SUPPLEMENTAL MATERIAL

BMX represses thrombin-PAR1-mediated endothelial permeability and vascular leakage during early sepsis

Expanded Materials and Methods

Online Figures I-VIII

EXPANDED MATERIALS AND METHODS

***Sepsis Model*.** Both male and female mice that were 8-10 weeks of age were used to select age- and sex-matched WT and BMX-KO mice for experiments. Sepsis was induced in mice by CLP with 19-gauge needle. Briefly, age- and weight-matched WT and BMX-KO mice were anesthetized with ketamine (80-100 mg/ kg body weight). The cecum was ligated at the designated position which depends on the desired severity grade, and then the ligated cecum was subjected to a single “through and through” puncture with 19-gauge needle. Sham-operated mice underwent the same procedure except for ligation and perforation of the cecum. Paraffin-embedded sections 5 μm in thickness prepared from the lungs were used for hematoxylin and eosin staining (HE), immunohistochemical staining (IHC) and immunofluorescence (IF). Inflammatory cells in the BALF were collected and measured by Complete Blood Count (CBC). Cytokines in the BALF were measured by enzyme-linked immunosorbent assay (ELISA).

Modified Mile Assay. Under anesthesia, mice tail veins were injected with Evans blue (30 mg/Kg; Sigma-Aldrich). And then Thrombin or PBS was injected into dorsal skin, respectively. About 20 min after treatment, mice were then euthanized and perfused; the skin was removed for image, over-dried at 55°C, and weighted. Evans blue was then extracted from the skin using 500 μl of formamide for 24 h at 55 °C. Evans blue extravasation into the skin was measured spectrophotometrically at 630 nm using a standard curve of Evans blue in formamide.

Cell culture, western blotting, immunofluorescence microscopy (IF) and immunoprecipitation (IP). Human umbilical vein ECs (HUVECs), obtained from the Yale Vascular Biology Program (VBT) Endothelial Cell Facility (see https://medicine.yale.edu/vbt/order/pricelist/), were isolated from human umbilical cord and maintained in M199 medium (20% FBS, 100 μg/ml ECGS, and 100 μg/ml heparin). Human Pulmonary Microvascular Endothelial Cells (HPMVEC or HMVEC-L, Cat# CC-2527), isolated from small vessels within normal lung tissues, were purchased from Lonza's and were cultured in medium from Lonza EGM-2MV (CC-3202). Human pulmonary epithelial cells (HPEpiC) or small airway epithelium cells (SAEC) were purchased from Lonza (CC-2547) and cultured in epithelium medium (Lonza CC-3118). All these primary cells were used for experiments at passages 3-5. Confluent cells were transfected with si-CT or si-BMX with RNAimax (Invitrogen 13778-150) for 3 days. Cells were then starved in EBM2 basal medium plus 1% FBS for 8 h followed by treatment with thrombin (1.5 u/ml) for indicated times.

For western blotting, the cells were lysed with RIPA buffer containing complete mini EDTA-free protease inhibitors (#11836170001, Roche) and phosphatase inhibitors (#04906837001, Roche). About 10 micrograms of total protein from each sample was resolved on a SDS-PAGE gel with Running Buffer and transferred onto nitrocellulose membranes (#162-0094, Bio-Rad). The blots were then probed with various antibodies. Chemiluminescence measurements were performed using SuperSignal West Femto Maximum Sensitivity Substrate (Thermo Fisher Scientific). For immunofluorescence staining, cultured primary endothelial cells were grown on 0.1% Gelatin–coated glass-bottomed dishes. After various treatment, the cells were fixed with 4% PFA in PBS for 20 min at 37 °C, then permeabilized with 0.1% Triton X-100 in PBS at room temperature for 5 min, and blocked with 5% normal horse serum (Vector laboratories, Inc.) at room temperature for 60 min. Cells were washed with PBS and incubated with primary antibodies (1:100 in 1% BSA) at 4°C overnight, washed three times with PBS, and incubated with diluted Alexa Fluor–conjugated secondary antibody (1:200) (Invitrogen) for 1 hour at room temperature. The dishes were then washed three times with PBS and mounted using Mounting medium with DAPI (Vector laboratories, Inc.). The cells were harvested with RIPA buffer, and then incubated with primary antibodies (2 ug) at 4 °C overnight. Later protein and antibody complex was immunoprecipitated with 50 μl Agarose A/G beads (Thermo Scientific) at 4 °C for 3 h. Western blotting was used to detect the immunoprecipitated protein.

CRISPAR/Cas9 Knockdown and lentivirus package. Specific gRNA for BMX was designed by http://chopchop.cbu.uib.no/. Then the sequence was cloned into lentiCRISPAR vector. After co-transfection with lentivirus helper plasmids into 293T cells, collected lentivirus medium and used it for transfection in HUVECs, and then used puromycin for selection BMX knockdown cells lines. Target gene cDNA was cloned into lentivirus vector. After co-transfection with lentivirus helper plasmids into 293T cells, collected lentivirus medium and used it for transfection in HUVECs.

***Cell surface biotinylation and internalization biotinylation*.** For cell surface biotinylation assay, after treatment by thrombin for various time, confluent HUVECs were immediately washed by ice cold PBS^{2+} supplemented with 1.5 mM $MgCl_2$, 0.2 mM $CaCl_2$, and then were incubated with fresh

1 ml/6-well 0.25 mg/ml EZ-Link Sulfo-NHS-SS-Biotin (Pierce) at 4 °C for 1 h. After washed by PBS^{2+}, the cells were quenched biotin with PBS^{2+} plus 100 mM glycine 4 °C for 30 mins. The cells were harvested with RIPA buffer, and then 200 μg of lysate were immuneprecipitated with 50 μl StreptAvidin beads (Thermo Scientific) at 4°C. For internalization biotinylation assay, Confluent HUVECs were washed by ice cold PBS^{2+} supplemented with 1.5 mM $MgCl_2$, 0.2 mM $CaCl_2$, and then were incubated with fresh 1 ml/6-well 0.25 mg/ml EZ-Link Sulfo-NHS-SS-Biotin (Pierce) at 4 °C for 1 h. After washed by PBS^{2+}, the cells were quenched biotin with PBS^{2+} plus 100 mM glycine 4 °C for 30 mins. The cells were changed with pre-warmed basal medium and incubated with thrombin (1.5 U/ml) for various time points. After washed by ice-cold PBS^{2+}, the cell incubated with stripping buffer at 4 °C for 30 mins; MesNa was quenched by ice-cold PBS^{2+}, and then the cells were incubated with 180 mM iodoacetamide (Sigma) in PBS^{2+} at 4 °C for 10 mins. After washed by PBS^{2+}, the cells were harvested with RIPA buffer, and then 200 μg of lysate was immunoprecipitated with 50 μl StreptAvidin beads (Thermo Scientific) at 4 °C.

Trans-endothelial electrical resistance (TEER) measurement by electric cell-substrate impedance sensing (ECIS) assay: The EC barrier function was assayed by measuring the resistance of cell-covered electrode by using an ECIS instrument (Applied BioPhysics). An 8W10E plate was incubated for 15 min with L-cysteine (10 mM) solution, followed by gelatin 0.1% for 30 min. Cells were plated on the electrode at $7x10^4$ cells per well. After 72 h incubation, ECs were exposed to thrombin, and the resistance was monitored.

Direct phosphorylation of PAR1 by BMX measured by an in vitro kinase assay. In vitro kinase assays were performed using BMX protein kinase and GST-PAR1-C tail (375-425 aa) fusion protein as a substrate. GFP-BMX-WT and GFP-BMX-KR vectors were transfected into 293T cells. BMX immunoprecipitates were immunoprecipitated by anti-GFP from 400 μg 293T lysates overexpressing BMX-WT or a kinase-inactive from (BMX-KR). Full-length BMX recombinant protein was purchased from Thermo Fisher Scientific (PV4074). For the kinase assay, the recombinant BMX (10 ng) or immunoprecipitates were mixed with 10 μg GST-PAR1-C suspended in the kinase buffer (20 mM Hepes, pH 7.6, 20 mM MgCl2, 25 mM β-glycerophosphate, 100 μM sodium orthovanadate, 2 mM DTT, 20 μM ATP). The kinase assay was performed at 25˚C for 30 min. The reaction was terminated by the addition of Laemmli sample buffer and the products were resolved by SDS-PAGE (12%) and the phosphorylated GST-PAR1-C was visualized by Western blotting with anti-phosphotyrosine. GST and BMX were detected as controls.

***Antibodies and Reagents*.** The primary and secondary antibodies used for Western blotting, immunofluorescence and immunohistochemistry are listed as follows:

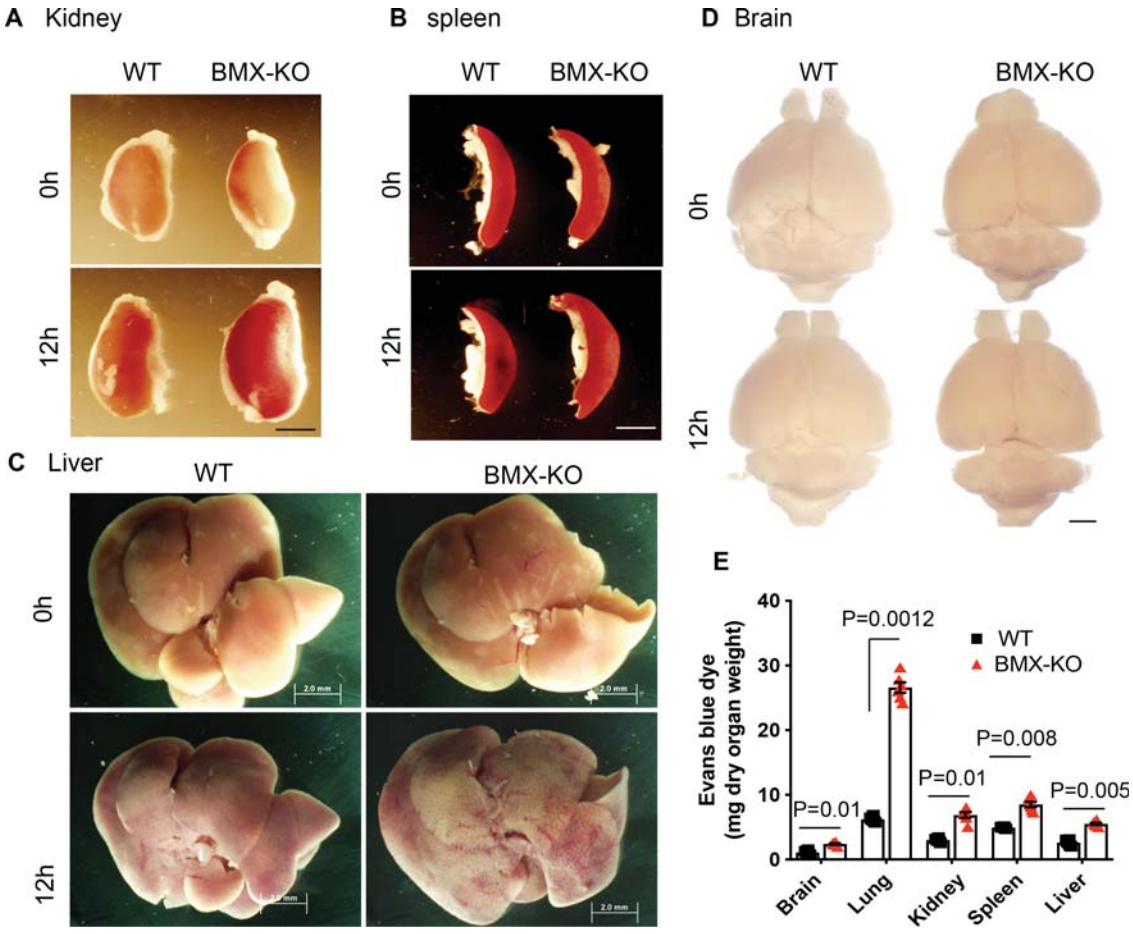
<u>The following antibodies were used for immunostaining:</u>

Antibody name	**Company**	**Cat#**	**Dilution/ conc.**

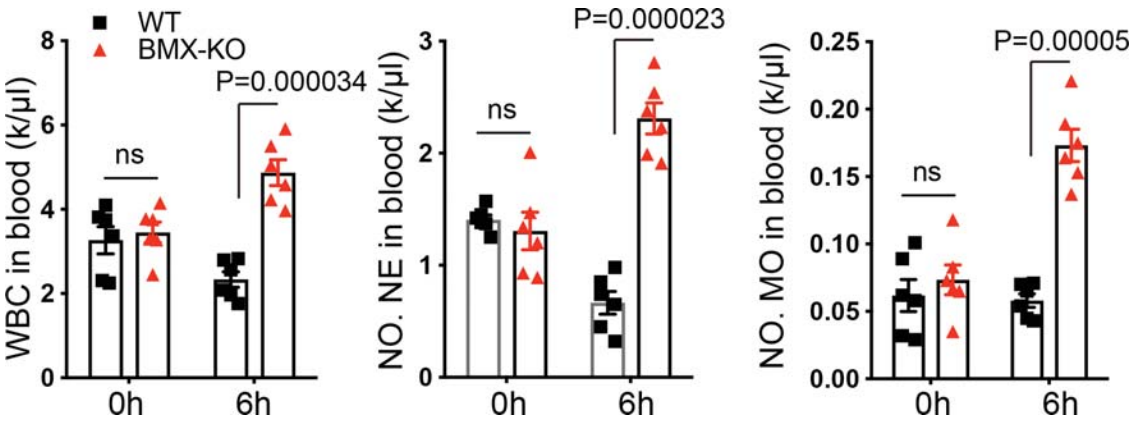
Angiopoietin-2, goat	R&D	AF623	1:100
Angiopoietin-2, rabbit	Abcam	ab8452	1:200
Angiopoietin-2, rabbit	Novus	NBP2-1538	1:100
CD31, rat	BD Pharmingen	553370	1:100
CD31, rabbit	Abcam	ab28364	1:100
Claudin-5, rabbit	Invitrogen	34-1600	1:100
iNOS, mouse	R&D	MAB9502	1:100
NG2, rabbit	Millipore	AB5320	1:100
Nitrotyrosine, mouse	Santa Cruz	Cs-32757	1:50
VE-cadherin, goat	Santa Cruz	sc-6458	1:100
VE-cadherin, rat	BD Pharmingen	555289	1:100
Alexa Flour 488 Donkey anti-Rat IgG	Invitrogen	A21208	1:200
Alexa Flour 488 Donkey anti-Goat IgG	Invitrogen	A11055	1:200
Alexa Flour 488 Donkey anti-Rabbit IgG	Invitrogen	A21206	1:200
Alexa Flour 488 Donkey anti-Mouse IgG	Invitrogen	A21202	1:200
Alexa Flour 594 Donkey anti-Rat IgG	Invitrogen	A21209	1:200
Alexa Flour 594 Donkey anti-Goat IgG	Invitrogen	A11058	1:200
Alexa Flour 594 Donkey anti-Rabbit IgG	Invitrogen	A21207	1:200
Alexa Flour 594 Donkey anti-Mouse IgG	Invitrogen	A21203	1:200

The following antibodies were used for Western blot: Phospho-p38, p38, phospho-p65, p65, phospho-MLC2 (#3674), phospho-Erk1/2, and GAPDH (Cell Signaling Technology), MLC2 (Millipore), BMX, and Erk1/2 (Santa Cruz Biotechnology), F2r (PAR1; Thermo Scientific), GFP, BMX and VE-Cadherin (Santa Cruz Biotechnology); β-actin (Sigma, A5316). Unconjugated IgG isotype controls: mouse IgG (#31903), rabbit IgG (#31235), rat (#31933), and goat IgG (#31245) were from Invitrogen and were used at the same concentration as the respective primary antibody when applied (0.1- 0.2 μg/ml). Horseradish peroxidase (HRP)-conjugated anti-rabbit (#7074), anti-rat (#7077), and anti-mouse secondary antibody (#7076) were from Cell Signaling Technology and were used at 0.2 μg/ml.

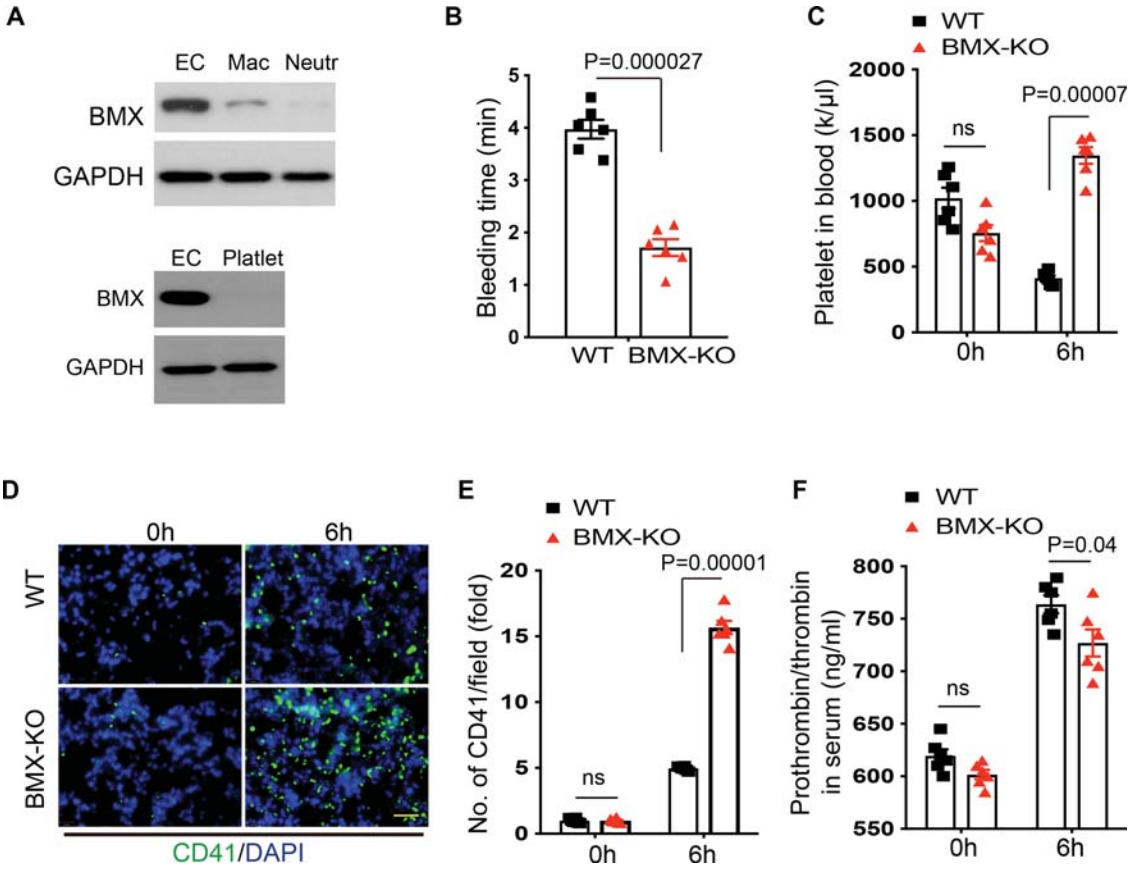
Other reagents: For co-immunoprecipitation assays, protein A/G magnetic agarose beads (Pierce 78609). ELISA kits were as follows: MCP1 (Sigma-Aldrich), TNFα and IL-6 (Millipore). Inhibitors were used as followed: PAR1 antagonist SCH79797 (Abcam), BMX inhibitor AG879 (Sigma), Src inhibitor PP2 and Erk1/2 inhibitor PD98059 (Calbiochem). Control siRNA and BMX siRNA (SI00605318 and SI00605325) were from Qiagen.



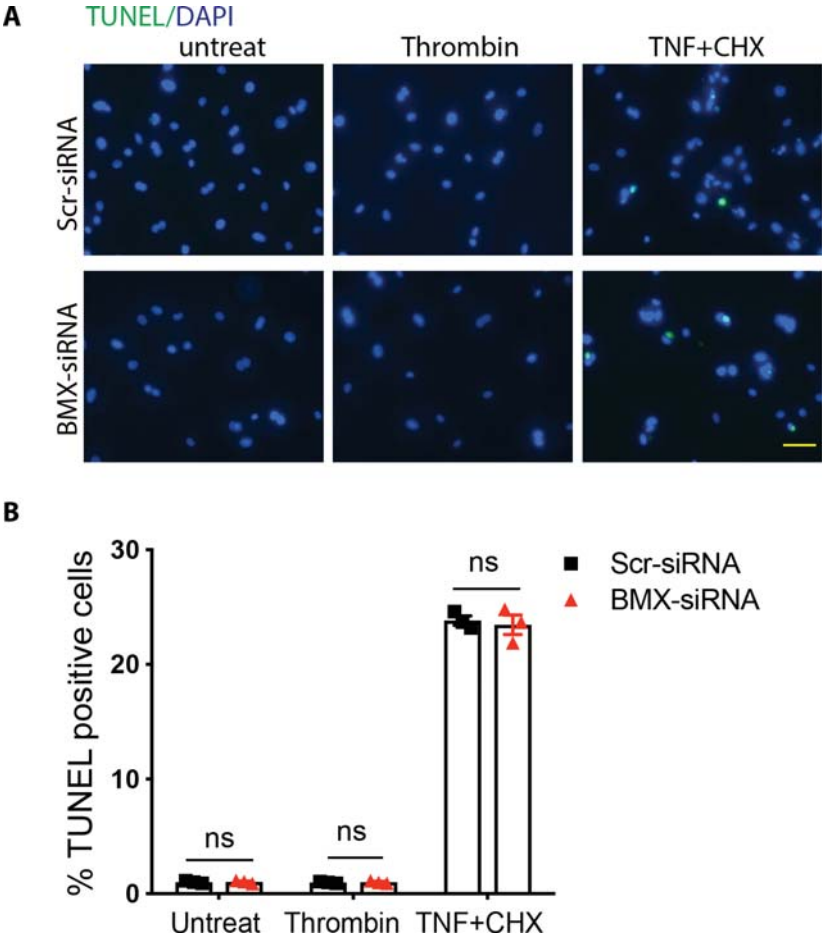
Online Figures I. Gross morphology of spleen, kidney and liver. Gross morphology of spleen (A), kidney (B), liver (C) and brain (D) tissues at indicated times after CLP-induced sepsis. (E) Evans blue dye (30 mg/ml) was injected into mice at 6 h post-CLP, and then mouse tissues were harvested for Evans blue dye measurements. The data were quantified as the relative fold of Evans blue dye per mg of the dry tissue weight by taking WT brain tissue as 1.0. n=6 mice in each group. Error bars represent mean±SEM. Data were analyzed between WT and BMX-KO in each tissue using unpaired, two tailed *t*-test. Scale bar: 2 mm.



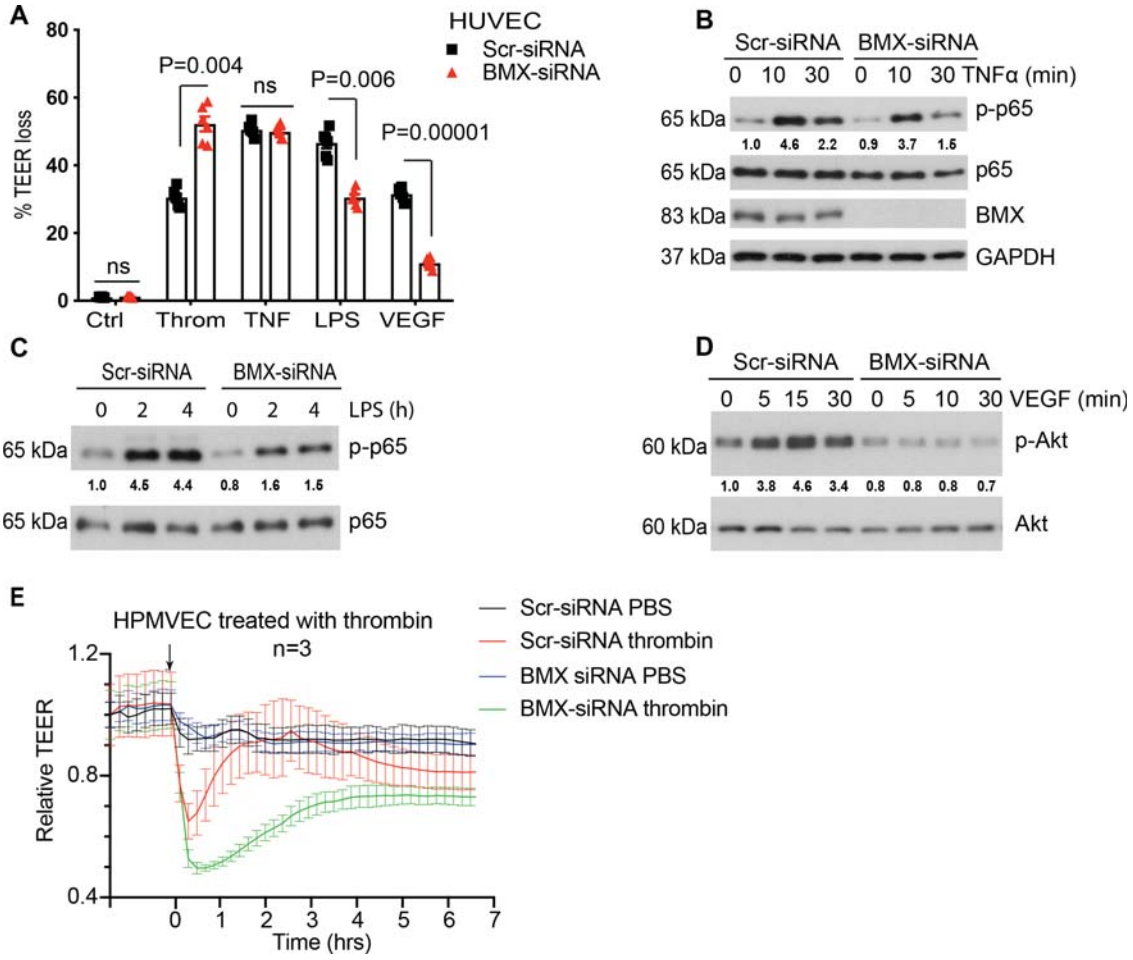
Online Figures II. **WBC, NE, MO and PLT numbers in mice serum.** CBC test for WBC, NE, MO and PLT numbers in mice serum at indicated times after CLP-induced sepsis; 6 mice in each group. Error bars represent mean±SEM. Data were analyzed between WT and BMX-KO using 2-way ANOVA and Bonferroni post-hoc multiple comparisons. ns, non-significant (P>0.05).



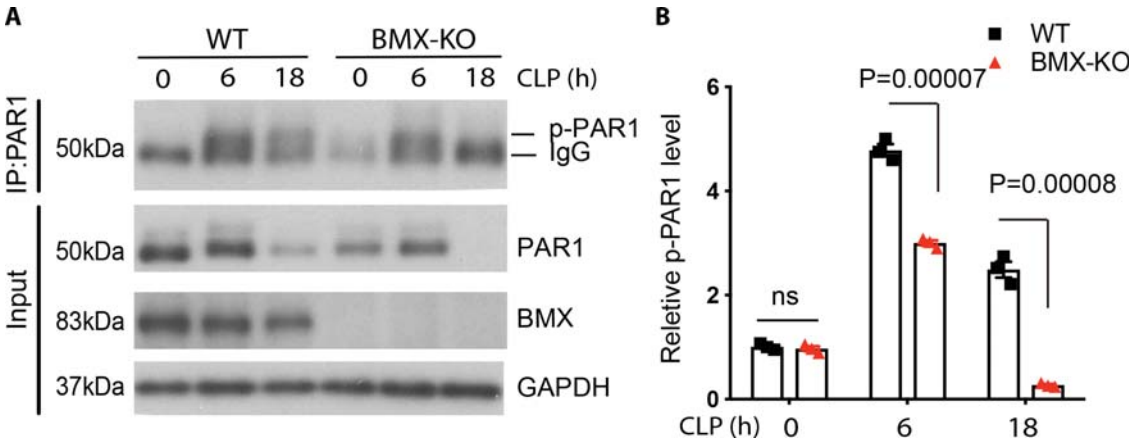
Online Figures III. Thrombosis phenotype in BMX-KO mice. (A) BMX protein levels in mouse lung microvascular endothelial cells, bone-marrow derived macrophages, bone-marrow derived neutrophils and blood platelets. (B) Mice tail bleeding time of WT and BMX-KO mice. (C) platelet in blood was detected in WT and BMX-KO mice at indicated times post-CLP. (D) Immunofluorescence about pulmonary platelet deposition at indicated time post-CLP. (E) Quantification of pulmonary deposited platelets at indicated time post-CLP. (F) ELISA test for total prothrombin and thrombin levels in mouse serum at indicated times post-CLP. N=6 mice in each group. Error bars represent mean±SEM. Data were analyzed between WT and BMX-KO using unpaired, two tailed *t*-test (B) or 2-way ANOVA and Bonferroni post-hoc multiple comparisons (C, E, F). ns, non-significant (P>0.05). Scale bar: 20 μm (D).



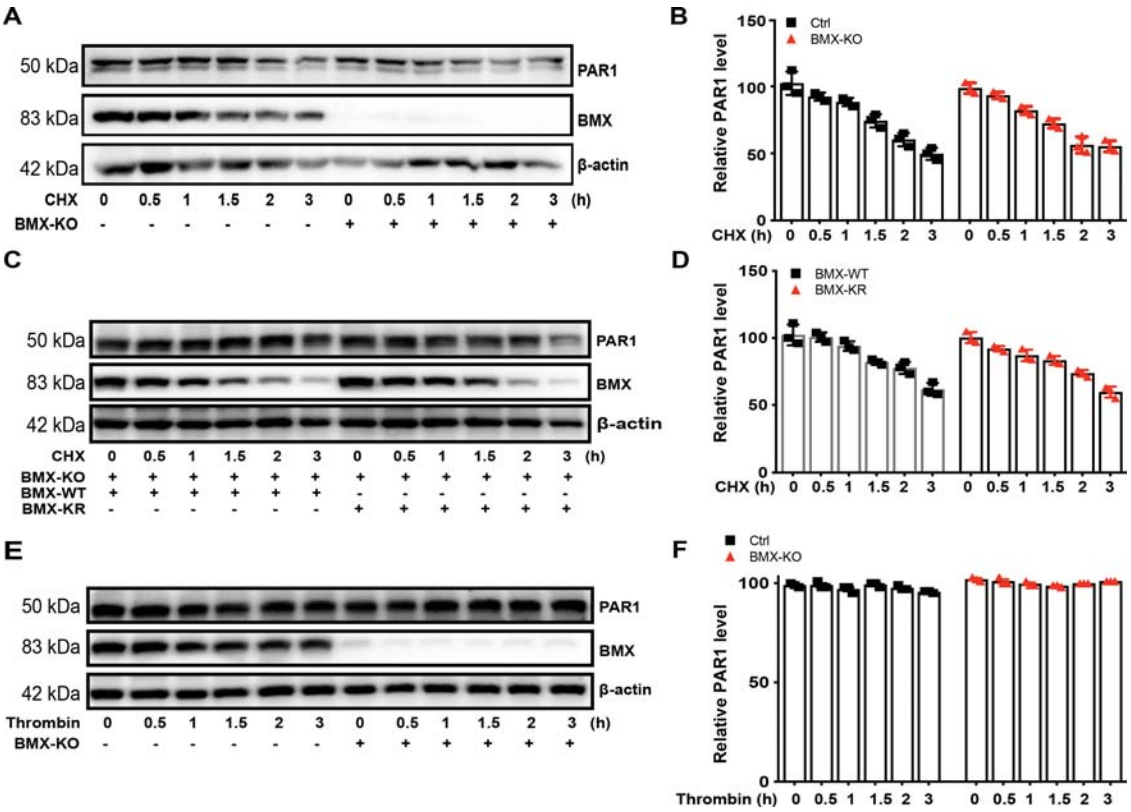
Online Figures IV. Effects of BMX knockdown on thrombin and TNF+CHX-induced HUVEC apoptosis. Scr-siRNA- or BMX-siRNA-transfect HUVECs were treated with thrombin (1.5 U/ml) or TNF (10 ng/ml) plus cycloheximide (10 mg/ml) for 8 h. (A) Cells were subjected to TUNEL staining. (B) Quantification of % apoptotic cells. All experiments were repeated three times with biological replicates. Error bars represent mean±SEM. No significance (ns; P>0.05) was detected between Scr-RNA and BMX siRNA, unpaired, two tailed *t*-test. Scale bar: 20 μm (A).



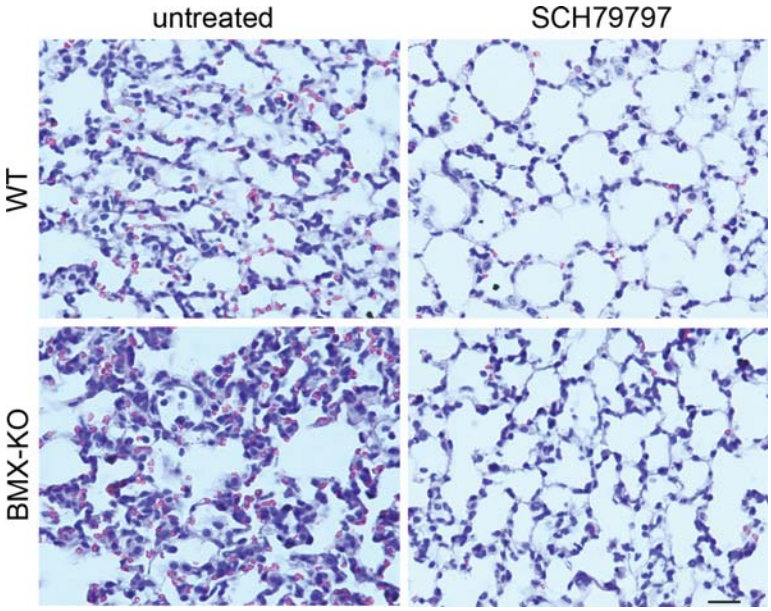
Online Figures V. Supplemental data for Figure 3. (A-D) Effects of BMX knockdown on thrombin, TNF, LPS and VEGF-induced HUVEC permeability. Post-confluent Scr-siRNA- or BMX-siRNA-transfect HUVECs were treated with thrombin (1.5 U/ml), TNF (10 ng/ml), LPS (100 ng/ml) and VEGF (10 ng/ml). (A) TEER was measured and % TEER losses at 1 h are presented. (B-D) The effects of BMX knockdown on TNF, LPS and VEGF signaling in HUVECs by Western blotting. (E) Thrombin-induced barrier dysfunction in HPMVECs. Post-confluent Scr-siRNA and BMX-siRNA-transfected HPMVECs were treated with thrombin (1.5 U/ml) as shown by the arrow, and then TEER was immediately measured for the indicated times. All experiments were repeated three times with duplicates. Error bars represent mean±SEM. Data were analyzed between the between Scr-RNA and BMX siRNA using 2 unpaired, two tailed *t*-test (A). ns, non-significant (P>0.05).



Online Figures VI. Effects of BMX-KO on CLP-induced PAR1 phosphorylation. WT and BMX-KO mice were subjected to CLP-induced sepsis surgery, and lung tissues were harvested at 0, 6 and 18 h post-injury. (A) Tissue lysates were then subjected to co-immunoprecipitation with anti-PAR1 followed by Western blotting with anti-phosphotyrosine. Input was determined for total PAR1, BMX and GAPDH proteins. (B) Relative phosphor-PAR1 was quantified. n=3 mice for each strain at each time point. Error bars represent mean±SEM. Data were analyzed between WT and BMX-KO using 2-way ANOVA and Bonferroni post-hoc multiple comparisons. ns, non-significant (P>0.05).



Online Figures VII. BMX siRNA had no effects on PAR protein stability. (A-B) BMX-KO HUVECs and control HUVECs were treated with CHX (10 μg/ml) to block translation for the indicated times. PAR1 and BMX were detected by western blot (A) with quantification of relative PAR1 levels in (B). (C-D) BMX-WT or BMX-KR re-expression in BMX-KO HUVECs and cells were treated with CHX (10 μg/ml) for the indicated times. PAR1 and BMX were detected by western blot (C) with quantification of relative PAR1 levels in (D). (E-F) Control and BMX-KO HUVECs were starved overnight and treated with thrombin (1.5 U/ml) for the indicated times. PAR1 and BMX were detected by western blot (E) with quantification of relative PAR1 levels in (F). All experiments were repeated three times with biological replicates. Error bars represent mean±SEM. No significance (P>0.05) was detected between Ctrl and BMX-KO or between BMX-WT and BMX-KR groups at various time points using 2-way ANOVA and Bonferroni post-hoc multiple comparisons.



Online Figures VIII. HE staining of lung tissue section at indicated times after CLP-induced sepsis. WT and BMX-KO mice were intraperitoneally injected with 100 μM SCH79797 or saline in each 12 hours within 3 day, and then sepsis surgery was performed, followed by HE staining of lung tissue section. Scale bar: 20 μm.