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Interaktion von Thrombozyten mit Gram-positiven Bakterien und die Rolle von Plättchenfaktor 4 (PF4) und PF4/Polyanion IgG in der Pathogenabwehr

Inaugural – Dissertation

zur

Erlangung des akademischen Grades

Doktor der Medizin

(Dr. med.)

der Universitätsmedizin

Greifswald

2022

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geb. am 31.08.1994

in Greifswald

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Tag der Disputation:	06.03.2023

Inhaltsverzeichnis

Abkürzungsverzeichnis..... 5

1 Einleitung.....7

2 Material und Methoden.....11

3 Ergebnisse.....16

3.1 Bindung von PF4 und monoklonalen und humanen anti-PF4/H Antikörpern an Gram-positive Bakterien.....16

3.2 Einfluss von Thrombozyten auf Gram-positive Bakterien.....19

3.2.1 Titration der Verhältnisse von Thrombozyten zu Bakterien.....19

3.2.2 Direkter Einfluss von Thrombozyten auf Gram-positive Bakterien.....20

3.2.3 Opsonierung Gram-positiver Bakterien mit PF4 und anti-PF4/H IgG und Hemmung des thrombozytären FcγRIIa.....21

3.3 Einfluss von Gram-positiven Bakterien auf Thrombozyten.....23

3.3.1 Aktivierung der Thrombozyten.....23

3.3.2 Induktion prokoagulatorischer Thrombozyten.....26

3.4 Einfluss von bakteriellen Toxinen auf Thrombozyten.....28

4 Diskussion.....32

5 Zusammenfassung.....38

6 Literaturverzeichnis.....39

7 Anhang.....46

7.1 Publikationsliste.....46

7.1.1 Zeitschriftenartikel.....46

7.1.2 Kongressbeiträge.....47

7.2 Eidesstattliche Erklärung 49

7.3 Danksagung 50

7.4 Publikationen 51

7.4.1 Orginalarbeit- Activated platelets kill Staphylococcus aureus, but not Streptococcus pneumoniae-The role of FcγRIIa and platelet factor 4/ heparin antibodies 53

7.4.2 Orginalarbeit- Pneumolysin induces platelet destruction, not platelet activation, which can be prevented by immunoglobulin preparations in vitro……. 63

7.4.3 Orginalarbeit: α-hemolysin of Staphylococcus aureus impairs thrombus formation 75

Abkürzungsverzeichnis

°C	Grad Celsius
µg	Mikrogramm
µl	Mikroliter
2E1	Humanisierter anti-PF4/Heparin Antikörper
5B9	Humanisierter anti-PF4/Heparin Antikörper
Abb.	Abbildung
ACD-A	Acid-Citrat-Dextrose-Anticoagulants
AG	Antigen
AK	Antikörper
Bcl-2	B-cell lymphoma 2
bPF4	Biotinylierter Plättchenfaktor 4
bzw.	beziehungsweise
ca.	circa
CD	Cluster of Differentiation
CD62p	P-Selektin
CFU	Colony forming units
E. coli	*Escherichia coli*
ELISA	Enzyme linked immunosorbent assay
FcγRIIa	Fcγ-Rezeptor IIA
FI	Fold-increase
FLICA	Fam-labeled inhibitors of caspases
GP	Glykoprotein
h	Stunde
HIPA	Heparin induced platelet activation assay
HIT	Heparin-induzierte Thrombozytopenie
Hly	Hämolysin
IgG	Immunglobulin G
kDa	Kilodalton
KKO	Muriner anti-PF4/Heparin Antikörper
min	Minuten
ml	Milliliter

MOI	Multiplizität der Infektion (multiplicity of infection)
MFI	Mittlere Fluoreszenzaktivität
NET	Neutrophil extracellular traps
ng	Nanogramm
PAC-1	Monoklonaler Antikörper gegen aktivierten Glyokoproteinkomplex IIb/IIIa
PBS	Phosphate buffered saline
PF4	Plättchenfaktor 4
PF4/H	Plättchenfaktor 4/Heparin
PFA	Paraformaldehyd
Ply	Pneumolysin
PMP	Platelet microbicidal proteins
PRP	Platelet rich plasma
rpm	Umdrehungen pro minute (revolutions per minute)
RTO	Muriner anti-PF4 Antikörper
s.	siehe
S. aureus	*Staphylococcus aureus*
S. pneumoniae	*Streptococcus pneumoniae*
TRAP-6	Thrombin receptor-activating peptide-6
u.a.	unter anderem
UFH	Unfraktioniertes Heparin
vWF	von Willebrand Faktor
z.B.	zum Beispiel

1 Einleitung

Thrombozyten sind kernlose Zellen mit einer essentiellen Rolle in der primären Hämostase. Sie entstehen durch Membranabschnürungen von Megakaryozyten. Der Prozess der Thrombozytopoese findet vornehmlich im Knochenmark statt, aber auch andere Orte, wie die Lunge, sind als primärer Bildungsort beschrieben worden.[1–3] Die Lebenszeit von Thrombozyten beträgt physiologsicherweise 7-12 Tage und kann beeinflusst werden durch Faktoren wie Infektion, Autoimmunität oder angeborene Thrombozyten-Veränderungen. Reguliert wird die Thrombozytopoese durch Zytokine wie Thrombopoetin, das wiederum von Thrombozyten internalisiert wird, wodurch ein negativer Rückkopplungsmechanismus entsteht. Aber auch Mechanismen wie die Modifizierungen der Glykokalyx (Desialylierung) oder Apoptose spielen eine wichtige Rolle bei der Regulierung ihrer Lebensspanne.[3–5]

Thrombozyten rücken zunehmend in den Fokus als Immunzellen. Die bereits identifizierten Rollen der Thrombozyten außerhalb von Thrombose und Hämostase sind vielfältig. Sie reichen von Funktionen des angeborenen Immunsystems, wie die Pathogenabwehr und Inflammation, über Angiogenese bis hin zu Modulation von Tumorwachstum.[1,6–10] Thrombozyten interagieren direkt und indirekt mit zellulären und humoralen Bestandteilen des Immunsystems wie Neutrophilen Granulozyten, B- und T-Lymphozyten und dem Komplementsystem. Sie besitzen eine Vielzahl an Rezeptoren. Dies spiegelt ebenfalls die Vielzahl der unterschiedlichen Aufgaben in Hämostase und in der Funktion als Immunzellen wider.[11,12]

Durch Bakterien versursachte Infektionen haben noch immer eine hohe Letalität. In ihrer Maximalform der Sepsis oder dem septischen Schock kann die Mortalität bis zu 40 % betragen.[13] Das verursachte klinische Bild der Infektionen durch die hier untersuchten Gram-positiven Erreger *Staphylococcus aureus* und *Streptococcus pneumoniae* umfasst eine große Bandbreite. Es reicht von Otitis media und Sinusitis, über Wund- und Weichteilinfektionen, Pneumonie, Endokarditis und Meningitis bis hin zum septischen Multiorganversagen.

Bei *S. aureus* handelt es sich um fakultativ anaerobe Haufenkokken, die über verschiedene Pathogenitätsfaktoren verfügen, wie das Immunglobulin-bindende Protein A, Clumping-Faktor und Enzyme wie Kollagenase und Lipase. Zudem kann *S. aureus* eine Reihe von Toxinen sekretieren, die wiederum Auslöser von Erkrankungen wie dem

Toxic Schock Syndrom sein können.[14–16] Von besonderem Interesse hierbei ist für diese Arbeit das porenformende Alpha-Hämolysin, welches zur Lyse von Erythrozyten und anderen Blutzellen führt.[17] Pneumokokken sind aerobe Diplokokken, die zu den Streptokokken gezählt werden. Zu den wichtigsten Pathogenitätsfaktoren zählt eine Polysaccharidkapsel, die Schutz vor Phagozytose und Bindung des Komplementsystems bietet.[18,19] Analog zu Staphylokokken verfügt *S. pneumoniae* über ein Hämolysin, welches unter dem Namen Pneumolysin bekannt ist.[20,21]

Thrombozyten und Bakterien interagieren miteinander, wobei es üblicherweise zu einer Aktivierung der Thrombozyten kommt, sowie zur Rekrutierung anderer Immunzellen durch Chemotaxis.[22–24] Zudem sind Thrombozyten durch Adhäsionsrezeptoren in der Lage Pathogene zu bündeln.[25] Die Interaktion von Thrombozyten und Bakterien kann direkt erfolgen, wie durch die Bindung an Membranrezeptoren (z.B. Toll-like-Rezeptoren) oder indirekt über Toxine oder Brückenproteine. So erlaubt beispielsweise die Bindung von Fibrinogen die Interaktion mit GP IIb-IIIa und die Bindung von vWF eine Interaktion mit GP Ib-V-IX.[26] Die pathogen-induzierte Aktivierung von Thrombozyten führt unter anderem zur Freisetzung thrombozytärer Granula, die eine Vielzahl von Proteinen und Gerinnungsfaktoren enthalten. Insbesondere in den Alpha-Granula finden sich in großer Dichte antimikrobieller Proteine (PMP) und Kinozidine. Aus dem Zytoplasma können Thrombozyten weitere antimikrobielle Peptide wie Defensine freisetzen.[27–30]

Von besonderem Interesse für diese Arbeit ist hierbei ein in den Alpha-Granula gespeichertes Protein, das Plättchenfaktor 4 (PF4) oder auch CXCL4 genannt wird und zur Gruppe der Chemokine zählt. Es liegt physiologischerweise als etwa 31,2 kDa großes Tetramer vor, das stark positiv geladen ist. Klinische Bedeutung kommt PF4 vor allem im Rahmen der Pathogenese der Heparin-induzierten Thrombozytopenie (Typ II) zu. Hierbei bindet PF4 aufgrund seiner starken positiven Ladung an Polyanionen wie Heparin. Dies führt zu einer Konformationsänderung von PF4 und daraus resultierenden Expression von Neoepitopen, an welche wiederum anti-PF4/Polyanion Antikörper binden.[31–33] Diese Immunkomplexe wiederum binden an den FcγIIa-Rezeptor [34] von Thrombozyten und führen zu einer Aktivierung von Thrombozyten und Expression von Gewebefaktor (Tissue factor) auf Monozyten und Endothelzellen.[35] Klinisch resultiert daraus ein Abfall der Thrombozytenzahl, der typischerweise zwischen Tag 5 und 10 nach Exposition mit Heparin auftritt. Paradoxerweise ist die resultierende Thrombozytopenie mit einem erhöhten Risiko für Thrombosen assoziiert.[36–38] Auffällig ist, dass die Erkrankung durch

Immunglobuline der Klasse IgG und nicht der Klasse IgM vermittelt wird wie man es bei einem primären Antigenkontakt vermuten würde. Vorarbeiten der Arbeitsgruppe haben gezeigt, dass nicht nur die Exposition gegenüber Heparin eine HIT auslösen kann. So konnte beobachtet werden, dass die Erkrankung beispielsweise nach orthopädischen Eingriffen oder nach einer bakteriellen Infektion bei Heparin-naiven Individuen auftrat.[39–43] Vorarbeiten von Krauel et al. konnten zeigen, dass PF4 ladungsbedingt nicht nur Heparin bindet, sondern auch an andere negativ geladene Moleküle wie Bestandteile der Bakterienzellwand oder Polyphosphate.[44–47] Die negativen Ladungen von Pathogenen vermitteln einen gewissen Schutz vor Phagozytose und erleichtern die Adhäsion. Aus diesen Beobachtungen ergab sich die Hypothese, dass es sich bei der HIT vermutlich um einen fehlgeleiteten anti-bakteriellen Abwehrmechanismus handelt und PF4 evolutionär genutzt wurde um Pathogene zu markieren für andere Zellen des Immunsystems. Die bei einer Infektion formierten Antikörper gegen die PF4/Bakterien-Komplexe können bei einer Re-Infektion mit demselben oder einem anderen Pathogen die Abwehr beschleunigen (Abb. 1).

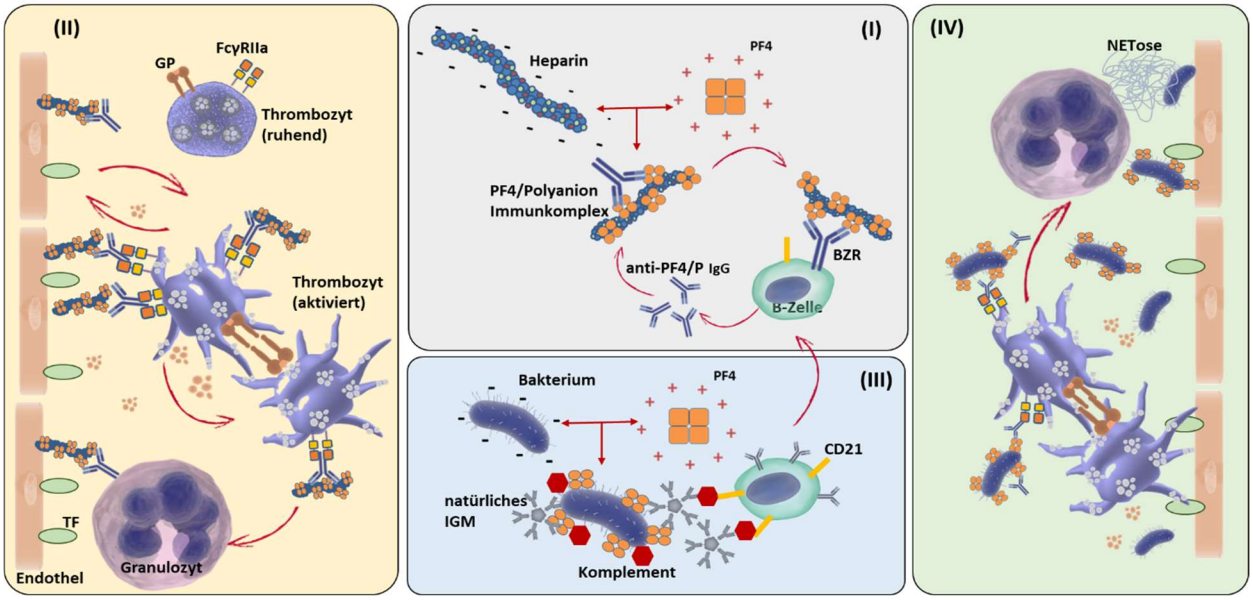


Abbildung 1: Schematische Darstellung der vermuteten Pathogenese der HIT. Nach Exposition mit Heparin kommt es ladungsbedingt zur Bindung von PF4 an Heparin. Durch die Konformationsänderung binden prä-formierte anti-PF4/P Antikörper und formen so einen Immunkomplex (I). Dieser aktiviert in Abhängigkeit vom FcγIIa-Rezeptor die Thrombozyten und wird durch Gewebefaktor (TF) und Rekrutierung von weiteren Thrombozyten und anderen Zellen verstärkt (II). Die Markierung von Bakterien mit PF4 und Bindung von Komplementfaktoren und natürlichem IgM erleichtert die Opsonierung und führt zur Bildung von anti-PF4/P Antikörpern durch B-Zellen (III). Die PF4/Bakterien-Immunkomplexe aktivieren FcγRIIa-abhängig Thrombozyten, die antimikrobielle Peptide freisetzen und führen zur Opsonierung der Pathogene für beispielsweise Granulozyten (IV).

Thrombozyten nehmen IgG-markierte Komplexe auf, [48] interagieren mit IgG-beladenen Bakterien und können diese abtöten.[49] Daran anlehnend haben Palankar et al. demonstriert, dass die Opsonierung mit PF4 und anti-PF4/Polyanion IgG zu einem Thrombozyten-vermittelten reduzierten Überleben des Gram-negativen Erregers *E. coli* führt. Dieser Mechanismus ist anhängig vom thrombozytären Rezeptor FcγRIIa.[50]

Aus dem Stand der Literatur und Vorarbeiten der Arbeitsgruppe ergaben sich folgende Fragestellungen, die in dieser Arbeit behandelt wurden:

1. Vermitteln Thrombozyten einen direkten schädigenden Effekt auf die Gram-positiven Bakterien *S. aureus* und *S. pneumoniae*?
2. Führt die Opsonierung mit PF4 und anti-PF4/Polyanion IgG FcγRIIa-abhängig zu einer Verstärkung des anti-bakteriellen Effektes und der Aktivierung der Thrombozyten?
3. Welche Effekte hat die Co-Inkubation mit den Gram-positiven Bakterien auf die Thrombozyten?

2 Material und Methoden

Aus Gründen der besseren Lesbarkeit sind die Methoden hier in ihren Grundprinzipien dargestellt. Details, wie Pufferzusammensetzung, Herkunft monoklonaler Antikörper, etc. finden sich in der Methodenbeschreibung der Originalarbeiten im Anhang. Zu jedem Abschnitt wird auf die korrespondierende Originalarbeit folgendermaßen verwiesen (z.B.: **Details s. Anhang 7.5.1**)

Für die vorliegende Arbeit wurden Modellstämme von *Staphylococcus aureus* und *Streptococcus pneumoniae* verwendet, bei welchen Virulenzfaktoren moduliert wurden, die eine Interaktion mit den anti-PF4/Polyanion Immunglobulinen beeinflussen. Der Wildtyp von *S. aureus* exprimiert Protein A, ein 40-60 kDa großes Zellwandprotein, welches die Fc-Teile von Immunglobulinen bindet. Daher wurde in dieser Arbeit der genetisch modifizierte Stamm *SA113Δspa* verwendet, welcher kein Protein A exprimiert. Bekapselte Stämme von *S. pneumoniae* gelten als besonders virulent. Jedoch erschwert die Polysacharid Kapsel die direkte Interaktion mit Plättchenfaktor 4 und Immunglobulinen. Daher wurde in dieser Arbeit der Stamm *S. pneumoniae D39 Δcps* verwendet. Die Stämme wurden freundlicherweise von der Abteilung Infektionsbiologie (Prof. Hammerschmidt) des Institutes für molekulare Genetik und Infektionsbiologie der Universität Greifswald zur Verfügung gestellt. *S. aureus* und *S. pneumoniae* wurden in allen Experimenten mit intakten Bakterien bis zur exponentiellen Wachstumsphase kultiviert. Die anschließende Inkubation mit PF4 und Antikörpern erfolgte auf Eis, um Metabolismus und Wachstum der Bakterien zu minimieren. Die darauffolgende Inkubation mit Thrombozyten erfolgte bei 37 °C unter regelmäßiger Agitation.

In Vorarbeiten wurde die Bindung von PF4 an Gram-positive und Gram-negative Bakterien bereits demonstriert. Der Nachweis der Bindung erfolgte hierbei durch die Bindung von biotinyliertem PF4 und fluoreszenzmarkiertem Streptavidin. Zum Beweis der erfolgten Konformitätsänderung wurde die Bindung von aufgereinigtem anti-PF4/Heparin-Komplex IgG aus Patientenseren nachgewiesen.[44] In dieser Studie erfolgte zum ersten Mal der Nachweis der Bindung von PF4 mit monoklonalen Antikörpern verschiedener Spezifitäten. Verwendet wurden ein anti-PF4-Antikörper (RTO), sowie zwei anti-PF4/H Antikörper (KKO, 5B9), welche nur nach erfolgter Konformitätsänderung von PF4 an den Komplex aus PF4 und Polyanionen binden. Vergleichend wurde die Bindung mit Streptavidin und aufgereinigtem anti-PF4/H IgG aus Patientenseren

nachgewiesen (Abb. 2). Die Inkubation erfolgte zunächst mit aufsteigenden Konzentrationen von PF4 (1-50 µg), um die optimale Konzentration für die Bindung zu titrieren und in weiteren Experimenten mit einer finalen Konzentration von 10 µg/ml bei *S. aureus* und 20 µg/ml bei *S. pneumoniae*. Nach einem Waschschritt wurden die PF4-beladenen Bakterien mit den monoklonalen Antikörpern inkubiert und nach einem weiteren Waschschritt mit sekundären Antikörpern fluoreszenzmarkiert. Der Nachweis der Bindung erfolgte mittels Durchflusszytometrie. Für die Bindung von biotinyliertem PF4 erfolgte analog die Inkubation mit bPF4 und Streptavidin-Atto 633 **(Details s. Anhang 7.5.1)**.

Die in der Arbeit verwendeten Seren bzw. die aufgereinigten Bestandteile stammen von Patienten, welche nachweislich klinisch und laborchemisch eine HIT aufwiesen mit einem positiven PF4/H ELISA sowie einem entsprechend positiven Funktionstest (HIPA).[51] Da sich mit hoher Prävalenz in der Bevölkerung Antikörper gegen *S. aureus* im Serum nachweisen lassen, erfolgte zunächst die Aufreinigung von Gesamt-IgG aus dem Serum von HIT Patienten mittels einer Protein G Säule. Anschließend wurde die anti-PF4/H IgG Fraktion durch eine PF4/Heparin Säule isoliert.[41] Die Proteinmenge wurde mittels BCA [52] quantifiziert und die Reaktivität der isolieren IgG Fraktion vergleichend zur korrespondierenden HIT-Serumprobe im PF4/H ELISA getestet.[51]

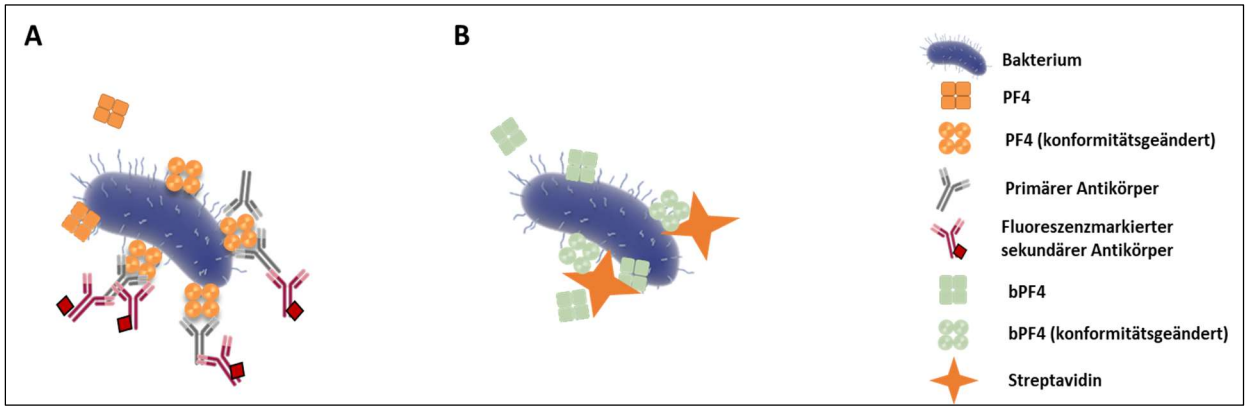


Abbildung 2: Schematische Darstellung Bindungsnachweis von PF4 an Bakterien mit monoklonalen Antikörpern [A] oder biotinyliertem PF4 und Streptavidin [B]. PF4 ändert bei Bindung an die Bakterienoberfläche seine Konformation und anti-PF4/H Antikörper (primäre AK) können binden. Durch die Bindung von fluoreszenzmarkieren sekundären Antikörpern kann die Bindung in der Durchflusszytometrie nachgewiesen werden [A]. Streptavidin bindet biotinylierte Substanzen. Dadurch erfolgt die Bindung an bPF4 unabhängig von der Konformation. Durch die Fluoreszenzmarkierung von Streptavidin kann die Bindung in der Durchflusszytometrie nachgewiesen werden [B].

Die Experimente erfolgten mit aus Vollblut isolierten Thrombozyten gesunder Spender, die keine Medikamente eingenommen hatten, welche die Thrombozytenfunktion hemmen.[53] Aufgrund der bekannten Aktivierung von Thrombozyten durch *S. aureus* und *S. pneumoniae* erfolgte eine Co-Inkubation von Thrombozyten und Bakterien in verschiedenen Verhältnissen. Die titrierten Verhältnisse von Thrombozyten : Bakterien werden nachfolgend als MOI (Multiplizität der Infektion) bezeichnet. Die getesteten MOI entsprachen 1:10, 1:5, 1:1, 5:1, 25:1, 50:1. Die Auswertung erfolgte nach 2 h Co-Inkubation über die Messung der Thrombozytenaktivierung in der Durchflusszytometrie mittels Quantifizierung der CD62p Expression auf der Thrombozytenoberfläche als Marker der Freisetzung thrombozytärer Alpha-Granula. Um den Effekt von PF4 und anti-PF4/H IgG zu untersuchen, wurden für nachfolgende Experimente jene MOI gewählt, die die geringste Thrombozytenaktivierung induziert hatten.

Das Überleben von *S. aureus* und *S. pneumoniae* wurde durch Zählen der keimbildenden Einheiten (colony forming units, CFU) bestimmt. Die Co-Inkubation erfolgte für 2 h mit und ohne Thrombozyten und mit und ohne vorherige Inkubation und Beladung mit PF4 und monoklonalen sowie humanen anti-PF4/H Antikörpern. Nach Erstellung entsprechender Verdünnungsreihen wurden die Organismen auf einer Blutagarplatte plattiert und für 12 h bei 37 °C inkubiert. Anschließend wurden die CFU ausgezählt und die CFU/ml berechnet. Für einige Experimente erfolgte die Inkubation mit dem Überstand von aktivierten Thrombozyten anstelle ganzer Zellen. Hierfür wurden die aus Vollblut isolierten Thrombozyten mit 20 µmol/l TRAP-6 und 5 µg Collagen unter Agitation aktiviert und der Überstand durch Zentrifugation gewonnen. Für die Blockade des thrombozytären FcγRIIa erfolgte die vorherige Inkubation mit dem aus Zellkultur gewonnenen IV.3 Antikörper für 45 min bei 37°C **(Details s. Anhang 7.5.1).**

Neben dem Überleben der Bakterien wurde auch der Einfluss der Co-Inkubation auf die Thrombozyten analysiert. Ausgewertet wurden die Thrombozyenaktivierung und deren weitere Aktivierbarkeit durch Agonisten, sowie die Expression von Phosphatidylserin auf der Thrombozytenoberfläche als Marker für prokoagulante oder apoptotische Thrombozyten. Ein schematischer Ablauf ist in Abbildung 3 zusammengefasst. Die Analysen erfolgten zu einem frühen Zeitpunkt der Co-Inkubation (nach 15 Minuten) und zum Ende der Co-Inkubation nach 2 Stunden. Die Thrombozytenaktivierung erfolgte durch Messung der CD62p Expression, als Marker der Freisetzung von Alpha-Granula, sowie durch Messung der Bindung des anti-PAC-1 Antikörpers, welcher an den

aktivierten Fibrinogen-Rezeptor bindet. Die Bindung der fluoreszenzmarkierten Antikörper wurde in der Durchflusszytometrie gemessen und die mittlere Fluoreszenzintensität (MFI) mit dem prozentualen Anteil CD62p/PAC-1 positiver Zellen multipliziert. Zusätzlich zur Messung der Grundaktivierung der Thrombozyten erfolgte eine Stimulierung mit einem starken Thrombozyten-Agonist (TRAP-6, 20 µmol/l) und die resultierende verbleibende Aktivierbarkeit wurde analysiert. Die PS-Exposition wurde durch Bindung von fluoreszenzmarkiertem Annexin V in der Durchflusszytometrie gemessen. Als positive Kontrolle dienten Thrombozyten, die zuvor mit einem TRAP-6 und Convulxin (20µmol/l, 100 ng/ml) oder 70% Ethanol inkubiert wurden **(Details s. Anhang 7.5.1).**

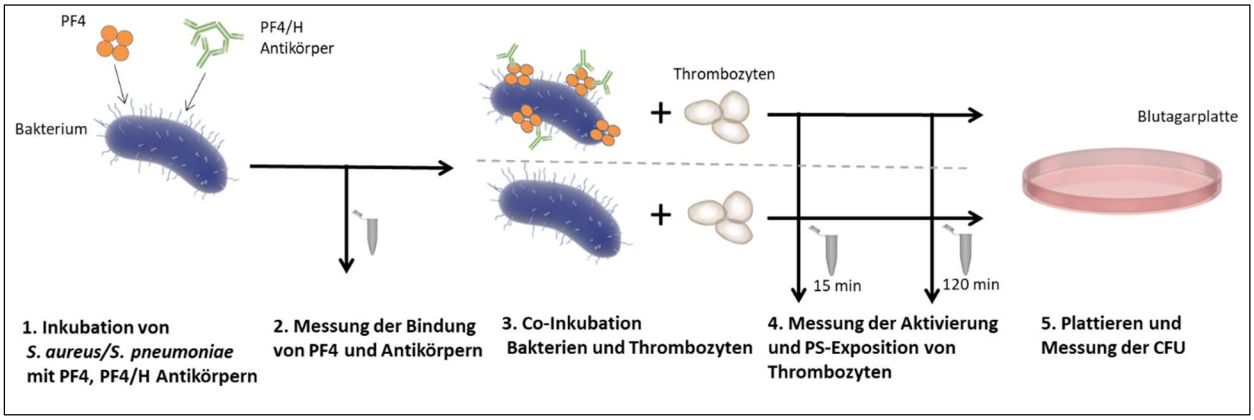


Abbildung 3: Schematische Darstellung des Versuchsablaufes bei der Co-Inkubation von Thrombozyten mit *S. aureus* und *S. pneumoniae* und PF4 sowie anti-PF4/H IgG.

In nachfolgenden Studien erfolgten Untersuchungen der Wirkung von Bakterientoxinen auf Thrombozyten. **(s. Anhang 7.5.2 und 7.5.3)**. Fokus waren hier das von *S. pneumoniae* sekretierte Pneumolysin und im Vergleich dazu das von *S. aureus* produzierte Hämolysin. Es ergab sich die Frage, ob diese Toxine eine Apoptose der Thrombozyten auslösen. Neben der Expression von Phosphatidylserin, welche auch bei prokoagulanten Thrombozyten auftritt [54], sind in der Literatur mehrere Methoden für den Nachweis für Apoptose bei Thrombozyten etabliert.[55] Die in dieser Arbeit untersuchten Zielstrukturen sind in Abbildung 4 hervorgehoben. Aus Vollblut isolierte Thrombozyten wurden mit aufsteigenden Konzentrationen von Pneumolysin (Ply, 3.0 – 300 ng/ml) oder Hämolysin (Hla, 0,02 – 20 µg/ml) inkubiert. Als positive Kontrolle dienten Thrombin (10 U/ml), TRAP-6 (20 µmol/l) und Convulxin (100 ng/ml), Ionophore (10 µM) oder vWF (20 µg/ml) mit Ristocetin (1.5 mg/ml). Für die Messung der Caspase-Aktivität wurde ein FAM-FLICA Caspase 3/7 Kit nach Herstellerangaben verwendet. Nach der Co-Inkubation mit

den Toxinen oder Kontrollen wurden die Thrombozyten mit FLICA-Lösung für 45 min bei 37°C inkubiert und nach einem Waschschritt mit dem Kit beiliegenden Puffer wurde die Aktivität der Caspase in der Durchflusszytometrie gemessen. Für die Messung des anti-apoptotischen Proteins Bcl-2 wurden die Thrombozyten nach der Co-Inkubation mit 0,5% PFA für 20 min fixiert und anschließend mit 0,5 % Saponin für 30 min permeabilisiert. Es folgte die Inkubation mit einem fluoreszenzmarkierten anti-Bcl-2 Antikörper und die Messung in der Durchflusszytometrie **(Details s. Anhang 7.5.3).**

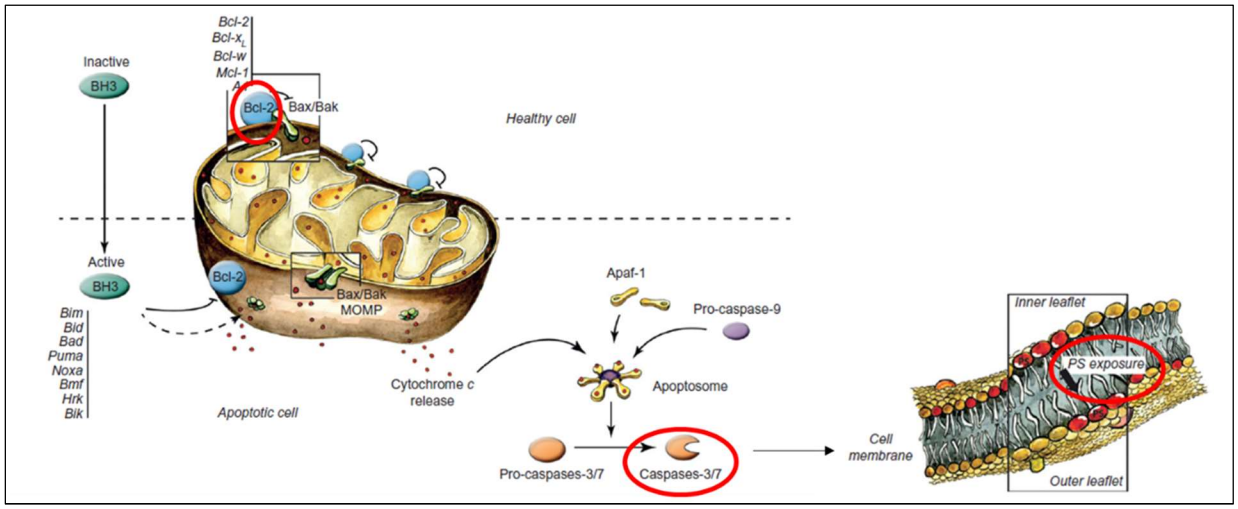


Abbildung 4: Schematische Darstellung relevanter Kaskaden der intrinsischen Apoptose bei Thrombozyten (modifiziert nach Josefsson et al.).[3] Die Untersuchten Zielstrukturen Bcl-2, Caspase 3/7 und Exposition von Phosphatidylserin sind mit einem roten Kreis hervorgehoben.

Die in dieser Arbeit verwendeten Thrombozyten wurden aus dem Vollblut von gesunden Blutspendern aufgereinigt, die ihr schriftliches Einverständnis entsprechend der Deklaration von Helsinki gegeben haben. Die verwendeten Seren von HIT-Patienten wurden freundlicherweise pseudonymisiert durch das Thrombozytenlabor der Transfusionsmedizin Greifswald zur Verfügung gestellt und verarbeitet. Die statistische Auswertung erfolgte unter der Verwendung der Software GraphPad Prism Version 7.04 und Signifikanz wurde definiert als $p < 0,05$.

3 Ergebnisse

3.1 Bindung von PF4 und monoklonalen und humanen anti-PF4/H Antikörpern an Gram-positive Bakterien

(Details siehe Anhang 7.5.1)

Humaner Plättchenfaktor 4 bindet an *S. aureus* und *S. pneumoniae*. Dies habe ich durch die Bindung von bPF4 als etablierter Nachweismethode, sowie auch durch die Bindung des monoklonalen Antikörpers RTO nachgewiesen. Darüber hinaus habe ich durch die Bindung der anti-PF4/H Antikörper KKO und 5B9 die Konformationsänderung von PF4 gezeigt, da diese spezifisch Komplexe aus PF4 und Polyanionen erkennen, bei denen PF4 seine Konformation geändert hat. Die Bindung der monoklonalen Antikörper ist abhängig von der PF4-Konzentration und zeigt eine Sättigung. Diese lag bei *S. aureus* bei etwa 10 µg/ml PF4, während eine Sättigung der Bindungskurve bei *S. pneumoniae* erst bei etwa 20 µg/ml PF4 auftrat (s. Abb. 5). Diese ermittelten Konzentrationen wurden für alle nachfolgenden Experimente verwendet.

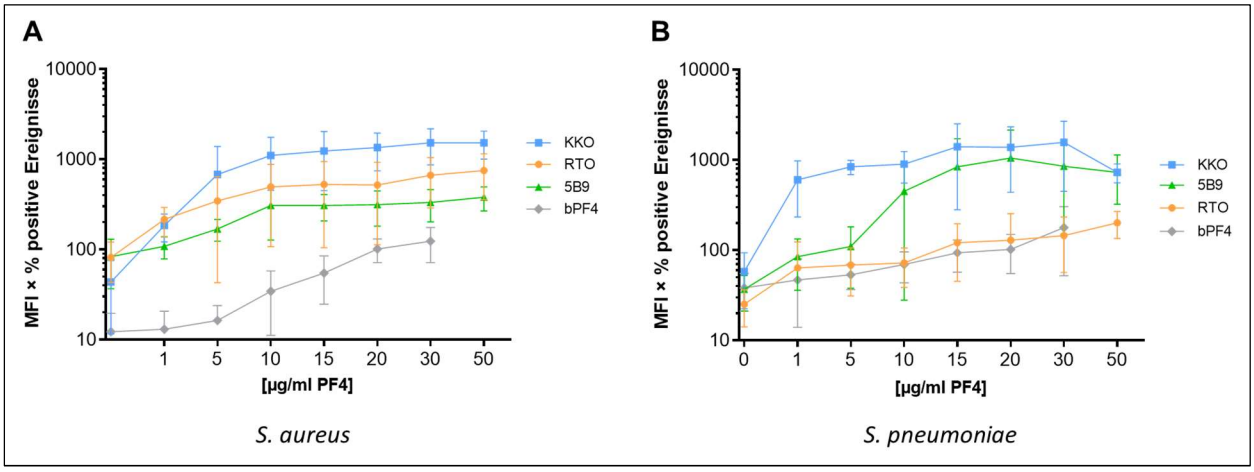


Abbildung 5: Bindungskurven monoklonaler anti-PF4/H Antikörper bei steigenden PF4-Konzentrationen im Vergleich zu bPF4. Dargestellt ist der Mittelwert mit Standardabweichung.

Nachfolgend wurde der vielfache Anstieg der Bindung (Fold-increase, FI) von Antikörpern an *S. aureus* und *S. pneumoniae* berechnet (Abb. 6). Verglichen wurde die Zunahme der gemessenen Bindung in der Durchflusszytometrie von Proben die in Abwesenheit von PF4 mit anti-PF4/H Antikörpern inkubiert wurden zu jenen, die in Anwesenheit von PF4 mit den Antikörpern inkubiert wurden. Hierbei zeigte sich bei *S. aureus* und

S. pneumoniae der stärkste Anstieg der Bindung für den monoklonalen Antikörper KKO. Für *S. aureus* zeigte sich im Median eine 23-fach erhöhte Bindung verglichen zur Kontrolle. Für *S. pneumoniae* war diese sogar 32-fach erhöht. Die geringste Bindung an die beiden Gram-positiven Bakterienspezies zeigten biotinyliertes PF4 und humanes anti-PF4/H IgG. Wobei hier zu vermerken ist, dass die humanen anti-PF4/H Antikörper im Vergleich zu den monoklonalen Antikörpern bereits ohne exogenes PF4 eine stärkere Bindung an *S. aureus* und *S. pneumoniae* zeigten. Ursächlich ist wahrscheinlich eine geringe Kontamination der IgG-Fraktion mit PF4 durch die Säure-Elution der PF4/Heparin-Säule. Der monoklonale Antikörper 2E1, der Eigenschaften von Antikörpern einer Autoimmun-HIT besitzt, hat sowohl an *S. aureus*, als auch an *S. pneumoniae* gebunden, wenn PF4 zu den Bakterien gegeben wurde.

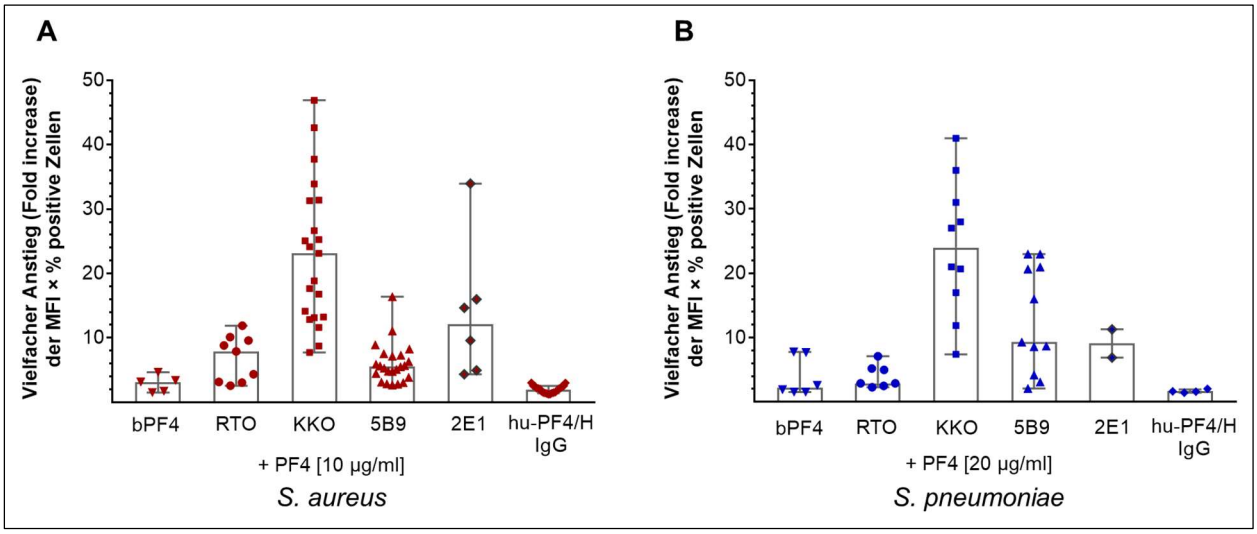


Abbildung 6: Vielfacher Anstieg der Bindung von anti-PF4/H Antikörpern an *S. aureus* (■) und *S. pneumoniae* (■) nach Zugabe von PF4 (modifiziert nach Wolff et al. 2020).[56] Der Fold-Increase wurde gebildet zwischen Proben ohne PF4 und mit PF4 Vorinkubation der Bakterien. Die stärkste Zunahme der Bindung ist bei *S. aureus* und *S. pneumoniae* für den monoklonalen Antikörper KKO zu sehen, die geringste Zunahme für humanes PF4/H IgG. Dargestellt ist der Median mit Minimum und Maximum.

Eine detaillierte Darstellung für die Bindung der einzelnen Antikörper an *S. aureus* (rot) und *S. pneumoniae* (blau) mit entsprechenden Kontrollen findet sich in Abbildung 7.

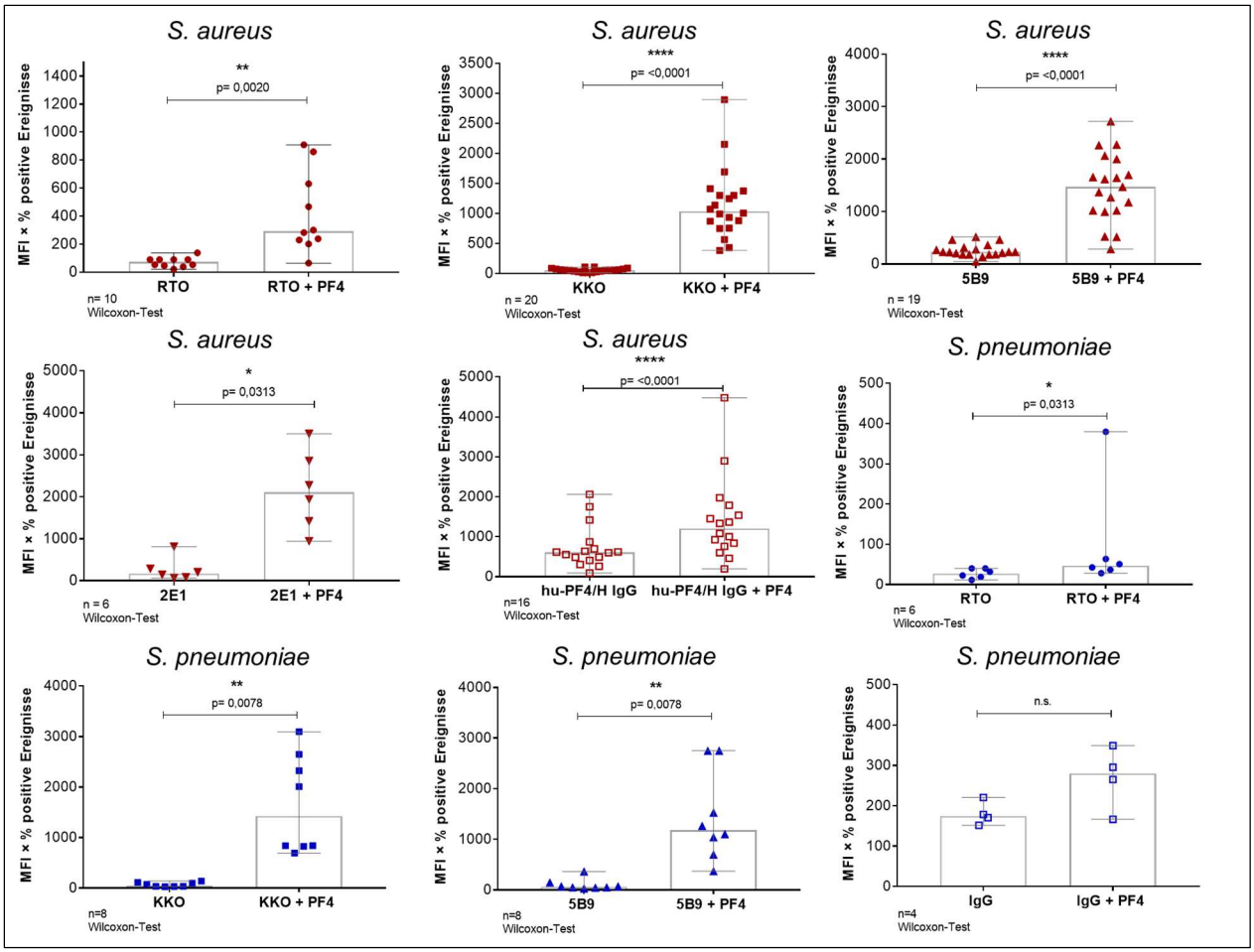
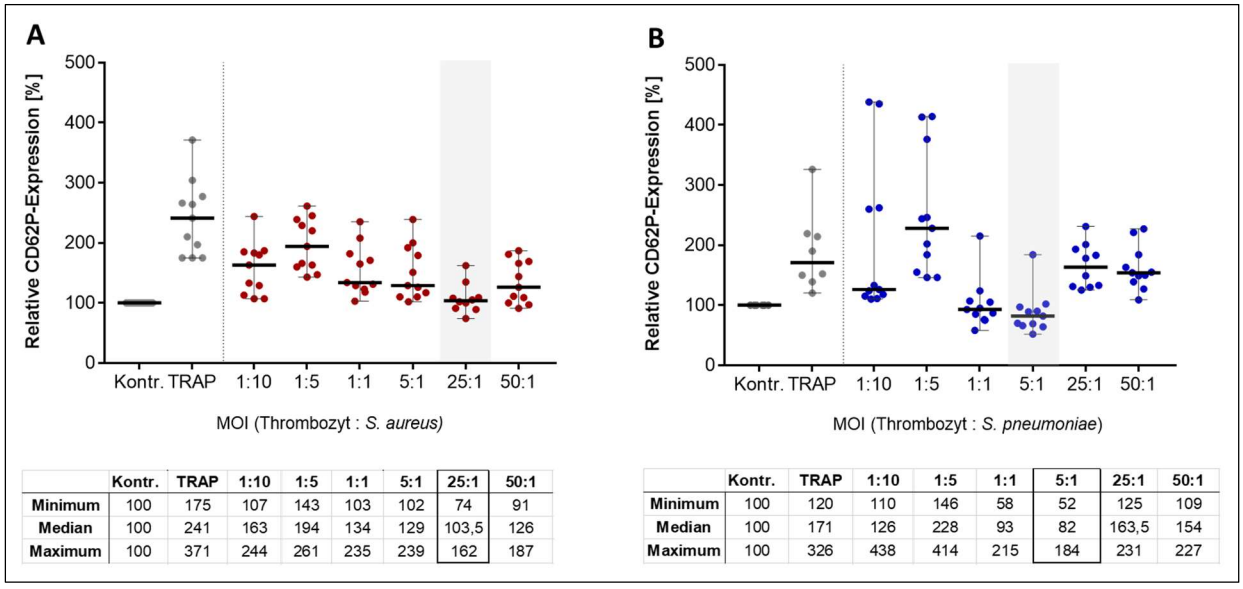


Abbildung 7: Bindung der monoklonalen und humanen anti-PF4 Antikörper an *S. aureus* (■) und *S. pneumoniae* (■). Dargestellt sind die Einzelergebnisse mit Angabe von Median, sowie Minimum und Maximum. Statistische Signifikanz wurde mit dem Wilcoxon-Test bestimmt.

3.2 Einfluss von Thrombozyten auf Gram-positive Bakterien

3.2.1 Titration der Verhältnisse von Thrombozyten zu Bakterien

Zunächst wurde nach 2 h Co-Inkubation die Thrombozytenaktivierung durch *S. aureus* und *S. pneumoniae* gemessen. Dargestellt ist die relative Zunahme der CD62p-Expression im Vergleich zu ruhenden Thrombozyten, die zwei Stunden unter gleichen Bedingungen inkubiert wurden. Als positive Kontrolle dienten TRAP-aktivierte Thrombozyten. Sowohl *S. aureus* als auch S. *pneumoniae* induzierten eine deutliche Aktivierung der Thrombozyten. Um die Wirkung von PF4 und PF4/H IgG in Folgeexperimenten messen zu können, sollten die Thrombozyten eine möglichst geringe bakterien-induzierte Voraktivierung zeigen. Diese lag bei einer MOI von 25:1 für *S. aureus* und 5:1 für S. *pneumoniae* (Abb. 8). Diese titrierten Verhältnisse wurden für alle folgenden Experimente verwendet.



	Kontr.	TRAP	1:10	1:5	1:1	5:1	25:1	50:1
Minimum	100	175	107	143	103	102	74	91
Median	100	241	163	194	134	129	103,5	126
Maximum	100	371	244	261	235	239	162	187

	Kontr.	TRAP	1:10	1:5	1:1	5:1	25:1	50:1
Minimum	100	120	110	146	58	52	125	109
Median	100	171	126	228	93	82	163,5	154
Maximum	100	326	438	414	215	184	231	227

Abbildung 8: Co-inkubation von Thrombozyten mit *S. aureus* (■) und *S. pneumoniae* (■) in verschiedenen Verhältnissen und Messung der resultierenden Thrombozytenaktivierung. Die Aktivierung wurde durch CD62p-Expression gemessen und als Median mit Minimum und Maximum grafisch und tabellarisch dargestellt. Das in weiteren Experimenten verwendete Verhältnis ist grau markiert.

3.2.2 Direkter Einfluss von Thrombozyten auf Gram-positive Bakterien

(Details siehe Anhang 7.5.1)

Die Co-Inkubation von Thrombozyten mit *S. aureus* führte zu einer signifikanten Reduktion der Kolonie-formierenden Einheiten (CFU) um fast 50 % von 5,5 x 10^4 auf 2,7 x 10^4 CFU/ml ($p < 0{,}0001$). Dieser Effekt ist nicht durch zelluläre, sondern sekretierte Anteile von Thrombozyten vermittelt. Die Inkubation mit dem Überstand TRAP und Collagen stimulierter Thrombozyten führte zu einer vergleichbaren Reduktion des Bakterienwachstums (2,9 x 10^4 CFU/ml; $p< 0{,}0001$), wie zuvor für intakte Thrombozyten beschrieben (Abb.9). Im Gegensatz dazu führte die Co-inkubation von Thrombozyten oder der Überstand aktivierter Thrombozyten mit *S. pneumoniae* nicht zu einer signifikanten Reduktion des Bakterienwachstums (Abb.9). Um auszuschließen, dass der beobachtete bakterizide Effekt auf *S. aureus* nicht nur bedingt ist durch eine 5-fach höhere Konzentration von Thrombozyten im Vergleich zu *S. pneumoniae*, wurden die Experimente zusätzlich mit einem Verhältnis von 25:1 durchgeführt. Auch hier zeigte sich kein signifikanter Effekt von Thrombozyten auf das bakterielle Wachstum. Zusammenfassend habe ich mit diesen Experimenten einen negativen Effekt von Thrombozyten auf das Überleben von *S. aureus* festgestellt. Im Gegensatz dazu haben Thrombozyten keinen Effekt auf das Überleben von *S. pneumoniae* vermittelt **(s. Fragestellung 1, S.10).**

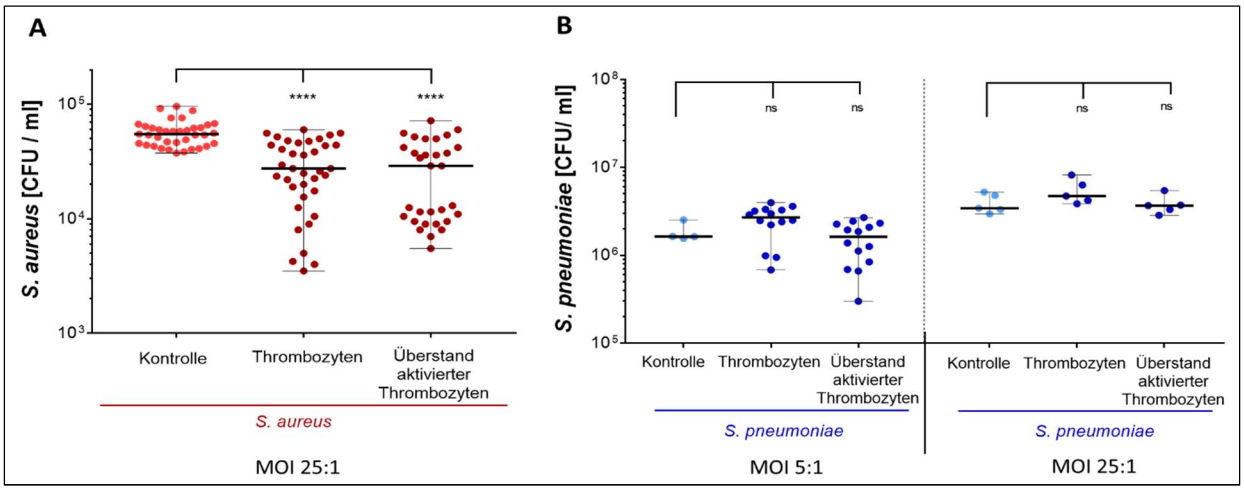


Abbildung 9: Einfluss der Co-Inkubation von Thrombozyten oder Überstand aktivierter Thrombozyten auf das Wachstum von *S. aureus* (■) und *S. pneumoniae* (■) (modifiziert nach Wolff et al. 2020).[56] Thrombozyten oder Thrombozytenüberstand senken signifikant das Wachstum von *S. aureus*, nicht jedoch von *S. pneumoniae*. Dargestellt ist der Median mit Minimum und Maximum. Statistische Signifikanz wurde mit dem Kruskal-Wallis Test, gefolgt von Dunn´s multiplen Vergleichstest bestimmt.

3.2.3 Opsonierung Gram-positiver Bakterien mit PF4 und anti-PF4/H IgG und Hemmung des thrombozytären FcγRIIa

(Details siehe Anhang 7.5.1)

Die zuvor beschriebenen Ergebnisse zeigten eine durch Thrombozyten vermittelte Reduktion des Wachstums von *S. aureus*. Hierbei habe ich mich zunächst auf *S. aureus* und nicht *S. pneumoniae* konzentriert, da die vorhergehenden Ergebnisse für *S. aureus* bereits eine deutliche Interaktion mit den Thrombozyten zeigten. Nachfolgend sollte die Frage adressiert werden, ob PF4 und anti-PF4/H IgG diesen bakteriziden Effekt verstärken, wie zuvor für *E. coli* gezeigt wurde.[50] Dargestellt ist das Wachstum von *S. aureus* in CFU/ml nach der Co-inkubation mit Bakterien und monoklonalen und humanen anti-PF4/H Antikörpern mit und ohne die Zugabe von PF4. Die bereits signifikante Reduktion der CFU/ml durch die alleinige Inkubation mit Thrombozyten konnte nochmals etwas verstärkt werden durch die Zugabe von PF4 oder monoklonalen und humanen anti-PF4/H Antikörpern. Die zusätzlich erreichte Reduktion ergab jedoch für die meisten getesteten Bedingungen nur numerische Unterschiede und erreichte keine statistische Signifikanz (Abb. 10 A). Hierbei ist zu beachten, dass zusätzlich zu dem exogen zugeführten PF4 auch endogenes PF4 durch Thrombozytenaktivierung frei wird und sich somit geringere Mengen an PF4 in allen Proben befinden, die Thrombozyten enthalten.

FcγRIIa spielt eine wichtige Rolle in der Pathogenese der Heparin-induzierten Thrombozytopenie und ist als wichtiger Bestandteil der Thrombozytenaktivierung bei solchen immunvermittelten Thrombozytenerkrankungen beschrieben worden. Durch die Vorinkubation der Thrombozyten mit dem IV.3 Antikörper wurde der Fcγ-Rezeptor und eine antikörpervermittelte Aktivierung der Thrombozyten blockiert. Thrombozyten mit blockiertem FcγRIIa zeigten die gleiche Wirkung auf das Wachstum von *S. aureus* wie Thrombozyten ohne Fcγ-Inhibition (Abb. 10 B).

Hieraus lässt sich ableiten, dass PF4, anti-PF4/H IgG und auch der FcγRIIa eine untergeordnete Rolle spielen, bei der bakteriziden Wirkung von Thrombozyten auf *S. aureus* **(s. Fragestellung 2, S.10).**

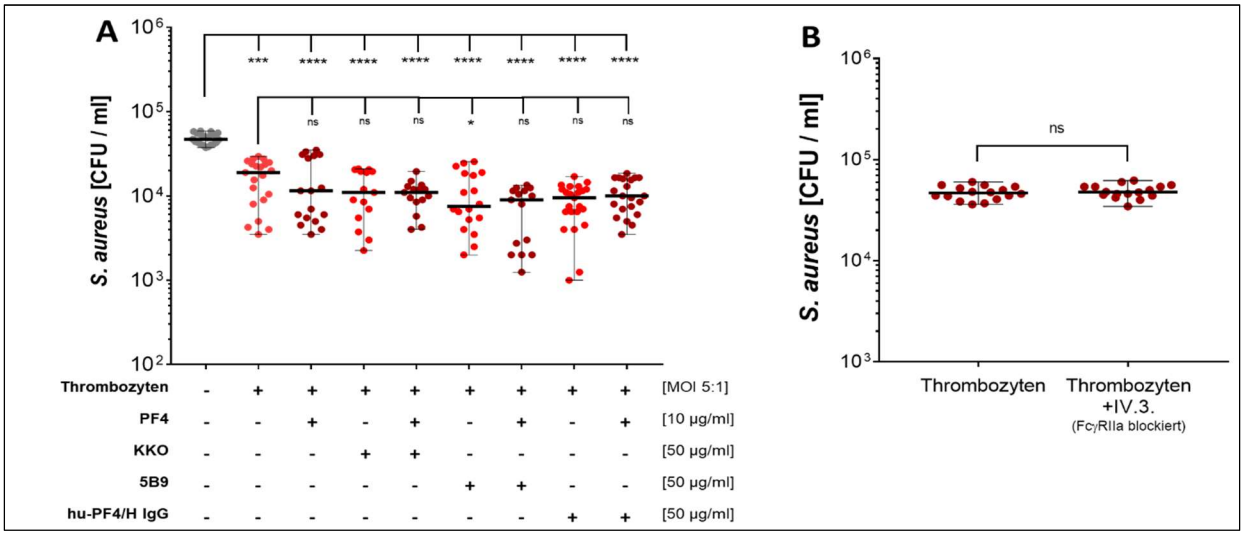


Abbildung 10: Einfluss von PF4, anti-PF4/H IgG und FcγRIIa auf Wachstum von *S. aureus* bei der Co-inkubation mit Thrombozyten (modifiziert nach Wolff et al. 2020).[56] Die Opsonierung mit PF4 und anti-PF4/H IgG hat die Inhibition des bakteriellen Wachstums nicht weiter verstärken [A]. Die Blockierung des Fcγ-Rezeptors IIa der Thrombozyten hatte keinen Effekt auf das Überleben von *S. aureus* [B]. Dargestellt ist der Median mit Minimum und Maximum. Statistische Signifikanz wurde mit dem Kruskal-Wallis Test, gefolgt von Dunn´s multiplen Vergleichstest für (A) und durch den Wilcoxon Test für (B) bestimmt.

3.3 Einfluss von Gram-positiven Bakterien auf Thrombozyten

3.3.1 Aktivierung der Thrombozyten

(Details siehe Anhang 7.5.1)

Nachdem ich den hemmenden Einfluss von Thrombozyten auf das Wachstum von *S. aureus* demonstrieren konnte stellte sich anschließend die Frage, welchen Einfluss die Co-Inkubation auf die Thrombozyten hat **(s. Fragestellung 3, S.10)**. Untersucht wurde zunächst die spontane pathogenvermittelte Aktivierung von Thrombozyten zu verschiedenen Zeitpunkten. In Abb. 11 ist die Expression von CD62p auf der Thrombozytenoberfläche dargestellt. *S. aureus* induzierte einen leichten Anstieg der CD62p Expression nach 120 Minuten. Hingegen war für *S. pneumoniae* eine deutliche Zunahme von CD62p bereits nach 30 min sichtbar und stieg stetig weiter an. Im Gegensatz dazu zeigte die Integrinaktivierung von Thrombozyten, dargestellt durch die Bindung von PAC-1, ein inverses Muster zur CD62p Expression. Die Bindung von PAC- 1 nach Inkubation mit *S. aureus* und *S. pneumoniae* war zunehmend rückläufig. Während bei *S. aureus*-inkubierten Thrombozyten nach 30 min keine Bindung mehr nachweisbar war, blieb diese bei *S. pneumoniae*-inkubierten Thrombozyten leicht erhöht (Abb. 12).

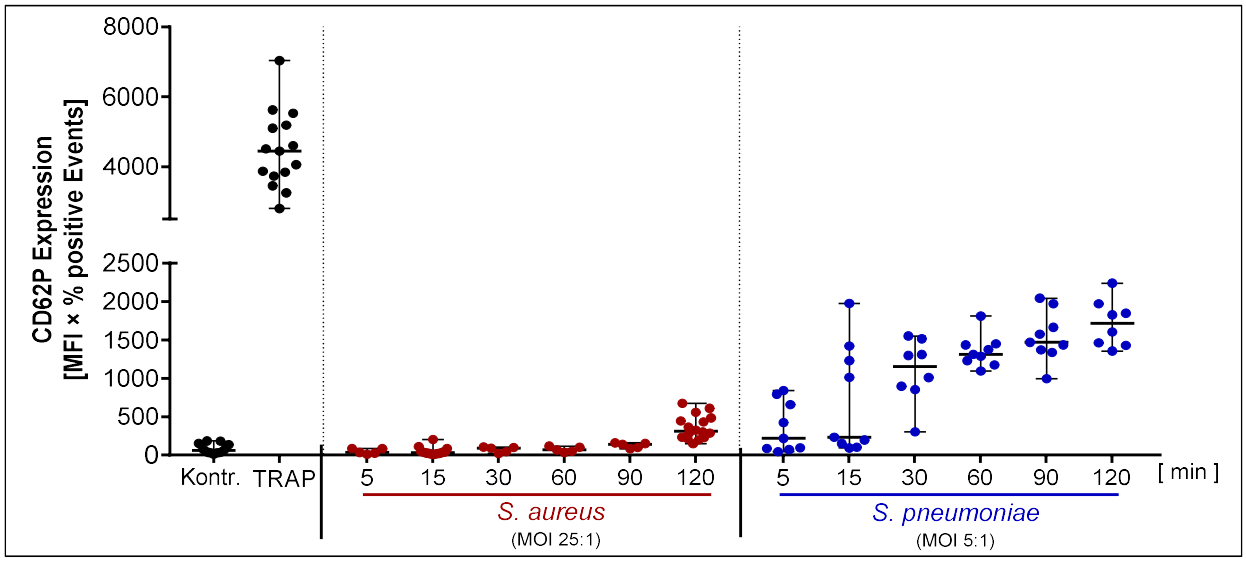


Abbildung 11: Zeitreihe CD62p Expression durch Inkubation von Thrombozyten mit *S. aureus* (■) und *S. pneumoniae* (■). Dargestellt ist der Median mit Minimum und Maximum.

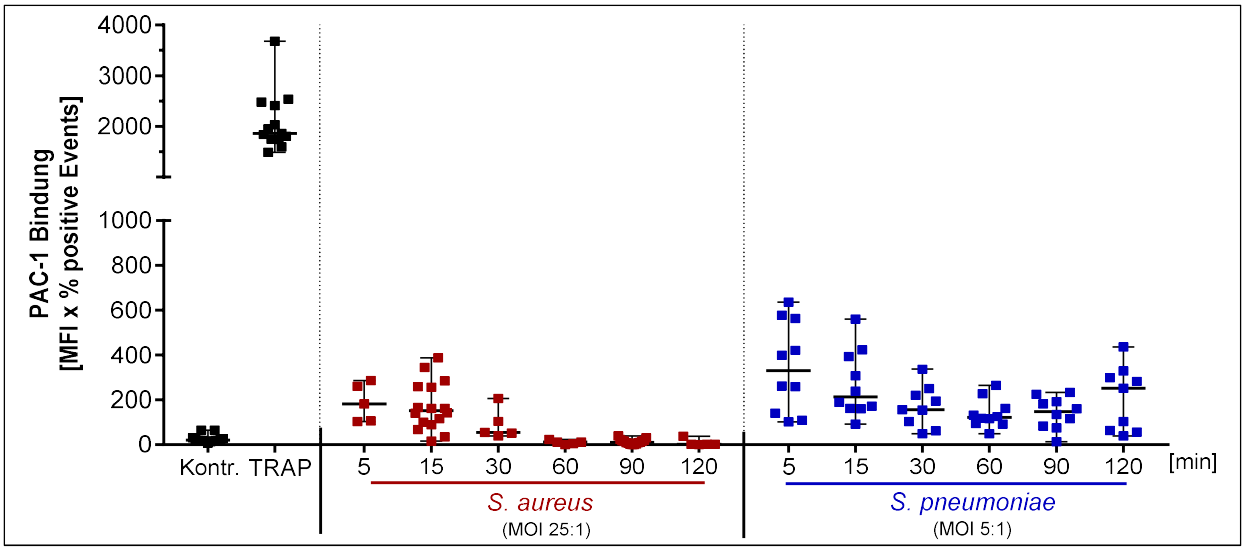


Abbildung 12: Zeitreihe Intergrinaktivierung (PAC-1 Bindung) durch Inkubation von Thrombozyten mit *S. aureus* (■) und *S. pneumoniae* (■). Dargestellt ist der Median mit Minimum und Maximum.

Als nächstes sollte die Frage geklärt werden, ob anti-PF4/H Antikörper die Aktivierung von Thrombozyten bei der Co-Inkubation zusätzlich verstärken **(s. Fragestellung 2, S. 10).** Dargestellt ist die CD62p-Expression und PAC-1 Bindung nach 15 und 120 min. Für *S. aureus*-inkubierte Thrombozyten zeigte sich wieder die erhöhte CD62p Expression, die jedoch durch die Zugabe von PF4 und anti-PF4/H IgG nicht verstärkt wurde (Abb. 13 A). Ebenso wurde die *S. aureus*-induzierte Integrinaktivierung durch PF4 und anti-PF4/H IgG nicht weiter verstärkt (Abb. 13 B). Vergleichbare Ergebnisse wurden für *S. pneumoniae* erhoben (Abb. 14).

Zusammenfassend kann festgestellt werden, dass die CD62p Expression von Thrombozyten durch Co-Inkubation mit *S. aureus* und *S. pneumoniae* mit der Zeit zunimmt, während die Integrin-Aktivierung abnimmt. PF4 und anti-PF4/H IgG verstärken die bakterieninduzierte Thrombozytenaktivierung nicht.

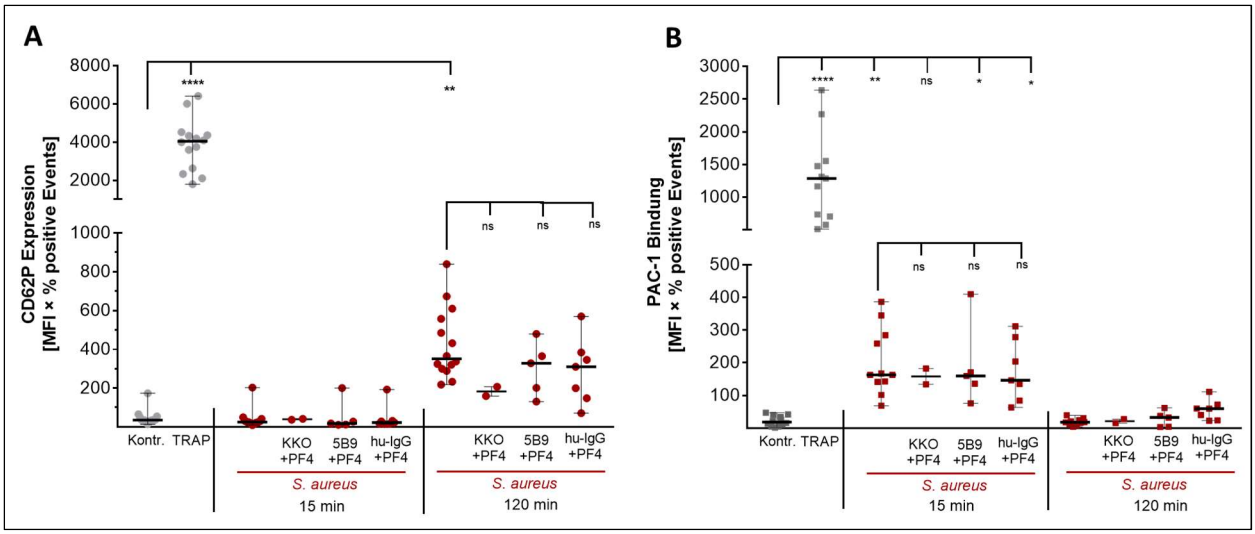


Abbildung 13: Effekt von PF4 und anti-PF4/H IgG auf die Aktivierung von Thrombozyten bei Inkubation mit *S. aureus* (modifiziert nach Wolff et al. 2020). [56] Die Thrombozytenaktivierung ist durch Opsonierung mit PF4 und anti-PF4/H IgG nicht beeinflusst. Dargestellt ist der Median mit Minimum und Maximum. Statistische Signifikanz wurde mit dem Kruskal-Wallis Test, gefolgt von Dunn´s multiplen Vergleichstest bestimmt.

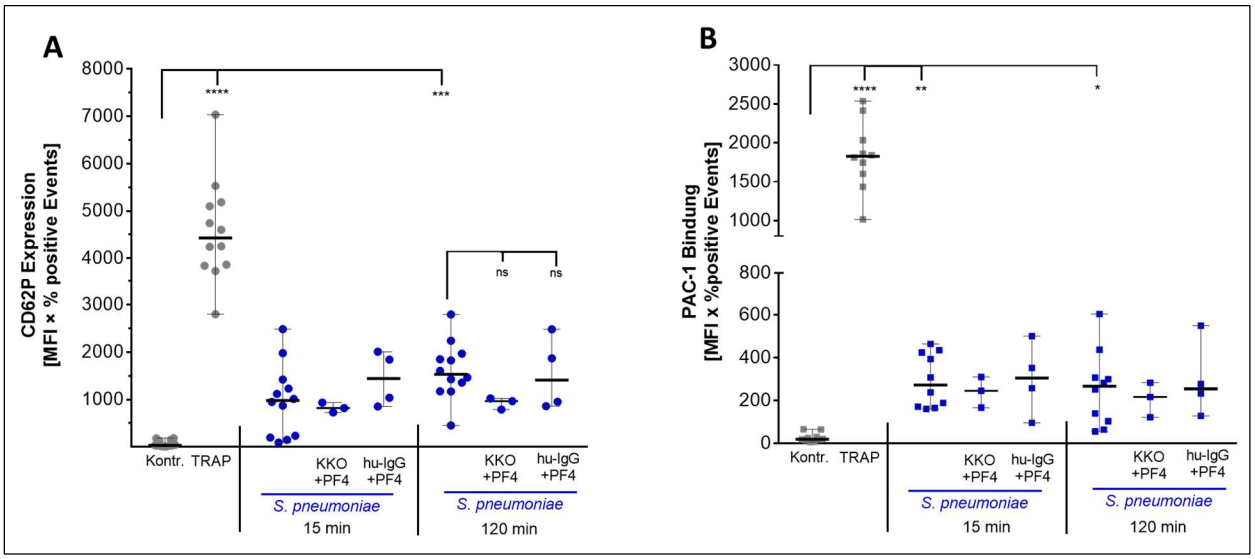


Abbildung 14: Effekt von PF4 und anti-PF4/H IgG auf die Aktivierung von Thrombozyten bei Inkubation mit *S. pneumoniae* (modifiziert nach Wolff et al. 2020).[56] Die Thrombozytenaktivierung ist durch Opsonierung mit PF4 und anti-PF4/H IgG nicht beeinflusst. Dargestellt ist der Median mit Minimum und Maximum. Statistische Signifikanz wurde mit dem Kruskal-Wallis Test, gefolgt von Dunn´s multiplen Vergleichstest bestimmt.

3.3.2 *Induktion prokoagulatorischer Thrombozyten*

(Details siehe Anhang 7.5.1)

Neben der spontanen Thrombozytenaktivierung durch die Gram-positiven Bakterien stellte sich die Frage nach der Induktion prokoagulatorischer Thrombozyten **(s. Fragestellung 3, S. 10).** Hierzu wurden Thrombozyten, die zuvor für 15 oder 120 Minuten mit *S. aureus* oder *S. pneumoniae* inkubiert wurden, mit TRAP-6 stimuliert. Die Messung mit diesem starken Thrombozytenagonist sollte physiologischerweise zu einer deutlichen CD62p Expression führen. Als Kontrolle wurden Thrombozyten unter gleichen Bedingungen mit PBS inkubiert. Die Inkubation mit *S. aureus* führte schon nach 15 min bei den Thrombozyten zu einer Reduktion der CD62p Expression nach TRAP-Stimulation, die sich nach 120 min nochmals halbierte. Dennoch zeigte sich ein signifikanter Anstieg der Aktivierung im Vergleich zur unstimmulierten Kontrolle. Im Gegensatz hierzu war bereits nach 15 min Co-Inkubation keine Erhöhung der CD62p Expression messbar bei den Thrombozyten, welche mit *S. pneumoniae* inkubiert wurden (Abb. 15).

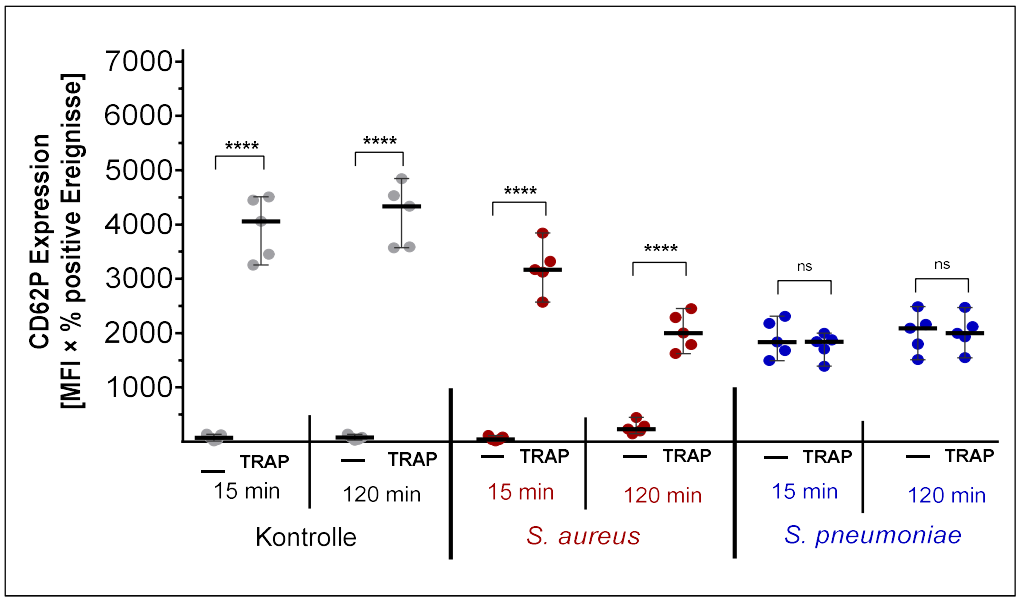


Abbildung 15: Aktivierbarkeit der Thrombozyten nach 15 und 120 min Inkubation mit *S. aureus* (■) und *S. pneumoniae* (■) (modifiziert nach Wolff et al. 2020).[56] Thrombozyten, die mit *S. aureus* inkubiert wurden zeigten eine deutliche Reaktion auf die TRAP-Stimulation, während Thrombozyten, die mit *S. pneumoniae* inkubiert wurden nicht auf TRAP-Stimulation reagierten. Statistische Signifikanz wurde bestimmt durch ANOVA gefolgt von Sidaks´s multiplen Vergleichstest.

Als Marker für prokoagulante Thrombozyten wurde die Expression von Phosphatidylserin auf der Thrombozytenoberfläche bestimmt. Dargestellt ist die PS-Exposition nach 15 und 120 min Inkubation mit *S. aureus* und *S. pneumoniae.* Als Kontrolle dienten Thrombozyten, die mit TRAP und Convulxin oder Ethanol inkubiert wurden. Während Thrombozyten, die mit *S. aureus* inkubiert wurden auch nach 120 min keinen signifikanten Anstieg der PS-Exposition zeigten, war bereits nach 15 min Co-Inkubation mit *S. pneumoniae* ein Anstieg der PS-positiven Ereignisse auf über 80 % zu verzeichnen (Abb.16).

Zusammengenommen zeigen diese Untersuchungen, dass *S. aureus* eine milde Funktionseinschränkung ohne Induktion von prokoagulatorischen Thrombozyten verursacht. Hingegen führt die Inkubation mit Pneumokokken zu deutlicher Störung der Thrombozytenaktivierung und Induktion von prokoagulatorischen Thrombozyten bereits nach 15 Minuten.

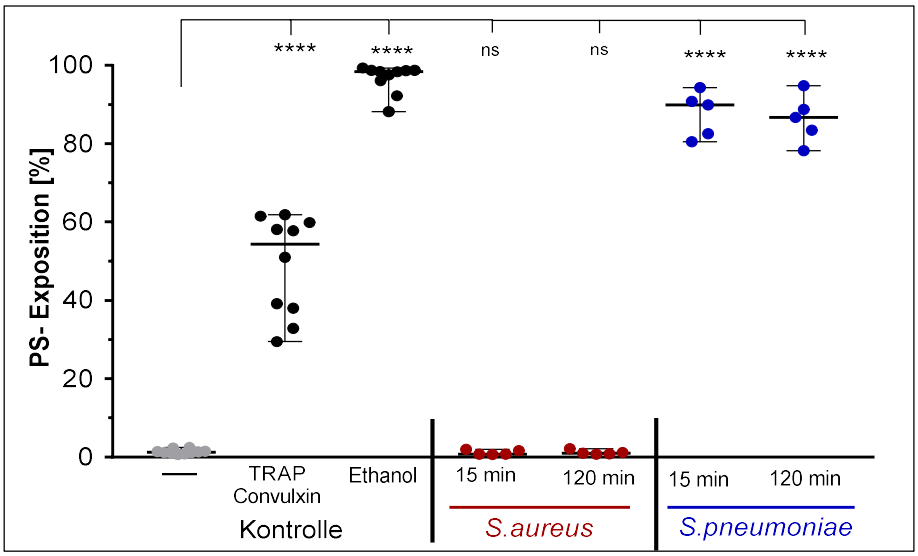


Abbildung 16: Exposition von Phosphatidylserin nach Inkubation mit *S. aureus* (■) und *S. pneumoniae* (■) (modifiziert nach Wolff et al. 2020).[56] *S. pneumoniae* induziert bereits nach 15 min eine deutliche PS-Exposition, während Thrombozyten nach Inkubation mit *S. aureus* keine erhöhte PS-Exposition zeigen. Statistische Signifikanz wurde bestimmt durch einen gepaarten t-Test.

3.4 Einfluss von bakteriellen Toxinen auf Thrombozyten

(Details siehe Anhang 7.5.2 und 7.5.3)

Mit den vorherigen Ergebnissen konnte ich zeigen, dass Thrombozyten einen schädigenden Effekt auf *S. aureus* ausüben, während bei der Interaktion mit *S. pneumoniae* die Thrombozyten geschädigt wurden. Darauf aufbauend wurden in parallellaufenden Arbeiten von Frau Kristin Jahn und Herrn Florian Aschenbrenner die Interaktion von Thrombozyten mit Proteinen *von S. pneumoniae* untersucht. Von besonderem Interesse der Untersuchungen war hierbei das porenbildende Toxin Pneumolysin. Dieses führt bereits in sehr geringen Konzentrationen zur Porenbildung in der Thrombozytenmembran und somit zur Destruktion der Zellen. Zudem konnte gezeigt werden, dass die in der Durchflusszytometrie gemessene Expression von CD62p durch Inkubation mit Pneumokokken oder Pneumolysin nicht durch Aktivierung der Thrombozyten bedingt ist, sondern durch intrazelluläre Bindung des Antikörpers nach Porenformation **(Details s. Anhang 7.5.2)**.

Bereits die oben im Detail beschriebenen Ergebnisse der Inkubation von *S. aureus* und *S. pneumoniae* mit Thrombozyten haben eine vermehrte Expression von PS nach Inkubation mit *S. pneumoniae* gezeigt. Dieses kann sowohl auf die Formation von prokoagulanten als auch apoptotischen Thrombozyten hinweisen. Nachfolgende Arbeiten von Frau Kristin Jahn konnten demonstrieren, dass die Toxine Pneumolysin und Hämolysin Thrombozyten schädigen **(Details s. Anhang 7.5.2 und 7.5.3)**.

Nachfolgend sollte in dieser Arbeit nun geklärt werden, ob durch Hämolysin (*S. aureus*) und Pneumolysin (*S. pneumoniae*) eine Induktion der Apoptose nachgewiesen werden kann (**s. Fragestellung 3 S. 10)**. Als Kontrolle dienten diverse in der Literatur beschriebene Induktoren der Thrombozyten-Apoptose.[55]

Gemessen wurde die Aktivität der Caspase 3/7 in der Durchflusszytometrie. Dargestellt ist die mittlere Fluoreszenzaktivität aktivierter Caspase. Während bei 3 ng/ml Pneumolysin noch keine Steigerung der Caspase-Aktivität zu beobachten war, stieg die Aktivität bereits ab 30 ng/ml Ply deutlich an. Bis zur höchsten getesteten Konzentration von Ply (300 ng/ml) konnte eine 20-fach höhere Caspase-Aktivität im Vergleich zur Kontrolle nachgewiesen werden. Die geringste getestete Konzentration von Hämolysin (0,02 µg/ml) zeigte keinen Anstieg der Caspase-Aktivität, während ein 14-facher Anstieg der Caspase-Aktivität bei 2 µg/ml Hly zu verzeichnen war (Abb. 17).

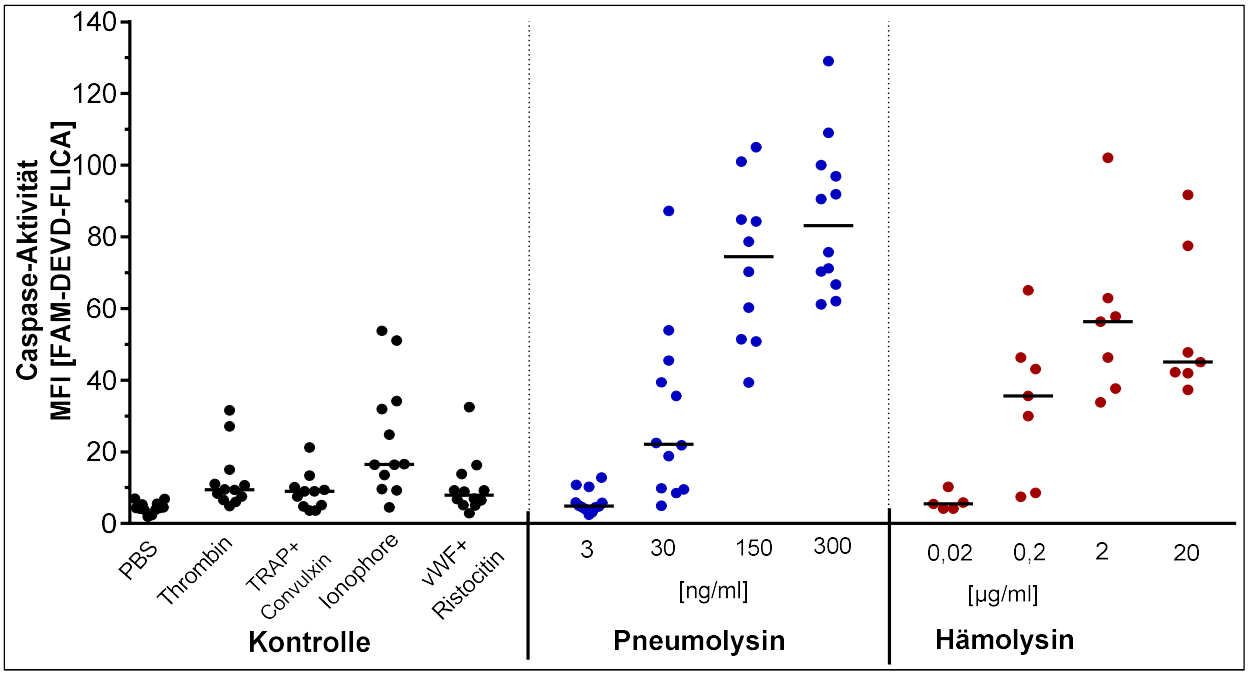


Abbildung 17: Aktivität der 3/7-Caspase bei Thrombozyten durch bakterielle Toxine (modifiziert nach Jahn et al. 2022).[57] Bereits geringe Konzentrationen von Ply und Hly führen zu einer gesteigerten Caspase-Aktivität. Dargestellt sind die Einzelmessungen mit Median.

Wie bereits zuvor für vitale Gram-positive Bakterien demonstriert, führt auch die Inkubation mit den korrespondierenden Toxinen Hämolysin und Pneumolysin zur gesteigerten Exposition von Phosphatidylserin auf der Thrombozytenoberfläche. Vergleichbar mit den Ergebnissen der Caspase-Aktivität, induzierten sehr niedrige Konzentrationen von Pneumolysin (3 ng/ml) und Hämolysin (0,02 µg/ml) keine PS-Exposition. Hingegen waren 30 ng/ml Ply und 0,2 µg/ml Hly bereits ausreichend um die Exposition von Phosphatidylserin auf gut 60 % bzw. 80 % zu steigern. Bis zur höchsten getesteten Konzentration beider Toxine konnte nochmals eine deutliche Steigerung der PS-Exposition nachgewiesen werden (Abb.18).

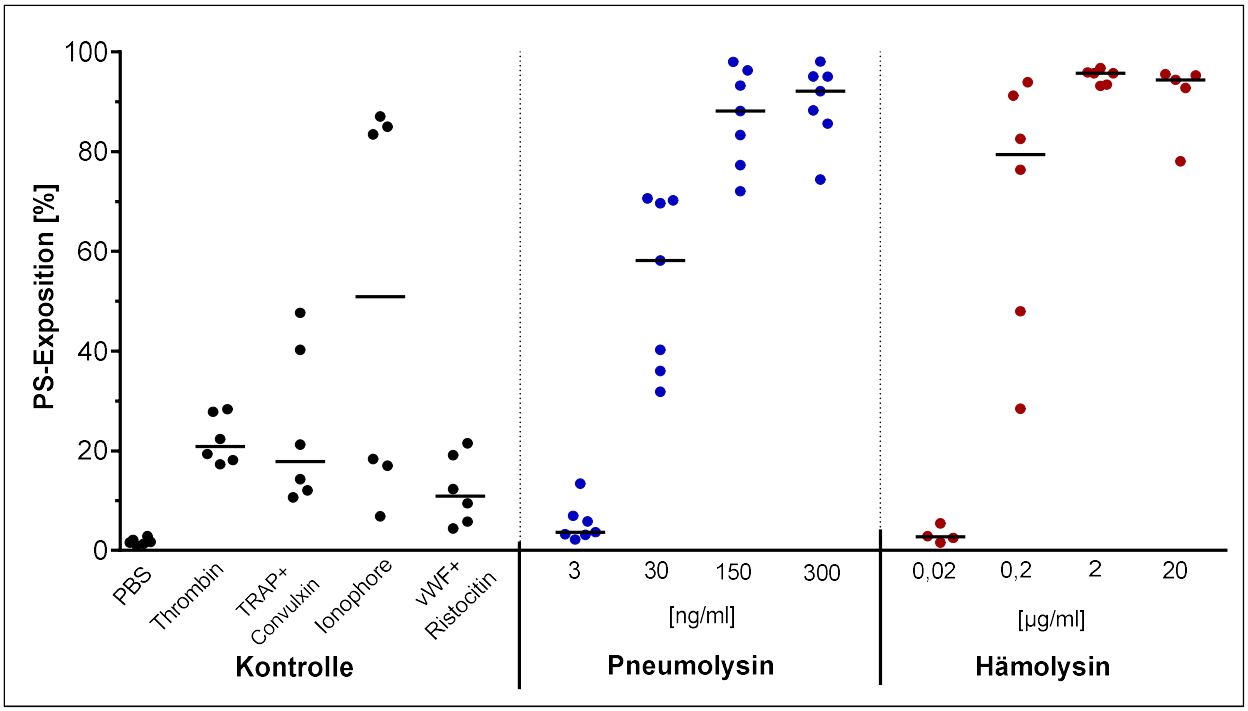


Abbildung 18: Exposition von Phosphatidylserin auf der Thrombozytenoberfläche durch bakterielle Toxine (modifiziert nach Jahn et al. 2022).[57] Bereits geringe Konzentrationen von Ply und Hly führen zu einer deutlich gesteigerten PS-Exposition >50 %. Dargestellt sind die Einzelmessungen mit Median.

Im Kontrast zu den beiden zuvor getesteten Markern gilt Bcl-2 als anti-apoptotische Zielstruktur. Nachfolgende pro-apoptotische Signalwege werden durch Proteine der Bcl2-Familie inhibiert. Dargestellt ist der vielfache Anstieg der Bindung des monoklonalen anti-Bcl-2 Antikörpers. Hierbei konnte zumindest numerisch eine leichte Reduktion der Bindung für alle getesteten Toxine festgestellt werden, was einer Hemmung der anti-apoptotischen Proteinfamilie entsprechen würde.

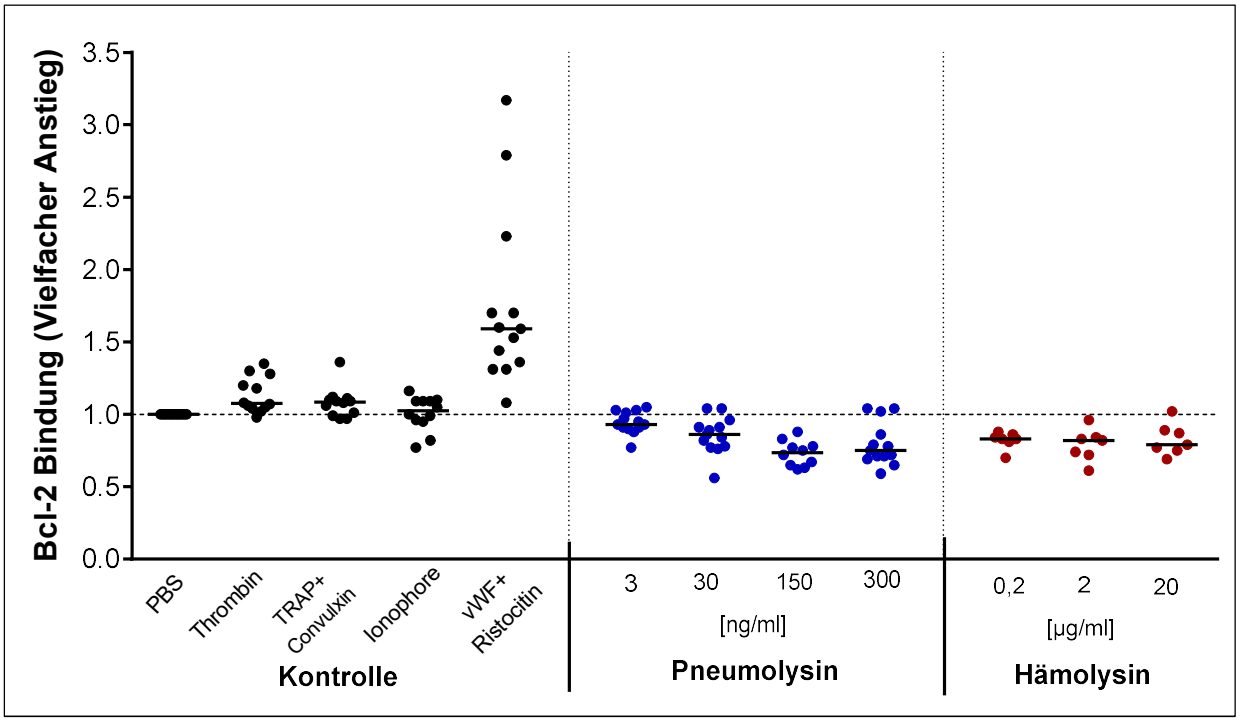


Abbildung 19: Induktion von Bcl-2 bei Thrombozyten durch bakterielle Toxine (modifiziert nach Jahn et al. 2022).[57] Ply, Hly und PVL führen zu einer leichten Reduktion von Bcl-2 ist Dargestellt ist der vielfache Anstieg der Bindung eines anti-Bcl-2 Antikörpers mit Einzelmessungen und Median.

Zusammenfassend zeigen die Untersuchungen, dass die porenformenden Toxine von *S. aureus* und *S. pneumoniae* eine Induktion der intrinsischen Apoptose von Thrombozyten verursachen. Hierbei ist zu vermerken, dass Pneumolysin bereits in deutlich geringeren Konzentrationen im Vergleich zu Hämolysin zu einer Induktion der Apoptose führt.

4 Diskussion

In der vorliegenden Arbeit habe ich gezeigt, dass

a) PF4 und monoklonale sowie humane anti-PF4/H Antikörper an Gram-positive Bakterien binden.
b) die Opsonierung mit PF4 und anti-PF4/H IgG nicht zu einer Verstärkung des anti-bakteriellen Effektes von Thrombozyten auf Gram-positive Bakterien führt und der FcγRIIa keinen relevanten Einfluss hat.
c) die Co-Inkubation von Thrombozyten und *S. aureus* zur Reduktion des Überlebens von *S. aureus* führt, während die Co-Inkubation keinen Einfluss auf das Überleben von *S. pneumoniae* hat.
d) es zur Aktivierung von Thrombozyten durch *S. aureus* kommt.
e) es zur Schädigung von Thrombozyten durch *S. pneumoniae* kommt.
f) *S. pneumoniae* die Exposition von Phosphatidylserin auf der Thrombozytenmembran erhöht.
g) die Exposition von Phosphatidylserin verursacht wird durch das porenbildende Toxin Pneumolysin, welches die Apoptose bei Thrombozyten induziert.

<u>Thrombozyten und *S. aureus*</u>

Neben der Aktivierung und Rekrutierung von anderen Immunzellen sind Thrombozyten in der Lage, das Überleben von Bakterien zu hemmen. Die Co-Inkubation von Thrombozyten mit *S. aureus* führte zu einer signifikanten Reduktion der gemessenen CFU (s. Ergebnisse 3.2.2). Diese Ergebnisse stehen im Einklang mit existierenden *in-vitro* und *in-vivo* Studien, in denen eine direkte antibakterielle Wirkung von Thrombozyten, sowie eine indirekte Wirkung durch die Aktivierung von Immunzellen wie Monozyten gezeigt wurde. Zudem wurde in einem Mausmodell gezeigt, dass bei einer *S. aureus* Bakteriämie die Depletion von Thrombozyten mit einer erhöhten Letalität assoziiert ist. [58–60]

Für *S. aureus* ist bekannt, dass er in der Lage ist, Thrombozyten zu aktivieren,[59,61] wobei hier das Verhältnis von Thrombozyten zu Pathogen eine entscheidenden Einfluss auf die Aktivierung von Thrombozyten und ihren vermittelten schädlichen Effekt hat. Für eine

ausreichende Aktivierung ist hierbei ein Verhältnis von >10 : 1 (Thrombozyt : Pathogen) notwendig.[59] Eine direkte Aktivierung der Thrombozyten durch *S. aureus* konnte auch in dieser Arbeit, bei einem Verhältnis von 25 : 1, demonstriert werden. Diese Aktivierung erhöht im Gegenzug die anti-bakterielle Wirkung von Thrombozyten. Der antibakterielle Effekt ist nicht auf vitale Thrombozyten beschränkt, denn auch der Überstand aktivierter Thrombozyten hemmt das Wachstum von *S. aureus*. Dies weist darauf hin, dass primär nicht zelluläre, sondern sekretierte Bestandteile von Thrombozyten den bakteriziden Effekt vermitteln. Welche Bestandteile der sekretierten Granula den Effekt vermitteln, wurde in dieser Arbeit nicht weiter analysiert und ist Gegenstand weiterer Untersuchungen. Es ist anzunehmen, dass humanes ß-Defensin-1 eine entscheidende Rolle spielt.[28,29] Es ist darauf hinzuweisen, dass einige Bakterienspezies wohl bereits eine Immunität gegenüber den von Thrombozyten sekretierten bakteriziden Substanzen entwickelt haben.[23]

Verschiedene Rezeptoren wie GP IIb/IIIa, P2X1, P2Y12 oder der FcγRIIa sind beschrieben worden die pathogeninduzierte Aktivierung und Aggregation zu verstärken.[59,61] Den letzteren Rezeptor habe ich in dieser Arbeit ebenfalls untersucht. Jedoch führte die Blockierung des thrombozytären Fcγ-Rezeptors nicht zu einem verbesserten Überleben von *S. aureus.* So ist anzunehmen, dass die FcγRIIa-abhängige Amplifikation für die Verstärkung der Thrombozytenaktivierung zu vernachlässigen ist.

Als weiterer Mechanismus der Hemmung des Bakterienwachstums durch Thrombozyten wird die Phagozytose der Bakterien durch Thrombozyten diskutiert,[62] nicht zuletzt aufgrund der beobachteten Fähigkeit von Thrombozyten FcγRIIa-abhängig IgG-markierte Partikel aufzunehmen.[48] Jedoch ist nicht sicher geklärt, ob Thrombozyten über Phagosomen verfügen und so kommt der Phagozytose vermutlich nicht die größte Bedeutung zu bei der direkten antibakteriellen Wirkung von Thrombozyten.[63] Wahrscheinlicher ist es, dass die Ingestion der Pathogene, ähnlich wie das beobachtete Bündeln der Pathogene, der Infektionseindämmung und Opsonierung für andere Immunzellen dient. [25,64] Durch die Aktivierung von Neutrophilen und die Freisetzung von NET´s sowie die Aktivierung der plasmatischen Gerinnung kommt es zur Bildung eines stabilen Thrombus um Pathogene. Dieser Mechanismus wird auch als Immunthrombose beschrieben.[65] Andererseits können Pathogene wie *S. aureus* gezielt Thrombozyten und auch die plasmatische Gerinnung aktivieren. So begünstigt die Bildung von Fibrin die Entwicklung von Biofilmen und die Interaktion mit Gerinnungsfaktoren ermöglicht es

Bakterien, sich vor Zellen des Immunsystems zu verbergen.[24,66,67] So ist die pathogenassoziierte Aktivierung der Hämostase oft als zweischneidiges Schwert beschrieben, das sowohl zur Eliminierung der Pathogene als auch deren Verbreitung beitragen kann. Daher ist es wenig verwunderlich, dass in kleineren Studien sowohl die Inhibition der plasmatischen als auch der thrombozytären Gerinnung diskutiert wurde als therapeutische Option einer bakteriellen Infektion.[65,67–70]

Thrombozyten und *S. pneumoniae*

Überraschenderweise habe ich in dieser Arbeit, im Gegensatz zu *S. aureus*, keinen bakteriziden Effekt der Thrombozyten auf *S. pneumoniae* festgestellt. Hier führte weder die Co-Inkubation mit vitalen Thrombozyten noch mit dem Überstand aktivierter Thrombozyten zu einer Reduktion des Wachstums von *S. pneumoniae* (Details s. Anhang 7.5.1). Im Vergleich zu *S. aureus* ist deutlich weniger bekannt über die Interaktion von Pneumokokken mit Thrombozyten. Bekannt ist, dass *S. pneumoniae* beispielsweise über Thrombospondin-1 an Thrombozyten binden kann und u.a. in Abhängigkeit von TLR-2 eine Aktivierung von Thrombozyten initiiert.[71–73] Auch in dieser Arbeit konnte durch die Co-Inkubation von Thrombozyten und Pneumokokken eine erhöhte CD62p Expression gemessen werden. Allerdings zeigten die mit *S. pneumoniae* inkubierten Thrombozyten keine Reaktion auf die nachfolgende Stimulation mit TRAP und exprimierten in hohem Maß Phosphatidylserin. Diese Beobachtung lässt eher darauf schließen, dass *S. pneumoniae* einen schädigenden Effekt auf Thrombozyten hat.

In einer Parallelarbeit in Kooperation mit der Funktionellen Genomforschung der Universität Greifswald hat Frau Kristin Jahn schließlich das porenbildende Toxin Pneumolysin als Ursache für die Pneumokokken-induzierte Thrombozyten-funktionsstörung identifiziert (Details s. Anhang 7.5.2). In der Literatur wird beschrieben, dass Pneumolysin in der Lage ist Thrombozyten zu aktivieren.[74,75] Dies basiert auf der Beobachtung einer erhöhten Expression von CD62p nach Inkubation von Thrombozyten mit Pneumokokken bzw. Pneumolysin, die ich ebenfalls nachweisen konnte. Eine erhöhte CD62p Expression war bereits nach etwa 30 Minuten sichtbar (s. Ergebnis 3.3.1). Allerdings kommt es durch die Inkubation mit Pneumokokken und dem sekretierten Pneumolysin nicht zu einer Aktivierung der Thrombozyten, sondern vielmehr zu deren Lysierung [76] (Details s. Anhang 7.5.2.). Die intrazelluläre Färbung von P-Selektin wird

ermöglicht durch die Bildung von 40-50 nm großen Poren, die eine Diffusion der Antikörper und Bindung an intrazelluläre Granula ermöglicht.[76] In weiteren Untersuchungen mit der Aggregometrie, Durchflusszytometrie und Mikroskopie konnte durch Frau Jahn weder eine Ply-induzierte Aggregation noch eine Aktivierung von Thrombozyten festgestellt werden.[76] Zudem konnte ich in dieser Arbeit zeigen, dass nicht nur Pneumokokken, sondern auch Pneumolysin zur Induktion der Apoptose von Thrombozyten führt. Hinzuzufügen ist hierbei, dass auch das von *S. aureus* produzierte Hämolysin in höheren Konzentrationen zu einer Induktion von Apoptose führen kann (s. Ergebnisse 3.4).

Zusammenfassend führt das von *S. aureus* sekretierte Hämolysin in geringen Konzentrationen zur Aktivierung von Thrombozyten und erst anschließend zur Lyse. Hingegen kommt es bei Pneumolysin direkt zu einer Lyse der Thrombozyten ohne vorherige Aktivierung. Dies wiederum entspricht meinen Ergebnissen, die ich bei der Inkubation mit vitalen *S. aureus* und *S. pneumoniae* erheben konnte (s. Ergebnisse 3.3.2 und 3.4).

Die Ergebnisse der thrombozytären Schädigung durch Pneumolysin sind von klinischer Relevanz, da *S. pneumoniae* ist einer der Haupterreger der ambulant erworbenen Pneumonie ist. [77,78] Auch bei der Pneumonie vermitteln Thrombozyten einen protektiven Effekt durch die Interaktion mit Zellen der angeborenen und adaptiven Immunabwehr und durch Erhaltung der Barrierefunktion des Endothels. Bei einem schweren Verlauf der Pneumonie kann es zu einer Thrombozytopenie kommen, was wiederum in mehreren Studien mit einer erhöhten Letalität assoziiert war. [79–82] Einerseits kann die Reduktion der Thrombozyten bedingt sein durch eine Aktivierung der Thrombozyten und daraus resultierenden Verbrauch. Andererseits kann ein direkter Einfluss der Pneumokokken angenommen werden.[83] So exprimieren Pneumokokken u.a. das Enzym Neuraminidase, das eine Abspaltung des endständigen Zuckers Sialinsäure auf der Glykokalyx der Thrombozyten verursacht und einen schnelleren Abbau der Zellen in der Leber vermittelt.[84] Als anderer Mechanismus kommt eine Schädigung durch Pneumolysin in Frage. Hierbei kann das Toxin sowohl eine schädigende Wirkung auf Endothelzellen [85] als auch auf Blutzellen wie Thrombozyten vermitteln und somit ein ARDS aggravieren (Details s. Anlage 7.5.2). In Folgearbeiten von Jahn et al. und Wiebe et al. konnte gezeigt werden, dass Pneumolysin durch intravenöses Immunglobulin (IVIG) inhibiert werden

kann. *In-vitro* zeigte sich eine wiederhergestellte Thrombozytenfunktion, die durch erste *in-vivo* Daten bei Patienten mit Pneumonie bestätigt werden konnten.[76,86,87].

Die Rolle von PF4 und PF4/H Antikörpern bei der Abwehr Gram-positiver Bakterien

Bei Gram-negativen Erregern wie *E. coli* spielt bei der direkten Pathogenabwehr durch Thrombozyten die Opsonierung der Bakterien mit PF4 und anti-PF4/H IgG eine wichtige Rolle.[50] Die IgG-abhängige Aktivierung der Thrombozyten über deren FcγRIIa verstärkt die Freisetzung bakterizider Inhaltsstoffe. In dieser Arbeit habe ich gezeigt, dass PF4 und anti-PF4/H IgG auch an Gram-positive Bakterien binden und konnte so Ergebnisse aus vorherigen Arbeiten bestätigen.[44,45] Die Untersuchungen mit humanen anti-PF4/H Antikörpern sind technisch schwierig, da fast alle Menschen in ihrem Serum Antikörper gegen Antigene von *S. aureus* haben, die an *S. aureus* binden könnten.[88] Trotz Affinitätsaufreinigung der anti-PF4/H Antikörper konnte ich eine Kontamination mit diesen anti-*S. aureus* Antikörpern nicht sicher ausschließen. Deshalb habe ich zusätzlich monoklonale anti-PF4 und anti-PF4/H Antikörper verwendet. Diese binden an PF4 auf der Oberfläche von *S. aureus* und *S. pneumoniae*. Da die Bindung der monoklonalen Antikörper KKO und 5B9 an PF4 nur erfolgt, wenn dieses seine Konformation geändert hat, nachdem es an Polyanion gebunden hat, beweist dies, dass PF4 nach Bindung an *S. aureus* und *S. pneumoniae* seine Konformation ändert.

Aufgrund der bereits diskutierten schädigenden Wirkung von Pneumokokken und Pneumolysin auf Thrombozyten, habe ich mich nachfolgend auf den Effekt von PF4 und anti-PF4/H IgG auf *S. aureus* konzentriert. Obwohl die Antikörper und PF4 an die Oberfläche von *S. aureus* binden, konnte im Gegensatz zu *E. coli* keine relevante Verstärkung des anti-bakteriellen Effektes oder der Thrombozytenaktivierung festgestellt werden. Im Einklang damit führte die Blockierung thrombozytären FcγRIIa nicht zu einer Änderung des Überlebens von *S. aureus*. Damit haben anti-PF4/Polyanion Antikörper wahrscheinlich keine besondere Relevanz für die direkte anti-bakterielle Wirkung von Thrombozyten auf *S. aureus.* Hierbei muss in Betracht gezogen werden, dass in dieser Arbeit isoliert die Effekte von Thrombozyten auf *S. aureus* untersucht wurden. Hierfür wurden aufgereinigte Thrombozyten und Bakterien in einem physiologischen Puffersystem inkubiert; ohne die Präsenz von Plasmafaktoren oder anderen Zellen der Blutgerinnung und des Immunsystems. Von besonderem Interesse könnte die

Untersuchung von Neutrophilen sein, da für diese erst kürzlich eine relevante Rolle in der Pathogenese der HIT identifiziert wurde. Anti-PF4/H IgG ist bekannt, NET´s zu induzieren. Wiederum stabilisiert die Bindung von PF4 diese NET´s und erleichtert die Bindung von anti-PF4/H Antikörpern, was die Resistenz gegenüber dem Verdau durch Nuclease erhöht.[89,90] Auf der Basis dieser Arbeit kann ich nicht ausschließen, dass anti-PF4/Polyanion Antikörper auf diesem Weg indirekt zur Abwehr von *S. aureus* beitragen. Ebenso denkbar wäre beispielsweise eine Aktivierung des Komplementsystems und eine dadurch vermittelte Verstärkung des antibakteriellen Effektes. [91]

Zusammenfassend vermittelt das von Thrombozyten freigesetzte PF4 vermutlich einen evolutionär konservierten Mechanismus der Pathogenabwehr. Die ladungsabhängige Bindung von PF4 an negativ-geladene Oberflächen erlaubt die Markierung von Pathogenen wie Bakterien und deren Opsonierung für phagozytierende Zellen des angeborenen Immunsystems. Die Bildung von anti-PF4/Polyanion Antikörpern stellt eine Verbindung zum adaptiven Immunsystem her und ermöglicht eine schnelle Immunantwort auf Pathogene (s. Abb. 1). Im Gegensatz zu Gram-negativen Bakterien haben PF4, anti-PF4/H Antikörper und der thrombozytäre Rezeptor FcγIIa für die Abwehr der Gram-positiven Bakterien *S. aureus* und *S. pneumoniae* jedoch nur eine untergeordnete Bedeutung.

5 Zusammenfassung

Thrombozyten haben neben ihrer Funktion in der Hämostase eine wichtige Rolle in der Immunabwehr. Sie interagieren hierbei mit Komponenten des angeborenen und des adaptiven Immunsystems und sind in der Lage, direkte anti-mikrobielle Einflüsse zu vermitteln. Die Interaktion von Thrombozyten mit Gram-positiven Bakterien unterscheidet sich von jener mit Gram-negativen Erregern. Bei beiden Gruppen von Bakterien scheint die Aktivierung von Thrombozyten und Freisetzung anti-mikrobieller Peptiden aus den Granula ein wichtiger Bestandteil der direkten Pathogenabwehr durch Thrombozyten zu sein. Hierbei führt die Interaktion mit *S. aureus* direkt zu einer starken pathogen-induzierten Thrombozytenaktivierung, während bei Gram-negativen Organismen wie *E. coli* eine Verstärkung durch die Opsonierung mit PF4 und anti-PF4/H IgG notwendig scheint. Vermutlich ist die Bindung von PF4 und anti-PF4/H IgG an Gram-positive Bakterien von größerer Bedeutung für die Opsonierung für andere Immunzellen als für den direkten bakteriziden Effekt der Thrombozyten.

Der Gram-positive *S. pneumoniae* führt durch Funktionsstörung und Exposition von Phosphatidylserin zu einer Schädigung der Thrombozyten. Dieser schädigende Effekt auf Thrombozyten durch *S. pneumoniae* wird unter anderem durch Pneumolysin, ein porenbildendes Toxin der Pneumokokken, vermittelt. Dieses induziert bereits in geringen Konzentrationen die Porenbildung in der Thrombozytenmembran und führt zur Induktion von Apoptose.

In der Arbeit konnten die initialen Fragestellungen folgendermaßen beantwortet werden:

1. Thrombozyten können einen direkten schädigenden Effekt auf Gram-positive Bakterien vermitteln.
2. PF4 und anti-PF4/Polyanion IgG spielen in der direkten Thrombozyten-vermittelten Pathogenabwehr bei Gram-positiven Erregern, trotz der Bindung an Gram-positive Bakterien, eine untergeordnete Rolle. Sie verstärken weder die Thrombozyten-aktivierung noch den anti-bakteriellen Effekt.
3. Die Auswirkung der Co-Inkubation mit Bakterien auf die Thrombozyten ist heterogen und abhängig vom untersuchten Bakterienstamm. Es kommt zur Aktivierung der Thrombozyten durch *S. aureus* und zur Schädigung der Thrombozyten durch *S. pneumoniae*.

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7 Anhang

7.1 Publikationsliste

7.1.1 Zeitschriftenartikel

- **Wolff M**, Handtke S, Palankar R, Wesche J, Kohler T P, Kohler C, Gruel Y, Hammerschmidt S, Greinacher A: Activated platelets kill Staphylococcus aureus, but not Streptococcus pneumoniae-The role of FcγRIIa and platelet factor 4/heparin antibodies, J Thromb Haemost. 2020;10.1111/jth.14814

- Jahn K, Handtke S, Palankar R, Weißmüller S, Nouailles G, Kohler TP, Wesche J, Rohde M, Heinz C, Aschenbrenner AF, **Wolff M**, Schüttrumpf J, Witzenrath M, Hammerschmidt S, Greinacher A.: Pneumolysin induces platelet destruction, not platelet activation, which can be prevented by immunoglobulin preparations in vitro Blood Adv. 2020; 10.1182/bloodadvances.2020002372

- Handtke S*, **Wolff M***, Zaninetti C, Wesche J, Schönborn L, Aurich K, Ulm L, Hübner NO, Becker K, Thiele T, Greinacher A: A flow cytometric assay to detect platelet-activating antibodies in VITT after ChAdOx1 nCov-19 vaccination. Blood. 2021 Jul 1;137(26):3656-3659
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- Zaninetti C, **Wolff M**, Greinacher A: Diagnosing Inherited Platelet Disorders: Modalities and Consequences. Hämostaseologie. 2021 Dec;41(6):475-488

- Greinacher A, Selleng K, Palankar R, Wesche J, Handtke S, **Wolff M**, Aurich K, Lalk M, Methling K, Völker U, Hentschker C, Michalik S, Steil L, Reder A, Schönborn L, Beer M, Franzke K, Büttner A, Fehse B, Stavrou EX, Rangaswamy C, Mailer RK, Englert H, Frye M, Thiele T, Kochanek S, Krutzke L, Siegerist F, Endlich N, Warkentin TE, Renné T: Insights in ChAdOx1 nCoV-19 vaccine-induced immune thrombotic thrombocytopenia. Blood. 2021 Dec 2;138(22):2256-2268

- Wiebe F, Handtke S, Wesche J, Schnarre A, Palankar R, **Wolff M**, Jahn K, Voß F, Weißmüller S, Schüttrumpf J, Greinacher A, Hammerschmidt S: Polyvalent Immunoglobulin Preparations Inhibit Pneumolysin-Induced Platelet Destruction. Thromb Haemost. 2021 Dec 16

- Jahn K, Handtke S, Palankar R, Kohler TP, Wesche J, **Wolff M**, Bayer J, Wolz C, Greinacher A, Hammerschmidt: Alpha-hemolysin of *Staphylococcus aureus* impairs thrombus formation J Thromb Haemost. 2022 Mar 18.

7.1.2 Kongressbeiträge

- GTH 2018, Abstract und Poster: **Wolff M**, Palankar R, Kohler T P, Gruel Y, Hammerschmidt S, Greinacher A: Anti-PF4/heparin antibodies bind to PF4 on Gram-positive Bacteria

- ISTH 2019, Abstract und Poster: **Wolff M**, Handtke S, Palankar R, Wesche J, Kohler T P, Kohler C, Gruel Y, Hammerschmidt S, Greinacher A: Activated platelets kill *S. aureus* but not *S. pneumoniae* by FcγRIIa-independent mechanisms, which are not enhanced by Platelet factor 4/heparin-antibodies

- ISTH 2019, Abstract und Poster Telle P, **Wolff M**, Handtke S, Palankar R, Kohler T P, Wesche J, Kohler C, Hammerschmidt S, Greinacher A: Interaction between large and small platelets with bacteria

- GTH 2020, Abstract und Poster: **Wolff M**, Handtke S, Palankar R, Wesche J, Kohler T P, Kohler C, Gruel Y, Hammerschmidt S, Greinacher A: Activated platelets kill Staphylococcus aureus, but not Streptococcus pneumoniae - The role of FcγRIIa and platelet factor 4/heparin-antibodies

- ISTH 2020, Abstract und e-Poster: **Wolff M**, Handtke S, Greinacher A, Thiele T: Towards the correlation of age with size in human platelets under steady state

- DGTI 2020, Abstract und Online-Vortrag: **Wolff M**, Handtke S, Greinacher A, Thiele T: Platelet apheresis as a human model for analysis of platelet subpopulations under increased platelet turnover

- GTH 2021, Abstract und Online-Vortrag: **Wolff M**, Handtke S, Greinacher A, Thiele T: In-vivo model to assess platelet age in humans: during increased platelet turnover large and small platelets are produced simultaneously

- GTH 2021, Abstract und Online-Vortrag: Zaninetti C, Rivera J Leinoe E, **Wolff M**, Freyer C, Greinacher A: Diagnosis of inherited platelet disorders: comparison between immunofluorescence analysis on the blood smear and genetic testing

- ISTH 2022, Abstract und Poster: **Wolff M**, Handtke S, Schwarz S, Greinacher A, Thiele T: Under increased platelet turnover large and small platelets are produced simultaneously

7.2 Eidesstattliche Erklärung

Hiermit erkläre ich, dass ich die vorliegende Dissertation selbstständig verfasst und keine anderen als die angegebenen Hilfsmittel benutzt habe.

Die Dissertation ist bisher keiner anderen Fakultät, keiner anderen wissenschaftlichen Einrichtung vorgelegt worden.

Ich erkläre, dass ich bisher kein Promotionsverfahren erfolglos beendet habe und dass eine Aberkennung eines bereits erworbenen Doktorgrades nicht vorliegt.

Datum Unterschrift

7.3 Danksagung

An erster Stelle möchte ich mich bei Herrn Prof. Andreas Greinacher bedanken, durch den ich die Freude und Interesse an wissenschaftlichem Arbeiten gefunden habe. Ich bedanke mich für die jahrelange intensive Betreuung, vielfältige Förderung und unermüdliche Ermunterung weiter zu machen und sich an größere Projekte und Ziele zu wagen. Es ist ganz außergewöhnlich mit jemandem zusammen arbeiten zu dürfen, dessen Motivation und Engagement fast jeden direkt begeistern kann. Rückblickend war die Promotionszeit für mich nicht nur geprägt von viel Arbeit, sondern auch von neuen Möglichkeiten und Perspektiven, die mir ein enormes Wachstum von Wissen und Persönlichkeit ermöglicht haben. Vielen Dank für das in mich gesetzte Vertrauen.

Zudem möchte ich mich bei den Mitarbeitern der Abteilung Transfusionsmedizin herzlich bedanken für die Unterstützung über all die Jahre und die herzliche Aufnahme in das Team, mit dem ich viele schöne Erinnerungen verbinde. Mein besonderer Dank geht an dieser Stelle an Herrn Jan Wesche für die geduldige Einarbeitung in die Methoden, die unermüdliche Hilfe beim Lösen von Problemen und Aufmunterung, wenn etwas mal nicht so gut lief. Ein besonderer Dank gilt auch Herrn Stefan Handtke für das Lesen zahlreicher Abstracts und Manuskripte und die schöne und lustige Zusammenarbeit in Labor bei verschiedenen Projekten. Auch Herrn Raghavendra Palankar möchte ich danken, für die Betreuung in der ersten Phase meiner Arbeit und die kontinuierlichen Ideen und Inspirationen, die mir mehr als einmal sehr geholfen haben. Zudem möchte ich mich bei Jessica Fuhrmann und Julia Klauke bedanken; für die technische und auch mentale Unterstützung während meiner Arbeit. Nicht zuletzt gilt mein Dank den Mitarbeitern des Thrombozytenlabors und der Blutspende, ohne die die Durchführung der Arbeit so nicht möglich gewesen wäre.

Weiterer Dank gilt auch den verschiedenen Kooperationspartnern, die diese Arbeit erst möglich gemacht haben. Namentlich möchte ich mich besonders bei Herrn Prof. Sven Hammerschmidt, Herrn Thomas Kohler und Frau Kristin Jahn für die Unterstützung in der mikrobiologischen Expertise bedanken.

Mein Dank gilt auch meinen Eltern, Großeltern und meiner Schwester, die mich über die Jahre in vielerlei Hinsicht stets unterstützt und ermutigt haben.

Zu guter Letzt gilt mein besonderer Dank meinem Partner Jan, für die Unterstützung über die ganze Zeit. Danke für die lieben Worte und das Spenden von Trost und Motivation. Ich bin glücklich und dankbar, dass du mich auf meinem Weg begleitest.

7.4 Publikationen

7.4.1 Orginalarbeit - Activated platelets kill Staphylococcus aureus, but not Streptococcus pneumoniae-The role of FcγRIIa and platelet factor 4/heparin antibodies (Seite 53-62)

7.4.2 Orginalarbeit - Pneumolysin induces platelet destruction, not platelet activation, which can be prevented by immunoglobulin preparations in vitro (Seite 63-74)

7.4.3 Orginalarbeit: α-hemolysin of Staphylococcus aureus impairs thrombus formation (Seite 75-86)

Received: 26 September 2019 | Accepted: 23 March 2020

DOI: 10.1111/jth.14814

ORIGINAL ARTICLE

jth

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

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Funding information
Deutsche Forschungsgemeinschaft, Grant/Award Number: 374031971 – TRR 240

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.

Manuscript handled by: Marcel Levi

Final decision: Marcel Levi, 23 March 2020

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

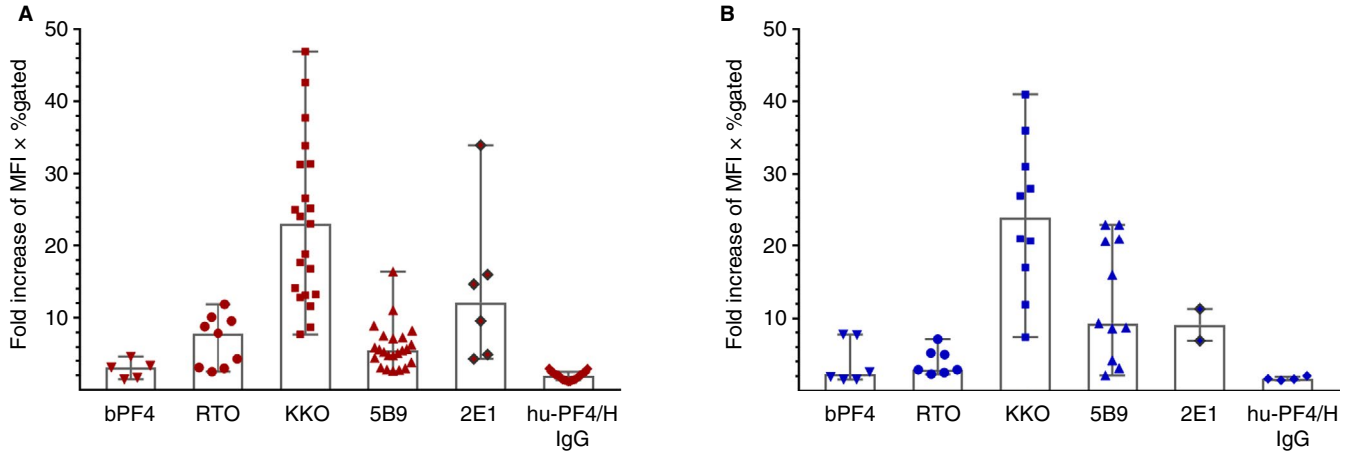

FIGURE 1 Binding of platelet factor 4 (PF4) and anti-platelet factor 4/heparin (PF4/H) antibodies to (A) *Staphylococcus aureus* and (B) *Streptococcus pneumoniae*. Biotinylated PF4 (bPF4) or non-labelled PF4 was used to measure PF4 binding to *S. aureus* (A) and *S. pneumoniae* (B). Binding of bPF4 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).

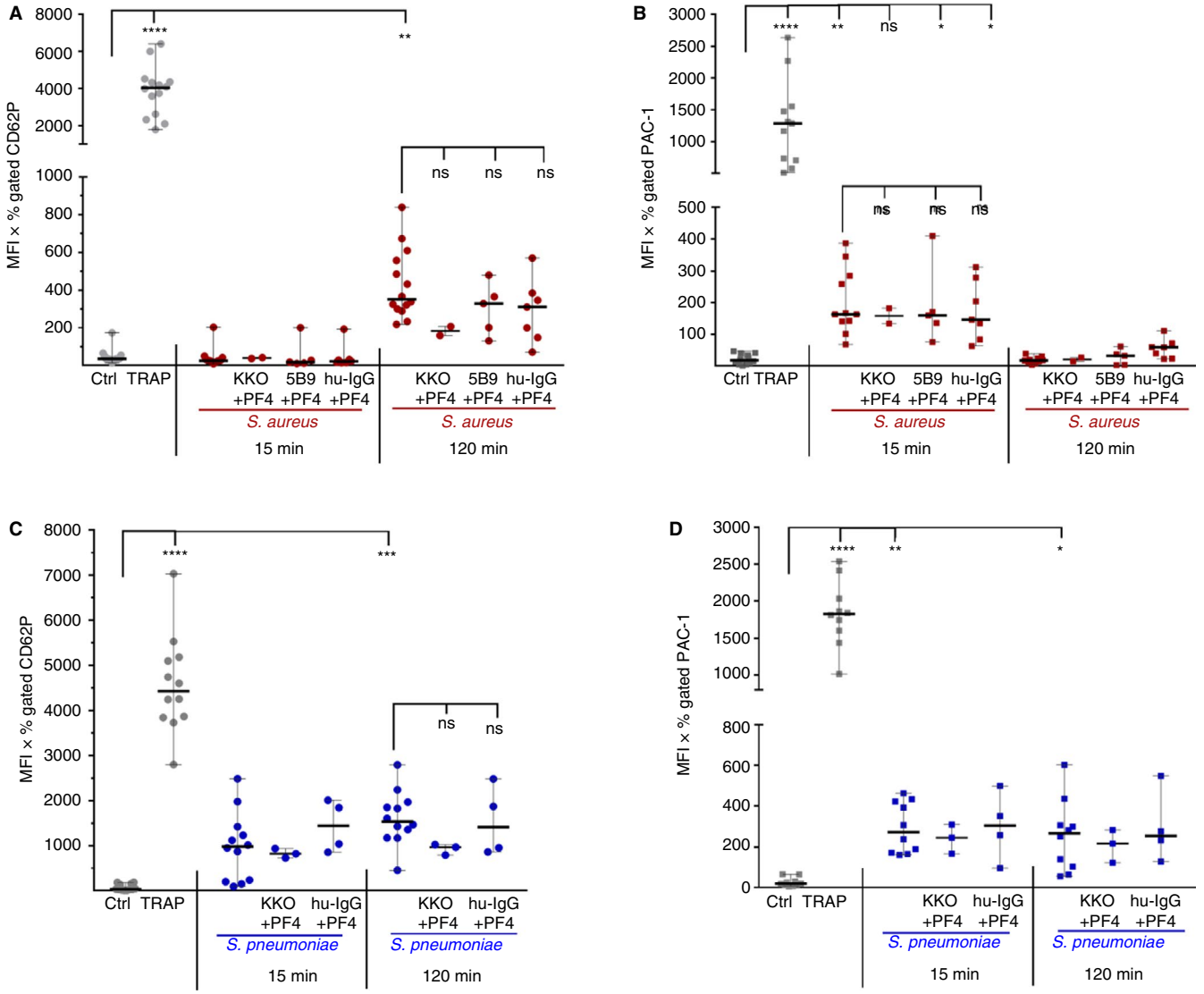

FIGURE 2 Activation of platelets by *Staphylococcus aureus* and *Streptococcus pneumoniae*. Platelet activation was measured by expression of CD62P and binding of PAC-1 after 15 and 120 minutes of incubation with *S. aureus* (A, B; red) or *S. pneumoniae* (C, D; blue). Compared to unstimulated platelets (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).

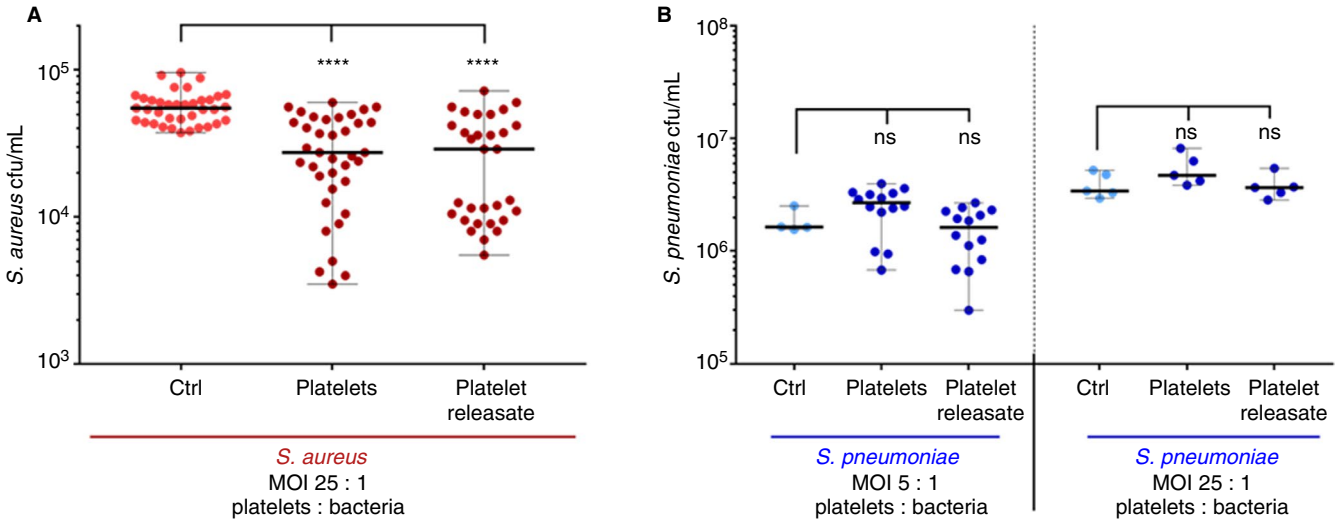

FIGURE 3 Colony-forming units (cfu) of *Staphylococcus aureus* and *Streptococcus pneumoniae* after incubation with platelets or platelet releasate. Cfu of *S. aureus* (A) and *S. pneumoniae* (B) were counted and cfu/mL calculated. Untreated bacteria served as negative control and were compared to bacteria 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

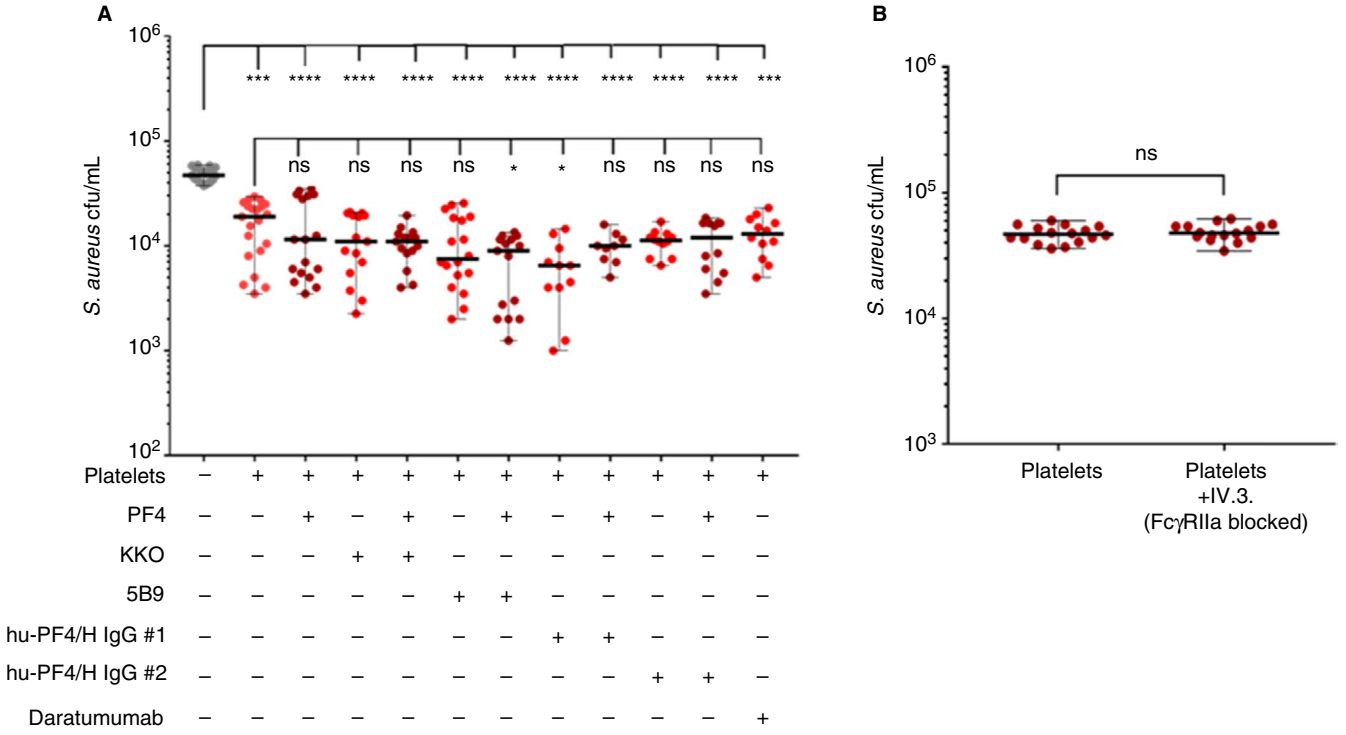

FIGURE 4 Killing of *Staphylococcus aureus* by platelets is not enhanced by platelet factor 4 (PF4) or platelet factor 4/heparin (PF4/H) antibodies and does not change after blocking of platelet FcγRIIa. Opsonisation of *S. aureus* with PF4 with or without addition of different monoclonal or polyclonal 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

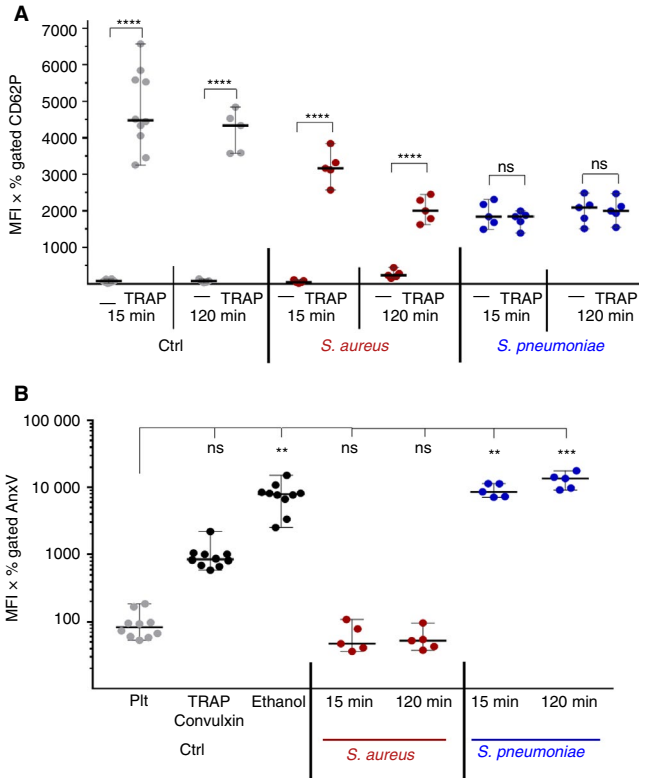

FIGURE 5 *Streptococcus pneumoniae* impairs platelet integrity, while platelets remain functional after incubation with *Staphylococcus aureus*. After 15 and 120 minutes of co-incubation with *S. aureus* or *S. pneumoniae* platelets were stimulated with TRAP-6 and CD62P-expression was compared with unstimulated 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.

ACKNOWLEDGMENTS

Funded by the Deutsche Forschungsgemeinschaft (DFG, German Research Foundation) – Projektnummer 374031971 – TRR 240.

CONFLICTS OF INTEREST

None of the authors has a conflict of interest to declare with regard to this manuscript. AG declares a conflict outside of the submitted work: personal fees and non-financial support from Maco Pharma, Boehringer Ingelheim, personal fees from ASPEN, personal fees from Bristol Myers Squibb, other from Bayer Healthcare, grants and other from Instrumentation laboratories. YG declares personal fees from LFB and Sanofi and research support from Stago.

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.

How to cite this article: Wolff M, Handtke S, Palankar R, et al. Activated platelets kill *Staphylococcus aureus*, but not *Streptococcus pneumonia*—The role of FcγRIIa and platelet factor 4/heparin-antibodies. *J Thromb Haemost*. 2020;18:1459–1468. https://doi.org/10.1111/jth.14814

REGULAR ARTICLE

blood advances

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

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Key Points

- Pneumolysin induces pores in platelets, which renders platelets nonfunctional.
- Polyvalent immunoglobulins prevent platelets from damage by pneumolysin.

Community-acquired pneumonia by primary or superinfections with *Streptococcus pneumoniae* can lead to acute respiratory distress requiring mechanical ventilation. The pore-forming toxin pneumolysin alters the alveolar-capillary barrier and causes extravasation of protein-rich fluid into the interstitial pulmonary tissue, which impairs gas exchange. Platelets usually prevent endothelial leakage in inflamed pulmonary tissue by sealing inflammation-induced endothelial gaps. We not only confirm that *S pneumoniae* induces CD62P expression in platelets, but we also show that, in the presence of pneumolysin, CD62P expression is not associated with platelet activation. Pneumolysin induces pores in the platelet membrane, which allow anti-CD62P antibodies to stain the intracellular CD62P without platelet activation. Pneumolysin treatment also results in calcium efflux, increase in light transmission by platelet lysis (not aggregation), loss of platelet thrombus formation in the flow chamber, and loss of pore-sealing capacity of platelets in the Boyden chamber. Specific anti-pneumolysin monoclonal and polyclonal antibodies inhibit these effects of pneumolysin on platelets as do polyvalent human immunoglobulins. In a post hoc analysis of the prospective randomized phase 2 CIGMA trial, we show that administration of a polyvalent immunoglobulin preparation was associated with a nominally higher platelet count and nominally improved survival in patients with severe *S pneumoniae*–related community-acquired pneumonia. Although, due to the low number of patients, no definitive conclusion can be made, our findings provide a rationale for investigation of pharmacologic immunoglobulin preparations to target pneumolysin by polyvalent immunoglobulin preparations in severe community-acquired pneumococcal pneumonia, to counteract the risk of these patients becoming ventilation dependent. This trial was registered at www.clinicaltrials.gov as #NCT01420744.

Introduction

Community-acquired pneumonia by primary or secondary infection with *Streptococcus pneumoniae* (*S pneumoniae*; the pneumococcus) is one of the most frequent severe infections associated with high mortality.[1,2] Patients are at risk of developing acute respiratory distress syndrome requiring mechanical

Submitted 18 May 2020; accepted 5 November 2020; published online 18 December 2020. DOI 10.1182/bloodadvances.2020002372.

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Requests for data may be e-mailed to the corresponding author, Andreas Greinacher.

The full-text version of this article contains a data supplement.

ventilation. A hallmark of acute respiratory distress syndrome is extravasation of protein-rich fluid into the pulmonary tissue, for example, when the pore-forming toxin pneumolysin alters the alveolo-capillary barrier.[3] In vivo concentrations of pneumolysin occurring during acute pneumonia or invasive infections have not been adequately determined. It can be assumed that the local concentrations differ greatly from circulating pneumolysin concentrations due to dilution in the flowing blood. In an experimental pneumococcal pneumonia mouse model, pneumolysin concentrations correlated with the number of bacteria and the highest concentration measured in the peripheral blood was ~1 ng/mL. This sublytic pneumolysin concentration induced tissue damage including cardiomyocyte injury and dysfunction, and was suggested to be involved in apoptosis of cells of the host immune system.[4,5] The only organ in which pneumolysin is not diluted by the blood flow is the cerebrospinal fluid in patients with pneumococcal meningitis. In this situation, pneumolysin concentrations of 0.85 ng/mL to 180 ng/mL or 10 μg/mL to 30 μg/mL have been measured in the cerebrospinal fluid.[6,7]

Platelets play a major role in maintaining the endothelial barrier.[8] We show that pneumolysin secreted by pneumococci renders platelets nonfunctional. These platelets can no longer seal gaps. Polyvalent immunoglobulins prevent this loss of platelet function. In addition, severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2)[9,10] and influenza virus are also causing severe virus-induced pneumonia. A subset of these patients might suffer from pneumococcal coinfections, which in part may add on to their ventilation needs.[11]

Methods

Ethics

The CIGMA study was conducted in accordance with the International Council for Harmonization, Good Clinical Practice standards, and the Declaration of Helsinki, and with the approval of local institutional review boards/independent ethics committees. All patients (or their representatives) provided written informed consent.

The use of whole blood and washed platelets from healthy adult individuals was approved by the Ethics Committee of the University Medicine Greifswald (BB 044/18). All volunteers gave written informed consent in accordance with the Declaration of Helsinki. All experiments were carried out in accordance with the approved guidelines.

Antibodies and reagents

We used the following antibodies and reagents: mouse monoclonal anti-pneumolysin (Abcam, Cambridge, MA), rabbit polyclonal anti-pneumolysin antibody (Davids Biotechnologie GmbH, Regensburg, Germany), mouse polyclonal anti-enolase antibody (routine immunization of mice with heterologously expressed enolase), IRDye 800CW goat anti-mouse immunoglobulin G (IgG) antibody (Abcam), IRDye 680RD goat anti-rabbit IgG antibody (Abcam), phycoerythrin (PE)-Cy5–labeled monoclonal mouse anti-human CD62P, fluorescein isothiocyanate (FITC)-labeled mouse PAC-1 antibodies recognizing activated $\alpha_{IIb}\beta_{III}$ (CD41/CD61), the RealTime-Glo MT Cell Viability Assay (Promega, Madison, WI), human polyvalent immunoglobulin preparations (pharmaceutical human IgG; IgG-enriched Privigen; CSL Behring, Marburg, Germany) and trimodulin [Biotest, Dreieich, Germany]), FITC-labeled mouse anti-human CD42a (BD Biosciences, Franklin Lakes, NJ), monoclonal mouse anti-human α-tubulin antibody (clone DM1A; Sigma-Aldrich, St. Louis, MO), ATTO 488–labeled Phalloidin (ATTO-TEC GmbH, Siegen, Germany), Arg-Gly-Asp-Ser (RGDS; Sigma-Aldrich), Alexa Fluor 647–labeled monoclonal mouse anti-human CD62P (P-selectin) antibody (clone AK4; BioLegend, San Diego, CA), Alexa Fluor 647-labeled goat anti-mouse IgG (GAMIG AF-647) (Abcam), and Triton X-100 (Sigma-Aldrich).

Platelet preparation

We purified platelets from acid citrate dextrose solution A (ACD-A) anticoagulated whole blood from healthy donors who did not take antiplatelet drugs or nonsteroidal anti-inflammatory drugs (NSAIDs) and used the platelets of the same volunteers for repeated experiments. We prepared platelets as described.[12] In brief, we washed platelet-rich plasma (PRP) twice with Tyrode buffer containing 0.35% bovine serum albumin (BSA), 0.1% glucose, 2.5 U/mL apyrase, 1 U/mL hirudin, pH 6.3; resuspended the final platelet pellet in a bicarbonate-based suspension buffer containing 0.35% BSA, 0.1% glucose, 0.212 M $MgCl_2$, 0.196 M $CaCl_2$, pH 7.2; and adjusted them to 300 000 platelets per microliter.[13]

Flow cytometry–based platelet-activation assay

We performed platelet-activation assays as described.[13] Briefly, we incubated washed human platelets in Tyrode buffer containing Ca^{2+} and Mg^{2+} with phosphate-buffered saline (PBS), 20 μM thrombin receptor activator peptide 6 (TRAP-6), pneumolysin, or pneumococci (D39, TIGR4, or their pneumolysin-free mutants D39Δ*ply* and TIGR4Δ*ply*).[14,15] We did grow bacteria to the mid-log exponential phase before incubating 1.8×10^6 bacteria with 9×10^6 platelets (ratio bacteria platelets 1:5) for 2 or 3 hours. We incubated platelets with pneumolysin at different concentrations or the pneumolysin mutants for 10 minutes. We measured CD62P expression using a mouse monoclonal PE-Cy5–conjugated CD62P antibody. In addition, we determined $\alpha_{IIb}\beta_{III}$ (CD41/CD61) activation using the FITC-labeled mouse PAC-1 antibody. After 10 minutes of incubation of platelets with the antibodies at room temperature (RT), we fixed platelets with paraformaldehyde (PFA)/PBS (pH 7.4) at a final concentration of 2% for 20 minutes at RT and measured them after 2 washing steps (700*g*, 7 minutes) using a FACSCalibur (Becton Dickinson) flow cytometer and CellQuest-Pro 6.0 or the Cytomics FC 500 (Beckman Coulter) and CXP 2.2 software. We then predefined by forward-sideward scatter a platelet gate based on measurements with $CD61^+$ platelets and analyzed in the gated region 20 000 events for fluorescence. We then calculated the value for platelet activation as the geometric mean fluorescence intensity (GMFI) of the gated population multiplied by the percentage of $CD62P^+$-labeled platelets.

Field emission scanning electron microscopy

We performed field emission scanning electron microscopy (FESEM) identical to the protocol described by Binsker et al.[13] Briefly, we incubated washed platelets with pneumococci (60 minutes) or pneumolysin proteins (10 minutes) followed by fixation with 1% formaldehyde at RT. Samples were then centrifuged at 2000*g* for 2 minutes, washed with TE buffer (20 mM Tris-HCl, 2 mM EDTA, pH 6.9), and the resulting pellet was resuspended in 50 μL of TE buffer. Fifty microliters of resuspended samples were placed

onto poly-L-lysine–coated cover slips (12 mm in diameter), fixed with 1% glutaraldehyde in TE buffer for 10 minutes, washed with TE buffer, critical point dried with acetone (CPD 300; Leica), and sputter coated with gold-palladium (SCD 500; Bal-Tec). For imaging in a field emission scanning electron microscope (Zeiss Merlin, Oberkochen, Germany), we used the Everhart-Thornley SE detector alone or together with the Inlens SE detector at a 75:25 ratio at an acceleration voltage of 5 kV and SmartSEM software 6.06 or 5.05.

Pneumolysin production, platelet treatment, and neutralization

For all platelet experiments, we used recombinant cytolytic active pneumolysin and mutants of pneumolysin, pneumolysin^{C428G} without cytolytic activity, and pneumolysin^{W433F} with ~10% cytolytic activity in PBS. We amplified the pneumolysin gene by polymerase chain reaction (PCR) using genomic DNA from *Streptococcus pneumoniae* TIGR4 using the forward primer 370 N-Ply 5′-CGGGATCCGCAAATAAAGCAGTAAATGAC-3′ and reverse primer 371 C-Ply 5′-GCGGTACCTAGTCATTTTCTA CCTGAG-3′. We ligated the *Bam*HI-digested PCR product into the *Bam*HI- and *Eco*RV-digested vector pASK-IBA5 (IBA). The resulting recombinant plasmid pKK2 was used for site-directed mutagenesis with the QuikChange XL site-directed mutagenesis kit (Agilent Technologies). For the amino acid exchange, the following primer combinations were used for an inverse PCR of pKK2: primer 453 PlyW433F+ CCGGGCTAGCCTTCGAATGGTGGCGTA CGG-3′ and 454 PlyW433F- 5′-CACCATTCGAAGGCTAGC CCGGTACACTCTC-3±′, 455 PlyC428G+ 5′-GAGAGGGTAC-CGGGCTAGCCTGGGAATGGTGGC-3′, and 456 PlyC428G-5′-CCCAGGCTAGCCCGGTACCCTCTCTAATTTTGA-3′. *Escherichia coli* DH5α was transformed with the resulting PCR products, after digestion with *Dpn*I to get rid of the template.

For protein production, we cultured *E coli* SCS1 containing the expression plasmids for pneumolysin in SB (super broth) medium at 30°C with shaking.[13] At OD_{600} 2.0, protein expression was induced for 3 hours at RT with 1 mM anhydrotetracycline. After cell lysis for protein purification, we performed affinity chromatography using a StrepTrap HP column according to the manufacturer's instructions (GE Healthcare). We dialyzed pneumolysin proteins against PBS (pH 7.4) overnight at 4°C and determined protein concentrations using a Bradford assay. We determined cytolytic activity of purified pneumolysin proteins by the hemolysis activity test as described.[14] In brief, we incubated ACD-A blood from healthy human volunteers with pneumolysin^{WT}, pneumolysin^{C428G}, and pneumolysin^{W433F} for 10 minutes at 37°C in a 96-well plate (U-bottom). After incubation, we centrifuged the plate and monitored formation of the erythrocyte sediment.

In platelet-activation assays with pneumolysin, we treated platelets for 4 minutes with 300 μg/mL, 3.0 μg/mL, 300 ng/mL, 30 ng/mL, or 3.0 ng/mL pneumolysin followed by 5-minute treatment with 20 μM TRAP-6. In neutralization experiments, we preincubated pneumolysin for 20 minutes at RT with 1 mg/mL human IV immunoglobulin (pharmaceutical human IgG) (IgG-enriched Privigen; CSL Behring, Marburg, Germany), 7.5 μg/mL mouse monoclonal anti-pneumolysin (Abcam), or 10 μg/mL rabbit polyclonal anti-pneumolysin antibodies.

Immunofluorescence staining of platelets

Three million platelets (300 000 cells per microliter) were incubated in Tyrode buffer (resting platelets) or treated with TRAP-6 (20 μM, control for activation), Triton X-100 (0.1%, control for detergent-induced pore formation) or pneumolysin (3, 5, 20, 30, 50, 100, 300 ng/mL) for 10 minutes at 37°C. Samples were fixed in 2% PFA for 20 minutes and subsequently spun on microscopy slides using the Cytospin system (Thermo Fisher). Slides were washed 3 times in PBS pH 7.2. Platelets were then incubated with anti-CD62P-AF647 (1:100) and phalloidin-ATTO 488 (20 pM) or monoclonal mouse anti-α-tubulin antibody (clone DM1A, 1:100) for 2 hours at RT in the dark. Afterward, slides were washed 3 times in PBS pH 7.2. Anti-CD62P-AF647 and phalloidin ATTO 488–stained platelets were then covered by 20 μL of fluorescent mounting medium (ROTI Mount FluorCare HP19; Carl Roth GmbH, Karlsruhe, Germany) and a coverslip. Platelets incubated with monoclonal mouse anti-α-tubulin antibody (used as control for a strictly intracellular protein) were stained with GAMIG-AF647 (1:750) secondary antibody and phalloidin-ATTO 488 (20 pM) for 2 hours at RT in the dark, again washed 3 times in PBS pH 7.2, and subsequently covered by 20 μL of fluorescent mounting medium and a glass coverslip. Confocal laser microscopy was performed on a Leica SP5 confocal laser scanning microscope (Leica, Wetzlar, Germany) equipped with HCX PL APO λ blue 40.0×/1.25 oil UV objective. For image acquisition, ATTO 488 and AF647 were excited by argon (488 nm) and helium-neon (HeNe; 633 nm) laser lines selected with an acousto-optic tunable filter (AOTF) and fluorescence emission was collected between 505 and 515 nm and 640 and 655 nm, respectively, on hybrid detectors (HyDs).

Assessment of CD62P immunofluorescence signal intensities and localization and α-tubulin staining was performed by measuring the line profile (5 μm length and 1 μm width) of nonsaturated grayscale fluorescence intensities (pixel values) of immunofluorescent probes across individual platelets (≥20) in confocal images. To provide further evidence for intracellular staining of CD62P, we performed confocal Z-stacks of platelets and created orthogonal views and 3-dimensional (3D) rendering.

Cell culture and Boyden chamber assays

We transferred 150 μL of washed platelets (300 000/μL) in Tyrode buffer containing 0.212 M $MgCl_2$, 0.196 M $CaCl_2$ into the upper well of a Boyden chamber (6.5-mm Transwell with 3.0-μm Pore Polycarbonate Membrane Insert; Corning). Then we added pneumolysin to the upper and lower chamber in the same concentration each (300 ng/mL, 30 ng/mL, and 3 ng/mL final) and incubated for 45 minutes at 37°C. Ca^{2+}, which can be taken up by cells and has been shown to be necessary for repair of pneumolysin-induced pores in eukaryotic membranes,[16] was present during the experiment. In a subset of experiments, a pneumolysin-inhibiting monoclonal mouse antibody (7.5 μg/mL), polyclonal rabbit anti-pneumolysin antibodies (10 μg/mL), and a pharmaceutical human IgG (IgG-enriched Privigen) preparation (1 mg/mL) were added. We then transferred the inserts into new wells containing 0.9% NaCl, and BSA-FITC (ThermoFisher) was pipetted into the upper chamber at a final concentration of 0.25 mg/mL and incubated for 10 minutes at RT in darkness. We determined platelet pore-sealing capacity by measuring the fluorescence signal of BSA-FITC in the flow-through using a Fluoroskan Ascent FL fluorimeter.

Light transmission aggregometry

We resuspended washed platelet-suspension buffer and added fibrinogen to a final concentration of 2.25 mg/mL. In some experiments, the following were added: RGDS peptides to a final concentration of 1.16 mM, or a pneumolysin-inhibiting monoclonal mouse antibody (7.5 μg/mL), polyclonal rabbit anti-pneumolysin antibodies (10 μg/mL), or a pharmaceutical human IgG preparation (1 mg/mL; Privigen, CSL Behring). After transfer to the aggregometer cuvette, different concentrations of pneumolysin were added to the platelet suspension after 15 seconds. We measured platelet aggregation as a decrease in turbidity of the medium with an APACT4 aggregometer at 500 rpm, 37°C (Haemochrom) applying APACT LPC software. In some experiments, we added 20 μM TRAP-6 after 240 seconds and continued measurement for a further 200 seconds.

Live/dead staining

For measurement of cell viability, we used the RealTime-Glo Cell Viability Assay (Promega) and measured viability for 30 minutes. We used the ability of a cell to reduce a substrate as a mean for viability. We mixed the assay substrate 1:1 with the twofold concentration of pneumolysin. In a subset of experiments, a pneumolysin-inhibiting monoclonal mouse antibody (7.5 μg/mL), polyclonal rabbit anti-pneumolysin antibodies (10 μg/mL), or a pharmaceutical human IgG preparation (1 mg/mL; IgG-enriched Privigen; CSL-Behring) were added. Then, we added the pneumolysin-substrate mixture to washed human platelets in a 96-well plate in duplicates. After 1 minute of incubation, we started shaking the plate with 300 rpm for 3 seconds and measured relative luminescence units (RLUs) using a microtiter plate reader. We repeated shaking and measurement of luminescence every 60 seconds until a total measurement time of 30 minutes. Sample values of luminescence represent the mean of the duplicates subtracted by blank (Tyrode buffer without platelets) of 6 independent experiments.

Release of intracellular calcium

We detected the release of Ca^{2+} from internal stores to the cytoplasm by fluorescent labeling of free intracellular Ca^{2+} using Fluo-4-AM (ThermoFisher). We resuspended platelets in PBS without $MgCl_2$ and $CaCl_2$ (pH 7.4), adjusted them to 150 000 platelets per microliter, and stained them with Fluo-4-AM for 30 minutes in the dark at RT. Buffers needed to be calcium free as diffusion of extracellular calcium or fluorescent dye through the pores would have caused artifacts. After a 1/2 dilution in PBS, we carried out baseline measurements for 15 seconds. Afterward, we stimulated platelets with a final concentration of 300 ng/mL, 30 ng/mL, and 3.0 ng/mL pneumolysin. In a subset of experiments, a pneumolysin-inhibiting monoclonal mouse antibody (7.5 μg/mL), polyclonal rabbit anti-pneumolysin antibodies (10 μg/mL), or a pharmaceutical human IgG preparation (1 mg/mL; IgG-enriched Privigen; CSL-Behring) were added. We measured free Ca^{2+} with a Fluoroskan Ascent FL fluorometer (ThermoFisher) over 7 minutes. In some experiments, we added TRAP-6 at a final concentration of 20 μM after 250 seconds and the measurement was carried out for another 200 seconds.

Thrombus formation under shear flow

We incubated hirudinized whole blood (1 mL) with 3 ng/mL, 30 ng/mL, and 300 ng/mL pneumolysin and pneumolysin^{C428G} (without cytolytic activity) and pneumolysin^{W433F} (with ≈10% cytolytic activity) at 300ng/mL final concentration in whole blood for 10 minutes. In a subset of experiments, pharmaceutical human IgG preparation (1 mg/mL; IgG-enriched Privigen; CSL-Behring) was added to hirudinized whole blood either in the absence (human IgG control) or in the presence of 300 ng/mL pneumolysin. We performed thrombus formation assays at a wall shear rate of 1000 s^{-1} on collagen-passivated surfaces (200 μg/mL HORM collagen type I from horse tendon; Nycomed) in a microfluidic parallel platelet flow chamber (on μ-Slide VI 0.1 with physical dimensions: 1 mm width, 100 μm height, and 17 mm length [Ibidi]). To visualize thrombus formation, prior to perfusion, platelets were labeled with monoclonal antibody (mAb) CD42a-FITC (0.125 μg/mL). We performed time-lapse confocal imaging at intervals of 10 seconds per image on a Leica SP5 confocal laser scanning microscope (Leica) equipped with a water immersion HC PL APO 20×/0.75 IMM CS2 objective. FITC was exited at 488 nm with an argon laser line selected with AOTF; fluorescence emission was collected between 505 and 515 nm on a HyD. We performed quantitative assessment of platelet adhesion and thrombus formation to obtain the percentage area covered by thrombi over time by computational image analysis using the surfaces creation wizard algorithm in Bitplane Imaris version 7.65. (Oxford Instruments, Abingdon, United Kingdom). Experiments were performed according to International Society on Thrombosis and Haemostasis Scientific and Standardization Committee (ISTH SSC) subcommittee on Biorheology recommendations.[14]

Hemolysis assay

To test the cytolytic activity of pneumolysin proteins, the hemolysis assay was performed as described recently.[15] In a subset of experiments, a pneumolysin-inhibiting monoclonal mouse antibody (7.5 μg/mL), polyclonal rabbit anti-pneumolysin antibodies (10 μg/mL), or a pharmaceutical human IgG preparation (1 mg/mL; IgG-enriched Privigen; CSL-Behring) were added.

To analyze the capacity of trimodulin to neutralize pneumolysin, we incubated different concentrations of trimodulin with 300 ng/mL pneumolysin for 30 minutes at 37°C. Afterward, we added 1.5% human whole blood in PBS for 60 minutes at 37°C. Following centrifugation, we measured the supernatant for hemoglobin content by spectrophotometry at 450 nm. As positive control, we used 1% Triton X-100; as negative control, we used Dulbecco PBS.

Quantification of pneumolysin in pneumococci and culture supernatants

S pneumoniae D39, D39Δ*ply*, TIGR4, and TIGR4Δ*ply* were grown until mid-log phase, harvested, and resuspended in PBS containing 30% Tyrode buffer (without BSA). Generation of the pneumolysin mutants has been described recently.[15] After 2 hours and 3 hours of incubation in PBS/Tyrode buffer at 37°C, pneumococci and supernatants were collected for immunoblotting. A total of 1×10^8 bacteria and the respective trichloroacetic acid precipitated supernatant of 1×10^8 bacteria were run on a 12% sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE). In addition, a serial dilution of recombinant pneumolysin protein was used as standard. The samples were blotted on a nitrocellulose membrane and, after blocking, polyclonal primary antibodies raised in rabbit or mouse and fluorescent-labeled secondary antibodies

were used to detect pneumolysin or enolase using the Odyssey CLx scanner (Li-Cor). Pneumolysin signals in the supernatants were normalized using the enolase signal of the respective bacterial lysate. Normalization was performed using Image Studio software. Calculation of the pneumolysin amount based on the pneumolysin standard curve was performed with Microsoft Excel (Office package 2016). After normalization and quantification, the immunoblot images were adjusted for brightness and contrast using Photoshop CS5.

Patient analysis

We investigated the effect of immunoglobulins on platelet numbers in patients with *S pneumoniae* infection with data generated in the CIGMA study (NCT01420744).[17] The patients were treated with trimodulin (182.6 mg/kg) or placebo for 5 consecutive days. Trimodulin is a human polyvalent immunoglobulin preparation containing 45 to 55 mg/mL human plasma immunoglobulin proteins; it is composed of ~23% IgM, ~21% IgA, and ~56% total IgG. In 100 of the 160 patients enrolled into the study, the causative pathogens (bacterial and/or viral) were identified.

Statistics

We performed statistical analysis using GraphPad Prism (version 5.01), unless otherwise indicated. We show the data as scatter plots and include median, minimal, and maximal values including median and interquartile range. We analyzed the data using the nonparametric Friedman test followed by a Dunn multiple comparison posttest. We considered $P < .05$ to be statistically significant.

Results

Pneumococci induce platelet staining for CD62P

We incubated platelets with wild-type and pneumolysin-deficient pneumococci. Wild-type pneumococci induced staining for the platelet activation marker CD62P (P-selectin; supplemental Figure 1). This was strongest for the strain TIGR4, less pronounced for strain D39, which produces less pneumolysin, and lowest for pneumolysin-deficient *ply* mutants. We therefore expected that pneumococci preactivate platelets and render them more reactive. However, in the presence of wild-type TIGR4 pneumococci, platelets were no longer reactive to costimulation with TRAP-6 (a potent platelet thrombin receptor agonist), whereas D39 and pneumolysin-deficient pneumococci still allowed additional platelet activation by TRAP-6. This suggested that pneumolysin interferes with platelet reactivity.

Pneumolysin induces pores in the platelet membrane, Ca^{2+} efflux, and platelet lysis

When we incubated platelets with purified pneumolysin, we observed a similar pattern as described in the previous paragraph for incubation of platelets with wild-type pneumococci (Figure 1A-C refers to experiments with neutralizing antibodies explained in detail below in "Antibodies and polyvalent immunoglobulins inhibit the effects of pneumolysin on platelets in vitro"). In contrast, pneumolysin proteins without or with low cytolytic activity failed to induce CD62P expression (Figure 1D-E) and integrin activation (supplemental Figure 2A-D) and platelets remained responsive to TRAP-6. To better understand the effects of pneumolysin, we visualized platelets incubated with wild-type or pneumolysin-deficient pneumococci by scanning electron microscopy.

We observed binding of both wild-type (supplemental Figure 3C-D) and pneumolysin-deficient pneumococci (supplemental Figure 3A-B) to platelets, but only wild-type pneumococci induced pores (supplemental Figure 3C-D). Controls are shown in supplemental Figure 3E-F. We observed pore formation with diameters of 40 to 50 nm in platelets when we added purified pneumolysin at concentrations of 300 μg/mL (supplemental Figure 4A), 300 ng/mL (Figure 1F; supplemental Figure 4B), 30 ng/mL (supplemental Figure 4C) and 3.0 ng/mL (supplemental Figure 4D), but not when we applied inactive pneumolysins (supplemental Figure 4E-F). The pneumolysin concentrations causing pores correspond to the concentrations causing hemolysis in erythrocytes (supplemental Figure 5A-B).

We then assessed the concentration of pneumolysin in bacterial culture supernatants by SDS-PAGE (supplemental Figure 5C). The intensities of the pneumolysin protein bands correspond to 2.677 ± 0.871 ng/mL pneumolysin for strain TIGR4 and 1.834 ± 0.261 ng/mL pneumolysin for strain D39 (supplemental Figure 5D) after 3 hours of incubation.

We next addressed the consequences of pore formation on platelet function. When we incubated washed platelets with 300 ng/mL pneumolysin, we observed an immediate release (2.5-fold) of Ca^{2+} (Figure 2A; supplemental Figure 6A), consistent with the findings of others,[18] and an increase in platelet aggregation (up to 50%) as measured by a change in light transmission of the platelet suspension (Figure 2B; supplemental Figure 6B). These effects were less pronounced when we used 30 ng/mL and were absent with 3.0 ng/mL pneumolysin or inactive pneumolysins (Figure 2A-B; supplemental Figure 6A-B), consistent with the platelet morphology by electron microscopy (Figure 1F; supplemental Figure 4C-F). We did not observe inhibition in the increase of light transmission induced by pneumolysin after adding 1.16 mM RGDS peptide. RGDS is a potent inhibitor of platelet aggregation (supplemental Figure 7). This indicates that the change in observed light transmission is caused by lysis of platelets rather than by aggregation. RGDS alone did not interfere with pore formation of pneumolysin in cell membranes (supplemental Figure 5A lower left wells). Furthermore, we found that platelets responded only to TRAP-6 either in the presence of very low concentrations of pneumolysin or inactive pneumolysins (Figure 2A-B; supplemental Figure 6A-B).

CD62P staining of platelets results from labeling intracellular CD62P

Because the pore formation and the functional experiments in the presence of pneumolysin suggested nonfunctional platelets, we asked why CD62P was upregulated on the platelet membrane, which typically requires platelet activation. By fluorescence microscopy, we visualized that pneumolysin treatment did not result in CD62P surface expression, but that intracellular CD62P was stained (Figure 3; supplemental Figure 8). Staining for CD62P in nonpermeabilized platelets increased with the concentration of pneumolysin (3, 5, 20, 30, 50, 100, 300 ng/mL). At very high pneumolysin concentrations of ≥100 ng/mL, platelets are completely destroyed and both CD62P and α-tubulin (supplemental Figure 9) immunofluorescence staining patterns appear outside in some of the platelets. This potentially results from the breakdown of the fragile platelet plasma membrane during preparation of slides for microscopy by cytospin and during coverslip mounting, which results in appearance of a "squeezing-out" effect of the labeled intracellular proteins from the damaged platelets. Figure 3B shows

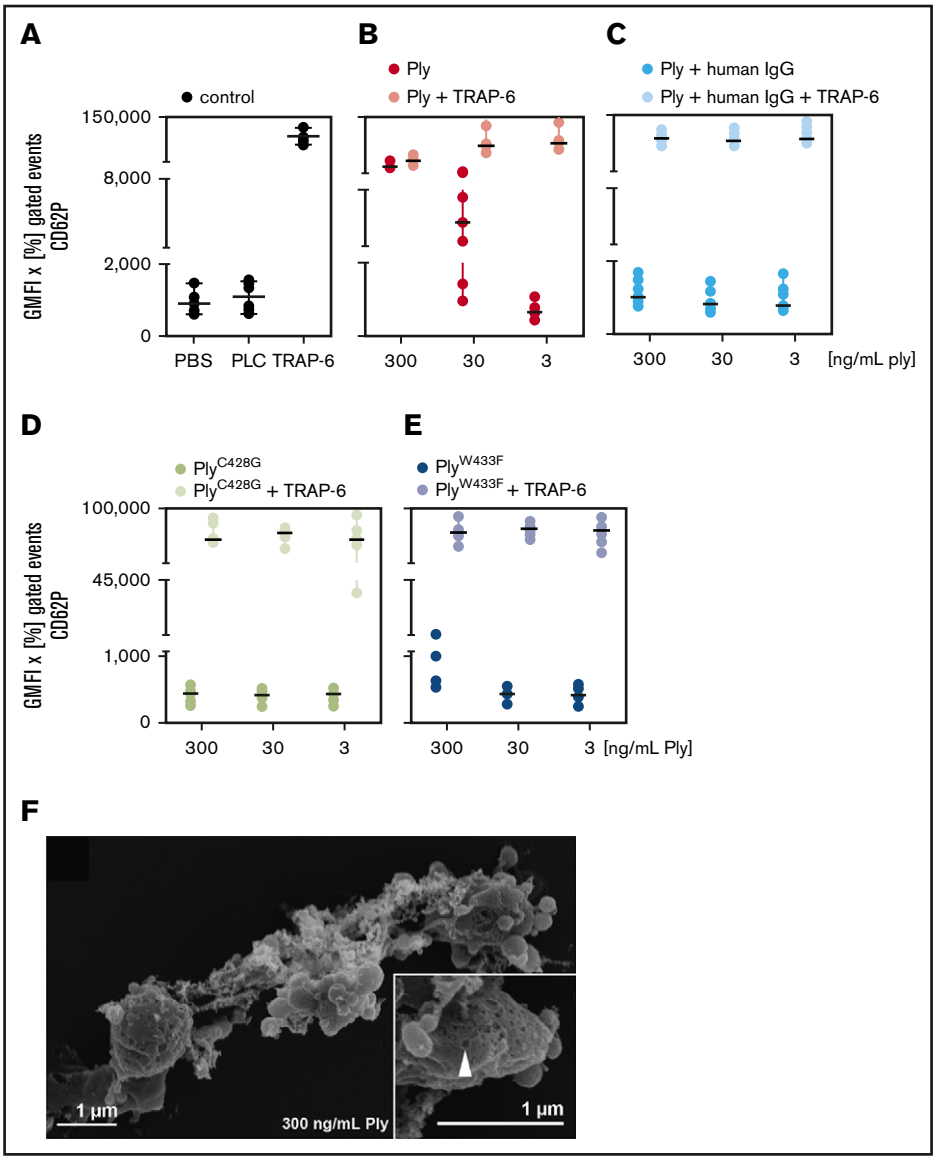

Figure 1. CD62P expression of platelets by pneumolysin is caused by pore formation. Washed platelets of a defined set of 6 donors were incubated with various concentrations of pneumolysin (Ply). CD62P was detected by flow cytometry using antibodies against CD62P (P-selectin). The data are presented as GMFI of the positive gated events multiplied with the percentage of positive gated events in the dot plots. (A) PBS (gray) and phospholipase C (PLC; gray) from *Staphylococcus aureus* known to not activate platelets[13] were used as negative controls and 20 µM TRAP-6 (gray) as positive control. (B) Pneumolysin (red; ng/mL) caused CD62P expression and dose-dependently inhibited an additional response to TRAP-6 (black). (C) Polyvalent human immunoglobulins (human IgG; Privigen) neutralized the effect of pneumolysin (pneumolysin plus human IgG = light blue) (to enable comparison with the experiments without immunoglobulins, the data are shown here, although they are presented in the text at the end of "Results"). (D) Pneumolysin^{C428G} without lytic activity (brown) did not activate platelets or impaired the response to TRAP-6 and (E) pneumolysin^{W433F} with ~10% lytic activity (purple) had a very minor effect only at 300 ng/mL. (F) Visualization of pore formation in the platelet membrane by pneumolysin by scanning electron microscopy. Platelets are altered in their shape and formed vesicles but not pseudopodias. At the left side, a platelet with pores can be seen. Inset, a higher magnification of the platelet indicating a pore by an arrow.

the cross-sectional fluorescence signal intensity (nonsaturated grayscale median values) for immunofluorescence localization of CD62P in individual platelets (from ≥20 single platelets). The most likely explanation is that pneumolysin-induced pores allowed for antibody penetration into platelets resulting in staining of intracellular CD62P. This is also shown by the control with Triton X-100, which induces pore formation in the platelet membrane. As additional control, we also immunostained for α-tubulin, a strictly intracellular cytoskeletal protein. Staining patterns (supplemental Figure 9A) and cross-sectional fluorescence signal intensities (supplemental Figure 9B; nonsaturated grayscale median values) were similar to the ones obtained for CD62P staining.

Thus, we explain the increase in CD62P staining by damaged platelets rather than by platelet activation.

Pneumolysin renders platelets nonfunctional

Consistently, platelets were no longer viable in the presence of 300 ng/mL pneumolysin, whereas 30 ng/mL pneumolysin showed an intermediate phenotype, and 3.0 ng/mL had no effect on viability as measured by an increase of relative luminescence units resulting from intracellular processing of a luminescent substrate. PBS and Triton X-100 were used as controls (Figure 4A; supplemental Figure 10).

We also demonstrate that platelets in the presence of pneumolysin dose-dependently lose their capability to form thrombi, when whole blood was flown over collagen at arterial shear (1000 s^{-1}) (Figure 4C; supplemental Figure 11). Figure 4D shows the area covered by thrombi in the presence of different concentrations of pneumolysin and inactive pneumolysin mutants. Wild-type but not the inactive pneumolysins inhibited thrombus formation.

One of the major functions of platelets is to seal gaps in the endothelium[8] evoked by acute infection.[19,20] Pneumolysin inhibits platelet function and thereby compromises their sealing function of the endothelium, increasing the risk of bleeding and fluid extravasation into the interstitial compartment.[21,22] We show

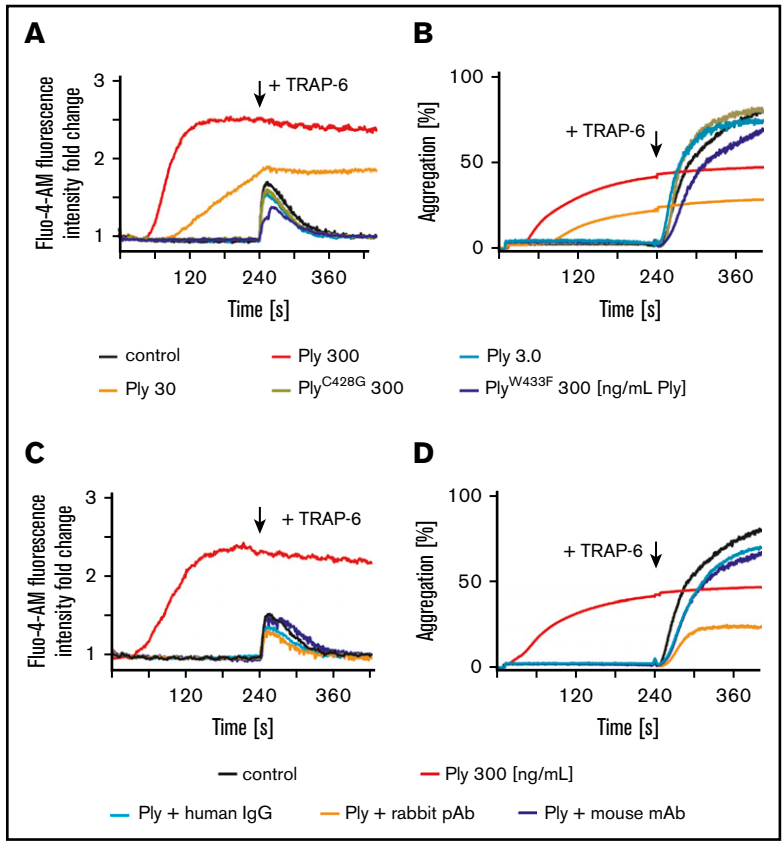

Figure 2. Loss of platelet function due to pneumolysin is prevented by immunoglobulins. (A) Prior to pneumolysin treatment intracellular Ca^{2+} of washed platelets was labeled with Fluo-4-AM for 30 minutes. After incubation with pneumolysin, the kinetics of Ca^{2+} release was measured and values are given as fold change compared with NaCl control. Different concentrations of pneumolysin (Ply) are color coded: 300 ng/mL (red); 30 ng/mL (orange); 3.0 ng/mL (light blue). Pneumolysin^{C428G} without lytic activity (brown) pneumolysin^{W433F} with ~10% lytic activity (blue) did not cause Ca^{2+} release. (B) Platelet aggregation is typically directly proportional to an increase in light transmission. Only pneumolysin 300 ng/mL (red) and 30 ng/mL (orange) induced an increase in light transmission, but platelets where no longer responsive to 20 μM TRAP-6. Light transmission did not change by addition of buffer, pneumolysin 3 ng/mL, or the mutant pneumolysins, but platelets were still responsive to 20 μM TRAP-6. (C-D) Polyvalent human immunoglobulin (human IgG (Privigen); 1 mg/mL; green), 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) in calcium influx (C) and platelet aggregation (D). In the presence of these immunoglobulins platelets became again responsive to 20 μM TRAP-6 (to enable comparison with the experiments without immunoglobulins, the data are shown here, although they are presented in the text at the end of "Results").

compromising of this important platelet function using Boyden chamber experiments. Platelets sealed the membrane pores of the Boyden chamber thereby preventing diffusion of fluorescently labeled BSA to the lower chamber. In the presence of pneumolysin, platelets no longer inhibited BSA diffusion (Figure 5).

Antibodies and polyvalent immunoglobulins inhibit the effects of pneumolysin on platelets in vitro

We then aimed to rescue platelet function in the presence of active pneumolysin by using antibodies neutralizing its cytolytic activity. A monoclonal mouse antibody (7.5 μg/mL), polyclonal rabbit antibodies (10 μg/mL), or a pharmaceutical human IgG preparation (1 mg/mL) completely restored the platelet phenotype and function.

For all impaired functions described in detail herein, we show that: namely, the CD62P expression response to TRAP-6 was restored (Figure 1C; supplemental Figure 12); integrin activation again occurred as measured by PAC-1 binding (supplemental Figure 13); calcium release no longer occurred (Figure 2C; supplemental Figure 14); no pseudoplatelet aggregation (= lysis) was observed (Figure 2D; supplemental Figure 14); thrombus formation in whole blood was again comparable to the normal control (Figure 4D,F; supplemental Figure 11); and platelet viability was rescued, no longer differing from the buffer control (Figure 4B; supplemental Figure 9). Finally, antibodies and immunoglobulins neutralize pneumolysin and platelets remain functional to seal membrane pores of the Boyden chamber (Figure 5).

A polyvalent immunoglobulin preparation was associated with a nominally higher platelet count and nominally reduced mortality in patients with *S pneumoniae*–induced severe community-acquired pneumonia

In addition to mAbs and pharmaceutical human IgG (IgG-enriched Privigen), the IgM/IgA-enriched immunoglobulin preparation trimodulin is able to effectively neutralize lysis of red cells by pneumolysin (Figure 6A). To translate our findings into clinical practice, we took advantage of an earlier performed phase 2 trial (CIGMA study) in 160 patients with severe community-acquired pneumonia requiring invasive mechanical ventilation. Patients were treated in addition to standard of care with trimodulin (182.6 mg/kg for 5 days) or placebo.[17] Infections with *S pneumoniae* were confirmed in 15 patients in the trimodulin group and in 18 patients in the placebo group. Platelet counts were higher (544 vs 361 platelets per nanoliter on day 14; Figure 6B) and mortality was nominally lower in the trimodulin group (2 of 15 [13.3%] vs 7 of 18 [38.9%]; Figure 6C). A limitation of this study was the small numbers of patients and therefore these preliminary observations warrant further investigation in larger clinical trials.

Discussion

In this study, we assessed the interaction of pneumococci and the toxin pneumolysin with platelets. Taken together, our data lead us to the following conclusions: pneumococci induce pores in the platelet membranes by secretion of pneumolysin. This renders

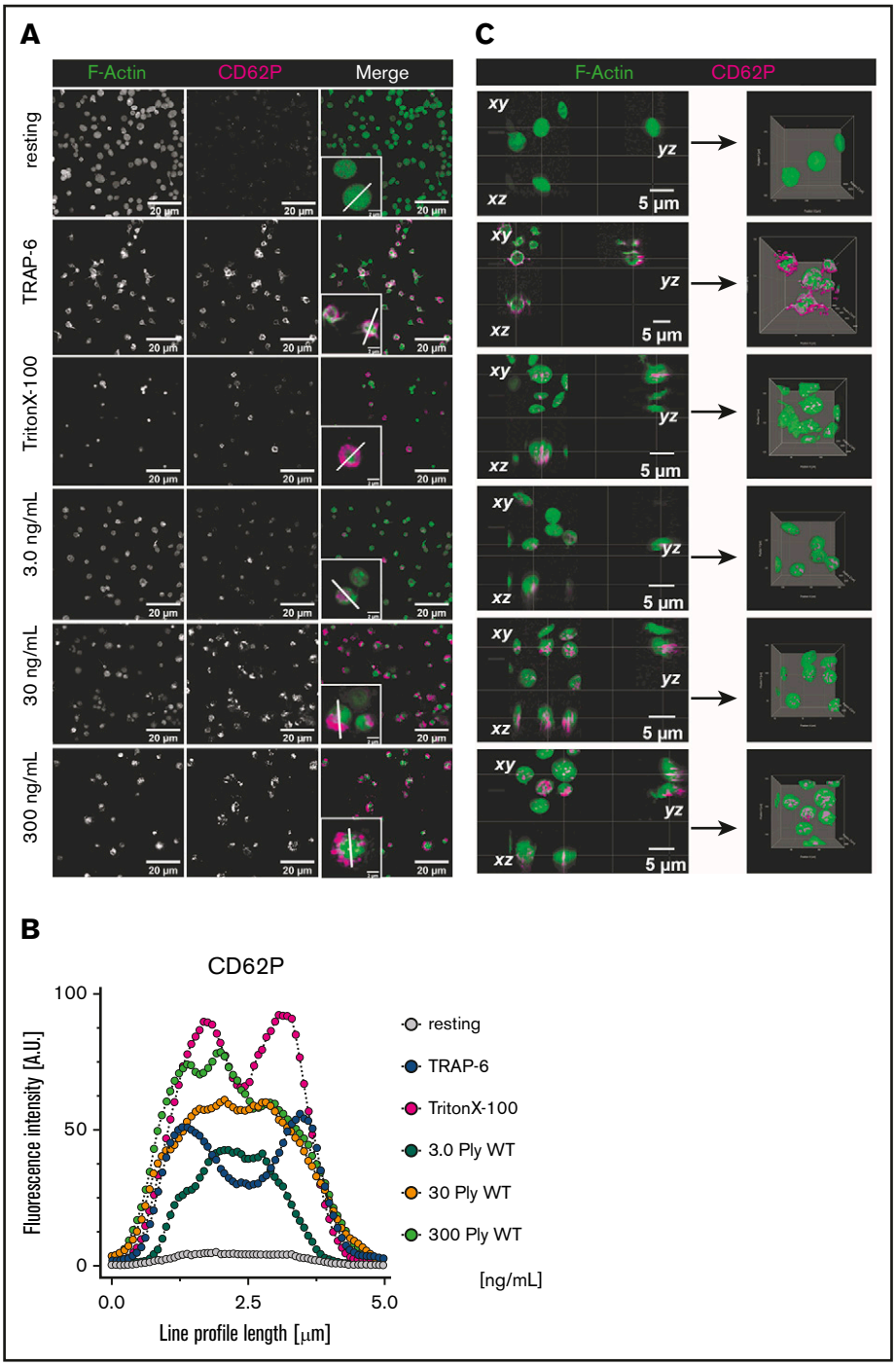

Figure 3. Fluorescence microscopy of pneumolysin-treated platelets. (A) Pneumolysin-treated platelets were stained for F-actin (green) and CD62P (magenta). Platelets were not permeabilized, with the exception of the Triton X-100 control. Insets, Single platelets at higher magnification and the line used for measuring fluorescence intensities shown in panel B. In the presence of 3.0 and 30 ng/mL pneumolysin, intracellular staining of CD62P and α-tubulin become visible. At 200 ng/mL pneumolysin, vesicles staining strongly for pneumolysin surround the platelets (compare Figure 1F). (B) Staining pattern of CD62P throughout single cells treated with pneumolysin was quantified to distinguish between cytoplasmic and only surface-associated CD62P staining. The pattern indicates that CD62P is stained intracellularly and not extracellularly. The different concentrations of pneumolysin used are color coded: 3.0 ng/mL (blue), 30 ng/mL (orange), 300 ng/mL (green). (C) Orthogonal views of confocal Z-stacks and 3D isosurface rendering of pneumolysin-treated platelets stained for F-actin (green) and CD62P (magenta). It shows distinct intracellular accumulation of anti-CD62P antibody in platelets treated with different concentration of pneumolysin and membrane permeabilization with Triton X-100 and surface expression of CD62P upon TRAP-6 stimulation.

platelets nonfunctional and inhibits platelet-thrombus formation in whole blood. Earlier reports indicating platelet activation by pneumolysin[18] are most likely caused by the artifact of anti-CD62P antibody diffusion through pores in the platelet membrane, which then stain intracellularly the activation marker CD62P. Local in vivo concentrations of pneumolysin in the lung are difficult to determine in the flowing blood obtained from patient veins, as the blood has to circulate from the lung through the entire arterial system and capillaries before sampling. Therefore, best estimates on local in vivo concentrations are likely obtained from pneumolysin concentrations in the cerebral fluid obtained from patients with pneumococcal meningitis. Here, pneumolysin concentrations reached up to 30 µg/mL, depending on the report.[4,6,7] As we did already observe major damage of platelets in a concentration of 0.03 µg/mL (30 ng/mL), the concentrations, which we have used for our in vitro experiments, are very likely within a clinically relevant range. Importantly, platelet damage depends on the incubation time: the shorter the incubation time, the higher the pneumolysin concentrations needed to damage platelets. In vivo pneumococci infections will last for hours or days and likely rather low

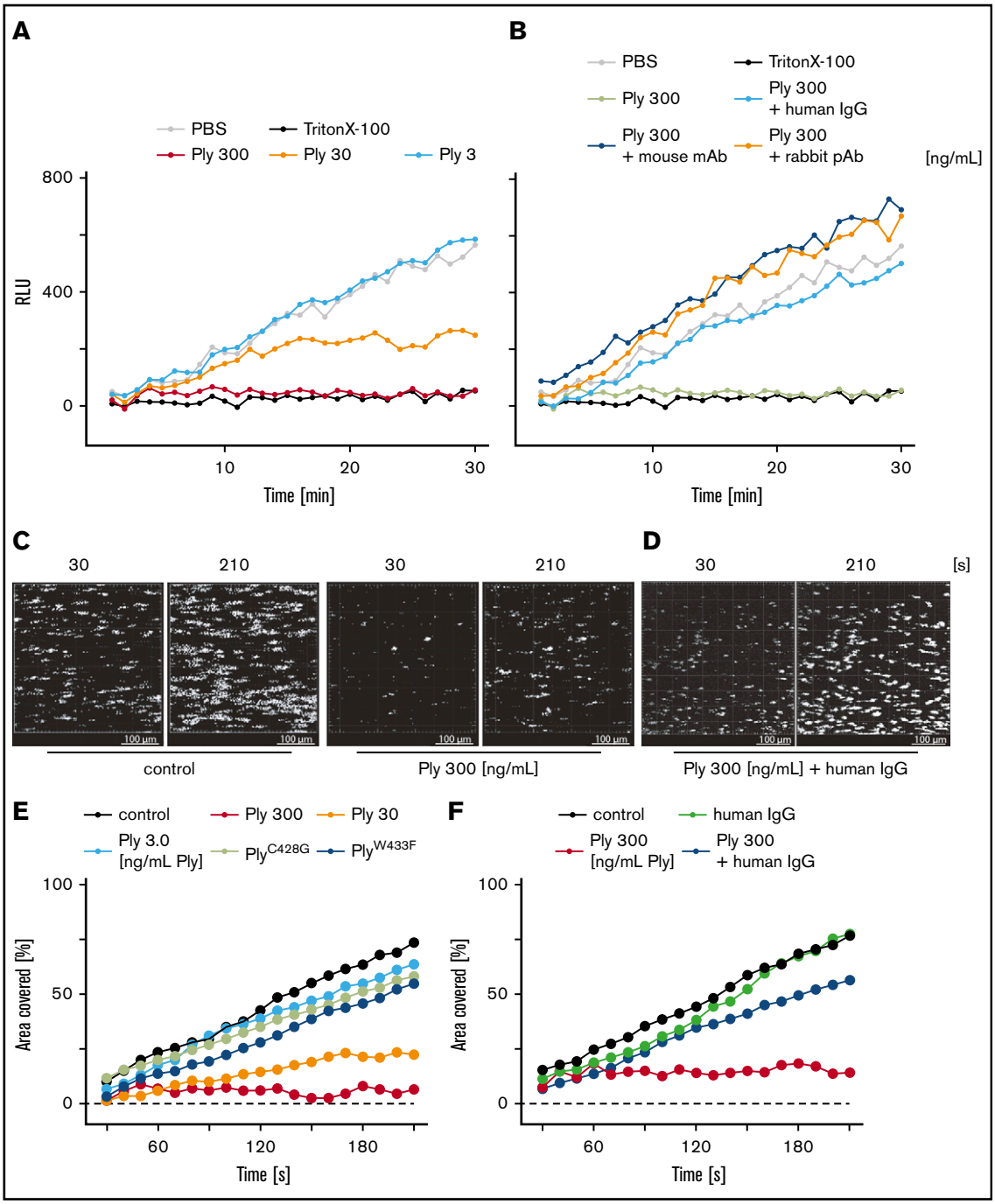

Figure 4. Pneumolysin induces platelet death. (A) Kinetics of platelet viability. PBS was used as viability control and Triton X-100 to induce platelet death. Pneumolysin in increasing concentrations induced platelet death measured by reduced substrate turnover. (B) Platelet viability was maintained in the presence of polyvalent human immunoglobulin (human Ig [Privigen]; 1 mg/mL), polyclonal rabbit anti-pneumolysin (rabbit pAb; 10 µg/mL), or a monoclonal mouse anti-pneumolysin antibody (mouse mAb; 7.5 µg/mL) despite a high concentration of pneumolysin (300 ng/mL). (C) Thrombus formation on collagen in a flow chamber in the absence of pneumolysin was monitored by image acquisition at an interval of 10 seconds by fluorescence microscopy at a shear stress of 1000 s^{-1}. In the presence of pneumolysin, thrombus formation was impaired. (D) Thrombus formation in the presence of pneumolysin was restored by polyvalent immunoglobulin. Human Ig (Privigen) alone had no effect on thrombus formation (supplemental Figure 10). (E) Quantification of the percentage of surface area covered over time by thrombi in the presence of pneumolysin in different concentrations or nonactive pneumolysin mutants. Different concentrations of pneumolysin (Ply) are color coded: 300 ng/mL (red); 30 ng/mL (orange); 3.0 ng/mL (green). Pneumolysin^{C428G} without cytolytic activity (brown); pneumolysin^{W433F} with ~10% cytolytic activity (blue). (F) Quantification of the effect of polyvalent immunoglobulin (human Ig (Privigen); 1 mg/mL) on restoring thrombus formation in the presence of pneumolysin (300 ng/mL). RLU, relative luminescence unit.

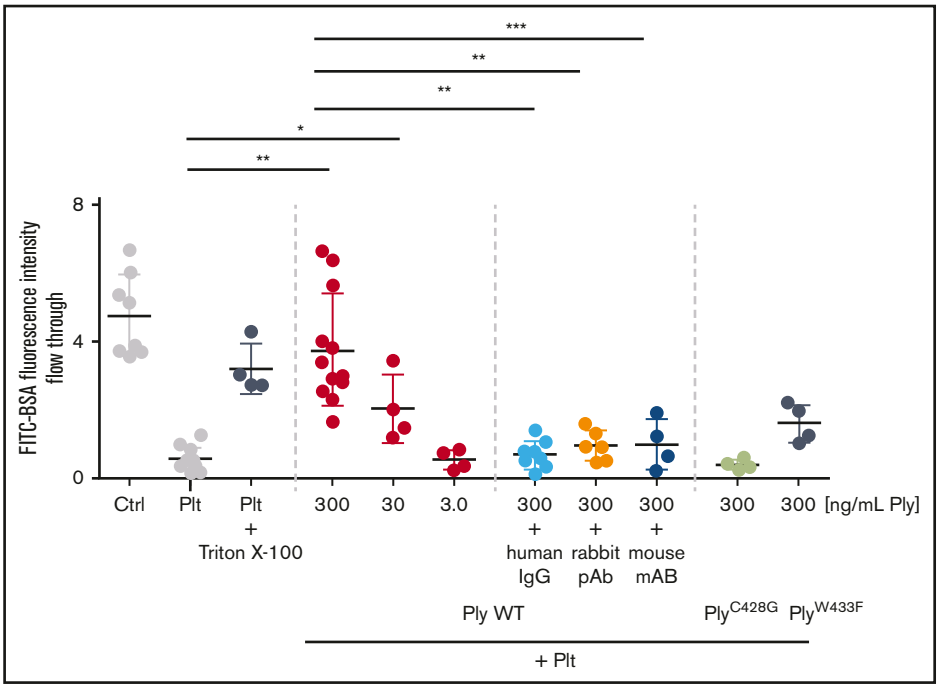

Figure 5. Sealing of Transwell membranes by platelets is impaired by pneumolysin. Platelets seal Transwell membranes with 3-μm pores. This was impaired in the presence of pneumolysin but not the pneumolysin mutants as indicated by the flow through of BSA-FITC to the lower chamber measured by fluorescence intensity (to enable comparison with the experiments without immunoglobulins, the data are shown here, although they are presented in the text at the end of "Results"). *$P < .05$; **$P < .01$; ***$P < .001$.

concentrations of pneumolysin can induce cell damage in the microenvironment.

We tried to exclude potential artifacts by showing that the increase in light transmission aggregometry was not inhibited by RGDS, performing experiments using washed platelets, platelets in plasma, or platelets in whole blood. All experiments showed consistent results. In this regard, an interesting question is why the many red cells in whole blood do not protect platelets from binding pneumolysin. The concentration of 30 ng/mL pneumolysin at which we observed impairment of thrombus formation in whole blood was the same concentration at which we observed inhibition of platelet function, increased staining of CD62P, or impaired sealing of holes in the Boyden chamber in the absence of red cells. We would have expected that the many red cells outnumbering platelets at least 10 times would quench the effect of pneumolysin on platelets.

It is known that Ca^{2+} is required to protect the cell membranes from damage by pneumolysin.[18] We therefore added Ca^{2+} in all experiments, but the ones in which we measured calcium release

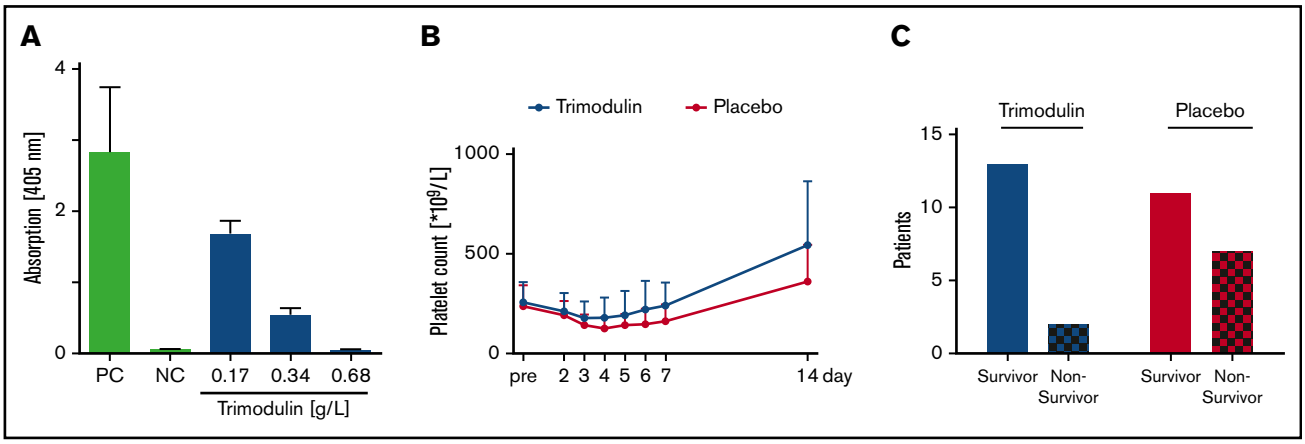

Figure 6. Neutralizaton of pneumolysin by trimodulin and relevance for severe community-acquired pneumonia patients. (A) In vitro pneumolysin neutralization assay measuring free hemoglobin as marker for cell lysis, using erythrocytes and different concentrations of trimodulin as indicated. Shown are results from 3 repeated measurements (mean plus or minus standard deviation.). (B) Platelet counts of patients in the CIGMA study with confirmed *S pneumoniae* infection were obtained before (pre), during (days 2-5), and after (days 6, 7, and 14) treatment with trimodulin or placebo. Pretreatment values were obtained from n = 15 (trimodulin group) and n = 18 (placebo group) and day 14 values obtained from n = 11 (trimodulin group, 1 missing value) and n = 12 patients (placebo group), respectively. (C) The 28-day mortality rate in patients with severe, confirmed *S pneumoniae* lung infection (n = 15 in trimodulin group, n = 18 in placebo group) was nominally lower in the trimodulin group compared with the placebo group (2 of 15 [13.3%] vs 7 of 18 [38.9%]). Due to small patient numbers, no statistical analysis has been performed. NC, negative control (PBS); PC, positive control (1% Triton X-100).

from platelets, because in the latter setting calcium in the buffer would cause artifacts.

Our results are likely clinically relevant. Here, we show that antibodies targeting pneumolysin can inhibit lysis and the loss of platelet function. With pharmaceutical immunoglobulin preparations approved for human use, a ready-to-use intervention is available to interfere with the platelet-damaging effect of pneumococci in patients with acute pneumonia. This may reduce the severity of acute respiratory distress syndrome in these patients. The standard dose of pharmaceutical human IgG is 1 g/kg body weight at 2 consecutive days. Pharmacokinetic studies of pharmaceutical human IgG given in this dose in patients with autoimmune disease show an increase in IgG even 2 weeks after infusion of 7.8 g/L (±5.6 g/L).[23]

This concept is supported by a post hoc analysis of the CIGMA trial. Although patient numbers with confirmed *S pneumoniae* infections in this trial are small (15 in the trimodulin vs 18 in the placebo group), the recovery of platelet numbers indicates a potential in vivo effect of immunoglobulins in *S pneumoniae*–induced pneumonia. However, confirmation of such clinical effects requires larger prospective randomized trials. In such a trial especially, the impact of trimodulin on mortality should be further assessed because we did see a nominally lower mortality in the trimodulin-treated group. In addition, larger patient numbers may also allow us to show the effect of pharmaceutical immunoglobulins at low platelet count levels. In the post hoc analysis, a significant difference between groups was only seen when platelet counts had already recovered to normal levels (544 000 vs 361 000 platelets per microliter). Trimodulin consisting of ~56% IgG, 23% IgM, and 21% IgA is dosed with a total dose of 0.9 g/kg body weight. Pharmacokinetic analysis from phase 1 testing in 6 healthy volunteers dosed with the same protocol as used in the CIGMA trial demonstrate a maximum plasma concentration of 6 g/L IgG, 1.7 g/L IgM, and 1.5 g/L IgA (S.W. and J.S., Biotest, unpublished data) and therefore plasma levels of IgG achieved by the standard treatment are above the pharmaceutical human IgG concentrations tested in our in vitro experiments.

Which additional role the IgM and IgA components might play in the context of pneumococcal pneumonia is not clear. Possibly, pneumolysin is neutralized by these secretory immunoglobulins already at the primary site of infection in the alveoli before causing larger platelet damage and entering the bloodstream. Previous studies with pharmaceutical human IgG and another IgM/IgA-enriched immunoglobulin preparation showed similar effects on rapid replenishment of platelets in patients and animal models with severe infections.[24-26] Interestingly, platelet numbers in these publications were replenished faster in patients or animals when treated with IgM/IgA-enriched preparations or pharmaceutical human IgG compared with controls.[25,26]

Although our in vitro and in vivo findings require analyses in larger randomized trials, our findings provide a rationale for targeting pneumolysin by use of polyvalent immunoglobulin preparations in severe community-acquired pneumococcal pneumonia to counteract the risk of these patients to become ventilation dependent.

Acknowledgments

The authors thank Marcus Gutscher, Dennis Riehl, and Katharina Heim for trimodulin assay development, and Alexander Staus for biostatistical analyses of the CIGMA trial at Biotest AG. They are further grateful to Katharina Passvogel, Ina Schleicher, Kristine Sievert-Giermann, and Gerhard Burchhardt for technical support.

This work was supported by the Deutsche Forschungsgemeinschaft (DFG [German Research Foundation] grant number 374031971–TRR 240).

Authorship

Contribution: K.J. performed flow cytometry and cell-viability experiments, contributed to electron microscopy, evaluated the data, prepared the figures and wrote the manuscript; S. Handtke performed platelet-function studies, evaluated the data, prepared figures, and edited the manuscript; R.P. designed and performed flow chamber experiments and platelet confocal microscopy, evaluated the data, prepared figures, and edited the manuscript; S.W., C.H., and J.S. contributed the data on trimodulin and CIGMA studies and edited the manuscript; G.N. and M. Witzenrath contributed to the conceptual design of the study and edited the manuscript; T.P.K. contributed to the flow cytometry experiments and preparation of electron microscopy samples, designed experiments, and edited the manuscript; J.W. contributed to flow cytometry experiments, platelet-function studies, managed healthy donors, and edited the manuscript; M.R. performed the electron microscopy, prepared the figures, and edited the manuscript; A.F.A. and M. Wolff performed the pneumococci-platelet interaction studies and edited the manuscript; S. Hammerschmidt and A.G. designed the project, were responsible for the funding of the project, supervised the project, evaluated the data, and wrote and edited the manuscript; and all authors reviewed the final version of the manuscript

Conflict-of-interest disclosure: A.G. reports grants and nonfinancial support from Aspen, Boehringer Ingelheim, Merck Sharp & Dohme (MSD), Bristol Myers Squibb (BMS), Bayer Healthcare, and Instrumentation Laboratory; personal fees from Aspen, MSD, Macopharma, BMS, Chromatec, and Instrumentation Laboratory; and nonfinancial support from Portola, Ergomed, and Biokit, outside of the submitted work. Charité (G.N. and M. Witzenrath) receives funding for research from Biotest AG. C.H., S.W., and J.S. are employees of Biotest AG. The remaining authors declare no competing financial interests.

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Received: 17 November 2021 | Accepted: 14 March 2022

DOI: 10.1111/jth.15703

ORIGINAL ARTICLE

α-hemolysin of *Staphylococcus aureus* impairs thrombus formation

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Funding information
This work was supported by the Deutsche Forschungsgemeinschaft (DFG, German Research Foundation; grant number 374031971-TRR 240 to A.G. and S.v.H.) and partially by the TR156 grant number 246807620 to C.W. The work also partially supported by infrastructural funding from the DFG Cluster of Excellence EXC 2124 "Controlling Microbes to Fight Infections."

Abstract

Background: Toxins are key virulence determinants of pathogens and can impair the function of host immune cells, including platelets. Insights into pathogen toxin interference with platelets will be pivotal to improve treatment of patients with bacterial bloodstream infections.

Materials and Methods: In this study, we deciphered the effects of *Staphylococcus aureus* toxins α-hemolysin, LukAB, LukDE, and LukSF on human platelets and compared the effects with the pore forming toxin pneumolysin of *Streptococcus pneumoniae*. Activation of platelets and loss of platelet function were investigated by flow cytometry, aggregometry, platelet viability, fluorescence microscopy, and intracellular calcium release. Thrombus formation was assessed in whole blood.

Results: α-hemolysin (Hla) is known to be a pore-forming toxin. Hla-induced calcium influx initially activates platelets as indicated by CD62P and αIIbβ3 integrin activation, but also induces finally alterations in the phenotype of platelets. In contrast to Hla and pneumolysin, *S. aureus* bicomponent pore-forming leukocidins LukAB, LukED, and LukSF do not bind to platelets and had no significant effect on platelet activation and viability. The presence of small amounts of Hla (0.2 μg/ml) in whole blood abrogates thrombus formation indicating that in systemic infections with *S. aureus* the stability of formed thrombi is impaired. Damage of platelets by Hla was not neutralized by intravenous immune globulins.

Manuscript Handled by: Katsue Suzuki-Inoue

Final decision: Katsue Suzuki-Inoue, 14 March 2022

Conclusion: Our findings might be of clinical relevance for *S. aureus* induced endocarditis. Stabilizing the aortic-valve thrombi by inhibiting Hla-induced impairment of platelets might reduce the risk for septic (micro-)embolization.

KEYWORDS
leukocidins, platelets, pneumolysin, *Staphylococcus aureus*, toxins, α-hemolysin

1 | INTRODUCTION

Platelets play an important role in hemostasis and vessel repair. They represent the smallest immune cells in humans and express, for example, Toll-like and complement receptors on their surface, thereby recognizing bacterial pathogens via pathogen-associated molecular patterns. Sensing of and interaction with bacteria leads to platelet activation and release of antimicrobial peptides.[1] Platelet activation can either be direct via secreted proteins or surface-associated bacterial proteins or indirect via bridging molecules of the extracellular matrix (ECM).[2-4]

Staphylococcus aureus and *Streptococcus pneumoniae* (pneumococci) are Gram-positive, facultative pathogens colonizing often asymptomatically the human upper respiratory tract. *S. aureus* is able to disseminate from the nasopharynx to other host compartments and can cause severe invasive diseases like pneumonia, infective endocarditis, and bacteremia, which can lead to organ damage and sepsis.[5,6] Similar, pneumococci can overcome the host epithelial barrier and invade deeper host compartments and enter the blood. This causes invasive diseases like pneumonia, septicemia, or meningitis. During dissemination via the bloodstream, bacteria get in close contact with circulating platelets. We and others have previously demonstrated the ability of *S. aureus* to activate platelets either directly via surface associated or secreted proteins (Eap, FLIPr, CHIPS, AtlA-1, α-hemolysin [Hla]) or indirectly, involving host ECM proteins.[3,7] Pneumococci were shown to at least indirectly activate platelets via ECM proteins.[8,9] Recently, we have shown that pneumococcal pneumolysin, a cholesterol-dependent cytolysin, does not activate but lyses platelets by oligomerization on the cell and formation of pores.[10] This may contribute to progression of pneumonia to acute respiratory distress syndrome.[10] Hla, released by *S. aureus*, is also a pore-forming toxin. Besides its role in disrupting epithelial barriers, Hla has been described to directly activate human platelets, leading to platelet aggregation.[3,11] Hla binds to the metalloprotease ADAM10, which is expressed on platelets.[12,13] In contrast to pneumolysin pores (diameter of 40–50 nm), pores formed by Hla are significantly smaller (diameter of 1–4 nm).[14] Besides Hla, *S. aureus* expresses further pore-forming toxins, the bicomponent pore-forming leukocidins LukSF, also referred to as Panton-Valentine Leukocidin (PVL), LukED, and LukAB (also known as LukGH).[15] These leukocidins multimerize after binding to the membrane of the respective target cell, which results in pore formation and finally host cell death. Neutrophils and other cells of the innate immune response have been shown to be the main targets of the Luk toxins.[15-18] So far, only indirect effects of leukocidins on platelets have been described and include the destruction of neutrophils and other leukocytes.[19] In this study, we investigated the effects of recombinant staphylococcal Hla and pore-forming leukocidins on platelet activation, aggregation, viability and clot stability and compared the results with effects caused by recombinant pneumococcal pneumolysin. Gaining further insight into how bacterial toxins interfere with platelet functions is essential to improve treatment of patients suffering from systemic bacterial infections.

ESSENTIALS

- Toxins are key virulence determinants interfering with platelet functions.
- *Staphylococcus aureus* α-hemolysin activates platelets but lyses platelets over time.
- Platelet lysis by α-hemolysin results in apoptosis, impaired thrombus formation and stability.
- Inhibiting α-hemolysin might be a relevant factor to mitigate the risk of dissemination of septic microthrombi.

2 | METHODS

2.1 | Ethics

The use of whole blood and washed platelets from healthy adult individuals was approved by the Ethics Committee of the University Medicine Greifswald (BB 044/18). All volunteers gave written informed consent in accordance with the Declaration of Helsinki. All experiments were carried out in accordance with the approved guidelines.

2.2 | Bacterial toxins

We used pneumococcal pneumolysin (Ply, 53 kDa) and *S. aureus* Hla (33 kDa) (kindly provided by Jan-Peter Hildebrandt,

University of Greifswald) recombinantly produced as described recently.[10,20] The components LukS (33 kDa) and Luk F (34 kDa) of the pore-forming bicomponent Panton-Valentine Leukocidin PVL were heterologously expressed in *Escherichia coli* BL21 pCG 94 LukS and *E coli* BL21 pCG142 LukF, respectively. To purify LukS and LukF Protino, Ni-TED 2000 columns were loaded with the *E coli* cell lysate, washed three times with 20 mM imidazole buffer and proteins were eluted with 500 mM imidazole buffer. After verification of purity by SDS-PAGE followed by Coomassie brilliant blue R-250 staining, the proteins were dialyzed against phosphate buffered saline (PBS). Luk A and Luk B were heterologously expressed and purified as described elsewhere.[21] Leukocidins E (ab190128) and D (ab190423) were purchased from Abcam (Berlin, Germany).

2.3 | Antibodies and reagents

We used the following antibodies: neutralizing mouse monoclonal anti-Hla IgG [8B7] (ab190467; Abcam, Cambridge, USA; using a rabbit red blood cell lysis assay half maximal effective concentration of ab190467 for neutralization of 0.3 μg/ml of Hla was determined to be 0.676 μg/ml), PE-Cy5-labelled monoclonal mouse anti-human CD62P, FITC-labelled mouse PAC-1 antibodies recognizing activated $\alpha_{IIb}\beta_{III}$ (CD41/CD61) (BD Bioscience, Franklin Lakes, USA), RealTime-Glo MT Cell Viability Assay (Promega, Madison, USA), FITC-labelled mouse anti-human CD42a (BD Biosciences, Franklin Lakes, USA), Alexa Fluor 647-labelled monoclonal mouse anti-human CD62P (P-Selectin) antibody (Clone AK4, BioLegend, San Diego, CA, USA), Alexa Fluor 647-labelled goat anti-mouse IgG (GAMIG AF-647) (Abcam, Cambridge) and human polyvalent immunoglobulin preparations (intravenous immunoglobulin [IVIG]; IgG-enriched Privigen; CSL Behring, Marburg, Germany). Mouse polyclonal anti-LukS and anti-LukF antibodies were generated by routine immunization of mice with heterologously expressed LukS or LukF. Female CD-1 mice (Charles River Laboratories, Sulzfeld, Germany) were immunized intraperitoneally with 100 μl of a 1:1 emulsion containing 50 μg recombinant protein LukS or LukF and incomplete Freund`s adjuvant (Sigma-Aldrich, Taufkirchen, Germany). Mice were boosted with an emulsion of protein and incomplete Freund´s adjuvant at day 14 and 28 and bled after 6 weeks. Specificity of polyclonal antibodies was verified by immunoblot analysis (data not shown). We also used the following reagents: FAM-FLICA caspase 3/7 assay kit from ImmunoChemistry (Hamburg, Germany), Thrombin (Sigma Aldrich, Darmstadt, Germany), Convulxin (Enzo Life Sciences, Lausen, Switzerland), Ionophore (Sigma Aldrich, Darmstadt, Germany), von Willebrand factor (VWF) (Merck, Darmstadt, Germany), Ristocetin (Mölab, Langenfeld, Germany), Annexin V (BioLegend, Koblenz, Germany), recombinant anti-Bcl-2 antibodies (AF647, Abcam, Berlin, Germany), and Triton -X-100 (Sigma-Aldrich, St. Louis, USA).

2.4 | Flow cytometry-based platelet activation assay, toxin treatment of platelets, and toxin neutralization

We performed all activation assays with washed platelets in Tyrode's buffer containing Ca^{2+} and Mg^{2+} with PBS using CD62P expression as an activation marker as described.[10] In platelet activation assays with toxins, we treated platelets for 4 min with 300 ng/ml of pneumolysin or 0.02, 0.2, 2.0, or 20 μg/ml of Hla, LukAB, LukED, or LukSF (for each pair, equimolar amounts of the single leukocidins were used) followed by 5 min of treatment with 20 μM TRAP-6. In neutralization experiments, we preincubated pneumolysin or Hla for 20 min at room temperature (RT) with 1 mg/ml human IVIG (pharmaceutical human IgG; Privigen; CSL Behring, Marburg, Germany) or increasing concentrations of a mouse monoclonal [8B7] antibody against Hla (ab 190467; Abcam).

We measured CD62P expression using a FACSCalibur (Becton Dickinson) flow cytometer and CellQuestPro 6.0. We then predefined by forward-sideways-scatter a platelet gate based on measurements with CD61-positive platelets and analyzed in the gated region 20 000 events for fluorescence. The value for platelet activation was calculated as the geometric mean fluorescence intensity of the gated population multiplied by the percentage of CD62P-positive labelled platelets.[10]

2.5 | Flow cytometry-based analysis of protein binding to human platelets

We incubated washed human platelets with human BD Fc Block (BD Biosciences) to prevent unspecific binding to platelet FcγRIIa; added increasing concentrations of pneumolysin, Hla, or LukSF for 10 min at 37°C, followed by fixation with PFA/PBS (pH 7.4) at a final concentration of 2% at RT for 20 min. Binding of toxins to platelets was measured using antibodies against pneumolysin (Streptavidin-Alexa Fluor 488, Dianova, Hamburg, Germany), Hla, PVL (1 h at RT), and with Alexa Fluor 488 conjugated secondary antibodies for Hla and PVL (30 min at RT); using a FACSCalibur (Becton Dickinson) flow cytometer and CellQuestPro 6.0.

2.6 | Platelet preparation, light transmission aggregometry, live/dead staining, release of intracellular calcium, immunofluorescence staining, thrombus formation assay, and Western blotting

We performed platelet preparation, light transmission aggregometry, LIVE/DEAD staining, detection of Ca^{2+} released from internal stores, immunofluorescence staining, *ex vivo* thrombus formation in whole blood under shear, and Western blotting as described.[10,22,23] Details are provided in the Supplementary material.

2.7 | Determination of apoptosis markers

We determined platelet caspase activity, expression of Bcl-2, and exposure of phosphatidylserine (PS) as apoptosis markers. Washed human platelets were incubated in a volume of 25 µl with thrombin (10 U/ml), TRAP-6 (20 µM) and convulxin (100 ng/ml), ionophore (10 µM) or VWF (20 µg/ml), and ristocetin (1.5 mg/ml) as controls as well as with increasing concentrations of pneumolysin (3.0–300 ng/ml), Hla (0.2–20 µg/ml), or PVL (0.2–20 µg/ml).

We determined caspase activity using the FAM-FLICA caspase 3/7 assay kit from ImmunoChemistry (Hamburg, Germany) according to the manufacturer's instructions. In brief, 0.8 µl of FLICA solution was added to the samples after toxin incubation and samples were then incubated for 45 min at 37°C in the dark. Afterwards, we added 100 µl of apoptosis wash buffer, incubated samples for 7 min, centrifuged (650*g*, 7 min at RT) and measured them by flow cytometry (Cytomics FC500, Beckman Coulter, USA) after resuspension in Tyrode´s buffer. To determine Bcl-2 expression, all samples were fixed with 0.5% PFA for 20 min at RT and then centrifuged (650*g*, 7 min at RT). Platelets were then permeabilized with 0.25% saponin for 30 min and stained using recombinant anti-Bcl-2 antibodies (AF647, Abcam, Berlin, Germany) for 30 min before being measured by flow cytometry. PS exposure was determined by Annexin V binding. We stained platelets with 5 µl Annexin V (BioLegend, Koblenz, Germany) in Annexin V binding buffer (BioLegend) containing 50 U/ml hirudin for 20 min (RT in the dark) and measured them by flow cytometry.

2.8 | Statistics

We performed statistical analysis using GraphPad Prism (version 5.01), unless otherwise indicated. We show the data as scatter plots and include median, minimal, and maximal values including median and interquartile range. We analyzed the data using the nonparametric Friedman test followed by a Dunn´s multiple comparison posttest. In kinetic curves, the area under the curve was calculated followed by one-way analysis of variance with Dunnett's posttest. We considered a p value <.05 to be statistically significant.

3 | RESULTS

3.1 | Pneumolysin and Hla but not PVL bind to human platelets

Binding assays showed that Hla and pneumolysin bound dose-dependently to platelets in the range of 0.02–20 µg/ml (Hla) or 0.003–3.0 µg/ml (pneumolysin), respectively (Figure 1A,B), whereas PVL (LukSF) did not (Figure 1C).

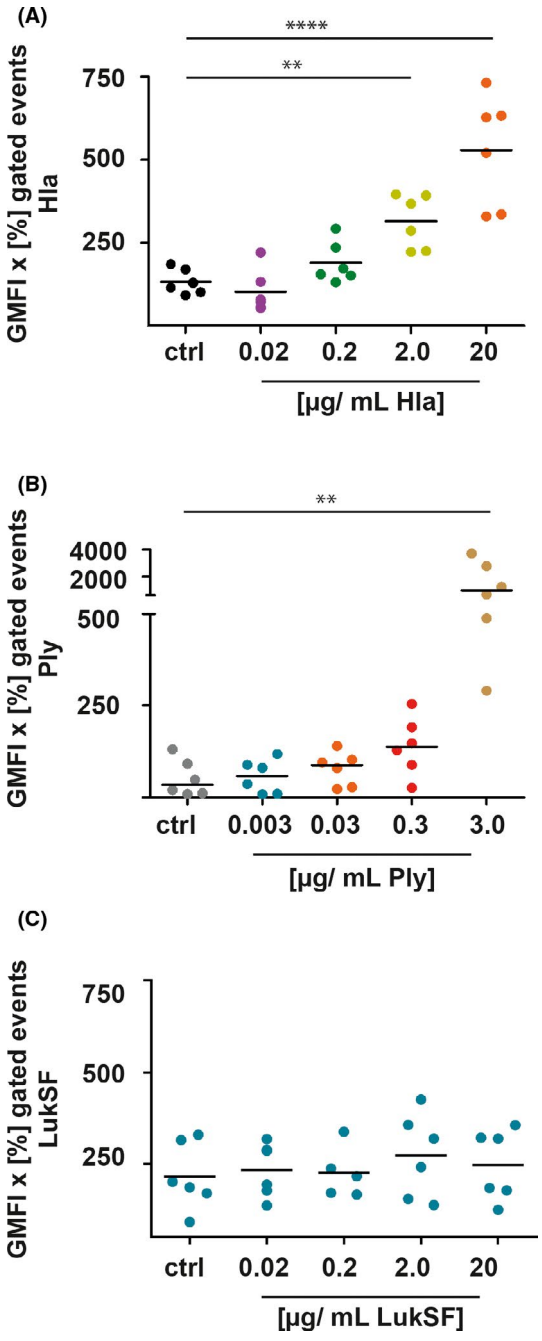

FIGURE 1 Pneumolysin and α-hemolysin bind directly to human platelets. Washed platelets of a defined set of healthy human donors were incubated with increasing concentrations of pneumolysin (Ply (0.3 to 300 ng/ml), α-hemolysin (Hla 0.02–20 µg/ml), and LukSF (PVL 0.02 to 20 µg/ml), fixed and stained with antibodies directed against the toxins (Hla, LukSF) or their Strep-tag (Ply). To exclude binding of the antibodies to the platelet Fcγ receptor, the receptor was blocked with Human BD Fc Block. Binding events were detected by flow cytometry. The data are presented as geometric mean of fluorescence intensity (GMFI) of the positive gated events multiplied with the percentage of positive gated events in the dot plots. (A) Platelets were treated with PBS (gray) or increasing concentrations of Hla for 10 min. PBS-treated platelets were used as a negative control. The staphylococcal Hla binds dose dependently to washed human platelets, starting at a concentration of 0.2 µg/ml. (B) Platelets were treated with PBS (gray) or increasing concentrations of pneumolysin for 10 min. PBS (gray) treated platelets were used as a negative control. Binding of pneumococcal pneumolysin to human platelets was detectable starting at a concentration of 30 ng/ml. (C) Platelets were treated with PBS (gray) or increasing concentrations of LukSF (PVL) for 10 min. PBS (gray)-treated platelets were used as a negative control

3.2 | Hla but not bicomponent leukocidins activate platelets

To investigate platelet activation by bacterial toxins, we treated washed human platelets with the toxins. After 10 min of incubation, Hla ≥2.0 μg/ml and Ply ≥30 ng/ml increased the CD62P signal of washed platelets. TRAP-6 stimulation did not further increase this CD62P signal. In contrast, PVL, LukAB, and LukED had no effects on platelet CD62P expression nor on platelet responsiveness to TRAP-6 stimulation (Figure 2A). Platelets incubated with >0.2 μg/ml Hla showed also an increased signal for αIIbβ3 integrin activation with reduced sensitivity to subsequent TRAP-6 stimulation (Figure 2A). Activation of platelets in the presence of Hla was not only dose- but also time-dependent. After 30 min of incubation, 0.2 μg/ml Hla were sufficient to induce increased levels of CD62P and integrin activation with responsiveness to subsequent TRAP-6 stimulation (Figure S1A and B). In addition, LukED also caused increased αIIbβ3 integrin activation at the highest tested concentration (Figure S1B).

At concentrations >0.2 μg/ml, Hla induced release of intracellular calcium (Figure 2B) and increased light transmission in the aggregometer (Figure 2C). Although the curves for Ca^{2+} release gradually increased (Figure 2B), a partly reversibly change in light transmission was observed in the aggregometer. We therefore measured the change in light transmission in the presence of RGDS, which inhibits platelet aggregation. Any change in light transmission measured in the presence of RGDS is caused by platelet lysis. Overlay of the curves reveals the following sequence of events (Figure 2C). Hla first induces platelet activation and aggregation (first peak of the curve) in parallel to calcium influx. Then platelets are destroyed by the toxin, start to disaggregate, and lysis occurs. The aggregation curve (solid line) overlays the curve of platelet lysis (dotted line) induced change of light transmission (measured in the presence of RGDS) after about 180 s for 2.0 μg/ml Hla and after about 400 s for 1.0 μg/ml Hla. Aggregate disintegration and lysis are also visible in the aggregometry cuvettes. In the presence of 20 μg/ml Hla, a turbid suspension without aggregates is visible, whereas aggregates are formed by TRAP-6-stimulated platelets (Figure 2D). LukSF, LukAB, or LukED did not induce calcium release or an increase in light transmission in the aggregometer (Figure 2B and data not shown).

3.3 | Platelets are lysed by prolonged exposure to Hla

Previously, we demonstrated that pneumolysin does not cause platelet activation but directly destroys platelets by formation of large pores (40–50 nm). The CD62P signal induced by pneumolysin results from antibody diffusion into the cytoplasm through the pores and intracellular CD62P staining instead of platelet activation.[10]

From the experiments described here, we concluded that the initial increase in CD62P and the first peak of an increase in light transmission in aggregometry of Hla-treated platelets represents platelet activation. However, like pneumolysin, Hla also forms pores in cell membranes, but the pore size is much smaller (1.5–2.0 nm) and theoretically too small for antibodies to pass through. We confirmed this by CLSM showing CD62P on the surface of platelets in response to Hla without intracellular staining (Figure 3A). In contrast, Triton X-100 (control for intracellular CD62P staining)-treated platelets were permeabilized and intracellular CD62P was stained. TRAP-6 (control for platelet membrane CD62P staining) incubated platelets showed, similar to Hla-treated platelets, CD62P on the surface (Figure 3A). However, Hla-treated platelets were enlarged and swollen compared with the TRAP-6 control, suggesting that Hla induces loss of platelet membrane integrity and subsequently loss of platelet function.

The platelet aggregometry experiment also indicated that, after initial activation, platelets are lysed. We therefore measured the viability of platelets exposed to different concentrations of toxins over 30 min. Pneumolysin was used as "cell death" control. Low concentrations of Hla (0.2 μg/ml) reduced platelet viability after 20 min. In contrast, higher Hla concentrations lysed platelets rapidly (Figure 3B). Only at ≤0.02 μg/ml Hla, platelet viability remained unaffected up to 60 min before platelet lysis occurred and RLU decreased (Figure S1B).

3.4 | Hla and pneumolysin induce apoptosis in human platelets

Because pneumolysin[10] and Hla differ in their initial effects on platelets, we asked whether these toxins differ in their capability and mechanism to induce cell death. Hla and pneumolysin strongly induced PS exposure on platelets. This signal was comparable to or even higher than the signal obtained for the positive controls ionophore and convulxin (Figure 4A). Both toxins, pneumolysin and Hla, dose-dependently increased caspase-3/7 activity (Figure 4B) in platelets, but did not increase Bcl-2 expression (Figure 4C). This suggests that both toxins induce apoptosis by activating effector caspases (Figure 4B). The toxin concentrations showing activation of cell death and apoptosis markers correspond to the concentrations inducing a loss of platelet function (Figures 2 and 3).

3.5 | Polyvalent immunoglobulin preparations did not inhibit platelet damage by Hla

Recently, we showed that IVIG or specific anti-pneumolysin antibodies prevent lysis of platelets by pneumolysin.[10] Based on these findings, we assumed that IVIG and a specific neutralizing monoclonal IgG antibody targeting Hla also have the potential to inhibit loss of platelet function and cell death. Both IVIG and a mouse anti-Hla antibody recognize purified Hla (Figure S2). However,

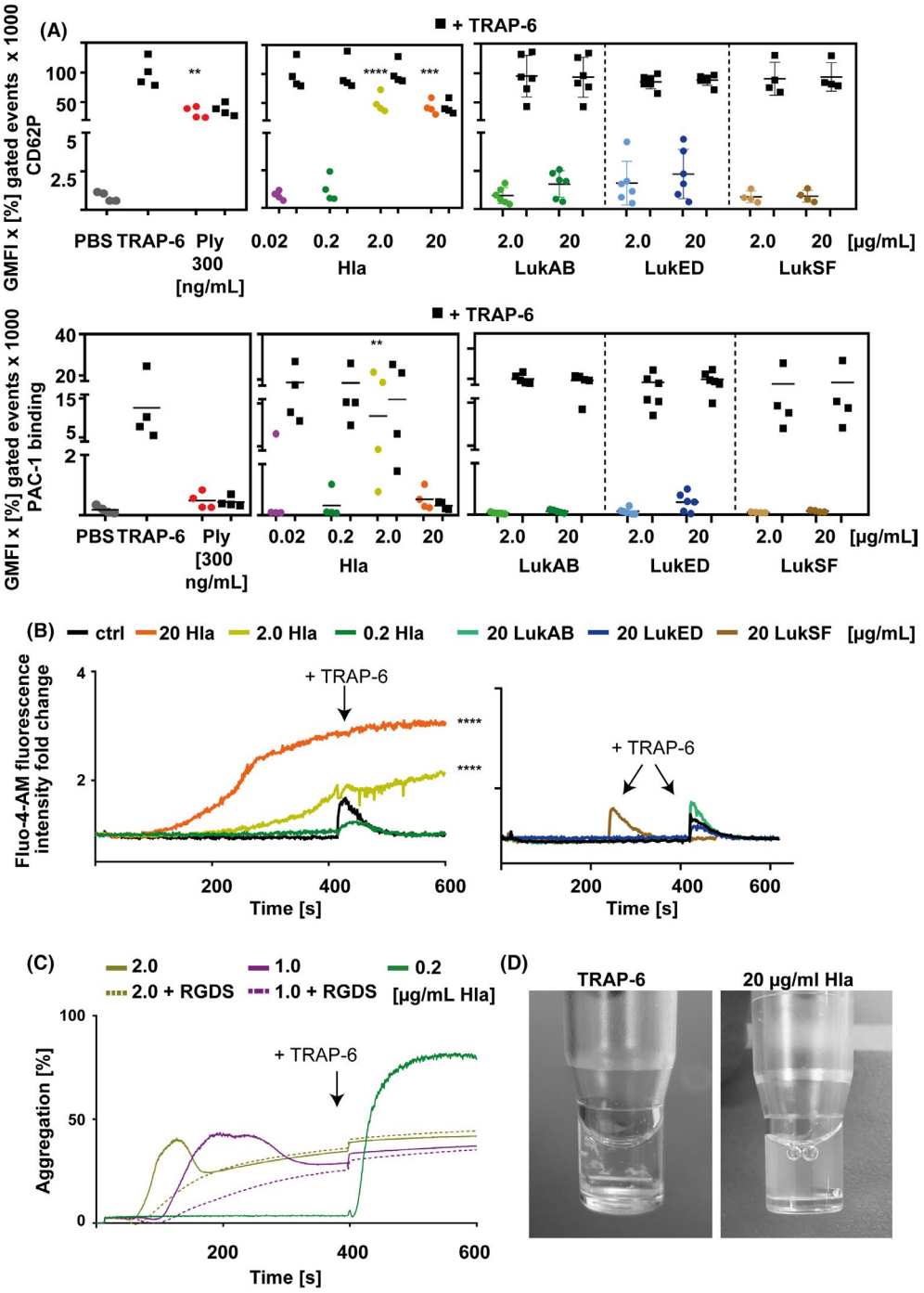

FIGURE 2 α-hemolysin and pneumolysin interfere with platelet function but with different mechanisms. Washed platelets of a defined set of healthy human donors were incubated with increasing concentrations of pneumolysin (Ply 0.3–300 ng/ml), α-hemolysin (Hla 0.02–20 g/ml) LukAB, LukED, and LukSF (PVL) for 10 min. (A) CD62P (upper panel) and PAC-1 binding (lower panel) were used as activation markers and detected by flow cytometry, using a PE-Cy5–labelled P-selectin antibody and a FITC-labelled anti-human GPIIbIIIa antibody (PAC-1). PBS was used as negative control and 20 µM TRAP-6 as a positive control. Platelets were incubated with the toxins for 10 min. Alternatively, after 5 min of incubation with the toxins, the platelets were additionally stimulated with 20 µM TRAP-6 for 5 min to proof functionality. The data are presented as geometric mean of fluorescence intensity (GMFI) of positive gated events multiplied with the percentage of positive gated events in the dot plots. (B) Before treatment with pneumolysin, Hla, LukA/B, LukD/E, or LukSF (PVL), intracellular Ca^{2+} of washed platelets was labelled with Fluo-4-AM for 30 min. After incubation with increasing concentrations of the indicated toxins, the kinetics of Ca^{2+} release were measured; values are given as fold change compared with NaCl control. (C) Platelet aggregation was measured using light transmission aggregometry. Hla concentrations ≥2.0 µg/ml induced an increase in light transmission, but platelets were no longer responsive to 20 µM TRAP-6, which was subsequently added after 6 min of incubation. (D) Visualization of aggregate formation after TRAP-6 treatment of platelets or treatment with 20 µg/ml Hla for 400 s in aggregometry cuvettes

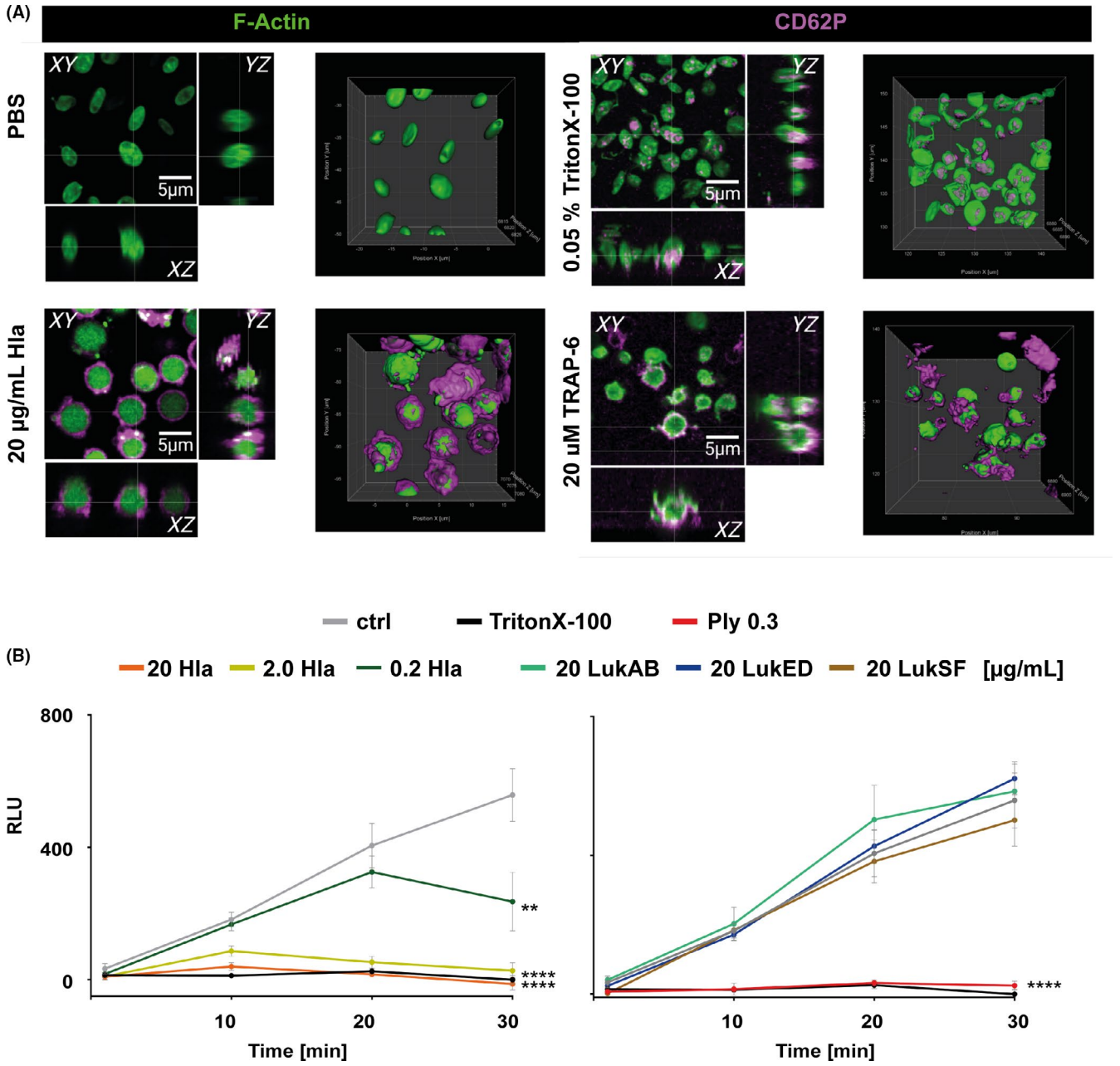

FIGURE 3 Treatment of human platelets with α-hemolysin leads to staining of surface associated CD62P (A) α-hemolysin (Hla) treated platelets were stained for F-actin (green) and CD62P (magenta). Platelets were not permeabilized. Orthogonal views of confocal Z-stacks and three-dimensional iso-surface rendering of platelets are shown. Platelets treated with 20 µg/ml Hla show distinct extracellular staining of CD62P comparable with only TRAP-6–treated platelets. TRAP-6 was used as control for surface associated CD62P and TritonX-100 as control for intracellular CD62P staining. (B) Kinetics of platelet viability measured with the RealTime-Glo MT Cell Viability Assay (Promega). PBS was used as viability control and Triton X-100 to induce platelet death. Increasing concentrations of Hla, LukA/B, LukD/E, LukSF (PVL), and 300 ng/ml pneumolysin were incubated for 30 min with washed platelets. One minute after mixing of platelets and toxins the measurement started

they neither prevent CD62P expression nor loss of platelet viability in response to Hla (Figure 5A,B; Figure S2). Only minor improving effects were observed in the presence of these antibodies. IVIG rescued the decrease in viability after 20 min of incubation with 0.2 µg/ml Hla and the specific monoclonal anti-Hla antibody showed a rescuing effect at 2.0 µg/ml Hla only at extremely high doses (500 µg/ml) (Figure 5A).

3.6 | Thrombus formation under shear is abrogated by α-hemolysin

To assess whether Hla impacts the capability of thrombus formation under shear, we next perfused whole blood in the absence or presence of Hla at different concentrations. Hla at the lowest concentration of 0.2 µg/ml significantly reduced

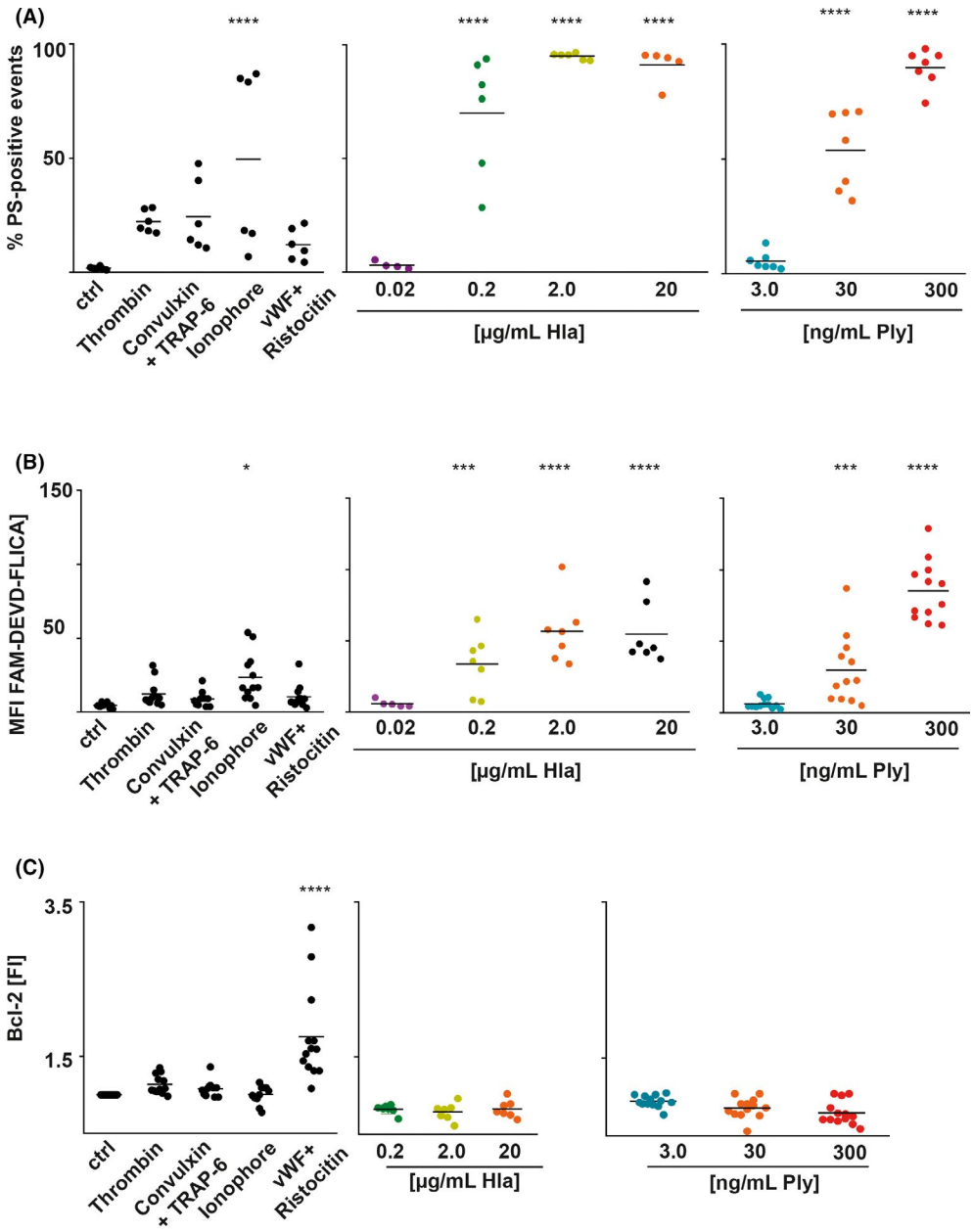

FIGURE 4 Platelets treated with α-hemolysin and pneumolysin are positive for apoptosis markers. Washed human platelets were incubated with increasing concentrations of α-hemolysin (Hla) and pneumolysin (Ply). The analysis of the apoptosis markers caspase activity, Bcl-2 expression, and phosphatidylserine (PS) exposure was measured using flow cytometry. For all experiments thrombin, convulxin/Trap-6, ionophore and VWF/ristocetin were used as positive controls. PBS was used as negative control. (A) PS exposure was determined by Annexin V binding. Values are given as percent of positive events. Treatment with Ply and Hla leads to PS exposure in a concentration-dependent manner. (B) Caspase activity was measured by fluorescent labelling of active caspase 3 and 7 in Ply- or Hla-treated human platelets. Values are given as mean fluorescent intensities and show a dose-dependent increase after pneumolysin or Hla treatment. (C) Bcl-2 expression was determined using a recombinant anti-Bcl-2 antibody. After treatment with Hla or Ply, platelets were fixed and analyzed for Bcl-2 expression using flow cytometry. Values are given as fluorescence intensities

thrombus formation and area covered by thrombi by more than 50% ($p < .001$) compared with the control (Figure 6A). Similarly, at higher concentrations (2.0 and 20 µg/ml), Hla strongly reduced the capacity of platelets to form stable thrombi. IVIG (1 mg/ml) failed to restore the ability of platelets to form stable thrombi under shear in the presence of Hla (Figure 6B).

4 | DISCUSSION

In this study, we show that the *S. aureus* toxin Hla directly activates but finally lyses platelets time and dose-dependently, whereas bi-component leukocidins have no direct effects on platelets.[15] We further indicate that Hla abrogates thrombus formation in whole blood and that Hla cannot be neutralized by IVIG.

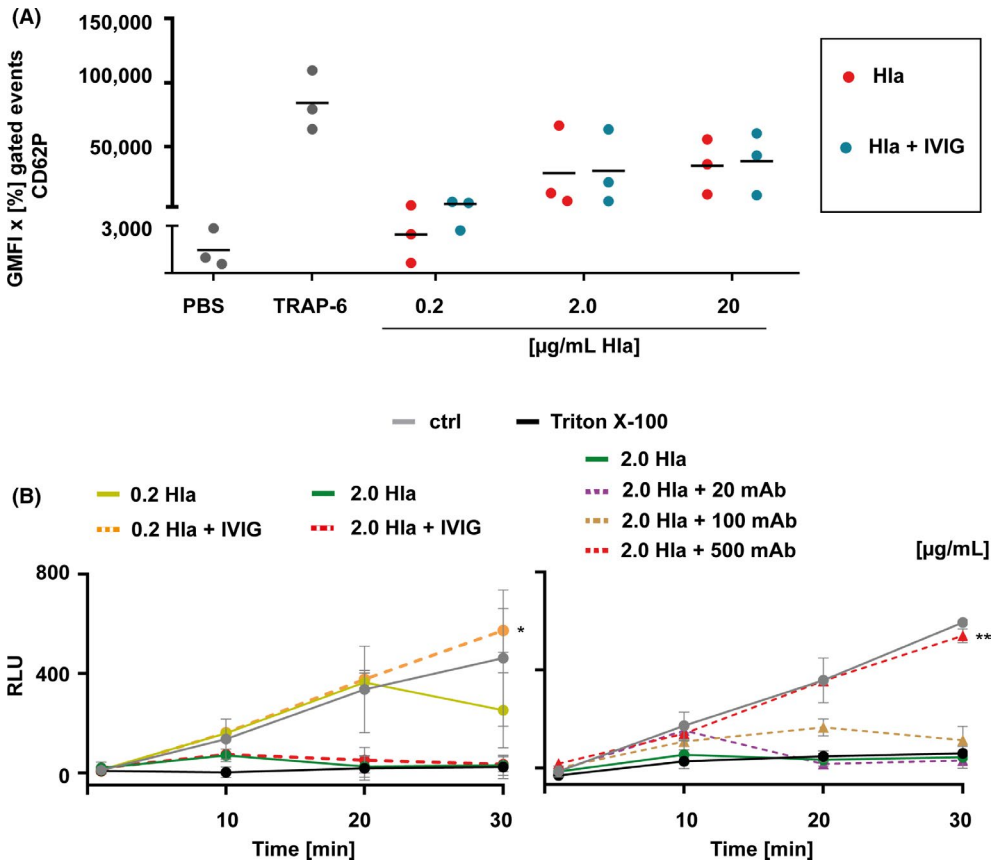

FIGURE 5 IVIG does not neutralize α-hemolysin. α-hemolysin (Hla) was treated with IVIG (1 mg/ml) or a specific mAb for 20 min before incubation with washed human platelets of healthy human donors. (A) Polyvalent human immunoglobulins (IVIG; 1 mg/ml; human IgG, Privigen) did not neutralize the increased CD62P signal after Hla treatment. The data are presented as geometric mean of fluorescence intensity (GMFI) of the positive gated events multiplied with the percentage of positive gated events in the dot plots. (B) Platelet viability was only barely improved by IVIG (1 mg/ml) or a specific monoclonal anti-Hla antibody (mAb). After treatment with 0.2 µg/ml α-hemolysin, IVIG rescued the decreasing platelet viability over time, and 500 µg/ml of the anti-Hla antibodies rescued platelet viability

Our binding assays demonstrate that Hla binds directly to platelets, whereas the bicomponent toxin LukSF (PVL) does not interact directly with platelets. LukSF is known to bind to complement receptors C5aR1 and C5aR2 on leukocytes.[15,24] Lack of platelet stimulation via LukSF can be explained by the lack of C5a receptor (C5aR) exposed on the platelet surface. This is supported by a recent study showing a C5aR transcript in platelets but the receptor protein was not detected by proteomics.[25] Similarly, LukED interacts with receptors CCR5, CXCR1, and CXCR2.[19,26,27] Also, the transcripts for CCR5, CXCR1, and CXCR2 were identified in platelets, whereas the protein was absent.[25] LukAB had also no effect on platelet activation and aggregation in our study. LukAB binds to CD11b,[28] which is not expressed by platelets. However, supernatants of neutrophils incubated with staphylococcal pore-forming leukocidins induce platelet activation and aggregation.[19] Our platelet-binding and activation data support the concept, that *S. aureus* bicomponent leukocidins only indirectly activate platelets via leukocyte activation.[19] The data also support that these toxins are highly receptor dependent and that the cognate receptors are not expressed on platelets obtained from healthy donors.

Staphylococcus aureus Hla forms pores of 1.5–2.0 nm diameters and is a major virulence determinant for staphylococcal infections.[20,29,30] Hla promotes blood coagulation via activation of human platelets. This phenomenon is independent of platelet lysis,[31-33] and consistent with the strong procoagulatory PS exposure on the platelet membrane induced by Hla. *In vivo*, intravenous injection of Hla in mice induced platelet aggregation and formation of microthrombi. The aggregates are retained in the liver sinusoids and kidney glomeruli, thereby causing multiorgan dysfunction.[11]

Our studies suggest that Hla acts in two steps on platelets. Hla induces calcium influx and initial platelet activation and aggregation, which is probably because of formation of small Hla pores on the platelet surface. Evidence for platelet activation is surface-exposed CD62P, αIIbβ3 integrin activation, and the ability of RGDS to block the initial aggregation peak. However, over time, platelets are finally lysed and thrombus formation in whole blood is abrogated. We hypothesize that this is caused by increasing pore formation. This explains, why Hla induced platelet activation and cell death are time and concentration dependent. Higher Hla concentrations (≥2.0 µg/ml Hla) strongly increased CD62P expression, abrogated sensitivity

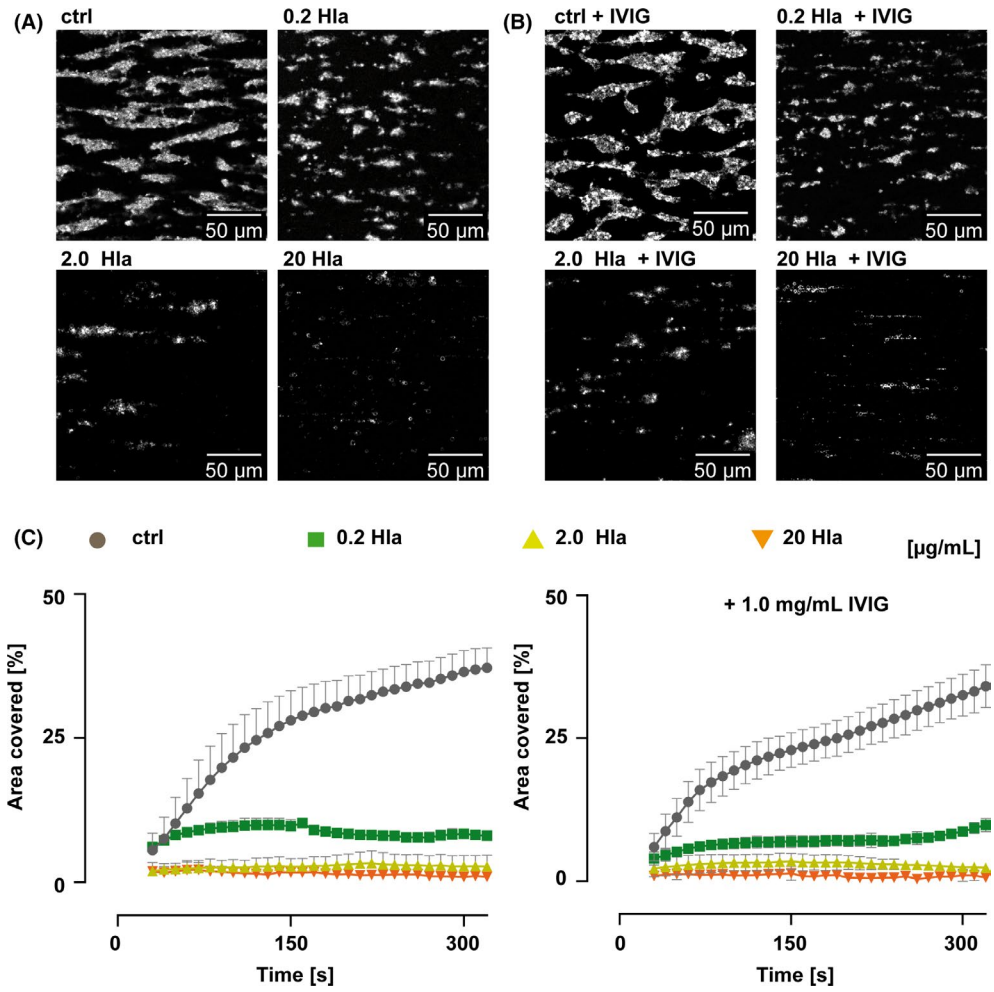

FIGURE 6 α-hemolysin induces abrogation of thrombus formation in whole blood under shear. Whole blood incubated with α-hemolysin (Hla) at 0.2, 2.0, and 20 µg/ml Hla was perfused over a collagen (shear 1000 s^{-1})-coated surface and thrombus formation was visualized by immunostaining of platelets with fluorescently labelled anti-CD61antibody. (A) Representative inverted grayscale images of the impact of Hla compared with the nontreated control show that Hla strongly inhibits thrombus formation under shear. (B) In the presence of IVIG (1 mg/ml), thrombus formation under shear is not restored. (C) Area covered by thrombi under shear in the presence of increasing concentrations of Hla in comparison to controls and after treatment with IVIG. The data show percentage of area covered by thrombi computed from three different regions of interest from n = 3 donors. Statistical analysis was performed with one-way ANOVA with Sidak multiple correction. $p < .05$ was considered significant.

to TRAP-6 stimulation and lysed platelets within 2–7 min, resulting in a total loss of platelet function. With these doses membrane disintegration by Hla also disturbed integrin activation, which was only observed at intermediate doses (2.0 µg/ml). Lower concentrations of Hla (≤0.02 µg/ml) lysed platelets earliest after 20 min of incubation. In contrast, pneumolysin forms large pores in the platelet membrane and thereby lyses platelets immediately without previous activation, even at very low concentrations.[10] Our data on diminished thrombus formation as well as only initial platelet aggregation in the presence of Hla contradict existing reports demonstrating that Hla induced platelet aggregation but not lysis. However, these studies were performed in plasma[31,34] or whole blood,[11] whereas anti-hemolysin antibodies might have blocked some of the effects.

We used much lower Hla concentrations (maximum of 0.56 µM = 20 µg/ml) compared with the Hla concentrations found in patient sera (up to 60 µM).[33] Although platelet activation by various *S. aureus* proteins like Clumping factor A (ClfA), SdrE, AtlA1, CHIPS, FLIPr, and Eap including Hla is well accepted, the consequences of platelet lysis by *S. aureus* has gained less attention.[3,35] Taken together, platelet activation by *S. aureus* is induced by the direct interplay of intermediate doses of released Hla as well as the presence of activating *S. aureus* surface proteins.

However, the role of platelet lysis and thrombus destabilization we observed with recombinant Hla *in vitro* requires further *in vivo* studies. Lysis of platelets might be clinically highly relevant. One of the most feared infections of *S. aureus* is endocarditis. The biggest risk in acute endocarditis is septic thrombi causing multiple occlusions of small arteries, especially in the brain. In this regard, our finding that Hla destabilizes thrombi has major implications. Based on our data, it can be hypothesized that thrombus stabilization by inhibition of Hla might reduce the risk of microthrombi dissemination from the infected aortic valve in *S. aureus*–induced endocarditis.

Next, we addressed the question of whether platelet lysis by Hla can be inhibited. Most individuals have anti-Hla IgG antibodies in their plasma. We therefore tested the potential neutralizing effect of the pharmaceutic immunoglobulin preparation IVIG on Hla, which, however, did not sufficiently abrogate platelet lysis by Hla. Besides IVIG, anti-Hla monoclonal antibodies might be an option. Although the monoclonal antibody tested in this study had nearly no effect on Hla-induced lysis of platelets, a humanized Hla neutralizing antibody (MEDI4893*)[36] inhibited organ damage in *S. aureus* sepsis in animal models[11] and is well tolerated in humans.[35] The antibody was not effective in preventing *S. aureus*–induced pneumonia in intensive care patients,[37] but its effects on thrombus stabilization has not been assessed before now.

The receptor for Hla on platelets is the widely expressed metalloprotease ADAM10.[13,25] Depletion of this receptor has been shown to prevent Hla-induced cellular damage and dysfunction.[38] Furthermore, inhibition of ADAM10 was shown to attenuate vascular injury during sepsis in mice.[39,40] However, because of incomplete mechanistic understanding of the regulation of metalloproteases, clinical trials with metalloprotease inhibitors have failed up to now.[41]

Finally, we addressed how Hla causes platelet death. Hla strongly increases caspase 3/7 activity, indicating apoptotic cell death. Bcl-2 as an antiapoptotic signal inhibiting caspase activity was not increased; however, other cell death mechanisms like necrosis could be involved. Platelets, which appear procoagulant, have morphological features like other nucleated mammalian cells undergoing necrosis such as permeabilization, ballooning, ruptured cytoskeleton, and PS exposure.[42,43] Because Hla itself disintegrates platelet membranes, direct measurement of permeabilization is not expedient.[42] Also, measurement of mitochondrial membrane potential or thrombin generation would not directly link the observed phenotype with necrosis. Future studies should address whether other cell death mechanisms are also involved, such as ferroptosis or necroptosis.

Taken together, we demonstrate that *S. aureus* Hla but not leukocidins interplay with platelets. Hla initially activates platelets as shown by induction of aggregation and increase in activation markers followed by rapid platelet lysis. Platelets undergo apoptosis, which leads to thrombocytopenia and impairment of thrombus stability. Inhibiting Hla might be a relevant factor to mitigate the risk of dissemination of septic microthrombi in *S. aureus* endocarditis.

ACKNOWLEDGMENT

The authors thank Peggy Stremlow (University of Greifswald) for technical support.

CONFLICT OF INTEREST

Andreas Greinacher reports grants and nonfinancial support from Aspen, Boehringer Ingelheim, MSD, Bristol Myers Squibb (BMS), Bayer Healthcare, and Instrumentation Laboratory; personal fees from Aspen, MSD, Macopharma, BMS, Chromatec, and Instrumentation Laboratory; and nonfinancial support from Portola, Ergomed, and Biokit outside the submitted work. All the other authors declare no conflict of interest.

AUTHOR CONTRIBUTIONS

Kristin Jahn performed binding experiments, flow cytometry and cell viability experiments, evaluated the data, prepared the figures and wrote the manuscript. Stefan Handtke performed calcium assays, aggregometry, and evaluated the data, prepared figures, and edited the manuscript. Raghavendra Palankar designed and performed platelet confocal microscopy, evaluated the data, prepared the figures, and edited the manuscript. Thomas P. Kohler contributed to the flow cytometry experiments, designed experiments, and edited the manuscript. Jan Wesche contributed to flow cytometry experiments, platelet function studies, managed healthy donors, and edited the manuscript. Martina Wolff performed apoptosis experiments. Janina Bayer purified leukocidins LuSF and LukAB. Christiane Wolz purified leukocidins LuSF and LukAB. Andreas Greinacher designed the project, funding of the project, supervised the project, evaluated the data, and wrote and edited the manuscript. Sven Hammerschmidt designed the project, funding of the project, supervised the project, evaluated the data, and wrote and edited the manuscript. All authors reviewed the final version of the manuscript.

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SUPPORTING INFORMATION

Additional supporting information may be found in the online version of the article at the publisher's website.

How to cite this article: Jahn K, Handtke S, Palankar R, et al. α-hemolysin of *Staphylococcus aureus* impairs thrombus formation. *J Thromb Haemost*. 2022;00:1–12. doi:10.1111/jth.15703