Supplemental Material and Methods

Backcrossing of the PAD4$^{-/-}$ mice

For backcrossing of the PAD4$^{-/-}$ line, only WT mice ordered directly from Jackson Laboratory were used. The mice used for this experiment were backcrossed between 8 and 9 generations to the C57Bl/6J background. Our lab maintains several lines of knockout mice for which we order WT controls from Jackson. Once a year our colonies undergo a full backcross to the Jackson C57Bl/6J substrain. For this, knockout mice are bred with WT mice from Jackson and the heterozygous offspring are used to generate future knockout breeders.

Surgical procedure for induction of myocardial ischemia/reperfusion injury

Mice were anesthetized using 2% isoflurane and placed in supine position on a heated operating table. All consecutive procedures were done under electrocardiogram (ECG) control using a 3-lead ECG system (Powerlab 4/30 with Labchat 7 software, AD Instruments). Mice were intubated and mechanically ventilated using a small animal ventilator (Harvard Apparatus) with 1.5% isoflurane. After a midline skin incision, a left parasternotomy was performed via a lateral incision along the left side of the sternum (cutting the second, third, and fourth ribs) to expose the heart and allow visualization of the left anterior descending branch (LAD) of the left coronary artery. An 8-0 polyamide suture (Aros Surgical) was passed underneath the LAD 1 to 3 mm from the tip of the left auricle. Both ends of the suture were passed through a 1 mm piece of PE-10 tubing (BD), and then, through the chest wall. After implantation of the occluding device, the chest was closed and the animal was allowed to recover for one week. After one week, the mouse was

reanesthetized with 2% isoflurane following LAD occlusion. The midline skin incision was reopened and the implanted 8-0 silk suture was pulled to acutely occlude the LAD, which was verified by ST-elevation on the electrocardiographic tracing. After 1 hour of LAD occlusion, blood flow was restored by releasing the 8-0 silk suture. Reperfusion was verified by ECG monitoring and the skin was closed again. The mouse was allowed to recover for the following 23 hours of myocardial reperfusion and then euthanized. Sham-operated mice underwent all of the steps described above, except for the pulling of the suture to occlude the LAD.

Determination of infarct size

As a result of TTC staining, viable myocardium becomes red and infarcted tissue appears white. The right ventricle was then excised and the left ventricle was weighed. Both basal and apical sides were photographed and the area of infarction was determined by planimetry using ImageJ 1.44o software (National Institutes of Health; http://rsb.info.nih.gov/ij/index.html). The amount of infarcted tissue per each four sections was calculated by using the average area of infarction, adjusted for the weight of the entire segment. The total weight of infarcted tissue of all four sections divided by the total amount of left ventricle tissue resulted in the percentage of infarction of the left ventricle for each heart. Analyses were performed by observers blinded to all experimental data and the treatment strategy.

Immunological assays and quantification analyses

For immunohistochemistry, Histofine Simple Stain Mouse MAX PO for rat (Nichirei Corporation) was applied as secondary antibody. Diaminobenzidine (DAB) substrate kit

(Nichirei Corporation) was used for visualization of staining. Finally, sections were counterstained with hematoxylin. No primary antibody was applied on the control negative staining. Five consecutive photographs of the ischemic area were taken from each section and the number of Gr-1-expressing cells (brown color) was quantified by an experimenter blinded to experimental design and data.

For immunofluorescence analysis, Alexa Fluor 488 donkey anti-rabbit IgG antibody (1:2000 dilution; Invitrogen) was applied as secondary antibody. Anti-rabbit IgG was used as primary antibody for negative control staining. DNA was counterstained with Hoechst 33342 (1:10000 dilution; Invitrogen). Photographs from three consecutive fields of the ischemic area of each section were taken and the number of H3cit-positive cells (yellow color) was quantified by an experimenter blinded to experimental design and data.

Double immunofluorescence staining for Gr-1 and H3cit antibodies was performed and fluorescent images were acquired using a Zeiss Axiovert 200 epifluorescence microscope connected to a monochrome camera (AxioCam MRm) using Axiovision software and Vivo Imaging System 3i with Slidebook 5.0 software for 3D image analysis. Nearest neighbors deconvolution analysis was performed for 3D images.

Sonoclot whole blood clotting measurement

Whole blood was drawn from the retro-orbital plexus, mixed with sodium citrate (3.8%) and 280 ml was immediately placed into a preheated cuvette (non-activated clotting test kit, Sienco). Saline or DNase I (45 mg/ml final concentration) was added and then blood was recalcified with 20 μL of calcium chloride (15 mM final concentration). Blood clotting was monitored using the Sonoclot analyzer with the following parameters as a readout: onset of

fibrin clot formation and velocity of fibrin polymerization (clotting rate). The dose of DNase I was chosen to mimic calculated DNase I concentration reached in plasma of a 25 g mouse in in vivo experiments assuming that no degradation occurred.

Platelet aggregation

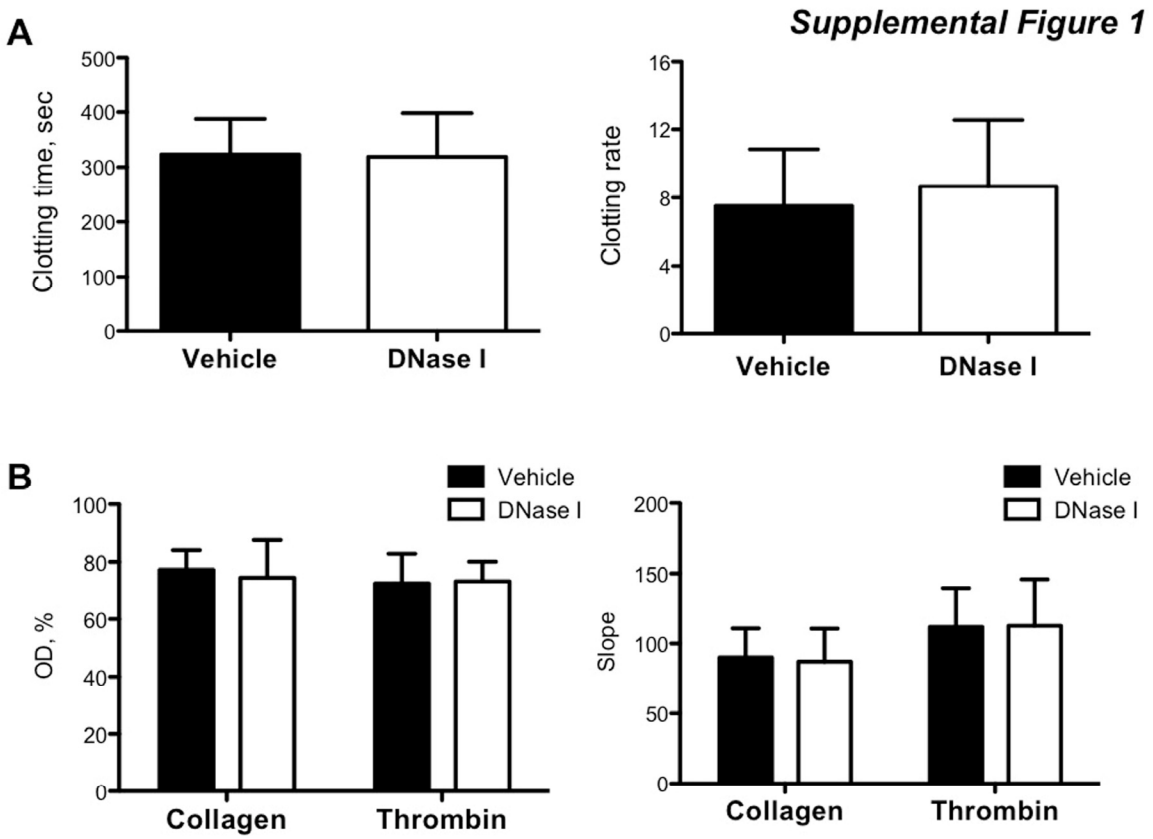
Whole blood was taken from the retro-orbital venous plexus and mixed with heparin (7.5 U/ml). Platelet rich plasma (PRP) was isolated by centrifugation (80 x g, 10 min), incubated with PGI2 (1 μg/ml, 5 min, 37°C) and platelets were pelleted by centrifugation (600 x g, 3.5 min). Then platelets were resuspended in Tyrode's-HEPES buffer (137 mM NaCl, 2 mM KCl, 12 mM NaHCO3, 0.3 mM NaH2PO4, 5.5 mM glucose, 5 mM HEPES, 0.35% bovine serum albumin) and used in experiment within 1 h. Washed platelets were incubated with DNase I (45 mg/ml final concentration, 5 min, 37°C), then thrombin (Sigma, 1 U/mL) or collagen (Nycomed, 5 μg/mL) were added and light transmission was monitored by a Chrono-Log 4-channel optical aggregation system (Chrono-Log) for 10 min. Maximal aggregation amplitude and aggregation rate (slope of the curve) were used as a readout.

Neutrophils isolation and treatment with rhADAMTS13

Peripheral blood neutrophils were isolated from male C57Bl/6J mice as previously described[16] with a purity of at least 90% as assessed by Wright Giemsa staining. 10,000 cells were plated per well in a glass-bottom 96-well plate in RPMI medium containing 10 mM HEPES and allowed to settle for 15 min at 37^{o}C in 5% CO_2. The equivalent of 3460 U/kg rhADAMTS13 (0.0346 U/μl) or an equal volume of vehicle was added to wells for 15 min. Cells were then stimulated using 4 μM ionomycin (Invitrogen) and incubated for 2 h. After

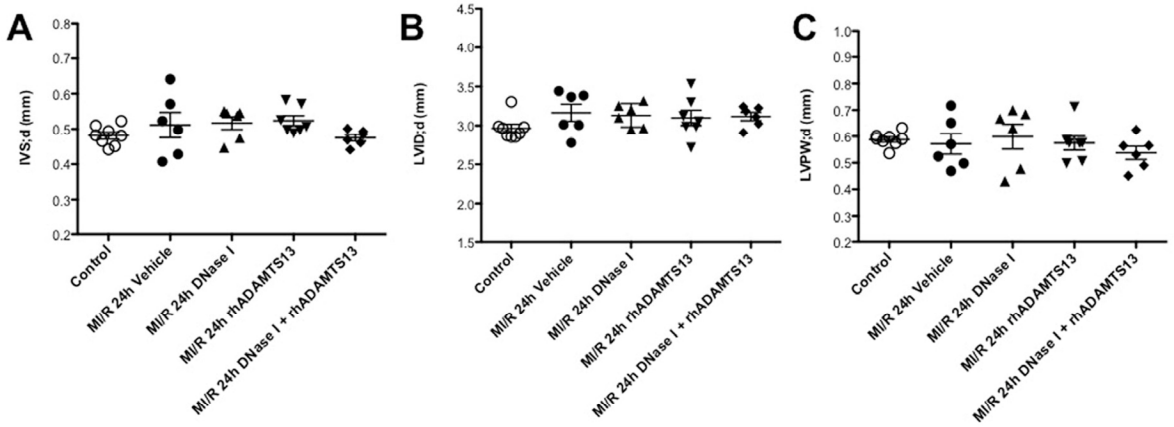
fixation in 2% paraformaldehyde for at least 30 min, cells were permeabilized using 0.1% Triton X-100 for 20 min at 4^{o}C and then incubated in 3% bovine serum albumin for 1 h at 37 ^{o}C and were immunostained using 0.5 μg/ml of anti-citrullinated histone H3 [H3cit] antibody overnight at 4^{o}C. After washing, AlexaFluor 488-conjugated anti-rabbit IgG was added (1.5 μg/ml, Invitrogen) as secondary antibody for 2 h at room temperature. After washing and counterstaining for DNA using Hoechst 33342 (1 μg/ml, Invitrogen), cells were imaged using an Axiovert 200 inverted epifluorescence microscope. Images were acquired with a Zeiss Axiocam MRm monochromatic CCD camera using Axiovision Software (Zeiss). Image analysis was performed using ImageJ software and NETs were manually counted as percentage of spread DNA structures from total cells. Quantification of NETs is presented as the percentage of NETs ± SD calculated from individual fields of view (5 per well taken from triplicate wells).

Supplemental Figure Legends:



Supplemental Figure 1. The effects of DNase I on coagulation and platelet aggregation ex vivo. (A) The addition of DNase I to citrated whole blood, followed by recalcification, did not affect either the onset (clotting time) or the velocity (clotting rate) of coagulation ex vivo, as compared to vehicle-treated blood. (**B)** Similarly, no significant changes were observed with respect to the aggregation amplitude and aggregation rate of platelets treated with DNase I or vehicle, and then stimulated by either thrombin or collagen.

Supplemental Figure 2



Supplemental Figure 2. Left ventricular dimensions in mice treated with vehicle, DNase I, rhADAMTS13 and combination of DNase I and rhADAMTS13. No significant changes were observed in the thickness of (**A**) interventricular septal wall (IVS;d), (**B**) the left ventricular internal dimension (LVID;d) and (**C**) the left ventricular posterior wall (LVPW;d) among all experimental groups during diastole.

Supplemental Figure 3

%NETs±SD (Mouse 1, Mouse 2)	- ionomycin	+ ionomycin
Vehicle	0.48±1.84, 0.89±1.13	24.13±9.54, 22.51±7.68
+rhADAMTS13	0.95±1.65, 1.04±1.92	28.58±9.99, 21.16±6.23

Supplemental Figure 3. The effect of rhADAMTS13 and vehicle (buffer) on NETosis in vitro. Quantification analysis of NETs produced from vehicle- or rhADAMTS13-treated isolated neutrophils is presented as the percentage of NETs ± SD calculated from individual fields of view (5 per well taken from triplicate wells, n=2).