SUPPLEMENTARY INFORMATION

Supplementary Materials and Methods

Apoptosis and necrosis controls for MPO-DNA ELISA. We tested neutrophils undergoing necrosis and apoptosis in our MPO-DNA ELISA as control experiments. Apoptosis was induced with TNF-α (100 ng/ml) for 6h and by incubating neutrophils in complete neutrophil medium for 24h. Apoptosis was confirmed using an Annexin V apoptosis detection kit (BD) and flow cytometry. Necrosis was induced by brief cell disruption using a hand-held sonicator (10 x 1s pulse, 30% or 50% of duty cycle, w380 model, Heat Systems) and necrosis was confirmed by Trypan blue uptake (1).

MNase treatment. MNase (Micrococcal nuclease, Thermo Scientific, 5 U/ml) was added 15 min after incubating neutrophils ± addition of platelets and TRAP and the cell culture supernatant was collected and tested with the MPO-DNA ELISA.

Human lung microvascular endothelial cell experiments. Human lung microvascular endothelial cells (HMVEC-L) (Lonza) were cultured in EGM™-2MV BulletKit™ (Lonza) supplemented with 10% FBS according to the manufacturer´s instructions and passages 5 to 8 were used for experiments. HMVEC-L were plated onto 24-well Transwell inserts (Corning; 0.4 μm pore size) and grown to confluency (48 hours). Neutrophils + PMA (25 nM) were added to the transwells and incubated at 37°C for 180 min. Neutrophils + platelets + TRAP (50μM) were added to the transwells and incubated at 37°C for 60 min. In selected experiments, we also pre-treated neutrophils with U0126 (10μM) for 10 min. We measured permeability across HMVEC-L monolayers by adding ^{125}I-labeled albumin (Iso-Tex Diagnostics) to the upper (Transwell) compartment and measuring the accumulation in the lower compartment after one hour.

Cytomix (TNFα, IL-1β, and INFγ) was used as a positive control (2) and in selected experiments LPS (2 μg/ml) was added to the HMVEC-L for 24h prior to the permeability assay.

TXB_2 measurements. TXB_2 levels were measured in cell supernatants (neutrophils or platelets) using a TXB_2 ELISA kit (Amersham) according to the manufacturer's protocol.

Flow cytometry for neutrophil-platelet aggregates in TRALI. To characterize neutrophil-platelets aggregates in the TRALI mouse model, lungs were removed at specific time points after injection of MHC I mAb, minced, and digested in RPMI supplemented with collagenase (Sigma, 1 mg/ml) and DNase1 (Sigma, 50 units/ml) for one hour in a shaking water bath at 37° C. The lungs were then filtered (100 μm filters) to prepare single cell suspensions for flow cytometry. Saturating concentrations of anti-CD41 (FITC), anti-CD11b (PE), and anti-Lys6G (APC) (all from R&D Systems) were then added and flow cytometric acquisition was performed using a FACScan (BD Biosciences) and data were analyzed using FlowJo software (Tree Star).

Electron Microscopy. Mouse lungs were instilled i.t. and then immersed in 2% glutaraldehyde and 4% formaldehyde (0.1 M PO4, pH 7.2). The samples were cut into ultrathin sections at 80 nM for transmission electron microscopy (TEM) and stained with 2% uranyl acetate. Sample examination and record micrographs used JEOL 1400 TEM.

Figure S1

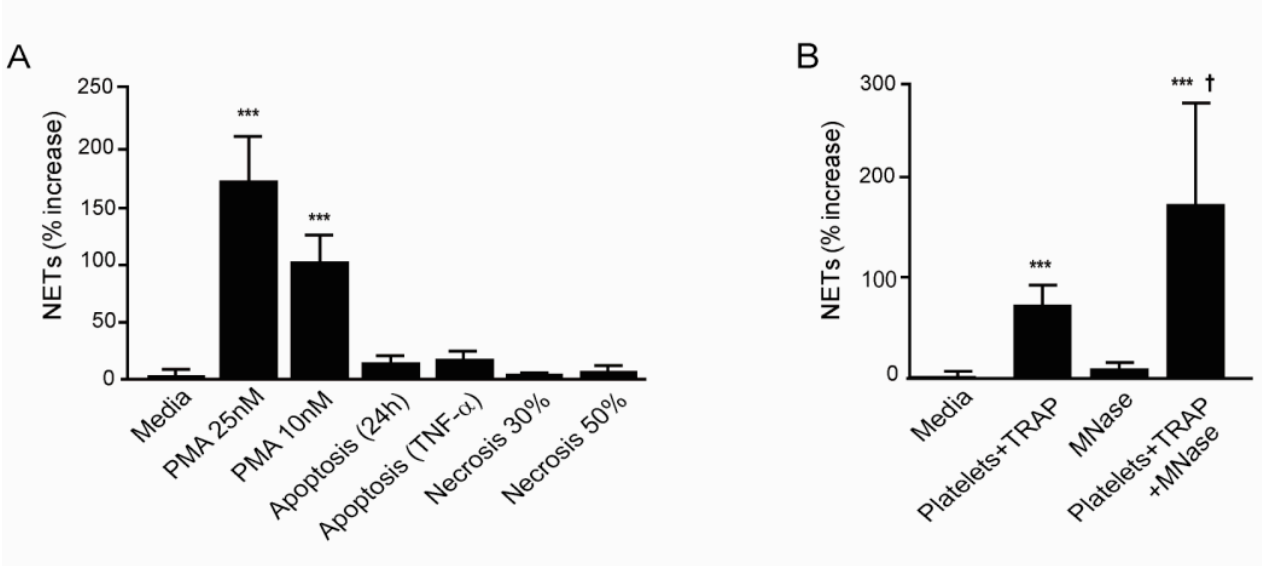

Figure S1
MPO-DNA ELISA is specific for NET formation. **(A)** Neutrophils were treated with PMA (25 nM and 10 nM, 3 hours) as positive control of NETosis, or with TNF-α (100 ng/ml, 6h) or culture for 24h in complete neutrophil media as controls for apoptosis. For apoptosis, ~60% of neutrophils (TNF-α) and ~40% of neutrophils (24h culture) were apoptotic by Annexin V staining. For necrosis, neutrophils were sonicated during 10 sec at either 30% or 50% duty cycle. Trypan blue staining confirmed that ~50% and ~80% of neutrophils were necrotic at the 30% and 50% duty cycle settings, respectively. MPO-DNA complexes increased in supernatants of neutrophils incubated with PMA, but not in necrotic or apoptotic neutrophil supernatants. Mean ± s.d. (*n* = 4 - 6). ***p<0.001 vs. media, apoptosis (TNF-α, 24h culture), and necrosis (30%, 50%). **(B)** Neutrophils were treated with TRAP-activated platelets for 1 hour and MNase (Micrococcal nuclease, 5U/ml) was added in selected experiments. MNase treatment produced an increase in soluble NET components when added to TRAP-activated platelets. ***p<0.001 vs. media. and †p<0.05 vs platelets+TRAP.

Figure S2

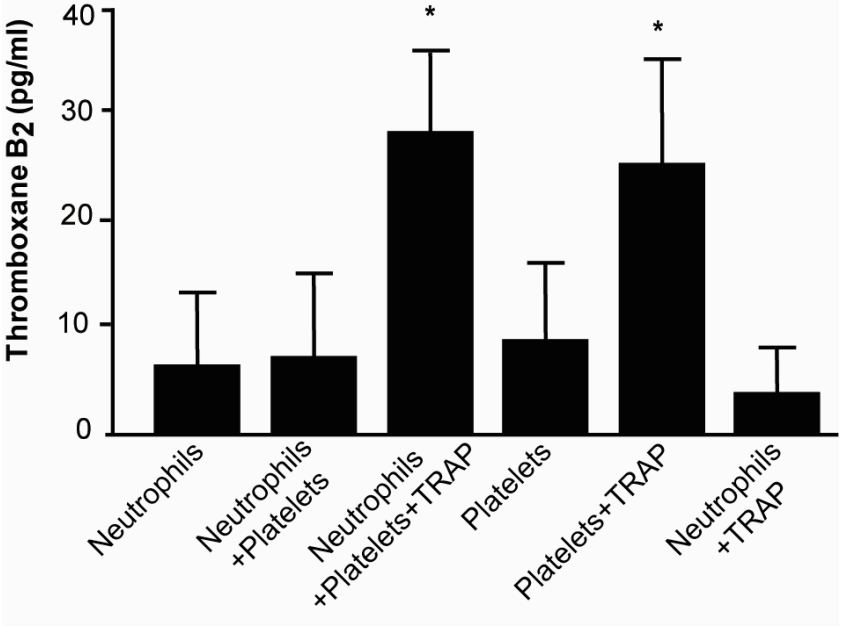

Figure S2
Platelets activated with TRAP release thromboxane B_2 (TXB_2)**.** TXB_2 production was measured by ELISA in cell supernatants and was increased after activation of platelets with TRAP (50 μM). Quantification of TXB_2 production is plotted as mean ± s.d. (*n* = 6).*$p<0.05$ vs. neutrophils alone.

Figure S3

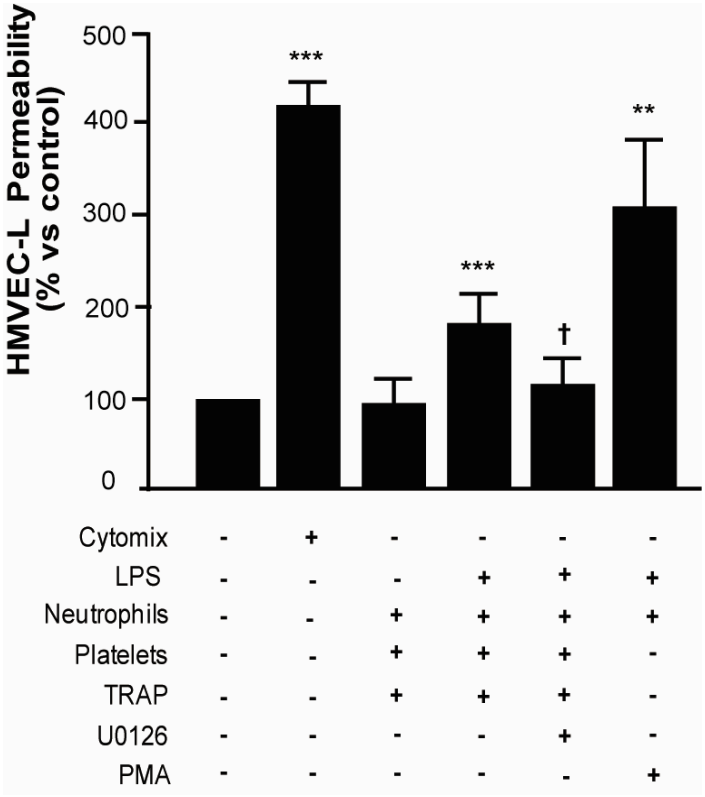

Figure S3
NET formation increases permeability of human lung microvascular endothelial cells (HMVEC-L). In selected experiments, HMVEC-Ls were primed with LPS (2 μg/ml) for 24h prior to the experiment. Permeability was measured by ^{125}I-albumin flux across endothelium over 1h and was increased only in cells treated with cytomix (0.5 ng/ml) or in LPS-primed endothelium + TRAP-activated platelets or PMA. U0126 (10 μM) was added in selected experiments. Mean ± s.d. (*n* = 3); **p<0.01 and ***p<0.001 vs. HMVEC-Ls without treatment. †p<0.05 vs. HMVEC-Ls + LPS + neutrophils + TRAP-activated platelets.

Figure S4

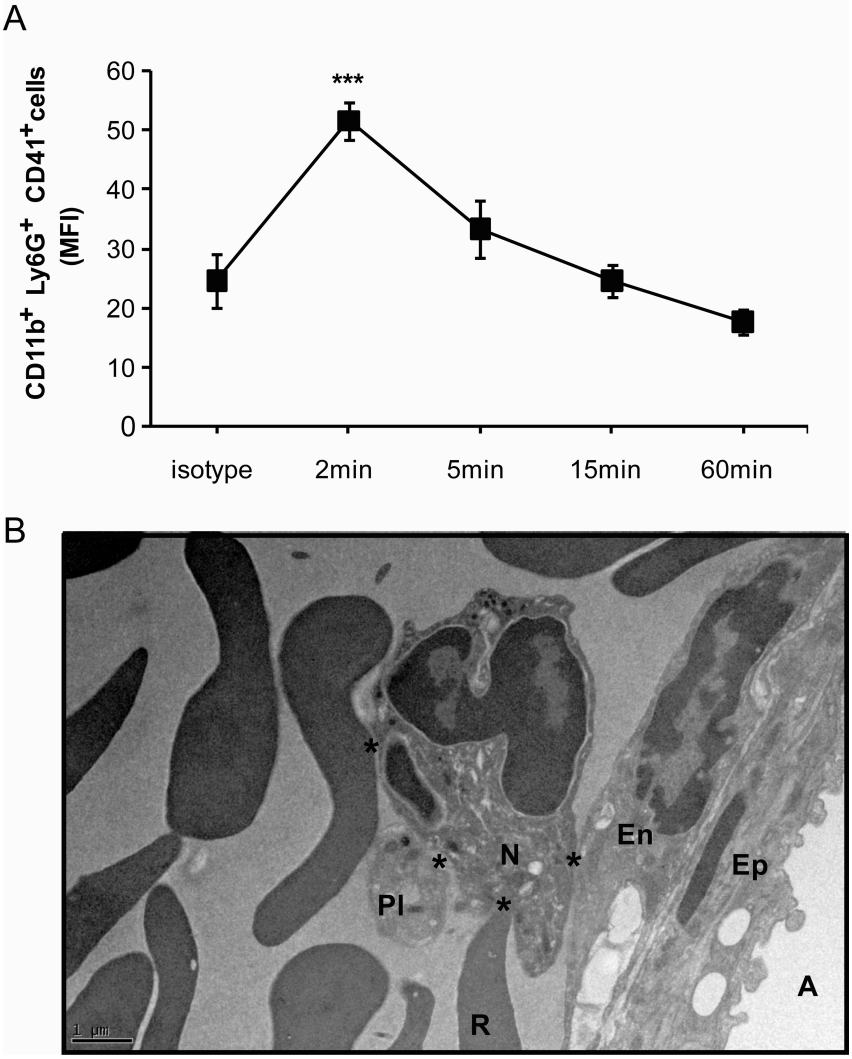

Figure S4
Neutrophil-platelet aggregates are increased in TRALI. **(A)** Neutrophil-platelet aggregates were measured by flow cytometry in digested mouse lungs after TRALI. Neutrophils were identified using Ly6G and CD11b mAbs and platelets were identified using a CD41 mAb. Compared with mice injected with isotype control mAb, there was an increase in neutrophil CD41 staining intensity (MFI) at 2 min after $H2K^d$ mAb injection. (*n* = 4 – 6 per group); ***p<0.01 vs. all other groups. **(B)** Transmission electron microscopy of a mouse lung after TRALI showing an intravascular neutrophil (N) adherent to an adjacent endothelial cell (En) and also adherent to a platelet (Pl) and a red blood cell (R). Ep = epithelial cell; A = alveolus. Asterisks denote areas of adherence to the neutrophil.

Supplemental References

1. Guzik, K., Skret, J., Smagur, J., Bzowska, M., Gajkowska, B., Scott, D.A., and Potempa, J.S. 2011. Cigarette smoke-exposed neutrophils die unconventionally but are rapidly phagocytosed by macrophages. *Cell Death Dis* 2:e131.
2. Fang, X., Neyrinck, A.P., Matthay, M.A., and Lee, J.W. 2010. Allogeneic human mesenchymal stem cells restore epithelial protein permeability in cultured human alveolar type II cells by secretion of angiopoietin-1. *J Biol Chem* 285:26211-26222.