Control of immunothrombosis through endogenous pathways of platelet inhibition

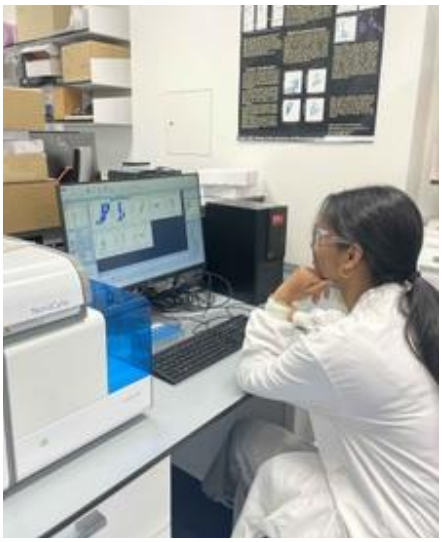
Student: Rithika Penugonda
Supervisor: Dr Paul Armstrong
Blizard Institute, Queen Mary University of London

Lay Summary:

Blood clotting is essential to prevent excessive blood loss after injury, and cells in the blood, called platelets, that stick together are central to this. However, unwanted clot formation can occur during diseases, such as sepsis, with grave consequences. We now realise the body's immune response and blood clotting is closely interlinked during this time. This process, 'immunothrombosis', involves close interactions between a type of immune cell, monocytes, and platelets. This increases the presence of a strong clotting protein called 'tissue factor'.

Under healthy conditions, stickiness of platelets is regulated by endothelial cells. These cells line blood vessels and naturally produce nitric oxide (NO) and prostacyclin (PGI2), preventing platelets from becoming sticky. However, we do not know how effective NO and PGI2 are at preventing immunothrombosis. This project therefore investigated whether strengthening these natural inhibitory pathways can reduce platelet-monocyte interactions and tissue factor expression.

We found that NO and PGI2 have strong effects in preventing immunothrombosis. Also, drugs to strengthen the effects of low levels of NO and PGI2, are also effective. This study highlights a potential therapeutic strategy to prevent unwanted clots, reinforcing the need for a better understanding of how different cell types and processes interact in disease.



Project Aim:

To investigate whether endogenous nitric oxide (NO) and prostacyclin (PGI2)-dependent inhibitory pathways regulate platelet activation, platelet-monocyte interactions and tissue factor expression, and whether pharmacological modulation of their downstream signalling could enhance their inhibitory effects.

Methods:

The project progressed through three sequential phases. Phase 1 established inhibitory conditions using platelet-aggregation assays. Platelet-rich plasma (PRP) was prepared from the whole blood of healthy donors, stimulated

with collagen-related peptide (CRP-XL). Increasing concentrations of treprostinil, a PGI2 analogue, and PAPA-NONOate, an NO donor, were investigated individually and in combination. Platelet aggregation was measured using a 96-well plate-based assay and spectrophotometry, allowing inhibitory concentrations to be characterised for subsequent experiments.

Phase 2 transferred selected conditions into whole blood to investigate platelet-monocyte interactions. Samples were treated with treprostinil and PAPA-NONOate before CRP-XL stimulation. Samples were then fixed, lysed and stained with CD14, CD41 and CD142 to identify monocytes, platelets and tissue factor, respectively. Flow cytometry quantified platelet-monocyte aggregates and tissue factor expression.

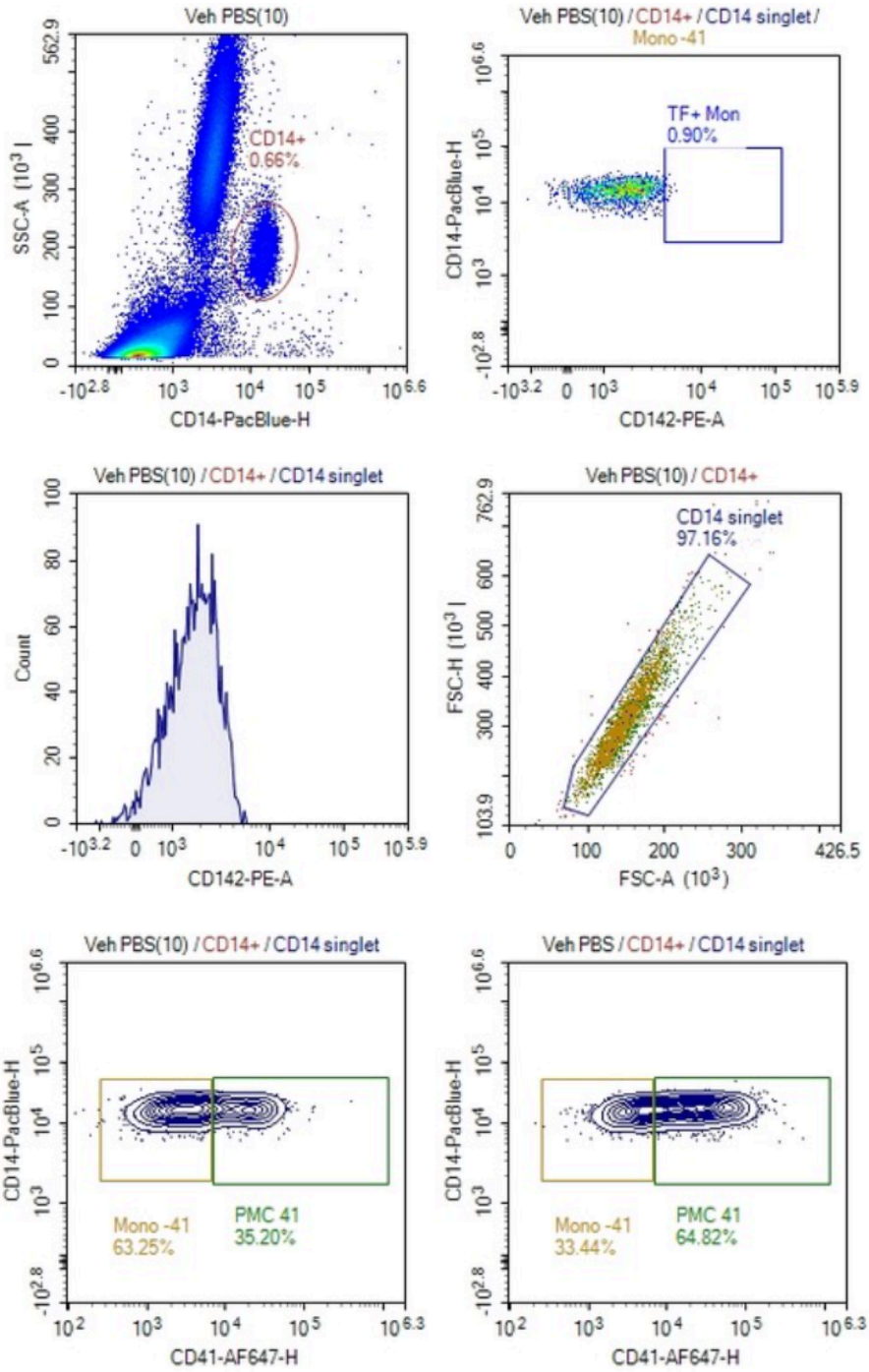


Figure 1. Gating strategy on 5000 CD14+ events, separating populations, and 1% TF+ monocytes

Phase 3 investigated pharmacological modulation using BAY-60-2770 and Cilostazol under conditions of reduced PGI2/NO inhibition. Platelet-monocyte aggregate formation and tissue factor expression were assessed using flow-cytometry.

Results:

Initial experiments demonstrated concentration-dependent platelet aggregation after ADP, TRAP-6 and U46619 stimulation. Responses reached ~70-85% and confirmed the reproducibility of the aggregation assay for this project.

The inhibitory effect of the PGI2 pathway was investigated using increasing concentrations of treprostinil across CRP-XL concentrations. Vehicle-treated platelets showed increasing aggregation with CRP-XL, reaching ~80%. Increasing treprostinil progressively reduced this response. While 1 nM PGI2 produced little difference from the vehicle, 5 nM produced modest inhibition and 10 nM resulted in greater inhibition, where aggregation reached ~ 45% at 0.1 µM CRP-XL compared with 80% in vehicle samples. Strongest inhibition was observed with 100 nM PGI2 as aggregation remained close to baseline.

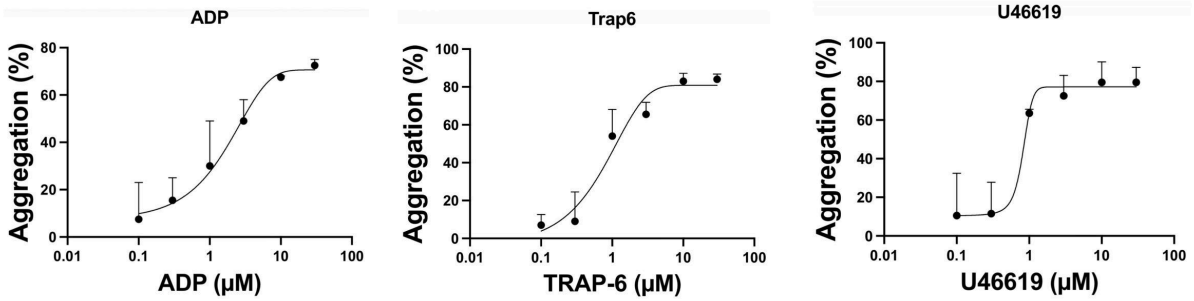


Figure 2a. Concentration-response curves showing platelet aggregation following stimulation with ADP, TRAP-6 and U46619

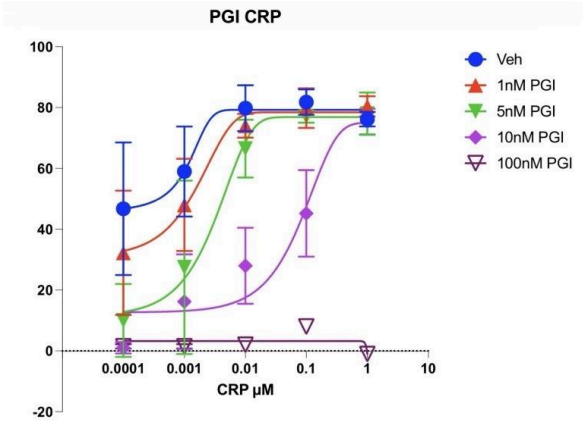


Figure 2b. Effect of increasing PGI2 concentrations on CRP-XL-induced platelet aggregation

Phase 2 examined whether these inhibitory conditions influence platelet-monocyte interactions in whole blood. Increasing PGI2 concentration from 1 to 10 nM reduced platelet-bound monocytes from 72% to 46% in vehicle-treated samples. With 100 µM PAPA-NONOate, platelet-bound monocytes decreased from 59% to 44%. Tissue factor MFI also decreased with increasing treprostinil in both CD14+ monocytes and CD14+CD41+ platelet-monocyte complexes, approaching the non-stimulated baseline at 10 nM.

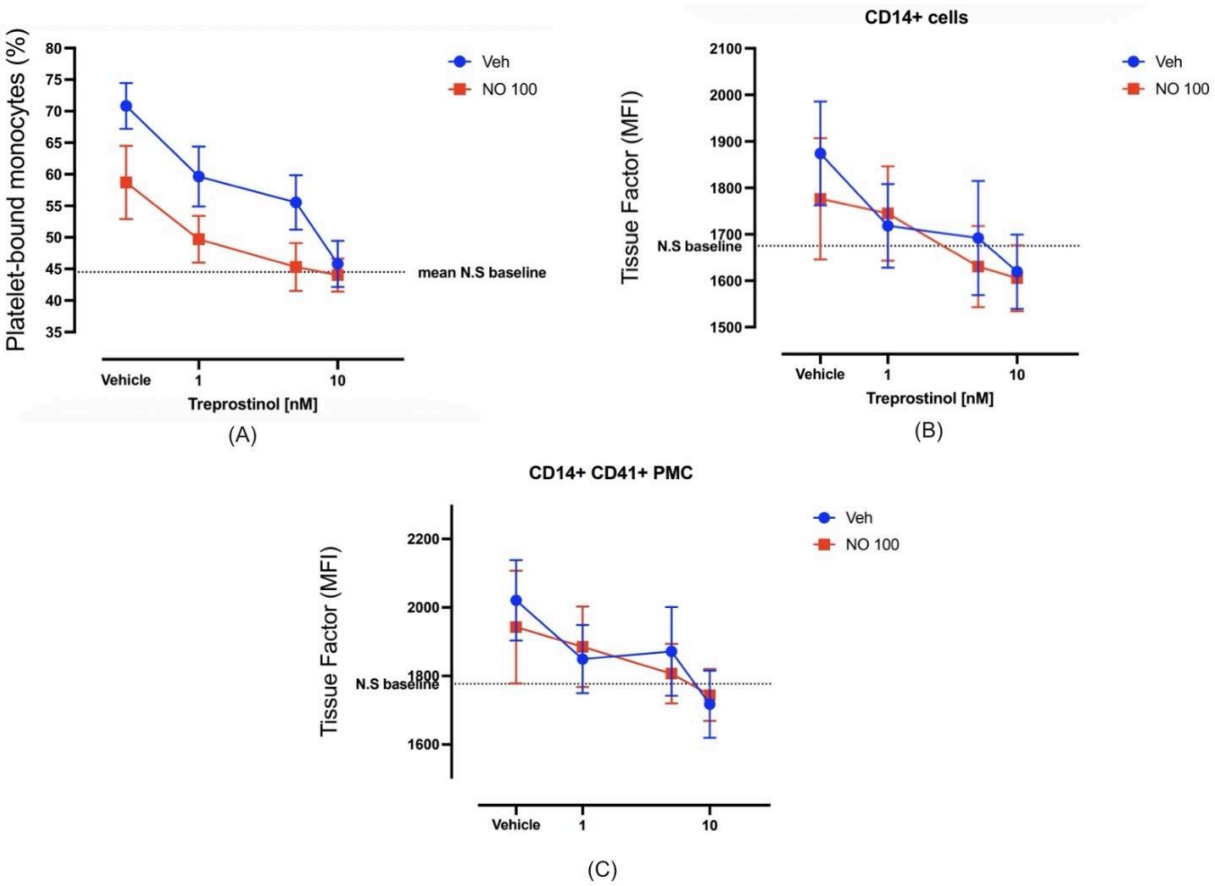


Figure 3. Effect of treprostinil and PAPA-NONOate on platelet-monocyte interactions and tissue factor expression in the presence or absence of 100 µM PAPA-NONOate on (A) platelet-bound monocytes. (B) tissue factor expression on CD14+ monocytes and (C) tissue factor expression on CD14+CD41+ platelet-monocyte complexes. Dotted line represents mean non-stimulated baseline.

Phase 3 investigated pharmacological modulation of cyclic-nucleotide signalling under sub-optimal PGI2/NO inhibition (PGI2 0.5 and NO 50). BAY 60-2770, with inhibitors, reduced platelet-bound monocytes to 38% compared with 49% in the vehicle, without inhibitors (p=0.0067). Cilostazol produced a similar reduction, with platelet-bound monocytes decreasing to approximately 34% with inhibitors versus 49% in the vehicle (p=0.0260). Cilostazol with inhibitors also reduced tissue factor MFI to 1660 compared with 2250 for vehicles without inhibitors (p=0.0204).

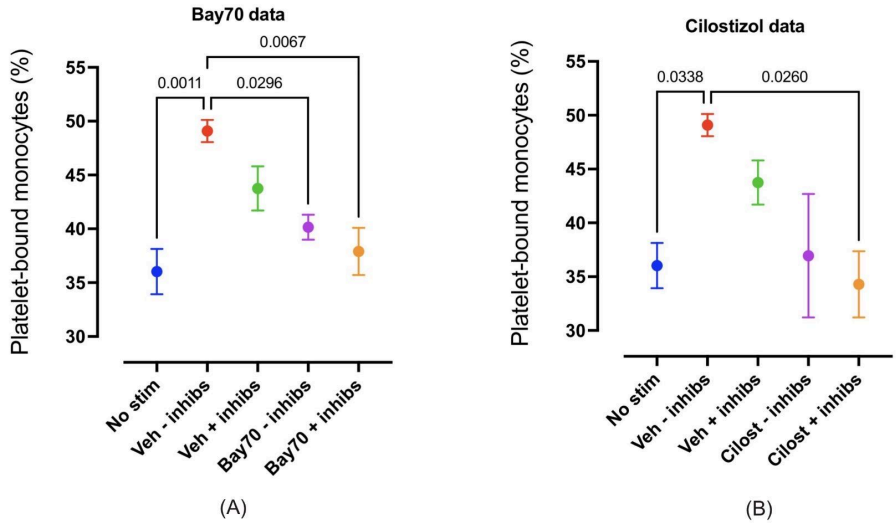


Figure 4. Pharmacological modulation of platelet-monocyte interactions and tissue factor expression. Effects of (A) BAY 60-2770 and (B) cilostazol on platelet-bound monocytes. Experiments performed under reduced PGI2/NO inhibitory conditions.

Overall, results showed that increasing inhibition of platelet activation was associated with reduced platelet-monocyte aggregation and tissue factor expression. Pharmacological modulation under reduced inhibitory conditions could further alter these responses.

What I gained from this project:

I began this studentship hoping to develop my laboratory and analytical skills, but it ultimately changed my understanding of what it means to conduct research. I gained hands-on experience in platelet preparation, aggregation assays and flow cytometry while becoming increasingly confident in carrying out experiments, assessing data reproducibility and interpreting findings rather than simply following established protocols. Studying endogenous platelet inhibition in the context of immunothrombosis also broadened my understanding of platelets, particularly their role in connecting haemostasis with inflammation and coagulation.

I also experienced the iterative nature of experimental research: reviewing existing literature, planning experiments, troubleshooting technical issues and adapting approaches when results were unexpected, taught me that research rarely progresses in a linear manner. I enjoyed the critical thinking involved in investigating why an experiment had produced a particular result and reflecting on how this should influence the next experiment. I leave this studentship having learnt new technical skills, but also a greater confidence in thinking critically, working independently in a laboratory environment and approaching scientific questions as a researcher.

How this project has influenced my long-term goals:

This project has undoubtedly strengthened my interest in pursuing a career in translational research within immunobiology, while also providing a more realistic understanding of what that involves. Before starting the project, I was primarily interested in pharmacology and intracellular signalling, areas I had encountered through my studies. Working on this project demonstrated how these concepts are translated into experimental questions and how much of scientific progress relies on careful experimental design, critical interpretation and the willingness to reconsider the method when data does not behave as expected.

I particularly enjoyed the problem-solving aspect of research: interpreting results and thinking about what they could and could not tell us about the underlying biology. Seeing experiments being conducted on patient samples from The Royal London Hospital gave me a clearer appreciation of the potential journey from mechanistic laboratory research towards clinically relevant applications and the impact of platelet research in improving treatments.

Following my Biochemistry degree, I hope to pursue postgraduate research through a MRes and PhD in pharmacology. In the future, I want to continue to contribute to platelet research and connect my understanding of disease mechanisms with the development of better therapeutic approaches.