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).

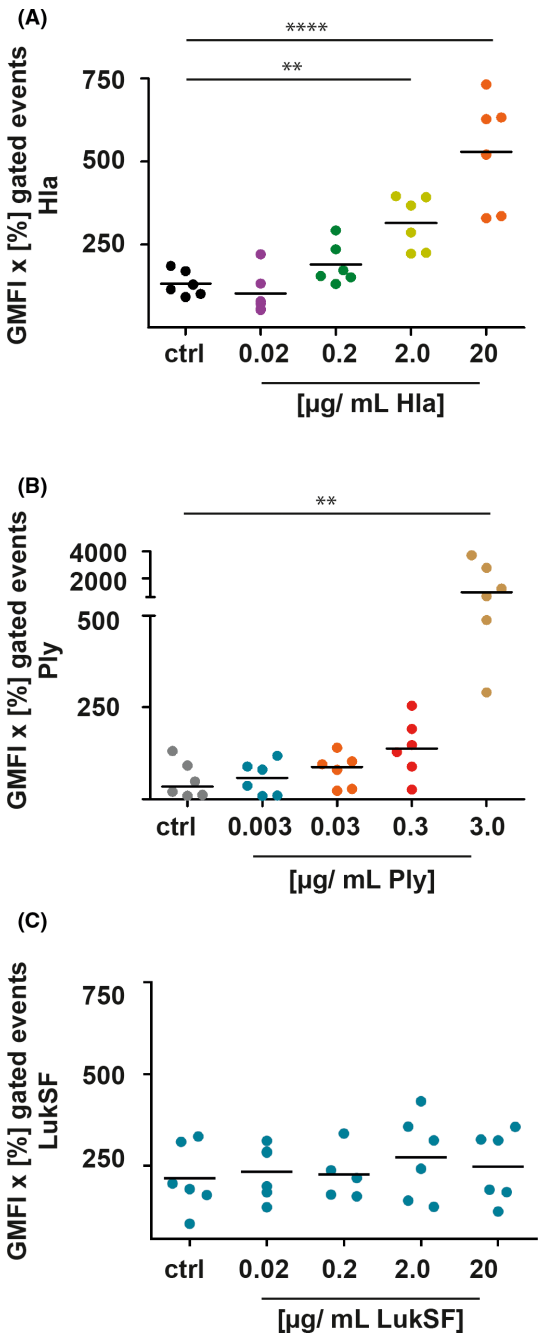

FIGURE 1 Pneumolysin and α-hemolysin bind directly to human platelets. Washed platelets of a defined set of healthy human donors were incubated with increasing concentrations of pneumolysin (Ply (0.3 to 300 ng/ml), α-hemolysin (Hla 0.02–20 µg/ml), and LukSF (PVL 0.02 to 20 µg/ml), fixed and stained with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

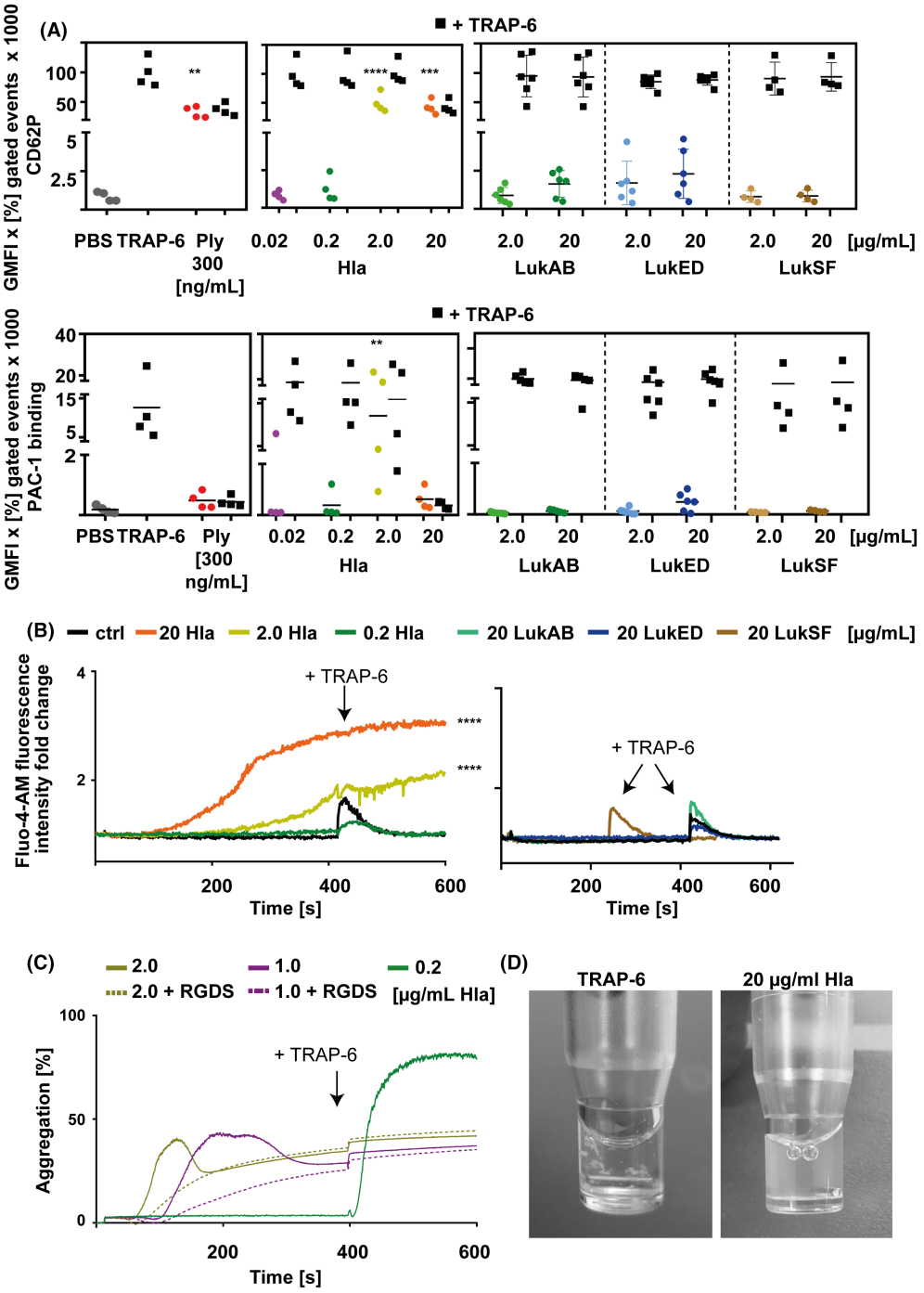

FIGURE 2 α-hemolysin and pneumolysin interfere with platelet function but with different mechanisms. Washed platelets of a defined set of healthy human donors were incubated with increasing concentrations of pneumolysin (Ply 0.3–300 ng/ml), α-hemolysin (Hla 0.02–20 g/ml) LukAB, LukED, and LukSF (PVL)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

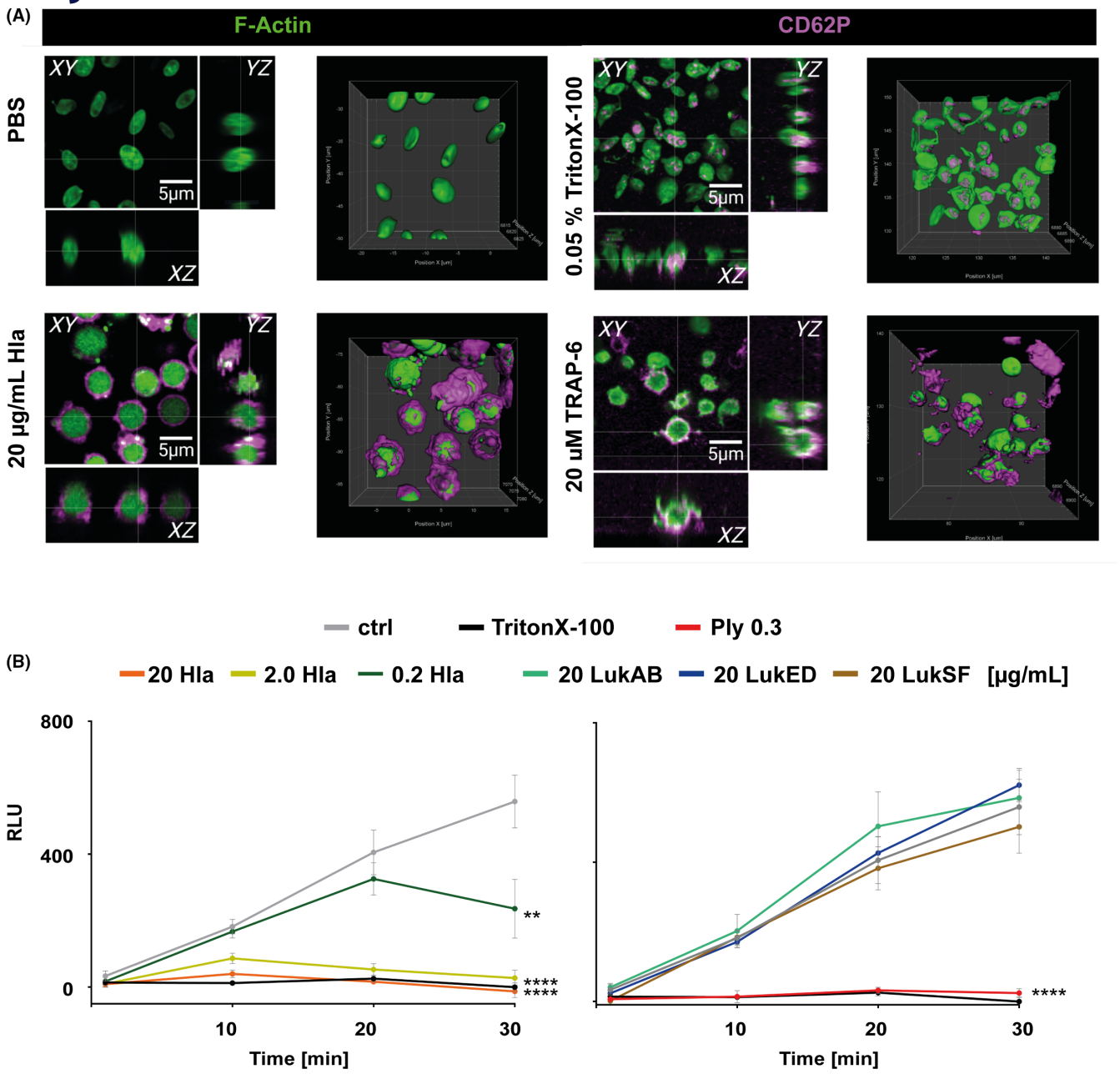

FIGURE 3 Treatment of human platelets with α-hemolysin leads to staining of surface associated CD62P (A) α-hemolysin (Hla) treated platelets were stained for F-actin (green) and CD62P (magenta). Platelets were not permeabilized. Orthogonal views of confocal Z-stacks and three-dimensional iso-surface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

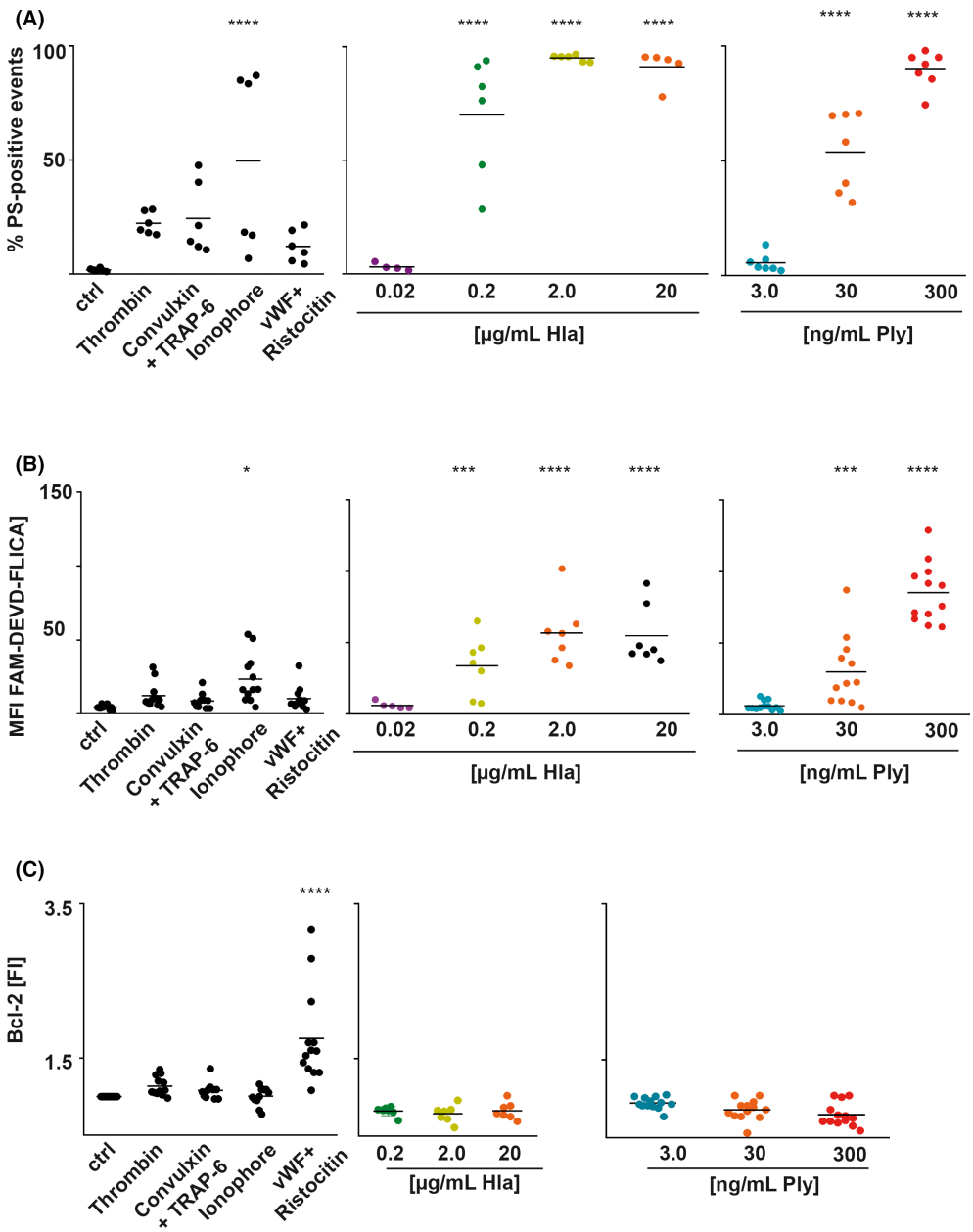

FIGURE 4 Platelets treated with α-hemolysin and pneumolysin are positive for apoptosis markers. Washed human platelets were incubated with increasing concentrations of α-hemolysin (Hla) and pneumolysin (Ply). The analysis of the apoptosis markers caspase activity, Bcl-2 expression, and phosphatidylserine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

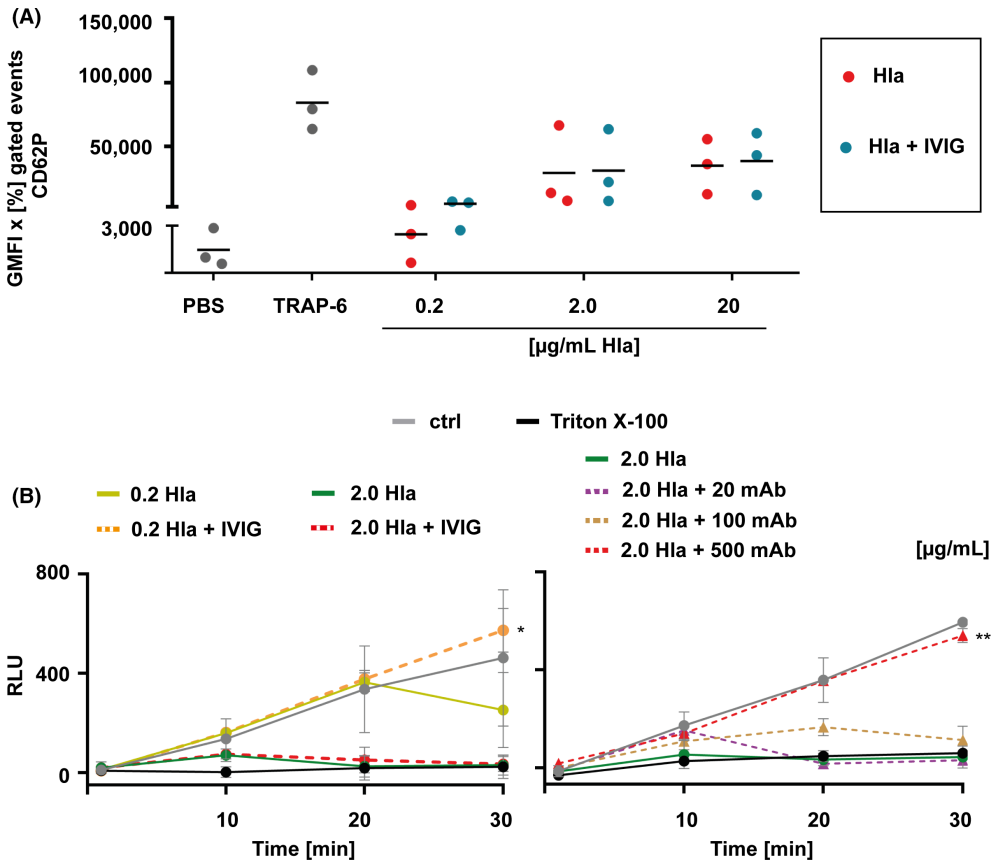

FIGURE 5 IVIG does not neutralize α-hemolysin. α-hemolysin (Hla) was treated with IVIG (1 mg/ml) or a specific mAb for 20 min before incubation with washed human platelets of healthy human donors. (A) Polyvalent human immunoglobulins (IVIG; 1 mg/ml; human IgG, Privigen) did not neutralize the increased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

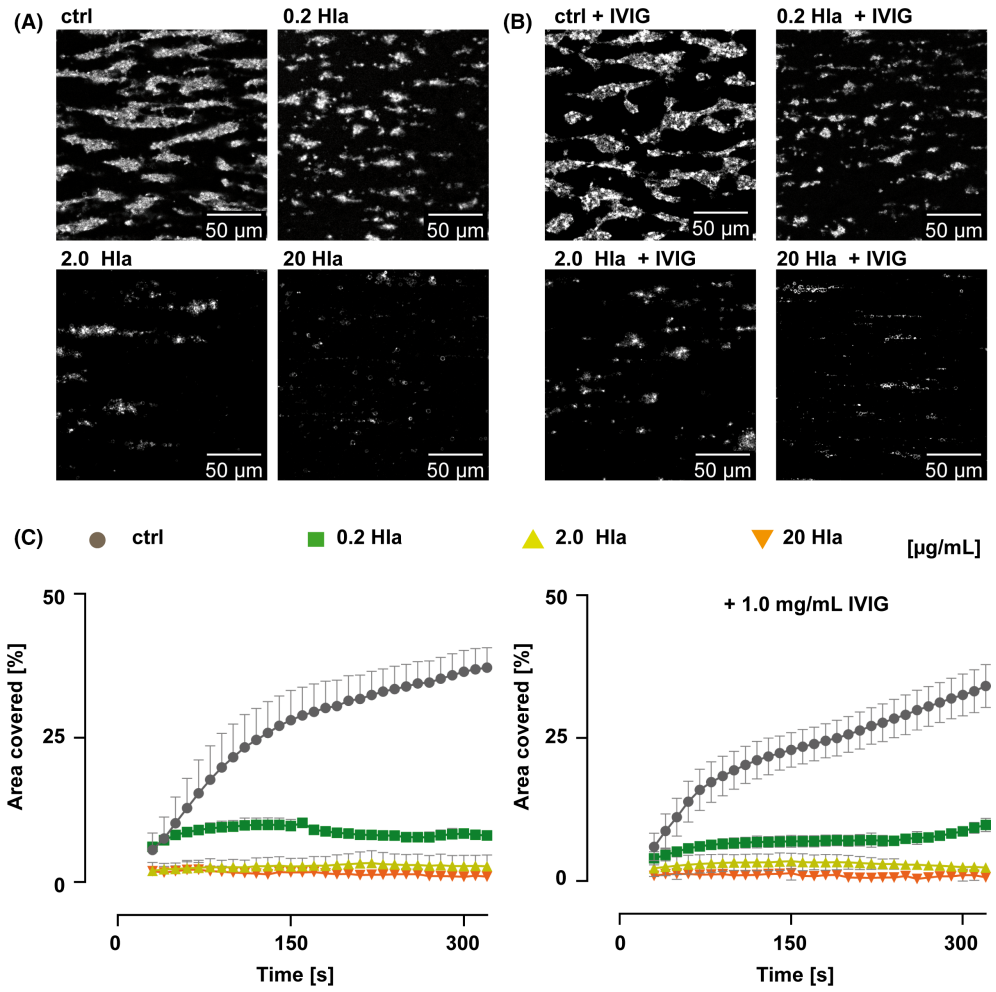

FIGURE 6 α-hemolysin induces abrogation of thrombus formation in whole blood under shear. Whole blood incubated with α-hemolysin (Hla) at 0.2, 2.0, and 20 µg/ml Hla was perfused over a collagen (shear 1000 s^{-1})-coated surface and thrombus formation was visualized by immunostaining of platelets with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

1. Portier I, Campbell RA. Role of platelets in detection and regulation of infection. *Arterioscler Thromb Vasc Biol*. 2021;41:70-78.
2. Watson CN, Kerrigan SW, Cox D, Henderson IR, Watson SP, Arman M. Human platelet activation by *Escherichia coli*: roles for FcγRIIA and integrin αIIbβ3. *Platelets*. 2016;27:535-540.
3. Binsker U, Palankar R, Wesche J, et al. Secreted immunomodulatory proteins of staphylococcus aureus activate platelets and induce platelet aggregation. *Thromb Haemost*. 2018;118:745-757.
4. Ali RA, Wuescher LM, Dona KR, Worth RG. Platelets mediate host defense against *Staphylococcus aureus* through direct bactericidal activity and by enhancing macrophage activities. *J Immunol*. 1950;2017(198):344-351.
5. Gordon RJ, Lowy FD. Pathogenesis of methicillin-resistant *Staphylococcus aureus* infection. *Clin Infect Dis*. 2008;46(Suppl 5):S350-S359.
6. Moreillon P, Que YA, Bayer AS. Pathogenesis of streptococcal and staphylococcal endocarditis. *Infect Dis Clin North Am*. 2002;16:297-318.
7. George NP, Wei Q, Shin PK, Konstantopoulos K, Ross JM. Staphylococcus aureus adhesion via Spa, ClfA, and SdrCDE to immobilized platelets demonstrates shear-dependent behavior. *Arterioscler Thromb Vasc Biol*. 2006;26:2394-2400.
8. Niemann S, Kehrel BE, Heilmann C, Rennemeier C, Peters G, Hammerschmidt S. Pneumococcal association to platelets is mediated by soluble fibrin and supported by thrombospondin-1. *Thromb Haemost*. 2009;102:735-742.
9. Binsker U, Kohler TP, Krauel K, Kohler S, Schwertz H, Hammerschmidt S. Pneumococcal adhesins PavB and PspC are important for the interplay with human thrombospondin-1. *J Biol Chem*. 2015;290:14542-14555.

10. Jahn K, Handtke S, Palankar R, et al. Pneumolysin induces platelet destruction, not platelet activation, which can be prevented by immunoglobulin preparations in vitro. *Blood Adv*. 2020;4:6315-6326.
11. Surewaard BGJ, Thanabalasuriar A, Zeng Z, et al. α-Toxin induces platelet aggregation and liver injury during *Staphylococcus aureus* sepsis. *Cell Host Microbe*. 2018;24:271-84.e3.
12. Lee H, Chae S, Park J, et al. Comprehensive proteome profiling of platelet identified a protein profile predictive of responses to an antiplatelet agent sarpogrelate. *Mol Cell Proteomics*. 2016;15:3461-3472.
13. Wilke GA, Bubeck WJ. Role of a disintegrin and metalloprotease 10 in *Staphylococcus aureus* alpha-hemolysin-mediated cellular injury. *Proc Natl Acad Sci USA*. 2010;107:13473-13478.
14. Song L, Hobaugh MR, Shustak C, Cheley S, Bayley H, Gouaux JE. Structure of staphylococcal alpha-hemolysin, a heptameric transmembrane pore. *Science*. 1996;274:1859-1866.
15. Spaan AN, van Strijp JAG, Torres VJ. Leukocidins: staphylococcal bi-component pore-forming toxins find their receptors. *Nat Rev Microbiol*. 2017;15:435-447.
16. Tromp AT, van Strijp JAG. Studying staphylococcal leukocidins: a challenging endeavor. *Front Microbiol*. 2020;11:611.
17. Rigby KM, DeLeo FR. Neutrophils in innate host defense against *Staphylococcus aureus* infections. *Semin Immunopathol*. 2012;34:237-259.
18. Spaan AN, Surewaard BG, Nijland R, van Strijp JA. Neutrophils versus *Staphylococcus aureus*: a biological tug of war. *Annu Rev Microbiol*. 2013;67:629-650.
19. Niemann S, Bertling A, Brodde MF, et al. Panton-valentine leukocidin associated with *S. aureus* osteomyelitis activates platelets via neutrophil secretion products. *Sci Rep*. 2018;8:2185.
20. Below S, Konkel A, Zeeck C, et al. Virulence factors of *Staphylococcus aureus* induce Erk-MAP kinase activation and c-Fos expression in S9 and 16HBE14o- human airway epithelial cells. *Am J Physiol Lung Cell Mol Physiol*. 2009;296:L470-L479.
21. Liu X, Pichulik T, Wolz OO, et al. Human NACHT, LRR, and PYD domain-containing protein 3 (NLRP3) inflammasome activity is regulated by and potentially targetable through Bruton tyrosine kinase. *J Allergy Clin Immunol*. 2017;140:1054-67.e10.
22. Mangin PH, Neeves KB, Lam WA, et al. Subcommittee on B. In vitro flow-based assay: from simple toward more sophisticated models for mimicking hemostasis and thrombosis. *J Thromb Haemost*. 2021;19:582-587.
23. Schindelin J, Arganda-Carreras I, Frise E, et al. Fiji: an open-source platform for biological-image analysis. *Nat Methods*. 2012;9:676-682.
24. Spaan AN, Henry T, van Rooijen WJM, et al. The staphylococcal toxin panton-valentine leukocidin targets human C5a receptors. *Cell Host Microbe*. 2013;13:584-594.
25. Huang J, Swieringa F, Solari FA, et al. Assessment of a complete and classified platelet proteome from genome-wide transcripts of human platelets and megakaryocytes covering platelet functions. *Sci Rep*. 2021;11:12358.
26. Reyes-Robles T, Alonzo F, Kozhaya L, Lacy DB, Unutmaz D, Torres VJ. Staphylococcus aureus leukotoxin ED targets the chemokine receptors CXCR1 and CXCR2 to kill leukocytes and promote infection. *Cell Host Microbe*. 2013;14(4):453-459.
27. Alonzo F, Kozhaya L, Rawlings SA, et al. CCR5 is a receptor for *Staphylococcus aureus* leukotoxin ED. *Nature*. 2013;493(7430):51-55.
28. DuMont AL, Yoong P, Day CJ, et al. *Staphylococcus aureus* LukAB cytotoxin kills human neutrophils by targeting the CD11b subunit of the integrin Mac-1. *Proc Natl Acad Sci USA*. 2013;110:10794.
29. Monecke S, Muller E, Buchler J, Stieber B, Ehricht R. *Staphylococcus aureus* in vitro secretion of alpha toxin (hla) correlates with the affiliation to clonal complexes. *PLoS One*. 2014;9:e100427.
30. Watkins RR, David MZ, Salata RA. Current concepts on the virulence mechanisms of methicillin-resistant *Staphylococcus aureus*. *J Med Microbiol*. 2012;61:1179-1193.
31. Bhakdi S, Muhly M, Mannhardt U, et al. Staphylococcal alpha toxin promotes blood coagulation via attack on human platelets. *J Exp Med*. 1988;168:527-542.
32. Arvand M, Bhakdi S, Dahlbäck B, Preissner KT. *Staphylococcus aureus* alpha-toxin attack on human platelets promotes assembly of the prothrombinase complex. *J Biol Chem*. 1990;265:14377-14381.
33. Meyer TC, Michalik S, Holtfreter S, et al. A Comprehensive view on the human antibody repertoire against staphylococcus aureus antigens in the general population. *Front Immunol*. 2021;12:651619.
34. Schubert S, Schwertz H, Weyrich AS, et al. *Staphylococcus aureus* alpha-toxin triggers the synthesis of B-cell lymphoma 3 by human platelets. *Toxins*. 2011;3:120-133.
35. Yu XQ, Robbie GJ, Wu Y, et al. Tolerability, and pharmacokinetics of MEDI4893, an investigational, extended-half-life, anti-staphylococcus aureus alpha-toxin human monoclonal antibody, in healthy adults. *Antimicrob Agents Chemother*. 2017;61:e01020-16
36. Cohen Taylor S, Hilliard Jamese J, Jones-Nelson O, et al. *Staphylococcus aureus* α toxin potentiates opportunistic bacterial lung infections. *Sci Transl Med*. 2016;8:329ra31.
37. Francois B, Jafri HS, Chastre J, et al. Efficacy and safety of suvratoxumab for prevention of *Staphylococcus aureus* ventilator-associated pneumonia (SAATELLITE): a multicentre, randomised, double-blind, placebo-controlled, parallel-group, phase 2 pilot trial. *Lancet Infect Dis*. 2021;21:1313-1323.
38. Powers Michael E, Becker Russell EN, Sailer A, Turner Jerrold R, Bubeck WJ. Synergistic action of *Staphylococcus aureus* α-toxin on platelets and myeloid lineage cells contributes to lethal Sepsis. *Cell Host Microbe*. 2015;17:775-787.
39. Powers ME, Kim HK, Wang Y, Bubeck WJ. ADAM10 mediates vascular injury induced by *Staphylococcus aureus* α-hemolysin. *J Infect Dis*. 2012;206:352-356.
40. Appel D, Hummel R, Weidemeier M, Endres K, Gölz C, Schäfer MKE. Pharmacologic inhibition of ADAM10 attenuates brain tissue loss, axonal injury and pro-inflammatory gene expression following traumatic brain injury in mice. *Front Cell Dev Biol*. 2021;9:661462.
41. Wetzel S, Seipold L, Saftig P. The metalloproteinase ADAM10: a useful therapeutic target? *Biochim Biophys Acta Mol Cell Res*. 2017;1864(11):2071-2081.
42. Hua VM, Abeynaike L, Glaros E, et al. Necrotic platelets provide a procoagulant surface during thrombosis. *Blood*. 2015;126:2852-2862.
43. Kholmukhamedov A, Jobe SM. Necrotic but Not apoptotic platelets are functionally procoagulant. *Blood*. 2018;132:2420.

SUPPORTING INFORMATION

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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;20:1464–1475. doi:10.1111/jth.15703