Material and Methods

Materials
All reagents, analytical grade, were obtained from Thermo Fisher Scientific (Waltham, MA) unless stated otherwise. Bovine serum albumin (BSA) was obtained from Sigma-Aldrich (St. Louis, MO). Clopidogrel was provided as 75 mg Plavix® tablets from Bristol-Myers Squibb/Sanofi Pharmaceutical partnership (New York, NY). Hank's balanced salt solution (HBSS) and PBS were purchased from Mediatech Inc. (Manassas, VA). BD FACS™ lysing solution, PE-conjugated rat anti-mouse CD41 (clone MWReg30), FITC-conjugated rat anti-mouse CD41 (clone MWReg30) and FITC-conjugated rat anti-mouse P-selectin (clone RB40.34) and were purchased from BD Bioscience (San Jose, CA). PE-conjugated rat anti-mouse CD11b (clone M1/70) and FITC-conjugated rat anti-mouse CD11b (clone M1/70.15) and the isotype control IgG2b were purchased from Invitrogen (Grand Island, NY). Isotype controls, $IgG_1\kappa$ and $IgG_1\lambda$, were obtained from BD Bioscience (San Jose, CA).

Animals and treatments
Animal procedures and handling adhered to National Institutes of Health standards and were approved by the Institutional Animal Care and Use Committee at Temple University School of Medicine (Philadelphia, PA, USA). Male wild-type and $P2Y_{12}$ deficient pathogen-free C57BL/6 mice (weight, 25-30 g) were obtained from Schering-Plough Corporation (Kenilworth, NJ)[1-4]. $P2Y_1$ deficient pathogen-free C57BL/6 male mice were generated by subcontract with Lexicon Genetics Inc. (Woodlands, TX) through knockout constructs as described previously [5-7]. Animals were housed in a climate-controlled facility and given free access to food and water.

The cecal ligation and double puncture (CLP) were performed on isoflurane-anesthetized animals as described previously [5-7]. Sham control animals underwent a laparotomy without ligation or double puncture. Experiments were performed in $P2Y_{12}$, $P2Y_1$ KO, and wild-type mice that were randomly assigned to one of four groups for wild-type or KO: wild-type and KO sham control group (6 animals per group); wild-type and KO undergoing CLP (CLP group, 6 animals per group).

Clopidogrel was orally administrated to wild-type and $P2Y_{12}$ KO (6 animals per group) with a loading dose of 30 mg/kg the day before surgery and a maintenance dose of 10 mg/kg two hours before surgery. Sham mice received the same doses of clopidogrel. After the procedure but prior to emergence, sham and CLP mice were fluid-resuscitated (1 ml/mouse sterile saline, subcutaneously).
At 24 hours post-surgery, mice were anesthetized and blood samples were collected by cardiac puncture (10:1 in 3.8% sodium citrate) for hematology studies (Hemavet® Multispecies Hematology System, Drew Scientific, Inc. Oxford, CT). All mice were euthanized by cardiac puncture and exsanguination. Lungs were collected and fixed or frozen immediately in liquid nitrogen.

Platelet-leukocyte aggregate formation and P-selectin expression in whole blood
Murine blood samples were incubated with either FITC-conjugated anti-mouse CD11b and PE-conjugated anti-mouse CD41 or with FITC-conjugated anti-mouse P-selectin for

20 minutes at 25 °C. The reaction was stopped by adding BD FACS™ lysing solution (1:10 in PBS). Samples were kept at 4°C prior to analysis. Flow cytometry was performed using a FACSCalibur analyzer and data were analyzed with FlowJo software. Platelet and neutrophil aggregates were discriminated by forward and side light scatter and identified by their positive staining for PE-CD41 or FITC-CD11b, respectively. Events double positive for PE and FITC identified platelet–neutrophil aggregates and were recorded as a percentage of a total of 10,000 gated neutrophils.

Lung histopathology

Lung sections were paraffin-embedded, cut into 5 μm sections, and stained with hematoxylin and eosin (H&E). Morphological analysis of random fields (n = 6) from each section (n = 3 sections/animal) was performed by a second independent, blinded observer using previously described methods [8]. Acute lung injury (ALI) was scored based on four parameters: a) alveolar capillary congestion, b) hemorrhage, c) infiltration or aggregation of neutrophils in the airspace or the vessel wall, and d) thickness of the alveolar wall. Each parameter was graded from 0–4 based on the damage present (0, no or little damage; 1, less than 25% damage; 2, 25–50% damage; 3, 50–75% damage; and 4, more than 75% damage). The degree of ALI was assessed by sum of scores of four parameters.

Myeloperoxidase peroxidation (MPO)

Lungs were also homogenized and sonicated. After centrifugation (10,000 for 10 minutes at 4°C), MPO levels were detected using a MPO assay kit (Cayman, USA).

Fluorescence microscopy

Murine lung tissue sections were deparaffinized, and antigen retrieval was achieved by microwaving the tissue slides for 4 min in citrate buffer pH 6.0. The slides were washed and incubated with FITC-conjugated rat anti-mouse CD41 [9] or with FITC-conjugated rat anti-mouse CD41 and PE-conjugated rat anti-mouse CD11b. The slides were mounted in Vectashield with DAPI (DNA stain) and imaged using fluorescence microscopy.

Cytokine profiles

Plasma aliquots from each animal were obtained by blood centrifugation (2,000g for 10 minutes) and utilized for detection of TNF-α, IL-6, IL-10 and MIP-1b plasma levels by the Luminex® System (Allied Biotech, Inc. Ijamsville, MD) [10].

Statistical analysis

Differences among groups were statistically analyzed using one-way ANOVA; Bonferroni's Multiple Comparison Test was used as post-test analyses. $P < 0.05$ was considered to be significant. Data are reported as mean ± standard error of the mean (S.E.M.) for each group.

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