Supplemental Information

Supplemental Data

Figure S1, Related to Figure 2

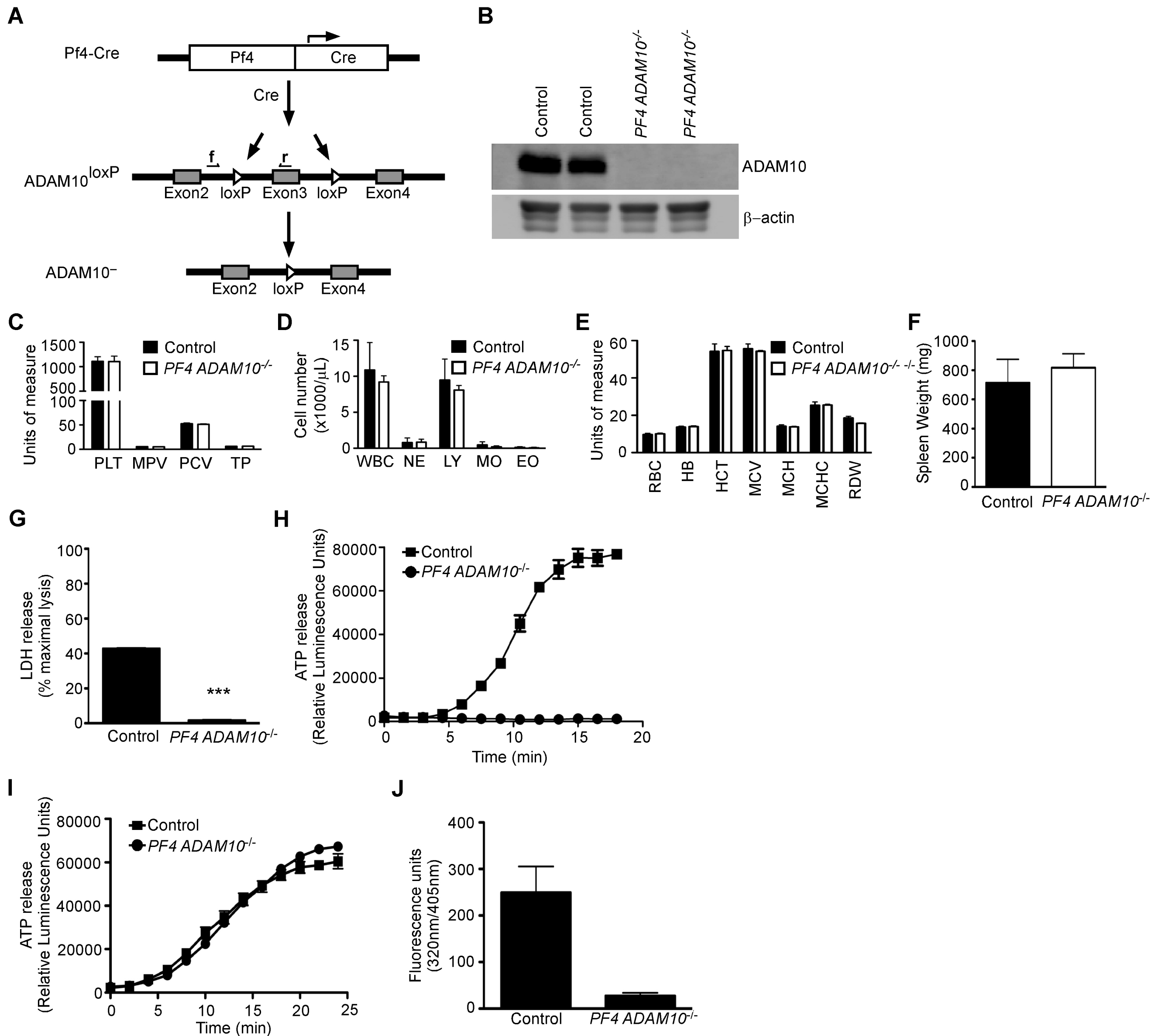

Figure S1. Characterization of platelet-specific ADAM10 knockout mice. (A) Genetic strategy to generate mice harboring a conditional deletion of *ADAM10* in platelets using a Platelet Factor 4 (Pf4) promoter-driven Cre recombinase. (B) Western blot analysis of platelet lysates ($2x10^6$) prepared from Control or *PF4 ADAM10$^{-/-}$* mice to examine ADAM10 expression (upper panel) or β-actin control (lower panel). (C) Complete blood counts from Control and *PF4 ADAM10$^{-/-}$* mice to analyze platelets (PLT,

K/μL), mean platelet volume (MPV, fL), packed cell volume (PCV, %), and total protein (TP, g/dL). (D) Peripheral blood leukocytes from Control and *PF4 ADAM10*$^{-/-}$ mice quantifying white blood cell count (WBC), neutrophils (NE), lymphocytes (L), monocytes (M), and eosinophils (E). (E) Red blood cell parameters in Control and *PF4 ADAM10*$^{-/-}$ mice quantifying red blood cell count (RBC, K/μL), hemoglobin (HB, g/dL), hematocrit (HCT, %), mean corpuscular volume (MCV, fL), mean corpuscular hemoglobin (MCH, Pg), mean corpuscular hemoglobin concentration (MCHC, g/dL), and red blood cell distribution width (RDW, %). (F) Spleen weight from Control and *PF4 ADAM10*$^{-/-}$ mice. Displayed results in (B) are one representative of at least 2 independent experiments, while results in (C-F) are representative of 5 mice per group. (G) Hla sensitivity of Control and *PF4 ADAM10*$^{-/-}$ platelets ($5x10^5$), quantified by LDH release assay where percent maximal lysis is calculated relative to detergent-lysed platelets. (H-I) Platelet activation was quantified by ATP release from the platelets isolated from Control or *PF4 ADAM10*$^{-/-}$ mice. Platelet-rich plasma (PRP) was exposed to subcytolytic Hla (H) or collagen (I) and platelet activation was quantified by ATP release assay (relative light units, RLU). For all time points ≥4 min, P≤0.05. (J) Toxin-induced metalloprotease activity in Control or *PF4 ADAM10*$^{-/-}$ platelets stimulated with Hla in the presence of a fluorogenic ADAM10 substrate for 30 min, ***P*≤0.01. Data are represented as mean ±SD.

Figure S2, Related to Figure 2

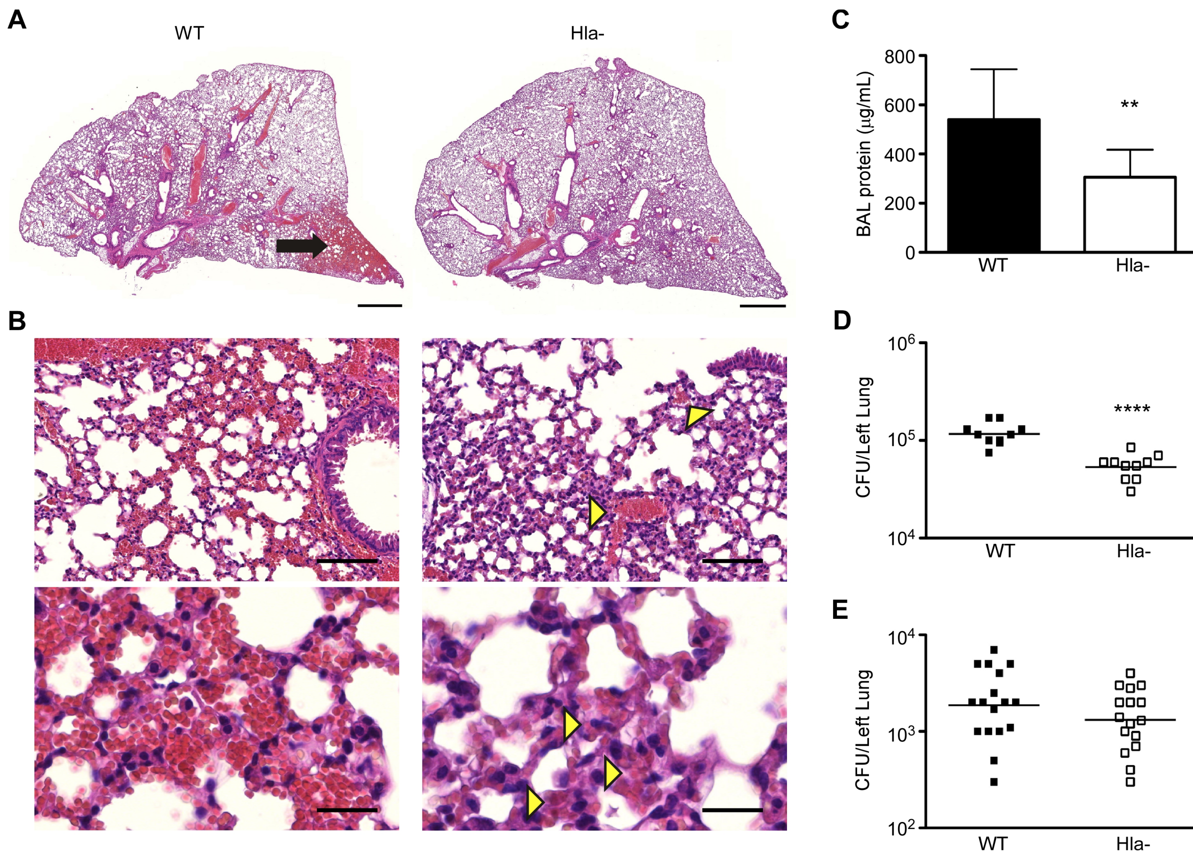

Figure S2. Hla contributes to sepsis-associated lung injury and bacterial dissemination. (A) Hematoxylin and eosin-stained whole lung sections harvested from wild-type C57Bl/6J mice 4 hours after intravenous infection with 5×10^7 wild-type (WT) or Hla-deficient (Hla-) *S. aureus* USA300 (black arrow, pulmonary hemorrhage). Scale bars denote 1000 μm, and images presented are representative of >3 mice per condition harvested from 2 independent experiments. (B) Higher magnification images of lung sections as in (A), where yellow arrows demonstrate red cells retained in the vasculature of Hla-infected mice. Scale bars denote 100 μm (upper panels) and 20 μm (lower panels). (C) Protein content in bronchoalveolar lavage (BAL) fluid harvested from mice (n = 9) infected with WT or Hla- *S. aureus* USA300 as in (A), **P≤0.01. Data are represented as mean ±SD. (D) *S. aureus* colony-forming unit (CFU) recovery from lung tissue 4 hours after intravenous infection of mice with WT (n = 9) or Hla deficient (n = 10) *S. aureus* USA300 as in (A), ****P≤0.0001. (E) CFU recovery from blood of mice (n = 15) infected as in (D), harvested 4 hours after infection.

Figure S3, Related to Figure 3

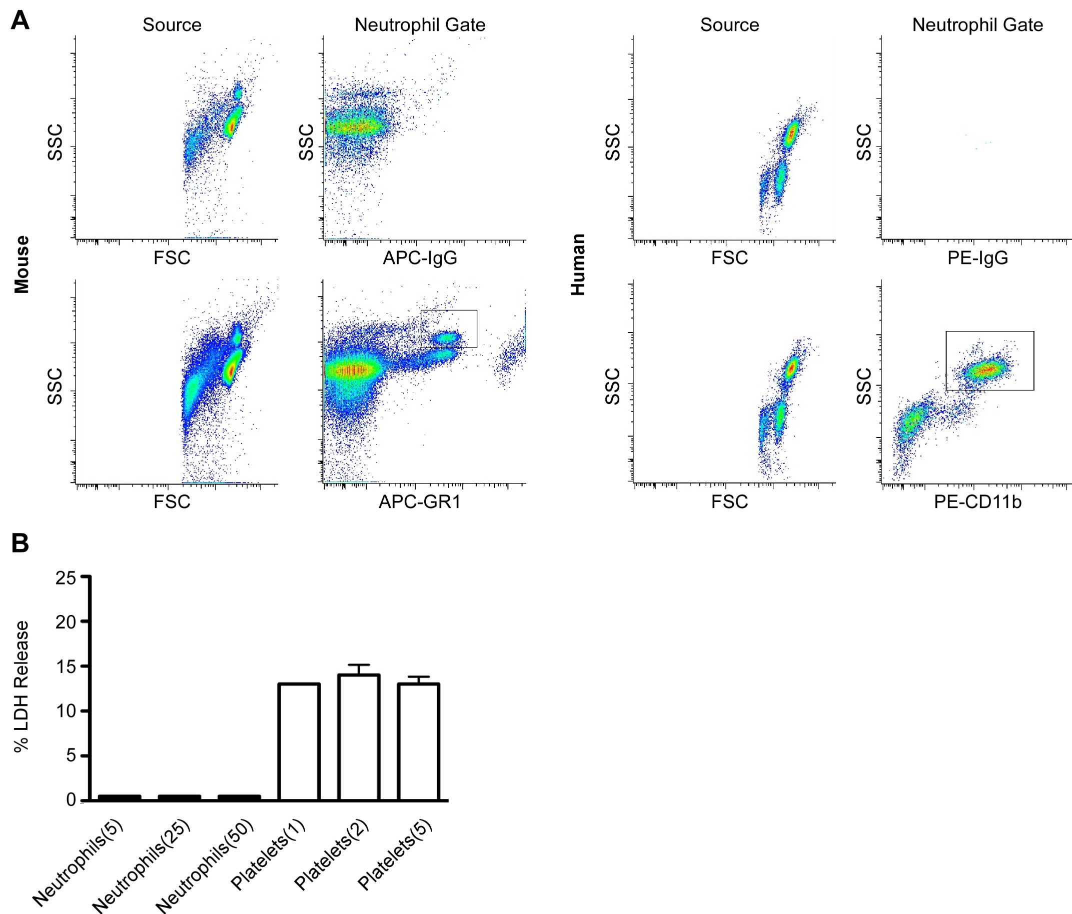

Figure S3. Identification strategy for neutrophil populations and comparative analysis of human neutrophils and platelet sensitivity to Hla. (A) Gating strategy of neutrophils in whole blood for identification of platelet-neutrophil aggregates. Gating occurs around the neutrophil population (top right cell population) in the FSC/SSC plot. Gated cell population examined for neutrophil-specific GR1 (mouse) or CD11b (human) staining. (B) Hla sensitivity of human neutrophils ($1.5x10^4$) and platelets ($5x10^5$), quantified by LDH release assay where percent maximal lysis is calculated relative to detergent-lysed cells.

Figure S4, related to Figure 4

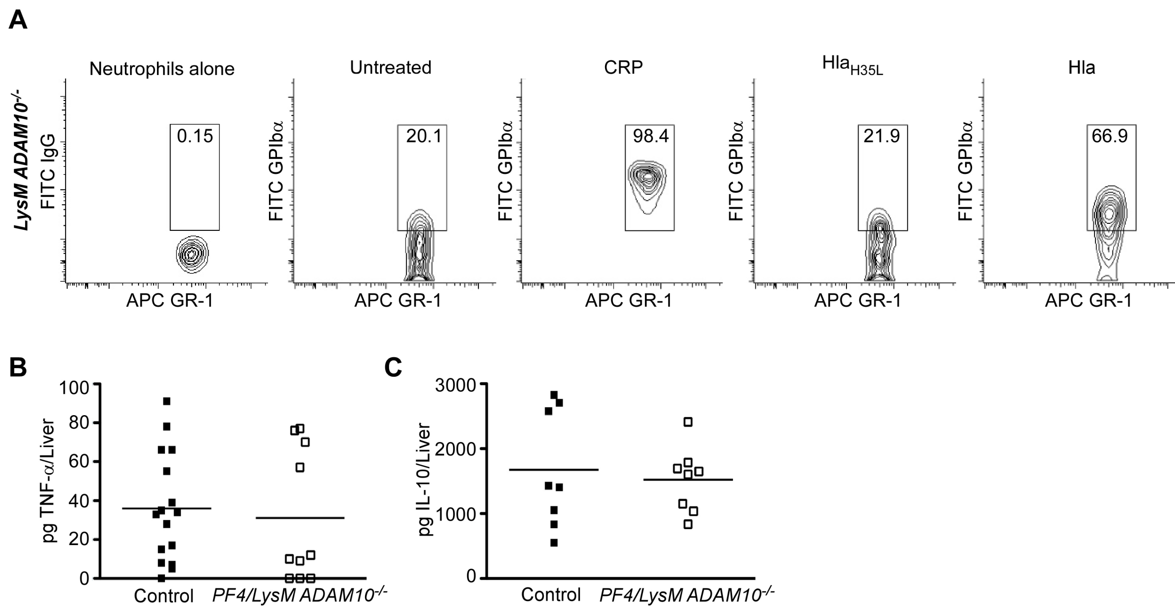

Figure S4. Platelet-neutrophil aggregate formation and liver TNF-α and IL-10 expression are not altered by selective ADAM10 myeloid lineage or platelet-myeloid lineage knockout, respectively. (A) Platelet-neutrophil aggregate formation ($GR1^{+}/GPIb\alpha^{+}$) in whole blood isolated from Control or *LysM ADAM10*$^{-/-}$ mice was quantified at baseline (untreated) or following treatment with collagen-related peptide (CRP), Hla$_{H35L}$, or Hla. (B-C) Control or *PF4/LysM ADAM10*$^{-/-}$ mice were infected with *S. aureus* as described, and livers were removed 72 hours post infection for quantification TNF-α and IL-10 in tissue homogenates.

Figure S5, Related to Figure 5

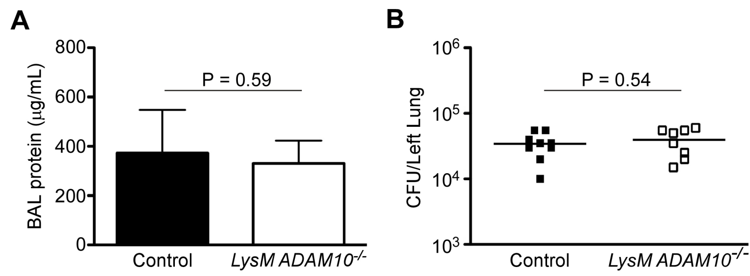

Figure S5. Myeloid-lineage specific ADAM10 knockout does not contribute to acute lung injury. (A-B) Protein content in bronchoalveolar lavage (BAL) fluid (A) and bacterial colony forming unit (CFU) recovery (B) in lungs harvested from Control and *LysM ADAM10*$^{-/-}$ mice infected with *S. aureus* USA300. Data are represented as mean ±SD.

Figure S6, Related to Figure 6

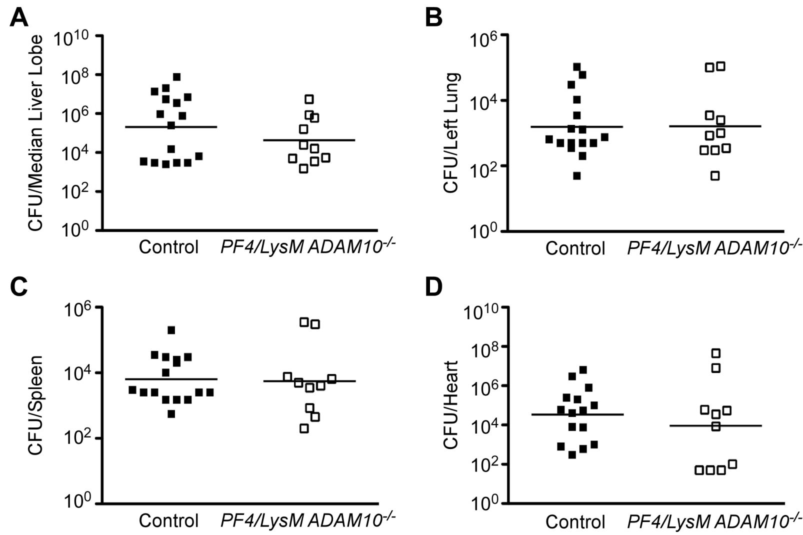

Figure S6. Tissue bacterial load in *PF4/LysM ADAM10$^{-/-}$* mice is unchanged relative to Control mice. (A-D) Control and *PF4/LysM ADAM10$^{-/-}$* mice were infected with *S. aureus* as described. 72 hours post-infection the liver (A), lung (B), spleen (C) and heart (D) were removed, homogenized and plated to quantify colony-forming units (CFU).

Figure S7, related to Figure 6

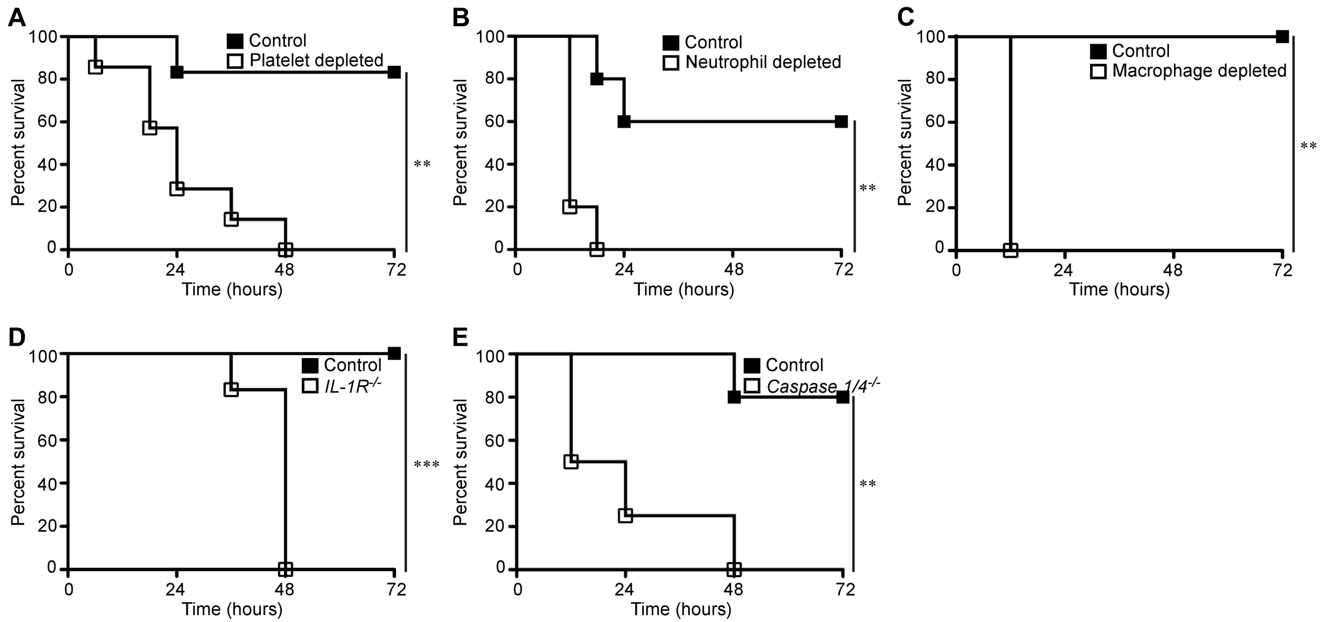

Figure S7. Innate immune cells and the inflammasome pathway provide immunologic control of *S. aureus* bloodstream infection. (A-E) platelet depleted (n = 7) (A), neutrophil depleted (n = 5) (B), macrophage depleted (n = 5) (C), *IL-1R*$^{-/-}$ (n = 5) (D) and *Caspase 1/4*$^{-/-}$ (n = 5) (E) or corresponding controls were infected with *S. aureus* as described and monitored for survival, **P≤0.01, ***P≤0.001.

Supplemental Experimental Procedures

Generation of platelet ADAM10$^{-/-}$ and platelet/myeloid ADAM10$^{-/-}$ conditional knockout mice. Platelet-specific knockout mice (*PF4 ADAM10*$^{-/-}$) were bred with myeloid lineage ADAM10 knockout mice (*LysM ADAM10*$^{-/-}$) to generate platelet and myeloid conditional double-knockout mice (*PF4/LysM ADAM10*$^{-/-}$). *PF4*^{cre} *ADAM10*$^{+/+}$ and *LysM*^{cre} *ADAM10*$^{+/+}$ transgenic C57Bl/6 mice were utilized as single knockout controls. *PF4*^{cre} *ADAM10*$^{+/+}$ and *LysM*^{cre} *ADAM10*$^{+/+}$ control mice were bred to generate double knockout control mice.

Analysis of ADAM10 expression. To determine ADAM10 expression on mouse platelets, 2 x 10^6 platelets were lysed in 15 µL lysis buffer. Lysates were suspended in non-reducing sample buffer, boiled for 5 min at 90°C and run on a 10% SDS-PAGE gel. Immunoblotting for ADAM10 or β-actin control was performed according to standard protocols and imaged with a LI-COR Imaging System for detection of Alexa-Fluor conjugated secondary antibodies.

Hematopoietic lineage analysis. For analysis of platelet, peripheral blood leukocyte and red blood cell parameters, blood from Control or *ADAM10*$^{-/-}$ (n=5) was isolated and sent to Comparative Clinical Pathology Services LLC. Spleens were isolated and weighed from Control or *PF4 ADAM10*$^{-/-}$ mice (n=5).

Tissue analysis. To analyze CFU recovery from the lungs 4 hours post-infection, lungs were perfused with 3 ml PBS delivered through a right ventricular cannula, excised and homgenized. To examine CFU on day 3 of infection, a lobe of the liver was taken and homogenized for serial dilution analysis plating. Homogenized tissues were also subjected to IL-1β, TNF-α and IL-10 ELISA or colorimetric myeloperoxidase activity assays as described (Cho et al., 2012). Analysis of serum alanine aminotransferase was performed by Charles River Laboratories.

Immunofluorescence microscopy and histochemical analysis. For lung-specific histopathologic studies, lungs were perfused in situ 4 hr post-infection with 2 mL of a 1:1 mixture of 0.5M sucrose and OCT. The right lung was excised, placed in OCT and frozen in liquid nitrogen. Sections (6µm) were cut using a Microm HM550 Cryostat (Thermo), placed onto glass coverslips and fixed for 20 min in methanol, followed by PBS rehydration. α-CD42 antibody was incubated with the sections overnight at 4° C prior

to staining with an Alexa-fluor 488-conjugated secondary antibody and mounting in Prolong Gold with Dapi. For liver-specific studies, formalin-fixed tissues were subjected to periodic acid-Schiff staining (Nationwide Histology) or staining with an anti-active caspase-3 for 24 hr at 4° C followed by detection with an Envision+ HRP detection kit (Dako).

Cellular analysis and reagents

Antibodies. Primary or conjugated antibodies were used according to the manufacturers' instructions. For human studies the following antibodies were utilized: von Willebrand Factor (Dako), GPVI (R&D Systems), activated GPIIb-IIIa (PAC-1 BD Biosciences), P-selectin (CD62P, BD Pharmingen), GPIbα (CD42b, BD Pharmingen), CD11b (Beckman Coulter), ADAM10 (R&D Systems). For mouse studies the following antibodies were utilized: GPVI (Emfret Analytics), activated GPIIb-IIIA (JON/A, Emfret Analytics), ADAM10 (R&D Systems), β actin (Abcam), P-selectin (CD62P, Emfret Analytics), neutrophil GR-1 (BioLegend), GPIb (CD42, Emfret Analytics), activated caspase 3 (Abcam) and CD-41 (BD Pharmingen). Alexa Fluor-conjugated secondary antibodies (Invitrogen) were utilized for detection in Western blot analysis and flow cytometric studies.

Reagents. Alexa-Fluor conjugated fibrinogen, calcein AM and cell-tracker red (Invitrogen), type-1 fibrillar collagen (Chrono-log, 100 μg/mL in 30% ethanol), human fibrinogen (Sigma, 250 μg/mL in water), and ELISA-based assays for detection of IL-1β, IL-18, TNF-α and IL-10 (R&D Systems) were used according to the manufacturer's protocols or previously described protocols (Nieswandt et al., 2007; Tomlinson et al., 2007). CHRONO-LUME® luciferin (Chrono-Log) was used for platelet ATP release assay according to the manufacturer's protocol. Cell lysis was measured using an LDH cell cytotoxicity kit (Roche). The ADAM10 inhibitor GI254023X was synthesized by OKeanos Tech., Ltd (Beijing, China) and applied to cells at 20 μM in complete media 16-18 hours prior to experimentation as previously described (Powers et al., 2012). The ADAM10 fluorescent substrate (Mca-PLAQAV-Dpa-RSSSR-NH_2, R&D Systems) was utilized as previously described (Inoshima et al., 2011; Inoshima et al., 2012; Powers et al., 2012). Clodronate (clodronateliposomes.org) was used to deplete mice of macrophages by injection of 200 μL intravenously. Platelets were depleted with 100 μL rabbit anti-

mouse thrombocyte serum (Cedarlane). Collagen-related peptide was produced by Dr. Richard W. Farndale (Cambridge, UK).

Buffers. Lysis buffer: 5% IGEPAL, 300 mM NaCl, 2 mM EDTA, 20 mM Tris pH 7.4 and protease inhibitors. Acid citrate dextrose: 85 mM sodium citrate, 69 mM citric acid, 111 mM glucose.

Platelet resuspension buffer: 140 mM NaCl, 3 mM KCl, 0.5 mM MgCl, 5 mM $NaHCO_3$, 10 mM glucose, 10 mM Hepes. Tyrodes buffer: 134 mM NaCl, 2.7 mM KCl, 12mM $NaHCO_3$, 200 mM Hepes, 0.34 mM Na_2HPO_4, 5 mM glucose, 0.35% bovine serum albumin. Metalloprotease assay buffer: 25mM Tris, 10μM fluorogenic peptide substrate (Mca-PLAQAV-Dpa-RSSSR-NH_2, R&D Systems), pH 8.0.

von Willebrand factor secretion. Human pulmonary artery endothelial cells (HPAECs) were exposed to Hla or designated mutants (5μg/ml) for 15 min, fixed in 4% PFA, stained with anti-von Willebrand factor antibody at room temperature for 1 hour. Following staining with an Alexa-fluor 594-conjugated secondary antibody, cells were visualized as described.

Platelet adhesion studies. HPAECs were stained with 1mM cell tracker red, plated in the wells of IBIDI μ-slide VI flow-chambers and allowed to adhere for 24 hours. Hla or Hla_{H35L} (5 μg/ml) in endothelial EBM-2 growth media was flowed over endothelial cells for 10 min at a fluid shear stress of 150 s^{-1}. Cells were washed and exposed to isolated human platelets stained with 4 μM calcein AM introduced to the flow system. Platelet adhesion to endothelial cells was visualized via fluorescence microscopy and images processed using ImageJ software (http://rsbweb.nih.gov/ij/). For binding to type-1 fibrillar collagen (100 μg/mL in ethanol) or human fibrinogen (250 μg/mL in water) coated IBIDI chambers, human platelets were stained with calcein AM as described. Toxin-exposed platelets were flowed at a shear rate of 150 s^{-1} and monitored for adherence by immunofluorescence microscopy. Unstained mouse platelets were visualized on collagen by differential interference contrast microscopy.

Platelet metalloprotease assay. Human or mouse platelets were washed in platelet resuspension buffer and treated with Hla or Hla_{H35L} in 25 mM Tris pH 8.0 for the time points indicated with 10μM ADAM10-specific fluorogenic peptide substrate in metalloprotease assay buffer. Fluorescence intensity was read on a BioTek Synergy HT plate reader.

Platelet surface protein expression. To analyze human GPVI expression, 2 x 10^6 platelets in 500μL platelet resuspension buffer were treated for 5-15 min with 1 μg/ml Hla or Hla_{H35L}. Lysates were pre-cleared with Protein G agarose and immunoprecipitated with an anti-GPVI antibody. Immunoblotting was performed according to standard protocols and imaged with a LI-COR Imaging System. For mouse and human platelet analysis by flow cytometry, 10 μL platelet rich plasma (PRP) prepared as described (Nieswandt et al., 2001) in 40μL Tyrodes buffer was incubated with active Hla or Hla_{H35L} (1 μg/ml) or collagen-related peptide (CRP, 5 mg/mL) for the time periods indicated, followed by the addition of the following detection reagents: GPVI - 5 μl FITC-conjugated anti-mouse GPVI antibody (JAQ-1) for 15 min; GPIIb-IIIa - 20μL FITC conjugated PAC-1 antibody (human) or 5 μL JON/A antibody (mouse); and P-selectin - 5 μL FITC-conjugated CD62P antibody was added for 15 min. Platelets were diluted in Tyrodes buffer and analyzed on a BD FACSCanto.

Platelet cytotoxicity assay. 5 x 10^5 Control or *PF4 ADAM10*$^{-/-}$ platelets were exposed to 5 μg/mL Hla for 1 hr and LDH release was measured using a cytotoxicity detection kit (Roche) according to the manufacturer's protocol and read with a BioTek Synergy HT plate reader.

Platelet activation assay. Platelet activation by Hla was monitored by ATP release. In brief, 90 μL platelet rich plasma (PRP) was exposed to 1 μg/mL Hla, Hla_{H35L}, or 5 μg/mL collagen-related peptide (CRP) and 10 μL luciferin, then monitored for luminescence on a Tecan Infinite 200 PRO plate reader.

Platelet-neutrophil aggregate (PNA) studies. Mouse or human whole blood (50 μL) was left untreated or treated with Hla or Hla_{H35L} (1 μg/mL, 30 min) or CRP (5 mg/mL, 10 min) then fixed with 50 μL 2% PFA. Following red cell lysis (400 μL H_2O, 30 sec), cells were pelleted at 400x*g* (5 min) and resuspended in 100 μL Tyrodes buffer. For cell subset analysis by flow cytometry, cell suspensions were treated with the following antibodies as indicated for 15 min: FITC-IgG control (5 μl), APC-conjugated-anti-GR-1 (1 μL) or PE-anti-CD11b (20 μL), and FITC-anti-GPIbα (mouse, 5 μL) or FITC-anti-GPIbα (human, 20 μL). Gated GR-1$^+$ cells were analyzed for control antibody staining or GPIbα to detect PNA. To examine PNA cytokine production, 1 x 10^5 neutrophils and 1 x 10^7 platelets were incubated in 200 μL RPMI/10% fetal bovine serum. Neutrophil-platelet suspensions were left untreated or treated with Hla (0.1 μg/mL)

for 4 hr to facilitate PNA formation. Suspensions were then treated with Hla (25 μg/mL) for 16 hours followed by supernatant IL-1β and IL-18 quantification by ELISA.

Visualization of neutrophil extracellular traps. 1 x 10^6 mouse neutrophils were seeded onto poly-L-lysine coated coverslips in RPMI with 10% FBS. Neutrophils were exposed to Hla alone (1 μg/mL), platelets (1 x 10^7), Hla and platelets, or PMA (20 mM) for 2 hours, fixed in 1% PFA and stained with Hoechst 33342.

Supplemental References

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