Supplementary Material

Clodronate Liposome Treatment

Clodronate liposomes and control liposomes were purchased from LIPOSOMA B.V. (Amsterdam, The Netherlands). C57BL/6 mice were intravenously injected with 0.1 mL/10 g of body weight of either Clodronate Liposome or Control Liposome approximately 48 hours after inferior vena cava (IVC) ligation and approximately every 48 hours after until harvest at 8 days.

Coagulation Parameters and Platelet Counts

The effect of diphteria toxoid (DTx) treatment on mouse platelet count and coagulation parameters was performed following isolation of whole blood via cardiac puncture in mice treated with either NaCl or DTx for 24 hours. Blood was preserved in ethylenediaminetetraacetic acid (EDTA) (for platelet count) and sodium citrate (for coagulation studies). Platelet count was determined via complete blood count (Element HT5 analyzer). Coagulation parameters prothrombin time (PT) and activated partial thromboplastin time (aPTT) were determined per manufacturer’s instructions utilizing Vet Scan VS pro.

Platelet Function Assays

The effect of DTx treatment on mouse platelets was determined by platelet aggregation and dense granule secretion assays, platelet surface P-selectin expression, and integrin αIIbβ3 activation in response to thrombin activation using isolated platelets from mice treated with vehicle or DTx (4 mice per/group, 3 independent experiments).

Isolation of Mouse Platelets

Blood was drawn via cardiac puncture of 8- to 12-week-old anesthetized mice following 24 hours of treatment with NaCl or DTx as described (4 mice/group) into a syringe containing sodium citrate (3.8%) and mouse platelets were isolated from whole blood by serial centrifugation as described.1 Briefly, mouse whole blood was centrifuged for 10 minutes at 200 g to collect the platelet-rich plasma (PRP). Pooled PRP was treated with acid citrate dextrose (2.5% sodium citrate tribasic, 1.5% citric acid, 2.0% D-glucose) and apyrase (0.02 U/mL) then centrifuged at 2,000 g. Platelets were resuspended to a final concentration of 3.0 x 10⁶ platelets/mL in Tyrode’s buffer (N-2-hydroxyethylpiperazine-N’-2-ethanesulfonic acid 10 mM, sodium bicarbonate 12 mM, sodium chloride 127 mM, potassium chloride 5 mM, monosodium phosphate 0.5 mM, magnesium chloride 1 mM, and glucose 5 mM) as determined by a complete blood cell counter using a Hemavet 950FS (Drew Scientific, Miami Lakes, Florida, United States).

Platelet Aggregation and Dense Granule Secretion Assays

Platelet aggregation was performed under stirring conditions (1,100 revolutions per minute) at 37°C using a Chrono-log Model 700D lumi-aggregometer ( Havertown, Pennsylvania, United States). Isolated platelets were incubated with Chrono-Lume reagent, an adenosine triphosphate (ATP)-sensitive dye, for 1 minute prior to aggregation assays. Platelet activation was then induced by thrombin (0.25 and 0.5 nM). Platelet aggregation and dense granule secretion was measured in real-time by recording the changes in light transmission and fluorescence following the ATP release as surrogate for dense granule secretion.

Flow Cytometry Assay

Isolated mouse platelets were stimulated with various doses of thrombin (0.25, 0.5, and 1 nM) in the presence of fluorescein isothiocyanate (FITC)-conjugated anti-mouse CD62P and PE-conjugated rat anti-mouse integrin αIIbβ3 (Clone JON/A, EMFRET Analytics, Eibelstadt, Germany) antibodies for 10 minutes followed by fixation using 2% paraformaldehyde (PFA) solution. Platelet surface P-selectin expression and integrin αIIbβ3 activation was evaluated by flow cytometry (Accuri C6) respectively as described.2

Polymerase Chain Reaction

Messenger ribonucleic acid (mRNA) expression was analyzed by quantitative real-time reverse transcription polymerase chain reaction (RT-PCR) using Applied Biosystems StepOne-Plus. Briefly, total RNA from mouse IVCs/thrombi were isolated using RNasy Mini Kit (cat#74106, Qiagen) extraction, 0.5 µg of total RNA was utilized for real-time RT-PCR analysis using RT2 quantitative Real-Time PCR System (Qiagen). Primers included interferon (IFN)-γ (PPM03121A-200 Qiagen), urokinase-type plasminogen activator (uPA) (PPM03121A-200 Qiagen), uPA receptor (uPAR) (PPM0305D-200 Qiagen), interleukin (IL)-10 (PPM03017C Qiagen), vascular endothelial growth factor (VEGF)-α (PPM03041F Qiagen), tumor necrosis factor (TNF)-α (PPM03113G, Qiagen), matrix metalloproteinase (MMP)-2 (PPM03642C-200, Qiagen), MMP-9 (PPM03661C-200, Qiagen), and β-actin (PPM02945B-200 Qiagen). The comparative ΔΔCt method was employed for relative mRNA quantification. Gene expression was normalized to the endogenous control, β-actin mRNA, and the amount of target gene mRNA expression in each sample was expressed relative to that control.
Supplementary Fig. S1 Blood coagulation parameters and circulating platelet count in mice treated with sodium chloride (NaCl, control) compared with diphtheria toxin (DTx).

Supplementary Fig. S2 Platelet function assays in control mice (NaCl) compared with those treated with diphtheria toxin (DTx). Platelet activation measured by P-selectin expression (A) and integrin αIIbβ3 activation (B) in response to thrombin shown in the left panel. Platelet aggregation (C) and dense granule secretion (D) shown in the right panel.

Supplementary Fig. S3 Inferior vena cava (IVC) thrombus weight-to-length ratio (g/cm) in mice treated with liposomes (control) and clodronate liposomes (clodronate).
Supplementary Fig. S4 Intrathrombus mediators of thrombus resolution at day 8 in CD11b-DTR mice treated with NaCl (control) or diphtheria toxin (DTx) (CD11b depleted). Gene expression of fibrinolytic mediators urokinase-type plasminogen activator (uPA) (A), uPA receptor (uPAR) (B), and protein levels of uPAR (C) shown in top row. Cytokine interleukin (IL)-10 gene expression (D) and relative thrombus protein levels of fibronectin (E) and laminin (F) shown in second row. Intrathrombus tumor necrosis factor (TNF)-α (G) gene expression and interferon (IFN)-γ gene expression (H) and protein (I) shown in third row. Gene expression of vascular endothelial growth factor (VEGF)-α (J), matrix metalloproteinase (MMP)-2 (K), and MMP-9 (L) shown in fourth row.
Supplementary Fig. S5  Representative western blot gel images of antigens and control proteins represented graphically in Supplementary Fig. S4.

References