Interplay between platelets and pathogenic bacteria

Inauguraldissertation

zur

Erlangung des akademischen Grades eines
Doktors der Naturwissenschaften (Dr. rer. nat.)
der
Mathematisch-Naturwissenschaftlichen Fakultät
der
Universität Greifswald

vorgelegt von
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Greifswald, 24.05.2022

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Tag der Promotion: 02.09.2022

Alice: “But I don´t want to go among mad people.”

Cat: “Oh, you can´t help, we´re all mad here, I´m mad.
You´re mad.”

Alice: “How do you know I´m mad?”

Cat: “You must be, or you wouldn´t have come here.”

Lewis Carrol, *Alice´s Adventures in Wonderland*

Content

I

SUMMARY – ZUSAMMENFASSUNG

Summary

Streptococcus pneumoniae (*S. pneumoniae*, pneumococci) and *Staphylococcus aureus* (*S. aureus*) belong to the Gram-positive, facultative pathogenic bacteria. They are typical commensals of the human upper respiratory tract and most people get colonized at least once during their life. Nevertheless, these potentially pathogenic bacteria are able to spread from the site of colonization to invade into deeper tissues and the blood circulation. Thereby, severe local and invasive infections like bacteremia and life-threatening sepsis can be caused. Once reaching the bloodstream, bacteria get in contact with platelets. Platelets are small, anucleated cells and the second most abundant cell type in the circulation. The role of platelets in hemostasis is well known. Circulating resting platelets sense vessel injury independent of its cause. Platelets bind to injured endothelium and exposed molecules of the underlying extracellular matrix, get activated and release intracellular adhesion proteins and different modulatory molecules. This in turn initiates activation and binding of nearby platelets resulting in closure of vascular injury by formation of small thrombi. Despite being pivotal in maintenance of the endothelial barrier they got increasingly recognized as cells with important immune functions. Platelets excert functions of the immune response by either, i) interacting with immune cells of different pathways of the immune response, ii) releasing immunomodulatory molecules stored in their granules or iii) interacting with invading pathogens via direct or indirect binding.

The basis for this study were results demonstrating direct binding of different *S. aureus* proteins to platelets resulting in platelet activation. The identified proteins in the mentioned study are the *S. aureus* proteins Eap, AtlA-1, CHIPS and FlipR. Severe invasive infections with *S. pneumoniae* are quite often associated with development of thrombocytopenia or disseminated vascular dissemination. This frequent observation hints towards either a direct or indirect interplay of platelets with pneumococci. Hence, this study aims to analyze potential interactions and aims to decipher involved factors on both the platelet- and bacterial site.

A screening of recombinant pneumococcal surface proteins identified proteins belonging to the group of lipoproteins, sortase-anchored proteins and choline-binding proteins to directly activate human platelets. Besides these surface proteins also the intracellular pneumococcal pneumolysin (Ply) induced highly increased values for the

platelet activation marker P-selectin. Since Ply is a major virulence factor of *S. pneumoniae* the primary focus was set on involvement of this pore forming toxin on platelet activation. Surprisingly, our data revealed Ply induced platelet activation to be a false positive result based on formation of large Ply pores in the platelet membrane. In fact, it was clearly demonstrated that Ply lyses platelets even at low concentrations and thereby rendering them non-functional. Lysis of platelets could be inhibited by the addition of pharmaceutical immunoglobulin preparations as well as antibodies specifically targeting Ply. Inhibition of Ply also resulted in fully rescued platelet function either in washed platelets or in whole blood as shown by thrombus formation. Next to pneumococci also *S. aureus* expresses pore forming toxins, namely α-hemolysin (Hla) and different pairs of bicomponent pore forming leukocidins. Whereas the different tested leukocidins did not affect platelets, Hla acted in a two-step mechanism on human platelets. The results confirm previous data on Hla induced platelet activation via Hla resulting in e.g., reversible platelet aggregation or surface expression of activation markers. Nevertheless, platelet activation by Hla is followed by dose- and time-dependent lysis of platelets resulting in loss of platelet function and abrogated thrombus formation. Platelet lysis by Hla could neither be rescued with specific monoclonal anti-Hla antibodies nor with pharmaceutical IgG preparations containing anti-Hla IgGs. Taken together, the presented data reveal new pathomechanisms involving disturbance of platelets by bacterial pore forming toxins. Platelet lysis as well as impaired platelet function play an important role in development of severe complications during invasive infections. In life threatening infections caused by *S. pneumoniae* the usage of antibody formulations containing antibodies targeting Ply might be a promising approach for the prevention or even intervention and improvement of clinical outcome.

Zusammenfassung

Streptococcus pneumoniae (*S. pneumoniae*, Pneumokokken) und *Staphylococcus aureus* (*S. aureus*) gehören beide zu Gram-positiven, fakultativ pathogenen Bakterien. Sie sind typische Kommensale der oberen Atemwege und die meisten Menschen werden im Laufe ihres Lebens mindestens einmal durch Pneumokokken oder Staphylokokken kolonisiert. Abgesehen von der meist asymptomatischen bakteriellen Kolonisierung können sich die Bakterien vom Ort der primären Besiedlung ausbreiten und dabei in tieferliegende Gewebe und die Blutzirkulation vordringen. In Folge dessen kann es zu lokalen und invasiven Infektionen bis hin zu lebensbedrohlichen Zuständen wie Bakteriämie und Sepsis kommen. Sobald Bakterien im Blutkreislauf sind, kommen sie in Kontakt mit zirkulierenden Blutzellen. Thrombozyten (Blutplättchen) sind kleine, kernlose Zellen und nach Erythrozyten der zweithäufigste Zelltyp im Blutkreislauf. Sie spielen eine entscheidende Rolle in der Blutgerinnung und damit dem Verschluss verletzter Blutgefäße. Thrombozyten zirkulieren in nicht-aktivierter Form mit dem Blutstrom und erkennen Gefäßverletzungen unabhängig von deren Ursache. Sie binden an das verletzte Endothel sowie an freiliegende Moleküle der darunterliegenden extrazellulären Matrix, werden aktiviert und schütten intrazelluläre Proteine und Moleküle aus den Granula aus. Dies bewirkt die Aktivierung von weiteren Thrombozyten im Bereich der Verletzung, die sich wiederum an das Endothel anlagern und mit anderen Thrombozyten aggregieren. Die Bildung kleiner Thromben führt in der Folge zum Verschluss der Gefäßverletzung. Neben ihrer zentralen Rolle in der Blutgerinnung wurden sie in den letzten Jahren zunehmend als Zellen mit wichtigen Immunfunktionen beschrieben. Thrombozyten üben Funktionen der Immunantwort aus, indem sie entweder i) mit verschiedenen Immunzellen interagieren, ii) immunmodulatorische Moleküle freisetzen, die in ihren Granula gespeichert sind oder iii) durch direkte oder indirekte Bindung mit eindringenden Pathogenen interagieren. Die Grundlage für die vorliegende Arbeit war eine vorangegangene Studie, die eine direkte Bindung verschiedener *S. aureus* Proteine an Thrombozyten nachweisen konnte. Die Interaktion von Thrombozyten mit den *S. aureus* Proteinen Eap, AtlA-1, CHIPS und FLIPr führte zu Thrombozytenaktiverung und Aggregation. Während schwerer invasiver *S. pneumoniae* Infektionen werden nicht selten eine Thrombozytopenie oder eine disseminierte vaskuläre Koagulopathie als Komplikation beobachtet. Dieser Umstand lässt auf eine direkte oder indirekte Interaktion von

Thrombozyten mit Pneumokokken schließen. Die vorliegende Studie hatte zum Ziel diese potentiellen Interaktionen zu analysieren und die beteiligten Faktoren auf Thrombozyten- und auf Bakterienseite zu identifizieren.

In einem Screening rekombinanter Pneumokokken-Oberflächenproteine konnten Proteine aus der Gruppe der Lipoproteine, Sortase-verankerten-Proteine und Cholin-bindenden Proteine als direkt Thrombozyten aktivierende Proteine identifiziert werden. Neben den genannten Oberflächenproteinen induzierte auch das intrazelluläre porenbildende Toxin Pneumolysin (Ply) einen starken Anstieg des gemessenen Aktivierungsmarkers P-Selektin der Thrombozyten. Da Ply einer der Hauptvirulenzfaktoren von *S. pneumoniae* ist, wurde nachfolgend das Hauptaugenmerk auf dessen Beteiligung an der Thrombozytenaktiverung gerichtet. Überraschenderweise zeigte sich, dass die bereits zuvor beschriebene Ply induzierte Thrombozytenaktiverung ein falsch positives Ergebnis ist, das durch die Bildung großer Poren in der Membran zustande kam. Vielmehr konnte eindeutig gezeigt werden, dass Ply die Thrombozyten bereits bei niedrigen Konzentrationen lysiert und sie dadurch funktionsunfähig macht. Die Lyse von Thrombozyten konnte sowohl durch pharmazeutische Immunglobulinpräparate als auch durch spezifische anti-Ply Antikörper gehemmt werden. Durch die Neutralisation der lytischen Wirkung von Ply konnte ebenso die Funktionalität der Thrombozyten vollständig erhalten werden. Nicht nur Pneumokokken, sondern auch *S. aureus* exprimiert porenbildende Toxine, so z.B. alpha-Hämolysin (Hla) und verschiedene Paare der Zweikomponenten-Porenbildenden Leukozidine. Während durch die Leukozidine keine messbare Veränderung der Thrombozytenfunktion beobachtet werden konnte, beeinträchtigte Hla humane Thrombozyten in einem zweistufigen Mechanismus. Die gezeigten Ergebnisse bestätigen Daten aus anderen Studien, dass Hla Thrombozyten aktiviert. In der hier vorliegenden Studie konnte jedoch zusätzlich eine nachfolgende Hla induzierte Lyse der Thrombozyten beobachtet werden. Dieser Effekt war sowohl Dosis- und Zeitabhängig und führte zum Verlust der Thrombozytenfunktion sowie der Thrombenbildung. Im Gegensatz zu Ply konnte Hla weder mit spezifischen Antikörpern noch mit einer pharmazeutischen IgG Präparation neutralisiert werden. Zusammengefasst weisen die vorgestellten Daten auf neue Pathomechanismen bakterieller porenbildender Toxine hin, die humane Thrombozyten stark beeinträchtigen. Sowohl die toxininduzierte Lyse der Thrombozyten als auch deren beeinträchtige Funktionalität könnten ein wichtiger Faktor bei der Entstehung

lebensbedrohlicher Komplikationen während invasiver Infektionen sein. Im Fall von invasiven Pneumokokkeninfektionen stellen Antikörper basierte Therapien, welche Immunglobuline gegen Ply enthalten, eine Interventionsmöglichkeit dar, um den schädigenden Effekt von Ply zu neutralisieren und damit den klinischen Verlauf zu verbessern.

II

BACKGROUND

Background – Content

1. *Streptococcus pneumoniae*

Epidemiology and Vaccination

Streptococcus pneumoniae (pneumococcus, pneumococci) was first isolated and cultivated by Louis Pasteur and George Miller Sternberg in 1881[1]. Pneumococci are Gram-positive, facultative anaerobic bacteria of lanceolate shape. They appear as pairs (diplococci) or in short chains and are further characterized by a α-hemolytic, oxidase- and catalase-negative and non-motile phenotype[2].
S. pneumoniae is a typical colonizer of the human upper respiratory tract where it can reside asymptomatically[3]. Nevertheless, aspiration of pneumococci into the lower respiratory tract as well as breaking through host barriers and dissemination to other organs can cause a variety of diseases. The clinical spectrum of pneumococcal diseases range from local, non-invasive diseases like otitis media and sinusitis to severe invasive diseases like meningitis, pneumonia and bacteremia[3]. As a consequence of a more weak immune system, children under the age of 5, the elderly as well as immunocomprimized individuals are at higher risk of developing infections[4]. Pneumococcal diseases contribute to approximately 1.6 million deaths annually. In developing countries, incidence and mortality are the highest. More than 500,000 children below the age of 5 die annually from pneumococcal pneumonia in low-income countries[5]. However, also in more developed countries incidences of pneumococcal diseases are high. Around 150,000 patients suffering from pneumococcal pneumonia are hospitalized in the United States annually. Up to 30% of these cases further develop bacteremia or meningitis and the overall fatal outcome is 5-7% of the cases[6]. In addition, there are up to 4,000 annual cases of bacteremia without a known site of primary infection in the United States. In children below the age of two, they account for about 70% of invasive pneumococcal diseases (IPD)[6]. But not just in the United States, but also in Europe more than 24,000 cases of IPD are reported annually, with the highest prevalence in the group of the elderly (≥65 years) and infants[7-8]. To combat pneumococcal disease, treatment can be either preventive by vaccination or therapeutic by prescription of antibiotics. Standard antibiotic care includes usage of tetracycline, chloramphenicol, penicillin, and erythromycin. However, due to excessive usage and misuse of antibiotics the prevalence of non-susceptible *S. pneumoniae* strains increased, leading to more frequent administration of penicillin and

erythromycin[8]. This development further highlights the importance of efficient vaccines against pneumococci. For *S. pneumoniae* 98 different serotypes, differing in virulence, prevalence, and drug resistance are currently known[9]. Serotypes are distinguished based on the composition of their capsule polysaccharides (CPS), which protect pneumococci from phagocytosis and antibody-mediated United States[10]. The CPS is highly immunogenic and essential for pneumococcal colonization and virulence, marking it as an optimal target for vaccination[11]. The currently used vaccines were designed to cover the most frequent occurring pneumococcal serotypes. Two different types of vaccines are available and approved. First, Pneumovax23 or 23-valent pneumococcal polysaccharide-based vaccine (PPV23) and second, pneumococcal conjugate vaccines with different formulations (PCV7, PCV10, PCV15, PCV13, PCV20). PPV23 (Pneumovax®, MSD) comprises purified CPS of the leading 23 serotypes being responsible for IPD in the United States and Europe at the time of licensing in 1983[12]. Although PPV23 was concluded to be effective in preventing IPD in healthy individuals below the age of 75, it has a few limitations[13]. Vaccine efficacy is limited to lesser severity of IPD but has no effect on pneumococcal carriage or non-invasive pneumococcal diseases[14]. Further, immunogenicity in younger children is reduced and the vaccine does not induce a sufficient immune memory[13, 15]. Pneumococcal conjugate vaccines were first licensed in 2000 with PCV7. The benefit of this vaccine type is the conjugation of carrier proteins to CPS, shifting immune response to a T-cell dependent response and higher immunogenicity[16]. The PCVs, which are currently on the market, are Prevnar 13 (PCV13, Pfizer), Vaxneuvance (PCV15, Merck Sharp & Dohme Cor), PHiD-CV (PCV10, GlaxoSmithKline plc.) and Prevnar 20 (PCV20, Pfizer). PCV13 is a 13-valent pneumococcal vaccine with diphtheria toxoid (CRM) as carrier protein[17]. In 2021, PCV 15 and PCV20 were licensed for adults above the age of 18. Both formulations contain all serotypes of PCV13 conjugated to CRM and additional 2 (PCV15) or 7 (PCV20) serotypes which account for up to 30% of IPD caused by non-PCV13 serotypes[18-19]. In contrast, PCV10 contains CPS of eight serotypes conjugated to *Haemophilus influenzae* carrier protein D, and additionally CPS of two serotypes conjugated to either tetanus toxoid or diphtheria toxoid[20]. Although development of PCVs led to increased efficiency in IPD and pneumonia prevention, some challenges aroused over the years. Obviously, protection against pneumococcal infections is limited to the included serotypes. Further, differences in geographic serotype distribution as well as age dependent

differences in disease causing serotypes are commonly observed[21-22]. Another problem is serotype replacement, which describes the effect of increasing prevalence of serotypes causing carriage and disease which are not included in the PCVs[23-25]. Serotype replacement as well as the increase in antibiotic resistance among serotype replacement strains clearly highlight the need for improved vaccines covering more than just a few serotypes. Therefore, ongoing work aims to include other conserved immunogenic proteins and structures of pneumococci to generate a serotype independent vaccination and therefore a broader protection against *S. pneumoniae*[11, 26-27].

Pneumococcal virulence factors

S. pneumoniae has the typical morphological characteristics of Gram- positive bacteria. The bacterial surface is enclosed by a bilipid membrane which is surrounded by the cell wall which comprises peptidoglycan (PGN) layers and teichoic acids[28]. The PGN is built up by highly cross linked strands of N-acetylglucosamine and N-acetylmuramic acid. Teichoic acids consist of a ribitol phosphate backbone with covalently attached phosphorylcholine (*P*Cho)[29]. They can be divided into PGN-anchored wall teichoic acids (WTA) and membrane-anchored lipoteichoic acids (LTA)[30-31]. The cell wall is further enclosed by the above described serotype specific CPS[32,33]. Proteins attached to the surface of pneumococci can be divided into four groups, based on their mode of anchoring: i) lipoproteins, ii) sortase-anchored proteins, iii) choline binding proteins and iiii) non-classical surface proteins. Lipoproteins represent the largest group of pneumococcal surface proteins with up to 37 predicted members[30-31]. Anchoring of lipoproteins onto the extracellular site of the cytoplasmic membrane is mediated by the lipoprotein diacylglyceryl transferase (Lgt) catalyzing deacetylation of lipoprotein precursors[30-31]. Most lipoproteins are predicted to function in adenosine triphosphate (ATP)-binding cassette (ABC) transporter systems in substrate binding, therefore determine substrate specifity (e.g. PiaA, AliA, PsaA)[34-37]. These ABC-transporters are important factors in bacterial fitness, because they are essential for nutrient uptake[31]. Other lipoproteins are involved in protein folding (e.g. SlrA)[38], colonization (e.g., PpmA)[39], cell wall biosynthesis (e.g., DacB)[40], resistance against oxidative stress (Etrx1 and Etrx2)[41], bacterial fitness as nucleoside transporters and pathogenicity (PnrA)[42]. Another group of pneumococcal surface proteins are

choline-binding proteins (CBPs) comprising strain dependent 13 to 16 proteins[43-44]. CBPs are non-covalently linked to the cell wall by binding of their N- or C- terminal choline binding domain to PCho moieties of LTA and WTA[41]. One of the best characterized CBPs is the highly conserved major autolysin A (LytA). LytA is a cell wall hydrolase and important for autolysis and virulence[45]. Different CBPs like CbpD, CbpG, and CbpL contribute to infection and invasion due to their adhesive properties. Further, a number of CBPs are described as pneumococcal virulence factors including PspC (CbpA), CbpC and PspA[46]. PspC is essential for colonization by binding to epithelial cells but also for pathogenesis via inhibition of opsonization and complement activation[46-47]. The third group of pneumococcal surface proteins are sortase-anchored proteins. Depending of the strain and serotype 13-19 proteins belong to this group. They contain an N-terminal signal peptide and C-terminal LPXTG amino acid sortase-recognition motif as cell wall sorting signal[48-49]. Sortase A with its transpeptidase activity recognizes this motif, cleaves it and anchors the protein to lipid II which is then integrated into the PGN of the cell wall[50-51]. Among this group is the exoglycosidase neuraminidase A (NanA), which promotes opsonophagocytosis and also plays a role in adhesion and invasion[52]. Additional members of this group are the Pneumococcal adherence and virulence factor B (PavB) and Pneumococcal serine-rich repeat protein (PsrP). PavB interacts with components of the extracellular matrix (ECM) like plasminogen and thrombospondin-1 (TSP-1) and is essential for adhesion to epithelial cells and pathogenesis[53-54]. PsrP belongs to the serine-rich repeat protein (SRRP) family and mediates adhesion to bronchial and alveolar cells and its homologue GspB in *Streptococcus gordonii* has been shown to bind to platelets[55-56]. The last group of pneumococcal surface proteins are the non-classical surface proteins or moonlighting proteins. They lack specific anchoring signals and can be found either intracellularly and/or on the surface[57]. Proteins of this group, like enolase or PavA where shown to act as adhesins[32-33].

Another major virulence factor of pneumococci is pneumolysin (Ply), which is a 471 amino acid long cholesterol dependent cytolysin with a molecular mass of 53 kDa[58]. Ply is produced as soluble monomer and oligomerizes with up to 50 subunits in cholesterol containing eukaryotic membranes. Membrane binding and oligomerization leads to formation of a prepore which subsequently is inserted into the membrane. Insertion takes place via conformational change in domain 2 of each monomer leading to formation of pores with a size of 35-45 nm in diameter[59-61]. Ply is an intracellular

toxin, because it lacks a N-terminal secretion signal. It was thought that Ply release depends on autolysin-dependent autolysis[62-63]. However, other studies demonstrated a secretion signal independent export of Ply and its extracellular association with the cell wall[64-65]. Ply excerts cytotoxic effects on many cell types. It damages cells by pore formation as seen in lung endothelial cells, tracheobronchial epithelial cells or cardiomyocytes[66-68]. Nevertheless, Ply can also indirect affect cells and tissues via targeting specific host factors. Ply leads to increased release of platelet activating factor (PAF), which further triggers inflammation and vascular permeability leading to acute lung injury[69]. Further, Ply also disturbs cellular function at sublytic concentrations. In cardiomyocytes, sublytic Ply concentrations induce cardiomycoyte dysfunction by Ca^{2+}-influx resulting in reduced contractility[66]. Similar, platelets have also been described to aggregate at sublytic Ply concentrations[70].

2. Staphylococcus aureus

Epidemiology

Staphylococcus aureus (*S. aureus*) is a Gram-positive, facultative anaerobe bacterium and was first described by Rosenbach in 1884. The bacterium has a sphere shape and appears in grape-like clusters. Further, it is catalase-, coagulase-, phosphatase-, and urease-positive and ferments mannitol to lactic acid[71]. *S. aureus* is a typical colonizer of human anterior nares and skin, but can also colonize other tissues like throat, perineum and intestine[72]. Up to 60% of the population are transiently colonized by *S. aureus* throughout their lifetime and about 30% are persistent carriers[73]. Carriers typically show no symptoms or have only minor local infections and transmiss the bacterium from person to person with special high transmission rates in healthcare facilities. Among colonization niches, nasal carriage bares the highest risk for subsequent infection[74]. *S. aureus* as an opportunistic pathogen can cause a variety of mild to severe infections and can affect any human tissue. Infections range from skin and soft tissue infections to life-threatening invasive diseases like pneumonia, toxic shock syndrome, endocarditis and sepsis[74]. Although not life-threatening, severe skin infections like wound infections, abscesses, and furuncles are also accompanied by significant morbidity and suffering. *S. aureus* is one of the most widespread bacterial

pathogens and the predominant causative agent in respiratory infections, surgical site infections and infective endocarditis[75]. The global prevalence of *S. aureus* induced severe infections can only be estimated and ranges up to millions of cases[76]. A study from 2017 reported 20,000 deaths upon *S. aureus* bacteremia only in the United States[77]. Next to high virulence, a further threat of *S. aureus* are widespread antibiotic-resistant strains, which develop upon excessive usage of antibiotics. The clinically most important ones are methicillin-resistant *S. aureus* (MRSA) strains. They are only susceptible to clindamycin, linezolid, daptomycin, vancomycin, and teicoplanin with the last two being antibiotics of last resort[78-79]. Earlier, MRSA were mainly detected in hospitals and health-care centers, but over the last decade, spread is also reported outside of hospitals. To date, community-associated MRSA became the leading cause for skin and soft tissue infections and sepsis in the US with the most prevalent MRSA strains USA300 (sequence type 8, ST8) and USA 400 (ST1) being the cause of over 60% of community infections[78, 80].

In contrast to *S. pneumoniae*, development of vaccines with high efficacy to prevent *S. aureus* infections was unsuccessful so far. To date, 12 *S. aureus* polysaccharide serotypes have been described with type 5 and type 8 (CP5/8) accounting for the majority of infections[81-82]. A promising vaccine candidate was StaphVAX®, which is in principle comparable to the PCVs against pneumococcal infections. StaphVAX® contains CP5/8 conjugated with recombinant non-toxic *Pseudomonas aeruginosa* exoprotein A and was the first vaccine tested in humans[83]. During a phase III clinical trial efficient protection by the vaccine was observed until week 40 post vaccination. However, until week 54 post vaccination the vaccine failed as measured by drop in efficacy to 26%[83]. A follow up study hypothesized a second round of vaccination at week 35 to boost immunity, but also two doses had no lasting protective effect[84]. Another vaccine candidate was V710 (Merck), a monovalent formulation targeting iron salvage protein (IsdB). Nevertheless, the phase IIB/III clinical trial had to be early terminated, because V710 was associated with increased mortality[85-86]. Very recently Pfizer developed the SA4Ag vaccine containing CP5/8 and additionally recombinant MntC and ClfA, each conjugated to a detoxified form of diphtheria toxin. But also this vaccine failed in a phase II clinical trial (NCT02388165)[87].

S. aureus virulence factors

S. aureus causes a broad range of infections. This is related to the expression of a wide variety of virulence factors, allowing the pathogen to adhere to host cells, invade host cell and escaping the immune system. Among these virulence factors are proteins which can be categorized into the groups of i) microbial surface component recognizing adhesive matrix molecules (MSCRAMMs) and ii) secretable expanded repertoire adhesive molecules (SERAMs).

MSCRAMMs are adhesins and share a similar structure. They contain two adjacent IgG-folded domains allowing binding of ECM proteins like fibrinogen, fibronectin, TSP-1 or van Willebrand factor (vWF)[88]. Members of this protein group contain a LPTXTG sortase-anchoring motif, which allows the covalent binding to the PGN of the cell wall by the transpeptidase sortase A[89]. One of the best described MSCRAMMs is clumping factor A (ClfA). It is the main fibrinogen binding protein of *S. aureus* and was found to be present in nearly all analysed isolates[90]. ClfA promotes attachment to host tissues and has been described to bind platelets resulting in platelet activation[91-92]. Two other well characterized members of this family are fibronectin-binding protein A and B (FnbpA/B), which are both expressed in most clinical isolates or singularly expressed in the minority of clinical isolates[93]. They have a N-terminal binding site for fibronectin, fibrinogen and elastin[94-95]. Both proteins are associated with biofilm formation of MRSA strains and contribute to inflammation and invasion[96-97] Another MSCRAMM is Staphylococcus protein A (SpA), which facilitates immune evasion by binding to the Fcγ proportion of IgG and thereby inhibition of opsonophagocytosis. Further, SpA mediates induction of apoptosis in B-cells by interacting with their Fab domain[98-99]. The second protein group of *S. aureus* are the SERAMs. One member is the extracellular adherence protein (Eap), which is expressed by nearly all tested clinical isolates[100]. Eap can bind to several ECM components like fibrinogen or TSP-1 and further interacts with the endothelial intercellular adhesion molecule 1 (ICAM-1) receptor thereby preventing leukocyte adhesion to the endothelium[101]. Eap is also involved in immune evasion via inhibition of complement pathways[102]. A protein important for abscess formation is the secreted coagulase (Coa). By interacting with prothrombin and fibrinogen, fibrin meshworks and clots are formed providing protection against host immune defence[103]. Another virulence factor is the major autolysin A (AtlA), which mediates hydrolysis of the PGN in the area of the septum, leading to cell division[104].

AtlA has binding sites for ECM proteins like e.g., fibronectin and has been shown to be crucial in biofilm formation especially on artificial substrates like medical implants[105-107]. Next to proteins with adhesive properties, *S. aureus* also expresses proteins with immune modulatory functions. Among them are the chemotaxis inhibitory protein (CHIPS) and the formyl peptide receptor-like 1 inhibitory protein (FLIPr). Both proteins bind human neutrophils and monocytes. Binding of CHIPS to the C5a receptor or the formyl peptide receptor 1 leads to inhibition of neutrophil recruitment to the site of infection whereas binding of FLIPr to FcγRIIa results in inhibition of opsonophagocytosis[108-109]. Further virulence factors expressed by *S. aureus* are pore forming toxins like alpha hemolysin (Hla) and the bicomponent pore forming leukocidins (BPFL). Hla is one of the major virulence factors of *S. aureus*. It is expressed by most disease associated clinical isolates and expression levels of Hla have been reported to correlate with disease severity[110]. Hla forms β-barrel transmembrane pores of 1-3 nm in diameter, allowing molecules up to 4 kDa in size to pass through[111-112]. By binding to the metalloprotease ADAM10 of host cells, *S. aureus* secreted Hla triggers proinflammatory signalling and disrupts cell-cell contacts supporting invasion of bacteria[113-114]. The BPFL like LukAB, LukSF (Panton valentine leukocidin, PVL) and LukED contain two protein components, which oligomerize in membranes of different cell types into a β-barrel pore[115]. They target specific receptors on different immune cells like neutrophils or phagocytes leading to proinflammatory signalling and cell death and therefore protection against the host's immune system[116-117].

3. Platelets

Origin and Morphology

Platelets are the second most abundant cell type in the circulation. They have a discoid shape and are of small size with 2-4 µm in diameter[118]. However, despite their small size, platelets are main players in hemostasis, vessel repair, infection and inflammation.

Platelets originate from megakaryocytes (MK), which are cells of up to 100 µm in diameter. MKs reside in bone marrow, but are also found in the lung circulation[119-120]. During maturation, MKs migrate to the bone marrows perivascular region and in

parallel their cytoplasm gets packed into long protrusions, so called proplatelets. Proplatelets further elongate and branch until they extend into sinusoid lumen[119]. Alongside those branched organelles and granules are transported in a microtubule dependent manner from the MK cell body into proplatelets and captured at the tips[121-122]. Platelets are finally formed at the distal end of proplatelets. Dependent of the shear and turbulent flow platelets finally are shedded into circulation[122-123]. Once they are released, platelets have an average life time of 5-9 days before they are cleared from circulation in liver and spleen[124-125]. Due to their origin, platelets are anucleated and translation is limited to stable MK derived mRNA[126]. Two membrane systems are found in platelets: the open canalicular system and the dense tubular system. The open canalicular system is connected to the plasma membrane and its channels provide membrane surface enlargement. The thereby provided increase in surface area is important for uptake of extracellular molecules but also shape change and granule release upon platelet activation[127]. In contrast, the dense tubular system is an internal smooth endoplasmic reticulum membrane system storing enzymes and ions like Ca^{2+}. Besides, platelet cytoplasm contains organelles like mitochondria, lysosomes and, of major importance for platelet function, different types of granules. Three types of granules can be distinguished: α-granules, dense granules, and lysosomal granules. Alpha granules are the most abundant ones with 50-80/human platelet[128] and contain mitogenic factors, coagulation factors (e.g. factor V, fibrinogen), adhesive proteins (e.g., P-selectin, von Willebrand factor (vWF)), angiogenic factors like angiogenin as well as different cytokines and chemokines[129]. Dense granule mainly store small molecules like adenosine diphosphate (ADP), adenosine triphosphate (ATP), serotonin, and ions (e.g. Ca^{2+}). Glycosidases being important for clot retraction and bactericidal proteins are stored in the lysosomal granules[130]. Upon platelet activation the granules undergo exocytosis and their content is released into circulation or rebound on the platelet surface for signal amplification[130].

Role of platelets in hemostasis

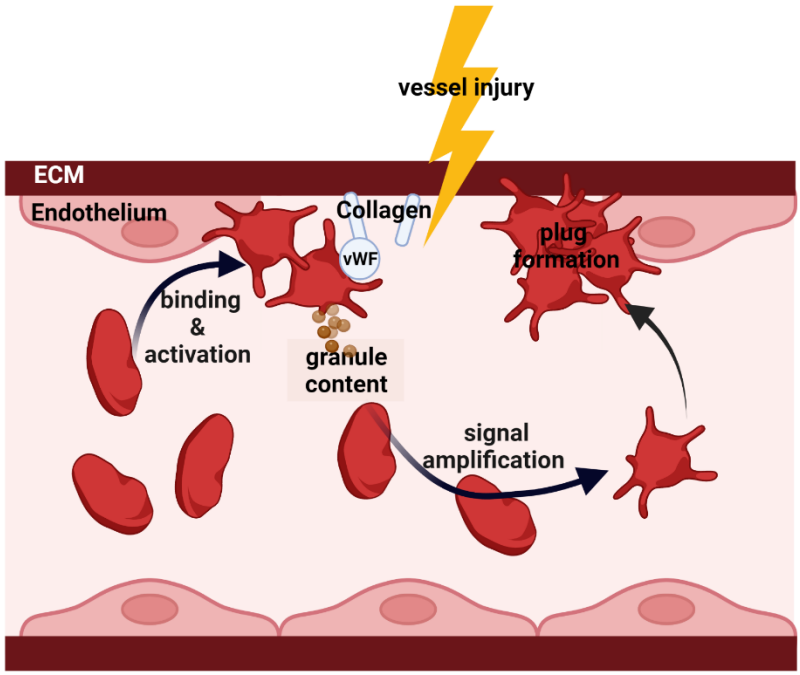

Figure 1: **Platelets sense and close vessel injury**. Circulating platelets sense exposed ECM molecules like collagen upon damage of the endothelial barrier. Binding to ECM molecules result in platelet activation and thereby release of granule content resulting in signal amplification. Subsequent activation and acquisition of nearby platelets results in thrombus formation and thereby closure of the endothelial gap. Created with BioRender.com

Platelets are essential for sensing and repairing injured vessels and therefore bleeding arrest. Accumulation of platelets at the site of damaged endothelium is described as the first wave of hemostasis. This process is followed by activation of the blood coagulation pathway as the second wave of hemostasis[131].

Upon vascular injury, matrix proteins of the subendothelium like collagen are exposed to components of the blood stream. Plasma vWF, which is derived from e.g., platelets and endothelial cells, binds to the exposed collagen. The interaction of platelet glycoproteins GPIb/IX/V with immobilized vWF initiates platelet tethering at the site of vessel injury under arterial high shear conditions[132,133] (Figure 1). Under venous low shear conditions GPIIb/IIIa binding to exposed fibrinogen and fibronectin are described to induce direct platelet adhesion[134]. For stable platelet adhesion GPVI and GPIa/IIa bind to collagen[135]. In addition, further integrins bind to ligands exposed on damaged vessels such as integrin α5β1 to fibronectin or GPIIb/IIIa to fibrinogen/fibrin[136]. Binding of platelet receptors to their ligands in the area of vessel injury leads finally to activation of platelets[137]. This in turn is followed by release of platelet granule content. Alpha

granules contain proteins being important for a stable adhesion and thrombus formation like fibrinogen, vWF and P-selectin among many others. Dense granules release small molecules like ADP supporting signal amplification. ADP is part of a positive feedback loop and rebinding of ADP to the platelet surface triggers cell based thrombin generation resulting in further platelet activation[138]. In parallel thromboxane A2 and reactive oxygen species are generated and phosphatidylserine (PS) is exposed on platelet surface providing a further signal for amplification of the activation reaction and recruitment of nearby platelets[139-140]. Besides adhesion, activation and aggregation, platelets also play a role in thrombin generation, a crucial step during coagulation. Thrombin converts fibrinogen to fibrin, the end product of the coagulation cascade[141]. PS exposure on the platelet surface provides a catalytic surface for thrombin generation. PS leads to a negative charged surface, thereby creating a high-affinity binding substrate for coagulation factors (e.g. prothrombin, FVII, FIX, and FX)[142-144]. Next to PS also rebound ADP and fibrin binding to GPVI potentiates thrombin generation[138, 145]. Further, thrombin is also part of a signal amplification loop. By cleaving protease-activated recpetors (PAR) and binding to GPIbα, further platelet activation, PS exposure and thrombin generation is stimulated[146-147].

Platelets as immune cells

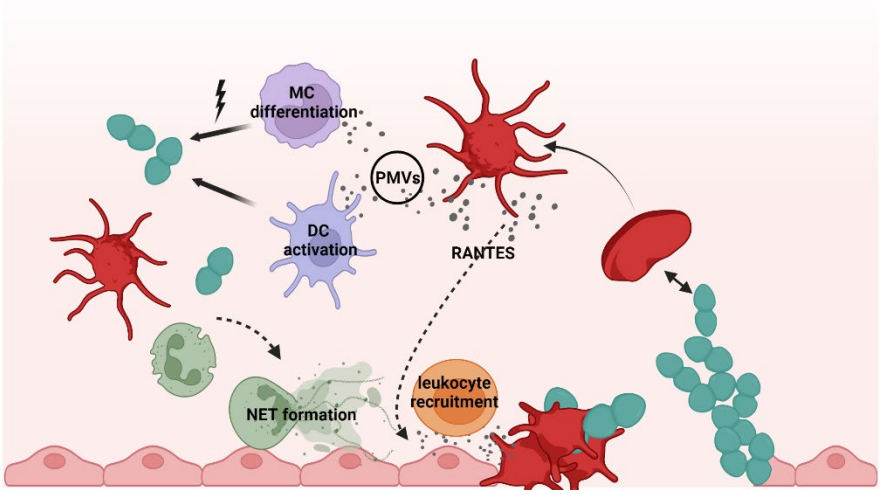

Figure 2: Role of platelets in the immune response. Platelets sense invasion of bacteria into the bloodstream as well as injured vessels and get activated resulting in release of their granule content and PMVs. Among many other proteins the releasate contains chemokines and cytokines which stimulates recruitment of leukocytes. Attracted neutrophils form NETs resulting in e.g. further platelet activation. PMVs modulate gene expression of monocytes and monocyte (MC)-derived cells such as dendritic cells (DC) to a phagocytotic phenotype. Source: Jahn *et a*l (see III.1)[148]

Despite their role in hemostasis platelets gain more and more interest regarding their function as immune cells. In fact, they are the most abundant cell type with immune function in the circulation. Platelets act either local at sites of acute processes like vessel injury or systemic via interaction with other immune cells or release of immune modulatory granule content[149]. Features making them part of the immune system are the expression of receptors being involved in inflammation, pathogen recognition and direct or indirect interaction and modulation of other immune cells like leukocytes (Figure 2). Interactions between leukocytes and activated platelets are generally categorized into adhesive and soluble mechanisms, although both mechanisms are closely coupled[150]. Adhesive interactions involve e.g., surface bound P-selectin (CD62P) on platelets, which binds P-selectin glycoprotein ligand-1 (PSGL-1) on leukocytes[151-152]. Binding triggers activation of neutrophils by a conformational change and clustering of the αMβ2-integrin Mac-1. This results in immobilization of neutrophils at the site of lesion and promotes transendothelial cell migration[152-154]. Another

adhesive mechanisms is dependent of platelet GPIbα binding to Mac-1 which stimulates inflammation and thrombosis in vascular injury[154]. Further interaction partners are platelet CD40L and leukocyte CD40, driving B-cell response, maturation of dendritic cells and inflammation[155].

Soluble interactions are mediated by platelet releasate. Platelet granules store more than 300 biological active proteins among them stimulators or modulators of different immune cells[156]. Alpha-granule derived proteins like CXCL4 (platelet factor-4), RANTES (Regulated upon Activation, Normal T Cell Expressed and Presumably Secreted) or fibrinogen act as proinflammatory chemokines or cytokines[157]. Release of e.g. CXCL4 or fibrinogen induces increased binding of neutrophils to the endothelium[158-159]. Small molecules with mainly modulatory function like ADP stimulates antigen endocytosis in dendritic cells[160]. Further, serotonin triggers differentiation of monocytes into dendritic cells and T-cell activation[161].

Next to various interactions with other immune cells, platelet microvesicles (PMVs) are of major importance for platelets as immune cells. PMVs are small lipid membrane vesicles (0,1-1 µm)[162] which store messenger RNA (mRNA), microRNAs, bactericidal proteins but also adhesion related platelet surface proteins[163]. They are part of physiological and pathophysiological cell-cell communication. Upon binding of PMVs to target cells, their content is released resulting in modulated gene expression[164] (Figure 2).

Platelets express a variety of receptors involved in immune functions and/or interacting with pathogens. Among them are integrins, G-protein coupled receptors, ADP receptors (P2Y), leucine-rich repeat glycoproteins (GP), Toll like receptors (TLRs), IgG superfamily receptors (GPVI, FcγRIIa) and tyrosine kinase receptors.

TLRs are transmembrane proteins being expressed on many cell types but best studied on immune cells. On the platelet surface TLR4, TLR1, TLR2, TLR7 and TLR9 are expressed[165-166]. TLRs recognise pathogen associated molecular patterns (PAMPS) via their leucine-rich β-sheets ectodomains and transduce this signal via an Toll-interleukin-1 receptor domain[167]. LPS recognition by TLR4 has been described multiple times, however the resulting activation of platelets is controvercially discussed. While Brown et al demonstrated LPS dependent platelet activation followed by shedding of proinflammatory IL-1β-rich microparticles[168], Binsker et al. observed platelet activation by LPS only in whole blood but not in washed platelets, indicating

an indirect effect[169]. During bacterial infections bacterial lipoproteins are recognized by TLR2 leading to formation of aggregates composed of platelets and neutrophils[170]. In addition, *S. pneumoniae* and Group B streptococci induce TLR2 dependent activation of the phosphoinositide-3 (PI3)-kinase pathway leading to platelet activation, aggregation and release of dense granules[171-172]. Another important toll like receptor during infection is TLR9, which is activated by bacterial DNA, subsequently leading to activation of the coagulation cascade[171]. Next to TLRs also the FcγRIIa receptor is of major importance for immune function. FcγRIIa receptors are expressed in high numbers on platelets (2000 – 3000 per platelet)[173] and mediate internalization of IgG covered particles like bacteria, resulting in platelet activation and aggregation[174]. In addition simultaneous stimulation of FcγRIIa receptor is needed for activation of e.g. GPIIb/IIIa by *E. coli* or *S. aureus*[175-176]. Further platelet receptors involved in direct interaction with bacteria are discussed in section 4.

Platelets in bacterial infections

As described above, platelets are involved in host defence against invading pathogens. Therefore, it is not surprising that involvement of platelets and disturbance of platelet function are frequently observed during bacterial infections.

A well described severe bacterial infection involving platelet interactions is infective endocarditis (IE) caused by *S. aureus*. Typically, the disease develops on the surface of heart valves and has a mortality rate of about 25%[177]. Structural abnormalities of the heart caused by preceeded conditions like congenital heart disease, rheumatic fever/rheumatic heart disease, prostethic valves or implanted devices like pacemakers increase susceptibility for developing IE[177]. Further risk factors are increasing age, intravenous drug abuse or dental procedures[177-180]. With 30% of the cases, *S. aureus* is the main causative agent of IE[177, 181], whereas pneumococci only account for ≤3% of the cases[182]. Typically, structural changes of heart tissue leads to sedimentation of fibrin and platelets thereby creating a colonization niche for bacteria[183-184]. Settled bacteria further acquire fibrin and platelets. Thereby, bacteria are shielding themselves from the immune system resulting in high bacterial densities[183,185]. However, *S. aureus* does not essentially needs preceding structural changes of heart valves, since it is capable of directly inducing an inflammatory environment which affects valvular endothelium[92]. This in turn triggers adherence of staphylococci to the subendothelial

matrix of the injured heart valves. Adherence is mediated by the interplay between staphylococcal surface proteins and ECM components and is followed by local cytokine release, platelet activation and fibrin deposition.

In contrast, interactions of platelets with pneumococci during invasive infections is rarely described. Most of the literature is focussed on platelets as a prognostic marker in pneumococcal infections, since development of thrombocytopenia and therefore low platelet counts correlate with increased mortality[186-187]. However, mechanistic insights into how infections with pneumococci lead to decreased platelet counts are missing.

One of the most frequent invasive pneumococcal diseases is community-acquired pneumonia (CAP). This life threatening disease is caused by different pathogens but predominantly by pneumococci. With 14% of hospitalized patients, the mortality rate is high and of special risk for severe courses are elderly, immunocomprimized patients or patient with comorbidities[10, 188]. Different symptoms and complications relate CAP to disturbed platelet hemostasis. Systemic platelet activation, a general coagulant phenotype and therefore dropping platelet counts are frequently observed during severe CAP[189]. In addition also formation of neutrophil extracellular traps (NETs) formation is observed in patients. NETosis is driven by platelet dependent mechanisms and elevated serum NET markers are associated with a higher risk of clinical instability and overall mortality[190].

A severe complication of invasive bacterial infections is the development of bacteremia and finally sepsis with mortality rates of up to 30%[191]. Due to massive coagulation and consumption of platelets, nearly half of all septic patients develop thrombocytopenia disseminated intravascular dissemination (DIC)[192]. During sepsis, neutrophil-platelet interactions are triggered resulting in formation of NETs[193]. NETs contain histones, defensins and neutrophil DNA all leading to amplification of platelet activation and therefore strong induction of a coagulant phenotype[194-195]. Uncontrolled coagulation finally leads to thrombocytopenia and DIC as one of the most severe conditions during sepsis[196]

4. Platelet bacteria interactions

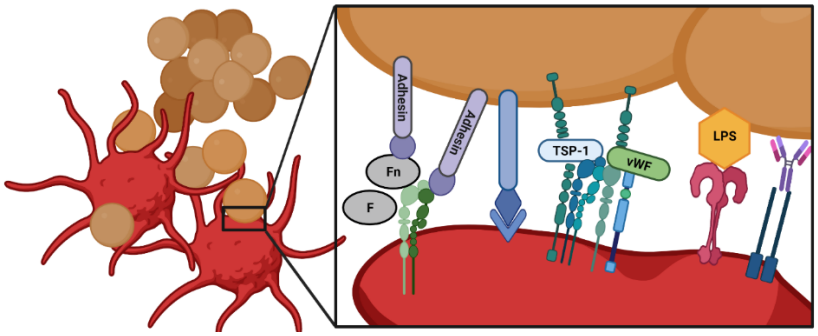

Figure 3: Platelet bacteria interactions. Scheme illustrating direct and indirect binding of bacteria to platelets and some of the involved receptors like TLRs (red), FcγRIIa (dark blue) or different Glycoprotein receptors (green, light blue). Source: Jahn et al (see III.1)[148]

During severe infections bacteria are able to spread from the site of infection and enter the bloodstream where they get in contact with circulating platelets. Interactions of bacteria with platelets can be categorized into: i) direct binding of bacteria to receptors on platelet membranes, like TLR2 or FcγRIIa, ii) indirect binding via bridging molecules of the ECM (e.g. fibrinogen or thrombospondin-1) and iii) interactions via released factors (e.g. Ply, Hla) (Figure 3). Binding of bacteria to platelets can result in platelet activation but also internalization of bacteria into platelets. The uptake of bacteria by platelets has been described for *Porphyromonas gingivales* and *S. aureus*, with the latter one being dependent of co-stimulation by e.g. ADP[197-198]. Further, adherent platelets are not static but migrate over their substrate. During this migration they engulf substrate bound particles like bacteria which leads to increased phagocytic activity[199]. Besides internalization mediated by direct binding of the pathogen, internalization dependent on IgG coating has been observed. IgG coated beads (0.5-1.5 µm) but also IgG preopsonized *E. coli* were reported to be internalized via platelet FcγRII receptor[174]. However, independent of the internalization mechanism, it remains unclear whether bacteria are killed intracellularly by granule derived antimicrobial peptides or whether internalization is an active immune evasion strategy. In the following sections interactions between *S. pneumoniae* and *S. aureus* with platelets are reviewed. Nevertheless, it is noteworthy that interactions with platelets have been described also for other bacteria. The serine-aspartate dipeptide repeat G (SdrG) of *Staphylococcus epidermidis* directly or indirectly via bridging of fibrinogen binds to platelets [200]. Other bacterial fibrinogen binding partners are e.g. the M1 protein of

Streptococcus pyogenes or lysine of *Streptococcus mitis*[201]. Further, several streptococci bind directly to sialic acids of platelet GPIbα via highly glycolsylated serine-rich proteins. To these belong the serine-rich protein A (SrpA) from *Streptococcus sanguinis*, the glycosylated streptococcal protein B (GspB) and the hemagglutinin salivary antigen (Hsa) from *Streptococcus gordonii* (*S. gordonii*)[202-203]. In addition, *S. gordonii* also expresses platelet adherence protein A (PadA), which mediates adherence to platelets[204].

S. pneumoniae interactions with platelets

So far, the knowledge about direct or indirect interactions between platelets and pneumococci is limited.

Since the first description of platelet aggregation by pneumococci in the 1970s, some contradictory data about how this activation was achieved appeared over the years. The first *in vitro* studies by Clawson *et al.* in 1971 showed serotype dependent platelet activation and aggregation, with serotype 8 inducing activation, wheras serotype 24 had no effect[205]. Some years later Keane *et al*. linked platelet aggregation to the presence of CPS on pneumococci, since non-encapsulated strains did not induce platelet activation. Further, this study reported this interaction to be TLR2 dependent[171]. However, the study of de Stoppelaar *et al.* revealed contradictory results. First, they could not provoke platelet activation by encapsulated *S. pneumoniae* strains and second, they observed platelet degranulation independent of TLR2 as confirmed in mice in the background of different TLR knockouts[206]. More recent studies demonstrated binding of pneumococci to platelets without regard to encapsulation or serotype. In the study of Niemann *et al.* formation of pneumococci-platelet aggregates was dependent on bridging molecules like soluble fibrin and TSP-1, which facilitated the interaction between pneumococci and platelets[207]. Further studies suggested the pneumococcal adhesins PspC and PavB bind to TSP-1, which thereby acts as a bridge to link the bacteria with platelet GPIIb/IIIa[53, 208].

S. aureus interactions with platelets

The interplay between *S. aureus* and platelets is well described. Staphylococci express a variety of proteins interacting with platelets via different mechanisms. Some of these

proteins belong to the family of MSCRAMMs. They mediate adherence to host ECM components and therefore bridging to platelets expressing receptors for ECM proteins[209]. Well described members of this family interacting with platelets are ClfA/ClfB and FnBPA/FnBPB. They bind to platelet GPIIb/IIIa utilizing fibrinogen and fibronectin as molecular bridges via their fibrinogen binding domains[210-211]. In addition, ClfA as well as serine-aspartate repeat-containing protein E (SdrE) are able to target directly GPIIb/IIIa, resulting in activation and aggregation of platelets[92]. Also members of other protein families like the iron-regulated surface determinant B (IsdB) or Eap interact with platelets[212-213]. Eap directly binds to protein disulfide polymerase (PDI) on the platelet surface, resulting in activation and aggregation of platelets[213]. Additionally Eap can utilize fibrinogen, vitronectin and TSP-1 as bridging molecules for binding to platelets[214]. Further, also secreted proteins of *S. aureus* were shown to directly activate platelets and trigger clotting in whole blood. Members of this group are AtlA, CHIPS and FLIPr[169]. Another staphylococcal protein interacting with platelets is the extracellular fibrinogen binding protein EfB. In contrast to the already mentioned proteins it does not trigger activation of platelets but binds to surface P-selectin of activated platelets and thereby competes with other binding partner of P-selectin like leukocytes[215-216]. Previously Hla has been reported to stimulate platelet activation and aggregation in plasma and whole blood. Additionally thrombus formation in small hepatic vessels was observed to be dependent on Hla expression of *S. aureus* in mice[112, 217-218].

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5. Objectives of the study

S. pneumoniae expresses various virulence factors and adhesins. During severe invasive pneumococcal disease reduced platelet counts are commonly observed, but except for the pore forming toxin pneumolysin no pneumococcal factors directly interacting with platelets are known. Nevertheless, there are sequence homologies of pneumococcal proteins with surface proteins of other bacteria known to activate platelets. Besides, with TLRs typical pattern recognition receptors for pathogens are expressed on platelets. Therefore it can be hypothesized, that in addition to pneumolysin other pneumococcal proteins may directly or indirectly interact with platelets. Here, a library of recombinant pneumococcal surface proteins was screened regarding their potential to directly activate human platelets. In addition, this study aimed to decipher the molecular mechanism of the previous reported platelet activation by pneumolysin and further show options to inhibition the mode of action of Ply on platelets.

Interactions of platelets with different *S. aureus* proteins resulting in platelet activation are well described. Similar to pneumococci, *S. aureus* expresses with alpha hemolysin and the two component leukocidins pore forming toxins. Platelet activation by alpha hemolysin has been reported several times, but for leukocidins only indirect effects on platelets have been described. Further, mechanistic understanding of the Hla platelet interaction beyond platelet activation *in vitro* is poor. Therefore this study aims to analyze the different pore forming toxins of *S. aureus* regarding their effect on human platelets in comparison to pneumococcal pneumolysin.

III

PUBLICATIONS

1.

Platelets, Bacterial Adhesins and the Pneumococcus

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Published in Cells, 2022 Mar, 25;11(7):1121. doi: 10.3390/cells11071121.

Author contributions:

Design of experiments: K.J., TP.K, LS.S and S.H.

Implementation of experiments: K.J., LS.S. S.W.

Experiments performed by K.J. shown in Fig. 2; 5A

Data analysis: K.J., LS.S. S.W.

Providing materials/reagents: S.H.

Writing the manuscript: K.J.

Revision of the manuscript: K.J., TP.K. and S.H.

Kristin Jahn Prof. Sven Hammerschmidt

cells

MDPI

Review

Platelets, Bacterial Adhesins and the Pneumococcus

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Abstract: Systemic infections with pathogenic or facultative pathogenic bacteria are associated with activation and aggregation of platelets leading to thrombocytopenia and activation of the clotting system. Bacterial proteins leading to platelet activation and aggregation have been identified, and while platelet receptors are recognized, induced signal transduction cascades are still often unknown. In addition to proteinaceous adhesins, pathogenic bacteria such as *Staphylococcus aureus* and *Streptococcus pneumoniae* also produce toxins such as pneumolysin and alpha-hemolysin. They bind to cellular receptors or form pores, which can result in disturbance of physiological functions of platelets. Here, we discuss the bacteria-platelet interplay in the context of adhesin–receptor interactions and platelet-activating bacterial proteins, with a main emphasis on *S. aureus* and *S. pneumoniae*. More importantly, we summarize recent findings of how *S. aureus* toxins and the pore-forming toxin pneumolysin of *S. pneumoniae* interfere with platelet function. Finally, the relevance of platelet dysfunction due to killing by toxins and potential treatment interventions protecting platelets against cell death are summarized.

Keywords: *Streptococcus pneumoniae*; platelet killing; platelet activation; pore formation; surface proteins; toxin; pneumolysin; *Staphylococcus aureus*; MSCRAMMs

Citation: Jahn, K.; Kohler, T.P.; Hammerschmidt, S.; Swiatek, L.-S.; Wiebe, S. Platelets, Bacterial Adhesins and the Pneumococcus. *Cells* **2022**, *11*, 1121. https://doi.org/10.3390/cells11071121

Academic Editor: Judith Cosemans

Received: 1 February 2022
Accepted: 22 March 2022
Published: 25 March 2022

1. Introduction

Platelets are anucleated, discoid shaped cells of the blood with a size of 2–4 µm in diameter [1]. Platelets are derived from megakaryocyte (MK) shedding in the bone marrow and the lungs [2,3]. Hence, their translation repertoire is limited to stable MK-derived mRNA [4]. Important platelet functions contribute to coagulation and closure of vascular damage occurring during, e.g., microbial infections. The main players during this processes are the surface expressed group of platelet glycoproteins. They are expressed in high numbers such as, e.g., integrin αIIbβ3, which is highly abundant on the surfaces of platelets and megakaryocytes and is involved in the crosslinking/aggregation of single platelets after activation [5]. Integrin αIIbβ3 is the fibrinogen receptor but also interacts with other extracellular matrix (ECM) proteins containing an RGD-like motif such as von-Willebrand-Factor (vWF), thrombospondin-1 (TSP-1), or fibronectin [6]. Platelet factors playing a role in hemostasis and infection are stored in different cytoplasmic granules. These granules undergo exocytosis upon platelet stimulation/activation and release their content into the circulation or granule proteins re-associate to the platelet surface [7]. Three types of granules can be distinguished: alpha-granules, dense granules, and lysosomal granules. Dense granules contain adenosine diphosphate (ADP), adenosine triphosphate (ATP), serotonin, histamine, and ions such as Ca^{2+}. Alpha granules contain platelet factor 4 (PF-4, CXCL4), P-selectin, coagulation factors such as factor V, mitogenic factors, adhesive glycoproteins

such as vWF, TSP-1, and fibrinogen, but also microbicidal proteins. Glycosidases, which are important for clot retraction, are mainly stored in the lysosomal granules [7].

However, in addition to their role in hemostasis, platelets have other important functions. In recent years, platelets have been increasingly recognized as immune and inflammatory cells. Several studies have highlighted the role of platelets in acute and chronic inflammatory processes such as stroke, myocardial infarction, infections, and sepsis [8–12]. Indeed, platelets are the most abundant circulating cell type with important immune functions. They display their function as immune cells either locally at sites of platelet activation or systemically by interacting with, e.g., leukocytes or via release of immune modulatory molecules [13,14]. Platelets store more than 300 proteins in their granules [15]. Besides the abovementioned proteins, granules also contain proteins acting as chemokines and cytokines such as, e.g., RANTES (Regulated upon Activation, Normal T Cell Expressed and Presumably Secreted) or CXCL12 (stromal cell-derived factor 1), and recruit and stimulate other cells of the immune system or induce endothelial inflammation [16]. Granule release also leads to changes in the platelet membrane composition. An increased number of integrin αIIbβ3 molecules and increased surface expression of P-selectin (CD62P) can be found on the surface after platelet activation. Exposure of P-selectin mediates initial interactions between platelets and leukocytes via immobilization of leukocytes at the site of lesion [17] and is used as a binding partner for initiation of complement activation [18]. In addition, platelets are a peripheral source of serotonin, stored in dense granules, which leads to differentiation of monocytes into dendritic cells (DCs) and also T-cell activation [19].

2. Platelets as Immune Cells in Infections

The first systemic response of the body to any kind of infection, tissue injury, or trauma is known as the acute phase response (APR). During APR, proinflammatory cytokines are released and acute phase proteins are produced [20]. By impairing microbial growth and promoting procoagulant activity to trap pathogens in local blood clots, platelets play a crucial role in the APR [21]. In addition, next to inflammatory and immune cells, platelets also produce interleukin 1-β (IL-1β) [21], which is not stored in granules as ready-to-use protein, but instead is translated from megakaryocyte (MK)-derived mRNA and released upon stimulation [22]. In mouse models of malaria, IL-1β has been demonstrated to play a major role in induction of the APR [21]. Another aspect that makes platelets part of the innate immune system is the expression of pattern recognition receptors (PRR) such as toll-like receptors (TLRs). TLR4, for example, is able to recognize bacterial lipopolysaccharides (LPS), which leads to platelet activation and release of IL-1β-rich microparticles, promoting interaction and activation with, e.g., endothelial cells [23]. However, others did not observe activation of washed platelets after incubation with LPS. Only incubation of whole blood with LPS led to increased P-selectin levels [24], indicating an indirect effect of LPS on platelets. During bacterial infections, TLR2 can be stimulated, leading to formation of platelet/neutrophil aggregates, which enhances adhesion of the aggregates to sites of injury or infection [25]. In addition, TLR2 stimulation of MKs is followed by increased maturation of MKs and elevated protein content, suggesting effects on platelet count, platelet function, and inflammation [26].

Besides their role in innate immunity, platelets are also pivotal for the acquired immune response. Platelets stimulate T-cell activation, trafficking, and also differentiation [27]. T cells are divided, with MHC class II recognizing $CD4^+$ cells and MHC class I recognizing cytotoxic $CD8^+$ cells. $CD4^+$ cells release cytokines regulating the activity of B cells and innate immune cells and can be divided into immune effector cells (Th1, Th2, Th17) and T-regulatory cells. Platelet-derived chemokines such as RANTES trigger activation and arrest of T cells at sites of infections. Upon release, RANTES is immobilized on the endothelium and triggers the arrest of monocytes and monocyte-derived cells at the endothelium but not on adherent platelets in a shear resistant manner (Figure 1) [28]. Adhesion of rolling monocytes on activated platelets as well as on the exposed endothelium is P-selectin-dependent, and this interaction further triggers expression and secretion of cytokines such

as TNF-α [29–31]. Furthermore, CXCL4 (PF-4) can mediate T-cell trafficking to sites of injury or infection by direct and indirect stimulation of CXCR3 expression on T cells [14]. In addition, T cells are also able to activate platelets via CD40L/CD40 interaction, leading to the release of chemokines such as RANTES and an increase in T cell recruitment [32,33]. Platelets further recruit and activate DCs via CD11b-JAM-C interactions, and the increased expression of CD80 and CD86 leads to enhanced T-cell response [34,35].

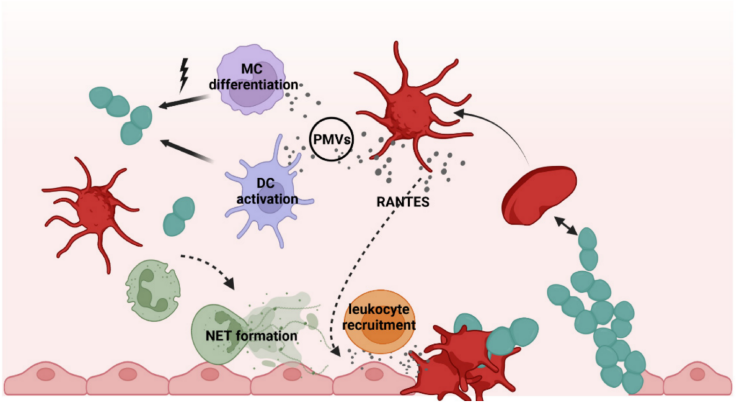

Figure 1. Scheme illustrating different platelet functions in the immune response. Platelets sense and bind invading bacteria and injured endothelium, resulting in platelet activation. Upon activation, platelets release chemokines and cytokines such as RANTES, triggering leukocyte recruitment and PMVs acting on gene expression of monocytes and monocyte (MC)-derived cells such as dendritic cells (DC). In addition, neutrophils are attracted, and NET formation occurs at the site of infection via platelet-dependent mechanisms. Created with BioRender.com (accessed on 21 March 2022).

Another immune cell function of platelets is displayed by the release of microvesicles. The so-called platelet microvesicles (PMVs) are lipid membrane vesicles with a size of 0.1–1.0 μm that are mediators of cell–cell communication. Elevated numbers of PMVs in the circulation are associated with inflammation and diseases such as arthritis [36], development of an acute coronary artery syndrome, and stroke [37,38]. PMVs carry adhesion molecules (CD62P, RANTES) that facilitate monocyte arrest at sites of deposited PMVs (vessel walls) and additional recruitment of activated platelets [39]. PMVs contain up to 250 different microRNAs (miRNAs, 20–22 nucleotides long). These miRNAs and also other stored molecules can be transferred to other cell types such as immune cells, vascular cells, and also smooth muscle cells, thereby changing their gene expression [40,41]. Changes in gene expression include phenotypic switches of monocytes and monocyte-derived cell lines such as macrophages towards a phagocytic phenotype (Figure 1) [42]. Furthermore, not only PMVs released upon platelet activation but also upon apoptosis of platelets have immunomodulatory effects, as shown by differentiation of monocytes into phagocytes [43]. In bacterial infections, platelets can contribute to clearance of bacteria via release of antimicrobial peptides present in PMVs or stimulation of immune cells via release of immunomodulatory molecules. PMVs can be distinguished in kinocidins, defensins, thymosins, and derivatives of antimicrobial peptides, which act against *Staphylococcus* aureus (*S. aureus*) and *Candida albicans* [44,45].

3. Interactions of Platelets with Bacteria

Bacteria are able to spread from the site of infection, thereby often crossing host barriers and entering the circulatory system, leading to bacteremia and sepsis. Complications of bacteremia associated with abnormal platelet functions are, e.g., infective endocarditis and disseminated intravascular coagulation (DIC) [46]. Interactions between platelets and

bacteria are characterized by direct (Figure 2) or indirect binding of bacteria to platelets or via released bacterial factors [24,46,47]. Indirect binding occurs via bridging molecules of the extracellular matrix (ECM), thereby linking bacterial surface proteins with platelet receptors [48]. The adhesive properties of bacteria towards platelets are essential for colonization of, e.g., cardiac valves during infective endocarditis [49,50]. Colonization can lead to local and/or systemic infections, and this might result in platelet activation in the bloodstream. Some bacterial infections cause severe thrombocytopenia without preceding bacteremia. To these belong, e.g., *Helicobacter pylori*-mediated immune thrombocytopenia driven by autoantibody-destroying platelets [51,52] or the hemolytic uremic syndrome (HUS) caused by Shiga toxin-producing *Escherichia coli* (*E. coli*) [52,53], which is due to amplification of the platelet activation process. Thrombocytopenia associated with bacterial infections appears as a secondary effect of systemic platelet activation, coagulation, and DIC, due to boosted platelet consumption [54].

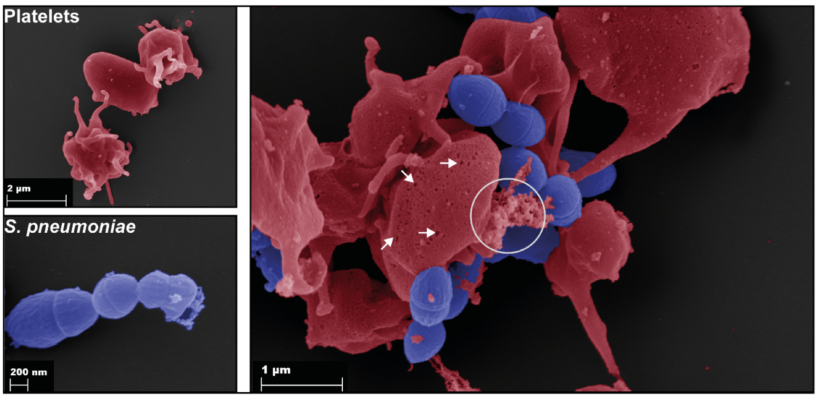

Figure 2. Binding of *S. pneumoniae* (blue) to platelets (red). Scanning electron microscopy of single platelets (upper left), single pneumococci (bottom left), and platelets incubated with the pneumococcal TIGR4 strain for 1 h (right). The right image shows binding of pneumococci to platelets. In addition, pneumolysin pores are formed in platelet membranes (arrows), and released granule content is visible (circle).

Besides platelet activation as a result of bacterial binding, another mode of bacteria–platelet interaction has been discovered, namely, internalization of bacteria by platelets. *S. aureus* was the first bacterium that was described to be internalized by platelets [55,56]. A prerequisite for internalization of *S. aureus* is a simultaneous platelet stimulation by ADP. Slightly differently, *Porphyromonas gingivales* was shown to be internalized by platelets without additional stimulation of platelets [57,58]. Furthermore, the platelet FcγRII receptor might also be able to initiate internalization of IgG–bacteria complexes as it has been shown for beads (0.5–1.5 µm in size) coupled with IgG or *E. coli* pre-opsonized with IgGs [59,60]. Other studies demonstrated a kind of searching and shuttling of invading bacteria by platelets. Adherent platelets can migrate over their substrate, collecting all substrate-bound material including bacteria, resulting in boosted activity of phagocytes [61]. In addition, platelets were shown to deliver the intracellular bacterium *Listeria monocytogenes* to dendritic cells [62]. However, the fate of the internalized bacteria is still unclear. On the one hand, they could be killed by antimicrobial substances of α-granules. On the other hand, the intracellular fate might help the bacteria to escape from the host immune system.

4. Platelet Receptors in Bacterial Infections and Bacterial Adhesins

Platelets express a large number of receptors involved in interactions with pathogens. This includes integrins, G-protein-coupled receptors, ADP receptors (purinergic receptors, P2Y), leucine-rich repeat glycoproteins (GP), Toll-like receptors (TLRs), IgG superfamily

receptors (GPVI, FcγRIIa), and tyrosine kinase receptors [63]. The FcγRIIa receptor is a low-affinity IgG receptor binding to the Fc part of immunogloblins [64]. About 2000 to 3000 FcγRIIa receptors are expressed on the surface of a single platelet [65], which enable binding and internalization of immune complexes containing IgGs (Figure 3) [59]. Binding of, e.g., IgG-covered pathogens leads to platelet activation and aggregation. Binding and activation of other platelet receptors by bacteria often requires simultaneous stimulation of FcγRIIa. For example, platelet aggregation induced by *E. coli* is dependent on simultaneous stimulation of integrin αIIbβ3 and FcγRIIa [66]. For *S. aureus* and *S. epidermidis*, a clustering of integrin αIIbβ3 or TLR with FcγRIIa is necessary for platelet activation [67]. Some pathogens such as *S. aureus* or *H. pylori* require plasma proteins such as fibrinogen or vWF to crosslink FcγRIIa with GPIb receptors for platelet activation [68].

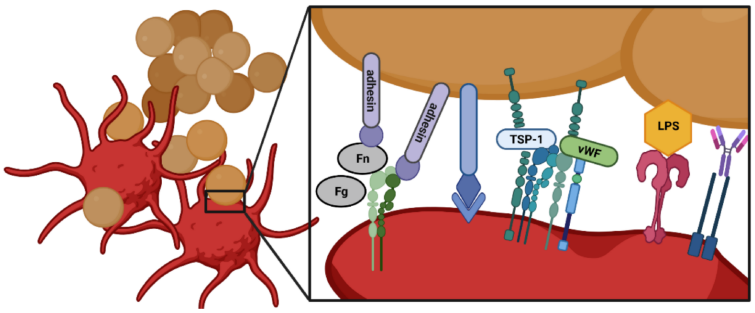

Figure 3. Binding of bacteria to platelets occurs either directly or indirectly. Bacterial adhesins with specific repeating units can utilize ECM proteins such as fibronectin (Fn), fibrinogen (Fg), TSP-1, or vWF as molecular bridges to bind to, e.g., integrin αIIβ3 or other complexes of glycoproteins. Furthermore, some bacterial factors can directly bind to integrins, TLRs, or other platelet surface proteins. Bacteria already covered by IgGs are recognized by FcγRIIa. Created with BioRender.com (accessed on 21 March 2022).

As mentioned above, integrin αIIbβ3 interacts with ECM proteins containing an RGD-like motif [6]. Some bacteria express surface proteins with domains rich in serine-aspartate repeats. This is highlighted by a family of *S. aureus* surface components called microbial surface components recognizing adhesive matrix molecules (MSCRAMMs). They exhibit sequence repeats mediating adherence to platelets or other host cell tissues as one of the first steps during infection [69]. Well-characterized members of this family are clumping factor A and B (ClfA and ClfB), fibronectin-binding protein A and B (FnBPA and FnBPB), and serine-aspartate repeat-containing protein E (SdrE). Fibrinogen bridges ClfA and ClfB via their fibrinogen binding domains to integrin αIIbβ3 [70], whereas fibrinogen or fibronectin is used by FnBPA and FnBPB for bridging [71,72]. In addition, integrin αIIbβ3 is also directly targeted by ClfA, but not ClfB, resulting in platelet activation (Figure 3) [73,74]. Further, integrin αIIβ3 can be directly bound by the *S. aureus* proteins iron-regulated surface determinant B (IsdB) [75]. Next to pneumococci, fibrinogen also bridges proteins of other bacteria such as serine-aspartate dipeptide repeat G (SdrG) protein of *S. epidermidis* to integrin αIIbβ3 [76]. A further platelet protein target of bacteria is the surface-associated protein-disulfide polymerase (PDI). The *S. aureus* extracellular adherence protein (Eap), a member of the SERAM (secretable expanded repertoire adhesive molecules) family, binds directly but also indirectly utilizes fibrinogen as a bridging molecule to PDI, resulting in platelet activation [77]. Direct activation and aggregation of platelets is also achieved by other secreted *S. aureus* proteins such as the chemotaxis inhibitory protein (CHIPS), the formyl peptide receptor-like 1 inhibitory protein (FLIPr), and the major autolysin (AtlA) (Figure 3) [24]. Upon activation of platelets, the extracellular fibrinogen-binding protein EfB of *S. aureus* binds to surface-exposed P-selectin and inhibits interactions between platelets and leukocytes [78,79].

GPIbα is a type-1 glycosylated membrane receptor that is highly abundant on the surface of platelets and megakaryocytes [80]. GPIbα is found in a complex with GPIbβ, GPIX, and GPV [81] and is the receptor for vWF but also for TSP-1, α-thrombin, and CD62P [80]. Different streptococcal species have been shown to bind to GPIbα via so-called glycosylated adhesins containing serine-rich repeats. This protein family was described in *Streptococcus gordonii* (glycosylated streptococcal protein B, GspB) [82] and *Streptococcus sanguinis* (serine-rich protein A, SrpA, and hemagglutinin salivary antigen, HSA) [83] and was shown to bind to sialic acid residues. In addition, vWF is used by bacteria for bridging to GPIbα as was shown for *S. aureus* surface protein A (SpA) and an uncharacterized *H. pylori* surface protein [68,84].

TLRs are type 1 transmembrane proteins widely expressed on eukaryotic cells and best characterized on immune cells such as macrophages and dendritic cells. The ectodomain of TLRs contains leucine-rich β-sheets interacting with PAMPs and a Toll-interleukin-1 receptor domain for signal transduction [85]. Platelets express TLR1, TLR2, TLR4, TLR7, and TLR9, with TLR4 being the most abundant [86,87]. Next to the LPS-recognizing TLR4, TLR2 is also important in bacterial infections. TLR2 contains a glycosylated N-terminal ligand-binding domain with leucine-rich repeats [88] and recognizes bacterial lipoproteins [89]. Platelet TLR2 has been shown to be a target of *S. pneumoniae* and group B streptococci (GBS). Binding induces activation of the phosphoinositide-3 (PI3)-kinase pathway, finally leading to platelet activation and aggregation [90,91]. The interaction between pneumococci and platelet TLR2 induces further activation of integrin αIIbβ3 as well as release of dense granules [90]. Furthermore, TLR9 is also involved in bacteria-induced platelet aggregation. TLR9 recognizes cell-free bacterial DNA, which is increased in the blood of septic patients, leading to activation of coagulation [92].

In addition to the above-mentioned platelet receptors, the zinc-dependent metalloproteinase ADAM10 is a receptor for the *S. aureus* α-hemolysin (Hla), and binding leads to cleavage of GPVI [93]. Hla is a β-barrel pore-forming toxin, forming pores of 1–3 nm in diameter in the lipid bilayer of eukaryotic cells. These pores allow molecules up to a size of 4 kDa to pass through [94,95]. Hla is expressed by most *S. aureus* clinical isolates, and expression levels have been reported to correlate with virulence and disease severity [96,97]. Interactions of platelets with Hla have been reported to cause platelet activation and aggregation [98,99]. In addition, we recently demonstrated that Hla-mediated platelet activation is followed by loss of platelet function, leading to impaired thrombus formation and reduced stability of formed thrombi [100].

5. Platelets in *S. pneumoniae* Infections

S. pneumoniae exhibits the typical characteristics of Gram-positive bacteria. Pneumococci are enclosed by a flexible bilipid membrane, which is surrounded by a thick, multi-layered peptidoglycan sacculus composed of highly cross-linked glycan strands [101]. Besides peptidoglycan, teichoic acids are a general constituent of the Gram-positive cell wall [102]. Pneumococci possess rather complex, structurally unique, peptidoglycan-anchored wall teichoic (WTA) and membrane-anchored lipoteichoic acids (LTA), which are built up of identical repeating sugar units, highly decorated with phosphorylcholine [103,104]. Pneumococci shield themselves from the environment by a thick polysaccharide capsule enveloping the cell wall, whose composition is serotype-specific [105]. The capsule is the main virulence factor of pneumococci and protects the bacteria effectively from opsonization and phagocytosis by the host immune system [106]. Furthermore, four classes of proteins can be found on the surface of pneumococci, which can be classified by their mode of anchoring. The largest group with 37 predicted members is the group of lipoproteins, which are anchored to the bacterial cell membrane via an N-acyl diacylglycerol group [107,108]. Most of the lipoproteins are predicted to be part of ABC-transporters, which are essential for nutrient uptake and therefore directly involved in bacterial fitness [109]. Other lipoproteins have been shown to have essential functions in protein folding, cell wall biosynthesis, stress response, or pathogenicity [110–113]. The second group of pneumococcal surface proteins is the

unique group of choline-binding proteins (CBPs). This group consists of 13–16 proteins (strain dependent), which contain N- or C-terminally a choline-binding domain composed of highly conserved choline-binding modules. CBPs are non-covalently bound to the phosphorylcholine moiety of the repeating units of WTA and LTA [114]. Well-characterized CBPs are the major autolysin LytA, which plays an important role in autolysis and virulence and the pneumococcal surface protein C (PspC), which is essential for pneumococcal colonization and pathogenesis [115–119]. The third class of pneumococcal surface proteins (about 13–19 members) contains a C-terminal cell wall-sorting signal beginning with a LPXTG amino acid motif [120,121]. The transpeptidase sortase A (SrtA) recognizes this motif; cleaves between the threonine and glycine residues; and anchors the protein to lipid II, which is subsequently incorporated into the peptidoglycan of the cell wall [122,123]. Known representatives from this group are, for example, the neuraminidase A (NanA) and the pneumococcal adhesion and virulence factor B (PavB), which were shown to be involved in pneumococcal adhesion and pathogenesis. The fourth group of pneumococcal surface proteins is the so-called moonlighting proteins, also known as non-classical surface proteins. This group includes enzymes that are actually ubiquitous intracellularly but can also be found on the surface of bacteria, where they play an additional role, often associated with virulence [124]. One example of such a protein is the pneumococcal enolase, which intracellularly converts 2-phosphoglycerate to phosphoenolpyruvate during glycolysis, but can also be found on the bacterial surface. Here, the enolase was shown to bind host plasminogen as well as the human complement inhibitor C4b-binding protein, leading to enhanced adherence to epithelial and endothelial cells and complement evasion [125,126].

5.1. Community Acquired Pneumonia (CAP)

S. pneumoniae is one of the leading causes of community-acquired pneumonia (CAP). CAP is a potentially life-threatening disease, with a mortality rate of up to 14% in hospitalized patients [127]. The highest risk for an infection with severe outcomes is in young children, the elderly, immunocompromised patients, and those with comorbidities [128]. During severe CAP, systemic platelet activation [129] and dropping platelet counts have been reported. The development of thrombocytopenia correlates with increased mortality [130,131]. As discussed in Section 5.4, *S. pneumoniae* directly and indirectly stimulates platelets, leading to activation and release of granule content, which is accompanied with the release of antimicrobial peptides (AMPs). However, although AMPs are released, *S. pneumoniae* is not affected by platelet releasates, but in turn destroys platelets themselves [132]. Neutrophils are one of the most important players in the progression of inflammation and also sepsis. In response to bacteria, neutrophils form NETs built up of neutrophil DNA, histones, and granular proteins such as defensins, thereby often leading to bacterial trapping and antimicrobial actions [133,134]. Neutrophil-derived defensins inhibit the synthesis of bacterial DNA, RNA, and proteins. In addition, lysozymes degrade the bacterial cell wall, and elastase cleaves bacterial surface virulence factors [135]. NETs and their extracellular histones mediate initiation and proceeding of platelet activation and coagulation, leading to a prothrombotic phenotype [136]. Pneumococci evade trapping in NETs by protective effects mediated by D-alanylation of LTA [137], blocking of NET binding via pneumococcal surface protein A [138], and by degrading the neutrophil DNA scaffold via endonuclease A [139]. Taken together, *S. pneumonia*e is able to evade the platelet induced immune response, and on the contrary triggers an inflammatory and coagulant phenotype of platelets during severe CAP.

5.2. Sepsis

Sepsis is a common complication of pneumococcal infections, with mortality rates of up to 30%. Sepsis leads to an activation of the coagulation cascade with consumption of circulating coagulation factors, platelets, and the generation of platelet–leukocyte complexes. As a result, patients develop thrombocytopenia and DIC, finally leading to hypoxic organ damage. Up to 50% of patients with severe sepsis develop DIC [140]. Several factors lead to

DIC. First, coagulation is activated via different pathways [134,141,142]. Second, there is an increase of endothelial adhesion molecules, leading to platelet adhesion to endothelial cells and exposed subendothelial collagen. During these processes, platelet activation, aggregation, microthrombus formation, and finally vessel occlusion are triggered [99,143–145]. In addition, neutrophils are attracted and affected during sepsis. High numbers of circulating immature forms of neutrophils in the peripheral, impaired migration but also prolonged presence of NETS are commonly observed in sepsis [146,147]. NET formation indirectly triggers tissue factor release of endothelial cells, which is then captured in NETs, further triggering coagulation [144,148]. Another factor is the massive amount of released reactive oxygen species (ROS). ROS favor vasoconstriction and have an activating effect on platelets [149]. Next to aggregation and thrombus formation, platelets also play a role in inflammatory processes, which are associated with sepsis. Platelet-derived soluble CD40L is increased in the circulation of septic patients [150–152] and plays a central role in activation and recruitment of neutrophils by activation of the β2 integrin Mac-1 of neutrophils and indirectly via macrophage inflammatory protein-2 and subsequent CXCR2 signaling [153,154]. Under septic conditions, activation of CXCL4 is also increased. This results in platelet degranulation, release of proinflammatory factors, and stimulation of the coagulation cascade [155]. Taken together, bacterially induced microthrombus and NET formation can cause uncontrolled coagulation and inflammation, leading to thrombocytopenia and DIC during sepsis.

5.3. Infective Endocarditis

Development of infective endocarditis is a complication of bacteremia caused by *S. pneumoniae*. In the pre-antibiotic era, *S. pneumoniae* caused about 15% of IE cases. Upon usage of penicillin or cephalosporine, the prevalence dropped to approximately 3% in the 1990s, and currently, prevalence data are missing [156,157]. IE caused by pneumococci mainly affects the aortic valves, and patients often also suffer from acute pneumonia; a common complication of pneumococcal IE is embolism [158]. Before bacteria can colonize the valves and cause IE, the surface structure of the endothelial valves has to be altered, as observed after previously occurred endocarditis or valve replacement [159]. On these structural changes, fibrin and platelets adhere, forming the so called non-bacterial thrombotic vegetation (NBTV) [160,161]. These NBTV serve as a niche for colonization in transient bacteremia or in the case of oral streptococci, after dental treatment [162–164], leading to further platelet aggregation on the surface and fibrin deposition [160]. By further acquisition of fibrin and platelets, bacteria are shielded from the immune system, allowing bacteria to reach very high densities [24,165].

5.4. Pneumococcal Interactions with Platelets

In contrast to *S. aureus*, little is known about the interaction between *S. pneumonia*e and platelets. The first studies describing platelet activation due to pneumococci were published in the 1970s. These studies showed that some pneumococcal serotypes induced platelet activation and aggregation in vitro, whereas other serotypes had no effect on platelet activation [166,167]. Later, platelet aggregation was shown to be induced only in interactions with encapsulated strains via an interaction with TLR2, but not with nonencapsulated strains [90]. However, other studies reported contradictory results. De Stoppelaar and colleagues did not observe platelet aggregation in encapsulated strains [168]. In addition, a TLR2-independent platelet degranulation was observed, which could be confirmed in mice with knockouts for several TLRs [168]. However, recent studies, including our own, demonstrated a direct binding of pneumococci to platelets. One study showed aggregate formation between platelets and pneumococci. This aggregate formation was dependent on the presence of soluble fibrin and the presence of TSP-1 derived from activated platelets [169]. The pneumococcal adhesins PavB and PspC are hypothesized to bind to platelet GPII/bIII via bridging of TSP-1 [170,171].

One of the major virulence factors of *S. pneumoniae* is pneumolysin (Ply), a cholesterol-dependent cytolysin that oligomerizes into the membrane after binding to the target cell, leading to pore formation [172]. Ply has been shown earlier to activate and aggregate platelets depending on Ca^{2+}-influx through pneumolysin pores [173,174]. However, our own data demonstrate platelet killing by pneumolysin (Figure 4) [47].

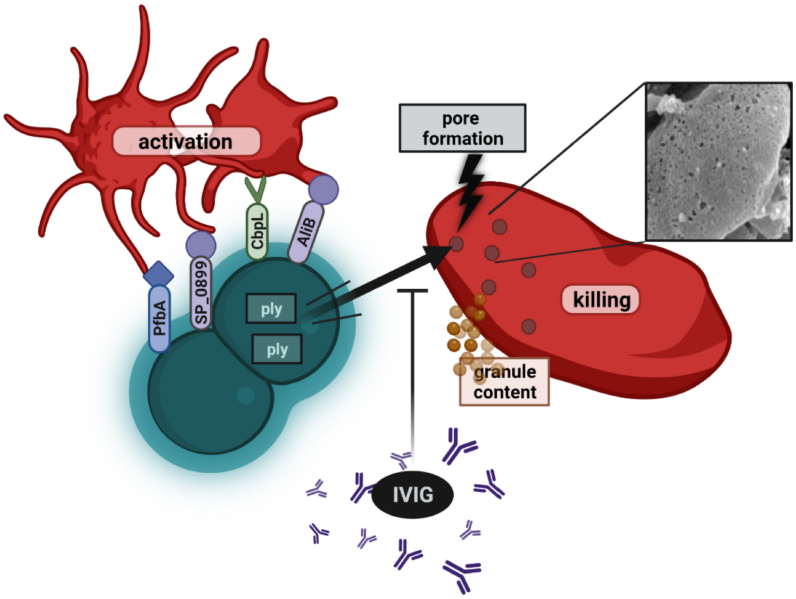

Figure 4. Scheme of different interactions between platelets and pneumococci. Individual pneumococcal surface proteins can induce direct activation of platelets. On the other hand, the intracellular pneumolysin (Ply) kills platelets by extensive pore formation in platelet membranes, as shown by the SEM image of a Ply-treated platelet on the right side. Pneumolysin is released in the circulation upon autolysis of pneumococci, and its action on platelets can be neutralized by the addition of pharmaceutical IgG preparations. Created with BioRender.com (accessed on 21 March 2022).

In accordance with other studies, we also observed highly increased P-selectin signals in flow cytometry, suggesting massive platelet activation. However, instead, as shown by confocal imaging, only intracellular stores of P-selectin were stained due to antibodies diffusing through the pneumolysin pores. In addition, the increase in light transmission of the platelet suspension after incubation with pneumolysin is due to cell lysis instead of aggregation. Platelet lysis occurred immediately after addition of pneumolysin, even at very low concentrations. A loss of platelet function was also observed in whole blood experiments [47]. Nevertheless, we also observed increased P-selectin signals in lysates of *ply* knockout strains, which appear independent of pneumococcal-derived H_2O_2, since P-selectin levels were similar in a *ply*-mutant and a Δ*spxB*Δ*ply* double mutant (Figure 5B). Therefore, we hypothesized that other surface-associated proteins of *S. pneumoniae* trigger platelet activation. A screening of our library of pneumococcal surface proteins (Table 1) in activation assays with washed platelets revealed candidate proteins, which directly activate platelets. Among them were SP_0899, a lipoprotein of thus far unknown function, and CbpL, a choline-binding protein and putative adhesion contributing to colonization [175]. In addition, AliB and SP_1833 also induced at least a slightly increased P-selectin staining. AliB is a lipoprotein and functions as a substrate-binding protein for oligopeptides [176], and SP_1833 (PfbA) is a sortase-anchored protein with plasmin- and fibronectin-binding capacity [177] (Figure 3A). All tested pneumococcal lipoproteins were heterologously expressed without the lipid moiety. The naturally occurring lipidation of lipoproteins

has been shown to trigger TLR2-dependent signaling in leukocytes [178,179]. Therefore, lipidated proteins were tested in comparison to their non-lipidated forms [179], but no activation leading to P-selectin surface staining was detected. Nevertheless, it is noteworthy to know that TLR expression levels in resting platelets is low, but they become upregulated, and levels increase on the platelet surface upon activation [87,180]. Since proteins with platelet activation potential were identified in all groups of pneumococcal surface proteins, mutants were applied in activation assays lacking pneumolysin or whole groups of surface proteins [107,177]. This was achieved by the deletion of genes encoding essential enzymes involved in the anchoring of proteins (prolipoprotein diacylglyceryl transferase Lgt for lipoproteins and SrtA for sortase-anchored proteins) to the bacterial surface. To remove CBPs from the surface of pneumococci, a *ply* mutant was treated with choline chloride. During severe invasive infections, antibiotic treatment autolysis as well as antibiotic-induced lysis occurs in the bloodstream. Therefore, lysates generated from these deletion or choline chloride-treated strains were also tested for their platelet activation potential. Independent of the genetic background, all lysates induced increased P-selectin signals at similar levels (Figure 1B), leading to the assumption that another factor, e.g., components of the cell wall could be responsible for platelet activation. However, isolated and structurally defined protein-free pneumococcal lipoteichoic acids as well as wall teichoic acids [181] had no impact on platelet P-selectin surface expression (Table 1). Taken together, besides the identified surface proteins, another thus far unknown factor might be able to induce direct platelet activation. Nevertheless, the lytic effect of pneumolysin on platelets, even at very low concentrations, probably overshoots any other effect of pneumococcal proteins on platelets in invasive infections (Figures 1 and 4).

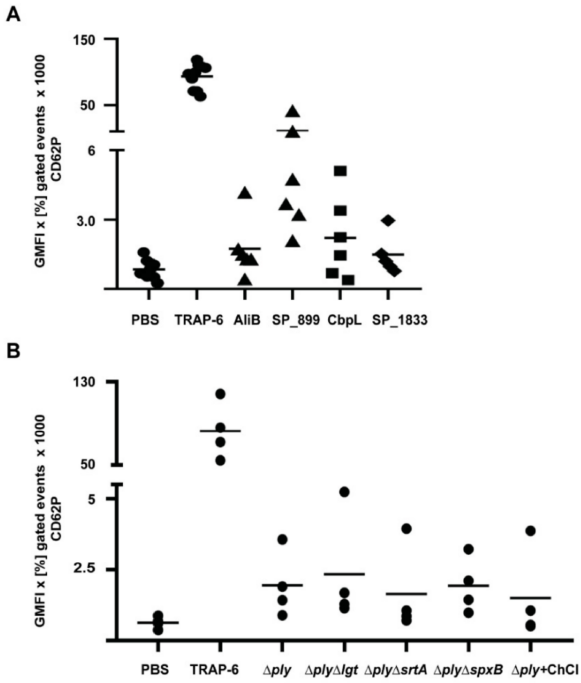

Figure 5. Individual pneumococcal proteins and pneumococcal lysates directly activate human platelets. Washed platelets of a defined set of donors were incubated with different concentrations of pneumococcal proteins (**A**) (Table 1) for 30 min or pneumococcal lysates (**B**) with the indicated genetic backgrounds for 60 min at 37 °C. CD62P was used as an activation marker and was detected

by flow cytometry, using a PE-Cy5-labelled P-selectin antibody. PBS was used as negative control, and 20 μM TRAP-6 was used as a positive control. 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.

Table 1. List of pneumococcal proteins and cell wall components, which were tested to activate platelets and led to CD62P expression. * The last column provides the highest tested concentration of each protein in the platelet activation assay. The molarities were chosen on the basis of previous publications determining platelet activating potential of bacterial proteins [24].

Protein Class	No.	Protein Name SP Number	Function	Activation of Washed Platelets	Protein Concentration (μM) *
Lipoproteins	1	AdcAII (SP_1002)	substrate-binding protein of ABC transporter for zinc(II) ions	-	4
	2	AliB (SP_1527)	substrate-binding protein of ABC transporter for oligopeptides	+	2
	3	AliC	substrate-binding protein of ABC transporter for oligopeptides	-	4
	4	AliD	substrate-binding protein of ABC transporter for oligopeptides	-	4
	5	AmiA (SP_1891)	substrate-binding protein of ABC transporter for oligopeptides	-	4
	6	DacB (SP_0629)	L,D-carboxypeptidase, peptidoglycan turnover	-	4
	7	Lipidated DacB	L,D-carboxypeptidase, peptidoglycan turnover	-	4
	8	Etrx1 (SP_0659)	extracellular thioredoxin protein 1	-	4
	9	Etrx2 (SP_1000)	extracellular thioredoxin protein 2	-	4
	10	MetQ (SP_0149)	substrate-binding protein of ABC transporter for methionine	-	4
	11	Lipidated MetQ	substrate-binding protein of ABC transporter for methionine	-	4
	12	PccL (SP_0198)	transport of small hydrophobic molecules such as siderophores	-	4
	13	PiaA (SP_1032)	substrate-binding protein of ABC transporter for iron	-	4
	14	PnrA (SP_0845)	substrate-binding protein of ABC transporter for nucleosides	-	4
	15	PpmA (SP_0981)	proteinase maturation protein, peptidyl-prolyl isomerase	-	4
	16	PsaA (SP_1650)	substrate-binding protein of ABC transporter for manganese	-	4
	17	SlrA (SP_0771)	streptococcal lipoprotein rotamase, peptidyl-prolyl isomerase	-	4
	18	GshT (SP_0148)	substrate-binding protein of ABC transporter for glutathione	-	4

Table 1. *Cont.*

Protein Class	No.	Protein Name SP Number	Function	Activation of Washed Platelets	Protein Concentration (μM) *
	19	SP_0191	unknown function	-	4
	20	SP_0899	unknown function	+++	2/4
	21	FusA (SP_1796)	substrate-binding protein of ABC transporter for fructo-oligosaccharides	-	2
	22	RafE (SP_1897)	substrate-binding protein of ABC transporter for multiple sugars	-	4
	23	PstS (SP_2084)	substrate-binding protein of ABC transporter for phosphate ions	-	-
	24	SP_1690	substrate-binding protein of ABC transporter	-	4
	25	MalX (SP_2108)	substrate-binding protein of ABC transporter for maltose/maltodextrin	-	4
	26	SatA (SP_1683)	substrate-binding protein of ABC transporter for sialic acid	-	4
CBPs	27	CbpC (SP_0377)	regulatory function for autolysis by inhibiting autolysin LytC	-	4
	28	CbpF (SP_0391)	putative adhesin	-	4
	29	CbpL (SP_0667)	putative adhesin	++	4
	30	Chimeric (PspA+PspC)	fusion of N-terminal domains of PspA and PspC	-	4
	31	PcpA (SP_2136)	adhesin	-	2
	32	PspA_QP2 (SP_0117)	virulence factor, binds lactoferrinand inhibits complement activation	-	4
	33	PspC_SH2 (SP_2190)	adhesion, IgA inactivation, major factor H–binding protein	-	4
Sortase–anchored proteins	34	PfbA (SP_1833)	plasmin- and fibronectin-binding protein	+	2
	35	PitB (spt_1059)	pilin of pneumococcal pilus-2, adhesin	-	2
	36	PsrP (SP_1772)	adhesion, biofilm formation	-	4
	37	RrgB (SP_0463)	pilus-1 anchorage protein	-	4
	38	RrgC (SP_0464)	pilus-1 backbone protein, pilin	-	4
	39	SP_1992	adhesin architecture, bind to collagen and lactoferrin in vitro	-	4
Cell wall components	40	lipoteichoic acids		-	4
	41	wall teichoic acids		-	40 μg/mL

6. Relevance of Findings for Disease

Thus far, there are only a few studies focusing on platelet activation during pneumococcal infections. One study showed that the expression of *pblB*, a phage-derived gene, was associated with increased platelet activation and mortality in hospitalized patients suffering from CAP caused by *S. pneumoniae* [129]. Another study showed increased

platelet activation and platelet hyperreactivity in a porcine model of invasive *S. pneumoniae* infections [182]. In a follow-up in vitro study, the authors demonstrated that desialylation of platelets by the pneumococcal neuraminidase A (NanA) results in hyperreactivity of platelets to ADP stimulation [183].

One approach to interfere with platelet dysfunction in pneumococcal infections is directly targeting and neutralizing the pore forming pneumolysin with antibodies. Promising candidates are the pharmaceutically available IgG preparation IVIG (Privigen, 98% IgG) or the mixed immunoglobulin preparation trimodulin (21% IgA, 23% IgM, 56% IgG). Both immunoglobulin preparations contain antibodies against pneumolysin and have been shown to efficiently inhibit platelet damage in vitro, as shown by rescued platelet function and viability even in the presence of high pneumolysin concentrations (Figure 4) [47,184]. The presence of IgM and IgA in trimodulin had no beneficial effect for pneumolysin neutralization. In fact, neutralization efficiency was dependent on IgG content in the immunoglobulin preparation [184]. In a phase 2 clinical trial (CIGMA study), patients with severe community-acquired pneumonia were treated with trimodulin in addition to standard care. In the trimodulin group, patients had higher platelet counts and a nominally lower mortality compared to the placebo group [47]. However, patients included in the study were only 160, and larger clinical trials are necessary to confirm this observation.

A major cause for thrombocytopenia is coagulopathy conditions, observed in 80% of septic patients, with DIC being the most severe form [185]. Therefore, treatment of coagulopathies is highly needed to reduce mortality rates and coagulation-associated tissue damage. The focus of ongoing research lies on inhibition of coagulation or the use of anti-platelet drugs. Anti-platelet therapy during sepsis or ARDS is not part of standard care, but several studies conclude this as a promising approach to reduce disease severity [186–189]. However, the benefit of anti-platelet drugs such as, e.g., acetylsalicylic acid during sepsis is under debate [189,190], and more stringent clinical studies are needed. The same accounts for inhibition of coagulation. Although some clinical studies conclude damped disease severity upon usage of, e.g., antithrombin, its benefit is controversial, and more research on this topic is needed [191,192].

7. Conclusions

In this review, we highlight interactions between platelets and *S. pneumoniae* or *S. aureus* in vitro and in disease. Multiple factors of both bacteria result in direct or indirect platelet activation and aggregation. However, the predominant effect seems to be exerted by the pore-forming toxins pneumolysin (pneumococci) and alpha-hemolysin (Hla, *S. aureus*). Whereas Hla first activates and later on lyses platelets, pneumolysin directly lyses platelets. The pathomechanisms of these toxins and their impact on platelet function are of high clinical importance because thrombocytopenia and DIC are typical complications in severe invasive infections caused by these pathogens. Future clinical studies targeting either bacterial components such as pneumolysin and Hla and their receptors or systemic coagulation as seen in dependence of Hla are highly needed to improve clinical outcomes.

Author Contributions: Conceptualization, S.H., K.J., T.P.K.; methodology, K.J., L.-S.S., S.W.; software, S.H.; validation, K.J., L.-S.S., T.P.K., S.H.; formal analysis, K.J., T.P.K., L.-S.S., S.W.; investigation, K.J., T.P.K., L.-S.S., S.W.; resources, S.H.; data curation, K.J., T.P.K., L.-S.S., S.W.; writing—original draft preparation, K.J.; writing—review and editing, S.H., K.J., T.P.K.; visualization, K.J.; supervision, S.H., T.P.K.; project administration, S.H.; funding acquisition, S.H. All authors have read and agreed to the published version of the manuscript.

Funding: This research was funded by the Deutsche Forschungsgemeinschaft (DFG, German Research Foundation), grant number 374031971–TRR 240 to S.H. and DFG HA 3125/5-2 to S.H.

Institutional Review Board Statement: The use of whole blood and washed platelets from healthy adult individuals was ap-proved by the Ethics Committee of the University Medicine Greifswald (BB 044/18).

Informed Consent Statement: All volunteers gave written informed consent in accordance with the Declaration of Helsinki. All experiments were carried out in accordance with the approved guide-lines.

Data Availability Statement: Data is contained within the article.

Acknowledgments: The authors thank Jan Wesche, Peggy Stremlow, and Jessica Fuhrmann for technical support. We further thank Manfred Rohde for electron microscopy image courtesy. We are grateful to Thomas Thiele (Transfusion Medicine, University Medicine Greifswald) for suggestions and critical reading.

Conflicts of Interest: The authors declare no conflict of interest. The funders had no role in the design of the study; in the collection, analyses, or interpretation of data; in the writing of the manuscript; or in the decision to publish the results.

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

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Published in Blood Advances,
2020 Dec 18. doi: 10.1182/bloodadvances.2020002372

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Experiments performed by K.J. shown in Fig. 1A-E; 4A,B; S2, S5 A-E, S10 A,B, S12, S13

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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

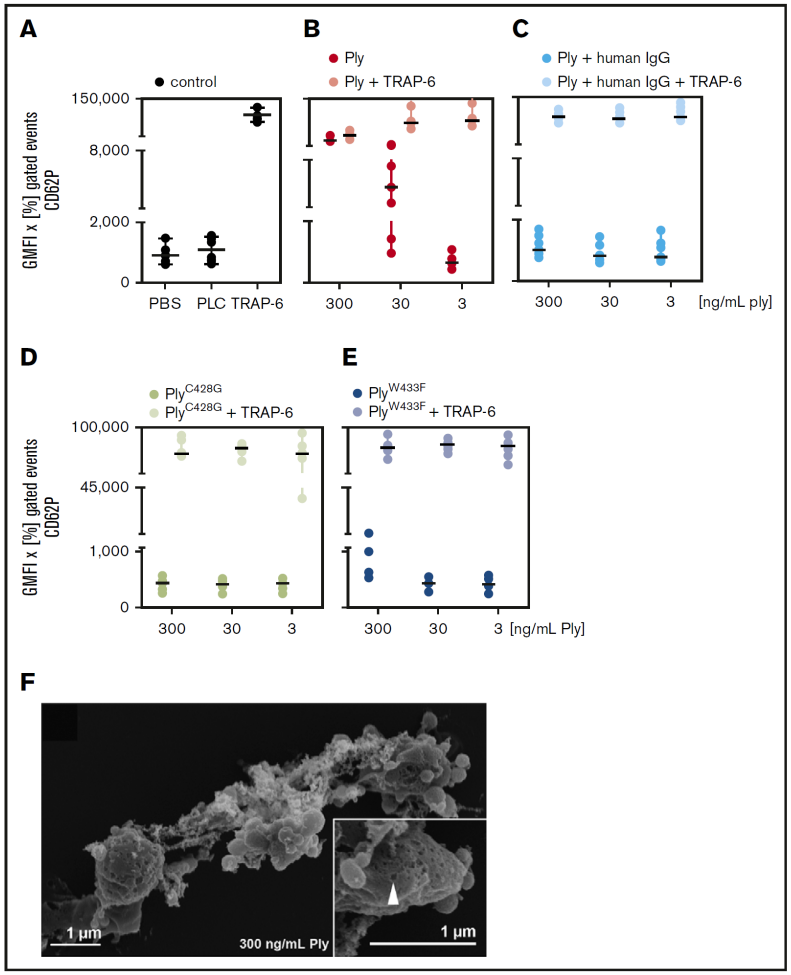

Figure 1. CD62P expression of platelets by pneumolysin is caused by pore formation. Washed platelets of a defined set of 6 donors were incubated with various concentrations of pneumolysin (Ply). CD62P was detected by flow cytometry using antibodies against CD62P (P-selectin). The data are presented as 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

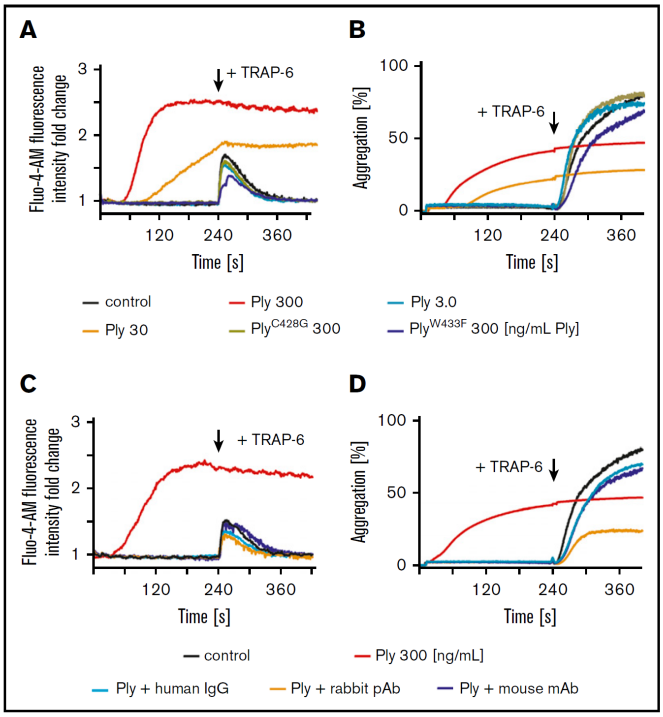

Figure 2. Loss of platelet function due to pneumolysin is prevented by immunoglobulins. (A) Prior to pneumolysin treatment intracellular Ca^{2+} of washed platelets was labeled with Fluo-4-AM for 30 minutes. After incubation with pneumolysin, the kinetics of Ca^{2+} release was measured and values are 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

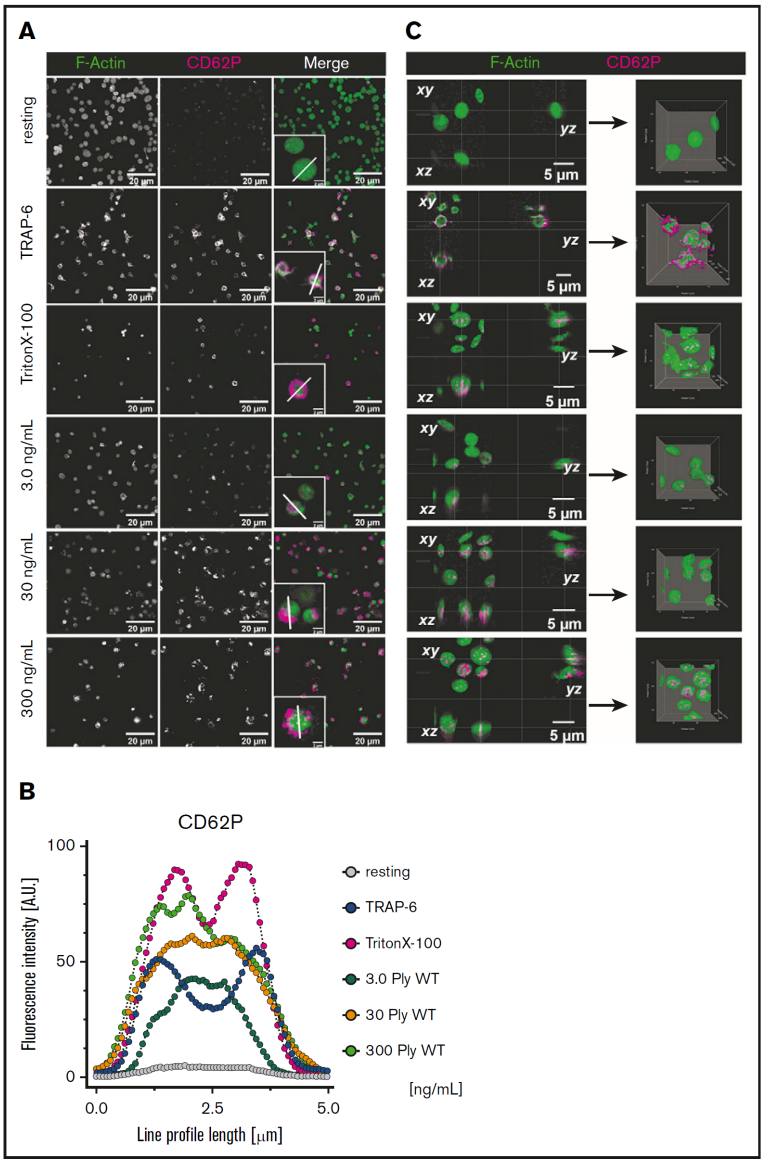

Figure 3. Fluorescence microscopy of pneumolysin-treated platelets. (A) Pneumolysin-treated platelets were stained for F-actin (green) and CD62P (magenta). Platelets were not permeabilized, with the exception of the Triton X-100 control. Insets, Single platelets at higher magnification and the line used 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

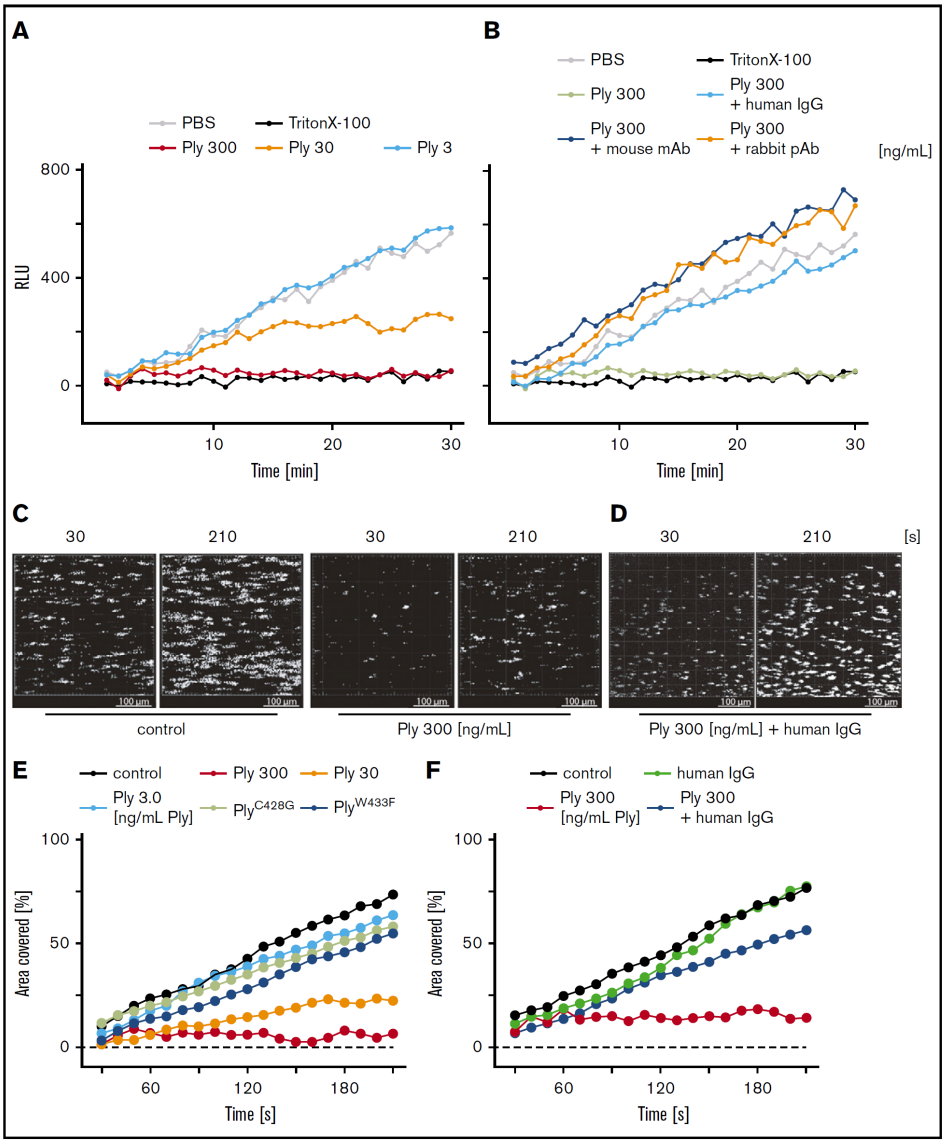

Figure 4. Pneumolysin induces platelet death. (A) Kinetics of platelet viability. PBS was used as viability control and Triton X-100 to induce platelet death. Pneumolysin in increasing concentrations induced platelet death measured by reduced substrate turnover. (B) Platelet viability was maintained 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.

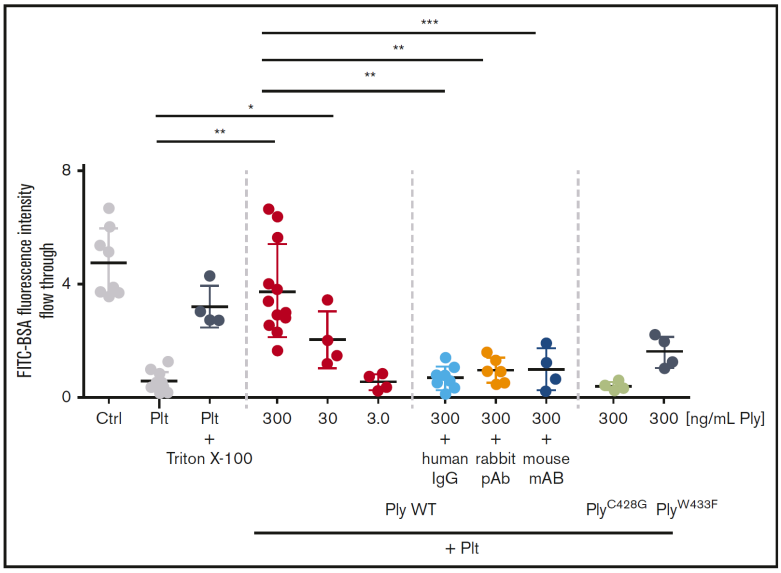

Figure 5. Sealing of Transwell membranes by platelets is impaired by pneumolysin. Platelets seal Transwell membranes with 3-μm pores. This was impaired in the presence of pneumolysin but not the pneumolysin mutants as indicated by the flow through of BSA-FITC to the lower chamber measured by fluorescence 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

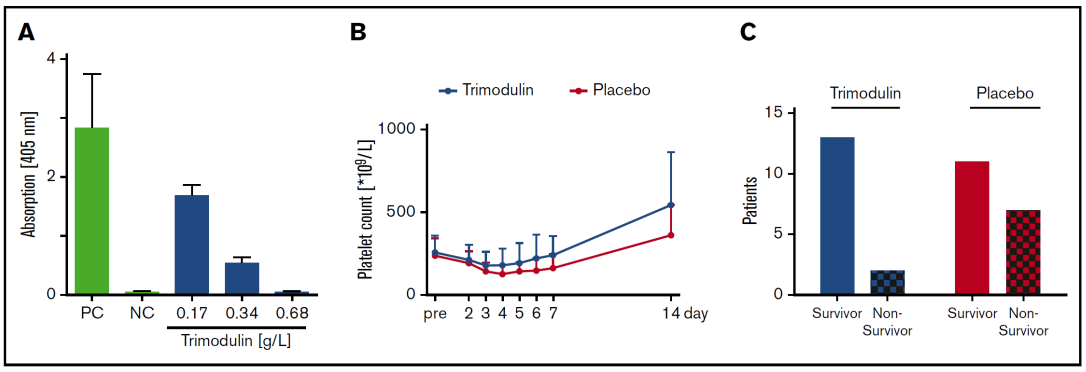

Figure 6. Neutralizaton of pneumolysin by trimodulin and relevance for severe community-acquired pneumonia patients. (A) In vitro pneumolysin neutralization assay measuring free hemoglobin as marker for cell lysis, using erythrocytes and different concentrations of trimodulin as indicated. Shown are 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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Supplemental Figures S1 – S14

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

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Figure S1

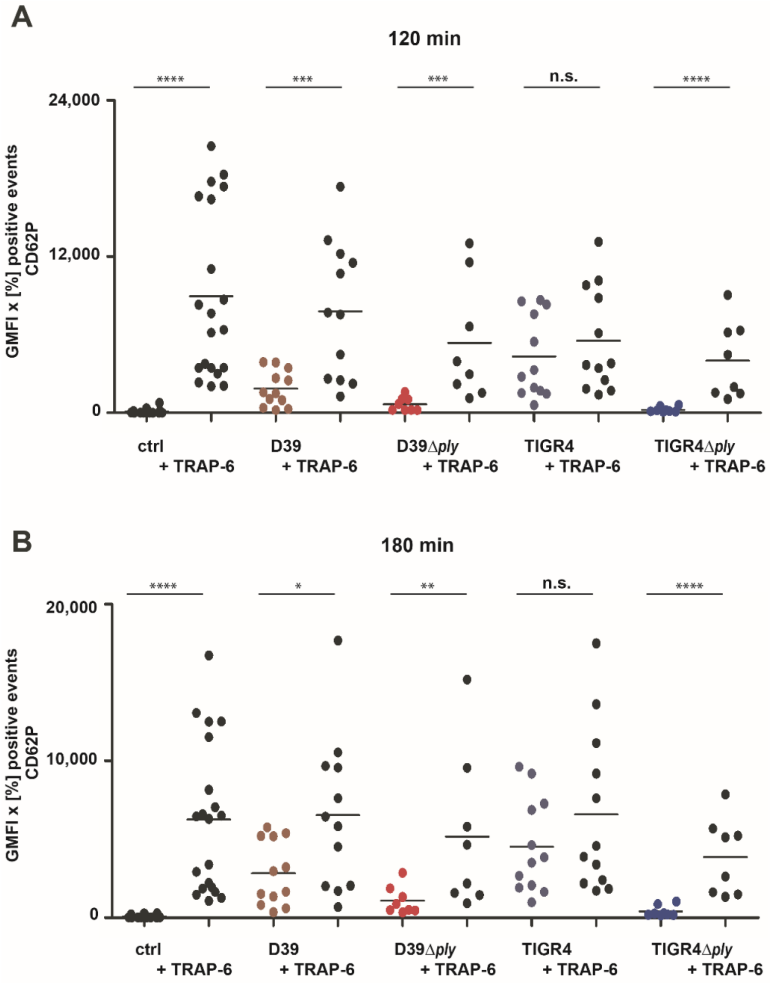

Figure S1. Platelets incubated with pneumococci producing pneumolysin stain positive for the activation marker CD62P

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

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

2

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

3

Figure S2

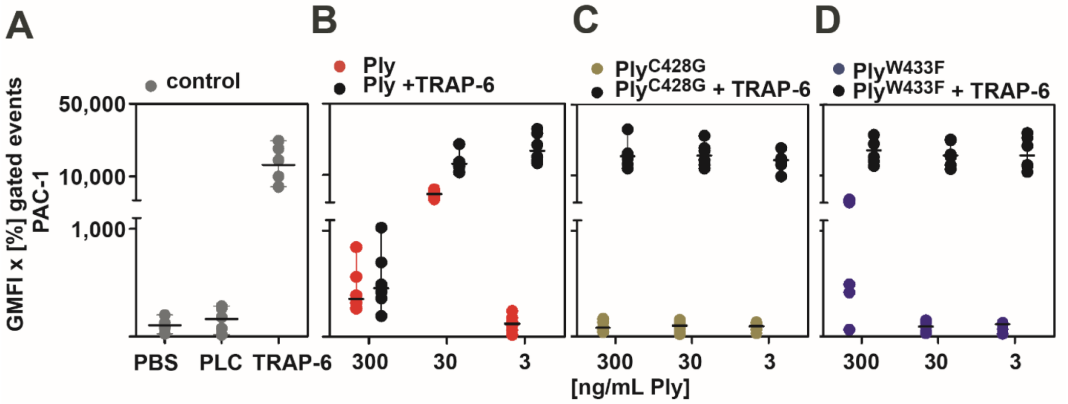

Figure S2: PAC-1 binding to platelets after activation with TRAP-6 is inhibited by high concentrations of pneumolysin

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

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

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

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

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

5

Figure S3

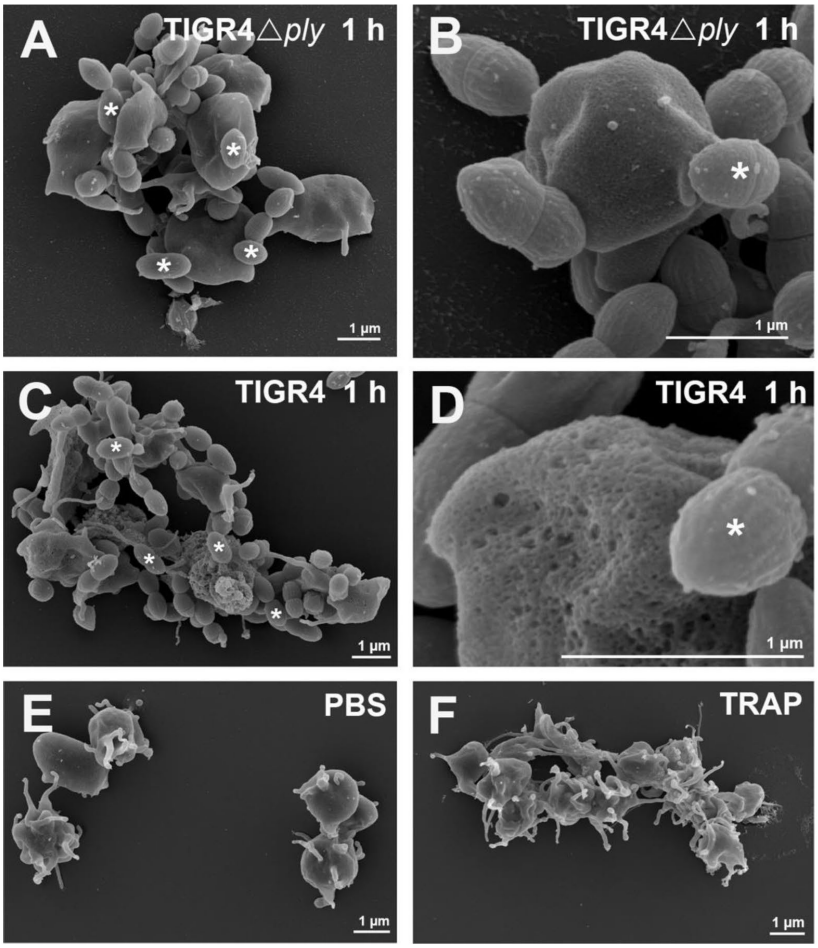

Figure S3. Visualization of pore formation in the platelet membrane by pneumococci by scanning electron microscopy.

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

Figure S4

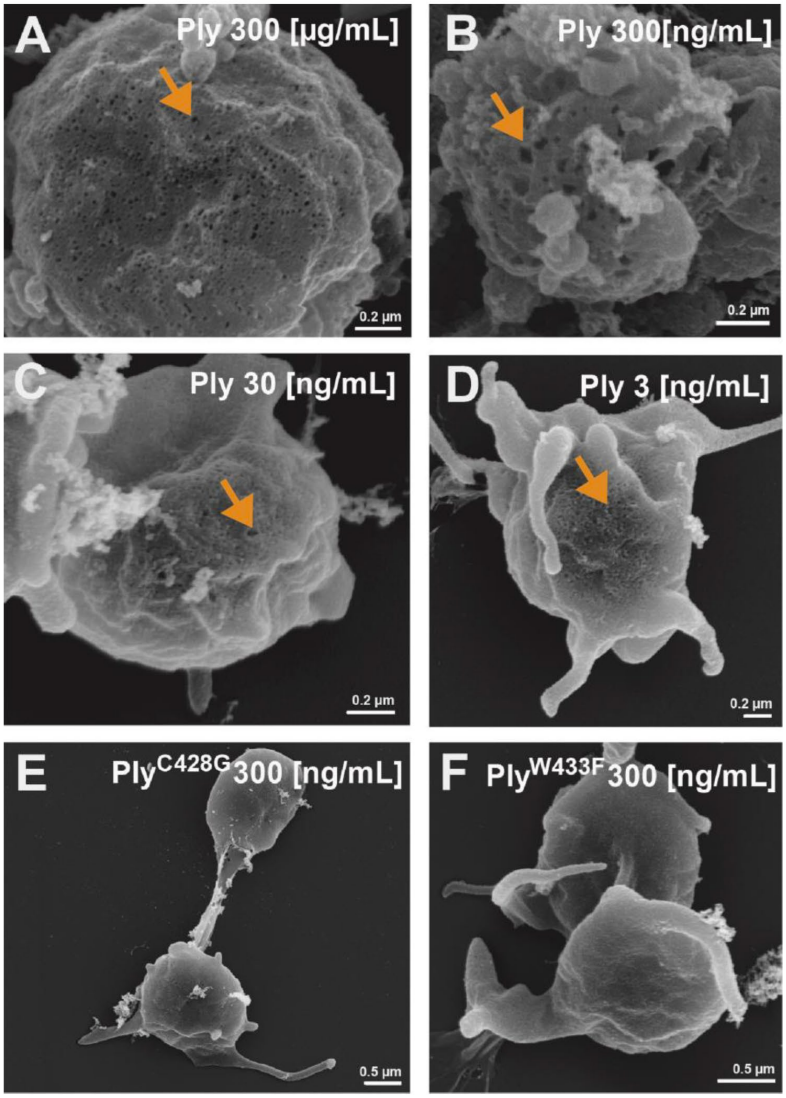

Figure S4. Visualization of pore formation in the platelet membrane by pneumolysin by scanning electron microscopy.

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

Figure S5

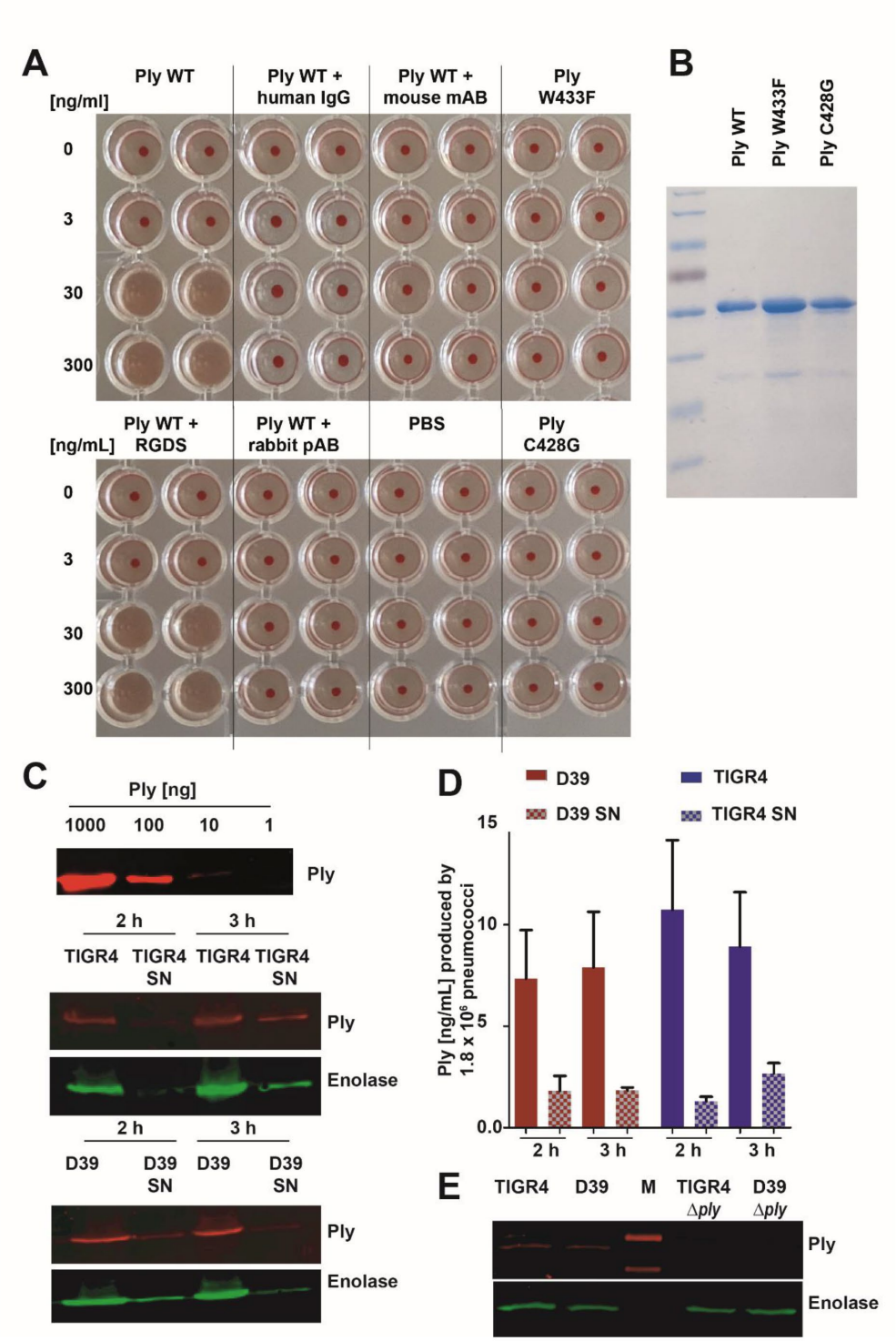

Figure S5. Pneumolysin activity test based on erythrocyte hemolysis and quantification of pneumolysin

8

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

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

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

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

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

Figure S6

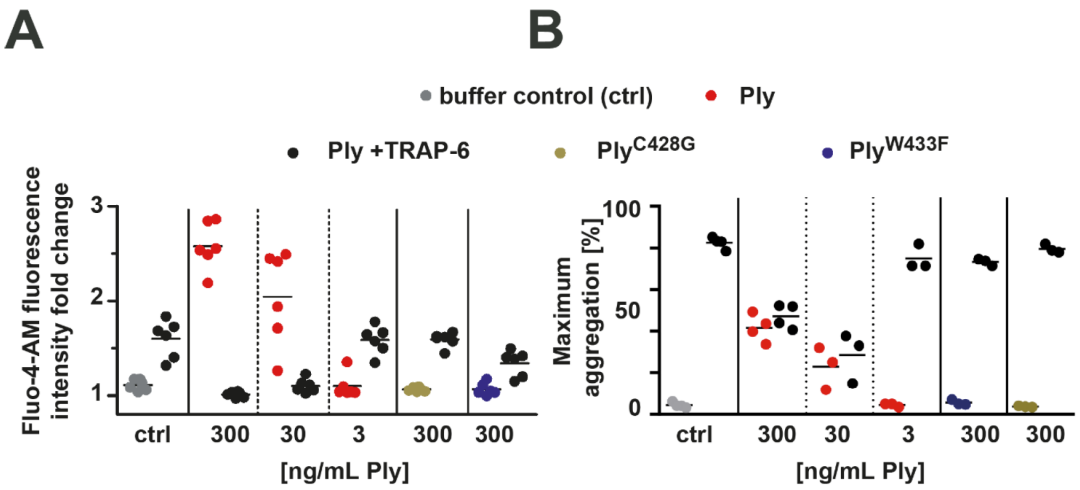

Figure S6. Pneumolysin impairs platelet function.

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

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

Figure S7

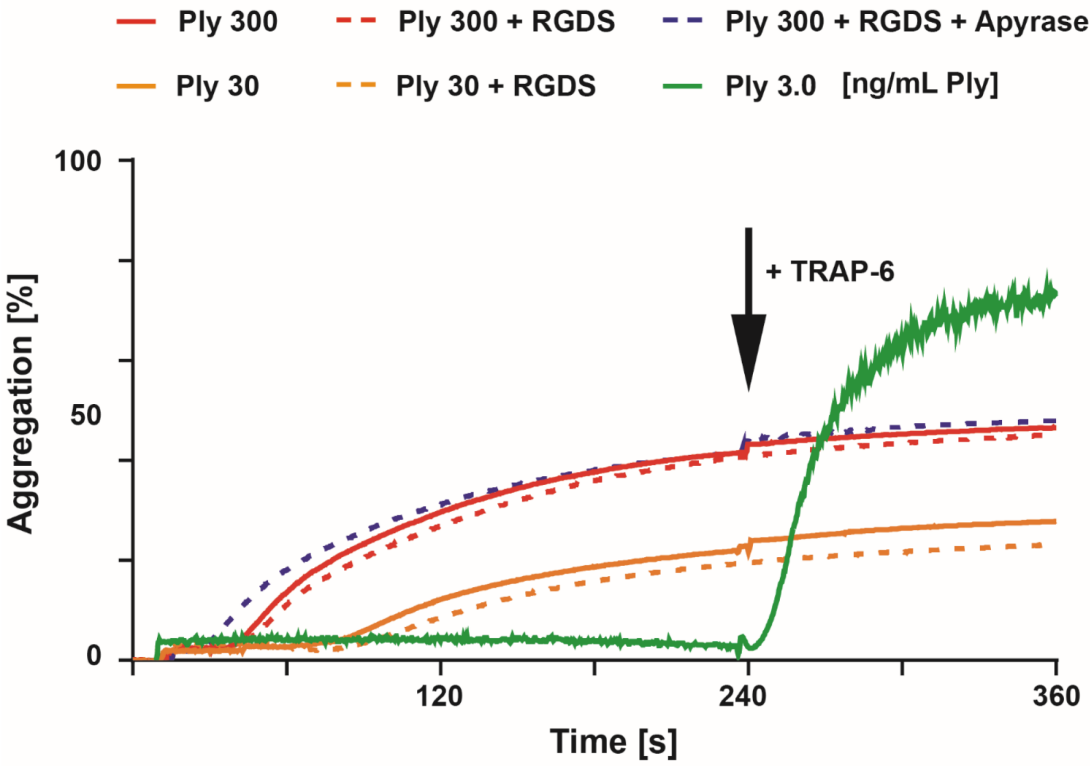

Figure S7. Blocking the platelet fibrinogen receptor αIIbβ3 binding site by RGDS does not inhibit increase in light transmission when platelets are incubated with pneumolysin.

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

Figure S8

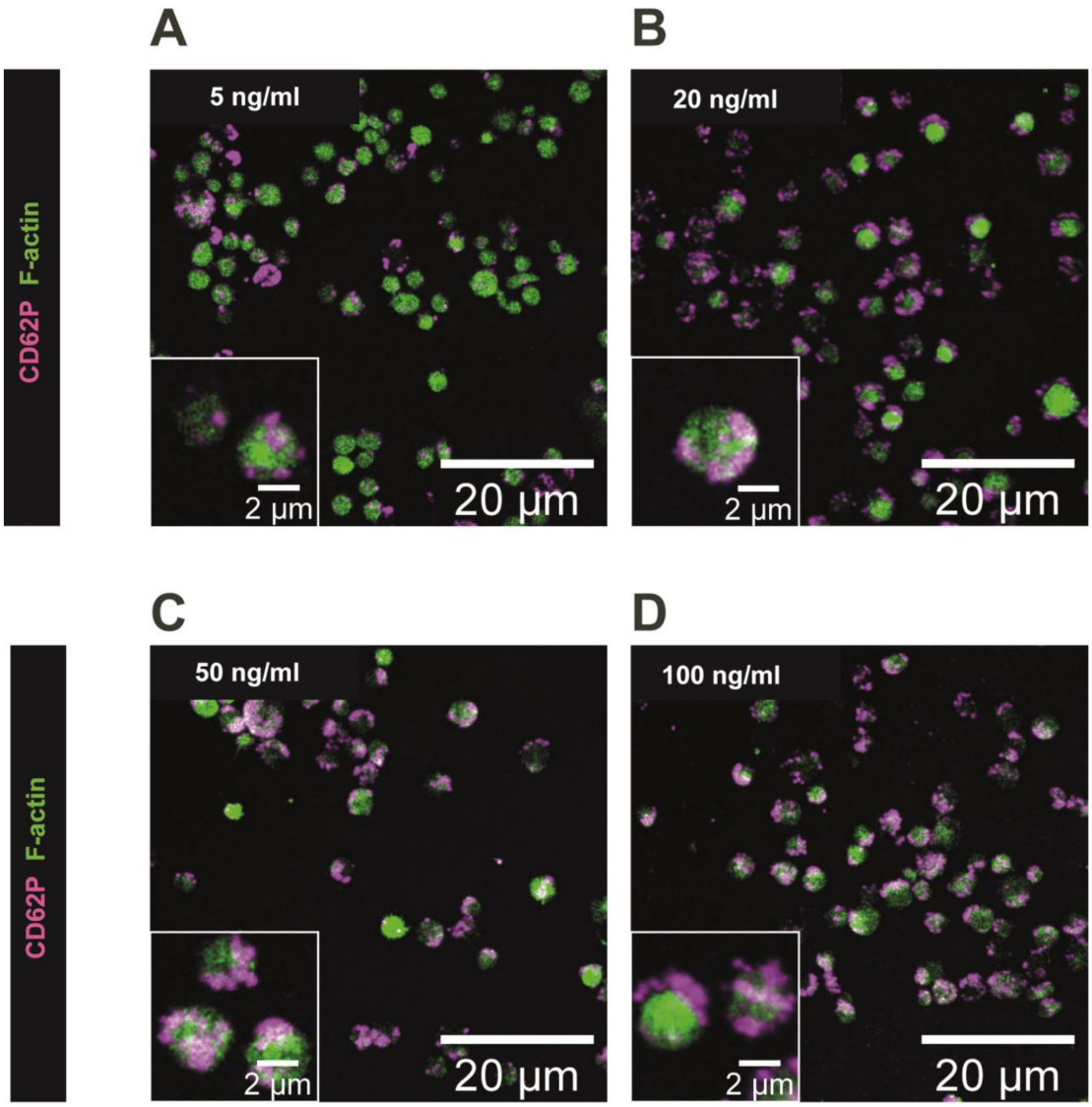

Figure S8. Pneumolysin induces staining of platelets for CD62P without inducing morphological features of platelet activation

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

Figure S9

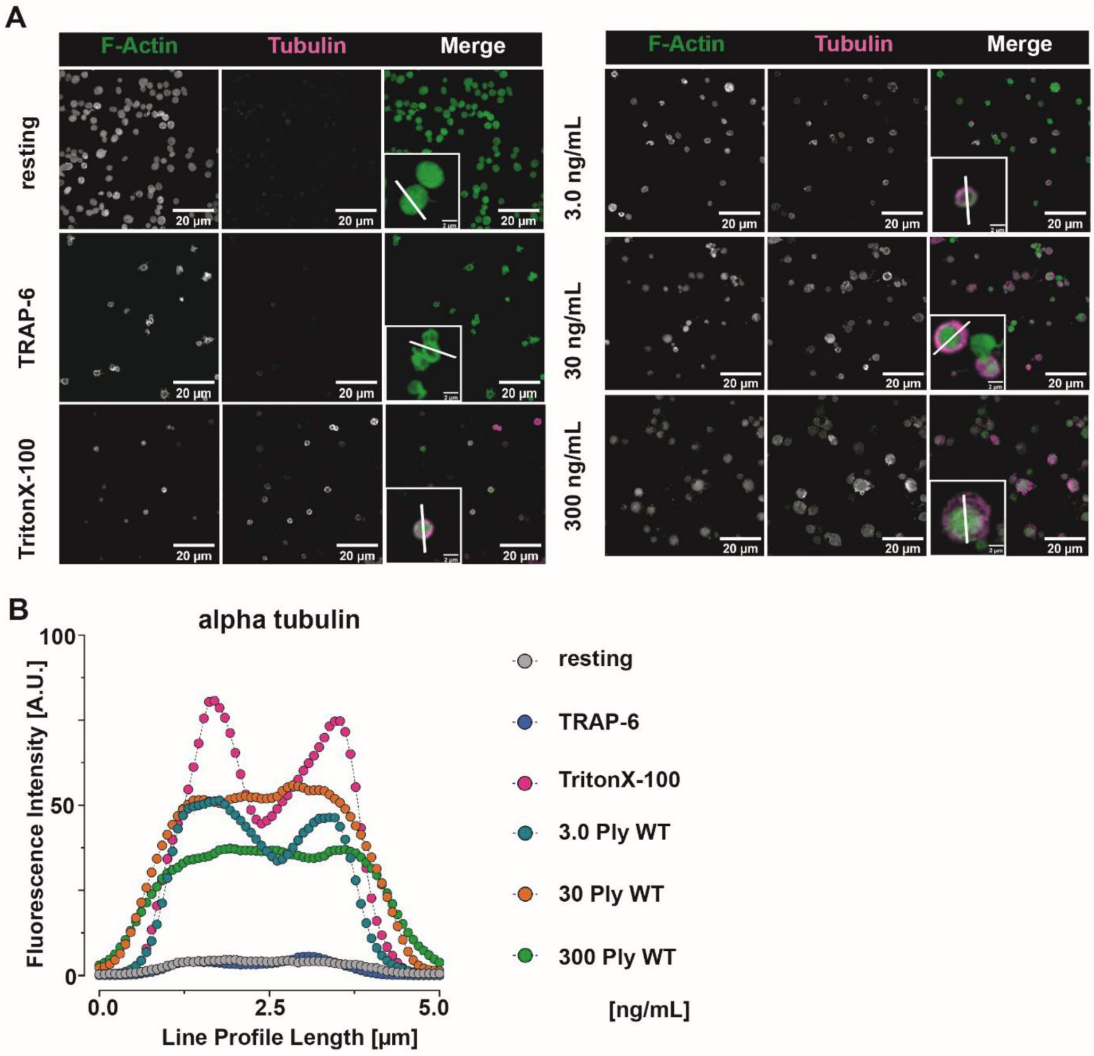

Figure S9. Fluorescence microscopy of pneumolysin treated platelets

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

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

Figure S10

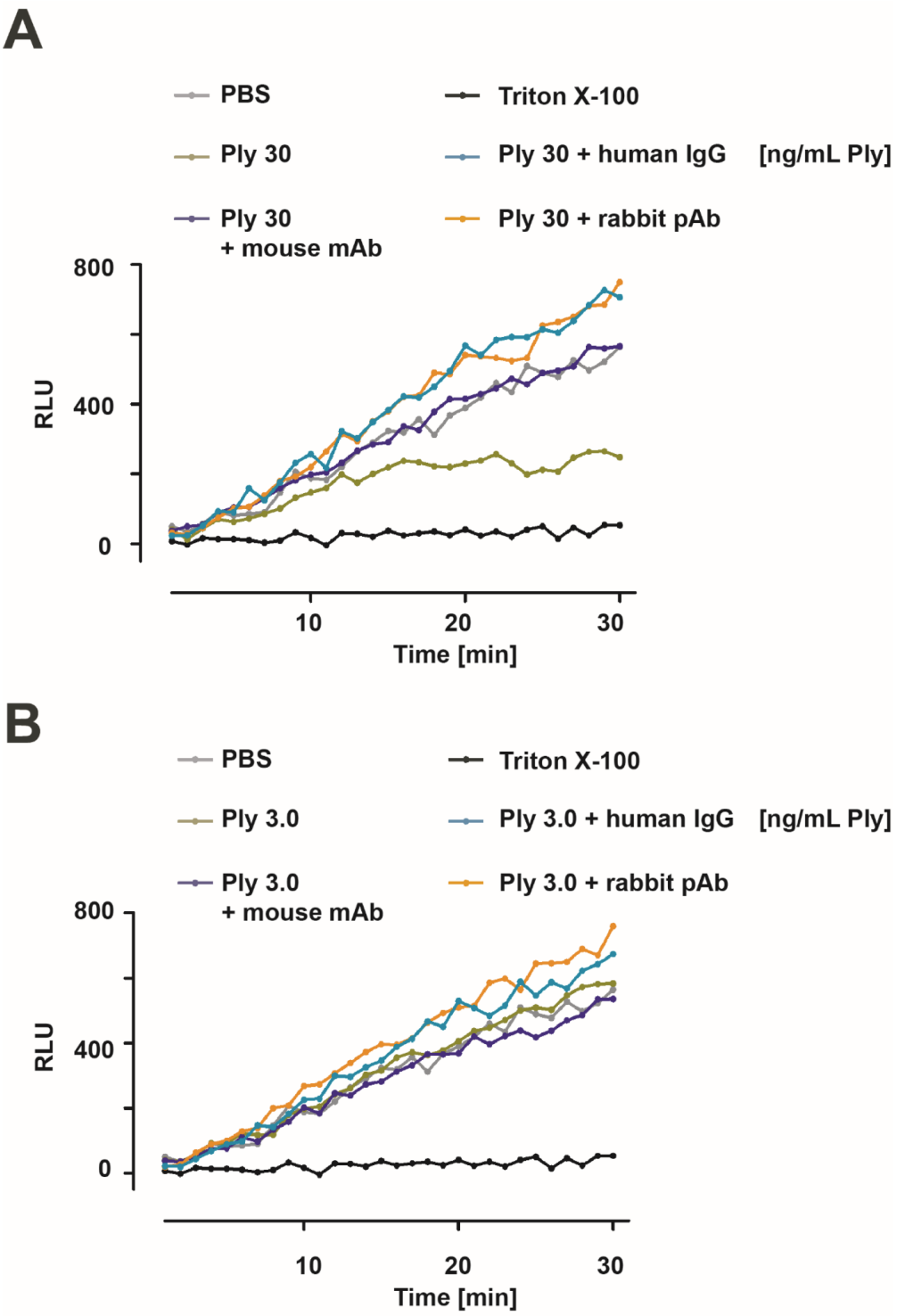

Figure S10. Platelet viability in the presence of pneumolysin is maintained by immunoglobulins

15

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

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

Figure S11

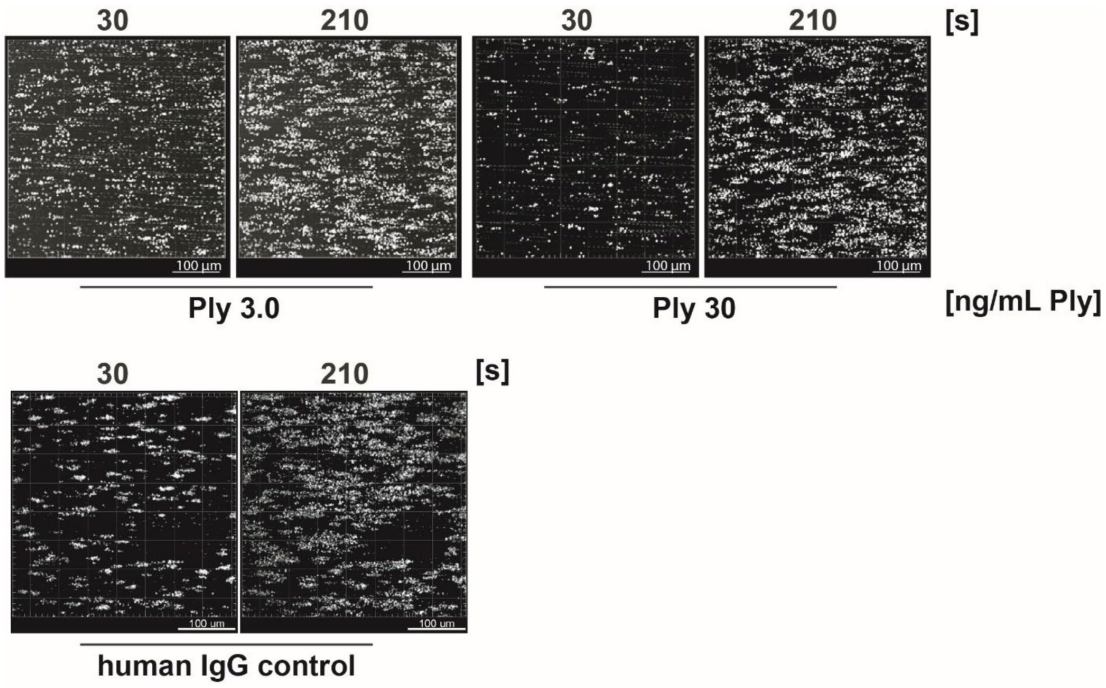

Figure S11. Effect of pneumolysin on platelet thrombus formation in flow chamber.

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

Figure S12

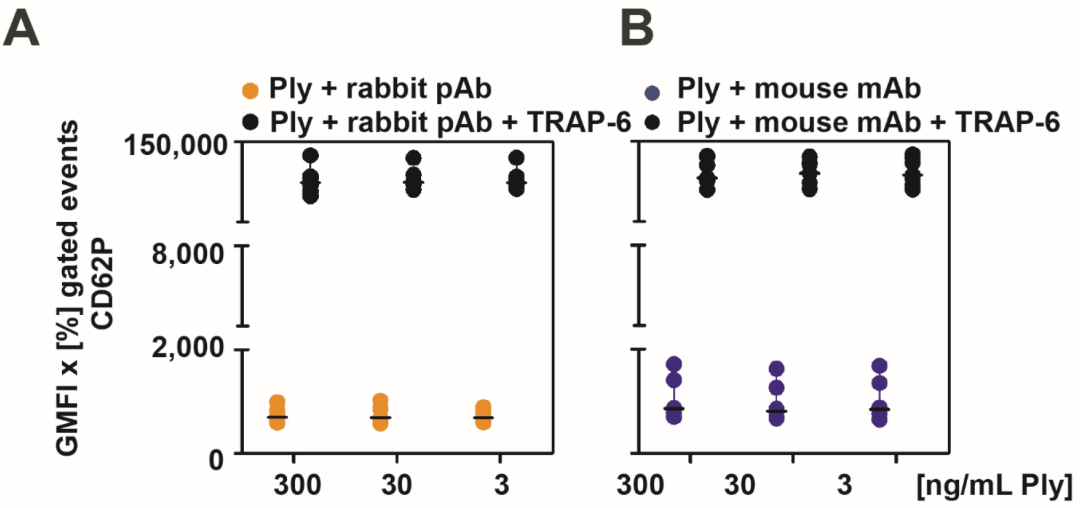

Figure S12. The effect of pneumolysin on CD62P expression on platelets is neutralized by antibodies

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

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

Figure S13

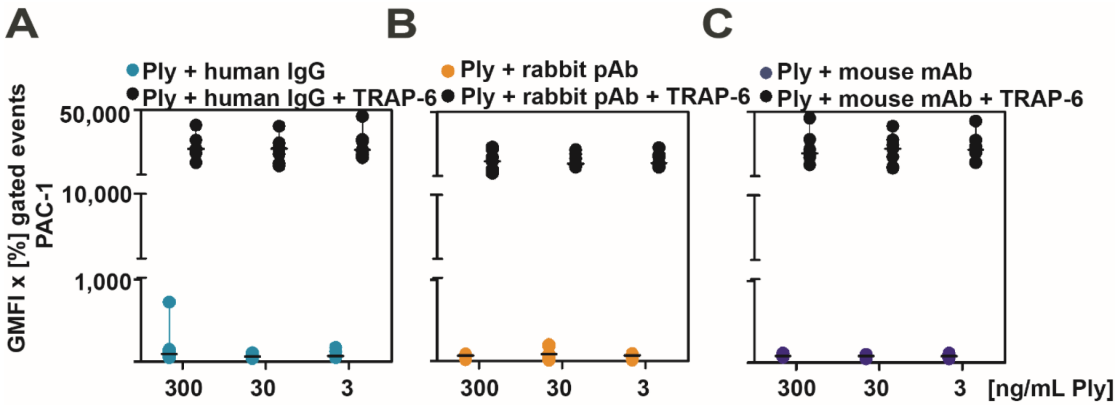

Figure S13. The effects of pneumolysin on PAC-1 binding to platelets is neutralized by antibodies

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

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

Figure S14

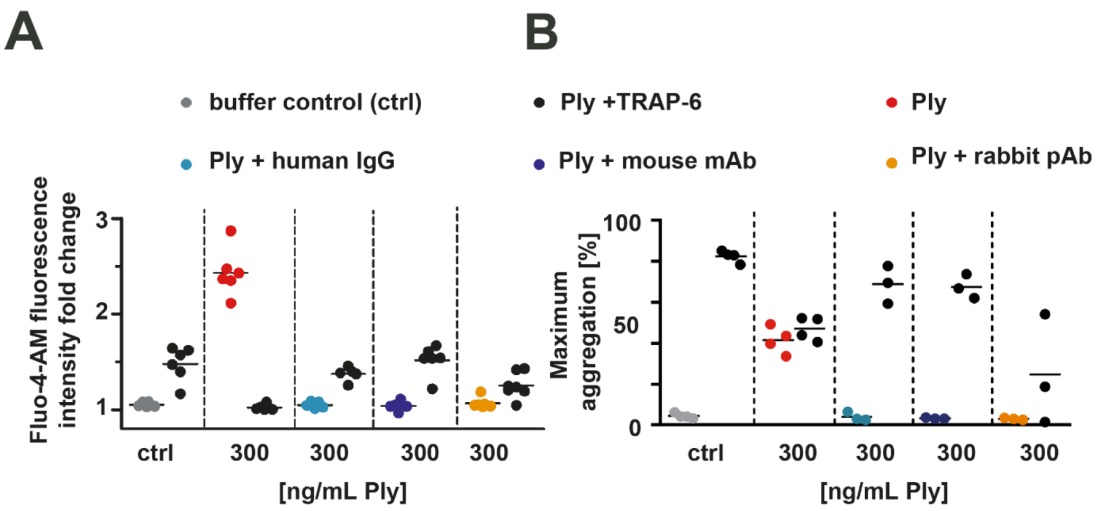

Figure S14. Immunoglobulins prevent pneumolysin impaired platelet function

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

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

3.

Polyvalent Immunoglobulin Preparations Inhibit Pneumolysin-Induced Platelet Destruction

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Published in Journal of Thrombosis and Haemostasis,
2021 Dec 16. doi: 10.1055/a-1723-1880.

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Implementation of experiments: F.W., A.S., R.P., M.W., St.H., K.J., F.V.
Experiments performed by K.J. shown in Fig. 3B
Data analysis: F.W., A.S., R.P., M.W., St.H., K.J., F.V.
Providing materials/reagents: S.H., A.G., S.W., J.S.
Writing the manuscript: F.W.
Revision of the manuscript: F.W., St.H., A.S., R.P., M.W., K.J., F.V., S.W., J.S:, A.G., S.H.

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Published online: 2021-12-16

Polyvalent Immunoglobulin Preparations Inhibit Pneumolysin-Induced Platelet Destruction

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Thromb Haemost

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Abstract

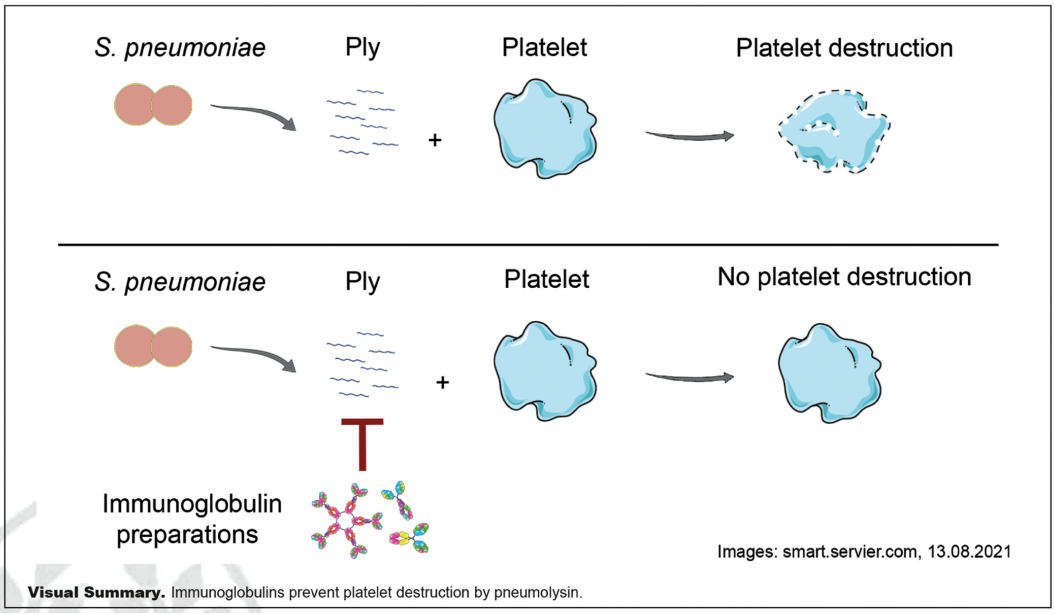
Platelets play an important role in the development and progression of respiratory distress. Functional platelets are known to seal inflammatory endothelial gaps and loss of platelet function has been shown to result in loss of integrity of pulmonary vessels. This leads to fluid accumulation in the pulmonary interstitium, eventually resulting in respiratory distress. *Streptococcus pneumoniae* is one of the major pathogens causing community-acquired pneumonia. Previously, we have shown that its major toxin pneumolysin forms pores in platelet membranes and renders them nonfunctional. In vitro, this process was inhibited by polyvalent intravenous immunoglobulins (IVIGs). In this study, we compared the efficacy of a standard IVIG preparation (IVIG, 98% immunoglobulin G [IgG]; Privigen, CSL Behring, United States) and an IgM/IgA-enriched immunoglobulin preparation (21% IgA, 23% IgM, 56% IgG; trimodulin, Biotest AG, Germany) to inhibit pneumolysin-induced platelet destruction. Platelet destruction and functionality were assessed by flow cytometry, intracellular calcium release, aggregometry, platelet viability, transwell, and flow chamber assays. Overall, both immunoglobulin preparations efficiently inhibited pneumolysin-induced platelet destruction. The capacity to antagonize pneumolysin mainly depended on the final IgG content. As both polyvalent immunoglobulin preparations efficiently prevent pneumolysin-induced platelet destruction and maintain platelet function in vitro, they represent promising candidates for clinical studies on supportive treatment of pneumococcal pneumonia to reduce progression of respiratory distress.

Keywords
- immunoglobulins
- platelets
- pneumolysin
- pneumonia
- *Streptococcus pneumoniae*

received
August 14, 2021
accepted after revision
November 10, 2021

DOI https://doi.org/10.1055/a-1723-1880.
ISSN 0340-6245.

Georg Thieme Verlag KG, Rüdigerstraße 14, 70469 Stuttgart, Germany



Visual Summary. Immunoglobulins prevent platelet destruction by pneumolysin.

Introduction

Community-acquired pneumonia (CAP) is frequent and can cause acute respiratory distress syndrome (ARDS),[1] a medical condition with high mortality.[2] One of the major pathogens causing CAP is *Streptococcus pneumoniae,* and its toxin pneumolysin contributes to the development of CAP and ARDS.[3–6] Recently, we have shown that pneumolysin induces platelet destruction and renders them nonfunctional.[7] Platelets play a role in lung diseases[8] and impaired platelet function leads to loss of vascular integrity,[9,10] also in the pulmonary vessels.[11,12] This results in fluid accumulation in the pulmonary interstitium and alveoli, eventually resulting in respiratory distress. Already in the 1990s, it has been shown that washed platelets prevent lung edema.[13,14] Prevention of pneumolysin-induced platelet destruction may represent a supportive therapeutic strategy in patients with pneumococcal CAP. Among other approaches,[15,16] inhibition of pneumolysin can be achieved by polyvalent immunoglobulin preparations pooled from human plasma. We have shown that a human immunoglobulin G (IgG) preparation (intravenous immunoglobulin [IVIG]; 98% IgG) prevents pneumolysin-induced platelet destruction in vitro.[7] Furthermore, the IgM/IgA-enriched immunoglobulin preparation trimodulin (23% IgM, 21% IgA, 56% IgG) reduced mortality in severe CAP (sCAP) patients who had high C-reactive protein and/or low IgM levels.[17] More targeted analysis revealed that trimodulin also reduced mortality in a small patient subgroup with pneumococcal microbiological etiology compared to placebo, and was shown to prevent lysis of erythrocytes by pneumolysin.[7]

In this study, we investigate the impact of trimodulin on pneumolysin-induced platelet destruction compared to an IVIG preparation in vitro.

Methods

Polyvalent Immunoglobulin Preparations

In all experiments, IVIG (98% IgG; Privigen, CSL Behring, United States) was used at a final concentration of 1 mg/mL. The IgM/IgA-enriched immunoglobulin preparation trimodulin (23% IgM, 21% IgA, 56% IgG; Biotest AG, Germany) was used in two final concentrations, 1 and 1.79 mg/mL. IVIG 1 mg/mL and trimodulin 1.79 mg/mL contain the same final concentration of IgG, whereas IVIG 1 mg/mL and trimodulin 1 mg/mL contain the same final overall concentration of human plasma immunoglobulin proteins.

Determination of Ply-Specific Antibodies Using the Luminex xMAP Technology

Human plasma samples to measure and compare Ply-specific antibody titers were either derived from the excellence network for Community Acquired Pneumonia (CAPNETZ) or the SHIP-TREND-0 cohort of the Study of Health in Pomerania (SHIP).[18] The observational CAPNETZ study includes more than 12,000 patients diagnosed with CAP from whom comprehensive data and biomaterials were collected (https://clinicaltrials.gov/ct2/show/NCT02139163?term=NCT02139163&draw=2&rank=1 or https://www.capnetz.de/html/capnetz/project?set-language-to=en). Samples from the SHIP-TREND-0 cohort were used as a healthy control group and comprised a stratified random sample of adults living in the region of Western Pomerania whose health status was assessed.[19] The study protocol of the CAPNETZ cohort was approved by the local ethics committee of the Medical School Hannover, Germany (registration number 301-2008). The study protocol of the SHIP-TREND-0 cohort was approved by the local ethics committee of the University of Greifswald (registration number BB39/08), with all participants providing written consent. A total of 138 human plasma

samples were included from each study, which were matched for age and gender. Samples: $n = 138$ CAPNETZ and $n = 138$ SHIP-TREND-0. Mean age in years: 54.3 years; men 54.3%, women 45.7%.

The determination of Ply-specific antibody titers in IVIG and human plasma samples was performed via a Luminex-based serological assay described recently.[20] In brief, recombinant pneumolysin was immobilized on xMAP MagPlex beads (Luminex, United States), which were incubated with fourfold serially diluted IVIG (98% IgG; Privigen, CSL Behring, United States) or plasma samples (seven dilutions ranging from 1:50 to 1:204,800). Detection of primary antibody binding was enabled by incubation with an R-Phycoerythrin-labeled anti-human IgG (Jackson ImmunoResearch Europe Ltd, Ely, United Kingdom) followed by measurement in the FLEXMAP 3D (Luminex, United States). The xMAPr analysis tool[21] was used for curve fitting and calculation of Ply-specific IgG response values.

Detection of Ply-Specific Antibodies Using Western Blot

Different concentrations of recombinant pneumolysin (0.125 , 0.25 , 0.5, and 1 µg/mL) were applied on a 12% SDS gel and blotted on a nitrocellulose membrane. After blocking in 5% skimmed milk a rabbit anti-pneumolysin polyclonal Ab (1:500) generated as described,[7] serum of a defined donor (1:500), IVIG (1 mg/mL), and trimodulin (1.79 mg/mL) were used as primary antibodies. After washing, the blots were incubated with HRP-labeled anti-human or anti-rabbit IgG. Detection was done with a ChemoCam (Intas, Science Imaging) with 15 second exposure time for all immunoblots. The immunoblot image was brightness-adjusted using Photoshop CS5 64 bit.

Platelet Preparation

Platelets were purified from ACD-A whole blood obtained from healthy volunteers matching the criteria for blood donation in Germany. All donors gave written and informed consent. Ethics were approved by the Ethics Committee of the Universitätsmedizin Greifswald (BB 044/18). Platelets were prepared as described.[7] Briefly, platelet-rich plasma was washed twice with Tyrode's buffer (0.35% BSA, 0.1% glucose, 2.5 U/mL apyrase, 1 U/mL hirudin, pH 6.3) and the final platelet pellet was resuspended in a bicarbonate-based suspension buffer (0.137 M NaCl, 0.027 M KCl, 0.012 M $NaHCO_3$, 0.42 mM NaH_2PO_4, 0.35% BSA, 0.1% glucose, 0.212 M $MgCl_2$, 0.196 M CaCl2, pH 7.2) and adjusted to 300.000 platelets/µL.

Pneumolysin

Purification of Strep-tagged pneumolysin and cytolytic activity assessment were performed as described.[7] Pneumolysin stock solution (0.296 mg/mL) was diluted in PBS (w/o Ca^{2+}/Mg^{2+}, pH = 7.0) to different concentrations, depending on the assay. Prior to the platelet assays, pneumolysin in different concentrations was incubated with IVIG or trimodulin (30 minutes, at room temperature [RT]). Pneumolysin concentrations were tested over a wide range (final concentrations of mainly 300–900 ng/mL) to test for potential differences in the efficacy of immunoglobulins.

Flow Cytometry (CD62P Staining)

To avoid artifacts of platelet activation caused by FcγRIIa-dependent platelet activation (e.g., due to blood group antibodies anti-A or anti-B IgG in the immunoglobulin preparations), platelets were preincubated with the monoclonal antibody (mAb) IV.3 (supernatant of cell culture, 45 minutes, 37°C in a 1:15 dilution) to block platelet FcγRIIa. Pneumolysin of different concentrations (final concentrations: 900, 600, 300, 30 ng/mL) was preincubated with IVIG (final concentration 1 mg/mL), trimodulin (final concentrations: 1 and 1.79 mg/mL) or buffer for 20 seconds, 60 seconds, 5 minutes, or 30 minutes and then incubated with platelets for 10 minutes at RT. To test remaining platelet functionality, samples were split and 50% were incubated with thrombin receptor activator peptide (TRAP-6, Hart Biologicals, United Kingdom; final concentration 20 µM) for 10 minutes at RT, the other 50% with PBS. Then, platelets were labeled with anti-human CD62P PE-Cy5-labelled antibody (BD Pharmingen, United States, 10 minutes, RT, 1:10 dilution), fixed with paraformaldehyde 2% (Morphisto, Germany, 15 minutes, RT), washed with PBS (650 g, 7 minutes), and assessed by flow cytometry (Cytomics FC500; Beckmann, United States; CXP 2.2 software). A platelet gate was predefined using the forward–sideward scatter followed by CD61-positive cells. Results are presented as mean fluorescence intensity of the CD62P-positive population multiplied by the percentage of gated events. The experimental setup is shown in ►**Supplementary Fig. S1** (available in the online version).

Comparison of Different IVIG and Trimodulin Batches

To test batch-to-batch variability, four different trimodulin batches (Lot 1, 2, 3, 4) and two different IVIG batches (Lot 1, 2) were assessed in the CD62P flow cytometry assay (performed as described above) and a hemolysis assay. For the hemolysis assay, ACD-A whole blood was washed with PBS four times (211 g, 10 minutes, brake 0), the pellet was resuspended and diluted in PBS (w/o Ca^{2+}/Mg^{2+}, pH = 7, 1:5). Samples were then incubated with pneumolysin or pneumolysin preincubated with IVIG or trimodulin (final concentrations: pneumolysin: 300, 600, 900 ng/mL; IVIG: 1 mg/mL; trimodulin: 1.79 mg/mL) in a 96-well plate for 10 minutes, 37°C. The plate was then centrifuged (10 minutes, 475 g, brake 0), and the supernatant was measured for absorption at 450 nm for free hemoglobin content (Tecan Infinite 200 PRO, Tecan Group, Switzerland). The experimental setup is shown in ►**Supplementary Fig. S2** (available in the online version).

Measurement of Intracellular Calcium Release

The release of calcium from intracellular stores was measured using the fluorescent calcium indicator FLUO-4 AM (ThermoFisher, United States). To avoid artifacts, platelets were washed and resuspended in a calcium-free Tyrode's buffer as described.[7] Platelets were stained with FLUO-4 AM (ThermoFisher, United States; 5.4 ng/µL), and FcγRIIa was

blocked by mAb IV.3 (1:15, 45 minutes, 37°C). Measurements were performed with Fluoroskan Ascent fluorometer (ThermoFisher, United States) applying Ascent software 2.6 as described.[7] In brief, pneumolysin (final concentrations: 900, 600, 300 ng/mL) preincubated with IVIG (final concentration 1 mg/mL), trimodulin (final concentrations: 1 and 1.79 mg/mL), or buffer was added after 15 seconds to the platelets and measurement continued for 225 seconds. Then, the remaining platelet functionality was tested by the addition of TRAP-6 (final concentration 20 µM). The amount of free intracellular calcium was normalized to the baseline measurement. The detailed experimental setup is shown in ►**Supplementary Fig. S3** (available in the online version).

Light Transmission Aggregometry (Platelet Lysis and Aggregation)

FcγRIIa of washed platelets was blocked using mAb IV.3 (1:15, 45 minutes, 37°C) and fibrinogen (Invitrogen, United States; 2.25 mg/mL) was added to allow platelet aggregation. Platelets were assessed for aggregation or platelet lysis using an APACT4 aggregometer (500 rpm, 37°C, Haemochrom, Germany) applying APACT LPC-software as described.[7] In brief, pneumolysin (final concentrations: 900, 600, or 300 ng/mL) preincubated with IVIG (final concentration 1 mg/mL), trimodulin (final concentrations: 1 and 1.79 mg/mL), or buffer was added to the samples after 15 seconds and change in light transmission was followed for 225 seconds, then TRAP-6 (final concentration 20 µM) was added to test the remaining platelet function. The detailed experimental set up is shown in ►**Supplementary Fig. S4** (available in the online version).

Platelet Viability

To determine platelet viability, RealTime-Glo Cell Viability Assay (Promega, United States) was used. RealTime-Glo reagents were prepared according to the manufacturer's protocol and mixed with platelets and pneumolysin (final concentrations: 900, 600, 300 ng/mL), which was preincubated with IVIG (final concentration 1 mg/mL) or trimodulin (final concentrations: 1 and 1.79 mg/mL) in a 96-well plate. Real-time luminescence measurement was performed for 30 minutes (FLUOstar Omega, BMG LABTECH, Germany) as described.[7] All samples were tested in duplicates. The mean of the relative luminescence units of duplicate measurements was subtracted by the blank and represents cell viability. The detailed experimental setup is shown in ►**Supplementary Fig. S5** (available in the online version).

Transwell Assay (Pore Sealing Capacity)

To determine the pore sealing capacity of platelets, transwell assays with fibronectin-coated membranes (Corning 6.5 mm Transwell with 3.0 µm Pore Polycarbonate Membrane Insert, Corning, United States) were performed as described.[7] Briefly, platelets were incubated with pneumolysin (final concentrations: 600, 300 ng/mL) preincubated with IVIG (final concentration 1 mg/mL), trimodulin (final concentration 1.79 mg/mL), or buffer for 45 minutes at 37°C in the inserts of the transwell plates. The supernatant of the inserts was discarded, and the inserts transferred into new wells containing PBS (w/o Ca^{2+}/Mg^{2+}, pH 7.0). FITC-labeled bovine serum albumin (BSA-FITC, ThermoFisher, United States; final concentration 0.25 mg/mL) was then added to the inserts (10 minutes, 37°C, darkness). The platelets' pore sealing capacity was determined by fluorometric measurement of BSA-FITC flow through in the liquid of the lower chamber (Fluoroskan Ascent FL, ThermoFisher, United States, Ascent Software 2.6). The detailed experimental setup is shown in ►**Supplementary Fig. S6** (available in the online version).

Thrombus Formation under Shear Flow

Platelets in hirudinized whole blood were stained with FITC-labeled anti-human CD61 antibody (BD Pharmingen, United States; 1:100) and then incubated with pneumolysin (final concentration: 600 ng/mL), which was preincubated with IVIG (1 mg/mL) or trimodulin (1 and 1.79 mg/mL) for 10 minutes at RT. Blood samples were flown over collagen (horse tendon, Nycomed, Germany; 200 µg/mL HORM collagen type I) coated channels of ibidi µ-slides VI 0.1 (ibidi, Germany) at a wall shear rate of 1,000 s^{-1}. Every 10 seconds, time-lapse images were captured on a confocal laser scanning microscope (Leica SP5, Leica, Germany) as described.[7] Quantitative assessment of thrombus formation was performed by image segmentation in Bitplane Imaris version 7.65. (Oxford Instruments, Abingdon, United Kingdom) using the surface creation wizard algorithm as previously described.[7] All flow experiments were performed according to International Society on Thrombosis and Haemostasis Scientific and Standardization Committee subcommittee on Biorheology recommendations.[22] The detailed experimental setup is shown in ►**Supplementary Fig. S7** (available in the online version).

Statistics

Statistical analysis was performed using GraphPad Prism 9. Data are shown as median, except where indicated. Non-normally distributed data were analyzed using the Friedman test followed by the uncorrected Dunn's test for multiple comparisons. Normally distributed data were analyzed using one-way repeated measures ANOVA (analysis of variance), followed by the Bonferroni method for multiple comparisons. A p-value <0.05 was considered to be statistically significant.

Results

Trimodulin and IVIG Inhibit Pneumolysin-Induced Platelet Damage

Permeabilization of Platelets

We have shown earlier that pneumolysin treatment of platelets results in intracellular CD62P staining due to pneumolysin-induced pore formation in the platelet membrane.[7] This allows anti-CD62P antibodies to pass the membrane and stain intraplatelet CD62P. Therefore, CD62P staining after pneumolysin treatment is a marker for platelet destruction.[7] In the presence of IVIG or trimodulin, pneumolysin-induced

increase in CD62P staining in platelets was reduced in a concentration-dependent manner (►**Fig. 1A**). Both immunoglobulin preparations significantly reduced CD62P staining induced by pneumolysin concentrations up to 300 ng/mL. At higher concentrations of pneumolysin (600–900 ng/mL), the values of CD62P staining in platelets in the presence of trimodulin 1.79 mg/mL and IVIG 1 mg/mL were similar. In contrast, CD62P staining in platelets was higher when only 1 mg/mL trimodulin was used (►**Fig. 1A**). Pneumolysin preincubations of >60 seconds with immunoglobulin preparations were sufficient to reduce CD62P values (►**Supplementary Fig. S8** [available in the online version]). As control, neither IVIG nor trimodulin increased CD62P staining in the absence of pneumolysin (►**Fig. 1A**).

Remaining Platelet Function

A similar pattern was observed for the remaining platelet functionality when the response to TRAP-6 was tested (►**Fig. 1B**). IVIG 1 mg/mL and trimodulin 1.79 mg/mL maintained TRAP-6 responsiveness up to 900 ng/mL pneumolysin, whereas trimodulin 1 mg/mL failed to do so when more than 300 ng/mL pneumolysin was used. As a control, neither IVIG nor trimodulin inhibited CD62P expression on platelets after activation with TRAP-6 in the absence of pneumolysin (►**Fig. 1B** and ►**Supplementary Fig. S8** [available in the online version]).

Batch-to-Batch Variability

To test batch-to-batch variability in the capacity to antagonize pneumolysin, e.g., due to different antipneumolysin immunoglobulin concentrations in different batches, four batches of trimodulin and two batches of IVIG were tested in the CD62P (►**Supplementary Fig. S9A** [available in the online version]) and hemolysis assay (►**Supplementary Fig. S9B** [available in the online version]). Overall, no relevant batch-to-batch variability in the capacity to antagonize pneumolysin was observed (►**Supplementary Fig. S9** [available in the online version]). Therefore, all other experiments were performed with only one batch per immunoglobulin preparation (IVIG lot 1 and trimodulin lot 1).

Calcium Release

Platelets physiologically mobilize calcium from intracellular stores upon activation over a short time, here demonstrated by TRAP-6 addition (►**Fig. 2A1**–**A3**). In contrast, calcium release upon pneumolysin treatment is permanent (►**Fig. 2A1**–**A3**).[7] Consistent with the flow cytometry results, both immunoglobulin preparations showed sufficient inhibition of calcium release and maintenance of TRAP-6 responsiveness up to 300 ng/mL pneumolysin (►**Fig. 2A1**). At 600 ng/mL pneumolysin, trimodulin 1.79 mg/mL and IVIG 1 mg/mL still showed sufficient inhibition of calcium release and maintenance of TRAP-6 responsiveness, whereas trimodulin 1 mg/mL did not (►**Fig. 2A2**). At 900 ng/mL pneumolysin, trimodulin 1.79 mg/mL showed a delay of pathological calcium release and only IVIG 1 mg/mL showed a reduction of calcium release and maintenance of TRAP-6 responsiveness similar to the control (►**Fig. 2A3**). In contrast, trimodulin 1 mg/mL was not sufficient to inhibit the 900 ng/mL pneumolysin effect (►**Fig. 2A3**). As a control, neither IVIG nor trimodulin inhibited calcium release of platelets after activation with TRAP-6 in the absence of pneumolysin (►**Supplementary Fig. S10A** [available in the online version]).

Platelet Aggregation

In aggregometry with functional platelets, increased light transmission upon activation (here following TRAP-6 stimulation) is a measure for platelet aggregation and represents the physiological platelet reaction (►**Fig. 2B1**–**B3**). In previous experiments we indicated that changes in light transmission upon pneumolysin treatment are not inhibited by the addition of RGDS, which blocks platelet aggregation.[7] In this case, increased light transmission upon pneumolysin treatment is not a measure for platelet aggregation but for platelet lysis.[7] Both immunoglobulin preparations inhibited platelet lysis and maintained TRAP-6 responsiveness up to 300 ng/mL pneumolysin (►**Fig. 2B1**). At 600 ng/mL pneumolysin, trimodulin 1 mg/mL showed an incomplete reduction of platelet lysis, and TRAP-6 responsiveness was lost, whereas trimodulin 1.79 mg/mL and IVIG 1 mg/mL showed a complete reduction of platelet lysis and maintenance of TRAP-6 responsiveness (►**Fig. 2B2**). At 900 ng/mL pneumolysin, trimodulin 1.79 mg/mL and IVIG 1 mg/mL inhibited platelet lysis, but only IVIG maintained TRAP-6 responsiveness completely (►**Fig. 2B3**). Neither IVIG nor trimodulin inhibited platelet aggregation after activation with TRAP-6 in the absence of pneumolysin (►**Supplementary Fig. S10B** [available in the online version]).

Antipneumolysin Antibody Levels in Immunoglobulin Preparations

To demonstrate that the rescue of platelet function by IVIG and trimodulin is due to neutralization of the toxin by specific antipneumolysin antibodies, the relative IgG antibody levels were determined in IVIG. These antibody levels were compared to antipneumolysin IgG levels of a healthy cohort (SHIP cohort) and a cohort of patients suffering on pneumonia (CAPNETZ cohort). The results showed high antipneumolysin IgG levels in IVIG (►**Fig. 3A**). In addition, the direct interaction of antibodies present in IVIG, trimodulin, and a healthy donor serum was indicated by immunoblot analysis with pneumolysin. Consistent with the findings of our functional experiments, IVIG was more reactive than trimodulin (►**Fig. 3B**).

Trimodulin and IVIG Prevent Loss of Platelet Viability, Pore Sealing Capacity, and Clot Formation upon Pneumolysin Treatment

After assessing the inhibition of pneumolysin-induced platelet damage and the maintenance of TRAP-6 responsiveness by trimodulin and IVIG, the maintenance of more complex platelet functions, which are likely relevant in the development of pulmonary vascular leakage and respiratory distress, was assessed.

Platelet Viability

Impaired platelet metabolism and enzymatic functionality have been shown to result in lung damage due to vascular

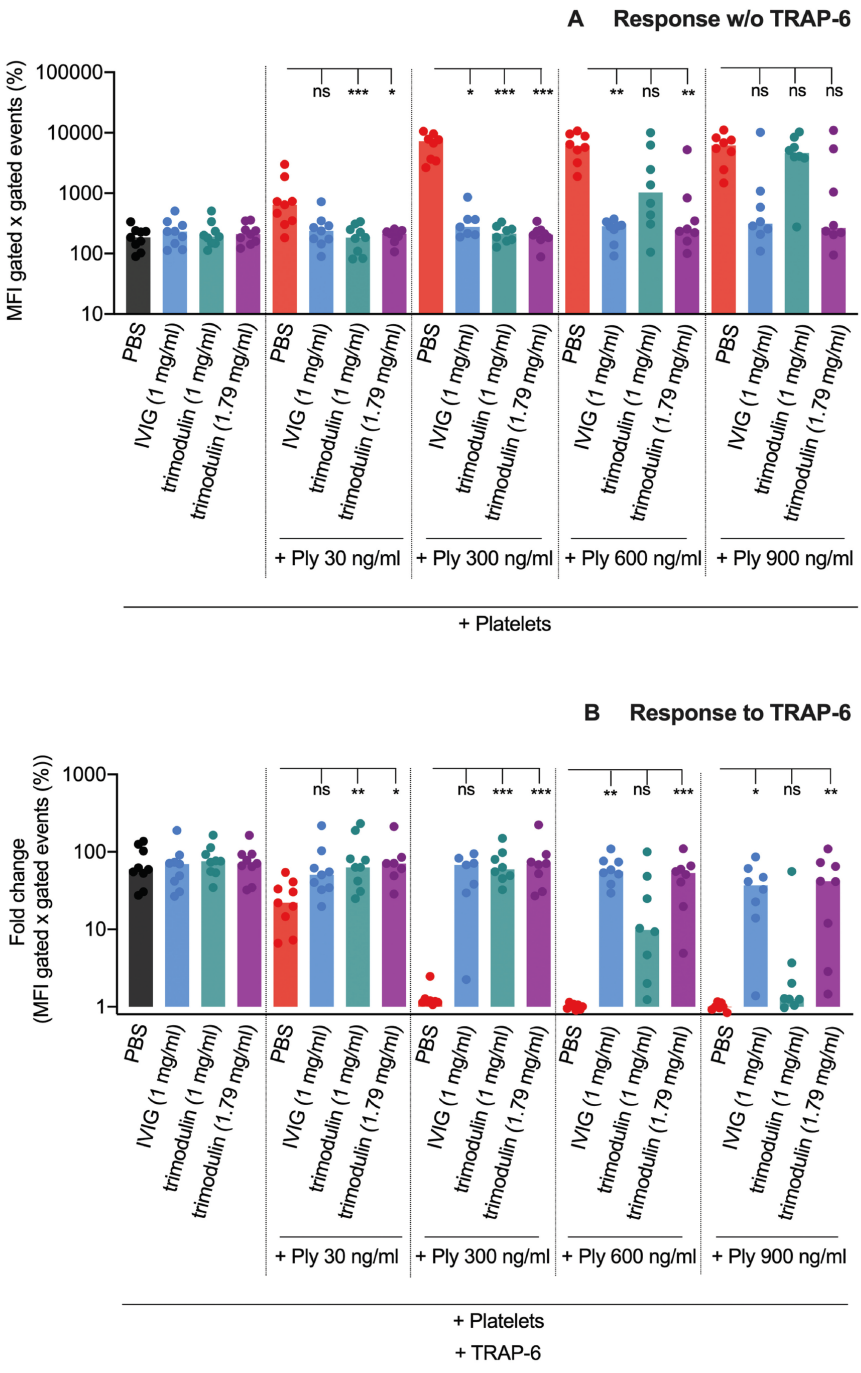


Fig. 1 CD62P staining in platelets upon pneumolysin treatment. (A) Increased CD62P staining upon treatment with pneumolysin (Ply) or pneumolysin preincubated with IVIG or trimodulin. CD62P staining is presented as mean fluorescence intensity (MFI) of CD62P-positive gated events multiplied with the percent of CD62P-positive gated events. As a control, samples were incubated only with PBS. (B) CD62P staining upon TRAP-6 (20 µM) addition after preincubation of platelets with pneumolysin or pneumolysin preincubated with IVIG or trimodulin. Response to TRAP-6 is presented as a fold change of the MFI of CD62P-positive gated events multiplied with the percent of CD62P-positive events compared to the PBS control. As a control, samples were incubated with PBS only. Statistical analysis was performed using the Friedman test followed by uncorrected Dunn's test for multiple comparisons. A p-value <0.05 was considered to be significant ($^{*}>0.033$, $^{**}>0.002$, $^{***}>0.001$). IVIG, intravenous immunoglobulin; PBS, phosphate-buffered saline.

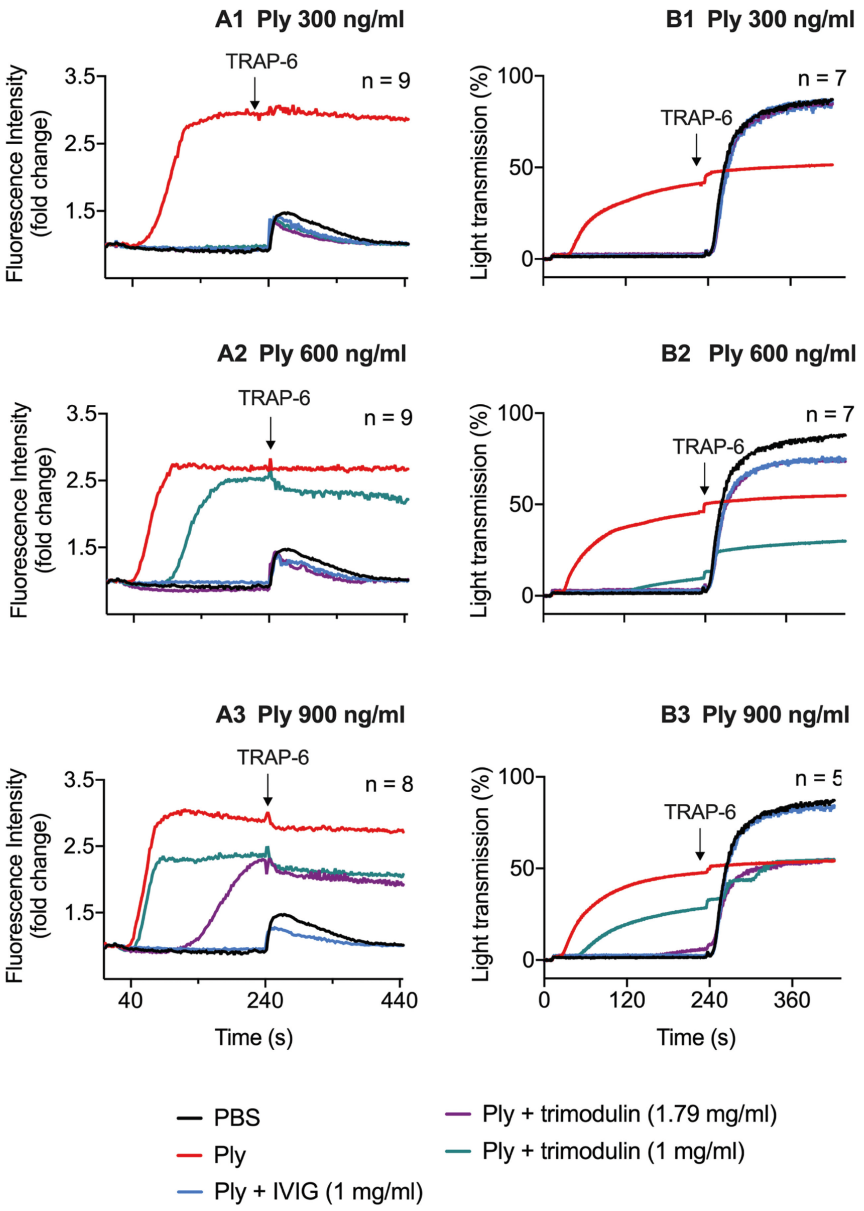


Fig. 2 Kinetics of (A) intracellular calcium release and (B) platelet aggregation/lysis upon pneumolysin treatment. (A) Intracellular calcium release in platelets upon treatment with different pneumolysin (Ply) concentrations (A1–A3) or pneumolysin preincubated with IVIG or trimodulin, respectively. Intracellular calcium was quantified by fluorescence measurement, resulting from the fluorescence intracellular calcium indicator FLUO-4-AM. After 240 seconds, platelet functionality was assessed by TRAP-6 (20 µM) addition. Curves represent the median of platelets of ≥8 donors. As a control, platelets were incubated with PBS. (B) Light transmission through platelet suspension upon treatment with different pneumolysin concentrations (B1–B3) or pneumolysin preincubated with IVIG or trimodulin in aggregometry, respectively. Increased light transmission upon pneumolysin treatment results from platelet lysis. After 240 seconds, platelet functionality was assessed by TRAP-6 (20 µM) addition. Increased light transmission upon TRAP-6 treatment results from platelet aggregation. Curves represent the median of at least five donors. As a control, platelets were incubated with PBS only. IVIG, intravenous immunoglobulin; PBS, phosphate-buffered saline.

leakage.[11,13] In platelet viability assays, metabolically active viable platelets converted the RealTime-Glo substrate to a luminescent product; metabolically nonactive dead platelets did not. Pneumolysin treatment impaired platelet viability and induced cell death (►**Fig. 4A–C**). Both immunoglobulin preparations inhibited cell death of platelets up to 300 ng/mL pneumolysin (►**Fig. 4A**). At 600 ng/mL pneumolysin, trimodulin 1.79 mg/mL and IVIG 1 mg/mL inhibited cell death

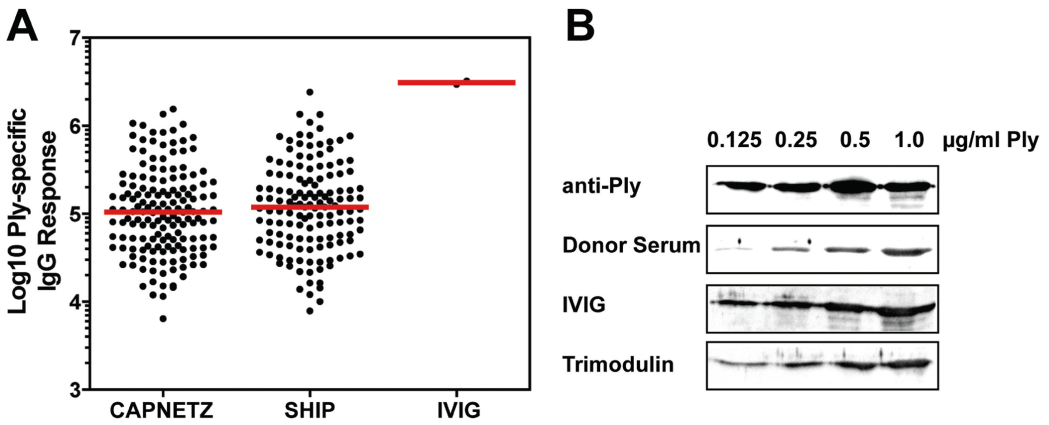


Fig. 3 Pneumolysin IgG antibody levels in human sera and immunoglobulin preparations. (**A**) Anti-pneumolysin IgG antibody levels in a cohort of pneumonia patients ($n = 138$), a healthy cohort ($n = 138$), and IVIG. The mean age was 54.3 years with 54.3% men and 45.7% women. The pneumolysin IgG antibody titers were determined by the Luminex xMAP technology and the xMAPr analysis tool was used for curve fitting and calculation of Ply-specific IgG response values. (**B**) Detection of IgG specific for pneumolysin by immunoblot analysis. Different amounts of pneumolysin (0.125, 0.25, 0.5, and 1 µg/mL) were separated by sodium dodecyl sulphate–polyacrylamide gel electrophoresis and incubated with a rabbit polyclonal antipneumolysin antibody (1:500, Davids Biotechnologie GmbH, Regensburg, Germany), a donor serum (1:500), IVIG (1 mg/mL), and trimodulin (1.79 mg/mL). Detection of bound IgG was conducted with a secondary HRP-labeled anti-human or anti-rabbit IgG. Detection was done with a ChemoCam (Intas, Science Imaging) and the immunoblot image was brightness-adjusted using Photoshop CS5 64 bit. IgG, immunoglobulin G; IVIG, intravenous immunoglobulin; HRP, horseradish peroxidase.

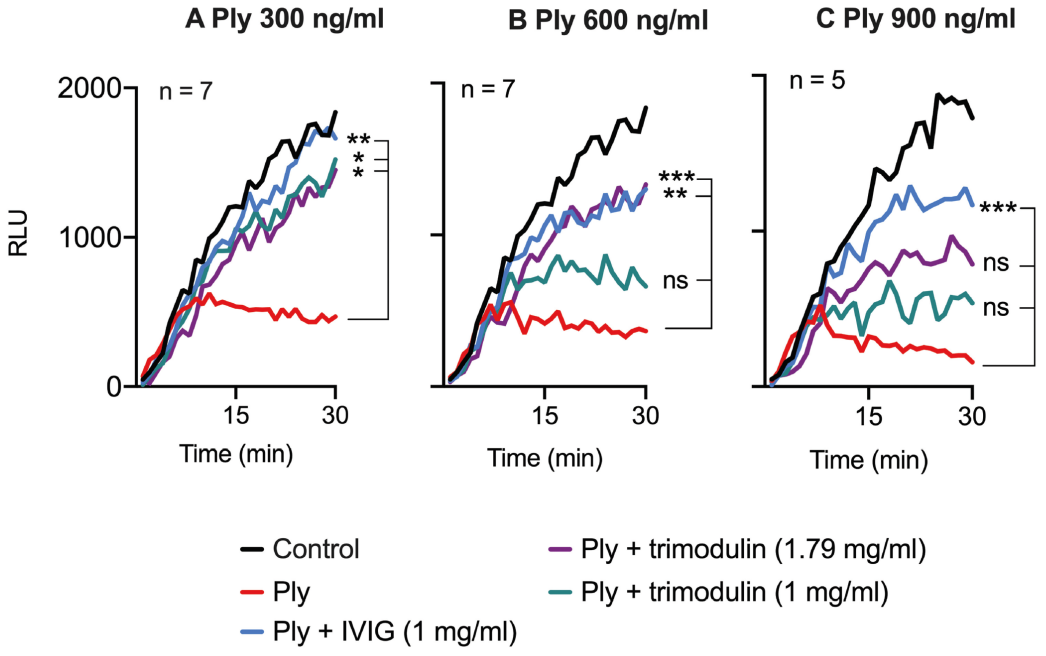


Fig. 4 Platelet viability upon pneumolysin treatment. Platelet viability upon treatment with different pneumolysin (Ply) concentrations (**A–C**) or with pneumolysin (Ply) preincubated with IVIG or trimodulin, respectively. Viability was assessed by real-time measurement of relative luminescence units (RLU) resulting from the enzymatic conversion of a substrate by viable platelets. Curves represent the median of platelets of ≥5 donors. As a control, platelets were incubated with PBS only. For statistical analysis, the measurement endpoints (RLU of the final minute) were compared applying the Friedman test, followed by uncorrected Dunn's test for multiple comparisons. A p-value <0.05 was considered to be significant ($^{*} > 0.033$, $^{**} > 0.002$, $^{***} > 0.001$). IVIG, intravenous immunoglobulin; PBS, phosphate-buffered saline.

(►**Fig. 4B**). At 900 ng/mL pneumolysin, only IVIG 1 mg/mL significantly inhibited cell death, and trimodulin 1 mg/mL inhibited cell death the least (►**Fig. 4C**). As a control, neither IVIG nor trimodulin inhibited platelet viability in the absence of pneumolysin (►**Supplementary Fig. S10D** [available in the online version]).

Pore Sealing Capacity of Platelets

In transwell assays, pneumolysin reduced the capacity of platelets to inhibit BSA-FITC diffusion through a perforated membrane. Such impaired pore sealing capacity of platelets could result in vascular leakage and fluid accumulation in the pulmonary interstitium during inflammation in vivo. In vitro, both immunoglobulin preparations maintained the platelet pore sealing capacity at 300 ng/mL pneumolysin (►**Fig. 5A**). At 600 ng/mL pneumolysin, only trimodulin 1 mg/mL was less efficient (►**Fig. 5B**). As control, neither IVIG nor trimodulin increased BSA-FITC flow through in the absence of pneumolysin to a relevant extent (►**Supplementary Fig. S9C** [available in the online version]).

Thrombus Formation

In whole blood, pneumolysin inhibited platelet adherence and subsequent thrombus formation (►**Fig. 6A**). Reduced platelet adherence and insufficient thrombus formation were preserved by trimodulin and IVIG (►**Fig. 6A**). The lower effect of trimodulin compared to IVIG is an artifact as the formulation buffer (without any immunoglobulins) of trimodulin reduced blood clot formation (►**Fig. 6B**). In vivo, agents contained in the formulation buffer will be rapidly diluted and cleared from the blood and therefore will likely have no effect on platelet function.

Discussion

This study aimed to provide further information about the impairment of platelets by pneumolysin and the impact of two immunoglobulin preparations, the IgM/IgA-enriched immunoglobulin preparation trimodulin and a standard IVIG, on pneumolysin-induced platelet destruction. We found that both pharmaceutical immunoglobulin preparations prevent the destruction of platelets by pneumolysin in vitro as indicated by flow cytometry, calcium release assay, and light transmission aggregometry. Further, the immunoglobulins maintain complex platelet functions as shown in in vitro viability and transwell assays and even in whole blood an effect can be observed as indicated by thrombus formation.

Importantly, the extent of prevention of pneumolysin-induced platelet damage depends mainly on the IgG content in the immunoglobulin preparation. When the total content of IgG was similar, IVIG and trimodulin showed similar in vitro effects. However, when the two preparations were adjusted to the same overall human plasma immunoglobulin concentration, trimodulin showed lower efficacy. This indicates that IgM and IgA antibodies do not contribute similarly to IgG to the inhibition of pneumolysin in the in vitro assays tested here. These data are strongly supported by the Luminex-based serological assay, which indicated high levels of antipneumolysin IgGs in IVIG. Although IVIG is diluted by

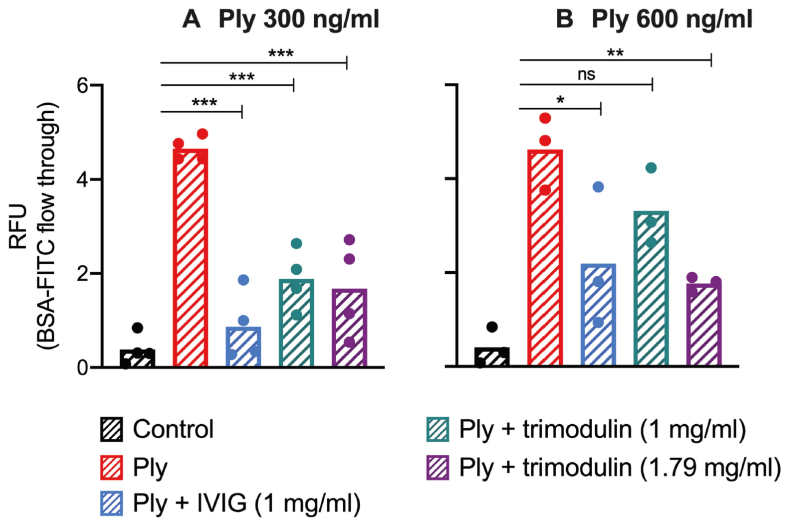


Fig. 5 Platelet's pore sealing capacity upon pneumolysin treatment. The pore sealing capacity of platelets upon treatment with different pneumolysin concentrations (**A**, **B**) or with pneumolysin preincubated with IVIG or trimodulin, respectively, was assessed by their capability to inhibit BSA-FITC diffusion through a perforated fibronectin-coated membrane. BSA-FITC diffusion was quantified by the measurement of relative fluorescence units (RFU). Bars represent the mean of platelets of at least three donors. As a control, platelets were incubated with PBS only. Statistical analysis was performed using the Shapiro–Wilk normality test and repeated measures one-way ANOVA. A p-value <0.05 was considered to be significant ($^{*}>0.033$, $^{**}>0.002$, $^{***}>0.001$). ANOVA, analysis of variance.

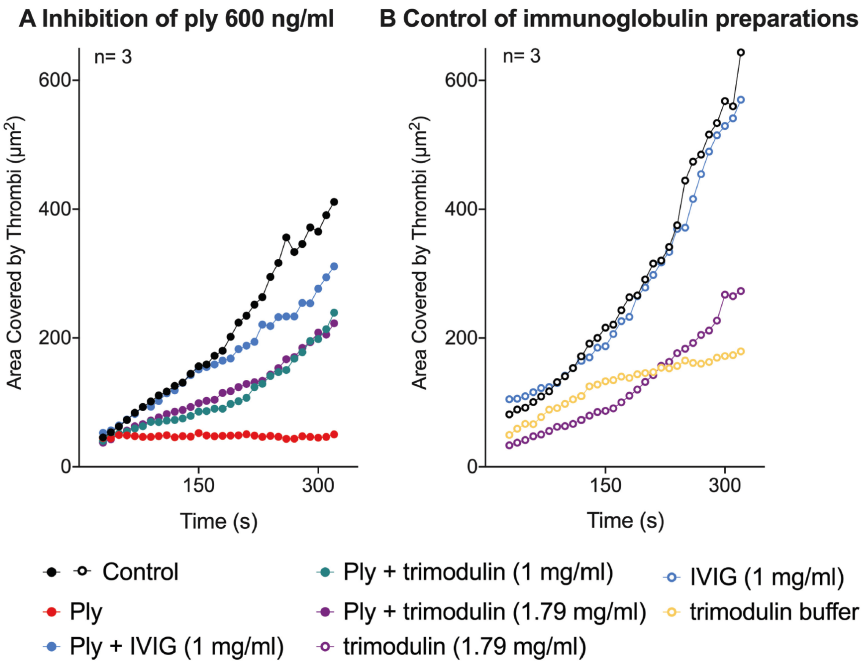


Fig. 6 Thrombus formation in whole blood. (**A**) Thrombus formation upon treatment with 600 ng/mL pneumolysin (Ply) or pneumolysin preincubated with IVIG or trimodulin. A quantitative assessment of thrombus formation was performed by analyzing the area of the collagen-coated microchannel slide that was covered by thrombi. Curves represent the mean of platelets of three donors. As a control, whole blood was incubated with PBS only. (**B**) Thrombus formation upon treatment with immunoglobulin preparations (without pneumolysin) to investigate their effects alone on the thrombus formation. A quantitative assessment of thrombus formation was performed by analyzing the area covered. Curves represent the mean of platelets of three donors. As a control, whole blood was incubated with PBS only. IVIG, intravenous immunoglobulin; PBS, phosphate-buffered saline.

patient blood after transfusion, the high levels of antipneumolysin will result in clinically meaningful concentrations of these inhibitory antibodies by IVIG and trimodulin. However, trimodulin reacted with a lower efficiency with pneumolysin compared to IVIG, which supports the observed differences on the in vitro neutralization of pneumolysin for IVIG and trimodulin.

All assays were done with IVIG at a concentration of 1 mg/mL and trimodulin at a concentration of 1 and 1.79 mg/mL. During treatment of patients with immunoglobulin preparations, immunoglobulin concentrations will be even higher. When trimodulin was given to patients with sCAP at a dose of 182.6 mg/kg body weight daily on 5 consecutive days, serum concentrations of up to 11.5 mg/mL IgG, 2.0 mg/mL IgM, and 4.1 mg/mL IgA were observed.[23] When IVIG was used in doses of 138.0 to 554.0 mg/kg body weight in patients with primary immunodeficiency, IgG concentrations of up to 16.6 mg/mL were reached 1 hour after infusion and 9 mg/mL IgG 2 weeks after infusion.[24] These pharmacokinetic properties indicate that the concentrations, which had been sufficient in our studies to prevent pneumolysin-induced platelet destruction in vitro, are even below those that can be reached in vivo using standard therapeutic regimens. The inhibitory effect of immunoglobulin preparations on pneumolysin is already measured after 60 seconds. Thus, treatment of patients suffering from pneumococcal pneumonia with immunoglobulin preparations will be clinically relevant, in particular in clinical settings where pneumolysin is released during therapeutic intervention.

Pneumolysin was used mainly at concentrations of 300 to 900 ng/mL in our experiments. In vivo data on the concentration of pneumolysin in pneumococci-induced pneumonia are difficult to obtain, because its local concentration in the lung is most likely much higher than the concentrations found in the venous blood taken from patients after it had circulated through the entire vessel system. The in vivo pneumolysin concentrations in humans, which probably come closest to the local concentrations of pneumolysin, are measured in cerebrospinal fluid from pneumococcal meningitis patients, where no dilution of pneumolysin occurs by the bloodstream. Reported concentrations range from 0.85 to 180 ng/mL,[25] with individual cases exceeding 600 ng/mL.[26] In a murine mouse model, pneumolysin concentrations in bronchoalveolar lavage were found up to 1 ng/mL.[4] Although there remains some uncertainty about in vivo pneumolysin concentrations at the local site of infection, the ratios of immunoglobulin preparations and pneumolysin used in our experiments are likely within a clinically relevant range.

Surprisingly, for the pneumolysin concentration of 900 ng/mL, differences between IVIG (1 mg/mL) and trimodulin with adjusted IgG content (1.79 mg/mL) were observed in aggregometry, calcium release (►**Fig. 2A, B**), and viability assay (►**Fig. 4**), but not in flow cytometry (►**Fig. 1**). This might be either a matter of different sensitivity of the assays to platelet damage or donor-specific reasons.

In addition, trimodulin buffer showed an effect itself on thrombus formation in the flow chamber assay (►**Fig. 6**). As the blood samples tested in the assay function as a closed system without any clearance, this effect is likely to be an experimental artifact. In vivo, agents contained in the trimodulin buffer will likely be rapidly diluted and cleared from the circulation.

The negligible impact of IgM in preventing pneumolysin-induced platelet destruction by trimodulin can very likely be explained by its origin. Trimodulin is prepared from pooled human plasma obtained from healthy blood donors. Blood donors with symptoms of acute infection within the last 2 weeks are typically excluded from blood donation, but antipneumolysin IgM concentrations in human plasma are highest during pneumococcal infection.[27] In addition, in contrast to antipneumococcal capsular polysaccharide IgM, antipneumolysin IgM titers remain relatively low, even during acute infection.[27]

An experimental limitation of our study is that pneumolysin was first preincubated with the immunoglobulin preparations for 30 minutes before platelets were added. In vivo, patients will already be infected with pneumococci, which release pneumolysin[28,29] before they are treated with immunoglobulins. However, pneumonia can progress to sCAP associated with sepsis and immunoglobulin treatment may prevent worsening of pulmonary leakage. Interestingly, in a clinical study in patients with sCAP due to confirmed *S. pneumoniae* infection, in those receiving trimodulin, platelet counts increased faster compared to placebo.[7] The underlying mechanisms remain unclear. Our experiments indicate that one potential explanation among others might be that neutralization of pneumolysin supports the faster increase of platelet counts. However, other studies indicate more pronounced immune modulation by trimodulin compared to IVIG.[30] Furthermore, trimodulin but not IVIG was found to interact with the complement system in an either activating or inhibiting way, depending on the concentration.[23] Which of these factors finally contributes to clinical efficacy in acute pneumonia is unresolved.

In conclusion, our results indicate that both IVIG and the IgM/IgA-enriched immunoglobulin preparation trimodulin represent promising candidates for supportive treatment of pneumococcal pneumonia. By preventing pneumolysin-induced platelet destruction and maintaining platelet functionality, immunoglobulin preparations could potentially prevent the worsening of respiratory distress. In pharmaceutical immunoglobulin preparations, IgG seems to be the determining component for the effective prevention of platelet destruction by pneumolysin in vitro.

What is known about this topic?

- Pneumolysin, the main toxin of *Streptococcus pneumoniae,* forms pores in platelets and renders them nonfunctional. Impaired platelet function plays a role in the development of respiratory distress, which occurs as a complication of severe community-acquired pneumonia (sCAP), often caused by S. *pneumoniae*.
- In vitro, a human IgG immunoglobulin preparation was shown to prevent pneumolysin-induced platelet destruction.
- In vivo, a human IgM/IgA-enriched immunoglobulin preparation was shown to reduce mortality in sCAP patients.

What does this paper add?

- Both IgG and IgM/IgA-enriched immunoglobulin preparations sufficiently inhibit pneumolysin-induced platelet destruction in vitro.
- Compared to IgG preparations, additional IgM and IgA do not increase the capacity to inhibit pneumolysin-induced platelet destruction in vitro. This indicates that IgG is the main component for an inhibition of pneumolysin-induced platelet destruction.

Funding

This study was funded by Deutsche Forschungsgemeinschaft, Grant/Award Number: Projektnummer 374031971-TRR240; Research support by Biotest. CAPNETZ is supported by the German Center for Lung Research (DZL).

Conflict of Interest

A.G. reports grants and nonfinancial support from Aspen, Boehringer Ingelheim, MSD, Bristol Myers Squibb (BMS), Bayer Healthcare, Instrumentation Laboratory; personal fees from Aspen, MSD, Macopharma, BMS, Chromatec, Instrumentation Laboratory, nonfinancial support from Portola, Ergomed, Biokit outside the submitted work. S.W. and J.S. are employees of Biotest AG. All other authors declare no conflict of interest.

Acknowledgement

The investigators of this scientific work acknowledge CAPNETZ STIFTUNG and the competence network CAPNETZ for project support with regard to using biomaterials and clinical data. CAPNETZ is a multidisciplinary approach to better understand and treat patients with CAP. The network has been made possible by the contributions of many investigators.

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Supplement

Figure S1

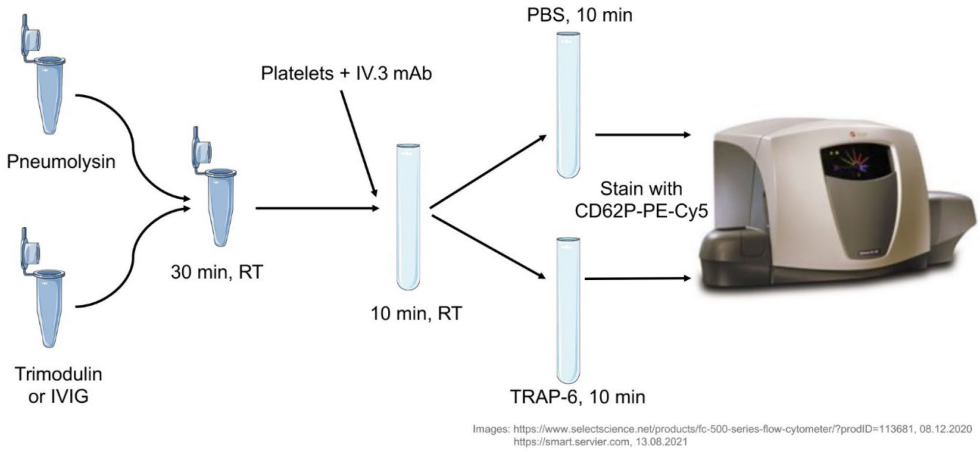


Figure S1: Experimental setup of CD62P assay.

Figure S2

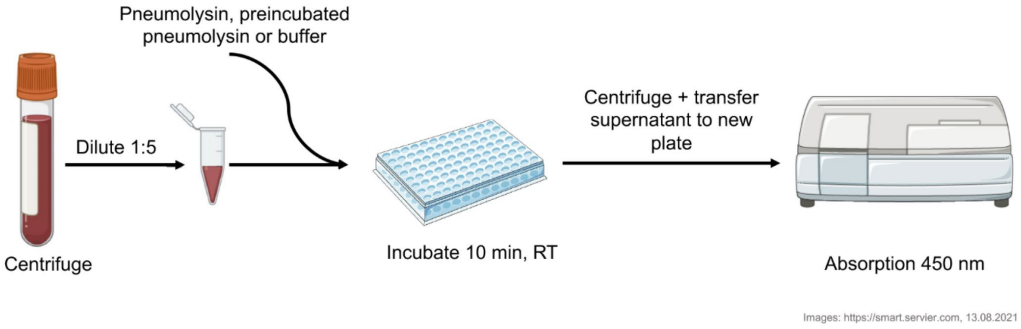


Figure S2: Experimental setup of the hemolysis assay.

Figure S3

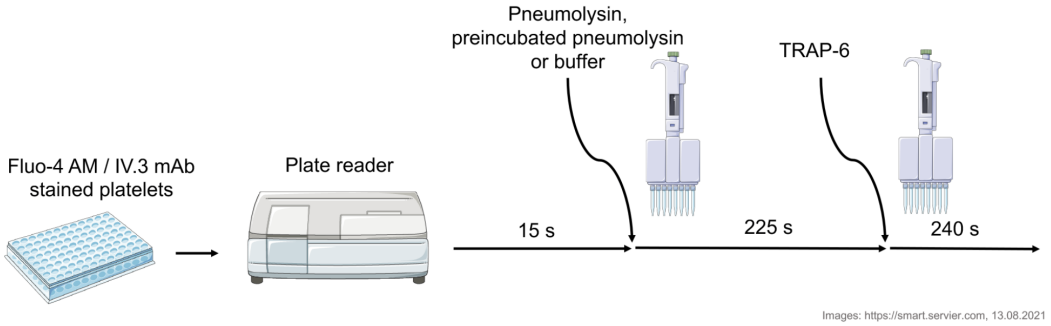


Figure S3: Experimental setup of the calcium release assay.

Figure S4

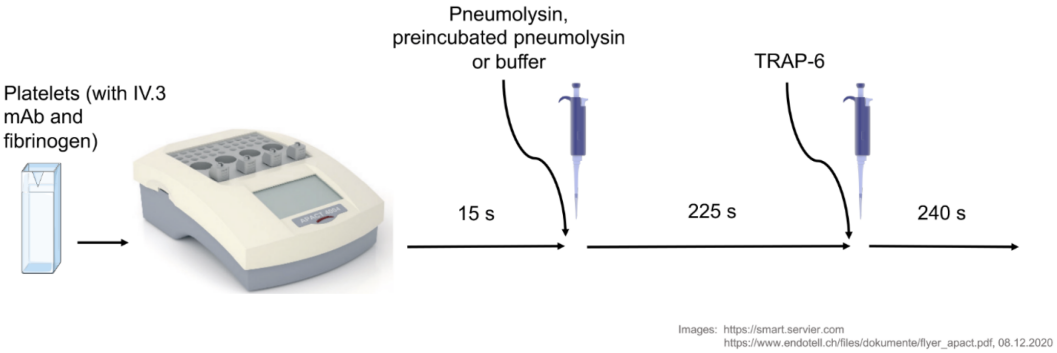


Figure S4: Experimental setup of aggregometry.

2

Figure S5

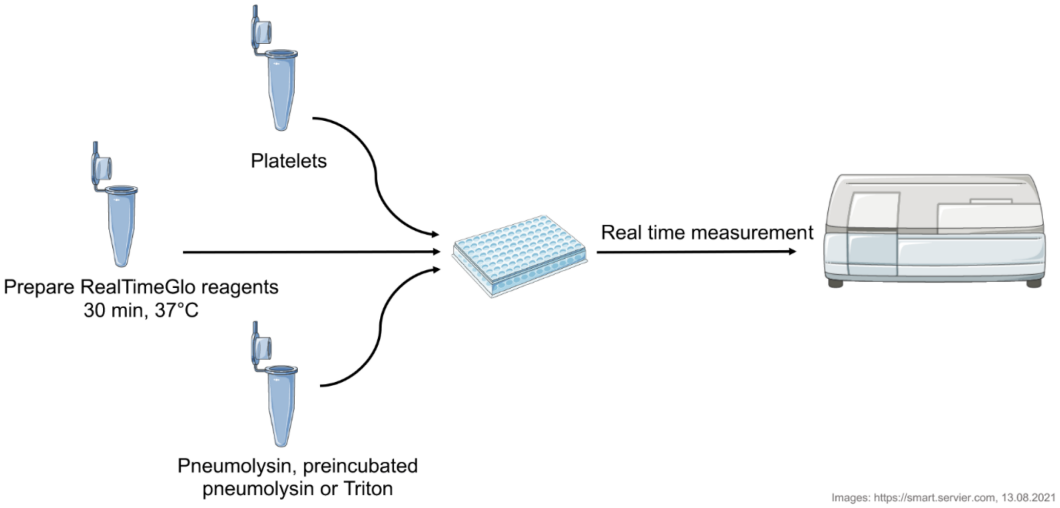


Figure S5: Experimental setup of the platelet viability assay.

Figure S6

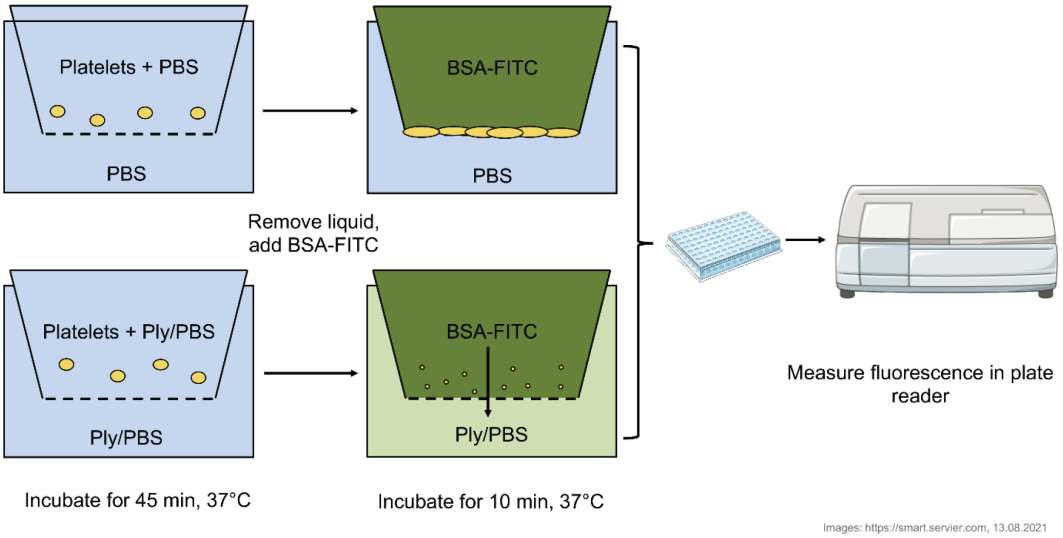


Figure S6: Experimental setup of the transwell assay.

3

Figure S7

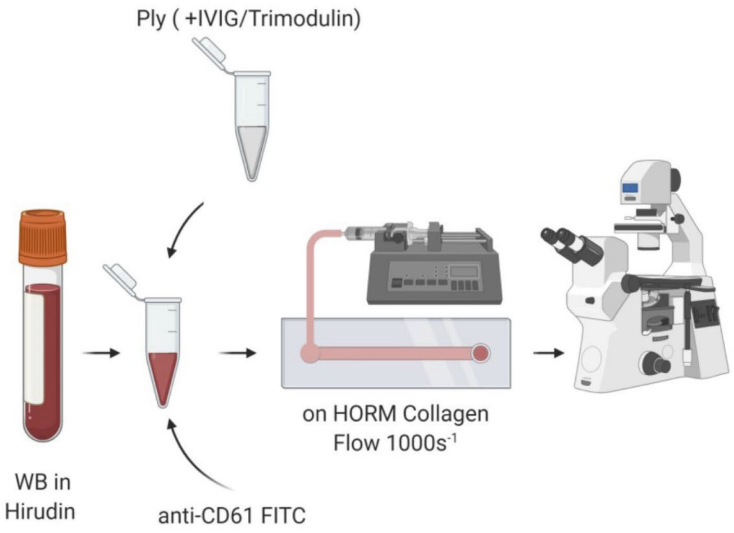


Figure S7: Experimental setup of the flow chamber assay.

Figure S8

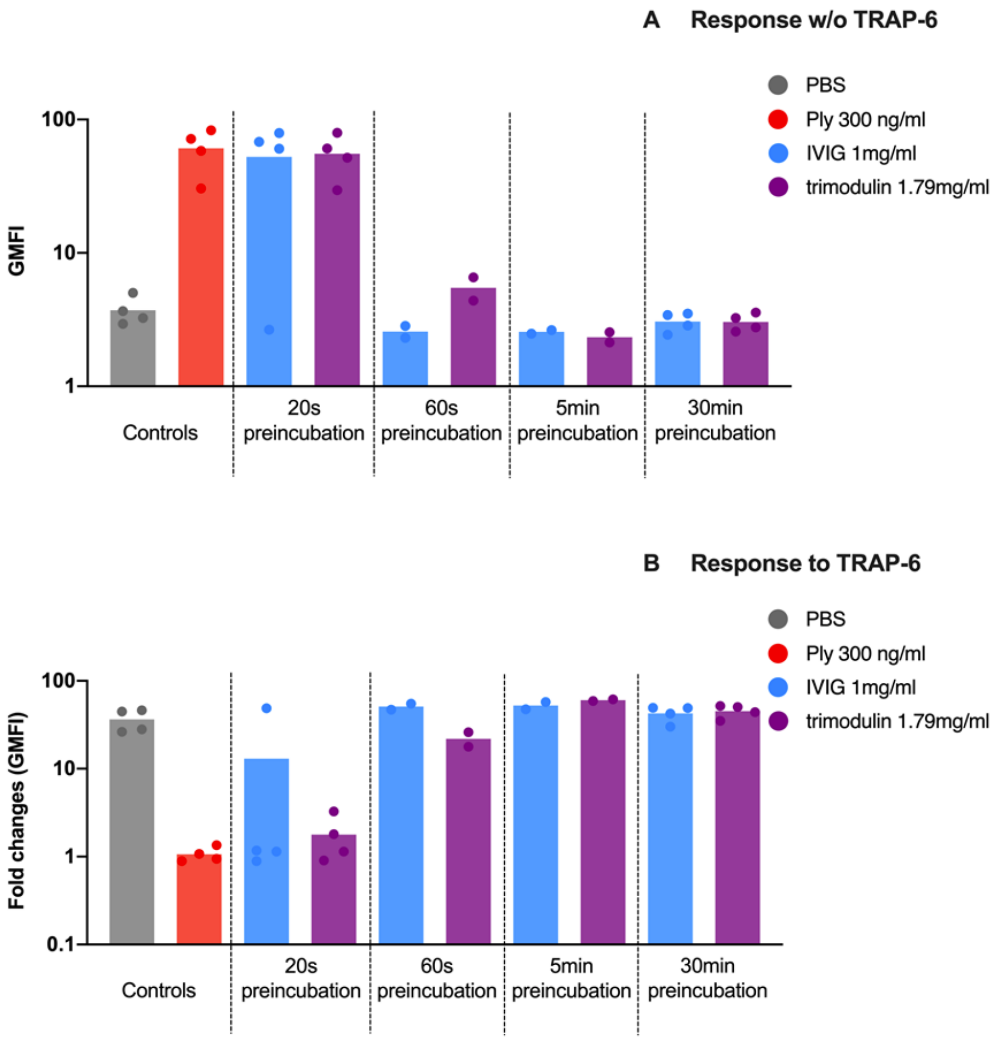
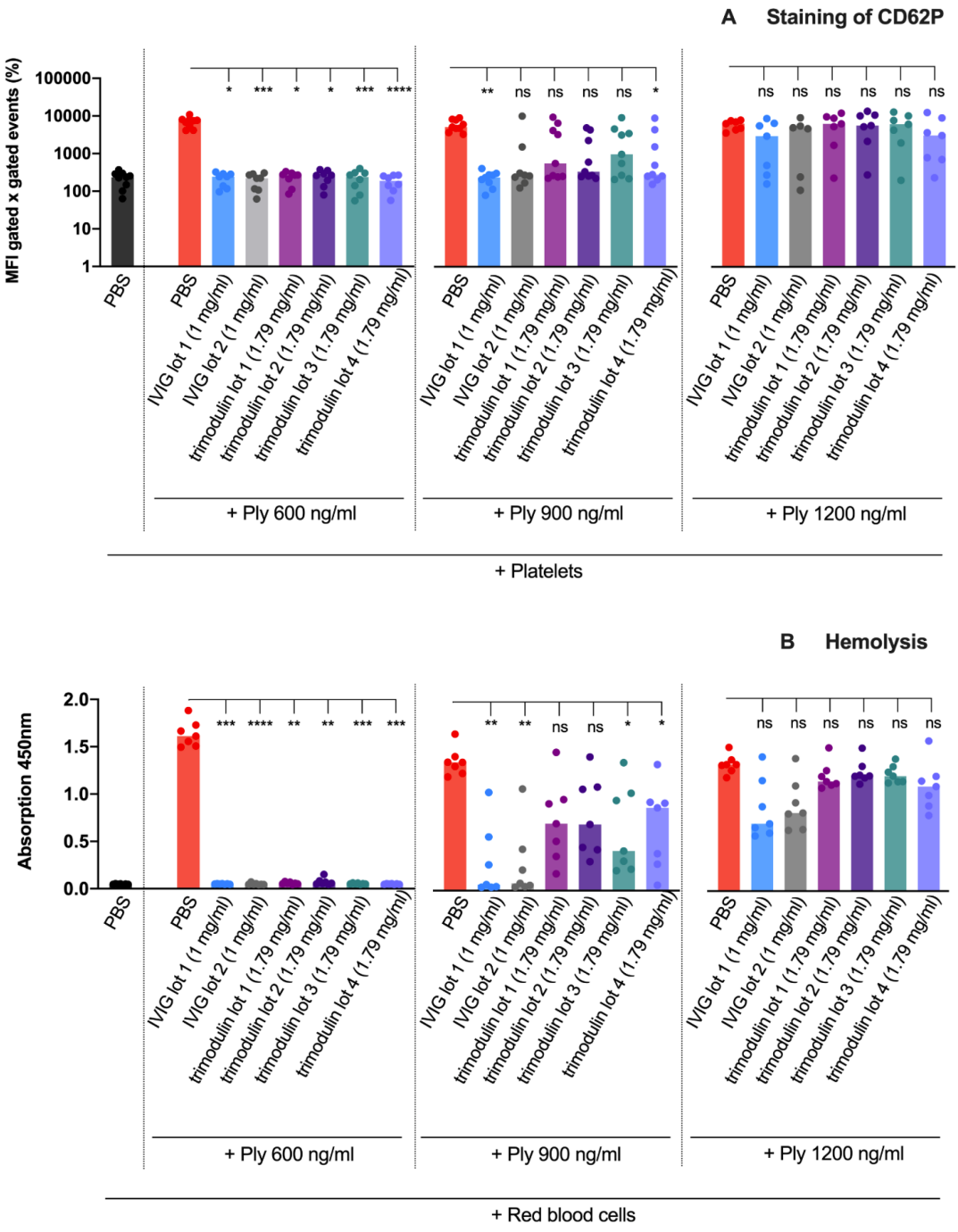


Figure S8: CD62P staining in platelets upon pneumolysin treatment.

(A) CD62P staining upon treatment with pneumolysin or pneumolysin preincubated with IVIG or trimodulin. CD62P staining is presented as geometric mean fluorescence intensity (GMFI) of CD62P-positive gated events. As a control, samples were incubated only with PBS.

(B) CD62P staining upon TRAP-6 (20 µM) addition after preincubation of platelets with pneumolysin or pneumolysin preincubated with IVIG or trimodulin. Response to TRAP-6 is presented as a fold change of the GMFI of CD62P-positive gated events. As a control, samples were incubated with PBS only.

Figure S9


A Staining of CD62P
MFI gated x gated events (%)
100000
10000
1000
100
10
1
PBS
IVIG lot 1 (1 mg/ml)
IVIG lot 2 (1 mg/ml)
trimodulin lot 1 (1.79 mg/ml)
trimodulin lot 2 (1.79 mg/ml)
trimodulin lot 3 (1.79 mg/ml)
trimodulin lot 4 (1.79 mg/ml)
+ Ply 600 ng/ml
+ Ply 900 ng/ml
+ Ply 1200 ng/ml
+ Platelets
B Hemolysis
Absorption 450nm
2.0
1.5
1.0
0.5
0.0
+ Red blood cells

4.

α- hemolysin of Staphylococcus aureus impairs thrombus formation

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Published in Journal of Thrombosis and Haemostasis.
2022 Mar 18. doi: 10.1111/jth.15703

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Experiments performed by K.J. shown in Fig. 1A-C; 2A; 3B; 5A,B; S1 A-C; S2
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Providing materials/reagents: S.H., A.G., C.W.
Writing the manuscript: K.J.
Revision of the manuscript: K.J., St.H., R.P, TP.K, C.W., A.G. S.H.

Kristin Jahn Prof. Sven Hammerschmidt

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).

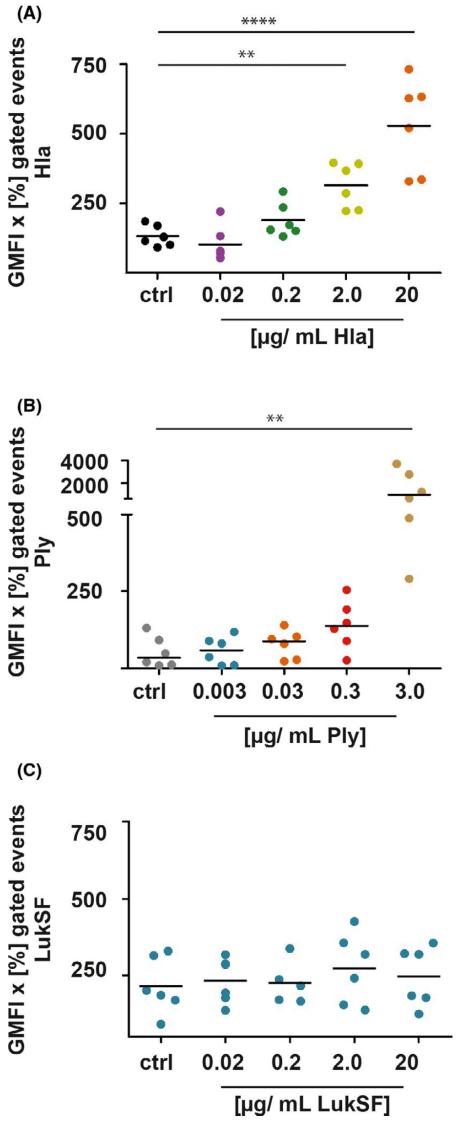

FIGURE 1 Pneumolysin and α-hemolysin bind directly to human platelets. Washed platelets of a defined set of healthy human donors were incubated with increasing concentrations of pneumolysin (Ply (0.3 to 300 ng/ml), α-hemolysin (Hla 0.02–20 µg/ml), and LukSF (PVL 0.02 to 20 µg/ml), fixed and stained with 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,

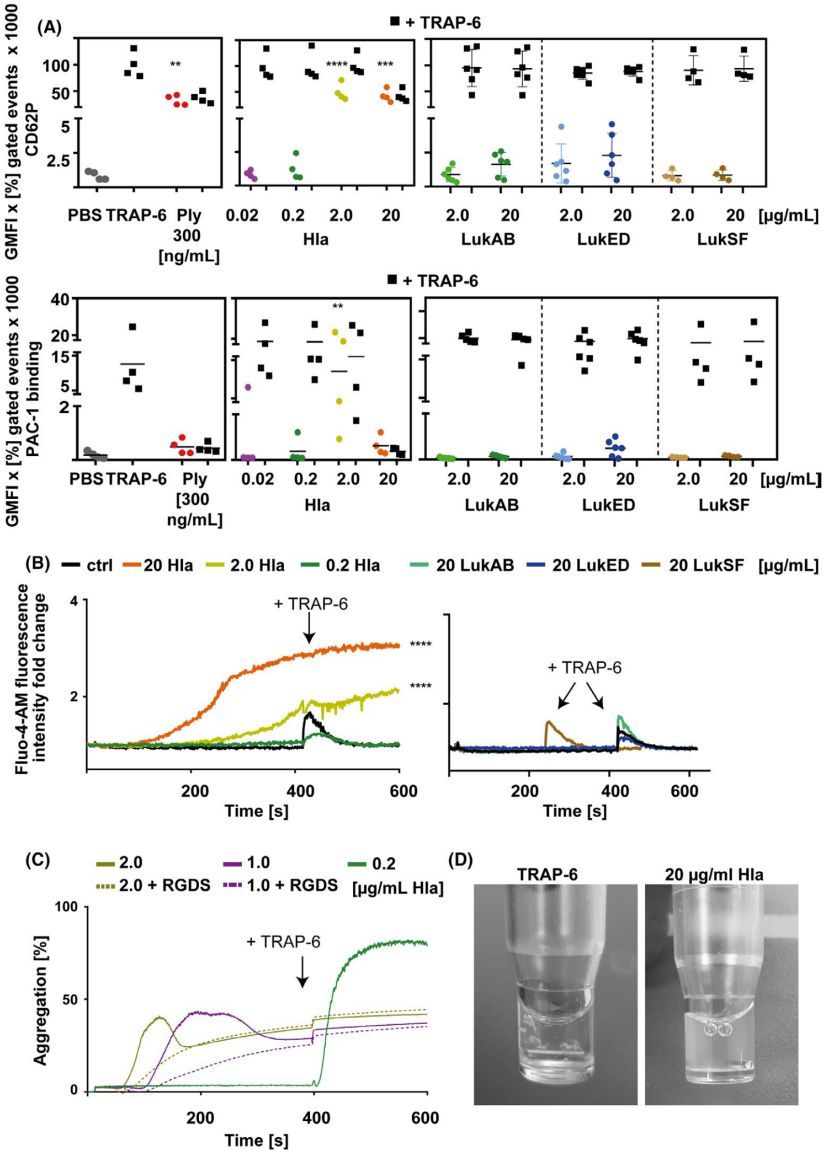

FIGURE 2 α-hemolysin and pneumolysin interfere with platelet function but with different mechanisms. Washed platelets of a defined set of healthy human donors were incubated with increasing concentrations of pneumolysin (Ply 0.3–300 ng/ml), α-hemolysin (Hla 0.02–20 g/ml) LukAB, LukED, and LukSF (PVL) 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

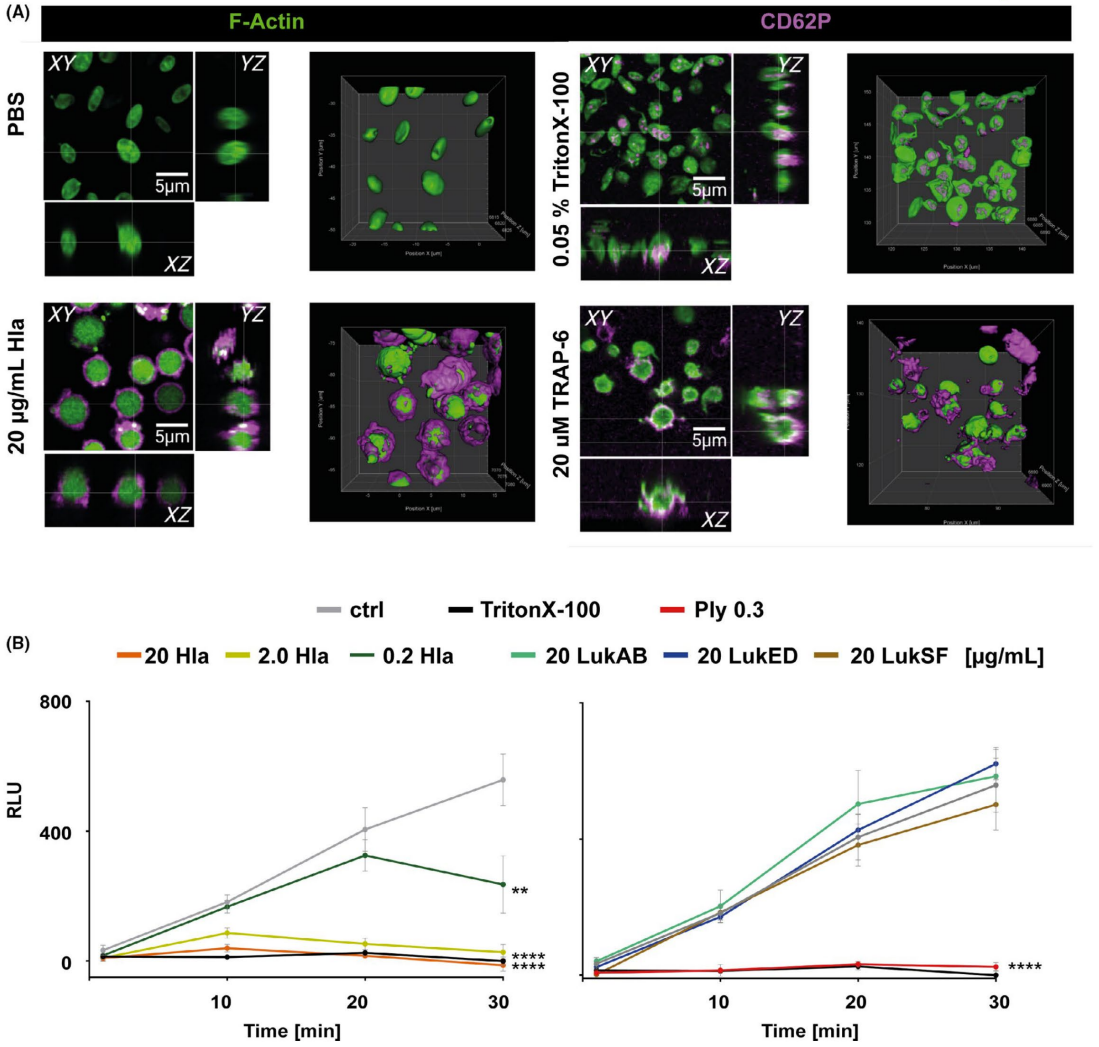

FIGURE 3 Treatment of human platelets with α-hemolysin leads to staining of surface associated CD62P (A) α-hemolysin (Hla) treated platelets were stained for F-actin (green) and CD62P (magenta). Platelets were not permeabilized. Orthogonal views of confocal Z-stacks and three-dimensional iso-surface 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

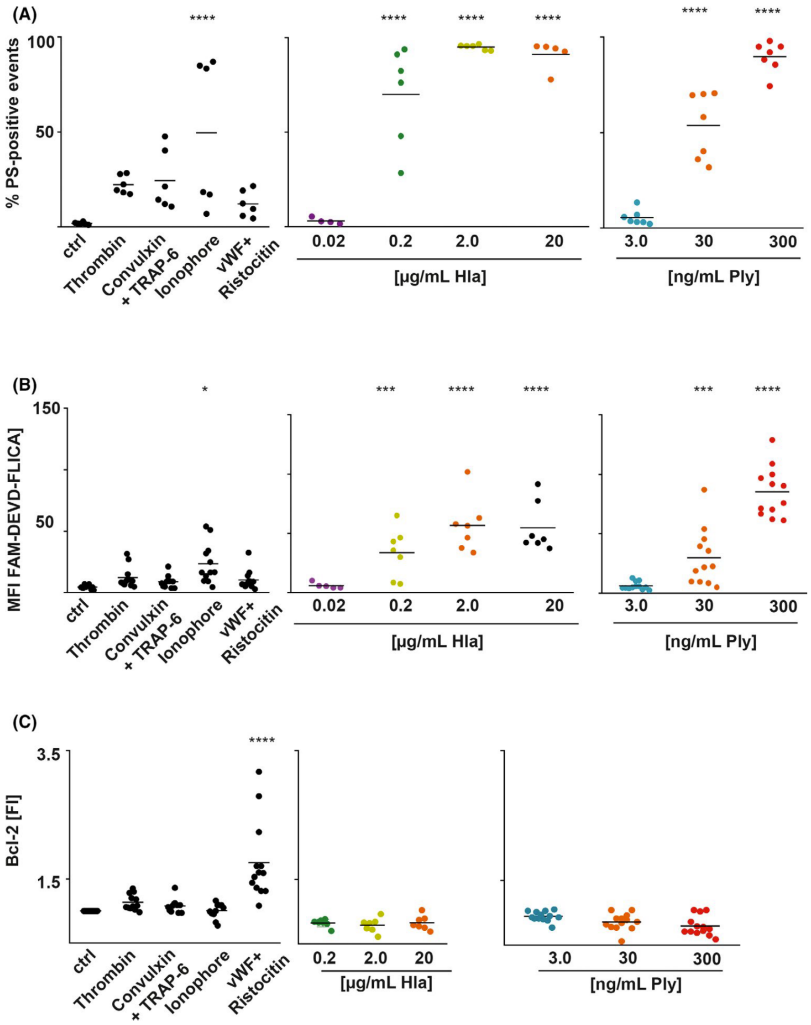

FIGURE 4 Platelets treated with α-hemolysin and pneumolysin are positive for apoptosis markers. Washed human platelets were incubated with increasing concentrations of α-hemolysin (Hla) and pneumolysin (Ply). The analysis of the apoptosis markers caspase activity, Bcl-2 expression, and phosphatidylserine (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.

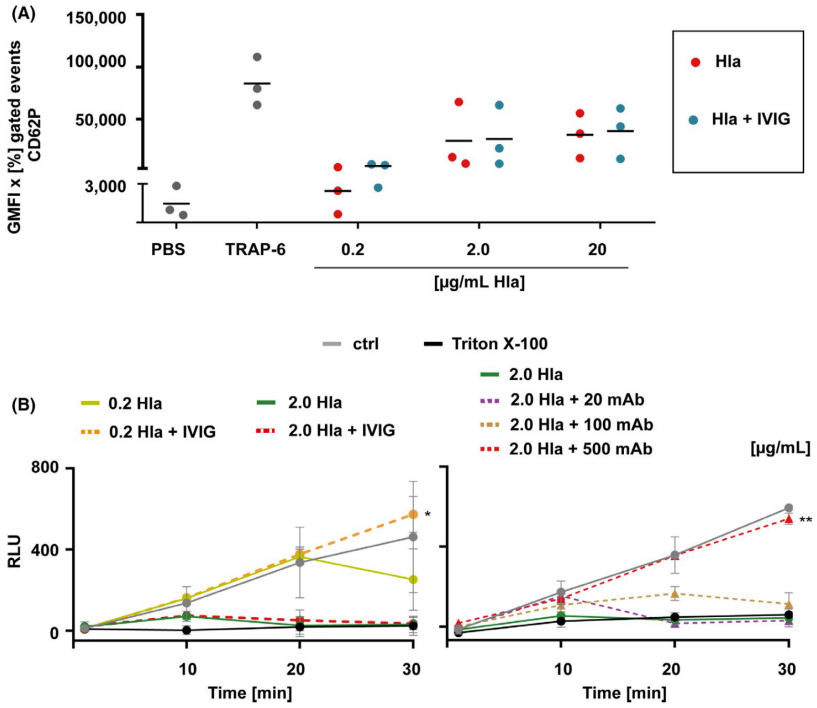

FIGURE 5 IVIG does not neutralize α-hemolysin. α-hemolysin (Hla) was treated with IVIG (1 mg/ml) or a specific mAb for 20 min before incubation with washed human platelets of healthy human donors. (A) Polyvalent human immunoglobulins (IVIG; 1 mg/ml; human IgG, Privigen) did not neutralize the increased 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

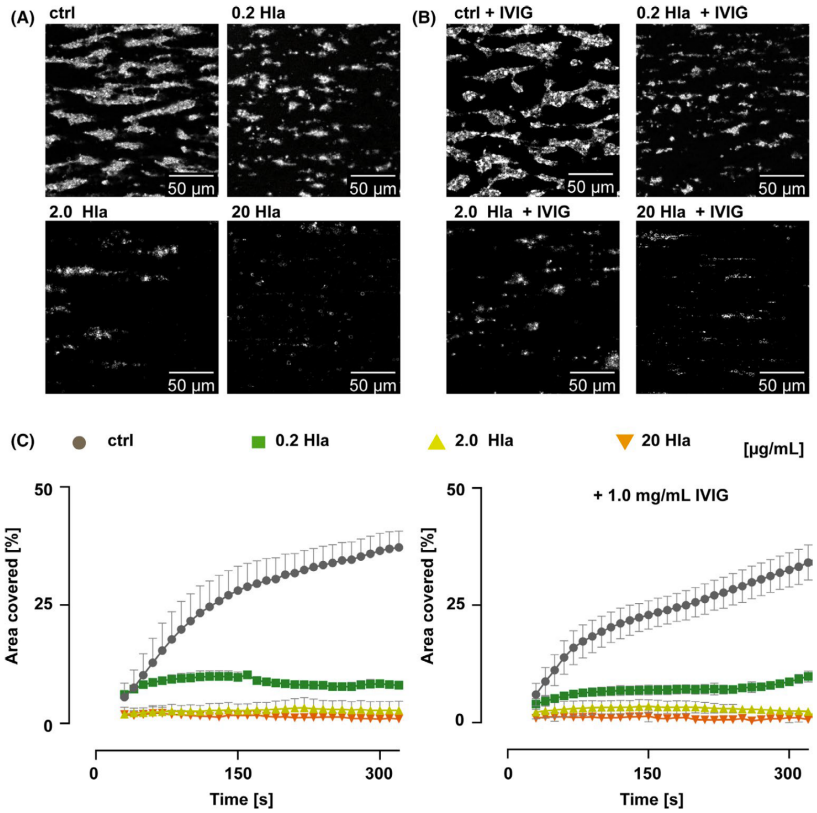

FIGURE 6 α-hemolysin induces abrogation of thrombus formation in whole blood under shear. Whole blood incubated with α-hemolysin (Hla) at 0.2, 2.0, and 20 µg/ml Hla was perfused over a collagen (shear 1000 s^{-1})-coated surface and thrombus formation was visualized by immunostaining of platelets with 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

Supplemental Methods

Supplemental Figures S1 and S2

α-hemolysin of *Staphylococcus aureus* impairs thrombus formation

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Methods

Platelet preparation

Platelets were purified from ACD-A anticoagulated whole blood from healthy donors, not taking any antiplatelet drugs or non-steroidal anti-inflammatory drugs (NSAIDs). We prepared platelets as described.[1] In brief, we washed platelet rich plasma (PRP) twice with Tyrode´s buffer containing 0.35% BSA, 0.1% glucose, 2.5 U/mL apyrase, 1 U/mL hirudin, pH 6.3 and resuspended the final platelet pellet in a bicarbonate based suspension buffer containing 0.35% BSA, 0.1% glucose, 2.14 mM $MgCl_2$, 2 mM $CaCl_2$, pH 7.2. Platelets were adjusted to 300,000 platelets/µL.[2]

Light transmission aggregometry

We resuspended washed platelets in suspension buffer and added fibrinogen to a final concentration of 2.25 mg/mL. In some experiments 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 human polyvalent immunoglobulin preparations (IVIG; IgG-enriched Privigen; CSL Behring, Marburg, Germany) were added. After transfer to the aggregometer cuvette, different concentrations of pneumolysin were added to the platelet suspension after 15 s. We measured platelet aggregation as a decrease in turbidity of the medium with an APACT4 aggregometer at 500 rpm, 37°C (Haemochrom, Germany) applying APACT LPC-software. In some experiments we added 20 µM TRAP-6 after 240 s and continued measurement for further 200 s.

Live/dead staining

For measurement of cell viability in the presence of pneumolysin, Hla and the leukocidins LukAB, LukSF (PVL) and LukDE, we used the RealTime-Glo™ Cell Viability Assay kit (Promega) and measured viability for 30 min. We mixed the assay substrate 1:1 with the 2-fold concentration of pneumolysin, Hla or LukSF. In a subset of experiments, a Hla inhibiting monoclonal mouse antibody, or IVIG (Privigen; CSL Behring, Marburg, Germany) was added. Then we added the toxin-substrate mixture to washed human platelets in a 96 well plate in duplicates. After 1 min of incubation, we started shaking the plate with 300 rpm for 3 s and measured relative luminescence units (RLU) using a microtiter plate reader. We repeated shaking and measurement of luminescence every 60 s until a total measurement time of 30 min. Sample values of luminescence represent the mean of the duplicates subtracted by blank (Tyrode's buffer without platelets) of six independent experiments.

Release of intracellular calcium

We detected the release of Ca^{2+} from internal stores to the cytoplasm by fluorescent labelling of free intracellular Ca^{2+} using Fluo-4-AM (ThermoFisher, USA). We resuspended platelets in PBS without $MgCl_2$ and $CaCl_2$ (pH 7.4), adjusted them to 150,000 platelets/µL and stained them with Fluo-4-AM for 30 min in the dark at RT. After a 1:2 dilution in PBS, we carried out baseline measurements for 15 s. Afterwards, we stimulated platelets with pneumolysin (3-300 ng/mL final), hla (0.2-20 µg/mL final) or LukSF (0.2-20 µg/mL final). We measured free Ca^{2+} with a Fluoroskan Ascent FL fluorometer (ThermoFisher, USA) over 7 min. In some experiments we added TRAP-6 at a final concentration of 20 µM after 250 s and the measurement was carried out for another 200 s.

Immunofluorescence staining of platelets

Immunofluorescence analysis of CD62P localization was done as described recently[3]. In brief, we incubated washed human platelets (300,000 cells/µL) in PBS (negative control), or 20 µM TRAP-6 (positive control), or 0.05% Triton X-100 (control for membrane disintegration) or 20 µg/mL Hla for 10 min at 37°C. After fixation with 2% PFA the samples were spun on microscopy slides using the Cytospin system (Thermo Fisher). We stained platelets with AF647 labelled CD62P antibody and 20 µM phalloidin ATTO 488. The samples were covered with 20 µL of Fluorescent Mounting Medium (ROTI Mount FluorCare HP19, Carl Roth GmbH Karlsruhe, Germany). Imaging was performed on a Leica SP5 confocal laser scanning microscope (Leica, Wetzlar, Germany) equipped with HCX PL APO lambda blue 40.0x/1.25 OIL UV objective. For detailed analysis of localization of CD62P we performed confocal Z-stacks of platelets and created orthogonal views and 3D rendering.

***Ex vivo* thrombus formation assay under shear**

Hirudinized whole blood (1 mL) was incubated with alpha-hemolysin at 0.2 µg/mL, 2 µg/mL and 20 µg/mL final concentration for 10 min.. In a subset of experiments, IVIG (Privigen; CSL Behring, Marburg, Germany) was added to hirudinized whole blood either in the absence or in the presence of alpha-hemolysin. Thrombus formation assays were performed (n=3 donors) 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]).[3-5] Platelets in whole blood were immunofluorescently labeled with monoclonal antibody CD61-

FITC (Cat. No. 561913, RRID: AB_396094 BD Biosciences, Franklin Lakes, USA, at a final concentration of 0.125 µg/mL) to facilitate visualization of thrombus formation. Time-lapse confocal imaging was performed at intervals of 10 seconds per image on a Leica SP5 confocal laser scanning microscope (Leica) equipped with a water immersion HCX PL APO Lambda blue 40×/1.25 UV 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. Assessment of platelet adhesion and thrombus formation to obtain the percentage area covered by thrombi (n=3 images measuring field of view 193.75 µm2 each from different regions of interest from per donor) was performed in Fiji. All flow chamber perfusion experiments were performed according to the International Society on Thrombosis and Haemostasis Scientific and Standardization Committee (ISTH SSC) subcommittee on Biorheology recommendations.

Western Blot

A total of 1 µg of pneumolysin, Hla and LukSF (PVL), respectively, was separated on a 12% SDS-PAGE. The samples were blotted using a semi-dry blotting system (Trans-Blot SD Cell, Bio-Rad) on a nitrocellulose membrane. After blocking with 5% skimmed milk powder (Roth, Karlsruhe) in TBS, we used IVIG (Privigen; CSL Behring, Marburg, Germany) and a HRP-conjugated secondary antibody to detect the toxins with a ChemoCam (Intas, Science Imaging). The Immunoblot images were adjusted for brightness and contrast using Photoshop CS5 64 bit.

Supplemental Figures:

Figure S1

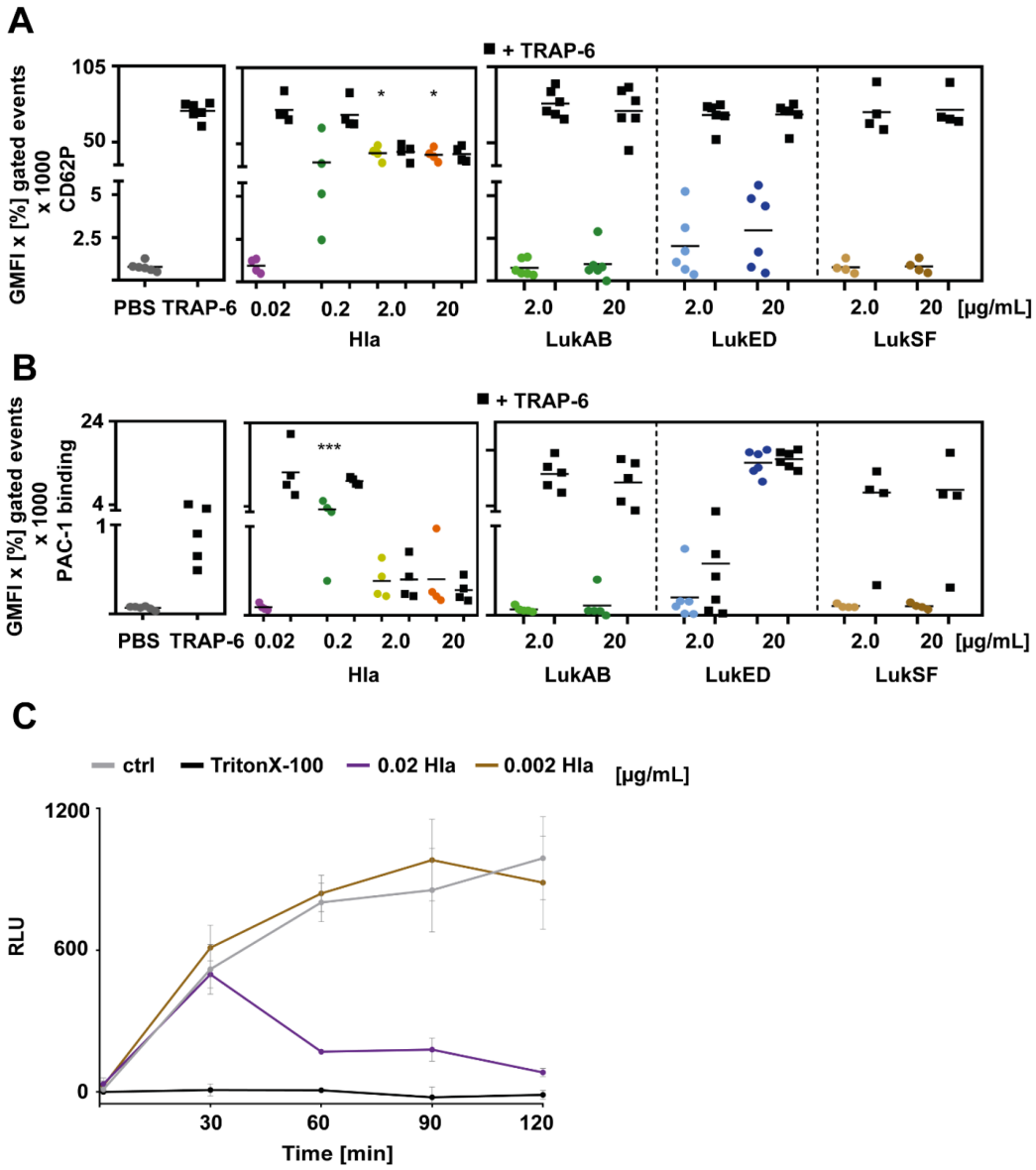

Figure S1. Hla induced CD62P expression and PAC-1 binding over time

Washed platelets of a defined set of healthy human donors were incubated with increasing concentrations of alpha-hemolysin (Hla, 0.02 to 20 g/mL), LukAB, LukED and LukSF (PVL) for 10 or 30 min.

(A) CD62P was used as activation marker and detected by flow cytometry, using a PE-Cy5 labelled P-selectin antibody. PBS was used as negative control and 20 µM TRAP-6 as a positive control. Platelets were incubated with the toxins for 30 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) PAC-1 binding was used as activation marker and detected by flow cytometry, using a FITC labelled anti-human PAC-1 antibody. PBS was used as negative control and 20 µM TRAP-6 as a positive control. Platelets were incubated with the toxins for 30 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. The decreased signals compared to 10 min of incubation is due to ongoing internalization of the activated GPIIb/IIIa.
(C) 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. Washed platelets were incubated for 120 min with 0.02 and 0.002 µg/ml Hla. One minute after mixing of platelets and toxins the measurement started.

Figure S2

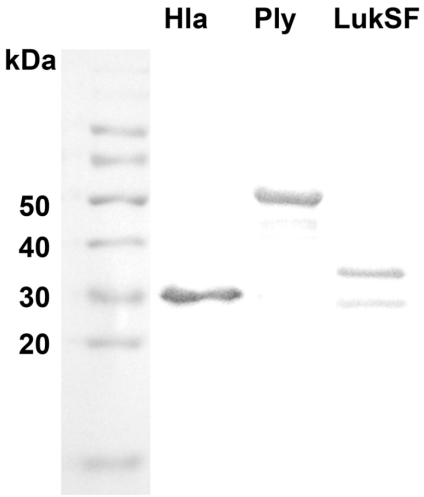

Figure S2. IVIG recognizes pneumolysin, Hla and LukSF

One µg of the indicated proteins or in the case of LukSF (PVL) protein mixture (equimolar ratio) were applied on a 12% SDS gel, blotted on a nitrocellulose membrane and stained with 1 mg/ml IVIG as primary antibody and a HRP coupled anti-human IgG as secondary antibody.

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5.

Group B Streptococcal Hemolytic Pigment Impairs Platelet Function in a Two-Step Process

Kristin Jahn, Patience Shumba, Phoenicia Quach, Mathias Müsken, Jan Wesche, Andreas Greinacher, Lakshmi Rajagopal, Sven Hammerschmidt and Nikolai Siemens

Published in Cells
2022 May 13. doi: 10.3390/cells11101637

Author contributions:
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Implementation of experiments: K.J., P.S., M.M., N.S.
Experiments performed by K.J. shown in Fig. 1A-C; 2A; 3B; 5A,B; S1 A-C; S2
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Communication

Group B Streptococcal Hemolytic Pigment Impairs Platelet Function in a Two-Step Process

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Abstract: Group B streptococci (GBS) cause a range of invasive maternal–fetal diseases during pregnancy and post-partum. However, invasive infections in non-pregnant adults are constantly increasing. These include sepsis and streptococcal toxic shock syndrome, which are often complicated by systemic coagulation and thrombocytopenia. GBS express a hyper-hemolytic ornithine rhamnolipid pigment toxin with cytolytic and coagulatory activity. Here, we investigated the effects of GBS pigment on human platelets. Infections of platelets with pigmented GBS resulted initially in platelet activation, followed by necrotic cell death. Thus, this study shows that GBS pigment kills human platelets.

Keywords: *Streptococcus agalactiae*; group B streptococcus; pigment; platelets

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Citation: Jahn, K.; Shumba, P.; Quach, P.; Müsken, M.; Wesche, J.; Greinacher, A.; Rajagopal, L.; Hammerschmidt, S.; Siemens, N. Group B Streptococcal Hemolytic Pigment Impairs Platelet Function in a Two-Step Process. *Cells* **2022**, *11*, 1637. https://doi.org/10.3390/cells11101637

Academic Editor: Judith Cosemans

Received: 22 February 2022
Accepted: 10 May 2022
Published: 13 May 2022

Publisher's Note: MDPI stays neutral with regard to jurisdictional claims in published maps and institutional affiliations.

1. Introduction

Streptococcus agalactiae, or group B streptococci (GBS), are a major cause of morbidity and mortality in neonates and pregnant women. Since the 1980s, reports of invasive infection in non-pregnant adults with underlying conditions have increased [1]. These include necrotizing skin and soft tissue infections (NSTIs), which are often accompanied by sepsis or streptococcal toxic shock syndrome (STSS) [1]. Sepsis, particularly STSS, is associated with excessive hyper-inflammation, leading to multi-organ failure [2]. Common additional complications include disseminated intravascular coagulation (DIC) and thrombocytopenia [3], which are associated with poor prognosis [4].

Platelets are the second most abundant cell type in the circulation. Despite their role in hemostasis, they also function as immune cells. Upon activation, platelets release their granule contents, comprising of bactericidal proteins, cytokines, coagulation factors, and immunomodulatory factors [4]. Researchers have reported that several bacterial species interact with platelets, resulting in platelet activation or damage [4]. So far, platelet activation has predominantly been reported by GBS strains isolated from septic patients [5]. Recently, an unusual case involving STSS, DIC, and *purpura fulminans* in an immunocompetent individual was reported [6]. The causative GBS strain was pigmented and hyper-hemolytic. The GBS pigment, which is a surface localized ornithine rhamnolipid also called Granadaene [7],

showed pro-inflammatory and coagulatory activity [6]. Here, we analyzed the impact of GBS pigment on human platelet function.

2. Materials and Methods

2.1. Ethics

The use of whole blood and washed platelets from healthy adults 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. Platelet Preparation

Washed human platelets were prepared as described previously [8]. In brief, platelet rich plasma (PRP) was prepared from ACD-A anticoagulated whole blood from a defined set of healthy volunteers who were not taking any anti-platelet or non-steroidal anti-inflammatory drugs. The PRP was washed two times in Tyrode's buffer containing 2.5 U/mL apyrase, 1 U/mL hirudin, 0.35% BSA, and 0.1% glucose, with a pH of 6.3. Lastly, the platelet pellet was resuspended in a bicarbonate-based suspension buffer supplemented with 2.12 mM $MgCl_2$, 1.96 mM $CaCl_2$, 0.35% BSA, and 0.1% glucose, with a pH of 7.2. The platelet count was adjusted to 300,000 platelets/µL.

2.3. Bacterial Strains and Pigment Purification

The following GBS strains isolated from NSTI and/or STSS patients were used: pigmented LUMC16 and STSS/NF-HH, and non-pigmented STSS/NF-LH [6,9]. The LUMC16Δ*cylX-K* mutant was constructed using methods described previously [7]. Briefly, the plasmid pHY304Δ*cylX-K* was electroporated into LUMC16, and selection and screening for the double crossover mutant was performed. PCR was used to verify the absence of *cylX-K* and the presence of Ω *km*-2, using primers for flanking genomic regions and internal gene primers as described previously [7]. The colonies were examined for the loss of hemolysis and pigmentation on blood agar and Granada media, respectively. All strains were cultured in Todd–Hewitt broth supplemented with 1.5% (*w*/*v*) yeast extract (Roth).

The GBS pigment from the STSS/NF-HH strain was purified as previously described [6]. Parallel extraction from the non-pigmented STSS/NF-LH strain was performed and used as a control. To confirm that the pigment was extracted, and for quantification, MALDI-FTICR-MS was performed [6,7]. The pigment was exclusively present in the samples from the STSS/NF-HH strain (m/z value of 677.3862), whereas no peak was detected in the extracts from the STSS/NF-LH strain. This extract was used in all experiments as a negative buffer control. A hemoglobin release assay was performed to confirm the hemolytic activity of the pigment [6].

2.4. Platelet Infections

All experiments were performed in Tyrode's buffer containing Ca^{2+} and Mg^{2+}. The platelets were infected with increasing multiplicity of infection (MOI) for LUMC16, LUMC16 Δ*cylX-K* (MOI 0.1, MOI 1.0, MOI 10), STSS/NF-HH, and STSS/NF-LH (MOI 0.1), or were incubated with increasing concentrations of isolated pigment or the respective buffer control (0.5, 1.0, 2.0 µM) for 30, 60, and 120 min. The platelet releasate was prepared by the activation of 2.1×10^7 platelets with 40 µM TRAP-6 and 30 µg/mL collagen for 10 min at 37 °C. The activated platelets were pelleted, and supernatants were used as a releasate. Washed platelets or platelet releasate were incubated with 2.1×10^6 CFU (MOI 0.1). The CFUs were determined by plating serial dilutions on blood agar plates.

2.5. Platelet Activation Assay and Assessment of Death

Activation assays were performed in Tyrode's buffer containing Ca^{2+} and Mg^{2+} as described previously [8]. The washed human platelets were infected as described above. The CD62P expression of CD42a-positive cells was measured using a FACSCalibur (Becton

Dickinson, Franklin Lakes, NJ, USA) flow cytometer and CellQuestPro 6.0, and was analyzed using FCS Express 7 Software (De Novo Software). The gating strategy is displayed in Figure S1A. In a subset of experiments, platelets were subsequently stimulated with 20 µM TRAP-6 5 min prior to the end of stimulations/infections. The following antibodies were used: PE-Cy5-labelled monoclonal mouse anti-human CD62P and FITC-labelled mouse anti-human CD42a (BD Biosciences, Franklin Lakes, NJ, USA).

Platelet viability was determined using a RealTime-Glo Cell Viability Assay kit (Promega, Walldorf, Germany) over a period of 120 min. The washed human platelets were infected with GBS or were stimulated with the pigment, as described above. One minute after mixing and shaking the plate (300 rpm; 3 s), luminescence was recorded every 180 s. PBS and TritonX-100 (Sigma-Aldrich, Taufkirchen, Germany) served as negative and positive controls, respectively. All experiments were performed in duplicates.

A FAM-FLICA caspase-3/7 assay kit (ImmunoChemistry, Davis, CA, USA), TMRE-mitochondrial membrane potential assay kit (Abcam), and Annexin V-FITC (BioLegend) were used to determine apoptotic and/or necrotic events after 120 min of infection or stimulation. All experiments were performed according to the manufacturer's instructions. The PBS and ABT-737 (10 µM, Hycultec, Beutelsbach, Germany), FCCP (20 µM), and Ply (300 ng/ml) stimulations served as negative or positive controls. All measurements were performed using a FACSCalibur (Becton Dickinson, Franklin Lakes, NJ, USA) flow cytometer and CellQuestPro 6.0.

2.6. Field Emission Scanning Electron Microscopy

Field emission scanning electron microscopy (FESEM) was performed as previously described [8]. Briefly, the infected platelets were fixed with 5% formaldehyde and 2% glutaraldehyde in 0.1 M Hepes buffer (0.1 M HEPES, 0.09 M sucrose, 10 mM $CaCl_2$, 10 mM $MgCl_2$, pH 6.9). The samples were centrifuged at 2000× *g* for 2 min, washed with TE buffer (20 mM Tris-HCl, 2 mM EDTA, pH 6.9), and resuspended in 50 µL of TE buffer. The cells were placed onto poly-l-lysine-coated cover slips, fixed with 1% glutaraldehyde in TE buffer for 10 min, washed with TE buffer, dehydrated with an increasing series of acetone, underwent critical point drying (CPD 300; Leica, Wetzlar, Germany), and were sputter-coated with gold–palladium (SCD 500; Bal-Tec, Los Angeles, CA, USA). For imaging via a field emission scanning electron microscope (Zeiss Merlin), Everhart–Thornley and In-lens SE detectors (at a ratio of 25:75 and an acceleration voltage of 5 kV) and SmartSEM software 6.06 were used.

2.7. Statistics

If not otherwise indicated, statistically significant differences were determined using Kruskal Wallis tests with Dunn's post-tests. Statistical analyses were performed using GraphPad Prism version 7 (GraphPad software). A *p*-value less than 0.05 was considered significant.

3. Results

3.1. Pigmented Hyper-Hemolytic GBS Strains Induce Initial Platelet Activation

To assess the impact of GBS pigment on platelet activation, the pigmented LUMC16 strain and its non-pigmented isogenic LUMC16Δ*cylX-K* mutant were used. Platelets were exposed to bacteria, and their activation was determined via CD62P-positive surface staining. Irrespective of the MOI, the platelets responded to stimulations with the LUMC16 strain within 30 min (Figure 1A,B and Figure S1B). However, no further activation was evident at later time points (60 and 120 min). To determine whether additional activation could be achieved, TRAP-6 (a thrombin receptor agonist) was added to the infections five minutes prior to termination of the experiment. Both the frequencies of CD62P-positive cells and CD62P expression increased after 30 and 60 min of infection when low MOIs (0.1 and 1.0) were used. The platelets remained unresponsive to TRAP-6 stimulation at a high

MOI of 10. In contrast, we did not observe a response to infections with the non-pigmented LUMC16Δ*cylX-K* strain (Figure 1A,B and Figure S1B).

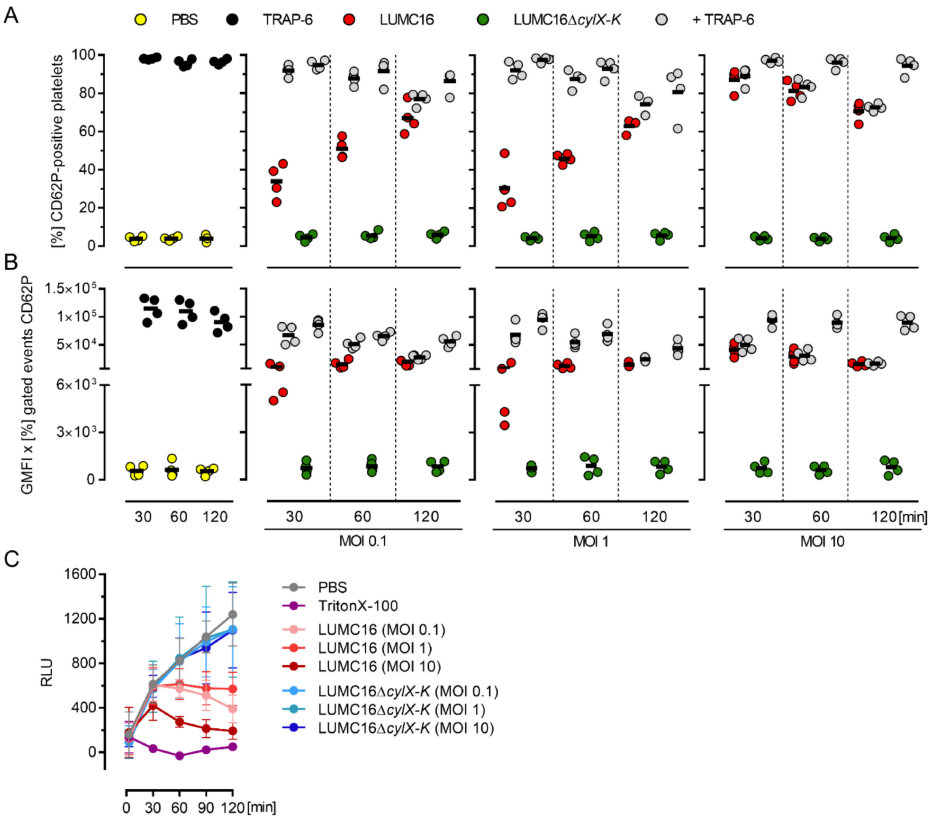

Figure 1. Pigmented GBS activate and kill human platelets. Washed human platelets were infected with the pigmented LUMC16 and the non-pigmented LUMC16Δ*cylX-K* GBS strains at MOI 0.1, MOI 1.0, and MOI 10. (A) Platelet activation was measured via flow cytometry using a PE-Cy5 labelled CD62P antibody. TRAP-6 (40 µM) and PBS were used as positive and negative controls, respectively. Alternatively, 5 min prior to the end of infection, TRAP-6 (40 µM) was added. The activation process was evaluated by assessing frequencies of CD62P-positive cells (**A**) as well as expression of CD62P (**B**). Each dot in A and B represents one independent experiment with washed platelets from one donor (n = 4). Horizontal lines depict mean values. (**C**) Kinetics of platelet viability. PBS and TritonX-100 were used as controls. Each dot represents the mean ± SD from four independent experiments (n = 4). Abbreviations (GMFI, geometric mean of fluorescence intensity; RLU, relative luminescence units).

In order to verify that activation was not only linked to the DIC/STSS case strain, two previously characterized GBS phenotypic variants, pigmented hyper-hemolytic and non-pigmented low-hemolytic variants (HH and LH, respectively) isolated from the same tissue culture of an STSS/NF case, were included [6,9]. All infections were performed with an MOI of 0.1. Again, both pigmented strains activated platelets within 30 min, and the cells maintained their responsiveness to the additional TRAP-6 stimulations for another 30 min (Figure 2A,B and Figure S1C). In contrast, the platelets remained unaffected in infections with non-pigmented strains, and responded exclusively to additive TRAP-6 stimulations (Figure 2A,B).

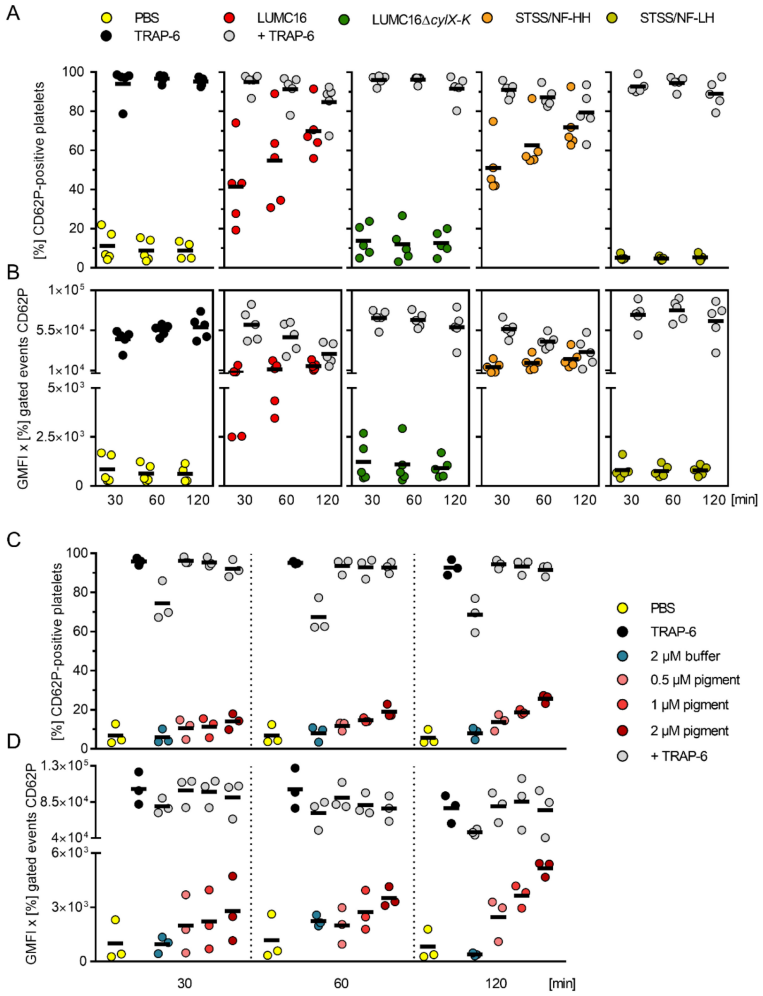

Figure 2. GBS pigment initially activates human platelets. Washed human platelets were infected with pigmented (LUMC16 and STSS/NF-HH) and non-pigmented (LUMC16Δ*cylX-K* or STSS/NF-LH) GBS strains at MOI 0.1 or were incubated with increasing concentrations of the pigment. Platelet activation was measured via flow cytometry using a PE-Cy5 labelled CD62P antibody. TRAP-6 (40 µM) and PBS were used as positive and negative controls, respectively. Alternatively, 5 min prior to the end of infection or pigment stimulation, TRAP-6 was added. The activation process was evaluated by assessing frequencies of CD62P-positive cells (**A**,**C**) as well as expression of CD62P (**B**,**D**). Each dot represents one independent experiment with cells from one donor (A and B, $n = 5$; C and D, $n = 3$). Horizontal lines depict mean values. Abbreviations (GMFI, geometric mean of fluorescence intensity).

Next, pigment extracts from the STSS/NF-HH and STSS/NF-LH (buffer control) strains were used. The dose- and time-dependent activation of platelets was observed in response to hemolytic pigment (Figure 2C,D and Figure S2A). A subpopulation of pigment-stimulated platelets responded to the additional TRAP-6 stimulation. In contrast, no response to extracts from the STSS/NF-LH strain was noted (Figure 2C,D and Figure S2A; "buffer").

3.2. Hemolytic GBS Pigment Causes Platelet Death

To assess the reason for limited platelet activation at later stages of infection, platelet viability was determined. The infection dose- and time-dependent killing of platelets by the pigmented GBS was observed (Figures 1C and 3A). In contrast, the cells remained viable in infections with non-pigmented strains (Figures 1C and 2A). Consequently, the platelets were stimulated with increasing concentrations of the pigment. The platelets were viable only for 30 and 60 min when high concentrations (1 and 2 µM) of the pigment were used (Figure 3A).

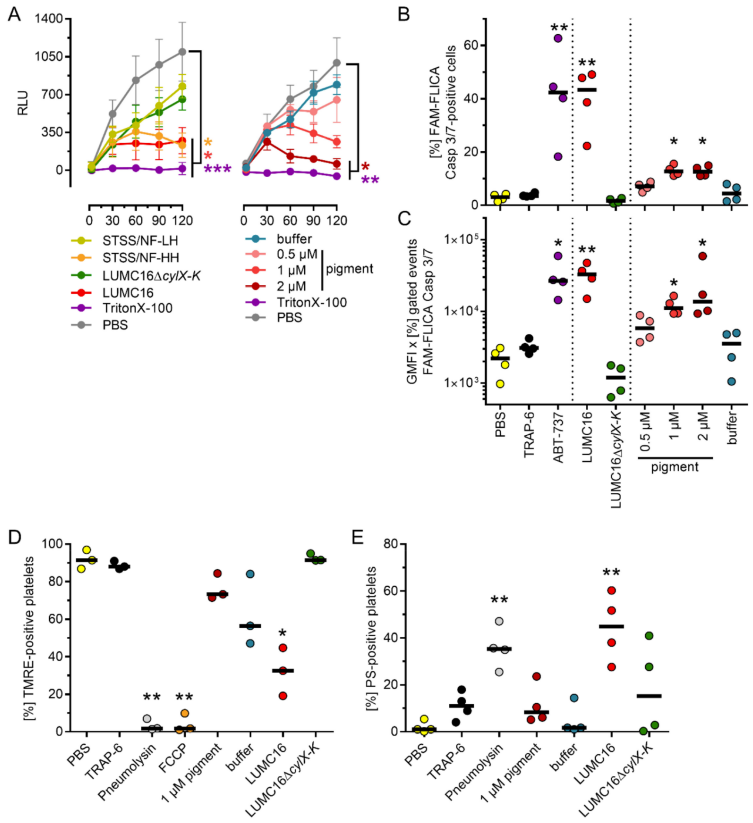

Figure 3. GBS pigment kills platelets. (**A**) Kinetics of platelet viability. PBS and TritonX-100 were used as negative and positive controls, respectively. (**B**,**C**) Caspase-3/7 activity in GBS-infected or pigment-treated platelets after two hours of exposure. The process was evaluated by assessing frequencies of Caspase 3/7-positive cells (**B**) as well as expression of Caspase 3/7 (**C**). PBS and ABT-737 (10 µM) were used as negative and positive controls, respectively. (**D**) Frequencies of TMRE-positive platelets after two hours of exposure to bacteria or pigment. Pneumolysin (300 ng/mL) and FCCP (20 µM) were used as controls. (**E**) Frequencies of PS-positive platelets after two hours of exposure to bacteria or pigment. Pneumolysin (300 ng/mL) was used as a control. Each dot in A represents the mean ± SD from four independent experiments (n = 4). Each dot in B-E represents one independent experiment with cells from one donor (B and C, n = 4; D, n = 3; E, n = 4). Horizontal lines depict mean values. The level of significance was determined using Kruskal-Wallis tests with Dunn's post-tests (*, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$). Abbreviations (GMFI, geometric mean of fluorescence intensity; RLU, relative luminescence units; TMRE, tetramethylrhodamine ethyl ester; FCCP, carbonyl cyanide 4-(trifluoromethoxy) phenylhydrazone; PS, phosphatidylserine).

The TRAP-6 unresponsiveness in activation assays, as well as the limited substrate utilization in viability experiments, showed that the pigment kills platelets at later stages of infection/stimulation. This is also supported by the increased PS-positivity of the cells, particularly in response to the pigmented LUMC16 strain (Figure 3 and Figure S2). To determine the type of cell death, platelets were exposed to bacteria or pigment for 120 min and caspase-3/7 activity was determined. The LUMC16 strain, as well as higher concentrations of the pigment (1 and 2 μM), induced caspase-3/7 activity in the platelets (Figure 3B,C). Only baseline levels of caspase-3/7 were detected in stimulation/infection when the pigment was not present. To differentiate between apoptotic and necrotic cell death, the inner mitochondrial membrane potential was measured via TMRE labeling of the mitochondria after 120 min of stimulation/infection. The stimulation of platelets with 1 μM pigment led to a slight decrease in mitochondrial membrane potential. In contrast, the infection of platelets with the LUMC16 strain resulted in diminished membrane potential. This was comparable to the FCCP control. Stimulation of platelets with the buffer control also resulted in reduced mitochondrial membrane potential without any evidence of platelet killing (Figure 3C and Figure S2). Infection with the non-pigmented LUMC16Δ*cylX-K* did not alter mitochondrial membrane potential.

The phenotypic assessment of platelets via electron microscopy confirmed the observations described above. The platelets showed a resting-like phenotype in infections with non-pigmented strains. In contrast, the platelets that were exposed to the pigmented strains or the pigment itself were characterized by ruptured membranes and surface-bound granule content (Figure 4).

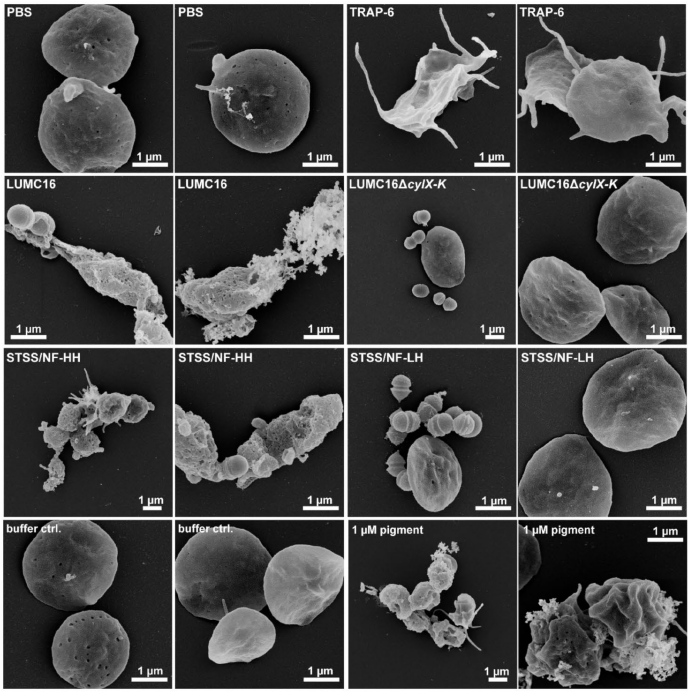

Figure 4. Platelet morphology in response to GBS infection or pigment stimulation. Washed human platelets were infected with indicated GBS strains or stimulated with pigment (1 μM) for 120 min and were visualized via FESEM. PBS and TRAP-6 were used as negative and activation controls, respectively. Representative images from three independent experiments are shown (n = 3).

Although platelets (especially platelet releasate) exert antimicrobial activity (e.g., against *S. aureus* [10]), dividing bacteria were detected via electron microscopy (Figure 4). Therefore, bacterial survival was assessed during the entire period of platelet or releasate exposure. All GBS strains survived regardless of the pigmentation (Figure 5).

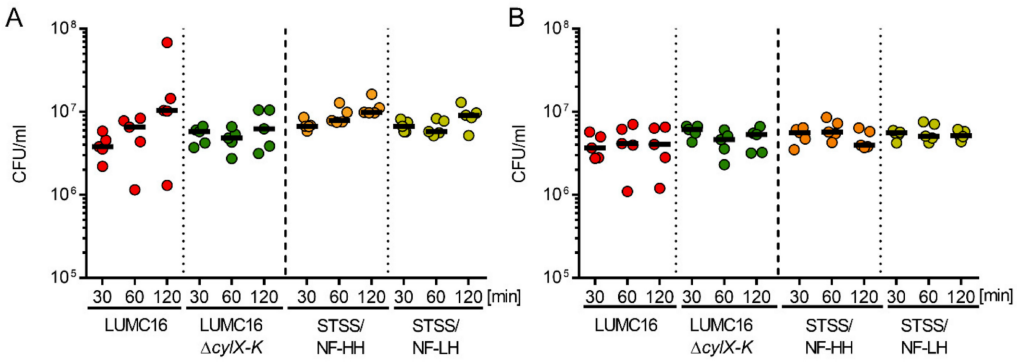

Figure 5. Human platelets do not kill GBS. CFU of the indicated GBS strains after 30, 60, and 120 min of platelet infection (**A**) or incubation with platelet releasate (**B**). Each dot represents one independent experiment with washed platelets from one donor (n = 5). Horizontal lines depict mean values. Abbreviations (CFU, colony-forming units).

4. Discussion

GBS pigment is one of the major virulence factors and has been associated with cytolytic injury to mast cells [11], CD4+ T cells, and B cells [12], pyroptosis in human macrophages [13], bacterial penetration of the human placenta [7], and invasion of the amniotic cavity and fetal injury [14]. Furthermore, pro-inflammatory and pro-thrombotic activities have been reported, including blood clotting and accelerated plasma clotting [6]. Here, we show that GBS pigment initially activates human platelets before killing them via necrosis.

The pathomechanisms of pigment-mediated cell damage are still not fully understood. GBS predominantly colonize the gastrointestinal and vaginal tracts. It was shown that pigment promotes the invasion of placental cells by GBS, disrupts amniotic barriers, and enables the dissemination of bacteria [7]. Subsequently, pigment contributes to inflammation, neutrophil infiltration, and mast cell degranulation in infected tissues [15]. Pigment synthesis is encoded by the *cyl*-operon [7]. Another key virulence factor is the α2,3-linked sialic acid (Sia) capsule, which protects GBS from phagocytic killing [16]. Both factors are oppositely regulated via the CovR/S two-component system, and clinical isolates of hyperhemolytic GBS (which harbor *covR/S* mutations) have reduced capsule levels [6,9,16]. We, and other researchers, have previously shown that: (i) The STSS/NF-HH and STSS/NF-LH variants exhibit low and high capsule expression, respectively. This phenomenon was exclusively linked to a deletion of three base pairs in the *covR* gene of STSS/NF-HH. (ii) Genes encoding for the pigment synthesis pathway are up-regulated in the LUMC16 strain to the same extent as in the STSS/NF-HH strain, although no mutations in *covR/S* were found [6,9]. (iii) GBS Sia blocks platelet activation [10]. On the basis of these findings, we hypothesized that reduced capsule levels and enhanced pigment expression will affect GBS–platelet interactions. Indeed, the infection of platelets with the pigmented strains resulted in dose- and time-dependent platelet activation, followed by a sequential loss of platelet viability. The activation phenotype was also observed after direct treatment with the pigment. In contrast, platelets retained the resting phenotype in infections with non-pigmented strains. These results indicate that GBS pigment is in fact the main driver of GBS-induced platelet interaction. However, the data also demonstrate that other GBS virulence factors contribute to platelet killing because GBS-mediated cytotoxicity towards platelets was more effective in

bacterial infections compared to the application of the toxin alone. Our results also confirm previous observations that GBS strains resist platelet-induced killing via the capsule [10]. However, the aforementioned study utilized a GBS strain without hyper-hemolytic activity. Furthermore, caspase-3/7 activation was noted. Activated caspase-3 is a key executioner in apoptosis and requires upstream activation via a diverse range of cell death receptors and cleavage by the initiator caspase-8 [17,18]. However, although platelets express effectors of the extrinsic apoptotic pathway, no cell death receptors of the TNFR family, including Fas, have been identified so far [19–21]. Therefore, pigment-induced platelet killing could also occur due to caspase 3/7-dependent secondary necrosis [22]. Necrotic platelets display a procoagulant phenotype and are phosphatidylserine-positive on their surface. In addition, the loss of mitochondrial membrane potential, ballooning, and rupture of the cytoskeleton are described as features of platelet necrosis [23,24]. Our results show that pigmented GBS induce a reduction in platelet inner mitochondrial membrane potential. These results demonstrate that pigmented GBS induce necrosis. However, the stimulation of platelets with purified pigment had only a minor effect. Of note, a subpopulation of the buffer-control-treated platelets were also characterized by slightly reduced TMRE staining, although cell death was not detected in these control stimulations. Extraction of the GBS pigment requires high molecular weight stabilizers such as starch. Traces of starch remain within the purified fraction. Traces of starch potentially induce mitochondrial dysfunction, resulting in reduced mitochondrial membrane potential, however this is speculative. In line with this, it was shown that hyperglycemia could induce changes in mitochondrial morphology in otherwise healthy cells [25].

Overall, the expression of hemolytic and cytolytic toxins is not unique to GBS. Other Gram-positive bacteria, including *Streptococcus pneumoniae* (pneumolysin, Ply), *Staphylococcus aureus* (alpha-hemolysin, Hla) or group A streptococci (streptolysin O, SLO, and streptolysin S, SLS), express toxins with lytic activity. In contrast to GBS pigment, these toxins are proteins/peptides. Although the lysis of a variety of cell types, including platelets, is a common feature of these toxins [8], they act via different mechanisms [26,27] and are able to induce pyroptotic, apoptotic, and necrotic cell death [28]. Ply and SLO are cholesterol-dependent cytolysins. They form pores in cholesterol-rich membranes and use glycans as cellular receptors [28]. Furthermore, mannose receptor C-type 1 (CD206) was identified as a Ply receptor on human phagocytic cells [29]. This interaction results in the suppression of co-stimulatory molecules, leading to impaired maturation of phagocytes and subsequently abrogated T cell activation [29,30]. In contrast, SLS is a small non-immunogenic peptide that mainly targets erythrocytes, platelets, and leukocytes by a mechanism that is not yet fully understood [31]. Furthermore, Hla-induced cell damage is mediated through Hla-ADAM10 interactions on the host cell surface [32,33]. Similar to GBS pigment, Hla initially activates and then kills human platelets over time [34].

5. Conclusions

In conclusion, GBS pigment directly affects human platelets, leading to the initial activation and subsequent killing of platelets over time. However, only pigmented GBS, and not pigment alone, induced necrosis. It is possible that bacteria itself or cargo molecules are required to deliver the pigment into the intracellular environment. Further studies are needed in order to determine the specific events that lead to pigment-mediated platelet cell death.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/cells11101637/s1, Figure S1: Washed human platelets were infected with the pigmented LUMC16 and the non-pigmented LUMC16ΔcylX-K GBS strains at MOI 0.1, MOI 1.0, and MOI 10 or with the pigmented STSS/NF HH and the non-pigmented STSS/NF HH at MOI 0.1.; Figure S2: Washed human platelets were infected with the pigmented LUMC16 and the non-pigmented LUMC16ΔcylX-K GBS strains at MOI 0.1 or treated with increasing concentration of pigment and/or the respective buffer control.

Author Contributions: Conceptualization, K.J., P.S. and N.S.; methodology, K.J., P.S., P.Q., M.M. and J.W.; software, M.M., A.G., L.R., S.H. and N.S.; validation, K.J., P.S. and N.S.; formal analysis, K.J., P.S., P.Q., M.M. and J.W.; investigation, K.J., P.S., P.Q., M.M. and J.W.; resources, M.M., A.G., L.R., S.H. and N.S.; data curation, K.J., P.S., P.Q., M.M. and J.W.; writing—original draft preparation, K.J. and N.S.; writing—review and editing, K.J., P.S., P.Q., M.M., J.W., A.G., L.R., S.H. and N.S.; visualization, K.J., M.M. and N.S.; supervision, A.G., L.R., S.H. and N.S.; project administration, A.G., L.R., S.H. and N.S.; funding acquisition, A.G., L.R., S.H. and N.S. All authors have read and agreed to the published version of the manuscript.

Funding: This research was funded by the National Institutes of Health (NIH, R01 AI112619, R01 AI152268, R01 AI145890 to L.R.), the German Research Foundation (DFG, 374031971 – TRR 240 to A.G. and S.H., 407176682 to N.S.), and The Swedish Society of Medicine (SLS-586271 to N.S.).

Institutional Review Board Statement: The study was conducted in accordance with the Declaration of Helsinki and was approved by the Ethics Committee of University Medicine Greifswald (protocol code BB044/18).

Informed Consent Statement: Informed consent was obtained from all subjects involved in the study.

Data Availability Statement: All data generated during this study are included in this article. Further enquiries can be directed to the corresponding authors upon reasonable request.

Acknowledgments: We would like to thank all volunteers for their blood donations. Ina Schleicher is acknowledged for expert technical assistance.

Conflicts of Interest: A.G. received research funding from Biokit, Boehringer Ingelheim, Rovi, Sagent, Macopharma, Portola, Biokit, Blau Farmaceutics, Prosensa/Biomarin, DRK-BSD NSTOB, DRK-BSD Baden-Württenberg/Hessen, travel support, speakers, and consulting fees from Roche, GTH e.V., Sanofi-Aventis, Macopharma, Chromatec, Instrumentation Laboratory, Bayer Vital, and Aspen. K.J., P.S., P.Q., M.M., J.W., L.R., S.H. and N.S. declare no conflicts of interest. The funders had no role in the design of the study; in the collection, analyses, or interpretation of data; in the writing of the manuscript; or in the decision to publish the results.

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Supplementary Material

Group B streptococcal hemolytic pigment impairs platelet function in a two-step process

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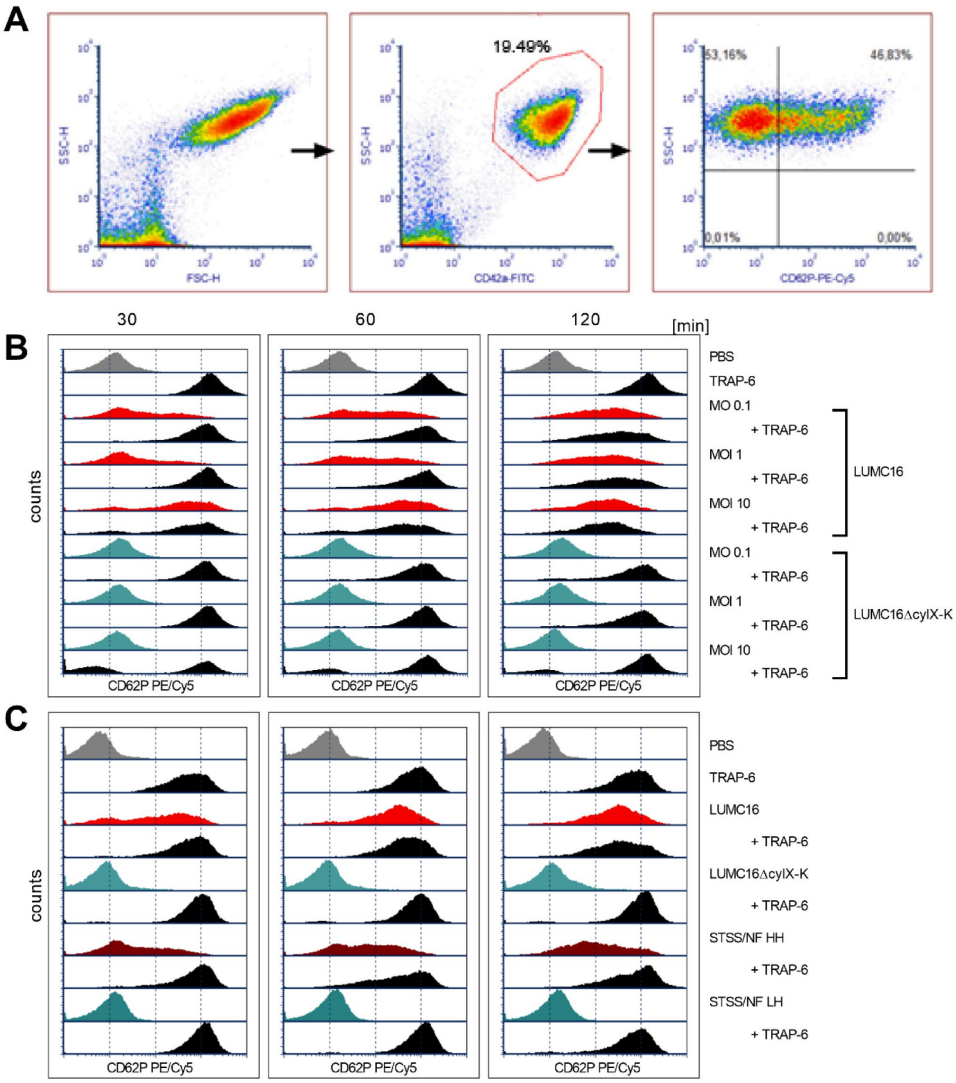

Figure S1. Washed human platelets were infected with the pigmented LUMC16 and the non-pigmented LUMC16Δ*cylX-K* GBS strains at MOI 0.1, MOI 1.0, and MOI 10 or with the pigmented STSS/NF HH and the non-pigmented STSS/NF HH at MOI 0.1. (A) Gating strategy of infected platelets. To exclude overlapping of bacteria and platelets in SSC/FSC scatter plot, platelets were labelled with CD42a. Activation of CD42a-positive cells was analyzed via CD62P PE/Cy5 staining. (B and C) Representative histograms for each platelet treatment/infection condition.

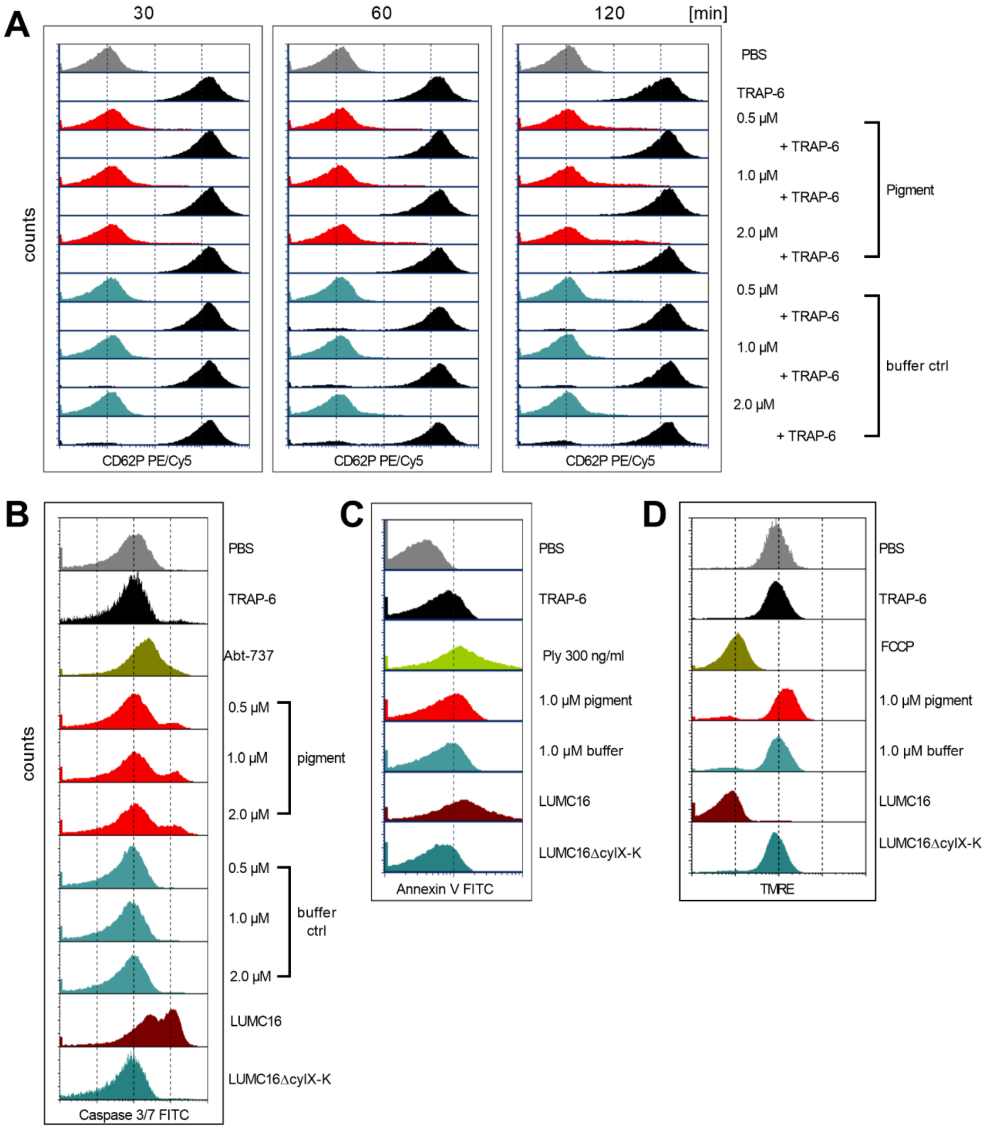

Figure S2. Washed human platelets were infected with the pigmented LUMC16 and the non-pigmented LUMC16Δ*cylX-K* GBS strains at MOI 0.1 or treated with increasing concentration of pigment and/or the respective buffer control. Shown are representative histograms for (A) CD62P dependent platelet activation, (B) caspase 3/7 activity, (C) phosphatidylserine positivity, and (D) changes of the inner mitochondrial membrane potential.

IV

RESULTS & DISCUSSION

1. Results

Human platelets are activated by different pneumococcal surface proteins

The aim of this study was first, to identify new pneumococcal factors involved in the interaction with human platelets and second, to analyze effect of this interaction on platelet side.

For the identification of bacterial proteins directly activating platelets, a library of recombinant pneumococcal surface proteins as well as purified cell wall components were screened. Platelets were incubated with different molarities of the proteins/components and afterwards analyzed regarding CD62P expression by flow cytometry. At least one protein of each group of pneumococcal surface proteins was identified to directly activate washed human platelets. SP_0899 and AliB, both lipoproteins as well as CbpL (choline binding protein) and SP_1833 (or PfbA, sortase anchored protein) all induced an increase in surface exposed CD62P (III.1). All recombinant expressed lipoproteins used in this screening lack their lipid moiety. Nevertheless, stimulation of platelets with lipidated lipoproteins did not increase CD62P expression on platelets compared to their non-lipidated counterparts. Based on these experiments pneumococcal mutants, deficient in anchoring of lipoproteins (Δ*ply*Δ*lgt*) or sortase-anchored proteins (Δ*ply*Δ*srtA*) and thereby releasing these proteins in the supernatant, were created. In addition a *ply*-mutant was treated with choline chloride to release CBPs into the medium. Incubation of enriched supernatants of these strains did not result in CD62P dependent platelet activation. However, lysates of the bacterial pellets resulted in increased CD62P signals, indicating involvement of other pneumococcal factors in platelet activation. In addition, purified cell wall components of pneumococci were tested regarding their ability to activate platelets, but with no positive result.

Pneumolysin kills, but not activates human platelets

One of the major pneumococcal virulence factors is the cholesterol-dependent pore forming toxin Ply. In the screening described above, incubation of Ply with human

platelets resulted in high levels of CD62P comparable to the positive control TRAP-6. This was in line with the literature stating platelet activation by Ply. However, further experiments revealed a different sight on the Ply-platelet interaction. Next to CD62P, PAC-1 was used in the studies as platelet marker indicating integrin activation. Surprisingly, incubation of human platelets with Ply did not result in integrin activation, independent of the Ply concentration. In addition, subsequent stimulation with 20 µM TRAP-6 did not result in integrin activation or a further increase of CD62P levels. In parallel, aggregation and calcium release of washed platelets incubated with Ply was measured. In both experimental setups a rapid and strong response to Ply was observed with loss of turbidity of platelet suspension and an immediate strong Ca^{2+} release. Nevertheless, also in these experiments platelets did not respond to subsequent TRAP-6 stimulation, indicating loss of platelet function in the presence of Ply. Only at low concentrations (≤ 30 ng/ml), when platelets did not responded with e.g., calcium release to Ply, they remained sensitive to TRAP-6. One of the main functions of platelets in the circulation is the formation of stable thrombi. When whole blood is flown at physiological shear rates through a collagen coated chamber typically numerous small thrombi are formed. In the presence of increasing concentrations of Ply, thrombus formation is impaired (≤30 ng/ml Ply) or completely abrogated (≥300 ng/ml Ply). The data on loss of platelet function are in line with data of viability measurements of Ply incubated platelets. In the presence of >30 ng/ml Ply, platelets were immediately lysed. Next, the question was addressed how increased signals for the platelet activation marker and immediate loss of platelet function after incubation with Ply fit together. Electron microscopy data of Ply incubated platelets clearly show numerous Ply pores of 40-50 nm in diameter on the platelet surface. This pore size would allow antibodies to pass through and stain intracellular stores of CD62P instead of activation typical surface rebound CD62P. Indeed, further localization studies of CD62P in 3D reconstructed confocal images showed only intracellular staining of CD62P similar to the permeabilization control Triton X-100 but contrary to the activation control TRAP-6.

Alpha-hemolysin acts in a two-step mechanism on platelets

S. aureus expresses with Hla and the bicomponent pore forming toxins LukAB, LukDE, and LukSF several disease associated pore forming toxins. As described earlier, the

pores formed by the staphylococcal toxins are much smaller in diameter compared to Ply pores. In addition, Hla has been previously described to activate and aggregate platelets whereas the leukocidins so far only have been described to excert indirect effects on platelets[218-220]. Therefore it was aimed to investigate the effects of recombinant Hla and leukocidins on platelet activation and platelet function in comparison to the effects caused by pneumococcal Ply. Similar to Ply, Hla bound dose-dependently to human platelets and induced highly increased CD62P levels with insensitivity to subsequent TRAP-6 stimulation. But contrary to Ply, Hla induced integrin activation (PAC-1 binding) at intermediate concentrations and the measured levels for both activation markers were time and concentration dependent. Opposed to Hla, none of the leukocidin pairs bound to platelets, induced strong expression of CD62P or rendered platelets insensitive to TRAP-6. A major difference between the mode of action of Ply and Hla was observed in aggregometry. Whereas Ply immediately lysed platelets resulting in a strong increase in light transmission, a partially reversibly change in light transmission was observed for incubation with Hla. Incubation of platelets with intermediate doses Hla (≥ 1 µg/ml) led to a strong peak in light transmission. Interestingly, this first peak could be blocked by addition of RGDS, an inhibitor of aggregation. Nevertheless, the aggregation curve gradually increases over time and finally overlays the curve with just Hla and platelets. In the presence of RGDS any measured change in light transmission reflects platelet lysis. Therefore, the first peak in light transmission after addition of Hla can be assumed to be real platelet aggregation which is followed by platelet lysis as observed by non-reversible increase in light transmission. Localization studies of CD62P support the assumption of initial platelet activation by Hla. In contrast to the predominant intracellular staining of CD62P after Ply treatment, incubation with Hla resulted in a strong surface associated staining of CD62P similar to the activation control with TRAP-6. However, platelet morphology was altered by Hla and they appeared enlarged and swollen, indicating loss of membrane integrity. Viability measurement of Hla treated platelets indeed revealed platelet lysis during the first minutes of incubation (≥2 µg/ml Hla) or later on (≤0.2 µg/ml Hla) in dependence of the used Hla dose. Although initial platelet activation could be observed, platelet lysis by Hla seems to be prevailing in the matter of platelet function. This is reflected by completely abrogated thrombus formation in whole blood in the presence of increasing concentrations of Hla. Hla and Ply induced platelet lysis occurs via apoptosis. After incubation with both toxins caspase 3/7 activity as well as exposure

of phosphatidylserine on platelet surface was highly increased. In contrast, the leukocidin pairs did not stimulate apoptosis.

Ply, but not Hla, can be neutralized by immunoglobulins

Based on the results, the obvious question erased how one could interfere with the devastating effect of Ply and Hla on human platelets. Next to specific antibodies against Ply and Hla also a pharmaceutical IgG preparation (IVIG, 98% IgG; Privigen, CSL Behring, United States) and an IgM/IgA enriched immunoglobulin preparation (21% IgA, 23% IgM, 56% IgG; trimodulin, Biotest AG, Germany) were included. Measurements of CD62P accessibility and platelet viability revealed inhibition of Ply induced platelet lysis by all included antibodies up to a concentration of 600 ng/ml Ply. Further, platelet function was fully restored in the presence of IgGs against Ply as shown by sensitivity to TRAP-6 and maintained thrombus formation after incubation with ≤300 ng/ml Ply. In the setting of disease the pharmaceutical immunoglobulin preparations IVIG and trimodulin are of high interest. Although both preparations equally neutralized pneumolysin up to a concentration of 300 ng/ml, at higher Ply doses trimodulin became less efficient compared to IVIG. In the presence of 600 ng/mL Ply, 1 mg/ml trimodulin only incompletely reduced platelet lysis, whereas 1 mg/ml IVIG was efficient in protecting platelets. The difference between both preparations is the reduced proportion of IgGs in trimodulin. Upon adjustment of both preparations to the same overall immunoglobulin concentration, Ply induced platelet lysis was similarly rescued by trimodulin as with IVIG. However, at concentration above 600 ng/ml trimodulin became again less efficient compared to IVIG.

In contrast to Ply, the damaging effect of Hla on platelets could not be rescued. Neither a specific monoclonal anti-Hla antibody, nor IVIG which contains anti-Hla antibodies neutralized Hla. IVIG as well as excessive amounts of the monoclonal antibody only minor improved the loss in platelet viability over time upon treatment with low doses of Hla. Nevertheless, this was not reflected in restored platelet function.

2. Discussion

The studies listed and described above can be differentiated in terms of content into i) interaction of pneumococcal proteins with human platelets with special focus on pneumolysin (III.1, III.2), ii) comparison of the pneumococcal pneumolysin with pore forming toxins of *S. aureus* (III.4) and iii) antibody mediated inhibition of pneumolysin and alpha-hemolysin as potential therapeutic approach during pneumonia and/or bacteremia (III.3, III.4).

The screening approach of heterologously expressed pneumococcal surface proteins revealed four new candidates interacting with platelets leading to CD62P expression. Among them were proteins of all classes of surface proteins. Two lipoproteins with SP_0899 (unknown function) and AliB (substrate-binding protein for oligopeptides[221]), a choline-binding protein, namely CbpL (putative adhesion, contribution to colonization[222]), and a sortase anchored protein , namely SP_1833 (PfbA, plasmin- and fibronectin-binding capacity[223]), induced increased CD62P expression (Figure 4). Of note, all lipoproteins included were heterologously expressed in *E. coli* without being lipidated. In pneumococci, lipoproteins contain a lipid moiety which triggers TLR2 dependent signalling in other cell types like leukocytes[224-225]. However, although platelets express TLR2 the included lipidated lipoproteins did not induce stronger CD62P expression compared to their non-lipidated counterparts. A potential explanation lies in the fact that expression of TLRs is activation dependent. Surface TLR expression on resting platelets is quite low, but upregulated upon stimulation[165, 226]. To rule out TLR dependent signalling induced by pneumococcal lipoproteins future studies should include a parallel stimulatory effect with e.g., low doses of ADP. During bacterial infections antibiotic treatment is part of standard care. Therefore, also intracellular and non-secreted pneumococcal proteins might become interacting partners for platelets. Lysates of Ply deletion strains additionally deficient in anchoring of whole groups of surface proteins induced increased CD62P expression, indicating intracellular proteins being involved in platelet activation. However, measured values were quite low und did not significantly differ between the different gene deletion strains. In contrast, the intracellular pneumolysin, which is mainly released upon pneumococcal autolysis, induced highly elevated CD62P levels in accordance with literature describing Ply-dependent platelet activation[70]. Therefore, detailed analysis of the impact of Ply on platelets was conducted. Taken together, instead of platelet

activation a lytic mode of action of Ply on platelets was discovered. Platelet lysis by Ply was shown in detail by pore formation in platelet membranes, predominant intracellular CD62P staining, loss of viability and loss of platelet function in washed platelets as well as in whole blood. Previous reports about Ply dependent platelet activation are most likely false positive based on formation of large pores allowing antibodies to pass through. The shown results might be of high relevance during invasive pneumococcal disease like CAP. A potential life-threatening complication of CAP is the development of an acute respiratory distress syndrome (ARDS). ARDS is defined as pulmonary syndrome, with acute development of lung injury and is often associated with sepsis, pneumonia or trauma. During ARDS the alveolar-capillary barrier is disrupted which leads to an entry of interstitial fluid into the alveolar space resulting finally in respiratory failure[227]. Under physiological conditions, platelets would seal gaps in the lung endothelium caused by e.g., bacteria and prevent flooding of the alveolar space. However, in the presence of Ply, barrier maintenance might be highly impaired. So far, *in vivo* concentrations of Ply during severe infections were only determined in enclosed areas without constant and strong flow like cerebrospinal fluid during meningitis. Ply concentration in those areas reached levels up to 30 µg/mL[228-230] which is 100-fold higher than the concentration needed to cause immediate platelet damage. However, due to constant blood flow in the lung released Ply is constantly diluted and local Ply concentrations are hard to determine. However, it is reasonable that in dependence of bacterial density and their accessibility to antibiotics local Ply concentrations reach levels sufficient in inducing cellular damage. Nevertheless, chapter III.2 demonstrates that Ply-dependent platelet damage is also caused over time. Even low Ply concentrations occurring during physiological bacterial autolysis, without further usage of antibiotics, led to intracellular CD62P accessibility in platelets after 120 min. Since severe bacterial infections last far more than 120 min and standard care involves antibiotic treatment, it can be assumed that Ply induced platelet damage is a disease relevant molecular pathomechanism. Overall, although surface proteins stimulating platelet activation have been identified, the lytic activity of Ply probably overshoots any effect of other proteins on platelets.

Similar to pneumococci, also *S. aureus* expresses with Hla and BPFL pore forming toxins. Taken together, the shown data demonstrate that Hla affects platelets in a two-step mechanism: First, platelets get activated and second, platelets are lysed within a time frame of minutes. In contrast, the BPFL had no major impact on platelet function

and viability. This is in line with existing literature demonstrating only indirect effects of BPFL on platelets by e.g. activation of leukocytes[219]. In contrast to Ply and Hla, LukSF did not bound to platelets. On leukocytes, LukSF has been demonstrated to bind to the receptors C5aR1 and C5aR2[116, 231]. Further, for LukED the receptors CCR5, CXCR1, and CXCR2 have been identified to be targets[232-233]. The mentioned receptors were not detected on protein level in platelets in a proteomics approach but the transcripts were[219, 232-234]. Nevertheless, data on stimuli or mechanisms leading to their translation are missing so far. In contrast, the LukAB receptor CD11b is neither present on protein level nor as transcript[235]. The receptor for Hla is the metalloprotease ADAM10, which is expressed by various cell types including platelets[234, 236]. Platelet activation as well as coagulation by Hla was reported several times[218, 220, 237]. However, subsequent platelet lysis was not noted so far. A reasonable explanation for this two different effects on platelets relies on the formation of small pores on the platelet surface. Small molecules like Ca^{2+} can diffuse through the pores leading to platelet activation. Over time the number of formed pores increase and platelets are lysed, resulting in diminished thrombus formation in whole blood. This assumption is supported by the time- and dose dependent effect of Hla on platelets. High doses of Hla resulted in an immediate increased CD62P signal but also a loss of viability after 2 to 10 min of incubation, whereas lower doses induced CD62P expression and a reduction of platelet viability at later time points. Platelet lysis by Hla might be of high clinical relevance especially with regard to circulating Hla concentrations during invasive diseases. Up to 60 µM Hla were reported to be found in sera of septic patients, which exceeds the highest concentration used in the study 100 fold (20 µM = 20 µg/ml)[237]. Recent *in vivo* studies correlate Hla dependent platelet aggregation to formation of microthrombi retained in small liver and kidney vessels[217] but data about stability of the formed thrombi are missing. Dissemination of septic thrombi, formed during e.g. infective endocarditis can result in multiple embolsims of small arteries of e.g. the brain. Based on the presented data it can be hypothesized that platelet lysis by Hla destabilizes thrombi thereby promoting dissemination. Further, stabilization of thrombi by interfering with Hla might reduce the risk of vessel occlusion caused by circulating microthrombi in the setting of infective endocarditis.

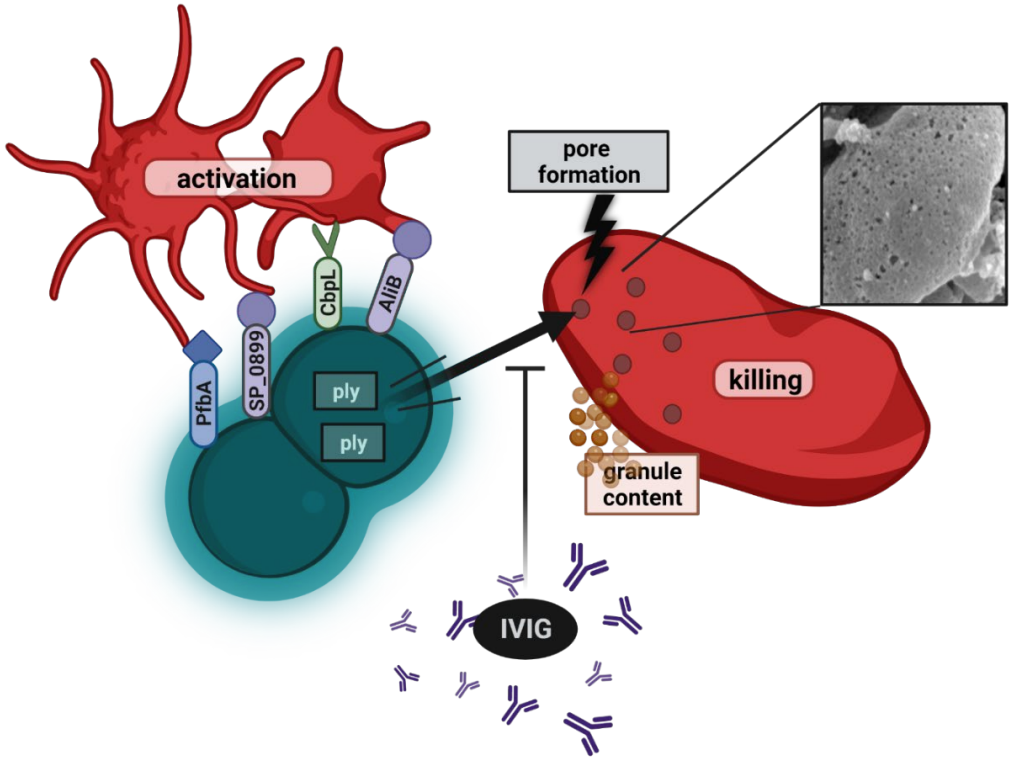

Figure 4: Model of *S. pneumoniae*-platelet interactions. Pneumococci can directly interact with human platelets via different surface proteins resulting in platelet activation. On the other hand, the intracellular pneumolysin forms large membrane pores on the platelet surface leading to lysis of platelets, release of granule content and diminished platelet function. Loss of platelet viability and platelet function by Ply can be rescued by usage of formulations containing antibodies against Ply, like IVIG. Source: Jahn et al (see III.1)[148]

The presented data indicate that the lytic activity of the pneumococcal Ply and the staphylococcal Hla might have major implications in the progression of invasive diseases. Therefore the question was addressed whether these toxins can be neutralized. Most people have measurable plasma titers of anti-Ply and anti-Hla IgG antibodies. Therefore, specific antibodies targeting Ply or Hla as well as pharmaceutical immunoglobulin preparations (IVIG, trimodulin) were tested regarding their neutralization efficiency against Hla and Ply. Two different specific anti-Ply antibodies as well as both immunoglobulin preparations successfully neutralized Ply. In the presence of those antibodies platelet viability and platelet function was completely rescued even at the highest tested concentration of Ply with 300 ng/ml. IVIG and trimodulin both are pharmaceutical available immunoglobulin preparations

and contain pooled immunoglobulins from healthy blood donors[238-239]. In contrast to IVIG which contains 98% IgG, trimodulin is a mixed immunoglobulin preparation containing 56% IgG, 23% IgM, and 21% IgA. Both formulations were further characterized *in vitro* regarding their Ply neutralization efficiency. Although both preparations successfully inhibited Ply induced platelet damage up to a Ply concentration of 300 ng/ml (Figure 4), upon usage of higher Ply doses trimodulin became less efficient compared to IVIG. The drop in efficacy of trimodulin could be equalized by adjustment of the IgG content in both preparations in the presence of 600 ng/ml Ply. However, upon further increase of Ply concentration, trimodulin became again less efficient and platelets got lysed. These results indicate that the presence of IgA and IgM in trimodulin has no beneficial effect on Ply inhibition and that Ply neutralization is based on Ply directed IgGs. Of note, IgA concentrations only peak during acute infections. However, recent infections lead to exclusion of donors carrying high IgA titers from blood donation[239]. Further, IgM levels remain unaltered independent of an acute infection[239]. Nevertheless, besides reduced efficacy of trimodulin at high Ply concentrations, both formulations sufficiently neutralized Ply at low and intermediate concentrations which are more likely to be found during invasive infections. The CIGMA clinical trial further supports the idea of interfering with pneumococcal infections by IgG preparations[240]. During this clinical study patients received trimodulin in addition to standard care. Although patient numbers with confirmed *S. pneumoniae* caused pneumonia were quite low, the data revealed a beneficial effect on platelet counts in the patients. These results mark both formulations as promising candidates as supportive treatment in severe invasive pneumococcal disease as prevention for development of complications like ARDS by dampening local peaks in Ply concentrations. Therefore, stringent studies using suitable animal models as well as clinical studies with sufficient patient numbers are needed.

On the contrary, neither platelet activation nor platelets lysis by Hla was efficiently prevented by specific antibodies or IVIG. Hla induced cellular damage is based on the interaction between Hla und its receptor ADAM10 and polyvalent IgGs might not block the respective binding sites necessary for binding. Therefore, also targeting ADAM10 would be an important approach. However, although sepsis associated vascular injury was dampened in mice upon inhibition of ADAM10, clinical trials targeting metalloproteases including ADAM10 failed so far[241]. A recent study demonstrated a non-commercial neutralizing Hla antibody (MEDI4893) to prevent Hla induced tissue

damage during sepsis in mice[242]. However, this antibody was not effective in preventing invasive disease in humans, but its effect on stabilizing thrombi might be of high interest. Taken together, the shown results highlight new pathomechanims driven by the pore forming toxins Ply and Hla. Both toxins represent promising targets in preventing severe complications during invasive pneumococcal or streptococcal diseases and future *in vivo* studies should concentrate on inhibition of these toxins and monitoring clinical outcomes.

V

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VI

APPENDIX

1. List of abbreviations

ABC	ATP-binding cassette
ADP	adenosine diphosphate
ARDS	acute respiratory distress syndrome
AtlA	autolysin A
BPFL	bicomponent pore forming leukocidins
CAP	community acquired pneumonia
CBP	choline binding protein
CHIPS	chemotaxis inhibitory protein
ClfA	clumping factor A
Coa	coagulase
CPS	capsule polysaccharide
CXCL4	platelet factor-4
DIC	disseminated intravascular dissemination
Eap	extracellular adherence protein
ECM	Extracellular matrix
FLIPr	formyl peptide receptor-like 1 inhibitory protein
FnBP	fibronectin-binding protein
GP	glycoprotein
Hla	alpha hemolysin
ICAM-1	intercellular adhesion molecule 1
IE	infective endocarditis
IPD	invasive pneumococcal diseases
IsdB	iron salvage protein
IsdB	iron-regulated surface determinant B
KO	knockout
Lgt	lipoprotein diacylglyceryl transferase
LTA	lipoteichoic acids
LytA	autolysin
MK	Megakaryocyte
mRNA	messenger RNA
MRSA	Methicillin-resistant *S. aureus*

MSCRAMM	microbial surface component recognizing adhesive matrix molecule
NanA	neuraminidase A
NET	neutrophil extracellular trap
PAF	platelet activating factor
PAMP	pathogen associated molecular pattern
PAR	protease-activated receptors
PCho	phosphorylcholine
PCV	pneumococcal conjugate vaccines
PDI	protein disulfide polymerase
PG	peptidoglycan
PI3K	phosphoinositid-3-kinase
Ply	pneumolysin
PMV	platelet microvesicles
PPV	pneumococcal polysaccharide-based vaccine
PS	phosphatidylserine
PSGL-1	P-selectin glycoprotein ligand-1
PVL	Panton valentine leukocidin
RANTES	Regulated upon Activation, Normal T Cell Expressed and Presumably Secreted
S. aureus	*Staphylococcus aureus*
S. pneumoniae, pneumococci	*Streptococcus pneumoniae*
SdrE	serine-aspartate repeat-containing protein E
SERAM	secretable expanded repertoire adhesive molecules
SpA	Staphylococcus protein A
TLR	Toll like receptor
TSP-1	thrombospondin 1
vWF	van Willebrand factor
WTA	wall teichoic acids

2. List of Figures

3. Eigenständigkeitserklärung

Hiermit erkläre ich, dass diese Arbeit bisher von mir weder an der Mathematisch-Naturwissenschaftlichen Fakultät der Universität Greifswald noch einer anderen wissenschaftlichen Einrichtung zum Zwecke der Promotion eingereicht wurde.

Ferner erkläre ich, dass ich diese Arbeit selbständig verfasst und keine anderen als die darin angegebenen Hilfsmittel und Hilfen benutzt und keine Textabschnitte eines Dritten ohne Kennzeichnung übernommen habe.

Greifswald,

Kristin Jahn

4. Publications and conference contributions

Peer-reviewed articles

Previously published papers

Hübner D., **Jahn K**., Sandra Pinkert S., Böhnke J., Jung M., Fechner H., Rujescu D., Liebert U.G., Claus C. (2017), Infection of iPSC Lines with Miscarriage-Associated Coxsackievirus and Measles Virus and Teratogenic Rubella Virus as a Model for Viral Impairment of Early Human Embryogenesis, *ACS Infectious Diseases*, doi: 10.1021/acsinfecdis.7b00103

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*contributed equally

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Jahn K.*, Handtke S.*, Palankar R.*, Weißmueller S., Nouailles G., Kohler T.P., Wesche J., Rohde M., Heinz C., Aschenbrenner A.F., Wolff M., Schuettrumpf J., Witzenrath M., Hammerschmidt S. and Greinacher A. (2020) Pneumolysin induces platelet destruction, not platelet activation, which can be prevented by immunoglobulin preparations in vitro, *Blood Advances*,
doi: 10.1182/bloodadvances.2020002372
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Presentations at conferences

Jahn K., Handtke S., Palankar R., Kohler T.P., Wesche J., Rohde M, Prucha J., Greinacher A., Hammerschmidt S. (2019), The interplay between platelets and pathogenic bacteria, annual retreat of the CRC/TR240, Neckarsulm, Germany (poster presentation)

Jahn K., Handtke S., Palankar R., Kohler T.P., Wesche J., Rohde M, Prucha J., Greinacher A., Hammerschmidt S. (2019), The interplay between platelets and pathogenic bacteria, European Meeting on the Molecular Biology of the Pneumococcus (Europneumo), Greifswald, Germany
(poster presentation)

Jahn K., Handtke S., Palankar R., Kohler T.P., Wesche J., Rohde M, Prucha J., Greinacher A., Hammerschmidt S. (2019), Streptococcus pneumoniae toxin Pneumolysin renders platelets unfunctional, 3rd Symposium platelets, Tübingen Germany
(poster presentation)

Jahn K., Handtke S., Palankar R., Kohler T.P., Wesche J., Rohde M, Prucha J., Greinacher A., Hammerschmidt S. (2020), *Streptococcus pneumoniae* toxin pneumolysin renders platelets non-functional, annual meeting of the International Society on Thrombosis and Haemostasis (ISTH), virtual congress
(poster presentation)

Jahn K., Handtke S., Palankar R., Kohler T.P., Wesche J., Rohde M, Prucha J., Greinacher A., Hammerschmidt S. (2020), The interplay between platelets and pathogenic bacteria, annual retreat of the CRC/TR240, online congress
(oral presentation)

Jahn K., Handtke S., Palankar R., Kohler T.P., Wesche J., Rohde M, Prucha J., Greinacher A., Hammerschmidt S. (2021), The pneumococcal pneumolysin destroys platelets and can be neutralized with pharmaceutical immunoglobulin preparations, , Annual Meeting of the German Society for Hygiene and Microbiology (DGHM), virtual congress
(poster presentation)

Jahn K., Handtke S., Palankar R., Kohler T.P., Wesche J., Rohde M, Prucha J., Greinacher A., Hammerschmidt S. (2022), Different modes of action of pneumococcal pneumolysin and Staphylococcus aureus alpha-hemolysin on platelets, annual meeting of the Association for General and Applied Microbiology (VAAM) , virtual congress
(oral presentation)

Jahn K., Handtke S., Palankar R., Kohler T.P., Wesche J., Rohde M, Prucha J., Greinacher A., Hammerschmidt S. (2022), Pneumococcal and staphylococcal pore forming toxins impair platelet function via different modes of action, European Meeting on the Molecular Biology of the Pneumococcus (Europneumo), Liverpool, united kingdom

(oral presentation)

5. Danksagung

Die vorliegende Arbeit, als Teilprojekt A11 des TR240, wurde in der Arbeitsgruppe und unter der Betreuung von Prof. Dr. Sven Hammerschmidt in der Abteilung Molekulare Genetik und Infektionsbiologie der Universität Greifswald angefertigt. In erster Linie möchte ich mich daher bei Prof. Dr. Sven Hammerschmidt bedanken, mir die Möglichkeit gegeben zu haben, an diesem interessanten und vielversprechendem Thema forschen und promovieren zu dürfen, ebenso wie für die kontinuierliche Unterstützung und die vielfältigen Ideen. Mein weiterer besonderer Dank gilt Prof. Dr. Andreas Greinacher, dem Leiter der Transfusionsmedizin in Greifwald sowie seiner gesamten Arbeitsgruppe. Ohne die enge Kooperation, dem regelmäßigen Austausch und der Implementierung klinischer Aspekte hätte das Projekt nicht so erfolgreich voranschreiten können. Weiterhin möchte ich Prof. Dr. Nikolai Siemens und Patience Shumba danken, durch die ein eher „spontanes Nebenprojekt“ sehr erfolgreich wurde.

Weiterhin möchte ich dem Zweitgutachter für die Übernahme des Gutachtens danken.

Mein besonderer Dank gilt Dr. Thomas Kohler, der mich von Tag eins an hervorragend betreut hat, hilfsbereit ist wie kein Zweiter und jederzeit für mich da war, unabhängig davon ob es um Wissenschaft oder den alltäglichen Wahnsinn ging.

Mein weiterer Dank gilt dem technischen Personal, insbesondere Peggy Stremlow und Kristine Sievert-Giermann, die jederzeit ein offenes Ohr hatten und mit Rat und Tat zur Seite standen.

Aber was wäre die Zeit als Doktorand ohne die Mit-Doktoranden. Ich danke euch allen, die ihr zu unterschiedlicher Zeit Büro- und Labornachbarn wart, für die vielen inhaltlich wertvollen, aber insbesondere für die wissenschaftlich weniger wertvollen aber umso unterhaltsameren Konversationen im und außerhalb des Labors. Ganz besonders herausheben möchte ich dabei Antje Paulikat, die mit ihrer Hilfsbereitschaft und guten Laune kaum zu übertreffen ist.

Natürlich möchte ich meiner Familie und Freunden an dieser Stelle danken. Danke, dass ihr mich auf dieser langen Reise begleitet habt, mir immer wieder Mut

zugesprochen habt und meine Launen in den etwas schwierigeren Phasen einfach ausgehalten habt. Ohne euren Rückhalt und immerwährende Unterstützung wäre diese Arbeit kaum möglich gewesen. Last but not least, Emma und Gismo. Ihr habt mich durch Höhen und Tiefen begleitet und mir jeden einzelnen Tag mindestens einmal ein Lächeln ins Gesicht gezaubert. Danke!