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# A novel FACS-based approach for studying platelet reactivity in murine whole blood

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Platelets are key to the processes of haemostasis. They are the target of anti-thrombotic therapies, notably aspirin and $P2Y_{12}$ receptor blockers, as well as playing central roles in certain bleeding disorders. The testing of platelet reactivities is therefore central to both clinical diagnoses and novel drug development. The most commonly used platelet reactivity assays have been developed for the testing of human samples and accordingly require a minimum of around 10-12 ml of blood. With increasing interest in the use of genetically modified mice as a tool to understand the link between genotype and phenotype, more efficient and lower volume approaches are required.

We have developed a miniaturised approach to whole blood aggregometry, using a 96-well plate format coupled with quantification by flow cytometric analysis of single platelet count (Alugupalli *et al.*, 2001). In brief, C57bl6 mice were anaesthetised by ketamine (100mg/kg, i.p.) plus xylazine (10mg/kg, i.p.). Blood was collected by venepuncture of the vena cava into hirudin (10µg/ml final). 35µl aliquots of blood were then placed into the individual wells of a 96-well plate, with or without the addition of test inhibitors and with or without agonist, and vigorously mixed (1200rpm, 37°C, 5min). At the end of this period 5µl samples were removed, diluted and labelled using a platelet specific antibody to allow single platelet counts to be established. Residual blood was treated with diclofenac (1mM) and centrifuged (2000*xg*, 10mins) to generate plasma which was then analysed for thromboxane (Tx) release (measured as $TxA_2$ stable metabolite $TxB_2$) by commercial ELISA. Platelet-platelet and platelet-neutrophil interaction was also confirmed by confocal imaging.

Concentration-dependent reductions in the numbers of single platelets were observed in blood samples incubated with collagen (log $EC_{50}$ -6.5±0.1 g/ml), U46619 (log $EC_{50}$ -6.8±0.1 M) or the PAR4 agonist (AYPGKF amide; log $EC_{50}$ -4.1±0.1 M; n=6 for all). Plasma thromboxane levels were markedly increased in samples stimulated with collagen (0.5µg/ml; 40±10ng/ml) compared to vehicle (1±0.5ng/ml; n=3 for both). The loss of single platelet coincided with the appearance of platelet-platelet and platelet-neutrophil interactions as confirmed by confocal imaging. These reductions were strongly inhibited by prostacyclin (2µg/ml; $p<0.05$, n=6). Moreover, the assay was sensitive to the effects of anti-platelet therapeutics. For instance, reductions in single platelet counts induced by collagen, U46619 and PAR4 agonist were strongly inhibited by prasugrel active metabolite (3µM; $p<0.05$, n=6). Furthermore, the reduction in single platelet count induced by arachidonic acid (0.1mM) was absent in blood from COX-1 knockout (KO) mice ($p<0.05$, n=3).

This assay allows the testing of platelet responses to a range of agonists, which interrogate the major platelet receptor signalling pathways, in small volumes of mouse

whole blood. The small sample volume also allows for the simultaneous testing of a wide range of conditions and concentrations, leading to a reduction in numbers of required mice. Furthermore, there is sufficient residual volume of blood from each test well to examine cell markers by flow cytometry as well as determining the levels of platelet releasates.

1) Alugupalli et al, Thromb Haemost 86:668, 2001.