Supplemental Figures S1 – S14

Pneumolysin induces platelet destruction, not platelet activation, which can be prevented by pharmacologic immunoglobulin preparations

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Figure S1

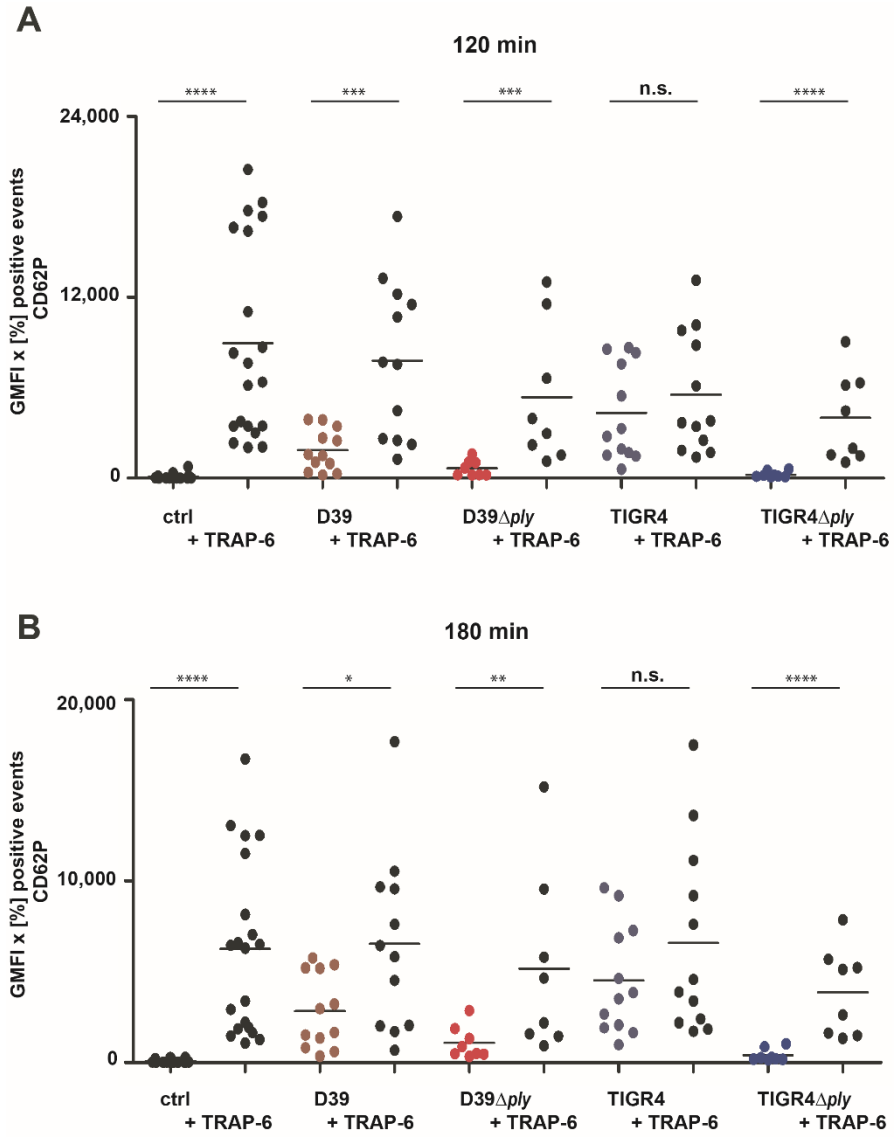

Figure S1. Platelets incubated with pneumococci producing pneumolysin stain positive for the activation marker CD62P

Columns 1 and 2: We incubated washed human platelets (300,000/µL) with PBS (column 1 ctrl) or 20 µM TRAP-6 (column 2) as baseline and maximal expression of CD62P.

We cultured pneumococci to the exponential growth phase before incubating 1.8x 10^6 bacteria with 9 x10^6 platelets (ratio bacteria to platelets 1:5).

Column 3 - 10: Platelets were incubated with pneumococci for (A) 120 minutes or (B) 180 min and CD62P expression was measured by flow cytometry in one aliquot. To the second aliquot 20 µM TRAP-6 was given to measure maximal CD62P expression. Gating for platelets was performed by forward-sideward scatter and CD41 staining. Columns 3 and 4 show the results for strain D39, producing low amounts of pneumolysin; columns 5 and 6 show the results for the pneumolysin-free mutant D39Δ*ply*; columns 7 and 8 show the results for strain TIGR4; and columns 9 and 10 for the pneumolysin-free mutant TIGR4Δ*ply*.

Figure S2

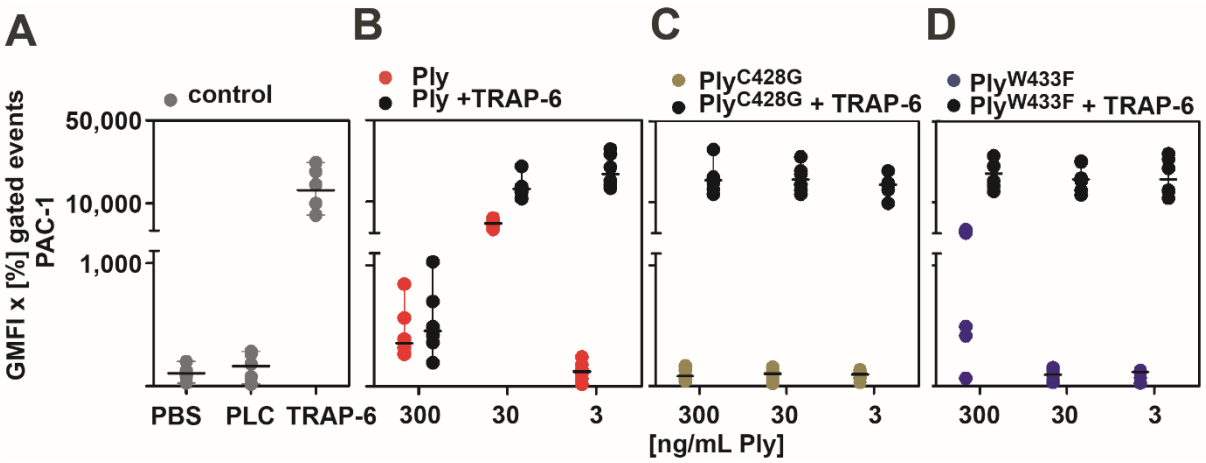

Figure S2: PAC-1 binding to platelets after activation with TRAP-6 is inhibited by high concentrations of pneumolysin

Washed platelets of a defined set of six donors were incubated with various concentrations of pneumolysin (Ply). Binding of the mAb PAC-1, which binds to the activation dependent fibrinogen binding site on platelet GP αIIbβ3 was detected by flow cytometry. The data are presented as geometric mean of fluorescence intensity (GMFI) of the gated population multiplied with the percentage of positive gated events in dot plots including median.

(A) Controls PBS (grey), TRAP (grey), and phospholipase C (grey) from *Staphylococcus aureus* known to not activate platelets[14] and 20 µM TRAP-6 were used as controls.

(B) Pneumolysin (red; ng/mL) caused expression of the PAC-1 binding site at 30 ng/mL, but PAC-1 binding was lower after incubation of platelets with 300 ng/mL. PAC-1 binding could not be increased by subsequent incubation with TRAP-6 indicating that platelets could no longer be activated. 3 ng/mL pneumolysin did not induce the

expression of the binding site for PAC-1 and platelets showed the same increase in PAC-1 binding after incubation with TRAP-6 is in the buffer control.

(C) Pneumolysin^{C428G} without lytic activity (brown) did not activate platelets and (D) pneumolysin^{W433F} with ~10% lytic activity (blue) had a minor effect only at 300 ng/mL.

Figure S3

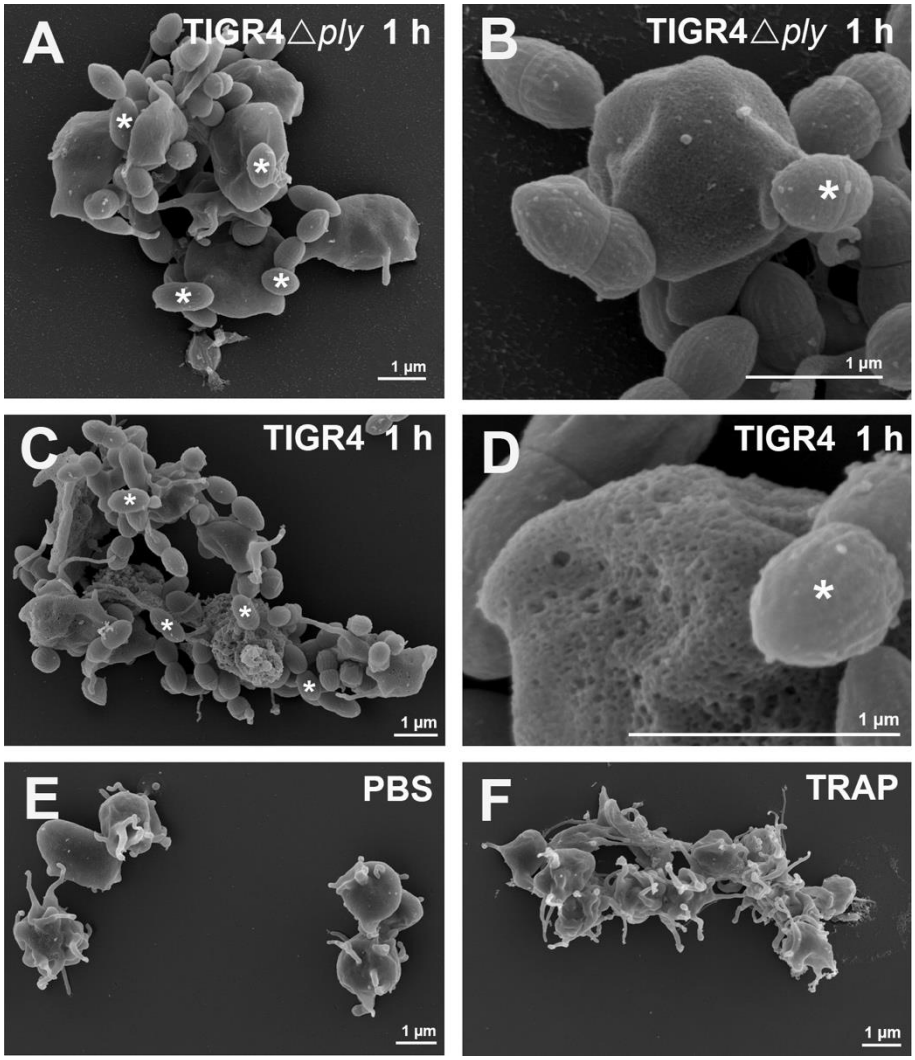

Figure S3. **Visualization of pore formation in the platelet membrane by pneumococci by scanning electron microscopy.**

The pneumococcal strains TIGR4Δ*ply* (A, B) and TIGR4 (C, D) bound to platelets. But only pneumolysin producing TIGR4 induced pores (C, D) in the platelet membrane after incubation for one hour, while TIGR4Δ*ply* did not (A, B). Panel E and F show the platelet morphology after incubation with PBS (E) or TRAP-6 (F). Some pneumococci are labelled by an asterisk.

Figure S4

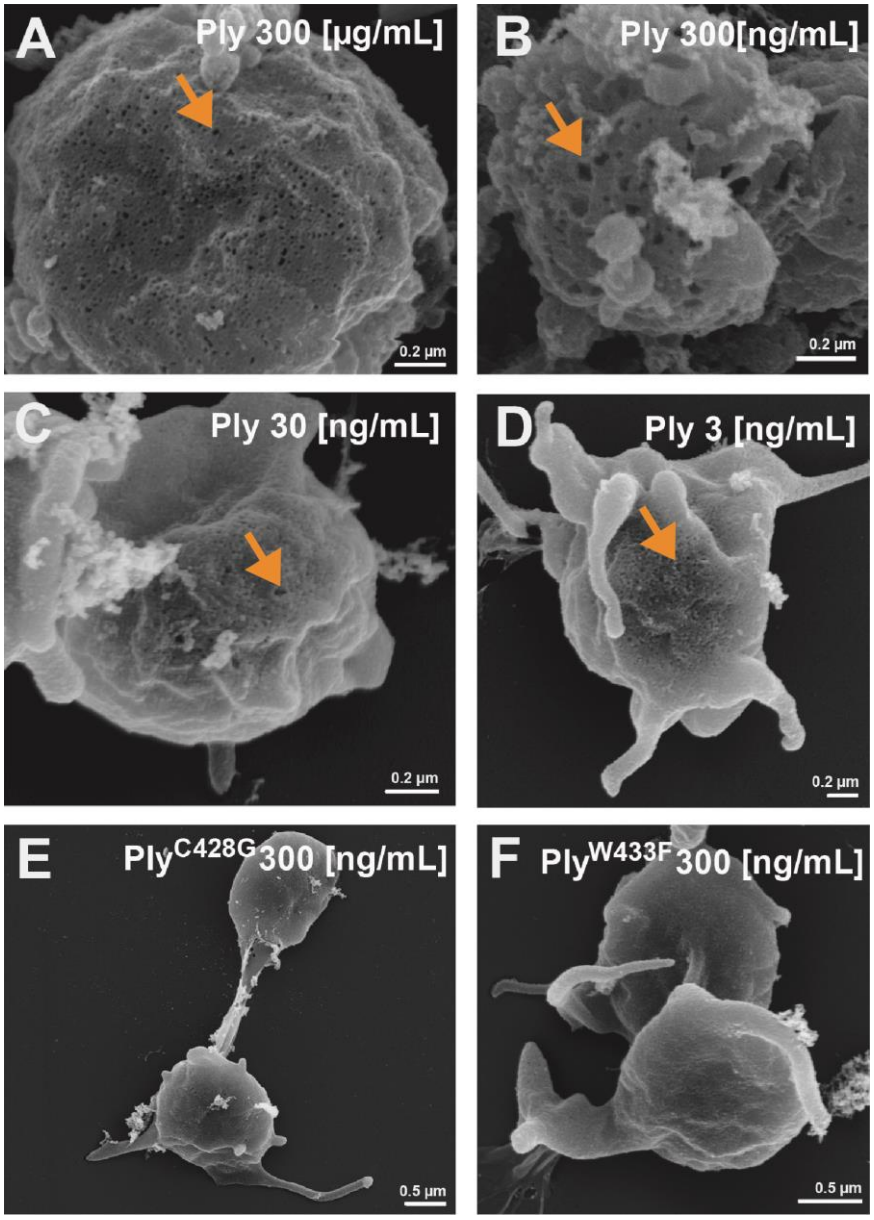

Figure S4. Visualization of pore formation in the platelet membrane by pneumolysin by scanning electron microscopy.

Pneumolysin at concentrations of 300 µg/mL (A), 300 ng/mL (B), 30 ng/mL (C) induced pores in the platelet membrane. Sporadic pores were formed at a pneumolysin concentration of 3 ng/mL (D), while pneumolysin^{C428G} (E) without lytic activity and pneumolysin^{W433F} (F) with ~10% lytic activity did not induce pore formation.

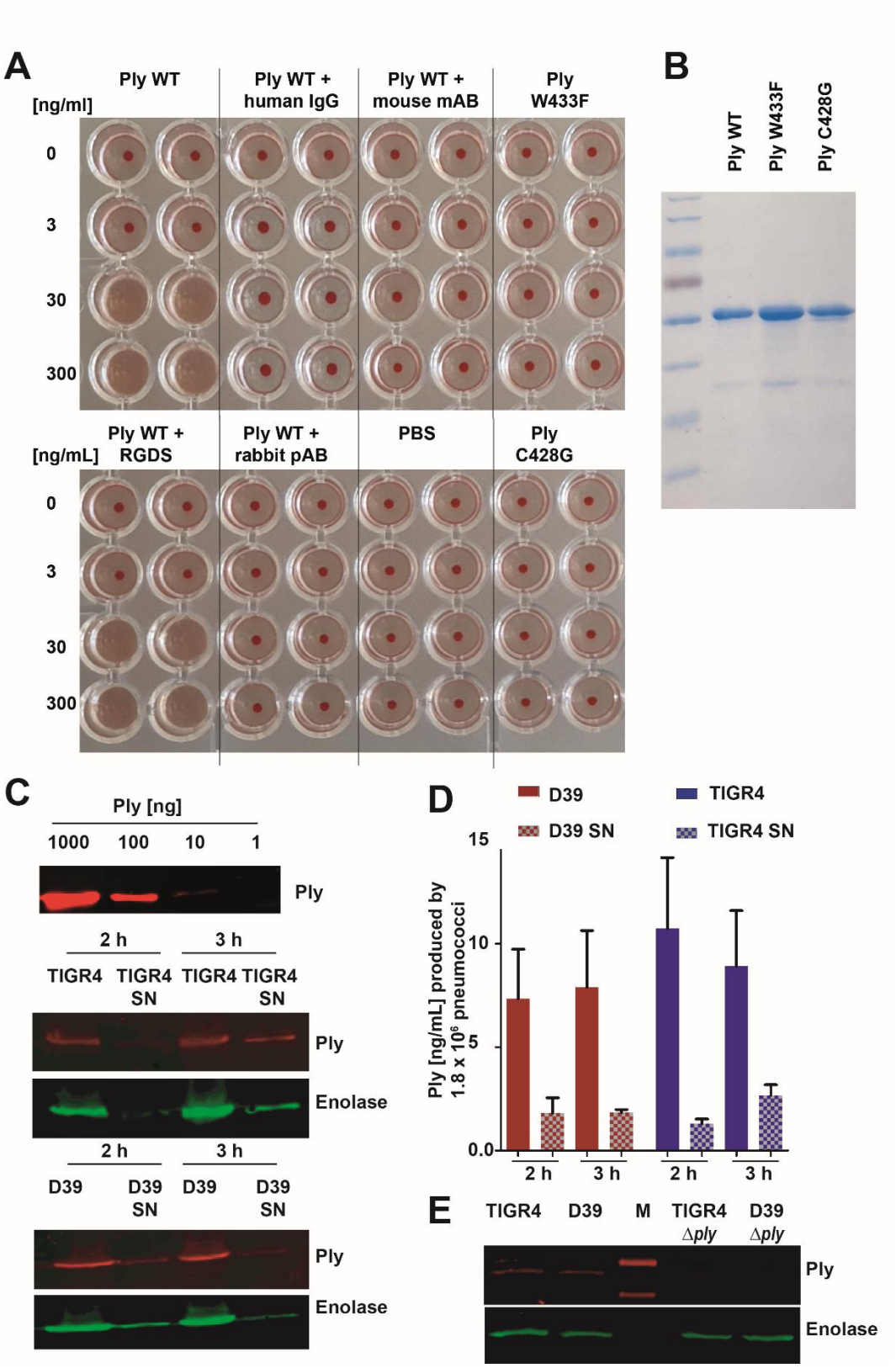

Figure S5. Pneumolysin activity test based on erythrocyte hemolysis and quantification of pneumolysin

(A) Washed red blood cells resuspended in PBS were incubated with increasing concentrations of Ply wild-type (WT), Ply^{W433F}, Ply^{C428G} or Ply WT preincubated with anti-pneumolysin antibodies (human IgG (Privigen), rabbit pAB, mouse mAB). We observed a dose-dependent increase of red blood cell hemolysis with Ply WT starting at 3.0 ng/mL and total cell lysis at 300 ng/mL. No lysis was observed with mutant pneumolysin proteins.

(B) Coomassie stained SDS-PAGE of recombinant Strep-tagged Ply WT, Ply^{W433F} and Ply^{C428G} with a molecular mass of ~56 kDa.

(C) Immunoblotting of D39 and TIGR4 total cell lysates and of the respective TCA (trichloroacetic acid) precipitated supernatants after incubation for 2 h and 3 h in PBS/Tyrodes buffer. Pneumolysin was detected using the antibodies described in Material and Methods. Enolase was used as loading control. For calculation of the Ply amount in the samples a standard curve of serially diluted recombinant Ply WT was used.

(D) Quantification of samples illustrated in C. The values are given as the Ply concentration in ng/mL being present in the experimental setup of Figure S1 (1.8×10^6 bacteria in 100 µL).

(E) Detection of pneumolysin in strains D39, D39Δ*ply*, TIGR4 and TIGR4Δ*ply* used for platelet activation assays. Enolase was used as a loading control.

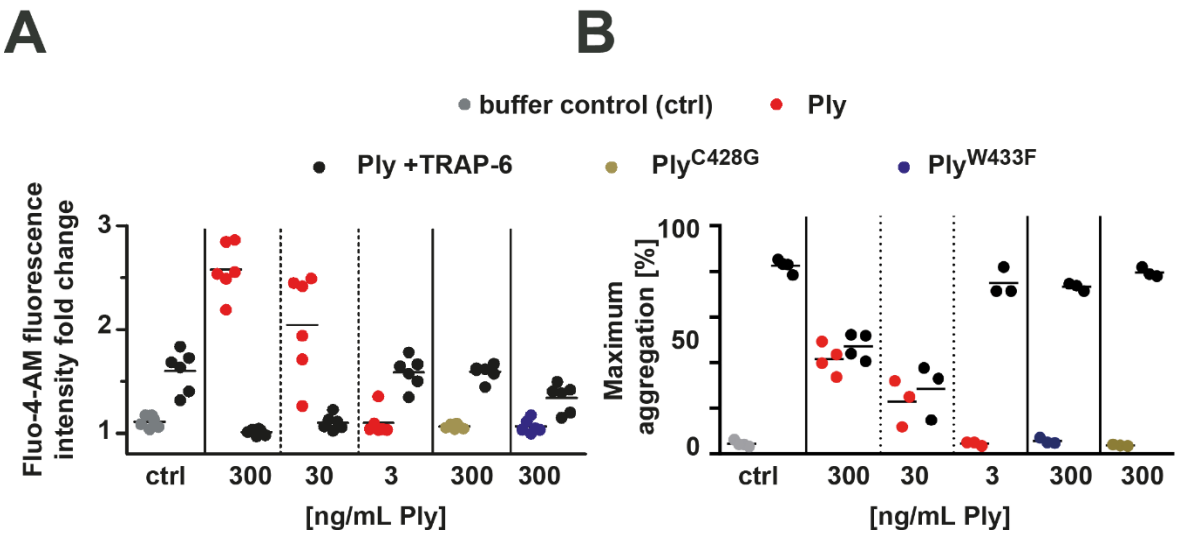

Figure S6. Pneumolysin impairs platelet function.

(A) Prior to pneumolysin treatment intracellular Ca^{2+} of washed platelets was labelled with Fluo-4-AM for 30 min. After incubation with pneumolysin the kinetics of Ca^{2+} release was measured and values are given as fold change compared to NaCl control. Pneumolysin (Ply) induced dose-dependently a Ca^{2+} release with strong release at 300 ng/mL pneumolysin, lower release at 30 ng/mL pneumolysin, and no release at 3 ng/mL. Pneumolysin^{C428G} without lytic activity (brown) pneumolysin^{W433F} with ~10% lytic activity (blue) did not cause Ca^{2+} release. When pneumolysin induced Ca^{2+} release, platelets were no longer responsive to TRAP-6. The response to TRAP-6 is shown in the right part of each column.

(B) Platelet aggregation is typically directly proportional to an increase in light transmission. Pneumolysin concentrations of 300 ng/mL and 30 ng/mL induced an increase in light transmission, but platelets were no longer responsive to 20 mM TRAP-6. Light transmission did not change by addition of buffer, pneumolysin 3.0 ng/mL, or the mutant pneumolysins and platelets were still responsive to 20 µM TRAP-6.

Figure S7

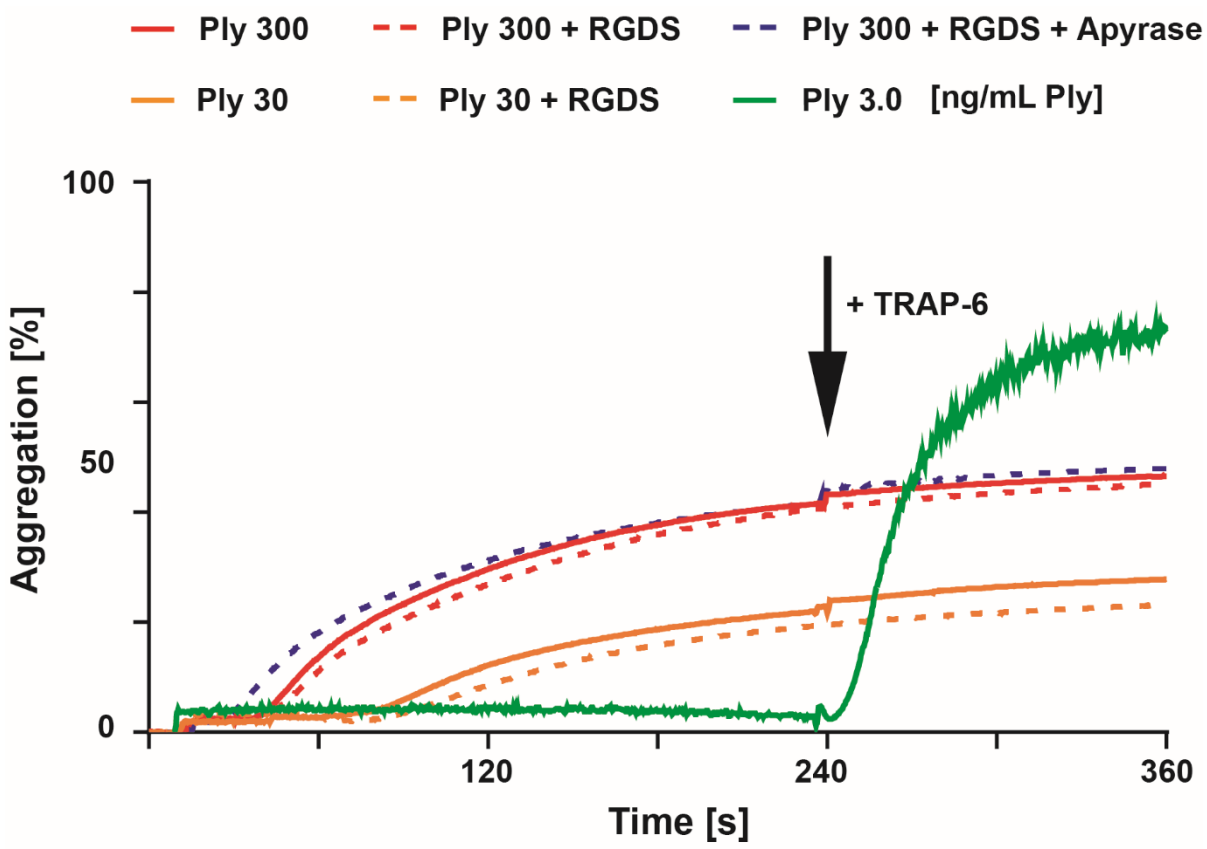

Figure S7. Blocking the platelet fibrinogen receptor αIIbβ3 binding site by RGDS does not inhibit increase in light transmission when platelets are incubated with pneumolysin.

RGDS is a peptide blocking the binding site of platelet fibrinogen receptor αIIbβ3 and hereby blocks platelet aggregation. Preincubation of platelets with RGDS did not reduce the increase in light transmission, which is used as a marker for platelet aggregation. Platelets were non-responsive to an additional dose of TRAP-6. This strongly indicates that the increase in light transmission is induced by lysis of platelets rather than by platelet aggregation.

Figure S8

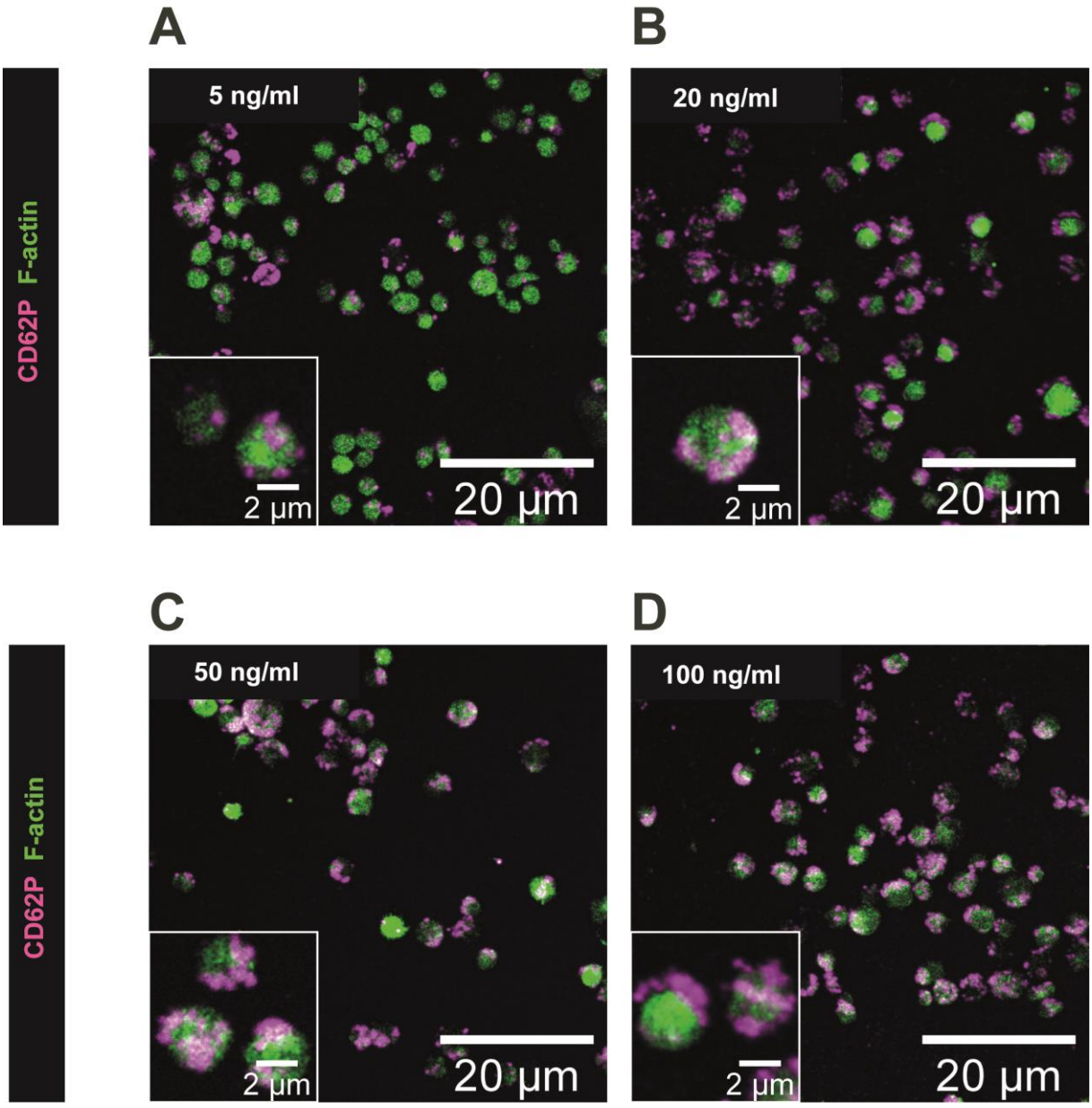

Figure S8. Pneumolysin induces staining of platelets for CD62P without inducing morphological features of platelet activation

Increasing concentrations of pneumolysin induced increasing staining of platelets for CD62P. The cytoskeleton protein F-actin is shown in green and CD62P is shown in magenta. Platelets were round and not strongly activated despite a major increase in CD62P staining. This indicates that intracellular CD62P is stained and CD62P is not expressed on the platelet membrane. Insets represent higher magnifications.

Figure S9

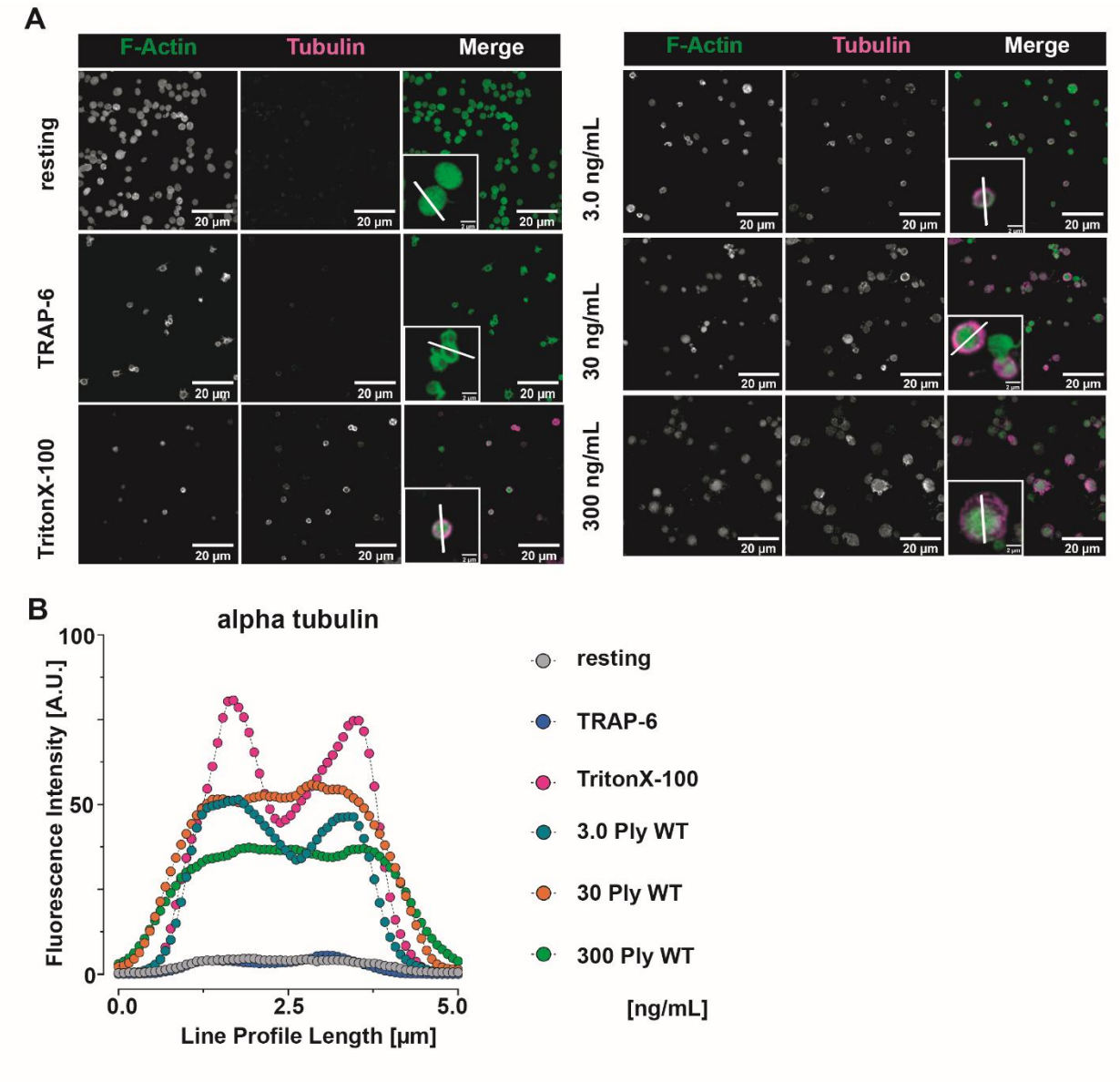

Figure S9. Fluorescence microscopy of pneumolysin treated platelets

(A) Pneumolysin treated platelets were stained for F-actin (green) and α-tubulin (magenta). Platelets were not permeabilized, with the exception of the TritonX-100 control. Inserts show single platelets at higher magnification and the line used for measuring fluorescence intensities shown in panel B. In the presence of 3.0 and 30 ng/mL pneumolysin, intracellular staining of α-tubulin becomes visible. At 300 ng/mL pneumolysin, vesicles staining strongly for pneumolysin surround the platelets. (B) Staining pattern of α-tubulin throughout single cells treated with pneumolysin was

quantified to distinguish between cytoplasmic and only surface associated α-tubulin staining. The pattern indicates that α-tubulin is stained intracellularly and not extracellularly. The different concentrations of pneumolysin used are colour coded, 3.0 ng/mL (blue), 30 ng/mL (orange), 300 ng/mL (green).

Figure S10

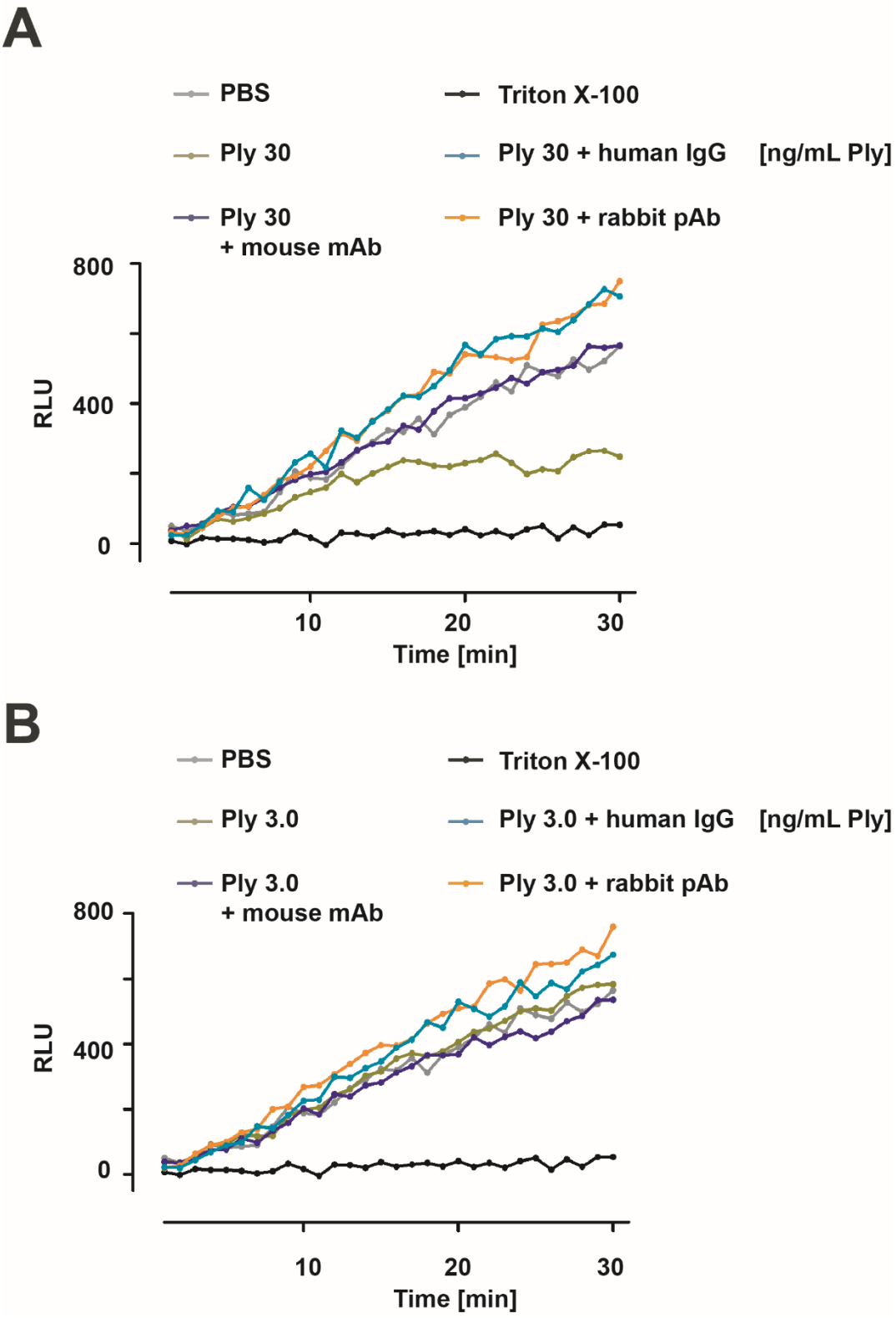

Figure S10. Platelet viability in the presence of pneumolysin is maintained by immunoglobulins

(A) Platelet viability was reduced in the presence of pneumolysin 30 ng/mL. Platelet viability was maintained in the presence of polyvalent human immunoglobulin (human IgG (Privigen)), polyclonal rabbit anti-pneumolysin or monoclonal mouse anti-pneumolysin antibody, despite the presence of 30 ng/mL pneumolysin. Triton X-100 was used as control to induce death of platelets.

(B) Pneumolysin at a concentration of 3.0 ng/mL had no effect on platelet viability.

Figure S11

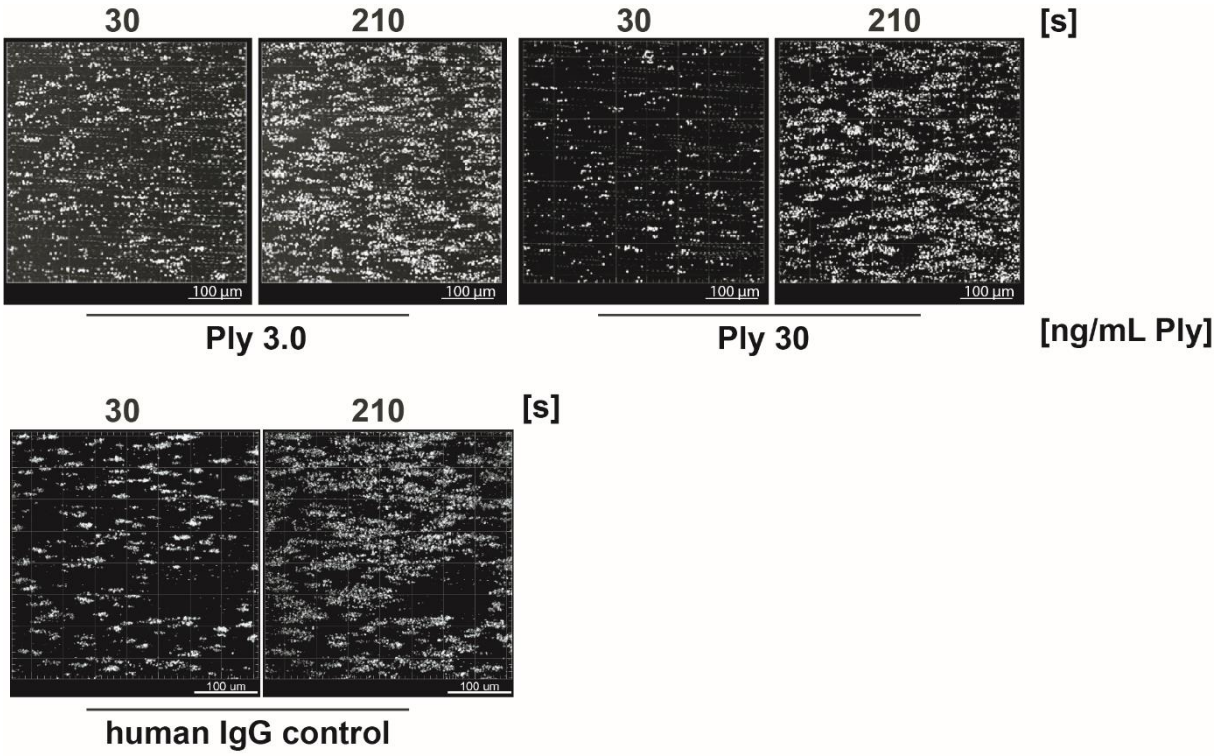

Figure S11. **Effect of pneumolysin on platelet thrombus formation in flow chamber.**

Thrombus formation on collagen in a flow chamber was monitored by image acquisition at an interval of 10 s by fluorescence microscopy under low and high shear stress. In the presence of 30 ng/mL pneumolysin thrombus formation was impaired, while 3.0 ng/mL pneumolysin had hardly any effect. Pharmaceutical human IgG alone had no effect on platelet thrombus formation. Buffer control and pneumolysin 300 ng/mL are shown in Figure 2.

Figure S12

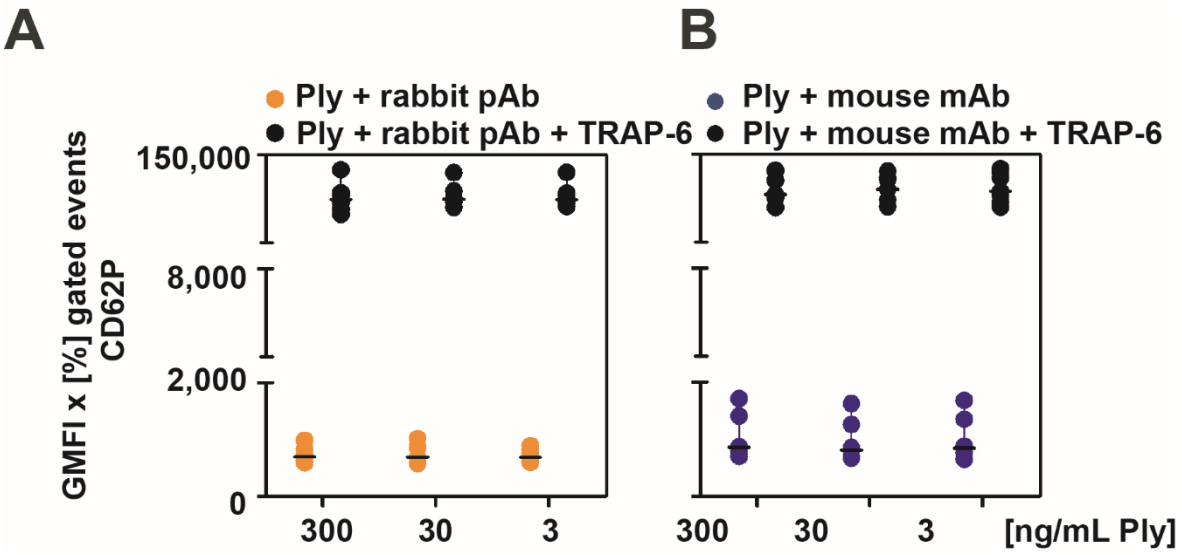

Figure S12. The effect of pneumolysin on CD62P expression on platelets is neutralized by antibodies

Pneumolysin caused P-selectin expression and dose-dependently inhibited an additional response to TRAP-6 (main text, Fig. 1A, B).

(A) Polyclonal rabbit anti-pneumolysin antibodies (10 µg/mL) and (B) a monoclonal mouse anti-pneumolysin antibody (7.5 µg/mL) neutralized the effects of pneumolysin.

Figure S13

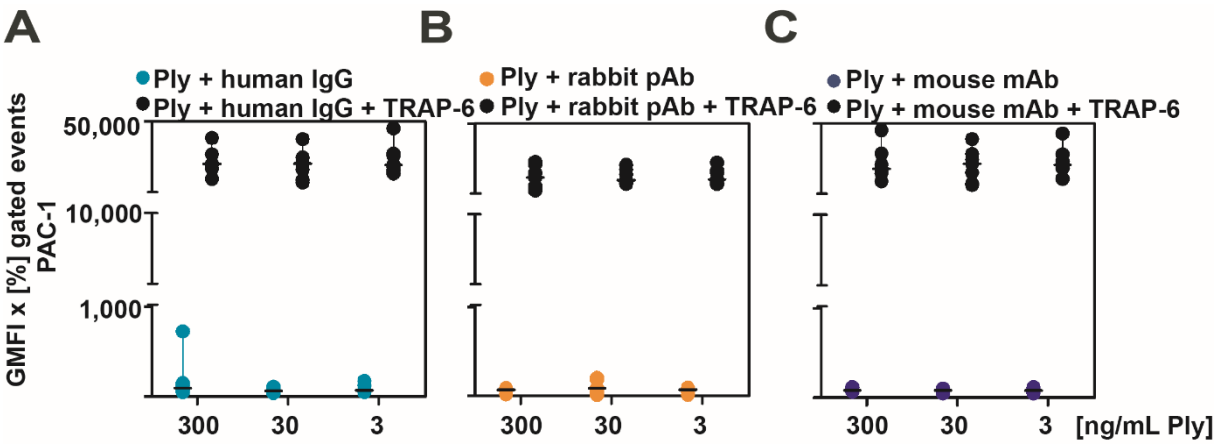

Figure S13. The effects of pneumolysin on PAC-1 binding to platelets is neutralized by antibodies

PAC-1 binding to platelets after activation with TRAP-6 is inhibited by high concentrations of pneumolysin (Fig. S2).

(A) Polyvalent immunoglobulins (human IgG (Privigen)), (B) a polyclonal rabbit anti-pneumolysin antibody, and (C) a monoclonal mouse anti-pneumolysin antibody neutralized the effects of pneumolysin on PAC-1 binding.

Figure S14

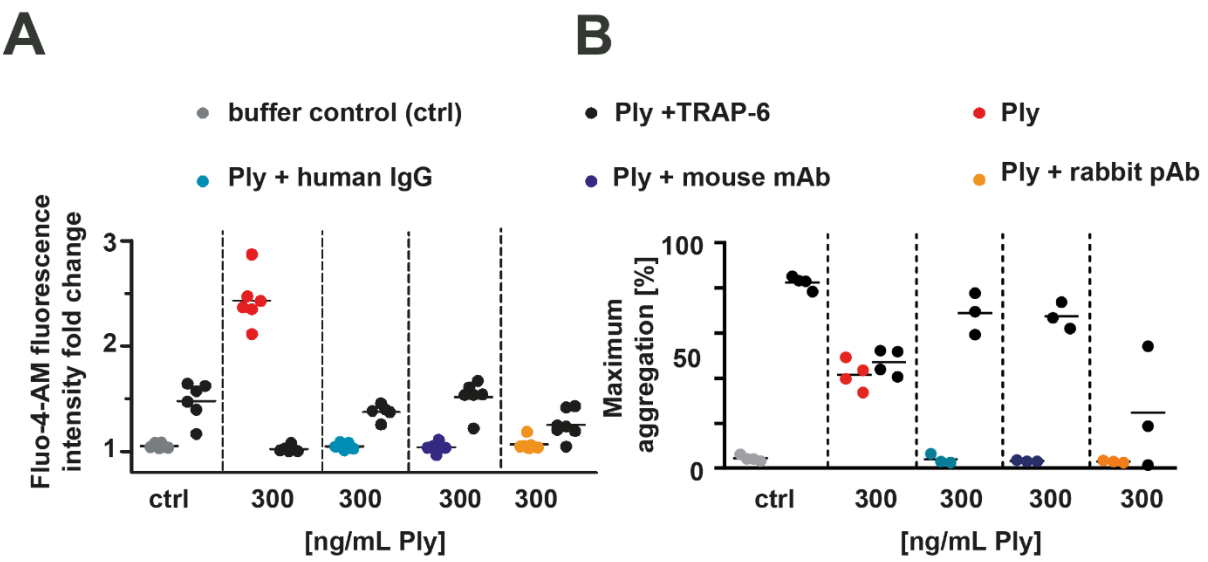

Figure S14. Immunoglobulins prevent pneumolysin impaired platelet function

(A) Polyvalent human immunoglobulin (human IgG (Privigen); 1mg/mL; green), polyclonal rabbit anti-pneumolysin (10 µg/mL; orange) and a monoclonal mouse anti-pneumolysin antibody (7.5 µg/mL; blue) prevented the Ca^{2+} release induced by pneumolysin (300 ng/mL; red) and subsequent unresponsiveness to TRAP-6.

(B) Polyvalent human immunoglobulin (human IgG (Privigen); 1mg/mL; light blue), polyclonal rabbit anti-pneumolysin (10 µg/mL; orange) and a monoclonal mouse anti-pneumolysin antibody (7.5 µg/mL; blue) prevented the effects of pneumolysin (300 ng/mL; red) on increase in light transmission (aggregation). In the presence of these immunoglobulins platelets became again responsive to 20 µM TRAP-6.