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BRIEF REPORT

Platelet glycoprotein VI cluster size is related to thrombus formation and phosphatidylserine exposure in collagen-adherent platelets under arterial shear

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Abstract

Background: Collagen-induced platelet activation is predominantly mediated by glycoprotein (GP) VI through formation of receptor clusters that coincide with the accumulation of signaling molecules and are hypothesized to drive strong and sustained platelet activation.

Objectives: To determine the importance of GPVI clusters for thrombus formation in whole blood under shear.

Methods: We utilized whole blood microfluidics and an anti-GPVI nanobody (Nb), Nb28, labeled with AlexaFluor 488, to assess the distribution of GPVI on the surface of platelets adhering to a range of collagen-like substrates with different platelet activation potentials.

Results: Automated analysis of GPVI surface distribution on platelets supported the hypothesis that there is a relationship between GPVI cluster formation, thrombus size, and phosphatidylserine (PS) exposure. Substrates that supported the formation of macroclusters also induced significantly bigger aggregates, with increased amounts of PS-exposing platelets in comparison to substrates where no GPVI clusters were detected. Furthermore, we demonstrate that only direct inhibition of GPVI binding, but not of downstream signaling, is able to disrupt cluster formation.

Conclusion: Labeled anti-GPVI Nb28 permits visualization of GPVI clustering under flow conditions. Furthermore, whilst inhibition of downstream signaling does not affect clustering, it does prevent thrombus formation. Therefore, GPVI macroclustering is a prerequisite for thrombus formation and platelet activation, namely, PS exposure, on highly GPVI-dependent collagen surfaces.

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Johan W. M. Heemskerk and Natalie S. Poulter contributed equally to this study and are Joint senior authors.

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KEYWORDS
glycoprotein VI, nanobody, platelet activation, receptor clustering, thrombus formation

1 | INTRODUCTION

Collagen-mediated platelet activation is dependent on 2 main receptors: glycoprotein (GP) VI, the major signaling receptor for collagen on platelets [1] and integrin α2β1, which mediates adhesion [2]. GPVI is associated with the immunoreceptor tyrosine-based activation motif (ITAM)–containing Fc receptor γ chain (FcRγ) at the platelet surface. Upon GPVI ligand binding, phosphorylation of the ITAM in FcRγ induces a signaling cascade involving phosphorylation of several downstream proteins including the tyrosine kinase Syk. This results in Ca^{2+} mobilization, cytoskeletal reorganization, granule secretion, and thrombus formation [3,4]. Strong, sustained signaling induces the exposure of phosphatidylserine (PS) on the platelet surface, increasing their procoagulant activity, which is linked to GPVI activation [5]. In spreading platelets, GPVI receptors undergo varying levels of clustering depending on the type of collagenous substrate, with the large and multi-ligand collagen-I fibers inducing higher levels of clustering [6,7]. These large "macro" GPVI clusters can be seen using diffraction-limited microscopy and are enriched in signaling molecules [7,8]. This suggests that the clustering of platelet GPVI is a prerequisite for sustained signaling in thrombus formation and thrombus stability under flow. However, this has not previously been directly demonstrated in a flow adhesion assay in which the extent of GPVI clustering can be controlled by the substrate used and a relationship between cluster size and platelet activation can be determined.

GPVI recognizes and binds to consecutive glycine-proline-hydroxyproline (GPO) motifs, which induce receptor engagement [9]. To investigate the GPVI-collagen interaction, GPO-enriched synthetic triple-helical collagen-like peptides have been developed [10]. Repeated GPO sequences induce a strong GPVI-dependent platelet activation. Integrin α2β1 can bind to glycine-phenylalanine-hydroxyproline-glycine-glutamicacid-arginine (GFOGER) sequences. Accordingly, GFOGER-GPO peptides activate platelets by engagement of both collagen receptors, while the GFOGER–glycine-proline-proline (GPP) peptides, in which hydroxyproline has been replaced by proline, only act via integrin α2β1 [11,12]. For a shear-dependent platelet interaction with collagen, von Willebrand factor (VWF) needs to be engaged. Therefore, a synthetic VWF-binding peptide (VWF-BP) is required in flow experiments involving collagen peptides, as they lack the natural VWF-binding sites [11,12].

We recently described the generation of camelid nanobodies (Nbs) against GPVI [13] and showed that Nb28 binds to GPVI without affecting collagen binding and can be used to visualize the clustering of GPVI under flow [14]. Therefore, we used whole blood microfluidics and AlexaFluor (AF)-488–labeled Nb28 to assess the surface distribution of GPVI on platelets adhering, under shear, to a range of collagen-like substrates with different platelet activation potentials [15] to mimic the first steps in thrombus formation. Our data support a relationship between GPVI cluster formation, thrombus size, and PS exposure. Furthermore, inhibition of GPVI binding, but not downstream signaling, is able to disrupt receptor clustering.

Essentials

- Fluorescently labeled Nb28 is a useful tool to investigate glycoprotein (GP) VI clusters in flow adhesion assays.
- GPVI macroclusters are only observed on surfaces with high platelet activation potential.
- Macroclusters coincided with increased thrombus formation and heightened procoagulant activity.
- GPVI clustering was disrupted by direct receptor inhibition, but not by inhibition of signaling.

2 | METHODS

2.1 | Reagents

Nbs were raised against the extracellular domain of GPVI by VIB Nanobody Core (VIB Nanobody Service Facility, Belgium) and expressed as described [13]. Collagen-related triple-helical peptides H-GPC$(GPP)_5$GFOGER$(GPP)_5$GPC-NH_2(GFOGER-GPP) and GPC$(GPO)_3$GFOGER$(GPO)_3$GPC-NH_2(GFOGER-GPO) and VWF-binding peptide H-GPC$(GPP)_5$GPRGQOGVMGFO$(GPP)_5$GPC-NH_2(VWF-BP) were from CambCol Laboratories [16]. Fibrillar collagen-I (Horm) was from Nycomed, human placenta-derived collagen-III (1230-01S) from Southern Biotechnology, PRT-060318 (PRT-318) from Bio-Connect, and EHT-1864 from AdooQ. 6F1 monoclonal antibody (mAb) was a gift from Barry Coller (Rockefeller University.

2.2 | Nb28 AF488 labeling

Nb28 was labeled with AF488 NHS-ester (ThermoFisher Scientific) at 1:40, (dye:protein) following manufacturer's instructions. Free dye was removed using a Pierce Dye Removal Column.

2.3 | Whole blood microfluidics

Blood was collected into 4% sodium citrate from drug-free healthy volunteers, after obtaining informed consent, in accordance with the Declaration of Helsinki and local ethical approval.

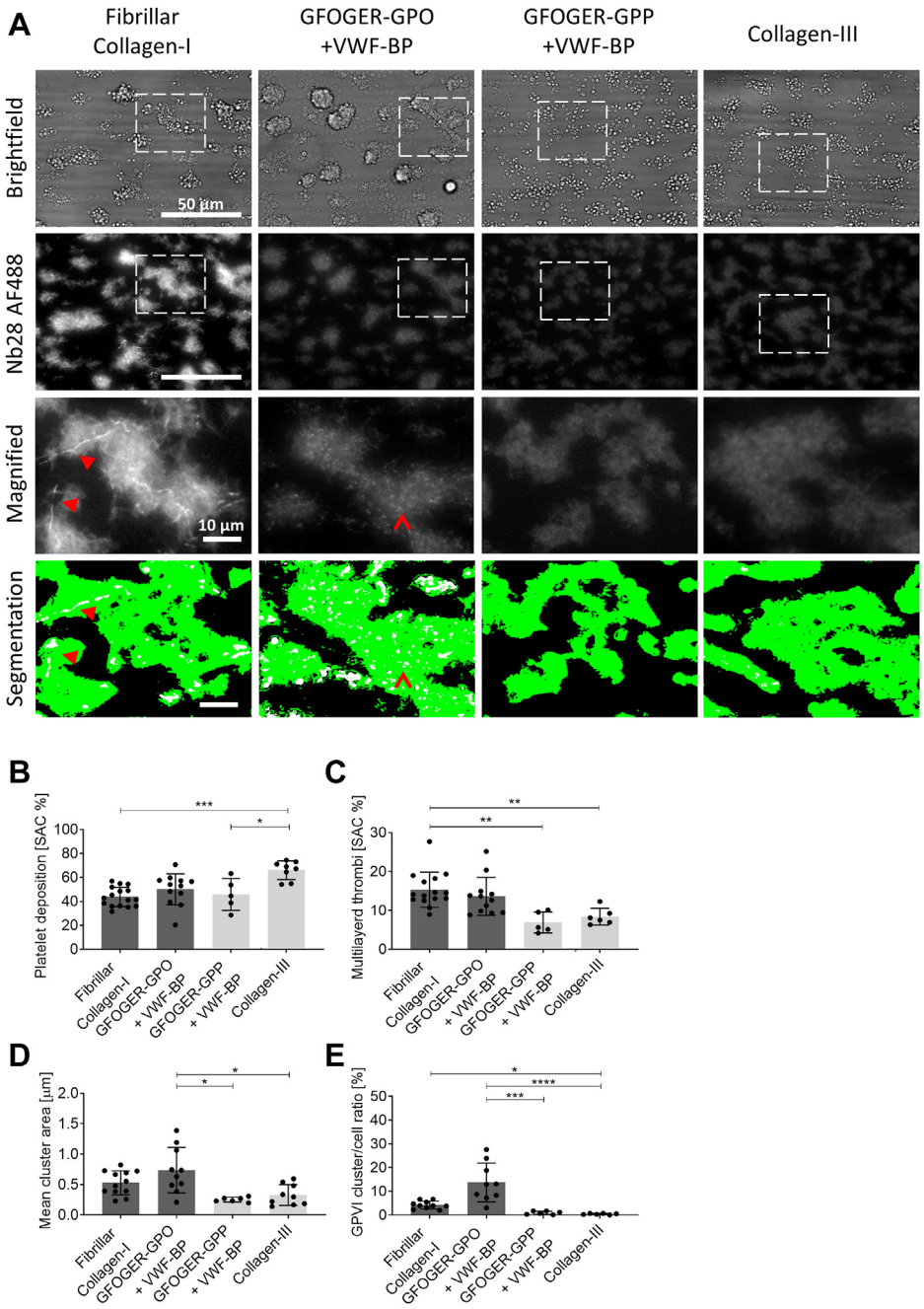

FIGURE 1 Nb28-AF488 assesses platelet GPVI cluster formation on collagenous substrates. Recalcified and thrombin-inhibited whole blood supplemented with 100 nM Nb28-AF488 perfused over fibrillar collagen-I, GFOGER-GPO + VWF-BP, GFOGER-GPP + VWF-BP, or collagen-III at arterial shear rates (1000/s). (A) Representative images of formed aggregates and GPVI clusters at focal plane of the substrates as well as the machine learning–based segmentation, where green indicates GPVI on platelet aggregates and white indicates segmented GPVI clusters. White box indicates area magnified in images below. Quantification of surface area covered by (B) platelets and (C) thrombi and (D) mean GPVI cluster size and (E) pixel ratio between GPVI in clusters and GPVI on platelets is shown. Red arrowheads indicate GPVI clusters (n = 5-16). All segmentation and quantitation was carried out on the raw images. Intensity of representative images in (A) was adjusted to aid visualization of receptor distribution. Scale bar = 50 µm in full image and 10 µm in magnified image. Mean ± SD. One-way analysis of variance, $^{*}p < .05$, $^{**}p < .005$, and $^{***}p < .0005$. AF, AlexaFluor; GFOGER, glycine-phenylalanine-hydroxyproline-glycine-glutamicacid-arginine; GP, glycoprotein; GPO, glycine-proline-hydroxyproline; GPP, glycine-proline-proline; Nb, nanobody; SAC, surface area coverage; VWF-BP, von Willebrand factor–binding peptide.

Glass coverslips were coated overnight at 4 °C with microspots containing 100 µg/mL of collagen (fibrillar collagen-I or collagen-III) or 250 µg/mL of peptides (GFOGER-GPO or GFOGER-GPP) + 100 µg/mL of VWF-BP. Whole blood was preincubated (10 minutes) with 500 nM Nb21, 20 µM PRT-318, 20 µg/mL 6F1 mAb, or 100 µM EHT-1864 before addition of 40 µM PPACK ($_{D}$-Phenylalanyl-$_{L}$-prolyl-$_{L}$-arginine chloromethyl ketone), 3.75 mM magnesium chloride, 7.5 mM calcium chloride, and 100 nM Nb28-AF488 and then perfused at a shear rate of 1000/s over the microspots in a parallel flow chamber [16,17]. After 3.5 minutes, 2 brightfield images were taken while flowing with label buffer [15] to stain for PS exposure (AF568-annexin A5, ThermoFisher) and CD62P expression (AF647 anti-CD62P mAb, BioLegend). Three fluorescence images were taken after perfusion of rinse buffer; all images were quantified for surface area coverage by semi-automated ImageJ scripts [17]. Images were taken on an EVOS-FL microscope (ThermoFisher) using an Olympus UPLSAPO 60× oil immersion objective [17].

2.4 | GPVI cluster quantification

A random forest-based pixel classifier was trained within Ilastik (1.3.3post3) [18] to split the collected GPVI fluorescence images into 3 classes: background (no platelets), platelets (diffuse GPVI signal), and receptor clusters (brighter, localized GPVI signal). Training images, selected from across conditions and replicates, were annotated within

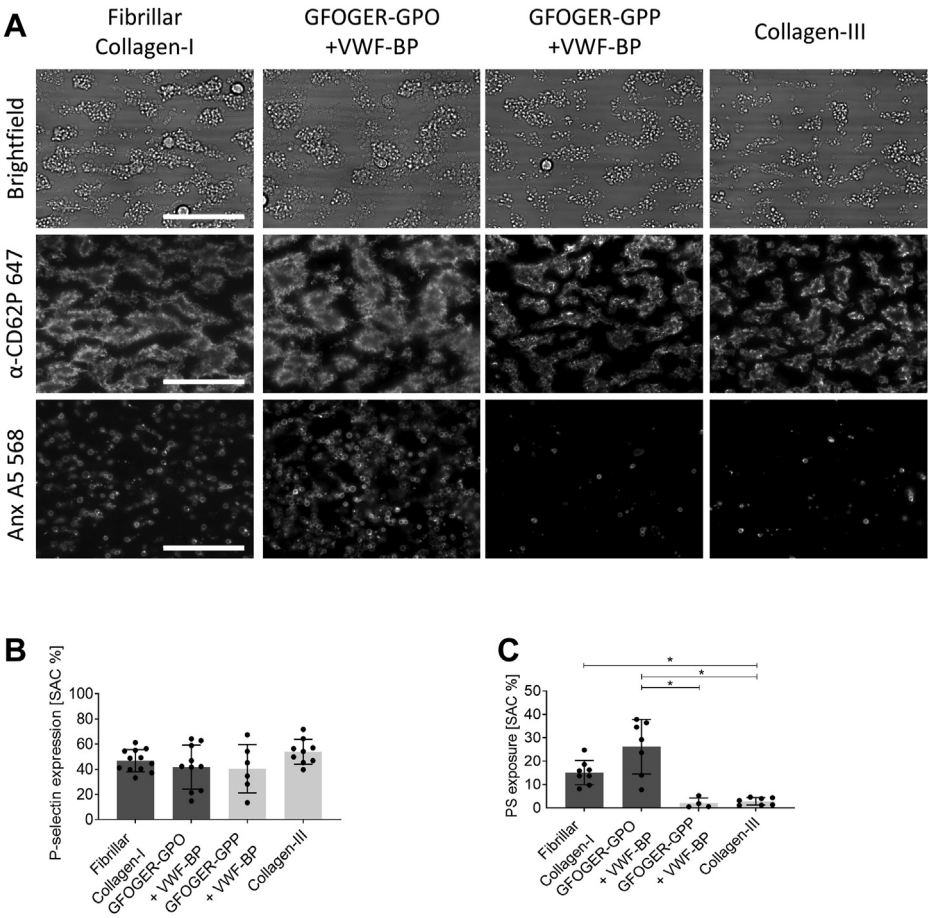

FIGURE 2 Relative presence of GPVI clusters correlates with number of PS-exposing platelets. Recalcified and thrombin-inhibited whole blood supplemented with Nb28-AF488 was perfused over indicated surfaces at 1000/s (Figure 1) and stained with AF647 anti-CD62P mAb (P-selectin) and AF-568 annexin A5 (PS exposure) at endpoint. (A) Representative images of thrombi and fluorescent staining. Quantification of surface area covered by *P*-selectin–positive (B) and PS-exposing platelets (C). Scale bar = 50 μm. Mean ± SD (n = 4-9). One-way analysis of variance, $^{*}p <$ .05. AF, AlexaFluor; Anx, annexin; GFOGER, glycine-phenylalanine-hydroxyproline-glycine-glutamicacid-arginine; GP, glycoprotein; GPO, glycine-proline-hydroxyproline; GPP, glycine-proline-proline; mAb, monoclonal antibody; Nb, nanobody; PS, phosphatidylserine; SAC, surface area coverage; VWF-BP, von Willebrand factor–binding peptide.

the Ilastik user interface. The classifier was then run on the full data set with a Fiji macro [19], which extracted the cluster and cell area parameters. For the calculation of mean cluster area, a minimum cluster size of 5 pixels was set. The macro is available at https://github.com/JeremyPike/ilastik-batch-clustering.

2.5 | Statistical analysis

Data are presented as mean ± SD. Statistical analyses were performed in GraphPad Prism V7 and are described in figure legends.

3 | RESULTS AND DISCUSSION

Nbs, the variable region of camelid antibodies, are target-specific antibody fragments. Their small size (12-15 kDa) and high affinity render them excellent imaging tools [20]. To assess platelet GPVI clustering in thrombus formation, whole blood was preincubated with AF488-labeled Nb28 and perfused over 2 substrates with high GPVI activation potential (fibrillar collagen-I and collagen-like peptide GFOGER-GPO) [15] and over 2 substrates with low GPVI activation potential (GFOGER-GPP and collagen-III) [15] (Figure 1). Fluorescence images of GPVI taken at the platelet-substrate focal plane revealed high-intensity labeling, representing focused GPVI clusters, that was only visible on the high activation substrates (white in segmentation images), with more diffuse labeling patterns (green in segmentation images) seen in the low GPVI activation substrates (Figure 1A).

All 4 substrates supported platelet adhesion and, to varying degrees, thrombus formation. Quantification of platelet deposition in brightfield images revealed no difference between fibrillar collagen-I, GFOGER-GPO, and GFOGER-GPP. There was increased platelet deposition on human collagen-III compared with fibrillar collagen-I and GFOGER-GPP (Figure 1B), but the thrombi formed on collagen-III were significantly less multilayered than those on collagen-I. Formation of multilayered thrombi was also significantly higher on fibrillar collagen-I than on integrin $\alpha 2\beta 1$-binding GFOGER-GPP peptide (Figure 1C). Although quantitation of the thrombi formed on GFOGER-GPO did not detect statistically significant differences in platelet deposition or multilayer compared with other collagens, the thrombi looked morphologically different in brightfield images, appearing tightly contracted (Figure 1A).

In immune cells, receptor clustering is crucial for generating strong and sustained signals [21,22]. Previous single-molecule super-resolution microscopy of platelets spreading on different collagenous substrates demonstrated that GPVI microclusters formed on all substrates tested, including collagen-III, but there are differences in the sizes of these microclusters depending on the collagen type [6].

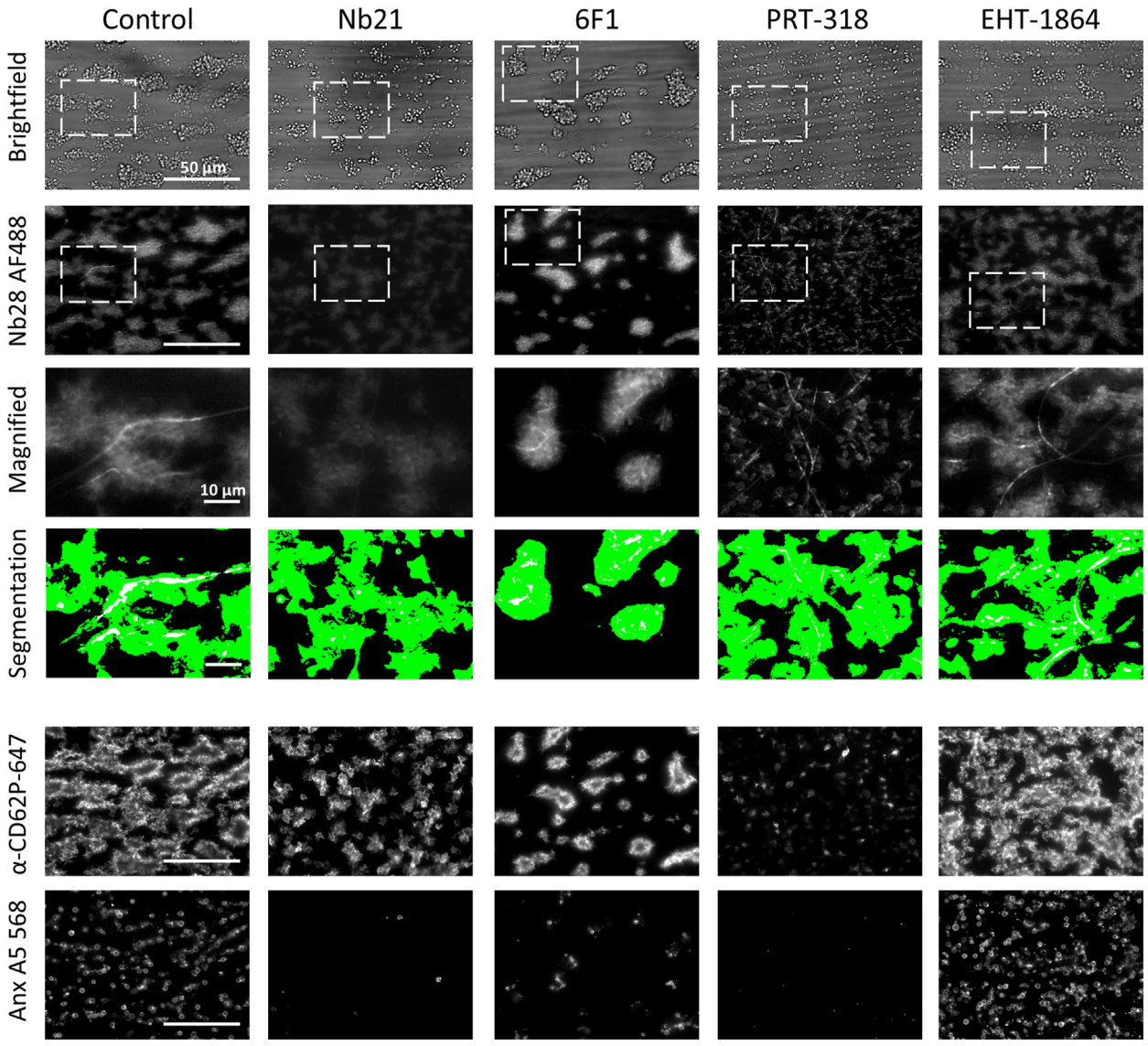

FIGURE 3 Inhibitor effects on parameters of thrombus formation on fibrillar collagen-I under flow. Recalcified and thrombin-inhibited whole blood supplemented with 100 nM Nb28-AF488 was perfused over fibrillar collagen-I in the presence of vehicle control medium, 500 nM Nb21 (GPVI receptor inhibition), 20 µg/mL of 6F1 (α2β1 inhibition), 20 µM PRT-060318 (Syk inhibition), or 500 µM EHT-1864 (Rac-1 inhibition). Representative images of (inhibited) thrombus formation and GPVI clusters, with magnified areas (white box) showing GPVI localization and the machine learning–based segmentation of platelets (green) and GPVI clusters (white). All segmentation and quantitation were carried out on the raw images. Intensity of representative GPVI images was adjusted to aid visualization of receptor distribution. Platelet activation was assessed by α-granule secretion measured by an antibody against P-selectin (α-CD62P), and PS exposure was detected by Annexin-V binding. Scale bar = 50 µm in full image and 10 µm in magnified image. *n* = 4 to 8. AF, AlexaFluor; Anx, annexin; GP, glycoprotein; Nb, nanobody; PS, phosphatidylserine.

However, it is the macroclusters, visible under diffraction-limited microscopy, that colocalize with the phosphorylated Syk proteins and, therefore, appear to be the hubs of sustained GPVI signaling [7]. Fibrillar collagen-I (Horm) forms large, visible fibers, whereas collagen-III, whilst fibrillar in nature, does not, suggesting that there are more GPVI binding sites in close proximity in our collagen-I. Indeed, Nb28-AF488 labeling of GPVI in flow over fibrillar collagen-I showed that macroclusters of GPVI formed and aligned with the visible collagen fibers (Figure 1A, closed arrowhead). The GFOGER-GPO peptide also induced visible, punctate GPVI macroclusters (Figure 1A, open arrowhead), whereas GFOGER-GPP and collagen-III did not.

GPVI clusters were segmented from the images using the machine learning–based, pixel classification software Ilastik [18], allowing automatic quantification of cluster parameters (Figure 1D, E). We observed increased values for mean cluster area and the proportion of the GPVI found in clusters (GPVI cluster:GPVI on platelet ratio) on the highly GPVI-dependent substrates. The GPVI cluster size and proportion in clusters was significantly larger for GFOGER-GPO than for

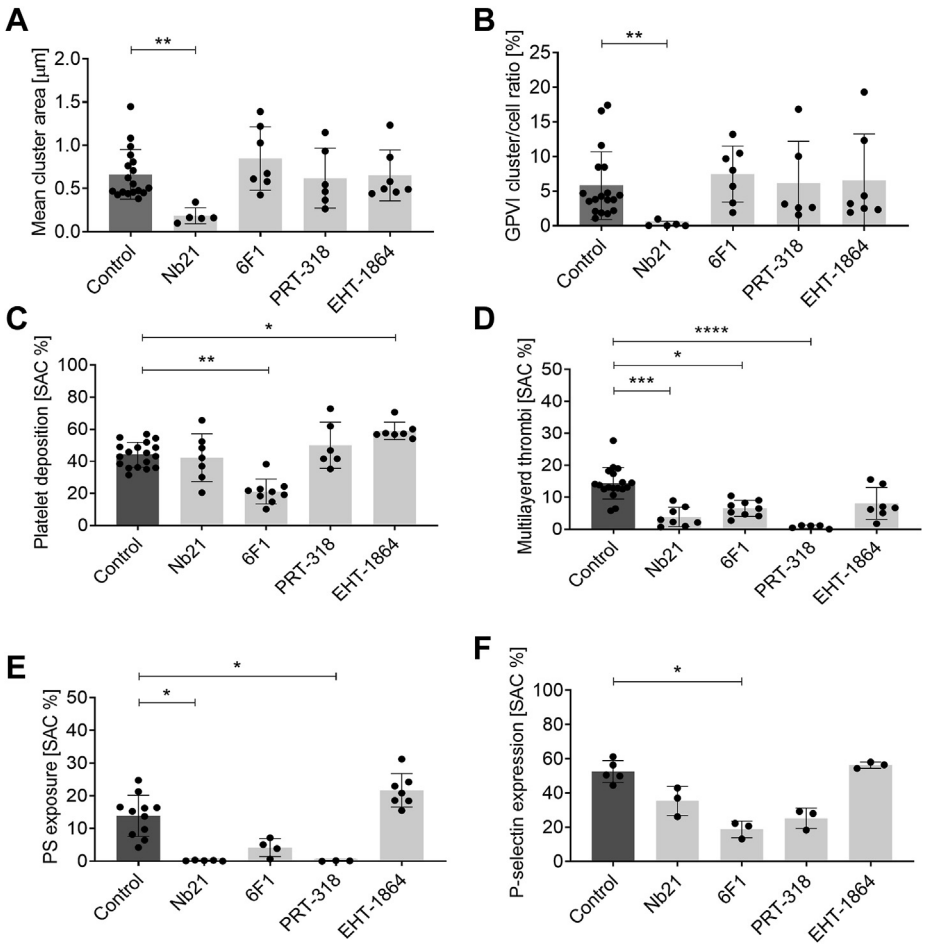

FIGURE 4 Inhibitor effects on GPVI clustering after blood perfusion over fibrillar collagen-I. Quantification of data presented in Figure 3, namely, quantification of GPVI cluster size (A) and GPVI cluster/platelet ratio (B). The surface area covered by platelets (C) and aggregates (D), platelet activation marker P-selectin (E), or PS exposure (F). Mean ± SD (n = 3-11). One-way analysis of variance, $^{*}p < .05$, $^{**}p < .005$, $^{***}p < .0005$. GP, glycoprotein; Nb, nanobody; PS, phosphatidylserine; SAC, surface area coverage.

GFOGER-GPP and collagen-III, whilst the proportion of receptor in clusters in platelets on fibrillar collagen-I was significantly greater than that in platelets adhering to collagen-III (Figure 1E). These results indicate there is a positive relationship between GPVI cluster size and the formation of larger platelet aggregates.

In addition to multilayered thrombus formation, microfluidic assays allow measurement of platelet activation by fluorescent labeling of P-selectin surface expression [16,17] (Figure 2A). Quantitation of the surface area covered by P-selectin demonstrated no difference in expression (Figure 2B), indicating that aggregate-forming platelets are active enough to secrete their α-granules, regardless of the level of GPVI activation mediated by the substrate. Platelet PS exposure is a well-established readout of overall GPVI activity [5,23], and we found significantly higher PS exposure on fibrillar collagen-I and GFOGER-GPO than that on collagen-III (Figure 2C). This indicates that on substrates with high GPVI activity (fibrillar collagen-I and GFOGER-GPO), GPVI forms large clusters and platelets exhibit a higher overall activation state under flow, as observed by the increased thrombus size and PS exposure. In contrast, no GPVI macroclusters are detected in platelets adhering to substrates with low GPVI activity (GFOGER-GPP and collagen-III), the thrombi are also less multilayered, and platelets are not so strongly activated.

To confirm this relationship between GPVI clustering and overall platelet activation in whole blood flow, we studied the effects of inhibitors. We utilized the inhibitory anti-GPVI Nb, Nb21 [13]; the inhibitory anti-α2β1 mAb 6F1 [24]; and small molecule drugs targeting downstream molecules, Syk kinase inhibitor PRT-318 [15] and EHT-1864, which inhibit the small GTPase Rac-1. Rac1 affects phospholipase Cγ2 activation, platelet spreading on collagen, and platelet aggregate stability under flow [25–28]. Representative images of the effects of these treatments are presented in Figure 3 with quantitation in Figure 4.

GPVI clustering was only disrupted by interfering with the GPVI-collagen interaction using Nb21 (Figure 4A, B). Whilst platelet deposition was unaffected (Figure 4C), as shown previously [13], Nb21 caused a substantial and significant decrease in thrombus size (Figure 4D) and PS exposure (Figure 4E) but did not affect P-selectin expression (Figure 4F). Blockade of the other platelet collagen receptor, integrin α2β1, did not affect GPVI clustering or PS exposure but decreased platelet deposition and multilayer thrombus formation, confirming previous data showing a role of integrin α2β1 in platelet adhesion to fibrillar collagen [29,30]. In addition, P-selectin expression was only reduced when integrin α2β1 was blocked, as shown before [14], likely due to the decreased platelet deposition.

Pallini et al. [7] showed that accumulation of signaling molecules is associated with GPVI clusters. Therefore, we inhibited downstream proteins Syk and Rac1 and assessed the effect. Neither intervention affected GPVI clustering (Figure 4A,B), but inhibition of Syk almost completely abolished the formation of aggregates (Figure 4D). These findings support previous data showing a significant role of Syk in thrombus formation [15]. Moreover, GPVI clustering was maintained in platelets adhered to collagen under flow, which has also been observed in PRT-318–treated spread platelets [6,7]. However, it inhibited GPVI-mediated PS exposure (Figure 4F). Thus, PRT-318, by inhibiting downstream Syk activity, has uncoupled GPVI clustering from thrombus formation and PS exposure. Conversely, Rac1 inhibition led to a significant increase in platelet deposition, reflecting its role in platelet aggregate stability, with inhibition resulting in embolism and a greater surface area coverage [26]. Furthermore, although depletion or inhibition of Rac1 has been shown to affect PLCγ2 activation in murine [25] and human platelets [28], in this study, Rac1 inhibition was not sufficient to cause any significant effect on thrombus formation or GPVI clustering. The latter is in line with clustering studies on spread platelets [28]. Of note, in the present experimental setup, endpoint images are acquired, and therefore, alterations in thrombus build up over time were not captured. This might explain the more minor effects of EHT-1864 observed here compared with the alterations in aggregate formation seen in Rac1 knockout mice [26].

Taken together, we provide evidence for a relationship between formation of platelet GPVI macroclusters under flow and larger aggregates containing more PS-exposing platelets. In addition, the formation and maintenance of GPVI clusters was independent of downstream signaling responses, only being disrupted by direct GPVI receptor antagonism and not by signaling inhibitors. These data support the hypothesis that GPVI macrocluster formation is needed to assemble signaling complexes to induce strong, sustained platelet activation, thrombus formation, and platelet PS exposure. The dynamics and timescales of clustering on collagen as well as GPVI-fibrin interaction within a thrombus and how this relates to thrombus formation are interesting avenues to pursue in future studies.

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AUTHOR CONTRIBUTIONS

N.J.J. designed and performed the experiments, analyzed data, prepared figures, and wrote the manuscript. C.W.S. performed the experiments and wrote the manuscript. J.A.P. developed the clustering analysis pipeline. R.W.F. supplied collagen-like peptides. Y.H., J.W.M.H., and S.P.W. acquired funding and supervised the study. N.S.P. designed the experiments, analyzed data, provided supervision and funding, and wrote the manuscript. All authors have read and approved the final paper.

DECLARATION OF COMPETING INTERESTS

S.P.W. and N.S.P. have a patent for the anti-GPVI nanobodies (WO2022/136457). R.W.F. is the Chief Scientific Officer of CambCol Laboratories Ltd. The other authors have no competing interests to disclose.

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