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Phenotyping platelet subpopulations using cell surface marker expression

Lay Summary

Platelets are small blood cells that primarily function to prevent bleeding by forming blood clots. Recent research suggests that there are distinct populations of platelets, characterised by differing cell surface markers, each with its own specific role in the blood clotting process. Using the relatively new technique of spectral flow cytometry, we are less limited by the overlap of fluorophores, allowing for the detection of more parameters than conventional flow cytometry. This expansion enhances our ability to characterise and sub-populate platelets based on their cell marker expression.

The inappropriate activation and aggregation of platelets can cause diseases such as a heart attack or stroke. Different platelet subpopulations may have distinct roles in the formation of a blood clot, and some may play a more significant role in the inappropriate aggregation of platelets. This information can then be used to help target and prevent the inappropriate activation of platelets while limiting the effect of the treatment on normal platelet activation in response to bleeding.

Aims

This study aimed to determine whether there are different subpopulations of platelets based on the expression of constitutive and activation markers in response to multiple platelet agonists. Using this knowledge to see whether different agonists, the concentration of agonist or platelet inhibitors affect these subpopulations.

Spectral flow cytometry was employed to determine whether we could detect different subpopulations of platelets and how these subpopulations change in response to different platelet agonists and inhibitors. A panel of three constitutive markers were selected: CD41-PE, CD36-RB613 and GPVI-RY586. This was coupled with five activation markers: CD63-BV786, CD62P-RB780, PAC-1-AF647, CD40L-RY703 and Annexin V-BV421. An optimal concentration of agonists was then selected and tested in the presence of five different platelet activation inhibitors: Aspirin, Cangrelor, Ibrutinib, Spironolactone and Carbenoxolone to determine whether these inhibitors affect the number or distribution of subpopulations.

Results

Optimisation

Before accurate data can be acquired for spectral flow cytometry, the fluorophore-conjugated antibodies need to be titrated to find an optimal concentration to provide high-resolution results. Table 1 shows the antibodies and the optimal concentrations from the titres, for all the antibodies except glycoprotein (GP)-VI, a saturating titre was used. However, the clone HY101 for the GPVI antibody can activate platelets, so it is best to be used at a subsaturated concentration to prevent this activation.

Table 1 – Details of the fluorophore-conjugated antibodies.

Antibody	Clone	Fluorophore	Final Concentration
Anti-CD36	FA6-152	RB613	0.25μg/ml
Anti-CD63	H5C6	BV786	-
Anti-CD62P	AK-4	RB780	0.75μg/ml
Anti-GPVI	HY101	RY586	0.375μg/ml
Anti-CD41/61	PAC-1	AF647	3μg/ml
Anti-CD41a	HIP-8	BV510	0.25μg/ml
Anti-CD154	TRAP1	RY703	1.5μg/ml
Annexin V	N/A	BV421	0.25μg/mL

During the process of optimising the flow cytometry, we needed to adapt by adding calcium (Ca2+) because annexin V binding to phosphatidylserine is Ca^{2+} dependent. This also posed a challenge, as the addition of Ca^{2+} can activate thrombin, which in turn activates platelets. To prevent this, D-phenylalanyl-L-prolyl-L-arginyl chloromethyl ketone (PPACK) and glycine-proline-arginine proline (GPRP) were added as they inhibit thrombin activation and fibrin polymerisation, respectively. Unfortunately, cluster of differentiation (CD)-41 also competed for binding with CD41/61 (PAC-1), which decreased the resolution of the PAC-1 data. This impacted the results, as our hypothesis was based on “pro-aggregatory” platelets being PAC-1+. Another limitation of the study was that CD63 conjugated to BV786 was not bright enough for accurate detection.

Dose-response to multiple platelet agonists

To ascertain the optimal concentration of agonist to use within the study and to validate that that the antibodies selected and spectral flow cytometry could reproduce similar results to conventional flow cytometry, the EC_{50} of adenosine diphosphate (ADP), collagen-related peptide (CRP) and thrombin receptor activator peptide 6 (TRAP-6) was determined by a dose-response relationship between a platelet agonist and surface CD62P, a marker for platelet activation. Figure 1a-c shows the relationship between differing concentrations of ADP, CRP and TRAP-6 and the release of CD62P to the surface of the platelet. The EC_{50} of ADP, CRP and TRAP-6 was calculated to be 8.7μM, 0.02μg/mL, and 0.38μM, respectively.

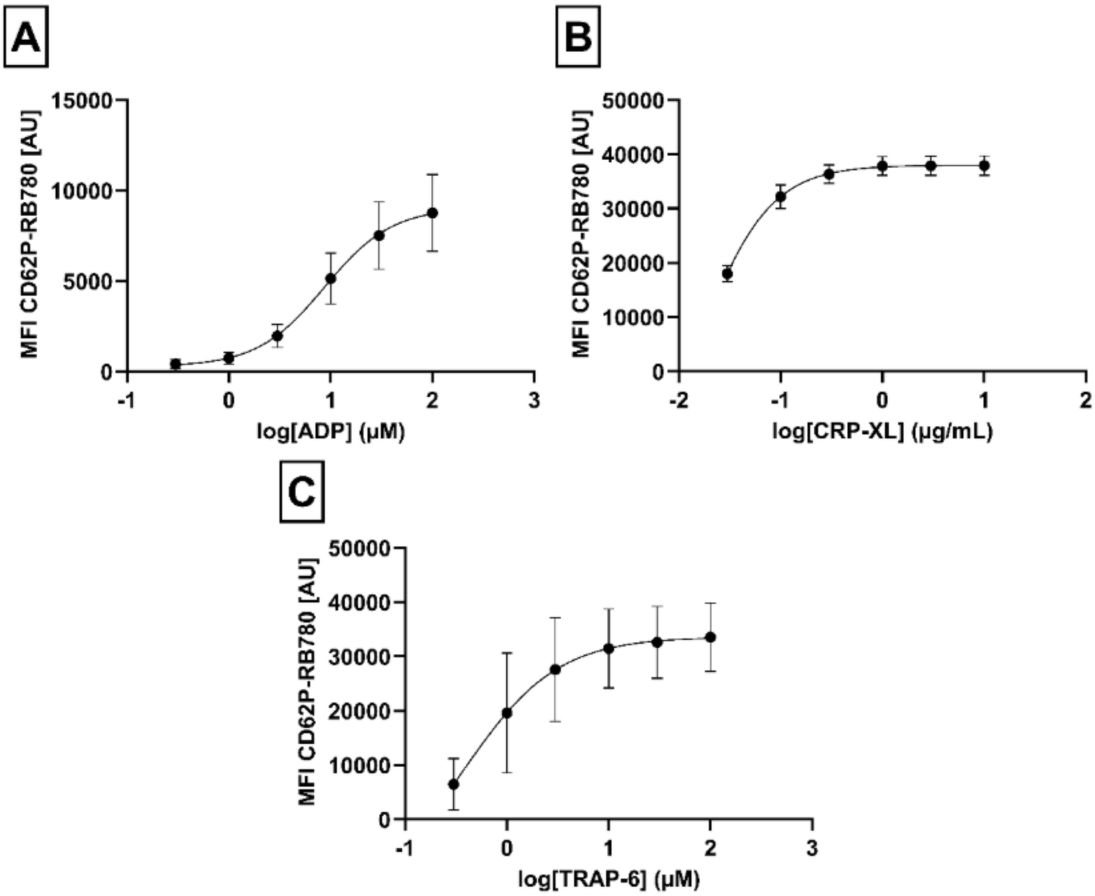

Figure 1 – Dose-response curves measuring the platelet activation by the surface marker (n=3), CD62P, against (A) ADP (0.3–100μM), (B) CRP (0.03–10μg/mL) and (C) TRAP-6 (0.3–100μM). Data is presented as median fluorescence intensity (MFI) ± standard deviation and analysed by GraphPad Prism's four-parameter curve function.

Workflow for phenotyping platelet subpopulations

Figure 2 demonstrates the workflow for phenotyping platelet subpopulations. Firstly, the blood needs to be stained with the fluorescently labelled antibodies and stimulated with a platelet agonist. Data for 10,000 platelet (CD41+) events are acquired and gated into positive and negative populations for each fluorophore. This can then be analysed using T-distributed stochastic neighbour embedding (tSNE), which helps to visualise high-dimensional data. This can then undergo clustering to find the different platelet subpopulations based on their marker expression.

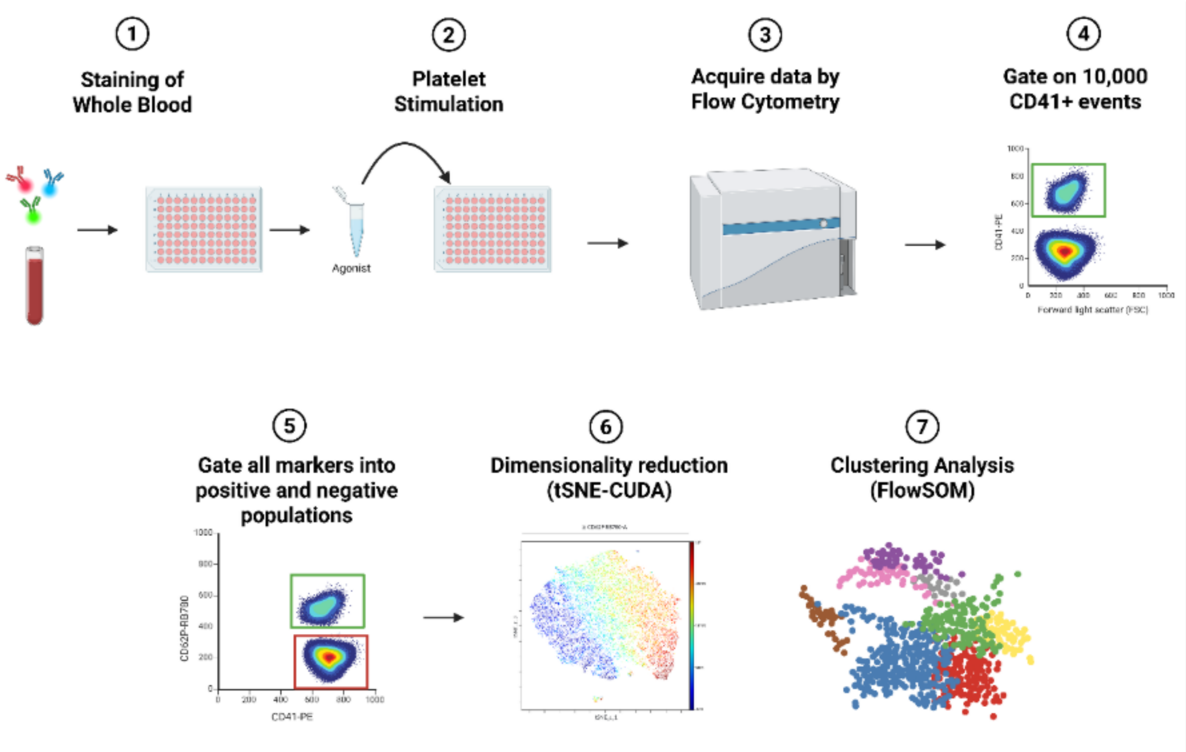

Figure 2 – Workflow for phenotyping platelet subpopulations.

Summary

To summarise, we optimised the spectral flow cytometry workflow and validated it using platelet-agonist dose-response curves. Future analysis of the data will include producing tSNE plots for each of the conditions and then using FlowSOM to cluster the data into distinct platelet subpopulations based on the cell surface marker expression. The subpopulation's differences in platelet signalling and response to agonists may then reveal novel methods for the treatment of platelet-related diseases.

Personal

During the course of this project, I really enjoyed working as part of a lab and learning new techniques. I learnt a variety of new techniques that help study platelets and their function, including making platelet-rich plasma, washed platelets, and using conventional and spectral flow cytometry. I have also learnt a lot about platelets and the biochemical processes that occur when they are activated, and how these processes change when stimulated with different agonists. I was also fortunate to have the opportunity to observe and learn about other aspects of the research conducted at Reading University, including fluorescent microscopy and intravital microscopy. Being at Reading University, I was able to participate in lab meetings and journal clubs, which allowed me to gain a better understanding of lab work and how to analyse scientific literature critically.

Currently, I am completing my master's degree and am looking to conduct my research project using spectral flow cytometry and platelets. I hope that this experience will enable me to pursue a PhD in platelet biology and, hopefully, allow me to utilise spectral flow cytometry. Researching platelets is very important as they form a large part of many cardiovascular diseases, which are one of the leading causes of death globally.