Received: 8 October 2021 | Accepted: 29 November 2021

DOI: 10.1111/jth.15613

ORIGINAL ARTICLE

A peptide from the staphylococcal protein Efb binds P-selectin and inhibits the interaction of platelets with leukocytes

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Funding information
British Heart Foundation, Grant/Award Number: FS/17/13/3269; Werner Otto Stiftung, Grant/Award Number: BN3/97; H2020 European Research Council, Grant/Award Number: 101025074

Abstract

Aims: P-selectin is a key surface adhesion molecule for the interaction of platelets with leukocytes. We have shown previously that the N-terminal domain of *Staphylococcus aureus* extracellular fibrinogen-binding protein (Efb) binds to P-selectin and interferes with platelet-leukocyte aggregate formation. Here, we aimed to identify the minimal Efb motif required for binding platelets and to characterize its ability to interfering with the formation of platelet-leukocyte aggregates.

Methods and Results: Using a library of synthetic peptides, we mapped the platelet-binding site to a continuous 20 amino acid stretch. The peptide Efb_{68-87} was able to bind to resting and, to a greater extent, thrombin-stimulated platelets in the absence of fibrinogen. Dot blots, pull-down assays and P-selectin glycoprotein ligand-1 (PSGL-1) competitive binding experiments identified P-selectin as the cellular docking site mediating Efb_{68-87} platelet binding. Accordingly, Efb_{68-87} did not bind to other blood cells and captured platelets from human whole blood under low shear stress conditions. Efb_{68-87} did not affect platelet activation as tested by aggregometry, flow cytometry and immunoblotting, but inhibited the formation of platelet-leukocyte aggregates (PLAs). Efb_{68-87} also interfered with the platelet-dependent stimulation of neutrophil extracellular traps (NETs) formation in vitro.

Conclusions: We have identified Efb_{68-87} as a novel selective platelet-binding peptide. Efb_{68-87} binds directly to P-selectin and inhibits interactions of platelets with leukocytes that lead to PLA and NET formation. As PLAs and NETs play a key role in thromboinflammation, Efb_{68-87} is an exciting candidate for the development of novel selective inhibitors of the proinflammatory activity of platelets.

KEYWORDS
leukocyte, neutrophil extracellular trap, platelet, platelet-leukocyte aggregate, P-selectin, thromboinflammation

Manuscript Handled by: X. Long Zheng

Final decision: X. Long Zheng, 29 November 2021

1 | INTRODUCTION

Our understanding of the role of platelets in vascular health has significantly progressed in recent years. In addition to platelets' established role in hemostasis and thrombosis, their participation in inflammatory responses has become evident.[1] The role of platelet pro-inflammatory activity for progression of cardiovascular disease has attracted considerable attention.[2] In addition to their ability to release pro-inflammatory cytokines[3] and modulate the release of cytokines by leukocytes,[4] platelets directly interact with leukocytes to form platelet-leukocyte aggregates (PLAs).[5] PLA formation has been shown to facilitate leukocyte homing and extravasation at the site of vascular injury, thus promoting inflammation.[6] Inflammation is a key factor in vascular complications and cardiovascular diseases.[7] PLAs are in fact increased in coronary syndromes, in the form of platelet-monocyte[8-12] and platelet-neutrophil complexes.[13] PLA formation also increases as a consequence of coronary surgical intervention.[14] Although CD40-CD40L interactions[15] and the binding of the leukocyte receptor CD11b/CD18 (Mac-1) with either platelet glycoprotein receptor GPIBα[16] or platelet integrin $\alpha_{IIb}\beta3$[17] participate in the formation of PLAs, the interaction of platelet P-selectin and its physiological ligand P-selectin glycoprotein ligand-1 (PSGL-1) on leukocytes is critical for the heterotypic aggregation of these cell types.[18] The interaction of platelets with neutrophils has particularly important pathophysiological consequences. Platelets can induce the formation of neutrophil extracellular traps (NETs).[19] While NETs have originally been described in host-defense processes,[20] their role in the onset and progression of venous and arterial thrombosis has been shown in animal models and clinical studies.[21-23] As for PLAs, the binding of P-selectin on platelets with PSGL-1 on leukocytes is a critical step in NET formation.[24]

Because of its role in the interaction of platelets with leukocytes, P-selectin has attracted increasing interest as a drug discovery target to develop pharmacobiological agents able to control inflammation and vascular degeneration via disruption or reduced formation of PLAs and NETs. The clinical potential of P-selectin inhibitors requires further investigation. We have shown previously that extracellular fibrinogen binding protein (Efb), a protein secreted by *Staphylococcus aureus* (*S. aureus*), directly binds P-selectin and inhibits its interaction with PSGL-1.[25] In contrast to numerous bacterial proteins that have been reported to positively modulate platelet function, Efb inhibits platelet activation and thrombus formation,[26,27] facilitating bacterial survival in the blood and aggravating infection.[28] Efb comprises an N-terminal secretion signal, a N-terminal domain lacking structural organization (Efb-N, residues Ser30-Thr104), and a tri-helical bundle C-terminal domain (Efb-C, residues Ile105-Lys165)[29] (Figure 1A). Efb-N includes two repeated motifs (residues Asn46-Pro67 and Asn77-Ala98) that are homologous to *S. aureus* coagulase repeats and that are part of fibrinogen binding sites comprising residues Ser30-Pro67 and Lys68-Ala98.[30] Efb-C plays an immunosuppressive role by interfering with the complement system.[29,31] In combination, Efb-N and Efb-C facilitate *S. aureus* escape from phagocytosis and increase *S. aureus* pathogenicity.[32] In fact, *S. aureus* infections are significantly exacerbated in vivo in the presence of Efb.[33]

Essentials

- Efb, a *Staphylococcus aureus* protein, binds P-selectin and inhibits the platelet/leukocyte interaction.
- Here, we aimed to identity and characterize the Efb motif responsible for P-selectin binding.
- Efb_{68-87} binds platelets in a P-selectin-dependent manner without affecting their activation.
- Efb_{68-87} inhibits the formation of PLAs and the platelet-dependent stimulation of NETs in vitro.

In this study, we mapped the P-selectin-binding site in Efb-N using a peptide scanning approach. A 20 aa-peptide located within Efb-N between Lys68 and Glu87 bound P-selectin. We show here that this peptide selectively binds platelets, without affecting their hemostatic function, and inhibits platelets' ability both to complex with leukocytes to form PLAs and to induce NET formation. The therapeutic potential of this Efb-derived peptide to control thromboinflammation in cardiovascular patients will require further investigation.

2 | METHODS

2.1 | Peptide synthesis and labelling

All peptides were synthesized by KareBay Bio with the following sequences (purity >98%, Figure S1), where (ctrl) designates the scrambled control version:

Efb_{30-45}	SEGYGPREKKPVSINH
Efb_{30-45} (ctrl)	SPGGKHPVNYKSERIE
Efb_{30-67}	SEGYGPREKKPVSINHNIVEYNDGTFKYQSRPKFNSTP
Efb_{30-67} (ctrl)	DTYKINSEYPPVRSPNPGGKQYKFSTINGKHESFRVEN
Efb_{46-67}	NIVEYNDGTFKYQSRPKFNSTP
Efb_{46-67} (ctrl)	RKVTPSFYQFNITDKNPGESNY
Efb_{68-76}	KYIKFKHDY
Efb_{68-76} (ctrl)	KIDKYYHKF
Efb_{68-87}	KYIKFKHDYNILEFNDGTFE
Efb_{68-87} (ctrl)	YKFEENLFGTNDKDFKIYHI
Efb_{68-99}	KYIKFKHDYNILEFNDGTFEYGARPQFNKPAA
Efb_{68-99} (ctrl)	KNFELAKAGYPQIAHKTNKPGFRFYDDNFIEY
Efb_{77-99}	NILEFNDGTFEYGARPQFNKPAA
Efb_{77-99} (ctrl)	PFYIAPLARAGTNDNEGFFQENK

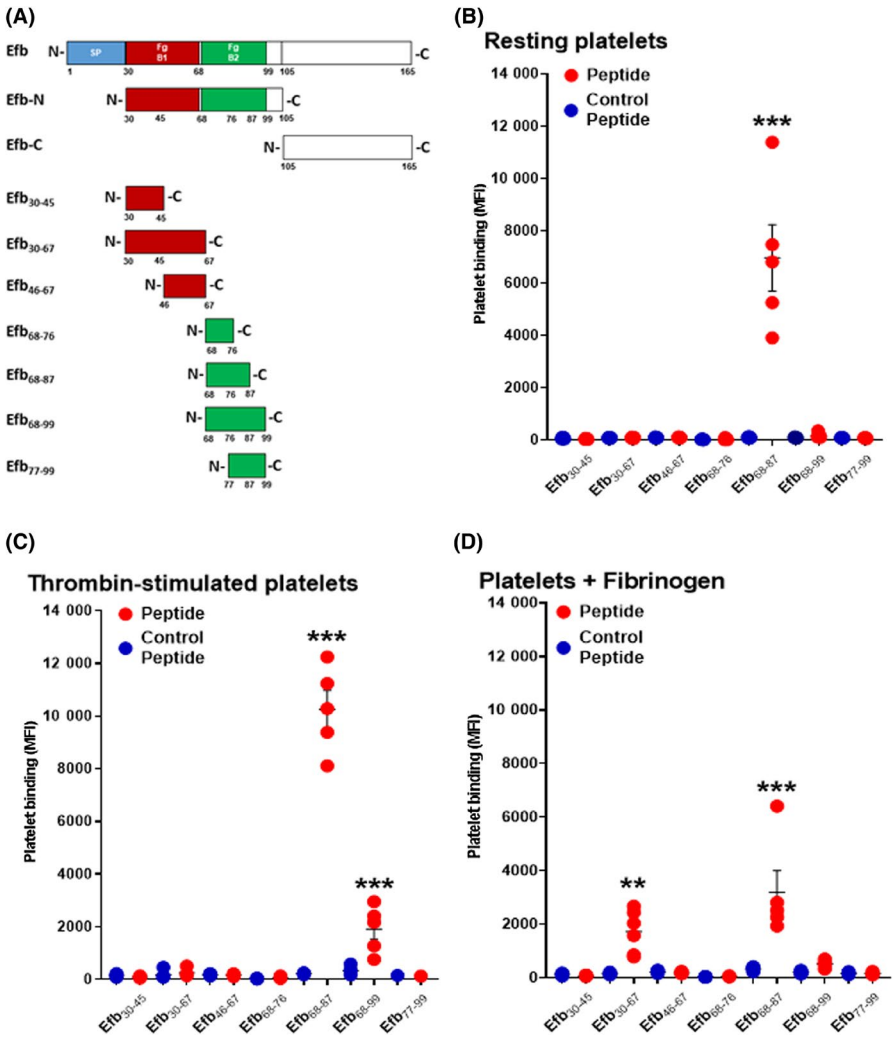

FIGURE 1 Synthetic Efb peptides bind human platelets. (A) Structure of Efb and the design of synthetic peptides representing different regions of the Efb N-terminal domain (amino acids 30–105). The signal peptide is shown in light blue, the first fibrinogen binding motif (Fg B1) in red, the second fibrinogen binding motif (Fg B2) in green. Efb peptide binding to resting (B), thrombin-stimulated (C) or fibrinogen-treated (D) platelets. 1 μM FITC-labelled Efb or scrambled control peptides were incubated with isolated human platelets and their binding was assessed by flow cytometry. In (C), thrombin stimulation was obtained with 0.1 μ/ml human thrombin for 10 min, while in (D) platelets were treated with 3 mg/ml fibrinogen for 10 min. Values are the median fluorescence intensity (MFI) from 10 000 events in five independent experiments (mean ± SEM). Statistical significance was assessed using one-way ANOVA with Bonferroni post-hoc test; $p < .05$ (*), $p < .01$ (**), $p < .001$ (***)

In order to fluorescently label lysine residues, synthetic peptides (or recombinant Efb-$N_{30\text{-}105}$) were coupled by direct reaction in 0.1 M sodium carbonate buffer with fluorescein isothiocyanate (FITC). The reaction mixture was incubated, with gentle agitation, at 4°C for 15 h. Free FITC was eliminated by dialysis in PBS using a 500 Da MWCO cut-off membrane.

2.2 | Blood collection and platelet isolation

Procedures using human blood conformed to the principles outlined in the Declaration of Helsinki. Human blood was collected at the Institute of Clinical Chemistry and Laboratory Medicine (University Medical Center Eppendorf - Hamburg) after informed volunteers' consent was given in written form. Sodium citrate (0.5% w/v) was used as an anticoagulant. Platelet-rich plasma (PRP) was separated from whole blood by centrifugation (250 g, 17 min) and platelets were separated from PRP by a second centrifugation step (500 g, 10 min) in the presence of prostaglandin E1 (PGE1, 40 ng/ml) and indomethacin (10 μM). All centrifugations were performed with soft deceleration settings. Platelets were resuspended in modified Tyrode's buffer at a density of 2×10^8 platelets/ml throughout the study.

2.3 | Flow cytometry (Efb binding)

For isolated platelet binding, 10^7 platelets/ml were incubated in the presence or absence of 3 mg/ml fibrinogen and 0.2 U/ml thrombin for 10 min at room temperature. 1 μM FITC-Efb peptide, FITC-scrambled Efb peptide or FITC-BSA was then added to the platelets followed by another 10 min incubation. Platelet binding was assessed by flow cytometry using a FACSAria III (BD Biosciences).

Alternatively, 10 μM FITC-conjugated Efb peptide was incubated for 30 min at room temperature in heparin-anticoagulated (5 U/ml) human blood with CD42b/PE (#561854, BD Biosciences) and CD45/APC (#555745, BD Biosciences) antibodies. Cells were then fixed with 1% paraformaldehyde (PFA) for 15 min and binding was assessed by flow cytometry using a FACSCanto II (BD Biosciences).

2.4 | Flow cytometry (activation/degranulation markers)

Heparin-anticoagulated (5 U/ml) human blood was preincubated with the relevant peptide for 15 min at 37°C. Platelets were activated with 1 U/ml thrombin for 15 min at 37°C. Samples were then labelled with anti-CD42b/APC (#551061, BD Biosciences), anti-CD62P/PE (#550561, BD Biosciences), and PAC-1/FITC (#340507, BD Biosciences) antibodies for 15 min at room temperature. Samples were then fixed with 4% PFA for 15 min at RT. Directly before measurement, the samples were diluted 1:1 with PBS and the fluorescence labelling was measured in 10,000 CD42b/APC-positive events, using a FACSCanto II flow cytometer (BD Biosciences). The mean fluorescence intensity (MFI) of CD42b/APC-positive events in the PE and FITC spectrum bands was analyzed.

2.5 | Isolated platelet fluorescence imaging

Coverslips were coated with 0.01% w/v poly-L-lysine and blocked with 0.1% w/v solution of BSA in PBS. Isolated platelets (resting, or activated in suspension with 10 μM TRAP6 peptide for 5 min at 37°C) were dispensed onto the prepared coverslips at 0.5×10^7 platelets/ml density and incubated for 1 h at RT. Platelets were then fixed for 15 min in 4% PFA and stained with 2 μM solution of FITC-labelled peptide ($Efb_{68\text{-}87}$ or $Efb_{68\text{-}87}$ (ctrl)) and, subsequently, with 1 U/ml phalloidin-rhodamine in PBS-Tween20 0.1% v/v. The coverslips were mounted onto the glass slides with Fluoromount mounting medium. Imaging was performed with a Leica TCS SP5 confocal microscope.

2.6 | Dot blots

200 ng of Fc-P-selectin (R&D Systems, # 137-PS-050) was dotted onto nitrocellulose membrane and air-dried. The membrane was then blocked with 5% w/v BSA in TBS-T for 30 minutes before being incubated with 1.0 μg/ml FITC-Efb peptides (labelling described above) for 45 minutes. Following three washes in TBS-T, imaging was performed with a Licor Odyssey CLx scanner.

2.7 | Pull-down experiments

$Efb_{68\text{-}87}$ or $Efb_{68\text{-}87}$ (ctrl) was immobilized on CarboxyLink™ Coupling Resin (Thermo Fisher Scientific), according to the manufacturer's instructions. Peptide-conjugated resin was incubated with platelet lysates prepared by sonication from isolated platelet solution. After binding overnight at 4°C, the peptide complexed to platelet proteins was eluted from the resin according to the manufacturer's instructions. Eluates were subsequently utilized for immunoblotting.

2.8 | Immunoblotting

Platelet suspensions prepared as described above were stimulated in the presence of 1 mM EGTA and lysed by adding lysis buffer (12.5 mM Tris, pH 8.3, 97 mM glycine, 2% sodium dodecyl sulphate (SDS), 0.5% dithiothreitol (DTT), 10% glycerol, and 0.01% bromophenol blue). Platelet proteins were separated by SDS-polyacrylamide gel electrophoresis (SDS-PAGE), transferred to polyvinylidene difluoride (PVDF) membrane, and analyzed by immunoblotting for P-selectin (Abcam, #ab182135), thrombospondin 1 (Thermo Fisher Scientific, #MA5-13398), multimerin 1 (Santa Cruz Biotechnologies, #sc-104427), actin (Merck Millipore #A5441), protein kinase C (PKC) phospho-substrates (#2261, Cell Signaling Technology) and phospho-Src (R&D Systems, # AF2685). Densitometry was performed using ImageJ 1.47v (Wayne Rasband, National Institute of Health, US) and presented as intensity ratio over actin staining (i.e. loading control).

2.9 | Platelet aggregation

Platelets resuspended in modified Tyrode's buffer at a density of 2×10^8 platelets/ml were stimulated using a Chrono-Log 490 4+4 aggregometer. Aggregation was induced with 0.1 U/ml human thrombin or 3 μg/ml Horm collagen. Absorbance was measured for 10 minutes and expressed as percentage change of absorbance.

2.10 | Platelet adhesion/thrombus formation under flow

Vena8-Fluoro + flow chambers were coated with 0.1 mg/ml collagen or $Efb_{68\text{-}87}$ or $Efb_{68\text{-}87}$ (ctrl). Blood was taken in 0.25% w/v citrate and 25 μM D-Phenylalanyl-Prolyl-Arginyl Chloromethyl Ketone (PPACK) and labelled with 1 μM 3,3'-dihexyloxacarbocyanine iodide (DiOC6) for 10 min at 37°C. As indicated in the text, the flow was set to 1000 sec^{-1} (40 μl/min) or 200 sec^{-1} (8 μl/min). Quantification of platelet adhesion after 5 min of flow was obtained by LED fluorescence microscopy (EVOS FI) and image analysis using ImageJ 1.47v (Wayne Rasband, National Institute of Health).

2.11 | PLA quantification

Heparin-anticoagulated (5 U/ml) human blood was treated with red blood cells (RBC) lysis solution (Miltenyi Biotec, Germany) and treated with $Efb_{68\text{-}87}$ or $Efb_{68\text{-}87}$ (ctrl) and collagen-related peptide (CRP) (1 μg/ml), TRAP6 (5 μM) and/or LPS from *Escherichia coli* (1 μg/ml) to stimulate platelet-leukocyte aggregation. Anti-CD42b/PE (#561854, BD Biosciences) and anti-CD45/APC (#555745, BD Biosciences) antibodies were used to stain platelets and leukocytes, respectively. Following fixation in 1% w/v PFA, all samples were

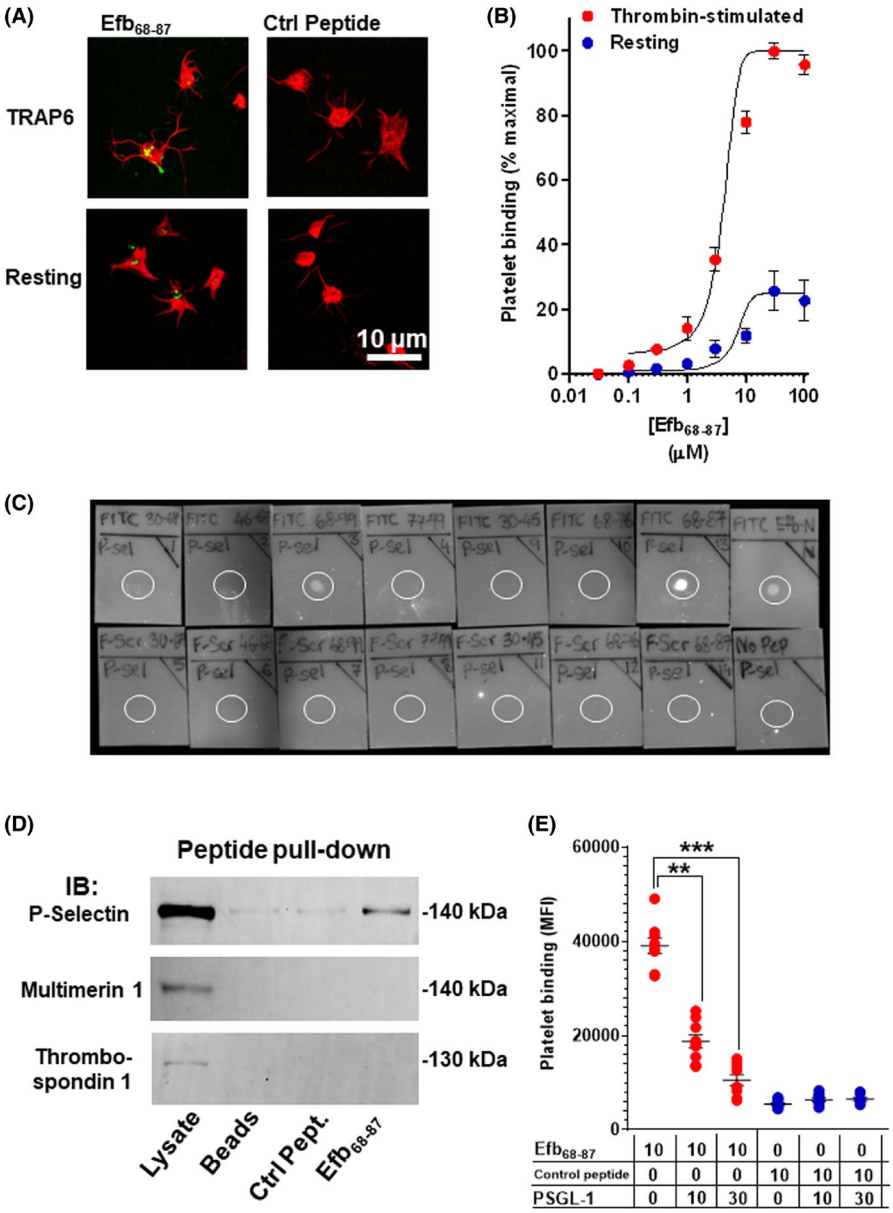

FIGURE 2 Efb_{68-87} binding to platelet P-selectin. (A) Phalloidin-rhodamine (red, 1 U/ml) and FITC-labelled Efb_{68-87} (green, 2 µM) were used to stain human platelets fixed either before or after stimulation in suspension with TRAP6. FITC-labelled scrambled Efb_{68-87} was used as control. Images are representative of five independent experiments. The scale bar indicated in white is 10 µm. (B) Using different concentrations of FITC-Efb_{68-87} (0.1–100 µM), Efb_{68-87} binding affinity to resting and thrombin-stimulated platelets was assessed by flow cytometry. Data are percentages of the maximal binding values and are mean ± SEM from five independent experiments. (C) The direct interaction of Efb_{68-87} and P-selectin was confirmed by dot blot. Fc-P-selectin was dotted onto individual nitrocellulose membranes and allowed to dry. After blocking with 5% w/v BSA, each FITC-Efb and FITC-scrambled Efb peptide (0.3 µg/ml of 68–87) was incubated on the membrane. Fluorescence images of the membranes were obtained with a ChemiDoc Scanner (Bio-Rad) at 488 nm_{exc}. The data are representative of six independent measurements. (D) Pull-down experiments performed with Efb_{68-87} conjugated to agarose beads confirmed P-selectin as the main candidate binding site for this peptide on platelets. Platelet lysate (lane 1), pull-downs with unconjugated beads (lane 2), pull-downs with beads conjugated with scrambled Efb_{68-87} (lane 3) and pull-downs with beads conjugated with Efb_{68-87} (lane 4) were compared. The immunoblotting was performed with antibodies for human P-selectin, multimerin-1 or thrombospondin-1. The data are representative of five independent experiments. (E) Binding competition by Fc-PSGL-1. As described for (B), the binding of FITC-Efb_{68-87} to platelets was assessed by flow cytometry in the presence of different concentrations of Fc-PSGL-1 (0, 10 or 30 µM). Values are the median fluorescence intensity (MFI) from 10 000 events. Statistical significance was assessed using one-way ANOVA with Bonferroni post-hoc test: $p < .05$ (*), $p < .01$ (**), $p < .001$ (***)

analyzed using a FACSCanto II flow cytometer (BD Biosciences). PLAs were quantified as events positively stained for both markers (see Figure 5A) and their density was expressed as number of double-positive events over 10 000 events.

2.12 | NET visualization and quantification

Neutrophils were isolated from human peripheral blood as previously described.[34] 5×10^4 neutrophils per well were seeded in 96-well

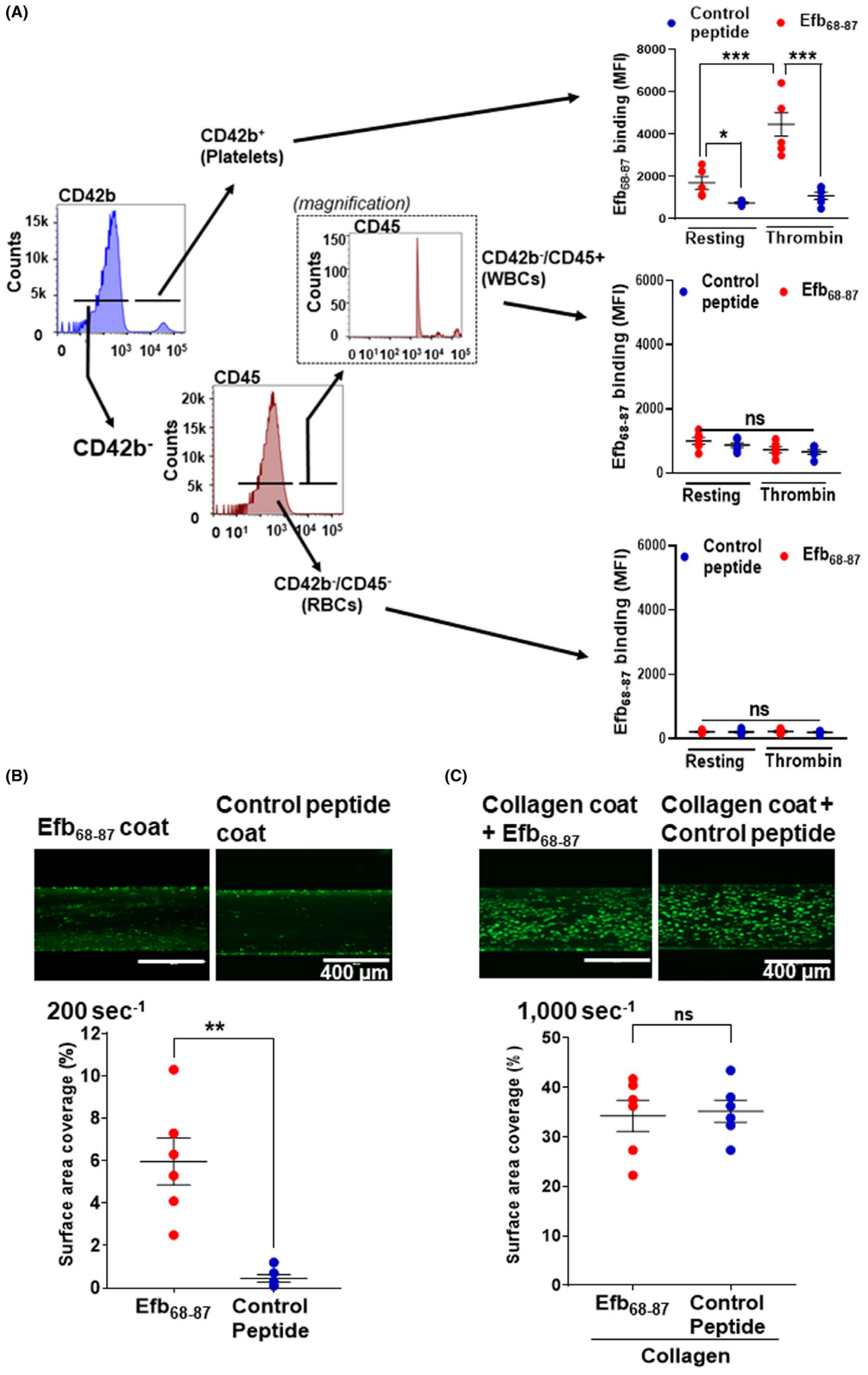

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FIGURE 3 Efb_{68-87} binds to platelets in whole blood without interfering with collagen-dependent thrombus formation. (A) CD42b/PE and CD45-APC antibodies were used to distinguish platelets ($CD42b^+$), leukocytes ($CD42b^-$, $CD45^+$) and red blood cells ($CD42b^-$, $CD45^-$) from human blood by flow cytometry. FITC-Efb_{68-87} binding to each cell population was tested by flow cytometry. Data are mean ± SEM of the MFI values from 10 000 events in six independent experiments. Statistical significance was assessed using one-way ANOVA with Bonferroni post-hoc test: $p < .05$ (*), $p < .01$ (**), $p < .001$ (***). (B) Platelet adhesion to Efb_{68-87} under physiological flow. Platelet adhesion to Efb_{68-87} was tested at shear stress 200 sec^{-1} (venous) and 1000 sec^{-1} (arterial, not shown). In addition, (C) the effect of Efb_{68-87} (5 μM) on platelet adhesion and thrombus formation on collagen was tested (1000 sec^{-1}). Platelets in whole blood were fluorescently labelled with $DiOC_6$ and their surface coverage was assessed by fluorescence imaging/densitometry analysis. Data presented are mean ± SEM of the surface coverage from 6 independent experiments. Statistical significance was assessed using the paired sample Student *t*-test (normality was assessed by Shapiro-Wilk test): $p < .05$ (*), $p < .01$ (**), $p < .001$ (***)

plates and incubated at 37°C for 1 h to allow cell adhesion. Platelet suspensions (1×10^7 platelets/ml) were incubated for 15 min with 5 μM Efb_{68-87} or Efb_{68-87} (ctrl) before incubation with TRAP6 (5 μM) for 15 min to obtain platelet stimulation. Platelet suspensions were then added to the neutrophil monolayers (1×10^6 platelets/well) and incubated for 16 h to induce NET formation. 0.1 μM phorbol-12-myristate-13-acetate (PMA) was added to positive control wells. Following fixation with 2% w/v PFA and staining with 1 μM Sytox Green (Life Technologies), NETs were visualized via fluorescence microscopy (Nikon ECLIPSE Ts2R with Nikon DS-Fi3 camera) and quantified using a microplate reader (Tecan Spark 10 M) using 485 nm_{exc}/535 nm_{em}.

2.13 | Statistical analysis

Data normality and homoscedasticity were tested with Shapiro-Wilk and Bartlett's tests, respectively. For dual comparisons (i.e. WT vs 3KO) of normal/homoscedastic data, statistical analysis was performed by unpaired Student's *t*-tests. Dual comparisons (i.e. WT vs 3KO) of non-normal/non-homoscedastic data were analyzed by non-parametric Mann–Whitney test. One-way ANOVA with Bonferroni post-test was used for multiple comparison tests after testing that data are normal and homoscedastic. The software package GraphPad Prism Version 8.1.0 for Windows 64 bit was used for all statistical analyses. For the K_D estimate, we used a sigmoidal regression model for one-site saturation binding within GraphPad Prism. Throughout the study, the results were expressed as the mean ± standard error (SEM). Differences were considered significant at $p < .05$ (*), 0.01 (**) or 0.001 (***).

3 | RESULTS

3.1 | Efb_{68-87} binds to platelets via P-selectin

Binding experiments identified Efb_{68-87} (Figure 1A) as the minimal Efb sequence required to interact with platelets in the absence of fibrinogen (Figure 1B). Platelet stimulation with thrombin increased platelet-binding of Efb_{68-87} (Figure 1C). Efb_{68-99}, a longer peptide including the newly-identified P-selectin-binding motif, displayed the ability to bind platelet upon platelet stimulation but not in unstimulated platelets. The addition of 3 mg/ml fibrinogen (i.e. physiological plasma concentration) stimulated binding of both Efb_{68-87} and Efb_{30-67} (Figure 1D), although it did not increase the level of Efb_{68-87} binding over the value for unstimulated platelets. Fluorescence microscopy (Figure 2A) and flow cytometry (Figure 2B) confirmed binding of FITC-Efb_{68-87} to unstimulated and stimulated platelets (TRAP6 and thrombin, respectively). The flow cytometry experiments showed that although the overall amount of bound Efb_{68-87} peptide is notably higher after platelet stimulation with thrombin (Figure 2B), the concentration-binding curve indicates a similar K_D for the binding of Efb_{68-87} to resting and thrombin-stimulated platelets (5.7 μM and 3.4 μM, respectively). The binding site for Efb_{68-87} on platelets was investigated by dot blot (Figure 2C). Fc-P-selectin, a chimeric construct that allows effective suspension of P-selectin in aqueous solution, comprising the extracellular domain of human P-selectin (i.e. residues 42–771) and human IgG1 residues 100–330, was immobilized on nitrocellulose membrane and treated with the library of FITC-labelled Efb peptides. As detected by fluorescence imaging of the membrane, Efb_{68-87}, Efb_{68-99} and Efb-N (i.e. Efb_{30-105}), bound to immobilized P-selectin. All other Efb- and scrambled control-peptides did not exhibit any binding activity to P-selectin. In addition, when immobilized on agarose beads, Efb_{68-87} pulled down P-selectin from platelet lysates, whereas it did not show any binding of thrombospondin-1 or multimerin-1, which were previously identified and confirmed as Efb-interacting proteins[25] (Figure 2D). The role of P-selectin in the binding of Efb_{68-87} was confirmed by competitive binding experiments using a recombinant chimeric construct of the physiological P-selectin ligand P-selectin glycoprotein ligand- (PSGL-1) (Figure 2E). In these experiments, the binding of FITC-Efb_{68-87} was competitively inhibited by Fc-PSGL-1 (R&D Systems, # 3345-PS-050), confirming that P-selectin is the binding site for Efb_{68-87} on platelets.

3.2 | Efb_{68-87} selectively binds platelets and captures them from blood under flow

Amongst blood cells, P-selectin is selectively expressed in platelets. Therefore, we assessed whether Efb_{68-87} selectively binds platelets or might interact with other blood cells. Antibodies for platelet-specific (CD42b) and leukocyte-specific (CD45) markers were utilized in flow cytometry to identify platelets and leukocytes (white blood cells (WBCs) and red blood cells (RBCs)) (Figure 3A). FITC-Efb_{68-87} exclusively bound to platelets, while binding of WBCs and RBCs was negligible. We then used an adhesion assay under physiological flow to probe the stability of Efb_{68-87} binding to platelets,

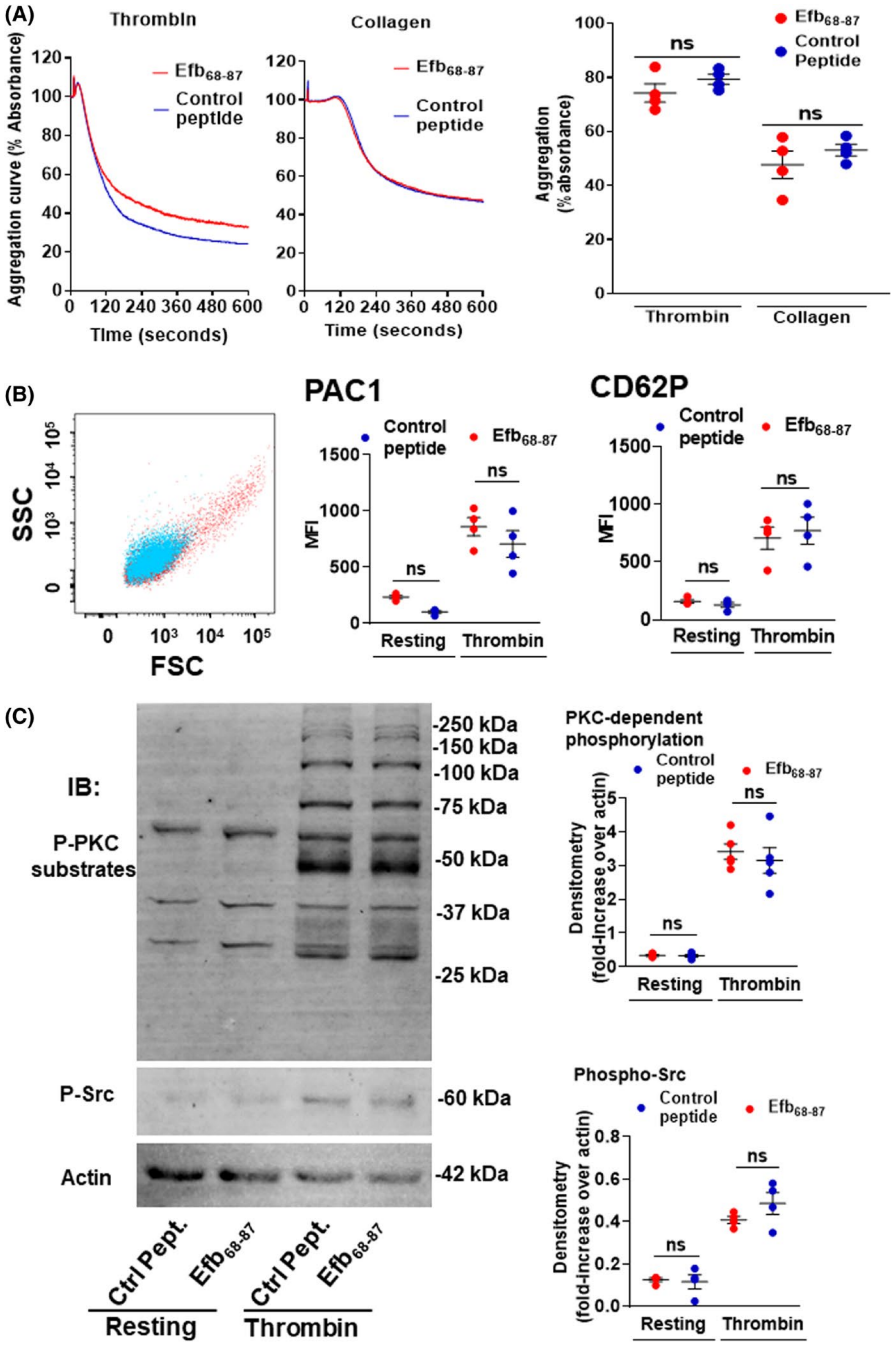

FIGURE 4 Efb_{68-87} does not interfere with the normal functions of platelets. (A) Platelet aggregation induced by thrombin (0.1 U/ml) and collagen (10 μg/ml) was tested in the presence of 5 μM Efb_{68-87} or scrambled Efb_{68-87}. Representative examples of aggregations from four independent experiments are shown and a bar graph shows mean ± SEM. No statistical significance was detected using the paired sample Student *t*-test. (B) Flow cytometry analysis of platelet activation and degranulation. Resting and thrombin-stimulated (1 U/ml) platelets were compared for degranulation (anti-CD42b/APC) and integrin $\alpha_{IIb}\beta_3$ activation (anti-PAC-1/FITC) in the presence of 5 μM Efb_{68-87} or scrambled Efb_{68-87}. A representative forward scattering (FSC)/side scattering (SSC) dot plot of isolated platelets in the presence of Efb_{68-87} at resting (blue) and thrombin-stimulated (red) is also shown. The statistical significance between Efb_{68-87} and the control peptide was assessed using one-way ANOVA with Bonferroni post-test; $p < .01$ (**), $p < .001$ (***), error bars represent mean ± SEM from four independent experiments. (C) Platelet signalling was studied by immunoblotting in the presence of Efb_{68-87} or Efb_{68-87} ctrl peptides (10 μM). Platelets were activated with 0.1 U/ml thrombin (in the presence of 1 mM EGTA to avoid platelet aggregation and so facilitate protein extraction). Following lysis and SDS-PAGE, lysates were immunoblotted for protein kinase C (PKC) phosphorylated substrates of classical protein kinase C (PKC) isoforms, phosphorylated Src and actin. Data were quantified by densitometry using ImageJ 1.47v, presented as intensity ratio over actin staining (i.e. loading control) and are mean ± SEM from five independent experiments. No statistical significance was detected using a paired sample Student *t*-test (normality was assessed by Shapiro-Wilk test)

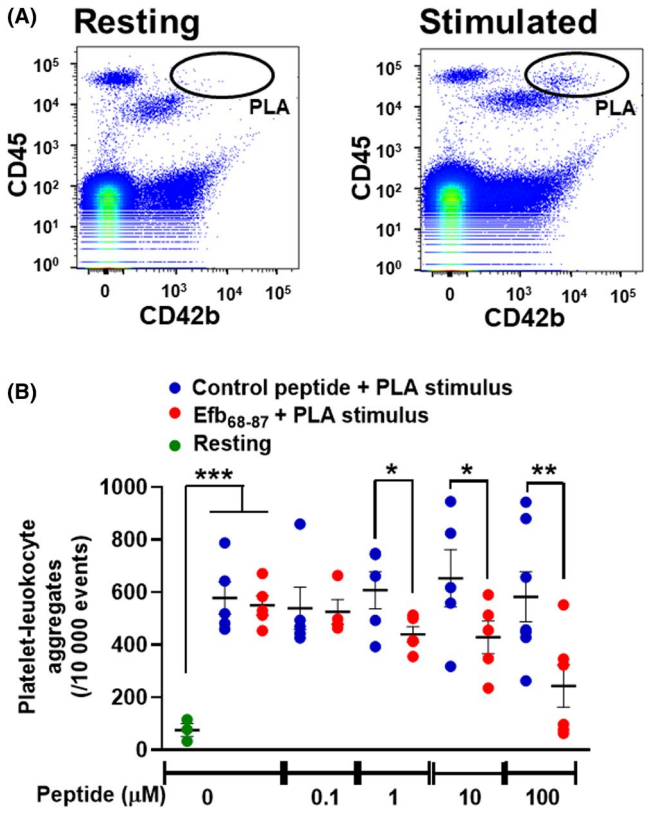

FIGURE 5 Efb_{68-87} inhibits the formation of PLAs in human whole blood. (A) Human blood was stimulated with CRP (1 μg/ml), TRAP6 (5 μM) and LPS (1 μg/ml) for 30 min. Anti-CD42b/PE and anti-CD45/APC antibodies were used to stain platelets and leukocytes. PLAs were quantified in the top right quadrant of the PE/APC plot. (B) Where indicated, 0.1–100 μM Efb_{68-87} (or control peptide) was co-incubated with the agonists. Data are the number of PLA per 10,000 events (i.e. blood cells) and represent mean ± SEM from five independent experiments. Statistical significance was assessed using one-way ANOVA with Bonferroni post-hoc test: $p < .05$ (*), $p < .01$ (**), $p < .001$ (***)

assessing whether Efb_{68-87} can be used to capture platelets from whole blood. Platelets bound to surfaces coated with Efb_{68-87} at venous (200 s^{-1}) (Figure 3B), but not arterial shear stress (1000 s^{-1}) (Figure S2). Interestingly, platelet adhesion and thrombus formation on fibrous collagen were not affected by Efb_{68-87} (Figure 3C), suggesting that Efb_{68-87} does not interfere with platelet function.

3.3 | Efb_{68-87} does not interfere with platelet activation and hemostatic function

We investigated functional implications of Efb_{68-87} for platelet function. Platelet aggregation in response to the agonists collagen and thrombin was not affected by platelet pre-treatment with Efb_{68-87} compared to Efb_{68-87} (ctrl) (Figure 4A). The aggregation in response to secondary agonists ADP and U46619 (a stable analogue of thromboxane A_2) was also unaffected by Efb_{68-87} (Figure 3). In addition, platelet activation and degranulation were tested by flow cytometry. Data showed that the presence of Efb_{68-87} did not significantly change integrin $\alpha_{IIb}\beta_3$ activation (PAC1 antibody) or platelet degranulation (P-selectin externalization) compared to Efb_{68-87} (ctrl) (Figure 4B). Finally, potential effect of Efb_{68-87} incubation on intracellular platelets signaling was tested by immunoblotting (Figure 4C). Basal (resting platelets) and activated (thrombin-stimulated platelets) levels of PKC activity and Src kinase activation in the presence of Efb_{68-87} and Efb_{68-87} (ctrl) were assessed using an anti-phospho PKC substrate antibody (detecting various proteins phosphorylated by classical platelet PKC isoforms α and β) and anti-phospho Src antibody (detecting Src in its active/phosphorylated form). Treatment with Efb_{68-87} did not affect activity of these two major platelet signaling pathways. Collectively, these results confirm that Efb_{68-87} binding does not interfere with platelet function.

3.4 | Efb_{68-87} inhibits formation of platelet-leukocyte aggregates and reduces formation of neutrophil extracellular traps

The formation of heterotypic cellular complexes between platelets and leukocytes (i.e. platelet-leukocyte aggregates or PLAs) is an important driver of thromboinflammation. Since PLA formation depends on binding of platelet P-selectin to leukocyte PSGL-1, we tested whether Efb_{68-87} can block the formation of PLAs in human whole blood. PLAs were detected by flow cytometry as an event highly stained by both anti-CD42b and anti-CD45 antibodies (Figure 5A). PLA levels were significantly increased by stimulation of whole blood with the GPVI receptor agonist CRP, but not the PAR1 agonist TRAP6 or the Toll-like receptor (TLR) agonist LPS used separately (Figure S4). The study of Efb_{68-87}'s effect on PLA formation was performed with combined stimulation by CRP, TRAP6 and LPS. Substantial inhibition of PLA formation was observed at 1 μM or higher concentrations of Efb_{68-87} (Figure 5B). Since NET formation is another process dependent on P-selectin binding to PSGL-1,[24] we next investigated whether Efb_{68-87} interferes with the stimulation of NET formation by platelets. Neutrophils isolated from human peripheral blood were allowed to adhere and were then treated with platelets. Resting platelets were compared to platelets treated with TRAP6 (5 μM). Stimulated platelets were able to induce a significant formation of NETs (Figure 6), which was inhibited by Efb_{68-87}, but not with the control peptide for Efb_{68-87}. The incubation of neutrophils with the agonist in the absence of platelets did not lead to formation of NETs. Direct stimulation of NETs by neutrophil treatment with PMA was used as positive control, which was not affected by Efb_{68-87} (data not shown).

4 | DISCUSSION

Discovery of their involvement in inflammatory responses and vascular degeneration has added an important new chapter in our understanding of the role of platelets in health and disease. In addition to the

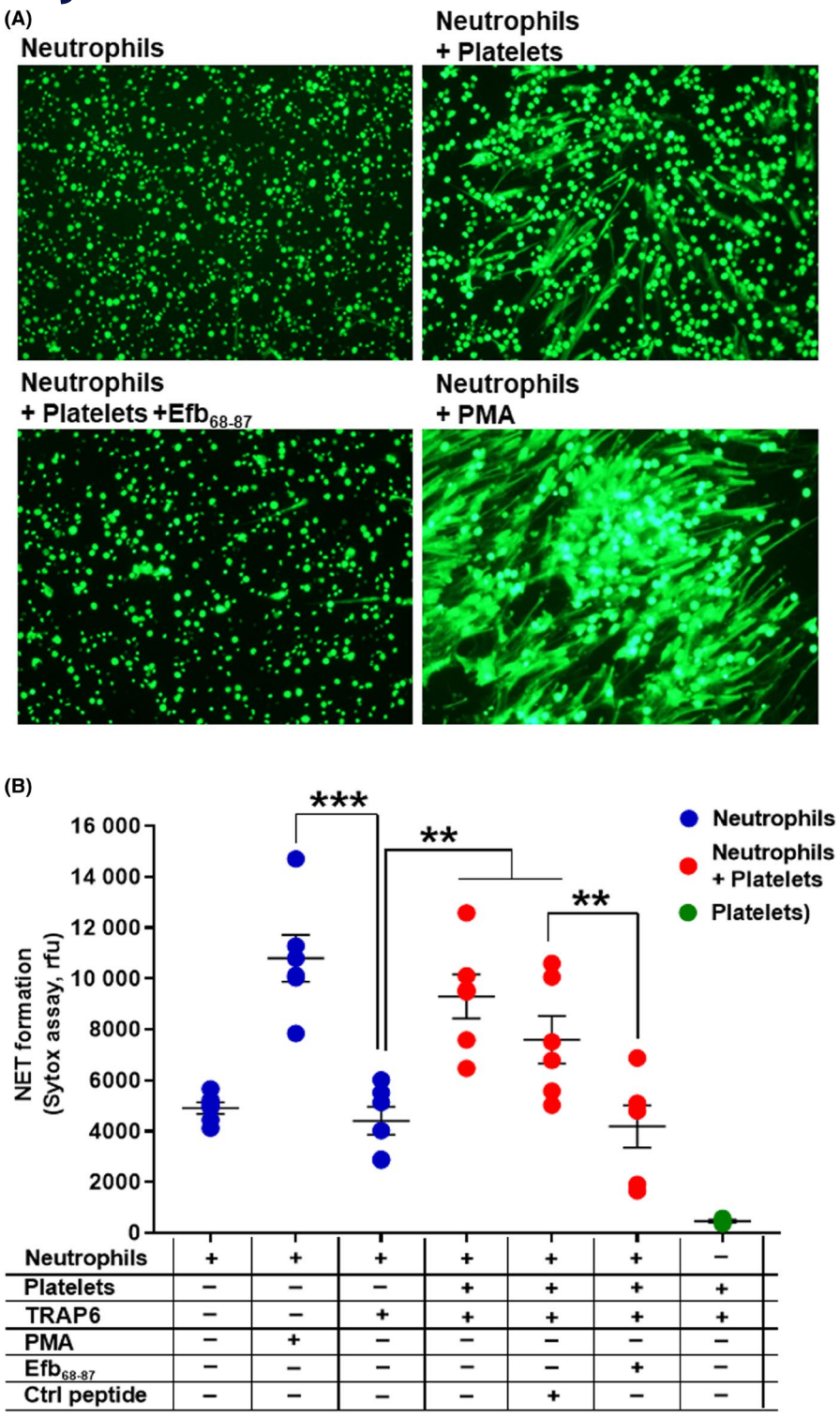

FIGURE 6 Efb_{68-87} inhibits the formation of platelet-induced NETs. 5×10^4/well neutrophils were seeded into 96-well plates and incubated at 37°C for 1 h. 5 μM Efb_{68-87}, scrambled control peptide and vehicle solution were added to platelets before stimulation with TRAP6 (5 M). 1×10^6 platelets were added to each well. Negative control wells were prepared by adding either only neutrophils or only platelets with TRAP6 (5 M). The positive control wells are neutrophils treated with 0.1 μM PMA. The plates were incubated for 16 h at 37°C with 5% CO_2, before fixation in PFA and staining of NETs with Sytox Green. (A) Representative images of selected conditions from five independent experiments were obtained by fluorescence microscopy and are shown in the top panels. (B) The quantification of NET release was obtained by staining of the cell supernatants by fluorescence microplate reading after addition of Sytox green. Data are the mean ± SEM from five independent experiments. Statistical significance was assessed using one-way ANOVA with Bonferroni post-hoc test: $p < .05$ (*), $p < .01$ (**), $p < .001$ (***)

release of pro-inflammatory cytokines,[35] the function of platelets in inflammatory processes involves the formation of PLAs, which mediate homing and extravasation of leukocytes at the site of vascular damage.[6,36] The interaction of P-selectin with PSGL-1 is the key molecular event for PLA formation.[4] Other key surface receptors involved in PLA formation are activated in response to P-selectin-PSGL-1 interaction. The activation of the leukocyte integrin $\alpha_M\beta2$ (macrophage-1 antigen or MAC-1) by P-selectin-PSGL-1 interaction, for example, is a key step in the formation of heterotypic complexes between platelets and leukocytes.[37] In addition to linking inflammation with platelet activation and thrombus formation, and supporting the concept of thromboinflammation,[38] the formation of PLAs has far-reaching consequences for cardiovascular health. Formation of heterotypic complexes between platelets and monocytes regulates the coagulation cascade through the upregulation of tissue factor,[39] which exacerbates the thrombotic risk for patients with a variety of conditions, including COVID-19.[40]

The P-selectin/PSGL-1 axis is therefore an attractive target for the development of novel anti-inflammatory and vasculoprotective drugs. Although some reduction of PLAs can be achieved by traditional antiplatelet therapy,[41] it is important to find strategies for achieving more comprehensive abatement of PLAs and avoiding the bleeding risk associated with existing antithrombotics. This makes the development of a safe and efficacious P-selectin antagonist an attractive therapeutic option in cardiovascular medicine. Although several P-selectin antagonists have been described and proposed as candidates for drug development, there is currently no P-selectin inhibitor validated for clinical use. Recombinantly expressed vimentin,[42] synthetic glycomimetics of the N-terminus of PSGL-1[43] and anti-P-selectin antibodies[44] have been characterized for their ability to compete with PLA formation *in vitro* and in vivo, while a promising small molecule failed to show any effect in vivo.[45] The cost and pharmacokinetic properties of full recombinant proteins, large post-translationally modified peptides or antibodies make the search for novel candidate antagonists for P-selectin a relevant, timely and unresolved challenge for vascular drug discovery. In this study, we therefore followed a different approach. Based on our previous work showing that the bacterial protein Efb binds directly to P-selectin, inhibits its interaction with PSGL-1 and interferes with PLA formation,[25] we set out to identify an Efb-derived peptide that retains the ability to bind P-selectin and inhibit PLA formation.

The peptide we identified is Efb_{68-87}, a twenty amino acid sequence from the N-terminal domain of Efb and part of one of two previously identified Efb binding sites for fibrinogen.[30] Efb_{68-87} binding to platelets is not increased by the addition of exogenous fibrinogen and is therefore fibrinogen-independent (Figure 1D). On the other hand, Efb_{30-67} interacts with platelets only in the presence of exogenous fibrinogen. Efb_{30-67} corresponds to the other previously identified fibrinogen binding site.[30] Interestingly, Efb_{68-87} shows similar binding affinity to resting and stimulated platelets, but the Efb_{68-87} binding capacity of platelets (i.e. amount of peptide bound per platelet) is significantly increased by stimulation (Figure 1B,C). This binding profile is compatible with a binding site that is present at a low level on the surface of resting platelets but that undergoes an activation-dependent increase in level. This is characteristic of P-selectin levels which increase on the surface of platelets as a consequence of stimulation-dependent degranulation (i.e. migration to the cell periphery and fusion with the plasma membrane of P-selectin-rich alpha granules).[46] These data were supported by dot blot experiments, which confirmed direct binding of Efb_{68-87} to P-selectin (Figure 2C). Efb_{68-99} was also able to bind to P-selectin in the dot blot experiments, although the level of staining was visibly lower. Further experiments would be required to assess whether the residues between Tyr88 and Ala99 may interfere with the binding of P-selectin. It is also noteworthy that contrarily to Efb_{30-105} (also known as Efb-N),[25] Efb_{68-87} does not interact with multimerin-1 and thrombospondin-1. Since the interaction of Efb-N with multimerin-1 and thrombospondin-1 was detected with the same approach used in this study (i.e. peptide conjugation and platelet protein pull-down), the most likely explanation is that the binding sites for these proteins map outside the Lys68-Glu87 portion of Efb-N. This observation emphasizes the modular and multifunctional nature of Efb, with separate or overlapping binding sites for multiple target proteins. The modular multifunctionality of Efb could facilitate further biotechnological applications based on the use of this protein.

Efb_{68-87} represents a promising candidate for the development of a novel pharmacological agent with anti-inflammatory or antithrombotic properties based on antagonism of P-selectin and inhibition of PLA formation. This approach would have the advantage of leaving unaffected the hemostatic function of platelets, which should reduce or abolish any bleeding side effects. The use of current antiplatelet drugs from acetylsalicylic acid (ASA) to P2Y12 antagonists (such as Clopidogrel, Prasugrel and Ticagrelor) to integrin inhibitors (such as Abciximab or Tirofiban) is associated with an increase in hemorrhagic risk for patients.[47,48] This is likely to be the consequence of targeting a molecular pathway normally required for hemostasis (i.e. cyclooxygenases, ADP receptors or integrins). By targeting a surface adhesion molecule such as P-selectin that is not involved in healthy hemostatic responses, Efb_{68-87} would selectively diminish or abolish the contribution of platelets to the onset and/or progression of thromboinflammation, with little or no risk of causing hemorrhagic complications.

We demonstrated in addition that Efb_{68-87} does not bind to other blood cells (Figure 3A) and that Efb_{68-87} does not interfere with the activation and function of platelets in response to physiological stimuli (Figure 4). Efb_{68-87} can therefore be used as a tool to selectively label platelets without affecting their responsiveness, which could find application in research and diagnostic laboratory practice. The ability to sequester platelets from whole blood (Figure 3B) could be investigated further to develop a novel platelet isolation method. Since Efb_{68-87} displayed increased binding to activated platelets (Figure 2B), it may be possible to develop a method to selectively capture activated platelets from whole blood. The selective reduction of the count of circulating activated platelets could find clinical application because an increase in circulating activated platelets is observed in several cardiovascular diseases and is proposed as a disease mechanism leading to thrombosis.[49,50]

In summary, we present here the identification and validation of Efb_{68-87}, a novel P-selectin antagonist derived from the N-terminal domain of the *S. aureus* protein Efb. In addition to confirming the selective binding to P-selectin, we have established the ability of Efb_{68-87} to interfere with PLA and platelet-induced NET formation in vitro without affecting platelet signaling and platelet functional responses. As the formation of PLAs and NETs is critical for the progression of vascular inflammation and its association with thrombotic complications, Efb_{68-87} has the potential to become a clinical tool for the treatment of conditions ranging from major blood vessel atherosclerosis to microcirculatory dysfunction. Future clinical studies on this peptide may lead to the development of a novel treatment to help in the battle against thromboinflammatory conditions.

ACKNOWLEDGEMENTS

The authors thank the DFG-funded Cytometry and Cell Sorting Core Unit at the UKE hospital (Hamburg). This work was funded by the

British Heart Foundation for the financial support of SW, SB and GP (FS/17/13/3269), the Werner Otto Foundation for the financial support of NW and GP (BN3/97) and the European Research Council for the support of GP (EU project 101025074). Open access funding enabled and organized by ProjektDEAL.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

AUTHOR CONTRIBUTIONS

SW, NW, HE, MP and AU performed experiments and part of the data analysis for this manuscript (the order reflects the level of involvement in this project). TR, IE and SB provided critical revisions of the manuscript. SB and GP designed the project and planned the experiments. GP wrote the manuscript.

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SUPPORTING INFORMATION

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How to cite this article: Wallis S, Wolska N, Englert H, et al. A peptide from the staphylococcal protein Efb binds P-selectin and inhibits the interaction of platelets with leukocytes. *J Thromb Haemost*. 2021;00:1–13. doi:10.1111/jth.15613