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ORIGINAL ARTICLE

Fibrin exposure triggers αIIbβ3-independent platelet aggregate formation, ADAM10 activity and glycoprotein VI shedding in a charge-dependent manner

Samantha J. Montague[1] | Sarah M. Hicks[1] | Christine S-M. Lee[1] | Lucy A. Coupland[1] | Christopher R. Parish[1] | Woei M. Lee[1,2] | Robert K. Andrews[1,3] | Elizabeth E. Gardiner[1]

[1]ACRF Department of Cancer Biology and Therapeutics, The John Curtin School of Medical Research, The Australian National University, Canberra, ACT, Australia

[2]Research School of Electrical, Energy and Materials Engineering, College of Engineering and Computer Science, The Australian National University, Canberra, ACT, Australia

[3]Australian Centre for Blood Diseases, Monash University, Melbourne, VIC, Australia

Correspondence
Elizabeth E. Gardiner, ACRF Department of Cancer Biology and Therapeutics, John Curtin School of Medical Research, The Australian National University, 131 Garran Rd, Canberra, ACT 2601, Australia.
Email: elizabeth.gardiner@anu.edu.au

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Abstract

Background: Collagen and fibrin engagement and activation of glycoprotein (GP) VI induces proteolytic cleavage of the GPVI ectodomain generating shed soluble GPVI (sGPVI). Collagen-mediated GPVI shedding requires intracellular signalling to release the sGPVI, mediated by A Disintegrin And Metalloproteinase 10 (ADAM10); however, the precise mechanism by which fibrin induces GPVI shedding remains elusive. Plasma sGPVI levels are elevated in patients with coagulopathies, sepsis, or inflammation and can predict onset of sepsis and sepsis-related mortality; therefore, it is clinically important to understand the mechanisms of GPVI shedding under conditions of minimal collagen exposure.

Objectives: Our aim was to characterize mechanisms by which fibrin-GPVI interactions trigger GPVI shedding.

Methods: Platelet aggregometry, sGPVI ELISA, and an ADAM10 fluorescence resonance energy transfer assay were used to measure fibrin-mediated platelet responses.

Results: Fibrin induced αIIbβ3-independent washed platelet aggregate formation, GPVI shedding, and increased ADAM10 activity, all of which were insensitive to pretreatment with inhibitors of Src family kinases but were divalent cation- and metalloproteinase-dependent. In contrast, treatment of washed platelets with other GPVI ligands, collagen, and collagen-related peptide caused αIIbβ3-dependent platelet aggregation and GPVI release but did not increase constitutive ADAM10 activity.

Conclusions: Fibrin engages GPVI in a manner that differs from other GPVI ligands. Inclusion of polyanionic molecules disrupted fibrin-induced platelet aggregate formation and sGPVI release, suggesting that electrostatic charge may play a role in fibrin/GPVI engagement. It may be feasible to exploit this property and specifically disrupt GPVI/fibrin interactions whilst sparing GPVI/collagen engagement.Fibrin engages GPVI in a manner that differs from other GPVI ligands. Inclusion of polyanionic

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molecules disrupted fibrin-induced platelet aggregate formation and sGPVI release, suggesting that electrostatic charge may play a role in fibrin/GPVI engagement. It may be feasible to exploit this property and specifically disrupt GPVI/fibrin interactions whilst sparing GPVI/collagen engagement.

KEYWORDS
fibrin, GPVI, ADAM10, receptor shedding, thrombosis

1 | INTRODUCTION

Glycoprotein (GP) VI is a platelet-specific receptor for collagen that has a critical role in maintaining vascular integrity[1] and emerging roles in arterial thrombosis and thrombus stability, potentially through collagen-independent mechanisms.[2,3] GPVI was recently shown by several groups to bind fibrin,[2,4-6] and this interaction is likely to be critical for thrombus stability under arterial shear rates because thrombi formed in the carotid artery of GPVI-deficient mice are remarkably unstable. Collagen binds to a binding pocket within the first immunoglobulin-like domain of GPVI[7] with strong preference for dimeric GPVI.[7,8] The site within GPVI-mediating fibrin interactions is still under investigation.[5,6] Recent studies suggest immobilized fibrinogen can also interact with GPVI,[5,9] although this remains controversial.[10] Collagen and fibrin binding to GPVI triggers an intracellular signalling cascade initiated by phosphorylation of the immunoreceptor tyrosine-containing activation motif (ITAM) within the GPVI-linked Fc receptor (FcR) γ-chain by Src family kinases,[2,6,11] leading to spleen tyrosine kinase (Syk) activation.[12] Notably, various charged and/or hydrophobic molecules including diesel exhaust particles, polysulfated sugars, and histones can also indirectly induce GPVI-dependent ITAM signalling.[13]

Fibrin is formed by the action of the serine protease thrombin on fibrinogen and is critical for insoluble clot formation to control blood loss from damaged blood vessels. Thrombin cleaves fibrinopeptides A and B from the N-termini of Aα and Bβ chains of fibrinogen, revealing a positively charged βN-terminal domain and allowing protofibril formation.[14] Lateral aggregation of protofibrils and interactions with αC regions between protofibrils leads to fibrin polymerization, and a fibrin network with pockets of positive charges. Fibrin is ultimately crossed-linked by activated factor XIII (FXIIIa),[15] creating a stable latticed mesh that both encapsulates the platelet aggregate[16] and forms at the base of a platelet aggregate[17] to promote stable thrombus formation, sealing a damaged vessel wall and initiating wound healing processes. In a ferric chloride injury model, GPVI-deficient mice displayed normal onset of thrombosis but delays in reaching occlusion and increased embolization,[18] implying that fibrin-GPVI binding might maintain thrombus stability in situations of minimal collagen exposure.

Platelet GPVI levels are stable under resting conditions but GPVI undergoes metalloproteolytic cleavage,[19] releasing soluble GPVI (sGPVI) into the plasma, when platelets are activated by exposure to elevated shear stress,[20] active FX,[21] or collagen, convulxin, and collagen-related peptide (CRP).[22] Proteolysis is mediated by A Disintegrin And Metalloproteinase (ADAM) family members, predominantly ADAM10, in human platelets. ADAM10 has a basal activity on resting platelets, which is increased following exposure of platelets to elevated shear stress, but not collagen or other classical GPVI ligands.[23] We have previously reported that polymerized fibrin, but not nonpolymerized fibrin or fibrinogen, triggered GPVI shedding, which did not require active Src or Syk signalling.[24] This contrasts findings made with collagen,[22] and suggests that fibrin and collagen engage GPVI via different mechanisms. We wanted to understand how fibrin induces GPVI shedding as this mechanism is likely to modulate thrombus size and stability with important implications for therapeutic intervention.

Essentials

- Platelet exposure to fibrin increases ADAM10 activity and GPVI shedding, without requirement for activated signalling pathways
- Negatively-charged molecules and metalloproteinase inhibitors reduce fibrin-mediated release of GPVI
- Collagen and fibrin treatment of platelets induces ADAM10-mediated GPVI shedding via different mechanisms

In this study, we demonstrate that fibrin exposure triggers αIIbβ3-independent platelet aggregation, P-selectin upregulation and increased ADAM10 activity, leading to sGPVI release. This was a specific fibrin response as fibrinogen exposure or activation of protease-activated receptors (PARs) did not induce GPVI shedding or increase ADAM10 activity. Fibrin-induced ADAM10 activity and GPVI shedding also did not require ITAM signalling or cytoskeletal rearrangement. An anti-GPVI antibody Fab fragment which blocked collagen-induced aggregation, did not disrupt fibrin-mediated aggregation or increase ADAM10 activity indicating differences in GPVI engagement with fibrin and collagen. Finally, we examined whether electrostatic interactions controlled fibrin-mediated platelet events and demonstrated that fibrin-induced aggregation, ADAM10 activity and GPVI shedding were disrupted by inclusion of small polyanionic molecules. We conclude that several properties of GPVI/fibrin responses

are unique and differ from collagen or CRP-mediated responses, thus providing an opportunity for specific therapeutic intervention.

2 | MATERIALS AND METHODS

2.1 | Reagents

A complete reagents list is described in Appendix S1. 1G5 mouse monoclonal antibody against human GPVI extracellular domain was affinity-purified using protein A-Sepharose CL-4B (GE Healthcare) as described.[25] 1G5 Fab fragment was generated and isolated using a Fab preparation kit (Thermo Fisher Scientific).[25] Small polyanions SPA.1 and SPA.2 are sulfated di- and trisaccharides, respectively, which were generated using standard laboratory methods; their complete characterization is the subject of a manuscript under review (Parish et al.).

2.2 | Platelet preparation

Human washed platelets were prepared as previously described.[23] Briefly, platelets were isolated from acid-citrate-dextrose (97 mM trisodium citrate, 111 mM glucose, 78 mM citric acid, 6:1 blood:acid-citrate-dextrose ratio) anticoagulated blood drawn from healthy donors after provision of informed consent using a protocol approved by the Australian National University Human Research Ethics Committee (2016/317).

2.3 | Light transmission aggregometry (LTA) and ADAM10 activity

LTA (Chrono-Log) was used to measure fibrin-induced platelet aggregation. Washed platelets (5×10^8/mL) in Tyrode's buffer containing 10 mM $CaCl_2$ and 5 μM $ZnSO_4$ were preincubated with 1 mM RGD peptide for 3 minutes at 37°C before agonist exposure. Other inhibitors or reagents were added 10 minutes before RGD addition. Washed platelets were placed in a cuvette and loaded into the aggregometer held at 37°C with constant stirring and 1 μM GPVI-Cy3 (ADAM10 substrate, see the following section) was added. After 1 minute, 100 μg/mL fibrinogen were added. After a further 3 minutes, 1 U/mL thrombin was added and αIIbβ3-independent aggregation was monitored for 20 minutes. Test agonists were also added 1 minute after substrate addition and aggregation monitored for 20 minutes. A total of 120 μL of each washed platelet suspension was mixed with 50 mM EDTA before assaying ADAM10 activity and sGPVI levels.

2.4 | Measurement of ADAM10 activity

ADAM10 activity was measured using an established fluorescence resonance energy transfer (FRET) assay[23] with a quenched GPVI-Cy3 peptide substrate comprising amino acid residues 236-248 of human GPVI that is known to be cleaved by ADAM10.[23,26] Fluorescence following cleavage of the peptide and quencher release at 37°C was detected by sample excitation at 540 nm and emission at 590 nm using a Tecan Infinite 200 Pro plate reader (endpoint or after 1 hour in assays using recombinant human ADAM10). Increases in relative fluorescence units represented increased ADAM10 activity.

2.5 | Quantification of sGPVI levels

sGPVI levels were measured in supernatant samples generated by fibrin or other agonists exposure of washed platelets. Supernatants were obtained after centrifugation at 2500 *g* for 15 minutes and levels of sGPVI quantified using an established ELISA as previously described.[24,27] Luminescence signal was detected with a Tecan Infinite 200 Pro plate reader.

2.6 | Statistical analysis

Data are represented as mean + standard deviation. GraphPad Prism version 8 was used for statistical analysis. Normal and log-normal distribution was tested using Anderson-Darling tests. One-way analysis of variance (ANOVA) with Tukey's multiple comparisons tests were performed if data were normally distributed, or Kruskal-Wallis analysis with Dunn's post multiple comparisons test for nonparametric data, unless stated otherwise.

3 | RESULTS

3.1 | Fibrin exposure triggers platelet aggregate formation and increases ADAM10 activity and sGPVI release

The interaction of fibrin with platelets has been shown by several groups to be mediated by engagement with GPVI[2,5,6,24]; however, the nature of this engagement and consequences for platelet function have not been fully defined. Initially washed platelets were exposed to polymerized fibrin that had been generated by adding thrombin to platelet suspensions that contained fibrinogen and inhibitors of αIIbβ3 function, such as RGD.[24] Fibrin exposure alone produced an αIIbβ3-independent aggregation trace with 84% maximal change in light transmission (maximal aggregation) ($P < .001$, Figure 1A) or 80% aggregation ($P < .05$) in samples pretreated with another αIIbβ3 inhibitor, GR144053 (Figure S1D). Platelet exposure to fibrin in the presence of RGD also elevated P-selectin exposure over resting samples ($P < .01$, Figure 1B) to levels equivalent to those quantified in platelets treated with collagen or calcium ionophore (A23187), suggesting fibrin-exposed platelets triggered platelet activation and degranulation.

Metalloproteolytic shedding of human GPVI ectodomain is predominantly controlled by ADAM10 and is a consequence of platelet

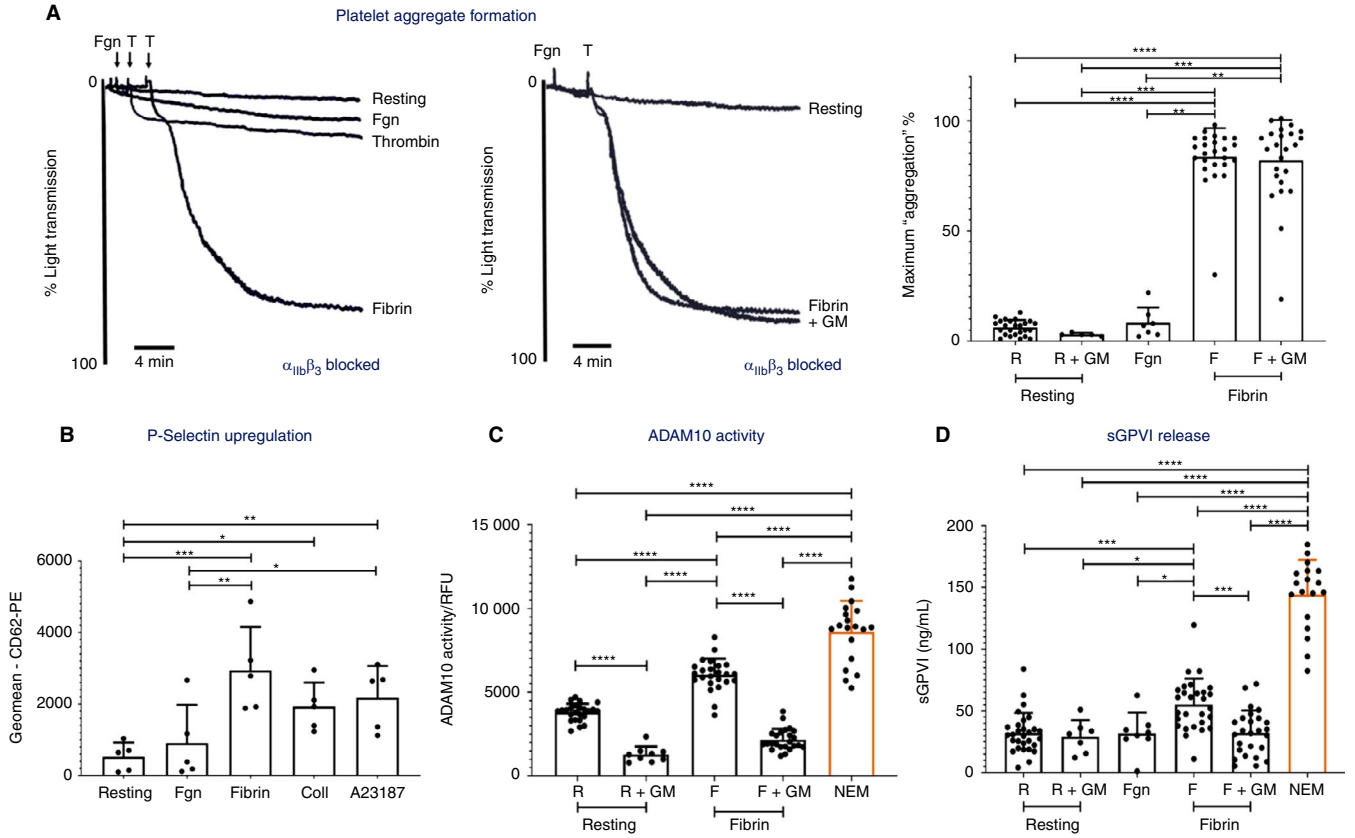

FIGURE 1 Fibrin exposure triggers αIIbβ3-independent platelet aggregation, increased P-selectin upregulation, increased ADAM10 activity, and sGPVI release. Washed platelets (5×10^8/mL) were pretreated with 1 mM RGD for 3 min before agonist exposure. Fibrin was generated when 1 U/mL thrombin (T; arrowed) was added 3 min after fibrinogen (Fgn; 100 µg/mL) under stirring conditions. In some cases, platelets were pretreated with 250 µM GM6001 (GM), a general metalloproteinase inhibitor, 10 min before agonist exposure. A, Platelet aggregation was monitored for 20 min. (Left = representative aggregation traces, right = quantitation). B, P-selectin upregulation was measured by flow cytometry following washed platelet exposure to vehicle (resting), fibrinogen (Fgn) or fibrin (as previously). 30 µg/mL collagen and 10 µM A23187 (calcium ionophore) stimulation for 1 h (static) were used as GPVI-ligand control and maximum degranulation control, respectively. CD62P-PE fluorescence measured as geomean. C, ADAM10 activity in fibrin-exposed washed platelets was measured as relative fluorescence units (RFU; endpoint value) using an established ADAM10 FRET assay (see methods). D, sGPVI levels in supernatants obtained following agonist exposure using a sGPVI ELISA. 5 mM NEM was used as a positive control for ADAM10 activity and sGPVI release. Aggregations = Kruskal-Wallis test with Dunn's multiple comparisons test performed. All other experiments = one-way ANOVA with Tukey's multiple comparisons. **P* < .05, ***P* < .01, ****P* < .005, *****P* < .001. Individual dots = individual donors (n = 3-29). Bars and error bars are mean + standard deviation (SD)

activation. Shedding can be triggered in the presence or absence of GPVI-ligand engagement and active signalling pathways.[23] We assessed the effect of fibrin exposure on platelet ADAM10 activity. Resting platelets have detectable ADAM10 activity that increases upon exposure to fluid shear stress or treatment of platelets with the thiol modifier *N*-ethylmaleimide (NEM), measured using an established ADAM10 FRET sensor tool.[23] Consistent with published data, Figure 1C (and Figure S1B) shows resting platelets had detectable ADAM10 activity that was increased by treatment with NEM and reduced by inclusion of the broad spectrum metalloproteinase inhibitor GM6001, with a similar result observed with the ADAM10-specific inhibitor GI254023X (Figure S2B).[23,28] Exposure of platelets to fibrin but not fibrinogen triggered increased ADAM10 activity (1.6-fold increase over resting, *P* < .01; Figure 1C and Figure S1B) that was reduced by inclusion of either GM6001 (64% reduction, *P* < .001) or GI254023X (39% reduction, *P* < .01) (Figure 1C and Figure S2B). Fibrin-mediated platelet aggregation was unaffected by pretreatment with the metalloproteinase inhibitors GM6001 or GI254023X (Figure 1A and Figure S2A), suggesting that metalloproteinase inhibition did not alter fibrin/GPVI engagement.

In human platelets, ADAM10 cleaves GPVI releasing sGPVI into the plasma.[26,27] We measured sGPVI levels released in the supernatants of fibrin-exposed platelets, and showed that fibrin but not fibrinogen exposure induced sGPVI release (*P* < .005; Figure 1D and Figure S1C), consistent with previous findings where fibrin exposure induced loss of intact GPVI and appearance of a 10-kDa remnant GPVI fragment, detected by western blotting.[24] Fibrin-mediated sGPVI release was blocked by GM6001 (Figure 1D) and reduced by treatment with GI254023X (Figure S2C), although this reduction did not reach statistical significance, confirming release was mediated

by metalloproteinases, with a contribution from ADAM10. To assess whether activation of PARs by thrombin during the fibrin generation step contributed to platelet aggregation, ADAM10 activity or GPVI release, RGD-treated platelets were mixed with either PAR-1 activating peptide (SFLLRN) or thrombin. Figure S1 shows that neither treatment induced αIIbβ3-independent platelet aggregation, increased ADAM10 activity or triggered sGPVI release. This suggested that the contribution of thrombin to fibrin-mediated aggregation and shedding was limited to its fibrinogen-cleaving properties, consistent with previous findings.[21,22,24]

3.2 | Fibrin and collagen activate platelet aggregation and GPVI shedding via different mechanisms

Using the same experimental design, we directly compared collagen- and fibrin-mediated platelet responses. Addition of 30 μg/mL collagen (an extremely high concentration) to RGD-treated platelets did not induce robust platelet aggregation (<40% maximum αIIbβ3-independent platelet aggregation compared with >80% maximum aggregation observed with fibrin exposure; Figure 2A). In line with previous studies using lower collagen concentrations,[23] 30 μg/mL collagen treatment did not increase ADAM10 activity above resting levels and was significantly less than fibrin-mediated ADAM10 activity ($P < .05$, Figure 2B), but did result in significant sGPVI release (Figure 2C). High concentration collagen-induced sGPVI levels were 2-fold greater than fibrin-induced levels, and approached levels achieved with NEM treatment. Taken together, these data suggest mechanistic and regulatory differences between collagen- and fibrin-induced platelet functions.

GPVI uses ITAMs within the associated FcRγ chain to trigger platelet aggregation in response to collagen exposure[3,11,29] and collagen-induced GPVI shedding also requires ITAM signalling.[22] Fibrin activation of GPVI also triggers ITAM signalling, and inhibition of Src and Syk reduces fibrin-mediated phosphorylation[6] and spreading on immobilized fibrin(ogen).[9] Fibrin-mediated GPVI shedding however was shown to be Src and Syk independent.[24] We therefore wanted to determine whether fibrin-mediated aggregation, ADAM10 activity, and sGPVI release was dependent on ITAM signalling. Figure 3 shows that inclusion of inhibitors of Syk (Bay61-3606) or Src (Src-1) did not reduce fibrin-mediated platelet aggregation, ADAM10 activity, or sGPVI release. These data indicate that fibrin interactions with GPVI to trigger GPVI shedding differ from collagen interactions with GPVI.

One explanation for the differences observed so far is that fibrin and collagen may interact with different sites within GPVI. To address this question, platelets were pretreated with 10 μg/mL of a Fab fragment generated from the anti-human GPVI monoclonal antibody 1G5.[25] This concentration of 1G5 Fab was

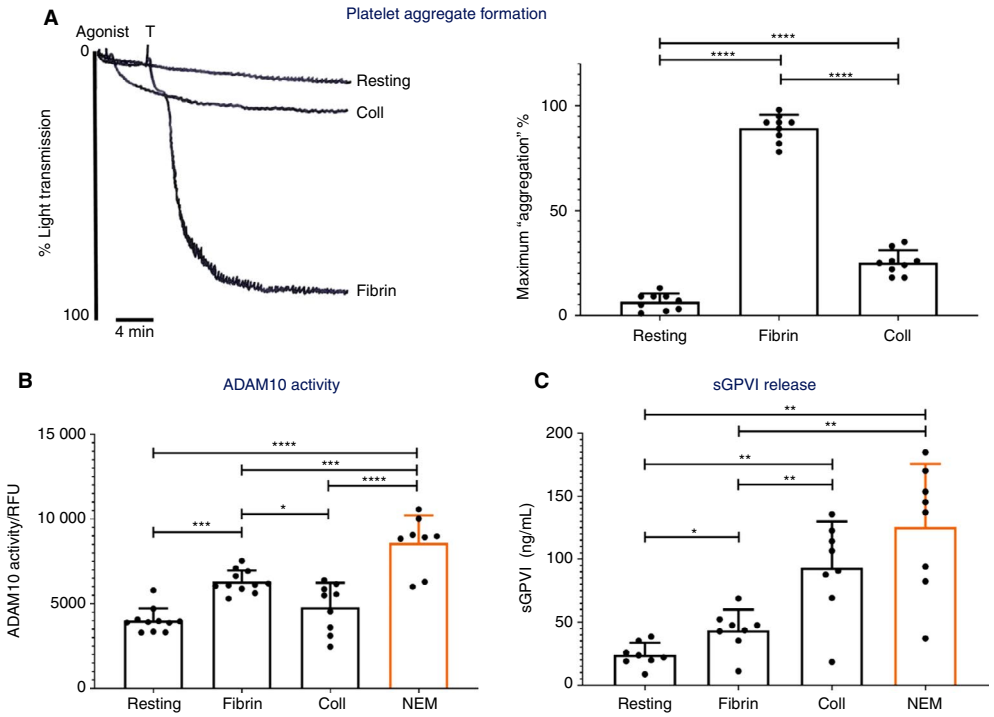

FIGURE 2 Fibrin-mediated GPVI shedding is different from collagen-induced GPVI shedding. RGD-treated washed platelets (5×10^8/mL) were treated with fibrin (fibrinogen: Fgn 100 μg/mL + thrombin: T 1 U/mL) or collagen (Coll; 30 μg/mL). A, Representative platelet aggregations after agonist exposure measured by LTA for 20 min. B, ADAM10 activity or C, sGPVI levels released from untreated platelets or platelets treated with 30 μg/mL collagen, or fibrin or 5 mM NEM. One-way ANOVA with Tukey's multiple comparisons, *$P < .05$, **$P < .01$, ***$P < .005$, ****$P < .001$. Individual dots = individual donors, n = 8-9. Bars and error bars are mean + SD

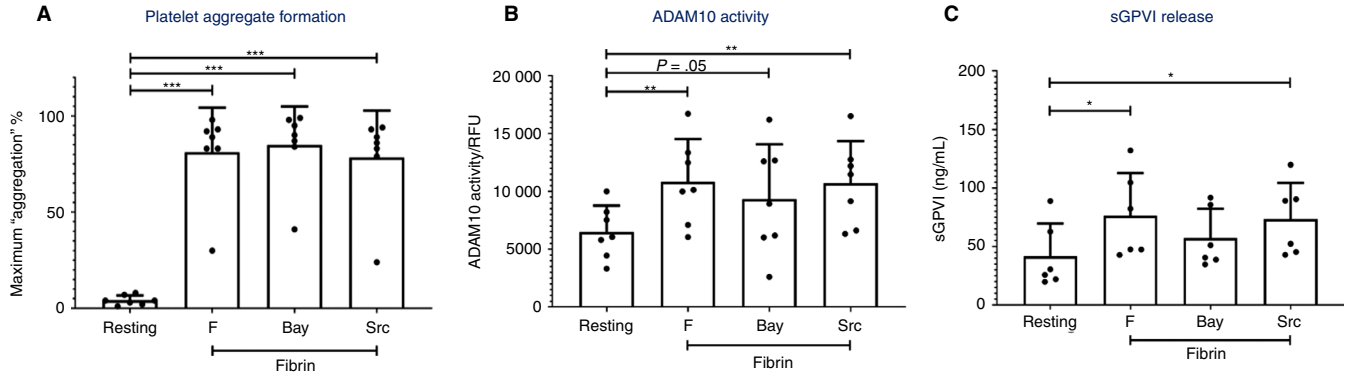

FIGURE 3 Fibrin-mediated increase in ADAM10 activity and GPVI release do not require ITAM signalling. Washed platelets were pretreated with 10 μM Bay61-3606 (Syk inhibitor) or 10 μM Src-1 (Src inhibitor) before exposure to fibrin and A, platelet aggregation; B, ADAM10 activity or C, sGPVI release were evaluated. One-way ANOVA with Tukey's multiple comparisons (for aggregations and ADAM10 activity), Friedman test with Dunn's multiple comparisons (for sGPVI release) *P < .05, **P < .01, ***P < .005. Individual dots = individual donors, n = 6-7. Bars and error bars are mean + SD

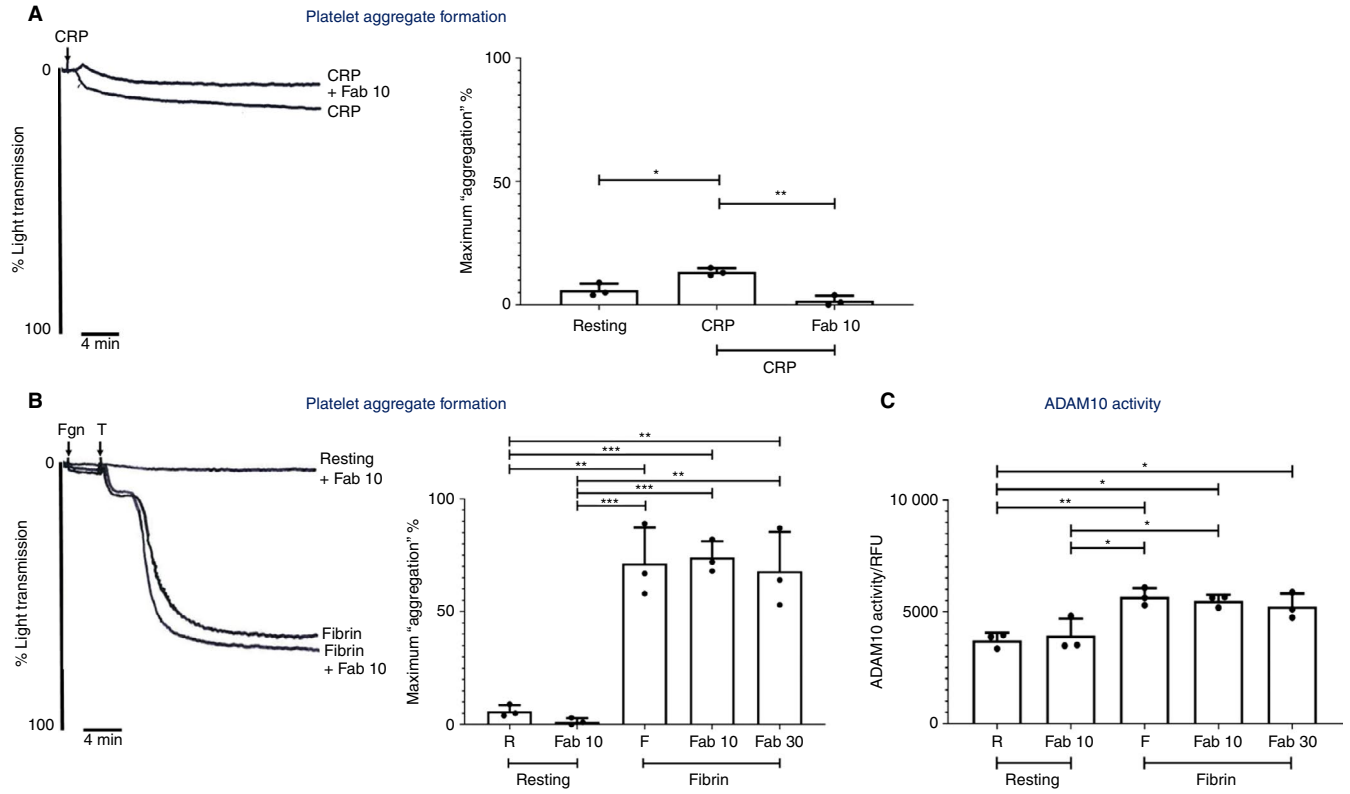

FIGURE 4 Anti-GPVI antibody 1G5 Fab reduces CRP-mediated aggregation but not fibrin-mediated aggregation or ADAM10 activity. A, RGD-treated washed platelets were preincubated with 10 μg/mL 1G5 Fab for 10 min before exposure to 25 μg/mL CRP. B, RGD- treated washed platelets were preincubated with 10 or 30 μg/mL 1G5 Fab 10 min before exposure to fibrin (Fgn 100 μg/mL + thrombin: T 1 U/mL). Aggregation measured for 20 min C, ADAM10 activity measured after aggregation. One-way ANOVA with Tukey's multiple comparisons test. *P < .05, **P < .01, ***P < .005. Individual dots = individual donors, n = 3-4, bars and error bars are mean + SD

sufficient to inhibit collagen- and CRP-mediated PRP aggregation (data not shown). However, inclusion of up to 30 μg/mL 1G5 Fab did not affect fibrin-induced αIIbβ3-independent aggregation in washed platelets (Figure 4B) or fibrin-mediated ADAM10 activity (Figure 4C). These data suggest that fibrin engagement of GPVI uses a binding site that is distinct from that shared by collagen and 1G5 Fab.

3.3 | Fibrin-mediated ADAM10 activity does not require intracellular Ca^{2+} flux

Because of the importance of Ca^{2+} flux in GPVI activation,[30] we assessed roles for calcium and cation fluxes in fibrin-mediated ADAM10 activity and GPVI shedding. Washed platelets were treated with RGD and 1,2-bis(o-aminophenoxy)ethane-N,N,N′,N′-tetraacetic

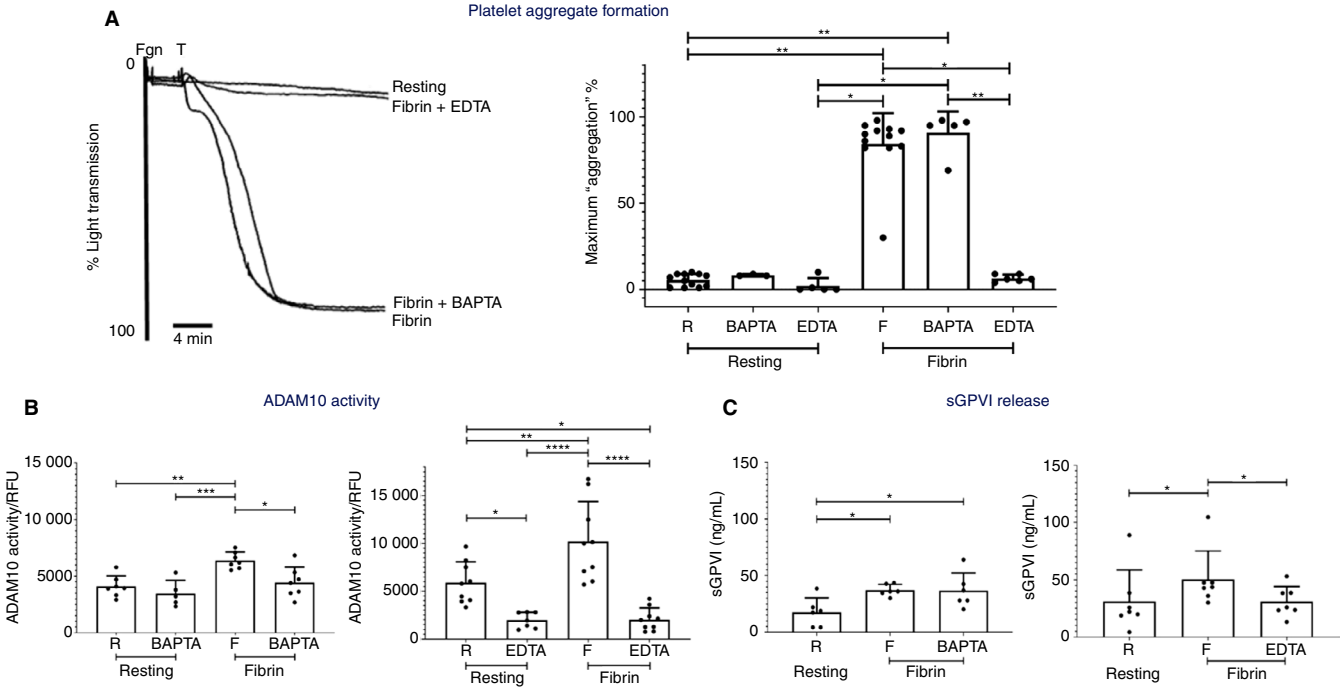

FIGURE 5 BAPTA-AM and EDTA reduces fibrin-mediated ADAM10 activity. Washed platelets were pretreated with vehicle or 50 μM BAPTA-AM or 50 mM EDTA then exposed to Fibrin (Fgn + T) and monitored for A, platelet aggregation (left = representative aggregation trace, right = quantification); B, ADAM10 activity or C, sGPVI release. Aggregations = Kruskal-Wallis with Dunn's multiple comparisons test. ADAM10 activity and sGPVI release = one-way ANOVA with Tukey's multiple comparisons test. *P < .05, **P < .01, ***P < .005, ****P < .001. Individual dots = individual donors. Bars and error bars are mean + SD

acid (BAPTA-AM), a membrane-permeable Ca^{2+}-specific chelator that inhibits intracellular Ca^{2+} flux,[31] then exposed to fibrin. EDTA, an extracellular divalent cation chelator and broad metalloproteinase inhibitor, was included as a control. Inclusion of EDTA but not BAPTA-AM to platelet suspensions reduced fibrin-induced platelet aggregation (Figure 5A). Treatment with BAPTA-AM did not disrupt resting ADAM10 activity and only mildly reduced fibrin-induced ADAM10 activity (Figure 5B), and had no effect on fibrin-induced sGPVI release (Figure 5C). Because ADAM10 catalytic activity requires Zn^{2+}, inclusion of EDTA inhibited both ADAM10 activity and sGPVI release induced by fibrin (Figure 5B,C) as expected. This effect of EDTA, and lack of major effect with BAPTA-AM was confirmed in an ADAM10 activity assay using 30 nM recombinant human ADAM10 (Figure S4A). We conclude that fibrin-induced platelet ADAM10 activity and release of sGPVI did not require intracellular calcium flux.

3.4 | Fibrin-mediated sGPVI release is reduced by negatively-charged molecules

Numerous electrostatically charged agents, including histones and diesel exhaust particles can activate platelets via GPVI and induce ITAM signalling, potentially through membrane perturbation and GPVI clustering.[13] Fibrinogen, a negatively charged molecule, is converted to fibrin by thrombin, creating a network of protein polymers with pockets of positive charges that can bind polyanions and negatively charged polyphosphates, and disrupt fibrin binding properties.[32,33] Because EDTA treatment disrupted fibrin-mediated platelet aggregation, we explored a role for electrostatic charge in contributing to fibrin-mediated responses. We assessed the ability of heparin, a negatively charged macromolecule of ~15 to 20 kDa and two small polyanions (SPA) termed SPA.1, a sulfated disaccharide and SPA.2, a sulfated trisaccharide, of approximately 0.9- to 1.5-kDa in size, respectively. The SPAs were evaluated at concentrations that were above levels shown to reduce histone toxicity in assays conducted in vitro and in vivo (manuscript under review). Whilst SPA.1 did not reduce fibrin-mediated platelet aggregation at any of the concentrations tested (Figure 6A), concentrations of 300 to 500 μg/mL of SPA.2 and 50 U/mL heparin reduced fibrin-mediated aggregation by 30% to 35% (Figure 6A).

We could not assess fibrin-induced ADAM10 activity in samples containing any of these charged molecules, as concentrations of 500 μg/mL SPA.1 and SPA.2 or 50 U/mL heparin interfered directly with cleavage of the ADAM10 substrate by recombinant ADAM10 (Figure S4A,B). However, the charged molecules did not interfere with NEM-induced sGPVI release (Figure S4C), indicating that the sGPVI ELISA could be used to evaluate the effect of these molecules on fibrin-induced sGPVI release. Each of these charged molecules did not affect platelet spreading on immobilized fibrin (Figure S5). Inclusion of SPA.1 or SPA.2 to fibrin-exposed platelet suspensions resulted in slight reduction (19% reduction SPA.1 and 20% reduction SPA.2, Figure 6B)

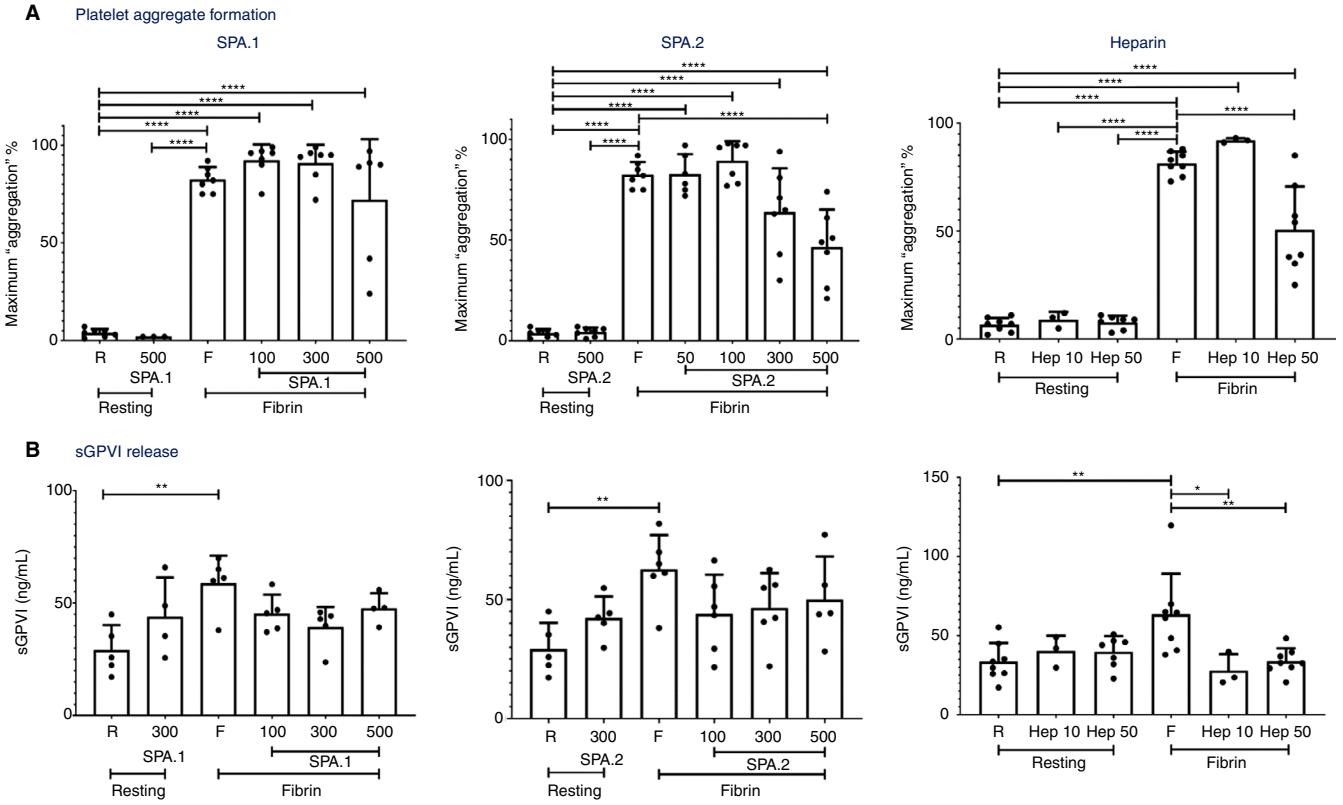

FIGURE 6 Fibrin-mediated platelet aggregation and sGPVI release is reduced by negatively-charged molecules. RGD-treated washed platelets (5×10^8/mL) were preincubated with 100-500 μg/mL SPA.1, 50-500 μg/mL SPA.2, or 10 or 50 U/mL heparin (corresponding to 67 and 333 μg/mL of heparin) for 10 min before exposure to fibrin. A, Platelet aggregations quantified from n = 3-8 donors; R = resting platelets, F = fibrin-exposed platelets. B, sGPVI release measured after fibrin-mediated aggregations. One-way ANOVA with Tukey's multiple comparisons test. **P* < .05, ***P* < .01, ****P* < .005, *****P* < .001. Individual dots = individual donors, n = 3-8. Bars and error bars are mean + SD

in fibrin-induced release of sGPVI that was not statistically significant. However, inclusion of 10 or 50 U/mL heparin did significantly reduce fibrin-mediated sGPVI release. Because heparin requires the presence of significant amounts of anti-thrombin III (unlikely to be present in our washed platelet preparations) to affect thrombin activity, we conclude that heparin interfered directly with the ability of fibrin to trigger shedding of GPVI, most likely via an electrostatic mechanism.

To further explore the effects of negatively charged molecules, we evaluated charged molecules, chondroitin sulfate (type A and C; 50-100 kDa) and dextran sulfate (45-50 kDa) to disrupt fibrin-mediated platelet effects on aggregation and sGPVI release. ADAM10 activity measurements again could not be validated because of charge molecules interference with ADAM10 substrate cleavage by recombinant ADAM10 (data not shown). Comparable with data gained using 50 U/mL heparin, pretreatment with 1000 μg/mL of chondroitin sulfate type A or C reduced fibrin-mediated sGPVI release (*P* = .037 and *P* = .046, respectively; Table S1), although aggregation was unaltered. In contrast, 200 and 1000 μg/mL of a 50-kDa (but not a 45-kDa) preparation of dextran sulfate reduced platelet aggregation (*P* < .01) but did not reduce sGPVI release (Table S1). These data indicate that fibrin-mediated platelet responses were disrupted by inclusion of polyanions and the extent of inhibition was determined by specific properties of each polyanion.

3.5 | Fibrin-mediated ADAM10 activity does not require cytoskeletal rearrangement but is inhibited by ATP hydrolysis

Platelet receptors including αIIbβ3 and the GPIb-IX-V complex are linked to the cytoskeleton, through actin and filamin A,[34] which enable platelet shape change[35,36] and platelet signalling through interactions with Syk.[37] Disruption of actin function using cytochalasin D (cytoD) to block actin polymerization was shown to disrupt GPVI dimerization and clustering.[8] To assess whether actin polymerization was required for fibrin-GPVI-mediated responses, we used 10 μM cytoD, a moderate concentration that reduced platelet spreading on collagen and immobilized fibrin (data not shown). Inclusion of cytoD to RGD-treated fibrin-exposed platelets did not disrupt αIIbβ3-independent aggregation (Figure 7A). Inclusion of cytoD also did not disrupt fibrin-mediated ADAM10 activity or sGPVI release (Figure 7B,C). This suggested that although fibrin can trigger platelet cytoplasmic events, fibrin mediates platelet aggregation and GPVI shedding via external effects on platelet receptors and metalloproteinase tertiary structure.

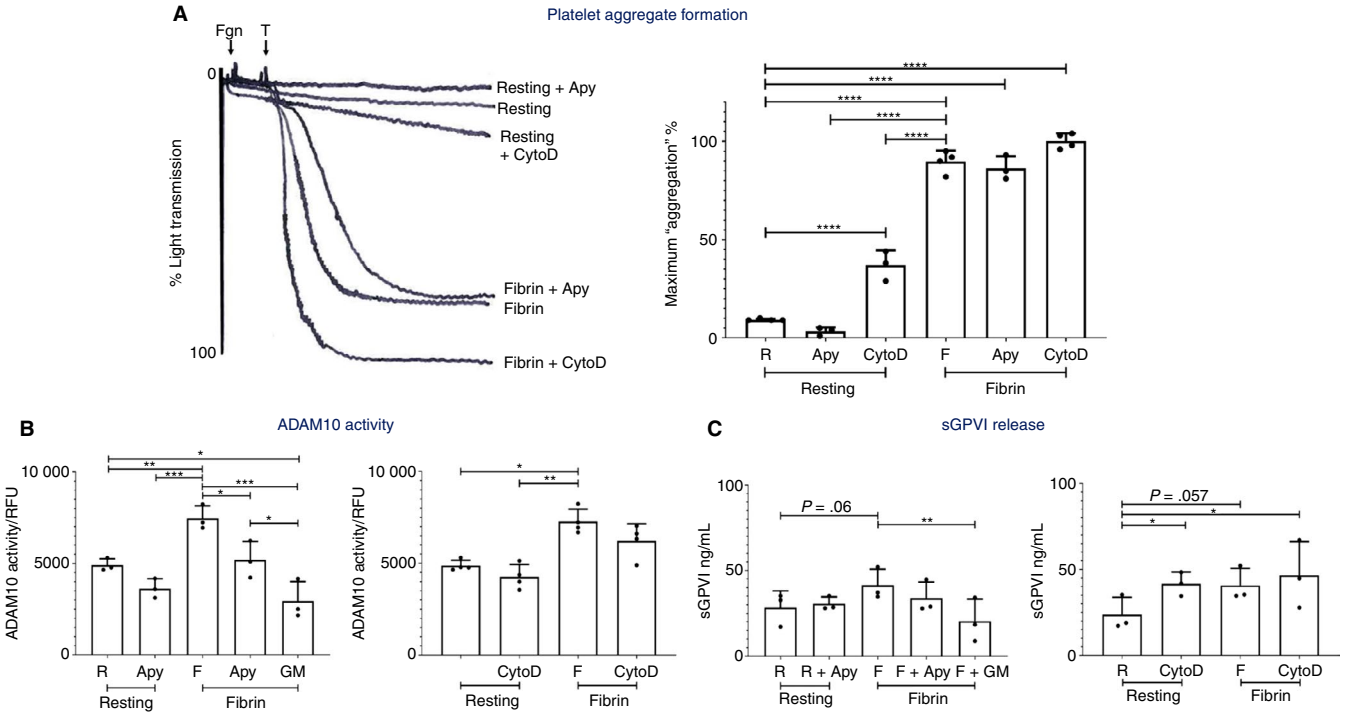

FIGURE 7 Fibrin-mediated ADAM10 activity does not require actin polymerization but is reduced with ADP scavenging. RGD-treated washed platelets were pre-treated with 2 U/mL apyrase (Apy) or 10 μM cytochalasin D (CytoD) or 250 μM GM6001 10 min before exposure to fibrin. A, Fibrin (Fgn + T) mediated platelet aggregation (left = representative aggregation traces; right = quantification). B, ADAM10 activity and C, sGPVI levels. One-way ANOVA with Tukey's multiple comparisons tests performed. **P* < .05 ***P* < .01, ****P* < .005, *****P* < .001. Individual dots = individual donors. n = 3-4. Bars and error bars are mean + SD

TABLE 1 Summary of fibrin- vs collagen-induced platelet responses

Event	Fibrin	Collagen
Binds GPVI and activates ITAM signalling pathways	Y	Y
Platelet αIIbβ3-independent aggregation	Y	N
Increased ADAM10 activity • Reduced with 1G5 Fab fragment	Y N	N Y
Induces GPVI shedding • Metalloproteinase-dependent • Src- and Syk-dependent	Y Y N	Y Y Y
Increases P-selectin surface levels	Y	Y
GPVI monomer/dimer binding	ND Charge-dependent	Dimeric GPVI

Note: Responses described in the current study or reported in other studies. Y = yes, N = no, ND = not determined.

We consistently observed a two-step aggregation trace following fibrin exposure. However, one donor had significantly ablated fibrin-induced platelet aggregation (Figure 1A), together with a reduction of adenosine diphosphate (ADP)-induced aggregation in PRP (not shown). We therefore examined whether release of the secondary mediator ADP, that is important for integrin α2β1-modulated collagen-GPVI activation,[38] contributed to fibrin-mediated ADAM10 activity or sGPVI release. Inclusion of the ADP scavenger apyrase did not disrupt fibrin-induced aggregation (Figure 7A), but reduced fibrin-mediated ADAM10 activity by 30% (*P* < .01, Figure 7B) and slightly (but not statistically significantly) reduced fibrin-induced sGPVI release (18% reduction; Figure 7C). Therefore, although apyrase interfered with fibrin-mediated ADAM10 activity, this was not sufficient for prevention of fibrin-mediated GPVI shedding.

4 | DISCUSSION

Hemostasis requires conversion of fibrinogen to fibrin to generate a distinctive fibrous network that mediates interactions with blood

cells and initiation of tissue repair processes. Fibrin is present at all stages of thrombus formation.[39] Recently, fibrin and fibrinogen have been identified as ligands for GPVI and shown to induce signalling in support of thrombus formation and stabilization.[2,4,5,9] In addition, platelet exposure to fibrin was shown to induce metalloproteolytic loss of intact GPVI by western blot.[24] In this present study, we now show key differences between collagen and fibrin engagement of GPVI (Table 1). First, 1G5 Fab blocks collagen- but not fibrin-mediated aggregation of platelets in suspension, indicating that under these experimental conditions, fibrin engages at least one site within GPVI that is distinct from collagen. Second, although fibrin engagement of platelets in suspension is able to trigger ITAM signalling responses, fibrin-mediated platelet aggregation did not require ITAM signalling, actin polymerization, intracellular calcium flux or active integrin αIIbβ3. Third, whereas both collagen and fibrin trigger shedding of GPVI, collagen does not achieve this via increased activation of ADAM10 activity whereas fibrin-treated platelets did elevate ADAM10 activity and induced sGPVI release. Blockade of fibrin-induced platelet aggregation by inhibitors of Syk and Src has been previously reported,[6] but integrin-independent aggregation by fibrin has not. Furthermore, we identified that the fibrin-mediated platelet aggregation and release of sGPVI involved an electrostatic charge component, as both of these outcomes could be disrupted by inclusion of small or large molecular weight polyanions.

From our data using the collagen-blocking anti-GPVI antibody 1G5, and also the work of others,[2,4,5,9] it is apparent that fibrin engages GPVI in a manner that differs from collagen, and is likely to involve charge–charge interactions. Despite an overall net negative charge, regions of fibrin such as the central E region has a net positive charge, predominantly conferred through three positively charged clusters of residues within the βN-domain. This domain, which is shielded within fibrinogen by fibrinopeptide B and exposed upon thrombin-mediated cleavage of fibrinopeptide B, is solvent-accessible in fibrin, and mediates interactions with a number of cell receptor ectodomains. GPVI is a sialomucin with a negatively charged membrane-proximal stalk region and it is conceivable that fibrin is able to electrostatically engage and cluster GPVI via this domain. Fibrin D-dimer contains fragment E when formed by the action of plasmin on fibrin in vivo. Recently a D-dimer preparation was reported to disrupt GPVI-fibrin interactions in vitro[6]; however, it is not clear whether fragment E was present in the commercial preparation used in that study. We were able to ablate fibrin effects by including high and low molecular weight polyanionic reagents. Consistent with our findings that heparin disrupts fibrin-GPVI effects, the βN-domain of fibrin also contains a heparin-binding site.[40,41]

Fibrin treatment also increased ADAM10 activity indicating that fibrin–platelet interactions were able to illicit changes to platelet ADAM10 that mimicked changes induced by exposure to elevated shear stress.[23] ADAM10 is active on the surface of resting platelets and collagen exposure does not increase ADAM10 activity, but is likely to increase GPVI shedding by altering the access of the catalytic domain of ADAM10 for the cleavage site within GPVI. How fibrin exposure increases the activity of platelet ADAM10 is not yet known, however it does not require platelet cytoskeletal rearrangement or intracellular signalling events. Interestingly, the βN-domain of fibrin has been shown to interact with a cysteine-rich domain within the very low density lipoprotein receptor on endothelial cells.[42] Cysteine-rich domains are present in both αIIbβ3 and ADAM10 and it is intriguing to speculate that fibrin elicits at least a portion of its platelet activity by interacting with these domains on the platelet surface. In other vascular systems, the positively charged amino terminus of the β chain of fibrin (but not fibrinogen) interacts with VE-cadherin[43,44] to orchestrate the transmigration of leukocytes across endothelium. Taken together, our data and the work of others suggest that fibrin has roles in the vasculature beyond maintenance of structural integrity of a thrombus.

Numerous reports using a variety of experimental conditions have concluded that thrombin treatment of platelets does not mediate direct release of GPVI.[21,22,24,45] We confirmed no role for thrombin activation of PARs in ADAM10 activity increase and sGPVI release because PAR-1 peptide or thrombin exposure alone did not increase ADAM10 activity or sGPVI release. Likewise, although platelets have been shown to spread on immobilized fibrinogen and invoke single-platelet Ca^{2+} signals through ITAM signalling,[9] inclusion of either the actin polymerization inhibitor cytochalasin D, or BAPTA-AM an inhibitor of intracellular Ca^{2+} flux did not interfere with fibrin-mediated platelet aggregation. It remains unclear why BAPTA-AM treatment reduced fibrin-mediated ADAM10 activity but GPVI shedding was not affected. It is possible that platelet Ca^{2+} flux facilitates an increase in ADAM10 activity as resting platelets become activated, but that fibrin engagement of GPVI also improves access of the ADAM10 catalytic site to the cleavage site within GPVI, resulting in no change to ADAM10 activity but increased GPVI shedding.

Finally, whether fibrin binds to monomeric[6] or dimeric GPVI[5] or mediates its effects on platelets via interaction with membrane or other platelet surface proteins remains to be resolved.[10] We provide evidence here that fibrin-GPVI engagement can be disrupted by negatively charged molecules, including heparin or small polyanionic molecules. GPVI pathways have previously been reported to be activated by several electrostatically charged molecules including diesel exhaust particles and histone proteins that trigger ITAM-dependent platelet signalling, and may work to trigger GPVI clustering,[13] either directly or via involvement with a co-associated receptor such as GPIb-IX-V,[46] or an adjacent molecule such as members of the tetraspanin family.[47]

As expected and consistent with published data, both collagen and CRP treatment of washed platelets induced GPVI shedding, and did not induce αIIbβ3-independent aggregation, or any increase in ADAM10 activity beyond resting levels. These findings strongly support the notion of molecular differences between collagen and fibrin engagement of GPVI. Fibrin-mediated effects were disrupted by inclusion of heparin, or small polyanions and higher concentrations of certain types of chondroitin sulfate and dextran sulfate molecules. Properties including charge density and stoichiometry of each of these molecules are likely to be important determinants of inhibitory capacity. Our data suggest that it will be possible in the

future to design and synthesis low molecular weight polyanions with appropriate conformation and chemical attributes for the targeted disruption of fibrin-GPVI interactions.

In conclusion, understanding mechanisms enabling fibrin-GPVI engagement and resultant effects on platelet activation and function, is of fundamental importance to our understanding of how a thrombus is formed and stabilized. Specifically targeting fibrin-GPVI engagement may be a therapeutic option that targets thrombus formation under conditions where there is minimal collagen exposure (sepsis, DIC, and other coagulopathy settings) or in situations where modulation of thrombus stability is important such as in atherothrombosis and stroke.

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CONFLICT OF INTEREST

The authors declare no competing financial interests.

AUTHOR CONTRIBUTION

Contributions: S.J. Montague designed, performed, and interpreted research and wrote the manuscript. S.M. Hicks and C.S-M. Lee interpreted data and processed samples. L.A. Coupland and C.R. Parish supplied crucial reagents and interpreted data. R.K. Andrews and W.M. Lee designed and interpreted research. E.E. Gardiner designed and interpreted research and wrote the manuscript. All authors reviewed the manuscript.

ORCID

Samantha J. Montague https://orcid.org/0000-0002-8934-3901
Sarah M. Hicks https://orcid.org/0000-0001-5712-124X
Christine S-M. Lee https://orcid.org/0000-0002-3851-9364
Lucy A. Coupland https://orcid.org/0000-0002-8912-9534
Christopher R. Parish https://orcid.org/0000-0001-7740-0430
Woei M. Lee https://orcid.org/0000-0002-3912-6095
Robert K. Andrews https://orcid.org/0000-0001-9577-8082
Elizabeth E. Gardiner https://orcid.org/0000-0001-9453-9688

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section.

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