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ORIGINAL ARTICLE

Activated platelets kill *Staphylococcus aureus*, but not *Streptococcus pneumoniae*—The role of FcγRIIa and platelet factor 4/heparinantibodies

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Abstract

Background: Heparin induced thrombocytopenia (HIT) is likely a misdirected bacterial host defense mechanism. Platelet factor 4 (PF4) binds to polyanions on bacterial surfaces exposing neo-epitopes to which HIT antibodies bind. Platelets are activated by the resulting immune complexes via FcγRIIA, release bactericidal substances, and kill Gram-negative *Escherichia coli*.

Objectives: To assess the role of PF4, anti-PF4/H antibodies and FcγRIIa in killing of Gram-positive bacteria by platelets.

Methods: Binding of PF4 to protein-A deficient *Staphylococcus aureus* (SA113Δ*spa*) and non-encapsulated *Streptococcus pneumoniae* (D39Δ*cps*) and its conformational change were assessed by flow cytometry using monoclonal (KKO,5B9) and patient derived anti-PF4/H antibodies. Killing of bacteria was quantified by counting colony forming units (cfu) after incubation with platelets or platelet releasate. Using flow cytometry, platelet activation (CD62P-expression, PAC-1 binding) and phosphatidylserine (PS)-exposure were analyzed.

Results: Monoclonal and patient-derived anti-PF4/H antibodies bound in the presence of PF4 to both *S. aureus* and *S. pneumoniae* (1.6-fold increased fluorescence signal for human anti-PF4/H antibodies to 24.0-fold increase for KKO). *Staphylococcus aureus* (5.5×10^4cfu/mL) was efficiently killed by platelets (2.7×10^4cfu/mL) or their releasate (2.9×10^4cfu/mL). Killing was not further enhanced by PF4 or anti-PF4/H antibodies. Blocking FcγRIIa had no impact on killing of *S. aureus* by platelets. In contrast, *S. pneumoniae* was not killed by platelets or releasate. Instead, after incubation with pneumococci platelets were unresponsive to TRAP-6 stimulation and exposed high levels of PS.

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Conclusions: Anti-PF4/H antibodies seem to have only a minor role for direct killing of Gram-positive bacteria by platelets. *Staphylococcus aureus* is killed by platelets or platelet releasate. In contrast, *S. pneumoniae* affects platelet viability.

KEYWORDS

aPF4/H antibodies, FcγRIIa, PF4, platelets, *Staphylococcus aureus*, *Streptococcus pneumoniae*

1 | INTRODUCTION

Platelets play an important role in hemostasis and their role in immune responses is increasingly noticed. They express a variety of receptors, usually found on cells of the immune system like Toll-like receptors, complement receptors or Fc-receptors.[1,2] An important role of the immune system is the defense against pathogens. Bacteria and platelets interact with each other[3-6] and bacteria can activate platelets directly via membrane receptors or indirectly through bridging proteins or secreted toxins.[7-10] Activated platelets consecutively secrete bactericidal substances stored in granules and non-granule compartments.[11-13] Platelets further contribute to defense against bacterial pathogens by mediating recruitment of other immune cells by chemotaxis. In addition, migrating platelets use adhesion receptors to mechanically probe their immediate microenvironment, thereby piling up bacteria and acting as cellular mechanical scavengers. The resulting higher density of bacteria boosts the activity of leukocytes.[14] Furthermore, platelets can directly kill bacteria.[12,15] We have recently shown that the FcγRIIa,[16] a low affinity IgG receptor, is involved in killing of Gram-negative *Escherichia coli* by platelets.[17] This is mediated by cross-linking of the platelet FcγRIIa receptor by antibodies bound to the chemokine platelet factor 4 (PF4),[18] which is bound to polyanions on the surface of bacteria.[19,20]

The mechanism how PF4 and anti-PF4/polyanion (PF4/H) antibodies are involved in platelet activation has been investigated in detail for the adverse drug effect heparin-induced thrombocytopenia (HIT).[21-23] PF4 bound to polyanions is the major antigen involved in the pathogenesis of HIT. Positively charged PF4 released from α-granules of platelets binds to negatively charged heparin, changing its conformation and exposing neoepitopes to which anti-PF4/H antibodies bind. These immune complexes lead to intravascular activation of platelets, monocytes, granulocytes, and endothelial cells via FcγRIIa and in consequence to thrombocytopenia and increased thrombin generation, which results in a major risk for new thrombosis.[24,25] According to our current concept the adverse drug effect HIT displays a misdirected antibacterial host defense mechanism.[26]

After binding to Gram-positive bacteria, PF4 undergoes similar conformational changes like after binding to *E. coli* and is then recognized by anti-PF4/H antibodies. The aim of this study was to determine whether anti-PF4/H antibodies are able to enhance killing of PF4 coated Gram-positive bacteria. As a source for anti-PF4/H antibodies we used human sera of patients who had developed HIT. To control for potential contamination of the affinity purified human anti-PF4/H antibodies with "anti-bacterial" antibodies we further used monoclonal antibodies with anti-PF4/H specificity. As prototypes for Gram-positive bacteria, we used a protein-A deficient strain of *Staphylococcus aureus* (*S. aureus*, SA113Δ*spa*)[19] and a non-encapsulated strain of *Streptococcus pneumoniae* (*S. pneumoniae*, D39Δ*cps*).[27,28] SA113Δ*spa* was used to avoid binding of IgG-Fc parts to protein-A and D39Δ*cps* to facilitate the interaction of *Spneumoniae* surface proteins with platelets and/or PF4, which would otherwise be shielded by the capsule.

We found that monoclonal and patient derived anti-PF4/H antibodies bind to Gram-positive bacteria. However, anti-PF4/H antibody opsonization has no major effects on killing *S. aureus* and *S. pneumoniae* by platelets. Strikingly, platelets had different effects on survival of *S. aureus* and *S. pneumoniae*. *Staphylococcus aureus* induced platelet activation and was killed by both, platelets and platelet releasate. In contrast *Streptococcus pneumoniae* was not killed by platelets or platelet releasate and seems to affect viability of platelets.

Essentials

- Platelet factor 4 (PF4) binds to bacteria and exposes neoepitopes to which PF4/H antibodies bind.
- *Staphylococcus aureus* is killed by platelets and platelet releasate independent of PF4/H antibodies.
- *Streptococcus pneumoniae* is not killed by platelets or releasate, instead they affect viability of platelets.

2 | MATERIAL AND METHODS

2.1 | Monoclonal antibodies

The monoclonal antibody RTO, which binds PF4 independent of heparin, was obtained from Thermo Fisher. Monoclonal antibodies (mAbs) binding to PF4/H complexes, mimicking human anti-PF4/H antibodies, were: KKO (obtained from Thermo Fisher); 5B9[29] and 2E1 (raised in the laboratory by one of the authors YG). MAb 5B9 behaves like a heparin dependent anti-PF4/H antibody, while mAb 2E1 binds to PF4 and activates platelets also in the absence of heparin. Goat anti-mouse IgG-Alexa Fluor 647 (Abcam) and goat anti-human IgG-Dylight 650 (Abcam) were used as secondary antibodies. Daratumumab (=Darazalex; Janssen Pharmaceutica), a humanized

anti-CD38-antibody, was used as a negative control for our monoclonal PF4/H antibodies.

2.2 | Purification of human PF4/H antibodies

Anti-PF4/H antibodies were isolated from sera of patients containing platelet activating anti-PF4/H antibodies, which was confirmed by the PF4/H enzyme immunosorbent assay (EIA)[30] and a functional assay using washed platelets, the heparin-induced platelet activation test (HIPA).[31]

Sera were diluted (1:30) in washing-buffer (20 mmol/L sodium phosphate buffer, pH 7.0) and added to a column containing protein G sepharose (GE Healthcare). After washing, antibodies were eluted with 0.1 mol/L glycine buffer (pH 2.7), pH adjusted to 7.4 by 1 mol/L Tris, and dialyzed in phosphate buffered saline (PBS; PAN-Biotech) using a 10 000 kDa membrane (Thermo Fisher). A column containing streptavidin sepharose (GE Healthcare) was coated with complexes of PF4, biotinylated PF4 (both Chromatec) and unfractionated heparin (UFH; Ratiopharm). Total IgG (purified from HIT patient serum by protein G affinity chromatography) was added, anti-PF4/H specific IgG eluted and pH neutralized.[32] The amount of protein was quantified by bicinchoninic acid assay (BCA, QuantiPro BCA-test; Sigma-Aldrich),[33] and reactivity tested by anti-PF4/H EIA and HIPA.

2.3 | Preparation of bacterial strains

Staphylococcus aureus SA113Δ*spa* (protein A–deficient strain[19]) was grown to mid-exponential phase at 37°C to an OD_{600} of 0.7-0.8 in basic nutrient broth (Sigma Aldrich). *Streptococcus pneumoniae* D39Δ*cps* (non-encapsulated mutant of serotype 2 strain D39)[28] was grown to mid-exponential phase at 37°C to an OD_{600} of 0.3-0.4 in Todd-Hewitt broth (Thermo Fisher) supplemented with 0.5% yeast extract. Bacteria were washed twice in PBS and adjusted to 1×10^9 colony forming units (cfu) per mL.

2.4 | Binding of IgG to bacteria

Bacterial strains were incubated with PF4 in increasing concentrations (1-50 µg/mL) to determine the optimal PF4 concentration for maximal PF4 binding (Figure S1 in supporting information). Briefly, bacteria (1×10^9 in 100 µL PBS) were incubated with PF4 (10 µg/mL final concentration for *S aureus*, 20 µg/mL for *S pneumoniae*) for 30 minutes at 4°C under agitation (40 rpm) in a 96-well plate and washed with PBS (2250 g; 5 minutes). Thereafter, 50 µg/mL of RTO, KKO, 5B9, 2E1, or human PF4/H-IgG were added and incubated for 30 minutes under agitation. After washing with PBS (2250 *g*; 5 minutes), samples were incubated (60 minutes; 4°C) with labeled secondary goat anti-mouse IgG-Alexa Fluor 647 or goat anti-human IgG-Dylight 650 and then fixed with 1% paraformaldehyde (PFA; Morphisto). Binding of biotinylated PF4 (bPF4) was tested as follows: bacteria were incubated with increasing concentrations of bPF4 (1-30 µg/mL), followed by washing with PBS and incubation with 12 µg/mL streptavidin-Atto 633 (Attotec) followed by fixation with 1% PFA. Binding of PF4 was measured by flow cytometry (Cytomics FC 500 Beckman Coulter).

2.5 | Preparation of platelets and assessment of platelet activation

Platelet rich plasma was isolated by differential centrifugation from anticoagulant citrate dextrose solution A (ACD-A) anticoagulated blood of healthy human donors, who gave written, informed consent. Platelets were washed with washing buffer, resuspended in Tyrode's buffer, containing bovine serum albumin and glucose (as described[31]), and incubated at pre-tested ratios with the bacteria. *S. aureus* was used with a multiplicity of infection (MOI) of 25:1 (platelets/bacteria) and *S. pneumoniae* with a MOI of 5:1 (platelets:bacteria) and samples were incubated for 2 hours at 37°C. Because bacteria can activate platelets directly, we identified the MOI ratios at which bacteria alone did not activate platelets (in the absence of PF4 and/or PF4/heparin antibodies) by incubating platelets and bacteria in increasing ratios and measuring CD62P expression on platelets (Figure S2 in supporting information).

Bacteria induced platelet activation was measured after 15 and 120 minutes assessing CD62P expression (CD62P-PE-Cy5; Becton Dickinson) and activated integrin αIIbβ3 (PAC-1 FITC; Becton Dickinson) by flow cytometry. TRAP-6 (20 µmol/L final concentration; Bachem) was used as control agonist. Gating of platelets in flow cytometry was based on size (forward scatter, FSC) versus granularity (side scatter, SSC) and was controlled by CD61 staining as shown in Figure S3 in supporting information.

2.6 | Inhibition of FcγRIIa and obtaining platelet releasate

To block FcγRIIa, washed platelets in Tyrode's buffer were incubated with 30 µg/mL mAb IV.3 for 45 minutes at 37°C.

To obtain the platelet releasate, washed platelets in Tyrode's buffer were co-stimulated with 20 µmol/L TRAP-6 and 5 µg/mL Horm collagen type I (Nycomed) for 15 minutes under agitation. After centrifugation (7 minutes, 650 *g*), the cell free supernatant was transferred to a new tube, centrifuged 5 minutes at 13 000 *g*, and the resulting supernatant was used as platelet releasate.

2.7 | Incubation of bacteria with platelets

Staphylococcus aureus pre-coated (with or without PF4, with or without anti-PF4/H antibodies) was incubated at a MOI of 25:1 (platelets:bacteria) with platelets or FcγRIIa-blocked platelets or platelet releasate obtained from the same amount of platelets, or

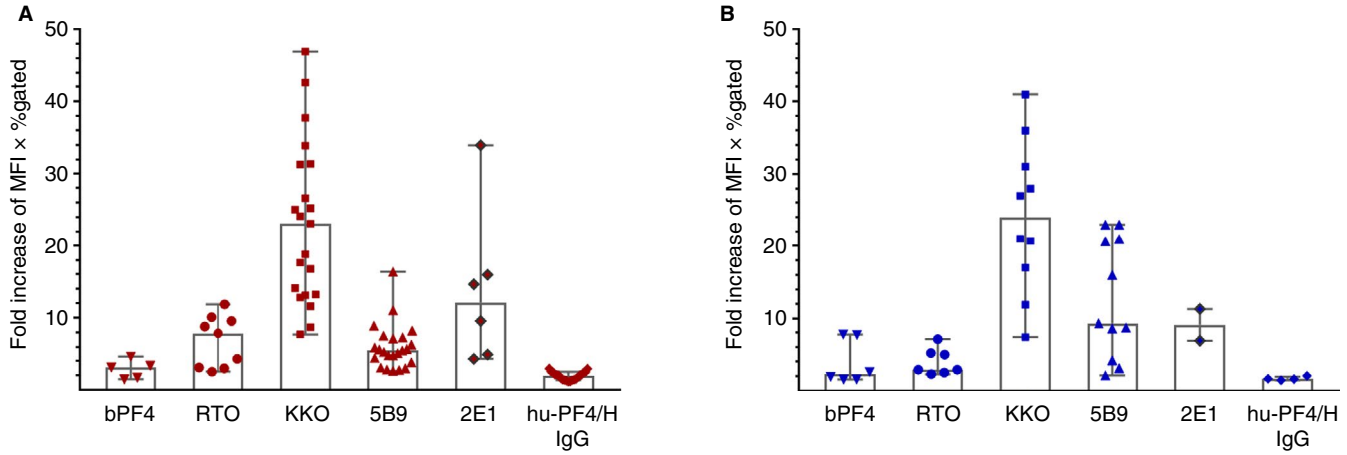

FIGURE 1 Binding of platelet factor 4 (PF4) and anti-platelet factor 4/heparin (PF4/H) antibodies to (A) *Staphylococcus aureus* and (B) *Streptococcus pneumoniae*. Biotinylated PF4 (bPF4) or non-labelled PF4 was used to measure PF4 binding to *S. aureus* (A) and *S. pneumoniae* (B). Binding of bPF4 was detected by Atto 488 conjugated streptavidin, while binding of non-labelled PF4 was detected using mAb RTO. Expression of the neoepitopes of PF4 was measured by the monoclonal antibodies KKO, 5B9, and 2E1, as well as human anti-PF4/H IgG. The mean fluorescence intensity (MFI) multiplied by percentage of gated events was determined and the increase in binding was calculated for bPF4 in comparison to streptavidin without bPF4 and for the antibodies by comparing antibody binding with and without addition of PF4. *S. aureus* data are shown in red and *S. pneumoniae* data are shown in blue in all Figures

the same volume of Tyrode's buffer (containing 0.35% bovine serum albumin and 0.1% glucose). Two hours after incubation the samples containing *S. aureus* were diluted 1:50, 1:100, 1:500, and 1:2000 in 0.9% NaCl and transferred to blood agar plates (Becton Dickinson). Cfu were determined via the drop plate method as described.[34] In brief, the agar plate was divided into quarters and 3 × 10 µL drops of each diluted sample were added (Figure S4 in supporting information). *Streptococcus pneumoniae* was incubated at a MOI of 5:1 with platelets, platelet releasate obtained from the same amount of platelets, or the same volume of Tyrode's buffer under the same conditions and 100 µL of serial dilutions were plated on blood agar plates. After incubation overnight at 37°C the cfu were determined and the survival rates (cfu/mL) calculated. To exclude that the lower MOI (platelets:bacteria) used for *S. pneumoniae* in comparison to *S. aureus* did cause a systematic bias, we also monitored survival rates of *S pneumoniae* at a MOI of 25:1 (platelets:bacteria).

2.8 | Platelet activation and phosphatidylserine exposure

Platelet activation (CD62P expression) and phosphatidylserine (PS) exposure (Annexin V binding) were measured by flow cytometry. Platelets were incubated with *S. aureus* (25:1) or *S. pneumoniae* (5:1) in 100 µL Tyrode's buffer, containing bovine serum albumin and glucose at 37°C and anti-CD62P-PECy5 was added. After 15 and 120 minutes samples were split and incubated again for 10 minutes. One sample was incubated with 20 µmol/L TRAP-6 and the other one with buffer. Samples for Annexin V binding were fixed with 0.5% PFA and stained for 20 minutes with Annexin V-APC in binding buffer (both Biolegend) supplemented with hirudin 5.4 U/mL (Canyon Pharmaceuticals). Control platelets were co-stimulated for 20 minutes at 37°C with 20 µmol/L TRAP-6 and 100 ng/mL Convulxin (Enzo Life Sciences), 70% Ethanol (Carl Roth) or PBS.

2.9 | Statistics

Statistical analyses were performed with GraphPad Prism version 7.04 software. Flow cytometry data were analyzed by multiplying the mean fluorescence intensity (MFI) with the percentage of positive gated events. Data are presented as median with range, except where indicated. Statistical significance was calculated by Mann-Whitney test and for multiple comparisons by Kruskal-Wallis test, except where indicated. Significance was set at $P < .05$. Outliers were identified by the ROUT method.[35]

2.10 | Ethics

The use of human sera with HIT antibodies and human platelets obtained from healthy volunteers was approved by the ethics board at the Universitätsmedizin Greifswald.

3 | RESULTS

3.1 | Monoclonal anti-PF4/H antibodies and patient derived human anti-PF4/H antibodies bind to *Staphylococcus aureus* and *Streptococcus pneumoniae*

Consistent with our previous findings,[19] we confirmed dose-dependent PF4 binding to bacteria with a saturation at 10 µg/mL for *S. aureus* and at 20 µg/mL for *S. pneumoniae* (Figure S1).

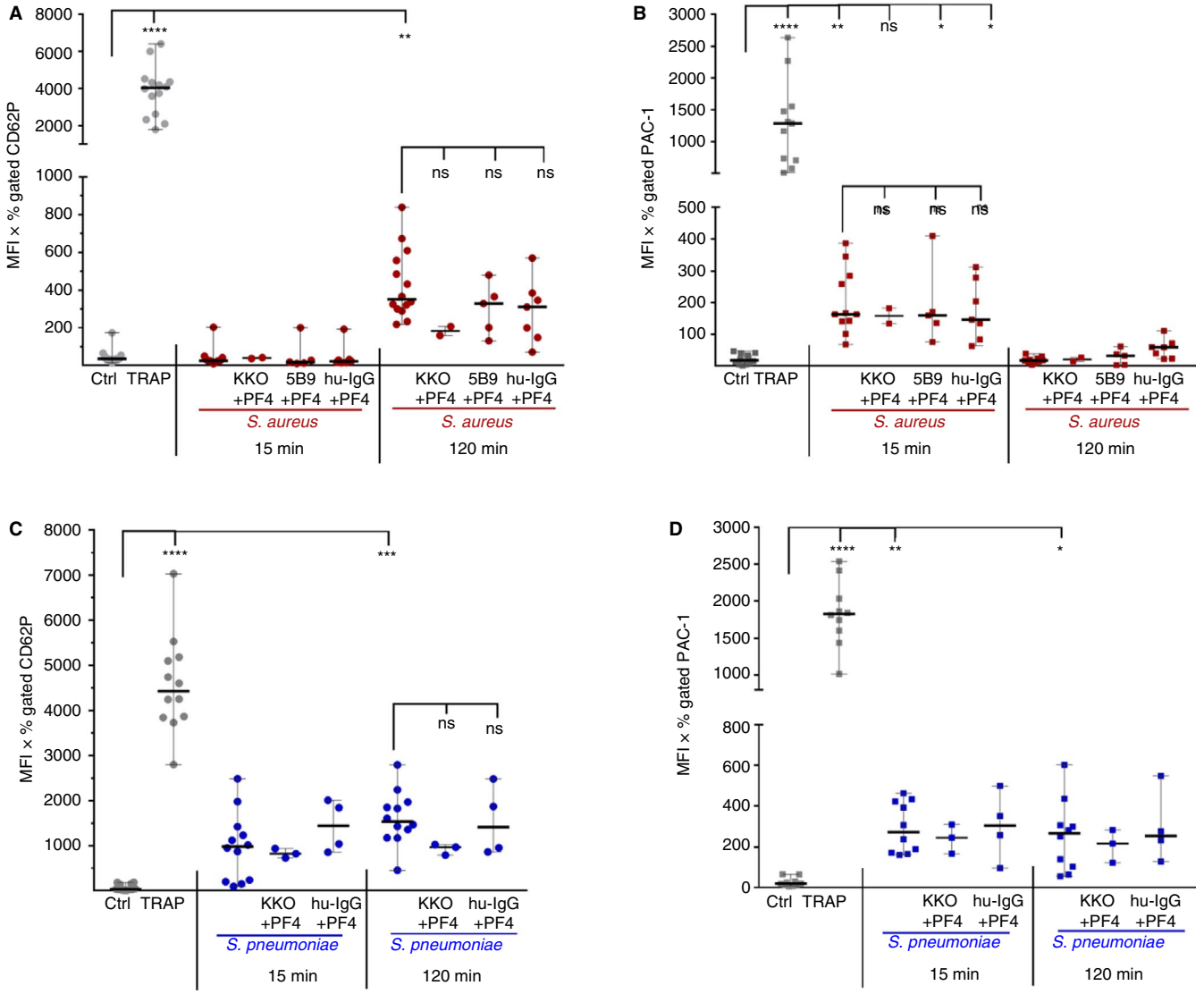

FIGURE 2 Activation of platelets by *Staphylococcus aureus* and *Streptococcus pneumoniae*. Platelet activation was measured by expression of CD62P and binding of PAC-1 after 15 and 120 minutes of incubation with *S. aureus* (A, B; red) or *S. pneumoniae* (C, D; blue). Compared to unstimulated platelets (see also Figure 5A), CD62P- and PAC-1 signals were significantly increased after incubation with *S. aureus* or *S. pneumoniae*. Addition of platelet factor 4 (PF4) and anti-platelet factor 4/heparin (PF4/H) antibodies did not enhance platelet activation. Reactivity of platelets to 20 µmol/L TRAP-6 is given as positive control. Mean fluorescence intensity (MFI) was multiplied by percentage of positive gated events. Statistical significance was calculated by Kruskal-Wallis test, followed by Dunn's multiple comparisons. $^{****}P < .0001$; $^{**}P < .01$; ns = $P > .05$

We showed earlier that PF4 exposes neoepitopes for anti-PF4/H antibodies when bound to bacteria.[19,20] This conformational change was confirmed here by binding of the mAbs KKO and 5B9, as well as by human anti-PF4/H antibodies recognizing specifically these neoepitopes. To determine the binding avidity of antibodies in the presence or absence of PF4 we calculated the fold increase (FI) of the product of MFI x positive gated events. Biotinylated PF4 and all tested mAbs and human anti-PF4/H antibodies bound to both bacterial strains albeit with different intensities. These ranged from 1.6-FI for human anti-PF4/H antibodies to 24.0-FI for KKO (median FI [range] as shown in Figure 1A were for *S. aureus*: bPF4 3.1 [1.5-4.7]; RTO 7.9 [2.5-11.9]; KKO 23.1 [7.7-46.9]; 5B9 5.3 [2.7-16.4]; 2E1 12.1 [4.3-33.9]; hu-anti-PF4/H-IgG 2.1 [1.2-3.0]; and for *S pneumoniae*: bPF4 2.3 [1.5-7.8]; RTO 2.9 [2.3-7.1]; KKO 24.0 [7.4-41.0]; 5B9 9.3 [2.1-23.0]; hu-anti-PF4/H-IgG 1.6 [1.5-2.0] as shown in Figure 1B).

3.2 | Gram-positive bacteria induce platelet CD62P expression and αIIbβ3 activation

To test platelet activation in the presence of Gram-positive bacteria, we incubated platelets with *S. aureus* or *S. pneumoniae* and measured activation markers by flow cytometry (median MFI x % gated events). Before incubation with *S. aureus*, resting platelets showed basal CD62P expression (median FI [range], 35.6 [14.0-173.6]) and

PAC-1 binding (18.2 [1.35-47.1]). TRAP-6 increased CD62P expression (4095.0 [1799.0-6736.0]) and PAC-1 binding (1298.0 [509.5-3126.0]). Activation of platelets by *S. aureus* was time dependent and differed for α-granule release (CD62P expression) and αIIbβ3 activation (PAC-1 binding). CD62P expression on platelets reached a maximum after 120 minutes (365.7 [217.9-839.3]) of incubation with *S. aureus* (Figure 2A). In contrast, αIIbβ3 was already activated after 15 minutes (167.0 [68.1-507.2]). However, PAC-1 binding returned to baseline levels after 2hrs (Figure 2B).

When we performed the same experiment with *S. pneumoniae*, activation of platelets was independent of the incubation time. Before incubation with *S. pneumoniae*, resting platelets showed basal CD62P expression (median FI [range], 32.7 [6.8-185.4]) and PAC-1 binding (19.8 [6.5-65.4]) and responded well to TRAP-6 (CD62P expression 4,428.0 [2800.0-7035.0]; PAC-1: 1826.0 [1016.0-2537.0]). Platelet activation markers were already increased after 15 minutes of incubation with *S. pneumoniae* and did not return to baseline after 120 minutes: CD62P at 15 minutes: 980.8 (93.7-2483.0) and 120 minutes: 1534.0 (452.1-2791.0); αIIbβ3 activation at 15 minutes: 307.7 (160.5-560.0) and 120 minutes: 282.4 (55.8-603.5). Platelet activation was not further enhanced by addition of PF4 or PF4/H antibodies at any condition tested (Figure 2C,D).

3.3 | Platelets and platelet releasate kill *Staphylococcus aureus* but not *Streptococcus pneumoniae*

We further analyzed the survival of *S. aureus* and *S. pneumoniae* post incubation with platelets. As a control, we determined the cfu/mL of untreated *S. aureus* (median cfu/mL: 5.5×10^4 [3.7×10^4-9.6×10^4]) and *S. pneumoniae* (median cfu/mL: 1.64×10^6 [1.56×10^6-2.5×10^6]) after 120 minutes' incubation with buffer. Incubation of *S aureus* with platelets or platelet releasate led to a significant reduction of cfu/mL by approximately 50% (2.7×10^4 [3.5×10^3-6.0×10^4] and 2.9×10^4 [5.5×10^3 –7.2×10^4], respectively; Figure 3A). In contrast, platelets (2.7×10^6 [6.8×10^5-3.9×10^6]) or platelet releasate (1.62×10^6 [3.0×10^5-2.7×10^6]) did not kill *S. pneumoniae* at a MOI of 5:1 or at a MOI of 25:1 (platelets:bacteria; Figure 3B).

3.4 | Killing of *Staphylococcus aureus* is independent of FcγRIIa and not enhanced by PF4 and anti-PF4/H antibodies

To test whether killing of *S. aureus* by platelets is dependent on FcγRIIa, we blocked the platelet FcγRIIa receptor with the monoclonal antibody IV.3 before incubating with *S. aureus*. Blocking of FcγRIIa did not reduce or enhance killing of *S. aureus* by platelets (Figure 4B), neither did addition of PF4 or anti-PF4/H. However, in the presence of mAb 5B9 and PF4 ($P = .033$) or human anti-PF4/H-IgG #1 ($P = .019$), killing of *S. aureus* was slightly enhanced, albeit with a large inter-donor variability among experiments (Figure 4A). However, in experiments using purified patient derived antibodies, we cannot exclude potential contamination of affinity purified anti-PF4/heparin antibodies with anti-*S. aureus* antibodies. Please note that platelets release PF4; therefore, these antibodies can bind without addition of exogenous PF4. Daratumumab (a humanized anti-CD38-antibody) was used as control for human antibodies and did not significantly increase killing of *S. aureus* (Figure 4A,B).

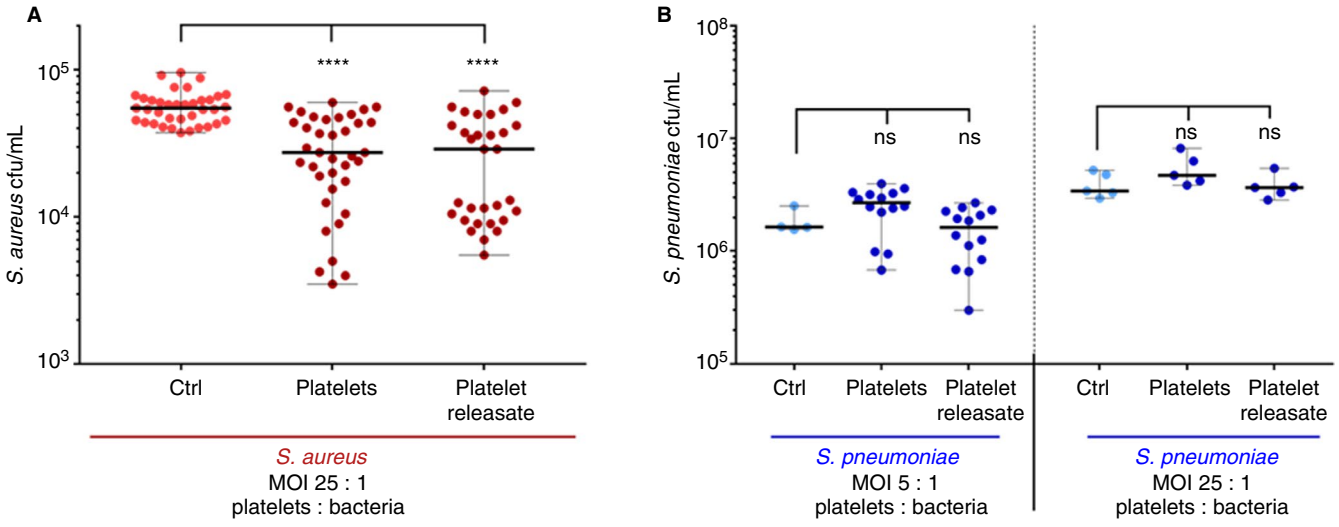

FIGURE 3 Colony-forming units (cfu) of *Staphylococcus aureus* and *Streptococcus pneumoniae* after incubation with platelets or platelet releasate. Cfu of *S. aureus* (A) and *S. pneumoniae* (B) were counted and cfu/mL calculated. Untreated bacteria served as negative control and were compared to bacteria incubated with platelets and platelet releasate. Incubation of platelets or platelet releasate with *S. aureus* led to a significant reduction of cfu/mL, while cfu of *S. pneumoniae* did not show major changes after incubation with platelets or platelet releasate in two tested multiplicity of infections (platelets:bacteria). Statistical significance was calculated by Kruskal-Wallis test, followed by Dunn's multiple comparisons test. ****$P < .0001$. *S. aureus* data are shown in red and *S. pneumoniae* data are shown in blue in all Figures

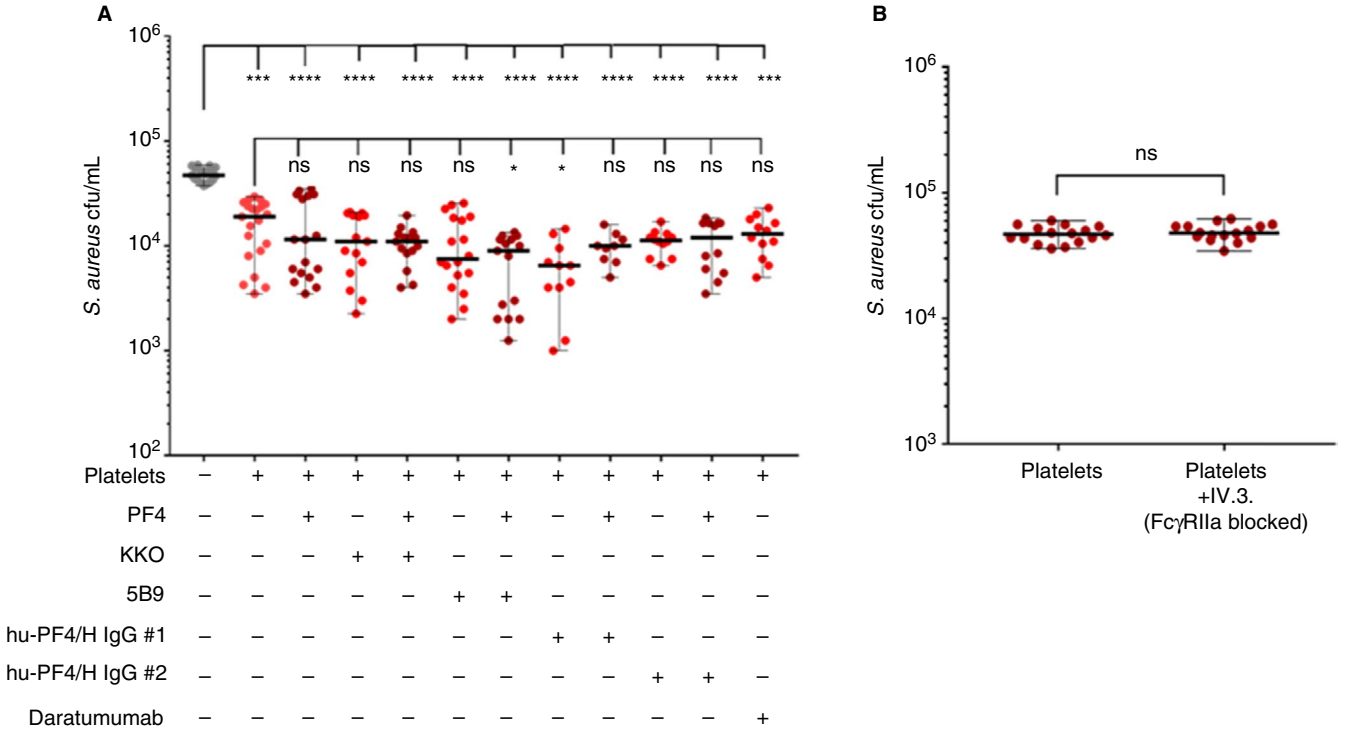

FIGURE 4 Killing of *Staphylococcus aureus* by platelets is not enhanced by platelet factor 4 (PF4) or platelet factor 4/heparin (PF4/H) antibodies and does not change after blocking of platelet FcγRIIa. Opsonisation of *S. aureus* with PF4 with or without addition of different monoclonal or polyclonal anti-PF4/H antibodies did not enhance killing with the exception of 5B9 + PF4 and human-anti-PF4/H IgG #1, which induced slightly increased killing of *S. aureus* (IgG #1/IgG #2 = human anti-PF4/H IgG). Blocking the FcγRIIa of platelets by monoclonal antibody IV.3 did not affect killing of *S. aureus* by platelets (B). Statistical significance was calculated by Kruskal-Wallis-test (A) Wilcoxon test (B). ****P < .0001; ***P < .001; **P < .01; *P < .05; ns = P > .05. *S. aureus* data are shown in red and *S. pneumoniae* data are shown in blue in all Figures

3.5 | Platelets incubated with *Staphylococcus aureus* remain functional, while *Streptococcus pneumoniae* increases PS exposure and renders platelets non-responsive to TRAP-6

We tested the viability of platelets after incubation with bacteria by measuring the binding of Annexin V and platelet response to TRAP-6 stimulation. Resting platelets showed low CD62P expression at baseline (72.8 [14.3-137.0]) and after 120 minutes' incubation with buffer (74.1 [27.7-136.3]), while TRAP-6 stimulated platelets showed high CD62P expression (4478 [3253-6573]).

After 15 minutes of incubation with *S. aureus*, median CD62P expression was still low (41.4 [12.1-111.9]) and platelets responded well to the TRAP-6 stimulation (median CD62P expression: 3168 [2568-3843]). After 120 minutes of incubation with *S. aureus*, platelets showed slightly increased CD62P expression (median 231.5 [147.9-443.7]) and response to TRAP-6 remained significantly higher (median CD62P expression: 2000 [1620-2453]; Figure 5A).

In contrast, platelets incubated for 15 minutes with *S. pneumoniae* showed higher CD62P expression (1837 (1491-2309]), compared to those incubated with *S. aureus*. However, these platelets could not be further stimulated by the addition of TRAP-6 (1842 [1390-1997]). This effect was also measured after 120 minutes of incubation with *S. pneumoniae* (without TRAP-6: 2088 [1510-2487]; with TRAP-6: 1996 [1544-2472]; Figure 5A). Consistently *S. aureus* did not induce PS-exposure (as measured by Annexin V binding) on platelets, while *S. pneumoniae* induced exposure of PS on platelet surface (Figure 5B). Resting platelets showed low PS exposure (83.2 [53.0-185.7]) compared to platelets co-stimulated with TRAP-6 and convulxin (848.9 [592.3-2184]) or platelets incubated with ethanol (7914 [2514-15 149]), which served as positive controls. Incubation with *S. pneumoniae* increased Annexin V binding to platelets 15 minutes (8579 [7108-11 415]) and 120 minutes (13 552 [9149-17 663]) post incubation. Annexin V binding after incubation with *S. pneumoniae* was comparable to controls that were stimulated with ethanol, and even higher compared to platelets stimulated with TRAP/convulxin.

4 | DISCUSSION

We demonstrated earlier that PF4 and anti-PF4/H antibodies enhance killing of Gram-negative *E. coli*.[17] In this study we show that PF4 and anti-PF4/H antibodies do not enhance killing of Gram-positive bacteria by platelets when using well characterized strains of *S. aureus* and *S. pneumoniae* as prototypes.

This difference in killing is not due to reduced binding of PF4 or PF4/H antibodies to Gram-positive bacteria. Consistent with previous findings,[19,20] and similar to *E. coli*, PF4 binds to the surface of *S. aureus* and *S. pneumoniae*. Hereby PF4 undergoes a

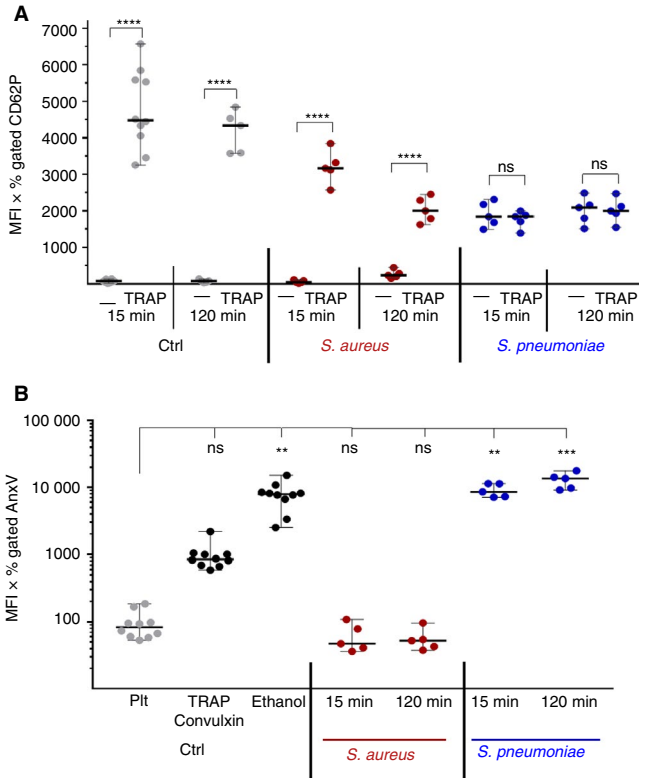

FIGURE 5 *Streptococcus pneumoniae* impairs platelet integrity, while platelets remain functional after incubation with *Staphylococcus aureus*. After 15 and 120 minutes of co-incubation with *S. aureus* or *S. pneumoniae* platelets were stimulated with TRAP-6 and CD62P-expression was compared with unstimulated controls. While platelets preincubated with *S. aureus* still responded to TRAP by an increase in CD62P expression, platelets preincubated with *S. pneumoniae* did not (A). Platelets preincubated with *S. aureus* did not show increased phosphatidylserin (PS)-exposure as measured by Annexin V (AnxV) binding. In contrast platelets preincubated with *S. pneumoniae* showed strongly increased Annexin V binding (B). Incubation of platelets with TRAP-6/convulxin or ethanol served as positive controls for (PS)-exposure/Annexin V binding. Statistical significance was calculated by analysis of variance test followed by Sidaks multiple comparisons (A) and Kruskal-Wallis test followed by Dunn's multiple comparisons (B). ****$P < .0001$; ***$P < .001$; **$P < .01$; *$P < .05$; ns = $P > .05$. *S. aureus* data are shown in red and *S. pneumoniae* data are shown in blue in all Figures

conformational change and expresses neoepitopes to which anti-PF4/H antibodies bind. This was demonstrated by binding of patient derived anti-PF4/H antibodies in the presence of PF4. A general problem of investigating antibody interactions with bacteria using human sera is the widespread prevalence and high titer of antibacterial antibodies in humans. Even when antibodies are purified from human sera by affinity purification, minor contamination with antibacterial antibodies (below detection levels) might be present. We therefore further investigated binding of monoclonal anti-PF4 and anti-PF4/H specific antibodies, mimicking human HIT-antibodies in order to avoid interference of non-PF4-specific antibodies. All PF4 and PF4/H specific mAbs bound to *S. aureus* and *S. pneumoniae* only in the presence of PF4. The present experiments therefore corroborate that PF4 undergoes a conformational change when bound to bacterial surfaces.

Platelets kill *E. coli* opsonized with PF4 and anti-PF4/H IgG involving the FcγRIIa receptor.[17] In contrast, anti-PF4/H antibodies do not enhance killing of Gram-positive *S. aureus*. In our experiments, the lack of killing was not due to protein A of *S. aureus*, which binds the Fc-part of IgG, because we used a protein A deficient strain. A plausible explanation is that *S. aureus* already activates platelets[1-5] in the absence of PF4 or anti-PF4/H antibodies (Figure 2), while *E. coli* does not.[17] Platelet granules contain a variety of bioactive molecules such as platelet microbicidal proteins (PMPs) or kinocidins, which are probably involved in killing of *S. aureus*.[12,36] As already shown earlier and confirmed here, CD62P was expressed on platelets after incubation with *S. aureus* indicating platelet activation and release of granules and granule stored antimicrobial peptides. As a result, bacterial growth of *S. aureus* was inhibited when co-incubated with platelets or the releasate of activated platelets. It is likely that also β-defensin 1 is involved, which is stored in a non-granule compartment of platelets and released after *S. aureus* induced platelet activation.[13] An interesting observation was the binding pattern of PAC-1 over time: 15 minutes after incubation of platelets with *S. aureus* we observed PAC-1 binding, while binding returned to baseline levels after 2 hours of incubation. This requires further studies. Platelets with activated αIIbβ3 might aggregate or fragment and are then no longer detected by flow cytometry. However, the forward sideward scatter of the flow cytometer histogram did not indicate that this has happened. Other causes might be that activation of αIIbβ3 is reversible, or PAC-1 binding was inhibited either by fibrinogen, released from activated platelets, or by bacterial compounds that bind to αIIbβ3. Finally, activated molecules of αIIbβ3 might have been internalized, or αIIbβ3 is deactivated because it has lost connection with the cytoskeletal proteins that support its active conformation.

FcγRIIa is discussed as a major player in platelet anti-bacterial response. The receptor has IgG-dependent functions, e.g. killing of IgG-opsonized *E. coli* [15] as well as IgG-independent functions by stimulating other platelet receptors like αIIbβ3 or GPIbIX.[4,37,38] Furthermore, crosslinking of FcγRIIa is known to play a major role in the pathogenesis of HIT by binding to immune complexes of PF4 and anti-PF4/H IgG resulting in platelet activation and clearance.[16] We therefore assumed that anti-PF4/H antibodies might also enhance platelet activation induced by Gram-positive bacteria and facilitate their killing in a FcγRIIa dependent manner. Surprisingly, neither addition of PF4 or anti-PF4/H antibodies, nor blocking of FcγRIIa by mAb IV.3 had an impact on *S. aureus* killing by platelets. Platelet activation by *S. aureus* via a FcγRIIa independent mechanism is probably already inducing release of bactericidal platelet substances[12,13,36] and additional FcγRIIa dependent mechanisms have therefore no or very limited additive effects (Figure 4B). There was a slight enhancement of killing by the combination of PF4 and 5B9 or anti-PF4/H IgGs derived from one of the patients (Figure 4A). Please note that platelets release upon activation endogenous PF4. Therefore, even without further addition of exogenous PF4, PF4 is present in the system. This might explain the

minor reduction in survival of *S. aureus* in the presence of only anti-PF4/H IgG. However, for the affinity purified human anti-PF4/H IgG we cannot exclude that they still contain small amounts of residual antibacterial antibodies.

Similar to *S. aureus*, *S. pneumoniae* is known to activate platelets through receptors or/and secreted toxins such as pneumolysin.[1,4,5,39] When incubating *S. pneumoniae* with platelets, we observed an increase in CD62P expression. In contrast to *S. aureus* this reaction reached its maximum already after 15 minutes. The released bactericidal agents of platelets seemed to have no effect on survival of *S. pneumoniae* (Figure 3).

To better understand the effects of bacteria on platelets, we further tested remaining platelet reactivity after incubation of platelets with Gram-positive bacteria by stimulating platelets with TRAP-6. Platelets pre-incubated with *S. aureus* showed strong enhancement of CD62P expression after additional stimulation with TRAP-6, while platelets pre-incubated with *S. pneumoniae* were unresponsive to TRAP-6. There was also a pronounced difference in PS exposure on the platelets incubated with *S. aureus* compared to *S. pneumoniae*. While PS exposure was not increased substantially in the presence of *S. aureus*, it was strongly enhanced on platelets pre-incubated with *S. pneumoniae*. PS exposure occurs during both: platelet activation resulting in strongly procoagulant platelets (COAT-platelets[40-42]), and in the case of platelet apoptosis. It is unclear why platelets co-incubated with *S. pneumoniae* show markers for both an increase in CD62P expression indicating active granule release and at the same time unresponsiveness to TRAP-6, which indicates dead platelets. This finding requires further studies. Clinically it would be highly relevant, whether infections with *S. pneumoniae* lead to a pro-coagulatory state in the case of expression of COAT platelets or to a pro-bleeding state in the case of apoptotic platelets.

In the present study, we focused on the specific interplay of platelets and Gram-positive bacteria and the possible role for anti-PF4/H antibodies for this interaction. To allow standardized experimental conditions, we used well-characterized strains of *S. aureus* and *S. pneumoniae* instead of clinical isolates. We therefore cannot exclude that clinical isolates show different effects due to their additional accessory genes.

We did also not consider the interaction with other immune cells like neutrophils or monocytes in our experimental settings. Gollomp et al showed that PF4 binds and stabilizes neutrophil extracellular traps (NETs), thereby facilitating binding of anti-PF4/H antibodies, further enhancing stabilization and DNase resistance. They hypothesized that NETs contribute in the pathogenesis of thrombosis in heparin-induced thrombocytopenia.[43] Recently, Perdomo et al confirmed that activated neutrophils and released NETs are a major promoter for thrombus formation in HIT. Moreover, anti-PF4/H antibodies were shown to induce release of NETs.[44] It is possible that PF4 and anti-PF4/H antibodies still enhance bacterial killing by inducing release of NETs, when granulocytes get activated by anti-PF4/H antibodies bound to PF4 on the bacterial surface.

While PF4 and anti-PF4/H antibodies seem to have only a minor role for direct killing of Gram-positive bacteria by platelets, their role in the interplay with other components of the innate and adaptive immune system requires further studies.

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CONFLICTS OF INTEREST

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AUTHOR CONTRIBUTIONS

M. Wolff, R. Palankar, S. Hammerschmidt, and A. Greinacher designed the experiments; M. Wolff performed the experiments; J. Wesche, T. P. Kohler, and C. Kohler supervised and assisted; M. Wolff, S. Handtke, R. Palankar, J. Wesche, S. Hammerschmidt, and A. Greinacher analyzed and interpreted the data; Y. Gruel provided the monoclonal antibodies; M. Wolff, S. Handtke, R. Palankar, Y. Gruel, S. Hammerschmidt, and A. Greinacher wrote the manuscript. All authors contributed to the manuscript, and read and approved the submitted version.

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section.

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