Effect of Toll-like Receptor agonists on platelet activation in Acute Coronary Syndromes

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Abstract

Introduction: Platelets express functional Toll-like receptors (TLRs), and numerous studies have found that certain TLR agonists and bacteria are capable of inducing thrombotic and inflammatory responses from isolated platelets. Few studies have examined the functionality of the platelet-expressed TLRs in the clinical context or in whole blood. Platelets and their thromboinflammatory functions are central to the pathogenesis of atherosclerosis and the acute coronary syndromes (ACS). As a first step to understanding the effects of TLR agonists on platelets in ACS, we aimed to develop a flow cytometry-based protocol to examine platelet activation responses to TLR agonist stimulation.

Methods: A flow cytometry-based protocol to measure the expression of platelet activation markers PAC-1, P-selectin (CD62p) and CD40-ligand (CD40L) in response to stimulation by TLR agonists was developed. Fourteen patients presenting to Wellington Regional Hospital with ACS pretreated with aspirin and clopidogrel (dual antiplatelet therapy, DAPT) were recruited prospectively. Fourteen age- and sex- matched healthy volunteers not on antiplatelet medications were recruited prospectively as controls. Whole blood samples from ACS patients and controls were each separately stimulated with agonists for TLR4 (lipopolysaccharide, LPS), TLR2/1 (Pam3CysSerLys4, PAM_3CSK_4) and TLR2/6 (fibroblast stimulating ligand-1, FSL-1). Thrombin Receptor Activating Peptide (TRAP) was used a positive control. Following stimulation, samples were analysed by flow cytometry.

Results: There were no differences in the platelet activation states between groups for the unstimulated samples. Stimulation of whole blood from the control group by the TLR agonists tested resulted in statistically significant increases in PAC-1 and CD62p. Stimulation of whole blood from the ACS patients on DAPT with TLR agonists did not result in statistically significant increases in the expression of the platelet-activation markers tested, although trends towards significance were noted at the higher concentrations of agonists tested. Platelet responses were lower in the ACS group relative to the controls. For the maximal LPS concentration used, the %-positive expression

differences between the control group and ACS group for PAC-1 and CD62p were 29.3 ($p = 0.002$) and 7.2 ($p = 0.02$), respectively. For the maximal concentration of PAM_3CSK_4, the differences for PAC-1 and CD62p were 16.4 ($p < 0.001$) and 8.4 ($p = 0.04$), respectively. For the maximal concentration of FSL-1, the differences for PAC-1 and P-selectin were 18.7 ($p < 0.001$) and 7.8 ($p = 0.04$), respectively.

Conclusions: TLR agonist stimulation increased platelet activation in healthy volunteers, although it was unclear whether this was due to a direct effect on the platelet surface-expressed TLRs or an indirect effect on platelets via leucocytes, erythrocytes or plasma proteins. DAPT appeared to inhibit TLR agonist-induced platelet activation in the ACS patients. Although we found no statistically significant platelet activation responses to the TLR agonists tested in the ACS patients on DAPT, this will require confirmation in larger sample sets as our study may have been underpowered. If so, current DAPT regimens may be insufficient to prevent ischaemic events in settings of infection for patients at risk of arteriothromboses involving platelets.

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1 Table of Contents

List of Tables

List of Figures

1 Chapter 1 – General Introduction

1.1 Introduction

Over the past two decades, it has become clear that inflammation, the immune system and platelets are central to the pathogenesis of atherosclerosis and the acute coronary syndromes (ACS) (1-5). Activated platelets play a crucial role in the development of atherosclerosis and ACS as the primary cells of thrombosis (2) and as mediators of immune and inflammatory responses (1, 6). Accordingly, the positive effect of antiplatelet agents may not solely be via inhibiting platelets' ability to aggregate, but may also be via modulating the significant roles platelets have in regulating immune responses (7). Because thrombotic events secondary to atherosclerotic disease continue to be the number one cause of death worldwide (8), we are interested in understanding the role of platelet activation in this process and, in particular, exploring the role of Toll-like receptors (TLRs) on platelet activation.

Platelets were recently found to express TLRs (9-15), a family of cellular sensors that upon activation by a variety of microbial pathogens and endogenous ligands play an important role in promoting and initiating inflammatory and immune responses (16-18). Under certain circumstances, stimulation of platelet-expressed TLRs by microbial pathogens (or by synthetic analogues) can promote the release of platelet-derived inflammatory mediators, such as soluble CD40-ligand (sCD40L) (19-21) and interleukin-1 beta (IL-1β) (22), and encourage platelet aggregation and thrombosis (14, 23, 24). Moreover, platelet-expressed TLRs can enhance the clearance of certain bacterial pathogens by the immune system (25, 26). On the other hand, aberrant activation of platelet-expressed TLRs significantly contributes to the pathophysiology of sepsis and disseminated intravascular coagulation, both of which are often fatal (27-29).

Whether activation of platelet-expressed TLRs by microbial pathogens contributes to the pathogenesis of other thrombotic and/or inflammatory conditions, such atherosclerosis and ACS, is

unclear (6, 9). As previously stated, atherosclerosis is now widely regarded as an inflammatory disease (1, 30). It is well recognised that activated platelets play important roles in the development and progression of both atherosclerosis and arteriothrombosis (1, 5, 30). Acute infections are associated with a transient increase in the risk of vascular thrombotic events (31, 32), and data from epidemiological studies associate acute infections with an increased risk of ACS (33). At the cellular level, coronary thrombi and atherosclerotic plaques sourced from ACS patients contain large numbers of platelets expressing high levels of TLRs (9).

This study aims to investigate the effect of TLR agonists on platelets from healthy volunteers and ACS patients. It therefore necessary to clearly define an ACS, explore the current knowledge of the role of platelets and the immune system in the pathogenesis of atherosclerosis and ACS, and review the literature on platelet-expressed TLRs.

1.2 Acute coronary syndromes

ACS encompass the spectrum of clinical presentations of acute myocardial ischemia resulting from coronary heart disease (CHD), namely myocardial infarction (MI) and unstable angina (UA) (34). A patient is defined as having had an ACS if their clinical presentation meets two of the three following criteria: symptoms typical of myocardial ischaemia, together with a significant rise in the serum biomarkers of cardiac damage, or new electrocardiogram (ECG) changes consistent with myocardial ischaemia (35). Once a patient has been identified with an ACS, their serum biomarker status and ECG findings are used to classify them into one of the three following diagnoses: ST-elevation myocardial infarction (STEMI), non-ST elevation myocardial infarction (NSTEMI) and unstable angina (UA) (35, 36).

Distinguishing between STEMI and NSTEMI/UA patients is important for informing clinical management. STEMI generally represents a significant degree of myocardial damage and requires emergent reperfusion therapy in the form of percutaneous coronary intervention (PCI), fibrinolysis, or coronary artery bypass graft (CABG) depending on the clinical circumstances (37, 38). By contrast, patients presenting with NSTEMI and UA do not necessarily always require urgent reperfusion therapy. For moderate-to-high risk NSTEMI/UA patients however, an early invasive approach with angiography within 48 hours and appropriate revascularisation have been demonstrated to lead to better outcomes than delayed angiography or conservative strategies (35, 39).

CHD is the leading cause of death worldwide and is the leading cause of death from a single condition in New Zealand (8, 40). According to WHO estimates, CHD was responsible for 7.3 million deaths worldwide in 2008 (8). Given the significant burden of CHD and ACS both in New Zealand and worldwide, obtaining a better understanding of the aetiology and underlying pathogenesis of these diseases will improve the ability for clinicians to treat these diseases effectively.

Reduced myocardial perfusion due to acute narrowing of a coronary artery by a thrombus derived from disruption of an atherosclerotic plaque is by far the most common cause of an ACS

(41). Disruption of an atherosclerotic plaque via rupture or erosion exposes platelets and the haemostatic system to the subendothelial matrix, containing a milieu of pro-thrombotic and platelet-activating substrates such as tissue factor, von Willebrand factor (vWF), laminin, fibronectin and thrombospondin (2, 42). The binding of local circulating platelets to these factors results in a cascade of platelet activation and recruitment to the site of plaque disruption, followed by the formation of platelet aggregates and platelet-plug over the site of plaque disruption (2, 42).

Activated platelets, through platelet-derived microparticle and calcium secretion, facilitate activation of the coagulation cascade (2, 43). Activation of the prothrombinase complex of the coagulation cascade by platelets results in the development of a fibrin-rich meshwork that functions to maintain the integrity of the platelet-plug (43). Together, this fibrin-rich meshwork and platelet-plug is known as a thrombus or clot (2). This thrombus may either immediately occlude the artery by narrowing or occluding the arterial lumen locally or via distal embolization, inducing myocardial ischaemia (44).

1.3 Atherosclerosis

Atherosclerosis is a chronic inflammatory disorder of medium to large-sized arteries, characterised by vascular remodelling of the intimal layer of arterial wall to consist of lipids, platelets, inflammatory, endothelial and smooth muscle cells (30, 45). Initially thought of as a lipid storage disease, there is a growing body of evidence implicating immune and inflammatory processes in the pathogenesis of atherosclerosis (1, 30). Chronic injury to the endothelium leads to endothelial dysfunction and the sequestration of lipids into the intimal space, which in turn elicits an inflammatory response from the endothelium (1, 30). This inflammatory response promotes the transmigration of monocytes and lymphocytes into the arterial wall that subsequently drive the vascular remodelling characteristic of an atherosclerotic plaque (1, 30). Activated platelets aid the initiation and the progression of atherosclerotic plaque development by promoting these endothelial inflammatory responses and by facilitating the penetration of monocytes and lymphocytes into the arterial wall (1).

1.4 Platelets

The main physiological role of platelets is to maintain the integrity of the vascular endothelium in a process involving the adhesion, activation, and formation of an aggregate of platelets and adhesive proteins over sites of endothelial injury (2). Since the discovery of platelets as the primary cells of haemostasis in the late 19th century, platelets have been found to have roles in cancer, angiogenesis, and inflammation (2, 46).

1.4.1 Platelet structure

Platelets are small (2-3µm in diameter), discoid shaped, anucleated fragments of cellular cytoplasm that circulate in the blood and are derived from megakaryocytes in the bone marrow (2). Platelets have a smooth surface punctuated by a collection of membrane invaginations that connect to a series of cytoplasmic membranes arranged into a network of tubules, collectively known as the open canalicular system (OCS) (47). The OCS provides platelets with a large reservoir of membrane surface area to allow platelets, upon platelet activation, to spread over areas of endothelial injury and act as a conduit for the secretion of platelet granules into the bloodstream (47-49).

1.4.1.1 Platelet granules

Platelets store a vast array of biologically active mediators in specialised granules that are released upon platelet activation (48, 49). There are four classes of platelet granules: α-granules, dense granules, lysosomes (48, 49) and T granules (15). α-granules contain adhesive and pro-inflammatory molecules, such as vWF, thrombospondin, sCD40L, Chemokine C-C motif ligand 5 (CCL5, otherwise known as RANTES), and P-selectin (CD62p). They also contain small plasma proteins (immunoglobulins and albumin), coagulation factors, protease inhibitors and stores of the $\alpha_{IIb}\beta_3$ integrin receptor. By contrast, dense granules contain high concentrations of small molecules capable of facilitating platelet activation, such as serotonin, adenosine diphosphate (ADP), magnesium and calcium. Lysosomes contain a variety of acid hydrolases capable of degrading glycoproteins, glycolipids and glycoaminoglycosans, which each may have roles in the elimination of

platelet aggregates (48, 49). T granules contain intracellular stores of TLR9, which are expressed on the platelet surface in response to platelet agonists, such as type IV collagen for example (15). Activation of platelet surface-expressed TLR9 by bacterial and viral oligodeoxynucleotides has been shown to induce platelet P-selectin expression and platelet-platelet aggregate formation (15).

1.4.1.2 Platelet cytoskeleton

The platelet cytoskeleton is primarily responsible for maintaining the shape of circulating platelets against the high shear force conditions encountered in the bloodstream (50). Upon platelet activation by thrombogenic stimuli, mobilisation of calcium from the dense tubular system into the cytosol activates actin (a contractile protein), resulting in rearrangement of the platelet cytoskeleton and a subsequent change in platelet shape – from discoid, to spheroid with pseudopodia (50, 51). This change in shape allows platelets to spread over sites of endothelial injury, more effective interaction with other platelets and adhesive proteins, and results in the release of biologically active mediators from platelet granules into the bloodstream (50, 51).

1.4.2 Platelet receptors

Platelets express an array of transmembrane receptors that determine how platelets interact with a variety of cells, adhesive proteins and soluble mediators. Classes of transmembrane receptors expressed on platelets include the integrins, leucine-rich repeated (LRR) receptors (glycoprotein [GP] GPIb/IX/V, Toll-like receptors), G-protein coupled receptors (GPCR) (PAR-1 and PAR-4 thrombin receptors, P2Y1 and P2Y12 ADP receptors, thromboxane A_2 [TXA_2] receptors]), tyrosine kinase receptors, receptors from the immunolglobulin superfamily (FcγRIIa), the C-type lectin receptors (P-selectin) and CD40L (42, 52). Of these receptors, the integrins, LRR receptors, GPCRs, FcγRIIa, P-selectin and CD40L are of importance in haemostasis and inflammation (42).

1.4.2.1 Integrins

The integrins are a family of transmembrane receptors involved in cell adhesive and signalling processes, and are present on many cell types, including platelets (47, 53). The integrins consist of

non-covalently bound heterodimers of α and β subunits that have large extracellular domains and comparatively smaller intracellular domains, with the function of linking external adhesive molecules and extracellular matrix proteins with components of the cellular cytoskeleton (53). The α and β subunits of integrins exist in two affinity states, high-binding and low-binding (47).

Platelets express six different integrins: $\alpha_2\beta_1$, $\alpha_5\beta_1$, $\alpha_6\beta_1$, $\alpha_1\beta_2$, $\alpha_{IIb}\beta_3$, and $\alpha_v\beta_3$ (54). Of these, only the $\alpha_{IIb}\beta_3$ (otherwise known as the GPIIb/IIIa complex) and the $\alpha_2\beta_1$ integrins are known to have roles in haemostasis (42). The $\alpha_{IIb}\beta_3$ integrin is unique to platelets and is the most abundantly expressed platelet receptor at approximately 80,000 receptors per platelet (54, 55). Activation of enough $\alpha_{IIb}\beta_3$ integrins into the high-affinity binding state during platelet activation is essential for the formation of platelet aggregates, and is the final common pathway for all platelet agonists (54).

1.4.2.2 Platelet leucine-rich repeated receptors

The LRR receptors function as mediators of protein-protein interactions and are characterised by horseshoe-shaped structural motifs rich in the amino acid leucine which recognise specific ligands (56). Platelets are currently known to express two sets of LRR receptors: the GPIb/IX/V receptor complex and the TLRs (52). Of these, the GPIb/IX/V receptor complex has a well-established role in haemostasis (2, 57). The GPIb/IX/V receptor complex binds vWF, and is essential for the initial tethering of platelets to sites of endothelial injury under high shear stress force conditions by allowing platelets to traverse in close proximity to the endothelial wall (2, 57). The platelet expressed TLRs will be discussed in more detail in section 1.5 of this thesis.

1.4.2.3 Platelet G-protein coupled receptors

Platelet-expressed GPCRs are involved in platelet activation, amplification of platelet responses, and the recruitment of platelets to sites of endothelial injury (58). Platelets bound to sites of endothelial injury activate and amplify other platelets by expressing a variety of auto- and paracrine mediators at sites of endothelial injury, including thromboxane A_2 (TxA_2), ADP, thrombin and serotonin (2, 58). Such mediators act upon platelet-expressed GPCRs to trigger a series of

intracellular signalling pathways that are critical in the in the formation of a stable platelet plug over sites of endothelial injury (58).

1.4.2.4 FcγRIIa receptors

The Fc family of receptors are expressed on a broad variety of cells, including neutrophils, macrophages, monocytes and platelets, and function to allow cells to bind the Fc region of antibodies (59). Accordingly, Fc receptors have central roles in infection and immunity (59). In platelets, the FcγRIIa receptor plays a critical role in bacteria-induced platelet aggregation (29).

1.4.2.5 P-selectin (CD62P)

P-selectin is a cell adhesion molecule expressed on the surface of activated platelets with functions in platelet-endothelial interactions and in the formation of platelet-leucocyte aggregates (60). Inactivated platelets express minimal levels of surface P-selectin (quiescent platelets store it in α-granules) (45, 48). Accordingly, platelet surface-expressed P-selectin is a marker of platelet activation (45). Increased levels of platelet surface-expressed P-selectin are observed in a variety of clinical conditions including ACS, diabetes mellitus, hypertension, congestive heart failure and stroke (61).

1.4.2.6 CD40L (CD154)

CD40L (CD154) is a pro-inflammatory and pro-thrombotic transmembrane protein belonging to the tumour necrosis factor (TNF) superfamily that is expressed by activated platelets (62, 63). Activated T cells, endothelial cells, smooth muscle cells, monocytes and macrophages also express CD40L (63, 64). Agonists such as thrombin and collagen are capable of inducting platelet surface-expression of CD40L (65, 66). Soon after CD40L is expressed on the platelet surface, surface CD40L is rapidly shed into the bloodstream as soluble CD40L (sCD40L) over a period of minutes to hours. sCD40L is implicated the release of inflammatory cytokines, chemokines and matrix metalloproteinases (MMPs) from leucocytes, and is also pro-thrombotic in its own right (64, 66).

1.4.3 Platelet activation by damaged endothelium

Damage to the vascular endothelium exposes the haemostatic system to an array of thrombogenic subendothelial matrix proteins and paracrine molecules capable of adhering and activating platelets at sites of endothelial injury (2, 42). These factors are detected by, and bind to, platelet transmembrane receptors that are generally expressed irrespective of the activation state of the platelet (2, 42, 52). Extracellular matrix proteins capable of facilitating platelet adhesion (and subsequent platelet activation) include collagen, tissue factor, proteoglycans, lamnin, fibronectin and thrombospondin (2, 42). Paracrine molecules released by damaged endothelium capable of facilitating platelet adhesion and activating local platelets include ADP, vWF and epinephrine (2, 42).

The initial tethering of platelets to the sites of damaged endothelium requires two sets of constitutively expressed platelet transmembrane receptors: the GPIb/IX/V complex, involved in the binding of vWF; and the integrin $\alpha_2\beta_1$ receptor with the GPVI receptor, involved in the binding of exposed collagen (2, 42). Once initially tethered, these platelets become rapidly activated by the aforementioned thrombogenic agonists present at the site of injury (2, 42, 67).

Activated platelets, by definition, have undergone a series of morphological and functional changes, including: granule secretion (leading to the release of auto- and paracrine mediators), shape change, expression of neoantigens, and changes in receptor conformation (most notably activation of the GPIIb/IIIa integrin) (2, 42, 67). Activated platelets are therefore capable of binding to and spreading over, recruiting, and activating other platelets, at sites of endothelial injury (2, 42). Autocrine and paracrine mediators released by activated platelets include ADP, thromboxane A2 (TxA_2), thrombin and vWF, all of which mediate further platelet binding and activation (2, 42, 67). Together, these processes are critical in the formation of a stable platelet aggregate over sites of vascular endothelial compromise, and for effective haemostasis (2, 42, 67).

1.4.4 Platelets aggregation and stabilisation of the platelet plug

Platelet aggregation is dependent on platelets expressing the GPIIb/IIIa integrin in its activated conformational state (2, 42, 54, 68). Activated GPIIb/IIIa forms crosslinks with fibrinogen and vWF, allowing platelets to form stable aggregates with one other (2, 42, 68). In addition, activated GPIIb/IIIa may also allow platelets to bind vitronectin, fibronectin, and thrombospondin, and therefore regulate the tethering of platelets to the subendothelial matrix (54). Furthermore, activated GPIIb/IIIa plays a critical role in the stabilisation of the platelet plug by facilitating platelet-fibrinogen binding, thereby allowing for the presentation of the prothombinase complex on the platelet surface for activation of the coagulation cascade (2, 42, 54, 67). Activation of the coagulation cascade by the prothrombinase complex results in the formation of a fibrin-rich meshwork over the platelet-plug, critical for maintaining the integrity of the plug (42). Since the activated GPIIb/IIIa complex forms the final common pathway for all platelet agonists and is crucial for platelet aggregation, it is a commonly used measure of platelet activation (54, 69).

1.4.5 Platelets as cells of the immune system

Platelets store and synthesise a broad array of pro-inflammatory mediators implicated in atherosclerosis, ACS and the defence against microbial infections (5, 6, 29, 70). Pro-inflammatory mediators stored and/or synthesised by platelets with roles in infection and immunity include histamine, TxA_2, IL-1β, thrombocidins 1 and 2, CD40L, and CCL5 (6, 70). These mediators are generally released upon platelet activation (6, 70).

1.5 Toll-like receptors

1.5.1 Toll-like receptors and the innate immune system

The innate immune system, in addition to consisting of a series of non-specific barriers to invasion, consists of cells capable of initiating non-specific and rapid inflammatory responses against pathogens. These cells (including neutrophils, macrophages and dendritic cells) rely on a collection of pathogen recognition receptors (PRRs) to detect structurally conserved molecular patterns associated with pathogenic invasion and cellular stress, known as pathogen-associated molecular patterns (PAMPs) (16) and danger-associated molecular patterns (DAMPs), respectively (71). The TLRs are a well-characterised family of membrane-spanning PRRs expressed by cells of the innate immune system that recognise a variety of PAMPs that mediate the production of cytokines and chemokines necessary for effective immune responses against infectious agents (16-18).

1.5.2 Toll-like receptor structure and ligands

The TLRs consist of a characteristic extracellular leucine-rich repeated receptor (LRR) domain for the recognition of PAMPs and an intracellular Toll/IL-1 receptor (TIR) domain for downstream signalling purposes. The human transcriptosome is currently known to express at least ten functional TLRs (TLRs 1 to 10) (16, 17).

TLRs are classified based on their cellular location and to what ligands/PAMPs they respond. TLRs 1, 2, 4, 5, and 6 are expressed on the cell surface and recognise extracellular PAMPs (lipids, lipoproteins, proteins), whilst TLRs 3, 7, 8 and 9 are expressed within intracellular vesicles and recognise intracellular PAMPs (mostly nucleic acids). Activation of a TLR by its appropriate ligand results in the recruitment of TIR domain adaptor molecules, which induce downstream signalling pathways and the secretion of cytokines, chemokines and antimicrobial peptides critical for effective inflammatory responses against pathogens from cells of the innate system (16, 17).

1.5.2.1 Toll-like receptor 4

The most well characterised human TLR is TLR4. TLR4 together with its co-receptor MD-2 (lymphocyte antigen 96), recognises lipopolysaccharide (LPS), a lipid-containing carbohydrate sourced from the outer membrane of gram-negative bacteria (72). In addition to MD-2, binding of LPS by TLR4 also requires lipoprotein binding protein (LBP) and CD14 (72). Activation of TLR4 by LPS can elicit strong innate immune responses from the human immune system and is one of the pathogenic mechanisms underlying septic shock (27-29). TLR4 is also capable of recognising antigens from the respiratory syncytial virus, *S. pneumoniae*, and modified LDL (72).

1.5.2.2 Toll-like receptor 2

TLR2 recognises a variety of triacylated and diacylated lipopeptides expressed by bacteria, fungi, parasites and viruses (72). The ability for TLR2 to recognise a variety of ligands is due to its unique ability to form functional heterodimers with either TLR1 or TLR6 (73, 74). The TLR2/1 and TLR2/6 heterodimers specifically recognise triacylated lipopeptides and diacylated lipopeptides, respectively (73, 74). Antigens recognised by TLR2 (or its heterodimers) include lipoarabinomannan from mycobacteria, zymosan from fungi, heat shock proteins, cardiac myosin and modified LDL (72).

1.5.2.3 Toll-like receptor 9

TLR9 is intracellularly expressed and belongs to the class of TLRs that recognise nucleic acids. TLR9 recognises unmethylated CpG (cytosine followed by guanine) sequences in DNA, which are motifs commonly expressed by bacteria and viruses but not human cells (17).

1.6 Infections, inflammation, platelets and thrombosis

There is a well-established link between inflammation, platelets and thrombosis. Inflammation and infections increase the risk of vascular thrombotic events (75-77). Platelets are cells involved in both haemostasis and immunity, and contribute to the pathogenesis of thromboinflammatory diseases such as atherosclerosis and disseminated intravascular coagulation (78).

1.6.1 Inflammatory cytokines in acute coronary syndromes

In ACS patients, coronary arterial blood contains more leucocytes with pro-inflammatory phenotypes and greater levels of pro-inflammatory cytokines (IL-1, IL6, IL-8, IL-12, IL-17, and CD40L) than aortic blood (4, 79). Furthermore, coronary thrombi leucocytes express more TLR2 and TLR4 than aortic blood leucocytes (79). These pro-inflammatory leucocytes and cytokines are thought to have pro-thrombotic roles by increasing platelet reactivity, enhancing tissue factor expression on epithelial cells, and by stimulating the generation of unusually dense fibrin (4).

1.6.1.1 Toll-like receptors in atherosclerosis and acute coronary syndromes

Endothelial cells, macrophages, T cells, smooth muscle cells and fibroblasts in atherosclerotic intima express higher levels of TLR1, TLR2 and TLR4 than in non-atherosclerotic intima (80). TLR4 expression is concentrated in the areas of atherosclerotic plaques most likely to rupture (81). Furthermore, TLR2 and TLR4 expression on macrophages and endothelial cells are correlated with the expression of MMPs and pro-inflammatory cytokines, both of which are associated in atherosclerotic plaque instability (80). Accordingly, monocytes from patients with ACS have been found to express significantly higher levels of TLR4 than monocytes from stable angina patients or healthy controls (82).

1.6.2 Chronic infections as a risk factor for acute coronary syndromes

Chronic infections with *Chlamydia pneumoniae*, periodontal pathogens such as *Aggregatibacter actinomycetemcomitans* and *Porphyromonas gingivalis*, cytomegalovirus, and

Epstein Barr virus are predictors of coronary artery disease (83-85) and have also been identified in atherosclerotic plaques (86, 87). These pathogens are thought contribute to the development of endothelial dysfunction and atherosclerotic plaque progression (78, 88, 89).

1.6.3 Acute infections as a trigger for acute coronary syndromes

Acute infections may trigger ACS by inducing inflammation of the coronary arteries and promoting atherosclerotic plaque instability (32). Atherosclerotic plaques from those with unstable coronary disease contain higher levels inflammatory cells (such as dendritic cells and activated macrophages) than those from stable coronary disease, for example (4, 76). Furthermore, influenza, acute respiratory tract, and urinary tract infections (UTIs) have each been associated with increased ACS risk (31, 33).

1.6.4 Infections as risk factors for adverse events following acute coronary syndromes

In addition to potentially triggering ACS, acute infections may also be a risk factor for adverse ischaemic events in patients who have already had an ACS. For example, influenza and UTIs are associated with increased recurrent myocardial infarction (33) and stent thrombosis (90) risk. Moreover, immunisation of patients who have had an ACS with the influenza vaccine has been demonstrated to reduce mortality and recurrent vascular ischaemic events in patients who have had an ACS (91, 92), and may even be more cost-effective than statins and beta-blockers in doing so (7).

1.6.5 Platelet receptor interactions with bacteria

Platelets can interact with bacteria through the GPIIb/IIIa and GPIbα integrins, the FcγRIIa receptor, and the platelet-expressed TLRs. Platelets have been demonstrated to become activated and/or aggregate via these receptors in the presence of the following bacteria: *Staphylococcus aureus*, *Streptococcus pneumoniae*, *C. pneumoniae*, and *P. gingivalis* (29, 78).

1.6.6 Platelets in atherosclerosis and acute coronary syndromes

Platelets have key roles in the initiation and progression of atherosclerosis. Examples of proinflammatory mediators released and/or expressed by activated platelets and platelets bound to

dysfunctional endothelium known to be pro-artherogenic and pro-thrombotic include IL-1β, CD40L, CCL5 and P-selectin (70, 88).

1.6.6.1 Platelet activation markers in atherosclerosis and acute coronary syndromes

Patients with atherosclerosis and ACS have higher levels of circulating activated platelets and platelets with pro-inflammatory phenotypes than healthy controls. Platelet activation markers increased in atherosclerosis and ACS patients include P-selectin, CD40L, platelet-monocyte/neutrophil (heterotypic) aggregates, and the activated GPIIb/IIIa integrin (93-95). Furthermore, these patients' platelets are more sensitive to stimulation by platelet agonists such as ADP, epinephrine, and thrombin receptor activator peptide (TRAP), than healthy controls (93, 95). Interestingly, in the setting of acute chest pain, circulating platelet-monocyte/neutrophil aggregates, CD40L and the activated GPIIb/IIIa may each be predictors of ACS (96). In STEMI patients, greater levels of platelet P-selectin expression are associated with increased myocardial damage (97). Finally, sCD40L may be predictive of adverse ischaemic events post-ACS (62).

1.6.7 Antiplatelet therapy in acute coronary syndromes

Platelet activation and aggregation are important in the pathogenesis of arteriothrombosis. Accordingly, antiplatelet therapies are commonly employed in clinical settings of arteriothrombotic ischaemic events, and involve the use of cyclooxygenase inhibitors, P2Y12 receptor inhibitors that block platelet responses to ADP, phosphodiesterase inhibitors, and thrombin inhibitors. Examples of these medications include aspirin, clopidogrel, dipyridamole and atopaxor, respectively (98).

Dual antiplatelet therapy (DAPT) in the form of aspirin and clopidogrel is a mainstay in the management of ACS, as it has been convincingly demonstrated to decrease mortality and prevent recurrent ischaemic events in these patients (99). Although the primary mechanism underlying the protective effects of DAPT in ACS is its ability to inhibit platelet activation and aggregation, there is a growing body of evidence to suggest that the modulation of platelet immune and inflammatory functions by DAPT may also contribute to its therapeutic effects (7).

1.7 Platelet-expressed Toll-like Receptors

1.7.1 Discovery and expression of platelet-expressed Toll-like receptors

It is now well established that human platelets express TLRs. TLRs known to be expressed on human platelets include TLRs 1, 2, 4, 6, and 9 (9, 11-15). TLR1 and TLR6 were first TLRs to be found on human platelets, and were discovered through a combination of real-time polymerase chain reaction (RT-PCR), flow cytometric and western blotting techniques (9). Flow cytometry and western blotting techniques have subsequently been used to confirm the expression TLRs 2, 4 and 9 (11-13) on human platelets, with immunopreciptation used to confirm the expression of TLR1 and TLR6 (14).

1.7.2 Functional significance of platelet-expressed Toll-like receptors

Given the central role of TLRs in inflammatory responses, the activation of platelet-expressed TLRs by pathogens may provide a bridging mechanism between platelets' immune and haemostatic functions. Studies investigating the functional significance of platelet-expressed TLRs have largely focused on the functionality of TLRs 2/1, 2/6, 4 and 9 in healthy subjects, with only a handful of studies investigating their expression and functionality in subjects with disease. Furthermore, there are inconsistencies in the literature on the functionality of platelet-expressed TLR2/1, 2/6 and TLR4 in different platelet systems (whole blood, platelet-rich plasma, and washed platelets).

1.7.2.1 Toll-like receptor 2/1

The platelet-expressed TLR2/1 heterodimer has pro-inflammatory and pro-thrombotic roles. Stimulation of washed platelets by the synthetic triacylated lipoprotein and TLR2/1 agonist, Pam3CysSerLys4 (PAM_3CSK_4) at concentrations of 5 µg/mL to 10 µg/mL, resulted in platelet activation (as measured by CD62p and PAC-1 expression), adhesion and aggregation (14, 24). At these concentrations PAM_3CSK_4 was also capable of stimulating platelet-platelet aggregation (24, 100), though not to the same degree as thrombin (a potent platelet agonist) (24). This response to PAM_3CSK_4 was dependent on the transformation of the GPIIb/IIIa integrin into its activated conformational state and the purinergic receptors P2X1, P2Y1 and P2Y12 (100). PAM_3CSK_4 has also

been demonstrated to be capable of stimulating the release of reactive oxidative species (14), intracellular calcium, and thromboxane B2 (TxB_2, inactive metabolite of TxA_2) from washed platelets (100).

Contrary to the studies investigating washed platelets, PAM_3CSK_4 appears to be unable to induce the formation of platelet-platelet aggregates or clots in whole blood (14, 24, 100). Similarly, stimulation by PAM_3CSK_4 at 10 ng/mL to 1 µg/mL concentrations appears to be unable to activate platelets (as measured by P-selectin expression and Ca^{2+} mobilisation) within platelet-rich plasma, even after priming of the platelet-rich plasma with submaximal levels of ADP (11).

In whole blood however, stimulation with 5 to 10µg/mL concentrations of PAM_3CSK_4 can induce the formation of platelet-neutrophil and platelet-monocyte (heterotypic) aggregates in a TLR2/1- and a P-selectin-dependent manner (14, 24, 25, 100). Interestingly, PAM_3CSK_4 was a more potent stimulator of heterotypic aggregates than thrombin (14). This observation may be secondary to some unknown effect(s) of TLR agonists on the propensity of neutrophils and/or monocytes to form platelet-leucocyte aggregates. Furthermore, the activation of the phosphoinositide 3-kinase (PI3K)/Akt signalling pathway shared by PAM_3CSK_4 and thrombin (14) appears to occur at different times and with different levels of phosphorylation for PAM_3CSK_4 and thrombin, which may also explain the difference between the two (24).

Platelet-expressed TLR2/1 also has roles in platelet-bacteria interactions. *S. pneumoniae*, a commensal bacteria present in the upper respiratory tract and the responsible pathogen for pneumococcal sepsis, can induce platelet-aggregation and dense-granule release in a platelet-expressed TLR2-dependent manner (101). The periodontal pathogens, *A. actinomycetemcomitans* and *P. gingivalis* have also been found to be capable of activating individual platelets in a TLR2/1-dependent manner, as measured by dense-granule release (25). Furthermore, platelet-neutrophil aggregates (formed in a TLR2-dependent manner) appear to be better than neutrophils alone at clearing of the periodontal pathogens *A. actinomycetemcomitans* and *P. gingivalis in vitro* (25).

1.7.2.2 Toll-like receptor 2/6

Stimulating platelets with Macrophage Activating Lipopeptide-2 (MALP-2), a synthetic TLR2/6 agonist, does not appear to result in platelet activation or platelet aggregation in washed platelets (100). Moreover, the presence of MALP-2 appears to nullify the positive effects of PAM_3CSK_4 on platelet activation and heterotypic aggregate formation in washed platelets and in whole blood (100). Another TLR2/6 agonist however, Pam2CysSerLys4 (PAM_2CSK_4), was found to be capable of inducing platelet aggregation in washed platelets, though to a lesser degree than the TLR2/1 agonist, PAM_3CSK_4 (14).

1.7.2.3 Toll-like receptor 4

While the results are somewhat divergent, the majority of the studies indicate that stimulating whole blood and platelet-rich plasma with LPS sourced from a variety of *E. coli* serotypes can activate platelets via TLR4, as measured by granule release (21, 23, 102), inflammatory cytokine release (19-22, 26), and the formation of platelet-leucocyte aggregates (23, 25). LPS by itself however, is not capable of inducing platelet-platelet aggregation in washed platelets and platelet-rich plasma (11, 27), but may be able to prime platelet aggregation responses to known platelet agonists such as collagen, epinephrine, thrombin and ADP (21, 23, 102).

An initial study found that stimulating platelet-rich plasma with 10 ng/mL to 1 µg/mL concentrations of LPS from *E. coli* serotype 0111:B4 had no impact on platelet P-selectin expression, Ca^{2+} mobilisation, or platelet aggregation (11). Moreover, LPS 0111:B4 was also unable to prime either platelet activation to stimulation by conventional agonists (such as ADP or platelet activating factor) or vice versa (11). In a similar study, stimulation of washed platelets with 5 µg/mL of LPS 0111:B4 alone had no impact on platelet P-selectin expression or platelet aggregation either (27).

Studies that are more recent suggest that a variety of *E. coli* serotypes of LPS (including serotype 0111:B4) are capable of inducing platelet activation via platelet-expressed TLR4. For example, stimulation of washed platelets by 100 ng/mL of LPS 0111:B4 in the presence of CD14 and

LBP was capable of inducing P-selectin expression on a subpopulation of platelets (21). In another study, stimulation of washed platelets by 1 μg/mL of LPS 0111:B4 for 30 minutes induced platelet P-selectin expression and dense granule release (102). Similarly, stimulation of washed platelets with 0.5 μg/mL to 2 μg/mL of LPS 0111:B4 for 15 minutes was also found to induce dense granule release (19). LPS 0111:B4 has also been found to up-regulate cyclooxygenase-2 splicing (22), induce the release of the proinflammatory cytokine interleukin-1β (IL-1β) on platelet-derived microparticles (21, 22), and promote the shedding of sCD40L (19, 20). Other *E. coli* serotypes of LPS, including O157:H7, O103:H2, 0111:HN, and 0121:H19, have also been demonstrated to be capable of activating washed platelets via platelet-expressed TLR4, as measured by the activation of the GPIIb/IIIa complex and increases in the surface expression of CD40L (21, 26).

It is not entirely clear what is responsible for the discrepancies in platelet activation responses to LPS stimulation between the studies, apart from possibly some unknown effect(s) of the use of different protocols in the preparation of platelet-rich plasma and washed platelets in each study.

In addition to having a role in platelet activation and platelet-platelet aggregation, stimulation of platelet-expressed TLR4 by LPS leads to the formation of heterotypic aggregates (25), which may allow platelets to aid the immune system in the detection and clearance of certain bacterial pathogens (25, 27). For example, stimulation of washed platelets with 5 μg/mL LPS 0111:B4 *in vitro* was demonstrated to lead to the formation of neutrophil extracellular traps (which aid in the killing of pathogens in settings of severe infection) in a platelet-expressed TLR4-dependent manner (27).

Two recently published papers demonstrated that stimulating washed platelets and platelet-rich plasma with LPS 0111:B4 could prime platelet aggregation to thrombin, collagen, and ADP (21, 102). Interestingly, Zhang et al. showed that the threshold concentration of LPS 0111:B4 required to prime platelet aggregation was significantly lower in platelet-rich plasma than in washed platelets, suggesting that a cofactor in plasma may be responsible for sensitising platelet aggregation (102).

1.7.2.4 Toll-like receptor 9

TLR9 is expressed in platelets within intracellular endosomes that are located in the periphery of the cell (15). Activated platelets express higher levels of TLR9 than quiescent platelets (12). Platelet surface expression of TLR9 can be induced by stimulating platelets with type IV collagen, or thrombin in the presence of Ca^{2+} (13, 15). Pre-incubation of resting washed platelets with type IV collagen followed by stimulation by the TLR9 agonist, type C CpG oligodeoxynucleotide (ODN), has been demonstrated to induce platelet P-selectin expression and platelet-platelet aggregation (15).

1.7.3 Summary

Platelets, the immune system and TLRs each have roles in the pathogenesis of atherosclerosis and ACS. Platelets express functional TLR2/1 (14, 24, 100), TLR2/6 (14), TLR4 (19-22, 25-27, 102) and TLR9 (15). Activation of these platelet-expressed TLRs by their appropriate ligands can lead to the expression of a number of platelet activation markers and cytokines relevant to inflammation, atherosclerosis and ACS, such as CD40L, P-selectin and IL-1β (70, 88). Furthermore, under some circumstances, activation of platelet-expressed TLRs can prime, or even lead directly to platelet-platelet and platelet-leucocyte aggregate formation (21, 23, 102).

Patients with atherosclerotic disease have increased platelet reactivity and greater levels of circulating activated platelets than healthy controls (93, 95). Patients with risk factors for atherosclerosis and acute myocardial infarction, such as smoking (103), hypertension (104) and diabetes mellitus (105), also have increased platelet reactivity and greater levels of circulating activated platelets. Activated platelets express greater levels of surface TLR2 and TLR9 and lesser levels of surface TLR4 than unstimulated platelets (12). Furthermore, the coronary environment of ACS patients has a more pro-inflammatory phenotype, with large numbers of platelets positive for TLR1 and TLR6 (9), than with those stable coronary disease or healthy controls (79).

Accordingly, the platelet-expressed TLRs may represent a mechanistic link between infection and thrombosis for diseases with dual infectious/inflammatory and thrombotic components. Given

the central role of platelets in atherosclerosis and ACS, we decided to examine platelet activation responses to TLR agonists in ACS patients and healthy controls

1.8 Hypothesis and Aims

1.8.1 Hypothesis

We hypothesised that stimulation by TLR agonists would lead to enhanced platelet activation, and that this response would differ amongst ACS patients and healthy controls.

1.8.2 Specific aims

1. Develop a flow cytometry based protocol to evaluate platelet-activation markers in whole blood;
2. Determine the appropriate concentration of TLR agonists for TLR2/1, TLR2/6 and TLR4, to elicit a platelet response in whole blood, and;
3. Examine and compare platelet responses to TLR agonists in whole blood from ACS patients and healthy, age- and sex- matched controls.

Chapters 2 of this thesis will describe the methods and results for the fulfilment of aims one and two, and Chapter 3 the methods and results from aim three of the study. Chapter 4 will discuss the results from the previous chapters, inspect the strengths and weaknesses of the study, and will examine how the results fit in the wider context of platelet-expressed TLRs.

2 Chapter 2 – Protocol Development

2.1 Introduction

2.1.1 Principles of flow cytometry

Flow cytometry is a powerful tool for the quantitative analysis of particles and cells and has numerous clinical and research applications that range from the diagnosis of cancer through to the measurement of cellular enzymatic processes. More detail on the principles of flow cytometry, including the principles of fluorescence, compensation and spectral overlap, can be found in Appendix 1.

2.1.2 Platelet flow cytometry

Flow cytometry can be used to assess the *ex vivo* function of circulating platelets and the *in vitro* reactivity of platelets to exogenous stimuli by measuring a variety of activation-dependent platelet markers and platelet-leucocyte aggregates. Previous studies have used flow cytometry based techniques to investigate a wide range of platelet related questions including the assessment of acquired and inherited platelet disorders, such as Bernard-Soulier syndrome and Glanzmann thrombasthenia; the monitoring of thrombopoesis and the efficacy of antiplatelet agents; and in a variety of blood bank applications including platelet concentrate quality control and platelet cross matching (45, 69, 106).

2.1.2.1 Advantages of platelet flow cytometry

There are a number of advantages to the flow cytometric analysis of platelets in comparison to other methods of measuring platelet function. One key advantage of platelet flow cytometry is that it allows for the detailed study of individual platelets, since flow cytometry can simultaneously assess an array of different platelet responses to a variety of putative *in vivo* and *in vitro* stimuli. This technique is distinct from platelet aggregometry or the measurement of soluble P-selectin for example, which only indirectly demonstrate whether a particular *in vivo* condition results in

increased platelet activation on a population level. Other advantages of flow cytometry include that platelets can be analysed in the physiological milieu of whole blood and that only very small amounts of blood (≈ 5 µL per sample) are required (45, 69, 106).

2.1.2.2 Disadvantages of platelet flow cytometry

Conversely, platelet flow cytometry also has a number of disadvantages. Firstly, platelets are particularly sensitive to *ex vivo* artifactual activation. Consequently, rapid access to a flow cytometer is required (within four hours of blood collection) to minimise artifactual platelet activation, which can be difficult to achieve in the clinical setting (45, 69, 106). Additionally, the method of blood collection, choice of anticoagulant, sample handling, processing times and method of cell fixation can each have an effect on platelet activation. Accordingly, particular care is required to minimise the factors that contribute to the *ex vivo* artifactual platelet activation when developing platelet flow cytometry protocols (45, 69, 106). Flow cytometers and the antibodies for flow cytometric analysis are expensive to maintain and purchase. Although flow cytometric analysis can be delayed to up to 24 hours if blood samples are fixed upon collection, not all of the antibodies used in platelet flow cytometry, especially PAC-1, are functional on fixed blood. Another limitation of platelet flow cytometry is that since the flow cytometry based methods of analysing platelets are limited to individual platelets, the presence of platelet-platelet and platelet-leucocyte aggregates can complicate the analysis and interpretation of the data (45, 69, 106).

2.1.2.3 Platelet flow cytometry in whole blood versus platelet-rich plasma

Platelets can be analysed by flow cytometry in whole blood or isolated in platelet-rich plasma. A key advantage of whole blood platelet flow cytometry is that it provides a more physiological depiction of *ex vivo* platelet-function than in platelet-rich plasma. This is because in whole blood, platelets are studied in the presence of erythrocytes and leucocytes, both of which are known to influence platelet function (45). Another advantage of whole blood platelet flow cytometry is that it involves the minimal manipulation of samples in comparison to platelet-rich plasma (69, 106).

Preparation of platelet-rich plasma involves centrifugation that can result in artifactual *ex vivo* platelet activation and a significant loss of platelets (in excess of 30% of the total platelet population can be lost in the preparation of platelet-rich plasma) (106). Consequently, the analysis of platelets in whole blood provides a better qualitative understanding of the potential effect(s) of a platelet agonist than the analysis of platelets in an isolated platelet system, at least for the clinical setting (69, 106).

By contrast, flow cytometric analysis of an isolated platelet system segregates platelets, and therefore allows examination of whether an agonist directly affects platelet function; rather than an indirect effect caused by the action(s) of this agonist on other cells (69, 106).

2.1.2.4 Platelet markers for flow cytometry

Platelets express a variety of surface markers suitable for flow cytometric analysis. These range from constitutively expressed surface glycoproteins unique to platelets used in the identification of platelet populations in whole blood, through to activation-dependent markers used for measuring platelet reactivity to a variety of *in vitro/ex vivo* stimuli.

Development of a flow cytometry based protocol for the analysis of platelets in whole blood requires the accurate differentiation of platelets from erythrocytes and leucocytes. Since platelets are fragments of cellular cytoplasm, they can be differentiated from other cell types based on their characteristic forward and side scatter properties (FSC and SSC) only. However, in order to ensure that other platelet-sized cells and particles do not contaminate this type of analysis, a pan-platelet marker can be used to accurately identify all of the platelets in the sample. This can be readily achieved using fluorescently-labelled monoclonal antibodies directed against constitutively-expressed surface glycoproteins unique to platelets (69, 106). Constitutively expressed surface glycoproteins commonly used to identify platelets in whole blood for flow cytometry include the GPIIb/IIIa complex (CD41/61), the GPIbα (CD42b) receptor and the GPIX (CD42a) receptor.

There are a variety of activation-dependent markers for use in the flow cytometric analysis of platelets. Initiation of the platelet activation process by agonists results in a series of morphological alterations and functional responses in platelets detectable by flow cytometry, including platelet degranulation, shape change, expression of neo-antigens, changes in the conformational state and the density of platelet glycoproteins/receptors, the initiation of pro-coagulant activity, and the formation of platelet-leucocyte aggregates. Consequently, platelet responsiveness to a variety of different agonists can be tested with antibodies directed against markers of platelet activation, and measuring how these activation-dependent markers change from baseline after stimulation (45, 69, 106).

Markers of platelet degranulation include P-selectin (α-granules), CD63 (dense granules) and Lysosomal-associated membrane protein 1 (lysosomes). CD40L (CD154) is an example of a neo-antigen expressed by activated platelets detectable by flow cytometry, while PAC-1 is an example of a monoclonal antibody directed against the GPIIb/IIIa receptor in its activated conformational state commonly used to identify activated platelets (45, 69, 106).

2.1.3 Toll-like receptor stimulation

2.1.3.1 Choice of Toll-like receptor agonists

The second aim of the thesis was to determine the appropriate concentrations of TLR agonists (LPS, PAM_3CSK_4, and FSL-1) to elicit platelet responses in whole blood. The TLR agonists selected for use in this study were Lipopolysaccharide from *E. coli* 0111:B4 strain (LPS), a TLR4 agonist; Pam3-Cys-Ser-Lys4 (PAM_3CSK_4), a synthetic TLR1/2 agonist; and fibroblast stimulating ligand-1 (FSL-1), a synthetic TLR2/6 agonist. These TLR agonists were selected because they are commonly used and are specific for their respective TLR receptors.

2.1.3.2 Concentration of Toll-like receptor agonists

Concentrations of serum endotoxin levels have been found to range from between 4 pg/mL and to 200 pg/mL in healthy subjects, and increase in response to infection, smoking and non-alcoholic fatty liver disease (107-109). It was not possible from the literature to ascertain what constituted a 'physiologically relevant' range of concentrations for PAM_3CSK_4 and MALP-2. Studies examining the reactivity of platelets to stimulation by TLR agonists *ex vivo* have used a range of TLR agonist concentrations, ranging from 10 ng/mL to 10 µg/mL for both LPS and PAM_3CSK_4 (11, 19, 21, 24, 26, 27, 110), however whether any of these concentrations are physiologically relevant *in vivo* is currently unclear. At the time of the writing of this thesis, no studies in the literature appeared to have used FSL-1, a synthetic TLR 2/6 agonist, for the *ex vivo* stimulation of platelets. MALP-2, also a synthetic TLR 2/6 agonist has been used to stimulate platelets *ex vivo* in concentrations ranging from 1 ng/mL to 4 µg/mL, however (100).

2.1.3.3 Incubation time

The length of time whole blood or platelet-rich plasma samples to be analysed by flow cytometry were incubated with TLR agonists also ranged between studies: 10 minutes (24), 15 minutes (19), 30 minutes (27), 45 minutes (26), 15 minutes to 1 hour (11, 110), and 1 hour to 3 hours

(21). The most common length of time samples were incubated with TLR agonists for across these studies was between 45 to 60 minutes.

2.1.4 Specific Aims

This following sections of this chapter will address the first and second aims of this study: 1) to develop a flow cytometry based protocol to evaluate platelet-activation markers, particularly those relevant to cardiovascular disease, in whole blood, and 2) to determine what concentrations of the aforementioned TLR agonists were capable of eliciting platelet responses for the above markers in whole blood.

2.2 Methods

2.2.1 Study participants and ethical approval

Samples for the development of the flow cytometric protocol were collected from four healthy adult volunteers with no history of platelet-function disorders and after they had given informed written consent to participate in the study. Ethics approval for this study was given by the Central Region Ethics Committee.

2.2.2 Blood collection

The act of drawing blood is potentially a source of artifactual platelet activation and aggregation, particularly if the draw results in red cell haemolysis, tissue thromboplastin contamination or stasis (45, 106, 111). Accordingly, a standardised protocol of blood collection, designed to minimise artifactual platelet activation and aggregation, was developed based on recommendations from the literature.

Blood was obtained from health adult volunteers by venepuncture from a vein in the antecubital fossa using a 21-gauge 'butterfly' needle (BD Bioscience) and the aid of a light tourniquet (if necessary) that was released immediately upon venous access. Blood was directly collected from the 'butterfly' system into three sterile vacuum tubes in the following order of draw: two 2.7 mL tubes containing 0.105 M buffered sodium citrate anticoagulant (BD Bioscience) that were used as discards, and one 3.0 mL tube containing 200 U/mL hirudin anticoagulant (Dynabyte, Munich, Germany) used for the flow cytometric analysis. Hirudin is a recommended anticoagulant for use in platelet flow cytometry (106).

If there was evidence of a poor blood draw (that is, intermittent or turbulent blood flow), blood samples were immediately discarded and another antecubital vein was accessed for venepuncture. Tubes containing satisfactory blood samples were gently inverted 3-4 times and left

for a maximum of 30 minutes at room temperature to sit before preparation for stimulation by agonists and then flow cytometric analysis.

2.2.3 Flow cytometer

The flow cytometer used was a Millipore guava easyCyte 8HT System (Millipore). It contains both a blue (488nm) and a red (640nm) laser and is capable of detecting six fluorescent channels with the following band pass filters: 525/30nm (green), 583/26nm (yellow), 690/50nm (red1), 785/70nm (NIR1), 661/19nm (red2) and 785/70nm (NIR2).

2.2.4 Choice of antibodies and fluorochrome selection for flow cytometry

The aforementioned platelet markers were chosen for analysis on the basis of their suitability for evaluating platelet-activation in whole blood and their relevance to cardiovascular disease.

a) CD42a (GPIX) receptor – a constitutively expressed platelet glycoprotein only found on platelets for the platelet-identification marker;
b) PAC-1 – the activated GPIIb/IIIa receptor essential for platelet aggregate formation;
c) P-selectin (CD62p) – a marker of α-granule degranulation; and
d) CD40L (CD154) – a pro-inflammatory and prothrombotic mediator expressed by platelets; it is implicated in the pathogenesis of atherosclerotic plaque rupture.

2.2.4.1 CD42a (GPIX) receptor

CD42a is a constitutively expressed platelet glycoprotein that forms a part of the GPIb/IX/V receptor complex and is involved in the binding of vWF and is only found on platelets. CD42a is theoretically preferred over the other platelet identification markers as it is not directly involved in ligand binding, does not change conformation upon platelet activation and resists cleavage by proteases (106). These characteristics make CD42a the ideal receptor for use in the context of this study, as neither the activation of platelets or leucocytes by TLR stimulation are likely interfere with CD42a receptor expression (and therefore the binding of anti-CD42a antibodies to the CD42a receptor).

2.2.4.2 PAC-1(activated GPIIb/IIIa receptor)

PAC-1 is a pentameric IgM that binds to the GPIIb/IIIa receptor only when it is in its activated conformational state, and is a widely used marker of platelet activation (68). As described above, activation of the GPIIb/IIIa receptor represents the final common pathway for all platelet agonists, and is essential for the formation of platelet aggregates (54, 69). PAC-1 is therefore an ideal marker for investigating the functional significance of putative platelet agonists and in quantifying the efficacy of antiplatelet agents in abrogating platelet aggregation responses to a variety of *in vivo* and *in vitro* stimuli (45, 68, 106). In coronary artery disease, increased PAC-1 expression may be associated with increased risk of transitioning from a clinically stable to an unstable phenotype (112).

2.2.4.3 P-selectin (CD62p)

P-selectin (CD62p) is a marker of platelet α-granule degranulation and has key functional roles in platelet-endothelial interactions and in the formation of platelet-leucocyte aggregates. It is one of the most widely studied flow cytometric markers of platelet activation and is capable of reliably quantifying platelet α-degranulation in response to a variety of *in vitro* stimuli (45). CD62p is a reliable marker of *in vitro* platelet α-degranulation as activation-dependent increases in platelet surface P-selectin expression *in vitro* have been demonstrated to remain constant over time (45). In the clinical setting, increased levels of platelet-expressed surface P-selectin have been observed in coronary artery disease and heart failure (96, 97, 113).

2.2.4.4 CD40L (CD154)

CD40L (CD154) is a pro-inflammatory and prothrombotic mediator expressed by activated platelets, and is therefore a commonly used marker of platelet activation (69, 106). Platelet-derived CD40L is implicated in the development of atherosclerosis and thrombosis (114). Activation of CD40 by CD40L results in the release of a variety of pro-inflammatory chemokines, cytokines and MMPs from a broad array of cell types including monocytes, fibroblasts, and endothelial cells (63, 64).

Furthermore, the CD40L-CD40 interaction has also been demonstrated to be pro-thrombotic by inducing the expression of tissue factor on macrophages and endothelial cells (62, 64). In the clinical setting, increases in platelet CD40L expression and levels of serum sCD40L have been observed in patients with coronary artery disease (94).

2.2.4.5 Fluorochrome selection

The selection of which conjugated fluorochrome to use for each antibody was based on an aim to minimise spectral overlap and to prevent staining populations of cells too brightly. Addressing these is important for ensuring that any loses in assay sensitivity and data resolution following compensation are minimised, and do not affect the reliability and reproducibility of the multicolour flow cytometry approach.

The selection of fluorochromes was limited by the specifications of the flow cytometer (the channels it was equipped with), and what fluorochrome-conjugated antibodies were commercially available.

2.2.5 Preparation of blood samples for flow cytometry

Blood for flow cytometry was collected as described above. Following blood collection, 90 µL of the hiruidin anticoagulated blood was added to a 0.5 mL eppendorf tube for stimulation by 10 µL of appropriately-titred agonist for stimulated samples, or 10 µL PBS for unstimulated samples. Following addition of agonist or PBS, the eppendorf tubes were capped and the blood samples were left undisturbed at room temperature to incubate for 30 minutes.

5 µL of this stimulated or unstimulated (where appropriate) blood was then added to a 0.5 mL eppendorf tube containing 45 µL of antibody staining mixture consisting of Phosphate Buffered Saline (PBS) and an appropriate concentration of antibody (or antibodies) to give a final staining volume of 50µL. This was done in a manner to ensure that the blood was delivered below the surface of the buffer. The sample was then mixed by gently agitating the eppendorf tube, and then left undisturbed in the dark at room temperature to stain for 30 minutes.

Platelets in whole blood were incubated with combinations of the following fluorochrome-conjugated monoclonal antibodies: Peridinin chlorophyll protein (PerCP)-conjugated mouse anti-human CD42a IgG antibody (Ab), clone Beb1 (Becton, Dickinson (BD) Bioscience); Fluorescein isothiocyanate (FITC)-conjugated mouse anti-human PAC-1 IgM Ab, clone SP2 (BD Bioscience); R-Phycoerythrin (PE)-conjugated mouse anti-human CD62p IgG Ab, clone AK4 (Biolegend, San Diego, CA, USA); and allophycocyanin (APC)-conjugated mouse anti-human CD154 IgG Ab, clone 24-31 (Biolegend, San Diego, CA, USA). The following isotype controls were used to set the negative control populations: PerCP-conjugated mouse IgG1 isotype control (BD Pharmingen), FITC-conjugated mouse IgM isotype control (BD Pharmingen), PE-conjugated mouse IgG1 isotype control (Biolegend, San Diego, CA, USA), and APC-conjugated mouse IgG1 isotype control (Biolegend, San Diego, CA, USA). Isotype controls were paired to each antibody based on their isotype and fluorochrome, and were purchased from the same supplier.

Following the 30 minute incubation period, the samples were fixed with the addition of a 450 µL of 0.2% paraformaldehyde in PBS. Then 50 µL of each sample was transferred into a 1.5 mL eppendorf tube and 950 µL of ice-cold 1% paraformaldehyde in PBS was added to dilute the sample to an appropriate concentration. The fixed and diluted samples were then stored at 4°C until analysis in the flow cytometer.

A schematic of the preparation of blood samples for flow cytometry is described in Figure 1.

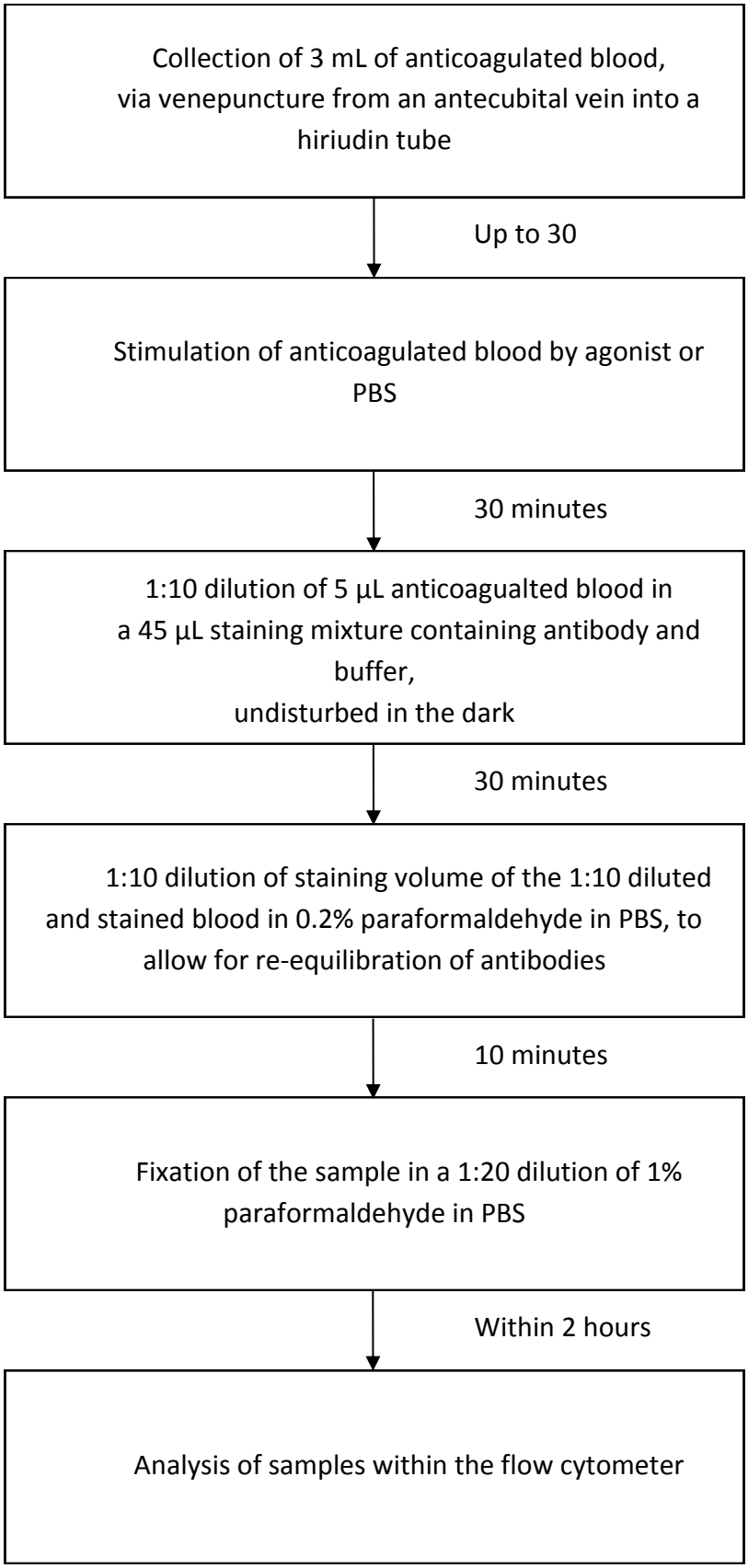


Figure 1. Schematic of the preparation of blood samples for flow cytometric analysis

2.2.6 Flow cytometry detector settings

Samples were run on the flow cytometer within two hours of fixation/storage, and at a low flow rate (0.24 µL/s) in order to minimise the number of coincident events.

Logarithmic amplification was selected for FSC and SSC gains. The FSC threshold was set to 10^1 in order to detect events in the size range of platelets. The gain control voltage settings were calibrated on unstained unstimulated blood samples (unstained negative control). Once appropriately adjusted, these calibrated gain control voltage settings were applied to every subsequent sample.

2.2.7 Antibody titrations

Titrations of antibodies was conducted in order to ensure the concentration of antibodies used in the flow cytometry based protocol were appropriate to prevent non-specific binding, and reduce antibody consumption.

2.2.7.1 Preparation of antibody titration

The optimal final concentration for each platelet monoclonal antibody (CD42a-PerCP, PAC-1-FITC, CD62p-PE and CD154-APC) and their respective isotype controls were determined by single stain titrations on whole blood. Titrations for each antibody and isotype control were conducted at the following dilutions: 1:5, 1:10, 1:20, and 1:40.

2.2.7.2 Preparation of blood samples for titration

Blood samples were prepared as previously described. For each platelet activation-dependent marker (PAC-1-FITC, CD62p-PE and CD154-APC), titrations were conducted on blood maximally stimulated by 20 µM ADP (final concentration in blood volume), a well-described and effective platelet agonist (69, 106), to act as a positive control. Titrations were also conducted on unstimulated blood, i.e. blood treated with PBS. Titrations were conducted on both maximally stimulated and unstimulated blood to facilitate the expression of these activation-dependent markers, and to check that the preparation of blood samples did not result in significant levels of artifactual platelet activation, respectively.

2.2.7.3 Gating strategy and identification of platelet subpopulations

For the CD42a-PerCP titrations, platelet populations were identified based on their characteristic FSC and SSC properties, gate R1 (Figure 2) (68). These settings were used to collect up to 7,500 events in the R1 gate. Isotype controls were used to set the negative controls for each antibody.

After determining the appropriate concentration for the CD42a-PerCP antibody by the titration, the platelet-identification gate R1 was altered so that the new gate (R2) contained at least

95% positive CD42a events with the aid of the IgG-PerCP isotype control (Figure 4, Figure 5). For each of the activation-dependent platelet markers, the relevant isotype control was used to set the negative control for each antibody.

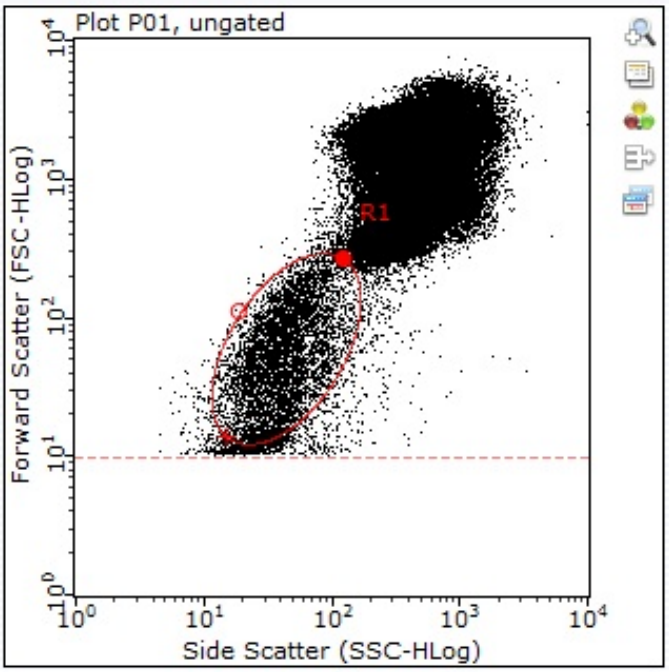


Figure 2. Gating on platelet population based on their FSC and SSC characteristics (gate R1)

2.2.8 Platelet multicolour flow cytometry panel

Following the single stain antibody titrations, the next step was to test a multicolour flow cytometry assay by using all of the appropriately titred antibodies together in a panel. A multicolour approach was selected for testing because it is generally considered more efficient and less time-consuming than single colour flow cytometry approaches.

2.2.8.1 Preparation of multicolour flow cytometry panel

The multicolour flow cytometry assay contained the following sets of antibody mixes (in a total staining volume of 45 µL): the antibody panel, consisting of one of each appropriately titred antibody; single stains for each antibody; single stain isotype controls for each antibody, with 10 µL of appropriately titred CD42a-PerCP to set the platelet population where appropriate, to set the negative controls; and single stains for each antibody on compensation beads to set the post-acquisition compensation settings (Table 1).

Fluorochrome	**FITC (green)**	**PE (yellow)**	**PerCP (red)**
Panel	PAC-1	CD62p	CD42a
Single stain PAC-1 (blood)	PAC-1	-	-
Single stain CD62p	-	CD62p	-
Single stain PerCP	-	-	CD42a
Single stain PAC-1 (compensation beads)	PAC-1	-	-
Single stain CD62p (compensation beads)	-	CD62p	-
Single stain PerCP (compensation beads)	-	-	CD42a
Isotype control	-	-	IgG-PerCP
Isotype control	IgM-FITC	-	CD42a
Isotype control	-	IgG-PE	CD42a

Table 1. Final panel for the flow cytometric assay of the function of individual platelets

2.2.8.2 Preparation of blood samples for multicolour flow cytometry panel

To test whether or not the antibodies comprising the panel interfered with each other, 5 µL of blood samples maximally stimulated by 20 µM ADP (final concentration in blood volume) or unstimulated by PBS were stained by the panel of appropriately titred antibodies. Unstimulated and stimulated samples were prepared in duplicate.

2.2.8.3 Gating strategy and identification of platelet subpopulations

To assess platelet populations, a gate (R1) was set up on the log FSC versus CD42a-PerCP positive dot plot to collect up to 7,500 CD42a-PerCP positive events with the aid of the IgG-PerCP isotype control (Figure 4). To assess platelet activation markers on individual platelets, a gate for analysis, R2, was set up over the CD42a-PerCP positive events based on the FSC and SSC characteristics of individual platelets (Figures 4 and 5) (68). Isotype controls were used to set the negative controls for each antibody, by setting a histogram marker R3 at the 5% level.

2.2.8.4 Compensation

Post-acquisition software compensation settings were set with the aid of single stains for each antibody on compensation beads (BD Bioscience). Compensation was conducted using compensation beads instead of platelets because the manner in which the platelet activation-dependent markers chosen for the study are expressed on platelets renders them unsuitable for use as compensation controls because they do not necessarily result in clearly distinct positive and negative populations (115). Furthermore, compensation beads are considered superior to cells as compensation controls (115).

Compensation control samples were prepared as follows. Single stains of 25 µL of compensation beads (BD Bioscience) in a total staining volume of 50 µL of FACS Buffer were conducted for each of CD42a-PerCP (BD Bioscience), PAC-1-FITC (BD Bioscience), and CD62p-PE (Biolegend, San Diego, CA, USA). Samples were gently agitated, and then left undisturbed in the dark at room temperature to stain for 30 minutes. The compensation beads were then diluted with 450

µL of FACS buffer, after which the compensation controls were analysed in the flow cytometer simultaneously with the blood samples. Post-acquisition compensation settings were calculated on the compensation beads using the InCyte (Millipore) analysis software bundled with the Millipore guava easyCyte 8HT System (Millipore).

2.2.9 Toll-like receptor stimulation in whole blood

2.2.9.1 Preparation of multicolour flow cytometry panel

The multicolour flow cytometry assay contained the following sets of antibody mixes (in a total staining volume of 45 µL): the antibody panel, consisting of one of each appropriately titred antibody; single stains for each antibody; single stain isotype controls for each antibody, with 10 µL of appropriately titred CD42a-PerCP to set the platelet population where appropriate, to set the negative controls; and single stains for each antibody on compensation beads to set the post-acquisition compensation settings (Table 1).

2.2.9.2 Preparation of blood samples for Toll-like receptor titration

Blood was stimulated with the following TLR agonists at the following concentrations (in the blood volume): Lipopolysaccharide from *E. coli* 0111:B4 strain (LPS), a TLR4 agonist (Invivogen, San Diego, USA) at 0.01 µg/mL, 0.1 µg/mL, 1 µg/mL; PAM_3CSK_4, a synthetic TLR1/2 agonist (Invivogen, San Diego, USA) at 0.1 µg/mL, 1 µg/mL, 10 µg/mL; and FSL-1, a synthetic TLR2/6 agonist (Invivogen, San Diego, USA) at 0.01 µg/mL, 0.1 µg/mL, 1 µg/mL.

Whole blood was incubated with TLR agonists for an initial 30 minutes, before 5 µL aliquots of each blood sample were stained with the antibody panel for another 30 minutes. This resulted in a total of 60 minutes of stimulation (Figure 1).

2.2.9.3 Gating strategy and identification of platelet sub populations

To assess platelet populations, a gate (R1) was set up on the log FSC versus CD42a-PerCP positive dot plot to collect up to 7,500 CD42a-PerCP positive events with the aid of the IgG-PerCP isotype control (Figure 18, Figure 19). To assess platelet activation markers on individual platelets, a gate for analysis, R2, was set up over the CD42a-PerCP positive events based on the on the FSC and SSC characteristics of individual platelets (Figure 19) (68). Isotype controls were used to set the negative controls for each antibody, by setting a histogram marker R3 at the 5% level (Figure 20, Figure 21).

2.2.9.4 Compensation

Post-acquisition software compensation settings were set with the aid single stains of each antibody on compensation beads (BD Bioscience). Post-acquisition compensation settings were calculated on the compensation beads using the InCyte (Millipore) analysis software bundled with the Millipore guava easyCyte 8HT System (Millipore).

2.2.10 Statistical Analysis

Graphs were produced using GraphPad, Prism v6.00 for Windows (GraphPad Software, San Diego California USA, www.graphpad.com). Data are expressed as the mean ± SD. Note that formal statistical analyses of the platelet activation markers in response to the TLR agonists were not conducted as we only wished to ascertain what appropriate concentration of TLR agonists for TLR2/1, TLR2/6 and TLR4 were capable of eliciting a response in whole blood.

2.3 Results

2.3.1 Antibody titrations

To identify the ideal concentration(s) of antibodies to use in a flow cytometry based protocol to evaluate platelet-activation markers in whole blood, single stain antibody titrations (1:5, 1:10, 1:20, and 1:40) were conducted for each of CD42a-PerCP, PAC-1-FITC, CD62p-PE and CD154-APC.

2.3.1.1 CD42a

Across all titrations, there appeared to be a distinct CD42a-PerCP positive population. There was a decrease in the PerCP fluorescence in a dose dependent manner (Figures 3a to 3d). The 1:10 dilution was selected as, it had a clear difference between the positive and isotype control populations but was also antibody sparing (Figure 3b). This titration was done using 5 µL of blood; therefore, all experiments using 5 µL of blood used a 1:10 dilution for CD42a.

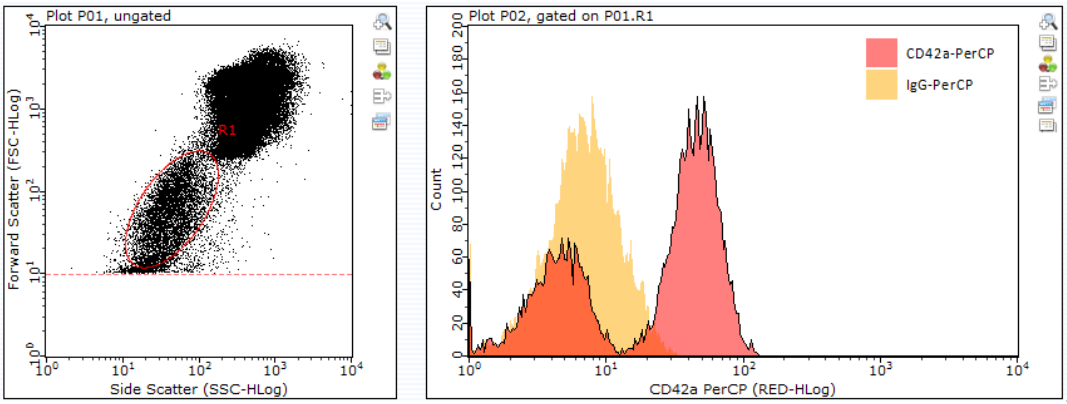


Figure 3a. CD42a-PerCP 1:5, unstimulated blood

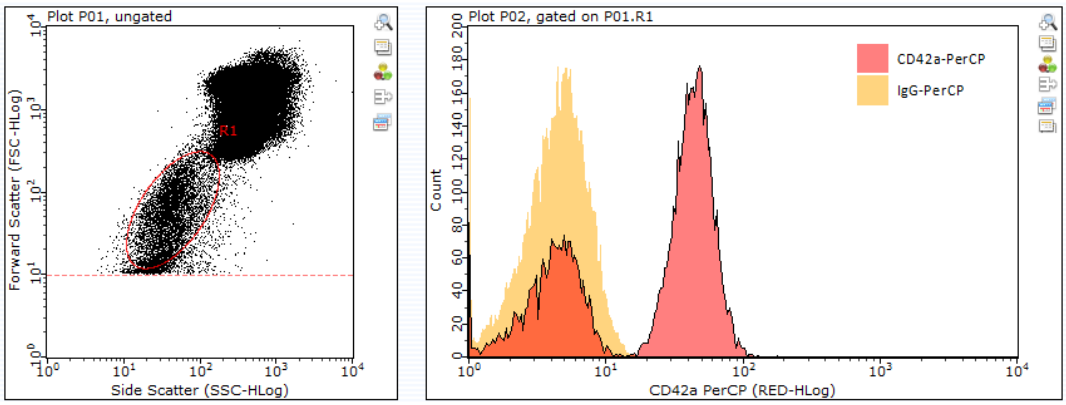


Figure 3b. CD42a-PerCP 1:10, unstimulated blood

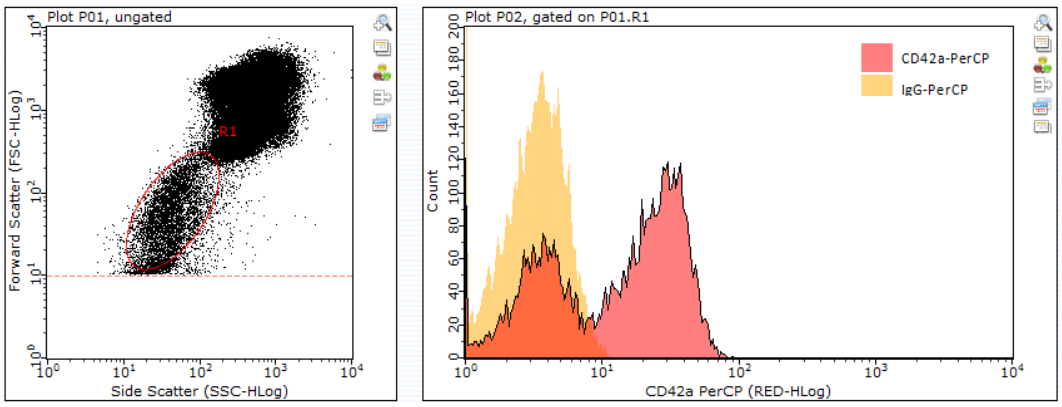


Figure 3c. CD42a-PerCP 1:20, unstimulated blood

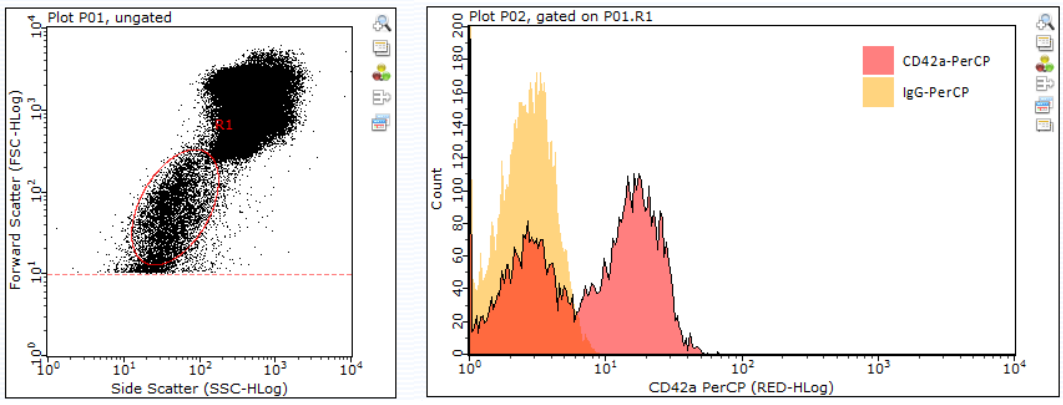


Figure 3d. CD42a-PerCP 1:20, unstimulated blood

2.3.1.2 Gating strategy for subsequent antibody titrations

After determining the appropriate concentration for the CD42a-PerCP antibody by titration, the platelet-identification gate R1 was altered so that the new R2 gate contained at least 95% positive CD42a-PerCP events based on the IgG-PerCP isotype control (Figure 4, Figure 5). For each of the activation-dependent platelet markers, its relevant isotype control was used to set the negative population for each antibody.

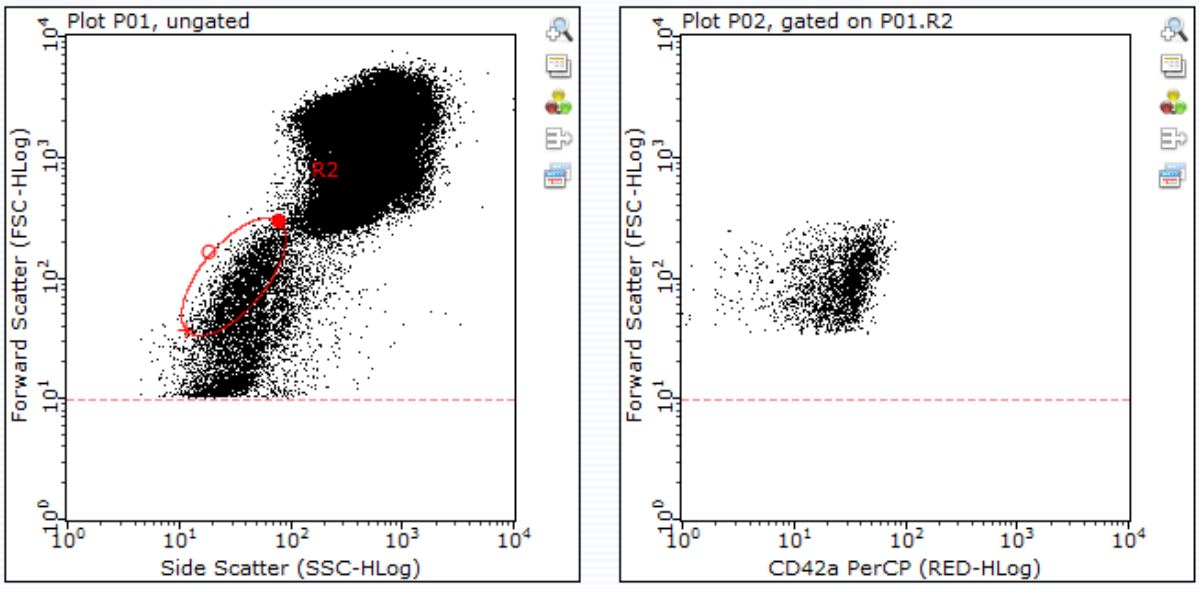


Figure 4. Dot plot for gating strategy to identify platelets by FSC and SSC

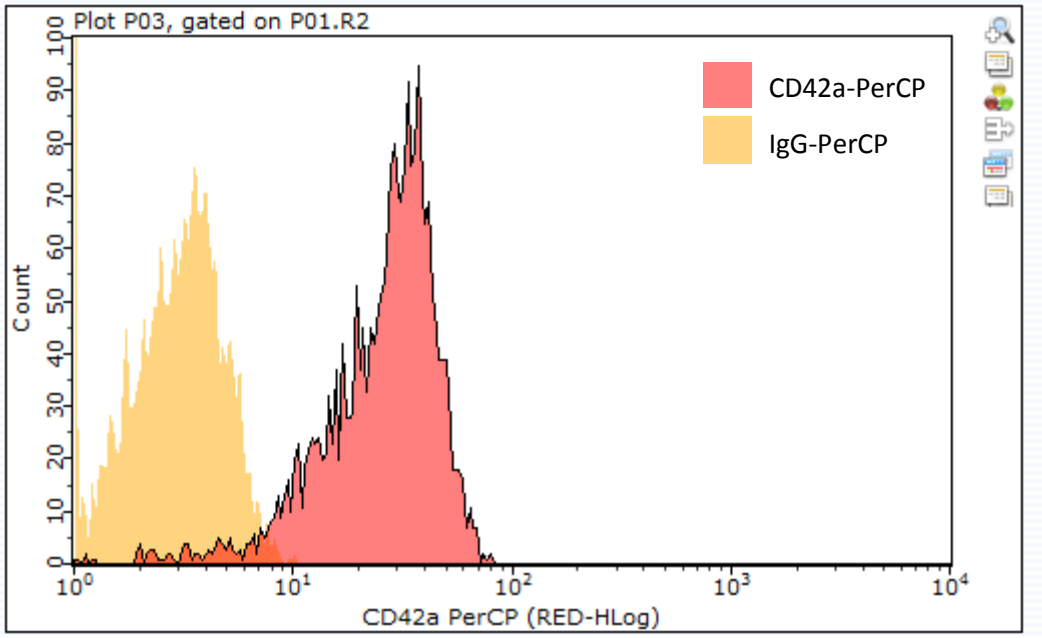


Figure 5. Histogram of above dot plot indicating that the R2 gate is CD42a-PerCP positive

2.3.1.3 PAC-1 (activated GPIIb/IIIa complex)

Across all the dilutions, there did not appear to be a distinctly PAC-1-FITC positive population (Figures 6 to 10). There was a decrease in the FITC fluorescence in a dose dependent manner, particularly between the 1:10 and the 1:20 dilutions. The 1:10 dilution was selected as it provided the best resolution between the unstimulated and the maximally stimulated blood samples and was antibody sparing (Figure 7). This titration was done using 5 µL of blood; therefore, all experiments using 5 µL of blood used a 1:10 dilution for PAC-1.

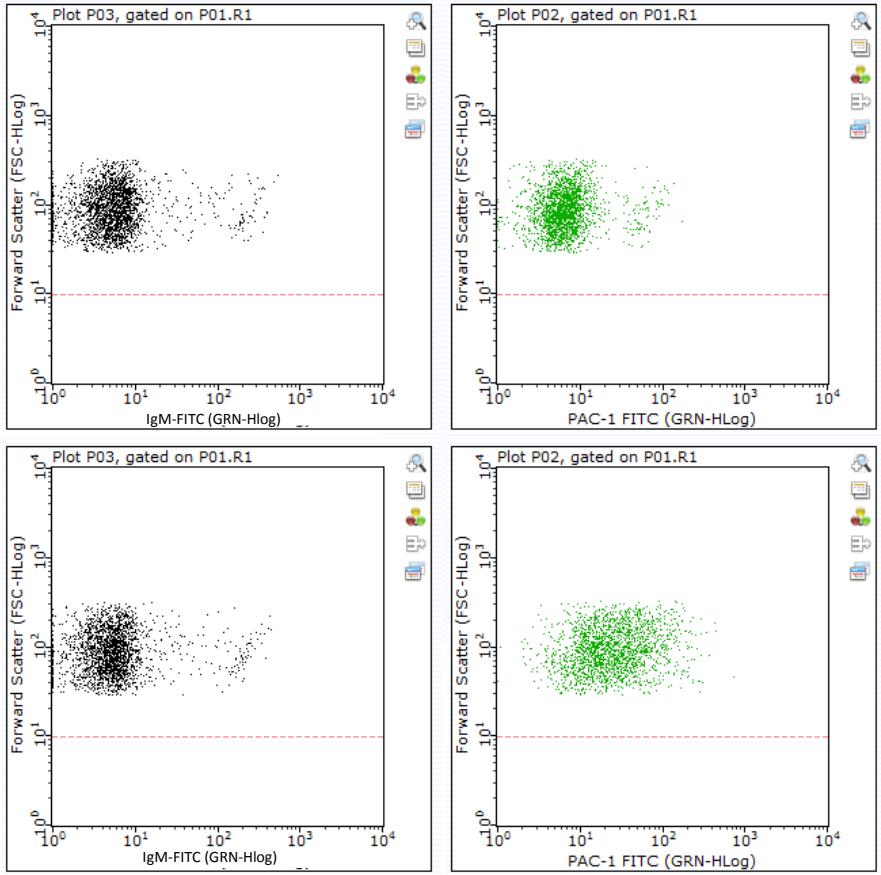


Figure 6. IgM-FITC (black) versus PAC-1-FITC (*green*) 1:5 unstimulated (top) versus stimulated with 20 µM ADP (bottom)

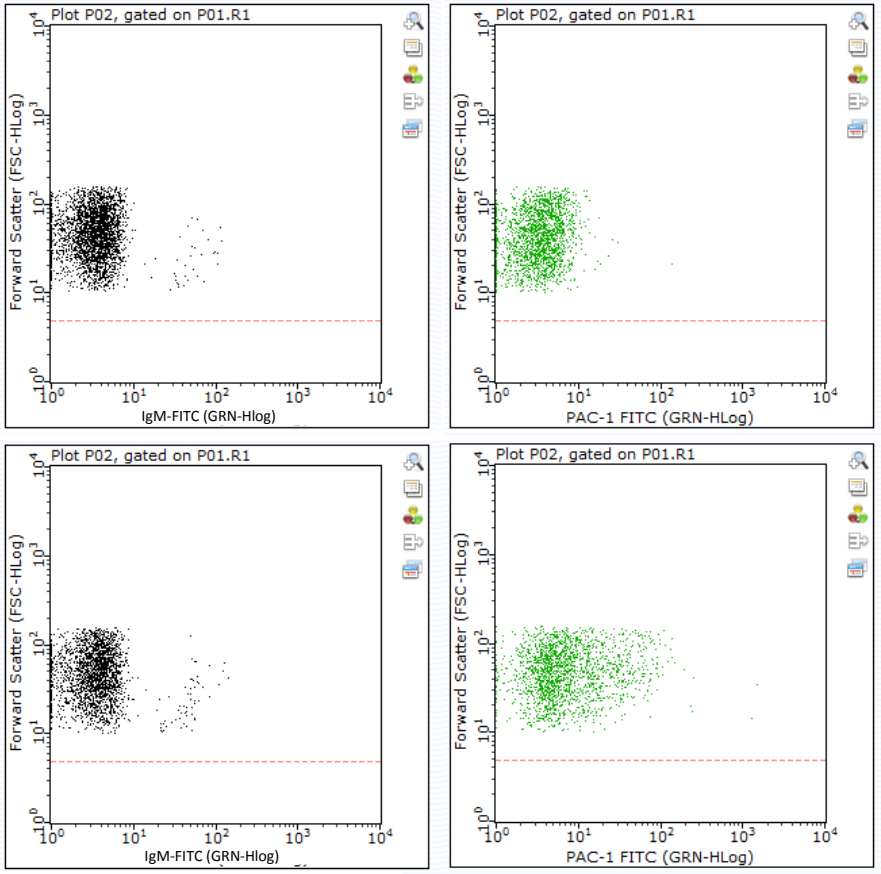


Figure 7. IgM-FITC (black) versus PAC-1-FITC (green) 1:10 unstimulated (top) versus stimulated with 20 µM ADP (bottom)

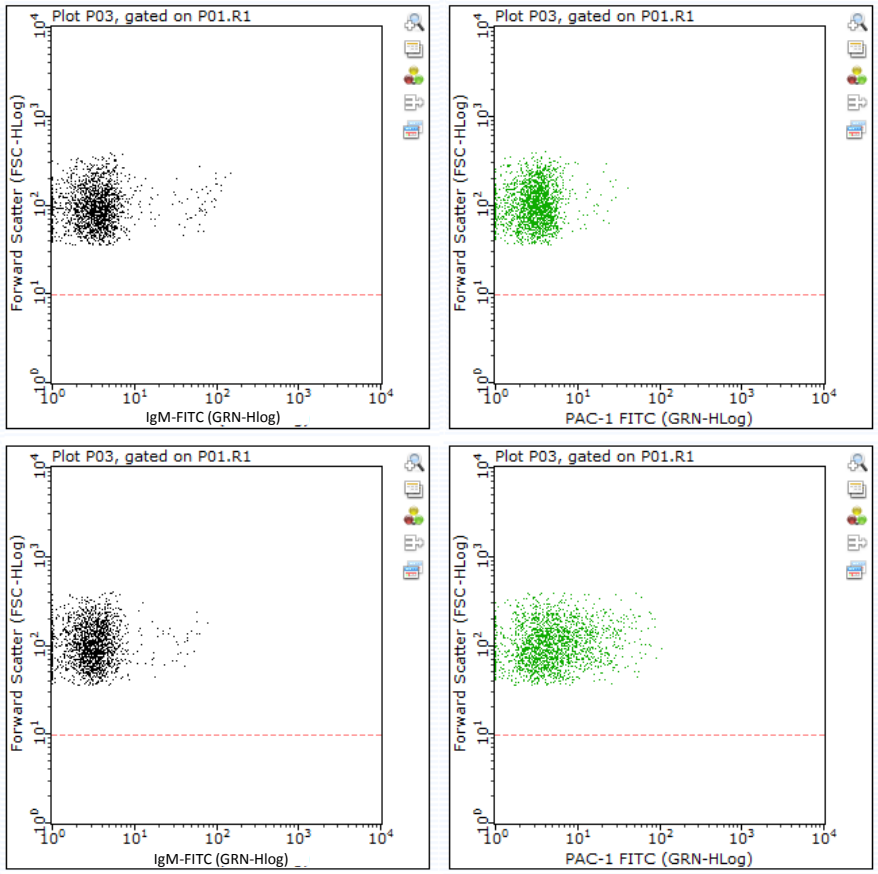


Figure 8. IgM-FITC (black) versus PAC-1-FITC (green) 1:20 unstimulated (top) versus stimulated with 20 µM ADP (bottom)

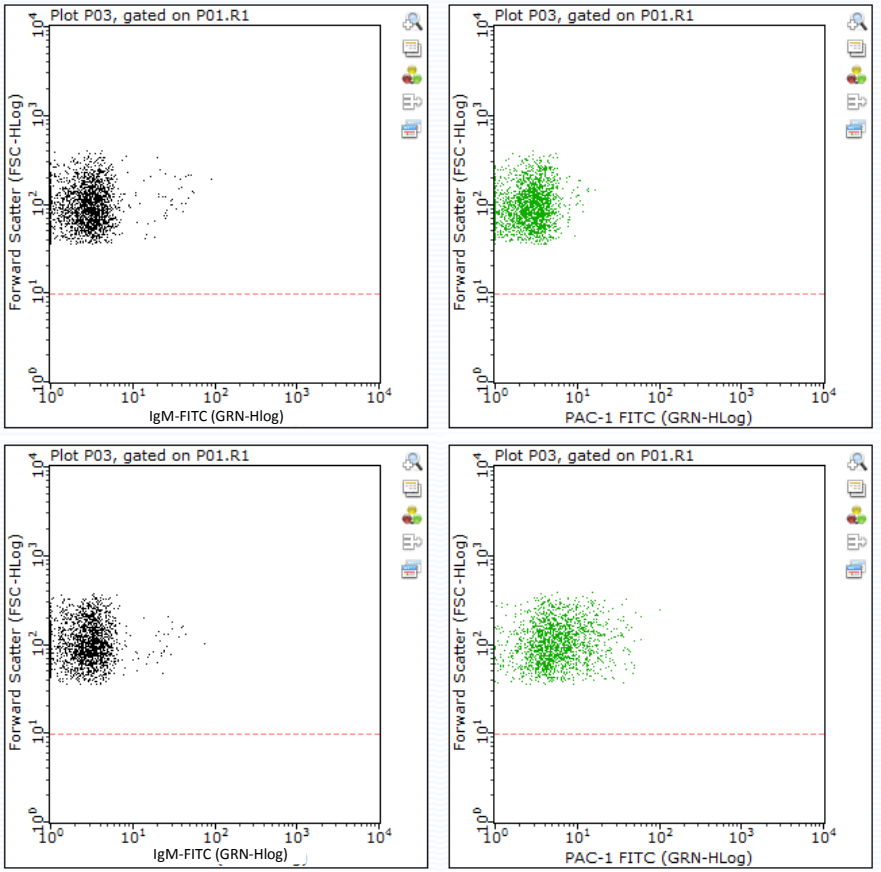


Figure 9. IgM-FITC (black) versus PAC-1-FITC (green) 1:40 unstimulated (top) versus stimulated with 20 µM ADP (bottom)

2.3.1.4 P-selectin (CD62p)

Across all the dilutions, there did not appear to be a distinctly CD62p-PE positive population, and there did not appear to be a significant dose dependent decrease in the PE fluorescence (Figures 10 to 13). The 1:20 dilution was selected as it provided the best resolution between the unstimulated and the maximally stimulated blood samples and was antibody sparing (Figure 12). This was done using 5 µL of blood; therefore, all experiments using 5 µL of blood used a 1:20 dilution for CD62p.

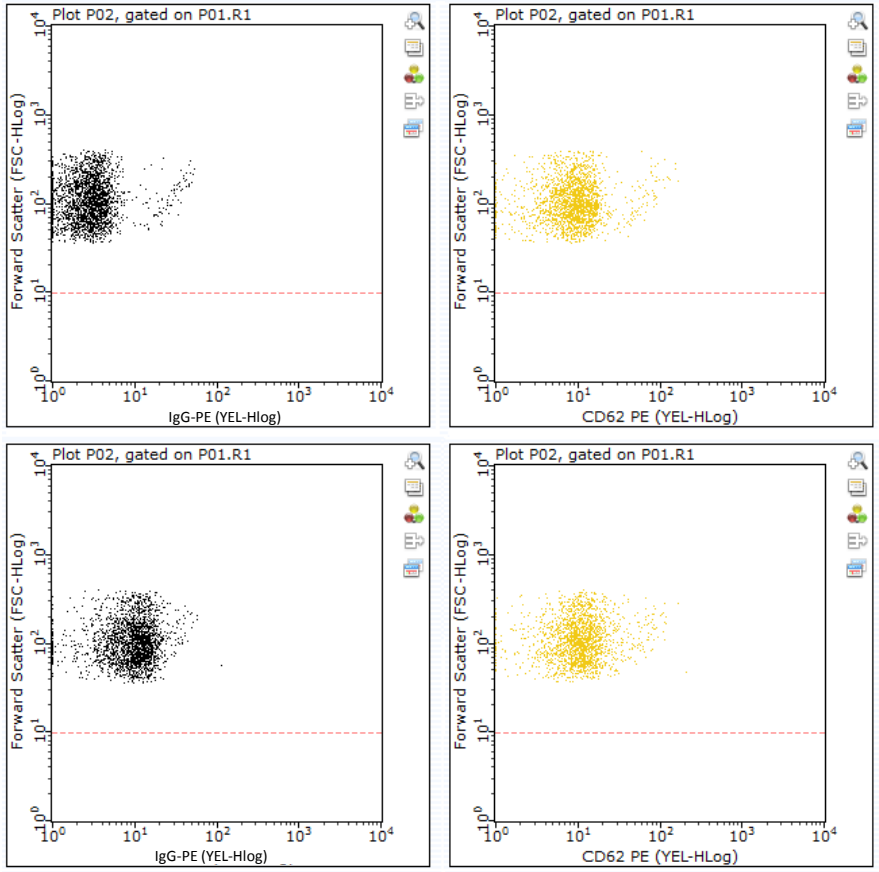


Figure 10. IgG-PE (black) versus CD62p-PE (yellow) 1:5 unstimulated (top) versus stimulated with 20 µM ADP (bottom)

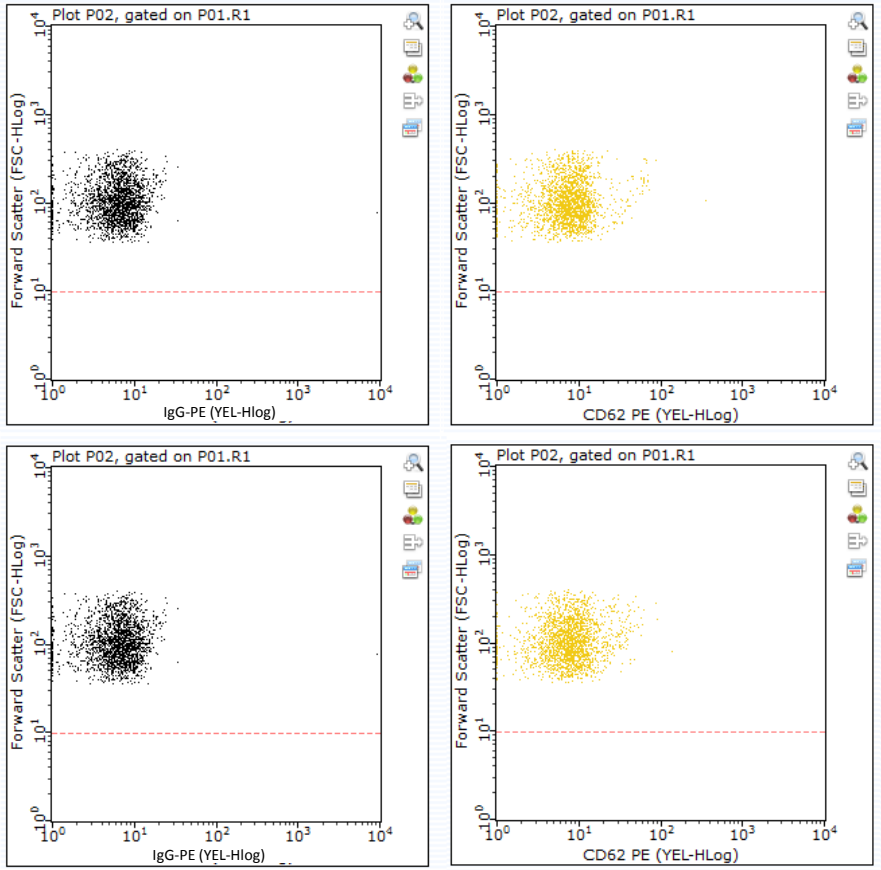


Figure 11. IgG-PE (black) versus CD62p-PE (yellow) 1:10 unstimulated (top) versus stimulated with 20 µM ADP (bottom)

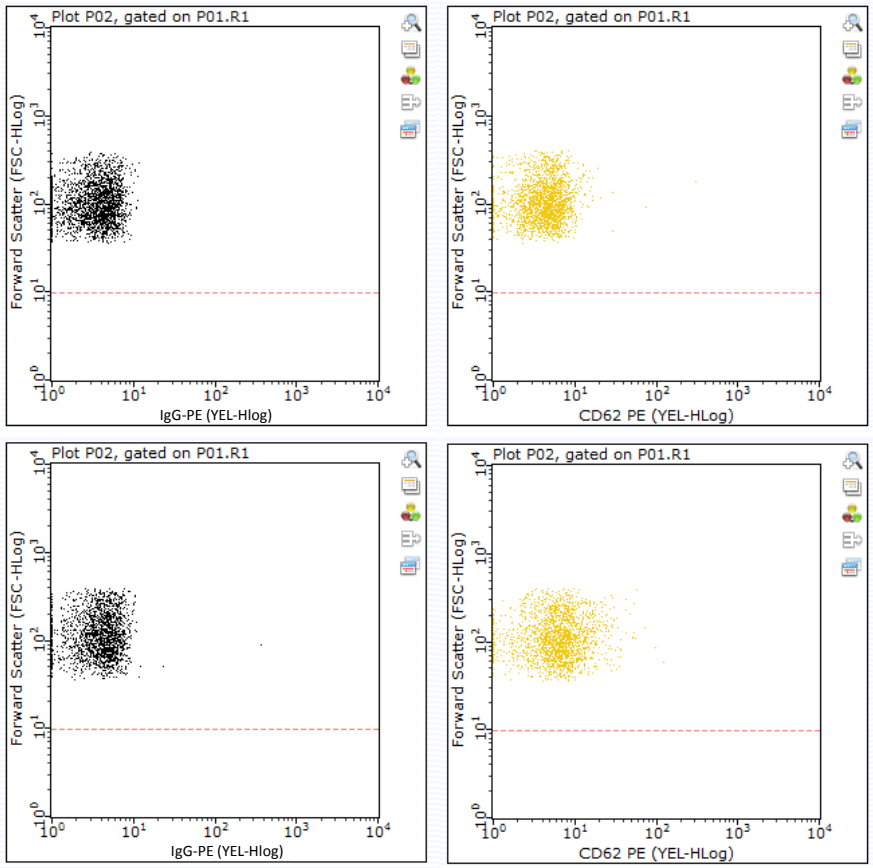


Figure 12. IgG-PE (black) versus CD62p-PE (yellow) 1:20 unstimulated (top) versus stimulated with 20 µM ADP (bottom)

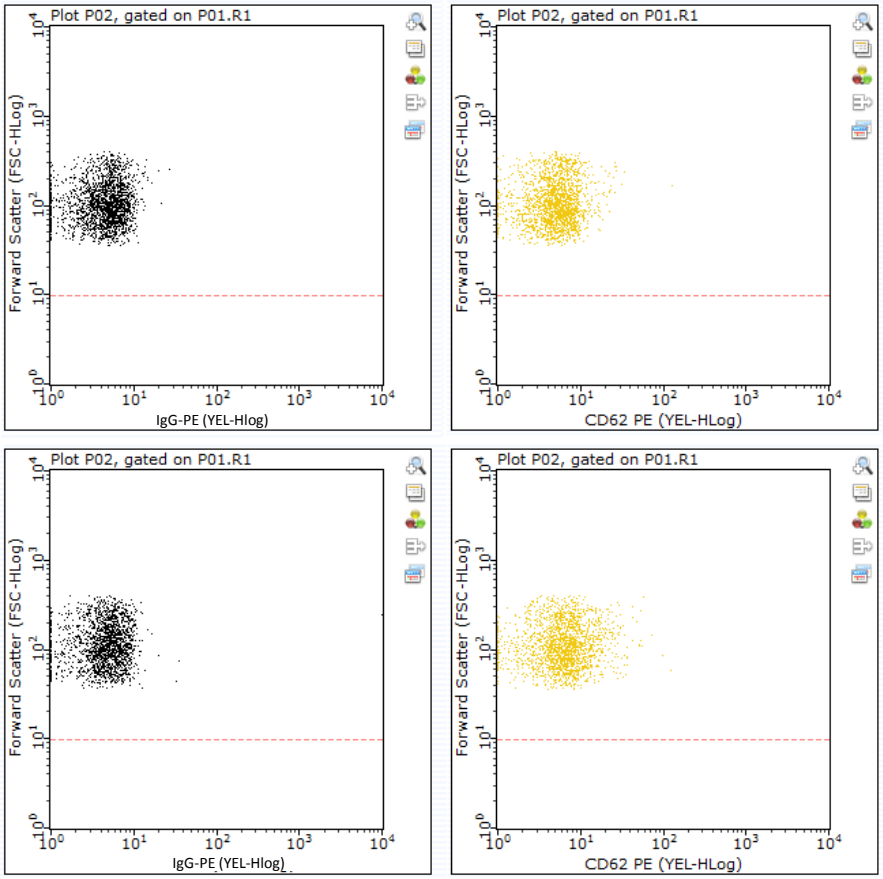


Figure 13. IgG-PE (black) versus CD62p-PE (yellow) 1:40 unstimulated (top) versus stimulated with 20 µM ADP (bottom)

2.3.1.5 CD40L (CD154)

Across all dilutions, there did not appear to be a distinctly CD154 positive population. It was not clear why this was the case, although it is possible that the antibody was not working. Because the CD154 was not showing a positive signal under any circumstances (even with the positive control ADP), we decided to proceed with the other two markers of platelet activation only (Figures 14 to 17).

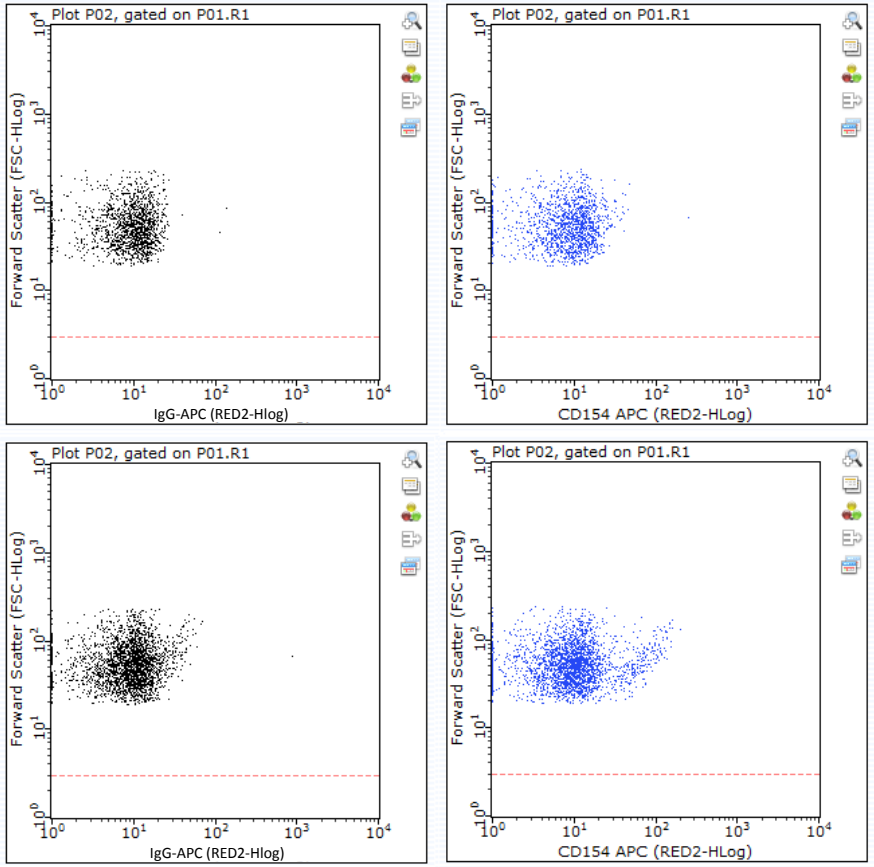


Figure 14. IgG-APC (black) versus CD154-APC (blue) 1:5 unstimulated (top) versus stimulated with 20 µM ADP (bottom)

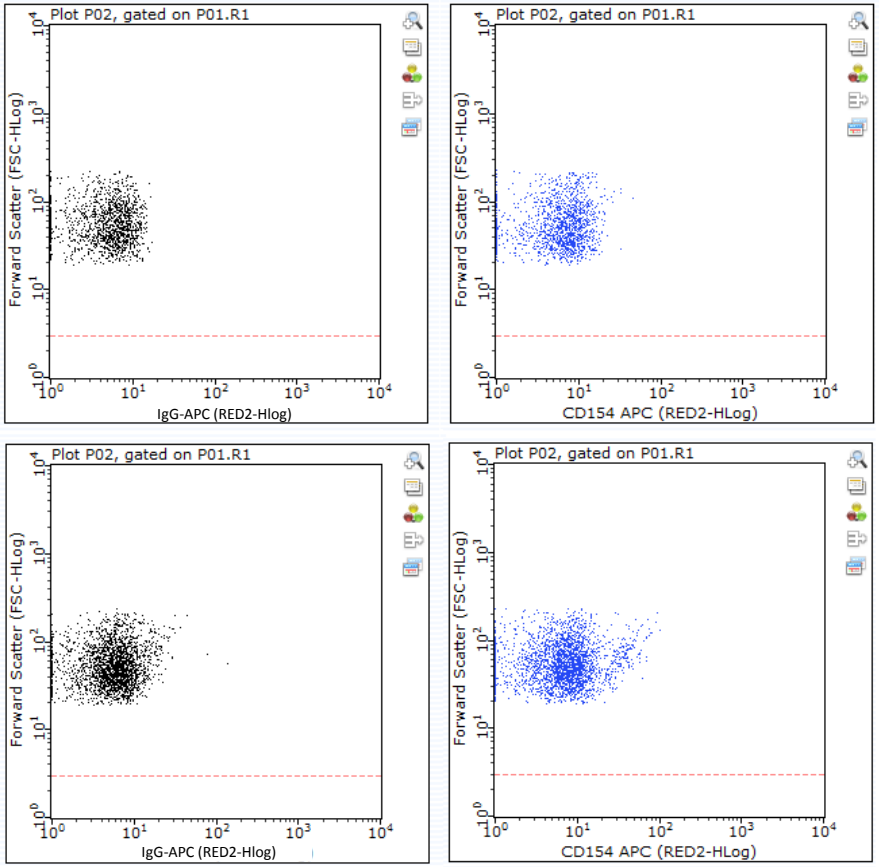


Figure 15. IgG-APC (black) versus CD154-APC (blue) 1:10 unstimulated (top) versus stimulated with 20 µM ADP (bottom)

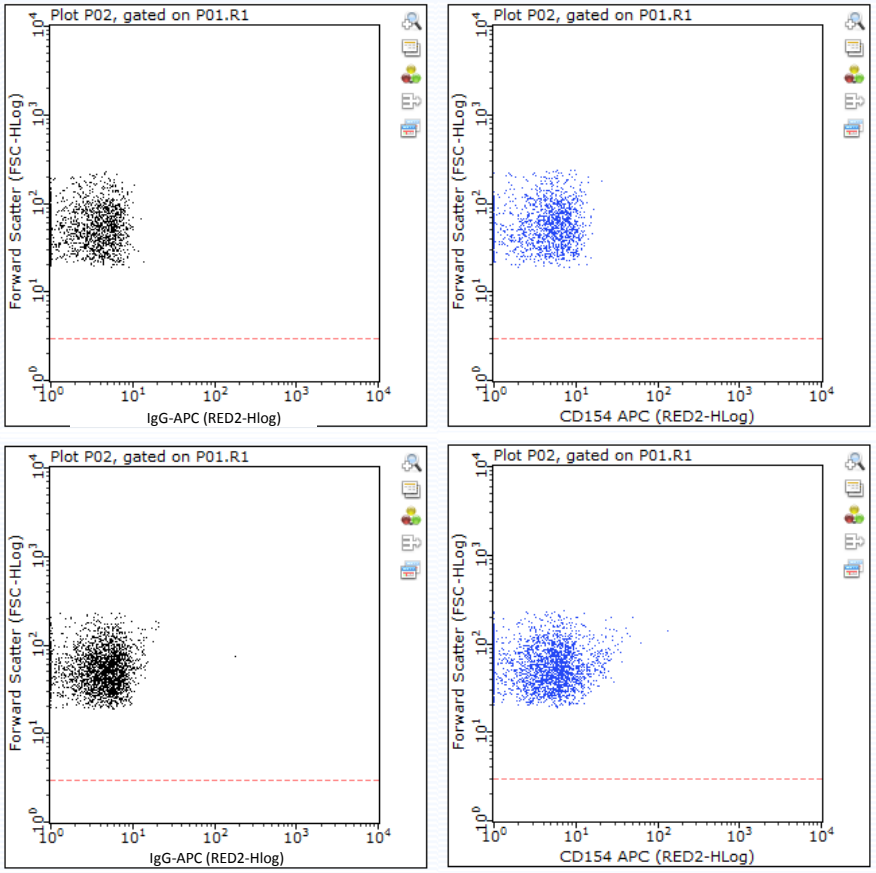


Figure 16. IgG-APC (black) versus CD154-APC (blue) 1:20 unstimulated (top) versus stimulated with 20 µM ADP (bottom)

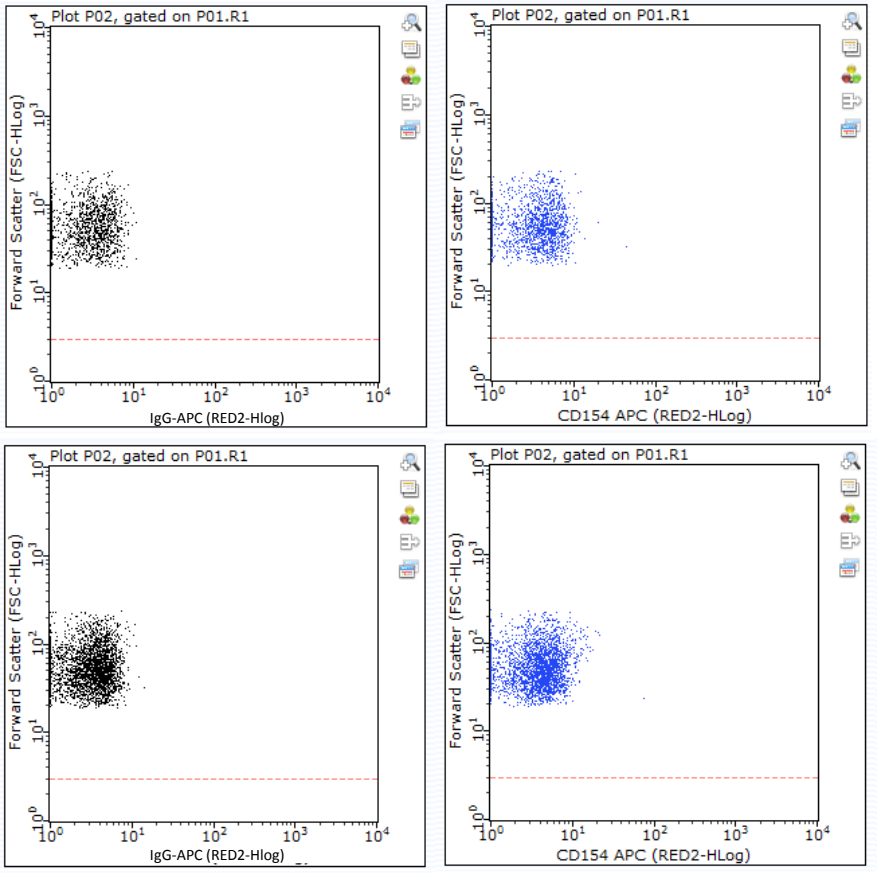


Figure 17. IgG-APC (black) versus CD154-APC (blue) 1:40 unstimulated (top) versus stimulated with 20 µM ADP (bottom)

2.3.2 Results for multicolour flow cytometry panel

To assess the feasibility of using all of the selected platelet antibodies for analysis together, a multicolour flow cytometry panel was tested protocol using all of the appropriately titred antibodies. Compensation settings were set on the basis of the fluorescent characteristics of the single stained (CD42a-PerCP, PAC-1-FITC, CD62p-PE and CD154-APC) compensation beads. The platelet population was identified on the basis of initial gating on CD42a expression (Figure 18), and subsequent gating on FSC and SSC of individual platelets as previously described (gate R2, Figure 19) (68).

A histogram statistic marker R3 was set at a 5% threshold for each isotype control for PAC-1-FITC, CD62p-PE and CD154-APC in order to set the negative population (Figure 20, Figure 21). The R3 histogram statistic marker was then used to calculate the %-positive number of cells for each of the activation-dependent platelet markers for each sample. For PAC-1-FITC, the average %-positive number of cells for the unstimulated samples was 21.7%, while the average %-positive number of cells for the samples maximally stimulated by 20 µM ADP was 86.0% (Figure 20). For CD62p-PE, the average %-positive number of cells for the unstimulated samples was 0%, while the average %-positive number of cells for the samples maximally stimulated by 20 µM ADP was 49.1% (Figure 21).

From these results, there appears to be some degree of platelet activation (either *in vivo* or artifactual) of the GPIIb/IIIa receptor, as indicated by the binding of anti-PAC-1 to the unstimulated blood samples. There appeared to be minimal levels of CD62p receptor activation. Stimulation of blood samples by 20 µM ADP elicited a clearly positive response from PAC-1 and CD62p.

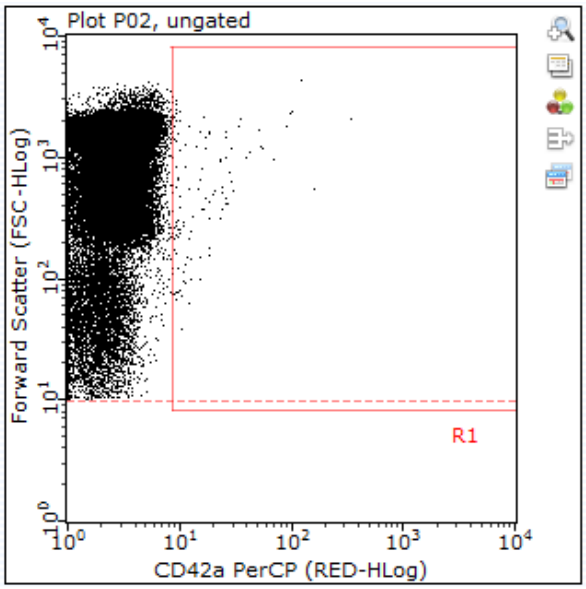


Figure 18. Gating strategy to collect CD42a-PerCP positive events in order to identify platelets

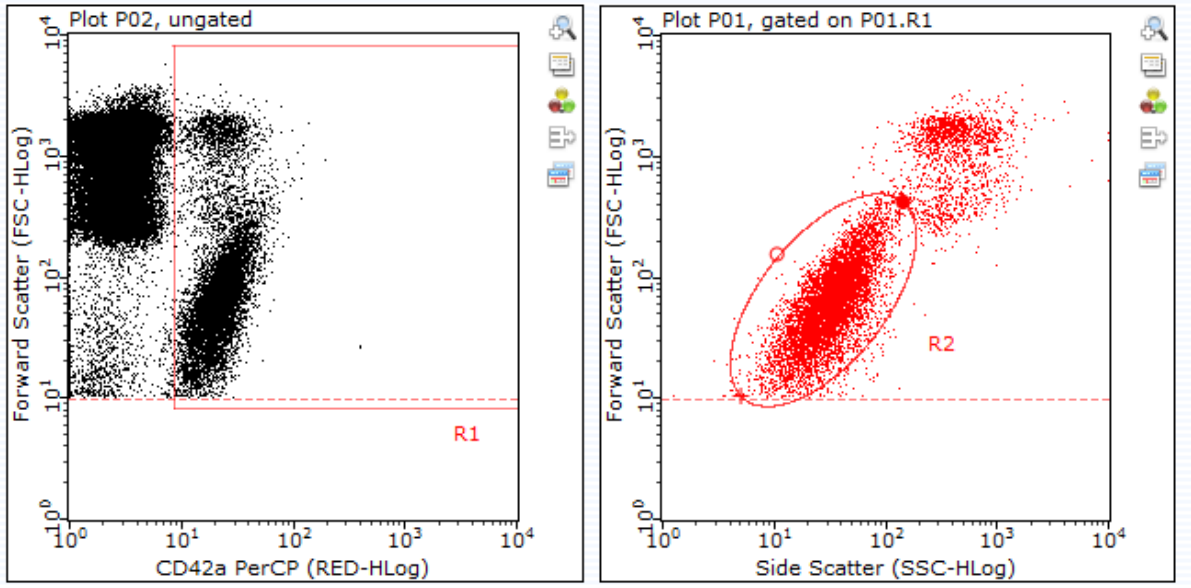


Figure 19. Gating strategy to identify individual platelets

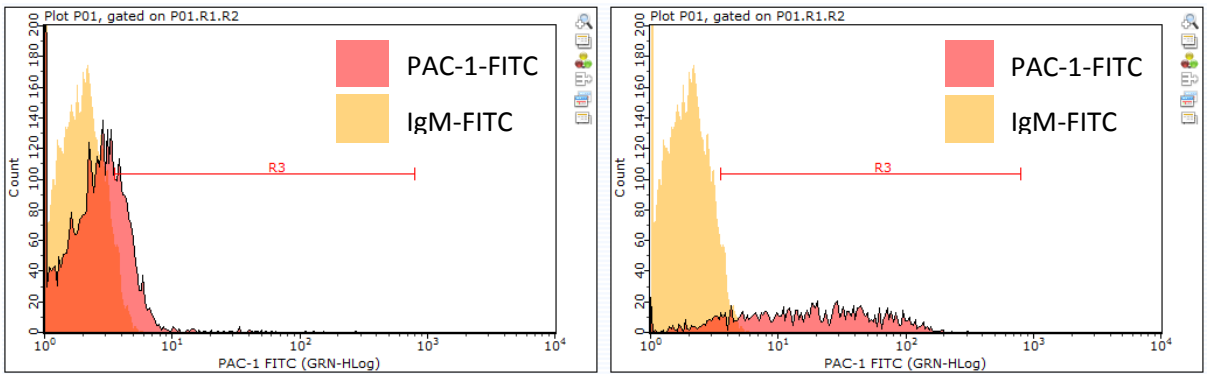


Figure 20. PAC-1-FITC unstimulated (left) versus maximally stimulated with 20 µM ADP (right)

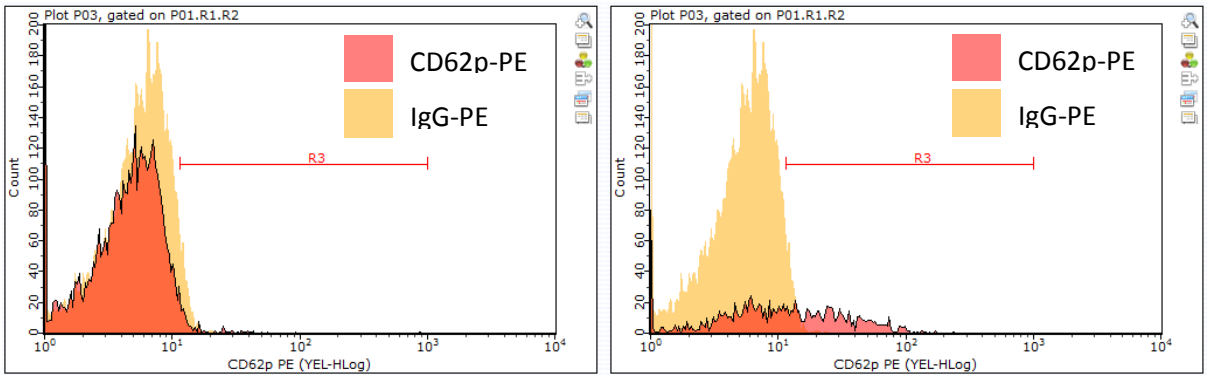


Figure 21. CD62p-PE unstimulated (left) versus maximally stimulated with 20µM ADP (right)

2.3.3 Toll-like receptor agonist stimulation in whole blood

In accordance with aim two of the study, which was to ascertain what concentrations of TLR agonists were capable of eliciting platelet responses in whole blood, samples from healthy volunteers (n = 4) were stimulated with differing concentrations of LPS, PAM_3CSK_4 and FSL-1 before flow cytometric analysis. Samples from healthy volunteers (n = 4) were also stimulated with ADP as a positive control.

2.3.3.1 Unstimulated versus ADP stimulated samples

Increases PAC-1 and CD62p expression were noted following stimulation with 20 µM ADP (Table 2, Figures 23, 24).

Table 2. %-positive expression of platelet activation markers induced by ADP

	ADP stimulation (n = 4)	
Marker	unstimulated	20 µM ADP
%PAC-1	20.4 ± 12.6	36.1 ± 11.1
%CD62p	4.2 ± 2.5	32.3 ± 11.4

Data are expressed as the mean ± SD

ADP = adenosine diphosphate

2.3.3.2 Unstimulated versus LPS stimulated samples

Increases in PAC-1, and CD62p expression were noted following stimulation of samples by each of the concentrations of LPS tested (Table 3).

Table 3. %-positive expression of platelet activation markers induced by differing concentrations of LPS

	LPS stimulation (n = 4)			
Marker	unstimulated	0.01 µg/mL	0.1 µg/mL	1 µg/mL
%PAC-1	20.4 ± 12.6	29.9 ± 15.5	30.33 ± 11.54	36.78 ± 18.1
%CD62p	4.2 ± 2.5	8.8 ± 5.4	10.0 ± 10.0	11.3 ± 4.9

Data are expressed as the mean ± SD

LPS = Lipopolysaccharide

2.3.3.3 Unstimulated versus PAM_3CSK_4 stimulated samples

Increases in PAC-1, and CD62p expression were noted following stimulation of samples by each of the concentrations of PAM_3CSK_4 tested (Table 4).

Table 4. %-positive expression of platelet activation markers induced by differing concentrations of PAM_3CSK_4

	PAM_3CSK_4 stimulation (n = 4)			
Marker	unstimulated	0.1 µg/mL	1 µg/mL	10 µg/mL
%PAC-1	20.4 ± 12.6	29.2 ± 8.6	29.3 ± 12.1	31.8 ± 13.1
%CD62p	4.2 ± 2.5	8.1 ± 6.9	5.2 ± 3.5	8.9 ± 6.2

Data are expressed as the mean ± SD

PAM_3CSK_4 = Pam3-Cys-Ser-Lys4

2.3.3.4 Unstimulated versus FSL-1 stimulated samples

Increases in PAC-1, and CD62p expression were noted following stimulation of samples by each of the concentrations of FSL-1 tested (Table 5).

Table 5. %-positive expression of platelet activation markers by differing concentrations of FSL-1

	FSL-1 stimulation (n = 4)			
Marker	unstimulated	0.01 µg/mL	0.1 µg/mL	1 µg/mL
%PAC-1	20.4 ± 12.6	32.9 ± 17.5	34.6 ± 19.5	35.4 ± 15.0
%CD62p	4.2 ± 2.5	8.7 ± 1.6	8.6 ± 1.5	11.0 ± 4.9

Data are expressed as the mean ± SD

FSL-1 = fibroblast stimulating ligand-1

3 Chapter 3 – Toll-like receptor agonists in acute coronary syndromes

3.1 Introduction

In Chapter 2 of this thesis, we developed a flow cytometry based protocol to evaluate the expression of the platelet-activation markers PAC-1 and CD62p in whole blood. We decided not to analyse CD154, as it was not showing a positive signal under any circumstances. We then used this protocol ascertain which the concentrations of the TLR agonists we used were capable of eliciting platelet responses for the aforementioned activation-dependent markers.

3.1.1 Platelets as cells of both inflammation and thrombosis

There is a growing body of evidence implicating platelets in both inflammation and thrombosis. Activated platelets for example, express a variety of pro-inflammatory and prothrombotic mediators, including CD40L and vWF, involved in the pathogenesis of atherosclerosis and ACS. Furthermore, platelets can activate and interact with leucocytes and endothelial cells following activation by certain pathogens, aiding the clearance of these pathogens by the immune system (10). Platelets have also been demonstrated to physically interact with bacteria resulting in direct platelet activation and aggregation, both *in vivo* and *in vitro* (25, 86, 116).

3.1.1.1 Platelet-expressed Toll-like receptors

As described earlier in the thesis, it is now well established that platelets express functional TLRs. Platelets have convincingly been demonstrated to express TLR1, TLR2, TLR4, TLR6 and TLR9, via real-time polymerase chain reaction, flow cytometry and western blotting (9, 12, 13, 117). Of these, only TLR 2/1, TLR 2/6 and TLR4 appear to be functional when activated on platelets. Activation of TLRs 2/1 and 2/6 by synthetic TLR agonists and various bacteria, including *S. pneumoniae* and periodontal pathogens, have been clearly demonstrated to lead platelet activation and aggregation, while TLR4 is thought to allow platelets to function as “immune sentinels” in response to infection

by enterohaemorrhagic *E. coli* and *Rickettsia africae* (23, 25, 26, 118). As such, platelet-expressed TLRs may provide a mechanistic link between platelets' inflammatory and haemostatic functions, and may therefore have pathophysiological roles in diseases with dual inflammatory and thrombotic components, such atherosclerosis and the ACS.

3.1.2 Specific aims

The specific aim of this chapter of the study was to examine and compare platelet responses to the TLR agonists LPS, PAM_3CSK_4 and FSL-1 in ACS patients and healthy controls.

3.2 Methods

3.2.1 Study participants and ethical approval

3.2.1.1 Patients

Fourteen patients presenting to Wellington Regional Hospital with an ACS, due to undergo coronary angiography and adequately pre-treated on dual antiplatelet therapy were included to be a part of the study. Patients were deemed to have had an ACS if they met two out of the three following criteria: symptoms of myocardial ischaemia, new ECG changes, and a troponin elevation. Symptoms of myocardial ischaemia were defined as follows: chest or epigastric pain or pressure, neck, jaw, shoulder or arm pain; nausea; shortness of breath; and diaphoresis (36). A new ECG change was defined as the presence of any one or combination of the following: ST-segment elevation or depression, T-wave inversion, Q-waves, and left bundle branch block. A rise in troponins was defined as a rise or fall in high sensitivity troponin-T (hsTnT) or high sensitivity troponin-I (hsTnI) test, with one value above the 99th percentile of the upper reference limit (36) equating to a hsTnT > 14 ng/L or hsTnI > 0.03 ng/L respectively. Adequate pre-treatment with dual antiplatelet therapy was defined as chronic therapy (greater than 7 days) with aspirin (≥75 mg) and clopidogrel (≥75 mg) and/or loading with aspirin ≥300 mg at least 2 hours prior and clopidogrel ≥300 mg at least 6 hours prior to enrolment in the study.

Exclusion criteria were the following: a platelet count of less than 100x10^{9}/L, a known platelet-function disorder, administration a fibrinolytic agent within 24 hours of enrolment or a GPIIb/IIIa antagonist within a week prior to enrolment, use of other antiplatelet agents within one month, pregnancy, or an inability to provide informed written consent.

3.2.1.2 Control subjects

Fourteen healthy volunteers were recruited to act as controls. Controls were age- and sex-matched to the patient population. Controls had no history (self-reported) of cardiovascular disease,

stroke, diabetes, peripheral artery disease, or platelet-function disorders. Controls were free from antiplatelet medication, and were not acutely unwell with an infection in the fourteen days prior to their enrolment into the study.

3.2.1.3 Ethical approval

All patients and volunteers included in the study provided informed written consent. The Central Region Ethics Committee gave ethical approval for this study.

3.2.2 Blood collection

Blood was collected prior to coronary angiography by venepuncture on the ward or via intra-arterial catheter prior to administration of heparin. Collection of blood samples via intra-arterial catheter does not appear to result in significant levels of artifactual activation of individual platelets (115, 119). After collection, tubes containing satisfactory blood samples were gently inverted 3-4 times and left for a maximum of 30 minutes at room temperature to sit before preparation for stimulation by agonists and then flow cytometric analysis.

3.2.2.1 Venepuncture

For the blood obtained via venepuncture, blood was collected as described in the protocol developed in Chapter 2. Briefly, blood drawn from a peripheral vein in the antecubital fossa using a 21-gauge butterfly needle (BD Bioscience) with the aid of a light tourniquet (if necessary) that was immediately released following venous access. Blood was immediately collected into sterile vacuum tubes containing 0.105 M buffered sodium citrate anticoagulant (BD Bioscience) as a discard tube, and finally 3.0 mL of blood into a tube containing 200 U/mL hirudin (Dynabyte, Munich, Germany) that was used for flow cytometry.

3.2.2.2 Intra-arterial catheter

For the blood obtained by intra-arterial catheter, 40 mL of blood was drawn from either the radial or femoral artery by cardiologists from Capital and Coast District Health Board into two 20 mL sterile, additive-free polypropylene tubes (BD Bioscience). Blood was immediately transferred from

these polypropylene tubes via a luer adapter device (BD Bioscience) into sterile vacuum tubes containing 0.105 M buffered sodium citrate anticoagulant (BD Bioscience) as a discard tube, and finally 3.0 mL of blood into a tube containing 200 U/mL hirudin (Dynabyte, Munich, Germany) that was used for flow cytometry.

3.2.3 Flow cytometer

The flow cytometer was a Millipore guava easyCyte 8HT System (Millipore).

3.2.4 Preparation of blood samples for flow cytometry

Blood was prepared as per the protocol described in Chapter 2. In addition, the hirudin anticoagulated blood was stimulated by 100µM thrombin receptor agonist peptide (TRAP), a potent platelet agonist, as a positive control (106). PBS was added to the hirudin anticoagulated blood as a negative control (unstimulated blood). Samples were prepared in duplicate for each agonist.

Each blood sample was stimulated with agonist for 30 minutes before incubation with the flow cytometry antibodies described in Chapter 2.

3.2.5 Flow cytometer detector settings

The detector settings for the flow cytometer were as described in Chapter 2.

3.2.6 Gating strategy and identification of platelet subpopulations

Platelet populations were identified using the gating strategy previously described in Chapter 2, and 7,500 CD42a-PerCP positive events were collected.

3.2.7 Compensation

Post-acquisition software compensation settings were set with the aid single stains of each antibody on compensation beads (BD Bioscience).Post-acquisition compensation settings were calculated on the compensation beads using the InCyte (Millipore) analysis software bundled with the Millipore guava easyCyte 8HT System (Millipore).

3.2.8 Statistical analysis

Graphs were produced using GraphPad, Prism v6.00 for Windows (GraphPad Software, San Diego California USA, www.graphpad.com). Statistics were also calculated using GraphPad Prism (GraphPad Software, San Diego California USA, www.graphpad.com).

Unless otherwise stated, the nonparametric Mann-Whitney *U* test was used for the statistical analyses. Accordingly, data are presented as median and (range), and differences between medians are expressed as Hodges-Lehmann differences. Nonparametric tests were selected for this study, as they are more robust than parametric tests when as is often the case in biomedical research, data departs from normality (120). Furthermore, nonparametric testing methods appear to be robust even when sample sizes are small (120). P-values < 0.05 were considered statistically significant.

3.3 Results

3.3.1 Patient and control demographics

The patient and control demographics are described in Table 6 below.

Table 6. Patient and control demographics

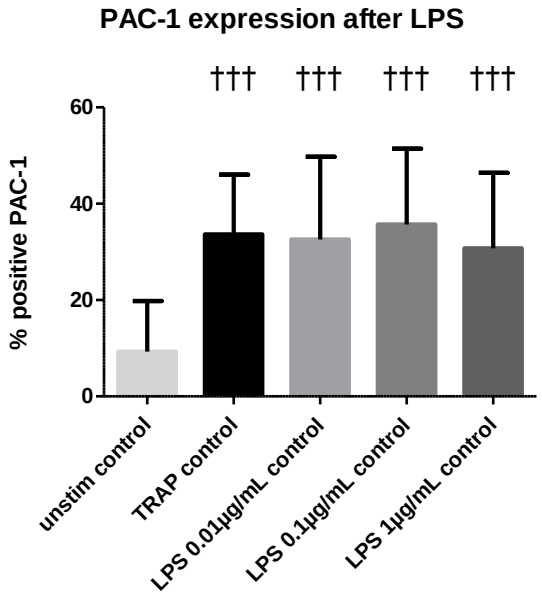
	Controls (*n = 14*)	**ACS patients** (*n = 14*)
Age (years)	57.3 ± 1.2	56.6 ± 1.6
Male sex	11 (78.5%)	11 (78.5%)
European ethnicity	14 (100%)	13 (92.9%)
Smoking	0	2 (14.3%)
Hypertension	0	4 (28.6%)
Dyslipidemia	0	7 (50.0%)
Diabetes	0	5 (35.7%)
Family history	0	10 (71.4%)
Previous MI	0	3 (21.4%)
NSTEMI	N/A	10 (71.4%)
STEMI	N/A	4 (28.6%)
Days between symptom onset and presentation	N/A	6.5 ± 0.83

Age and days between symptom onset both expressed as mean ± SEM

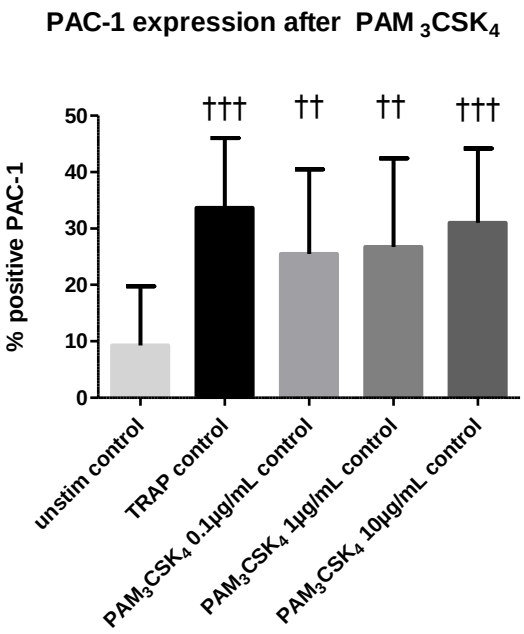
3.3.2 Response to Toll-like receptor agonists in healthy controls

As expected, healthy controls expressed low levels of platelet activation markers, with the median %-positive value for PAC-1 and CD62p 4.2% and 9.2%, respectively (Tables 7, 8). As expected, stimulation of the healthy control blood samples by the positive-control TRAP resulted in significant increases in the expression of PAC-1 and CD62p in comparison to the unstimulated blood samples ($p < 0.001$) (Table 7, Table 8; Figure 22, Figure 23).

There were no significant differences in PAC-1 expression between TRAP-stimulated blood and blood stimulated with each of LPS, PAM_3CSK_4, and FSL-1. Furthermore, there was no evidence of any dose-response relationship in PAC-1 expression for each of the TLR agonists tested (Tables 7, 9, 11; Figure 22). By contrast, there was a differential response in CD62p expression between TRAP-stimulated and the TLR-stimulated blood samples in the healthy controls. Blood samples stimulated by TRAP resulted in a statistically significant higher expression of CD62p in comparison to unstimulated samples than the samples stimulated by each of the TLR agonists tested (TRAP versus: LPS, $p < 0.001$; PAM_3CSK_4 $p < 0.01$; FSL-1 $p < 0.01$). Similar to PAC-1, there was no evidence of any dose-response relationship in %CD62p expression for each of the TLR agonists tested (Tables 8, 10, 12; Figure 23).

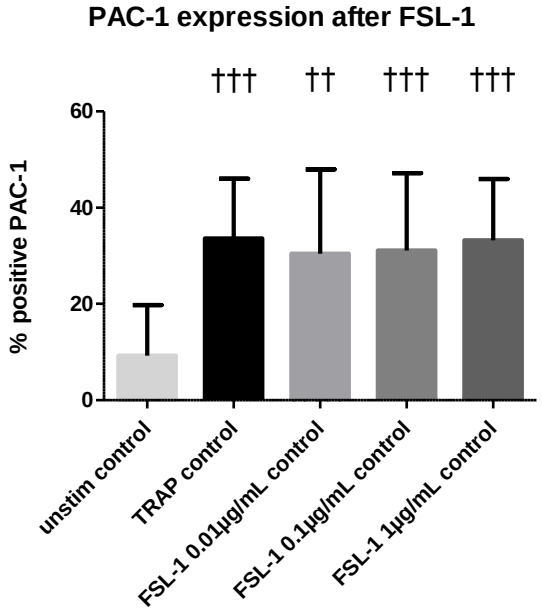


††† p <0.001 vs unstimulated



††† p <0.001 vs unstimulated

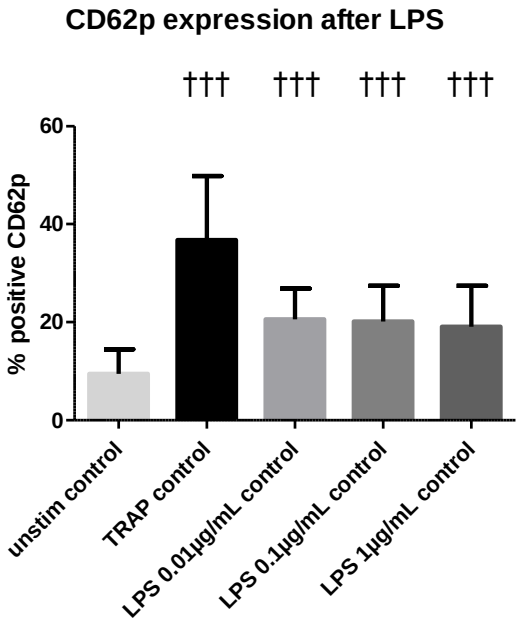
†† p < 0.01 vs unstimulated



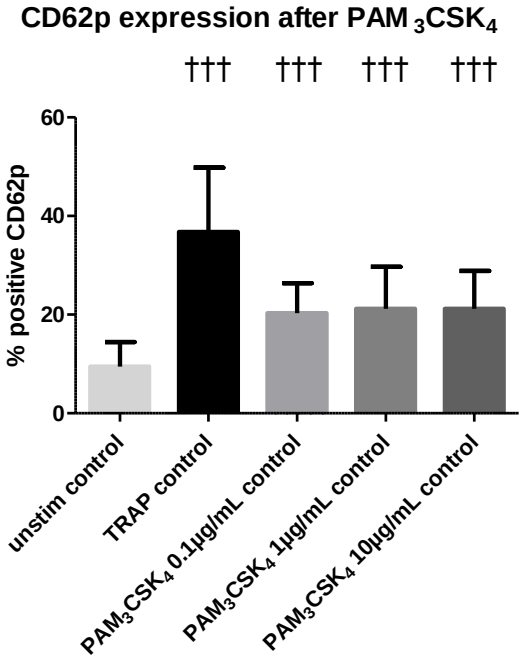
††† p <0.001 vs unstimulated

†† p < 0.01 vs unstimulated

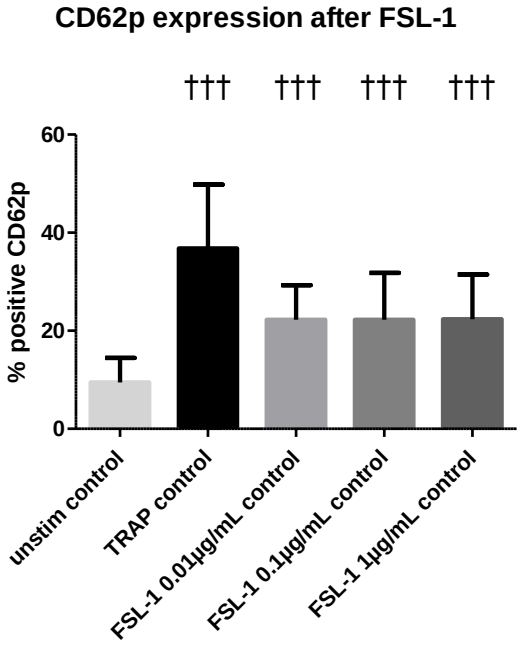
Figure 22. PAC-1 expression in response to stimulation with TLR agonists, in healthy controls



††† p <0.001 vs unstimulated



††† p <0.001 vs unstimulated



††† p <0.001 vs unstimulated

Figure 23. CD62p expression in response to stimulation with TLR agonists, in healthy controls

3.3.3 Response to LPS stimulation in controls versus acute coronary syndrome patients

The baseline expression of PAC-1 and CD62p for the unstimulated blood samples was similar across the healthy controls and ACS patients (Tables 7, 8). For PAC-1 expression, the ACS patients on DAPT responded significantly less than the healthy controls after stimulation by TRAP (difference 18.6%, $p < 0.001$) (Table 7; Figure 24). No significant differences in CD62p expression in response to TRAP were observed between ACS patients on DAPT and healthy controls however (Table 7; Figure 24).

The levels of PAC-1 and CD62p expression in response to stimulation by LPS were greater in the healthy controls than the ACS patients on DAPT (Table 7, Table 8). For samples stimulated with 1 µg/mL LPS, the median PAC-1 expression in the healthy controls was 35.0% (5.0% – 52.3%, $p < 0.001$) compared to 11.5% (0.4% – 18.7%, $p = 0.370$) in the ACS patients on DAPT, representing an absolute difference of 23.3% ($p = 0.002$) (Table 7, Figure 24). The median CD62p expression in the healthy controls was 17.0% (7.6% – 52.3%, $p < 0.001$) compared to 10.7% (0.8% – 26.8%, $p = 0.192$) in the ACS patients on DAPT in response to stimulation with 1 µg/mL LPS, representing an absolute difference of 7.2% ($p = 0.02$) (Table 8, Figure 24).

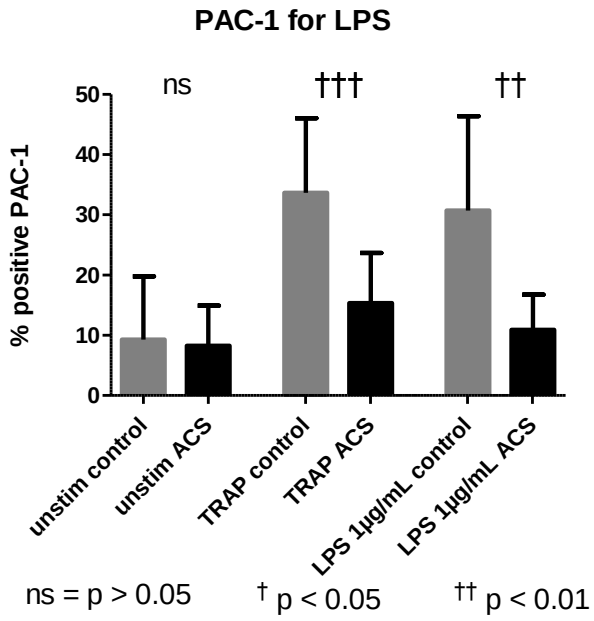
Table 7. %PAC-1 expression in controls versus ACS patients for LPS

	%PAC-1 expression			
Agonist	Controls (n= 14)	ACS (n = 14)	Differences	*P* (controls vs. ACS)
Unstimulated	4.2 (0 – 27.5)	6.3 (0.0 – 19.0)	0.0	0.81
TRAP 100 µM	34.9 (14.5 – 51.4) ***	18.3 (1.4 – 26.6) *	18.6	<0.001
LPS 0.01 µg/mL	35.4 (7.7 – 65.2) ***	11.4 (0.2 – 21.2)	22.5	<0.001
LPS 0.1 µg/mL	32.6 (14.1 – 64.5) ***	13.2 (0.0 – 28.7)	20.1	<0.001
LPS 1 µg/mL	35.0 (5.0 – 52.3) ***	11.5 (0.4 – 18.7)	23.3	0.002

Table 8. %CD62p expression in controls versus ACS patients for LPS

	%CD62p expression			
Agonist	Controls (n= 14)	ACS (n= 14)	Differences	*P* (controls vs. ACS)
Unstimulated	9.2 (1.9 – 18.3)	7.3 (0 – 22.4)	1.6	0.52
TRAP 100 µM	35.7 (17.3 – 56.7) ***	32.1 (7.1 – 65.3) ***	5.4	0.33
LPS 0.01 µg/mL	20.0 (8.0 – 31.2) ***	11.0 (2.1 – 27.3)	8.5	0.004
LPS 0.1 µg/mL	21.8 (4.3 – 30.5) ***	11.9 (2.7 – 26.7)	8.5	0.006
LPS 1 µg/mL	17.0 (7.6 – 35.3) ***	10.7 (0.8 – 26.8)	7.2	0.02

Data are expressed as median (range) *p*-values were calculated by the Mann-Whitney *U* test, difference between medians are expressed as Hodges-Lehmann differences
* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ refers to the comparison of TRAP and/or LPS concentrations versus the unstimulated samples for that cohort



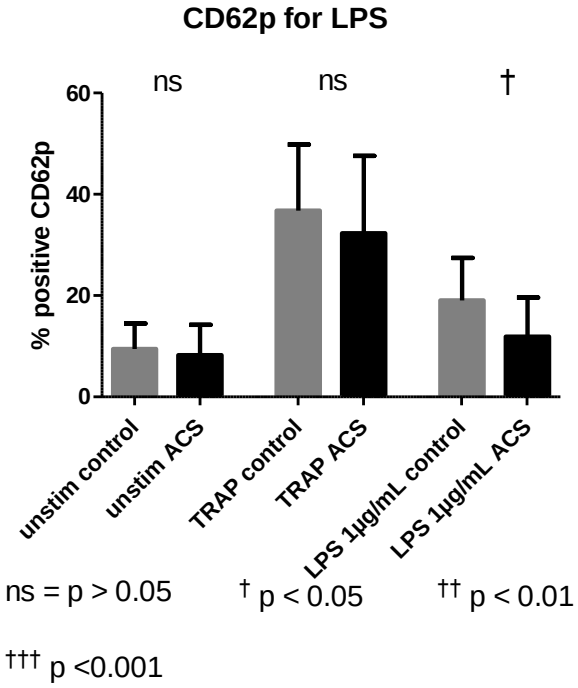


Figure 24. Platelet responses to LPS stimulation in healthy controls versus ACS patients

3.3.4 Response to PAM_3CSK_4 stimulation in controls versus acute coronary syndrome patients

A similar pattern of responses was observed for the samples stimulated by PAM_3CSK_4 for PAC-1 and CD62p expression as for the samples stimulated by LPS. The levels of PAC-1 and CD62p expression following PAM_3CSK_4 stimulation were greater in the healthy controls than the ACS patients on DAPT (Table 9, Table 10). For samples stimulated with 10 µg/mL PAM_3CSK_4, the median PAC-1 expression in the healthy controls was 28.2% (10.8% – 58.3%, $p < 0.001$) compared to 12.2% (0.0% - 30.0%, $p = 0.150$) in the ACS patients on DAPT, representing an absolute difference of 23.3% ($p < 0.001$) (Table 10; Figure 25). For the CD62p response to stimulation with 10 µg/mL PAM_3CSK_4, the median CD62p expression in the healthy controls was 20.3% (9.7% – 36.8%, $p < 0.001$) compared to 11.4% (2.2% – 28.7%, $p = 0.051$) in the ACS patients on DAPT, representing an absolute difference of 8.4% ($p = 0.04$) (Table 10; Figure 25).

Levels of CD62p expression in response to stimulation by 10 µg/mL PAM_3CSK_4 (median 11.4%, range 2.2% – 28.7%) and 1 µg/mL PAM_3CSK_4 (median 11.9%, range 2.7% – 24.1%) each appeared to trend towards significance when compared with unstimulated samples (median 7.3%, range 0.0% – 22.4%) in the ACS patient group on DAPT ($p = 0.051$ and $p = 0.076$, respectively) (Table 10).

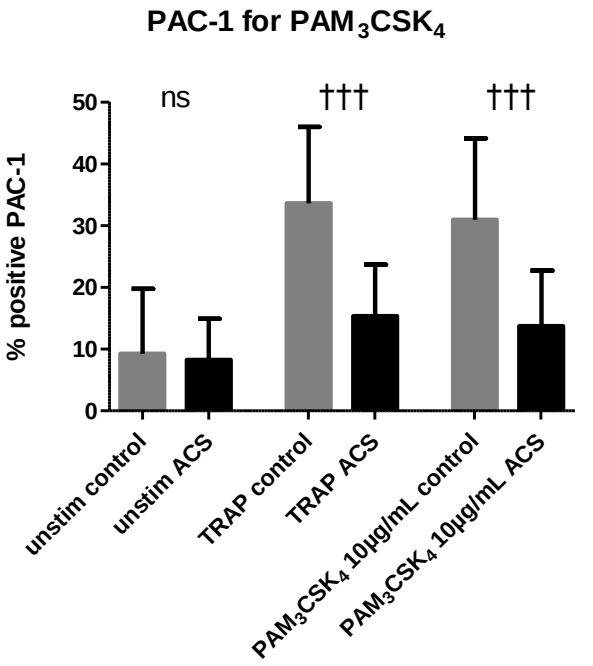
Table 9. %PAC-1 expression in controls versus ACS patients for PAM_3CSK_4

	%PAC-1 expression			
Agonist	Controls (n= 14)	ACS (n= 14)	Differences	*P* (controls vs. ACS)
Unstimulated	4.2 (0 – 27.5)	6.3 (0.0 – 19.0)	0.0	0.81
TRAP 100 µM	34.9 (14.5 – 51.4) ***	18.3 (1.4 – 26.6) *	18.6	<0.001
PAM_3CSK_4 0.1 µg/mL	24.4 (5.7 – 48.2) **	10.2 (0.0 – 26.0)	13.2	0.03
PAM_3CSK_4 1 µg/mL	26.0 (2.1 – 52.8) **	11.0 (0.0 – 23.1)	14.8	0.008
PAM_3CSK_4 10 µg/mL	28.2 (10.8 – 58.3) ***	12.2 (0.0 - 30.0)	16.4	<0.001

Table 10. %CD62p expression in controls versus ACS patients for PAM_3CSK_4

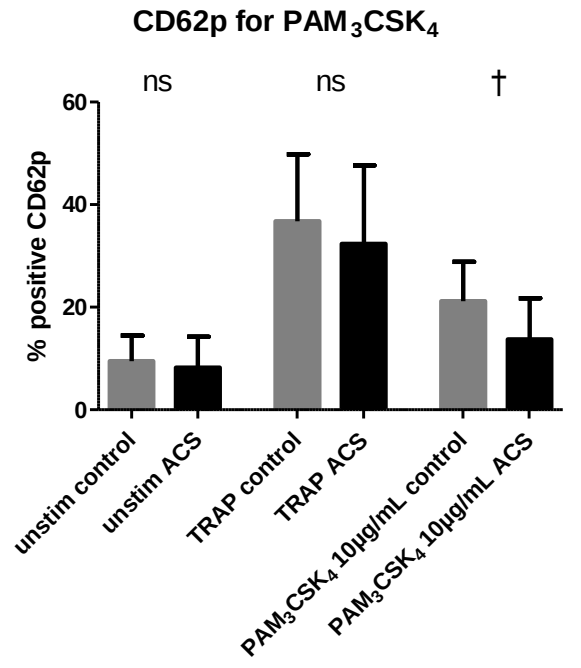
	%CD62p expression			
Agonist	Controls (n= 14)	ACS (n= 14)	Differences	*P* (controls vs. ACS)
Unstimulated	9.2 (1.9 – 18.3)	7.3 (0 – 22.4)	1.6	0.52
TRAP 100 µM	35.7 (17.3 – 56.7) ***	32.1 (7.1 – 65.3)***	5.4	0.33
PAM_3CSK_4 0.1 µg/mL	20.6 (10.1 – 32.0) ***	10.6 (1.9 – 24.0)	9.1	0.001
PAM_3CSK_4 1 µg/mL	21.0 (11.0 – 34.9) ***	11.9 (2.7 – 24.1)	8.8	0.007
PAM_3CSK_4 10 µg/mL	20.3 (9.7 – 36.8) ***	11.4 (2.2 – 28.7)	8.4	0.04

Data are expressed as median (range) *p*-values were calculated by the Mann-Whitney *U* test, difference between medians are expressed as Hodges-Lehmann differences
* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ refers to the comparison of TRAP and/or PAM_3CSK_4 concentrations versus the unstimulated samples for that cohort



ns = p > 0.05 † p < 0.05 †† p < 0.01

††† p <0.001



ns = p > 0.05 † p < 0.05 †† p < 0.01

††† p <0.001

Figure 25. Platelet responses to PAM_3CSK_4 stimulation in healthy controls versus ACS patients

3.3.5 Response to FSL-1 stimulation in controls versus acute coronary syndrome patients

The changes in PAC-1 and CD62p expression in response to FSL-1 stimulation were similar to those observed in the samples stimulated with PAM_3CSK_4 and LPS. The levels of PAC-1 and CD62p expression in response to FSL-1 were greater in the healthy controls than the ACS patients (Table 11, Table 12). For samples stimulated with 1 µg/mL FSL-1, the median PAC-1 expression in the healthy controls was 31.3% (12.0% – 54.5%, p <0.001) compared to 13.2% (0.4% - 30.4%, p = 0.075) in the ACS patients, representing an absolute difference of 18.7% (p < 0.001) (Table 11; Figure 26). In response to stimulation with 1 µg/mL FSL-1 for CD62p expression, the median in the healthy controls was 22.9% (7.5% – 39.5%, p < 0.001) compared to 15.8% (0.0% – 25.9%, p = 0.051) in the ACS patients, representing an absolute difference of 7.8% (p = 0.04) (Table 12; Figure 26).

Levels of PAC-1 expression in response to stimulation by 1 µg/mL FSL-1 (median 13.2%, range 0.4% – 30.4%) and 0.1 µg/mL FSL-1 (median 13.7%, range 0.0% – 46.1%) each appeared to trend towards significance when compared with unstimulated samples (median 6.3%, range 0.0% – 19.0%) in the ACS patient group on DAPT (p = 0.069 and p = 0.077, respectively) (Table 11).

Levels of CD62p expression in response to stimulation by 1 µg/mL FSL-1 (median 15.8%, range 0.0% – 25.9%) appeared to trend towards significance when compared with unstimulated samples (median 7.3%, range 0.0% – 22.4%) in the ACS patient group on DAPT (p = 0.054) as well (Table 12).

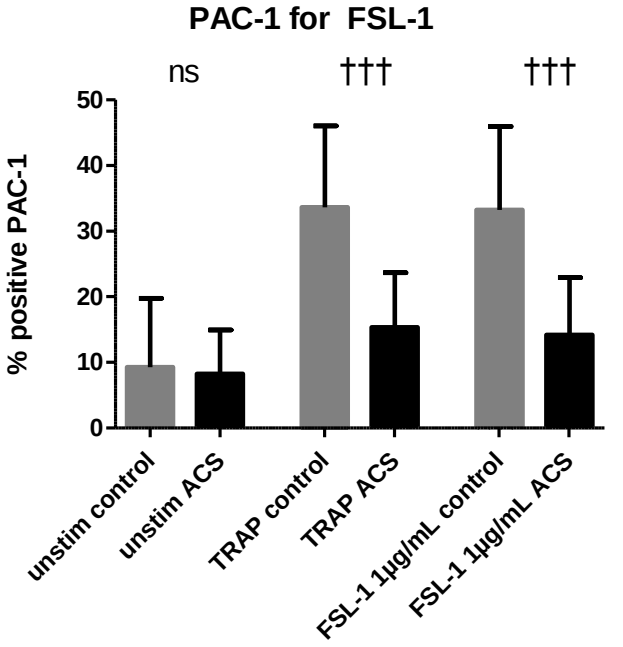
Table 11. %PAC-1 expression in controls versus ACS patients for FSL-1

	%PAC-1 expression			
Agonist	Controls (n= 14)	ACS (n= 14)	Differences	*P* (controls vs. ACS)
Unstimulated	4.2 (0 – 27.5)	6.3 (0.0 – 19.0)	0.0	0.81
TRAP 100 µM	34.9 (14.5 – 51.4) ***	18.3 (1.4 – 26.6) *	18.6	<0.001
FSL-1 0.01 µg/mL	35.2 (5.2 – 56.0) **	9.5 (0.0 – 33.8)	21.0	<0.001
FSL-1 0.1 µg/mL	28.9 (4.1 – 54.9) ***	13.7 (0.0 – 46.1)	16.6	<0.001
FSL-1 1 µg/mL	31.3 (12.0 – 54.5) ***	13.2 (0.4 – 30.4)	18.7	<0.001

Table 12. %CD62p expression in controls versus ACS patients for FSL-1

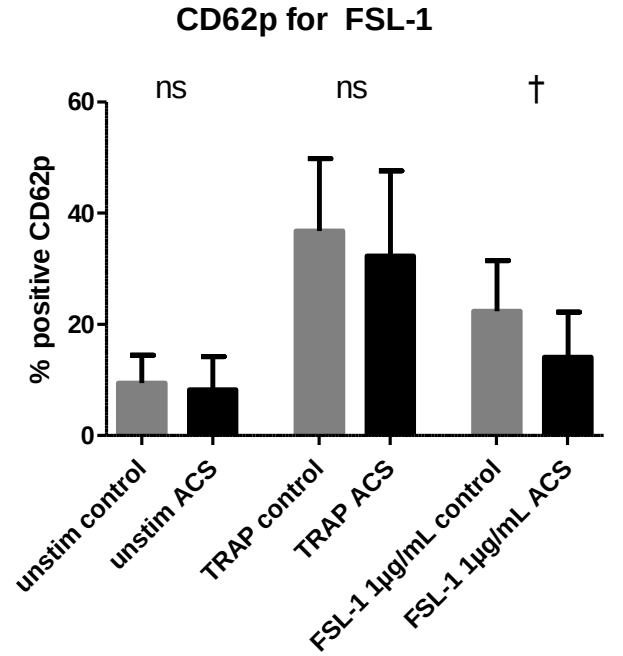
	%CD62p expression			
Agonist	Controls (n= 14)	ACS (n= 14)	Differences	*P* (controls vs. ACS)
Unstimulated	9.2 (1.9 – 18.3)	7.3 (0 – 22.4)	1.6	0.52
TRAP 100 µM	35.7 (17.3 – 56.7) ***	32.1 (7.1 – 65.3)***	5.4	0.33
FSL-1 0.01 µg/mL	20.9 (11.1 – 35.9) ***	13.5 (1.4 – 28.5)	9.1	0.008
FSL-1 0.1 µg/mL	21.0 (8.3 – 46.1) ***	11.2 (2.8 – 32.8)	9.8	0.005
FSL-1 1 µg/mL	22.9 (7.5 – 39.5) ***	15.8 (0.0 – 25.9)	7.8	0.04

Data are expressed as median (range) *p*-values were calculated by the Mann-Whitney *U* test, difference between medians are expressed as Hodges-Lehmann differences
* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ refers to the comparison of TRAP and/or FSL-1 concentrations versus the unstimulated samples for that cohort



ns = p > 0.05 † p < 0.05 †† p < 0.01

††† p <0.001



ns = p > 0.05 † p < 0.05 †† p < 0.01

††† p <0.001

Figure 26. Platelet responses to FSL-1 stimulation in healthy controls versus ACS patients

4 Chapter 4 – Discussion

4.1.1 Summary

We demonstrated that stimulating whole blood from healthy controls with TLR agonists to TLR4, TL2/1 and TLR2/6 (LPS, PAM_3CSK_4 and FSL-1, respectively) resulted in significant levels of platelet activation, as measured by the activation of the GPIIb/IIIa complex and platelet surface expression of P-selectin. There were no statistically significant responses to the TLR agonists we tested in the ACS patients on DAPT group, although there were a number of trends towards statistical significance with the higher concentrations of PAM_3CSK_4 and FSL-1 tested. At baseline, there were no significant differences in responses between the groups for the unstimulated samples. Accordingly, we found that the platelet responses to TLR agonist stimulation were significantly lower in the ACS patients on DAPT group relative to the healthy controls.

Stimulating isolated platelet systems with TLR agonists would clarify the degree to which the direct binding of TLR ligands to their corresponding platelet-expressed TLR may activate platelets. Investigating ACS patients who are naïve to DAPT, on aspirin only, and on a P2Y12-inhibitor only, would clarify the relative contributions of the TxA_2 and P2Y12 purinergic signalling pathways to TLR-agonist induced platelet activation. Given the large variability of platelet responses observed in the ACS patients on DAPT group, it is possible our study was insufficiently powered to detect the appropriate effect. Consequently, our findings will require confirmation in a larger sample set of ACS patients on DAPT.

4.2 Effects of stimulation of whole blood by Toll-like receptor agonists in healthy controls

4.2.1 Platelets activate in response to whole blood stimulation by Toll-like receptor agonists

Ex vivo stimulation of whole blood sourced from healthy controls by each of the TLR agonists tested (LPS, PAM_3CSK_4 and FSL-1) produced platelet activation, as measured by increases in the surface expression of PAC-1 and CD62p on individual platelets. Our observations therefore corroborate and extend the findings of previous studies that have examined platelet activation responses to stimulation by TLR2/1 (14, 24) and TLR4 agonists (21, 102) in isolated platelet systems to whole blood.

We are the first to demonstrate a potential role for the TLR2/6 agonist, FSL-1, as platelet agonist in whole blood. *Ex vivo* stimulation of whole blood with FSL-1 produced platelet activation, as measured by the surface expression of PAC-1 and CD62p on individual platelets. A previous study however, found that MALP-2 (another synthetic TLR2/6 agonist) did not activate washed platelets (100). This discrepancy may be due to a number of factors: FSL-1 may be a more potent TLR2/6 agonist than MALP-2, washed platelets may be less reactive to stimulation by TLR agonists than platelets in whole blood, or the effect may be reliant on FSL-1 acting on components of whole blood absent from isolated platelet systems.

Whether the responses to LPS, PAM_3CSK_4 and FSL-1 are predominantly dependent on platelets is unclear given that we analysed platelets in the presence of leucocytes and erythrocytes, both of which are capable of influencing platelet function (45, 121). Regarding LPS and PAM_3CSK_4 however, there is a wealth of evidence from studies in isolated platelet systems to suggest that these agonists do indeed activate platelets by binding directly to platelet surface-expressed TLR4 and/or TLR2/1 (14, 26, 102). Furthermore, there is strong evidence in the literature to suggest that the binding of certain bacteria (such as *S. pneumoniae*, *A. actinomycetemcomitans* and *P. gingivalis*) to platelet

surface-expressed TLR2/1 and/or TLR4 is also capable of directly activating platelets (14, 25, 101). Accordingly, we think it is plausible to conclude that at least a proportion of the platelet activation responses observed in our study were due to a direct effect of the TLR agonists we tested on their corresponding platelet surface-expressed TLRs.

4.2.2 In whole blood, Toll-like receptor agonists may simultaneously act on leucocytes and platelets to lower the threshold for platelet activation

In our study, *ex vivo* stimulation of whole blood by PAM_3CSK_4 at concentrations of 0.1 µg/mL, 1 µg/mL and 10 µg/mL activated platelets without an appreciable dose-response relationship, as measured by CD62p and PAC-1 expression (Table 7, Table 8). This contrasts with studies that have investigated platelet responses to TLR agonists in isolated platelet systems (with divergent findings). In one study, stimulation of washed platelets with increasing concentrations of PAM_3CSK_4 (5 µg/mL to 10 µg/mL) increased the surface expression of CD62p in a dose-dependent manner (14). In another study, stimulation of platelet-rich plasma with LPS and PAM_3CSK_4 at concentrations of 0.01 µg/mL, 0.1 µg/mL and 1 µg/mL was not capable of increasing the surface expression of CD62p (11).

The lack of dose-response relationship observed in our study with the concentrations of TLR agonists we tested raises the possibility that lower concentrations of TLR agonists may be capable of inducing platelet activation in whole blood relative to platelet-rich plasma and washed platelets. This may be due to a sensitising effect of plasma proteins, leucocytes or erythrocytes on platelet reactivity to TLR agonists; or a consequence of the separating and washing processes involved in the preparation of platelet-rich plasma or washed platelets.

We speculate that the TLR agonists we used may have had an indirect effect on platelets by activating leucocytes. Activated leucocytes are capable of influencing platelet activation: they release a variety of factors capable of inducing both platelet activation and aggregation, including cathespin G, superoxide reactive oxidative species, elastases and platelet activating factor (45, 121). Furthermore, inactivated neutrophils have been shown to inhibit platelet activation and aggregation

through the release of neutrophil-derived nitric oxide and ADPase (121). Importantly, neutrophils express functional TLRs 1 to 10 (122), while monocytes express functional TLRs 1, 2, 4, 5 and 8 (123, 124). Finally, the concentrations of TLR utilised in our study have been shown to be capable of activating leucocytes (122, 125, 126).

TLR agonists may therefore act simultaneously on leucocytes and platelets to lower the threshold concentration of TLR agonists required for platelet activation in whole blood. For example, TLR-activated leucocytes may indirectly activate platelets (as described above) while the TLR agonists simultaneously activate platelets by binding directly to their corresponding surface-expressed TLR. In addition to this effect, plasma cofactors may sensitise platelets to TLR agonists (102), further lowering the threshold concentration required for platelet activation.

Together, these processes may have a synergistic effect and may explain why we did not observe a dose-response relationship in our study while the study by Blair et al. did (14) with similar concentrations of PAM_3CSK_4. Furthermore, these processes may also explain why we were able to show significant platelet activation responses in whole blood with concentrations of PAM_3CSK_4 and LPS shown to be unable to do so (0.01 µg/mL, 0.1 µg/mL and 1 µg/mL) in platelet-rich plasma (11). None of these processes however satisfactorily explain how the study by Zhang et al. was able to demonstrate an increase in platelet P-selectin expression with 1 µg/mL of LPS in washed platelets (102).

Consequently, it is not possible to conclude from either our data or the literature, the degree to which the platelet activation responses observed in our study were mediated by platelets, TLR-activated leucocytes, erythrocytes, or plasma cofactors. Measuring platelet responses using the same concentrations of the TLR agonists we tested across whole blood, platelet-rich plasma, and washed platelets would therefore help clarify the relative degree to which each of these different factors drove the platelet activation responses we observed in whole blood.

4.2.3 Platelets respond differently to Toll-like receptor agonists in comparison to thrombin

The surface expression of the activated GPIIb/IIIa complex in response to stimulation by each of the TLR agonists tested were of a similar magnitude to the activated GPIIb/IIIa complex response observed in the samples stimulated by the positive control TRAP. By contrast, the surface expression of P-selectin induced by TLR agonists tested were significantly less than the surface expression of P-selectin induced by TRAP. These observations suggest that TLR agonists are potentially are less potent platelet activators than thrombin, and are consistent with the literature. Furthermore, they provide support for the hypothesis that platelets respond differently to thrombotic and infectious/inflammatory stimuli (14, 24, 25, 100).

4.2.4 Expression of CD154 in response to adenosine diphosphate and thrombin receptor activating peptide

We were unable to demonstrate significant increases in the platelet surface of expression of CD154-APC (Biolegend, San Diego, CA, USA) over its appropriate isotype control, in response to stimulation by either ADP or TRAP. This did not resolve even after we conducted titrations for the staining concentration of CD154-APC (Biolegend, San Diego, CA, USA). It is well documented that stimulation of platelets with either ADP or TRAP is capable of inducing platelet surface expression of CD154 (69, 106, 113). It was not clear why we were unable to identify a CD154 positive population; it is however possible there was an issue with the particular CD154 antibody we selected given that our antibody titrations were unsuccessful. Because the CD154 antibody was not showing a positive signal under any circumstances, we removed it from our flow cytometric panel.

4.2.5 Clinical relevance of platelet responses to Toll-like receptor stimulation of whole blood

Irrespective of the uncertainty whether our observations are predominantly platelet- or leucocyte- driven, the finding that the TLR2/1, TLR2/6 and TLR4 agonist stimulation of whole blood were each capable of inducing platelet activation in whole blood has some clinical relevance.

The observation that concentrations of TLR agonists incapable of activating platelets in isolated platelet systems were capable of doing so in whole blood is clinically relevant, as it provides a potential mechanism by which mild infections, such as influenza or lower respiratory tract infections, may contribute to increased thrombotic risk (32). In washed platelets, platelet aggregation and activation are induced at concentrations of TLR agonists (0.1 µg/mL to 10 µg/mL) (14, 19, 21, 24, 102) consistent with severe sepsis (102, 127). Mild infections may, like severe infections, activate platelets to assume an inflammatory phenotype (heterotypic aggregates, increased sCD40L release) in a TLR-mediated fashion (either directly on platelets and/or indirectly via leucocytes), thereby promoting the progression of atherosclerosis and potentially increasing the risk an atherothrombotic event (128, 129).

We do not know the physiological relevance of the higher concentrations of the synthetic TLR agonists PAM_3CSK_4 and FSL-1 we tested. Regarding LPS, plasma levels of the concentrations we employed to stimulate platelets are only found in the clinical setting in patients *in extremis* with sepsis (127). However, it is possible that the local concentrations of TLR agonists/pathogens within the coronary vasculature in mild infections are many orders of magnitude higher than the systemic plasma concentrations.

We made a conscious decision to conduct the flow cytometric analysis of platelets in whole blood. Whole blood has a greater physiological and clinical relevance than isolated platelet systems because platelets do not exist in the human body as platelet-rich plasma or as washed platelets. Moreover, leucocytes and erythrocytes are capable of influencing platelet function (45, 121).

Finally, the preparation of isolated platelet systems can introduce artifactual *in vitro* platelet activation and the loss of platelet populations that can corrupt the flow cytometric analysis of platelets (45, 69). Therefore, the manner in which we analysed our platelets in whole blood arguably has greater clinical relevance relative to platelets analysed in isolated platelet systems.

Our novel finding that stimulation of whole blood with a synthetic TLR2/6 agonist is capable of inducing platelet activation is clinically relevant because heterodimerization of TLR2 with TLR6 enables TLR2 to recognise a wide variety of antigens (73, 74). As described in the introduction, examples of specific antigens recognised by TLR2 (or TLR2 heterodimers) include lipoarabinomannan from mycobacteria, zymosan from fungi, heat shock proteins, cardiac myosin, and many more (72). Consequently, our observations potentially extend the range of antigens capable of inducing platelet activation in whole blood, albeit potentially via an indirect effect on leucocytes, to those antigens recognised by TLR2/6. Whether a number of these putative antigens are *bona fide* TLR2 (or TLR2 heterodimer) agonists is contentious, as there is evidence to suggest that the reported activity of some of these agonists could be secondary to contamination by other biologically active lipoproteins (130).

4.3 Effects of stimulation of whole blood by Toll-like receptor agonists in acute coronary syndrome patients

4.3.1 Platelets did not activate in response to whole blood stimulation by the concentrations of Toll-like receptor agonists employed

Stimulation of whole blood sourced from ACS patients on DAPT with TLR agonists did not result in statistically significant increases in the expression of the platelet-activation markers relative to the unstimulated samples, for each of the concentrations of agonists we tested.

A number of trends towards significance for PAC-1 and CD62p expression were noted for samples stimulated with the higher concentrations of PAM_3CSK_4 and FSL-1 relative to the unstimulated samples. As described in the results, trends to significance were noted for 10 µg/mL and 1 µg/mL PAM_3CSK_4 versus unstimulated for CD62p ($p = 0.051$ and $p = 0.076$, respectively), 1 µg/mL and 0.1 µg/mL FSL-1 versus unstimulated for PAC-1 ($p = 0.069$ and $p = 0.077$, respectively), and 1 µg/mL FSL-1 versus unstimulated for CD62p ($p = 0.054$). Moreover, the median levels of PAC-1 and CD62p expression in response to the higher concentrations of PAM_3CSK_4 and FSL-1 consistently appear to be 1.5 to 2 times greater than the levels of these markers in the unstimulated samples (Tables 9-12).

In summary, these observations raise the possibility that our study did not have enough power to detect differences of this magnitude in the ACS patients on DAPT group. As was the case with the healthy controls, we cannot be sure from our data the relative degree to which the platelet responses we did observe in the ACS patient group were driven by leucocytes, erythrocytes, plasma proteins and platelets.

4.3.2 Differences in platelet responses to stimulation by Toll-like receptor agonists between healthy controls and acute coronary syndrome patients

The expression of platelet activation markers was not significantly different between the ACS patient on DAPT group and the healthy control group for the unstimulated samples. This was an unexpected finding because baseline levels of PAC-1 and CD62p have consistently been found to be elevated in ACS patients (96, 97, 112, 113). In response to TLR stimulation, both PAC-1 and CD62p expression were significantly lower in the ACS patient group on DAPT than the healthy control group, overall.

4.3.2.1 Dual antiplatelet therapy almost completely inhibited platelet responses to Toll-like receptor stimulation in the acute coronary syndrome patient group

In the present study, the ACS patient group was loaded on DAPT in the form of aspirin and clopidogrel (7 days with aspirin and clopidogrel ≥75 mg and and/or loading with aspirin ≥300 mg at least 2 hours prior and clopidogrel ≥300 mg at least 6 hours) prior to enrolment. These levels of platelet therapy are in accordance with current best clinical practice (35).

Dual antiplatelet therapy decreases the reactivity of platelets a variety of platelet agonists (7, 131-133). A combination of aspirin and clopidogrel appears to be more effective than either aspirin or clopidogrel alone in inhibiting platelet reactivity and decreasing levels of circulating activated platelets (131, 132). In patients with atherosclerotic disease, Klinkhardt et al. demonstrated that aspirin and clopidogrel was more effective than either aspirin or clopidogrel alone in inhibiting increases in platelet CD62p and PAC-1 expression and platelet-monocyte aggregate formation in response to ADP and TRAP stimulation (131). Similarly, Xiao et al. found that the addition of clopidogrel to ACS patients already on aspirin significantly decreased CD62p expression and platelet-monocyte and platelet-neutrophil aggregate formation in response to ADP and TRAP stimulation (132).

Loading with dual antiplatelet therapy in the ACS patient group, particularly with clopidogrel, prior to testing (131, 132) therefore provides a reasonable explanation for why the ACS patient group did not have a higher baseline level of platelet reactivity relative to the controls as we had expected. Accordingly, it also explains why we did not observe statistically significant differences in PAC-1 and CD62p expression between the groups for the unstimulated samples.

The cyclooxygenase-TxA_2 pathway and the P2Y12 receptor appears to be involved in platelet-expressed TLR-induced platelet activation and aggregation (100, 101). Aspirin has been shown to inhibit PAM_3CSK_4-induced platelet aggregation, ATP release, calcium mobilisation and TxB_2 production, in washed platelets (100). Furthermore, a P2Y12 receptor antagonist has been shown to inhibit PAM_3CSK_4-induced platelet aggregation and ATP release, but not calcium mobilisation or TxB_2 production (100). Finally, clopidogrel but not aspirin was shown to significantly reduce *S. pneumoniae* induced platelet aggregation, suggesting that TLR2-induced platelet aggregation is predominantly dependent on ADP but not TxA_2 (101).

The literature regarding the role of aspirin and purinergic P2Y12 antagonists in inhibiting TLR2-mediated platelet activation and aggregation provides another explaination (100, 101) for the lower levels of platelet responses to TLR agonism observed in the ACS patients on DAPT group. Our study extends the findings of studies examining the effects of aspirin and P2Y12 receptor antagonism on platelet TLR-signalling into whole blood and the clinical context of ACS patients.

It is unclear whether some, at this stage unknown, 'factor(s)' uniquely associated with the disease process of an ACS has contributed to our observations, as our study design was unable to assess the potential impact(s) of TLR agonism independent from the presence of DAPT. It is clear from a number of studies however that the ACS are associated with increased levels of platelet reactivity (134, 135), even at an mRNA transcriptional level (136).

4.3.2.2 *Clinical relevance of the lack of complete inhibition of platelet activation in response to stimulation by high concentrations of Toll-like receptor agonists*

Although we found no statistically significant platelet activation responses to the TLR agonists we tested in the ACS patient on DAPT group, this will require confirmation in larger sample sets because we observed a number of trends towards statistical significance for the higher concentrations of PAM_3CSK_4 and FSL-1 we tested.

If the observed trends towards statistical significance do indeed represent a 'true' population effect upon further testing in larger samples, it raises the possibility that the presence of DAPT may be insufficient reduce the risk of arteriothrombotic events involving platelets in settings of infection. We think that this is a possibility given the growing body of evidence implicating immune and infectious processes on platelets in the pathogenesis of atherosclerosis and arteriothromboses (32). Both chronic and acute infections may contribute to endothelial dysfunction and the progression of atherosclerosis by facilitating local inflammatory responses (78, 88, 89). Furthermore, infections appear to be risk factors for vascular thrombotic events (32, 83, 84), and are associated with post-ACS adverse events such as cardiovascular death and stent thrombosis (33, 78, 90, 137). Moreover, immunisation of ACS patients with the influenza vaccine has been demonstrated to reduce the risk of death and ischaemic events for up to one year (91, 92). Nevertheless, we cannot be sure from our study whether the observed increases in the platelet activation markers (even if they do represent a true effect) are of a great enough magnitude to be clinically significant.

If DAPT is indeed insufficient to reduce the risk of arteriothrombotic events involving platelets in settings of infection, therapies based upon TLR antagonism during times of infection for ACS patients may have clinical utility. Care would need to be taken when using such agents however, as TLRs are expressed on a broad variety of immune cells and are important for effective innate immune responses (16-18). Accordingly, the use of such therapy risks compromising host responses to infections. Alternatively, higher doses of antiplatelet therapy could be employed instead in

settings of infection in an attempt to overcome the negative impacts of pathogens on platelet activation.

4.4 Study limitations

A considerable limitation of our study was that it might have been poorly powered to detect mild to moderate increases in expression of platelet activation markers following stimulation by TLR agonists. In retrospect, this is unsurprising given the significant variability of the platelet activation responses amongst individuals within both the groups of our study (Tables 7-12). Moreover, it has been well demonstrated that platelet activation responses to ADP and other platelet agonists can vary considerably between individuals (134, 135, 138). Consequently, we cannot be certain as to whether the trends to statistical significance observed in the ACS patient on DAPT group represent a 'true' population effect, without conducting the same experiments in a larger sample set.

Analysing platelets in whole blood only meant that we were unable to determine whether our observations were predominantly platelet-mediated or mediated by other cells or components present in the blood. This was a limitation as with the whole blood analysis alone, we were unable to ascertain whether the TLR agonists we used were acting directly on platelets or on platelets via some secondary effect by leucocytes/erythrocytes or plasma proteins.

Investigating patients with a confirmed ACS who were aspirin and clopidogrel naïve would have been useful, as it would have allowed us to examine the impacts of the TLR-stimulation of platelets in ACS patients independently from the presence of dual antiplatelet therapy. This would have been difficult to achieve, however, given that a large number of patients with ACS are treated with at least a loading dose of aspirin, either in the ambulance or at the general practitioner, before presenting to hospital. Furthermore, it would be unethical to withhold or unnecessarily delay aspirin and clopidogrel therapy to patients given that prompt dual antiplatelet therapy in ACS patients improves patient outcomes and is standard clinical practice (98, 99).

Our flow cytometric protocol only analysed individual circulating platelets. In doing so, we ignored the subpopulations of platelets encompassing platelet-platelet aggregates, and platelets bound to leucocytes/erythrocytes. This is a limitation because these platelet subpopulations tend to

express different phenotypes to individual circulating platelets (45, 69). Moreover, analysis of the individual circulating platelets subpopulation can make it difficult to interpret platelet-surface P-selectin expression in this group, as a large proportion of the population of P-selectin expressing platelets in the individual circulating platelet subpopulation may have been lost to the platelet-leucocyte subpopulation (platelets interact with leucocytes via the P-selectin/PSGL-1 interaction) (60).

Another limitation of the flow cytometric protocol utilised was that we did not analyse platelet-leucocyte aggregates. Platelet-leucocyte aggregates are a more sensitive marker of *in vivo* platelet activation and platelet reactivity than P-selectin (45, 139), and are pro-inflammatory and pro-thrombotic in their own right (128, 129). Accordingly, platelet-leucocyte aggregates appear to have important roles in the pathogenesis of the progression of atherosclerosis and plaque rupture (95, 128, 129).

4.5 Future directions

As alluded to previously, our study may not have been sufficiently powered to detect small levels of platelet activation in response to stimulation by TLR agonists. Since we now have a better indication of the potential effect size and variation of platelet responses to TLR agonists, it may now be possible to conduct informed *a priori* power calculations to provide an estimate of what sample sizes we might need to observe a reasonable effect size. Consequently, our negative findings regarding platelet responses to TLR agonist stimulation in the ACS patients on DAPT group will probably require confirmation in a larger sample set.

The analysis of platelet responses to Toll-like receptor stimulation in whole blood only is a significant limitation of this study. Accordingly, it would be useful to examine the platelet responses to the same TLR agonists we tested in platelet-rich plasma and compare the responses in platelet-rich plasma with the responses in whole blood. This would clarify the degree to which the platelet responses in whole blood were dependent on the components of whole blood not present in platelet-rich plasma, such as leucocytes and erythrocytes which are both are capable of influencing platelet function (45, 121). It would also help test the hypothesis that the effects of TLR agonism in blood (for example on leucocytes) lowers the threshold concentration of TLR agonists required to activate platelets in isolated platelet systems.

Examining ACS patients that are aspirin and clopidogrel naïve, or at least naïve to clopidogrel only would help clarify the impact of having had an ACS on the reactivity of platelets to TLR-stimulation. In doing so, the sampling of blood from such patients would need to be done without overly delaying their access to life-saving treatment.

It would be interesting to examine the expression of platelet-TLRs in those with stable coronary disease and patients with risk factors for atherosclerosis and acute myocardial infarction, and compare the correlation between TLR-expression and response to TLR agonists in these patients. Activated platelets express higher levels of TLR2 and TLR9 but less of TLR4 than quiescent

platelets (12, 13). Patients with atherosclerotic disease have increased platelet reactivity and increased levels of circulating activated platelets than healthy controls (77, 79). Therefore, comparing platelet-expressed TLR expression and platelet reactivity to TLR agonists between these patient groups and healthy controls would provide information into the functionality of the platelet-expressed TLRs as a potential mechanism underlying the association between acute and chronic infections and ACS in these at risk patient groups (32, 33). Another way to do so would be to compare the platelet responses to TLR agonists in healthy patients before and after the priming of platelets with submaximal doses of ADP (in order to increase the expression of the platelet-expressed TLRs).

There is a growing body of evidence specifically linking influenza with increased risk for ACS (32). A recent study demonstrated that the intracellular platelet-expressed TLR9, capable of recognising CpG DNA sequences, is functional on platelets (15). TLR9, by virtue of being an intracellularly expressed, is capable of detecting certain single-stranded DNA and RNA from influenza viruses (140). Thus, examining and comparing the function of platelet-expressed TLR9 in ACS patients and healthy controls might provide insights into the observed association between influenza and ACS risk (32, 137). Furthermore, it would also be interesting to examine the function of platelet-expressed TLR9 in those receiving the influenza vaccine as well, given protective effects of the influenza vaccine on ACS risk and post-ACS adverse ischaemic events (32, 91, 92, 137).

To clarify the impact of TxA_2 and P2Y12 antagonism separately on TLR signalling, it would be useful to measure platelet reactivity to the TLR agonists we tested in healthy controls, in the following four groups: free from antiplatelet therapy, on aspirin only, on ticagrelor only, and on both aspirin and ticagrelor. Ticagrelor is an oral, reversibly binding, P2Y12 antagonist that, unlike clopidogrel, does not require metabolising into its active form (141, 142). Consequently, using ticagrelor would circumvent the confounding effects of variation in clopidogrel metabolism in the interpretation of the effects of P2Y12 receptor antagonism on TLR signalling. Of note, ticagrelor has

been shown in a large randomised controlled trial to significantly reduce mortality from vascular causes, myocardial infarction and stroke without an increase in major bleeding risk in ACS patients as compared with clopidogrel (141).

4.6 Conclusions

In this study, we investigated platelet responses to stimulation of TLR4, TLR2/1 and TLR2/6 using the following TLR agonists, LPS, PAM_3CSK_4 and FSL-1, respectively, in healthy controls and ACS patients on DAPT, with flow cytometry.

TLR agonist stimulation significantly increased the surface expression of the activated GPIIb/IIIa complex and P-selectin in the healthy control group. By contrast, TLR agonist stimulation did not increase platelet activation in the ACS patients on DAPT group. There were no statistically significant differences in the levels of platelet activation between the groups for the unstimulated samples. Accordingly, responses to each of the TLR agonists we tested were significantly lower in the ACS patient group relative to the healthy control group. We hypothesise that the mostly likely reason for this difference are the effects of DAPT in the ACS patient group.

For the ACS patients on DAPT group, trends towards statistically significant increases in the expression of platelet activation markers were observed at the higher concentrations of PAM_3CSK_4 and FSL-1 we employed. Whether or not this represents a 'true' population effect is unclear, as it is possible our study was insufficiently powered to detect, statistically significantly, the effect size we observed.

Interestingly, we found that we could induce platelet activation in whole blood using concentrations of TLR agonists lower than those capable of doing so in isolated platelet systems. From our study, it is unclear what pathways other than the direct stimulation of surface-expressed TLRs, or the role of the platelet TxA_2 and P2Y12 purinergic signalling pathways (blocked by aspirin and clopidogrel, respectively) are, contribute to our observations. Nevertheless, lower, and possibly more clinically relevant, concentrations of TLRs agonists than those we tested may be capable of activating platelets in whole blood. This is of clinical significance as it suggests that more common, mild infections may be capable of inducing aberrant platelet activation, thereby increasing the risk of arteriothromboses involving platelets.

Though we found no statistically significant platelet activation responses to TLR agonists we tested in the ACS patient on DAPT group, this will require confirmation in a larger sample set given the possibility our study was underpowered. Analysing platelet responses to TLR agonist stimulation in whole blood, platelet-rich plasma and in washed platelets would help clarify the relative contributions of platelets, leucocytes, erythrocytes and plasma proteins towards our observations in whole blood. Investigating TLR agonist responses in subjects who were naïve to DAPT, on aspirin only, and on a P2Y12-inhibitor only, would help clarify the relative contributions of the TxA_2 and P2Y12 purinergic signalling pathways, respectively, in platelet-expressed TLR signalling.

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6 Appendices

6.1 Appendix 1 – Principles of Flow Cytometry

6.1.1 Flow cytometry

Flow cytometry uses the principles of light-scattering, light excitation and the emission properties of fluorochromes to measure multiple different physical characteristics of individual particles and cells. The physical characteristics of individual particles measured by flow cytometry include relative particle size, granularity and fluorescence intensity. Analysis of individual particles and cells is achieved using fluidics systems to focus particles and cells to flow single file, within a fluid stream, past a beam of focused light (typically a laser beam) for interrogation, at a rate of thousands of particles/cells per second (106).

6.1.2 Principles of flow cytometry

As individual particles pass single file through a beam of focused light, light is scattered and any attached fluorochromes can become excited and fluoresce. A system of lenses, beam splitters and filters then collects this scattered and fluorescent light, and steers it to a series of specific detectors called channels. Flow cytometers contain channels specific for the measurement of diffractive and refractive light scatter, as well as channels specific for the detection of certain fluorescent emission spectrum peaks, or colours, for any number of excited fluorochromes. Each channel translates the optical signals it receives into electronic voltages proportional to the intensity of the light entering the channel. These voltages are then converted into data sets for each particle or cell, now termed an 'event', on the basis each particle or cell's measured light scatter and fluorescence properties (106).

6.1.3 Forward and side scatter

Interception of incident light by a particle or cell results in diffractive and refractive light scatter. The extent to which a particle or cell diffracts and refracts light is dependent on its size and

granularity respectively. In flow cytometry, forward-scattered light (FSC) is a measure of diffractive light scatter and corresponds to size or surface area, while side-scattered light (SSC) is a measure of refractive light scatter and corresponds granularity (internal complexity of the particle/cell) (106).

6.1.4 Fluorescence and flow cytometry

Pre-treatment of samples with fluorescently-conjugated monoclonal antibodies against antigens of interest adds another dimension to flow cytometry by allowing for the identification of particular cell types and cell subpopulations, based on the specific surface marker antigens expressed on the cell. Thus, the addition of multiple fluorescently-conjugated antibodies to flow cytometry assays allows for the independent measurement of multiple different antigens per cell simultaneously.

Fluorescent compounds absorb light energy over a specific range of wavelengths that are characteristic to the compound. When a fluorescent compound is stimulated by an appropriate wavelength of light, the absorbed energy excites an election to a higher (and more unstable) energy state. As this electron falls back to a lower energy state, it releases this excess energy in the form of photons of light with an emission spectrum, often with one or more intensity peaks, that is unique to the specific fluorescent compound. These fluorescent emission peaks can in turn be measured by fluorescence channels specific for these spectrum peaks or colours and translated into data, with the intensity of the fluorescent light measured within a channel being directly proportional to the number of fluorescent antibodies bound to the particle or cell (143, 144).

6.1.5 Multicolour flow cytometry and spectral overlap

The use of multiple fluorescently-conjugated antibodies per sample in flow cytometry is commonly referred to as multicolour flow cytometry. Multicolour flow cytometry allows for the analysis of several independent antigens simultaneously, and is considered to be more efficient and less time-consuming than single colour (fluorochrome) flow cytometry. Multicolour flow cytometry is however limited by the specifications of the flow cytometer used for the analysis; that is, what

lasers and detectors (and therefore what fluorescent channels) the cytometer is equipped with. Technical issues, relating to the spectral overlap of fluorochromes measurable by more than one fluorescent channel, also limit multicolour flow cytometry by introducing error into the analysis (119, 143).

When two or more fluorescent conjugates are present in a flow cytometry assay, their emission spectra can overlap one another and/or spillover into one or several other fluorescence channels, resulting in false positive signals in the affected channels. Consequently, the intensity of the fluorescence measured in a channel affected by spillover may not be truly representative of the fluorescence of the cell population that channel was designated to measure. The degree to which the emission spectrum of a fluorochrome spills over into another channel is termed its 'spillover coefficient' (119, 143, 144).

6.1.5.1 Minimising the deleterious effects of spectral overlap

Spectral overlap introduces artifactual measurement error to multicolour flow cytometry, which increases for every extra fluorescently conjugated antibody added to the assay. As such, spectral overlap can have deleterious effects on the reliability and the reproducibility of multicolour flow cytometry assays. This error can be ameliorated in three ways: a) applying compensation, a process where spectral overlap between fluorochromes is mathematically eliminated; b) pairing fluorochromes with bright staining indices with lowly-expressed antigens; and c) choosing a panel of fluorochromes with low levels of spectral overlap when used together (119, 143, 144).

6.1.5.2 Compensation

Compensation is the process by which the spillover from one or more fluorochromes into any number of affected channels designated for data collection is mathematically corrected. Proper compensation is achieved when, on average for a population of cells, there is no contribution of fluorescent spillover (from any given fluorochrome) into the output data for any of the channels

used for data collection. In other words, proper compensation ensures that the output data from any one channel contains information from only a single fluorochrome (115, 119).

Compensation controls are samples used to set the compensation settings that correct for fluorescent spillover into the channels used for data collection. They can either be cells or specialised 'beads' capable of binding fluorochrome-conjugated antibodies. Selection of appropriate compensation controls is critical for ensuring these compensation settings are calculated correctly; 'compensation beads' are preferred because they provide more standardised fluorescence staining profiles than cells do (115).

Compensation can be conducted either pre- (hardware) or post- (software) acquisition. Pre-acquisition compensation occurs after signal detection but before data digitalisation, and relies upon the instrumentation of the flow cytometer to remove spillover before data analysis. By contrast, post-acquisition compensation occurs after data digitalisation. Post-acquisition compensation is recommended over pre-acquisition compensation in flow cytometry experiments employing four or more fluorochromes, as above this threshold the complexity of the pairwise corrections required for proper compensation is generally beyond the capacity of most hardware systems (119).

6.1.5.3 Pairing of fluorochromes

Pairing of fluorochromes with a high stain index that are 'brightly staining' to lowly-expressed antigens can minimise losses in assay sensitivity and data resolution when conducting compensation for spectral overlap (though compensation does not increase error in the sample per se). This arises from the observations that brighter staining populations tend to have greater variances than dimly staining populations, and that compensating for spectral overlap from a channel does not correct for the variance of any staining population removed by compensation. In other words, though the spectral overlap from a brightly staining population may have been compensated, its variance remains in the now compensated channel. Variance decreases the resolution of fluorescence signals within a channel and makes distinguishing dimly staining events from background noise and/or

autofluorescence difficult. Pairing high stain index fluorochromes to lowly-expressed antigens reduces the brightness (and the variance) of staining populations, thereby improving the reliability and the reproducibility of the flow cytometry assay (115, 143, 144).

6.1.5.4 Choosing fluorochromes with low levels of spectral overlap

Selection of panels of fluorochromes with low levels of spectral overlap minimises the degree to which samples need to be compensated. Reducing the degree of compensation required is beneficial because it reduces losses in assay sensitivity and data resolution associated with compensation (115, 144).

6.1.6 Titration of antibodies

Titration of antibodies for flow cytometry serves three specific purposes: prevention of the non-specific binding of cells to unbound antibodies that can occur upon sample fixation, minimisation of the error associated with high background staining secondary to excessively high concentrations of antibody, and spares antibodies (86, 143).