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ORIGINAL RESEARCH

LPS-induced Lung Platelet Recruitment Occurs Independently from Neutrophils, PSGL-1, and P-Selectin

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Abstract

Platelets are recruited to inflammatory foci and contribute to host defense and inflammatory responses. Compared with platelet recruitment in hemostasis and thrombosis, the mechanisms of platelet recruitment in inflammation and host defense are poorly understood. Neutrophil recruitment to lung airspaces after inhalation of bacterial LPS requires platelets and PSGL-1 in mice. Given this association between platelets and neutrophils, we investigated whether recruitment of platelets to lungs of mice after LPS inhalation was dependent on PSGL-1, P-selectin, or interaction with neutrophils. BALB/c mice were administered intranasal LPS (O55:B5, 5 mg/kg) and, 48 hours later, lungs were collected and platelets and neutrophils quantified in tissue sections by immunohistochemistry. The effects of functional blocking antibody treatments targeting the platelet–neutrophil adhesion molecules, P-selectin or PSGL-1, or treatment with a neutrophil-depleting antibody targeting Ly6G, were tested on the extent of LPS-induced lung platelet recruitment. Separately in Pf4-Cre × mTmG mice, two-photon intravital microscopy was used to image platelet adhesion in live lungs. Inhalation of LPS caused both platelet and neutrophil recruitment to the lung vasculature. However, decreasing lung neutrophil recruitment by blocking PSGL-1, P-selectin, or depleting blood neutrophils had no effect on lung platelet recruitment. Lung intravital imaging revealed increased adhesion of platelets in the lung microvasculature which was not associated with thrombus formation. In conclusion, platelet recruitment to lungs in response to LPS occurs through mechanisms distinct from those mediating neutrophil recruitment, or the occurrence of pulmonary emboli.

Keywords: platelets; neutrophils; recruitment; lungs; LPS

Clinical Relevance

This research highlights that lung platelet recruitment occurs as a result of infection that is not due to alterations in hemostasis and is independent of the recruitment of neutrophils.

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Platelet and neutrophil recruitment to lungs are critical to host defense, but when excessive or inappropriate, can also contribute to tissue damage in a range of inflammatory diseases, including acute respiratory distress syndrome, chronic obstructive pulmonary disease, and respiratory infections (1, 2). Research suggests that platelets can exert a controlling influence on neutrophil recruitment, particularly in acute inflammation in the lung, where responses are dependent upon the presence of platelets displaying adhesion molecules that interact with counter ligands expressed on neutrophils (3–5), signaling in platelets through the purinergic receptors, $P2Y_1$ and $P2Y_{14}$ (6, 7), P-Rex1 and Vav guanine nucleotide exchange factors (8), and the release of CCL5, CXCL4, and CXCL7 chemokines (9), and serotonin (10), from platelets. These data provide evidence of platelet-dependent signaling mechanisms by which platelets influence neutrophil recruitment. Associations between platelets and neutrophils are further observed in acute lung inflammation by the presence of heightened circulating blood platelet–neutrophil complexes (11), and platelet dependence of neutrophil rolling, intravascular crawling, and early diapedesis (12). Furthermore, platelets can sometimes be found associated with extravascular neutrophils in inflamed lungs (11, 13–15).

How leukocytes are recruited from blood to tissues to act as major effectors of host defense and inflammation has become increasingly well characterized (2, 16). The involvement of platelets in leukocyte recruitment in inflammation, therefore, adds a further point of control for coordinating inflammatory responses (1, 17).

Blood platelet recruitment to lesions in blood vessel walls is also becoming increasingly understood at a molecular level in the context of hemostasis and thrombosis. Similarly to leukocytes, platelets can independently respond to pathogen-associated molecular patterns, and, importantly, can be recruited from blood to accumulate in tissues after infection or different types of inflammatory insult to mediate temporally and spatially restricted signaling (1). This platelet recruitment in host defense and inflammation can occur in the absence of any overt vascular trauma or thrombus formation through mechanisms that are incompletely understood, but which are distinct from those required for hemostasis and thrombosis (1, 18).

Intriguingly, given the involvement of platelets in neutrophil recruitment into lung airspaces, platelet recruitment to lungs after inflammatory responses originating elsewhere in the body, such as LPS-induced peritonitis (19), or in the bloodstream after transfusion with anti–major histocompatibility complex I antibodies (20), has shown evidence of neutrophil dependence. Neutrophils have been identified as effector cells in mediating the responses to inhaled LPS (9, 21), but the requirements for platelets in this context have not yet been explored. Understanding responses, such as lung neutrophil and platelet recruitment, and how these are regulated, may provide important information leading to new targets for the development of novel antiinflammatory drugs.

The platelet adhesion molecules involved in the recruitment of platelets to lungs in host defense and inflammation have not yet been fully characterized. Potential candidates for this role are P-selectin, PSGL-1, a counter ligand of P-, E-, and L-selectins, and ESL-1, a counter ligand to E-selectin (3, 5, 8, 22–24). Study of these adhesion molecules offers potential identification of similarities and differences between platelet and neutrophil recruitment in inflammation.

We therefore developed methods for imaging lung platelet recruitment after LPS inhalation in mice, and then investigated the effects of blockade of P-selectin or PSGL-1, or the selective depletion of neutrophils, on this response.

Parts of this work have been presented in abstract form at the Winter Meeting of the British Pharmacological Society (London, United Kingdom), December 14, 2016 (25), and at the European Respiratory Congress (Milan, Italy), September 10, 2017 (26).

Methods

Readers are directed to the data supplement for methodology related to: collection of mouse lungs for histology; immunohistochemistry using fluorescent reporters; immunohistochemistry using bright-field 3,3′diaminobenzidine (DAB) reporter; analysis of images of lung sections; flow cytometry analysis; experiments involving dabigatran etexilate; leukocyte and platelet counts from blood microsamples; and BAL collection.

Mice

Mice (BALB/c strain, female, 6–12 wk of age) used at King's College London were obtained from Charles River and housed in nonbarrier facilities. Animal experiments were conducted according to Animal Research: Reporting of In Vivo Experiments (ARRIVE) guidelines, and the Animals (Scientific Procedures) Act, 1986, and 2012 amendments, after local ethics approval. Terminal anesthesia was achieved using urethane (3 g/kg intraperitoneally). Weight changes were monitored as an index of general health status (Figure E1B in the data supplement), and it was noted that intervention studies outlined below (e.g., blockade of PSGL-1 and P-selectin, and neutrophil depletion) had no effect on this parameter (Figures E2I and E3E in the data supplement).

Experiments performed at the University of California, San Francisco were approved by the Institutional Animal Care and Use Committee. Mice were bred in specific pathogen-free barrier facilities at University of California, San Francisco. Pf4-Cre × mTmG mice (27) were generated from crosses of Pf4-Cre mice (C57BL/6-Tg(Pf4-iCre)Q3Rsko/J; Jackson Laboratories), which express Cre recombinase largely restricted to the megakaryocyte and platelet lineage (28), with mTmG mice (*Gt(ROSA)26Sor*$^{tm4(ACTB-tdTomato,-EGFP)Luo}$/J; Jackson), in which a membrane targeted tandem dimer of tomato fluorescent protein (tdTomato, mT) is constitutively globally expressed, unless Cre recombinase is expressed, where tdTomato gene expression is stopped and expression of membrane-targeted enhanced GFP (eGFP, mG) is activated (29). Male mice at 8–12 weeks of age were used for intravital microscopy experiments due to their larger size. Terminal anesthesia was induced with 125 mg/kg ketamine/12.5 mg/kg xylazine (intraperitoneal).

Intranasal Challenge with LPS

To induce lung inflammation, mice were briefly anesthetized with inhaled isoflurane, then challenged with LPS (from *Escherichia coli*, O55:B5 serotype, 5 mg/kg; Sigma-Aldrich), or PBS vehicle control, by intranasal delivery. Samples (lungs, BAL fluid) were collected 48 hours later, as previously described (8, 11).

Administration of Blocking or Depleting Antibodies and Detection of Circulating Platelet–Neutrophil Complexes

Neutrophil depletion was achieved with three doses of monoclonal anti-Ly6G antibody (clone 1A8, 25 mg/kg

intraperitoneally; BioXCell) at −24, −1, and +24 hours relative to intranasal challenge based on previously reported work (30). For experimental blockade of adhesion molecule function, either an anti–P-selectin monoclonal antibody (clone RB40.34; BD Biosciences) or an anti–PSGL-1 monoclonal antibody (clone 4RA10; BioXCell) was administered (4 mg/kg intravenous in PBS) at −1 and +24 hours relative to intranasal LPS challenge, with dose and frequency selected based on previously published work investigating the effects of LPS inhalation in mice (3) and other reports (14, 31). Equivalent doses of

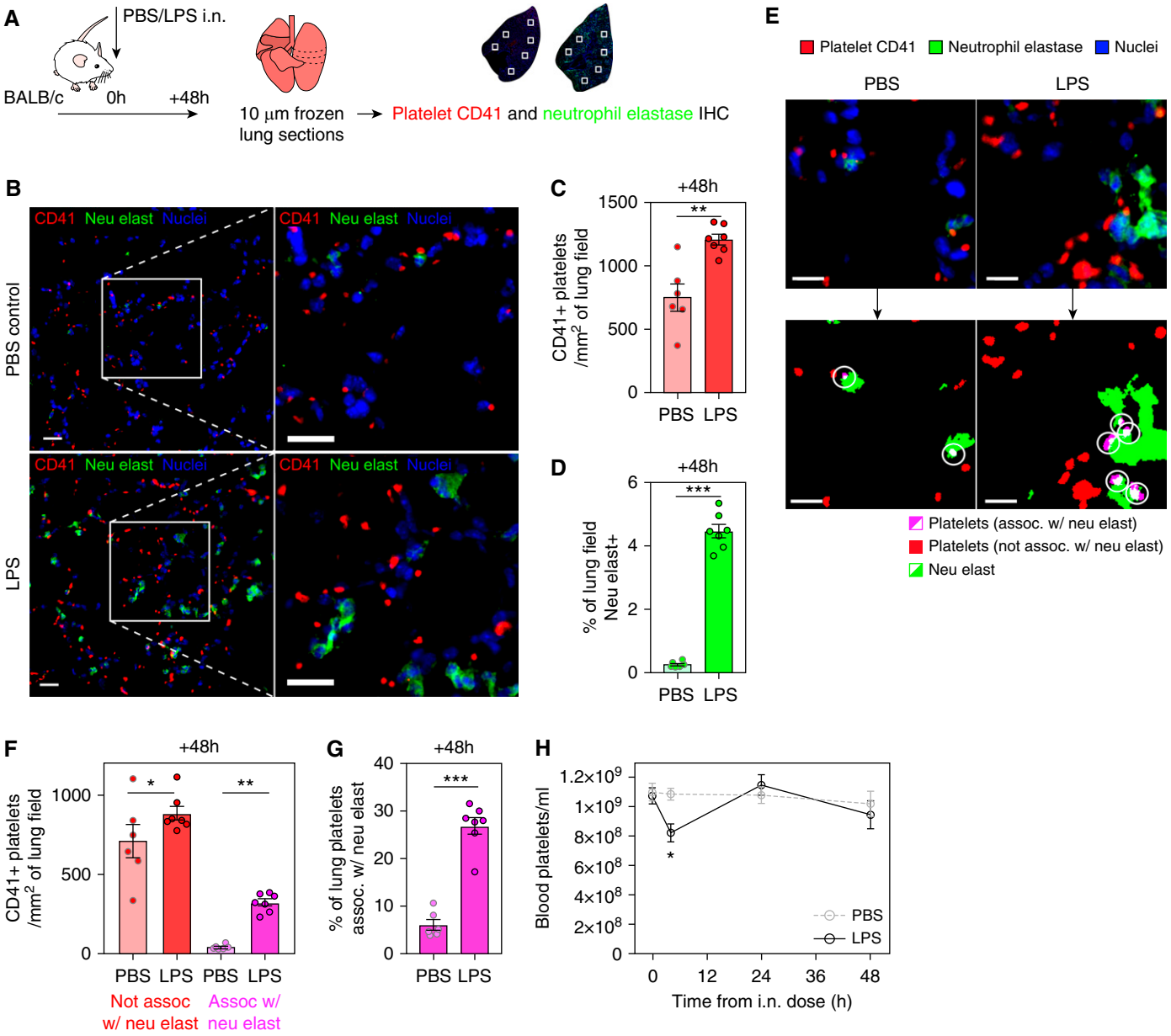

Figure 1. LPS inhalation causes platelet and neutrophil recruitment to the lungs, increased spatial associations of platelets in lungs with neutrophil elastase, and a transient decrease in blood platelet counts. (*A*) Mice were administered PBS or LPS (5 mg/kg) intranasally, and lungs collected at 48 hours. (*B*) Platelet CD41 and neutrophil elastase were stained in frozen lung sections using immunofluorescence. $CD41^+$ platelets are in red, neutrophil elastase staining is in green, and Hoechst-stained nuclei are in blue. (*C*) Quantification of number of $CD41^+$ platelets/mm^2 lung tissue and (*D*) neutrophil elastase staining. (*E*) Identification of visibly neutrophil elastase–associated platelets and platelets not associated with neutrophil elastase, with output from Cell Profiler shown below input images (neutrophil elastase–associated platelets recolored in magenta, with overlap in white, highlighted with white circles). (*F*) Quantification of platelets not visibly spatially associated, and those spatially associated with neutrophil elastase, and (*G*) percentage of platelets visibly spatially associated with neutrophil elastase. (*H*) Blood platelet counts from serial tail blood microsamples. Scale bars: 20 μm. Mean ± SE. Imaging: PBS group, $n = 6$; LPS group, $n = 7$; blood counts, both $n = 8$; unpaired *t* test or two-way ANOVA with repeated measures and Holm's test for LPS effects, Flow cytometric analysis was conducted with $n = 4$ per group, and analyzed using one-way ANOVA with Dunett's test between groups, $^*P < 0.05$, $^{**}P < 0.01$, and $^{***}P < 0.001$. IHC = immunohistochemistry; i.n. = intranasal; neu elast = neutrophil elastase.

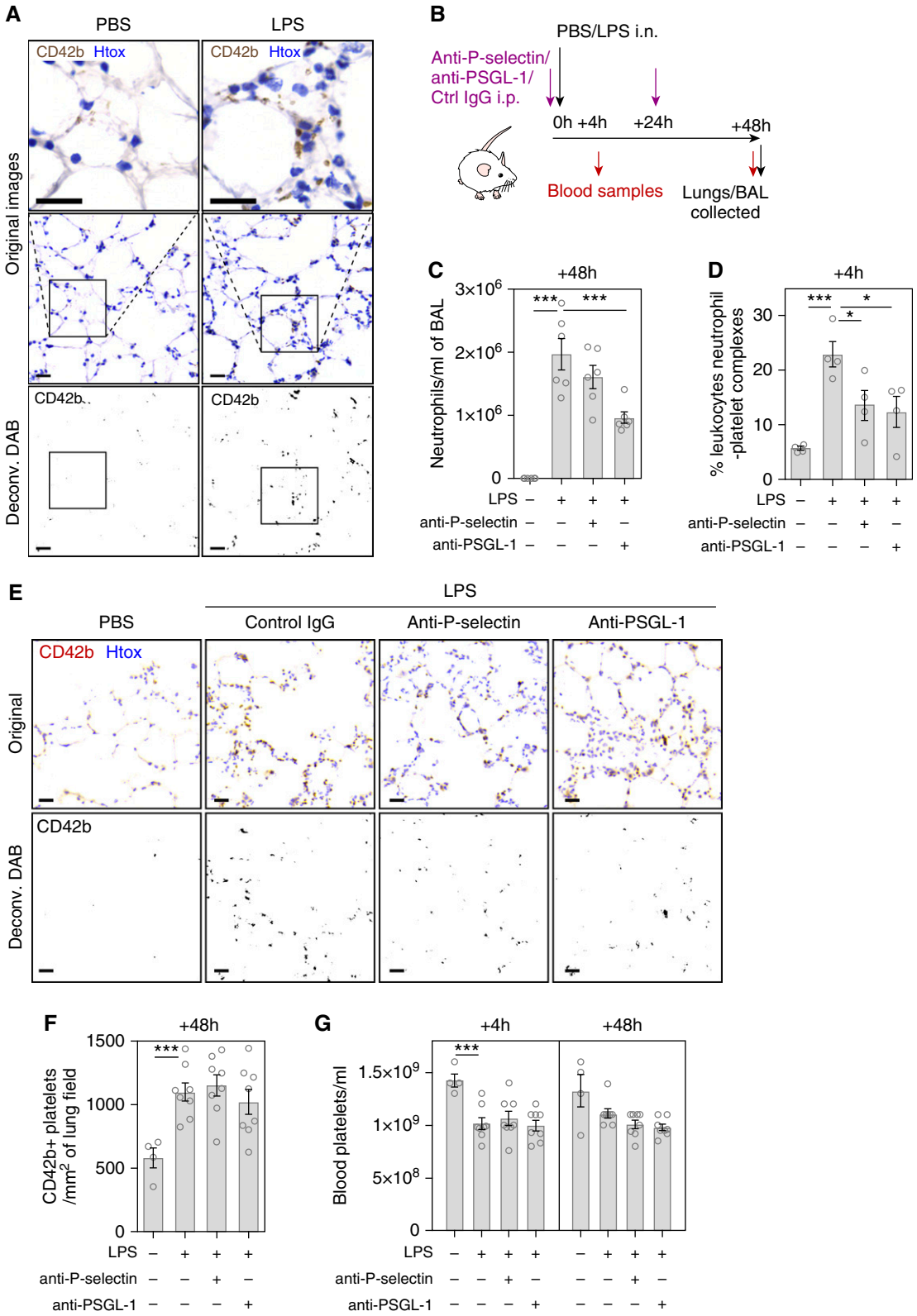

Figure 2. PSGL-1 blockade suppresses LPS-induced lung neutrophil recruitment but does not affect LPS-induced lung platelet recruitment. (*A*) Bright-field staining of platelet CD42b (brown, black in quantification output in bottom row) with nuclei counterstain (hematoxylin [Htox], blue). (*B*) Mice were administered control IgG, anti–P-selectin blocking antibody (RB40.34), or anti–PSGL-1 blocking antibody (4RA10) at 4 mg/kg intravenously at −1 hour and +24 hours in relation to intranasal administration of PBS or LPS. (*C*) BAL neutrophil counts. (*D*) In some mice, a cardiac puncture performed 4 hours

monoclonal anti-trinitrophenol (clone 2A3; trinitrophenol is not present in mammals; BioXCell) were administered to control mice. At 4 hours after challenge, some groups of mice were terminally anesthetized and whole blood collected via cardiac puncture into citrated syringes for flow cytometric analysis of circulating platelet–neutrophil complexes.

Intravital Microscopy

At 48 hours after PBS or LPS inhalation, Pf4-Cre × mTmG mice were anesthetized. The dorsal left ribcage was shaved, the trachea cannulated, and the mouse attached to a Mini Vent ventilator (Harvard Apparatus) with isoflurane and oxygen supplied at 130 × 250 μl breaths/min with 2 cm H_2O of positive end-expiratory pressure. Skin over the shaved region was removed, and an approximately 1-cm-long intercostal incision was made to expose the ventilated left lung and allow the surface of the visceral pleura to fall away from the parietal pleura. Body temperature was maintained using a heated stage (37°C) and mice administered PBS (0.5 ml intraperitoneally) as fluid support.

As previously reported (27, 32), a custom-made thoracic window was then inserted to sit between two ribs, secured onto a 90° angle post clamp attached to the stage, and 3–3.5 kPa negative pressure was applied between the visceral pleura of the lung and the inner surface of the window using an Amvex vacuum regulator to immobilize the lung and to remove air from the pleural cavity.

After around 10 minutes of stabilization, the lung was brought into focus on a custom Nikon A1R multiphoton microscope coupled with a MaiTai DeepSee infrared excitation laser tuned to 920 nm for simultaneous excitation of eGFP and tdTomato, using a ×25 objective Nikon water immersion lens, and emission filters set for tdTomato at 570–620 nm and for eGFP at 500–550 nm. Image capture was controlled using NIS Elements AR software (Nikon) with high-resolution bidirectional Galvano scanning over 512 × 512 pixels.

The internal surface of the final generation of alveoli, a densely networked capillary bed, was brought into focus, and viability of the mouse confirmed by observation of blood flow in the pulmonary vessels. Then, using a Z drive, the imaging plane was moved 10 μm deeper into the lung to establish an imaging plane at a consistent depth into the lung. Video recordings at the maximum speed possible using Galvano scanning (0.91 frames/s) were then made to produce datasets where platelet adhesion in the lung microvasculature could be quantified. For each mouse, eight videos of 230 × 230 μm fields with a duration of 5 minutes were sampled at different sites across the window, and videos were screened in a blinded fashion and excluded from analyses if stable imaging was not maintained, or if large blood vessels or megakaryocytes were in the sample region.

Analysis of Platelet Adhesion Using Intravital Image Sequences

Intravital image sequences were analyzed using NIS Elements 4.5 tracking. Platelets were identified by size and eGFP fluorescence intensity, and then platelet adhesion events were quantified by tracking platelets that did not move more than 2 μm/s between consecutive frames, whereas free-flowing or transiently adhesive platelets ("nonadhesive") were observed to appear and disappear between frames (Video E1).

Experimental Design and Analysis

For experiments using blocking or depleting antibody treatment interventions, the experimenter was blinded, and randomization within cage blocks was used to assign mice into treatment groups. Other experiments were performed unblinded, with blinding applied for analysis of samples or images.

Comparisons between two independent groups were made using two-tailed unpaired Student's *t* tests. Correlations were analyzed using Pearson's test to derive the r^2 value describing goodness of fit, with an *F* test for the probability that r^2 values from experimental data would be generated from randomly generated datasets with no correlation. Where appropriate, single- or repeated-measures one-way ANOVA, two-way ANOVA, three-way ANOVA, or three-way analysis of covariance with baseline values fit as covariates were used.

P values reported are from *t* tests, *F* tests on Pearson's correlations, or multiplicity-adjusted *P* values (Dunnett's or Holm's adjustments, where appropriate). The threshold for statistical significance was set at *P* less than 0.05.

Data are graphed and reported as means (±SE). Group sizes (*n*) are listed in the figure legends. Excel 2016 (Microsoft Office), InVivoStat 3.6 (InVivoStat), and Prism 6.0 (Graphpad Software).

Results

Platelets and Neutrophils Are Recruited to the Lungs after LPS Administration in Mice

To record platelet and neutrophil recruitment in the same lung tissue sections of mice administered intranasal LPS, we undertook histological analysis of frozen lung sections stained for CD41 (integrin α-IIb, platelet marker), and neutrophil elastase using immunofluorescence (Figures 1A and 1B). Quantitative analysis of tissue sections revealed a significant increase in pulmonary platelet recruitment in mice administered LPS compared with PBS-administered control mice (Figure 1C), as well as an increase in lung neutrophil elastase staining (Figure 1D). Platelets in lung sections appeared larger with LPS inhalation, although the majority of platelets in sampled fields resembled single platelet events, and did not display the morphology of megakaryocytes or thrombi (Figure E1A).

LPS inhalation was previously shown to decrease blood platelet counts (8), and LPS challenge in the present study also decreased blood platelet counts at 4 hours after challenge, but this thrombocytopenia was transient, returning to levels observed in control mice at 24 hours after challenge

Figure 2. (Continued). after LPS administration to measure, via flow cytometric analysis, the percentage of leukocytes identified to be neutrophils bound to platelets. (*E*) Platelet CD42b immunostaining with CD42b DAB reporter positivity (brown in top row, with Htox counterstain in blue) and deconvolution of DAB signal (black in bottom row, from same images as top row) for image analysis. Scale bars: 20 μm. (*F*) Quantification of $CD42b^+$ platelets in lung tissue. (*G*) Blood platelet counts from serial tail microsamples. Means ± SE, (*F* and *G*) PBS group, $n = 4$; LPS groups, $n = 8$; (*C*) PBS group, $n = 8$; LPS groups, $n = 16$. (*D*) PBS group, $n = 4$; LPS groups, $n = 4$. (*C*, *D*, and *F*) One-way ANOVA, or (*G*) two-way ANOVA, with Dunnett's or Holm's test for difference versus LPS + control IgG group, $^*P < 0.05$ and $^{***}P < 0.001$. Ctrl = control; DAB = 3,3′diaminobenzidine; i.p. = intraperitoneal.

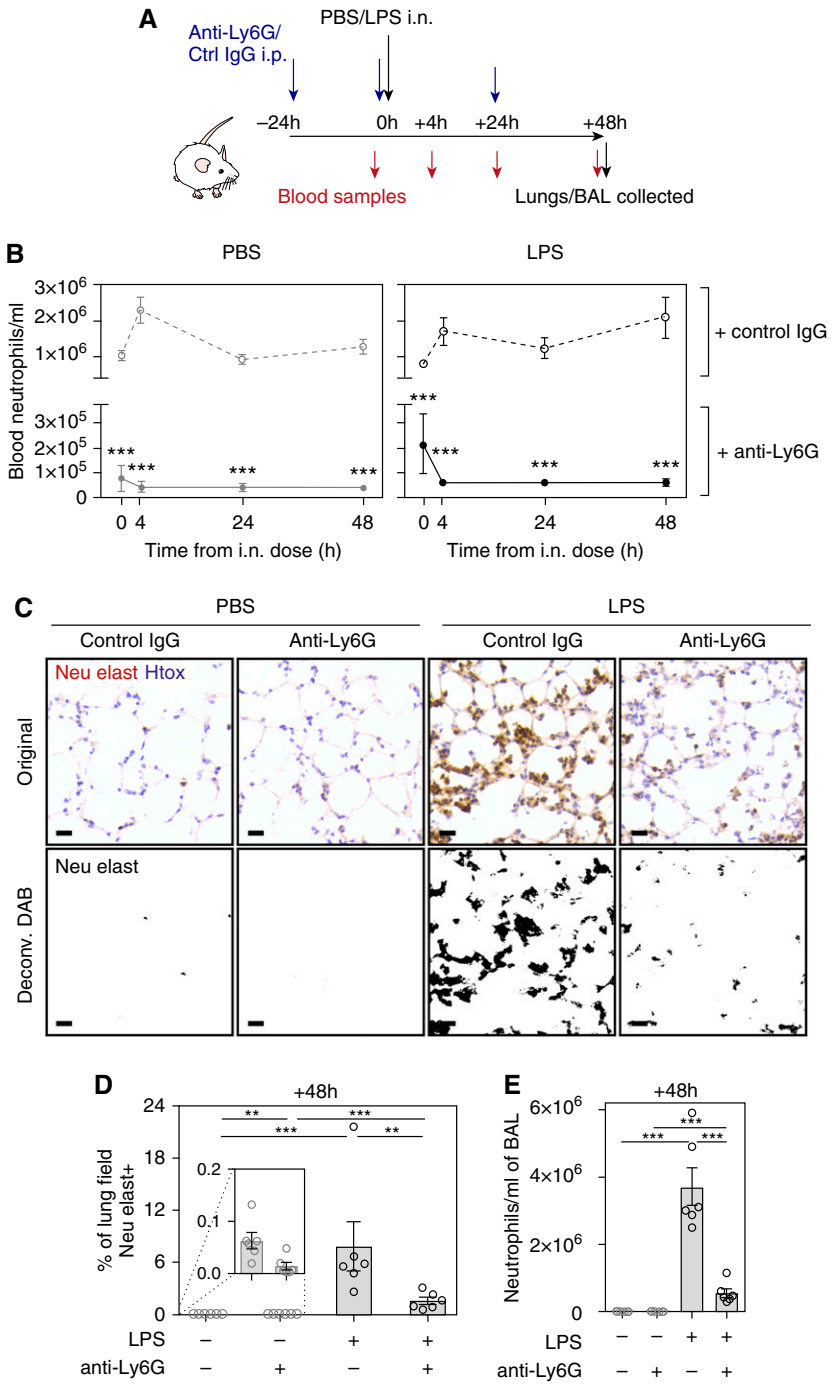

Figure 3. Treatment with anti-Ly6G antibody depletes neutrophils in blood and lungs. (*A*) Mice were administered either control IgG or anti-Ly6G antibody at −24 hours, −1 hour, and +24 hours in relation to intranasal administration of PBS or LPS (5 mg/kg). (*B*) Serial tail blood microsamples were used to quantify blood neutrophils immediately before (0 h), and at +4, +24, and +48 hours after PBS or LPS administration (note break in axis). (*C*) Neutrophil elastase was immunostained in lung sections, and in sample fields, neutrophil elastase DAB reporter positivity (brown in top row) was deconvoluted (black in bottom row, from same images as top row) for image analysis. Scale bars: 20 μm. (*D*) Quantification of neutrophil elastase in lung sections. (*E*) Other groups of mice were killed at 48 hours and BAL neutrophils were quantified. Means ± SE, (*A*) $n = 12$, (*D* and *E*) $n = 6$. Data analyzed via: (*A*) three-way ANOVA with repeated measures and Holm's test for LPS and anti-Ly6G treatment effects within time points, or (*D* and *E*) two-way ANOVA with Holm's test for LPS and anti-Ly6G treatment effects, $^{**}P < 0.01$ and $^{***}P < 0.001$.

(Figure 1H), providing evidence that changes in platelet numbers in lung sections containing blood at 48 hours after LPS were due to platelet recruitment to lungs rather than changes in numbers of circulating blood platelets.

Immunofluorescence staining allowed for identification of spatial associations of platelets with neutrophil elastase within lung sections (Figure 1E). LPS inhalation increased the quantity of platelets both associated with and not associated with neutrophil elastase in the lungs (Figure 1F), with an increase in the percentage of platelets that were neutrophil elastase associated (Figure 1G). Interventional studies were therefore performed to further investigate whether interactions of platelets with neutrophils were a causative factor in LPS-induced lung platelet recruitment.

P-Selectin and PSGL-1 Are Dispensable for LPS-induced Lung Platelet Recruitment

Previous observations of the increased presence of neutrophil-associated platelets in the lungs after LPS administration in lung sections (Figures 1F–1H), using intravital lung imaging (8, 11), and the requirement of platelets and contact-dependent PSGL-1 interactions for LPS-induced pulmonary neutrophil recruitment (3) led us to speculate that platelet recruitment might be dependent on the formation of platelet–neutrophil conjugates mediated through PSGL-1. A bright-field method was developed for higher-throughput quantification of the platelet marker, CD42b (GP1bα; Figure 2A), as well as for neutrophil elastase (Figure E1C), and the involvement of P-selectin and PSGL-1 in LPS-induced lung platelet recruitment was tested by administering functional blocking antibodies to mice (Figure 2B).

As reported previously (3), the administration of an anti–PSGL-1 blocking antibody led to significant and selective reduction in pulmonary neutrophil recruitment to lung airspaces (Figures 2C and Figures E2A and E2B). Indeed, 4 hours after LPS exposure, the percentage of events that are neutrophils attached to platelets in the circulating leukocyte population was significantly increased in LPS compared with PBS administered mice ($P < 0.001$, Figure 2D). The administration of an anti–PSGL-1 blocking antibody or anti–P-selectin blocking antibody led to a significant reduction in the percentage of

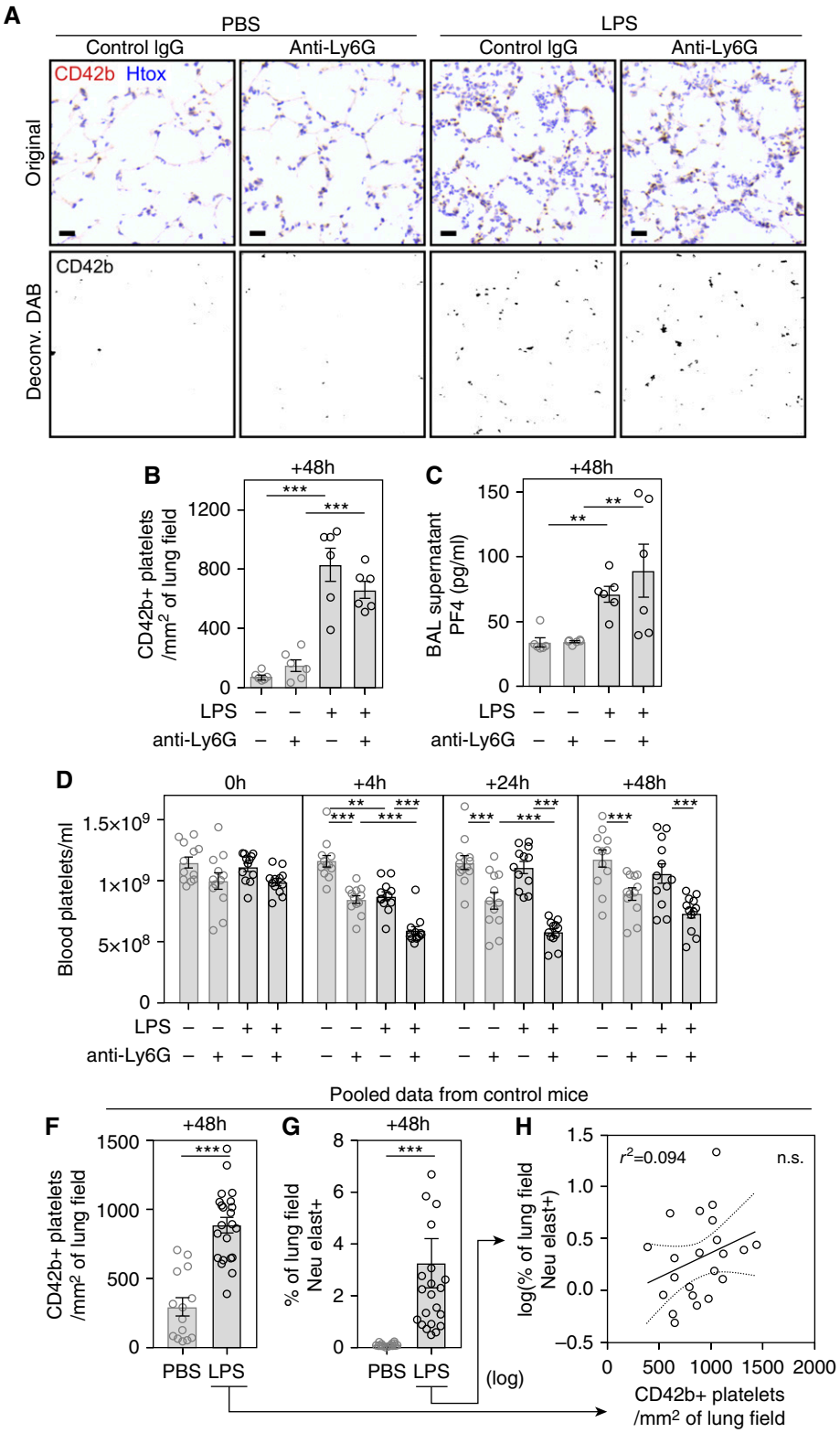

Figure 4. LPS-induced lung platelet recruitment is unaffected by neutrophil depletion. Mice were administered either control IgG or anti-Ly6G antibody at −24, −1, and +24 hours in relation to intranasal administration of PBS or LPS (5 mg/kg). (*A*) Platelet CD42b was immunostained in lung sections, and in sample fields CD42b DAB reporter positivity (brown in top row) was deconvoluted (black in bottom row, from same images as top row) for image analysis. Scale bars: 20 μm.

events that are neutrophils attached to platelets in the circulating leukocyte population attached to platelets ($P < 0.05$, Figure 2D). In contrast, the presence of platelets in the lungs was not reduced in mice administered either an anti–P-selectin or anti–PSGL-1 blocking antibody (Figures 2E and 2F). Together, these data suggest that platelet recruitment to lungs after LPS inhalation occurs via a distinct mechanism compared with the mechanisms required for neutrophil recruitment, which show dependence on PSGL-1.

In addition, neither P-selectin nor PSGL-1 blockade affected the decrease in blood platelet counts at 4 hours after LPS inhalation (Figure 2G), whereas PSGL-1 blockade decreased blood mononuclear cell counts at 4 hours, and blood neutrophil counts at 48 hours; P-selectin blockade increased blood neutrophil counts 48 hours after LPS inhalation (Figures E2C–E2E). The latter effect is a potential mechanism for increases in neutrophil elastase staining in lung sections with anti–P-selectin antibody treatment (Figures E2G and E2H).

LPS-induced Platelet Recruitment to Lungs Is Not Affected by Neutrophil Depletion

To investigate whether pulmonary platelet recruitment was dependent on neutrophils, mice were depleted of circulating neutrophils using an anti-Ly6G antibody (Figure 3A). Anti-Ly6G antibody administration depleted neutrophils from circulating blood in both PBS control mice and in LPS-challenged mice in which inflammation drives greatly enhanced neutrophil production after LPS inhalation over the entire 48 hours (Figures 3B and 3E). The anti-Ly6G antibody treatment also reduced neutrophil elastase staining in both control and LPS-challenged lungs (Figures 3C and 3D), and reduced neutrophil recruitment to lung airspaces (Figure 3E), with monocyte recruitment to lung airspaces unaffected (Figures E3A and E3B). Circulating blood mononuclear cells were not affected by the administration of anti-Ly6G antibody, apart from 4 hours after LPS administration (Figures E3C and E3D), possibly due to the involvement of mononuclear cells in antibody-mediated neutrophil clearance (33).

The depletion of neutrophils had no detected effect on lung platelet recruitment (Figures 4A and 4B). Furthermore, LPS administration resulted in an increase in the platelet granule protein, Pf4, in BAL supernatant, which was not affected by the

depletion of neutrophils (Figure 4C). The immunodepletion of neutrophils resulted in a reduction in circulating blood platelet counts, irrespective of, and in addition to the effects of, LPS administration at 4, 24, and 48 hours (Figure 4D), which could be anticipated to bias toward a finding of decreased lung platelet recruitment in lung sections containing blood. There was a complete absence of CD42b events (platelets) in the lungs of mice depleted of platelets using an anti–platelet-depleting antibody, or using the staining protocol in the absence of the primary detection antibody, to signify the robustness of the imaging system and quantification methods in the absence of a PSGL-1–, P-selectin–, or neutrophil-dependent effect (Figure E4).

We also hypothesized that, if platelet recruitment was dependent on neutrophil recruitment, mice with high LPS-induced neutrophil recruitment to lungs would have high LPS-induced lung platelet recruitment. Lung platelet (CD42b) and neutrophil elastase staining quantifications from serial sections from all LPS-treated mice were combined (pooled from control mouse data in Figures 2E, 3D, and B4B, and eight additional mice [data shown in Figures E5A and E5B, shown together in Figures 4F and 4G), and tested for correlation. No significant correlation between extent of neutrophil and platelet recruitment was observed (Figure 4H). In addition, the extent of lung platelet (CD41) and neutrophil elastase staining showed no evidence of any correlation (Figure E5C, from data in Figures 1C and 1D), again providing no evidence supportive of neutrophil dependence of LPS-induced platelet recruitment.

Imaging LPS-induced Platelet Recruitment in Living Lungs Reveals Increased Platelet Adhesion in the Lung Microvasculature without Platelet Aggregation

To further understand the nature of the interactions of platelets with the pulmonary vasculature, and to directly image the effects of LPS inhalation on platelets in the living lung microcirculation, two photon intravital microscopy video recordings were made in lungs of Pf4-Cre × mTmG mice at 48 hours after intranasal exposure to LPS or PBS. The effect of LPS inhalation on the quantity of lung platelet–leukocyte interactions imaged using intravital microscopy has been previously explored using simultaneous genetic labeling of platelets and leukocytes in mice (11), but total platelet recruitment has not previously been measured. Two-photon intravital microscopy in Pf4-Cre × mTmG mice, reporter mice with high eGFP fluorophore density on platelet membranes, enabled improved imaging of single platelets passing through the lung microvasculature (Figure 5A), as previously reported (27). Image sequences were captured at 0.91 frames/s for 5 minutes from several fields across the visible lung, and tracking software was used to isolate adhesive platelets, which did not move faster than 2 μm/s over three or more frames, from platelets which were free flowing in the bloodstream that appeared and disappeared with each frame (Figure 5B and Video E1). LPS inhalation increased the number of adhesive platelets detected in lungs, and no time effect was detected, indicating stable levels of platelet adhesion during acquisition of images (Figures 5C and 5D). The mean duration of platelet adhesion events increased, although not significantly, after LPS inhalation (Figure 5E). The imaged microvasculature received blood flow during imaging, and nonadhesive platelets were observed in quantities that did not differ between mice challenged with LPS or PBS controls (Figures 5F and 5G). It was confirmed that mice used for imaging had raised inflammatory responses to LPS (Figure 5H).

Live imaging revealed that platelet recruitment to the lungs appeared to require vascular adhesion, and platelets did not appear to be attached to neutrophils, although, in the microvasculature, leukocytes were not easily discriminated from other Pf4− tdTomato+ cells in the lung. Furthermore, platelets appeared as single platelets or small aggregates, no gross platelet aggregation was evident in the lung microvasculature, and the LPS-induced platelet adhesion events were visually distinct from thromboemboli induced by intravenous infusion of 100 IU/kg human α-thrombin (Enzyme Research Laboratories; Figure 5I and Video E2), or hemostatic thrombosis occurring as a result of incision into the lung surface during intravital window insertion (Figure 5J and Video E3). Furthermore, the administration of the direct thrombin inhibitor, dabigatran etexilate, at a dose sufficient to significantly increase tail bleeding time (Figure E6A) and suppress *ex vivo* platelet aggregation toward thrombin from pooled platelet-rich plasma (Figure E6B), did not suppress platelet recruitment to the lungs in response to LPS (PBS + vehicle, 320.1 ± 180.0 platelets/mm^2; LPS + vehicle, 806.6 ± 314.1 platelets/mm^2; LPS + dabigatran, 757.7 ± 490.2 platelets/mm^2; Figure E6C, data not significantly different between groups) or event size (Figure E6D, data not significantly different between groups). This suggests that platelet adhesion was not likely to be dependent on fibrin deposition in the lungs as a result of thrombin activation.

As previously reported (27), occasional megakaryocyte thrombopoeisis events were observed that had an appearance distinct from adhesive platelets, with these large cells captured in small arterioles and moving over minutes to extend proplatelet projections (Figure 5K and Video E4), although sample regions and durations required for platelet adhesion tracking were insufficient for quantification of megakaryocyte thrombopoiesis.

Discussion

This is the first report investigating the relationship of neutrophils with lung platelet recruitment in response to bacterial LPS administered via inhalation. We show that,

Figure 4. (Continued). (*B*) Quantification of platelets in lung sections. (*C*) Separate groups of mice were killed at 48 hours after intranasal challenge for BAL for the measurement of platelet factor 4 (PF4) via ELISA. (*D*) Blood platelet counts from serial tail blood microsamples. (*F*) CD42b$^+$ lung platelet counts and (*G*) extent of lung neutrophil elastase staining data pooled from all PBS and LPS-challenged control mice and additional mice (Figures E4A and E4B) were used for correlation measurements of extent of neutrophil elastase staining versus lung platelet staining from mice administered with LPS with line of best fit and residuals. One neutrophil elastase reading of 21.65% coverage (Figure 3D) is not shown in (*G*) for scaling reasons but was included in analyses, and exclusion did not affect the conclusion of the analysis. Means ± SE, (*B* and *C*) $n = 6$ or (*D* and *E*) $n = 12$. Analysis was (*B* and *C*) two-way ANOVA or (*D*) three-way ANOVA with repeated measures, all followed by Holm's test for LPS and anti-Ly6G treatment effects. (*F* and *G*) Unpaired *t* test and (*H*) Pearson's correlation, r^2 and result of *F* test for significantly non-zero slope shown, $^*P < 0.05$ and $^{***}P < 0.001$. n.s. = not significant.

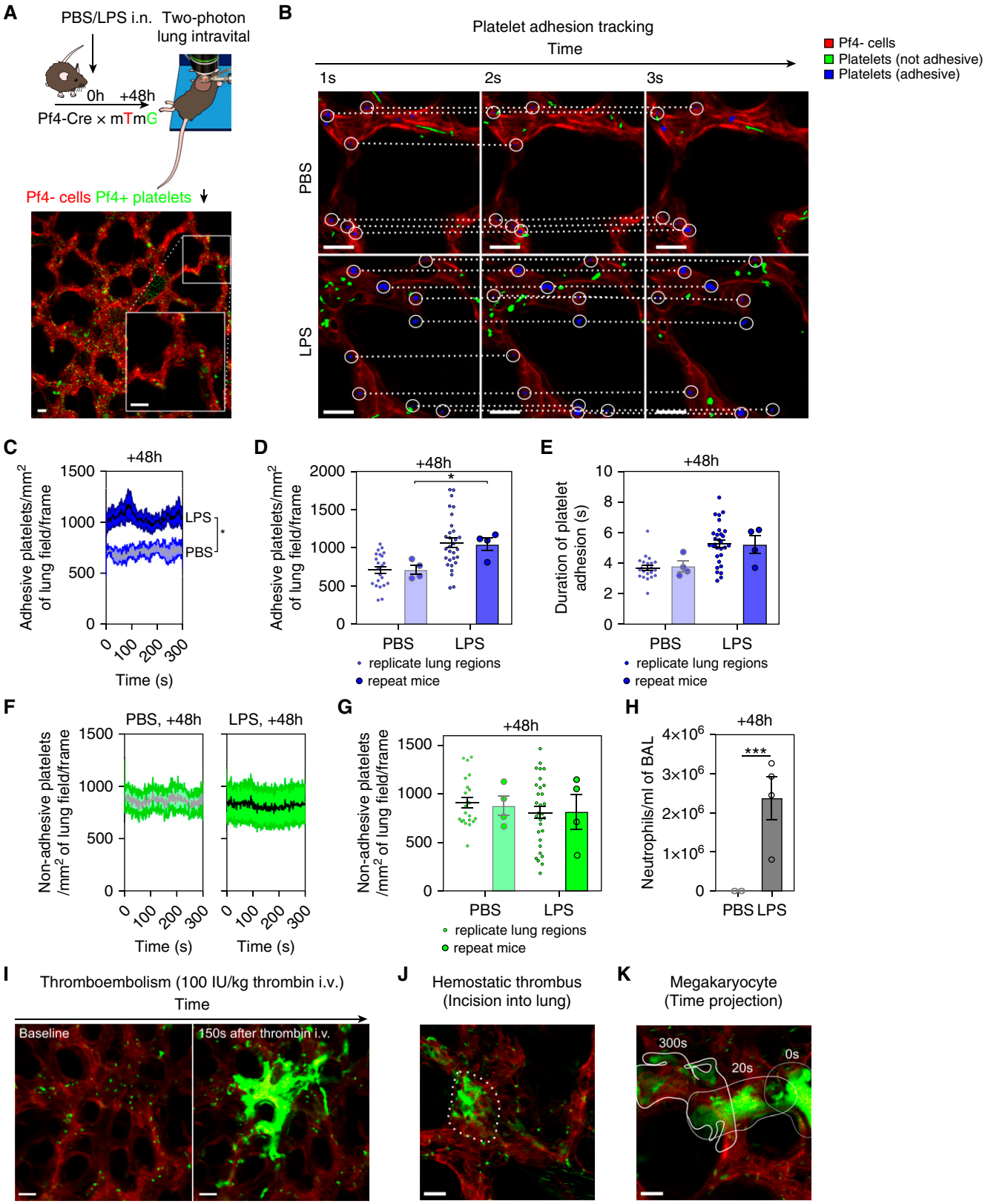

Figure 5. LPS inhalation increases platelet adhesion in lungs, which is distinguishable from lung hemostatic thrombosis and thromboembolism. (*A*) Pf4-Cre × mTmG mice were administered PBS or LPS (5 mg/kg) intranasally, and 48 hours later were prepared for two-photon lung intravital microscopy to image tdTomato$^+$ lung cells (red) and enhanced GFP (eGFP)$^+$ platelets (green). (*B*) Diagram showing the identification of adhesive platelets (blue)

despite the close association of recruited platelets with neutrophils in the lungs, ultimately, platelet recruitment showed no dependence on the corecruitment of neutrophils, nor P-selectin– or PSGL-1–mediated interactions classically associated with platelet-dependent neutrophil recruitment. It is possible that platelet interaction with the vascular endothelium after LPS administration is dependent on other adhesive mechanisms that are attributable to platelet adhesion to damaged vessel walls during trauma, for example, platelet GPIb interacting with von Willebrand factor, or platelet GPIIb/IIIa binding to fibrinogen on the vessel wall (34–36). However, the administration of a direct thrombin inhibitor (dabigatran etexilate) in this LPS model did not affect platelet recruitment, suggestive that fibrin was not an adhesive substrate for platelets under these conditions. Furthermore, the fact that the bleeding time is not different between mice administered LPS and those administered PBS suggests that disseminated intravascular coagulation does not occur in this model.

The relationship between platelets and neutrophils during the innate immune response is complex and involves multiple processes. An overriding theme of this relationship is the requirement for platelets to increase the efficiency of neutrophil recruitment to areas of inflammation or infection. Selectin, and integrin-mediated events, provide an increase in platelet–neutrophil circulating complexes in blood (3), increased neutrophil rolling and adhesion events on the endothelial surface (12), increased neutrophil intravascular crawling (14), and increased neutrophil diapedesis into tissue (15). Furthermore, platelets are sometimes found associated with extravascular neutrophils (11, 13). Interestingly, although no evidence was found in the present study for requirement of neutrophils of pulmonary platelet recruitment, we still observed a highly significant increase in the spatial association of platelets with neutrophils in the lungs after LPS administration. Platelets influence neutrophil effector processes in a contact-dependent manner; for example, free-radical formation and phagocytosis (37), transcellular metabolism of proinflammatory lipids (38), and involvement in NET formation (39–41). It is not known if these neutrophil activation processes occur due to a different population of platelets accessing lung tissue that are not initially associated with intravascular neutrophil recruitment events, but this is something worthy of follow-up experiments.

These findings contrast with previous reports studying the effects of anti-Gr1 antibody–mediated neutrophil depletion on lung recruitment of radiolabeled platelets after intraperitoneal injections of LPS (19), or after priming with intraperitoneal administration of LPS, followed by intravenous anti–major histocompatibility complex I antibody administration in a model of transfusion-related acute lung injury (20). This contrast may be explained by differences between anti-Ly6G (1A8) and anti-Gr1 (RB6-8C5) antibodies, with the latter depleting $Ly6C^+$ inflammatory monocytes in addition to $Ly6G^+$ neutrophils (30), a difference that can have functional consequences in some bacterial infection models where monocytes, but not neutrophils, are critical for host defence (42). In addition, different routes of LPS administration can produce differing inflammatory responses. For example, in "organ-on-a-chip" models, platelet recruitment to human lung endothelial cells requires epithelial rather than endothelial exposure to LPS (43), intraperitoneal LPS administration promotes lung neutrophil recruitment as a distant organ affect away from the inflammatory focus in the peritoneum, and the two-hit model of transfusion-related acute lung injury, where LPS acts as a priming agent, involves a rapid intravascular response in the lung in which injury is not dependent on PSGL-1 (20). Furthermore, unlike previous reports, we were unable to detect pulmonary platelet recruitment at early time points (4 h after administration of LPS; data not shown) (19). However, platelet sequestration might be occurring in different tissues at this earlier time point in our model, made evident by thrombocytopenia at 4 hours after LPS administration.

Intravital imaging experiments in living lungs provide further evidence that platelet activation and recruitment in inflammation contrasts with that in hemostasis and thrombosis (18, 44), as platelet adhesion to the pulmonary vascular endothelium occurred as single events in the absence of platelet aggregation. Intravital microscopy experiments have recently demonstrated a novel immune function of platelet adhesion in which platelets migrate on endothelial cells in the infected liver endothelium to accumulate fibrinogen-bound bacteria. This intravascular crawling of lone platelets was shown to still occur after neutrophil and monocyte depletion with anti-Gr1 (45). However, the efficiency of platelet intravascular crawling in these circumstances is difficult to determine (45). The convoluted three-dimensional anatomy of the lung capillary network, lung movements during ventilation, and the sterile LPS model prevented measurements of directed platelet migration in the lungs from imaging reported in the present study, but the platelet adhesion observed in lungs after LPS inhalation is indicative that a similar platelet migration response may occur in the inflamed lung. Indeed, inhalation of LPS (11), bacteria (13), and other inflammatory insults (46) can promote migration of platelets into airspaces, and platelets are important for limiting lung infection by *Klebsiella pneumoniae* (47), *Streptococcus pneumoniae* (48), and *Pseudomonas aeruginosa* (13). The distinct recruitment of platelets from other recruited inflammatory cells might suggest a role for platelets in the inflammatory response

Figure 5. (Continued). compared with the temporary sightings of platelets free flowing in blood (green) over 3 seconds, with white dotted lines demonstrating between-frame platelet tracking (*see* Video E1). (*C*) Quantification of adhesion events over 300-second sample image sequences, and (*D*) mean summary data showing data from each video and mean data from each mouse used for statistical comparisons. (*E*) Mean adhesive platelet adhesion duration data from each field of lung sampled with means from each mouse. (*F*) Quantification of platelet events, which were not tracked between frames over time (either freely flowing in blood or making interactions too transient for tracking), and (*G*) mean summary data from each field of lung sampled with means from each mouse. (*H*) Neutrophil counts in BAL from mice used in intravital experiments, sampled after completion of imaging. (*I*) Pulmonary thromboembolism induced by injection of 100 IU/kg of thrombin intravenously during imaging. (*J*) Appearance of hemostatic platelet thrombi 20 minutes after incision into lung surface (highlighted with white dotted line, *see* Video E2). (*K*) Appearance of megakaryocyte trapping and thrombopoeisis in the lung (highlighted with white line, *see* Video E3). Scale bars: 20 μm. Means ± SE; $n = 4$; two-way ANOVA with repeated measures or unpaired *t* tests, $^*P < 0.05$ and $^{***}P < 0.001$. i.v. = intravenous.

in their own right. Although it is not yet evident whether direct platelet microbicidal activity is important for host defense, platelets do contain potent microbicidal activity both *in vitro* and *in vivo* (41, 49).

Interventional studies reported here revealed that neutrophil depletion and subsequent LPS inhalation did not cause shock and mortality, as reported with intravenous LPS or TNF-α priming followed by intravenous anti-Gr1 (RB6–8C5) or anti-Ly6G (1A8) antibodies (50, 51). However, modest decreases in body weight and platelet counts in anti-Ly6G–treated mice in both PBS- and LPS-treated mice suggested that some milder adverse effects were present, and were in addition to LPS effects. It is possible that these effects in LPS-challenged mice relate to a requirement for neutrophil myeloperoxidase to limit inflammation after exposure to high doses of LPS (52).

The findings that blockade of P-selectin increased, while PSGL-1 blockade decreased, blood neutrophil counts in the context of lung inflammation are of note for several reasons. First, they agree with knockout mouse studies in which genetic deletion of P-selectin increased steady-state blood neutrophil counts (53), but contrast with data from PSGL-1 knockout mice in which baseline neutrophil counts are elevated (54). Second, these findings implicate P-selectin and PSGL-1 as potential molecular mediators of blood neutrophil homeostasis under pressure from acute inflammation. Finally, it is possible that anti–PSGL-1 antibodies, which have been characterized as having a blocking function, may potentially deplete circulating neutrophils during inflammation (14, 55).

To conclude, we report, for the first time, the lack of neutrophil-dependence of platelet recruitment in lung inflammation after LPS inhalation, a model where lung airspaces are the inflammatory focus and neutrophils are the major cellular infiltrate. The lung platelet recruitment did not depend on P-selectin– or PSGL-1–mediated adhesive interactions, and involved platelet adhesion in the absence of platelet aggregation. These findings provide further support for a role for platelets in host defense and inflammation in the lungs that is mechanistically separate from neutrophil recruitment, and distinct from platelet adhesion in thrombosis and hemostasis. ■

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