Platelets inhibit apoptotic lung epithelial cell death and protect mice against infection-induced lung injury

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Key Points

• Platelets protect against lung injury from pathogen-secreted factors that promote host cell death even in the absence of overt infection.
• Neutrophil airspace influx does not potentiate lung injury caused by pathogens in the susceptible host with thrombocytopenia.

Thrombocytopenia is associated with worse outcomes in patients with acute respiratory distress syndrome, which is most commonly caused by infection and marked by alveolar-capillary barrier disruption. However, the mechanisms by which platelets protect the lung alveolar-capillary barrier during infectious injury remain unclear. We found that natively thrombocytopenic Mpl−/− mice deficient in the thrombopoietin receptor sustain severe lung injury marked by alveolar barrier disruption and hemorrhagic pneumonia with early mortality following acute intrapulmonary Pseudomonas aeruginosa (PA) infection; barrier disruption was attenuated by platelet reconstitution. Although PA infection was associated with a brisk neutrophil influx, depletion of airspace neutrophils failed to substantially mitigate PA-triggered alveolar barrier disruption in Mpl−/− mice. Rather, PA cell-free supernatant was sufficient to induce lung epithelial cell apoptosis in vitro and in vivo and alveolar barrier disruption in both platelet-depleted mice and Mpl−/− mice in vivo. Cell-free supernatant from PA with genetic deletion of the type 2 secretion system, but not the type 3 secretion system, mitigated lung epithelial cell death in vitro and lung injury in Mpl−/− mice. Moreover, platelet releasates reduced poly (ADP ribose) polymerase cleavage and lung injury in Mpl−/− mice, and boiling of platelet releasates, but not apyrase treatment, abrogated PA supernatant–induced lung epithelial cell cytotoxicity in vitro. These findings indicate that while neutrophil airspace influx does not potentiate infectious lung injury in the thrombocytopenic host, platelets and their factors protect against severe pulmonary complications from pathogen-secreted virulence factors that promote host cell death even in the absence of overt infection.

Introduction

Acute respiratory distress syndrome (ARDS) is estimated to underlie 10% of intensive care unit admissions worldwide and cause 74,500 deaths annually in the United States.1,2 The most common risk factor for ARDS is infection, primarily pneumonia and sepsis.1,3 Platelet deficiency has consistently been associated with worse outcomes in ARDS, and a missense genetic variant in LRRC16A/CARMIL1 that limits reductions in platelet counts during ARDS is associated with improved patient survival.4-8 However, it remains uncertain whether platelets can directly provide protection during lung injury.9,10 Platelets potentiate injury in mouse models of acid aspiration and antibody-mediated transfusion-related
lungs injury in part through platelet–neutrophil interactions.\textsuperscript{11,12} However, diffuse lung injury can occur even in severe neutropenia,\textsuperscript{13–15} and a major clinical trial failed to show significant benefit from aspirin therapy in patients with lung injury.\textsuperscript{16} In contrast to acid-aspiration and antibody-mediated injury models, platelets limit alveolar hemorrhage during bacterial lipopolysaccharide (LPS)–induced injury and reduce lung bleeding in pneumonia models.\textsuperscript{17,18} Alveolar hemorrhage frequently results from disruption of the alveolar–capillary barrier,\textsuperscript{19,20} which is a pathologic hallmark of lung injury.\textsuperscript{21}

Invading pathogens, particularly those with toxins and protease virulence factors,\textsuperscript{22,23} can cause lung epithelial cell death.\textsuperscript{24,25} 

\textit{Pseudomonas aeruginosa} (PA) is a common cause of hospital-associated pneumonia in critically ill patients and complication of ARDS.\textsuperscript{26,27} PA virulence is mediated in part by the type 2 secretion system (T2SS), which secretes products into the extracellular space, and the type 3 secretion system (T3SS), which forms a needle and pore system to directly inject toxins into the host cytoplasm.\textsuperscript{28} Notably, the T3SS is not required for virulence, as the PA T2SS causes lethal pneumonia in the absence of the T3SS.\textsuperscript{29} Further, T2SS products trigger lung cell death in vitro\textsuperscript{24,30,31} and other non-T3SS products can cause hemorrhagic pneumonia.\textsuperscript{22,29,32} 

Lung epithelial cell death contributes to alveolar–capillary barrier disruption.\textsuperscript{33–36} Platelets have been shown to support lung microvascular integrity independent of their classic hemostatic pathways.\textsuperscript{17,37,38} Platelets possess numerous factors that may promote cell survival, support the endothelial barrier,\textsuperscript{39,40} and counter programmed cell death pathways.\textsuperscript{41–43} Importantly, platelets are not only present in the vascular space during lung inflammation but also enter the alveolar space during experimental lung injury.\textsuperscript{44,45} Therefore, we hypothesized that platelets protect against PA mediated lung injury in part by counteracting epithelial cell death.

**Materials and methods**

**Animals**

\textit{Mpl}\textsuperscript{−/−} mice were obtained from the Walter and Eliza Hall Institute in Melbourne, Australia.\textsuperscript{46} \textit{Mpl}\textsuperscript{−/−} mice were backcrossed 12 generations to C57Bl/6 mice at the University of Pittsburgh before establishing a founder colony. C57Bl/6J mice were used for experiments when litters were unavailable. All experimental procedures were performed in age- (8-16 weeks old) and sex-matched mice in randomized fashion. A technician blinded to the experimental hypothesis performed the in vivo experiments. Depending upon mouse availability, experiments were conducted with precalculated sample size as one single experiment or divided into independent experiments. The University of Pittsburgh Institutional Animal Care and Use Committee approved the animal protocol. Resilience studies were conducted separately from pneumonia studies, and mice were monitored carefully and euthanized when they met predefined criteria.

**Experimental bacterial pneumonia and cell-free bacterial SN model**

PA14\textsuperscript{47} was grown per protocol.\textsuperscript{48} In-frame deletion mutants PA14:jexoTUY and PA14:jcpQ were used in select experiments.\textsuperscript{48,49} Intratracheal (IT) inoculations were performed as previously described.\textsuperscript{48,50,51} PA cell-free bacterial supernatant (SN) was prepared from pelleted PA by careful aspiration of the SN followed by passage through a 0.22 \textmu m sterile filter. The absence of bacterial growth was confirmed by plating filtered SN directly on LB agar. \textit{Klebsiella pneumoniae} (KP) strain 43816 serotype 2 (American Type Culture Collection) was handled as previously described.\textsuperscript{51}

**Mouse necropsies, MPO content, and lung tissue histology**

Mice were euthanized 20 hours following PA inoculation with isoflurane overdose followed by exsanguination. Mouse necropsy, lung tissue processing, bronchoalveolar lavage (BAL), and myeloperoxidase (MPO) activity were performed as previously described.\textsuperscript{48,50,52,53} In dedicated experiments, hematoxylin and eosin staining was performed on lung specimens as previously described.\textsuperscript{55}

**BAL hemoglobin, platelet counts, OD\textsubscript{540}, and IgM measurements**

BAL fluid was cataloged by digital photography and BAL optical density at 540 nm (OD\textsubscript{540}) was measured immediately using 100-\textmu L aliquots. BAL hemoglobin and platelet counts were measured in 1 mL BAL fluid by Hemavet 950 (Drew Scientific) as described in a prior report.\textsuperscript{54} BAL total protein concentration was determined after centrifugation by Pierce BCA Protein Assay. BAL immunoglobulin M (IgM) was determined following 1:10 dilution according to the manufacturer’s instructions (#E90-101, Bethyl Labs).

**Evans blue extravasation in the lungs**

Pulmonary microvascular permeability was measured using the Evans blue dye extravasation technique\textsuperscript{55,56} by measuring the absorbance of the formamide extract of lung at 620 nm and 740 nm with correction for heme; corrected absorbance = OD\textsubscript{620} – (1.426 \times OD\textsubscript{740} + 0.03).\textsuperscript{55,57}

**Antibody depletion of platelets and neutrophils**

To deplete circulating neutrophils, \textit{Mpl}\textsuperscript{−/−} mice were administered 200 \textmu g anti-Ly-6G antibody (clone 1A8 rat IgG2a, BioLegend) or isotype control (RTK2758 rat IgG2a, \kappa, BioLegend) by intraperitoneal injection 24 hours prior to bacterial exposure. Wild-type (WT) mice were administered 75 \textmu g anti-GP1b-\alpha (R300 Rat IgG, Emfret) or nonimmune IgG control (C301, Emfret) antibody by retro-orbital injection after isoflurane anesthesia to deplete circulating platelets 24 hours prior to bacterial SN inoculation. Blood counts were performed using Hemavet 950.

**Immunofluorescence of mouse lungs**

Mouse lungs were perfused with 5 mL normal saline via the right ventricle then inflated via the trachea with 2% paraformaldehyde at 20 cm H\textsubscript{2}O for 5 minutes prior to preservation. Frozen sections were stained with 4',6-diamidino-2-phenylindole, terminal deoxynucleotidyltransferase-mediated dUTP nick end labeling (TUNEL), pro–surfactant protein C (pro-SPC), and CD31. Confocal images were collected using a Nikon A1R equipped with GaP detectors and a 20 \times (0.75 numerical aperture) objective. TUNEL-positive cells were identified using segmentation analysis (NIS Elements).

**Murine lung epithelial cells**

MLE 12 cells (CRL-2110, ATCC) were grown in RPMI 1640 supplemented with 5% heat-inactivated fetal bovine serum, 1%
penicillin-streptomycin, and 1% L-glutamine at 37°C in 5% CO₂.59,60 Passages 4 to 10 were used for transfusion. Cytotoxicity was measured by lactate dehydrogenase assay according to the manufacturer’s instructions (#JM-K313-500, MBL International). Manufacturer-supplied cell lysis buffer was used to establish 100% cytotoxicity. For cell death inhibition studies, cells were incubated with carbobenzoxy-valyl-alanyl-aspartyl-[O-methyl]-fluoromethylketone (Z-VAD-fmk) or Nec-1s (Millipore) for 1 hour prior to the addition of PA SN.

Live-cell imaging

MLE 12 cells cultured on 35-mm glass microwell dishes (MatTek) were imaged in a closed, thermo-controlled (37°C) incubator (Tokai Hit) atop the motorized stage of an inverted Nikon TiE fluorescent microscope (Nikon). Annexin V and propidium iodide dyes (V13241, ThermoFisher) were added with initiation of imaging. Dyes were excited using a diode-pumped light engine (SpectraX, Lumencor) and detected by ORCA-Flash 4.0 sCMOS camera (Hamamatsu Corporation) with Chroma Technology excitation/emanison filters. Time-lapse large-area images (5 x 5 fields) were collected every 15 minutes using a 20x dry optic (0.75 numerical aperture) and stitched using NIS Elements (Nikon).

Flow cytometric-based caspase-3/7 cleavage assay

After experimental exposure, MLE 12 cells were incubated with a fluorogenic caspase-3/7 substrate (C10740, Invitrogen) per the manufacturer’s instructions. Flow cytometry was performed immediately on a FACSCalibur machine (BD Biosciences) and analyzed with FlowJo V10.

Western blots

MLE 12 cells were lysed with ice-cold Pierce cell lysis buffer with complete protease and phosphatase inhibitors (Roche). Lung tissues were lysed with ice-cold lysis buffer #6 (R&D Systems) and then homogenized and sonicated on ice. Cleaved caspase-3 (#9664), total caspase-3 (#9662), cleaved PARP (#94885), or β-actin (#4970) antibodies were purchased from Cell Signaling Technology. Membranes were developed with chemiluminescent substrate (34095, ThermoFisher) using an Amersham 600 imager (General Electric).

Isolation and transfusion of mouse platelets

Blood was collected into 10% vol/vol citrate phosphate dextrose mixed with 2 μg/mL prostaglandin L2 (PGI₂)63 and allowed to sit for 10 minutes before centrifugation at 200 g for 5 minutes without brake to obtain platelet-rich plasma (PRP). PRP was gently transferred to a new tube using wide-orifice pipette tips and taking care to avoid the buffy coat. An additional 2 μg/mL PGI₂ was added to PRP prior to centrifuging at 5 minutes without brake to pellet platelets, which were gently re-suspended in modified Tyrode’s buffer (137 mM NaCl, 0.3 mM Na₂HPO₄, 2 mM KCl, 12 mM NaHCO₃, 5 mM N-2-hydroxyethylpiperazine-N’-2-ethanesulfonic acid, and 5 mM glucose, pH 7.27) with 0.35% vol/vol sterile-filtered endotoxin-free bovine serum albumin. Platelets were normalized to desired count in 100 μL buffer for transfusion via retro-orbital vein after isoflurane. All steps were performed at room temperature.

Platelet releasates

Platelet releasates (PR) were prepared from platelets isolated from WT mice or 4 healthy human volunteers (University of Pittsburgh #IRB0410173) as above without PGI₂. Platelets were stimulated with 1 U/mL of thrombin, incubated at 37°C for 20 minutes, and centrifuged at 1000 g. The clear SN (releasates) was stored at ~80°C.

Statistical analysis

A 2-tailed Mann-Whitney U test was used when comparing 2 groups, and a Kruskal-Wallis test with Dunn’s post hoc test were used when comparing >2 groups, unless otherwise indicated. P < .05 was considered significant. In one experiment, a linear bootstrap regression model was applied to identify a statistical outlier, which was removed from subsequent analysis. All statistics were performed using GraphPad Prism V7 (La Jolla, CA) and Stata V15 (College Station, TX).

Results

Thrombocytopenic Mpl−/− mice sustain severe lung injury after IT PA infection

To evaluate the role of platelets during pathogen-triggered lung injury, we exposed natively thrombocytopenic Mpl−/− mice to acute lung infection with the reference PA strain, PA14. Mpl−/− mice exhibit normal erythrocyte and leukocyte counts in circulation,62 and no obvious defects in host defense have been reported.46,63 After IT inoculum of 10⁵ colony-forming units (CFU) of PA, Mpl−/− mice demonstrated rapid mortality; 8 of 8 Mpl−/− mice died within 48 hours, whereas 7 of 8 littermate Mpl+/+ mice survived to 7 days (Figure 1A). Given the rapid mortality of Mpl−/− mice, we used a 20-hour time point to evaluate mechanisms of enhanced susceptibility in the acute PA lung infection model. Mpl−/− mice showed severe lung injury with brisk neutrophilic airspace infilter (Figure 1B) and increased BAL protein concentrations (Figure 1C). Notably, platelets have roles in host defense against invading pathogens,16,64-66 and as others have shown,67 platelets appear to support control of lung bacterial burden in acute PA pneumonia (Figure 1D). However, we did not identify significant dissemination of PA to liver or spleen (supplemental Figure 1A-B) to suggest extrapulmonary sepsis that would account for early Mpl−/− mortality. Mpl−/− mice showed lung hepatization on gross examination (Figure 1E) and diffuse hemorrhage on representative hematoxylin and eosin-stained lung sections (Figure 1F).

Mpl+/+ mice exhibited ~20% of the baseline circulating platelet counts of WT mice (Figure 1G; Mpl+/− median, 123 x10⁹ platelets/L; WT median, 621 x10⁹ platelets/L). We noted striking BAL hemorrhage, a hallmark of alveolar–capillary barrier disruption,19,20 after lung injury in Mpl−/− mice (Figure 1H) that was attenuated in platelet-sufficient WT mice. We found that BAL fluid OD₅₄₀, a spectrophotometric peak of hemoglobin,16,69 strongly correlated (Spearman r = 0.98; supplemental Figure 1C) with BAL hemoglobin content (Figure 1I-J).34 Supporting OD₅₄₀ as an acceptable, rapid surrogate for BAL hemoglobin. In addition, Evans blue dye infusion confirmed increased lung microvascular permeability in Mpl−/− mice when compared with WT mice (Figure 1K). Moreover, adoptive transfer of platelets from WT mice into Mpl−/− mice 1 hour following acute PA infection mitigated lung injury, as demonstrated by reduction in BAL OD₅₄₀ (Figure 1L). Therefore, Mpl−/− mice exposed to acute PA bacterial infection model demonstrate rapid mortality marked by lung injury and alveolar hemorrhage that is attenuated by partial platelet reconstitution.
Pathogenic KP does not induce hemorrhagic lung injury, but the cell-free SN of PA is sufficient to induce neutrophil airspace influx and lung injury in thrombocytopenic Mpl<sup>-/-</sup> mice

Alveolar hemorrhage after intrapulmonary LPS administration has been observed in models of severe antibody-mediated thrombocytopenia. Given the phenotype of hemorrhagic lung injury during acute PA pneumonia in Mpl<sup>-/-</sup> mice, we wondered whether another gram-negative bacteria, KP, caused a similar phenotype as LPS is a major KP virulence factor. We used KP serotype 2, a hypervirulent strain previously shown to cause extra-pulmonary dissemination and death in mice. Others have shown lung hemorrhage during acute KP pneumonia with platelet counts an order of magnitude lower than Mpl<sup>-/-</sup> mice. However, we did not observe gross alveolar hemorrhage in Mpl<sup>-/-</sup> mice nor significant differences in survival, BAL cell counts, BAL neutrophils, BAL protein, or lung CFU counts between Mpl<sup>-/-</sup> and their platelet-sufficient WT littermates 48 hours following KP infection (Figure 2A-F), when KP serotype 2 is known to disseminate. Given the discrepancy between alveolar injury between PA and KP infection models, we wondered whether PA-secreted bacterial products may cause lung injury in the absence of live bacteria. Filtered cell-free PA SN was...
sufficient to cause alveolar–capillary barrier disruption in Mpl$^{-/-}$ mice compared with vehicle (LB broth), as demonstrated by gross alveolar hemorrhage (Figure 2G), BAL neutrophil influx (Figure 2H), and increased BAL protein (Figure 2I). Collectively, these findings suggest that platelet deficiency enhanced susceptibility to PA infection that is characterized by prominent alveolar injury, but not to KP infection characterized by extrapulmonary sepsis.

Neutrophil depletion does not significantly attenuate PA-triggered alveolar barrier disruption in thrombocytopenic Mpl$^{-/-}$ mice

Prior reports have shown that platelets repair holes created by neutrophils exiting the vasculature during immune complex–mediated skin inflammation. We therefore examined whether brisk neutrophil airspace influx during acute PA pneumonia contributes to alveolar–capillary barrier disruption. We depleted circulating neutrophils in Mpl$^{-/-}$ mice with Ly-6G antibody 24 hours prior to IT PA inoculation (Figure 3A). The antibody-depletion strategy resulted in >60% fewer circulating neutrophils (Figure 3B) at the time of PA inoculation compared with isotype control IgG2a antibody. Given the essential role of neutrophils in host defense against PA, it was not surprising that neutrophil depletion increased lung bacterial burden (Figure 3C). Antibody depletion reduced neutrophil airspace recruitment by 85% compared with isotype-control during acute PA infection (Figure 3D; anti-Ly-6G group median, 3.2 $\times$ 10$^4$ neutrophils/mL). Despite neutrophil reduction, there was no significant difference in the gross appearance of BAL fluid, BAL OD$_{540}$, or BAL IgM, an independent measure of alveolar–capillary barrier disruption, in either live bacteria (Figure 3D-G) or bacterial SN (Figure 3H-K) models. Notably, there was a 90% reduction in neutrophil airspace recruitment with a second antibody treatment compared with isotype control antibody.
These findings suggest that bacterial-secreted factors rather than neutrophil influx are the primary contributor to alveolar injury observed in thrombocytopenic Mpl$^{-/-}$ mice.

PA SN induces lung injury after antibody-mediated platelet depletion of WT mice, and PA SN-induced injury can be attenuated by partial reconstitution of platelet counts in thrombocytopenic Mpl$^{-/-}$ mice.

To mitigate concerns that compensatory defects in Mpl$^{-/-}$ mice caused enhanced susceptibility, we examined PA SN in a second model of thrombocytopenia by depleting platelets in WT C57Bl/6J mice using anti-glycoprotein1bα (GP1bα) antibody (Figure 4A; anti-GP1bα group median, $118 \times 10^9$ platelets/L; control group median, $607 \times 10^9$ platelets/L). PA SN administration recapitulated hemorrhagic lung injury in anti-GP1bα-treated WT mice as demonstrated by gross alveolar hemorrhage (Figure 4B) as well as significantly increased BAL OD$_{540}$, BAL protein, and BAL IgM concentration (Figure 4C-E). Interestingly, BAL neutrophil counts did not significantly differ between groups (Figure 4F). We then returned to the Mpl$^{-/-}$ mouse model system to demonstrate that partial platelet reconstitution (Figure 4G; platelet recipient median, $160 \times 10^9$ platelets/L; vehicle recipient median, $83 \times 10^9$ platelets/L) mitigated PA SN-triggered alveolar–capillary barrier disruption (Figure 4H-I). We therefore concluded that platelets protect against secreted bacterial products that were sufficient to induce lung injury in platelet-deficient mice.
PA exoproducts are sufficient to induce lung epithelial cell apoptosis, in part through a secreted T2SS protein.

PA T2SS products have been shown to induce cell death in vitro, and lung epithelial cell death may lead to alveolar–capillary barrier disruption. We found increased TUNEL-positive cell counts in the alveolar space of mice 6 hours after PA SN exposure compared with baseline (Figure 5A-B), suggesting that PA SN can cause lung cell death in vivo. Notably, TUNEL positivity frequently costained with pro-SPC, suggesting that type 2 alveolar epithelial cells are susceptible to PA SN-triggered programmed cell death. Therefore, we used a murine type 2 alveolar epithelial cell line (MLE 12) to better characterize PA SN-triggered cell death. We found that PA SN caused lung epithelial cell death in a time-dependent manner in vitro (Figure 5C), and live-cell imaging of MLE cells exposed to PA SN revealed cellular blebbing and early annexin V staining, consistent with apoptosis, that later transitioned to propidium iodide staining (Figure 5D). MLE cell death was inhibited by Z-VAD-fmk, a pan-caspase inhibitor, but not the RIP1-kinase inhibitor Nec-1s that targets necroptosis, even after prolonged PA SN exposure (Figure 5E). Our finding of apoptotic cell death was corroborated by flow cytometry demonstrating MLE caspase-3/7 activation (Figure 5F; supplemental Figure 1D) and by immunoblot of MLE cell lysates, which identified caspase-3 cleavage, following PA SN exposure (Figure 5G). We found that boiling PA SN prior to MLE exposure decreased cytotoxicity by a
median of 87% (Figure 5H), suggesting that protein products in PA SN mediate its cytotoxic effect. We then compared lung epithelial cell death following exposure to SN from WT PA14, PA14 ΔxcpQ (deletion mutant lacking the outer membrane protein XcpQ that controls T2SS exoproduct efflux),28 or PA14 ΔexoTUY (deletion mutant lacking the T3SS exotoxins T, U, and Y).49 Deletion of the T2SS secretory protein XcpQ decreased lung epithelial cell death, whereas deletion of T3SS exotoxins T, U, and Y did not (Figure 5I).

These findings indicate that PA SN can induce apoptotic lung epithelial cell death, in part through T2SS protein exoproducts.

**Genetic deletion of PA T2SS, but not T3SS exotoxins, attenuates alveolar–capillary barrier disruption in thrombocytopenic Mpl−/− mice**

Given that lung epithelial cell death in vitro was attenuated after PA14ΔxcpQ SN exposure in contrast to PA14 ΔexoTUY SN, we
wondered whether this phenomenon could account for alveolar–capillary barrier disruption in vivo. Mpl\(^{-/-}\) mice administered PA\(\Delta xcpQ\) showed attenuated lung injury compared with the vehicle-treated or parent PA SN-treated mice as evidenced by BAL fluid appearance, BAL OD\(_{540}\), BAL protein, and BAL IgM (Figure 6A-D). Notably, there were similar neutrophil counts in the airspaces and lungs of the SN-treated groups (Figure 6E-F), reinforcing the concept that neutrophils do not potentiate lung injury in this model. As expected, the cell-free SN of PA\(\Delta exoTUY\) did not attenuate lung injury compared with vehicle-treated or PA SN-treated Mpl\(^{-/-}\) mice as measured by BAL fluid appearance, BAL OD\(_{540}\), and BAL protein (Figure 6G-I). Therefore, platelets provide protection against lung injury mediated by bacterial secreted products of PA T2SS even in the absence of live pathogen or cell–cell contact and suggest a role for platelets as defenders against lung epithelial cell death.

**PR attenuates PA SN-triggered lung cell death and limits PA SN-mediated lung injury in thrombocytopenic Mpl\(^{-/-}\) mice**

Several studies have suggested that platelets enter the alveolar space in response to experimental lung injury in mice, and platelet products have been noted in BAL fluid from patients with diffuse lung injury. We found platelet accumulation in the BAL fluid of WT mice following acute PA IT infection (Figure 7A). Thrombin–antithrombin complexes (TATc) have been noted in BAL from patients with ventilator-associated pneumonia, and TATc was increased in BAL fluid from both WT and Mpl\(^{-/-}\) mice after PA infection.
SN exposure (Figure 7B). We next evaluated whether releasates from thrombin-stimulated platelets could counter PA SN-induced lung epithelial cell death in vitro. PR dose-dependently protected MLE 12 cells from PA SN-mediated cytotoxicity when compared with vehicle-treated cells (Figure 7C), and protection was markedly attenuated by boiling PR (Figure 7D), suggesting that platelet proteins mediate the cytoprotective effect. PR inhibited caspase-3 cleavage in PA SN-treated MLE 12 cells (Figure 7E). Notably, there was no significant impact on cytotoxicity from apyrase treatment (Figure 7F). Moreover, human PR also reduced MLE cytotoxicity by a median of 94% after PA SN (Figure 7F), suggesting conserved platelet proteins may underlie their protective effect. Finally, delivery

| Panel | Description |
|-------|-------------|
| **A** | WT mice BAL platelet counts (10^9/L) at baseline (0 hours, n=5) and 20 hours after IT 10^6 CFU PA (n=8). (B) BAL thrombin–antithrombin complexes (nM) in WT and Mpl^-/- mice at 0 and 4 hours after IT PA SN (n=4 each group). In separate experiments using MLE cells, fold change in MLE cytotoxicity 6 hours after PA SN in the presence or absence of pooled thrombin-stimulated PR from WT mice (C) (n=4 trials, where 3 of the 4 trials included 10 µg group) or denatured PR (D). Cleaved caspase-3, total caspase-3, and β-actin expression in MLE cells treated with PA SN in the presence or absence of increasing PR (100 and 300 µg total protein) at 4 hours (E), fold change in MLE cytotoxicity 6 hours after PA SN in the presence or absence of apyrase (0.2 U) or PR from WT mice (F), or thrombin-stimulated platelets pooled from healthy volunteers (G) (n=4). For panels C-D and F-G, each point represents the median cytotoxicity of 3 to 4 technical replicates from a single trial, relative to PA SN and vehicle treatment cytotoxicity, which is set at 1.0. In separate experiments with Mpl^-/- mice 20 hours after IT PA SN with either vehicle or 100 µg PR, lung tissue expression of cleaved PARP and β-actin is shown, n=3 in each group (H). Fold change in BAL protein concentration, n=14 (vehicle) and n=17 (PR-treated group) from 3 separate experiments (I). Each point represents an individual mouse, and the group median is displayed. Statistical comparison by Mann-Whitney U test (*) or Kruskal-Wallis test with Dunn’s test for multiple comparisons (lines represent post hoc analysis; *P < .05 (A-B,I) or ***P < .001) (A-B,I) or Kruskal-Wallis test with Dunn’s test for multiple comparisons (lines represent post hoc analysis; *P < .05 (C-D,F)).

SN exposure (Figure 7B). We next evaluated whether releasates from thrombin-stimulated platelets could counter PA SN-induced lung epithelial cell death in vitro. PR dose-dependently protected MLE 12 cells from PA SN-mediated cytotoxicity when compared with vehicle-treated cells (Figure 7C), and protection was markedly attenuated by boiling PR (Figure 7D), suggesting that platelet proteins mediate the cytoprotective effect. PR inhibited caspase-3 cleavage in PA SN-treated MLE 12 cells (Figure 7E). Notably, there was no significant impact on cytotoxicity from apyrase treatment (Figure 7F). Moreover, human PR also reduced MLE cytotoxicity by a median of 94% after PA SN (Figure 7F), suggesting conserved platelet proteins may underlie their protective effect. Finally, delivery
of PR to Mpl<sup>−/−</sup> mice reduced cleavage of PARP, a marker of caspase-3 activation, in lung tissue and decreased BAL protein concentrations when compared with vehicle-treated Mpl<sup>−/−</sup> mice (Figure 7H-I). Taken together, platelet factors appear to play mitigating roles against infection-induced lung injury beyond hemostasis alone.

**Discussion**

The major finding of our study is that platelets attenuate pathogen-induced lung injury and provide protection against lung epithelial cell apoptosis. We show that platelet deficiency is associated with severe alveolar–capillary barrier disruption in both live bacteria and bacterial exoproduct models of acute PA intrapulmonary infection. Barrier disruption is attenuated by the partial reconstitution of platelet counts via transfusion. Based on our findings, we propose that in addition to their role in hemostasis at the alveolar–capillary barrier, platelets can also enter the airspace during infection and release protective factors that may contribute to host protection by limiting alveolar epithelial cell death and attenuating further lung injury. Significant work remains to characterize platelet factors that mitigate PA-induced lung injury in our model system. Our findings suggest conserved platelet products mediate the effect and candidate factors could include mitogens, thrombospondin-1, defensins.

The second major finding of our study is that neutrophils do not potentiate lung injury following acute PA pneumonia. Neutrophils have been implicated as mediators of acute lung injury, and this is supported by evidence from several animal models. Furthermore, neutrophil transmigration across the endothelium is critical to vascular injury in models of immune complex and UV light–mediated skin injury during thrombocytopenia. However, neutrophils are not required for acute lung injury in humans as neutropenic patients can develop ARDS. We show that antibody-mediated neutrophil depletion does not substantially protect against alveolar–capillary barrier disruption in Mpl<sup>−/−</sup> mice during both acute live bacteria and PA SN infection models. Furthermore, BAL neutrophil influx in our KP pneumonia model (without associated alveolar hemorrhage) was similar in magnitude to our anti-Ly-6G treatment. We further show that the cell-free SN of PA is sufficient to cause lung injury in thrombocytopenic mice, which can be attenuated by the genetic deletion of the T2SS protein XcpQ that enables secretion of several factors with cell-damaging properties. Interestingly, pathogen with cytotoxic and proteolytic virulence mechanisms, such as PA, cytomegalovirus, and invasive fungi such as Aspergillus and Mucor, are common causes of diffuse lung injury in neutropenia. Notably, in patients diagnosed with idiopathic pneumonia syndrome who were later found to harbor pathogen, there was increased mortality in those patients with evidence of cytotoxic pathogens in their BAL. Therefore, we suggest that cytotoxic pathogen virulence factors can mediate lung injury independent of neutrophils and that this effect is accentuated in the thrombocytopenic host.

Finally, our study identifies a potential role for platelet granule factors in protection against pathogen-triggered lung injury. We show that PR attenuates lung epithelial cell death in vitro and disruption of the lung vascular barrier in platelet-deficient mice, independent of whole platelets. Consistent with our findings, others have shown that platelet granules are crucial to preventing inflammatory bleeding during thrombocytopenia. In contrast, the double knockout of platelet α and dense granule secretion did not modify lung vascular barrier function in an LPS model of lung inflammation in mice with normal platelet counts. However, invading pathogens may pose a distinct challenge to the alveolar–capillary barrier beyond the presence of LPS alone, and this challenge may be accentuated during platelet deficiency. Although we did not observe alveolar hemorrhage during acute KP pneumonia in the Mpl<sup>−/−</sup> mouse, others have shown significant lung hemorrhage during KP pneumonia after antibody-mediated reduction of platelet counts an order of magnitude lower than Mpl<sup>−/−</sup> mice. Therefore, platelet count may provide important context; we show that even partial reconstitution of platelet counts can significantly mitigate alveolar–capillary barrier disruption, which is consistent with another report. Therefore, both platelet number and the nature of the challenge posed by invading pathogens may influence the platelet role in safeguarding the lung vascular barrier. Platelet granule factors may be dispensable for alveolar–capillary barrier integrity during LPS injury if platelet counts are normal, yet they may be required to protect against cytotoxic and proteolytic damage mediated by invading lung pathogens, including during thrombocytopenia.

In conclusion, secreted bacterial products are capable of inducing lung epithelial cell apoptosis and fatal lung injury during acute PA pneumonia marked by severe breakdown of the alveolar–capillary barrier that can be attenuated by genetic deletion of the PA T2SS, partial reconstitution of platelet counts, or administration of platelet factors. Our findings enforce the concept that thrombocytopenia identifies a host susceptible to pathogen-related complications in the lung and that platelet factors can provide direct protection against lung epithelial cell death mediated by pathogen products even in the absence of live bacteria or cell–cell contact. Further study is warranted to identify the mechanisms by which platelet factors modulate infection-triggered lung epithelial injury and cell death pathways.

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**Authorship**

Contribution: W.B. performed the experiments, analyzed the data, interpreted the data, and wrote the manuscript; T.O., M.Y., Y.Q., M.H., Z.X., and H.L. performed the experiments and analyzed the data; Z.C., R.M.Q.S., and C.S.C. provided critical reagents, interpreted the data, and revised the work for important intellectual
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