Acid sphingomyelinase plays a critical role in LPS- and cytokine-induced tissue factor procoagulant activity

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Supplemental Information

Materials and methods

Materials

Rabbit polyclonal antibodies against human acid sphingomyelinase (A-SMase), which also recognize murine ASMase,[1] were from Cell Signaling Technology (Danvers, MA, USA). Rat anti-murine TF mAb (1H1) was provided by Daniel Kirchhofer (Genentech Inc., CA). TF9C3 hybridoma was kindly provided by James H. Morrissey, University of Michigan, Ann Arbor, MI, and 9C3 mAb was purified from mouse ascites fluid using the Affi-Gel Protein A MAPS II Kit from Bio-Rad (Hercules, CA, USA). Goat polyclonal antibodies against human TF were raised in-house and characterized earlier.[2] Alexa Fluor (AF) 488 and 546 conjugated secondary antibodies were obtained from Thermo Fisher Scientific (Waltham, MA). siRNAs for ASMase (sense strand, AACUCCUUGGAUGGGCCUGG(dTdT); anti sense AAUGCUACUGGCUGGUGGACC (dTdT)) and control scrambled siRNA (scRNA) were obtained from Sigma Aldrich Corp. (Woodlands, TX). Macrophage colony stimulating factor (MCSF) was from Peprotech (Rocky Hill, NJ). Cell culture medium DMEM and RPMI 1640 were from GIBCO (Life Technologies, Grand Island, NY) and fetal bovine serum (FBS) was from Atlanta Biologicals (Flowery Branch, GA). Recombinant human FVIIa was provided by the late Walter Kisiel, the University of New Mexico Health Science Center, Albuquerque, NM, USA. Purified human clotting factors X, Xa, and prothrombin were purchased from Enzyme Research Laboratories (South Bend, IN). Purified human alpha-thrombin and factor Va were obtained from Hematologic Technologies, Inc. (Essex Junction, VT).

Isolation of murine PBMCs

Murine PBMCs were isolated by density gradient centrifugation using Ficoll-Paque (GE Healthcare, Pittsburg, PA; density 1.0776 ± 0.001 g/mL). Briefly, one mL of whole blood was diluted two-fold with saline and carefully loaded on a five mL of Ficoll gradient and centrifuged at 400 x g for 40 min at room temperature to separate mononuclear cells and plasma. PBMCs were washed twice by suspending them in HEPES buffer based Tyrode's solution (10 mM HEPES, 135 mM NaCl, 10 mM glucose, 5.4 mM KCl, 1 mM

$MgCl_2$, and 1.8 mM $CaCl_2$, pH 7.5) and sedimenting the cells by brief centrifugation (400 x g for 10 min). PBMCs were suspended in 0.5 mL of Tyrode's solution.

Isolation of MVs from murine plasma

Platelet-poor plasma was centrifuged at 21,000 x g for 60 min at 4°C to sediment MVs. MVs were resuspended in HEPES buffer based Tyrode solution to the original volume of plasma.

Inhibition of ASMase by siRNA

HUVECs were transfected with a control transfection reagent (mock transfection), scrambled oligonucleotide (scRNA) as a control, or ASMase siRNA (100 nM) using Lipofectamine RNAiMax transfection reagent (Thermo Fisher Scientific, Waltham, MA). Briefly, cells were cultured in 24-well culture plates and grown to ~ 70% confluence. Before the transfection, the spent medium was replaced with fresh serum-containing growth medium (450 µL/well). siRNA and RNAiMax transfection reagent were diluted in serum-free medium and mixed to prepare the transfection mixture. After allowing the mixture to stand for 5 min at room temperature, 50 µL of the transfection mixture was added into each well. The final concentration of scRNA or siRNA was 100 nM. The cells were further cultured for 48 h at 37°C and 5% CO_2 in a humidified incubator. A similar protocol was used to transfect MDMs.

Induction of TF activity in human monocyte-derived macrophages (MDMs) and HUVEC

MDMs constitutively express low levels of TF activity. To induce TF expression, MDMs were treated with LPS (1 µg/mL) for 4 h in serum-containing RPMI medium. To induce TF expression in endothelial cells, confluent monolayers of HUVEC were treated with a combination of TNFα and IL-1ß, 10 ng/mL each, for 6 h in serum-containing EBM-2 medium.

Determination of TF antigen levels

Unstimulated or TNFα+IL-1β-stimulated HUVEC were incubated with ^{125}I-labeled human TF mAb (9C3, 10 nM) for 2 h at 4°C. Levels of the radioligand bound to cells were determined as described earlier.[6]

Evaluation of sphingomyelin levels in PBMCs by lysenin binding

To label SM in PBMCs, cells were fixed first in 4% paraformaldehyde and then incubated with 0.5 µg/mL lysenin (Peptide Institute Inc., Osaka, Japan) in 2% BSA/PBS for 60 min. After washing cells to remove the unbound lysenin, the cells were incubated with rabbit polyclonal anti-lysenin antiserum (Peptide Institute Inc., Osaka, Japan; 200 x dilution) for 60 min. After removing the unbound primary antibodies by washing the cells twice with PBS by brief centrifugation, the cells were incubated with DAPI (5 µg/mL) and AF546

donkey anti-rabbit IgG (2 µg/mL) for 90 min and washed again. A drop of stained cell suspension was placed on a glass side, covered with a coverslip, and subjected to confocal microscopy. A similar protocol was used to stain SM in endothelial cells.

Determination of ASMase levels in plasma

ASMase levels in plasma were measured using the Amplex^{TM} Red Sphingomyelinase Assay Kit (Molecular Probes) following instructions provided by the manufacturer. The assay was performed in a two-step assay at a lower pH (pH 5.0) to exclude the activity of other SMases in plasma. To measure secretory ASMase activity, plasma samples were diluted in reaction buffer (0.1 M Na acetate, pH 5.0) supplemented with $ZnCl_2$ (0.1 mM). Each plasma sample was analyzed in the presence and absence of fluorescence substrate to correct for auto-fluorescence.

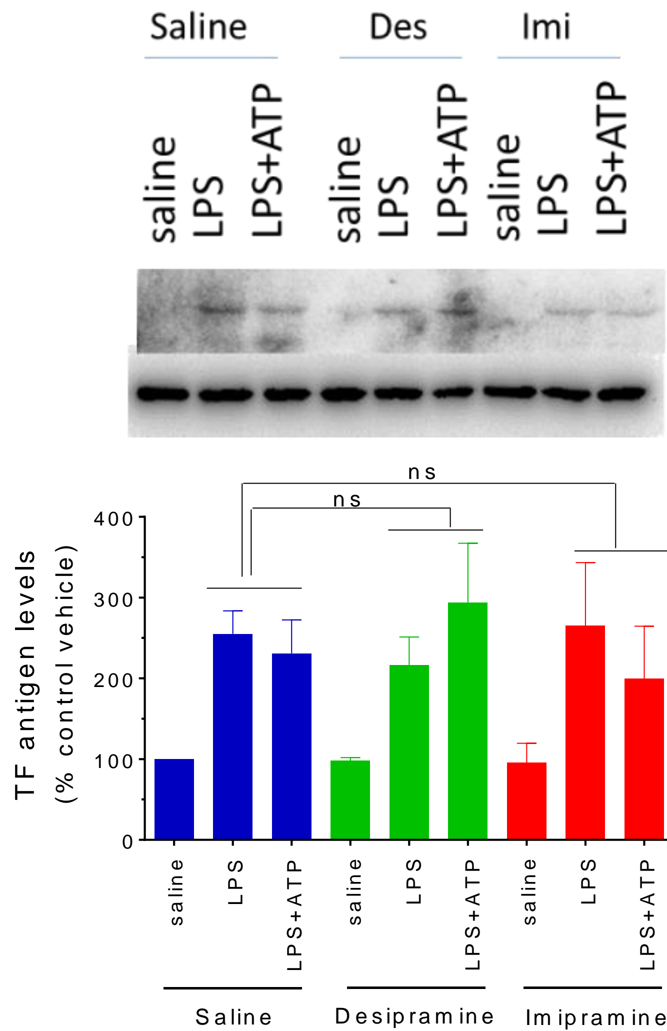
FACS analysis

Murine PBMCs were fixed with 4% paraformaldehyde at room temperature for one hour. Fixed cells were blocked with 10% FBS in Tyrode solution for one hour and then labeled with control IgG or antibodies against murine TF (rat anti-murine TF mAb 1H1, 10 µg/mL) or ASMase (rabbit anti-human ASMase, 2 µg/mL) for overnight at 4°C. After washing cells thrice with Tyrode solution, cells were incubated with fluorescence-conjugated secondary antibodies (donkey anti-rat IgG AF 488 and donkey-anti rabbit IgG AF546, 2 µg/mL each) and DAPI (5 µg/mL) for 90 min. Monocytes were gated from the rest of the PBMCs by forward scattering and expression of TF and ASMase in monocytes were analyzed using Attune NxT flow cytometer (Thermo Fisher Scientific, Waltham, MA).

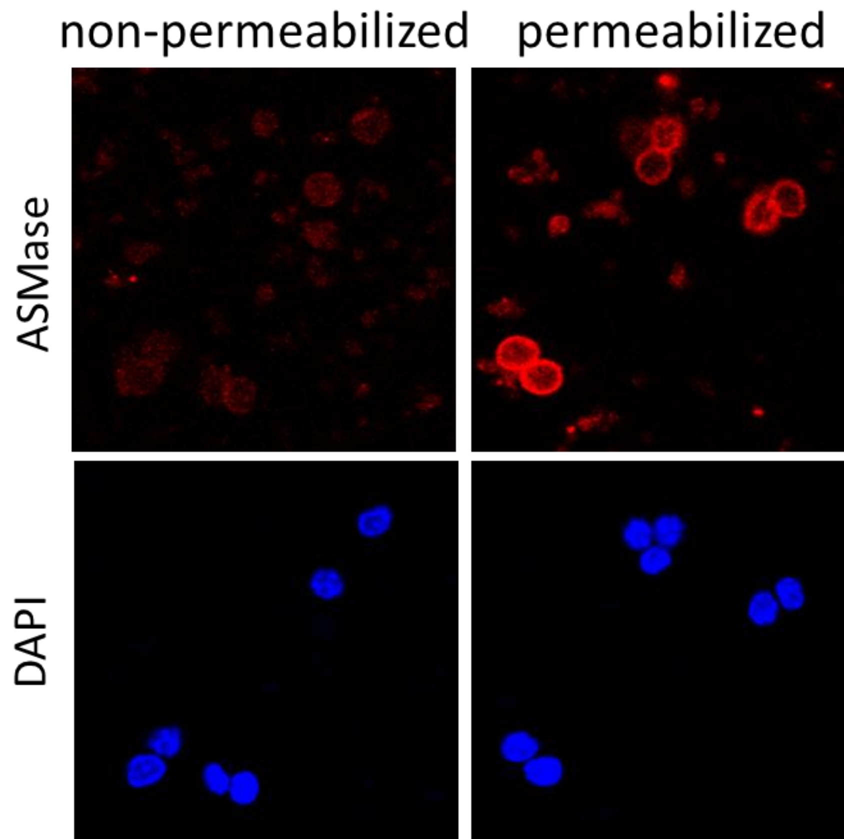
Immunofluorescence microscopy, image acquisition, scoring, and colocalization

Immunofluorescence confocal microscopy, image acquisition, and colocalization analysis were performed essentially as described in our earlier publications.[3,7] Briefly, the confocal images were obtained using an LSM 510 Meta confocal system (Carl Zeiss) equipped with an inverted microscope (Axio Observer Z1; Carl Zeiss). Immunostained cells were viewed using a Plan-APOCHROMAT 633/1.4 NA oil objective lens. Zen 2009 software (Carl Zeiss) was used for the image acquisition, quantification of fluorescence signals, and determining colocalization. The fluorescence intensity was also quantified using FIJI software (ImageJ2, Wayne Rasband, National Institute of Mental Health).

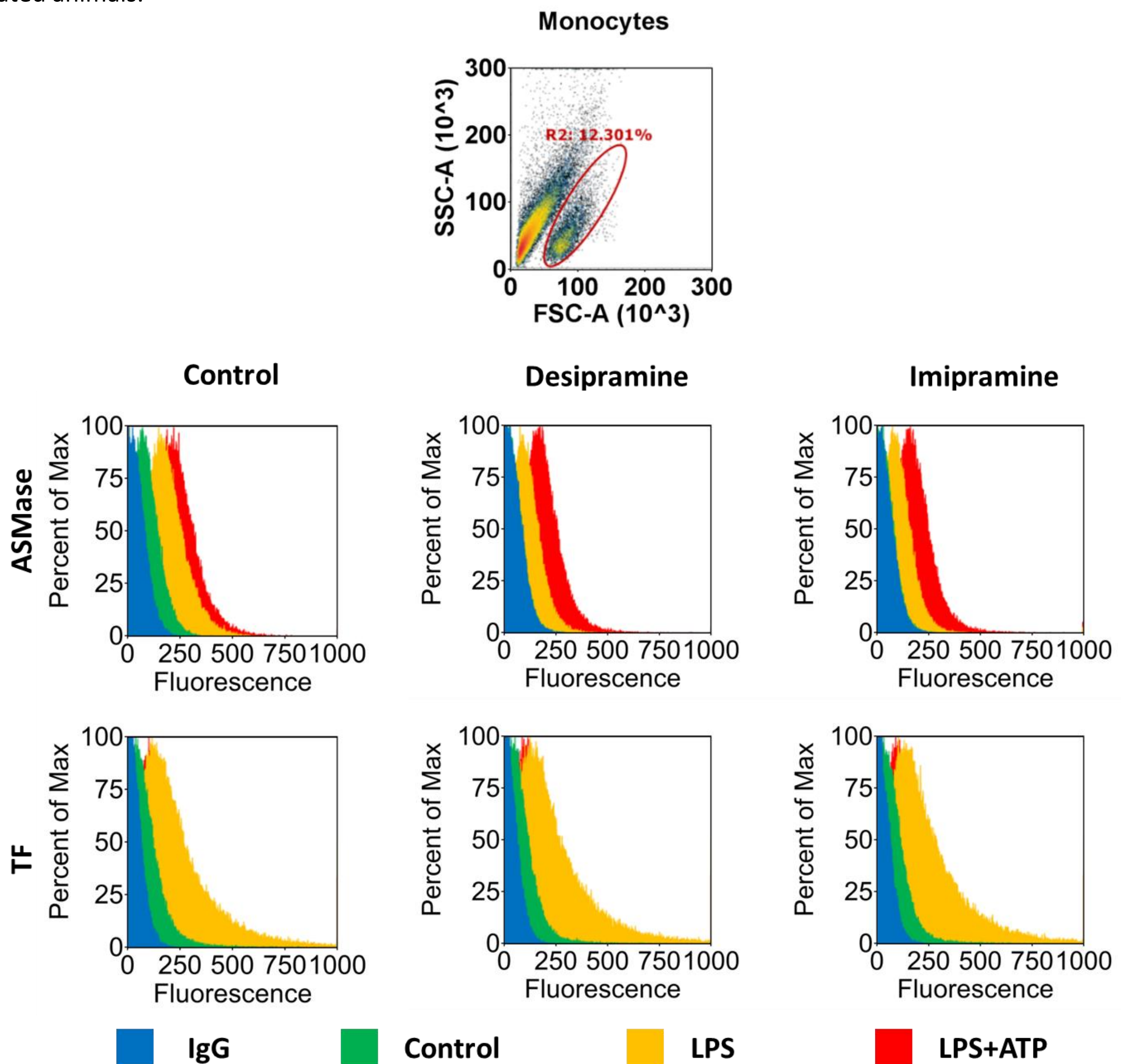
Supplemental Figure 1. Treatment of mice with ASMase inhibitors do not affect LPS-induced TF protein expression in monocytes. Wild-type (C57BL/6J) mice were treated with saline or ASMase inhibitors - desipramine (Des) or imipramine (Imi),10 mg/kg b.w in 100 µL saline, - daily by intraperitoneal injection (i.p.) for 7 days. After 7 days, mice were challenged with saline or LPS (*E coli* O111:B4, 5 mg/kg) by injecting them i.p. in 100 µl volume. In a sub-group of mice, four hours following the LPS challenge, bz-ATP (50 mg/kg in 100 µl) was administered. Fifteen minutes following ATP administration, animals were killed, and blood was drawn into citrate anticoagulant by cardiac puncture. PBMCs were separated and subjected to western blot analysis for TF antigen. Top panel, a representative image of western blot; bottom panel, semi-quantification of TF antigen levels by densitometry analysis of TF band (n = 4). ns, not a statistically significant difference between the groups.



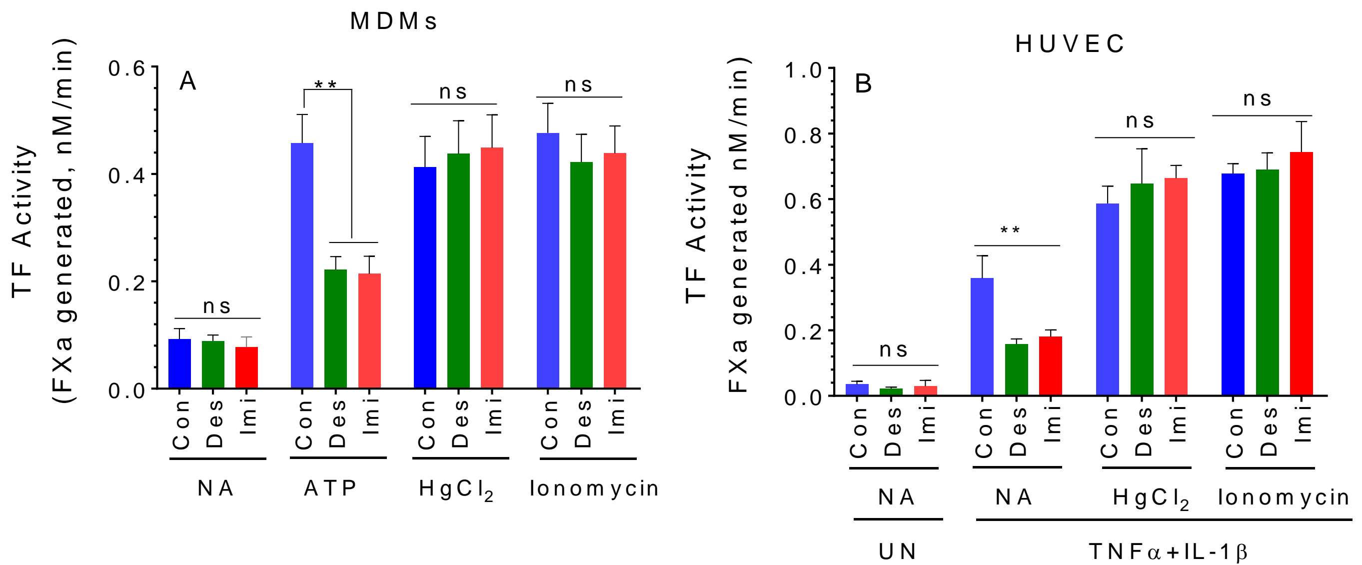
Supplemental Figure 2. ASMase staining in murine PBMCs. PBMCs isolated from the blood of wild-type mouse (C57BL/6J) were fixed, and either non-permeabilized or permeabilized with 0.01%Triton X-100 for 10 minutes, and then stained with rabbit anti- ASMase (recognizes human, murine and rat ASMase), followed by AF546 donkey anti-rabbit IgG. DAPI was used for nuclear staining. Larger size cells are monocytes. DAPI staining of other PBMCs, which are much smaller than monocytes, is not prominent and thus these cells are not clearly visible in DAPI staining.



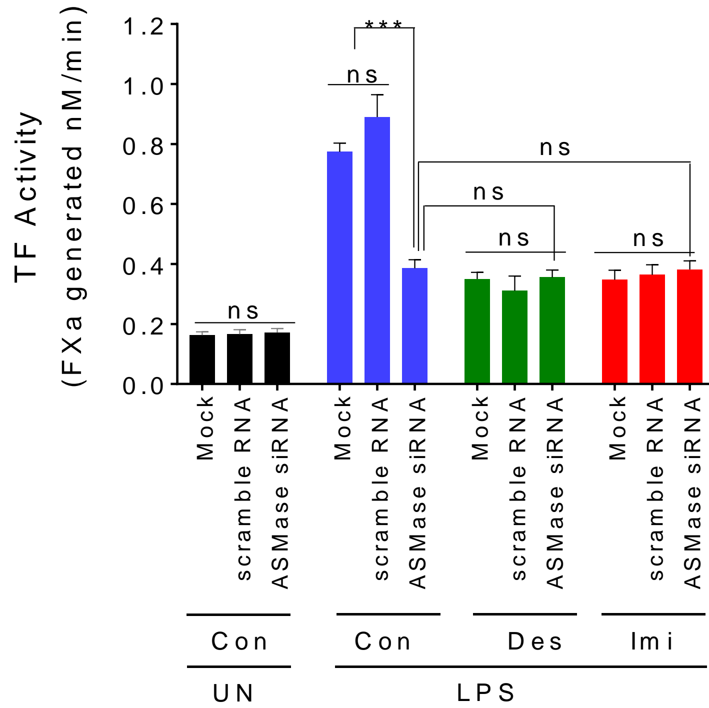
Supplemental Figure 3. Flow cytometry analysis of ASMase and TF expression in monocytes from mice treated with ASMase inhibitors and challenged with LPS or LPS followed by ATP. Wild-type mice were injected with saline (control) or ASMase inhibitors - desipramine or imipramine (10 mg/kg b.w in 100 µL saline) - daily by i.p. for 7 days. On the 7th day, mice were challenged with saline (control) or LPS (*E. coli* O111:B4, 5 mg/kg) by injecting them i.p. (in 100 µl volume). In a sub-group of mice, four hours following the LPS challenge, bz-ATP (50 mg/kg in 100 µl) was administered. Fifteen minutes following ATP administration, animals were killed and blood was drawn into citrate anticoagulant by cardiac puncture. PBMCs were separated, fixed, stained with rabbit anti-human ASMase and rat anti-murine TF mAb (1H1), and subjected to flow cytometry. Monocyte population identified by forward scattering (top row) was analyzed for expression of ASMase (second row) and TF (third row). Green, monocytes from mice challenged with saline (control); yellow, monocytes from mice challenged with LPS; red, monocytes from mice challenged with LPS and ATP. The flow chart for TF expression in LPS and LPS+ATP were very similar, therefore, the staining of TF expression in LPS+ATP treated animals was masked by TF expression in LPS treated animals.



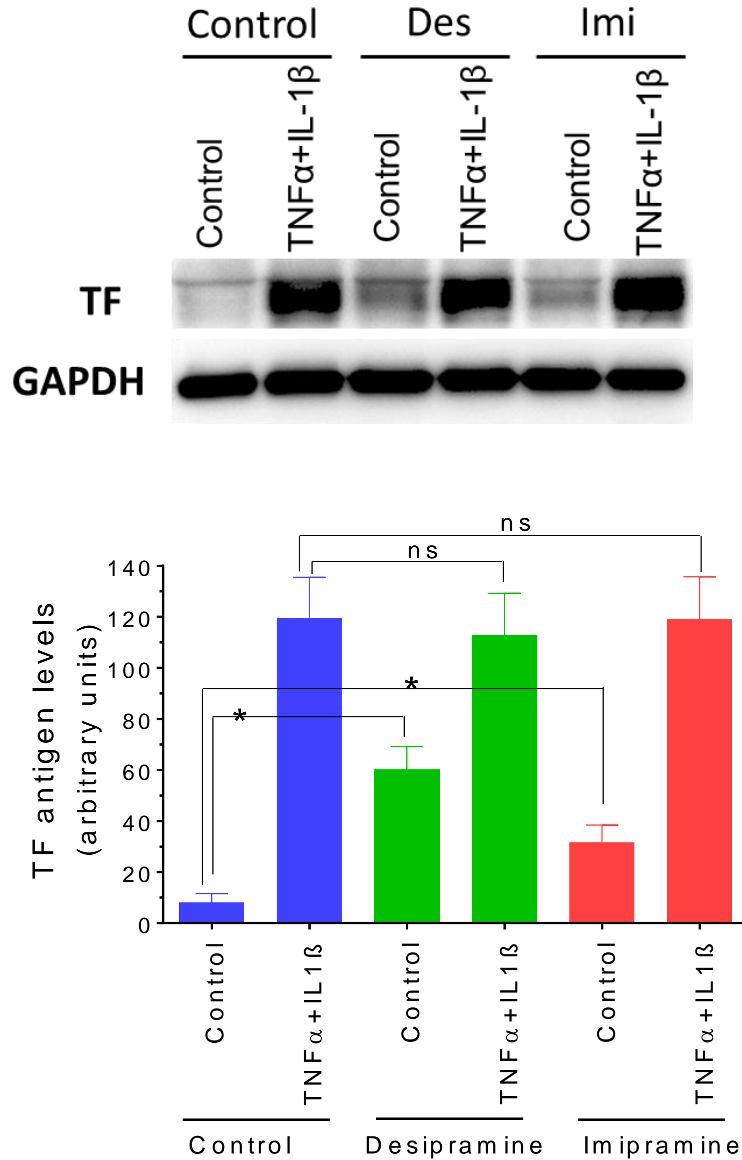
Supplemental Figure 4. ASMase inhibitors do not block $HgCl_2$- or ionomycin-induced TF activation. (A) MDMs were pre-treated with a control vehicle saline (Con), desipramine (Des, 10 µM) or imipramine (Imi, 10 µM) for 1 h. Then, the cells were left alone (not activated, NA) or treated with bz-ATP (200 µM) for 15 min, $HgCl_2$ (100 mM) for 5 min or ionomycin (10 µM) for 15 min. Following the treatments, the cells were washed and the cell surface TF activity was measured as its ability to support FVIIa activation of FX. (B) Monolayers of confluent endothelial cells were pre-treated with a control vehicle saline (Con), desipramine (Des, 10 µM) or imipramine (Imi, 10 µM) for 1 h. Then, the cells were left unstimulated (UN) or stimulated with TNFα+IL-1ß (10 ng/mL, each) for 6 h. At the end of 6 h, TNFα+IL-1ß-stimulated cells were left alone (not activated, NA) or treated with $HgCl_2$ (100 mM) for 5 min or ionomycin (10 µM) for 15 min. Following the treatment, the cells were washed and the cell surface TF activity was measured as its ability to support FVIIa activation of FX. **, $p < 0.01$; ns, not statistically significant difference.



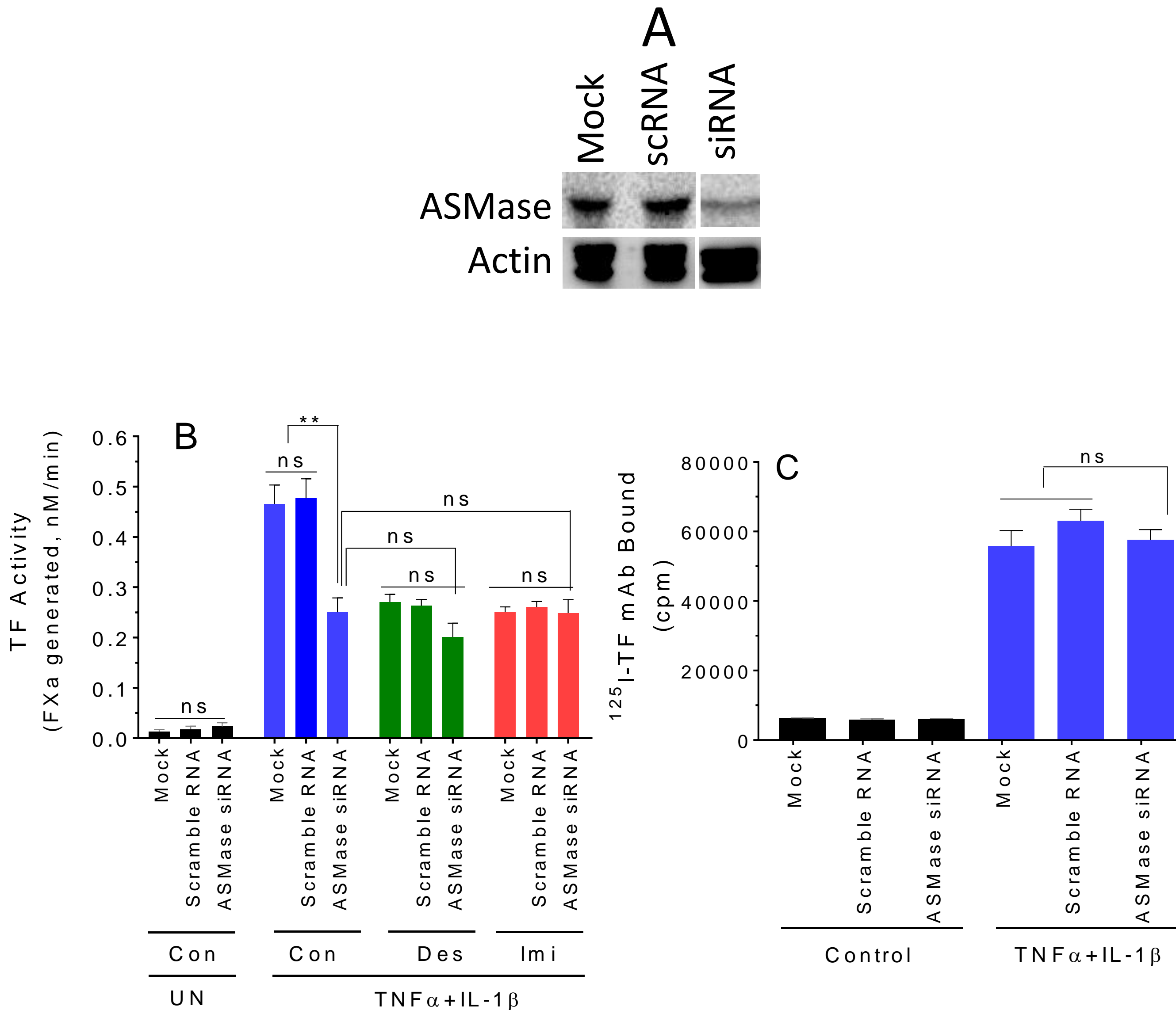
Supplemental Figure 5. ASMase inhibitors do not further reduce LPS-induced TF activity in ASMase silenced cells. MDMs were transfected with transfection reagent alone (Mock), scramble RNA (100 nM) or ASMase siRNA (100 nM). Two days post-transfection, cells were treated with saline (Con), desipramine (Des, 10 μM) or imipramine (Imi, 10 μM) for 1 h. Then the cells were left unstimulated (UN) or stimulated with LPS (1 μg/mL) for 4 h. At the end of 4 h, the cells were washed, and the cell surface TF activity was measured as its ability to support FVIIa activation of FX. ***, p <0.001; ns, not statistically significant difference.



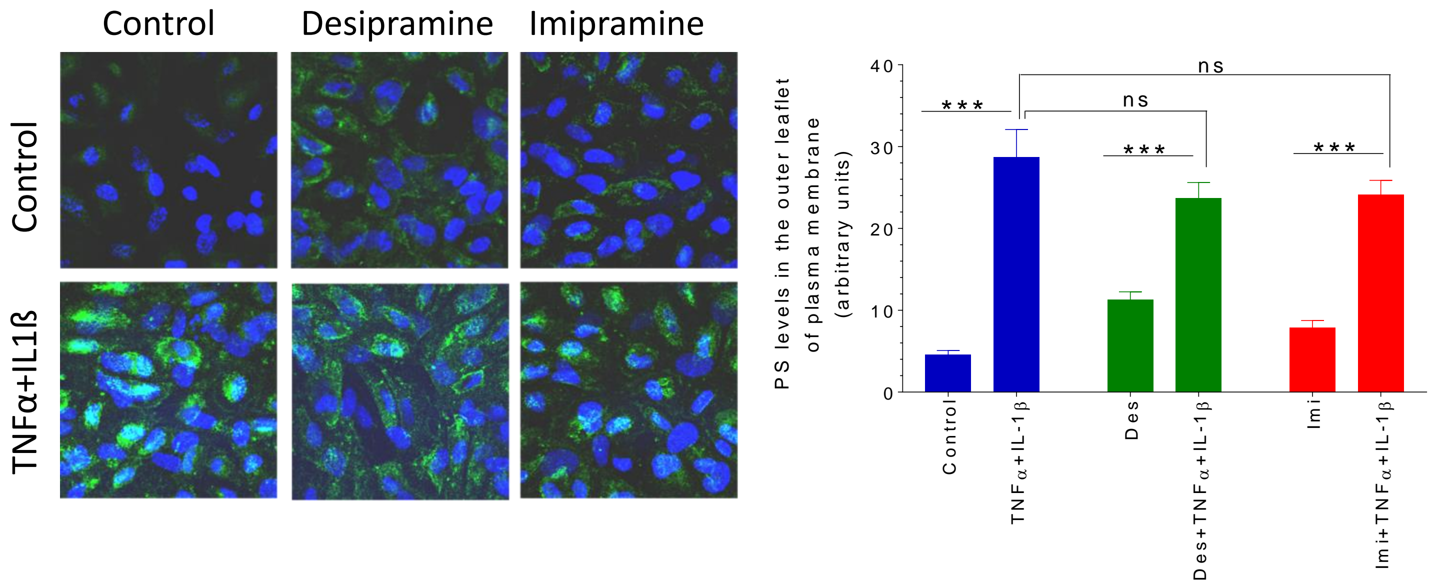
Supplemental Figure 6. ASMase inhibitors do not reduce TNFα+IL-1β-induced TF antigen levels in endothelial cells. HUVECs were treated with desipramine (Des 10 µM) or imipramine (Imi, 10 µM) for 1 h, stimulated with TNFα and IL-1β (10 ng/mL each) for 6 h. Cells lysates were subjected to western blot analysis to analyze TF antigen levels. Top panel, a representative image of western blot; bottom panel, quantification of TF antigen levels by densitometry analysis of TF band (n=3). ns, not statistically significant difference; *, p <0.05.



Supplemental Figure 7. ASMase knock-down diminishes cytokine-induced TF procoagulant activity in endothelial cells. HUVEC were subjected to mock transfection or transfection with a control scramble RNA or ASMase siRNA (100 nM each). Two days post-transfection, ASMase levels in cells were analyzed by western blot analysis (A). (B) HUVEC transfected with mock, scramble or ASMase siRNA were treated with a control vehicle saline (Con), desipramine (Des, 10 μM) or imipramine (Imi, 10 μM) for 1 h. The cells were left either unstimulated (UN) or stimulated with TNFα plus IL-1ß (10 ng/mL each) for 6 h. Folowing the stimulation, cell surface TF activity as measured its ability to support FVIIa activation of FX. (C) Binding of ^{125}I-TF mAb (9C3) to the cell surface of unstimulated and TNFα+IL-1ß-stimulated HUVEC that were subjected to mock transfection or transfection with a control scramble RNA or ASMase siRNA as described in panel A. **, *p*<0.01; ns, not statistically significant difference.



Supplemental Figure 8. Effect of ASMase inhibitors on TNFα+IL-1ß-mediated externalization of phosphatidylserine on endothelial cells. HUVECs were treated with desipramine (Des, 10 µM) or imipramine (Imi, 10 µM), or saline (control) for 1 h, and then stimulated or not with TNFα and IL-1β (10 ng/mL each) for 6 h. Cells were fixed, stained with AF488-annexin V and subjected to fluorescence microscopy. Left panel, a representative micrograph of annexin V staining; right panel, quantification fluorescence staining of annexin V. ns, not statistically significant difference; ***, p <0.05.



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