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Research Article

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There is emerging evidence that platelets are major contributors to inflammatory processes through intimate associations with innate immune cells. Here, we report that activated platelets induce the formation of neutrophil extracellular traps (NETs) in transfusion-related acute lung injury (TRALI), which is the leading cause of death after transfusion therapy. NETs are composed of decondensed chromatin decorated with granular proteins that function to trap extracellular pathogens; their formation requires the activation of neutrophils and release of their DNA in a process that may or may not result in neutrophil death. In a mouse model of TRALI that is neutrophil and platelet dependent, NETs appeared in the lung microvasculature and NET components increased in the plasma. We detected NETs in the lungs and plasma of human TRALI and in the plasma of patients with acute lung injury. In the experimental TRALI model, targeting platelet activation with either aspirin or a glycoprotein IIb/IIIa inhibitor decreased NET formation and lung injury. We then directly targeted NET components with a histone blocking antibody and DNase1, both of which protected mice from TRALI. These data suggest that NETs contribute to lung endothelial injury and that targeting NET formation may be a promising new direction for the treatment of acute lung injury.

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# Platelets induce neutrophil extracellular traps in transfusion-related acute lung injury

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**There is emerging evidence that platelets are major contributors to inflammatory processes through intimate associations with innate immune cells. Here, we report that activated platelets induce the formation of neutrophil extracellular traps (NETs) in transfusion-related acute lung injury (TRALI), which is the leading cause of death after transfusion therapy. NETs are composed of decondensed chromatin decorated with granular proteins that function to trap extracellular pathogens; their formation requires the activation of neutrophils and release of their DNA in a process that may or may not result in neutrophil death. In a mouse model of TRALI that is neutrophil and platelet dependent, NETs appeared in the lung microvasculature and NET components increased in the plasma. We detected NETs in the lungs and plasma of human TRALI and in the plasma of patients with acute lung injury. In the experimental TRALI model, targeting platelet activation with either aspirin or a glycoprotein IIb/IIIa inhibitor decreased NET formation and lung injury. We then directly targeted NET components with a histone blocking antibody and DNase1, both of which protected mice from TRALI. These data suggest that NETs contribute to lung endothelial injury and that targeting NET formation may be a promising new direction for the treatment of acute lung injury.**

## Introduction

Almost 200,000 persons in the US develop acute lung injury (ALI) every year (1) from a variety of causes, including sepsis, bacterial pneumonia, aspiration of gastric contents, and epidemic viruses, such as H1N1 and SARS. ALI mortality remains high, approaching 40% (1), and despite extensive research into the pathogenesis of ALI and many clinical trials testing new therapeutics, there remains no effective pharmacotherapy to treat patients with this syndrome. More research is desperately needed to identify novel pathways that can be targeted with new treatment approaches.

Blood transfusions are one of the well-recognized causes of ALI. This syndrome, which has been termed transfusion-related ALI (TRALI), is the leading cause of death from transfusion therapy in the US (2) and a major cause of transfusion morbidity, but its pathogenesis is poorly understood (3). TRALI is an especially troubling condition for health care providers, since it is an unintended and unpredictable consequence of physician-directed care. A generally agreed upon threshold or multiple-event model for the pathogenesis of TRALI posits that susceptible patients develop TRALI after receiving a blood transfusion containing antibodies to human leukocyte antigens (HLAs), human neutrophil antigens, or other bioactive mediators (4). We have studied the antibody theory of TRALI using a 2-event model that primes mice with low-dose LPS, followed by challenge with cognate MHC class I antibody (5, 6). This model strongly resembles human TRALI, and it produces severe, neutrophil-mediated ALI within minutes of antibody challenge (5).

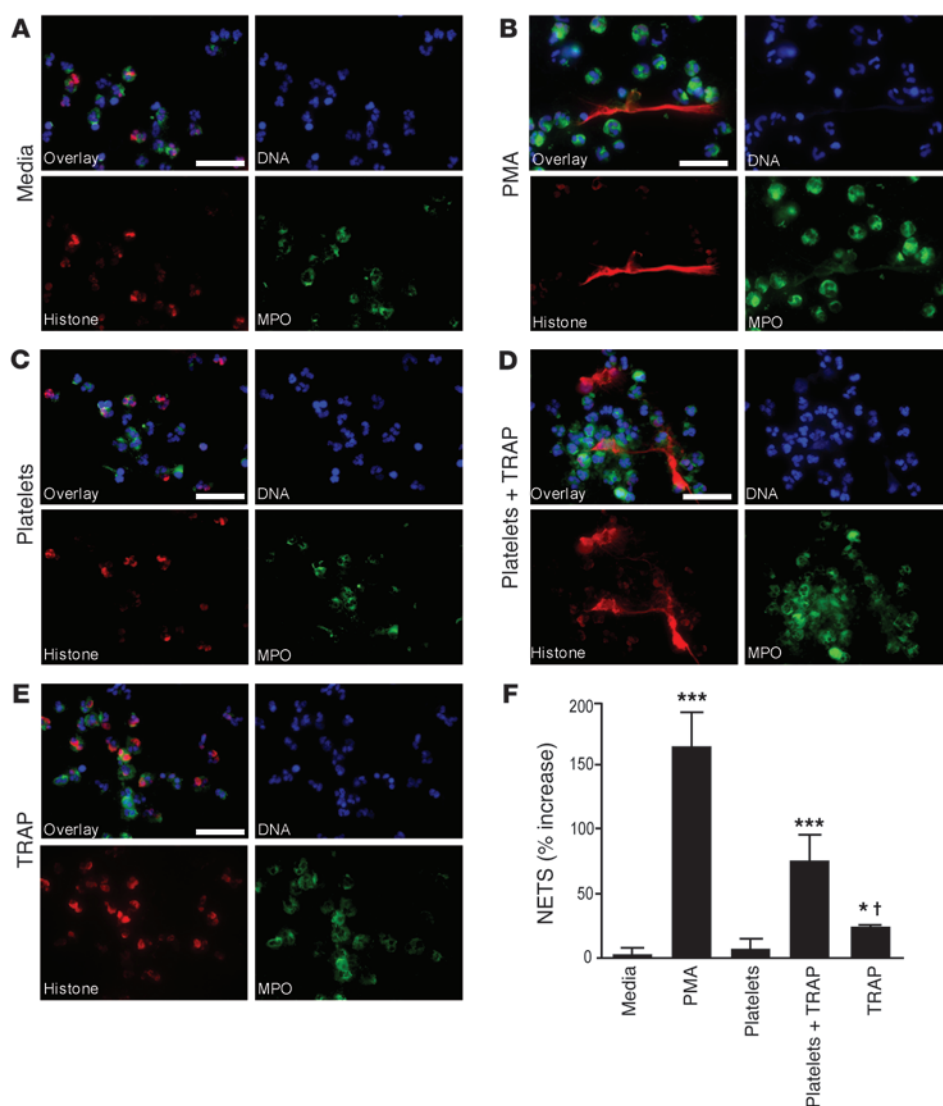
Evidence is accumulating that platelets are major contributors to acute inflammation and injury (7) in conditions such as rheumatoid arthritis (8) and cerebral malaria (9) as well as

ALI (10). Indeed, we have shown that the murine TRALI model is critically dependent on platelets (5). Platelets become sequestered in the lung microcirculation in a neutrophil-dependent process, and platelet depletion protects mice from severe lung injury and mortality. When platelet activation is blocked with aspirin, plasma thromboxane production decreases, as do lung injury and mortality (5). However, the mechanisms by which either neutrophils or platelets injure the lung endothelium, and the potential critical interactions between neutrophils and platelets, are unknown.

Here, we focused on neutrophil extracellular traps (NETs) as a potential explanation for the neutrophil- and platelet-dependent lung damage in TRALI. Activated neutrophils have recently been described to undergo NETosis, a unique type of cell death that is distinct from apoptosis and necrosis, in which neutrophil nuclear DNA is released in long chromatin filaments that form web-like structures decorated with granular proteins, called NETs (11). These NETs have been shown to exhibit antimicrobial functions by trapping and killing extracellular pathogens in blood and tissues during infection (12). However, NETs are not exclusively produced during severe infections. They have also been observed in inflammatory diseases such as preeclampsia (13), small-vessel vasculitis (14), and systemic lupus erythematosus (15). The molecular mechanisms underlying NET formation are poorly understood, but it has been recently shown that platelets activated by LPS can induce NET formation (16). We hypothesized that in experimental TRALI, NET formation occurs in the lungs and is driven by interactions between activated platelets and neutrophils. We reasoned that NETs could produce lung endothelial injury mediated by exposed extracellular histones, neutrophil granular proteins, and by a tangled web of extracellular DNA that could potentially provide a template for trapping of platelets and thrombus formation (17–19) in the lung microcirculation.

**Conflict of interest:** The authors have declared that no conflict of interest exists.

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**Figure 1**

Activated platelets induce NET formation in human neutrophils. (A–E) Representative images from direct immunofluorescence staining of DNA (blue), histones (red), and MPO (green) on (A) normal neutrophils incubated in media, (B) showing NET formation in neutrophils treated with PMA (25 nM; as positive control of NETosis) and (D) with platelets activated by TRAP (50  $\mu$ M). (C) No NET formation was apparent from treatment with platelets alone. (E) Treatment with TRAP alone produced less NET formation than in B or D.  $n = 6$ . Scale bar: 10  $\mu$ m. (F) MPO-DNA ELISA was used to quantify NETs in neutrophil supernatants and is expressed as percentage increase above control (media); mean  $\pm$  SD ( $n = 6$ ). \* $P < 0.05$  versus media and platelets groups; † $P < 0.05$  versus PMA and TRAP-activated platelets groups; \*\*\* $P < 0.001$  versus media and platelets groups.

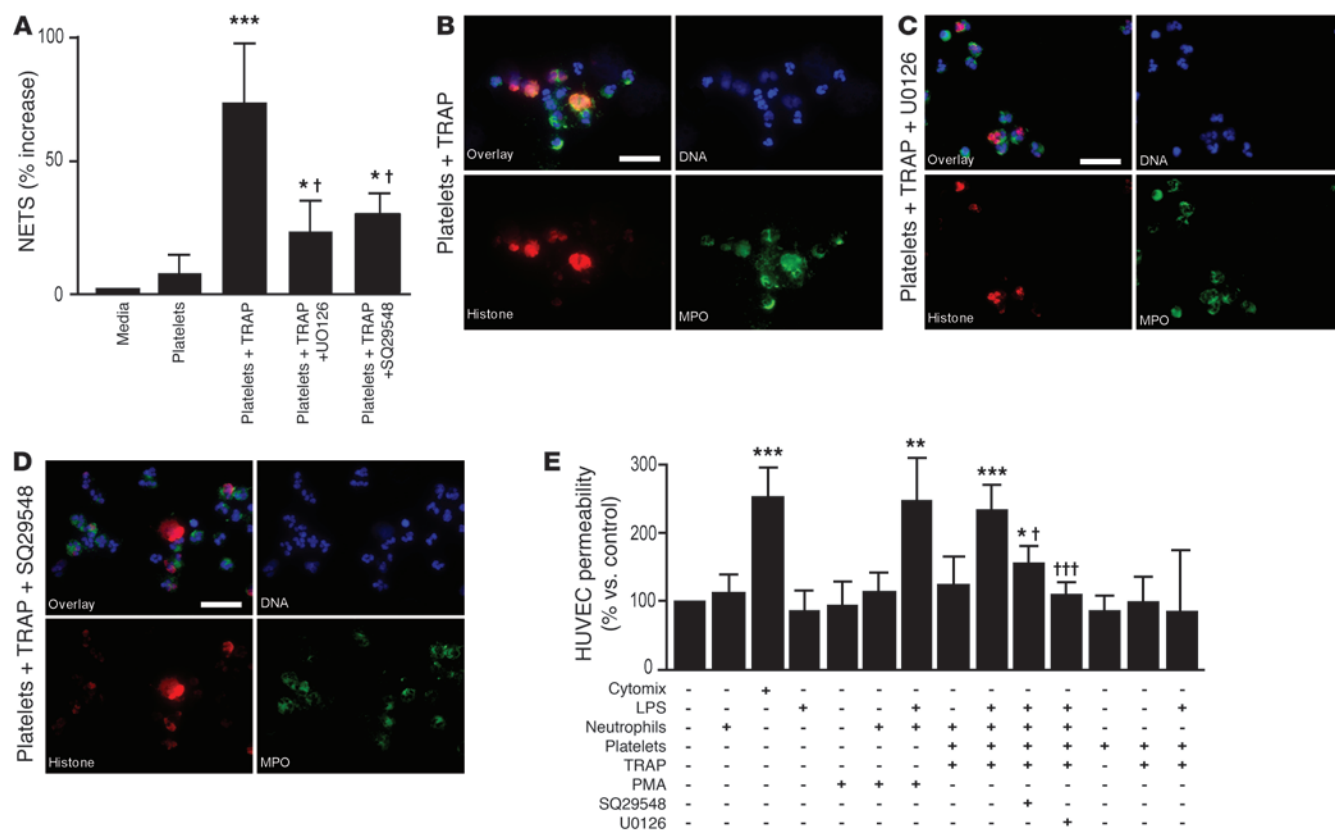
In this study, we show that activated platelets induce NET formation and that NETs can increase the permeability of endothelial monolayers. NETs were found in the lungs in both experimental and clinical TRALI. Finally, preventing platelet activation or interfering with NET constituents results in marked lung protection in experimental TRALI, suggesting that NETs may serve as a novel therapeutic target to treat patients suffering from this severe condition.

## Results

**Activated platelets induce NET formation in human neutrophils.** We used neutrophils isolated from normal human volunteers (Figure 1A) to assay for NET formation determined by the colocalization of extracellular DNA, extracellular histone protein, and extracellular myeloperoxidase (MPO). Phorbol 12-myristate 13-acetate-treated (PMA-treated) neutrophils were used as a positive control for NET formation (Figure 1B). When platelets were added to neutrophils, the cells maintained condensed nuclei, without NET formation (Figure 1C). However, when platelets were activated with the PAR-1 agonist, thrombin receptor-activating peptide (TRAP)

(20), and then were added to neutrophils, there was robust NET formation (Figure 1D). The addition of TRAP alone to neutrophils also induced rare NET formation (Figure 1E), which was surprising, since human neutrophils do not express PAR-1 (21). It is known, however, that approximately 10%–20% of neutrophils circulate as neutrophil-platelet aggregates (ref. 22 and data not shown). Therefore, NET formation induced by TRAP alone may be explained by preexisting neutrophil-platelet aggregates present in the neutrophil preparation. In separate experiments, we also stimulated platelets with thrombin (0.2 U/ml) or LPS (2  $\mu$ g/ml), and when added to neutrophils, we observed strong NET induction (data not shown).

To further corroborate our fluorescence microscopy experiments and to quantify NET formation, we developed a capture ELISA that detects MPO-associated DNA, similar to an assay previously used in a study of NETs in small-vessel vasculitis (14). To validate this ELISA, we tested the supernatants of neutrophils treated with PMA (NET control) or TNF- $\alpha$  (apoptosis control) (23), neutrophils incubated for 24 hours (apoptosis control) (24), sonicated neutrophils (necrosis control) (25),



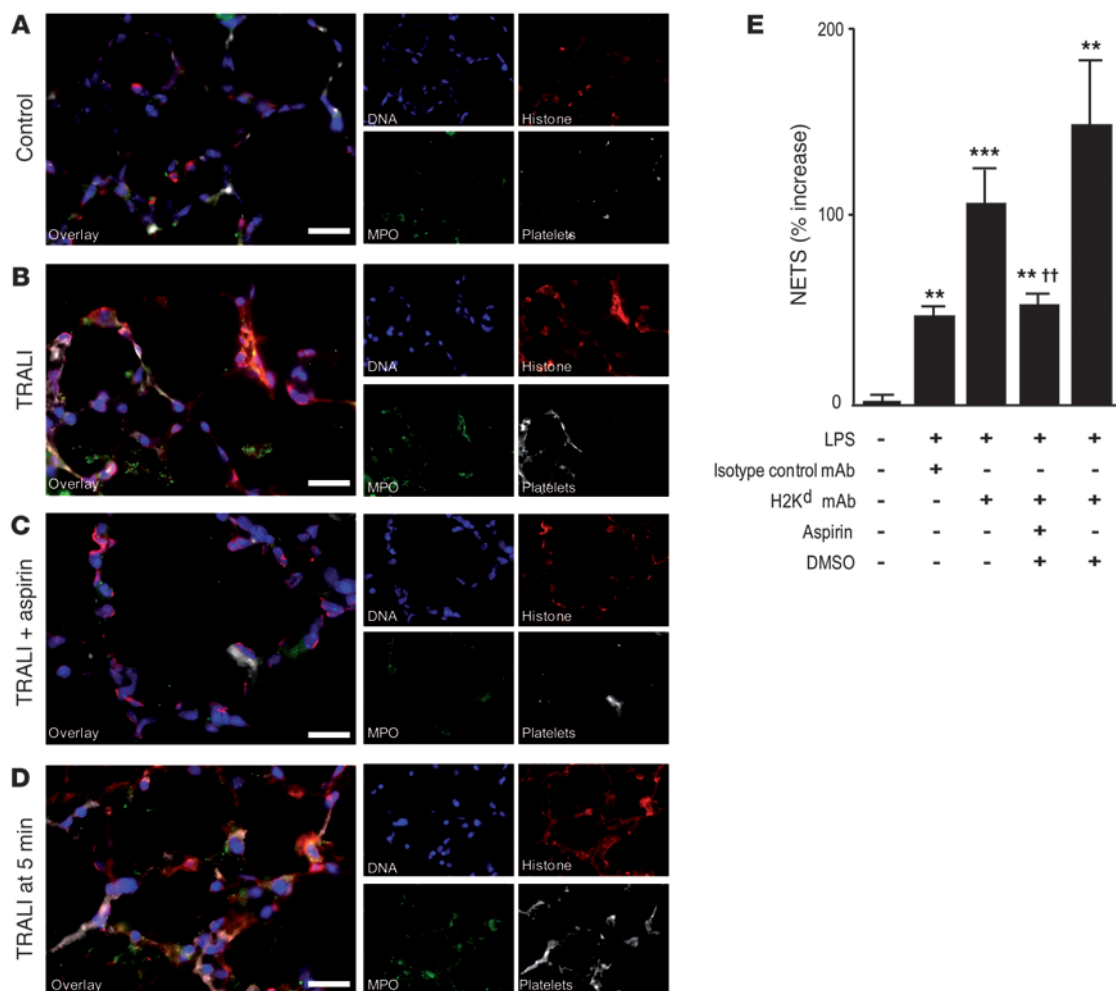
**Figure 2**

NET formation is dependent on thromboxane production and MEK signaling and increases endothelial permeability. **(A)** Quantification of NET release in cell supernatant (MPO-DNA ELISA). Neutrophils were pretreated with the MEK inhibitor (U0126, 10  $\mu$ M) or a thromboxane receptor antagonist (SQ29548, 10  $\mu$ M) for 10 minutes before the addition of platelets and TRAP. Pretreatment of neutrophils with U0126 or SQ29548 inhibited NET formation compared with that in neutrophils treated with TRAP-activated platelets. Mean  $\pm$  SD ( $n = 6$ );  $^*P < 0.05$ ,  $^{***}P < 0.001$  versus media and platelets;  $^{\dagger}P < 0.05$  versus TRAP-activated platelets. **(B–D)** Representative images from direct immunofluorescence staining of DNA (blue), histone (red), and MPO (green), showing NET formation in neutrophils treated with **(B)** TRAP-activated platelets and less NET formation in neutrophils pretreated with **(C)** U0126 or **(D)** SQ29548.  $n > 6$ ; Scale bar: 10  $\mu$ m. **(E)** Permeability of endothelial cell monolayers (HUVEC) measured in a Transwell system. In selected experiments, HUVECs were primed with LPS (2  $\mu$ g/ml) for 24 hours prior to the experiment. Permeability was measured by  $^{125}$ I-albumin flux across endothelium over 1 hour and was increased in cells treated with cytomix (0.5 ng/ml) or in LPS-primed endothelium with TRAP-activated platelets or PMA. Mean  $\pm$  SD ( $n = 12$ );  $^*P < 0.05$ ,  $^{**}P < 0.01$ ,  $^{***}P < 0.001$  versus HUVECs without treatment.  $^{\dagger}P < 0.05$ ,  $^{\dagger\dagger}P < 0.001$  versus HUVECs treated LPS and with neutrophils and TRAP-activated platelets.

and unstimulated neutrophils (negative control). The optical density measurements were normalized to those of the negative control and are reported as the percentage of NET formation. With dose dependency (10 nM vs. 25 nM PMA), PMA-treated neutrophils produced a large increase in MPO-DNA complexes, whereas the apoptosis and necrosis controls produced only minimal increases (Supplemental Figure 1A; supplemental material available online with this article; doi:10.1172/JCI61303DS1). Using this NET-specific assay, we found that neutrophils increased NET production in the presence of PMA or TRAP-treated platelets but not in the presence of nonactivated platelets (Figure 1F). In parallel to the fluorescence microscopy experiments (Figure 1E), neutrophils treated with TRAP or thrombin (data not shown) showed a small increase in NET production (Figure 1F). The addition of micrococcal nuclease to the cocubation of neutrophils and TRAP-activated platelets more than doubled the soluble NET components detected by the MPO-DNA ELISA (Supplemental Figure 1B).

The canonical Raf/MEK/ERK signaling pathway is essential for PMA-induced NET formation (26). We therefore tested the importance of this pathway in activated platelet-induced NET formation. In the presence of a specific MEK inhibitor (U0126), activated platelet-induced NET formation was substantially diminished (Figure 2, A and C). We have previously reported that thromboxane  $B_2$  (TXB $_2$ ) levels increased in the plasma of mice with TRALI and that blocking TXA $_2$  production with aspirin treatment reduces lung injury and mortality (5). We determined whether thromboxane produced by TRAP-activated platelets is a potential mediator in NET formation. TRAP-activated platelets released TXA $_2$ , which we measured by assaying for its degradation product, TXB $_2$  (Supplemental Figure 2). Pretreatment of neutrophils with a selective thromboxane receptor antagonist (SQ29548) prior to the addition of TRAP-activated platelets reduced the production of NETs (Figure 2, A and D).

*NETs increase the permeability of primed endothelial cells.* A hallmark of ALI is increased lung endothelial protein permeability. Therefore, we next explored the relationship between NET for-



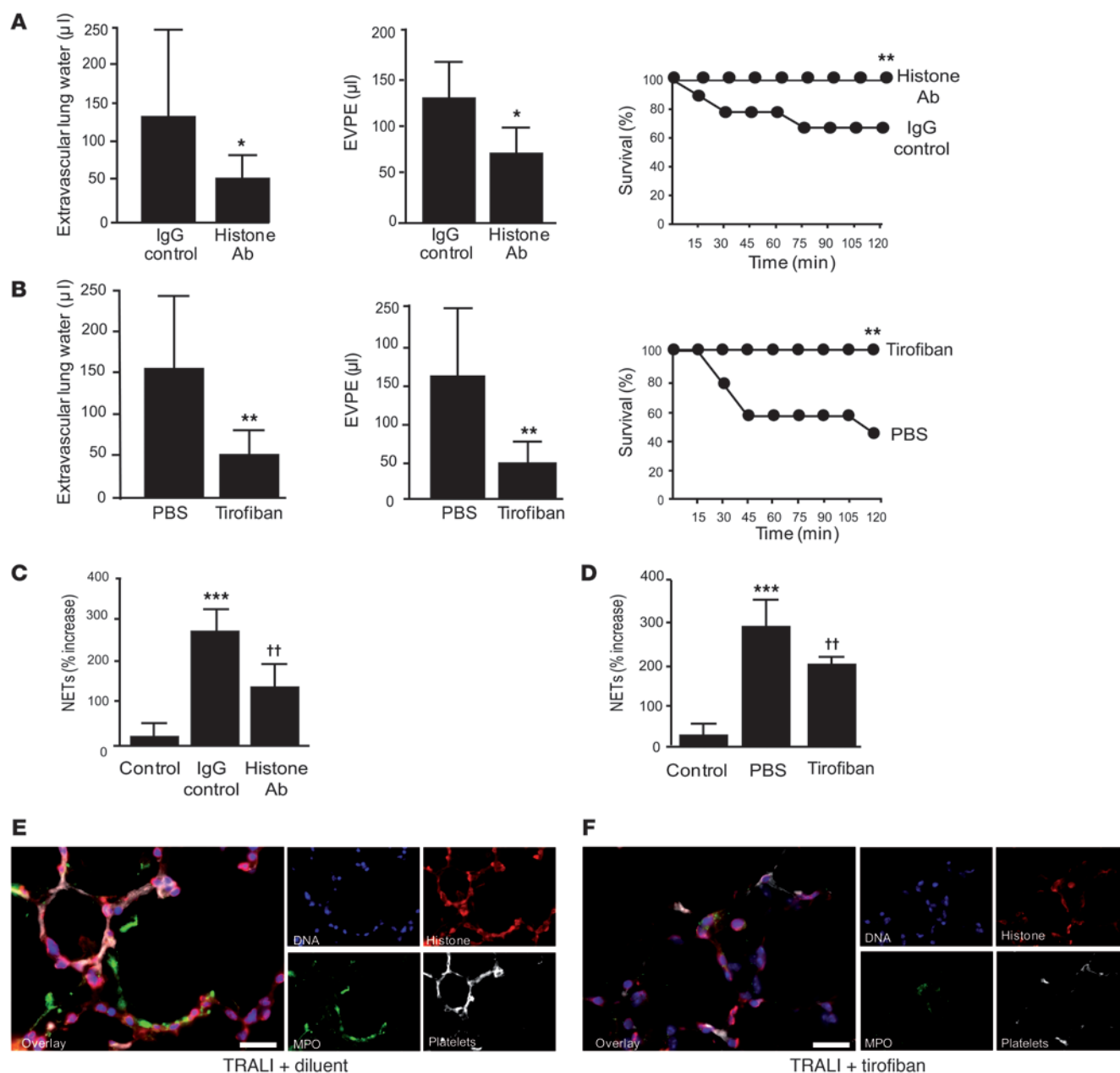
**Figure 3** NETs are present in TRALI mouse lungs, and aspirin decreases NET formation. (A–D) Representative images of NET formation detected by immunofluorescence in the lung microcirculation of (A) control mice (LPS plus isotype control mAb) and (B) mice with TRALI (LPS plus H2K<sup>d</sup> mAb, 1.0 mg/kg), (C) with or without aspirin treatment at 2 hours after mAb injection. Platelet (CD41) staining was increased in TRALI mice and localized to areas of NET formation. Aspirin treatment decreased platelet staining to levels observed in control mice. (D) Mice treated with a higher dose of H2K<sup>d</sup> mAb (4.5 mg/kg) had NET formation at 5 minutes after mAb injection. Scale bar: 20  $\mu$ m. (E) NET formation was quantified (MPO-DNA ELISA) in mouse plasma and plotted as mean  $\pm$  SD ( $n > 4$ ). \*\* $P < 0.01$ , \*\*\* $P < 0.001$  versus group receiving no treatment; †† $P < 0.01$  versus TRALI plus DMSO group.

mation and endothelial cell permeability in a transwell model. HUVECs were grown to confluency on transwell inserts suspended in cell culture media, and the permeability across this endothelial monolayer was measured. Cytomix (TNF- $\alpha$ , IL-1 $\beta$ , and IFN- $\gamma$ ) induced a 2- to 3-fold increase in endothelial permeability, but the addition of neutrophils, platelets, or neutrophils activated to produce NETs failed to induce permeability changes (Figure 2E). However, when the endothelial cells were primed with LPS for 24 hours, NETs induced by PMA or by activated platelets produced an increase in permeability similar to that produced by cytomix (Figure 2E). Additionally, the permeability changes induced by NETs were attenuated by the MEK inhibitor (U0126) and the thromboxane receptor antagonist (SQ29548) (Figure 2E). Similar results were also obtained using human lung microvascular endothelial cells in which NETs (treated with a combination of LPS, neutrophils, platelets, and TRAP)

induced protein permeability changes that were attenuated with MEK inhibition (Supplemental Figure 3). We conclude from these experiments that NETs increase permeability in LPS-primed endothelial cells.

*NETs are produced in TRALI.* Using the mouse model of TRALI, we previously demonstrated that neutrophils and platelets are required for injury development (5, 6). Since both neutrophils and platelets become sequestered in the lung microcirculation during TRALI (5, 6), we reasoned that intimate platelet-neutrophil interactions are produced that lead to NET formation. We prepared single-cell suspensions of lung tissue from mice in which TRALI was induced and used flow cytometry to detect neutrophil-platelet aggregates. In lungs removed only 2 minutes after injection of the H2K<sup>d</sup> mAb, there was a greater than 2-fold increase in the staining intensity for the platelet-specific marker, CD41, on the surface of CD11b<sup>+</sup>Ly6G<sup>+</sup> cells (Supplemental Figure 4A). Using transmission



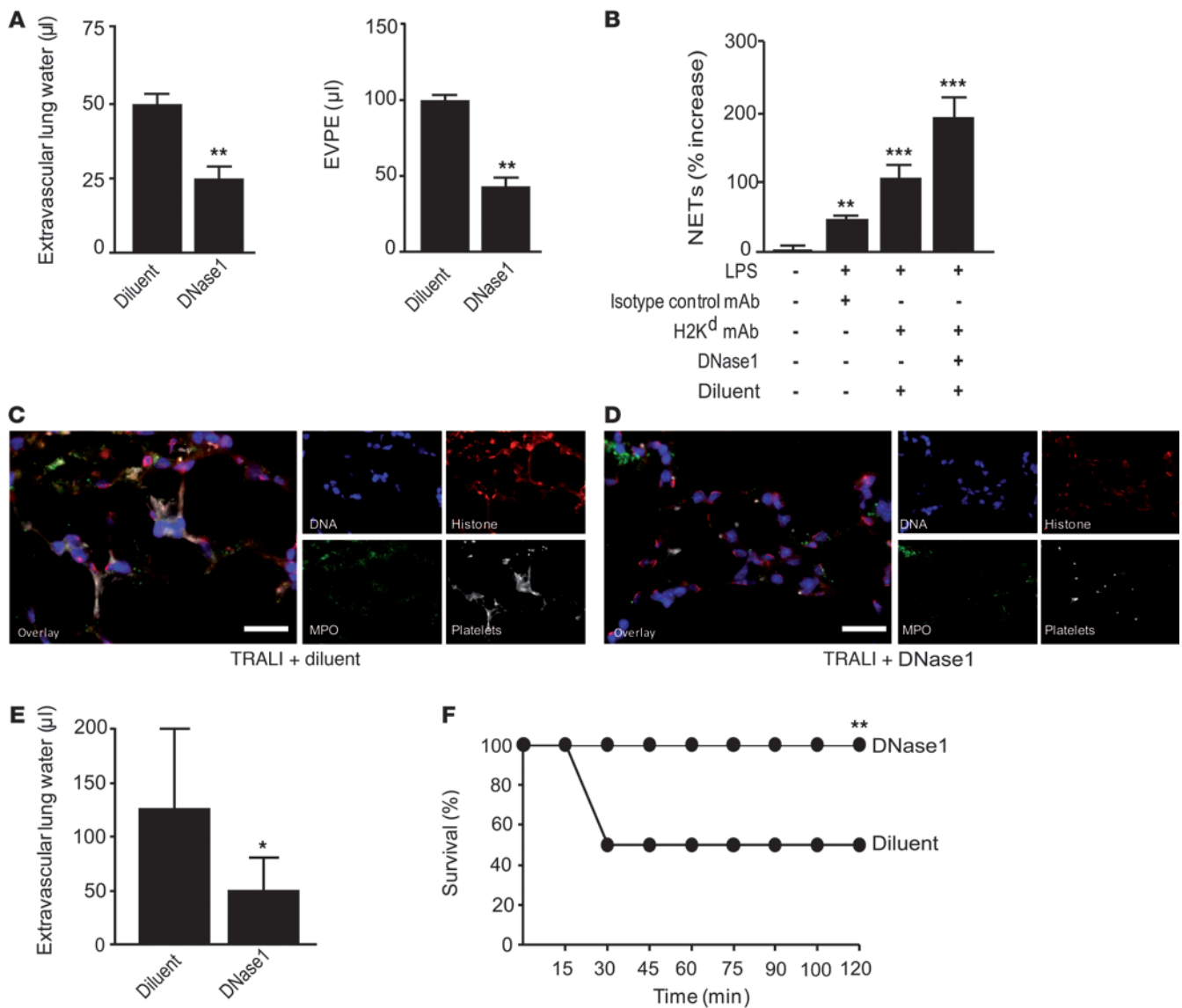


**Figure 4**

Platelets and extracellular histones are therapeutic targets in TRALI. **(A)** Mice with TRALI (LPS plus H2K<sup>d</sup> mAb) administered BWA3 mAb (10 mg/kg, i.v.) or IgG control (10 mg/kg) given immediately prior to H2K<sup>d</sup> mAb. BWA3 mAb decreased extravascular lung water and lung vascular permeability to <sup>125</sup>I-labeled albumin and decreased mortality compared with that in the IgG control group. Mean  $\pm$  SD ( $n = 9$ ). \* $P < 0.05$  versus IgG control group. \*\* $P < 0.01$ . **(B)** Mice with TRALI (LPS plus H2K<sup>d</sup> mAb) administered tirofiban (0.5  $\mu$ g/g, i.v.) or PBS given immediately prior to H2K<sup>d</sup> mAb. Tirofiban decreased extravascular lung water and lung vascular permeability to <sup>125</sup>I-labeled albumin and decreased mortality compared with that in the PBS group. Mean  $\pm$  SD ( $n = 7-9$ ). \*\* $P < 0.01$  versus PBS group. **(C and D)** MPO-DNA ELISA was used to quantify NET formation in the plasma of mice treated with **(C)** BWA3 mAb or **(D)** tirofiban compared with that in treatment controls and normal mouse plasma. Mean  $\pm$  SD ( $n = 6$ ). \*\*\* $P < 0.001$  versus control group; †† $P < 0.01$  versus IgG control and PBS groups. **(E and F)** Lung sections stained for NET formation (DNA, histone, and MPO) and for platelets (CD41). Representative images of mice with **(E)** TRALI given diluent (PBS) compared with those with **(F)** TRALI administered tirofiban. Mice treated with tirofiban have decreased NET formation and associated platelet sequestration compared with the PBS group. ( $n = 6$ ). Scale bar: 20  $\mu$ m.

electron microscopy, we detected neutrophils that were simultaneously adherent to lung endothelial cells, platelets, and red blood cells, providing further evidence for the spatial interactions among these cells in TRALI (Supplemental Figure 4B).

Previously, we demonstrated that aspirin treatment decreases platelet sequestration in the lung, decreases lung vascular permeability and edema, and increases survival in experimental TRALI (5). We therefore hypothesized that aspirin treatment would decrease



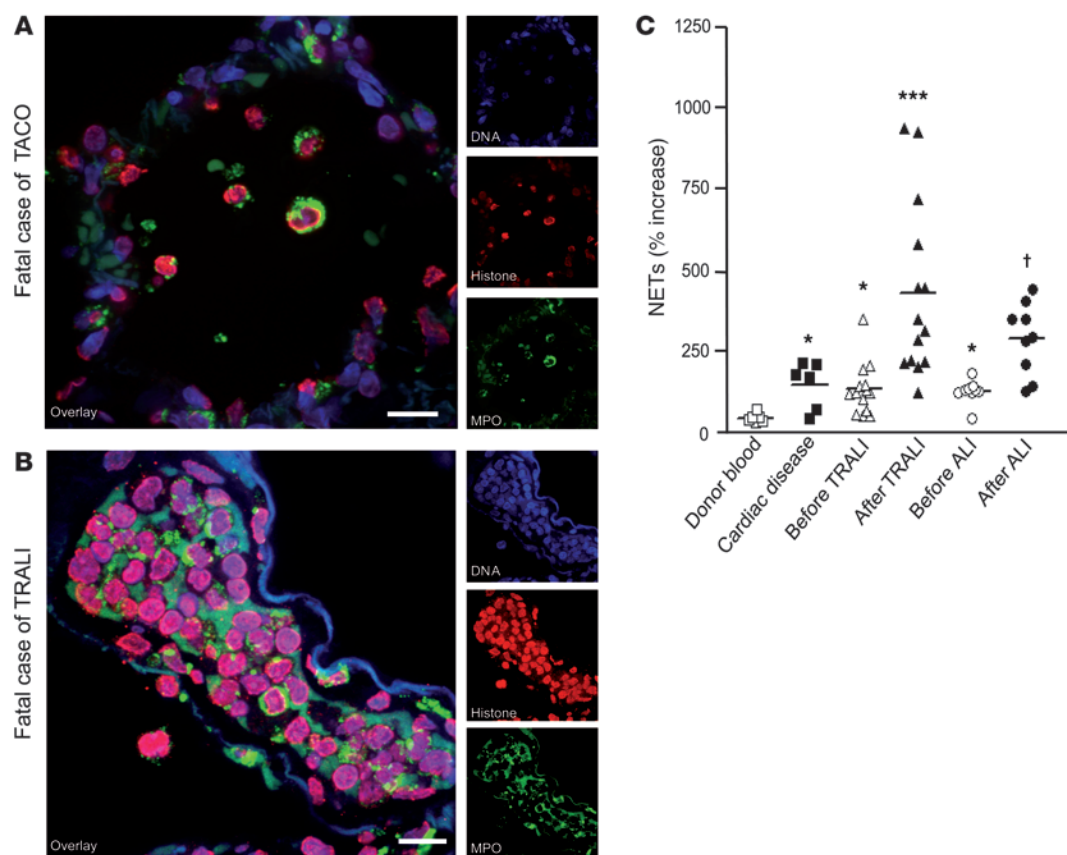
**Figure 5**

DNase1 treatment decreases TRALI and NETs. **(A)** Mice treated with DNase1 (10 mg/kg, i.v.) or diluent immediately prior to H2K<sup>d</sup> mAb injection are protected from TRALI with decreased extravascular lung water and decreased vascular permeability to <sup>125</sup>I-labeled albumin (EVPE). Mean  $\pm$  SD ( $n = 7$ ). \*\* $P < 0.01$  versus diluent group. **(B)** MPO-DNA ELISA was used to quantify NET formation in the plasma of mice pretreated with DNase1 or diluent compared with that in normal mouse plasma. Mean  $\pm$  SD ( $n = 6$ ). \*\* $P < 0.01$ , \*\*\* $P < 0.001$  versus control group. **(C and D)** Lung sections stained for NET formation (DNA, histone, and MPO) and for platelets (CD41). Representative images of mice with TRALI given **(C)** diluent or **(D)** DNase1. Mice treated with DNase1 have decreased NET formation and associated platelet sequestration compared with the diluent-treated group. ( $n = 3$ ). Scale bar: 20  $\mu$ m. **(E and F)** Mice treated with DNase1 (10 mg/kg, i.v.) or diluent 5 minutes after H2K<sup>d</sup> mAb injection are protected from TRALI with **(E)** decreased extravascular lung water and **(F)** decreased mortality compared with that in the diluent group. Mean  $\pm$  SD ( $n = 8$ ). \* $P < 0.05$ , \*\* $P < 0.01$  versus diluent group.

NET formation by decreasing platelet activation. We found that NETs were present in abundance in the lung microcirculation of mice with TRALI (compare Figure 3A with Figure 3B), and soluble NET components were higher in the peripheral blood (Figure 3E). Treatment of mice with aspirin prior to the induction of TRALI decreased NET formation (Figure 3, C and E). In addition, platelet sequestration increased in areas of the lung in which NETs were present (Figure 3B), and aspirin treatment decreased NET-associated platelets (Figure 3C). The speed by which NETs formed after

H2K<sup>d</sup> mAb injection was remarkable. With higher doses of mAb (4.5 mg/kg), we detected NET formation and associated platelet trapping only 5 minutes after mAb injection (Figure 3D).

*NETs are therapeutic targets in TRALI.* We next took aim at NET components as a therapeutic target in TRALI. Extracellular histones are directly toxic to primary human endothelial cells (HUVECs), and, in a mouse model of endotoxemia, the neutralization of extracellular histones with a blocking mAb decreases mortality (27). We hypothesized that extracellular histones may medi-



**Figure 6**

NETs are present in human TRALI lungs and plasma. **(A and B)** Human lung paraffin sections were stained for histone (red), MPO (green), and DNA (blue) and analyzed by confocal microscopy. **(B)** In the TRALI fatality case, we observed clumps of NET-forming neutrophils in the intravascular compartment. **(A)** In the TACO fatality case, neutrophils were found in mainly intra-alveolar locations, but no NET formation was detected. Scale bar: 10  $\mu$ m. **(C)** MPO-DNA ELISA was used to quantify NET components in the plasma of patients, and the mean optical density of plasma obtained from normal, human blood donors ( $n = 6$ ) was used as the control. Plasma from individuals with cardiac disease ( $n = 6$ ), individuals before TRALI and after TRALI (paired samples,  $n = 14$ ), and individuals before ALI and after ALI (paired samples,  $n = 9$ ) are compared. Horizontal bars represent the mean; symbols represent individual samples. \* $P < 0.05$  versus donor blood group; \*\*\* $P < 0.001$  versus donor blood, cardiac disease, and before TRALI groups;  $^{\dagger}P < 0.05$  versus donor blood, cardiac disease, and before ALI groups.

ate the increased endothelial permeability observed in our HUVEC (Figure 2E) and human lung microvascular endothelial cell (Supplemental Figure 3) experiments and in the TRALI mouse model. Accordingly, we administered a histone-blocking mAb (BWA3) that recognizes histone 2A (H2A) and histone 4 (H4) (28) to mice with TRALI (immediately prior to H2K<sup>d</sup> mAb injection). We observed a decrease in lung edema, lung vascular permeability to protein, and mortality (Figure 4A), compared with that after a control antibody injection. This treatment strategy also reduced NET formation detected in plasma samples (Figure 4C), which implies that extracellular histones may propagate NET formation.

Since platelets bind to extracellular histones (29), we reasoned that the increase in platelet sequestration in areas of NETosis could reflect this binding. Moreover, the binding of platelets to histones may lead to platelet activation and propagation of platelet-platelet binding around NETs, much like the platelet aggregation that occurs with thrombosis. Therefore, we tested the effect of tirofiban, an inhibitor of the platelet glycoprotein IIb/IIIa receptor that mediates platelet-platelet binding through fibrinogen (30). Mice that were injected with tirofiban at the time of H2K<sup>d</sup> mAb

challenge were protected from lung edema and lung vascular permeability to protein (Figure 4B), and were also completely protected from mortality (Figure 4B). Tirofiban treatment also decreased soluble NET components (Figure 4D), NETs detected in lung tissue (Figure 4, E and F), and platelet sequestration in areas of NET formation (Figure 4, E and F). These results indicate that NET-induced platelet aggregation is an important feature of TRALI.

In vitro studies have demonstrated that NETs may be dismantled using DNase1, which helps to untangle the web of extracellular chromatin (31). Extracellular DNA of NETs could damage the lung microcirculation by impeding blood flow and potentially creating zones of ischemia. To test this hypothesis, we treated mice undergoing TRALI with DNase1, using a dose similar to that used in experimental studies of autoimmune kidney disease (32). When we initiated the treatment simultaneously with H2K<sup>d</sup> mAb injection, we found that the pretreatment strategy with DNase1 protected mice from lung edema and lung vascular permeability (Figure 5A) and reduced NET formation and also platelet sequestration in the lung (Figure 5, C and D). DNase1 actually increased soluble NET components detected in the peripheral blood compared with the dilu-





ent control (Figure 5B), indicating that DNase1 cleaves extracellular DNA in the lung microcirculation that then enters the systemic circulation. Injection of DNase1 5 minutes after mAb injection, when NETs were already forming (Figure 3D), also reduced lung edema and protected mice from mortality (Figure 5, E and F).

**NETs in human TRALI and ALI.** To establish the clinical significance of our findings, we sought evidence of NET formation in patients with TRALI and ALI. TRALI autopsy reports are rare, as are available tissue samples, but we were able to test lung tissue from a fatal case of HLA class I antibody-associated TRALI (33). For a control, we used a case of fatal transfusion-associated circulatory overload (TACO), which is characterized by hydrostatic rather than permeability pulmonary edema. In the TRALI case, we detected areas in lung vessels in which many neutrophils probably were undergoing NETosis, with large, decondensed nuclei and abundant extranuclear histones and extracellular MPO (Figure 6B). In the TACO control case, we found intra-alveolar neutrophils, but we detected no NET formation (Figure 6A).

Finally, we used the MPO-DNA ELISA to test plasma samples for NET formation in patients with TRALI and other causes of ALI. Plasma samples were obtained from a case-control study of TRALI and ALI in which blood was collected before and after the development of lung injury (34). There was a striking increase in NETs in the plasma samples after development of TRALI and, to a lesser degree, in samples after development of ALI, compared with that in normal human plasma and with patients with acute cardiac syndromes (Figure 6C).

## Discussion

In this study, first, we have identified that platelet activation can induce NET formation in the absence of infection and that NETs increase endothelial permeability. Second, we found that NETs are present in abundance in the lungs of mice undergoing experimental TRALI, and NETs and circulating NET components are strongly increased in clinical cases of TRALI. Third, inhibiting platelet activation reduced NET formation and ALI in vivo. Fourth, by targeting NETs directly with a histone blocking antibody or dismantling NETs with DNase1 in preinjury or postinjury models, there was strong protection from lung endothelial injury in TRALI. The discovery of the important role of platelet-induced NET formation in ALI represents a paradigm shift that may lead to new therapeutic approaches.

NETs were described less than a decade ago (11), but there is a growing list of diseases that are associated with NET formation. NETs have classically been linked with severe infections, such as sepsis, in which the release of chromatin decorated with neutrophil granular proteins serves as an additional weapon of the innate immune system against circulating bacteria. However, NETs are also found in noninfectious diseases in which the presence of NETs may be a maladaptive response that leads to tissue injury. We now add ALI to the list of diseases that involve NETs, and indeed, in our model of noninfectious ALI, TRALI, we cannot postulate an obvious beneficial role of NET formation.

A major mechanism by which platelets promote inflammation and injury is through critical interactions with neutrophils. For example, in sepsis, activation of platelet TLR4 is a potent stimulus for neutrophils residing in the sinusoids of the liver and other capillary beds to release NETs (16). We also found that activation of platelets with PAR-1 agonists (TRAP, thrombin) was equally efficient in producing NETs, which suggests that a variety of platelet agonists

may be capable of promoting NET formation. The mechanism by which platelets promote NETosis is still not clear, but we propose that platelet activation primes neutrophils for NET formation. It is known that neutrophil-platelet aggregates have greater adhesive capacity, greater reactive oxygen species production, and phagocytic potential (35). Reactive oxygen species production is tied closely to NET formation (36). Experiments have shown that MEK signaling, which is upstream of the NADPH oxidase, is critical to NET formation resulting from PMA-induced activation (26). Interruption of MEK signaling in our platelet-activation experiments also efficiently decreased NET formation, suggesting that MEK signaling is a common pathway through which different stimuli can induce NETosis. We also found that inhibition of TXA<sub>2</sub> signaling in our system reduced NET formation, even though neutrophils are not known to possess thromboxane receptors. However, TXA<sub>2</sub> has been previously shown to augment the respiratory burst of neutrophils, and this effect can be blocked with aspirin (37).

We found that in TRALI, within minutes after antibody challenge, there was an increase in platelet aggregation on neutrophils in the lungs, which supports the hypothesis that critical neutrophil-platelet interactions are promoting NET formation. The speed by which these interactions develop after MHC class I antibody injection is remarkable and is proximal to the onset of overt ALI in our model. We also observed ultrastructural evidence of neutrophil-platelet aggregates in the lung microcirculation that included close interactions with the lung endothelium and red blood cells. Similar findings have been reported in TRALI and are dependent on polarized domains of CD11b/CD18 (38). However, using the same MHC class I mAb, another group has recently reported the observation of neutrophil- and platelet-independent lung injury (39). This report contrasts with our findings of neutrophil- and platelet-dependent ALI and our current findings of platelet-mediated NET formation. There are fundamental differences in the animal models that likely explain the divergent results, including (a) production and purification of mAb, (b) dosing of mAb, and (c) recipient immune status. Delivery of large doses of mAb in mainly unprimed animals (39) could concentrate mAb on endothelial surfaces, which potentially could produce a more complement- and monocyte-dependent lung injury. Our results showing intense neutrophil and platelet sequestration in the lung, intimate neutrophil-platelet interactions, abrogation of injury with nonantibody-based platelet inhibitors (aspirin, tirofiban) and inhibitors of NETs (DNase1) or NET components (histone antibody), and the presence of NETs in human TRALI cases all strongly point to the importance of platelets and neutrophils in TRALI. Additionally, recent results from a prospective TRALI case-control study found that thrombocytopenia before transfusion (platelet count < 50 × 10<sup>9</sup>/l) was associated with protection from TRALI, and there was a trend for neutropenia before transfusion also being protective (34).

We found abundant NET formation in the lung microcirculation and plasma in experimental TRALI. A postulated function of NETs is to skim the plasma for intravascular pathogens; however, other intravascular components may also be efficiently trapped. We found intense platelet sequestration in areas of NET formation. Platelets may bind especially well to NETs through interactions with charged extracellular histones (29). Platelet accumulation on NETs may perpetuate platelet aggregation and activation and promote coagulation in the lung microcirculation that could have ischemic consequences (18). However, using fibrinogen immunostaining, we were unable to detect increased clot formation in NET-containing areas of TRALI lungs (data not shown),



but ischemic conditions could still be induced by NETs through physical trapping of platelets, red blood cells, and other leukocytes. Consistent with the role of TXA<sub>2</sub> in our *in vitro* experiments, treatment of mice with aspirin decreases plasma TXB<sub>2</sub> levels, ALI, and mortality (5). Here, we found decreased NET formation in the lung microcirculation and plasma and decreased lung deposition of platelets with aspirin treatment. When we blocked platelet-platelet interactions with a glycoprotein IIb/IIIa inhibitor, mice were also protected from TRALI and mortality.

NETs likely induce injury to the lung through direct toxicity to endothelial cells, as we showed in our *in vitro* model of endothelial permeability, perhaps by concentrating histones and granular proteins to reach high local concentrations that enhance their toxicity. We also propose that there is a substantial contribution from the DNA scaffold that may trap cells and impede forward flow, leading to microcirculatory injury. However, to move the field forward beyond the association of NETs with disease, evidence is needed showing that NET-specific treatment approaches ameliorate disease manifestations.

Effective therapeutic agents for ALI are desperately needed. We propose that NETs are a new target for pharmaceutical development in ALI. One treatment strategy focuses on inhibiting the initial formation of NETs; we propose that antiplatelet agents (aspirin, tirofiban) are effective approaches. Targeting NET components is an alternative approach and potentially more clinically feasible, since NETs may be formed early in diseases like ALI and sepsis and already producing injury upon initial clinical presentation. We targeted NET components using a blocking antibody against H2A and H4 histones and also by disrupting the NET scaffold with DNase1. Both strategies were effective in reducing lung injury and mortality. DNase1 was also effective when given 5 minutes after initiation of TRALI when NETs are already present and lung injury is developing. DNase1 is naturally occurring in human blood (40) and is also produced as a defense mechanism by bacteria attempting to escape entanglement by NETs (41). Intrapulmonary DNase is already an effective therapy in cystic fibrosis, targeting the extracellular DNA that interferes with mucociliary clearance (42). Delivering therapeutic agents to patients with TRALI will be a challenge, given the abrupt onset of the condition, but targeting NET components (histones, DNA) is an attractive option that is downstream of the events that initiate transfusion-induced lung injury.

In conclusion, NETs are present and pathogenic in TRALI, and targeting platelet activation or NET components (histones, DNA) strongly protects mice from lung injury. Therapeutic advances in ALI will likely come from a better understanding of pathogenesis, and we propose that NETs are a worthy target for future preclinical and clinical testing.

## Methods

***In vitro* NET experiments.** Whole blood collected from normal volunteers was used to isolate platelets by centrifugation (43) and to isolate neutrophils using Polymorphprep (Axis-Shield) following the manufacturer's protocol. Neutrophil purity was routinely approximately 95%. RPMI 1640 plus 1% FBS was used as the culture medium for all reactions. Red blood cells were lysed with the red blood lysis buffer (8.3 g NH<sub>4</sub>Cl, 1.0 g KHCO<sub>3</sub>, 0.5 M EDTA). At a concentration of 25 nM, PMA served as a positive control of NET formation. Neutrophils ( $2 \times 10^4$  to  $5 \times 10^4$ ) were treated with PMA, platelets ( $2 \times 10^5$  to  $5 \times 10^5$ ), or platelets activated with TRAP (Sigma-Aldrich; 50  $\mu$ M), and, in selected experiments, neutrophils were pretreated with inhibitors (U0126, SQ29548) for 10 minutes. After 60 minutes of treatment at 37°C

and 5% CO<sub>2</sub> (except for PMA for which cells were treated for 180 minutes), neutrophils were fixed, permeabilized, and blocked for immunofluorescent staining. Cells were incubated with antibodies against MPO (Dako, catalog no. A0398), histones (BWA3 clone, gift from M. Monestier), and CD41 (clone MWReg30, BD Biosciences) and followed by species-specific secondary antibodies coupled with Alexa Fluor Dyes (Invitrogen). DNA was stained using Hoechst 33342, and the cells were mounted in Vectashield Mounting Media (Vector Laboratories) for imaging with a Nikon 6D fluorescent microscope and image acquisition using NIS Elements (Nikon Imaging Center).

**MPO-DNA ELISA.** To quantify NETs in cell culture supernatant and in mouse and human plasma, we developed a capture ELISA based on MPO associated with DNA. For the capture antibody, 5  $\mu$ g/ml anti-MPO mAb (Upstate, catalog no. 07-496) was coated onto 96-well plates (dilution 1:500 in 50  $\mu$ l) overnight at 4°C. After washing 3 times (300  $\mu$ l each), 20  $\mu$ l of samples was added to the wells with 80  $\mu$ l incubation buffer containing a peroxidase-labeled anti-DNA mAb (Cell Death ELISA<sup>PLUS</sup>, Roche; dilution 1:25). The plate was incubated for 2 hours, shaking at 300 rpm at room temperature. After 3 washes (300  $\mu$ l each), 100  $\mu$ l peroxidase substrate (ABTS) was added. Absorbance at 405-nm wavelength was measured after 20 minutes incubation at room temperature in the dark. Values for soluble NET formation are expressed as percentage increase in absorbance above control.

**HUVEC experiments.** HUVECs (Lonza) were cultured in EBM-2 complete medium (Lonza) with 10% FBS according to the manufacturer's instructions, and passages 4 to 7 were used for experiments. HUVECs were plated onto 24-well Transwell inserts (Corning; 0.4- $\mu$ m pore size) and grown to confluency. PMA-treated neutrophils (25 nM) were added to the transwells and incubated at 37°C for 180 minutes. TRAP-activated platelets and neutrophils (50  $\mu$ M) were added to the transwells and incubated at 37°C for 60 minutes. In selected experiments, we also preincubated neutrophils with U0126 (10  $\mu$ M) or SQ29548 (10  $\mu$ M) for 10 minutes. We measured permeability across HUVEC monolayers by adding <sup>125</sup>I-labeled albumin (Iso-Tex Diagnostics) to the upper (Transwell) compartment and measuring the accumulation in the lower compartment after 1 hour. Cytomix (TNF- $\alpha$ , IL-1 $\beta$ , and IFN- $\gamma$ ; 0.5 ng/ml) was used as a positive control (2), and in selected experiments LPS (2  $\mu$ g/ml) was added to the HUVECs for 24 hours prior to the permeability assay. The radioactivity was measured with a gamma counter (Packard 5000 Series).

**TRALI experiments.** BALB/c wild-type mice (H2K<sup>d</sup>) were purchased from Charles River Laboratories. All experimental procedures were performed in 8- to 12-week-old male mice housed under specific pathogen-free conditions in the Animal Barrier Facility at UCSF. A 2-event TRALI model was used as previously described by our group (5). Briefly, after being primed with LPS (0.1 mg/kg, i.p.) for 24 hours, anesthetized mice were challenged with MHC I mAb (H2K<sup>d</sup>; IgG<sub>2a</sub>,  $\kappa$ ; 0.5–4.5 mg/kg) or isotype control mAb injected into the jugular vein. Mice were euthanized when they appeared moribund or at 2 hours after MHC I mAb injection. To quantify pulmonary edema, bloodless, extravascular lung water was measured as previously described (5, 6). We also measured lung vascular permeability to protein by instilling mice with i.v. <sup>125</sup>I-labeled albumin. The radioactivity in the blood and the bloodless lung was measured with a gamma counter (Packard 5000 Series) and the ratio was used to calculate the lung extravascular plasma equivalents (EVPEs) (5, 6).

**TRALI treatment experiments.** For the aspirin experiments, aspirin tablets were crushed and dissolved in DMSO and diluted in PBS for i.p. injection. Aspirin (100  $\mu$ g/g) or DMSO control was delivered i.p. 30 minutes prior to LPS priming and again 2 hours prior to MHC I mAb challenge. Tirofiban (Medicure) or PBS control was administered i.v. (0.5  $\mu$ g/g) at the same time as MHC I mAb challenge. We generated antibody to histones H2A/H4 from a hybridoma (BWA3 clone) as previously described (28). BWA3 mAb (10 mg/kg) or IgG control was injected at the same time as MHC I mAb. DNase1



(Sigma-Aldrich; 10 mg/kg) or diluent control was administered either at the same time of MHC I mAb injection or 5 minutes after MHC I mAb injection.

**Identification of NETs in mouse and human lung sections.** OCT-embedded frozen mouse lungs were sectioned (7  $\mu$ m), fixed in PFA 4%, blocked with PBS containing 1% donkey serum and 3% BSA, and then permeabilized with PBS/Triton 0.01%. Sections were incubated with CD41 antibody (clone MWReg30, BD Biosciences), MPO antibody (Abcam, catalog no. ab45977), histone antibody (BWA3), and then with species-specific secondary antibodies coupled with Alexa Fluor Dyes. DNA was stained using Hoechst 33342, and sections were mounted in Vectashield Mounting Media.

Human paraffin-embedded lung samples from fatal cases of TRALI and TACO were provided by M.B. Marques, and 5- $\mu$ m sections were prepared and mounted on glass slides. After antigen retrieval with citrate buffer, specimens were treated with blocking buffer and subsequently with primary antibodies against MPO (Dako, catalog no. A0398) and histone (BWA3), followed by species-specific secondary antibodies. DNA was stained by using Vectashield Mounting Medium with DAPI (Vector Laboratories). We used a Zeiss Axiovert 200 M spinning disk confocal microscope, and images were acquired with a Plan APOCHROMAT 63 $\times$ /1.4 oil DIC objective (Zeiss) using Micromanager software and processed with ImageJ.

**Human plasma samples.** P. Toy provided ALI and TRALI blood samples (before and after event) from the UCSF and Mayo Clinic TRALI case-control study (39). Blood was also collected from normal human volunteers and from patients admitted to the UCSF emergency department with acute cardiac conditions. All blood samples were refrigerated as soon as possible and centrifuged at 800 g for 10 minutes, and the plasma fraction was aliquoted and frozen at -80°C.

**Statistics.** Results are reported as both individual data points and mean  $\pm$  SD. To determine significance, 2-tailed Student's *t* test, ANOVA, and Fisher's exact test were used as appropriate (GraphPad PRISM version 3.0). *P* values of less than or equal to 0.05 were determined to be significant.

**Study approval.** All experiments were approved by the Institutional Animal Care and Use Committee at UCSF, the Committee on Human Research at UCSF, and the University of Alabama at Birmingham, Birmingham, Alabama, USA.

**Additional methods.** Detailed methodology is described in the Supplemental Methods.

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