

## ORIGINAL ARTICLE

# Multidimensional flow cytometry reveals novel platelet subpopulations in response to prostacyclin

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## Funding information

The authors would like to thank Heart  
Research UK (RG2659) and the British  
Heart Foundation (RG/16/5/32250 and  
FS/18/75/33978).

## Abstract

**Background:** Robust platelet activation leads to the generation of subpopulations characterized by differential expression of phosphatidylserine (PS). Prostacyclin (PGI<sub>2</sub>) modulates many aspects of platelet function, but its influence on platelet subpopulations is unknown.

**Objectives and Methods:** We used fluorescent flow cytometry coupled to multidimensional fast Fourier transform-accelerated interpolation-based t-stochastic neighborhood embedding analysis to examine the influence of PGI<sub>2</sub> on platelet subpopulations.

**Results:** Platelet activation (SFLLRN/CRP-XL) in whole blood revealed three platelet subpopulations with unique combinations of fibrinogen (fb) binding and PS exposure. These subsets, PS<sup>lo</sup>/fb<sup>hi</sup> (68%), PS<sup>hi</sup>/fb<sup>lo</sup> (23%), and PS<sup>hi</sup>/fb<sup>hi</sup> (8%), all expressed CD62P and partially shed CD42b. PGI<sub>2</sub> significantly reduced fibrinogen binding and prevented the majority of PS exposure, but did not significantly reduce CD62P, CD154, or CD63 leading to the generation of four novel subpopulations, CD62P<sup>hi</sup>/PS<sup>lo</sup>/fb<sup>lo</sup> (64%), CD62P<sup>hi</sup>/PS<sup>lo</sup>/fb<sup>hi</sup> (22%), CD62P<sup>hi</sup>/PS<sup>hi</sup>/fb<sup>lo</sup> (3%), and CD62P<sup>lo</sup>/PS<sup>lo</sup>/fb<sup>lo</sup> (12%). Mechanistically this was linked to PGI<sub>2</sub>-mediated inhibition of mitochondrial depolarization upstream of PS exposure. Combining phosphoflow with surface staining, we showed that PGI<sub>2</sub>-treated platelets were characterized by both elevated vasodilator-stimulated phosphoprotein phosphorylation and CD62P. The resistance to cyclic AMP signaling was also observed for CD154 and CD63 expression. Consistent with the functional role of CD62P, exposure of blood to PGI<sub>2</sub> failed to prevent SFLLRN/CRP-XL-induced platelet-monocyte aggregation despite reducing markers of hemostatic function.

**Conclusion:** The combination of multicolor flow cytometry assays with unbiased computational tools has identified novel platelet subpopulations that suggest differential regulation of platelet functions by PGI<sub>2</sub>. Development of this approach with increased surface and intracellular markers will allow the identification of rare platelet subtypes and novel biomarkers.

## KEYWORDS

cAMP, flow cytometry, mitochondria, platelets, procoagulant, subpopulations

## Essentials

- The influence of prostacyclin (PGI<sub>2</sub>) on platelet subpopulations is unknown.
- Multiparameter flow cytometry was combined with fast Fourier transform-accelerated interpolation-based t-stochastic neighborhood embedding to identify novel PGI<sub>2</sub>-responsive platelet subpopulations based on phosphatidylserine (PS) exposure.
- PGI<sub>2</sub> differentially regulates platelet functions to remodel agonist-induced procoagulant subpopulations.
- PGI<sub>2</sub> preserves mitochondrial function to prevent PS exposure.

## 1 | INTRODUCTION

Platelets are crucial for efficient hemostasis through their ability to rapidly accumulate at sites of vascular trauma.<sup>1,2</sup> The activation of platelets under physiological conditions is controlled primarily through endothelial secreted prostacyclin (PGI<sub>2</sub>) acting through cyclic-AMP (cAMP) and protein kinase A (PKA), and nitric oxide (NO) acting through cyclic-GMP (cGMP) and protein kinase G (PKG).<sup>3</sup> Elevations in cAMP are linked to reduced Ca<sup>2+</sup> mobilization,<sup>4</sup> dense granule secretion, spreading,<sup>5</sup> integrin  $\alpha_{IIb}\beta_3$  activation, and aggregation *in vitro*,<sup>6</sup> and diminished platelet accrual at sites of vascular injury *in vivo*.<sup>3,7</sup> A loss of platelet sensitivity to PGI<sub>2</sub> in diseased states is also linked to increased and uncontrolled activation.<sup>8–10</sup> In other cells cAMP can differentially modulate distinct aspects of cell function through the selective coupling of cAMP signaling complexes to specific substrates or regions within the cell.<sup>11</sup> However, the relationship between cAMP signaling and the control of distinct platelet functions and subpopulations is still unclear.<sup>12</sup>

Potent platelet activation stimulating both protease activated receptor (PAR) and glycoprotein VI (GPVI) receptors leads to the generation of two subpopulations that may enact distinct functional roles.<sup>13</sup> The two groups are typically characterized as proaggregatory and procoagulant,<sup>13</sup> where procoagulant platelets are characterized through  $\Delta\Psi_m$  depolarization,<sup>14</sup> sustained calcium flux,<sup>15</sup> and the expression of phosphatidylserine (PS), which binds to coagulation factors to drive thrombin generation.<sup>16</sup> The second group of platelets is characterized by activated integrin  $\alpha_{IIb}\beta_3$ , and these platelets primarily support platelet aggregation and mechanical clot retraction.<sup>17</sup> Beyond the characteristic expression of PS on procoagulant cells, these subpopulations have also been determined by physical characteristics; it is predominantly smaller platelets that are enriched in PS.<sup>18</sup> These subpopulations likely localize within specific regions of the thrombus, with the procoagulant platelets being highly activated at the thrombi core through their close proximity to both collagen and sites of thrombin generation allowing activation of both GPVI and PAR receptors.<sup>19</sup> Importantly procoagulant platelets have been described in the circulation of trauma patients *ex vivo* confirming they are not an artifact of *in vitro* stimulation.<sup>22</sup>

Previously procoagulant platelets have been identified *in vitro* by fluorescent flow cytometry,<sup>18</sup> fluorescence microscopy,<sup>16</sup> and *ex vivo* flow<sup>20</sup> and imaging flow cytometry.<sup>21</sup> Advances in flow cytometry have increased the number of parameters that can be measured on each cell, allowing subset characterization<sup>23</sup> and when these multiparameter assays are coupled to computational analysis it can allow unprecedented insights into platelet heterogeneity. Recently, elegant mass cytometry (CyTOF) studies showed how an unbiased approach to surface marker characterization suggested the possibility of novel platelet subpopulations.<sup>24–26</sup> With this in mind we combined whole blood multiparameter fluorescent flow cytometry, with a variant of t-stochastic neighborhood embedding (t-SNE), fast Fourier transform-accelerated interpolation-based t-SNE (Fit-SNE) to examine platelet subpopulation formation.<sup>27</sup> This approach allowed us define novel platelet subpopulations after activation, and further demonstrated that under conditions of elevated cAMP the platelet population was remodeled, revealing a population of cells that are principally defined by expression of CD62P and other markers of granule secretion with reduced integrin  $\alpha_{IIb}\beta_3$  activation and PS exposure.

## 2 | METHODS

### 2.1 | Venipuncture

Blood was drawn from healthy adults with informed consent. Venipuncture was performed with a 21G butterfly needle into citrate vacutainers,<sup>28</sup> and the first-drawn tube was discarded to minimize artifactual activation. Some samples were treated with calcium (1.8 mM) to allow annexin V binding<sup>29</sup> and GPRP<sup>30</sup> (500  $\mu$ M) to prevent fibrin polymerization in the presence of calcium.

### 2.2 | Flow cytometry

Samples were run on a Beckman Coulter CytoFLEX RUO flow cytometer with two lasers (488 nm 50 mW and 638 nm 50 mW) and five filters (525/40 BP, 585/42 BP, 660/10 BP, 690/50 BP, and 712/25 BP). Cytometer settings were optimized with a LED pulser (quantiFlash).<sup>31</sup>

Automatic compensation was performed with VersaComp antibody capture beads and CytExpert (v2.1). Antibodies were used at optimal titers, and assays were designed according to platelet flow cytometry guidelines.<sup>26,32,33</sup>

## 2.3 | Activation panels

In all activation panels, whole blood was diluted 1:9 in assay buffer to minimize aggregate formation, then incubated with agonists and antibodies for 20 min at 37°C before fixation with paraformaldehyde solution (0.9%).<sup>34</sup> In some cases, whole blood was pre-incubated with PGI<sub>2</sub> for 2 min before the addition of agonists; PGI<sub>2</sub>-induced inhibitory signaling was shown to last for the duration of the experimental time course by direct measurement of cAMP and pVASPser<sup>157</sup>. Several multicolor panels were developed (Table S1 in supporting information). Platelets were gated on SSC/CD42b, excluding debris and doublets (Figure S1 in supporting information), and 10,000 CD42b+ events were recorded. Gates were set on matched isotypes and/or internal negative (EDTA) controls.

## 2.4 | Surface staining and phosphoflow

A novel application of platelet phosphoflow was designed for this study that allowed the measurement of intracellular markers alongside surface antigens that would otherwise be unable to be measured by a standard phosphoflow protocol. We applied a stain (CD62P), fix, perm, stain (pVASPser<sup>157</sup>) method, unlike routine protocols that measure CD42b and pVASP by fix, perm, and stain.<sup>35</sup> Staining for CD62P prior to fixation and permeabilization ensured that only surface CD62P (and no intracellular CD62P) was detected. Citrated whole blood was incubated with SFLLRN in the presence or absence of PGI<sub>2</sub>. CD62P-PE was co-incubated with agonists (20 min) prior to fixation with BD Phosflow Lyse/Fix Buffer (10 min), permeabilization with Triton X-100 (0.1%/phosphate-buffered saline [PBS], 10 min), primary staining with pVASPser<sup>157</sup> antibody (4°C, 30 min), and secondary staining with Alexa Fluor 488 and CD42b-APC (4°C, 30 min), with PBS washes (1000 g, 10 min) between steps. Platelets were gated on SSC/CD42b, and 10,000 CD42b+ events were recorded. Gates were set on matched isotype controls.

## 2.5 | Data and statistical analysis

Flow cytometry standard (FCS) files were analyzed with CytExpert (v2.1), Cytobank (v7.2), and/or FlowJo (v10). Statistical *T*-tests and two-way analysis of variance with Dunnett's multiple comparisons (>95% confidence) were performed on GraphPad Prism (v9).

## 2.6 | Data sharing statement

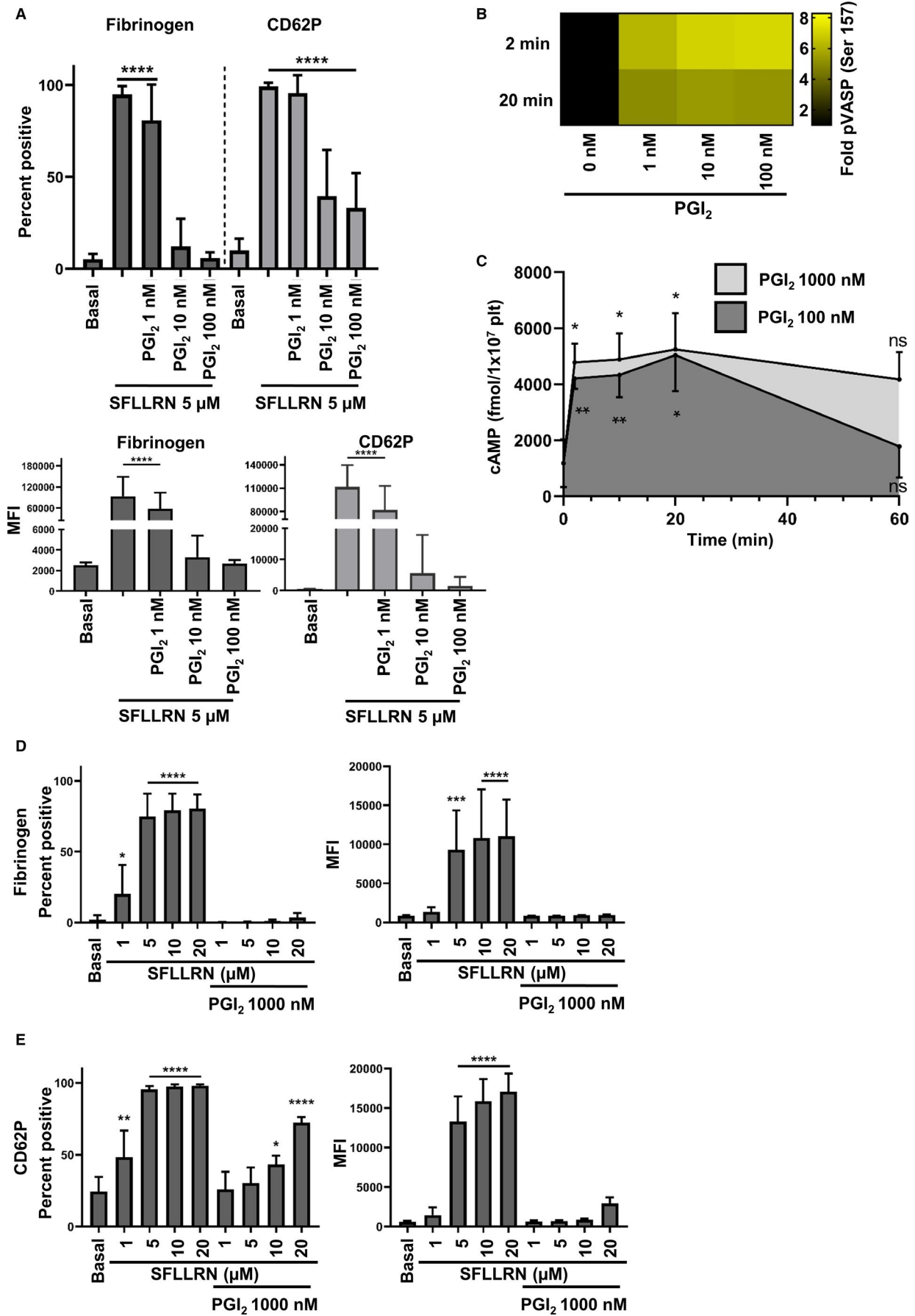
For FCS files, please contact k.naseem@leeds.ac.uk.

# 3 | RESULTS

## 3.1 | Surface markers of platelet activation show differential sensitivity to PGI<sub>2</sub>

Prostacyclin can inhibit multiple aspects of platelet function in a cAMP-dependent manner.<sup>3,6</sup> However, it is unclear if changes in the expression of surface markers that are required for platelets to fulfil their distinct hemostatic and immunological functions are differentially regulated by cAMP signaling. We first tested the effects of PGI<sub>2</sub> on fibrinogen binding and CD62P expression using a three-parameter whole blood flow cytometry assay. SFLLRN (5 μM) treatment induced a significant increase in fibrinogen (fb) binding (94.8 ± 5% positive) and CD62P expression (99.2 ± 2% positive; Figure 1A). Pre-incubation with PGI<sub>2</sub> (1–100 nM) led to the dose-dependent inhibition of both fibrinogen binding and CD62P expression. However, fibrinogen binding was more sensitive to inhibition by PGI<sub>2</sub> than CD62P expression. Using PGI<sub>2</sub> (100 nM), fibrinogen binding was reduced to basal levels (5.9 ± 3%) while surface CD62P remained significantly elevated (33.1 ± 18%, *P* < .0001 compared to basal). This observation was confirmed by similar experiments performed with the monoclonal antibody PAC-1, which recognizes activated integrin α<sub>IIb</sub>β<sub>3</sub>, and which demonstrated a significant inhibition of integrin α<sub>IIb</sub>β<sub>3</sub> activation in response to PGI<sub>2</sub>, while CD62P remained elevated (Figure S2 in supporting information). To ensure that the differences between the two markers were not due to time-dependent variations in cAMP signaling or a degradation of inhibitory signal, we measured phosphoVASPser<sup>157</sup> with whole blood

**FIGURE 1** Differences in sensitivity to inhibition of fibrinogen binding and CD62P expression by prostacyclin (PGI<sub>2</sub>). A, Whole blood was stimulated with SFLLRN (5 μM) in the presence and absence of PGI<sub>2</sub> (1–100 nM). Fibrinogen binding (dark grayscale) and CD62P (light grayscale) were measured simultaneously on CD42b-positive cells using flow cytometry. Data are expressed as % positive cells and mean fluorescence intensity (MFI) for the individual markers. Comparisons were made against basal (mean ± standard deviation [SD]; *n* = 42). B, pVASPser<sup>157</sup> was measured in whole blood by phosphoflow cytometry in paired samples to (A) at matched doses of PGI<sub>2</sub> (1–100 nM) at 2 and 20 min poststimulation and data expressed as fold pVASPser<sup>157</sup> over basal (*n* = 42). C, Washed platelets (1 × 10<sup>8</sup> platelets/ml) were treated with PGI<sub>2</sub> (100 and 1000 nM) for up to 60 min before lysis and measurement of cyclic AMP (cAMP). Data are expression as fmol cAMP/1 × 10<sup>8</sup> platelets (mean ± SD; *n* = 3 [100 nM] and *n* = 5 [1000 nM]). (Two-way analysis of variance [ANOVA] with multiple comparisons vs. basal; ns=non-significant, \*\*\*<0.005, \*\*\*\*<0.0001.) D, Fibrinogen binding measured as in (A) except platelets were treated with varying concentration of either SFLLRN (1–20 μM) +/- PGI<sub>2</sub> (1000 nM), comparisons made with basal (mean ± SD; *n* = 5). E, as in (D) except CD62P was measured (mean ± SD; *n* = 5). F, As in (D/E) except CD42b was measured (mean ± SD; *n* = 5). (Two-way ANOVA with multiple comparisons; \*<0.05, \*\*<0.01, \*\*\*<0.005 and \*\*\*\*<0.0001)



phosphoflow as well as cAMP concentrations in washed platelets. In donor-matched whole blood, pVASPser<sup>157</sup> was phosphorylated by PGI<sub>2</sub> (1–100 nM) in a dose-dependent manner and was sustained for up to 20 min, which was the timepoint used in all assays (Figure 1B). In washed platelets, PGI<sub>2</sub> (100 and 1000 nM) caused a significant increase in cAMP. After 20 min the concentrations of cAMP were diminished in comparison to the 5 min peak but remained significantly elevated over basal ( $P < .0001$ ; Figure 1C). These two sets of data confirm that cAMP signaling was sustained throughout the experimental timeline of 20 min and that observations were not due to a loss of inhibitory signaling.

To further characterize the differential regulation of fibrinogen binding and CD62P expression by PGI<sub>2</sub>, we used increasing concentrations of SFLLRN (1–20  $\mu$ M) with a supra-physiological dose of PGI<sub>2</sub> (1000 nM). Without the inhibitors present SFLLRN caused a concentration-dependent increase in platelet activation with similar dose-dependent kinetics in both fibrinogen binding and CD62P expression. However, treatment with PGI<sub>2</sub> (1000 nM) prevented fibrinogen binding (Figure 1D), while CD62P expression remained elevated (Figure 1E). To explore the potential for a dichotomy in inhibitory responses between fibrinogen binding and CD62P we examined the ability of PGI<sub>2</sub> to reverse platelet activation by adding PGI<sub>2</sub> (1000 nM) 20 min after the addition of agonists. Here trends of inhibition similar to when PGI<sub>2</sub> was added before the agonists were observed, with the prostanoid causing a modest reversal of fibrinogen binding (Figure S12Ai in supporting information) and PAC1 (Figure S12Bi), while CD62P again remained insensitive to the inhibitory effects of PGI<sub>2</sub> (Figure S12Aii). Together, these data suggest a dichotomy in PGI<sub>2</sub>-mediated regulation of fibrinogen binding and CD62P expression independent of sustained PGI<sub>2</sub>-cAMP inhibitory signaling.

### 3.2 | Multicolor flow cytometry identifies novel platelet subsets

Several studies have previously identified distinct procoagulant or proaggregatory subsets of platelets, characterized primarily by elevated surface PS expression.<sup>16,18,20,21</sup> To better characterize these subpopulations, we developed a four parameter whole blood assay for the simultaneous measurements of fibrinogen binding, PS exposure, and CD62P and CD42b expression. The data were analyzed using FIt-SNE to cluster phenotypically similar platelets in two-dimensional space.<sup>24,25</sup> Under basal conditions, most platelets were low in markers of activation but high in CD42b (Figure 2A, upper panel). We first demonstrated that dual stimulation with SFLLRN (20  $\mu$ M) and CRP-XL (10  $\mu$ g/ml) induced PS exposure (Figure S3 in supporting information), consistent with other studies.<sup>18</sup> FIt-SNE analysis differentiated platelets into three distinct subsets, PS<sup>lo</sup>/fb<sup>hi</sup>/CD62P<sup>hi</sup> (68%), PS<sup>hi</sup>/fb<sup>lo</sup>/CD62P<sup>hi</sup> (23%), and PS<sup>hi</sup>/fb<sup>hi</sup>/CD62P<sup>hi</sup> (8%; Figure 2A lower panel and Figure 2B). The PS<sup>hi</sup> subpopulation was differentiated into two subsets based on differences in fibrinogen binding. CD62P was expressed on all stimulated platelets independent of

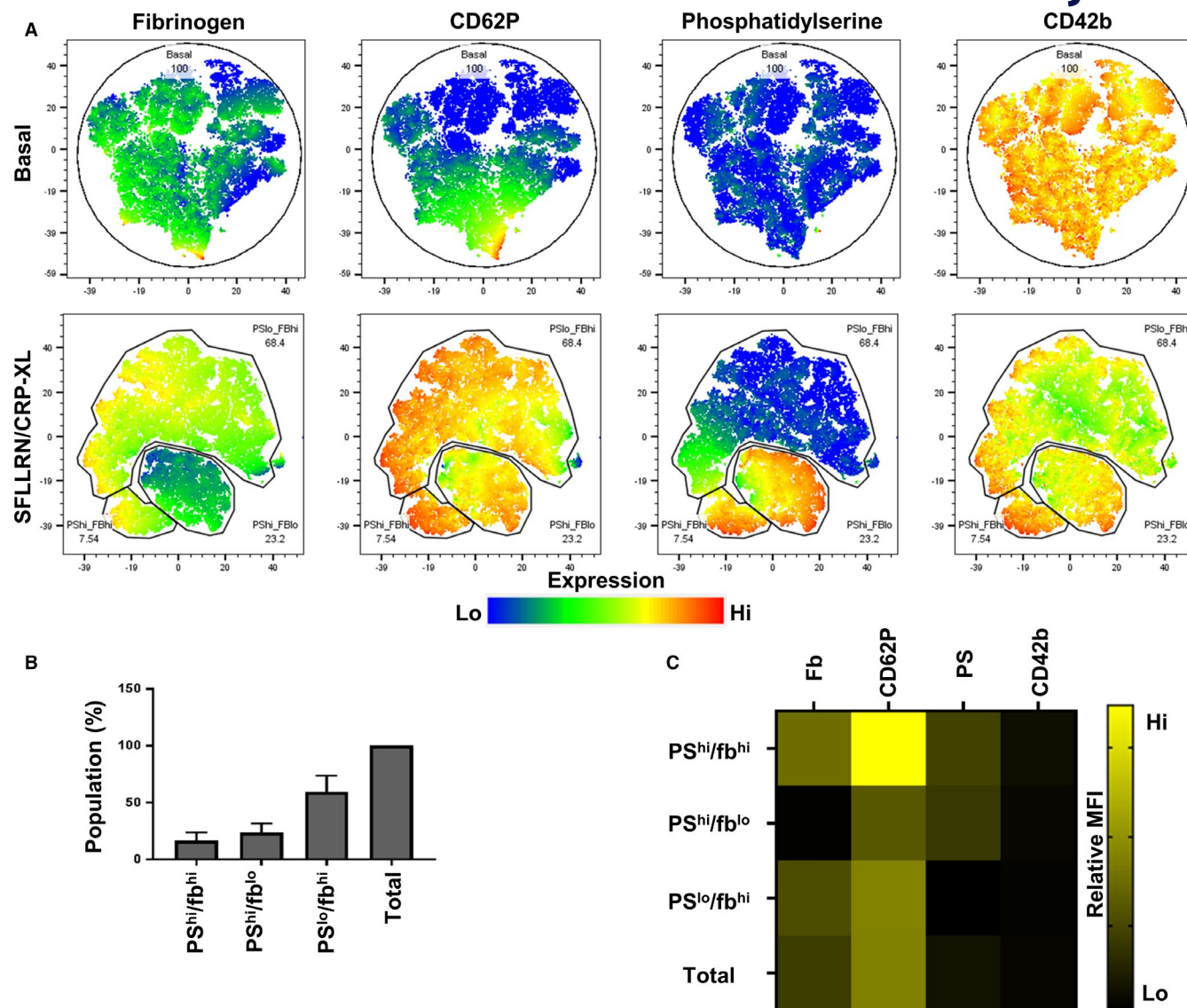
their respective subset, but it was particularly high on the PS<sup>hi</sup>/fb<sup>hi</sup> subset (Figure 2C and Figure S4 in supporting information), possibly suggesting a hyper-responsive subset of cells. In addition to this, we observed that the surface expression of CD42b was reduced on all cells, more so on the proaggregatory PS<sup>lo</sup>/fb<sup>hi</sup> subset (Figure 2C). In order to validate our findings, the anti-fibrinogen antibody was substituted for PAC-1, and consistent with the previous experiments the PS<sup>hi</sup> subpopulation was differentiated based on PAC-1 (integrin  $\alpha_{IIb}\beta_3$  activity; Figure S5 in supporting information). To confirm our findings by FIt-SNE, we also performed manual gating,<sup>32</sup> and consistent with earlier data we identified the same subsets (Figure S6 in supporting information). Together these data suggest that PS<sup>hi</sup> platelets can be differentiated into subsets based on differential binding of fibrinogen to two unique subsets.

### 3.3 | Elevated cAMP prevents formation of procoagulant platelets and creates CD62P+ subsets on activation

We next explored if the differential inhibition of platelet functions by PGI<sub>2</sub> shown in Figure 1 was shared with other markers, with emphasis on how PGI<sub>2</sub> affects platelet subpopulations defined by PS exposure. Activation of whole blood after pretreatment with PGI<sub>2</sub> (100 nM) led to the generation of four platelet subpopulations upon stimulation with SFLLRN (20  $\mu$ M) and CRP-XL (10  $\mu$ g/ml). PGI<sub>2</sub>, as anticipated, restricted fibrinogen binding, although it also restricted PS exposure, while CD62P was retained on the surface (Figure 3A, lower panel and Figure 3B). Consistent with prior observations CD62P expression remained significantly elevated over basal (Figure 3A, lower panel) whether analyzed by mean fluorescence intensity (MFI; Figure 3B,C) or percent positive cells (Figure S7 in supporting information), while other markers of fibrinogen binding and PS exposure were inhibited. As a result of elevated cAMP signaling, several CD62P<sup>hi</sup> subsets emerged, CD62P<sup>hi</sup>/PS<sup>lo</sup>/fb<sup>lo</sup> (64%), CD62P<sup>hi</sup>/PS<sup>lo</sup>/fb<sup>hi</sup> (22%), CD62P<sup>hi</sup>/PS<sup>hi</sup>/fb<sup>lo</sup> (3%), and CD62P<sup>lo</sup>/PS<sup>lo</sup>/fb<sup>lo</sup> (12%). Therefore, under conditions of elevated cAMP and dual agonist stimulation, platelet subsets are remodeled, with most cells expressing CD62P (Figure 3C).

Depolarization and loss of mitochondrial membrane potential has been implicated as a precursor to PS exposure.<sup>14,15,18</sup> Because we had observed an inhibition of PS exposure in response to PGI<sub>2</sub> we performed a two-parameter washed platelet assay measuring PS exposure and mitochondrial membrane potential (tetramethylrhodamine ethyl ester [TMRE]). Basal washed platelets stained positive for TMRE (86%), indicating mitochondrial polarization, which was coupled to low PS exposure (5%). Platelet activation led to the generation of PS-positive cells (78%), which were negative for TMRE indicative of mitochondrial depolarization (Figure 3D and Figure S8 in supporting information). Importantly, pretreatment with PGI<sub>2</sub> significantly reduced the number of PS-positive cells (30%) and was correlated with elevated levels of platelet TMRE, where mitochondrial polarization was no longer significantly different to basal





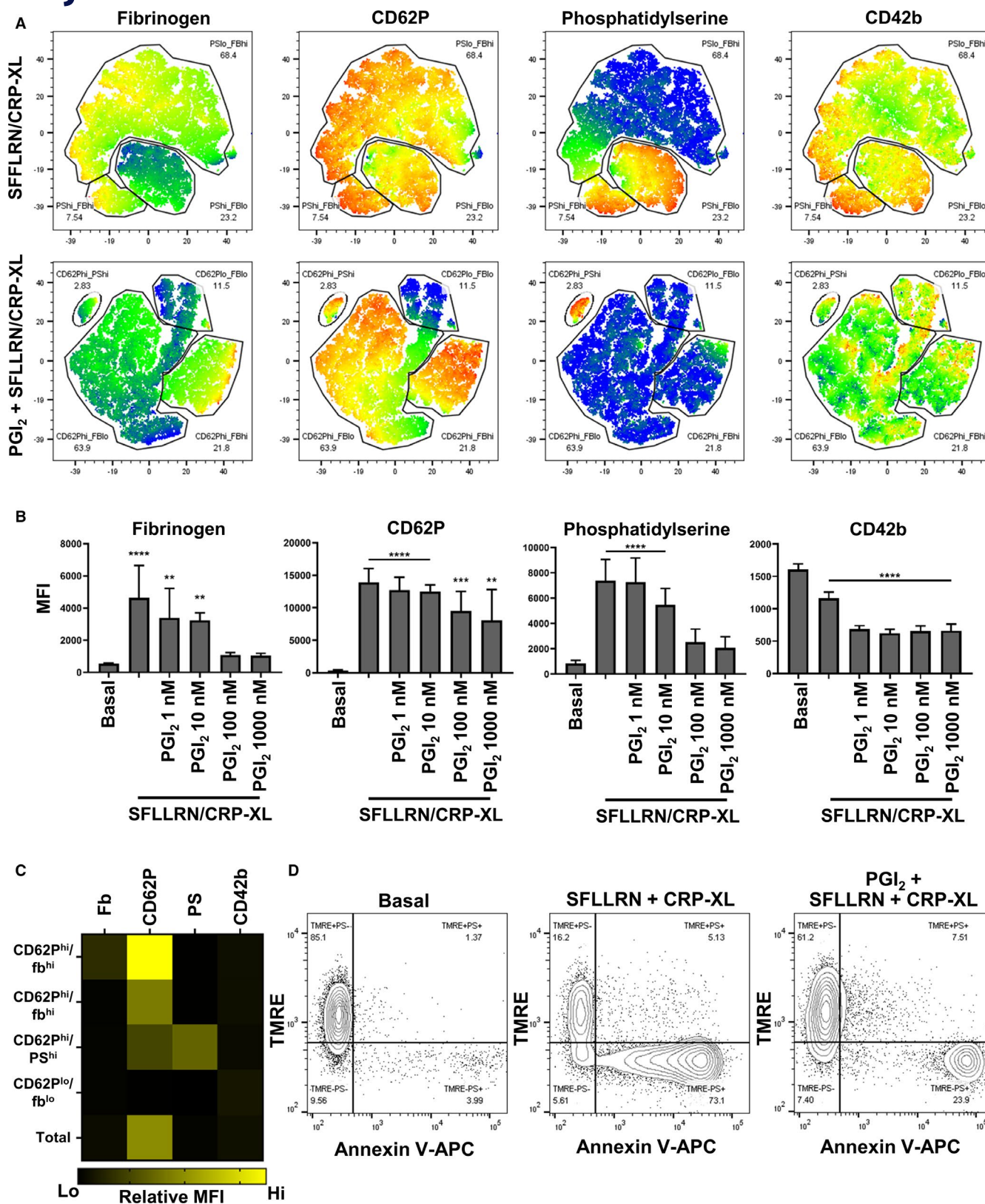
**FIGURE 2** The platelet procoagulant subsets characterized by multidimensional analysis. A, Whole blood was stimulated with SFLLRN (20  $\mu$ M) and CRP-XL (10  $\mu$ g/ml) for 20 min before fixation and staining with anti-Fibrinogen-FITC, annexin V-APC, CD62P-PE, and CD42b-BB700. Data were analyzed by fast Fourier transform-accelerated interpolation-based t-stochastic neighborhood embedding (Fit-SNE) and plots pseudocolored for marker expression (concatenated data,  $n = 3$ ). B, Percentage of platelets within each identified subset from separate non-concatenated Fit-SNE analysis is compared for platelets treated as in A (mean  $\pm$  standard deviation;  $n = 3$ ). C, Heatmap of individual marker expression, mean fluorescence intensity (MFI), within each identified subset from concatenated Fit-SNE analysis of platelets stimulated as in A;  $n = 3$

(Figure 3D and Figure S8). In the presence of PGI<sub>2</sub>, the cells that would have previously expressed PS were maintained in a resting mitochondrial phenotype. These data both confirm that mitochondrial membrane depolarization is associated with PS exposure and suggest that cAMP signaling preserves mitochondrial function, which contributes to inhibiting PS exposure.

### 3.4 | CD62P expression is resistant to cAMP signaling

While we previously demonstrated that the dichotomy in inhibition was not altered by time-associated loss of cAMP signaling (Figure 1B)

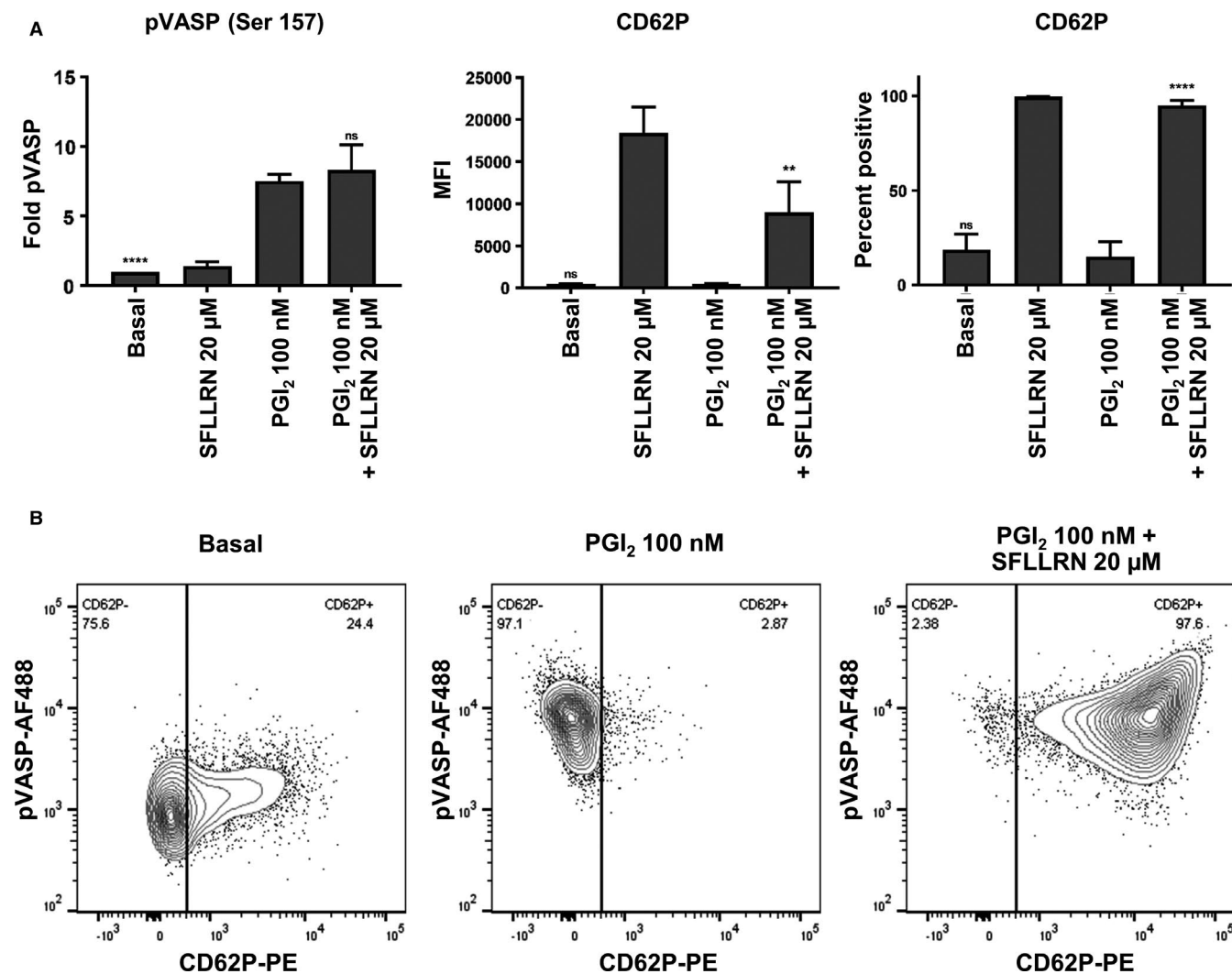
or cAMP concentrations (Figure 1C), we next explored whether this was due to different sensitivities of the PGI<sub>2</sub>-cAMP pathway within the platelet population. We adapted our phosphoflow protocol to create a novel assay that allowed the simultaneous measurement of both functional surface CD62P and CD42b as well as intracellular signaling marker pVASPser<sup>157</sup>. PGI<sub>2</sub> (100–1000 nM) treatment of whole blood led to increases in pVASPser<sup>157</sup>, which remained unchanged in the presence of SFLLRN (20  $\mu$ M) at the higher and supra-physiological dose (100 and 1000 nM, respectively; Figure 4A and Figure S9 in supporting information). At all doses of PGI<sub>2</sub> co-treated with SFLLRN, CD62P remained elevated in both MFI and percent positive cells (>90%). The data presented as biaxial plots showed that CD62P was highly expressed in conjunction with high levels of



pVASPser<sup>157</sup> on all cells with no subsets of cells that were less or more sensitive to inhibition or activation (Figure 4B). Together these demonstrate that, despite elevated pVASPser<sup>157</sup> indicative of active inhibitory signaling, CD62P expression on the same populations of cells was maintained after stimulation with potent agonists.

To further validate and explore the phenotype where agonist-induced CD62P expression was independent of aspects of cAMP signaling, we measured other markers of platelet granule secretion. A new four-parameter whole blood panel was developed to allow simultaneous assessment of CD154 ( $\alpha$ -granule), CD62P ( $\alpha$ -granule),

**FIGURE 3** CD62P-enriched platelet subsets predominate under conditions of elevated cyclic AMP (cAMP). A, Whole blood was stimulated with SFLLRN (20  $\mu$ M) and CRP-XL (10  $\mu$ g/ml) in the presence and absence prostacyclin (PGI<sub>2</sub>; 100 nM) before fixation and staining with anti-fibrinogen-FITC, annexin V-APC, CD62P-PE, and CD42b-BB700. Data were analyzed by fast Fourier transform-accelerated interpolation-based t-stochastic neighborhood embedding (Fit-SNE) and plots pseudocolored for marker expression (concatenated data,  $n = 3$ ). B, Data for (A) shown for increasing concentrations of PGI<sub>2</sub> expressed as mean fluorescence intensity (MFI; mean  $\pm$  standard deviation;  $n = 5$ ). (Analyzed by two-way analysis of variance with multiple comparisons against basal; \*\* $<0.01$ , and \*\*\*\* $<0.0001$ .) C, Heatmap of individual marker expression, MFI, within each identified subset from concatenated Fit-SNE analysis of platelets stimulated as in (A;  $n = 3$ ). D, Washed platelets ( $4 \times 10^7$ /ml) were stimulated with SFLLRN (20  $\mu$ M) and CRP-XL (10  $\mu$ g/ml) in the presence and absence PGI<sub>2</sub> (100 nM) and stained for tetramethylrhodamine, ethyl ester (TMRE) and annexin V-APC. Representative biaxial contour plots for TMRE and Annexin V-APC ( $n = 3$ )

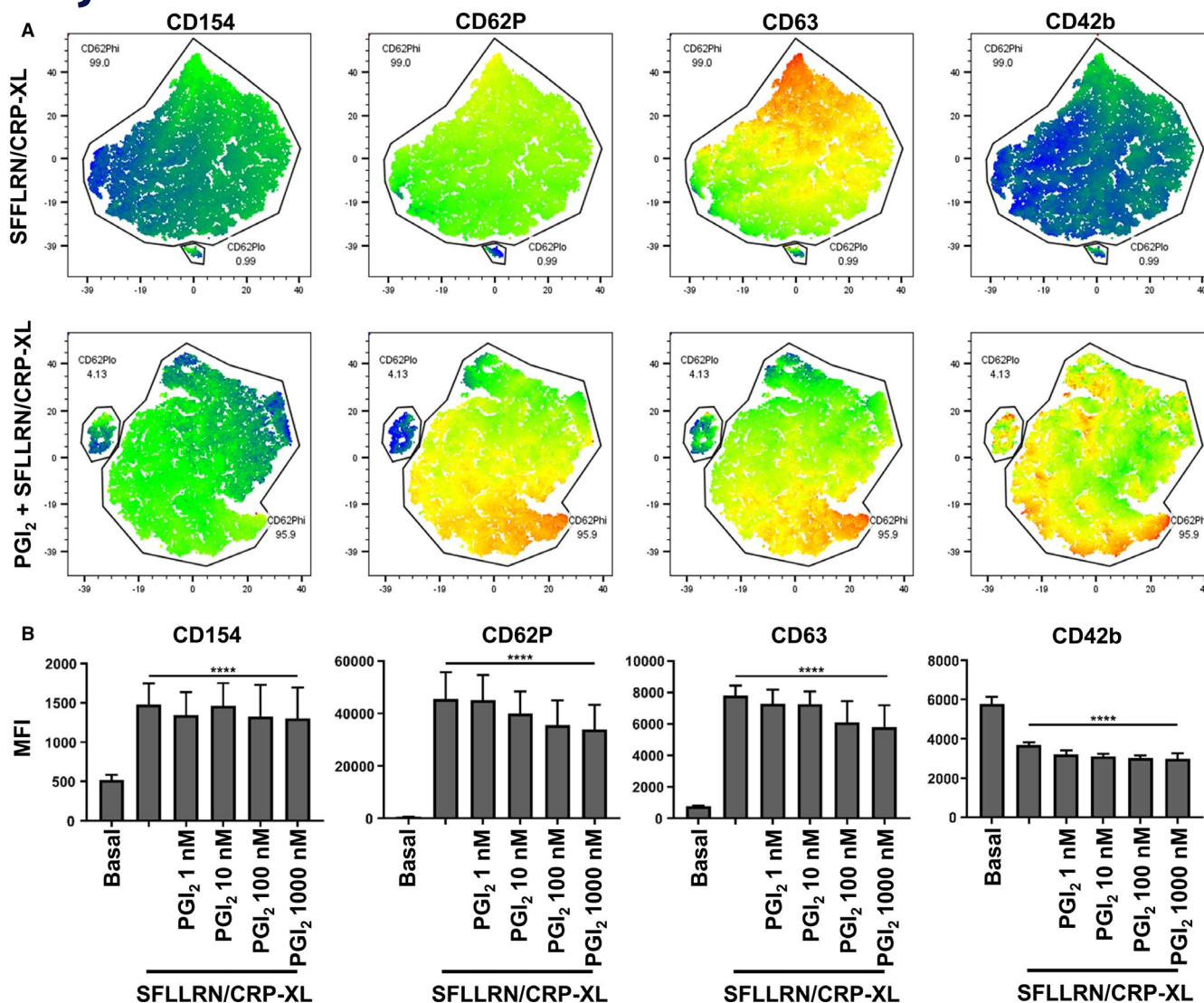


**FIGURE 4** Simultaneous assessment of intracellular cyclic AMP (cAMP) signaling and platelet CD62P expression in whole blood. A, Whole blood was stimulated with SFLLRN (20  $\mu$ M) in the presence and absence of prostacyclin (PGI<sub>2</sub>; 100 nM) before fixation; permeabilization; and staining with anti-CD62P-PE, anti-pVASP<sup>157</sup>, and CD42b-BB700. Data are expressed as fold increase in mean fluorescence intensity (MFI) for pVASP<sup>157</sup> or MFI and percent positive cells for CD62P (mean  $\pm$  standard deviation;  $n = 3$ ). (Two-way analysis of variance with multiple comparisons as 100 nM vs. 100 nM + SFLLRN; \* $<0.05$ , \*\* $<0.01$ , \*\*\*\* $<0.0001$ .) B, Biaxial contour plots of population fluorescence for pVASP<sup>157</sup> and CD62P (representative plots of  $n = 3$ )

CD63 ( $\delta$ -granule), and CD42b expression. Fit-SNE analysis of strongly activated platelets (SFLLRN [20  $\mu$ M] and CRP-XL [10  $\mu$ g/ml]) showed that all granule markers increased when platelets were stimulated with dual agonists, with no differentiation between the individual markers (Figure 5A, upper panel). After treatment with PGI<sub>2</sub> (1–1000 nM), all three markers of degranulation remained

significantly elevated on the platelet surface (Figure 5B and Figure S10 in supporting information) and continued to co-localize when analyzed by Fit-SNE (Figure 5A, lower panel). This indicates that the entire platelet population shares a phenotype of granule secretion independent of PGI<sub>2</sub>-cAMP signaling in the context of robust activation.





**FIGURE 5** Granule secretion is not prevented in the presence of inhibition. A, Whole blood was stimulated with SFLLRN (20  $\mu$ M) and CRP-XL (10  $\mu$ g/ml) for 20 min in the presence and absence of prostacyclin (PGI<sub>2</sub>; 1–1000 nM) before fixation and staining with anti-CD154-FITC, CD62P-PE, CD63-EF660, and CD42b-BB700. Data were analyzed by fast Fourier transform-accelerated interpolation-based t-stochastic neighborhood embedding and plots pseudocolored for marker expression (concatenated data,  $n = 3$ ). B, As in (A) with data expressed as mean fluorescence intensity (MFI; mean  $\pm$  standard deviation;  $n = 5$ ); two-way analysis of variance with multiple comparisons vs. basal; \*\*\*\* $<0.0001$ )

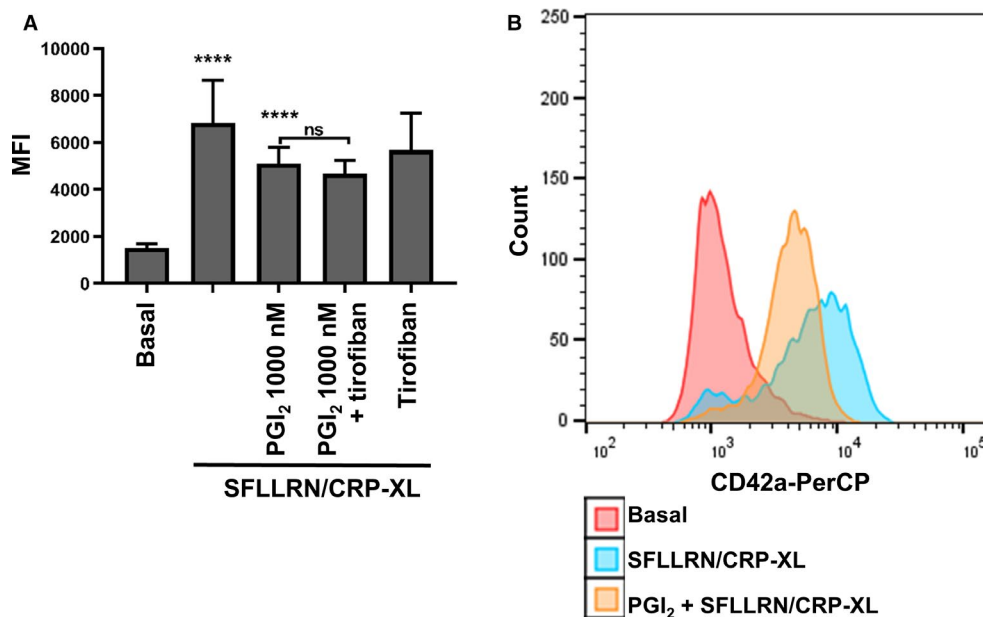
### 3.5 | Platelet-monocyte aggregation occurs in the presence of cAMP

Given that CD62P and CD154 are well described as key mediators of platelet-leukocyte interactions,<sup>36,37</sup> we assessed the effect of PGI<sub>2</sub> on platelet-monocyte aggregation (PMA), reasoning that it may be independent of PGI<sub>2</sub>-cAMP signaling under the previously explored experimental conditions. Here, monocytes were characterized as CD14<sup>+</sup> cells (Figure S11 in supporting information), and CD42a was used as a marker of platelet adhesion to monocytes. The co-stimulation of whole blood with SFLLRN (20  $\mu$ M) and CRP-XL (10  $\mu$ g/ml) led to a significant increase in PMA. Preincubation of the blood with a supra-physiological concentration of PGI<sub>2</sub> (1000 nM), which we have shown inhibits aspects of platelet activation and elevates

cAMP, did not prevent the formation of these heterotypic cell aggregates (Figure 6A,B). The  $\alpha_{IIb}\beta_3$  integrin blocker Tirofiban did not potentiate PGI<sub>2</sub> (1000 nM)-induced inhibition and mimicked the effects of PGI<sub>2</sub> alone, suggesting that PGI<sub>2</sub> is blocking PMA regulated by fibrinogen binding (Figure 6A). Together these results suggest that the previously observed PGI<sub>2</sub>-cAMP resistant platelet granule secretion allows PMA to form independent of platelet-fibrinogen interactions.

## 4 | DISCUSSION

Numerous studies have suggested that activated platelets are a heterogeneous population of cells differentiated into PS-enriched procoagulant and integrin  $\alpha_{IIb}\beta_3$ -enriched subpopulations with



**FIGURE 6** Platelet monocyte aggregates continue to form in the context of inhibition and are not dependent on fibrinogen. A, Platelet monocyte aggregates are significantly increased in the presence of SFLLRN (20  $\mu$ M) and CRP-XL (10  $\mu$ g/ml) and blunted with the addition of prostacyclin (PGI<sub>2</sub>; 1000 nM). There was no significant additive inhibition on top of PGI<sub>2</sub> alone when co-treated with tirofiban (1  $\mu$ g/ml; mean  $\pm$  standard deviation;  $n = 5$ ). (Two-way analysis of variance with multiple comparisons vs. basal and 1000 nM vs. 1000 nM +tirofiban where indicated; ns=non-significant and \*\*\*\*<0.0001.) B, Representative histogram of data presented in (A)

distinct functional capabilities.<sup>13</sup> This functional heterogeneity may allow platelets to assume distinct roles in the thrombotic process *in vivo*.<sup>38</sup> Where aggregatory platelets facilitate platelet-platelet interactions and clot retraction,<sup>17</sup> procoagulant platelets support coagulation.<sup>2</sup> The complexity of any model of platelet activation is increased by PGI<sub>2</sub> and NO, which act as a tonic inhibitors of platelets in circulation.<sup>3</sup> In this study we concentrated on the effects of PGI<sub>2</sub>, although previous work has suggested that NO-mediated signaling may also dictate heterogeneity in platelet function.<sup>39</sup> In the whole blood model used here, the use of NO donors would require micromolar doses to induce a response,<sup>40</sup> due to the quenching effects of hemoglobin, making it less compatible with this approach than PGI<sub>2</sub>, although the effects of NO on platelet subpopulations should be explored in the future. The aim of the present study was to apply multiparameter fluorescent flow cytometry and multidimensional data analysis to further characterize these subsets and how they may be controlled by the endothelial inhibitor PGI<sub>2</sub>. Our key findings include: (1) platelet activation reveals three subsets that can be differentiated by unique combinations of PS, fibrinogen binding, and CD62P; (2) under conditions of elevated cAMP platelet activation reveals four distinct subsets, which primarily express markers of granule secretion with low levels of PS exposure and bound fibrinogen; and (3) platelet granule secretion after robust stimulation in whole blood shows resistance to the inhibitory effect of PGI<sub>2</sub>-cAMP.

One of our key aims was to harness new cytometry analysis tools to obtain a deeper understanding of platelet heterogeneity, which cannot be obtained using traditional low-content manual gating

approaches. The development of a four-parameter assay allowed measurement of CD62P and CD42b expression alongside PS exposure and fibrinogen binding. This approach combined with multidimensional Flt-SNE analysis allowed us to identify three distinct subpopulations, which we termed PS<sup>hi</sup>/fb<sup>hi</sup>, PS<sup>hi</sup>/fb<sup>lo</sup>, and PS<sup>lo</sup>/fb<sup>hi</sup>. The two procoagulant subsets were differentiated primarily by their capacity to bind fibrinogen (PS<sup>hi</sup>/fb<sup>hi</sup> and PS<sup>hi</sup>/fb<sup>lo</sup>). The existence of a PS<sup>hi</sup>/fb<sup>hi</sup> subset has been controversial and has been suggested to be artifactual measurement of doublet events.<sup>14,41</sup> In order to assess this possibility we analyzed the platelet cloud for both characteristic increases in pulse width (FSC-W) and the fluorescent markers (CD42b) that would be indicative of doublets and showed doublets were not included in our analysis, confirming that PS<sup>hi</sup>/fb<sup>hi</sup> is a novel subset of procoagulant human platelets. Our analysis of forward scatter pulse also supports previous studies that it is predominantly smaller platelets that become procoagulant and larger that become aggregatory.<sup>18</sup> Given the functional dichotomy of procoagulant and aggregatory platelets, the role of the intermediate subset (PS<sup>hi</sup>/fb<sup>hi</sup>) that expresses both markers is unclear. However, it may represent a group of cells that acts to integrate the two arms of platelet function (aggregation and coagulation) or a hyperactivated subset of platelets.<sup>2</sup> As the assay allowed us to measure four markers simultaneously, CD42b was shown to be reduced in expression on stimulation, primarily on the PS<sup>lo</sup> subsets, potentially due to sheddase activity<sup>42</sup> or receptor internalization.<sup>43</sup> We also observed that CD62P was expressed on all platelets. Therefore, a more complete description of each of these subsets is CD62P<sup>hi</sup>/PS<sup>hi</sup>/fb<sup>hi</sup>/CD42b<sup>hi</sup>, CD62P<sup>hi</sup>/PS<sup>hi</sup>/fb<sup>lo</sup>/CD42b<sup>hi</sup>, and CD62P<sup>hi</sup>/PS<sup>lo</sup>/fb<sup>hi</sup>/CD42b<sup>lo</sup>. This understanding

of the subsets formed under conditions of robust activation may allow for further detailed analysis of their function including the roles these subsets may play in the growing thrombi. Furthermore, given that an increased presence of procoagulant platelets (PS<sup>hi</sup>) is associated with an increased risk of stroke,<sup>44</sup> transient ischaemic attack (TIA),<sup>45</sup> and has been associated with trauma,<sup>22</sup> our assay could be deployed to understand how functional subpopulations, or indeed PS<sup>hi</sup> subsets, change in disease. Given that a previous study has shown a subset of platelets that are packaged with tissue factor to facilitate coagulation, whether these same cells also go on to become procoagulant has not yet been explored although it seems likely given the shared function, that these same cells may go on to become procoagulant.<sup>46</sup> Undoubtedly there are further biomarkers to be described and this may be where the platform of CyTOF supersedes fluorescent flow cytometry as many more parameters can be simultaneously screened.<sup>24–26</sup> However, where single-parameter platelet fluorescent flow cytometry is performed diagnostically this could be expanded to include panels of markers described here, allowing further aspects of platelet function to be examined within the clinical setting without requiring the additional complexity of CyTOF.<sup>47</sup>

In this study we were able to observe a dichotomy in inhibition of different aspects of platelet function. We found that PGI<sub>2</sub> failed to inhibit agonist-induced CD62P despite reducing fibrinogen binding in the same platelets. In support of this observation, a recent study demonstrated agonist-dependent platelet sensitivity to PGI<sub>2</sub>, where CD62P expression had far greater resistance to PGI<sub>2</sub>-mediated inhibition than fibrinogen-mediated aggregation in paired samples that were stimulated with CRP-XL.<sup>48</sup> Exploring this observation further with our new assay and analysis, we show for the first time that PGI<sub>2</sub> can reduce the generation of procoagulant subsets. The formation of procoagulant platelets has been shown to require a sustained increase in Ca<sup>2+</sup> and subsequent mitochondrial depolarization.<sup>13</sup> To further this we demonstrate that PGI<sub>2</sub> blocks agonist-induced mitochondrial membrane depolarization, suggesting that cAMP likely blocks Ca<sup>2+</sup> flux which in turn protects the mitochondria from depolarization and prevents downstream PS exposure. In support of this theory our previous work has demonstrated that elements of Ca<sup>2+</sup> signaling show resistance to supraphysiological concentrations of PGI<sub>2</sub>,<sup>4</sup> and could suggest that under conditions of robust platelet activation cAMP signaling preferentially targets pathways leading to PS exposure and integrin activation. However, as the platelet subpopulations are not separated until the activated fixed cells have been acquired and Fit-SNE analysis has been performed, the measurement of calcium signaling in this setting remains currently impractical.

Further exploration of the activation of platelets in the presence of elevated cAMP signaling led to the generation of four populations of platelets that are dominated by CD62P expression and show an overall reduction in fibrinogen binding and PS exposure. We supported these findings by examining platelet inhibition in the context of reversal of activation, as opposed to prevention. Previously the role of reversal of platelet activation has been explored *in vitro*<sup>5,49</sup>

and *in vivo*;<sup>19,50</sup> here in whole blood we demonstrated that fibrinogen binding, and its precursor integrin  $\alpha_{IIb}\beta_3$  activation, were both sensitive to postactivation inhibition, whereas CD62P remained insensitive. To fully demonstrate the resistance of CD62P to inhibition by PGI<sub>2</sub>, in many experiments we increased the dose of PGI<sub>2</sub> to a supraphysiological level (1000 nM) to confirm this resistance, although where included experimentally results were consistent with those at a lower dose of PGI<sub>2</sub> (100 nM). Further investigation using a separate multiparameter assay for independent validation showed that the context-dependent CD62P-positive population was also enriched in other granule markers CD154 and CD63. To understand the wider physiological relevance of these findings we measured platelet-monocyte adhesion in whole blood, as it is established to occur through CD62P<sup>36</sup> and CD154.<sup>37</sup> In these experiments, PMA was not significantly inhibited by PGI<sub>2</sub>. This suggests that cAMP-independent CD62P on the platelet surface is competent to support heterotypic cell-cell interactions in whole blood, while under the same conditions procoagulant and aggregatory subsets are diminished. This may drive a platelet phenotype in which these cells are inhibited in their potential to interact with other platelets, but remain highly reactive in terms of association with both endothelium and leukocytes.

In many cells, cAMP modulates distinct aspects of cell function through the selective coupling of its signaling complexes to specific substrates.<sup>12</sup> Previously we have shown evidence of compartmentalization of cAMP signaling,<sup>12,51</sup> with individual PKA isoforms targeting distinct components of activatory machinery, although we are unable to comment on this here as non-imaging flow cytometry cannot reveal the localization of signaling molecules.<sup>23</sup> However, we were able to confirm that cAMP signaling in platelets, as evidenced by increased pVASPser<sup>157</sup>, was elevated in all platelets that were CD62P positive. While other studies have examined the role of cAMP in platelet function, much of the data regarding this, including CD62P expression,<sup>52</sup> has been gained from *in vitro* studies using mimetics that act as global cAMP modulators or bypass adenylyl cyclase, as well as pharmacological inhibitors that have off-target effects.<sup>52,53</sup> This means that physiological nuances in the regulation of function by PGI<sub>2</sub> may well be lost as several steps in the signaling pathway are avoided. We chose to focus on using PGI<sub>2</sub> as opposed to synthetic ligands as this represents the physiological messenger found in the blood that may drive the subcellular compartmentalization needed for differential regulation of platelet function. However, how this targeting of cAMP to distinct platelet functions is achieved is unknown and requires further investigation.

In summary, we have developed new fluorescent flow cytometry assays for whole blood platelet analysis linked to computational tools to demonstrate increasing levels of heterogeneity within platelet subpopulations. Further development of these approaches using greater numbers of markers will allow the high throughput identification and characterization of rare platelet populations that may impact disease processes and lead to the translation of novel biomarkers into a clinical setting.

## ACKNOWLEDGMENTS

We thank Dr. Kate Downes (University of Cambridge) for access to blood donors and we thank Prof. Robert Ariens (University of Leeds) for donating reagents.

## CONFLICT OF INTEREST

The authors declare no competing interests.

## AUTHOR CONTRIBUTIONS

MSH designed the research, performed experiments, analyzed the data, and wrote the manuscript. BEJS designed and performed experiments and co-wrote the manuscript. LTC and BAW performed experiments. KMN designed the research and wrote the manuscript.

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## SUPPORTING INFORMATION

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

**How to cite this article:** Hindle MS, Spurgeon BE, Cheah LT, Webb BA, Naseem KM. Multidimensional flow cytometry reveals novel platelet subpopulations in response to prostacyclin. *J Thromb Haemost*. 2021;19:1800-1812. <https://doi.org/10.1111/jth.15330>