Single platelet variability governs population sensitivity and initiates intrinsic heterotypic responses

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Investigations into the nature of platelet functional variety and consequences for homeostasis require new methods for resolving single platelet phenotypes. Here we combine droplet microfluidics with flow cytometry for high throughput single platelet function analysis. A large-scale sensitivity continuum was shown to be a general feature of human platelets from individual donors, with hypersensitive platelets coordinating significant sensitivity gains in bulk platelet populations and shown to direct aggregation in droplet-confined minimal platelet systems. Sensitivity gains scaled with agonist potency (convulxin > TRAP-14>ADP) and reduced the collagen and thrombin activation threshold required for platelet population polarization into pro-aggregatory and pro-coagulant states. The heterotypic platelet response results from an intrinsic behavioural program. The method and findings invite future discoveries into the nature of hypersensitive platelets and how community effects produce population level responses in health and disease.
Understanding cellular diversity and interactions provides the key to elucidating system behaviour. It becomes meaningful to investigate cellular diversity and identify even potentially rare phenotypes when amplification mechanisms exist in the system and when there is good reason to predict large-scale variety. Classically, cancer1–3, immunology4–8 and stem cells9,10 with associated cell expansion have been the focus of the large majority of single-cell studies.

In this work we turn our attention to platelets, dispersed sentinels which patrol the vasculature to detect breaches and respond in a coordinated manner using rapid and potent paracrine signalling to collectively form a thrombus. Platelets are also inherently variable9, originating from the fragmentation of heterotopic9 megakaryocytes resulting in variously small subcellular compartments (60% volume CV)10 with dissimilar contents and biochemistry11–13 and, without a nucleus, having limited repair capabilities during ageing14,15 before clearance. Therefore, platelet activation represents an ideal system for investigating cellular diversity and consequences for homeostatic control. Indeed, the nature and functional consequences of platelet diversity has been a matter of enquiry for almost half a century5,10,11. More recently, the discovery that dual stimulation with collagen and thrombin16,17 polarises platelets into distinct pro-coagulant and pro-aggregatory phenotypes8,16,18–21 has renewed interest on the topic of platelet diversity. In particular, pro-coagulant platelets have been further characterised22–27, revealing diverse functions that either represent multiple procoagulant subpopulations or a unified, yet versatile pro-coagulant subpopulation21. The bifurcation of the platelet population into the two phenotypes further creates debate regarding intrinsic versus extrinsic functional programming8. Allied to this, subjects harboured a single to multiple ratio of 42. The droplet microfluidic circuit used in this study is shown in Fig. 1c and was used to generate 25-μm-diameter droplets (Fig. 1d, e) at 10.4 kHz for single platelet packaging (352 Hz). This allowed >100,000 platelets to be encapsulated in the 5 min collection timeframe. To demonstrate the Poisson distribution effect, high platelet input concentrations were used to observe the relationship between singlet and multiple occupancy events (Fig. 1f). Coupled with the kHz measurement capabilities of flow cytometry the analytical pipeline enables the functional diversity of large-scale platelet populations to be readily mapped. The complete sampling to microfluidics and flow cytometry methodology is illustrated in Supplementary Fig. 2.

To evaluate single platelet sensitivity differences, a dose response experiment involving stimulating droplet-confined single platelets with convulxin (a GPVI receptor agonist) was undertaken and compared with the stimulation of platelet collectives. Using αIIbβ3 activation (inside-out signalling) as an analytical end-point the platelet collectives produced a sigmoidal response curve emerging at 0.1 ng/mL and saturating at 1 ng/mL concentrations. The signal intensity distribution of the collective population indicates continuous functional variety. In comparison, a higher activation threshold is evident with singularly stimulated platelets, with activation emerging at 1 ng/mL and saturating at 10 ng/mL levels (Fig. 2a). The similar signal distribution to platelet collectives indicates that droplet confinement does not downregulate platelet activation. Extending the analysis to a different pathway, the P-selectin exposure end-point for alpha granule secretion, the same increased activation threshold for singularly stimulated platelets was observed (Fig. 2b). Activation and aggregation density plots for platelets stimulated at 3 ng/mL are shown in Fig. 2c and highlight the hypersensitive behaviour of the collective response, the correlation between the two endpoints and the bimodal distribution for singularly stimulated platelets undergoing population-level transition. Importantly, the hypersensitive subpopulation was not observed by platelet collective dilution (up to a further 100-fold dilution), demonstrating the merit of the droplet microfluidics approach for single platelet analysis. To measure the significance in the response differences between single and collective platelets, the relative risk was considered (Fig. 2d, e). At low and high agonist concentrations the relative risk score is ~1.0, showing no effect, and at 3 ng/mL rises to 53 for αIIbβ3 activation and 6 for P-selectin exposure endpoints, highlighting the significantly (p value <1×10−5) distinct hypersensitivity of collectively stimulated platelets.

During confined platelet stimulation with convulxin degranulation results in the accumulation of stimulatory molecules in the droplets and this may lead to enhanced activation (Supplementary Fig. 3). This observation deserves confirmation using
inhibitors but nevertheless at the activation transition with a 3 ng/mL convulxin stimulation in droplets a clear bimodal distribution is evident with the activated population having a higher αIIbβ3 activation signal than platelet collectives also undergoing activation transition (0.3 ng/mL).

The sensitivity gains emerging from collective platelet behaviour were reproducible, with equivalent dose responses, both single and collective, obtained from the same donor three times over a 9-month period (Supplementary Fig. 4). When the study was extended to a cohort of eight healthy yet diverse donors (gender, age, BMI, smoking) the same pattern was observed, confirming the generality of the hypersensitive collective response, and allowing an efficacy model to be generated. For both αIIbβ3 activation and P-selectin endpoints, the collective convulxin response had an EC50 value of 0.4 ng/mL, whereas the single platelet EC50 was 7.5 ng/mL (Fig. 2f, g). The 19-fold median sensitivity gains demonstrates the importance of hypersensitive platelets and their cooperative influence.

To confirm that the molecular αIIbβ3 activation and P-selectin endpoints represent functional behaviour the dose study was extended to larger droplets (65 µL; ø50 µm) packaging 0–15 platelets. Here the presence of hypersensitive platelets was predicted to result in aggregation at moderate convulxin concentrations. At low concentrations (0.01 ng/mL) P-selectin negative platelets are observed as multiple, spatially distinct entities within each droplet. At moderate concentrations (1 ng/mL) this droplet case is observed along with the other case in which droplets contain a single platelet aggregate. These stain positive for P-selectin are typically large and, dictated by the Poisson statistic, must generally contain multiple, co-localised platelets (Fig. 2h). Overall, this points to the existence of hypersensitive platelets in a large fraction of droplets when treated with 1 ng/mL convulxin. At maximal concentrations (100 ng/mL) all droplets contain platelet aggregates. Plotting the flow cytometry data shows a closer similarity with the collective platelet response (Fig. 2i). However, a distinct bimodal distribution still results when using 1.0 ng/mL convulxin. Elevated P-selectin signals in droplets relative to collective conditions are observed at 10 and 100 ng/mL convulxin. This could indicate autocrine and paracrine signalling resulting from the accumulation of degranulation products within the droplets, but again requires confirmation using inhibitors. This experiment demonstrates the functional consequence of broad-spectrum sensitivity with cooperation and that minimalistic platelet cooperation models can be used to understand transition states and the linkage between probabilistic molecular events and collective functional outcomes.

Collective sensitivity gains are attributed to the existence of low abundance hypersensitive platelets which, upon activation, degranulate to activate platelets in the vicinity that were insensitive to the initial stimulus. These modes of paracrine signalling produce a spatiotemporal corolling effect that drives platelet cooperation to deliver the collective response. Nevertheless, sufficient numbers of activated platelets are required to polarise the entire platelet population into an activated response (e.g. Fig. 2a; collectives with 0.1 ng/mL convulxin). Our experiment involved platelets diluted to approximately 1/50th of in vivo concentrations, suggesting digital activation may well occur under physiological conditions with insufficient volume to disperse paracrine signals. Platelet cooperation is mediated through the secretion of alpha granules as evidenced by P-selectin exposure, but also ADP and serotonin secretion from dense granules as evidenced by CD63 presentation (Supplementary Fig. 5), a marker for dense granule and lysosome fusion with the membrane.

The dense granule secretion pathway had a higher activation concentration than the alpha granule secretion pathway, although upon activation the kinetics of dense granule secretion are faster which may allow the timely augmentation of pathways for specialised platelet activation.
The study was extended to other agonists; the peptide TRAP-14 functional motif was used in place of thrombin to activate the PAR-1 receptor and as before α_{IIb}β_{3} activation and P-selectin exposure endpoints were measured. The median activation threshold was again increased for single platelets stimulated in droplets, indicating that coordination by low abundance hypersensitive platelets reduces the activation threshold for platelet collectives. The emergence of a bimodal population distribution with singularly stimulated platelets was also observed for both collectives. The emergence of a bimodal population distribution

**Fig. 2 Broad-spectrum response to convulxin stimulation and hypersensitive collective behaviour.** Violin plots comparing the activation of single platelets with platelet collectives using a convulxin dose response experiment, with PAC-1 binding to activated α_{IIb}β_{3} (a) and P-selectin exposure (b) endpoints (relative risk; **>2, ***>5, ****>10). Contour plot and density plots of the emergence of hypersensitive single platelets at 3 ng/mL convulxin concentrations, while the collective population is fully activated (c). Relative risk analysis was used to determine the significance of the ~20-fold differences between the single and collective platelet responses using PAC-1 (d) and P-selectin (e) endpoints with confidence intervals determined by the Koopman asymptotic score. The E_{max} model was used to show a consistent difference between single and collective platelet behaviour across a diverse cohort (age; gender; smoking; BMI; exercise) of eight healthy donors using PAC-1 (f) and P-selectin (g) endpoints. Droplet volume scaling to 65 µL produces minimal collectives (0–15 platelets with ~500 × 10^{6} platelet/mL inputs) to allow aggregation responses to be investigated. Triple fluorescent imaging (P-selectin, CD63 and CD42b) with brightfield overlay of minimal platelet collectives stimulated with 1 ng/mL convulxin. Droplets containing aggregates are indicated by a green ring and those with multiple separate platelets by a white ring (h). Resulting dose response violin plots of minimal platelet collectives compared with bulk platelet collective responses (i). To measure aggregates by flow cytometry, doublet(+) gating was removed, thereby increasing the signal spread by the inclusion of various aggregate scales (2–15 in the case of minimal collectives) along with signals from active single platelets and inactivate single platelets. For each single platelet condition, n = 10,000–36,000 platelet events were measured, and n ≈ 48,000 for the collective conditions.
exploits inherent plateletvariability, thereby bypassing the need for functionally uniform platelets. Whether this diversity model involving community cross-talk for the transition from a dispersed state to localised recruitment and responsiveness can be generalised to other scenarios such as immune infiltration remains to be seen.

Functional variety is a common feature of cellular systems enabling powerful system responsiveness and control. This research shows that broad and continuous sensitivity distributions of single platelets interfaced via paracrine signalling produces robust collective sensitivity gains. During dual stimulation with collagen and thrombin, platelets are known to polarise into two distinct populations: pro-coagulant and pro-aggregatory phenotypes. The intrinsic or extrinsic nature of this heterogeneity is a matter of debate. Again using droplet confinement we sought to resolve this debate and also to question the role of collective hypersensitivity in the emergence of the heterotypic response.

A dual stimulation dose response experiment was undertaken requiring the addition of calcium to unwashed platelets to enable membrane inversion for annexin V binding to phosphatidylserine moieties and also the addition of rivaroxaban to prevent additional thrombin formation by factor Xa and gly-pro-arg-pro (GPRP) to limit the formation of fibrin fibres. The responses of single and collective platelet populations are compared using violin plots (Supplementary Fig. 7) and show that that the different conditions and integration of the two activation pathways alters system sensitivity (see Fig. 2a), with platelet collective pro-coagulant (annexin V high; α_{IIb}β_{3} low) and pro-aggregatory (annexin V low; α_{IIb}β_{3} high) heterotypic states emerged with a 100 ng/mL convulxin and 1.0 U/mL thrombin stimulation and is consistent with the literature. At the same concentrations droplet-confined, single platelet populations do not fully polarise, with an unresponsive third population (Fig. 4a). Again this demonstrates the need for cooperation to enhance system sensitivity to activate all platelets. At higher dual stimulation doses (300 ng/mL convulxin and 3.0 U/mL thrombin) single platelet populations fully polarise into pro-coagulant and pro-aggregatory states. By excluding paracrine cross-talk, this confirms the intrinsic origins of heterogeneity. Indeed, removal of paracrine cooperative effects produces a fully digital pro-coagulant or pro-aggregatory response (Fig. 4b). Importantly, these findings are made possible by single platelet confinement, advocating the use of droplet microfluidics to accurately delineate intrinsic single platelet phenotypes. In contrast to droplet-confined stimulation, the heterotypic distribution of platelet collectives involves some platelets with graded intermediate states. This implies the role of extrinsic effects for the generation of subtler phenotypes likely required to enable more sophisticated functionality throughout the thrombus.

In this study, collective sensitivity gains are shown to be a general feature of human platelet biology. To gain further insights into this behaviour, these platelets were gated for characterisation (Supplementary Fig. 8). Their forward and side scatter properties are indistinguishable from other, insensitive platelets. The CD42b signal (monomer component of the Von Willebrand factor receptor, GPIb-IX-V) for the hypersensitive platelets is similar albeit slightly reduced as a consequence of matrix metalloprotease excision upon activation. Further investigations involving large-scale antibody panels for highly multiplexed cytometry or more global proteomic and even transcriptomic screens following platelet sorting will be needed to determine the composition of the hypersensitive platelet subpopulation. While this holds promise for the identification of elements governing system behaviour and potential hubs for therapeutic intervention, caution is required for extension to in vivo contexts in which multiple cues integrated in time and space mediate responses that allow highly robust homoeostatic control.

Discussion

In summary, the microfluidics and cytometry methodology was used to identify a broad-scale sensitivity continuum containing hypersensitive platelets which coordinate collective sensitivity gains
Fig. 4 Intrinsic heterotypic states in response to dual stimulation. Stimulation of platelet collectives with 100 ng/mL convulxin and 1 U/mL thrombin produces pro-coagulant (annexin V high; αMβ2 low) and pro-aggregatory (annexin V low; αMβ2 high) states. With the same stimulation, single platelets produce a third unresponsive population (annexin V low; αMβ2 intermediate) indicating the requirement for paracrine cooperation to achieve complete population activation (a). Single platelets stimulated with higher 300 ng/mL convulxin and 3 U/mL thrombin concentrations drives platelets exclusively to functionally distinct pro-coagulant or pro-aggregatory states (b). Cooperation in platelet collectives at both dual stimulations concentrations directs some platelets into graded, intermediate activation states. For each single platelet condition, $n = 14,000-26,000$ platelet events were measured, and $n = 48,000$ for the collective conditions.
Flow cytometry. Platelets were stimulated with convulxin (Enzo Life Sciences), a snake venom which activates the GPIVI receptor, TRAP-14 (Bachem AG) an agonist of the PAR-1 receptor or ADP (Sigma Aldrich) an agonist of the P2Y12 receptor. The dual agonist experiment involved stimulation with convulxin and thrombin (Sigma) in the presence of 2.5 mM CaCl₂. Here, coagulation was pre-
vented using 0.5 μM rivaroxaban (Advanced ChemBlocks Inc.) and 100 mM H-Gly-Pro-Arg-Pro-OH (GPRP, Bachem) added to the HEPES platelet dilution buffer. Fluorescent antibodies and selective stains were used to detect biomarkers: fluororescin isothiocyanate (FITC)-conjugated PAC-1 (PAC-1 clone at 1.25 μg/μL), allophycocyanin (APC)-conjugated CD62P (P-selectin, AK-4 clone at 0.63 μg/μL), FITC-conjugated anti-CD63 (HS56 clone at 2.0 μg/μL), R-phycocerythrin (PE)-conjugated CD42b (HIP1 clone at 1.25 μg/μL) and Annexin V at 0.8 μg/μL were obtained from BD Biosciences. Following treatments, antibody incubation and fixation, samples were diluted in PBS and measured using an Accuri C6 flow cytometer (BD Biosciences). Platelets were identified using a gate on CD42b-PE intensity, with doublets and non-platelet-sized events removed by gating. An overview of the gating procedure is provided in Supplementary Fig. 9. Importantly, platelet dilution and short 15 min incubations without stirring of the collective samples did not increase the gating out of platelet doublet/aggregate events, thereby suggesting the population without bias. In the case of the 50 μm droplets, CD42b-PE, CD63-FITC and CD62P-APC antibodies were used and incubated for 60 min prior to fixation and emulsion breaking. Here, doublet gating was not applied to retain platelet aggregates in the analysis and PAC-1 staining was not used to avoid interference with aggregation.

Statistics and reproducibility. Droplet images were analysed using ImageJ (NIH) and flow cytometry data using R. All flow cytometry data were tested for normality using the Shapiro–Wilks test (GraphPad Prism). To compare single and collective platelet responses from a single donor, the relative risk statistic was used to quantify the association between stimulation and response (R, epitoids). Violin plots were made using the 1–99 percentile (R, ggplot2). The overall cohort response difference between single and collective platelets was plotted using an efficacy maxima (Eₚₑₙ) sigmoidal model generated in GraphPad Prism. The cohort was diverse, with five male and three female volunteers, aged between 20 and 60 and with one smoker. To ascertain reproducibility, some donors were measured three or more times. For the single platelet flow cytometry analyses, an average of ~30,000 platelet events were measured for each condition and ~48,000 platelet events for each of the collective platelet conditions.

Reporting summary. Further information on research design is available in the Nature Research Reporting Summary linked to this article.
