 2004;37:167–215. doi: 10.1007/978-1-4757-5806-1_5
56. Krishna A, Prakash S, Sengupta D. Sphingomyelin effects in caveolin-1 mediated membrane curvature. *J Phys Chem B.* 2020;124:5177–5185. doi: 10.1021/acs.jpcb.0c02962
57. Kaltenegger M, Kremser J, Frewein MPK, Ziherl P, Bonthuis DJ, Pabst G. Intrinsic lipid curvatures of mammalian plasma membrane outer leaflet lipids and ceramides. *Biochim Biophys Acta Biomembr.* 2021;1863:183709. doi: 10.1016/j.bbamem.2021.183709
58. McMahon HT, Boucrot E. Membrane curvature at a glance. *J Cell Sci.* 2015;128:1065–1070. doi: 10.1242/jcs.114454
59. Mallik S, Prasad R, Das K, Sen P. Alcohol functionality in the fatty acid backbone of sphingomyelin guides the inhibition of blood coagulation. *RSC Adv.* 2021;11:3390–3398.
60. McGovern MM, Wasserstein MP, Giugliani R, Bembi B, Vanier MT, Mengel E, Brodie SE, Mendelson D, Skloot G, Desnick RJ et al. A prospective, cross-sectional survey study of the natural history of Niemann-Pick disease type B. *Pediatrics.* 2008;122:e341–e349. doi: 10.1542/peds.2007-3016
61. McGovern MM, Lippa N, Bagiella E, Schuchman EH, Desnick RJ, Wasserstein MP. Morbidity and mortality in type B Niemann-Pick disease. *Genet Med.* 2013;15:618–623. doi: 10.1038/gim.2013.4
62. Munzer P, Borst O, Walker B, Chmid E, Feijge MA, Cosemans JM, Chatterjee M, Schmidt EM, Schmidt S, Towhid ST, et al. Acid sphingomyelinase regulates platelet cell membrane scrambling, secretion, and thrombus formation. *Arterioscler Thromb Vasc Biol.* 2014;34:61–71. doi: 10.1161/ATVBAHA.112.300210
63. Fujii Y, Taniguchi M, Nagaya S, Ueda Y, Hashizume C, Watanabe K, Takeya H, Kosaka T, Okazaki T. A novel mechanism of thrombocytopenia by PS exposure through TMEM16F in sphingomyelin synthase 1 deficiency. *Blood Adv.* 2021;5:4265–4277. doi: 10.1182/bloodadvances.2020002922
64. Guo Y, Chang L, Zhang G, Gao Z, Lin H, Zhang Y, Hu L, Chen S, Fan B, Zhang S, et al. The role of Sphingomyelin synthase 2 (SMS2) in platelet activation and its clinical significance. *Thromb J.* 2021;19:27. doi: 10.1186/s12959-021-00282-x
65. Mohammed BM, Monroe DM, Gailani D. Mouse models of hemostasis. *Platelets.* 2020;31:417–422. doi: 10.1080/09537104.2020.1719056
66. Ay C, Hisada Y, Cooley BC, Mackman N. Factor XI-deficient mice exhibit increased bleeding after injury to the saphenous vein. *J Thromb Haemost.* 2017;15:1829–1833. doi: 10.1111/jth.13766
67. Kretz CA, Vaezzadeh N, Gross PL. Tissue factor and thrombosis models. *Arterioscler Thromb Vasc Biol.* 2010;30:900–908.
68. Manly DA, Boles J, Mackman N. Role of tissue factor in venous thrombosis. *Annu Rev Physiol.* 2011;73:515–525. doi: 10.1146/annurev-physiol-042210-121137 [doi]
69. Jenkins RW, Canals D, Hannun YA. Roles and regulation of secretory and lysosomal acid sphingomyelinase. *Cell Signal.* 2009;21:836–846. doi: 10.1016/j.cellsig.2009.01.026
70. Eilertsen KE, Osterud B. Tissue factor: (patho)physiology and cellular biology. *Blood Coagul Fibrinolysis.* 2004;15:521–538. doi: 10.1097/00001721-200410000-00001
71. Munzer P, Borst O, Walker B, Schmid E, Feijge MA, Cosemans JM, Chatterjee M, Schmidt EM, Schmidt S, Towhid ST, et al. Acid sphingomyelinase regulates platelet cell membrane scrambling, secretion, and thrombus formation. *Arterioscler Thromb Vasc Biol.* 2014;34:61–71. doi: ATVBAHA.112.300210
72. Deguchi H, Yegneswaran S, Griffin JH. Sphingolipids as bioactive regulators of thrombin generation. *J Biol Chem.* 2004;279:12036–12042. doi: 10.1074/jbc.M302531200 [doi];M302531200 [pii]
73. Neumann A, Brogden G, Jerjomiceva N, Brodesser S, Naim HY, von Kockritz-Blickwede M. Lipid alterations in human blood-derived neutrophils lead to formation of neutrophil extracellular traps. *Eur J Cell Biol.* 2014;93:347–354. doi: 10.1016/j.ejcb.2014.07.005