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SUPPLEMENTARY MATERIALS

Supplementary Methods

Platelet Isolation. Mouse platelets were prepared from the whole blood obtained by terminal inferior vena cava phlebotomy. Human platelets were prepared from the whole blood drawn from the antecubital vein of healthy volunteers after providing informed consent in accordance with University Hospitals Case Medical Center Institutional Review Board-approved protocol. Platelets were isolated, as described (1). Briefly, platelet-rich plasma was prepared by centrifugation (200g for 15 min for human; 2300g for 20 sec for mouse) of blood collected in 1:6 (v/v) acid citrate dextrose solution [2.5% (w/v) sodium citrate tribasic, 1.5% citric acid monohydrate, and 2.0% D-glucose]. Platelet-rich plasma was centrifuged (1000g for 10 min for human; 2300g for 3 min for mouse) and platelets suspended in Tyrode's buffer (130 mM sodium chloride, 5.0 mM potassium chloride, 1.0 mM magnesium chloride, 0.4 mM sodium phosphate, 5.0 mM D-glucose, 12 mM sodium bicarbonate, and 10 mM HEPES, pH 7.4). Platelet suspensions were adjusted to final density after counting particles >3-fl using a Z1 series Coulter Counter (Beckman Coulter, Fullerton, CA) equipped with a 50 μ M aperture or were measured as part of a complete blood count (CBC) of sodium citrate anti-coagulated mouse blood on a HEMAVET 950FS system in the Case Comprehensive Cancer Center, Case Western Reserve University School of Medicine.

Activated partial thromboplastin time. The activated partial thromboplastin time (aPTT) was performed using Amelung KC4 coagulation analyzer (Sigma, St. Louis, MO), as described previously (2). Briefly, 100 μ L of sodium citrate-anticoagulated plasma was incubated with 50 μ L of a PTT reagent (Siemens, Washington DC) at 37°C

for 5 minutes. Fifty μL of 30 mM calcium chloride was then added and the time to clot formation was recorded.

Thrombin generation. Tissue factor-induced thrombin generation time (TGT) was performed, as previously described (3). Briefly, a 1:2 dilution of mouse plasma in 25 mM HEPES, 175 mM NaCl_2 , containing 5 mg/mL bovine serum albumin, pH 7.7, was incubated with ~ 3 pM tissue factor (3 μL of 1:60 dilution of stock Innovin, Siemens) and 0.42 mM Z-Gly-Gly-Arg-AMC. The reaction was initiated with the injection of 0.16 M calcium chloride, final concentration 16 mM. Substrate hydrolysis was measured on a fluorescent plate reader (NOVOstar, BMG Labtech). The TGT data are expressed as an arbitrary rate of fluorescent accumulation as determined by the second derivative of the raw fluorescent values. The lag time, peak height, and total area under the curve were calculated using Prism software (Graphpad, San Diego, CA).

Photochemical carotid artery thrombosis. Male WT and *Mrp14*^{-/-} mice (age 7-9 weeks) were anesthetized by intraperitoneal injection with sodium pentobarbital (62.5mg/kg) and placed in the supine position on a dissecting microscope (Nikon SMZ-2T, Mager Scientific, Inc., Dexter, MI). A midline surgical incision was made to expose the right common carotid artery and a Doppler flow probe (MC 0.5PSL Nanoprobe, Model 0.5 VB, Transonic Systems, Ithaca, NY) was placed under the vessel. The probe was connected to a flowmeter (Transonic Systems Model TS420) and was interpreted with a computerized data acquisition program (Windaq, DATAQ Instruments, Arkron, OH). Rose Bengal at a concentration of 10 mg/mL in phosphate-buffered saline was then injected into the tail vein to administer a dose of 50 mg/kg (4, 5). The mid portion of the common carotid artery was then illuminated with a 1.5-mW green light laser

source (540 nm; Melles Griot, Carlsbad, CA) 5 cm from the artery. Blood flow was monitored continuously from the onset of injury. The time to occlusion, determined only after the vessel remained closed with a cessation of blood flow for 10 min, was recorded. In a separate group of animals, purified, recombinant human MRP-8, MRP-14, or MRP-8/14 (0.08 µg/g mouse or 0.4 µg/g mouse in 100 µL) was also infused into *Mrp14*^{-/-} mice via tail vein injection to determine the effect of extracellular MRP-8/14 on thrombosis.

Laser-induced injury to the cremaster microcirculation using intravital microscopy. Thrombus formation in vivo after laser-induced injury to the arteriolar wall in the cremaster microcirculation of WT (**Video 1**) was compared with that of *Mrp14*^{-/-} (**Video 2**) mice using intravital microscopy (VIVO, 3I Inc.) was performed as described previously (6). Platelets were labeled in vivo using a FITC-conjugated rat anti-mouse CD41 antibody.

Platelet α-Granule Release and GPIIb/IIIa Activation. Mouse platelets (5.0 X 10⁵ in 20 µl of Tyrode's buffer containing 2.0 mM calcium chloride) were stimulated for 10 minutes at room temperature with agonist diluted in 5 µl Tyrode's buffer. The Wug.E9 and JON/A antibodies (5 µl each) were added to detect the expression of P-selectin (CD62P) and activated GPIIb/IIIa, respectively. After 20 minutes, platelets were fixed and diluted for FACS analysis by addition of 365 µl of 1% formaldehyde. Platelets were distinguished on the basis of side- and forward-light scatter, and the mean fluorescence intensity (MFI) of 10,000 platelets was measured per condition using FACSDiva LSR II (Becton Dickinson) and analyzed using Winlist.

Platelet aggregation and secretion. *Mrp14*^{-/-} and WT platelet-rich plasma was washed by centrifugation (7). Washed platelets were labeled with [¹⁴C] 5-hydroxytryptamine for 30 min at 37°C for dense granule secretion studies, as described previously (8). Briefly, at the conclusion of the incubation, the samples were treated with imipramine (2 μM). Washed platelets were incubated with collagen (2.5 μg/mL, BioData Corporation) or α-thrombin (2.5 nM, Hemetech) for aggregation studies in a Chronolog Model 440-VS dual channel aggregometer. Samples of activated platelets (0.2 mL) were removed and placed in a microcentrifuge tube containing 0.05 mL of 135 μM formaldehyde, 5 mM EDTA solution. After 5 min centrifugation at 12,000 g, the supernatants were collected for the degree of loss of the [¹⁴C] 5-hydroxytryptamine from the labeled platelets.

Platelet adhesion. Platelet adhesion assays were performed as previously described (7). Briefly, 96 well plates were coated with 100 μL of GFOGER peptide (10 μg/mL) or vWF (30 μg/mL) overnight at 4°C. Washed platelets (1 x 10⁸ /mL) in the presence of 1 mM Ca²⁺ on GFOGER or botrocetin (1 mg/mL) on vWF were incubated on the coated plates for 1 h at 37°C. After washing with PBS, adherent platelets were quantified based on their alkaline phosphatase activity (9).

Platelet spreading. Platelet spreading assays were performed as previously reported (7). Briefly, MatTek culture dish was coated with GFOGER (10 μg/mL) or vWF (30 μg/mL) overnight at 4°C. After blocking with 3 mg/mL BSA, washed platelets (3 x 10⁷ /mL) were allowed to spread on GFOGER or vWF for 30 min at 37°C in the presence of Ca²⁺ (1 mM) or botrocetin (1 μg/ml), respectively. Adherent platelets were fixed with 3% paraformaldehyde, permeabilized with 0.1% Triton X-100, and stained

with Alexa-Fluor 568-conjugated phalloidin (25 µg/ml) for filamentous actin. Surface area was calculated in pixels using ImageJ software (NIH).

Immunofluorescence microscopy. Coverslips were coated in 24-well plates with 0.5 ml fibrinogen (1mg/ml) for 24 h at 4°C and blocked with 5% bovine serum albumin (BSA) for 15 min at room temperature. Platelets (0.5 ml) adjusted to 1×10^7 platelets/ml in Medium 199 were adhered to the coverslips at 37°C. After 30 min, an additional 0.5 ml of Medium 199 containing 2 nM α -thrombin (i.e., final 1 nM α -thrombin), and platelets were cultured 6 h at 37°C. Platelets were fixed for 10 min by the addition of 1 ml of 8% formaldehyde (i.e., final 4% formaldehyde), permeabilized for 20 min with 0.5% Triton X-100, and blocked with 1% BSA and 40 µg/ml non-immune human IgG. Primary and non-immune species-specific IgG control antibodies (2 µg/ml) were diluted in blocking solution and applied for at least 1 h at room temperature. The mouse monoclonal antibody to MRP-8 and MRP-14 (5.5, Abcam) was used for human platelets. Alexa Fluor 488-conjugated, species- and isotype-specific secondary antibodies (10 µg/ml) diluted in 1% BSA were applied in the dark for 1 h at room temperature. In some experiments, platelet GPIX was counterstained after extensive washing with Alexa Fluor 647-conjugated mouse anti-human CD42a. Coverslips were mounted on standard glass slides using Vecta shield mounting medium. Images were captured using a Leica microscope (DM2500) and captured with a RETIGA EXi Fast 1394 camera (QIMAGING, Surrey, BC, Canada).

Immunoblotting. Protein samples were denatured by boiling in sodium dodecyl sulfate (SDS) sample buffer, run on 4% to 20% reducing SDS-polyacrylamide gel

electrophoresis (PAGE), and transferred to nitrocellulose. The membrane was then blotted with indicated antibody, and the bands visualized with horseradish peroxidase-conjugated secondary antibody followed by the enhanced chemiluminescence Western blotting detection system (PerkinElmer Life and Analytical Sciences, Waltham, MA). Anti-tubulin mouse antibody was used as an internal control for protein loading.

ELISA Assay. Human platelets (4.0×10^8 platelets/mL) suspended in Tyrode's buffer and stimulated with 1.0 nM α -thrombin for 2 minutes. Following centrifugation at 10,000g for 10 min at 4°C, the supernatant was collected and stored at -20°C. The concentration of MRP-8/14 in the supernatant was determined by ELISA assay (Buhlmann Laboratories, Schonenbuch, Switzerland) according to the manufacturer's protocol.

CD36 platelet signaling. Gel-filtered human platelets (2×10^8 /ml) containing 2 mM CaCl_2 and 1 mM MgCl_2 were incubated with 50 $\mu\text{g/mL}$ oxLDL, 1 $\mu\text{g/mL}$ MRP-14, or the combination for 10 min. Platelets were lysed with buffer containing protease and phosphatase inhibitors and lysates were then analyzed by immunoblot with anti-phospho-VAV (source) and phospho-JNK (source) antibodies. The membranes were then stripped and reprobed with antibodies to the total VAV (source) or JNK (source) protein and actin.

Plate Binding Assay. High-protein binding plate (Nunc, Thermo Scientific) was coated with purified CD36 (Abcam) or BSA (10 $\mu\text{g/mL}$) overnight at 4°C. After blocking with 10% BSA at RT for 2 hours, purified human MRP-14 protein (0-2.5 $\mu\text{g/mL}$) was added and incubated for 2 hours at RT. After washing, plate was incubated with anti-human MRP-14 antibody for one hour followed by washing and incubation with HRP-

conjugated secondary antibody that was detected with TMB substrate (Thermo Scientific) at 450nm.

Histology and immunohistochemistry of tissue samples. At various time points following carotid artery photochemical injury, anesthesia was administered, the chest cavity opened, and the animals sacrificed by right atrial exsanguination. A 22-gauge butterfly catheter was inserted into the left ventricle for *in situ* pressure perfusion at 100 mm Hg with 0.9% saline for 1 min followed by fixation with 4% paraformaldehyde in 0.1 M phosphate buffer, pH 7.3, for 10 min. The carotid arteries were excised and immersed in buffered paraformaldehyde, embedded in paraffin, sectioned (5 μ m), and stained with hematoxylin and eosin or Masson's trichrome. For immunohistochemistry, standard avidin-biotin procedures for mouse MRP-14 (R&D Systems) and mouse platelets (anti-GPIIb, BD Biosciences) were used. For each antibody, controls included species-specific non-immune IgG as well as omission of the primary antibody. A histologist blinded to genotype analyzed staining using a microscope equipped with a charge-coupled device camera (Zeiss AxioCam MRc5, Oberkochen, Germany) interfaced to a computer.

Human coronary artery thrombus was obtained using a thrombectomy catheter (Medtronic Export catheter) at the time of percutaneous coronary intervention for ST-segment elevation myocardial infarction prior to balloon angioplasty and stent deployment. Thrombus fragments were immersed in formalin, embedded in paraffin, sectioned (5 μ m), and stained by immunofluorescence microscopy using the identical antibodies described for human platelets above.

Supplementary Figure Legends

Supplementary Figure 1: Hematologic and coagulation assays in WT and *Mrp14*^{-/-} mice. **A**, Platelet count. **B**, Activated partial thromboplastin time, aPTT (sec). **C**, Tissue factor-induced thrombin generation. For each parameter data represent mean $\pm$ SD, n=3-5 per group.

Supplementary Figure 2: Platelet expression of MRP-8/14. MRP-8/14 expression was investigated using 2-color flow cytometry on gel-filtered human platelets that were permeabilized, fixed, and then stained with platelet (anti-GPIIb/IIIa), leukocyte (anti-CD45), and anti-MRP-8/14 antibodies.

Supplementary Figure 3: Platelet activation in WT and *Mrp14*^{-/-} platelets. Flow cytometric analysis of P-selectin expression (**A**, **C**, **E**) and GPIIb/IIIa activation using the JON/A antibody (**B**, **D**, **F**) was assessed following stimulation of washed platelets from WT (black bars) and *Mrp14*^{-/-} (white bars) mice with α -thrombin (0-3 nM), arachidonic acid (0-800 μ M), or ionomycin (0-400 nM) (n=3-5 per group).

Supplementary Figure 4: Platelet functions in WT and *Mrp14*^{-/-} platelets. **A**, Platelet expression of GPIb α , GPVI, and β_1 integrin by flow cytometry (mean MFI $\pm$ SD). **B**, Platelet adhesion to the collagen peptide GFOGER. Platelet adhesion to (**C**) and spreading on vWF (**D**). Collagen (2.5 mg/mL) - and α -thrombin (2.5 nM)-stimulated platelet dense granule secretion (**E**) and aggregation (**F**). ADP-induced fibrinogen

binding in WT and *Mrp14*^{-/-} platelets (**G**) or WT platelets incubated with and without purified 1 µg/mL MRP-8/14 (**H**).

Supplementary Figure 5: Platelet expression of MRP-8/14 in human coronary artery thrombi. Coronary artery thrombi were obtained from 3 additional patients presenting to the cardiac catheterization laboratory with acute ST-segment elevation myocardial infarction. For each patient panel, immunofluorescence staining of coronary artery thrombus with anti-MRP-8/14 and anti-platelet GPIIb antibodies, and DAPI for nuclei. Co-localization of MRP-8/14 and platelets are depicted in the overlay.

Supplementary Videos: Thrombus formation in vivo after laser-induced injury to the arteriolar wall in the cremaster microcirculation of WT mice (**Video 1**) was compared to that of *Mrp14*^{-/-} mice (**Video 2**) mice using intravital microscopy. Platelets were labeled in vivo using an FITC-conjugated rat anti-mouse CD41 antibody.

Supplementary References

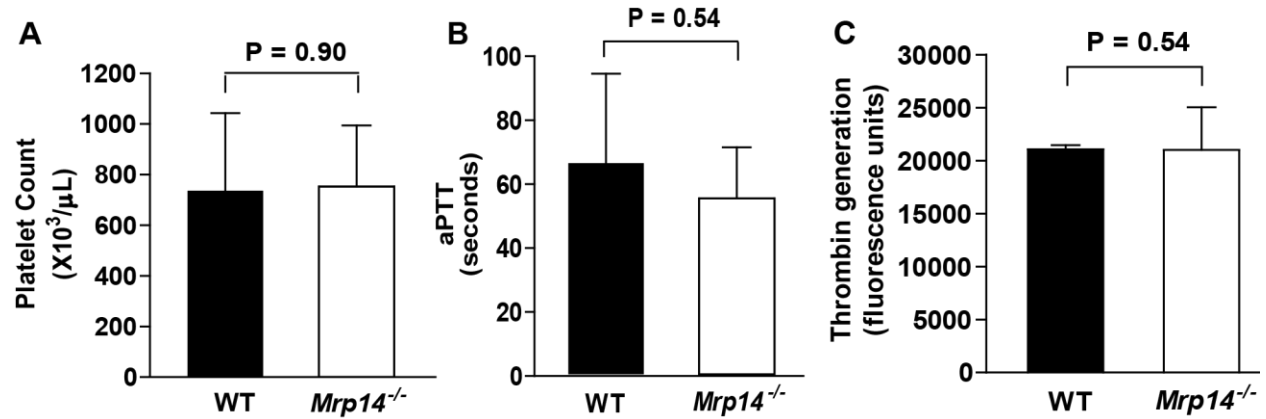
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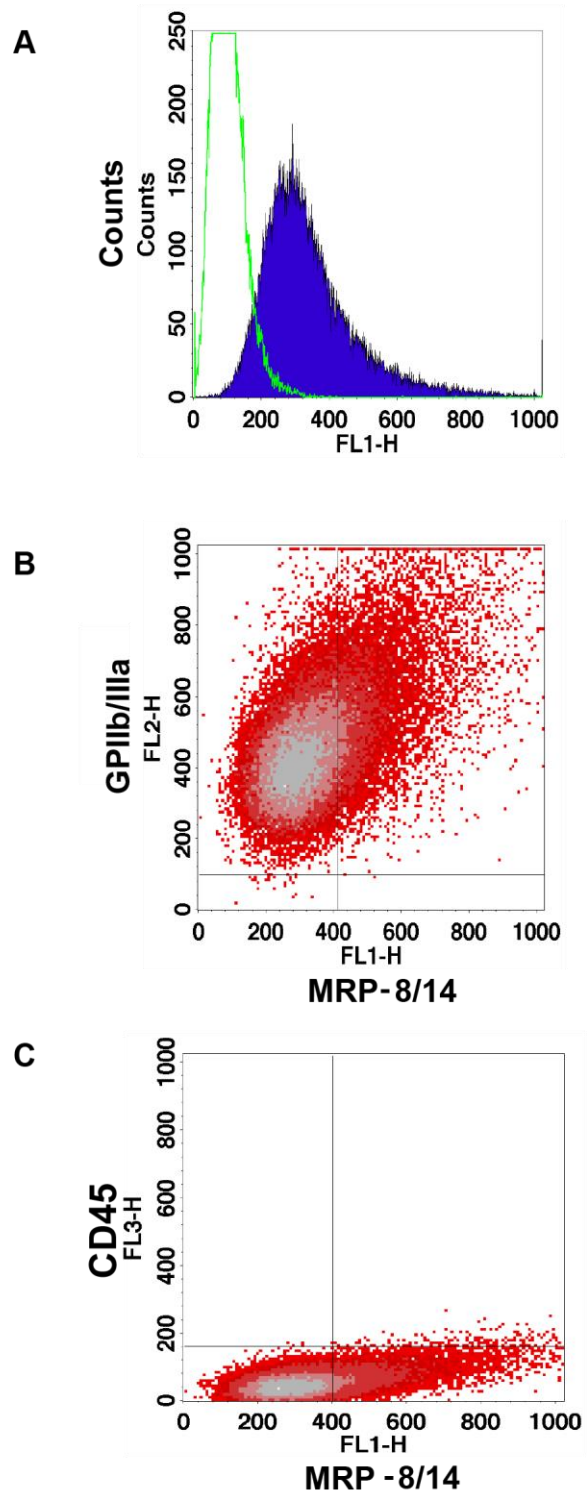
Supplementary Figures and Figure Legends

Supplementary Figure 1



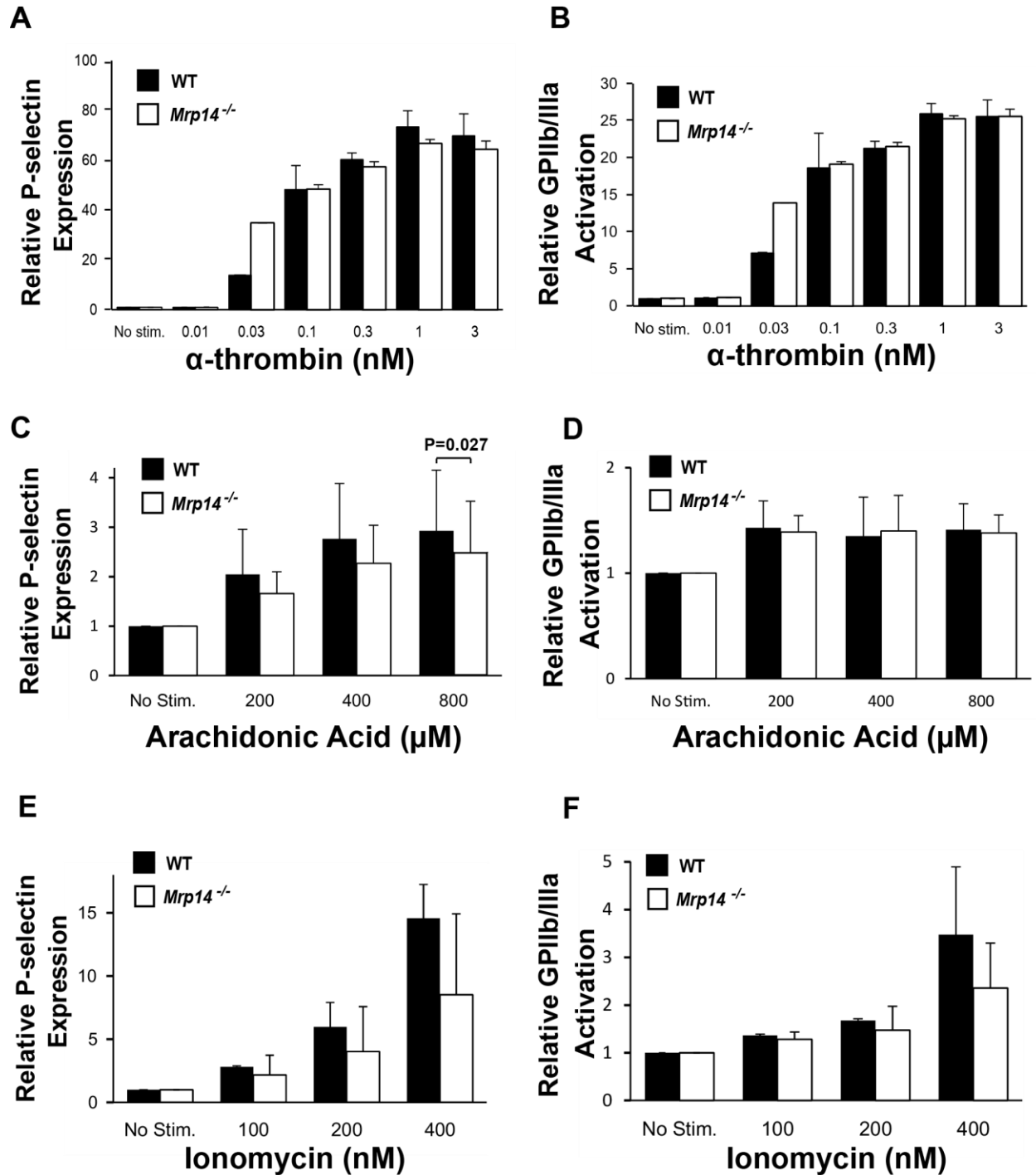
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Supplementary Figure 2



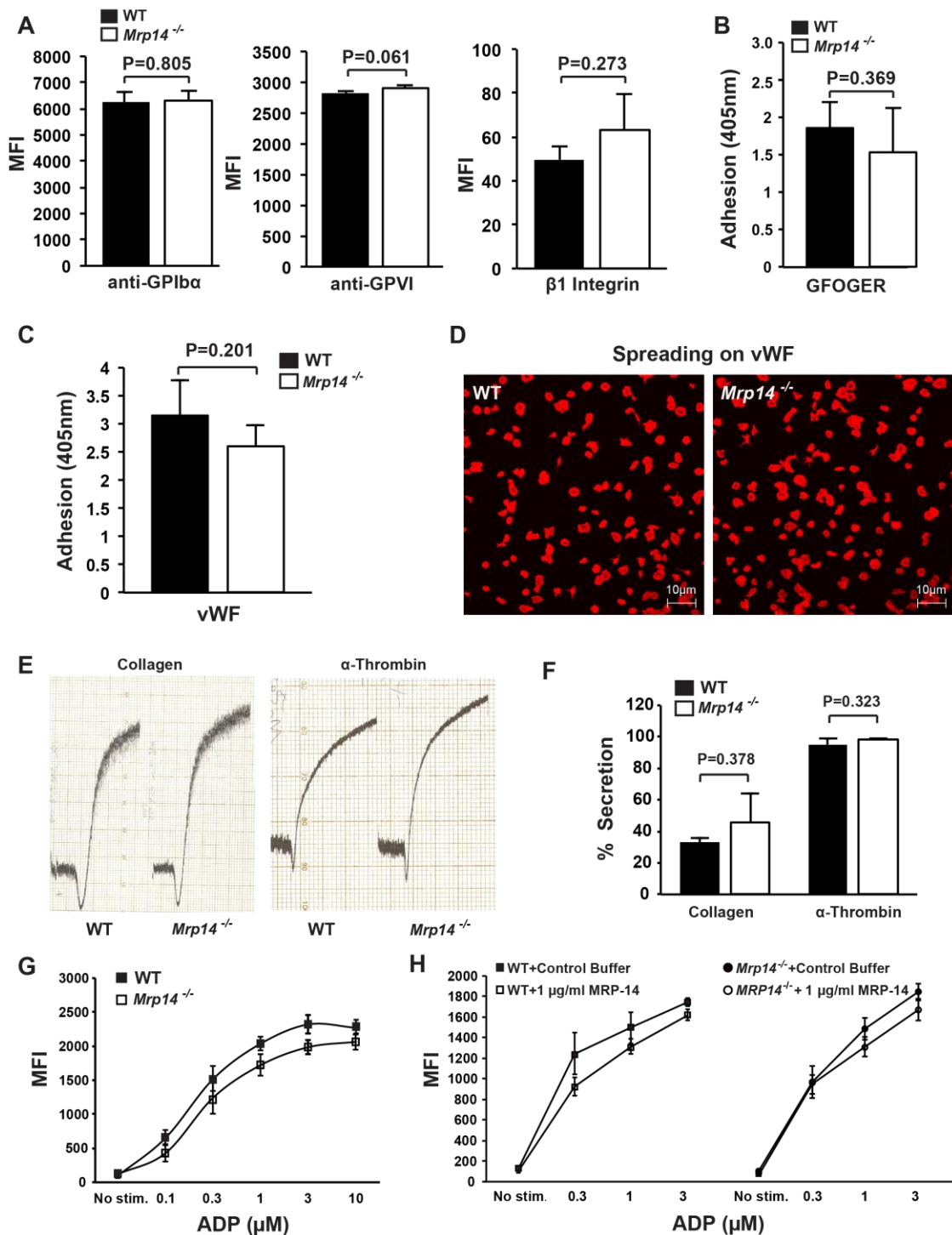
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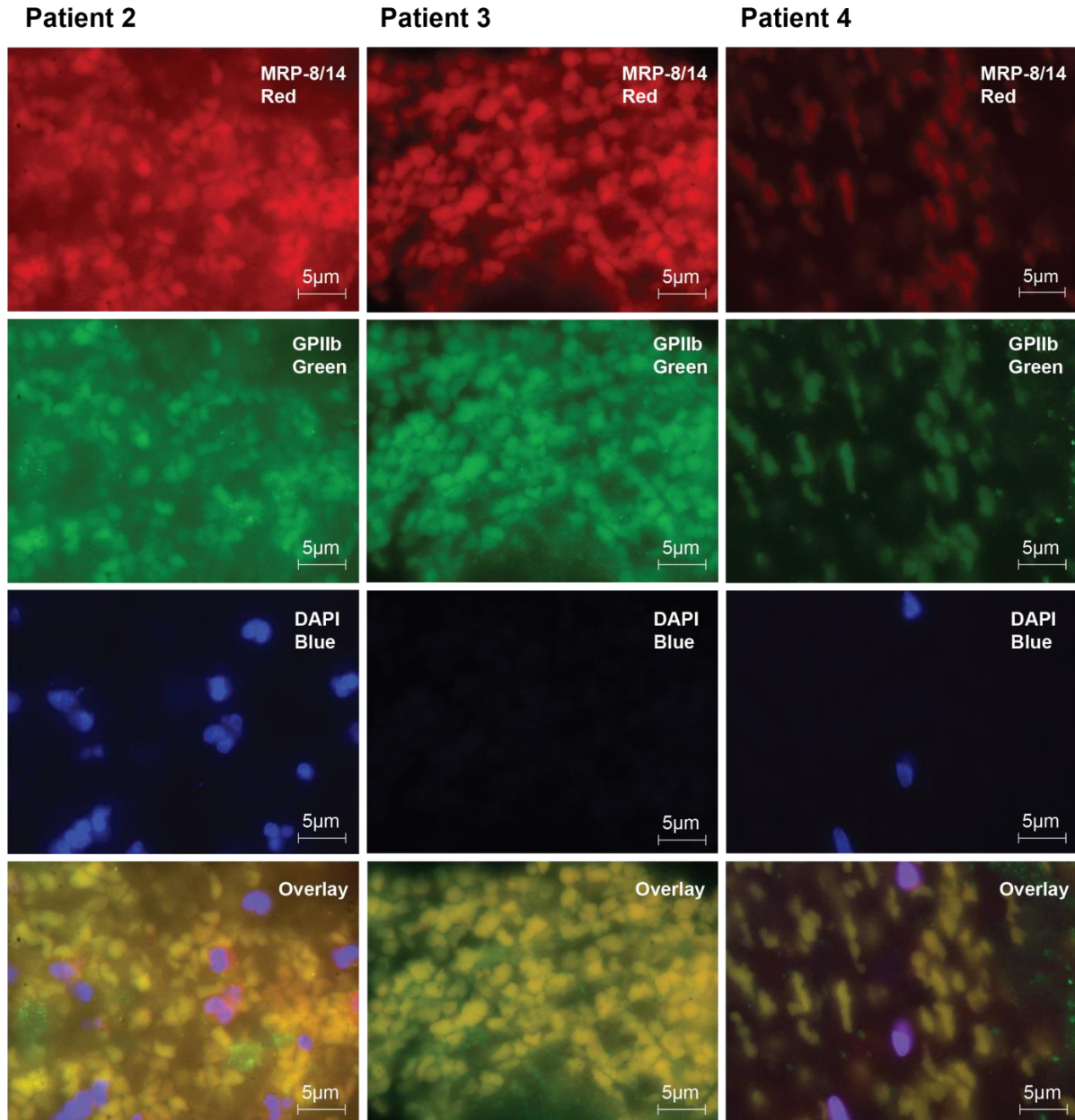
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Supplementary Figure 5



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