The Effect of Antiplatelet Medications on Innate Immune Activation

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Abstract

Clinical studies suggest that platelet $P2Y_{12}$ inhibitors reduce mortality from sepsis, although the underlying mechanisms have not been clearly defined *in vivo*. It was hypothesised that platelet $P2Y_{12}$ inhibitors may improve survival from sepsis by suppressing systemic inflammation and its prothrombotic effects. It was therefore determined whether clopidogrel and the novel, more potent $P2Y_{12}$ inhibitor, ticagrelor modify these responses in an experimental human model.

In a randomised controlled trial, thirty healthy volunteers were allocated to ticagrelor (n=10), clopidogrel (n=10) or no antiplatelet medications (controls; n=10) for 1 week. Systemic inflammation was then induced by intravenous injection of *E.coli* endotoxin. Both $P2Y_{12}$ inhibitors significantly reduced platelet-monocyte aggregate formation and peak levels of major pro inflammatory cytokines, including TNFα, interleukin-6 and chemokine (C-C motif) ligand 2. In contrast to clopidogrel, ticagrelor also significantly reduced peak levels of interleukin-8 and granulocyte colony-stimulating factor and increased peak levels of the anti-inflammatory cytokine IL-10. Both $P2Y_{12}$ inhibitors suppressed D-dimer generation and scanning electron microscopy revealed that ticagrelor also suppressed prothrombotic changes in fibrin clot ultrastructure. There was a marked 6-fold expansion of the intermediate monocyte population at 24 hours after endotoxin administration. Platelet $P2Y_{12}$ inhibitors potentiated the increase in intermediate monocyte count, suggesting that platelet-monocyte interactions play an important role in regulating intermediate monocyte mobilization. Ticagrelor, but not clopidogrel, also inhibits cellular uptake of adenosine and thereby increases extracellular levels of adenosine. *In-vitro* experiments showed that this mechanism potentiates the stimulatory effect of low concentrations of adenosine on neutrophil phagocytosis. Additionally, this mechanism potentiated the suppressive effect of high concentrations of adenosine on FMLP-induced leukocyte activation.

Potent inhibition of multiple inflammatory and prothrombotic mechanisms by $P2Y_{12}$ inhibitors demonstrates critical importance of platelets as central orchestrators of systemic inflammation induced by bacterial endotoxin. This provides novel mechanistic insight into the lower mortality associated with $P2Y_{12}$ inhibitors in patients with sepsis in clinical studies.

Publications arising from this thesis

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Abbreviations

ACS	Acute coronary syndrome
ADAM	A disintegrin and metalloproteinase
ADP	Adenosine diphosphate
AMP	Adenosine monophosphate
ANOVA	Analysis of variance
APC	Allophycocyanin
ATP	Adenosine triphosphate
cAMP	Cyclic adenosine monophosphate
CI	Confidence interval
CCL	CC chemokine ligand
CCR	CC chemokine receptor
CD	Cluster of differentiation
COX-1	Cyclooxygenase-1
CRP	C-reactive protein
CXCL	Chemokine (C-X-C motif) ligand
DAMP	Damage associated molecular pattern
DMSO	Dimethyl sulfoxide
ENT1	Equilibrative nucleoside transporter 1
FCS	Fetal calf serum
FITC	Fluorescein isothiocyanate
FMLP	N-Formylmethionine-leucyl-phenylalanine
G-CSF	Granulocyte colony stimulating factor
GP	Glycoprotein
HMGB1	High-mobility group protein 1
HR	Hazard ratio
ICAM-1	Intercellular adhesion molecule-1

IL	Interleukin
LPS	Lipopolysaccharide
MCP-1	Monocyte chemotactic protein-1
MIP	Macrophage inflammatory protein
MMP	Matrix metalloproteinase
MPO	Myeloperoxidase
NET	Neutrophil extracellular trap
NOX	Nicotinamide adenine dinucleotide phosphate oxidase
NSTEMI	Non-ST-elevation myocardial infarction
PAI-1	Plasminogen activator inhibitor-1
PAMP	Pathogen associated molecular pattern
PAR	Protease-activated receptor
PE	R-phycoerythrin
PECAM-1	Platelet endothelial cell adhesion molecule-1
PerCP	Peridin chlorophyll protein
PF4	Platelet factor 4
PGH_2	Prostaglandin H_2
PLATO	PLATelet inhibition and patient Outcomes study
PRP	Platelet-rich plasma
PSGL-1	P-selectin glycoprotein ligand-1
RANTES	Regulated upon activation, normal T-cell expressed and secreted
RPMI	Roswell Park Memorial Institute medium
STEMI	ST-elevation myocardial infarction
TLR	Toll-like receptor
TNFα	Tumour necrosis factor α
TREM-1	Triggering receptor expressed on myeloid cells 1
VCAM-1	Vascular cell adhesion molecule-1

VSMC	Vascular smooth muscle cell
VWF	Von Willebrand Factor

1 Introduction

1.1 Cardiovascular Disease

Coronary artery disease continues to be the commonest cause of death worldwide (World Health Organization, 2012). Acute coronary syndrome (ACS) refers to a group of conditions that arise when there is acute obstruction to blood flow in the coronary arteries, leading to an impairment of the blood supply to the myocardium of the heart. ACS is subdivided on the basis of electrical abnormalities detected on the electrocardiogram and detection of myocardial proteins that are indicative of myocardial infarction (Roffi et al., 2015). Partial obstruction often leads to unstable angina or non-ST-elevation MI (NSTEMI), whereas total obstruction of the larger coronary arteries normally leads to ST-elevation myocardial infarction (STEMI).

It is well-established that platelets play a central role in the formation of a thrombus in the coronary arteries after atherosclerotic plaque rupture or erosions. Platelets, which are discoid-shaped fragments derived from bone marrow megakaryocytes, have a major physiological role in maintaining vascular integrity and haemostasis. They are released under the regulation of thrombopoietin and circulate for approximately 7-10 days (Kaushansky, 2005). At rest, the human body produces approximately 200 billion platelets per day (Harker & Finch, 1969). Platelets can synthesise a limited number of proteins, since they contain messenger RNA (mRNA) but not a nucleus and therefore do not contain DNA (Italiano & Shivdasani, 2003). Platelets do, however, contain a vast number of pre-formed megakaryocyte-derived molecules in their granules that can be released upon activation.

The recognition of the importance of platelets in ACS has led to some of the most successful pharmacological therapies in cardiovascular medicine. However, it is becoming increasingly clear that platelets also have a major role in inflammation (Semple et al., 2011). This is relevant to coronary artery disease, which is a chronic inflammatory disease, but may also be of consequence in infections, particularly sepsis, which is defined by systemic inflammation.

1.2 Platelets in Acute Coronary Syndromes

Coronary artery disease may not cause symptoms for decades and the formation of an intracoronary thrombus by platelets is the pivotal moment that initiates ACS, which can be fatal (Falk et al., 2013). Platelets are involved in advanced atherosclerosis and their role intensifies following atherosclerotic plaque rupture or erosion. This exposes platelets to multiple thrombogenic substrates, such as collagen, von Willebrand factor (VWF) and fibronectin, to which platelets adhere, mediated by glycoprotein (GP) Ib-V-X, GPIa/IIa and GPVI (Li et al., 2010). This initiates platelet activation and cross-linking of fibrinogen between platelet GPIIb/IIIa receptors, which causes platelet aggregation. Platelet activation is amplified by platelet release of dense granules and interactions with the coagulation cascade and leukocytes. Of these responses, platelet release of adenosine diphosphate (ADP) from dense granules and subsequent activation of platelet $P2Y_{12}$ receptors has a particularly important role in amplifying the response of platelets to other agonists (Storey et al., 2000).

Aspirin was the first medication to demonstrate the dramatic benefits of antiplatelet therapy in patients with ACS, which has led to the now routine strategy of dual antiplatelet therapy (Grove et al., 2015). However, patients with ACS continue to have increased risk of mortality, highlighting the need for further refinement of treatment strategies.

1.2.1 Platelets and the Pathogenesis of Acute Coronary Syndromes

Platelets appear to have a limited role in early atherogenesis (West et al., 2014), but play an increasingly important role during the lead up to atherosclerotic plaque rupture (Li et al., 2012) (Figure 1.1). The development of an advanced atherosclerotic plaque is characterised by chronic inflammation, driven primarily by macrophages and T-cells (Falk et al., 2013). There is increasing evidence that rupture and erosion of advanced plaques are relatively frequent and do not result in ACS unless other thrombogenic factors are present (Arbab-Zadeh et al., 2012). Multiple asymptomatic plaque ruptures and erosions appear to lead to an increase in plaque volume and the development of high-grade coronary artery stenosis (Arbab-Zadeh et al., 2012). Throughout these processes, platelets promote the underlying inflammatory processes

by direct interactions with the cells involved or by releasing pro-inflammatory cytokines.

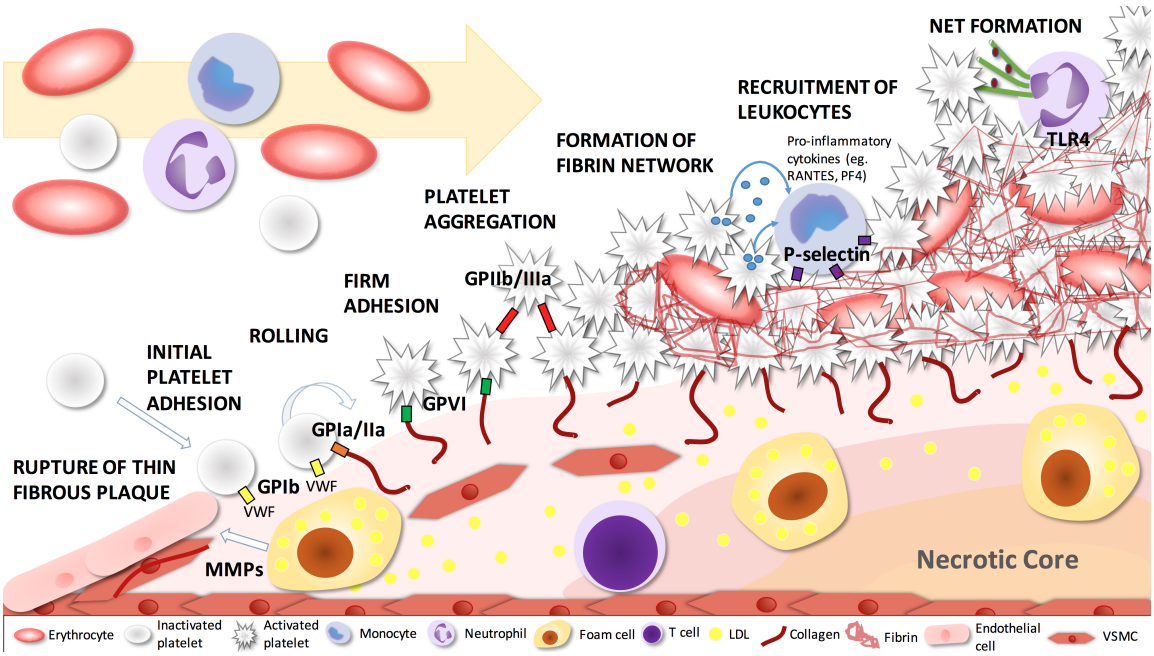

Figure 1.1 The role of platelets in forming a thrombus following atherosclerotic plaque rupture

1.2.1.1 Platelet-Endothelial Interactions Promote Monocyte Recruitment

Although platelets do not normally adhere to endothelium that is intact, they are capable of adhering to activated endothelium (Massberg et al., 2002). Many conditions related to atherosclerosis, such as hypercholesterolaemia, smoking and oxidative stress, induce endothelial dysfunction and activation, resulting in expression of adhesion molecules, such as vascular cell adhesion molecule (VCAM)-1, intercellular adhesion molecule (ICAM)-1 and E-selectin (Liao, 2013). Additionally, atherosclerosis increases endothelial VWF, further promoting platelet adhesion and activation (De Meyer et al., 1999). Adhesion of platelets to these adhesion molecules and the endothelium is mediated by a variety of corresponding platelet adhesion molecules and receptors, including P-selectin, GPIbα, GPVI and GPIIb/IIIa (Bültmann et al., 2010; Huo et al., 2003; Massberg et al., 2002). Depletion of these adhesion receptors reduces leukocyte accumulation within the arterial vessel wall and reduces plaque formation in animal models (Bültmann et al., 2010; Huo et al., 2003; Massberg et al., 2002), demonstrating an important role of platelet adhesion in the pathophysiology of advanced atherosclerotic plaques.

Adherent platelets form bridges between the endothelium and leukocytes, which facilitates the recruitment of monocytes to the atherosclerotic plaque where they take up cholesterol and become a type of macrophage known as a foam cell (Randolph, 2014). Activated platelets release cytokines, including platelet factor (PF) 4 and Regulated on Activation Normal T Expressed and Secreted (RANTES), which are released from α–granules and also act synergistically to promote recruitment of monocytes to the endothelium (Hundelshausen et al., 2005). Platelets are a major source of both PF4 and RANTES and mice that are deficient in either PF4 or RANTES demonstrate attenuated development of atherosclerosis and reduced macrophage infiltration into atherosclerotic plaques (Koenen et al., 2009). Activated platelets also promote leukocyte recruitment by upregulating endothelial expression of ICAM-1 and release of monocyte chemotactic protein (MCP)-1/CCL2 (Gawaz et al., 2000). In addition, platelets facilitate T cell adhesion to the endothelium and $CD4^+$ T cells actually appear to be incapable of sustaining adhesion with the endothelium under arterial flow conditions in the absence of platelets (Solpov et al., 2006). This enhancement of lymphocyte adhesion by platelets is mediated by P-

selectin, GPIIb/IIIa and CD40L (Spectre et al., 2012). Platelets interact directly with monocytes and macrophages that are present in the atherosclerotic plaque to alter their phenotype and function. Platelet-derived PF4 prevents monocyte apoptosis and induces a phenotype in macrophages that is functionally distinct to both M1 and M2 macrophages and displays altered matrix metalloproteinase (MMP) gene expression (Gawaz et al., 2000). Platelets are activated by OxLDL, mediated by CD36 and nicotinamide adenine dinucleotide phosphate oxidase (NOX)-2 (Magwenzi et al., 2015). This induces the formation of platelet-monocyte aggregates, which in turn induces phenotypic changes in monocytes, promoting monocyte extravasation and enhancing foam cell formation (Badrnya et al., 2014).

1.2.1.2 Platelets are the Central Mediators of the Progression from Ruptured Atherosclerotic Plaque to Subsequent Thrombus Formation

ACS is largely a stochastic event and it is therefore particularly difficult to make physiological assessments immediately prior to the onset of ACS to determine possible triggers. However, various different triggers have been described, such as physical activity, emotional stress and infections. It has been suggested that the underlying mechanisms of these triggers may include autonomic activation, vasoconstriction and systemic inflammation, which are all mediated by platelets and point to possible triggers of platelet activation (Boyle et al., 2003). Vulnerable plaques that are prone to rupture are associated with a thin fibrous cap, high leukocyte content and a large necrotic core (Silvestre-Roig et al., 2014). Apoptosis of vascular smooth muscle cells (VSMC) in the fibrous cap destabilizes the plaque and is promoted by macrophage release of tumour necrosis factor (TNF)-α (Boyle et al., 2003) and MMP-mediated degradation of collagen within the fibrous cap (Silvestre-Roig et al., 2014). In addition, CD40L, which is derived from T-cells and platelets, also promotes macrophage production of collagen-degrading MMPs (Libby, 2013a). Although platelets may play a role in these processes (Thomas & Storey, 2015b; Libby, 2013a; Seizer & May, 2013), direct evidence for a role of platelets in plaque rupture is limited. However, it is clear that platelets have a central role in mediating a pro-thrombotic state, which appears to be the key requirement for progression of plaque rupture or erosion towards thrombus formation and subsequent ACS.

1.2.2 Platelet Adhesion in Acute Coronary Syndromes

Following atherosclerotic plaque rupture or erosion, circulating blood is exposed to the highly prothrombotic necrotic core of the plaque and subendothelial components, such as VWF, collagen, fibronectin and laminin (Falk et al., 2013). Exposed macrophages express tissue factor, which is a potent stimulus for thrombin generation and activation of the extrinsic coagulation cascade. Platelet interactions with mononuclear phagocytes further upregulates tissue factor expression (Lindmark et al., 2000), thus increasing thrombin generation. Atherosclerotic plaques also narrow the arterial lumen, thereby inducing haemodynamic disturbances and increasing shear stress (Crea & Liuzzo, 2013). Under these conditions, platelet adhesion to exposed subendothelial components is critically dependent upon adhesion to VWF and collagen.

1.2.2.1 Initial Adhesion to the Vessel wall is Mediated by the GPIb-V-IX complex

The initial adhesion of platelets to exposed subendothelial components is primarily mediated by the platelet GPIb-V-IX complex, which binds to exposed and immobilized VWF (Delaney et al., 2012) (Figure 1.1). Downstream signaling, mediated by Src family kinases (Figure 1.2), results in activation of GPIIb/IIIa, which causes platelet aggregation (Delaney et al., 2012). VWF-mediated pulling of the GPIb unit induces the unfolding of a mechanosensitive domain, which induces platelet activation in response to high levels of sheer stress (Zhang et al., 2015). Platelet GPIb also adheres to VWF that has bound to immobilized collagen or fibrinogen, which allows platelets to bind to collagen and fibrinogen under high shear stress (Crúz et al., 2013). In addition, GPIb-V-IX signaling can be initiated by a direct action of thrombin on the GPIb-V-IX complex, which is synergistic with thrombin's activation of protease activated receptor (PAR)-1 (Estevez et al. 2015).

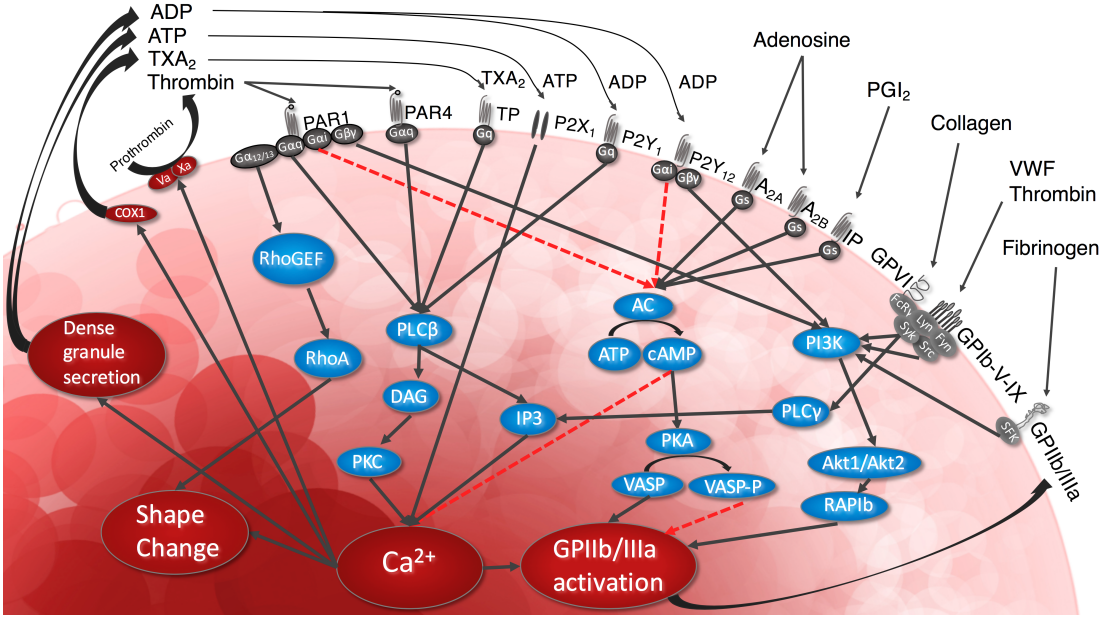

Figure 1.2 Intracellular signaling of platelets

1.2.2.2 GPIa/IIa Mediates Early Adhesion to Collagen

The integrin GPIa/IIa is present in a low affinity state on resting platelets. Following initial adhesion, interaction of platelet GPIb with VWF mediates activation of GPIa/IIa (Crúz et al., 2005), which facilitates platelet adhesion and initiates rolling. Even in its inactive form, GPIa/IIa contributes towards adhesion by binding to collagen, although inside-out signaling changes its conformational shape, thereby increasing its affinity for collagen (Lecut et al., 2004; Z. Wang et al., 2003). GPIa/IIa then acts synergistically with GPVI to reinforce collagen-mediated platelet activation (Kuijpers et al., 2003; Jarvis et al., 2002). GPIa/IIa deficiency in mice does not affect the bleeding time and GPIa/IIa deficient platelets can still aggregate and adhere, although this is delayed, demonstrating that GPIa/IIa is not essential in the adhesion process (Nieswandt et al., 2001). However, in the absence of both GPIa/IIa and GPVI, platelet adhesion to collagen under flow is completely abolished (Sarratt, 2005).

1.2.2.3 Firm Adhesion and Platelet Activation is Mediated by GPVI

In distinction to many platelet receptors that are G-protein-coupled receptors, GPVI belongs to the immunoglobulin superfamily (Clemetson et al., 1999) and its downstream signaling pathways involve Src family kinases (Figure 1.2). GPVI is thought to be exclusive to platelets and megakaroycytes and it has been estimated that there are approximately 9,600 copies per platelet (Burkhart et al., 2012). GPVI is the major receptor for collagen, but is also activated by other endogenous ligands, including laminin (Inoue et al., 2006) and adiponectin (Riba et al., 2008), and the exogenous ligand collagen-related peptide (Knight et al., 1999). GPVI also potentiates platelet activation in response to thrombin by mechanisms that are independent of Src kinases and Syk (Hughan et al., 2007). Platelets that lack either GPVI or a functional FcRγ-chain display severely impaired thrombus formation at high shear stress (Best et al., 2003), suggesting that it is likely to have an important role during the high-sheer conditions of ACS. GPVI is present as a monomer on the resting platelet membrane and becomes a dimer on platelet activation, thereby increasing its affinity for collagen (Loyau et al., 2012). There is an increase in levels of the GPVI dimer in response to ADP and VWF and the levels of GPVI dimers correlates with P-selectin expression (Loyau et al., 2012). It has recently been demonstrated that GPVI is also a receptor for fibrin, which explains why deficiency

of GPVI increases time to occlusion following arterial injury as well as inhibiting initiation of thrombus formation in animal models (Alshehri et al., 2015). Binding of GPVI to fibrin promotes thrombin generation and thus amplifies platelet activation (Mammadova-Bach et al., 2015), which demonstrates an important role of GPVI in thrombus growth and stability. Inhibition of GPVI also decreases platelet responses to ADP, thrombin and epinephrine by approximately 20% (Boylan et al., 2006), which suggests that GPVI is involved in amplifying platelet responses to these agonists.

GPVI appears to also have an important role in maintaining vascular integrity during inflammation, whereas G protein-coupled receptors, such as the ADP receptors and TPα appear to be dispensable in this process in animal models (Boulaftali et al., 2013). However, this role of GPVI appears complex and in some respects paradoxical. Platelet GPVI appears to enhance injury of the vessel wall caused by neutrophils, but also supports subsequent platelet adhesion, promoting repair of neutrophil-induced vascular breaches in animal models (Gros et al., 2015).

1.2.3 Platelet Activation and Aggregation in Acute Coronary Syndromes

Following initial adhesion to the endothelium, platelets become activated by a number of specific intracellular signaling pathways (Figure 1.2), resulting in activation of the GPIIb/IIIa receptor on the surface of platelets (Figure 1.3). Fibrinogen then cross-links activated GPIIb/IIIa receptors on adjacent platelets, which causes platelet aggregation. There are a number of mechanisms by which the platelet activation is amplified, involving the release of dense granules and interactions with leukocytes and the coagulation system.

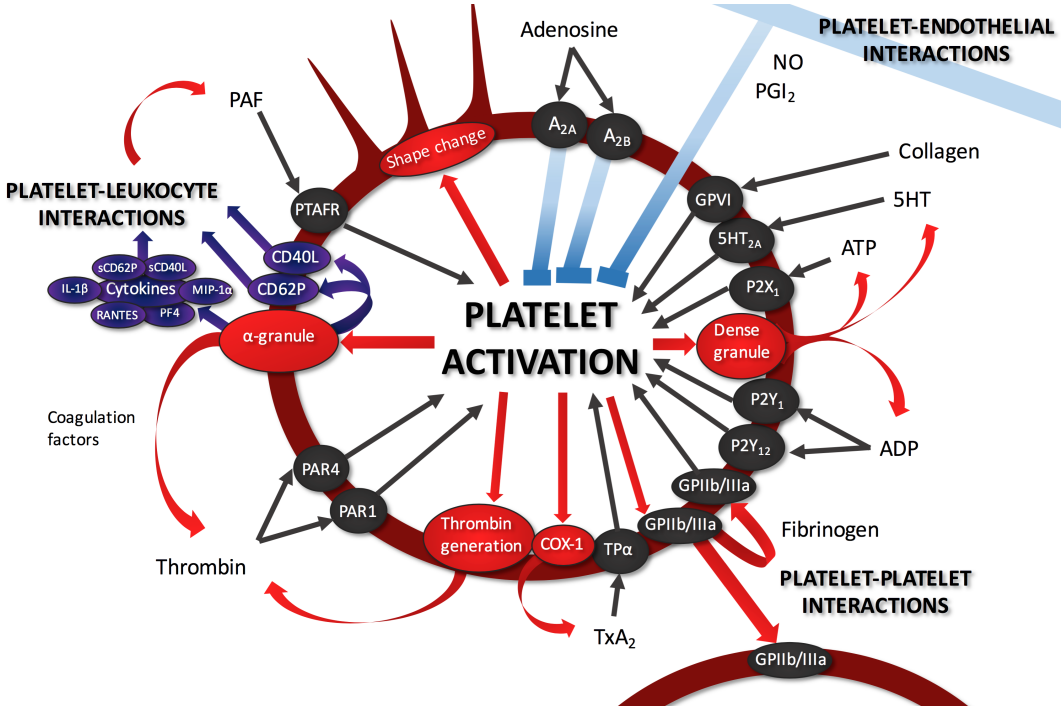

Figure 1.3 Platelets are activated by multiple agonists, which act on surface receptors, leading to platelet activation, secretion of granules and generation of thrombin and thromboxane A_2.

1.2.3.1 GPIb and GPVI Initiate Platelet Activation During Acute Coronary Syndromes

Following plaque rupture, platelet adhesion to collagen is a potent stimulus for platelet activation. Activation of GPVI by collagen initiates platelet activation and induces conformational changes in GPIa/IIa and GPIIb/IIIa that increases their affinity for adhering to collagen and other platelets respectively (Lecut et al., 2004). VWF and soluble agonists induce dimerization of GPVI and thereby increase its affinity for collagen, further potentiating platelet activation (Loyau et al., 2012). The GPVI receptor is upregulated in response to pathological sheer and by components of the coagulation cascade, particularly factor Xa (Naitoh et al., 2015; Al-Tamimi et al., 2012; 2011). The receptor is shed upon platelet activation, mediated by a disintegrin and metalloproteinase (ADAM) family of metalloproteinases, which also mediate the shedding of many other platelet membrane receptors (Bender et al., 2010). Surface expression of the collagen receptor GPVI is upregulated during ACS and correlates with expression of platelet P-selectin (Bigalke et al., 2006) suggesting that it may be a marker of platelet activation.

1.2.3.2 Platelet Activation Leads to GPIIb/IIIa Activation and Platelet Aggregation

GPIIb/IIIa is an adhesion receptor that belongs to the integrin superfamily and is a heterodimer composed of an α (α_{IIb}) and a β (β_3) transmembrane subunit (Coller & Shattil, 2008). In the resting state there are approximately 40,000 to 80,000 complexes present on the platelet membrane and GPIIb/IIIa is only able to weakly bind fibrinogen, which is sufficient to allow uptake of fibrinogen into α-granules (Coller & Shattil, 2008). The number of GPIIb/IIIa receptors on the surface membrane rapidly increases following platelet activation, which also induces conformational changes in the receptor allowing it to bind fibrinogen, VWF and fibronectin (Coller & Shattil, 2008). Fibrinogen molecules have two binding sites for GPIIb/IIIa and act as bivalent ligands, forming cross-links between activated GPIIb/IIIa receptors on different platelets, resulting in platelet aggregation (Coller & Shattil, 2008). This inside-out signaling has been described as the final common pathway of platelet activation as it is downstream of all other platelet activation signaling pathways and mediates platelet aggregation (Coller & Shattil, 2008). In addition, platelet GPIIb/IIIa receptors are activated by ligand binding, resulting in

outside-in signaling, which is mediated by Src family kinases (Figure 1.2) and causes platelet activation (Coller & Shattil, 2008).

Intravenous platelet GPIIb/IIIa inhibitors are potent inhibitors of platelet aggregation and have been used for the treatment of ACS (Grove et al., 2015). However, platelet inhibition during ACS has increasingly focused on the use of platelet $P2Y_{12}$ inhibitors (Roffi et al., 2015). As GPIIb/IIIa antagonists increase the risk of major bleeding, their use is now largely limited to high-risk situations during PCI such as primary PCI for STEMI or as a "bailout" therapy for procedure-related thrombus formation (Grove et al., 2015).

1.2.3.3 Platelet Release of ADP and Subsequent Activation of Platelet $P2Y_{12}$ Receptors is a Central Amplification Process

Platelet response to ADP is mediated by two ADP receptors, namely $P2Y_1$ and $P2Y_{12}$. $P2Y_1$ is coupled to G_q (Figure 1.2) and initiates calcium mobilization, platelet shape change and transient platelet aggregation in response to ADP (Gachet, 2012). Few attempts have been made to target $P2Y_1$ receptors pharmacologically since they are expressed in many tissues throughout the human body, including the heart, blood cells, neural tissues and other organs, making off-target effects very likely (Gachet, 2012). Platelet $P2Y_{12}$ receptors are coupled to G_i and inhibit adenylyl cyclase activity (Figure 1.2), thereby decreasing levels of cyclic adenosine monophosphate (cAMP) (Gachet, 2012). $P2Y_{12}$ also activates the phosphoinositide 3-kinase signaling pathway, which mediates robust platelet aggregation (Cosemans et al., 2006). Platelet activation in response to ADP triggers release of platelet dense granules, which also contain ADP. The released ADP has autocrine effects that amplify the response of the activated platelet and paracrine effects that stimulate other platelets (Gachet, 2012). Amplification of platelet activation by the $P2Y_{12}$ receptor has a central role in amplifying the response of platelets to other agonists (Storey et al., 2000). In addition, $P2Y_{12}$ receptors are expressed in few cell types other than platelets (Gachet, 2012), which makes them an attractive target for antiplatelet pharmacotherapy. Many successful platelet $P2Y_{12}$ inhibitors have been developed and now rank amongst some of the most commonly used medications worldwide (Storey, 2011). Platelet $P2Y_{12}$ inhibitors are capable of inhibiting the response of platelets to ADP and multiple other agonists, as they inhibit a central amplification pathway (Storey et al., 2000). The potent $P2Y_{12}$ inhibitors prasugrel and ticagrelor are currently

recommended as first-line treatments for patients with ACS, in addition to aspirin (Roffi et al., 2015).

1.2.3.4 The Platelet Agonist Thromboxane A_2 is Synthesized upon Platelet Activation

Arachidonic acid is formed by phospholipase A_2, which hydrolyses membrane phospholipids. Arachidonic acid is then rapidly converted by cyclooxygenase (COX-1) in platelets into prostaglandin G_2, which is then converted into prostaglandin H_2 (PGH_2) by peroxidase (Patrono et al., 2005). Thromboxane synthase converts PGH_2 into thromboxane A_2 (TxA_2), which can diffuse out through the platelet membrane to then act upon platelet TP receptors to amplify platelet activation (Patrono et al., 2005). Aspirin inhibits COX-1, thereby reducing the synthesis of TxA_2, which strongly inhibits arachidonic acid-induced platelet aggregation and moderately inhibits collagen-induced platelet aggregation (Storey et al., 2000). In citrate-anticoagulated platelet-rich plasma, aspirin also inhibits ADP- and adrenaline-induced platelet aggregation (Storey et al., 2000). However, these effects are not seen at physiological levels of calcium, which suggests that the arachidonic acid pathway has a limited role in platelet activation under physiological conditions (Heptinstall & Mulley, 1977).

The antiplatelet properties of low-dose aspirin are of great benefit in patients with ACS (Antithrombotic Trialists' Collaboration, 2002). Since the successful introduction of aspirin, it is now used routinely in combination with platelet $P2Y_{12}$ inhibitors (Storey, 2011). Further studies are therefore needed to determine optimal usage of aspirin when it is used on a background of potent $P2Y_{12}$ inhibition (Thomas & Storey, 2014b).

1.2.3.5 Amplification of Platelet Activation by Protease-activated Receptors

Platelets express two different receptors for thrombin, namely PAR1 and PAR4 (Leger et al., 2006). Thrombin is the most potent activator of platelets and is thought to exert the majority of its effects through PAR1 (Leger et al., 2006). PARs have an unusual mechanism of action, in that a cryptic ligand within the receptor is unmasked by proteolytic cleavage and the tethered ligand then interacts with the receptor moieties (Leger et al., 2006). PAR1 activates $G_{12/13}$, resulting in marked shape change

and formation of pseudopodia, as well as dense granule release (Figure 1.2). PAR1 is also linked to G_q, which increases intracellular calcium and activates GPIIb/IIIa (Leger et al., 2006). Whilst PAR1 has a high affinity for thrombin and is activated at subnanomolar concentrations of thrombin, PAR4 has a lower affinity and is cleaved more slowly (Leger et al., 2006). Despite this, PAR4 is able to mediate robust increases in intracellular calcium and platelet aggregation, mediated by G_q (Leger et al., 2006).

The PAR1 receptor antagonist vorapaxar initially showed great promise in early phase studies as it appeared to reduce platelet aggregation without adversely affecting haemostasis. However, in patients with ACS, vorapaxar increased the risk of bleeding and only showed a trend for cardiovascular benefits (Tricoci et al., 2012). Vorapaxar did significantly reduce the incidence of adverse cardiovascular events in patients with stable coronary artery disease with a prior history of MI and has recently been licensed for this indication (Morrow et al., 2012).

1.2.4 Platelet-Leukocyte Interactions

There are a number of mechanisms by which activated platelets interact with leukocytes and the endothelium. Activated platelets express the adhesion molecule P-selectin on their surface membrane, which interacts with its corresponding ligand, P-selectin glycoprotein ligand (PSGL)-1, on monocytes, neutrophils and eosinophils (Semple et al., 2011). This mediates the formation of platelet-leukocyte aggregates and circulating levels of platelet-monocyte aggregates have been shown to be increased in patients with ACS (Michelson et al., 2001). Increased platelet expression of P-selectin despite antiplatelet therapy has been shown to be associated with a higher incidence of adverse cardiovascular events in patients with ACS (Thomas et al., 2014). Inhibition of P-selectin reduces the formation of platelet-monocyte aggregates *in vivo* (Tardif et al., 2013). A novel specific antibody against P-selectin, inclacumab, lowers levels of soluble P-selectin and shows trends towards reduced myocardial damage in patients with ACS, presumably mediated by reducing platelet-leukocyte interactions (Tardif et al., 2013).

Platelets also express CD40L on their surface membrane when activated. CD40L has a similar structure to TNFα and has effects that are similar (Henn et al., 1998). Platelet CD40L acts on monocyte CD40 to increase monocyte expression of tissue

factor, which exerts prothrombotic effects by activating the extrinsic coagulation cascade (Lindmark et al., 2000). CD40L is also an important mediator of adaptive immune responses and the regulation of T-cells in particular (Elzey et al., 2008). Patients with ACS have an increased plasma level of soluble CD40L, which is thought to derive from platelets since they are the main source (Blanchet et al., 2014). Reflecting high levels of platelet activation, elevated levels of CD40L are associated with an increased risk of mortality in patients with ACS (Blanchet et al., 2014).

1.2.4.1 Platelets Interact with Monocytes, Promoting the Release of Pro-Inflammatory Cytokines

As well as containing many mediators of coagulation, platelet α-granules contain a large number of inflammatory mediators that serve no role in haemostasis. Upon activation, platelets release cytokines such as interleukin (IL)-1β, chemokine (C-X-C motif) ligand (CXCL) 1, PF4, CXCL5, CXCL7, IL-8, CXCL12, macrophage inflammatory protein (MIP)-1α and RANTES (Semple et al., 2011). PF4, RANTES and MIP1α are important promoters of monocyte chemotaxis and adhesion to the endothelium and have been directly implicated in atherogenesis (Maurer & Stebut, 2004). Levels of PF4, RANTES and MIP1α are increased during ACS (Blanchet et al., 2014) and high levels of RANTES are associated with more rapid subsequent progression of atherosclerotic plaques (Blanchet et al., 2014). Increased levels of RANTES and MIP1α are also both associated with a three-fold increase in risk of mortality (Blanchet et al., 2014; de Jager et al., 2012).

The formation of platelet-monocyte aggregates increases monocyte release of pro-inflammatory cytokines, such as TNFα, CCL2 and IL-8 (Bournazos et al., 2008; Neumann et al., 1997), which represents a mechanism by which platelets amplify systemic inflammation during ACS. Patients with ACS who have high levels of baseline inflammatory markers have an increased risk of subsequent adverse cardiovascular events and mortality (Blanchet et al., 2014). Platelet $P2Y_{12}$ inhibitors have been shown to reduce systemic inflammation and also lower levels of inflammatory markers during ACS, which may contribute to their clinical benefit (Gurbel et al., 2006).

1.2.4.2 Platelets Interact with Neutrophils, Resulting in the Formation of Neutrophil Extracellular Traps

Neutrophil counts are often raised in patients with ACS and there is evidence for a rapid burst of neutrophil activation and release of myeloperoxidase (MPO) in the first few hours of ACS, which is associated with platelet-neutrophil aggregate formation (Maugeri et al., 2012). Platelets interact with neutrophils to promote the formation of neutrophil extracellular traps (NETs), mediated by the platelet TLR4 receptor (Clark et al., 2007). NETs are normally extruded by neutrophils as a means of ensnaring bacteria and consist of cytoplasmic proteins and nuclear contents (Brinkmann & Zychlinsky, 2012). Whilst this may be beneficial to aid the clearance of bacteria, it is becoming increasingly clear that these NETs also play a significant role in pathological inflammation and thrombosis. In patients with STEMI, it has recently been demonstrated that a significant proportion of coronary thrombus is actually made up of NETs in close relation to platelets (Maugeri et al., 2014). Furthermore, it has also been shown that coronary NET burden is a predictor of subsequent infarct size (Mangold et al., 2015). Deoxyribonuclease hydrolyzes the DNA scaffold of NETs and has been shown to accelerate the lysis of coronary thrombi *ex vivo*, suggesting that NETs could be a pharmacological target for the treatment of ACS (Mangold et al., 2015).

1.2.4.3 Platelets Release Microparticles, which are Important Mediators of Inflammation and Coagulation and also Contain microRNA

Platelets release small membrane vesicles upon activation, known as microparticles (Biasucci et al., 2012). Platelets are a major source of microparticles, but other cell types, including endothelial cells, leukocytes and VSMCs all release different types of microparticles as well (Vajen et al., 2015). Microparticles mediate pro-inflammatory and pro-coagulant interactions between many different cell types and have effects that include increasing the thrombogenicity of platelets and promoting monocyte interactions with the endothelium. Levels of platelet-derived microparticles are increased during ACS (Biasucci et al., 2012), suggesting their potential use as a biomarker or as a pharmacological target in patients with ACS.

miRNA are small non-coding RNA, which function as translational repressors to modulate gene expression (Willeit et al., 2013). They have a diverse range of effects

across a range of cell types and have been shown to have important roles in modulating endothelial cell function and inflammatory responses. Interestingly, platelet microparticles appear to be a predominant source of miRNA in the plasma (Willeit et al., 2013). MiR-126 and miR-223 are particularly abundant within platelets and their release can be inhibited by platelet $P2Y_{12}$ inhibitors and aspirin (Willeit et al., 2013). In patients with ACS, levels of miR-126 and miR-223 both correlate with indices of platelet reactivity, which suggests that they may be useful as a novel marker of platelet activation in plasma (Mayr et al., 2014).

1.2.5 Platelet Mediated Formation of a Stable Thrombus

In their resting state, platelets have an asymmetrical phospholipid membrane that is maintained by translocase. Upon activation, increased levels of cytoplasmic calcium inhibit translocase and activate scramblase, which enhances the expression of negatively charged phosphatidylserine on the outer membrane. This allows factor Xa and Va to form a prothrombinase complex on the surface membrane, which converts prothrombin into thrombin (Solum, 1999). The produced thrombin activates platelet PARs and also leads to fibrin formation, which binds the developing clot together. In addition, platelets synthesise large quantities of plasminogen activator inhibitor (PAI)-1 and release this from α-granules upon activation (Brogren et al., 2004). The balance between clot formation and lysis is closely regulated by the fibrinolytic system and tissue plasminogen activator (tPA) in particular plays an important role on clot lysis. PAI-1 inhibits the fibrinolytic action of tPA and thereby increases resistance to clot lysis, particularly in arterial thrombosis (Brogren et al., 2004). High plasma concentrations of PAI-1 have been demonstrated in patients with ACS and increased levels are associated with an increase in mortality (Collet et al., 2003). Platelet-monocyte aggregate formation has also been linked with altered fibrinolytic status during ACS, which may be related to the intermediate monocyte phenotype in particular (Shantsila et al., 2012). In patients with ACS there is a dynamic cycle between prothrombotic and prothrombolytic processes, which occur simultaneously (Falk et al., 2013). This leads to intermittent flow obstruction and distal embolization, which may contribute towards myocardial microvascular obstruction and is more common following plaque erosion rather than rupture (Falk et al., 2013).

1.2.6 Platelet Contribution Towards Subsequent Adverse Cardiovascular Events

After atherosclerotic plaque rupture, the thrombus rapidly grows in size and may obstruct, or partially obstruct, the coronary artery, leading to myocardial ischaemia that may be fatal. Acute total occlusion of a coronary artery by thrombus usually results in STEMI and contemporary management of this revolves around emergency primary percutaneous coronary intervention (PCI) to re-open the affected artery. The aim is to achieve this as quickly as possible, to minimize the duration of myocardial ischaemia. However, even when successful primary PCI is prompt and potent antithrombotic agents are administered, myocardial ischaemia persists in a significant proportion of patients due to microvascular obstruction further downstream of the initial obstruction (Niccoli et al., 2015). Platelets contribute towards some of the main mechanisms of microvascular obstruction, which involve ischaemia, reperfusion injury and distal embolization (Niccoli et al., 2015). Platelet-neutrophil aggregates damage the endothelium of the microvasculature by releasing inflammatory mediators and vasoconstrictors (Niccoli et al., 2015). Additionally, platelet NOX-2 is upregulated during microvascular obstruction, which exacerbates the underlying pathophysiology by promoting phospholipase A_2 activity and generating TxA_2, a potent vasoconstrictor and platelet agonist (Niccoli et al., 2013). Patients with STEMI that is complicated by microvascular obstruction have higher levels of platelet-monocyte aggregates, platelet-neutrophil aggregates and activated GPIIb/IIIa than those without microvascular obstruction (Zalewski et al., 2012). Furthermore, levels of platelet-derived microparticles have been shown to be increased at the site of coronary plaque rupture and higher levels are associated with microvascular obstruction in patients with STEMI (Porto et al., 2012). In animal models, neutrophils release NETs during myocardial ischaemia-reperfusion, which contributes to the pathophysiology of the condition (Brinkmann & Zychlinsky, 2012). Interestingly, deoxyribonuclease is able to degrade the backbone of NETs and is effective at reducing the ischaemic region and infarct size in this situation (Ge et al., 2015).

Following atherosclerotic plaque rupture, there is evidence to suggest that platelets play an important role in progression of atherosclerotic plaques. By forming a thrombus over the site of atherosclerotic plaque rupture, platelets may contribute

towards atherosclerosis by releasing pro-inflammatory mediators and enhancing the recruitment of monocytes (Libby, 2000). In an animal model, $P2Y_{12}$-deficient mice displayed a significant reduction in thrombus and neointima formation following arterial injury (Evans et al., 2009). Further bone marrow transplantation experiments suggested that the reduction in neointima formation was primarily due to platelet $P2Y_{12}$ deficiency rather than VSMC $P2Y_{12}$ deficiency (Evans et al., 2009). This demonstrates a mechanism by which platelet $P2Y_{12}$ inhibitors may reduce restenosis following PCI in patients with ACS. In addition, platelet activation and secretion of dense granules releases ATP, which may act on VSMC P2X receptors, thereby promoting VSMC proliferation and migration (Erlinge, 1998).

Treatment with a $P2Y_{12}$ inhibitor, in addition to aspirin, is recommended for at least a year after ACS (Roffi et al., 2015). The more recent and potent $P2Y_{12}$ inhibitors ticagrelor and prasugrel are recommended as first-line treatments for patients with ACS (Roffi et al., 2015). It has become clear that approximately a third of patients do not display adequate inhibition of platelet reactivity whilst on treatment with the older $P2Y_{12}$ inhibitor clopidogrel (Aradi et al., 2015). The reasons for this poor response to clopidogrel are multifactorial and incompletely understood, but in part relate to genetic polymorphisms (Thomas & Storey, 2014a) and drug-drug interactions amongst other factors (Thomas & Storey, 2011). High platelet reactivity to ADP despite treatment with clopidogrel is associated with an increased risk of subsequent adverse cardiovascular events in ACS patients (Aradi et al., 2015). Studies have investigated whether this poor response to clopidogrel can be ameliorated by increasing the dose or changing to prasugrel, but unfortunately have not shown any significant benefit so far (Collet et al., 2015). However, even with the use of potent platelet $P2Y_{12}$ inhibitors, patients with ACS continue to have an increased risk of mortality, demonstrating the need for novel developments in antithrombotic strategies.

1.2.7 Summary

Platelets are central to all stages of the pathophysiology of ACS (Table 1.1). Platelets release many pro-inflammatory mediators, such as PF4, RANTES and sCD40L, which have been implicated in the progression of advanced atherosclerotic plaques. Following plaque rupture or erosion, platelets bind to exposed VWF and collagen,

mediated by GPIb, GPIa/IIa and GPVI. Initial platelet activation is then amplified by platelet release of soluble agonists, such as ADP, TxA_2 and thrombin, which stimulates further platelet activation in an autocrine and paracrine manner. Fibrinogen cross-links activated platelet GPIIb/IIIa receptors, causing platelet aggregation and the pro-coagulant activities of platelets drive the development of a stable fibrin-bound clot. Emerging evidence also suggests that platelet-leukocyte interactions have an important role in clot formation and microvascular obstruction. Aspirin and $P2Y_{12}$ inhibitors have made a tremendous impact on improving outcomes from ACS, which paves the way for novel antiplatelet strategies to further benefit patients with ACS by targeting currently unexploited mechanisms.

Table 1.1 Summary of the Major Roles of Platelets in the Pathophysiology of ACS

Process	Platelet involvement
Progression of advanced atherosclerotic plaque	Activated platelets promote leukocyte recruitment to the endothelium by upregulating expression of ICAM-1 and release of CCL2 Platelet release of PF4, RANTES and CD40L promotes macrophages to infiltrate atherosclerotic plaques, change phenotype and produce collagen-degrading MMPs (Libby, 2013b; Hundelshausen et al., 2005; Gawaz et al., 2000) OxLDL induces the formation of platelet-monocyte aggregates, which promotes monocyte extravasation and enhances foam cell formation (Badrnya et al., 2014)
Platelets adhere to exposed subendothelial components, initiating thrombus formation	Platelet GPIb initiates platelet adhesion and activation by binding exposed VWF (Delaney et al., 2012) Platelet GPIa/IIa mediates adhesion by binding exposed collagen (Kuijpers et al., 2003; Jarvis et al., 2002) Platelet GPVI acts synergistically with GPIa/IIa to facilitate adhesion and potently activates platelets in response to collagen (Kuijpers et al., 2003; Jarvis et al., 2002)
Activated platelets aggregate at the site of plaque rupture	Fibrinogen cross-links activated GPIIb/IIIa receptors on adjacent platelets, causing aggregation (Coller & Shattil, 2008)
Platelet activation is amplified in an autocrine and paracrine manner	Platelets release ADP from their dense granules, which activates platelet $P2Y_{12}$ ADP receptors, causing further activation (Storey et al., 2000) Platelets synthesise thromboxane A_2, which acts on platelet TP receptors (Patrono et al., 2005)
Stable thrombus occludes, or partially occludes, the coronary lumen	Platelets activation induces the formation of a prothrombinase complex on their surface membrane, which converts prothrombin into thrombin (Solum, 1999) Thrombin activates platelet PAR receptors and also promotes fibrin generation, which consolidates clot formation (Leger et al., 2006)
Inflammation induced by ACS is mediated by platelet-leukocyte interactions	Platelet-monocyte aggregate formation promotes monocyte release of pro-inflammatory cytokines (Thomas & Storey, 2015b) Platelet-neutrophil interactions induces NET formation and may play an important role in the pathophysiology of microvascular obstruction

1.3 The Role of Platelets in Inflammation

Platelets were traditionally considered to purely have a role in the maintenance of haemostasis. However, there is growing recognition that they also have a critical role in inflammation and immune responses. Indeed, some have even argued that this role may be as important as their role in haemostasis (Semple et al., 2011) (Figure 1.4 and Table 1.2). Interest in the role of platelets in inflammation and immune responses has recently returned to the forefront. Findings from the PLATelet inhibition and patient Outcomes (PLATO) study suggested that the novel antiplatelet medication ticagrelor might reduce the incidence of pulmonary infections and infection-related deaths compared to clopidogrel, the previous standard treatment for patients with acute coronary syndromes (ACS) (Varenhorst et al., 2014; Storey et al., 2014; Varenhorst et al., 2012). Epidemiological evidence also supports the hypothesis that antiplatelet medications affect host immunity, since a recent review of observational studies has suggested that antiplatelet medications are associated with a reduction in mortality from sepsis, without causing an excess of bleeding (Akinosoglou et al., 2014). There are many potential mechanisms for a clinical benefit of antiplatelet medications in systemic inflammation related to infection, which justifies detailed examination of the role of platelets in inflammation and immune responses. The purpose of this section is therefore to summarise the role of platelets in inflammation, with a focus on their role in innate immune responses.

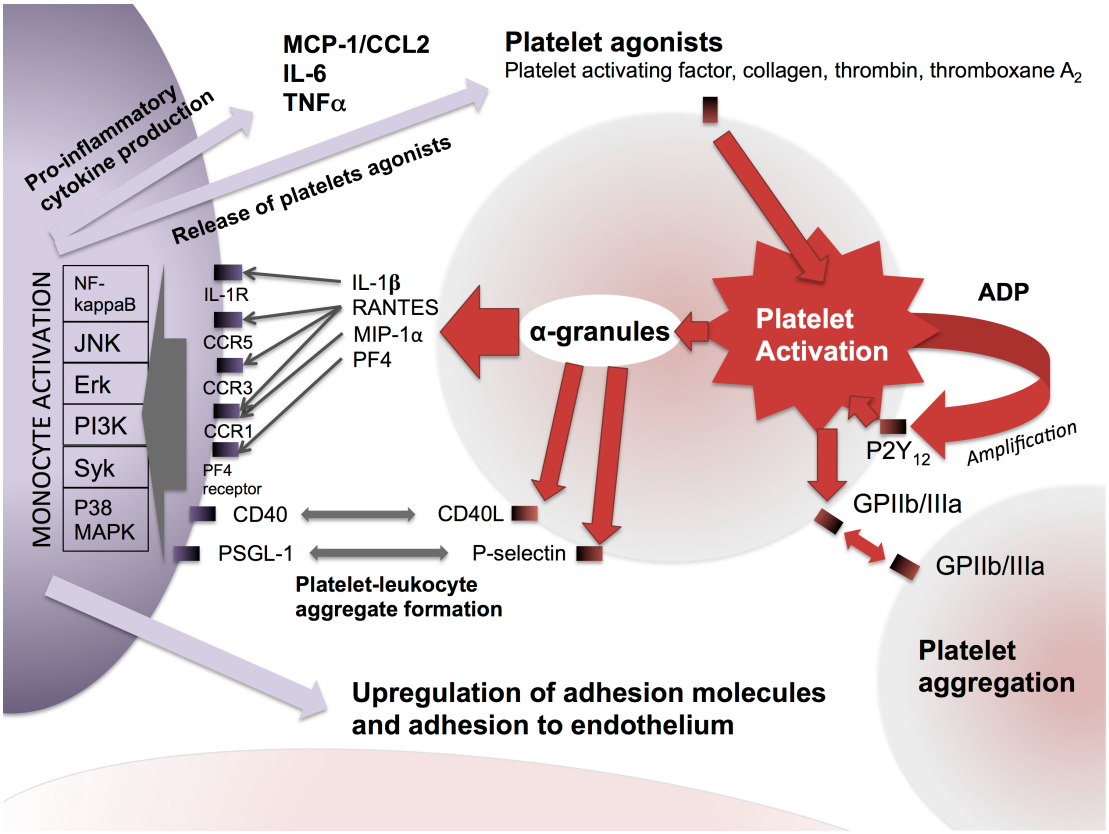

Figure 1.4 Platelet-monocyte interactions.

After platelet activation, platelets release α-granules containing soluble P-selectin, soluble CD40L, RANTES, MIP-1α and IL-1β, which act on monocyte receptors to upregulate proinflammatory functions. In addition, platelet expression of P-selectin mediates formation of platelet-monocyte aggregates, thereby upregulating monocyte release of pro-inflammatory cytokines and adhesion to the endothelium.

Table 1.2 Summary of the major platelet mechanisms that modulate inflammation and immunity

Mechanism	Main actions of mechanism
Platelet α-granule release	Platelet α-granules contain multiple mediators of inflammation (see Table 1), which have a diverse range of mostly pro-inflammatory effects.
Platelet P-selectin expression	Platelet P-selectin interacts with leukocyte PSGL-1, which is critical to the formation of platelet-leukocyte aggregates. Additionally, forms cross-links between leukocytes and the endothelium, thereby facilitating adhesion (Ma et al., 2004).
Platelet-leukocyte aggregate formation	Upregulates a wide range of pro-inflammatory functions of leukocytes, including release of pro-inflammatory cytokines, reactive oxygen species production, phagocytosis and endothelial adhesion (Semple et al., 2011).
Platelet expression of CD40L	Interacts with leukocyte CD40 and induces monocyte expression of tissue factor and activation of the coagulation system (Lindmark et al., 2000). Influences several important T cell functions, including antigen presenting cell activation.
Platelet TLR4-mediated NET formation	Emerging evidence suggests an important role of platelets in the formation of NETs (Clark et al., 2007), which aid the clearance of bacteria. However, NETs also have prothrombotic effects and contribute to the scaffold of a thrombus (Mangold et al., 2015; Maugeri et al., 2014).
Platelet P-selectin-mediated activation of the complement system	Platelets releases chondroitin sulfate, which activates the complement system (Hamad et al., 2008). This may have an important role in the clearance if microbes, but may also contribute towards vascular inflammation.
Platelet release of HMGB1	Platelets present HMGB1 to neutrophils, which induces the formation of NETs (Maugeri et al., 2014). Additionally, HMGB1 is a potent inflammatory stimulus that activates MAP kinases and NF-κB (Orlova et al., 2007).
Platelet activation in response to binding of GPIb and GPIIb/IIIa by bacteria	Platelets aggregate, in response to bacteria directly or indirectly binding GPIb and GPIIb/IIIa (Cox et al., 2011). This may contribute towards thrombocytopenia and may allow bacteria to become surrounded by platelets and inaccessible to leukocytes.
Platelet expression of TREM1 ligand	Mediates leukocyte activation and engagement of leukocyte TREM1 induces secretion of IL-8, TNFα and CCL2 (Bouchon et al., 2000)
Platelet release of microparticles	Presents IL-1β and RANTES (Mause et al., 2005) to the endothelium. Plays a key role in signaling between platelets and the innate immune system (Vajen et al., 2015; Italiano et al., 2010)

1.3.1 Platelet Granules

Supporting the important role of platelets in inflammation, activated platelets secrete a vast number of inflammatory mediators that have no identifiable role in haemostasis (Coppinger et al., 2007). Platelets possess three major types of storage granules: dense granules, lysosomes and α-granules, of which the α-granules are the most abundant. Dense granules contain small non-protein molecules that have important roles in the amplification of platelet responses, such as ADP, ATP and serotonin. Recently platelet serotonin has been shown to have an important role in neutrophil rolling and adhesion to the endothelium (Duerschmied et al., 2013). Platelet lysosomes contain proteases, glycosidases and other proteins that have a bactericidal effect (Rendu & Brohard-Bohn, 2009). Platelet α-granules contain a large number of varied proteins that are released during platelet activation and act on thrombosis and haemostasis, inflammation, host defenses and atherosclerosis amongst other effects (Blair & Flaumenhaft, 2009).

1.3.1.1 α-granules

There are approximately 50-80 α-granules per platelet, which have heterogeneous contents consisting of membrane-bound proteins that are either expressed on the platelet surface or released upon activation (Blair & Flaumenhaft, 2009). Of the membrane-bound proteins, most are already present on the resting membrane, whilst others, such as the adhesion molecule P-selectin, are only minimally expressed prior to platelet activation.

Many of the proteins contained within platelet α-granules have an important role in haemostasis. However, α-granules also have a significant role in innate immunity, mostly either by modulating the expression of platelet adhesion receptors that interact with leukocytes or by releasing cytokines that affect leukocyte function. Detail of the full contents of α-granules is incomplete, but they are known to contain a diverse range of chemokines, including CXCL1, platelet factor 4 (PF4; also known as CXCL4), CXCL5, CXCL7, interleukin (IL)-8 (also known as CXCL8), CXCL12, macrophage inflammatory protein (MIP)-1α (also known as CCL3) and RANTES (also known as CCL5) (Blair & Flaumenhaft, 2009). The predominant effect of these cytokines is to regulate leukocyte movement, migration from the vasculature into the tissues and other pro-inflammatory functions, such as phagocytosis and generation of

reactive oxygen species (Table 1.3). This affects the recruitment of leukocytes to sites of inflammation and mostly upregulates their pro-inflammatory functions. In addition, α-granules also contain small, cationic microbicidal proteins that can directly disrupt the membrane of *S. aureus* (Yeaman et al., 1998).

Table 1.3 Main platelet-derived mediators of inflammation

Mediator (Also known as)	Type	Main Source	Main interactions	Main role in inflammation
P-selectin (CD62P)	Adhesion molecule	α-granules Expressed on surface membrane or released in soluble form	PSGL-1 on: Monocytes Neutrophils	Formation of platelet-leukocyte aggregate (Evangelista, Manarini, Sideri, Rotondo, Martelli, Piccoli, Totani, Piccardoni, Vestweber, de Gaetano, & Cerletti, 1999a) Formation of bridges between leukocytes and endothelium (Mine et al., 2001)
CD40L (CD154)	Member of TNF family	α-granules Expressed on surface membrane or released in soluble form	CD40 on: T cells B cells Monocytes Dendritic cells	Important mediator of T cell immune responses (Elzey et al., 2008) Link between innate and adaptive immune responses (Elzey et al., 2008) Promotes leukocyte recruitment to the endothelium (Henn et al., 1998)
Platelet factor 4 (CXCL4)	CXC chemokine	α-granules	CXCR3 on: Monocytes Neutrophils	Induces leukocyte pro-inflammatory cytokine release, phagocytosis, chemotaxis, generation of ROS (Kasper et al., 2007; Hundelshausen et al., 2005; Scheuerer et al., 2000) Inhibits leukocyte apoptosis (Scheuerer et al., 2000)
MIP-1α (CCL3)	CC chemokine	α-granules	CCR1 on: Monocytes and macrophages	Promotes monocyte and macrophage migration (Maurer & Stebut, 2004) Upregulates monocyte and macrophage release of pro-inflammatory mediators (Maurer & Stebut, 2004)
RANTES (CCL5)	CC chemokine	α-granules	CCR1, CCR3 and CCR5 on: Monocytes and macrophages T cells	Promotes monocyte, macrophage and T cell chemotaxis and recruitment to the endothelium (Mause et al., 2005; la Motte et al., 2004)
IL-1	Cytokine	α-granules	Wide range of effects on leukocytes and endothelium	Central to pro-inflammatory cytokine cascade and vascular inflammation (Lindemann et al., 2001)
Microbicidal proteins	Cationic proteins	α-granules	Bacteria	Disrupt cell membrane (Yeaman et al., 1998)

1.3.1.2 Major platelet-derived cytokines

Many of the major platelet-derived cytokines affect monocytes in particular (Table 1.3 and Figure 1.4). PF4 is one of the most abundant proteins contained in platelet α-granules. PF4 is a CXC chemokine which shares sequence similarities with the chemokine IL-8, albeit with different functional effects (Nesmelova et al., 2005). As well as having a role in thrombosis and haemostasis, PF4 has a broad range of activities related to innate immunity, including effects on monocyte and neutrophil chemotaxis (Deuel et al., 1981). PF4 promotes neutrophil granule release and adhesion to endothelial cells, mediated by L-selectin and leukocyte function-associated molecule 1 (LFA-1) (Petersen et al., 1998). In addition, PF4 prevents monocyte apoptosis, promotes monocyte differentiation into macrophages and induces phagocytosis and generation of reactive oxygen species (Scheuerer et al., 2000). PF4 induces monocyte release of cytokines (including CXCL8, CXCL3, IL-1α, IL-1β, IL-6, IL-19, tumour necrosis factor (TNF) -α, CCL2, CCL3 and CCL22) (Kasper et al., 2007). In combination with regulated on activation, normal T cell expressed and secreted (RANTES), PF4 also promotes monocyte arrest on the endothelium (Hundelshausen et al., 2005) and may upregulate endothelial E-selectin expression (Yu et al., 2005). RANTES is a chemokine that has a role in atherosclerosis and is found in large quantities in platelet α-granules. Platelets either directly release RANTES or form microparticles containing RANTES, which can be immobilized on activated endothelium and promote monocyte recruitment, mediated by P-selectin (Mause et al., 2005). T cells that express CD40L can also induce platelet RANTES release, which promotes T cell recruitment to the endothelium in a process that is amplified by RANTES (la Motte et al., 2004).

Activated platelets release the pro-inflammatory cytokine IL-1β *in vitro* (Lindemann et al., 2001), although it has been suggested that this may be at least partly dependent on contaminating leukocytes (Pillitteri et al., 2007). IL-1 is central to the cytokine cascade and has a major role in vascular inflammation (Loppnow & Libby, 1990). Activated platelets induce IL-6 and IL-8 release from vascular smooth muscle cells, mediated by release of IL-1 (Loppnow et al., 1998). In addition, IL-1 is known to mediate vascular NO production (Ignarro et al., 2001) and promote neutrophil adhesion to endothelial cells (Bevilacqua et al., 1985). Activated platelets have also been shown to induce CCL2 secretion and intercellular adhesion molecule (ICAM-1;

also known as CD54) expression in endothelial cells in a process mediated by IL-1 (Gawaz et al., 2000). CCL2 is the major chemokine that regulates monocyte and macrophage chemotaxis and acts on monocyte CCR2 receptors (Deshmane et al., 2009).

1.3.1.3 Adhesion molecules

One of the key constituents of platelet α-granules is P-selectin (also known as CD62P), which has a key role in linking thrombosis and haemostasis and inflammation. P-selectin and P-selectin ligand-1 (PSGL-1) are a receptor and its respective ligand that have a central role in the interaction between platelets, leukocytes and endothelial cells (Blann, 2003). α-granules also contain other adhesion molecules, such as platelet endothelial cell adhesion molecule-1 (PECAM-1), GPIIb/IIIa and vWF (Rendu & Brohard-Bohn, 2009).

1.3.2 Direct Platelet-Leukocyte Interactions

1.3.2.1 P-selectin and PSGL-1

P-selectin is contained within the α-granules of platelets and is expressed on the surface membrane upon platelet activation (Stenberg et al., 1985). Monocytes, neutrophils, eosinophils and haematopoietic progenitor cells have all been shown to possess the corresponding ligand, PSGL-1 (Spertini et al., 1996; Symon et al., 1996). P-selectin cross-links platelets and leukocytes and is therefore a major mediator of platelet-leukocyte aggregate formation and its action is described in more detail in the following sections.

1.3.2.2 CD40 and CD40L

CD40 and CD40L (also known as CD154) are a receptor and its respective ligand that are important mediators of interactions between lymphocytes and antigen-presenting cells (Grewal & Flavell, 1998) and have also been shown to have a role in atherothrombotic disease (Anand et al., 2003). Activated platelets express CD40L, which has a similar structure to TNF-α and a similar effect. Platelet CD40L expression induces monocyte expression of tissue factor, which in turn initiates the extrinsic coagulation cascade (Lindmark et al., 2000). Platelet-expressed CD40L has also been shown to affect dendritic cells as well as B lymphocytes and T lymphocytes, suggesting that it provides a communicative link between innate and

adaptive immunity (Elzey et al., 2008). In addition to its direct effects on leukocytes, platelet-expressed CD40L also interacts with CD40 on endothelial cells to promote secretion of chemokines, such as IL-8 and CCL2, and express adhesion molecules, such as E-selectin (also known as CD62E), vascular cell adhesion molecule 1 (VCAM-1; also known as CD106) and ICAM-1 (Henn et al., 1998). This promotes migration of leukocytes to the site of vascular injury and subsequent adhesion. As well as expressing CD40L on their surface membrane, platelets also release soluble CD40L, which can induce vascular cells to express E-selectin and P-selectin and release IL-6 (Henn, 2001; Déchanet et al., 1997). Indeed, it has been suggested that activated platelets are the predominant source of soluble CD40L (Lim, 2004; Henn, 2001). A role of CD40 and CD40L in ACS has been suggested by reports of increased levels in patients with ACS (Garlichs et al., 2001; Aukrust et al., 1999).

1.3.2.3 TREM1

Platelets interact with triggering receptor expressed on myeloid cells 1 (TREM1) that is primarily expressed on monocytes and neutrophils, although the natural ligand of TREM1 is unknown (Klesney-Tait et al., 2006). Platelets have been shown to express a ligand for TREM1, which potentiates lipopolysaccharide (LPS) -induced neutrophil respiratory burst and IL-8 release but does not mediate platelet-leukocyte aggregate formation (Haselmayer et al., 2007).

1.3.3 Platelet-Leukocyte Aggregate Formation

1.3.3.1 Binding of Platelets to Leukocytes

The initial binding of platelets to leukocytes is mediated by platelet P-selectin binding to leukocyte PSGL-1. This is followed by firm adhesion, which is mediated either by leukocyte CD11b/CD18 binding to platelet GPIb or platelet-bound fibrinogen, or by platelet ICAM-2 binding to leukocyte CD11a/CD18 (Evangelista et al., 1999a). The initial capture and rolling of leukocytes on the endothelium is largely mediated by endothelial selectins. In addition to the direct effect of platelet P-selectin on promoting leukocyte adhesion to endothelium, endothelium-bound immobilised platelets can also bind leukocytes via P-selectin, acting as a bridge and further promoting leukocyte adhesion (Mine et al., 2001). Therefore, P-selectin/PSGL-1 interaction has a functionally important role in leukocyte rolling and adhesion to platelets and endothelium, which are critical steps in the process of leukocyte

extravasation (Moore et al., 1995; Yeo et al., 1994; Buttrum et al., 1993; Doré et al., 1993).

1.3.3.2 Effect of Platelet-Leukocyte Aggregate Formation on Leukocyte Function

The interaction between platelet P-selectin and PSGL-1 increases the adhesive properties of monocytes, by promoting expression of beta integrins and adhesion to fibronectin, VCAM-1 and ICAM-1, and promotes transendothelial migration (da Costa Martins et al., 2006). Similarly, P-selectin interaction with neutrophil PSGL-1 increases the adhesive properties of neutrophils by upregulating CD11b/CD18 and promoting adhesion to fibrinogen and ICAM-1 (Ma et al., 2004; Blanks et al., 1998). Neutrophils that have formed platelet-neutrophil aggregates shed L-selectin, show increased phagocytic activity and produce more reactive oxygen species (Peters et al., 1999). Interaction between P-selectin and PSGL-1 also regulates monocyte cytokine production. P-selectin potentiates monocyte secretion of CCL2 (also known as monocyte chemotactic protein-1) and TNFα in response to platelet activating factor (PAF), possibly by upregulating nuclear translocation of nuclear factor- κB (Weyrich et al., 1995). Monocytes and neutrophils roll over long, negatively-charged platelet flow-induced protrusions (FLIPRs) in a P-selectin/PSGL-1 dependent manner, which induces leukocyte CD11b and L-selectin shedding; the leukocytes retain fragments of the FLIPRs as microparticles on their surface (Tersteeg et al., 2014). Monocyte-platelet aggregate formation also appears to induce a change in monocyte phenotype to the $CD14^{++}$ $CD16^{+}$ intermediate monocyte phenotype (Passacquale et al., 2011).

1.3.3.3 In-vivo Sequelae of Platelet-Leukocyte Interactions

The innate immune system is essential for the resolution of microbial infection. However, in sepsis, excessive innate immune activation can cause excessive collateral tissue damage and, in particular, neutrophils have been implicated in the microvascular pathology that results in multi-organ failure (Brown et al., 2006). Platelet P-selectin has been shown to mediate leukocyte recruitment into post-ischaemic tissue, due to its effects on transendothelial migration (Salter et al., 2001), and blocking platelet P-selectin reduces post-ischaemic neutrophil infiltration of kidneys and subsequent acute kidney injury (Singbartl et al., 2001). Platelets appear to have a particularly prominent role in pulmonary neutrophil sequestration. In an animal model, blocking P-selectin reduces pulmonary neutrophil recruitment during

abdominal sepsis (Asaduzzaman et al., 2009). Acute lung injury also appears to be mediated by P-selectin in a murine model; inhibiting P-selectin expression and the subsequent formation of platelet-neutrophil aggregates improves gas exchange, decreases neutrophil recruitment and improves survival (Zarbock et al., 2006).

In ACS, plaque rupture induces the formation of platelet-monocyte aggregates and platelet-neutrophil aggregates by both P-selectin- and non-P-selectin-dependent mechanisms, although the physiological role of these aggregates is not clearly established (Sarma et al., 2002). In patients with ACS, the platelet $P2Y_{12}$ inhibitor clopidogrel reduces levels of CRP and TNFα (Gurbel et al., 2006), which may be related to a reduction in $P2Y_{12}$-mediated platelet-leukocyte interactions or may be related to reduced myocardial necrosis secondary to the antithrombotic effects of clopidogrel. Patients with ACS who have high platelet reactivity, as shown by increased expression of platelet P-selectin despite treatment with clopidogrel, have been shown to have a higher risk of adverse cardiovascular events (Thomas et al., 2014). At antiplatelet doses, aspirin also has anti-inflammatory effects that appear to be mostly related to its inhibition of platelet thromboxane A_2 synthesis, by inhibiting COX-1 (Hohlfeld & Schrör, 2015). Since thromboxane A_2 is a potent platelet agonist, this may indirectly reduce inflammation that results from thrombosis. Additionally, thromboxane A_2 and other thromboxane prostanoid receptor agonists mediate vascular inflammation (Capra et al., 2014), which may also be to some extent inhibited by aspirin. Even antiplatelet doses of aspirin may have additional anti-inflammatory effects mediated by COX-2, especially when used at higher doses, as reviewed by Hohlfeld and colleagues (Hohlfeld & Schrör, 2015). Stable coronary artery disease and atherosclerosis are also associated with low grade inflammation, which in turn is associated with an increase in platelet reactivity, as reviewed by Larsen and colleagues (Larsen et al., 2015). It has also been suggested that platelet-leukocyte interactions, mediated by platelet-expressed P-selectin and CD40L, may contribute to the pathogenesis of a number of other inflammatory conditions (Schrottmaier et al., 2015). In addition, cross-talk between monocytes, neutrophils and platelets activates the extrinsic coagulation cascade, which is a critical mediator of the initiation and propagation of deep vein thrombosis (Brühl et al., 2012).

1.3.4 Role of Platelets in Sterile Inflammation

Tissue damage and cell death are major initiators of sterile inflammation, which is central to a number of pathological processes, including ischaemia, atherosclerosis, gout and Alzheimer's disease (Rock et al., 2010). Cell death, particularly necrosis, initiates the release of damage-associated molecular patterns (DAMPS), which act on monocyte/macrophage intracellular and extracellular receptors, thereby triggering an inflammatory response. The most clearly characterized DAMPs include high-mobility Group Box-1 (HMGB1), IL-1α, S100 proteins, heat shock proteins (HSPs), dsDNA and uric acid (Zheng et al., 2011). HMGB1 is released extracellularly upon cell necrosis, but it has also been shown that platelets contain HMGB1 and express it on their outer membrane upon activation (Cognasse et al., 2015). HMGB1 is a highly potent inflammatory mediator that acts on the receptor for advanced glycation endproducts (RAGE), which activates a number of signaling pathways, including MAP kinases, and activates NF-κB (Orlova et al., 2007). It has recently been shown that activated platelets present HMGB1 to neutrophils, which induces the formation of neutrophil extracellular traps (NETs) (Maugeri et al., 2014), which are discussed in more detail in later paragraphs. Platelets also express receptors for other DAMPS, including the heat shock protein Gp96 (Hilf et al., 2002). These receptors appear to have an important role in mediating dendritic cell activation, although the mechanism is unclear and appears to be independent of soluble platelet factors and cell-to-cell contact (Hilf et al., 2002).

1.3.5 Response of Platelets to Pathogens

1.3.5.1 Bacterial Interaction with Platelets

The immediate activity of platelets at the site of a wound also means that they are ideally located to act as first-responders to invading microbes. Bacteria interact directly with platelets causing platelet activation, which allows platelets to release pro-inflammatory mediators and act as sentinel cells. However, this also directly contributes to the pathology of disseminated intravascular coagulation and infective endocarditis (Cox et al., 2011). Bacteria, such as *Streptococcus pyogenes*, bind fibrinogen that can then interact with platelet GPIIb/IIIa to trigger platelet activation (Shannon et al., 2007), whilst other bacteria such as *Streptococcus epidermidis* are capable of interacting directly with platelet GPIIb/IIIa causing platelet activation

(Brennan et al., 2009). Similarly, *Staphylococcus aureus* can bind VWF, which can then interact with platelet GPIbα (O'Seaghdha et al., 2006), whereas some species of streptococcus interact directly with platelet GPIbα to cause platelet activation (Plummer et al., 2005). The knowledge-base regarding platelet signalling after the adherence of bacteria is relatively incomplete (Cox et al., 2011). Platelet activation by *Streptococcus sanguinis* and *Streptococcus gordonii* results in platelet aggregation and release of α-granules (containing RANTES, PF4, sCD40L, soluble P-selectin and platelet-derived growth factor (PDGF)-AB) and dense granules (containing ADP and ATP) (McNicol et al., 2011; Herzberg & Krishnan, 1993). Thrombocytopenia is common in patients with sepsis and is associated with increased mortality (Baughman, 1993). Patients with sepsis have higher levels of platelet P-selectin expression and leukocyte CD11b and there is some evidence that levels of these markers correlate with severity of sepsis (Russwurm et al., 2002).

1.3.5.2 Platelet TLR

Toll-like receptors (TLR) are a major family of receptors that recognise pathogen-associated molecular patterns (PAMPs). It has been demonstrated that platelets possess TLR4 (Aslam et al., 2006), as well as TLR2, TLR3, TLR7, TLR8 and TLR9 (Koupenova et al., 2014). TLR4 mediates leukocyte response to the bacterial product lipopolysaccharide (LPS), which is a classical initiator of innate immune responses (Lu et al., 2008). However, LPS has not been consistently demonstrated to have a clear effect on traditional aspects of platelet function, such as platelet aggregation and P-selectin expression or cytosolic concentrations of calcium (Ward et al., 2005). Conversely, other studies have shown that activation of platelet TLR4 by LPS may increase platelet adhesion to fibrinogen (Andonegui, 2005) and the formation of neutrophil extracellular traps (NETs) that ensnare bacteria in sepsis (Clark et al., 2007). NETs, which are composed of cytoplasmic proteins and nuclear contents, including DNA, are expelled by activated neutrophils into the extracellular space, possibly as a last resort to control bacterial infections (Brinkmann & Zychlinsky, 2012). Important roles of NETs have been identified in a number of pathological processes, including autoimmune disorders, chronic lung diseases and vascular disorders (Kaplan & Radic, 2012). Recent studies have demonstrated an additional platelet-dependent component of NET formation that is mediated by platelet chemokines, including RANTES and PF4 (Rossaint et al., 2014; McDonald et al.,

2012; Caudrillier et al., 2012). There is also an increasing recognition of a major role of NETs in the pathophysiology of thrombosis, particularly DVT (Martinod & Wagner, 2014). However, very recent studies also demonstrate a major role of NETs in coronary thrombosis (Mangold et al., 2015; Maugeri et al., 2014). Coronary thrombus from patients with acute myocardial infarction (MI) contains numerous neutrophils and high levels of NETs, which contribute to the thrombus scaffold (Maugeri et al., 2014). Additionally, there are increased levels of NETs at the site of coronary plaque rupture during ST-segment elevation myocardial infarction and the NET burden predicts infarct size (Mangold et al., 2015).

1.3.6 Conclusion

Platelets have a major role in coordinating inflammation and immune responses. Platelet P-selectin expression and subsequent formation of platelet-leukocyte aggregates upregulates leukocyte pro-inflammatory functions. In addition, platelet α-granules contain a wide range of cytokines that have a predominantly pro-inflammatory effect (Table 1.3). It is known that platelet $P2Y_{12}$ inhibitors reduce platelet P-selectin expression, platelet-leukocyte aggregate formation and release of α-granule contents. However, the effect of this on host immunity is not yet established. The major role of platelets in inflammation and immune responses (Table 1.2) provides a clear rationale for further studies to determine whether modulation of platelet function can improve patient outcomes in inflammatory disorders, particularly inflammation related to infection.

1.4 Effect of $P2Y_{12}$ inhibitors on inflammation and immunity

1.4.1 Introduction

Platelet $P2Y_{12}$ inhibitors are now some of the most commonly used medications worldwide, due to their established benefit in the management of atherothrombosis (Grove et al., 2015). The PLATO study showed that ticagrelor reduces the incidence of adverse cardiovascular events in patients with ACS compared to clopidogrel, which was previously the standard treatment (9.8% vs. 11.7%; HR 0.84; $p<0.001$) (Wallentin et al., 2009). Ticagrelor provides faster, greater and more consistent platelet $P2Y_{12}$ inhibition than clopidogrel (Storey et al., 2010) and so the observed reduction in recurrent myocardial infarction was predictable. However, ticagrelor was also associated with an even larger reduction in all-cause mortality compared to clopidogrel (HR 0.78; 95% CI 0.69-0.89; $p<0.001$). This was unexpected and prompted speculation that some of the benefit of ticagrelor may be due to mechanisms unrelated to $P2Y_{12}$ receptor inhibition. Although ticagrelor is now recommended in ACS patients as first line antiplatelet therapy by the European Society of Cardiology (Roffi et al., 2015), it remains of great importance that its mechanisms of action are fully elucidated in order to guide the development of new antithrombotic strategies.

In PLATO, ticagrelor was associated with significantly fewer pulmonary infections and deaths related to infection than clopidogrel (Varenhorst et al., 2014; Storey et al., 2014; Varenhorst et al., 2009). In distinction to clopidogrel, ticagrelor additionally inhibits cellular uptake of adenosine by inhibition of equilibrative nucleoside transporter 1 (ENT1) (Armstrong et al., 2014). ENT1 is almost ubiquitously expressed in human cells, although its abundance varies between tissues (Baldwin et al., 2004). The main role of ENT1 is to transport nucleosides, such as adenosine, into and out of cells (Baldwin et al., 2004). A number of inhibitors of ENT1 have been identified and it has been shown that they increase extracellular levels of adenosine, which has a wide range of modulatory effects on inflammation as well as potentially cardioprotective properties (Baldwin et al., 2004; Cattaneo et al., 2014). There are therefore many mechanisms by which ticagrelor and clopidogrel could differ in their effects on inflammation and immune responses. This section of the introduction summarises the current evidence for the effects of $P2Y_{12}$ inhibitors on inflammation.

Of the currently used platelet $P2Y_{12}$ inhibitors, clopidogrel has been available for the longest. Therefore the majority of the currently available evidence regarding the effects of $P2Y_{12}$ inhibition on inflammation derives from studies of clopidogrel.

1.4.2 Effect of $P2Y_{12}$ inhibition on inflammation

$P2Y_{12}$ inhibitors are amongst some of the most commonly prescribed medications worldwide. The platelet $P2Y_{12}$ receptor has a central role in amplification of platelet activation in response to a number of different agonists (Storey et al., 2000). Platelet activation increases levels of cytosolic calcium and activates specific signaling pathways, which leads to the release of platelet α-granule contents. These granules contain many mediators of thrombosis, inflammation and host defenses (Rendu & Brohard-Bohn, 2009). By inhibiting platelet reactivity to ADP and a broad range of other agonists, $P2Y_{12}$ inhibitors reduce the release of pro-inflammatory mediators from platelet α-granules (Xiao & Theroux, 2004). The pro-inflammatory mediators contained within platelet α granules are not exclusive to platelets, but some, such as CD40L, are thought to mostly derive from platelets (Henn, 2001). Consequent platelet expression of platelet P-selectin also mediates the formation of platelet-leukocyte aggregates (Evangelista et al., 1999b). It is well established that $P2Y_{12}$ inhibitors inhibit these platelet-leukocyte interactions (Storey et al., 2000) but it is less well known how this affects more downstream inflammatory pathways. Inhibition of platelet $P2Y_{12}$-mediated platelet-leukocyte interactions is generally thought to be one of the main mechanisms by which $P2Y_{12}$ inhibitors affect inflammation. However, other cell types, including dendritic cells and vascular smooth muscle cells, have also been shown to express $P2Y_{12}$ (Gachet, 2012) and it is also therefore possible that $P2Y_{12}$ inhibitors may also affect inflammation by direct effects on these cell types, which will be covered in later pararaphs.

1.4.3 Clopidogrel

Clopidogrel is a second-generation thienopyridine, the active metabolite of which covalently binds to the platelet $P2Y_{12}$ receptor. Clopidogrel, in addition to aspirin, has been shown to reduce the incidence of adverse cardiovascular events compared to placebo in patients with non-ST elevation ACS (NSTE-ACS) (Yusuf et al., 2001), ST-elevation MI (STEMI) (Sabatine et al., 2005) and following PCI (Steinhubl et al., 2002). In patients with atherosclerotic disease there is considerable variation in

response to clopidogrel, in part due to polymorphisms of *CYP2C19*, which encodes for the main enzyme responsible for its metabolism (Thomas & Storey, 2014a), and in part due to drug-drug interactions (Thomas & Storey, 2011), age, weight and co-morbidities such as diabetes mellitus (Bonello et al., 2010). Interestingly, it has also been shown that cytochrome p450s are downregulated by inflammation, which may reduce the formation of the active metabolite of clopidogrel (Aitken & Morgan, 2007).

1.4.3.1 Effect of clopidogrel on inflammation related to atherothrombosis

Inflammation is, at all stages, an important component of the pathophysiology of coronary artery disease and atherothrombosis, including the initial development of a coronary plaque and during acute coronary syndromes (Libby, 2002; Mulvihill & Foley, 2002). Antiplatelet medications may influence inflammation relatively directly, by modulating leukocyte responses for example, but are also likely to indirectly influence inflammation by reducing myocardial necrosis, due to their antithrombotic effect.

1.4.3.2 Effect of clopidogrel on mediators that are contained within platelet α granules

In patients with ACS, it has been shown that soluble P-selectin and CD40L levels decrease after a loading dose of clopidogrel (Xiao & Theroux, 2004). Whilst a higher clopidogrel loading dose (600 or 900 mg) further decreased platelet aggregation responses, it did not appear to further lower levels of sCD40L, PAI-1 or vWF compared to a 300 mg loading dose in the ALBION study (Montalescot et al., 2006). Clopidogrel treatment prior to PCI decreases platelet P-selectin and CD40L expression after PCI (Vivekananthan et al., 2004). Heitzer et al also showed that clopidogrel, in addition to aspirin, reduced levels of sCD40L and RANTES in stable coronary artery disease patients (Heitzer, 2006). However, clopidogrel monotherapy did not appear to change levels of soluble P-selectin, sCD40L, transforming growth factor (TGF)-β or CCL2 compared to aspirin monotherapy in stable coronary artery disease patients in the ASCET study (Solheim et al., 2003).

1.4.3.3 Effect of clopidogrel on pro-inflammatory cytokines and CRP

Ruptured plaques contain activated leukocytes and, in particular, monocytes/macrophages have been implicated in mediating systemic inflammation related to ACS (Libby et al., 2009; Mulvihill & Foley, 2002). In addition, platelet-monocyte aggregates form after ACS (Michelson et al., 2001) and upregulate monocyte pro-inflammatory functions, such as the release of pro-inflammatory cytokines including TNFα, interleukin (IL)-1β, IL-8 and CCL2 (Bournazos et al., 2008; Weyrich et al., 1995). Pro-inflammatory cytokines such as these induce CRP production by the liver, leading to increased levels (Yeh, 2004). Clopidogrel has been shown to reduce markers of systemic inflammation, including TNFα and CRP, in patients with ACS (Table 1.4, Table 1.5 and Table 1.6) Levels of TNFα are significantly higher in patients with ACS than in healthy volunteers (Chen, Xu, et al., 2006). Clopidogrel, in addition to aspirin, significantly decreases levels of TNFα and CRP compared to aspirin alone in patients with ACS (Chen, Xu, et al., 2006). An increased maintenance dose of 150 mg of clopidogrel, compared to 75 mg, in addition to aspirin, led to approximately 50% lower levels of CRP at 1 week in STEMI patients, although this was not statistically significant ($p=0.06$) (Palmerini et al., 2010). Neither the CLEAR-PLATELETS (Gurbel et al., 2006) nor the ALBION study showed an effect of increasing the loading dose of clopidogrel on subsequent levels of CRP (Montalescot et al., 2006), perhaps suggesting that higher levels of $P2Y_{12}$ inhibition must be sustained to achieve a reduction in CRP. The CADET study showed that clopidogrel monotherapy does not appear to significantly differ in its effects on CRP compared to aspirin monotherapy in patients with ACS (Woodward et al., 2004).

It is apparent from these studies that changes in levels of CRP at the time of ACS are more subtle than might be anticipated. To show an effect of clopidogrel on these small changes, it appears it was necessary to use clopidogrel in conjunction with aspirin, whereas replacing aspirin with clopidogrel does not have a significant effect. Studies may also need a large sample size to demonstrate significant differences in these small changes in CRP. It is possible that more upstream mediators of inflammation, such as TNFα, may be more able to demonstrate an effect of $P2Y_{12}$ inhibition. Pretreatment with clopidogrel before PCI is associated with significantly lower subsequent levels of IL-1α, IL-2, IL-6, IL-13 and TNFα than no pretreatment

(Antonino et al., 2009). Whilst this study only showed a non-significant trend towards lower levels of CRP, another study has shown that pre-treatment with clopidogrel prior to PCI was associated with lower levels of CRP (Vivekananthan et al., 2004). In addition, in the CLEAR-PLATELETs study, an increased loading dose of clopidogrel significantly reduced subsequent levels of TNFα in PCI patients (Gurbel et al., 2006). Of course, it is unknown whether these changes reflect a direct effect of clopidogrel on inflammation or an indirect effect through reduction in periprocedural MI.

Compared to ACS and PCI, the inflammation related to chronic atherosclerosis involves distinct molecular and cellular pathways (Libby, 2012), with a less well recognised role of platelet $P2Y_{12}$. Despite this, in patients with stable coronary artery disease, and hence no significant myocardial necrosis, high levels of $P2Y_{12}$ reactivity during treatment with clopidogrel correlates with increased levels of CRP and WBC (Bernlochner et al., 2010). Heitzer et al. also showed that levels of CRP, sCD40L and RANTES reduced after the initiation of clopidogrel, in addition to aspirin, in patients with symptomatic stable coronary artery disease (Heitzer, 2006). The ASCET study did not show any difference between clopidogrel monotherapy and aspirin monotherapy on levels of CRP, TNFα, IL-6, IL-10 and CCL2 in patients with stable coronary artery disease (Solheim et al., 2006).

Table 1.4 Effect of clopidogrel on CRP

Study population	Treatment	Timing of measurements	Difference between treatment groups
Chen et al. (Chen, Xu, et al., 2006) 115 NSTE-ACS patients	Clopidogrel + aspirin vs. aspirin alone	Baseline, 1 week and 1 month	Significantly lower: 2.40 vs 3.49 at 30 days ($p<0.05$)
DOUBLE study (Palmerini et al., 2010) 54 STEMI patients	Clopidogrel maintenance dose: 150 mg vs. 75 mg	Baseline, 1 week and 1 month	Trend for lower levels: 1.7 vs 3.1 at 1 week ($p=0.06$)
ALBION study (Montalescot et al., 2006) 103 NSTE-ACS patients	Clopidogrel loading dose: 900 mg vs. 600 mg. vs 300 mg	Baseline, 6 hours and 24 hours post PCI	NS
CLEAR-PLATELETS study (Gurbel et al., 2006) 60 elective PCI patients	Clopidogrel loading dose: 600 mg vs. 300 mg	Baseline and 18-24 hours post PCI	NS
CADET study 184 ACS patients	Clopidogrel vs. aspirin	Baseline, 1 month, 3 months and 6 months	NS
Vivekananthan et al. (Vivekananthan et al., 2004) 833 PCI patients	Clopidogrel pretreatment vs. no clopidogrel pretreatment	Baseline, immediately after PCI and 18-24 hours after PCI	Significantly lower: change from baseline of 0.15 vs. 0.43 ($p=0.03$)
ASCET study (Solheim et al., 2006) 206 SCAD patients	Clopidogrel vs. aspirin	Baseline, 1 month and 1 year	NS
Heitzer et al. (Heitzer, 2006) 103 SCAD patients	Clopidogrel + aspirin vs. aspirin alone	Baseline and 5 weeks	CRP decreased in patients treated with clopidogrel but did not in those treated with aspirin alone ($p<0.01$)
Azar et al. (Azar et al., 2006) 73 SCAD patients	Clopidogrel + aspirin vs. aspirin alone	Baseline and 8 weeks	NS

Table 1.5 Effect of clopidogrel on TNFα

Study population	Treatment	Timing of measurements	Difference between treatment groups
Chen et al. (Chen, Xu, et al., 2006) 115 NSTE-ACS patients	Clopidogrel + aspirin vs. aspirin alone	Baseline, 1 week and 1 month	Significantly lower: 44 vs 63 at 30 days ($p<0.05$)
CLEAR-PLATELETS (Gurbel et al., 2006) 60 elective PCI patients	Clopidogrel loading dose: 600 mg vs. 300 mg	Baseline and 18-24 hours post PCI	Significantly lower: approx. 35% decrease from baseline vs. 30% increase from baseline ($p<0.001$)
ASCET study (Solheim et al., 2006) 206 SCAD patients	Clopidogrel vs. aspirin	Baseline, 1 month and 1 year	NS

Table 1.6 Effect of clopidogrel on IL-6

Study population	Treatment	Timing of measurements	Difference between treatment groups
Quinn et al. (Quinn et al., 2004) 74 PCI patients	Clopidogrel pretreatment vs. no clopidogrel pretreatment	Baseline, immediately after PCI and 18-24 hours after PCI	NS

1.4.4 Effect of clopidogrel in other types of inflammation

Bacterial lipopolysaccharide (LPS), also known as endotoxin, is a potent activator of the innate immune system. During LPS-induced inflammation in mice and rats, clopidogrel appears to have a number of beneficial effects, including a reduction in levels of IL-6 and TNFα and a reduction in liver and lung injury (Hagiwara et al., 2011; Winning et al., 2009). However, in a pig model, clopidogrel did not significantly affect LPS-induced increases in levels of IL-6 or TNFα (Lipcsey et al., 2005). A study of pigs whose coronary arteries were injured by angioplasty or irradiation showed that three months of clopidogrel treatment was associated with development of significantly fewer proliferative cells and inflammatory cells compared to 1 month of treatment (Pels et al., 2009).

Ticlopidine, the first thienopyridine $P2Y_{12}$ inhibitor to be used in clinical practice, was associated with neutropaenia, which limited its use. Clopidogrel, a second generation thienopyridine, can also rarely cause neutropaenia, which, according to a case report, resolves after switching to ticagrelor (Shah et al., 2014). This suggests that the neutropaenia may not be mediated by platelet $P2Y_{12}$ receptors. In the PLATO study, neutrophil counts appeared to increase slightly 1 month after discontinuing clopidogrel (Storey et al., 2014). This was not seen in ticagrelor-treated patients and suggests a subtle suppression of neutrophil counts by clopidogrel that is likely to be independent of its effects on platelet $P2Y_{12}$ receptors.

Interestingly, there are also at least 9 case reports of clopidogrel inducing a characteristic form of arthritis associated with a rash (Kanadiya et al., 2011). Similarly, a rat model has also previously shown that clopidogrel may potentiate peptidoglycan polysaccharide-induced arthritis (Garcia et al., 2011). If clopidogrel does rarely cause arthritis, it would appear that prasugrel may not have the same effect (Kanadiya et al., 2011). This suggests that this is not due to $P2Y_{12}$ inhibition or the active metabolite of clopidogrel, which is structurally very similar to the active metabolite of prasugrel.

1.4.4.1 Summary of the effect of clopidogrel on inflammation

In patients with ACS and stable coronary artery disease, clopidogrel reduces levels of CRP when it is used in addition to aspirin compared to aspirin alone (Heitzer, 2006; Chen, Xu, et al., 2006). Increasing the maintenance dose of clopidogrel in patients

with STEMI has also previously shown a trend towards lower levels of CRP (Palmerini et al., 2010). However, the balance of evidence does not suggest additional anti-inflammatory effects of clopidogrel monotherapy compared to aspirin monotherapy. There is also limited evidence that clopidogrel may have off-target effects on inflammation that are not mediated by $P2Y_{12}$.

1.4.5 Prasugrel

Prasugrel is a third-generation thienopyridine that inhibits platelet $P2Y_{12}$ receptors more potently than clopidogrel (Michelson et al., 2009). Prasugrel has been shown to reduce the incidence of adverse cardiovascular events compared to clopidogrel in patients with ACS who are planned to undergo PCI (Wiviott et al., 2007).

1.4.5.1 Effect of prasugrel on markers of platelet-leukocyte interactions

Prasugrel active metabolite potently inhibits ADP-induced platelet P-selectin expression and platelet-leukocyte aggregate formation *in vitro* (Totani et al., 2012; Judge et al., 2008; Frelinger et al., 2007) (Table 1.7). Inhibition of platelet-neutrophil aggregate formation by prasugrel active metabolite has been shown to decrease neutrophil activation, as demonstrated by lower expression of Mac-1 (Totani et al., 2012). Prasugrel has a greater inhibitory effect on platelet CD40L and P-selectin expression than clopidogrel in patients undergoing PCI (Serebruany, 2006). Prasugrel, in addition to aspirin, reduces ADP-induced platelet P-selectin and platelet-monocyte aggregate formation compared to clopidogrel in addition to aspirin in patients with stable coronary artery disease (Braun et al., 2008).

Table 1.7 Effect of prasugrel on inflammatory markers

Study population	Treatment	Timing of measurements	Difference between treatment groups
JUMBO study (Serebruany, 2006) 9 PCI patients	Prasugrel + aspirin vs. clopidogrel + aspirin	Baseline, 4 and 24 hours after PCI	Platelet expressed sCD40L and P-selectin significantly lower at 4 and 24 hours

1.4.6 Effect of prasugrel in other types of inflammation

In mice, prasugrel attenuates LPS-induced increases in TNFα and thromboxane B_2 (Totani et al., 2012). In a human model of LPS administration, prasugrel inhibited platelet reactivity according to a number of measures; however there was a suggestion that VWF release after endotoxin administration might antagonize the inhibitory effect of prasugrel (Spiel et al., 2012). There has also been a suggestion that prasugrel metabolites may directly affect neutrophil function, in a process that is not mediated by platelet $P2Y_{12}$ receptors (Liverani et al., 2013).

1.4.6.1 Summary of the effect of prasugrel on inflammation

Prasugrel inhibits platelet-leukocyte interactions more potently than clopidogrel, as would be expected from its greater inhibition of platelet $P2Y_{12}$ receptors. It remains to be established whether this has an additional anti-inflammatory effect during ACS or other models of inflammation.

1.4.7 Possible mechanisms for effects of $P2Y_{12}$ inhibitors on inflammation

1.4.7.1 Platelet $P2Y_{12}$-mediated mechanisms

As has been previously been discussed, the predominant mechanism of effect of $P2Y_{12}$ inhibitors on inflammation is generally presumed to be mediated by a reduction in platelet $P2Y_{12}$-mediated platelet-leukocyte interactions (Figure 1.5). Inhibition of platelet $P2Y_{12}$ reduces the release of pro-inflammatory cytokines from platelet α granules and reduces the formation of platelet-leukocyte aggregates (Storey et al., 2002; 2000). α granules contain the cytokines soluble P-selectin, soluble CD40L, PF4, RANTES, IL-1β and MIP-1α amongst others, which generally upregulate a wide range of pro-inflammatory functions of leukocytes, particularly monocytes and macrophages. P-selectin-mediated platelet-leukocyte aggregate formation also upregulates leukocyte release of pro-inflammatory cytokines IL-1β, IL-8, TNFα and CCL2 (Neumann et al., 1997; Weyrich et al., 1995) and expression of adhesion molecules (da Costa Martins et al., 2006).

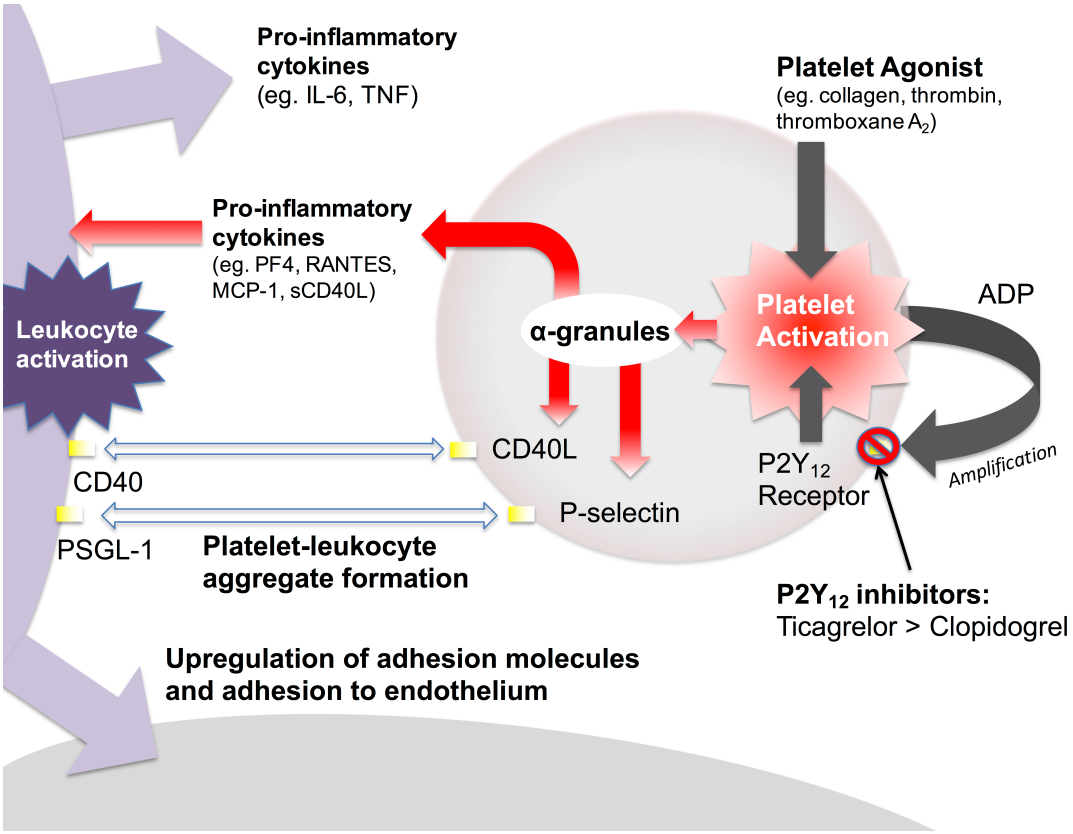

Figure 1.5 Effect of platelet $P2Y_{12}$ inhibitors on platelet-leukocyte interactions

1.4.7.2 Non-platelet $P2Y_{12}$-mediated mechanisms

When $P2Y_{12}$ was originally cloned in humans in 2001, it was thought to be predominantly expressed on platelets, with a lesser expression in certain brain cells (Hollopeter et al., 2001). It has since become apparent that $P2Y_{12}$ receptors are also expressed on murine dendritic cells, which are antigen-presenting cells that are related to monocytes (Ben Addi et al., 2010). The function of dendritic cell $P2Y_{12}$ is not well defined, but may regulate dendritic cell endocytosis and IL-12 production (Ben Addi et al., 2010; Schnurr et al., 2005; Marteau et al., 2004). It has also been shown that lymphocytes may contain mRNA for $P2Y_{12}$ although the functional consequence of this is unknown and could be related to possible platelet contamination (Wang et al., 2004). So, whilst these studies show that dendritic cells and lymphocytes may express $P2Y_{12}$, they do not appear to play as important a role as platelet $P2Y_{12}$.

Vascular smooth muscle cells (VSMC) also express $P2Y_{12}$ and ADP stimulation causes vasoconstriction in rat tails, which can be inhibited by ticagrelor, but not clopidogrel or prasugrel treatment (Grzesk et al., 2012). The mechanism for this has not been established, but ticagrelor has a longer half-life than the active metabolites of clopidogrel and prasugrel (Siller-Matula et al., 2010) and may therefore have a greater effect on nucleated cells that can potentially recover from $P2Y_{12}$ inhibition. The active metabolite of prasugrel is able to inhibit ADP-induced CCL2 expression by VSMCs in culture, which is also mediated by $P2Y_{12}$ (Satonaka et al., 2015). In addition, vascular $P2Y_{12}$, as opposed to platelet $P2Y_{12}$, has a role in atherogenesis and transplant arteriosclerosis in mouse models (West et al., 2014a; Harada et al., 2011). However, despite this clear role of vessel wall $P2Y_{12}$ in atherogenesis, administration of $P2Y_{12}$ inhibitors failed to inhibit atheroma formation.

There is also accumulating evidence that $P2Y_{12}$ receptors play a regulatory role in pulmonary inflammation induced by leukotriene (LT) E_4, which is a potent pro-inflammatory lipid mediator involved in asthma (Cameron, 2012). In particular, clopidogrel and platelet depletion reduce LTE_4 mediated expression of mRNA encoding IL-13 and MUC5AC and accumulation of airway eosinophils (Paruchuri et al., 2009) in mice. In humans, however, prasugrel was associated with a slight reduction in airway reactivity in patients with asthma that was not statistically significant (Lussana et al., 2015).

1.4.8 Effect of Combined $P2Y_{12}$ and ENT1 inhibition on Inflammation

1.4.8.1 Clinical effects of ticagrelor

Ticagrelor is a novel class of antiplatelet medication that potently inhibits platelet $P2Y_{12}$ receptors and also inhibits cellular uptake of adenosine by inhibiting ENT1 (Armstrong et al., 2014). In the PLATO study, ticagrelor reduced the incidence of adverse cardiovascular events in patients with ACS compared to clopidogrel (Wallentin et al., 2009). However, ticagrelor also unexpectedly reduced all-cause mortality to a greater degree than would be expected from previous trials of $P2Y_{12}$ inhibitors. This has raised the question as to whether or not ticagrelor has additional beneficial pleiotropic effects, such as modulation of host immunity (Storey et al., 2014).

In PLATO, ticagrelor was unexpectedly associated with fewer pulmonary infections and deaths related to infection than clopidogrel (Varenhorst et al., 2014; Storey et al., 2014; Varenhorst et al., 2012). Also, surprisingly, ticagrelor was associated with slightly higher levels of IL-6 and CRP at discharge than clopidogrel in PLATO, converse to what would be expected from a more potent $P2Y_{12}$ inhibitor (Storey et al., 2014) (Table 1.8). This demonstrates a differential effect of the medications on inflammatory responses, although the mechanisms have not yet been fully explained. It is likely that the smaller DISPERSE 2 study was underpowered to demonstrate these subtle effects and the differences in levels of CRP, IL-6, MPO and CD40L in ACS patients treated with ticagrelor or clopidogrel were not significant in this study (Husted et al., 2010).

Table 1.8 Effect of ticagrelor on inflammatory markers

Study population	Treatment group comparison	Timing of measurements	Difference between treatment groups
PLATO study (Storey et al., 2014) 18,421 ACS patients	Ticagrelor + aspirin vs. clopidogrel + aspirin	Baseline, discharge, 1 month and 6 months	CRP significantly higher at discharge: mean 28 vs 26 ($p<0.001$) IL-6 significantly higher at discharge: mean 5.4 vs 4.9 ($p<0.001$)
DISPERSE 2 study (Husted et al., 2010) 990 NSTE-ACS patients	Ticagrelor + aspirin vs. clopidogrel + aspirin	Baseline, discharge and 1 month	No significant difference in CRP, IL-6, MPO or sCD40L Median CRP at discharge: 14 vs. 11 Median IL-6 at discharge: 5.8 vs. 5.0

1.4.8.2 Potential adenosine-mediated effects of ticagrelor on inflammation

The adenosine-mediated effects of ticagrelor are still relatively unexplored, since this mechanism has only been identified relatively recently (Armstrong et al., 2014; van Giezen et al., 2012). Ticagrelor has now also been shown to increase extracellular levels of adenosine in patients with ACS (Bonello et al., 2014). This appears to have clinically relevant effects, since ticagrelor potentiates adenosine-induced coronary vasodilation and dyspnoea (Alexopoulos et al., 2013; Wittfeldt et al., 2013).

Adenosine is a major modulator of inflammation and innate immune responses that acts on 4 different receptors (A_1, A_{2A}, A_{2B} and A3), which are differentially expressed in different cell types (Haskó & Cronstein, 2013). Adenosine is a degradation product of ATP, ADP and AMP that is leaked into the extracellular space in pathological conditions that exert cellular stress, such as ischaemia and infection (Eltzschig et al., 2012). Possible adenosine-mediated effects of ticagrelor are complex and may be technically difficult to characterise. At low concentrations, adenosine predominantly acts on high affinity leukocyte A_1 receptors (Figure 1.6) (Barletta et al., 2012; Haskó & Pacher, 2012). This has mostly pro-inflammatory effects, including potentiation of neutrophil chemotaxis and phagocytosis and macrophage phagocytosis (Barletta et al., 2012; Haskó & Pacher, 2012). At higher concentrations, adenosine predominantly acts on lower affinity leukocyte A_{2A} and A_{2B} receptors (Figure 1.6). This has mostly anti-inflammatory effects, such as downregulation of the release of pro-inflammatory cytokines, including IL-6 and TNFα (Barletta et al., 2012; Haskó & Pacher, 2012). In keeping with this, dipyridamole, another inhibitor of cellular uptake of adenosine, inhibited the release of IL-6 and TNFα in a model of human sepsis (Ramakers et al., 2011). This may limit excessive innate immune activation during conditions of severe cellular stress, such as sepsis, which may prevent collateral damage from innate immune responses. In summary (Figure 1.6), possible adenosine-mediated effects of ticagrelor on inflammation may be pro-inflammatory or anti-inflammatory, depending on the context.

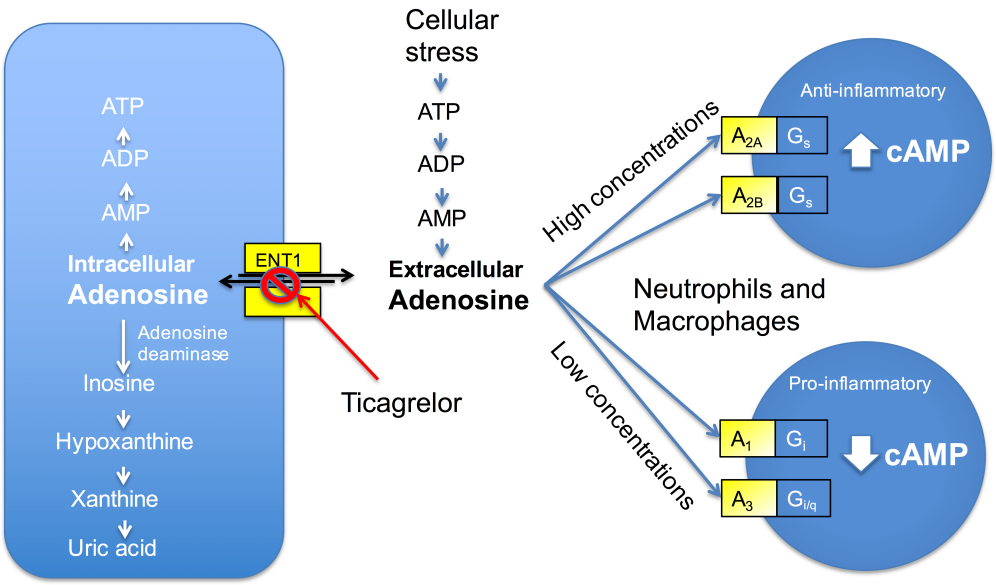

Figure 1.6 Adenosine-mediated effects of ticagrelor on leukocytes

1.4.8.3 Summary of the effect of ticagrelor on inflammation

The possible effects of ticagrelor on inflammation are complex, due to its dual inhibition of platelet $P2Y_{12}$ receptors and ENT1. PLATO showed that ticagrelor was associated with slightly higher levels of IL-6 and CRP than clopidogrel at discharge and the mechanism underpinning this remains to be established. PLATO suggests that the relative difference between ticagrelor and clopidogrel on inflammation might be associated with a beneficial effect on host immunity however.

2 Hypotheses and Objectives

2.1 Summary of evidence and unanswered questions

It has been established that platelet $P2Y_{12}$ inhibitors reduce platelet-leukocyte interactions *in vitro*. However, innate immune activation in humans is complex and dynamic and it has not previously been demonstrated whether platelet $P2Y_{12}$ inhibitors have an important direct effect on systemic inflammation *in vivo*. Whilst studies of patients with ACS have suggested anti-inflammatory effects of $P2Y_{12}$ inhibitors, these studies are confounded by the antithrombotic effects of the medications. Although it has now been established that ticagrelor is a weak inhibitor of adenosine uptake, the effects of this on inflammation have not yet been characterized.

2.2 Hypotheses

Ticagrelor and clopidogrel differ in their effect on innate immune activation in humans, due to $P2Y_{12}$ and non-$P2Y_{12}$ dependent mechanisms.

2.3 Objectives

1.) Determine the *in-vivo* effect of ticagrelor and clopidogrel on systemic inflammation in an experimental human model.

2.) Determine the effect of ticagrelor and clopidogrel on the prothrombotic effects of systemic inflammation in an experimental human model.

3.) Determine the effect of ticagrelor and clopidogrel on activation and mobilization of classical, intermediate and non-classical monocytes during systemic inflammation in humans.

4.) Determine the modulatory adenosine-mediated effects of ticagrelor on leukocyte activation *in-vitro*.

3 Methods

3.1 Study of the effect of ticagrelor and clopidogrel on the immune response of healthy volunteers

The results of this study are presented in Chapter 4 *Platelet P2Y12 inhibitors reduce systemic inflammation and its prothrombotic effects in an experimental human model* and Chapter 5 *Platelet P2Y12 Inhibitors Potentiate the Expansion in Intermediate Monocyte Population that Occurs after Endotoxaemia.*

3.1.1 Study design

This was a randomized, single-centre, open-label, parallel-group study assessing the modulatory effect of ticagrelor and clopidogrel on the immune response of healthy volunteers. In total, there were 3 groups: ticagrelor (n=10), clopidogrel (n=10) and no antiplatelet medication (control; n=10).

3.1.2 Plan of investigation

Healthy volunteers received a loading dose of antiplatelet medication followed by maintenance therapy for a total of 7 days of treatment. The study used the well-established method of intravenous injection of *E.coli* lipopolysaccharide (LPS) into healthy volunteers to induce systemic inflammatory response syndrome (SIRS) (Suffredini et al., 1999). The study was approved by the Sheffield Research Ethics Committee (UK) and the Medicines and Healthcare products Regulatory Agency (UK) and was conducted in accordance with Good Clinical Practice guidelines. Subjects provided written informed consent. The study was registered at https://clinicaltrials.gov (unique identifier NCT01846559).

3.1.3 Study visit schedule

Visit 1: Screening (Day -7 to -21)

Visit 2: Initiation of randomized treatment (ticagrelor, clopidogrel or control) – Day 1

Visit 3: Intravenous endotoxin challenge – Day 7

Visit 4: Follow up and repeat blood test at 24 hours – Day 8

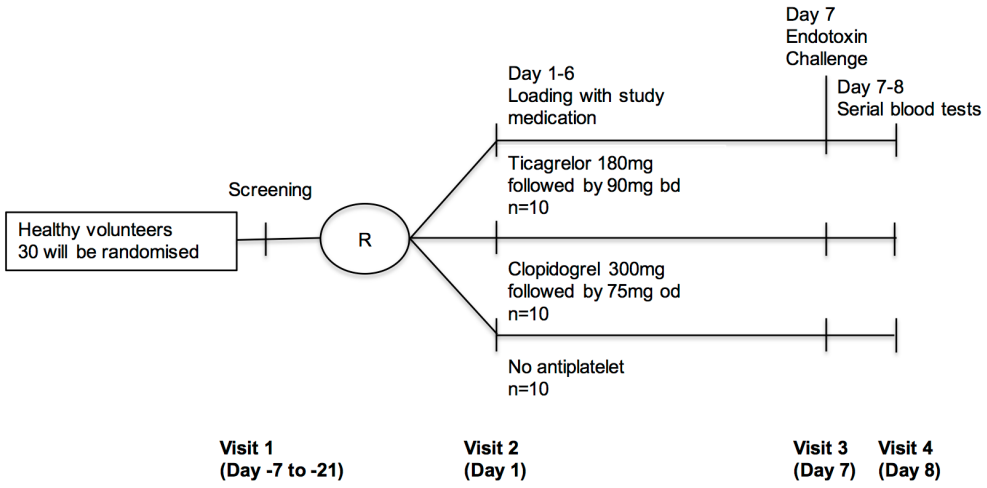

Figure 3.1 Study flow chart

3.1.4 Study population

Subjects were recruited from the area local to the Northern General Hospital, Sheffield. This included members of staff and students from universities in Sheffield and from Sheffield Teaching Hospitals. Volunteers were given at least 24 hours to consider taking part in the study prior to signing informed consent. Subjects who withdrew from the study prior to endotoxin were replaced.

3.1.4.1 Inclusion criteria

- Healthy male subjects, or female subjects not of childbearing potential (either surgically sterile or post menopausal)
- Age between 18 and 65 years inclusive
- Non smokers
- Body mass index (BMI) between 18 and 28 kg/m^2 inclusive, with a body weight between 60-100 kg
- Subjects are to be in good health as determined by a medical history, physical examination, vital signs and clinical laboratory test results including renal and liver function and full blood count
- Subjects have given their informed consent before any trial-related activity

3.1.4.2 Exclusion criteria

- In the opinion of the investigator, subjects with, or a history of, cancer, diabetes or clinically significant cardiovascular, respiratory, metabolic, renal, hepatic, gastrointestinal, haematological, dermatological, neurological, psychiatric, or other major disorders
- Subjects with a history of significant multiple drug allergies or with a known allergy to the study drugs or a medicine chemically related to the trial product
- Subjects who have had a clinically significant illness within 4 weeks of dosing
- Subjects taking regular medicines including NSAIDs, antibiotics, aspirin or anticoagulant therapy
- Any clinically significant abnormal laboratory test results at screening
- Subjects who have a supine blood pressure at screening, after resting for 5 minutes, higher than 150/90 mmHg or lower than 105/65 mmHg
- Subjects who have a supine heart rate at screening, after resting for 5 minutes, outside the range of 50-100 beats/min
- Subjects who have received any prescribed systemic or topical medication within two weeks prior to the start of dosing. Limited use of paracetamol or

non-steroidal anti-inflammatory drugs (NSAIDs) prior to the initiation of the study will not necessarily require exclusion unless there is an ongoing requirement for these medications.

- Subjects who have received an investigational medicinal product within the previous four months (new chemical entity) or three months (licensed product) or subjects who have received a vaccine within three months preceding the start of dosing
- Subjects who have donated any blood or plasma in the month preceding the start of dosing
- Subjects who have a history of alcohol or drug abuse
- Subjects with mental incapacity or language barriers which preclude adequate understanding
- Subjects with a contraindication to ticagrelor (as listed in the SmPC – hypersensitivity to the active substance or any of its excipients, active pathological bleeding, history of intracranial hemorrhage, moderate to severe hepatic impairment and co-administration with strong CYP3A4 inhibitors)
- Subjects with a contraindication to clopidogrel (as listed in the SmPC – hypersensitivity to the active substance of any of its excipients, severe hepatic impairment, active pathological bleeding such as peptic ulcer or intracranial haemorrhage)

3.1.5 Dietary and fluid restrictions

Whilst in the study, subjects were allowed to drink freely and received a light breakfast and light lunch on the day of the endotoxin challenge. Subjects were required to avoid consumption of caffeine for the duration of the study as it is an adenosine receptor blocker. Volunteers received a total of 750 ml of 0.9% saline during the endotoxin challenge.

3.1.6 Experimental protocol

Volunteers were randomized to receive one week of ticagrelor 90 mg twice daily (n=10), clopidogrel 75 mg once daily (n=10) or no antiplatelet medication (controls; n=10). Ticagrelor and clopidogrel-treated subjects received loading doses of 180 mg and 300 mg respectively. All studies were started between 08:00 and 09:00 AM in a clinical research facility with full resuscitation facilities. One venous cannula was inserted into an antecubital vein in each arm. One cannula was used for blood sampling and the other for administration of LPS and intravenous fluid (250 ml 0.9% saline over 30 minutes prior to LPS administration, then 500 ml 0.9% saline over 4

hours after LPS administration). 2 ng/kg *E. coli* O:113 LPS (Clinical Center Reference Endotoxin, National Institutes of Health, Bethesda, MD) was administered over 1 minute at $t = 0$ hours. Venous blood samples were collected at baseline (prior to any randomized medication), prior to LPS administration and at the following time points after LPS administration: 5, 15 and 30 minutes and 1, 1.5, 2, 4, 6 and 24 hours. All laboratory measurements were performed by staff blinded to treatment allocation.

3.1.7 Plasma levels of cytokines

Blood samples for isolation of plasma were collected into tubes containing trisodium citrate dihydrate (3.13% w/v), centrifuged immediately at 1,500 *g* for 10 minutes and the supernatant stored at -80°C. Plasma levels of cytokines were measured by cytometric bead array at the University of Sheffield Core Facility using standardised kits (BD™ Cytometric Bead Array, Becton Dickinson [BD], Oxford, UK). High-sensitivity C-reactive protein (hsCRP) was measured by nephelometry according to manufacturer's intrstructions using a Siemens BN II Nephelometer (Siemens, UK).

3.1.8 Platelet aggregation

Blood was collected into tubes containing trisodium citrate dihydrate (3.13% w/v) for measurement of platelet aggregation. Platelet-rich plasma (PRP) was prepared by centrifugation of whole blood at 200 *g* for 10 minutes at room temperature and removal of supernatant. Platelet aggregation induced by 20 µM ADP was assessed in PRP by light transmission aggregometry (LTA) using a BioData PAP-8E optical aggregometer. Maximum and final platelet aggregation after 6 minutes were recorded.

3.1.9 Platelet P-selectin expression

Blood was collected into trisodium citrate dihydrate (3.13% w/v) for measurement of platelet P-selectin expression by flow cytometry. Forty µl of citrate-anticoagulated whole blood was added to a combination of saline or ADP (final concentration 30 µM), allophycocyanin (APC)-conjugated CD61 (104316, BioLegend, London, UK) and PE-Cy5-conjugated CD62P (551142, BD, UK) and incubated in the dark for 20 minutes. Platelets were gated on morphological characteristics and expression of CD61 and median fluorescence of CD62P was used to determine platelet P-selectin

expression. All flow cytometric analysis was performed with an Accuri C6 multi-color flow cytometer (BD, UK).

1. Gated based on FSC/SSC morphological characteristics

2. Platelets identified on the basis of the expression of CD61 (a platelet marker)

3. Median fluorescence of CD62P (p-selectin) recorded. % positivity of P-selectin determined by comparison to isotype.

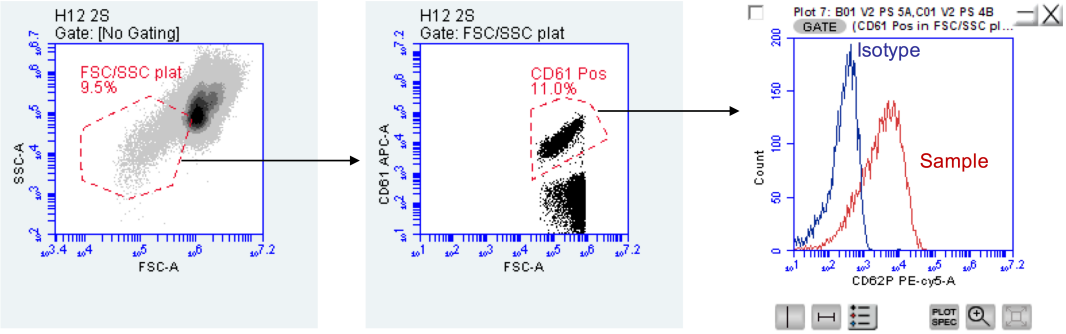

Figure 3.2 Gating strategy for platelet P-selectin measurement

3.1.10 Platelet-leukocyte aggregate formation

Blood was collected into trisodium citrate dihydrate (3.13% w/v) for measurement of platelet-leukocyte aggregate formation by flow cytometry. 480 µl of citrate-anticoagulated whole blood was added to saline or ADP (final concentration 30 µM) and stirred for 10 minutes. Two ml diluted FACSlyse solution (BD, UK) was then added to 180 µl of blood to simultaneously lyse erythrocytes and fix the leukocytes. This was centrifuged at 300 *g* for 5 minutes and the pellet was resuspended in 100 µl PBS + 10% bovine serum albumin. This suspension was then stained with PE-conjugated CD14 (555398, BD, UK) and fluorescein isothiocyanate (FITC)-conjugated CD42a (558818, BD, UK). Monocytes were gated based on morphological characteristics and expression of CD14. Neutrophils were gated based on morphological characteristics and exclusion of monocytes. Platelet-leukocyte aggregate formation was determined by monocyte or neutrophil median fluorescence of the platelet marker CD42a. Samples were processed for analysis by flow cytometry immediately after blood was sampled. Samples from all treatment groups were analysed within the same time frame. Flow cytometry was performed with an Accuri C6 (BD, UK) multi-color flow cytometer and data analysis was performed using FlowJo software (Oregon, USA).

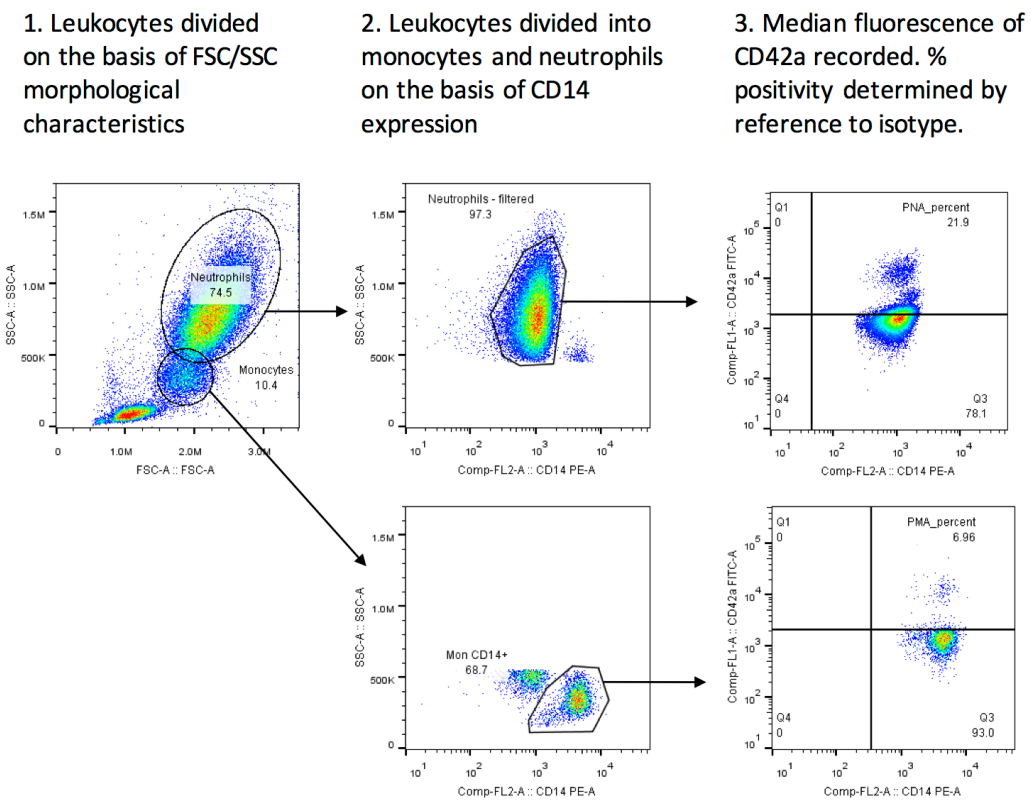

Figure 3.3 Gating strategy for measurement of platelet-leukocyte aggregate formation

3.1.11 Leukocyte count and differential count

Blood was collected into EDTA anticoagulant tubes prior to cell counting according to manufacturer's instructions using an automated Sysmex cell counter (XN-9000, Sysmex, Milton Keynes, UK).

3.1.12 Fibrin clot structure and D-dimer

Blood samples for isolation of plasma were collected into tubes containing trisodium citrate dihydrate (3.13% w/v), centrifuged immediately at 1,500 *g* for 10 minutes and the supernatant stored at -80°C. Fibrin clot characteristics were studied in each individual at 4 time points using plasma and a validated high-throughput turbidimetric assay (Carter et al., 2007). In duplicate in a 96-well plate, 25 µl of plasma was added to 75 µl of assay buffer (0.05 mol/L Tris-HCl, 0.1 mol/L NaCl, pH7.4). At 10 second intervals, 50 µl of activation mix (final concentrations: 0.03 U/ml thrombin [Calbiochem] and 7.5 mmol/L Ca diluted in assay buffer) was added to each column of the 96-well plate. The 96-well plates were then shaken and optical density at 340 nm was recorded every 12 seconds for 1 hour in a BIO-TEK ELx-808 microplate reader. Maximum absorbance was recorded as a measure of the density of the fibrin clot that is formed.

To further visualise fibrin networks, fibrin clots were prepared from pooled plasma of 10 volunteers from each treatment group, as previously described (Hooper et al., 2012). Fibrin clot structure was assessed using scanning electron microscopy. All clots were prepared in duplicate at 4 different time points and photographed at x5,000, x10,000 and x30,000 magnifications in 4 different areas using a field-emission scanning electron microscope (Quinta 200F FEG ESEM, FEI company, Netherlands). In each of these photographs, fiber diameter (n=40) was determined using image analysis software (ImageJ 1.48; National Institutes of Health, USA). Fibrin network density was also determined using ImageJ, by converting all images to binary using a fixed threshold and calculating the percentage of white pixels. To exclude bias, all clots were viewed by 2 operators blinded to the type of sample.

D-dimer was measured by a Sysmex 2100i (Sysmex, UK) using the INNOVANCE D-dimer assay.

3.1.13 Monocyte phenotype and expression of adhesion molecules, chemokine receptors and toll-like receptors

Blood was collected in tubes containing citrate anticoagulant (3.13% w/v) and monocyte phenotype was assessed by flow cytometry according to recognised conventions (Ziegler-Heitbrock et al., 2010). 100 µl of blood was added to the following antibodies; FITC-conjugated anti-human CD14 (Biolegend, UK; 325604), R-phycoerythrin (PE)-conjugated anti-human CD11b (Biolegend, UK; 301306), peridin chlorophyll protein (PerCP)-conjugated anti-human CCR2 (Biolegend, UK; 357204) and APC-conjugated anti-human CD16 (Biolegend, UK; 302012). Another 100 µl was added to the following antibodies; FITC-conjugated anti-human CD14 (Biolegend, UK; 325604), PE-conjugated anti-human CXCR2 (Biolegend, UK; 320706) and PerCP-conjugated anti-human CD16 (Biolegend, UK; 302030). A further 100 µl was added to the following antibodies; FITC-conjugated anti-human CD14 (Biolegend, UK; 325604), PE-conjugated anti-human TLR4 (Biolegend, UK; 312806), PerCP-conjugated anti-human CD16 (Biolegend, UK; 302030) and Alexa Fluor 647-conjugated anti-human TLR2 (Biolegend, UK; 400234). In separate tubes, blood was also added to matched isotypes (Biolegend, UK). The blood was incubated in the dark for 20 minutes. Then 1 ml of FACSLyse (BD) was added to each tube and each tube was vortexed twice. The samples were then centrifuged at 300 *g* for 5 minutes and the pellet was resuspended in FACSFix or PBS prior to analysis by flow cytometry. Samples were processed for analysis by flow cytometry immediately after blood was sampled. Samples from all treatment groups were analysed within the same time frame. Flow cytometry was performed with an Accuri C6 (BD, UK) multi-color flow cytometer and data analysis was performed using FlowJo software (Oregon, USA).

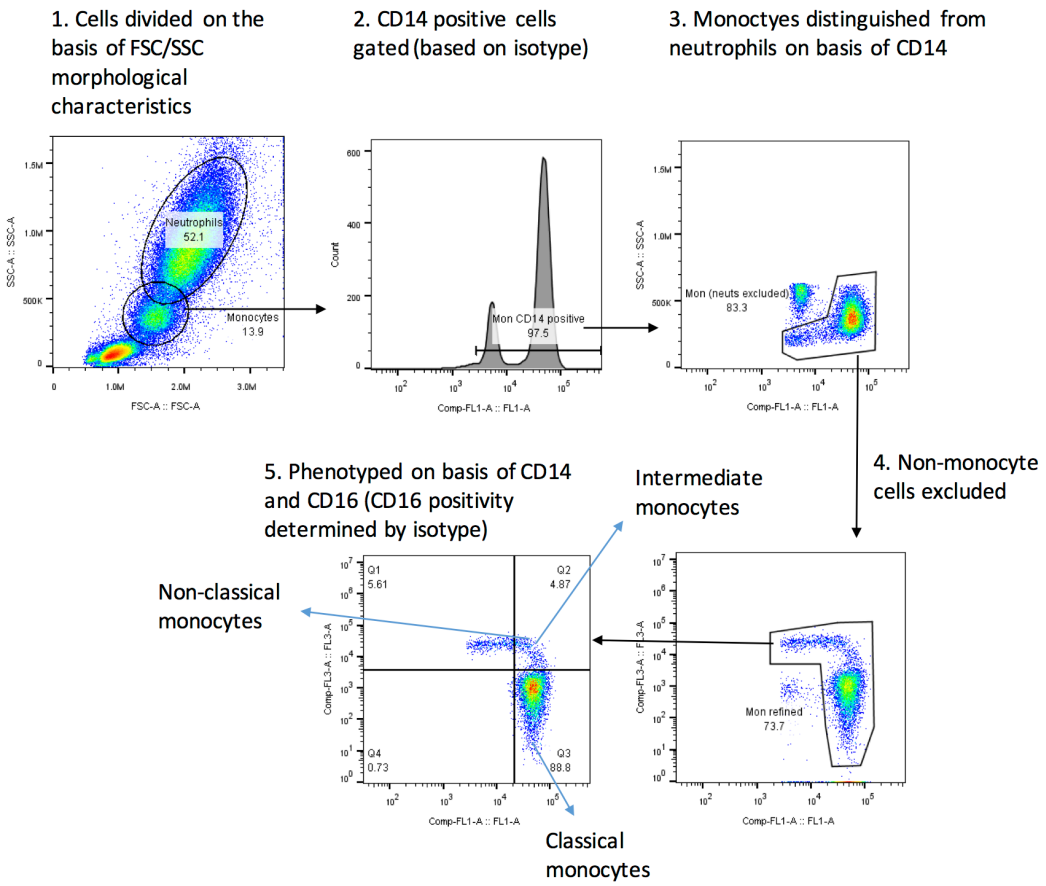

Figure 3.4 Gating strategy for assessment of monocye phenotype and expression of CD14, CD16, CD11b, CCR2, CXCR2, TLR4 and TLR2

3.1.14 Effect of ticagrelor and cangrelor on classical, intermediate and non-classical monocyte expression of CD14, CD16, CCR2, CD11b, CXCR2, TLR4 and TLR2 *ex vivo*

Blood was collected in tubes containing citrate anticoagulant (3.13% w/v). The blood was incubated at 37°C for 30 minutes with the following: 0.04 % DMSO (vehicle control), 1 μM ticagrelor, 10 μM ticagrelor or 1 μM cangrelor. The blood was then incubated with PBS (vehicle control) 1 ng/ml or 100 ng/ml LPS for 1 hour at 37°C. In experiments where CD14, CD11b and CD16 were measured, 100 μl of blood was added to the following: FITC-conjugated anti-human CD14 (Biolegend, UK; 325604), PE-conjugated anti-human CD11b (Biolegend, UK; 301306) and APC-conjugated anti-human CD16 (Biolegend, UK; 302012). In experiments where CD14, CD16, CCR2, CD11b, CXCR2, TLR4 and TLR2 were measured, blood was added to 3 different tubes containing combinations of antibodies. 100 μl of blood was added to the following antibodies; FITC-conjugated anti-human CD14 (Biolegend, UK; 325604), PE-conjugated anti-human CD11b (Biolegend, UK; 301306), PerCP-conjugated anti-human CCR2 (Biolegend, UK; 357204) and APC-conjugated anti-human CD16 (Biolegend, UK; 302012). Another 100 μl was added to the following antibodies; FITC-conjugated anti-human CD14 (Biolegend, UK; 325604), PE-conjugated anti-human CXCR2 (Biolegend, UK; 320706) and PerCP-conjugated anti-human CD16 (Biolegend, UK; 302030). A further 100 μl was added to the following antibodies; FITC-conjugated anti-human CD14 (Biolegend, UK; 325604), PE-conjugated anti-human TLR4 (Biolegend, UK; 312806), PerCP-conjugated anti-human CD16 (Biolegend, UK; 302030) and Alexa Fluor 647-conjugated anti-human TLR2 (Biolegend, UK; 400234). In separate tubes, blood was also added to matched isotypes (Biolegend, UK). The blood was incubated in the dark for 20 minutes. Then 1 ml of FACSLyse (BD) was added to each tube and each tube was vortexed twice. The samples were then centrifuged at 300 *g* for 5 minutes and the pellet was resuspended in FACSFix or PBS prior to analysis on an Accuri C6 flow cytometer.

3.1.15 Statistical analysis

Parametric data are presented as mean ± standard error of the mean (SEM) and non-parametric data are presented as median (interquartile range). An independent

statistician designed the statistical analysis plan prior to the commencement of the study. Area under the curve of hsCRP over a 24-hour period following LPS administration was compared between treatment groups using ANOVA. More complex variables, such as WBC, were compared using repeated measures two-way ANOVA with Dunnett's correction or Bonferroni correction for multiple comparisons as appropriate. The specific tests that have been used are described in each figure legend. $P<0.05$ was considered to be statistically significant. Analyses were performed using SPSS 21 (Chicago, Illinois) and GraphPad Prism 6 (San Diego, CA).

The state-of-the-art statistical method Random Forests (Breiman, 2001) was used to investigate the relationship between the intermediate monocyte count at 24 hours and the other variables in the dataset. The advantage of this method over traditional methods is that it is robust at determining non-linear, non-parametric relationships between variables and inherently accounts for complex interactions (Breiman, 2001). This validated method has been shown to be as accurate or more accurate than traditional regression models in the context of data from the life sciences (Hsich et al., 2011; Ishwaran et al., 2009; 2008). Analyses were performed using R (R foundation for statistical computing, version 3.2.2) and the randomForests package. P-values were ascertained by permutation testing using the rfPermute and rfUtilities packages. The R code and corresponding R markdown document are provided in the appendix.

3.2 Study of the effects of ticagrelor on innate immune activation *in vitro*

The results of this study are presented in Chapter 5 *Platelet P2Y12 Inhibitors Potentiate the Expansion in Intermediate Monocyte Population that Occurs after Endotoxaemia* and Chapter 6 *Adenosine-mediated effects of ticagrelor on innate immune activation in vitro*.

3.2.1 Healthy volunteers

The study had ethics approval from the University of Sheffield Ethics Committee and was conducted in accordance with Good Clinical Practice guidelines. All volunteers for blood donation provided written consent and had no regular or recent use of medications.

3.2.2 Neutrophil and erythrocyte isolation

Blood was collected in tubes containing trisodium citrate dihydrate (3.13% w/v). The blood was centrifuged at 260 *g* for 20 minutes at room temperature and PRP was then discarded. Dextran (6%) was added to the blood for 30 minutes at room temperature to sediment erythrocytes. The resultant leukocyte-rich plasma was then withdrawn and layered gently over 15 ml Histopaque 1077 (Sigma, UK), prior to centrifugation at 400 *g* for 25 minutes at room temperature. The supernatant was discarded and 25 ml of hypotonic saline (0.2% NaCl) was added to lyse residual erythrocytes. 25 ml of hypertonic rescue buffer (1.6% NaCl and 0.1% glucose) was then added to correct the concentration of saline to 0.9%. The suspension was then centrifuged at 280 *g* for 7 minutes at room temperature. The supernatant was removed and the resultant isolated neutrophils cells were resuspended in RPMI buffer (Life Technologies Ltd, UK) with 10% fetal calf serum (FCS) (Sigma, UK).

Erythrocytes were isolated in parallel. The blood was centrifuged at 260 *g* for 20 minutes at room temperature and PRP was then discarded. Instead of dextran, normal saline (0.9%) was added to the blood for 30 minutes at room temperature. The blood was then layered gently over 15 ml Histopaque 1077 (Sigma, UK), prior to centrifugation at 400 *g* for 25 minutes at room temperature. The supernatant was discarded and 50 ml of normal saline (0.9%) was added. The suspension was then

centrifuged at 280 *g* for 7 minutes at room temperature. The supernatant was removed and the resultant erythrocytes were resuspended in RPMI buffer (Life Technologies Ltd, UK) with 10% fetal calf serum (FCS) (Sigma, UK).

3.2.3 Preparation of heat-killed opsonized *Streptococcus pneumoniae*

Heat-killed *S.pneumoniae* (D39 strain) were centrifuged at 6,000 rpm for 3 minutes and the bacteria were then resuspended in RPMI with 10% serum from donors who had been vaccinated against pneumococcus. The bacteria were then shaken for 30 minutes.

3.2.4 Assessment of the effect of adenosine on neutrophil phagocytosis

Neutrophils were isolated as described in 3.2.2 and resuspended in RPMI (10% FCS) at a concentration of 5 x 10^6/ml (to provide a final concentration of 2.5 x 10^6/ml) and then added to a 96-well plate. Adenosine (final concentrations: 10^{-8} M or 10^{-5} M, in RPMI) or RPMI were then added to each of the wells immediately prior to the addition of opsonized pneumococcus that had been prepared according to the protocol described above. The cells were then incubated for 30 minutes (37°C, 5% CO_2). A Cytospin machine (Shandon, Thermo Scientific, Waltham, MA) was then used to prepare cytocentrifuge slides from the cell suspension. The cells were stained using modified Giemsa-based stains (Differentiation-Quik, Reagena, Toivala, Finland). The percentage of neutrophils that had phagocytosed bacteria was calculated by assessment of 300 neutrophils by light microscopy. The phagocytic index of the neutrophils was calculated using the following formula: (total number of phagocytosed bacteria / total number of counted neutrophils) x (number of neutrophils containing phagocytosed bacteria / total number of counted neutrophils) (Sano et al., 2003).

3.2.5 Assessment of the effect of adenosine on LPS-induced changes in isolated neutrophil expression of CD11b, CXCR1 and CD16

Neutrophils were isolated as described in 3.2.2. The neutrophils were resuspended in 4 ml RPMI (10% FCS) at 2.5 x 10^6/ml. Then 490 µl of neutrophils were added to 10 µl of RPMI (vehicle control) or adenosine for 4 minutes at the following final concentrations: 10^{-9} M, 10^{-8} M, 10^{-7} M, 10^{-6} M, 10^{-5} M or 10^{-4} M. Then 90 µl of neutrophils were incubated with 10 µl of PBS (vehicle control) or LPS (1, 10 or 100 ng/ml) for 30 minutes at 37°C in a water bath. Then 75 µl of the cell suspension was added to the following antibody combination: PE-conjugated anti-human CD11b (Biolegend, UK; 301306). APC-conjugated anti-human CXCR1 (Biolegend, UK; 320612) and PerCP-conjugated anti-human CD16 (Biolegend, UK; 302030). In separate tubes, blood was also added to matched isotypes (Biolegend, UK). The cells were incubated in the dark for 20 minutes. Then 1 ml of FACSLyse (BD) was added to each tube and each tube was vortexed twice. The samples were then centrifuged at 300 *g* for 5 minutes and the pellet was resuspended in FACSFix or PBS prior to analysis on an Accuri C6 flow cytometer.

3.2.6 Assessment of the effect of adenosine on FMLP-induced changes in isolated neutrophil expression of CD11b, CXCR1 and CD16

Neutrophils were isolated as described in 3.2.2. The neutrophils were resuspended in 4 ml RPMI (10% FCS) at 2.5 x 10^6/ml. Then 490 µl of neutrophils were added to 10 µl of RPMI (vehicle control) or adenosine for 4 minutes at the following final concentrations: 10^{-9} M, 10^{-8} M, 10^{-7} M, 10^{-6} M, 10^{-5} M or 10^{-4} M. Then 90 µl of neutrophils were incubated with 10 µl of PBS (vehicle control) or FMLP (10^{-8} M, 10^{-7} M or 10^{-6} M) for 30 minutes at 37°C in a water bath. Then 75 µl of the cell suspension was added to the following antibody combination: PE-conjugated anti-human CD11b (Biolegend, UK; 301306). APC-conjugated anti-human CXCR1 (Biolegend, UK; 320612) and PerCP-conjugated anti-human CD16 (Biolegend, UK; 302030). In separate tubes, blood was also added to matched isotypes (Biolegend, UK). The cells were incubated in the dark for 20 minutes. Then 1 ml of FACSLyse (BD) was added to each tube and each tube was vortexed twice. The samples were

then centrifuged at 300 *g* for 5 minutes and the pellet was resuspended in FACSFix or PBS prior to analysis on an Accuri C6 flow cytometer.

3.2.7 Assessment of the effect of erythrocytes on the effect of adenosine on FMLP-induced changes in isolated neutrophil expression of CD11b, CXCR1 and CD16

Neutrophils and erythrocytes were isolated as described in 3.2.2. The neutrophils were resuspended in (10% FCS) at 2.5 x 10^6/ml either without erythrocytes or with erythrocytes at 12.5 x 10^6/ml or 2,500 x 10^6/ml (5:1 and 1000:1 erythrocyte to neutrophil ratio respectively). Then 490 µl of the cell suspensions was added to 10 µl of RPMI (vehicle control) or adenosine for 4 minutes at the following final concentrations: 10^{-8} M and 10^{-5} M. Then 90 µl of the cell suspension was incubated with 10 µl of PBS (vehicle control) or FMLP (10^{-7} M) for 30 minutes at 37°C in a water bath. Then 75 µl of the cell suspension was added to the following antibody combination: PE-conjugated anti-human CD11b (Biolegend, UK; 301306). APC-conjugated anti-human CXCR1 (Biolegend, UK; 320612) and PerCP-conjugated anti-human CD16 (Biolegend, UK; 302030). In separate tubes, blood was also added to matched isotypes (Biolegend, UK). The cells were incubated in the dark for 20 minutes. Then 1 ml of FACSLyse (BD) was added to each tube and each tube was vortexed twice. The samples were then centrifuged at 300 *g* for 5 minutes and the pellet was resuspended in FACSFix or PBS prior to analysis on an Accuri C6 flow cytometer.

3.2.8 Assessment of the effect of dipyridamole on adenosine-mediated changes in FMLP-induced neutrophil expression of CD11b, CXCR1 and CD16 in whole blood

Blood was collected in tubes containing trisodium citrate dihydrate (3.13% w/v). Then 2.94 ml of whole blood was incubated with 60 µl of 0.1% DMSO (vehicle control) or dipyridamole (10 µM final concentration) for 5 minutes at room temperature. 980 µl of this blood was incubated with 20 µl of PBS (vehicle control) or adenosine (10^{-5} M) 4 minutes prior to incubation with FMLP or immediately prior to incubation with FMLP. 95 µl of blood was incubated with 5 µl of PBS (vehicle control) or FMLP (10^{-7} M final concentration) for 5 or 30 minutes. Then 75 µl of the

cell suspension was added to the following antibody combination: PE-conjugated anti-human CD11b (Biolegend, UK; 301306) and APC-conjugated anti-human CXCR1 (Biolegend, UK; 320612). In separate tubes, blood was also added to matched isotypes (Biolegend, UK). The cells were incubated in the dark for 20 minutes. Then 1 ml of FACSLyse (BD) was added to each tube and each tube was vortexed twice. The samples were then centrifuged at 300 *g* for 5 minutes and the pellet was resuspended in FACSFix or PBS prior to analysis on an Accuri C6 flow cytometer.

3.2.9 Assessment of the effect of timing of pre-incubation of adenosine on subsequent response to FMLP in the presence of ticagrelor in whole blood

Blood was collected in tubes containing trisodium citrate dihydrate (3.13% w/v). Then 2.94 ml of whole blood was incubated with 60 µl of 0.1% DMSO (vehicle control) or ticagrelor (10 µM final concentration) for 10 minutes at room temperature. 95 µl of this blood was then incubated with PBS (vehicle control) or adenosine (10^{-5} M final concentration) for 0, 15, 30 or 60 seconds prior to incubation with FMLP (10^{-7} M final concentration) for 5 minutes at 37°C. Then 75 µl of the cell suspension was added to the following antibody combination: PE-conjugated anti-human CD11b (Biolegend, UK; 301306) and APC-conjugated anti-human CXCR1 (Biolegend, UK; 320612). In separate tubes, blood was also added to matched isotypes (Biolegend, UK). The cells were incubated in the dark on ice for 30 minutes. Then 1 ml of FACSLyse (BD) was added to each tube and each tube was vortexed twice. The samples were then centrifuged at 300 *g* for 5 minutes and the pellet was resuspended in FACSFix or PBS prior to analysis on an Accuri C6 flow cytometer.

3.2.10 Assessment of the modulatory effect of ticagrelor, cangrelor and dipyridamole on the effect of adenosine on response to FMLP in whole blood

Blood was collected in tubes containing trisodium citrate dihydrate (3.13% w/v). Then 2.94 ml of whole blood was incubated with 60 µl of 0.1% DMSO (vehicle control) or ticagrelor (10 µM final concentration), cangrelor (1 µM final concentration) or dipyridamole (10 µM final concentration) for 10 minutes at room

temperature. 95 µl of this blood was then incubated with PBS (vehicle control) or adenosine (10^{-5} M final concentration) at the same time as PBS (vehicle control) or FMLP (10^{-7} M final concentration) for 5 minutes at 37°C. Then 75 µl of the cell suspension was added to the following antibody combination: PE-conjugated anti-human CD11b (Biolegend, UK; 301306) and APC-conjugated anti-human CXCR1 (Biolegend, UK; 320612). In separate tubes, blood was also added to matched isotypes (Biolegend, UK). The cells were incubated in the dark on ice for 30 minutes. Then 1 ml of FACSLyse (BD) was added to each tube and each tube was vortexed twice. The samples were then centrifuged at 300 *g* for 5 minutes and the pellet was resuspended in FACSFix or PBS prior to analysis on an Accuri C6 flow cytometer.

3.2.11 Statistical Analysis

Data are presented as mean ± SEM. Comparisons between samples are made by one- or two-way ANOVA as appropriate. Multiple comparisons were corrected for by Dunnett's correction or Bonferroni correction as appropriate. The specific statistical tests that have been used are described in the figure legends. Analyses were performed using GraphPad Prism 6 (San Diego, CA).

4 Platelet $P2Y_{12}$ inhibitors reduce systemic inflammation and its prothrombotic effects in an experimental human model

4.1 Introduction

Sepsis is one of the most devastating clinical syndromes in medicine and severe sepsis still has a mortality rate of 20-30% and remains resistant to specific pharmacological therapy (Angus & van der Poll, 2013). Sepsis is characterized by dysregulated systemic inflammatory response to bacterial components, such as endotoxin (lipopolysaccharide; LPS) (Angus & van der Poll, 2013). Excessive innate immune activation causes a pro-inflammatory cytokine storm, extravasation of activated neutrophils and disturbances of the coagulation system, leading to collateral host tissue damage and increased mortality (de Stoppelaar et al., 2014; Angus & van der Poll, 2013). A number of pathological processes, such as sepsis, involve the formation of platelet-leukocyte aggregates. These platelet-leukocyte interactions have a potentially important role in the pathogenesis of inflammation as they augment leukocyte production of pro-inflammatory cytokines, leukocyte recruitment and activation of coagulation (Semple et al., 2011). However, the overall magnitude of the contribution of platelets to systemic inflammation and the pathophysiology of human sepsis is not well defined.

Platelet $P2Y_{12}$ inhibitors, such as clopidogrel and ticagrelor, inhibit a central ADP-mediated amplification pathway and therefore blunt a broad spectrum of platelet functions (Storey et al., 2000). It is well established that the antithrombotic effect of this is beneficial for patients with atherothrombosis, which has led to $P2Y_{12}$ medications becoming some of the most commonly prescribed medications worldwide. However, in addition, this inhibits the formation of platelet-leukocyte aggregates, which is primarily mediated by inhibition of platelet expression of the adhesion molecule P-selectin (Semple et al., 2011). A very recent cohort study of 683,421 patients with sepsis has shown that current use of antiplatelet therapy is independently associated with a significant reduction in mortality from sepsis (odds ratio [OR] 0.78; 95% confidence interval [CI] 0.76 – 0.79; $p < 0.001$) (Tsai et al., 2015). This also corresponds with previous observational studies that suggest that

clopidogrel reduces mortality from sepsis (Akinosoglou et al., 2014; Gross et al., 2013). However, the mechanisms underpinning this reduction in mortality have not been clearly demonstrated *in vivo*, since animal models of sepsis conflict regarding the immunomodulatory effects of clopidogrel, which may be species dependent (Liverani et al., 2014; Winning et al., 2011; Hagiwara et al., 2011; Lipcsey et al., 2005).

Ticagrelor is a novel $P2Y_{12}$ inhibitor that causes more potent and consistent $P2Y_{12}$ inhibition than clopidogrel (Storey et al., 2010) and also weakly inhibits cellular uptake of adenosine (Bonello et al., 2014). In the PLATO study of over 18,000 patients with ACS, ticagrelor reduced all-cause mortality compared to clopidogrel (HR 0.78; $p<0.001$), which was out of proportion to its incremental cardiovascular benefit (Wallentin et al., 2009). Intriguingly, ticagrelor was associated with lower mortality related to infection (HR 0.67; $p<0.05$) (Varenhorst et al., 2014; 2012) and fewer deaths following sepsis and pulmonary infections than clopidogrel (Storey et al., 2014).

The mechanistic impact of $P2Y_{12}$ inhibitors was determined on pathophysiological processes that are central to sepsis responses in humans. It was hypothesized that $P2Y_{12}$ inhibitors may reduce mortality from sepsis by suppressing systemic inflammation and its prothrombotic effects, mediated by inhibition of platelet-leukocyte interactions. It was hypothesized that the more potent $P2Y_{12}$ inhibitor, ticagrelor, suppresses these responses more potently than clopidogrel. To test these hypotheses in humans, a well-established model of systemic inflammation was used, which involves intravenous injection of *E.coli* endotoxin (lipopolysaccharide; LPS) into healthy volunteers (Suffredini et al., 1999). The particular strength of this unique model is that it allows direct assessment of dynamic cellular and molecular pathways that are also major mediators of the pathophysiology of sepsis in humans.

4.2 Results

Thirty healthy volunteers underwent LPS administration (see CONSORT flowchart Figure 4.1). To avoid any possibility of administering intravenous *E.coli* LPS to a pregnant female, volunteers were only included if they were not of childbearing potential. No eligible female subjects volunteered and so all recruited volunteers were male. Compliance was assessed from a diary and pill-count and all subjects were >90% compliant. There were no unexpected adverse reactions.

Baseline characteristics were comparable in all of the treatment groups (Table 4.1). The median age was 22.5, 21 and 21.5 in the control, clopidogrel and ticagrelor groups respectively. The mean duration of treatment was 7.1 ± 0.1 days in the clopidogrel group and 6.7 ± 0.3 in the ticagrelor group respectively (Table 4.1).

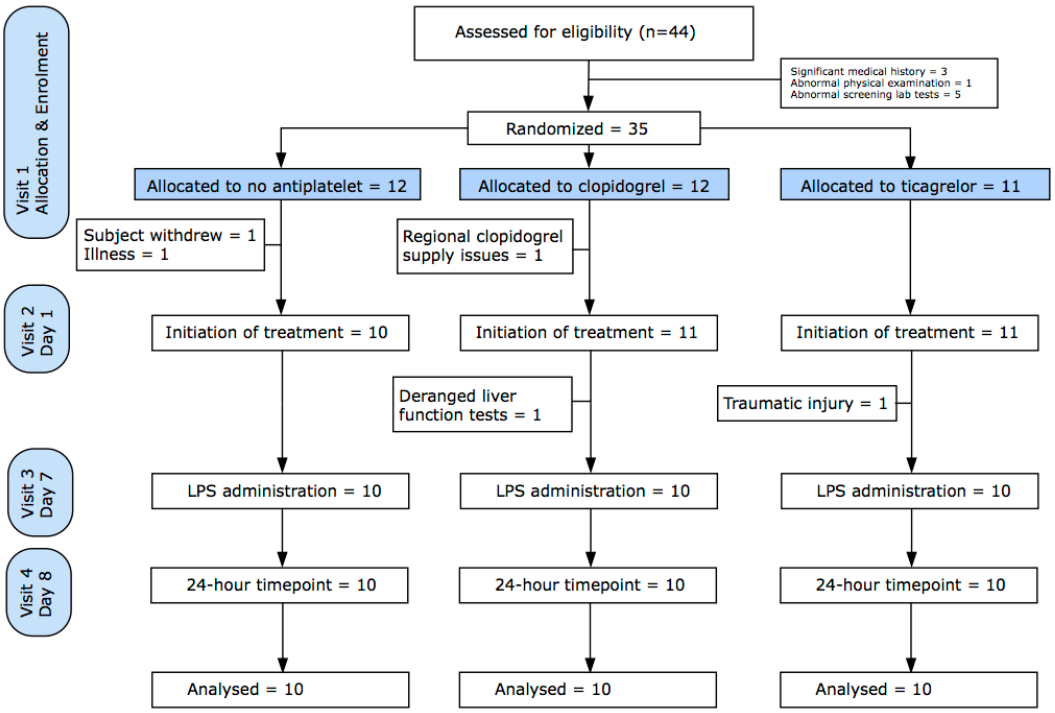

Figure 4.1 CONSORT flow diagram

Presenting the enrolment, intervention allocation, follow-up and data analysis with number of subjects for each group.

Table 4.1 Baseline and treatment characteristics

	Control n=10	Clopidogrel n=10	Ticagrelor n=10
Age - median years (interquartile range)	22.5 (21.0 – 24.25)	21.0 (20.50 – 22.50)	21.5 (20.0 – 22.0)
Male sex – no./total no. (%)	10/10 (100)	10/10 (100)	10/10 (100)
Weight (kg) – mean (SEM)	76.4 (2.7)	75.0 (3.2)	74.2 (2.5)
Body mass index (kg/m^2) – mean (SEM)	23.0 (0.6)	22.7 (0.5)	23.2 (0.8)
Race – no./total no.			
White (%)	10/10 (100)	8/10 (80)	9/10 (90)
Black (%)	0/10 (0)	1/10 (10)	0/10 (0)
Asian (%)	0/10 (0)	1/10 (10)	1/10 (10)
Duration of clopidogrel treatment – days (SEM)	0 (0)	7.1 (0.1)	0 (0)
Duration of ticagrelor treatment – days (SEM)	0 (0)	0 (0)	6.7 (0.3)

There were no significant differences in baseline characteristics between groups.

4.2.1.1 Haemodynamic effects

After LPS administration, all subjects developed anticipated flu-like symptoms and signs of sepsis that peaked at 90 - 180 minutes and resolved within 6 hours (Table 4.2). After LPS administration, there was a significant increase in the heart rate in each of the treatment groups from approximately 70-74 bpm to approximately 93-94 bpm (all $p < 0.05$), which was not significantly different in each of the treatment groups. There was a numerical reduction in the mean arterial blood pressure, but this was not statistically significant and did not differ between treatment groups.

Table 4.2 Haemodynamic parameters before randomized treatment, after treatment and 3 hours after LPS administration

	Baseline	Before LPS	3 Hours After LPS
Heart rate (bpm)			
Control	72±4	70±2	94±4*
Clopidogrel	70±5	68±4	94±4*
Ticagrelor	74±2	71±3	93±3*
Mean arterial blood pressure (mm Hg)			
Control	87±2	88±3	84±2
Clopidogrel	89±3	88±3	82±3
Ticagrelor	87±3	87±3	80±2
Temperature °C			
Control	36.1±0.1	35.9±0.1	37.9±0.2*
Clopidogrel	36.0±0.1	36.0±0.1	38.1±0.2*
Ticagrelor	36.0±0.1	36.0±0.1	37.7±0.2*

Data are mean ± SEM. * = P<0.05 compared to value before LPS administration.

4.3 Both Ticagrelor and Clopidogrel Reduce Peak Levels of IL-6, TNFα and CCL2, whilst Ticagrelor Additionally Reduces Peak Levels of IL-8 and G-CSF and Increases Peak Levels of IL-10

Systemic inflammation in response to LPS administration was assessed by measuring the release of major pro-inflammatory cytokines and the modulatory effects of $P2Y_{12}$ inhibitors were determined. Plasma levels of interleukin (IL)-6, TNFα, IL-8, chemokine (C-C motif) ligand (CCL)-2, granulocyte colony stimulating factor (G-CSF) and high sensitivity C-reactive protein (hsCRP) significantly increased after LPS administration (all $p<0.001$). Compared to control, both $P2Y_{12}$ inhibitors had a marked effect on the pro-inflammatory cytokine response, reducing peak levels of TNFα (66% reduction [$p<0.001$] and 60% reduction [$p<0.001$] respectively; Figure 4.2A), IL-6 (47% reduction [$p<0.001$] and 28% reduction [$p=0.001$] respectively; Figure 4.2B), and CCL2 (38% reduction [$p<0.001$] and 19% reduction [$p=0.049$] respectively; Figure 4.2C). In addition, ticagrelor, but not clopidogrel, significantly reduced peak levels of G-CSF (51% reduction; $p<0.001$; Figure 4.2D) and IL-8 (29% reduction; $p=0.001$; Figure 4.2E) compared to control. Ticagrelor, but not clopidogrel, also significantly increased peak levels of the anti-inflammatory cytokine IL-10 compared to control (54% increase; $p=0.02$; Figure 4.2F). Neither drug significantly modified the hsCRP response (Figure 4.2G).

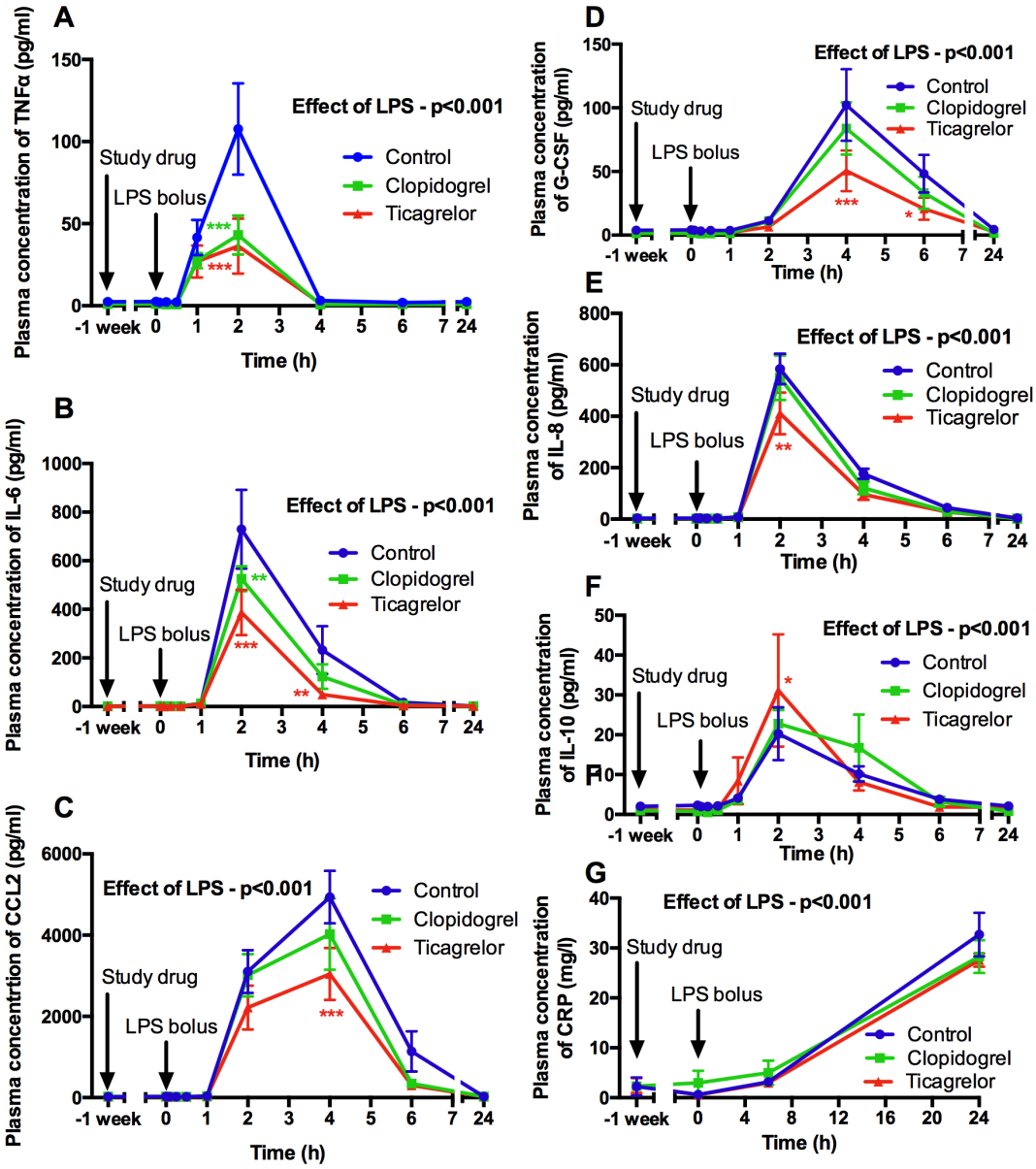

Figure 4.2 Levels of cytokines TNFα (A), IL-6 (B), CCL2 (C), G-CSF (D), IL-8 (E), IL-10 (F) and hsCRP (G)

Before and after 1 week of antiplatelet treatment and following LPS administration (t = 0 hours). Data expressed as mean ± SEM (n=10 in each group). The overall effect of LPS and the effect of ticagrelor and clopidogrel (both compared to control at each time point) determined using 2-way ANOVA with Dunnett's correction for multiple comparisons for the cytokines (*p<0.05, **p<0.01 and ***p<0.001). For hsCRP, the effect of ticagrelor and clopidogrel compared to control was determined using ANOVA of AUC.

4.4 Ticagrelor Inhibits LPS-induced Platelet-Monocyte Aggregate Formation

Formation of platelet-leukocyte aggregates (defined as leukocyte expression of the platelet marker CD42a) amplifies leukocyte release of pro-inflammatory cytokines (Suffredini et al., 1999). It was therefore investigated whether this is a mechanism by which $P2Y_{12}$ inhibitors reduce systemic inflammation. Ticagrelor significantly reduced formation of platelet-monocyte aggregates compared to control (21% vs 36%; $p<0.001$) that occurred 6 hours after LPS administration (Figure 4.3A). Clopidogrel also significantly reduced the formation of platelet-monocyte aggregates compared to control (23% vs 36%; $p=0.04$; Figure 4.3A). A similar pattern of effect of LPS and modulation by the antiplatelet medications was seen in platelet-neutrophil aggregate formation, but the effects on platelet-neutrophil aggregate formation were not statistically significant (Figure 4.3B). Platelet P-selectin expression did not significantly change after LPS administration (Figure 4.3C).

Inhibition of platelet $P2Y_{12}$ ADP receptors was also assessed by measuring platelet aggregation, platelet-leukocyte aggregate formation and platelet P-selectin expression in response to ADP added *ex vivo*. Ticagrelor and clopidogrel inhibited ADP-induced platelet aggregation, platelet-monocyte aggregate formation, platelet-neutrophil aggregate formation and platelet P-selectin expression compared to control at all time points (all $p<0.001$; Figure 4.3). After randomized treatment, platelet aggregation responses following 5 minutes exposure to ADP (final platelet aggregation response) were 2±1%, 14±6% and 70±11% in the ticagrelor, clopidogrel and control groups respectively. This indicates that clopidogrel achieved satisfactory $P2Y_{12}$ inhibition, in contrast to its effect on patients, which is variable and often incomplete (Breet et al., 2010). Final platelet aggregation responses did not significantly change following LPS administration in any of the treatment groups (all $p>0.05$).

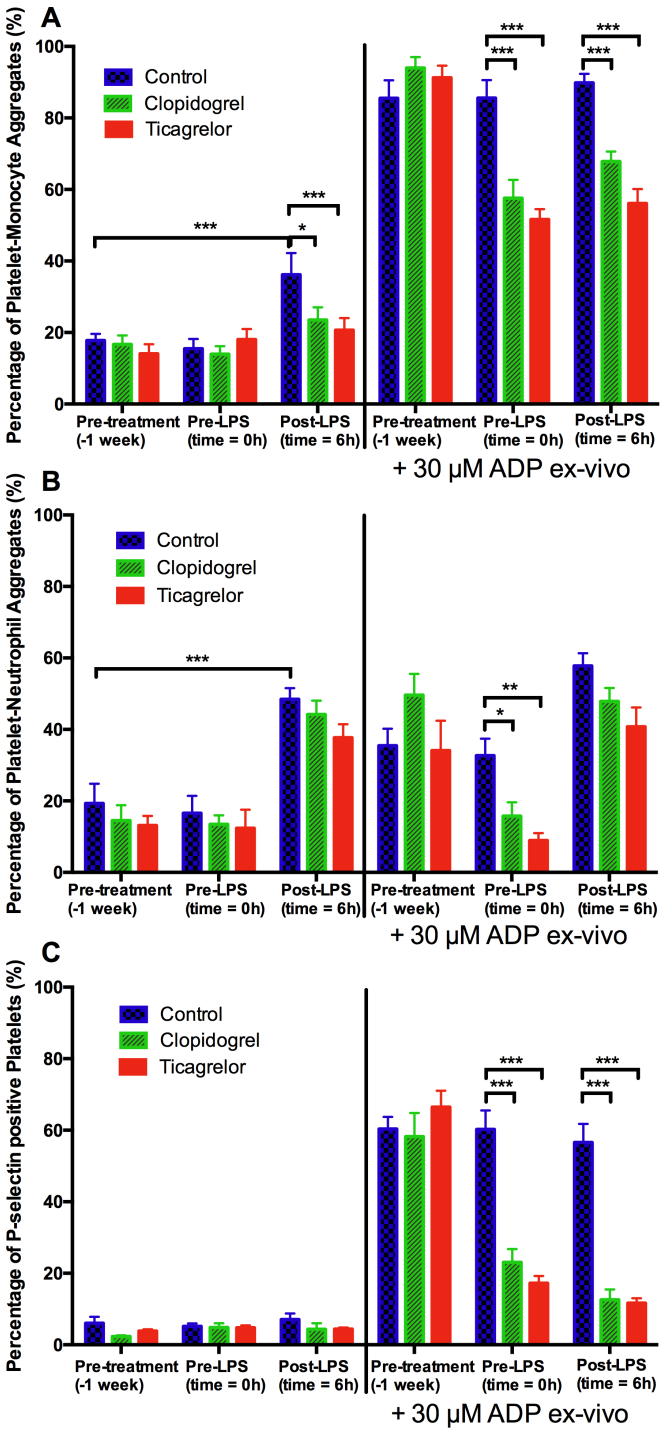

Figure 4.3 Platelet-monocyte (A) and platelet-neutrophil (B) aggregate formation and platelet P-selectin expression (C)

At baseline, immediately before LPS administration and 6 hours after LPS administration, in unstimulated samples and samples stimulated by 30 µM ADP *ex vivo*. Data expressed as mean ± SEM (n=10 in each group). The overall effect of LPS and the effect of ticagrelor and clopidogrel (both compared to control at each time point) were determined using 2-way ANOVA with Dunnett's correction for multiple comparisons (*p<0.05, **p<0.01 and ***p<0.001).

4.5 Ticagrelor Increases Neutrophil Counts and Alters Monocyte Dynamics During Systemic Inflammation

Since the formation of platelet-leukocyte aggregates facilitates adhesion of leukocytes to the endothelium and subsequent extravasation, it was investigated whether inhibition of these processes by $P2Y_{12}$ inhibitors affects leukocyte trafficking. Ticagrelor potentiated the increase in neutrophil count, which was significantly higher than controls 2-4 hours after LPS administration ($p<0.05$; Figure 4.4B) and may have been due to inhibition of non-specific sequestration of neutrophils. Clopidogrel did not have a significant effect (Figure 4.4B). Similarly, subjects receiving ticagrelor showed altered monocyte dynamics. Transient monocyte sequestration was observed after LPS administration in all volunteers, but recovery from this was significantly greater in the ticagrelor and clopidogrel groups (Figure 4.4C). Neither $P2Y_{12}$ inhibitor significantly affected the decrease in platelet count that occurred after LPS administration (Figure 4.4D).

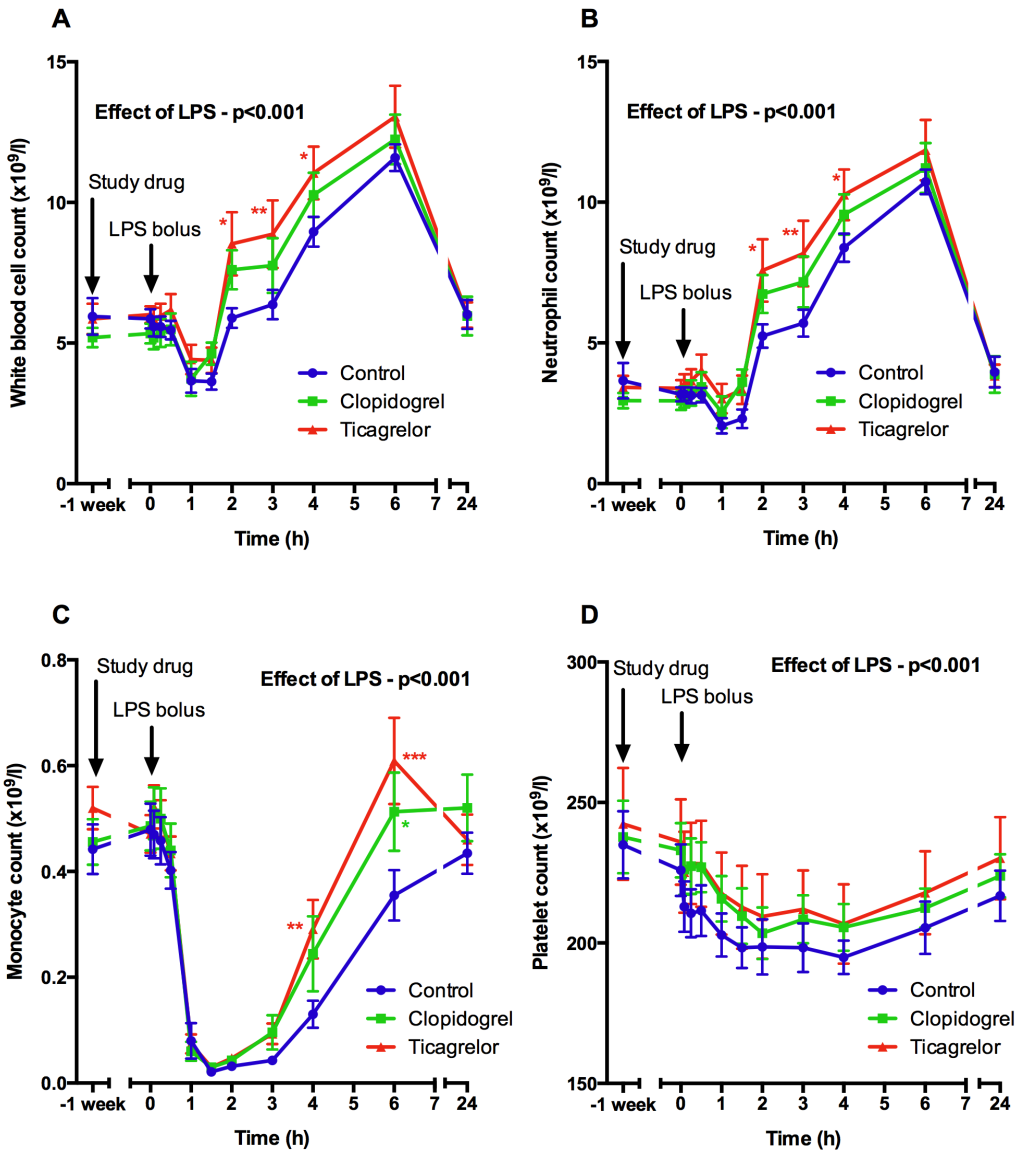

Figure 4.4 Leukocyte (A), neutrophil (B), monocyte (C) and platelet (D) counts

Before and after 1 week of antiplatelet treatment and following LPS administration (t = 0 hours). Data expressed as mean ± SEM (n=10 in each group). The overall effect of LPS and the effect of ticagrelor and clopidogrel (both compared to control at each time point) were determined using 2-way ANOVA with Dunnett's correction for multiple comparisons (*p<0.05, **p<0.01 and ***p<0.001).

4.6 LPS Induces Prothrombotic Changes in the Fibrin Network that are Attenuated by Ticagrelor

The development of a stable fibrin clot represents the critical final stage of thrombosis and has the potential to be modified by systemic inflammation. Turbidimetric assays of individual samples showed that fibrin clot maximum absorbance (a measure of clot density) and lysis area (a complex measure that assesses both clot formation and lysis) increased after LPS administration ($p<0.001$) (Figure 4.5). Ticagrelor significantly reduced the rise in maximum absorbance after LPS administration compared with control (percentage increase from baseline of 33% vs. 109%; $p=0.02$; Figure 4.5A). Similarly, ticagrelor also reduced the increase in lysis area after LPS administration compared with control (percentage increase from baseline of 46% vs. 147%; $p=0.02$; Figure 4.5B). Clopidogrel had a similar, less potent effect that was not statistically significant (Figure 4.5).

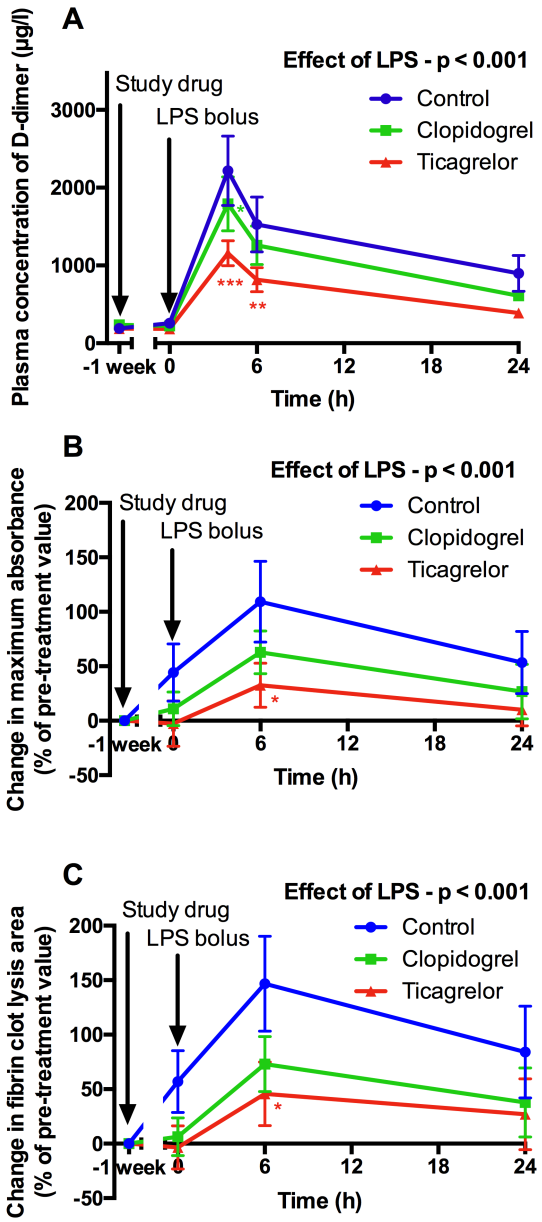

Figure 4.5 Levels of D-dimer (A) and fibrin clot maximum absorbance (B) (a measure of clot density) and lysis area under the curve (C) (a complex measure that assesses both clot formation and lysis) determined by turbidimetry (expressed as percentage change from baseline value) following treatment and LPS administration.

Data expressed as mean ± SEM (n=10 in each group). The overall effect of LPS and the effect of ticagrelor and clopidogrel (both compared to control at each time point) were determined using 2-way ANOVA with Dunnett's correction for multiple comparisons (*p<0.05, **p<0.01 and ***p<0.001).

More detailed analysis of clot ultrastructure using scanning electron microscopy of pooled plasma samples demonstrated that LPS administration resulted in more compact clot formation (Figure 4.6), shown by a significant increase in fibrin clot density ($p=0.02$) and a decrease in fibrin fibre diameter ($p=0.01$) (Figure 4.7). These changes have been shown to increase clot stability and confer resistance to fibrinolysis, both of which contribute to a prothrombotic state (Undas & Ariens, 2011). Ticagrelor significantly reduced LPS-induced changes in fibre density and fibre diameter (Figure 4.7), whereas clopidogrel had a similar less potent effect that was not statistically significant. LPS induced a marked increase in D-dimer (Figure 4.5), which peaked at 4 hours ($p<0.001$). Ticagrelor significantly reduced peak levels of D-dimer by 48% compared to control ($p<0.001$) and clopidogrel significantly inhibited peak levels by 19% compared to control ($p=0.01$).

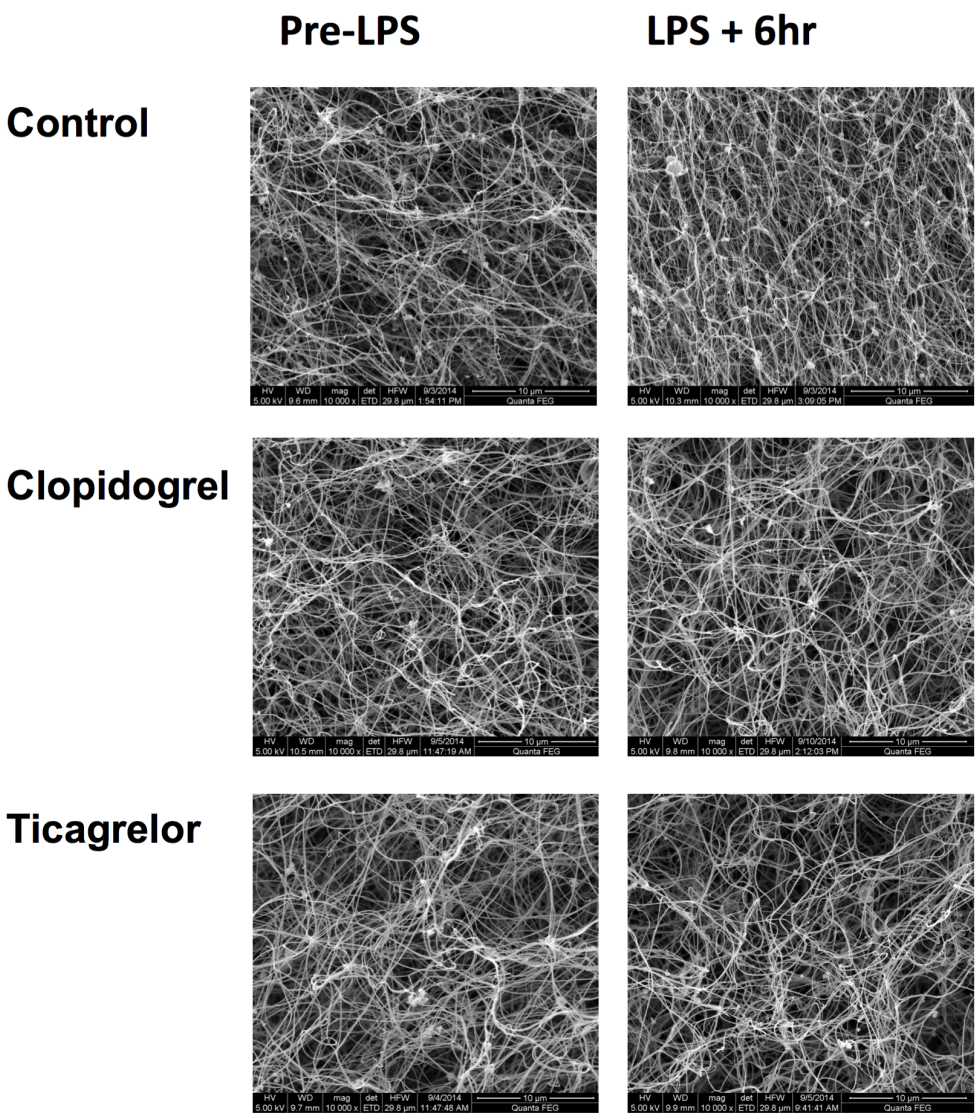

Figure 4.6 Representative electron microscope images of fibrin clots formed from plasma *ex vivo*.

in each treatment group immediately before and 6 hours after LPS administration. Clots were prepared in duplicate and 4 photographs were taken of each clot at each time point. In the control group, there is an increase in fibrin network density following LPS whereas in the clopidogrel and ticagrelor groups this is not apparent.

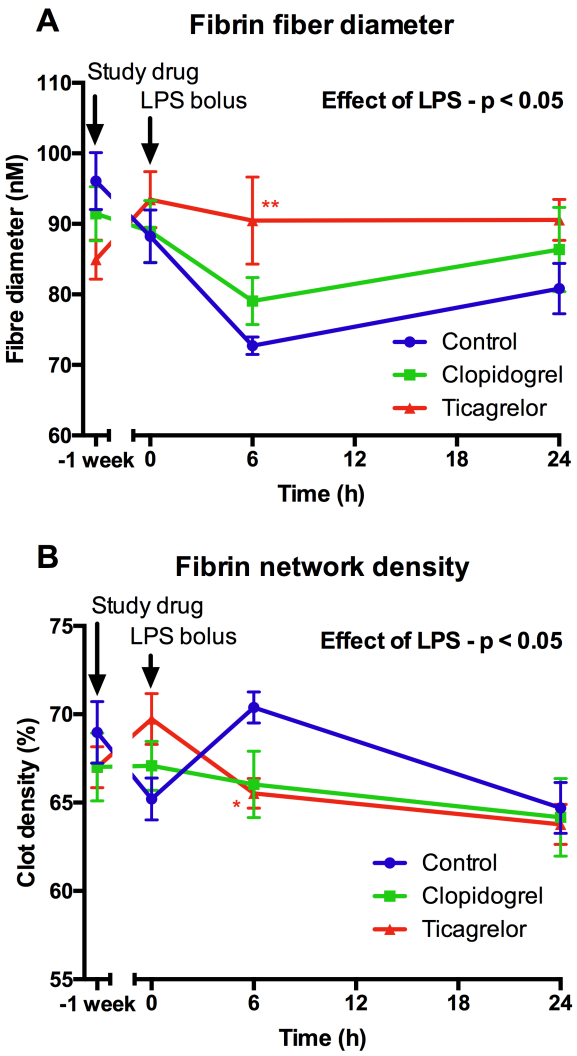

Figure 4.7 Fibrin fibre diameter (A) and fibrin network density (B) (determined by electron microscopy) following LPS administration.

Data expressed as mean ± SEM (n=8 in each group). The overall effect of LPS and the effect of ticagrelor and clopidogrel (both compared to control at each time point) were determined using 2-way ANOVA with Dunnett's correction for multiple comparisons (*p<0.05, **p<0.01 and ***p<0.001).

4.7 Discussion

Sepsis is a devastating syndrome for which therapeutic options remain limited. In addition to the immediate collateral host tissue damage and mortality caused by sepsis, there is a 20-fold increase in risk of myocardial infarction and stroke following sepsis by mechanisms that are not fully understood (Dalager-Pedersen et al., 2014). Data from clinical studies of platelet $P2Y_{12}$ inhibitors suggest that their use improves mortality from sepsis (Tsai et al., 2015; Akinosoglou et al., 2014; Gross et al., 2013). However, animal models of sepsis have conflicted regarding the immunomodulatory effect of platelet $P2Y_{12}$ inhibition on sepsis responses (Liverani et al., 2014; Winning et al., 2011; Hagiwara et al., 2011; Lipcsey et al., 2005), which may be species dependent. The mechanistic impact of $P2Y_{12}$ inhibitors on key molecular and cellular pathways that are central to sepsis responses in humans was determined. Effects of $P2Y_{12}$ inhibitors on leukocyte responses, interactions with platelets that may govern such interactions, and with activation of the coagulation system were determined. These data point to a substantial modulatory effect of ticagrelor in particular and place the regulation of platelet activation at the heart of systemic inflammation induced by LPS in humans.

To our knowledge, this is the first study to demonstrate marked suppression of response to bacterial endotoxaemia by platelet $P2Y_{12}$ inhibitors. Both $P2Y_{12}$ inhibitors potently reduced peak levels of D-dimer and major pro-inflammatory cytokines, including IL-6, TNFα, CCL2. In contrast to clopidogrel, ticagrelor also significantly reduced peak levels of IL-8 and G-CSF and increased the peak level of IL-10 compared to control. Additionally, ticagrelor reduced platelet-leukocyte aggregate formation, altered leukocyte trafficking and suppressed prothrombotic changes in fibrin clot ultrastructure. Since these changes in fibrin clot structure have been shown to shift the haemostatic balance towards thrombosis (Undas & Ariens, 2011), this represents a novel mechanism by which ticagrelor inhibits the prothrombotic consequences of systemic inflammation.

Inhibition of platelet-monocyte aggregate formation demonstrates a mechanism by which platelet $P2Y_{12}$ inhibition reduced systemic inflammation, as the formation of platelet-monocyte aggregates amplifies monocyte release of pro-inflammatory cytokines, including TNFα, CCL2 and IL-8 (Bournazos et al., 2008; Neumann et al.,

1997). Levels of residual platelet $P2Y_{12}$ reactivity before endotoxin administration significantly correlated with subsequent inflammatory and prothrombotic responses, suggesting that the responses were $P2Y_{12}$-mediated. Ticagrelor and clopidogrel belong to different chemical classes, inhibit platelet $P2Y_{12}$ receptors by different mechanisms, and do not have any structural similarities or shared metabolites. From a pharmacological perspective, shared non-$P2Y_{12}$-mediated effects are therefore unlikely. $P2Y_{12}$ receptors were originally identified to be almost exclusive to platelets, although they have now also been identified on a limited number of other cell types (Gachet, 2012). The extent to which leukocytes express $P2Y_{12}$ still remains unclear, particularly as platelets often contaminate isolated leukocyte preparations. In mice, dendritic cells express $P2Y_{12}$, which appears to mediate the secretion of certain cytokines, such as IL-12 (Gachet, 2012). This offers an additional mechanism by which $P2Y_{12}$ may mediate inflammatory responses, although it has not been established whether dendritic cells function in the same way in humans. Recent studies have demonstrated that vascular smooth muscle cells (VSMC) also express $P2Y_{12}$, which mediates CCL2 release (Satonaka et al., 2015). This may have contributed to the modulatory effect of $P2Y_{12}$ inhibition on inflammation, although it has been shown that clopidogrel does not inhibit VSMC $P2Y_{12}$ in rats, possibly due to the potential for nucleated cells to regenerate $P2Y_{12}$ (Grzesk et al., 2012). Ticagrelor is also a weak inhibitor of cellular uptake of adenosine, which increases extracellular levels of adenosine (Bonello et al., 2014). Since adenosine can increase macrophage production of IL-10, which is associated with a reduction in TNFα and IL-6, this offers a further mechanism by which ticagrelor may modify systemic inflammation (Haskó & Pacher, 2012).

LPS administration induced platelet-leukocyte aggregate formation, which was inhibited by ticagrelor in particular. Platelet-monocyte aggregate formation potentiates monocyte release of pro-inflammatory cytokines, including TNFα, CCL2 and IL-8, mediated by NF-κB (Bournazos et al., 2008; Neumann et al., 1997). Additionally, platelet-neutrophil aggregates have been implicated in the pathophysiology of acute lung injury (Zarbock et al., 2006) Therefore greater inhibition of the formation of platelet-leukocyte aggregates represents a mechanism by which ticagrelor may have conferred greater protection against excessive innate immune activation during sepsis compared to clopidogrel in the PLATO study. Our

data show that LPS administration induced an initial neutropaenia and monocytopaenia, which is held to be due to rapid sequestration of leukocytes to the endothelium (Coughlan, 1994). This was then followed by a rapid increase in neutrophil count due to bone marrow mobilization. It is notable that neutrophil counts were consistently higher in the ticagrelor group compared to the control group, despite lower overall levels of pro-inflammatory cytokines. This was apparent from very early time points onwards, which suggests that this was due to inhibition of leukocyte sequestration, rather than increased bone marrow mobilization, which would tend to have a more delayed effect. It is logical that inhibition of platelet-leukocyte aggregate formation reduces leukocyte sequestration, as formation of platelet-leukocyte aggregates upregulates leukocyte expression of adhesion molecules and facilitates adhesion to the endothelium (da Costa Martins et al., 2006).

In this study, and in other studies, platelet-expressed P-selectin was not increased at 30 minutes or 6 hours after LPS administration. This is likely to be due to the transient nature of platelet expression of P-selectin, which is shed by degranulated platelets, although they continue to function normally and aggregate (Michelson et al., 1996). It has been asserted that the formation of platelet-monocyte aggregates is therefore a more reliable marker of platelet activation *in vivo* (Michelson et al., 2001). The precise cause of thrombocytopenia related to systemic inflammation has still not been clarified and the relative contribution of platelet activation is unclear. Neither ticagrelor nor clopidogrel significantly attenuated the LPS-induced reduction in platelet count. The findings of our study therefore suggest that thrombocytopenia is not entirely mediated by platelet activation or the formation of platelet-monocyte aggregates, as $P2Y_{12}$ inhibitors inhibit these processes. The formation of platelet-neutrophil aggregates, or other processes where platelet $P2Y_{12}$ has a less prominent role, may therefore have a greater contribution towards thrombocytopenia.

This study provides a number of novel insights into potential mechanisms for increased risk of atherothrombotic events following bacteraemia and sepsis (Dalager-Pedersen et al., 2014). Although a prothrombotic state is well recognized in sepsis (Donzé et al., 2014), the underlying mechanisms are incompletely understood and the relative role of platelets has not been well defined. For the first time, this study demonstrates that exposure to bacterial LPS directly causes prothrombotic changes in the fibrin network that increase clot stability and confer resistance to fibrinolysis,

both of which shift the haemostatic balance towards thrombosis (Undas & Ariens, 2011). Platelet $P2Y_{12}$ inhibitors inhibited these prothrombotic effects of LPS and these results suggest that this may have been due to a reduction in levels of pro-inflammatory cytokines, as TNFα in particular has been shown to be a potent activator of the coagulation system *in vivo* (van der Poll et al., 1990). The greater overall effect of ticagrelor on these pathways compared to clopidogrel suggests a mechanism by which ticagrelor reduced cardiovascular death following infection in the PLATO study. The combined effect of ticagrelor, in particular, on leukocyte production of cytokines, leukocyte sequestration, platelet-leukocyte aggregate formation and subsequent changes in fibrin clot ultrastructure point to a substantial role for platelets in orchestrating the innate immune response to LPS. This suggests potential for timed platelet $P2Y_{12}$ inhibition in patients with infection to modify the risk of sepsis and associated thrombotic complications. In this study and in observational clinical studies, subjects were already taking $P2Y_{12}$ inhibitors at the onset of systemic inflammation and sepsis respectively. However, no studies have established the effect of administration of $P2Y_{12}$ inhibitors to patients with established sepsis. The greater infectious and inflammatory burden of established sepsis presents a different balance of risks and benefits and it is critical that any studies that investigate these medications in the context of sepsis carefully address the optimal timing of administration. In sepsis, excess fibrin deposition and impaired anticoagulant mechanisms lead to exhaustion of the coagulation cascade, causing coagulopathy and bleeding (Angus & van der Poll, 2013). Although antiplatelet medications normally exacerbate bleeding, this may therefore be counter-balanced by attenuating the prothrombotic state that drives the development of coagulopathy during sepsis. In support of this, antiplatelet medications are not associated with excess bleeding in patients with sepsis in observational studies (Akinosoglou et al., 2014). However, it is important to recognize that patients with established coagulopathy may have their antiplatelet medications discontinued in these studies and are less likely to benefit from these agents.

Ticagrelor and clopidogrel are some of the most commonly prescribed medications worldwide, due to their established benefit in the management of atherothrombosis. The results of this study therefore have potentially important clinical implications for millions of patients who are currently treated with these medications. In addition,

there is great interest in the use of specific immunomodulatory therapy for the treatment of acute coronary syndromes (Ridker & Luscher, 2014). The results of this study elucidate the background anti-inflammatory effects of medications that are already used for ACS. This is crucial information for determining the most appropriate inflammatory targets in the design of novel treatment strategies.

A limitation of this study is that clopidogrel has a less potent inhibitory effect in patient populations compared to its effects in healthy volunteers (Breet et al., 2010). This is not the case for ticagrelor, which has a consistently potent effect in patient populations (Gurbel et al., 2009). Therefore the results of this study may actually underestimate the additional efficacy of ticagrelor compared to clopidogrel in suppressing systemic inflammation in patients. Whilst this study demonstrates the key cellular and molecular pathways by which platelet $P2Y_{12}$ inhibitors could reduce mortality from sepsis, further randomized human studies are needed to determine whether this improves outcomes in patients.

In conclusion, this study demonstrates for the first time that clopidogrel and ticagrelor have a marked effect on multiple critical mechanisms involved in the pathophysiology of sepsis. This suggests a promising line of investigation for novel applications of $P2Y_{12}$ inhibitors in a syndrome that has proved elusive to almost all previous pharmacological strategies. The greater overall effect of ticagrelor compared to clopidogrel also provides critical mechanistic insight into the lower mortality following sepsis observed in the ticagrelor group compared to the clopidogrel group in the PLATO study.

5 Platelet $P2Y_{12}$ Inhibitors Potentiate the Expansion in Intermediate Monocyte Population that Occurs after Endotoxaemia

5.1 Introduction

Monocytes originate from the bone marrow and have major roles in inflammation and atherosclerosis. They replenish tissue macrophage and dendritic cell populations and also have phagocytic activity, release cytokines and are involved in antigen presentation and tissue repair (Ziegler-Heitbrock et al., 2015). Classical monocytes (CM) constitute approximately 80-90% of the monocyte population and have high expression of the LPS co-receptor CD14 but do not express the FcγIII receptor CD16 ($CD14^{++}CD16^{-}$) (Ziegler-Heitbrock et al., 2010). In contrast, intermediate and non-classical monocytes both express CD16. However, intermediate monocytes have high expression of CD14 ($CD14^{++}CD16^{+}$), whereas non-classical monocytes have low expression of CD14 ($CD14^{+}CD16^{+}$). Gene expression profiling and functional investigations have now identified distinct and often opposing roles of intermediate and non-classical monocytes (Wong et al., 2011). Prior to the revised definition in 2010 (Ziegler-Heitbrock et al., 2010), older studies defined monocytes only on the basis of whether they expressed CD16 or not and did not distinguish between intermediate and non-classical monocytes. This has led to many contradictory reports in the literature. Although still controversial, more recent evidence is converging to support roles of intermediate monocytes in producing the anti-inflammatory cytokine interleukin (IL) -10 (Shantsila et al., 2011) and non-classical monocytes in producing pro-inflammatory cytokines, such as TNFα (Mukherjee et al., 2015; Wong et al., 2011; Belge et al., 2002).

Specific expansion of the intermediate monocyte population has been identified in sepsis and other disorders involving systemic inflammation, which may be due to their mobilization from the marginal pool (Ziegler-Heitbrock, 2015; van Furth & Sluiter, 1986). Thrombotic conditions, such as acute coronary syndromes, are also associated with increased levels of intermediate monocytes (Tapp et al., 2012). Although levels of intermediate monocytes have been associated with tissue damage

and mortality (Rogacev et al., 2012; Tapp et al., 2012), it is unclear whether their role is deleterious or whether they may in fact be bystanders involved in tissue repair (Wong et al., 2012).

In addition to their central role in thrombosis, it is increasingly recognised that platelets also have a major role in systemic inflammation and innate immunity (Semple et al., 2011). Acute coronary syndromes, sepsis and many other inflammatory disorders induce the formation of platelet-monocyte aggregates. Platelet-monocyte interactions have a potentially important role in limiting monocyte mobilization from the marginal pool as they facilitate leukocyte adhesion to endothelium and upregulate monocyte expression of adhesion molecules (da Costa Martins et al., 2006). Platelet $P2Y_{12}$ receptor antagonists, such as clopidogrel and the more potent agent ticagrelor, inhibit platelet-monocyte interactions by down-regulating a central amplification pathway in platelets (Thomas & Storey, 2015a; Storey et al., 2000). The functional consequences of interactions between platelets and monocytes can therefore be investigated by using platelet $P2Y_{12}$ inhibitors. This offers potential insight into the pathophysiology of a rapidly increasing number of diseases where intermediate monocytes have been identified to play an important role, including sepsis, ACS, heart failure and a number of autoimmune conditions.

It was hypothesized that platelet-monocyte interactions regulate intermediate monocyte mobilization. Platelet-monocyte interactions were inhibited using platelet $P2Y_{12}$ inhibitors and the effects of this on intermediate monocyte mobilization were investigated during systemic inflammation in humans. A well-established experimental human model was used, which involves intravenous injection of *Escherichia coli* endotoxin (lipopolysaccharide; LPS) into healthy volunteers. To our knowledge, the effect of LPS on intermediate monocyte populations has not previously been established.

5.2 Marked expansion of the intermediate monocyte population following administration of LPS

Firstly, the effect of LPS administration on intermediate monocyte trafficking was characterized in subjects that did not receive antiplatelet medications (Figure 5.1). At 90 minutes there was a marked reduction in the total monocyte count and monocytes were almost undetectable in the peripheral blood, which is related to non-specific sequestration to the endothelium (Figure 5.1D). At six hours after LPS administration, the classical and intermediate monocyte counts returned to baseline levels, whereas there was still a significant reduction in the non-classical monocyte count (0.007 vs. 0.027 x 10^6/L; p=0.01; Figure 5.1D). At 24 hours, there was a marked 6-fold increase in the number of intermediate monocytes compared to baseline, 0.176 vs. 0.029 x10^6/L; p<0.001; Figure 5.1C and D).

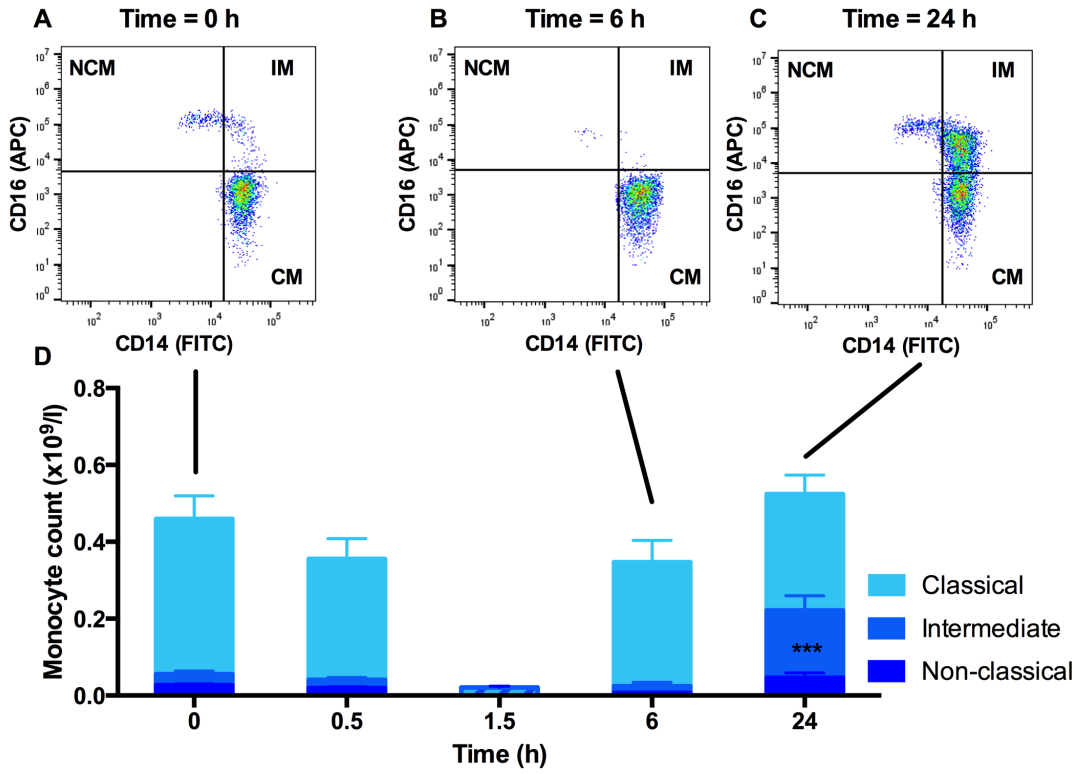

Figure 5.1 Monocyte phenotype and count over a 24-hour period after LPS administration.

Data expressed as mean ± SEM (n=10 in each group). Monocyte phenotype was not available at 1.5 hours and the total monocyte count as determined by the Sysmex automated cell counter is displayed instead. The overall effect of LPS and the effect of ticagrelor and clopidogrel (both compared to control at each time point) determined using 2-way ANOVA with Dunnett's correction for multiple comparisons (*p<0.05, **p<0.01 and ***p<0.001

5.3 Expanded population of intermediate monocytes has similar expression of adhesion molecules, chemokine receptors and toll-like receptors compared to the baseline population

Next, phenotypic differences between intermediate monocytes and classical and non-classical monocytes at baseline were determined in the subjects who had not received antiplatelet therapy. Intermediate monocytes expressed significantly lower levels of the chemokine receptors CCR2 and CXCR2 than classical monocytes (6,386 vs. 18,340 MF; $p<0.001$; Figure 5.2A and 3,068 vs. 6,916 MF; $p<0.001$; Figure 5.2B respectively). Intermediate monocytes expressed higher levels of the adhesion molecule CD11b and the toll-like receptor TLR2 than classical monocytes (25,250 vs. 14,713 MF; $p=0.01$; and 23,739 vs. 17,161 MF; $p=0.04$ respectively; Figure 5.2). Non-classical monocytes displayed significantly lower levels of CCR2, CXCR2, CD11b and TLR2 than intermediate monocytes (all $p<0.05$; Figure 5.2).

It was then demonstrated that the newly expanded intermediate monocyte population at 24 hours displayed similar expression of adhesion molecules, chemokine receptors and toll-like receptors as the baseline intermediate monocyte population (no significant difference in levels of CCR2, CXCR2, TLR2, TLR4, CD11b, CD14 and CD16).

By definition, if classical monocytes express CD16 at a higher level than the isotype threshold, then they are classified as intermediate monocytes. After LPS administration, there was a slight, but significant, overall increase in median fluorescence of CD16 in classical monocytes that were below the isotype threshold (Figure 5.1D). This level of CD16 expression was by definition still far lower than seen in intermediate monocytes, but may indicate that classical monocytes had started to express CD16 to some extent.

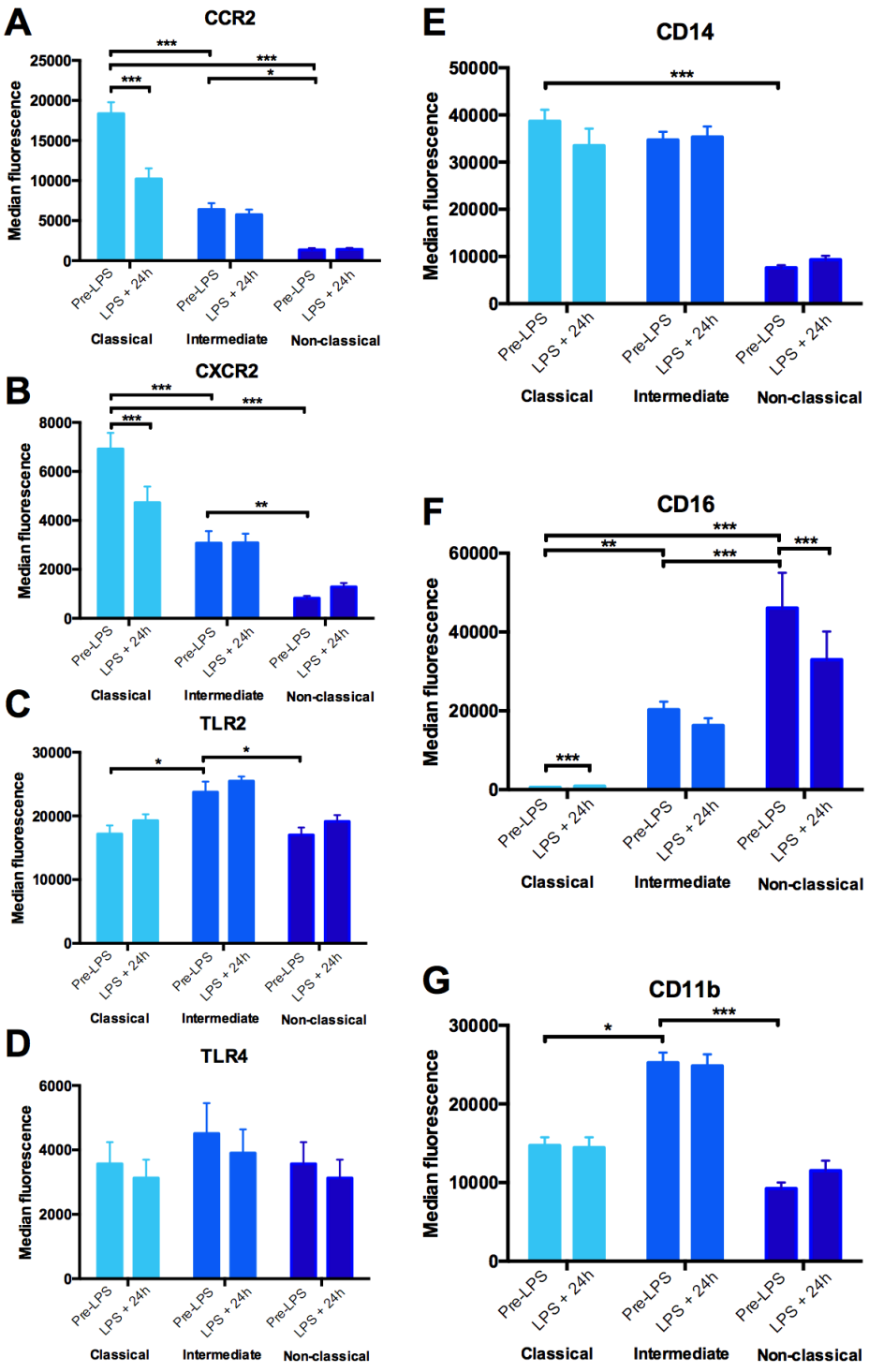

Figure 5.2 Monocyte expression of CD14, CD16, CD11b, CCR2, CXCR2, TLR2 and TLR4 before and 24 hours after LPS administration.

Data expressed as mean ± SEM (n=10 in each group). The effect of LPS and the differences between monocyte phenotypes were determined using 2-way ANOVA with Bonferroni correction for multiple comparisons (*p<0.05, **p<0.01 and ***p<0.001).

5.4 Platelet $P2Y_{12}$ inhibition augments the expansion of the intermediate monocyte population

Platelet-monocyte interactions potentiate endothelial adhesion of monocytes by forming P-selectin-mediated bridges between monocytes and the endothelium and by upregulating monocyte expression of adhesion receptors. Since platelet $P2Y_{12}$ receptors have a central role in platelet activation and platelet-monocyte interactions, it was investigated whether platelet $P2Y_{12}$ inhibitors affected the expansion of the intermediate monocyte population. At 24 hours after LPS administration, there was a striking 9-fold increase in the intermediate monocyte population in subjects treated with clopidogrel (0.344 vs. 0.038 x 10^6/L; $p<0.001$; Figure 5.3) and a 7-fold increase in subjects treated with ticagrelor (0.279 vs. 0.038 x 10^6/L; $p<0.001$; Figure 5.3) compared to the baseline timepoint. These increases were significantly greater than seen in the group that did not receive $P2Y_{12}$ inhibitors ($p<0.001$ and $p=0.005$ for clopidogrel and ticagrelor respectively; Figure 5.3).

In the clopidogrel group, 24 hours after LPS administration, there was a slight, but significant increase in classical monocyte expression of CD16 (Table 5.1), although this was by definition far lower than the level of CD16 expression in intermediate monocytes (Table 5.1). There were no other significant effects of clopidogrel or ticagrelor on classical, intermediate and non-classical monocyte expression of CD14, CD16, CD11b, CCR2, CXCR2, TLR4 or TLR2 (all $p > 0.05$; Table 5.1 and Table 5.2).

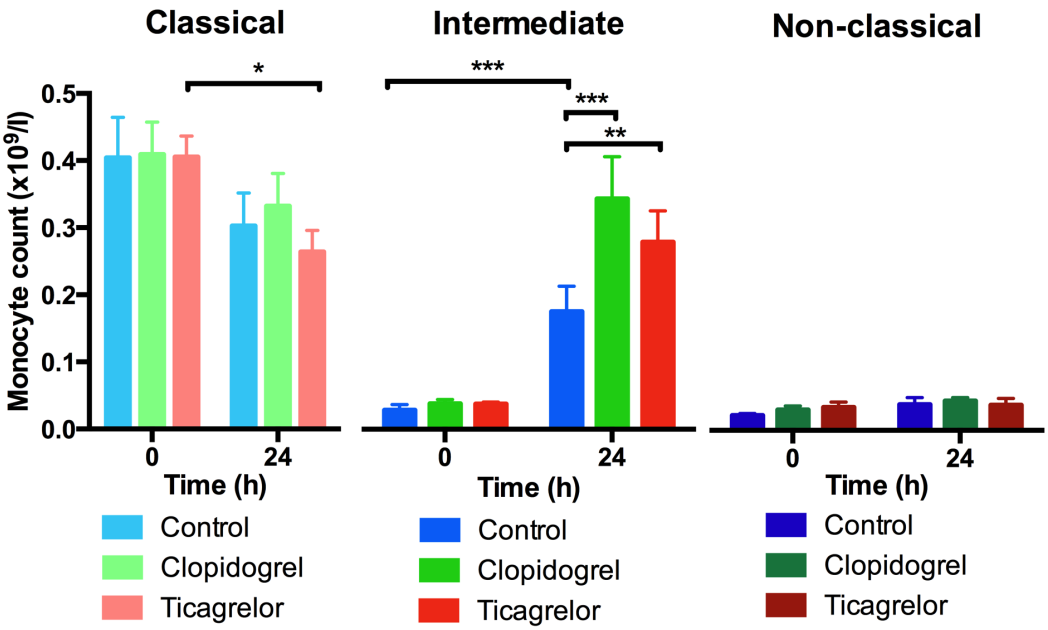

Figure 5.3 Effect of platelet $P2Y_{12}$ inhibitors, clopidogrel and ticagrelor, on monocyte phenotype and count before and 24-hours after LPS administration.

Data expressed as mean ± SEM (n=10 in each group). The overall effect of LPS and the effect of ticagrelor and clopidogrel (both compared to control at each time point) were determined using 2-way ANOVA with Dunnett's correction for multiple comparisons (*p<0.05, **p<0.01 and ***p<0.001).

Table 5.1 Effect of clopidogrel and ticagrelor on classical, intermediate and non-classical monocyte expression of CD14, CD16 and CD11b 24 hours after LPS administration.

	Control	Clopidogrel	P value for clopidogrel vs. control	Ticagrelor	P value for ticagrelor vs. control
Classical CD14 (MF)	33498 ± 3588	34943 ± 1778	0.91	36906 ± 2100	0.60
Intermediate CD14 (MF)	35299 ± 2255	32955 ± 1521	0.71	35887 ± 1997	0.98
Non-classical CD14 (MF)	9323 ± 841	9688 ± 519	0.87	9074 ± 427	0.93
Classical CD16 (MF)	862 ± 82	1073 ± 46	0.0057	921 ± 72	0.61
Intermediate CD16 (MF)	16257 ± 1868	23272 ± 1765	0.12	22285 ± 2748	0.20
Non-classical CD16 (MF)	32980 ± 7168	54819 ± 731	0.21	54767 ± 10760	0.21
Classical CD11b (MF)	14449 ± 1306	15841 ± 1521	0.89	13929 ± 1246	0.98
Intermediate CD11b (MF)	24829 ± 1486	25919 ± 2148	0.96	21251 ± 2077	0.63
Non-classical CD11b (MF	11506 ± 1286	12963 ± 939	0.85	10759 ± 973	0.96

Table 5.2 Effect of clopidogrel and ticagrelor on classical, intermediate and non-classical monocyte expression of CCR2, CXCR2, TLR4 and TLR2 24 hours after LPS administration.

	Control	Clopidogrel	P value for clopidogrel vs. control	Ticagrelor	P value for ticagrelor vs. control
Classical CCR2 (MF)	10195 ± 1337	12457 ± 1263	0.37	12927 ± 1197	0.24
Intermediate CCR2 (MF)	6386 ± 659	6583 ± 536	0.99	5521 ± 910	0.74
Non-classical CCR2 (MF)	1414 ± 176	1469 ± 172	0.99	2025 ± 735	0.46
Classical CXCR2 (MF)	4721 ± 663	5377 ± 410	0.64	5684 ± 361	0.40
Intermediate CXCR2 (MF)	3083 ± 373	2703 ± 148	0.76	2856 ± 155	0.91
Non-classical CXCR2 (MF)	1281 ± 163	1128 ± 88	0.97	1351 ± 213	0.99
Classical TLR4 (MF)	3125 ± 575	3490 ± 465	0.89	2415 ± 570	0.64
Intermediate TLR4 (MF)	3905 ± 730	4115 ± 467	0.97	3073 ± 841	0.64
Non-classical TLR4 (MF)	3125 ± 575	3490 ± 465	0.89	2415 ± 570	0.64
Classical TLR2 (MF)	19264 ± 1008	19553 ± 662	0.98	18416 ± 1207	0.87
Intermediate TLR2 (MF)	25468 ± 725	25185 ± 968	0.99	22898 ± 1584	0.49
Non-classical TLR2 (MF)	19144 ± 998	21360 ± 807	0.42	17909 ± 1536	0.75

5.5 Higher intermediate monocytes counts at 24 hours were associated with higher neutrophil and non-classical monocyte counts and lower levels of platelet reactivity

It was then determined whether the increase in intermediate monocyte count was associated with alterations in dynamics of other leukocytes and whether this was related to distinct changes in cytokine release or platelet reactivity. As described in the previous chapter, LPS administration significantly increased levels of IL-6, IL-8, IL-10, TNFα, G-CSF and CCL2, which peaked between 2-4 hours and also increased levels of CRP, which peaked at 24 hours. It was demonstrated that platelet $P2Y_{12}$ inhibitors reduce platelet-monocyte interactions, which is associated with reduced pro-inflammatory cytokine release and an attenuation of the prothrombotic response to LPS.

The dataset is complex as there are a large number of variables at multiple timepoints. A state-of-the-art statistical method, known as random forests, was therefore used to identify relationships between the rise in intermediate monocyte count and changes in the other variables. The strengths of this approach involve the ability to robustly assess complex non-linear relationships and complex interactions between variables in hundreds or thousands of variables, even when the sample size is limited as in this case. Random forests showed that the following were the most important associations with the intermediate monocyte count at 24 hours: neutrophil count at 6 hours after LPS ($p < 0.001$; Figure 5.4A), non-classical monocyte count at 24 hours ($p = 0.001$; Figure 5.4B), neutrophil count at 4 hours ($p = 0.003$) and platelet-monocyte reactivity to ADP assessed at baseline ($p=0.01$; Figure 5.4C). In the random forests model, these 4 variables accounted for 57% of the variation in the intermediate monocyte count at 24 hours ($p < 0.001$). The random forest model was validated by training on the first 80% of subjects and ensuring accuracy of predictions in the final unseen 20% ($R^2 = 0.66$; $p = 0.01$; see Appendix). Standard Spearman correlation also confirmed significant positive correlation between the intermediate monocyte count at 24 hours and the neutrophil count at 6 hours ($\rho = 0.48$; $p = 0.007$; Figure 5.4A) and non-classical monocyte count at 24 hours ($\rho = 0.42$; $p = 0.02$; Figure 5.4B). In contrast, there was an inverse relationship between platelet reactivity at baseline (as assessed by the formation platelet-monocyte

aggregates in response to ADP *ex vivo*) and the subsequent levels of intermediate monocytes at 24 hours after LPS administration ($\rho = -0.39$; $p=0.04$).

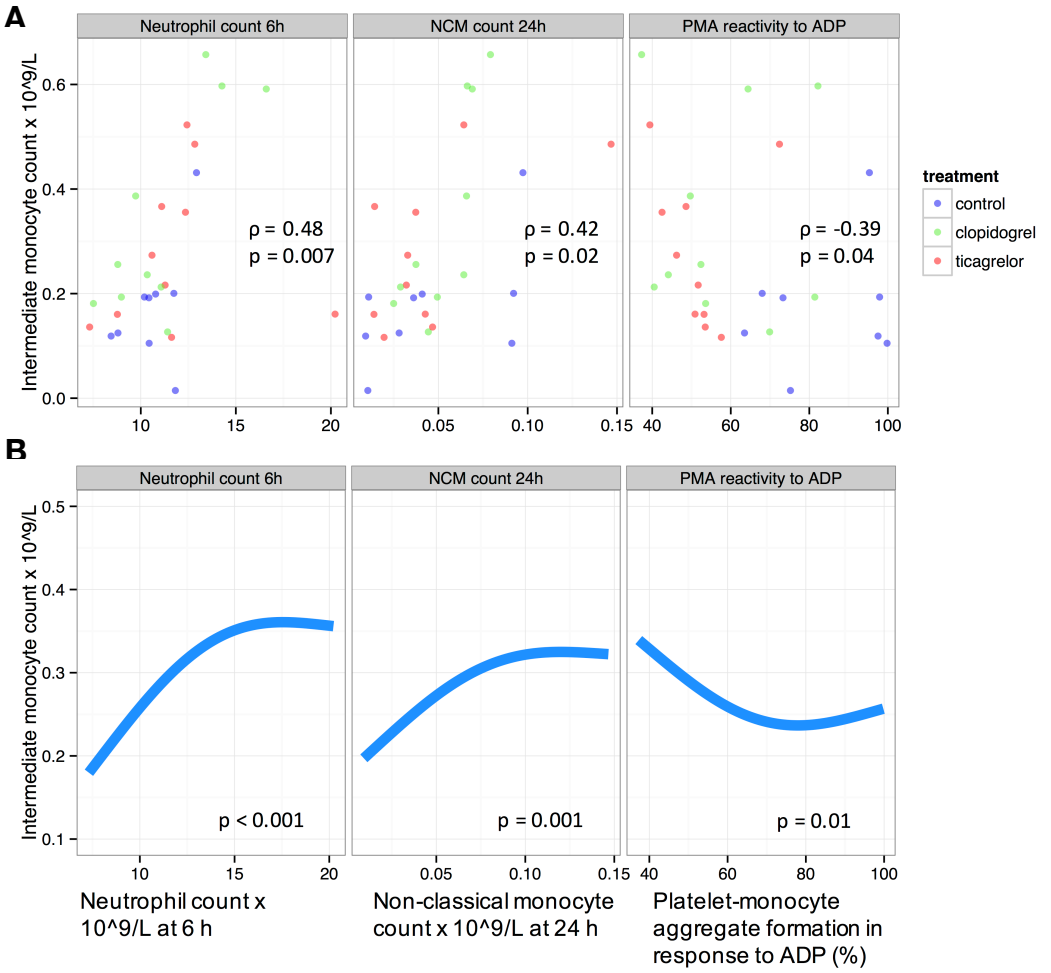

Figure 5.4 Correlation (A) between intermediate monocyte count at 24 hours and the neutrophil count, non-classical monocyte count and formation of platelet-monocyte aggregates in response to ADP at baseline. Random forest partial dependence plots (B) showing partial dependence of intermediate monocyte count at 24 hours on neutrophil count, non-classical monocyte count and formation of platelet-monocyte aggregates in response to ADP at baseline.

Correlation co-efficient determined using Spearman's rank. Random forest analyses performed as detailed in methods section and appendix.

5.6 LPS has differential effects on classical, intermediate and non-classical monocytes *in vitro*

Monocyte expression of CD14, CD16 and CD11b were determined after 1 hour of stimulation with 10 and 100 ng/ml of LPS *in vitro*, in order to assess the direct effects of LPS on each of the monocyte phenotypes (see 3.1.14). In the samples that had been pre-incubated with the vehicle control (DMSO), classical monocyte expression of CD14 significantly increased following exposure to 10 ng/ml LPS compared to PBS (73,997 ± 4726 MF vs 58795 ± 7831; $p = 0.009$; Figure 5.5). There was no additional effect of 100 ng/ml LPS compared to 10 ng/ml LPS and there were no modulatory effects of ticagrelor (all $p > 0.05$; Figure 5.5). In the DMSO samples, 10 ng/LPS also increased intermediate monocyte expression of CD14 compared to PBS (60,234 ± 9,400 vs. 48,973 ± 6679; $p = 0.007$; Figure 5.5). Again, there was no further increase with 100 ng/ml LPS and there were no modulatory effects of ticagrelor. Non-classical monocyte CD14 expression was lower at baseline and did not significantly increase following exposure to LPS (Figure 5.5).

In the DMSO samples, there was a low-level significant increase in classical monocyte median fluorescence of CD16 following exposure to 10 ng/ml LPS compared to PBS (926 ± 150 vs. 658 ± 86; $p = 0.03$; Figure 5.5). There was no additional effect of 100 ng/ml LPS and no modulatory effect of ticagrelor (Figure 5.5). In contrast, in the DMSO samples, there was a significant reduction in intermediate monocyte expression of CD16 16,767 ± 4,043 vs. 7,511 ± 1,284; $p = 0.003$; Figure 5.5). There was no additional effect of 100 ng/ml LPS and no significant modulatory effect of ticagrelor. In the DMSO samples, 10 ng/ml LPS also decreased non-classical monocyte expression of CD16 compared to PBS (44,678 ± 3062 vs. 15,496 ± 1,682; $p < 0.001$; Figure 5.5). There was no additional effect of 100 ng/ml LPS and no significant effect of ticagrelor (Figure 5.5).

In the DMSO samples, 10 ng/ml LPS significantly increased classical and intermediate monocyte expression of CD11b compared to PBS (117,0000 ± 8,892 vs. 61,2444 ± 13,446; $p < 0.001$; Figure 5.5). There was no additional effect of 100 ng/ml and no significant modulatory effects of ticagrelor. In the DMSO samples, 10 ng/ml LPS also significantly increased intermediate monocyte expression of CD11b compared to PBS (110,538 ± 5,310 vs. 55,658 ± 11,646; $p < 0.001$; Figure 5.5).

Similarly, there was no additional effect of 100 ng/ml LPS and no modulatory effect of ticagrelor. LPS did not increase non-classical monocyte expression of CD11b and there were no significant modulatory effects of ticagrelor (Figure 5.5).

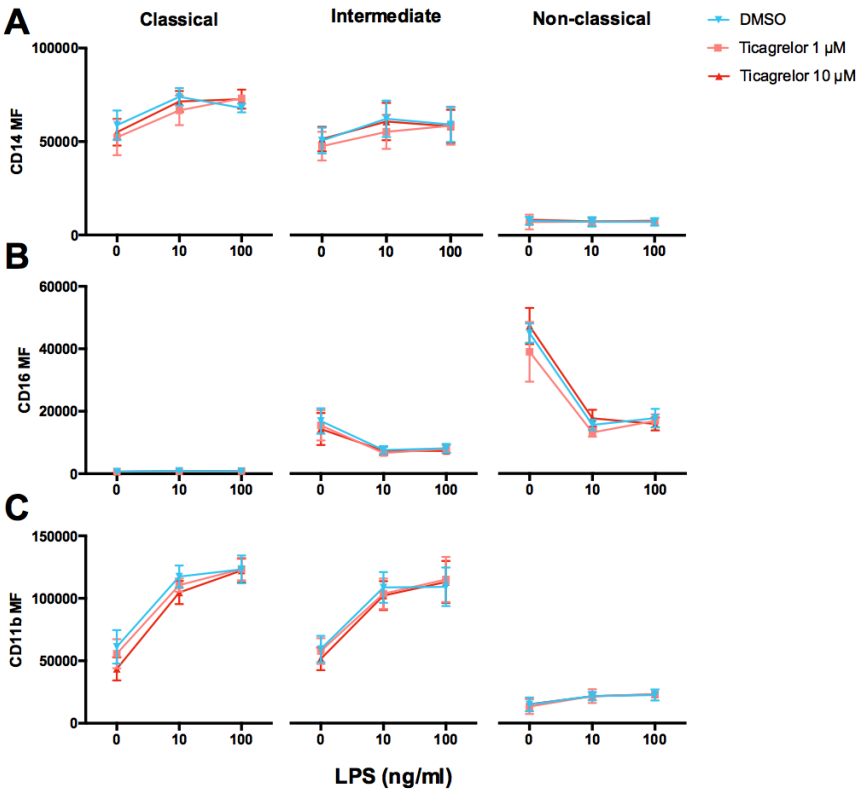

Figure 5.5 Dose-dependent effects of LPS and the impact of ticagrelor on monocyte expression of CD14 (A), CD16 (B) and CD11b (C).

Data expressed as mean ± SEM (n=4). Effect of LPS and ticagrelor (both compared to control) determined using 2-way ANOVA with Dunnett's correction for multiple comparisons (*p<0.05, **p<0.01 and ***p<0.001).

5.7 Effect of LPS on monocyte adhesion molecules, chemokine receptors and toll-like receptors is not directly influenced by $P2Y_{12}$ inhibition *in vitro*

Whole blood was incubated with LPS (100 ng/ml) for 1 hour at 37° C and classical, intermediate and non-classical monocyte expression of adhesion molecules, chemokine receptors and toll-like receptors was investigated by flow cytometry. The modulatory effects of ticagrelor and cangrelor were investigated (see 3.1.14).

Changes in monocyte expression of CD14, CD16 and CD11b were similar to the results described in the previous section (Figure 5.6). There were no significant modulatory effects of ticagrelor or cangrelor.

In the unstimulated DMSO samples, classical monocyte expression of CCR2 was higher than in intermediate monocytes (18,342 ± 6,859 vs. 8,835 ± 4,115; $p = 0.005$; Figure 5.7) and non-classical monocytes (18,342 ± 6,859 vs. 3,288 ± 639; $p < 0.001$; Figure 5.7). LPS decreased classical monocyte expression of CCR2 compared to PBS (13,394 ± 4,497 vs. 18,342 ± 6,859; $p = 0.006$), but had no significant effect on intermediate or non-classical monocytes (Figure 5.7). There were no significant modulatory effects of ticagrelor or cangrelor (Figure 5.7). Levels of expression of CXCR2 were similar on classical, intermediate and non-classical monocytes and there was no effect of LPS or modulatory effect of ticagrelor of cangrelor (Figure 5.7).

In the unstimulated DMSO samples, intermediate monocyte expression of TLR4 was higher than in classical monocytes (10,877 ± 1,004 vs. 5,309 ± 1,532; $p = 0.007$; Figure 5.8). There was no significant change in TLR4 expression in response to LPS and there was no modulatory effect of ticagrelor (Figure 5.8). In the unstimulated DMSO samples, intermediate monocytes had higher expression of TLR2 than classical monocytes, which had higher expression than non-classical monocytes (each comparison $p < 0.05$; Figure 5.8). In the DMSO samples, LPS significantly increased classical monocyte TLR2 compared to PBS (54,686 ± 8006 vs. 41,935 ± 9007; $p = 0.02$; Figure 5.8). LPS did not significantly affect intermediate or non-classical monocyte TLR2 and there were no modulatory effects of ticagrelor and cangrelor (Figure 5.8).

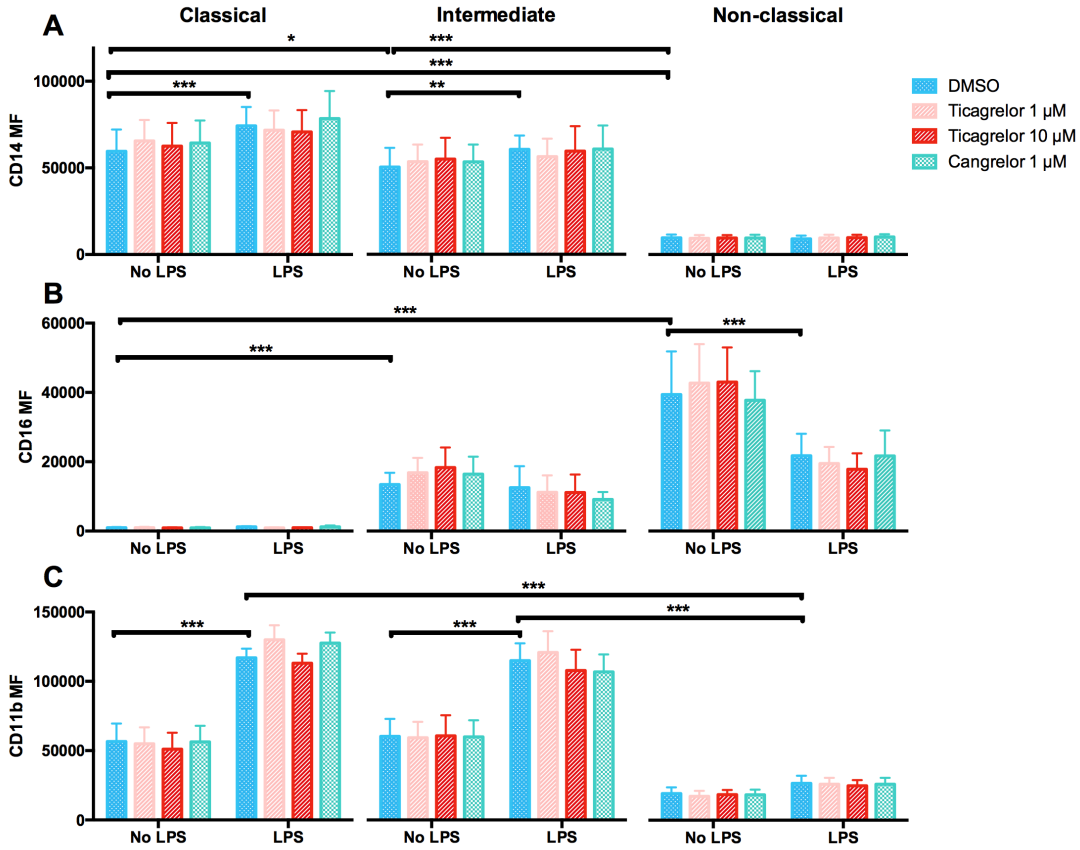

Figure 5.6 Effect of LPS on classical, intermediate and non-classical monocyte expression of CD14 (A), CD16 (B) and CD11b (C).

Data expressed as mean ± SEM (n=7). Effect of LPS and cangrelor and ticagrelor (each compared to their respective control) determined using 2-way ANOVA with Dunnett's correction for multiple comparisons (*p<0.05, **p<0.01 and ***p<0.001).

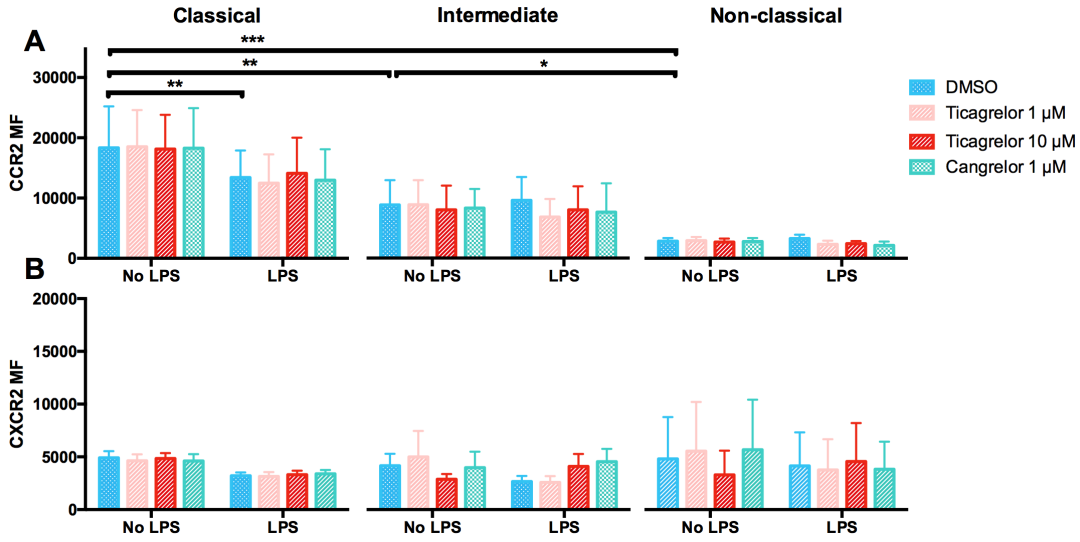

Figure 5.7 Effect of LPS on classical, intermediate and non-classical monocyte expression of the chemokine receptors CCR2 (A) and CXCR2 (B).

Data expressed as mean ± SEM (n=4). Effect of LPS and cangrelor and ticagrelor (each compared to their respective control) determined using 2-way ANOVA with Dunnett's correction for multiple comparisons = (*p<0.05, **p<0.01 and ***p<0.001).

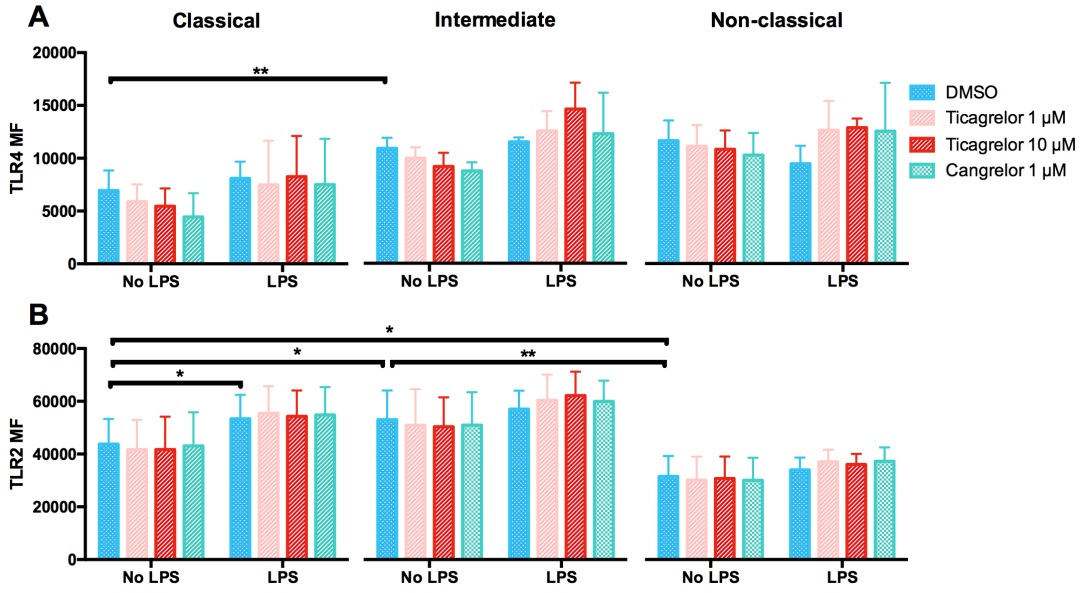

Figure 5.8 Effect of LPS on classical, intermediate and non-classical monocyte expression of the toll-like receptors TLR4 (A) and TLR2 (B).

Data expressed as mean ± SEM (n=3). Effect of LPS and cangrelor and ticagrelor (each compared to their respective control) determined using 2-way ANOVA with Dunnett's correction for multiple comparisons (*p<0.05, **p<0.01 and ***p<0.001).

5.8 Discussion

The intermediate monocyte population has recently been shown to increase in number during sepsis, ACS and heart failure (Mukherjee et al., 2015; Wrigley et al., 2013; Tapp et al., 2012). It has been suggested that this increase in intermediate monocyte count may be due to mobilization from the marginal pool of monocytes that reside in close association with the endothelium in the spleen, lungs and liver (Ziegler-Heitbrock, 2015; Ginhoux & Jung, 2014).

TLR4-mediated response to LPS is central to the pathophysiology of gram negative sepsis (Schouten et al., 2008). It was hypothesized that mobilization of the intermediate population during sepsis is due to response to LPS. The effect of LPS on intermediate monocyte mobilization was investigated by intravenous injection of LPS into healthy volunteers. Platelet-monocyte interactions increase monocyte expression of adhesion molecules and immobilized, endothelium-bound platelets directly facilitate monocyte adhesion to the endothelium. It was therefore also hypothesized that platelet-monocyte interactions may limit monocyte mobilization from the marginal pool. Intermediate monocyte mobilization was investigated in the presence and absence of the $P2Y_{12}$ inhibitors clopidogrel and ticagrelor, which inhibit platelet-monocyte interactions as demonstrated in Chapter 4.

The results of this chapter provide several novel findings relating to the mobilization of intermediate monocytes in response to systemic inflammation induced by LPS in humans. LPS caused a marked expansion of the intermediate monocyte population and this was potentiated by platelet $P2Y_{12}$ inhibitors, which inhibit platelet-monocyte interactions. At 24 hours after LPS administration there was a 6-fold rise in the intermediate monocyte count in subjects that had not received $P2Y_{12}$ inhibitors. In addition to differences in CD14 and CD16, intermediate monocytes express greater levels of TLR2 and CD11b and lower levels of the chemokine receptors CCR2 and CXCR2 than classical monocytes. The greatly expanded population of intermediate monocytes appeared phenotypically similar to the baseline intermediate monocytes as they expressed similar levels of CD14, CD16, CD11b, CCR2, CXCR2, TLR2 and TLR4. The much higher proportion of intermediate monocytes at 24 hours after LPS may therefore modify subsequent inflammatory responses by altering the balance of

expression of adhesion molecules, toll-like receptors and chemokine receptors compared to classical monocytes.

The increase in intermediate monocyte population at 24 hours after LPS administration was associated with higher neutrophil and non-classical monocyte counts. This likely reflects that a similar physiological process underlies mobilization of intermediate monocytes, neutrophils and non-classical monocytes. The platelet $P2Y_{12}$ inhibitors clopidogrel and ticagrelor significantly potentiated the increase in intermediate monocyte count. Furthermore, the increase in intermediate monocyte count was greatest in subjects who displayed reduced platelet-monocyte aggregate formation in response to ADP. Platelet-monocyte interactions increase the adhesiveness of monocytes to endothelium, which may limit monocyte mobilization from the marginal pool. Inhibition of platelet-monocyte interactions therefore represents a mechanism by which platelet $P2Y_{12}$ inhibition may potentiate mobilization of intermediate monocytes. Interestingly though, clopidogrel was associated with a non-statistically significant greater number of intermediate monocytes at 24 hours than ticagrelor, despite lower levels of platelet $P2Y_{12}$ inhibition than ticagrelor. This difference between the drugs was not statistically significant and is therefore difficult to interpret. Ticagrelor increases plasma levels of adenosine, whereas clopidogrel does not (Bonello et al., 2014). As adenosine is recognised to alter monocyte differentiation (Merrill et al., 1997), it is therefore possible that this mechanism may cause additional non-$P2Y_{12}$ mediated effects on monocyte dynamics. Although the marginal pool is an established source of CD16+ monocytes, it is largely unknown whether intermediate monocytes in humans can be derived from classical monocytes or whether they are produced directly by the bone marrow. *In-vitro* experiments did not reveal any clear direct effects of either LPS or ticagrelor on monocyte phenotype.

It is well-established that both clopidogrel and ticagrelor reduce the incidence of adverse cardiovascular events in patients with ACS (Grove et al., 2015). Additionally, platelet $P2Y_{12}$ inhibitors have been associated with a reduced risk of mortality during sepsis (Tsai et al., 2015). Further work is therefore needed to establish whether platelet $P2Y_{12}$ inhibitors alter intermediate monocyte populations in these diseases and whether this contributes to their benefit or whether it represents an adverse off-target effect. In addition, further work is needed to understand the

functional role of intermediate monocytes in these conditions. Although initial reports from *in vitro* experiments with isolated cells suggested that intermediate monocytes may produce pro-inflammatory cytokines, such as TNFα, more recent investigations in whole blood have demonstrated that intermediate monocytes may actually produce anti-inflammatory cytokines such as IL-10 (Mukherjee et al., 2015). Higher levels of intermediate monocytes are associated with increased mortality in patients with ACS, but it is not known whether this simply reflects a greater ischaemic and inflammatory insult leading to a confounding increase in mortality. In fact, higher levels of intermediate monocytes have been associated with reduced left ventricular function in particular (Stansfield et al. 2015), which raises the possibility that they may be involved in myocardial repair.

In conclusion, there is a marked expansion of the intermediate monocyte count following bacterial endotoxaemia and this is potentiated by platelet $P2Y_{12}$ inhibitors, which inhibit platelet-monocyte interactions. Further work is needed to clarify the role of intermediate monocytes in sepsis, ACS and other inflammatory conditions.

6 Adenosine-mediated effects of ticagrelor on innate immune activation *in vitro*

6.1 Introduction

The main mechanism of action of ticagrelor is potent platelet $P2Y_{12}$ inhibition. However, in addition, ticagrelor is also known to weakly inhibit cellular uptake of adenosine (van Giezen et al., 2012), which has been shown to increase extracellular levels of adenosine (Bonello et al., 2014). Although ticagrelor causes relatively weak inhibition of adenosine uptake (Armstrong et al., 2014), the effects appear to be clinically significant, because ticagrelor potentiates the effects of adenosine on coronary blood flow and adenosine-induced dyspnoea in patients (Alexopoulos et al., 2013; Wittfeldt et al., 2013). Furthermore, in animal models, ticagrelor reduces myocardial infarct size and improves subsequent remodelling, mediated by adenosine (Ye et al., 2015; Nanhwan et al., 2014).

Ticagrelor may therefore have additional non-$P2Y_{12}$ mediated effects on innate immune activation due to the effect of adenosine. Adenosine is produced from the degradation of ATP and is released during cellular stress, such as during hypoxia, ischaemia and inflammation. The effects of adenosine are mediated by 4 G-protein-coupled adenosine receptors, namely A_1, A_{2A}, A_{2B} and A_3, which are ubiquitously expressed (Haskó & Pacher, 2012). A_1 and A_3 receptors are coupled to G_i and decrease intracellular levels of cAMP, whereas A_{2A} and A_{2B} are generally considered to be coupled to G_s and G_q and increase intracellular levels of cAMP (Barletta et al., 2012; Haskó & Pacher, 2012). A_1 and A_3 receptors have high affinity and are activated at nanomolar concentrations of adenosine, whereas A_{2A} and A_{2B} receptors have lower affinity and are activated at micromolar concentrations of adenosine (Barletta et al., 2012; Haskó & Pacher, 2012). To further complicate matters, leukocytes can produce adenosine and the expression of adenosine receptors is also dynamic (Barletta et al., 2012; Haskó & Pacher, 2012). The effects of adenosine are therefore complex and adenosine can either have stimulatory or suppressive effects depending on the concentration of adenosine in the microenvironment (Barletta et al., 2012; Haskó & Pacher, 2012).

At nanomolar concentrations, adenosine has predominantly pro-inflammatory effects and upregulates a number of monocyte/macrophage and neutrophil pro-inflammatory functions, including phagocytosis and chemotaxis (Table 6.1 and Table 6.2). At higher concentrations, adenosine has predominantly anti-inflammatory effects and limits excessive innate immune activation by downregulating phagocytosis, chemotaxis and release of TNFα (Table 6.1 and Table 6.2).

Erythrocytes rapidly take up adenosine by ENT1, resulting in a very short half life of less than 10 seconds in the blood. However, ENT1 is also expressed by a wide range of cell types and this mechanism therefore also regulates adenosine levels within tissues. As ticagrelor inhibits ENT1 and therefore inhibits erythrocyte uptake of adenosine, it was hypothesized that this would preserve the effects of adenosine on leukocytes in the presence of erythrocytes. The impact of adenosine uptake inhibition on leukocytes was therefore investigated by studying the effect of adenosine on leukocytes in the presence and absence of erythrocytes in the presence or absence of ticagrelor and another more potent ENT1 inhibitor, dipyridamole (Armstrong et al., 2014). Work in our department established that adenosine potentiates chemotaxis in isolated neutrophils and that this effect is abolished in the presence of erythrocytes (Alsharif et al., 2015). The effect of adenosine is preserved in the presence of ticagrelor or dipyridamole however (Alsharif et al., 2015). The work included in this chapter demonstrates the effects of this mechanism on phagocytosis of isolated neutrophils. In addition, the effects of this mechanism on leukocyte activation were also investigated by measuring neutrophil and monocyte expression of the adhesion molecule CD11b in response to the leukocyte agonists LPS and FMLP.

Table 6.1 Influence of adenosine receptors on neutrophil function

	A_1	A_3	A_{2A}	A_{2B}
Phagocytosis	↑ (Zalavary et al., 1994; Salmon & Cronstein, 1990)		↓ (Zalavary et al., 1994; Salmon & Cronstein, 1990)	
Chemotaxis	↑ (Cronstein et al., 1990; Rose, 1988)	↑ (Chen, Corriden, et al., 2006)	↓ (Cronstein et al., 1990)	
Apoptosis			↓ (Yasui et al., 2000; Walker et al.)	
Adhesion	↑ (Cronstein et al., 1992)	↓ (S. Armstrong & Ganote, 1994)	↓ (Sullivan et al., 2004)	
Transmigration				↓ (Wakai et al., 2001)
Superoxide production	↑ (Salmon & Cronstein, 1990)		↓ (Visser et al., 2000; Sullivan et al., 1999; 1995; Stewart & Harris, 1993; Kubersky et al., 1989)	↓ (van der Hoeven et al., 2011)
Degranulation		↓ (Bouma et al., 1997)	↓ (Sullivan et al., 2004; Visser et al., 2000; Bouma et al., 1997; Richter, 1992)	

Pro-inflammatory effects indicated by a red arrow and anti-inflammatory effects indicated by a blue arrow.

Table 6.2 Influence of adenosine receptors on mononuclear phagocyte function

	A_1	A_3	A_{2A}	A_{2B}
Differentiation into giant cells	↑ (Merrill et al., 1997)		↓ (Merrill et al., 1997)	
Phagocytosis	↑ (Salmon et al., 1993)		↓ (Salmon et al., 1993)	
Chemotaxis	↑ (Schnurr et al., 2004)		↓ (Schnurr et al., 2004)	
Superoxide generation			↓ (Elliott & Leonard, 1989)	
Nitric oxide release	↓ (Haskó, Szabó, Nemeth, Kvetan, Pastores, & Vizi, 1996a)		↓ (Haskó, Szabó, Nemeth, Kvetan, Pastores, & Vizi, 1996b)	
IL-12 release			↓ (Haskó et al., 2000)	
TNFα release	↓ (Haskó, Szabó, Nemeth, Kvetan, Pastores, & Vizi, 1996a)	↓ (Haskó, Szabó, Nemeth, Kvetan, Pastores, & Vizi, 1996a)	↓ (Haskó et al., 2000; Haskó, Szabó, Nemeth, Kvetan, Pastores, & Vizi, 1996a)	↓ (Kreckler et al., 2006)
MIP-1α release		↓ (Szabó et al., 1998)	↓ (Szabó et al., 1998)	
IL-10 release	↑ (Haskó, Szabó, Nemeth, Kvetan, Pastores, & Vizi, 1996a)	↑ (Haskó, Szabó, Nemeth, Kvetan, Pastores, & Vizi, 1996b)	↑ (Haskó, Szabó, Nemeth, Kvetan, Pastores, & Vizi, 1996b)	

Pro-inflammatory effects indicated by a red arrow and anti-inflammatory effects indicated by a blue arrow.

6.2 Ticagrelor potentiates adenosine-induced stimulation of neutrophil phagocytosis

The effect of adenosine, at low (10^{-8} M) and high concentrations (10^{-5} M), was determined on phagocytosis of opsonized *S.pneumoniae (*pneumococcus) by neutrophils over a 30 minute period (see 3.2.4).

In isolated neutrophils, a nanomolar concentration of adenosine (10^{-8} M) significantly increased the percentage of neutrophils phagcoytosing *S.pneumoniae* compared to the vehicle control RPMI (51 ± 4% vs. 43 ± 2%; p = 0.005; Figure 6.1) and significantly increased the phagocytic index (98 ± 21 vs. 60 ± 10; p = 0.04). In contrast, a micromolar concentration of adenosine (10^{-5} M) had no significant effect (Figure 6.1).

The effect of adenosine was then investigated in the presence of erythrocytes (red blood cells [RBC]). It was necessary to dilute the concentration of erythrocytes to allow assessment of neutrophil phagocytosis be microscopy. In samples that had been treated with the vehicle control (DMSO), neither a nanomolar or micromolar concentration of adenosine had any effect on the percentage of neutrophils phagocytizing or the phagocytic index (Figure 6.2A and B). However, in samples that had been pre-treated with ticagrelor (10 μM), the stimulatory effect of a nanomolar concentration of adenosine was preserved. In these samples, adenosine (10^{-8} M) significantly increased the percentage of neutrophils phagocytizing compared to control (16 ± 2% vs. 11 ± 2%; p = 0.002; Figure 6.2A and B).

The mechanism by which ticagrelor potentiated the effect of adenosine was then investigated by the use of the specific A_1 receptor antagonist DPPX. Ticagrelor no longer preserved the effect of adenosine when DPCPX was present, demonstrating that the stimulatory effect was mediated by adenosine A_1 receptors (Figure 6.2C and D)

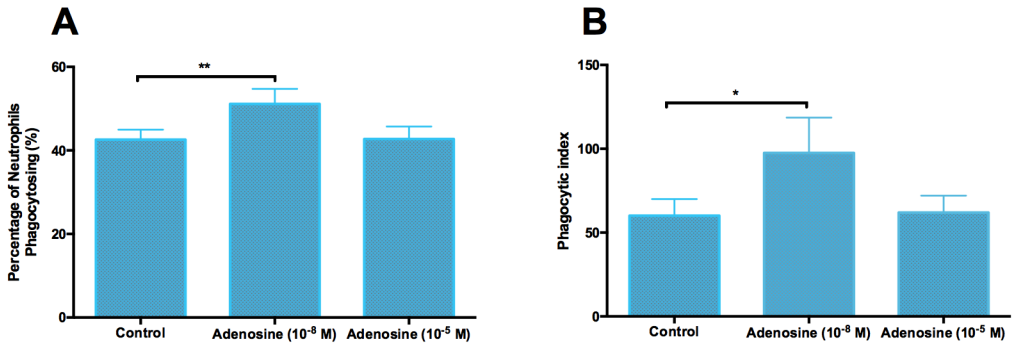

Figure 6.1 Effect of adenosine on percentage of neutrophils phagocytosing (A) and the phagocytic index (B) of isolated neutrophils with no erythrocytes present

Data expressed as mean ± SEM (n=8). Effect of adenosine determined using 1-way ANOVA with Dunnett's correction for multiple comparisons (*p<0.05, **p<0.01 and ***p<0.001).

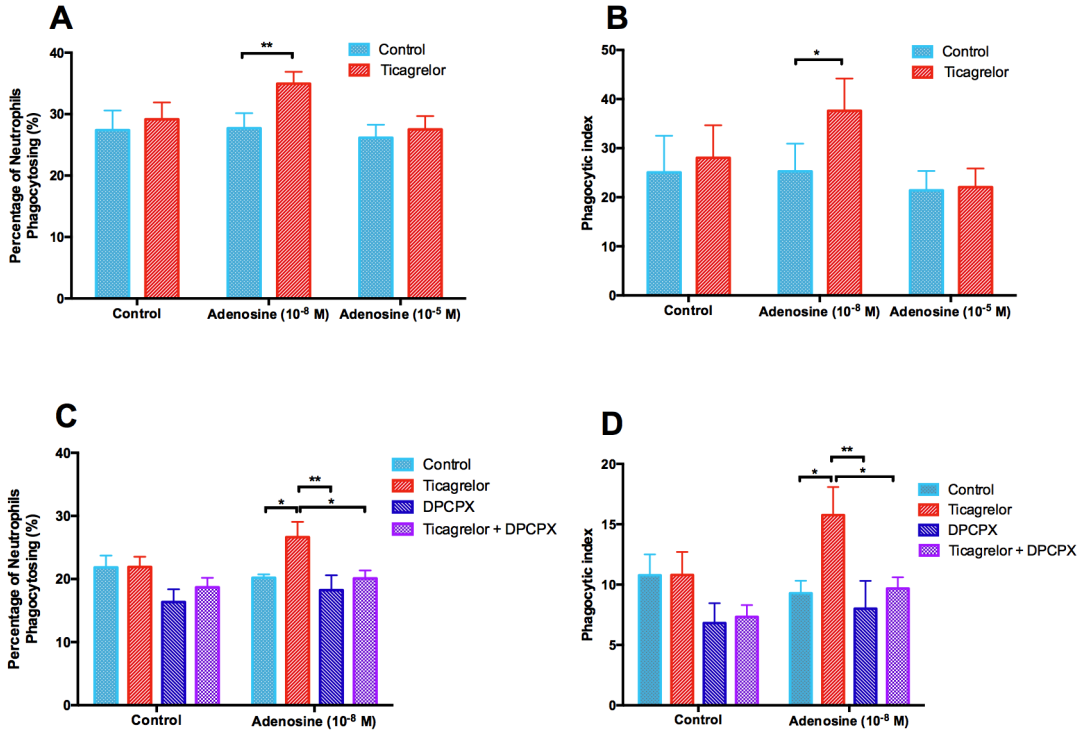

Figure 6.2 Modulatory effect of ticagrelor on A1-mediated effects of adenosine on percentage of neutrophils phagocytosing (A and C) and the phagocytic index (B and D) of isolated neutrophils in the presence of erythrocytes.

Data expressed as mean ± SEM (n=8 [A and B] and n=5 [C and D]). Effect of LPS and cangrelor and ticagrelor (each compared to their respective control) determined using 2-way ANOVA with Dunnett's correction for multiple comparisons (*p<0.05, **p<0.01 and ***p<0.001).

6.3 Adenosine has minimal effect on LPS-induced changes in isolated neutrophil expression of CD11b, CXCR1 and CD16

Isolated neutrophils were then pre-incubated with adenosine and their reactivity to LPS over 30 minutes was determined by measurement of CD11b, CXCR1 and CD16 using flow cytometry (see 3.2.5). Neutrophil CD11b expression is upregulated in response to LPS and has a critical role in neutrophil adhesion (Zhou et al., 2005). The chemokine receptor CXCR1 is activated by IL-8 and plays a critical role in neutrophil chemotaxis (Sabroe et al., 2005). Neutrophil expression of CXCR1 is downregulated in response to LPS (Sabroe et al., 2005). The FcγRIII receptor CD16 has an important role in response to immunoglobulin-opsonized bacteria and phagocytosis (Bredius et al., 1994) and neutrophil expression of CD16 is downregulated in response to LPS (Wagner et al., 2003).

A high concentration of adenosine (10^{-4} M) reduced expression of CD11b in unstimulated isolated neutrophils (Figure 6.3A). Compared to PBS vehicle control, LPS caused a dose-dependent increase in isolated neutrophil expression of CD11b (Figure 6.3A). Nanomolar concentrations of adenosine did not significantly affect LPS-induced CD11b expression (Figure 6.3A). Higher concentrations of adenosine (10^{-5} M and 10^{-4} M), did significantly reduce LPS-induced CD11b expression but only by approximately 10-15% (Figure 6.3A).

Neutrophil expression of CXCR1 was downregulated in a dose-dependent manner by LPS, but this was not significantly affected by any of the concentrations of adenosine (Figure 6.3B).

Neutrophil expression of CD16 was downregulated in response to LPS (Figure 6.3C). A high concentration of adenosine (10^{-4} M) reduced neutrophil expression of CD16 in unstimulated and LPS stimulated samples, but only by approximately 10-15% (Figure 6.3C).

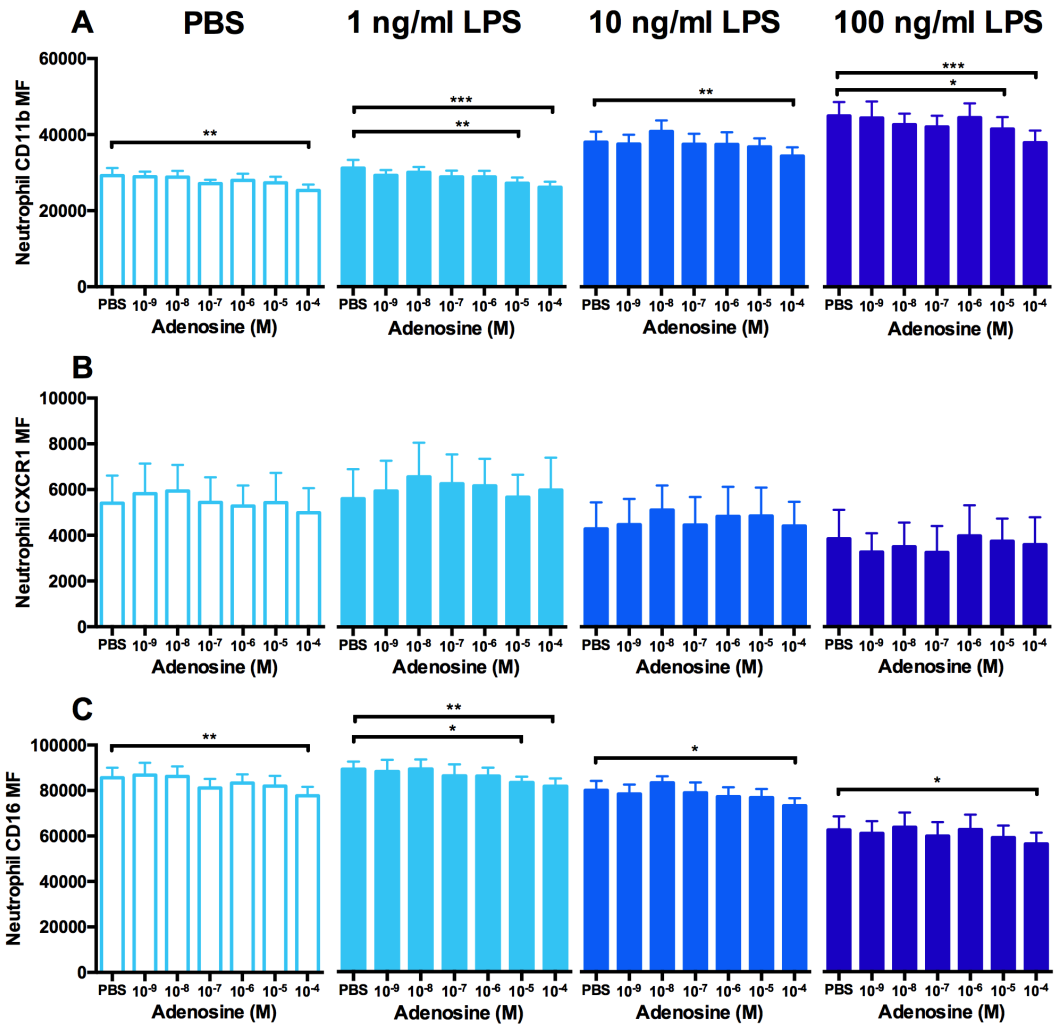

Figure 6.3 Effect of adenosine on LPS-induced expression of CD11b (A), CXCR1 (B) and CD16 (C) on isolated neutrophils

Data expressed as mean ± SEM (n = 6). The effect of LPS and the effect of adenosine (both compared to their respective controls) determined using 2-way ANOVA with Dunnett's correction for multiple comparisons (*p<0.05, **p<0.01 and ***p<0.001).

6.4 Adenosine dose-dependently modifies FMLP-induced changes in isolated neutrophil expression of CD11b, CXCR1 and CD16

FMLP is a naturally occurring bacterial peptide that stimulates neutrophils through the FMLP receptor (Torres et al., 1993). Isolated neutrophils were stimulated with FMLP for 30 minutes and the modulatory effect of pre-incubation with adenosine was determined (see 3.2.6). FMLP at concentrations of 10^{-8} M, 10^{-7} M and 10^{-6} M increased neutrophil expression of CD11b. Compared to PBS (vehicle control), 10^{-4} M adenosine significantly reduced CD11b expression in response to FMLP at concentrations of 10^{-8} M, 10^{-7} M and 10^{-6} M (all $p < 0.01$; Figure 6.4A). Adenosine 10^{-9} M significantly potentiated isolated neutrophil expression of of CD11b in response to FMLP 10^{-7} M compared to control (61,188 ± 6,515 vs. 51,548 ± 3,059; p=0.002; Figure 6.4A). Adenosine at 10^{-5} M significantly reduced neutrophil expression of CD11b compared to control in response to this concentration of FMLP (44,217 ± 1,803 vs. 51,549 ± 3,059; p = 0.03; Figure 6.4A).

In unstimulated samples, 10^{-4} M adenosine significantly reduced neutrophil expression of CXCR1 compared to the PBS vehicle control (4,990 ± 502 vs. 5,871 ± 687; p = 0.003; Figure 6.4B). FMLP reduced neutrophil expression of CXCR1 and this was inhibited by higher concentrations of adenosine. Adenosine at concentrations of 10^{-7} M, 10^{-6} M, 10^{-5} M and 10^{-4} M significantly reduced the decrease in neutrophil expression of CXCR1 that was induced by FMLP at concentrations of 10^{-7} M and 10^{-6} M (all p <0.001; Figure 6.5B). For example, 10^{-5} adenosine significantly reduced the decrease in neutrophil expression of CXCR1 in response to 10^{-7} M FMLP, resulting in a higher level of CXCR1 expression compared to samples without adenosine (3,192 ± 430 vs. 1,838 ± 443; $p < 0.001$; Figure 6.4B).

In unstimulated samples, 10^{-4} M adenosine significantly reduced neutrophil expression of CD16 compared to PBS vehicle control (87,574 ± 4,097 vs. 97,558 ± 5,782; p = 0.003). Neutrophil expression of CD16 significantly decreased following stimulation by FMLP at concentrations of 10^{-8} M, 10^{-7} M and 10^{-6} M (all $p < 0.05$; Figure 6.4C). Although this decrease was significantly attenuated by higher

concentrations of adenosine, adenosine only modified levels of CD16 expression by approximately 10% or less (Figure 6.4C).

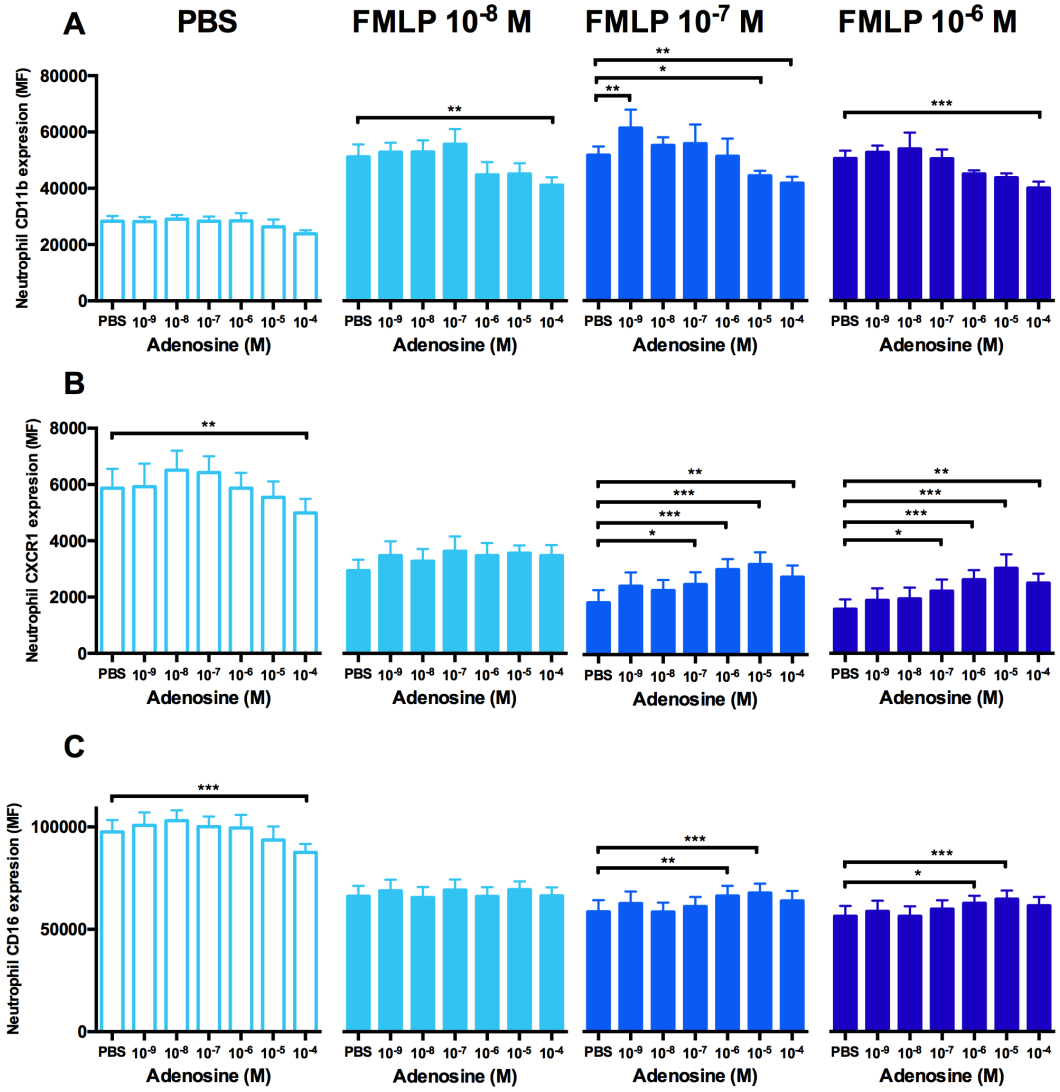

Figure 6.4 Effect of adenosine on FMLP-induced expression of CD11b (A), CXCR1 (B) and CD16 (C) on isolated neutrophils.

Data expressed as mean ± SEM (n = 8). The effect of FMLP and the effect of adenosine (both compared to their respective controls) determined using 2-way ANOVA with Dunnett's correction for multiple comparisons (*p<0.05, **p<0.01 and ***p<0.001).

6.5 Erythrocytes abolish the effect of adenosine on FMLP-induced changes in neutrophil expression of CD11b, CXCR1 and CD16

Next, it was investigated whether the effects of adenosine are abolished in the presence of erythrocytes, which take up adenosine. It was investigated whether the effects of adenosine persisted for 5 and 30 minutes of stimulation of neutrophils with 10^{-7} M FMLP (see 3.2.7). Neutrophil expression of CD11b was significantly increased by 5 and 30 minutes of exposure to FMLP (all $p < 0.001$; Figure 6.5A). In isolated neutrophils, 10^{-5} M adenosine significantly reduced neutrophil expression of CD11b in response to 5 minutes stimulation with FMLP (37,575 ± 4,890 vs. 56,308 ± 5,917; $p < 0.001$; Figure 6.5A).

A), whereas 10^{-8} M adenosine did not have a significant effect. Even after 30 minutes stimulation with FMLP, 10^{-5} M adenosine still significantly reduced CD11b expression ($p < 0.001$). The addition of erythrocytes in a 5:1 ratio with neutrophils did not modify the effect of adenosine. However, 10^{-5} M adenosine no longer significantly reduced FMLP-induced CD11b expression in the presence of erythrocytes at 1000:1 ratio with neutrophils.

In isolated unstimulated neutrophils, 10^{-8} M adenosine significantly increased expression of CXCR1 ($p < 0.001$; Figure 6.5B), whereas 10^{-5} M adenosine had no effect. In the presence of erythrocytes, both 10^{-8} M and 10^{-5} M adenosine reduced neutrophil expression of CXCR1 however (all $p<0.001$; Figure 6.5). 10^{-5} M adenosine significantly attenuated the FMLP-induced decrease in CXCR1 expression and this effect was abolished by the addition of erythrocytes in 1000:1 ratio with neutrophils but not at the lower concentration of erythrocytes (Figure 6.5B).

30 minutes stimulation with FMLP induced a significant decrease in neutrophil expression of CD16, which was significantly attenuated by 10^{-5} M adenosine ($p = 0.02$ (Figure 6.5C). This effect was abolished by the addition of erythrocytes in 1000:1 ratio with neutrophils but not by the lower concentration of erythrocytes (Figure 6.5C).

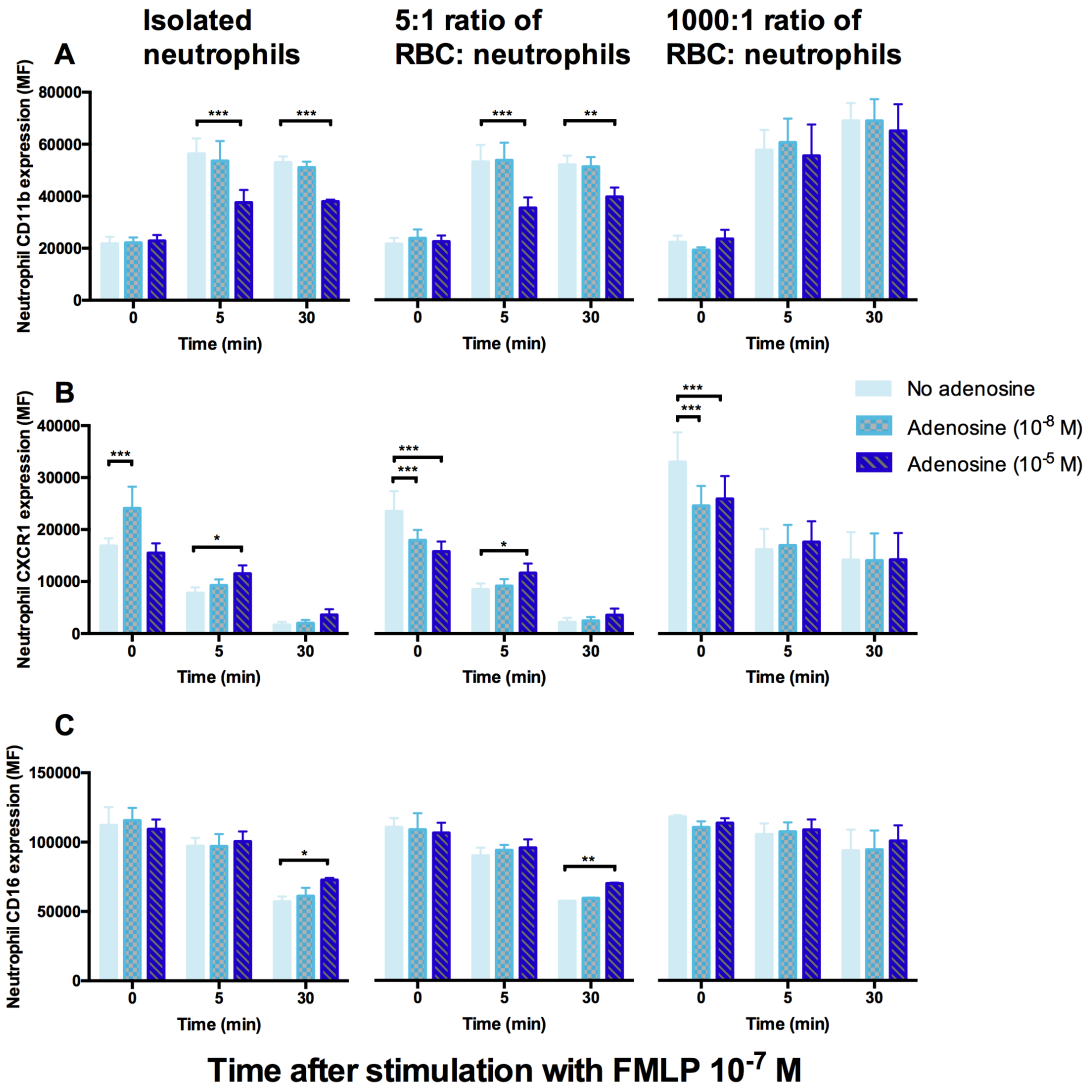

Figure 6.5 Effect of adenosine on the expression of CD11b (A), CXCR1 (B) and CD16 (C) in isolated neutrophils following 5 and 30 minutes of stimulation with FMLP.

Data expressed as mean ± SEM (n = 4). The effect of FMLP and the effect of adenosine (both compared to their respective controls) determined using 2-way ANOVA with Dunnett's correction for multiple comparisons (*p<0.05, **p<0.01 and ***p<0.001).

6.6 Inhibition of erythrocyte uptake of adenosine preserves its effects on FMLP-induced changes in neutrophil expression of CD11b

Dipyridamole is a well-established potent inhibitor of adenosine uptake (Gresele et al., 1986). The previous section's results demonstrated that erythrocytes take up adenosine, thereby abolishing its effect. It was next investigated whether inhibiting erythrocyte uptake of adenosine preserves the effects of adenosine in whole blood (see 3.2.8). 5 and 30 minutes of stimulation with 10^{-7} M FMLP significantly increased neutrophil expression of CD11b compared to PBS vehicle control (all $p < 0.001$). The inhibitory effect of 10^{-5} adenosine was preserved by dipyridamole and this was most marked when adenosine was added at the same time as the FMLP, rather than when adenosine was added 4 minutes before FMLP (Figure 6.6A).

Although 5 and 30 minutes stimulation with FMLP also significantly increased monocyte expression of CD11b ($p < 0.001$) and significantly decreased neutrophil expression of CXCR1, no modulatory effects of adenosine or dipyridamole were seen (Figure 6.6B and C).

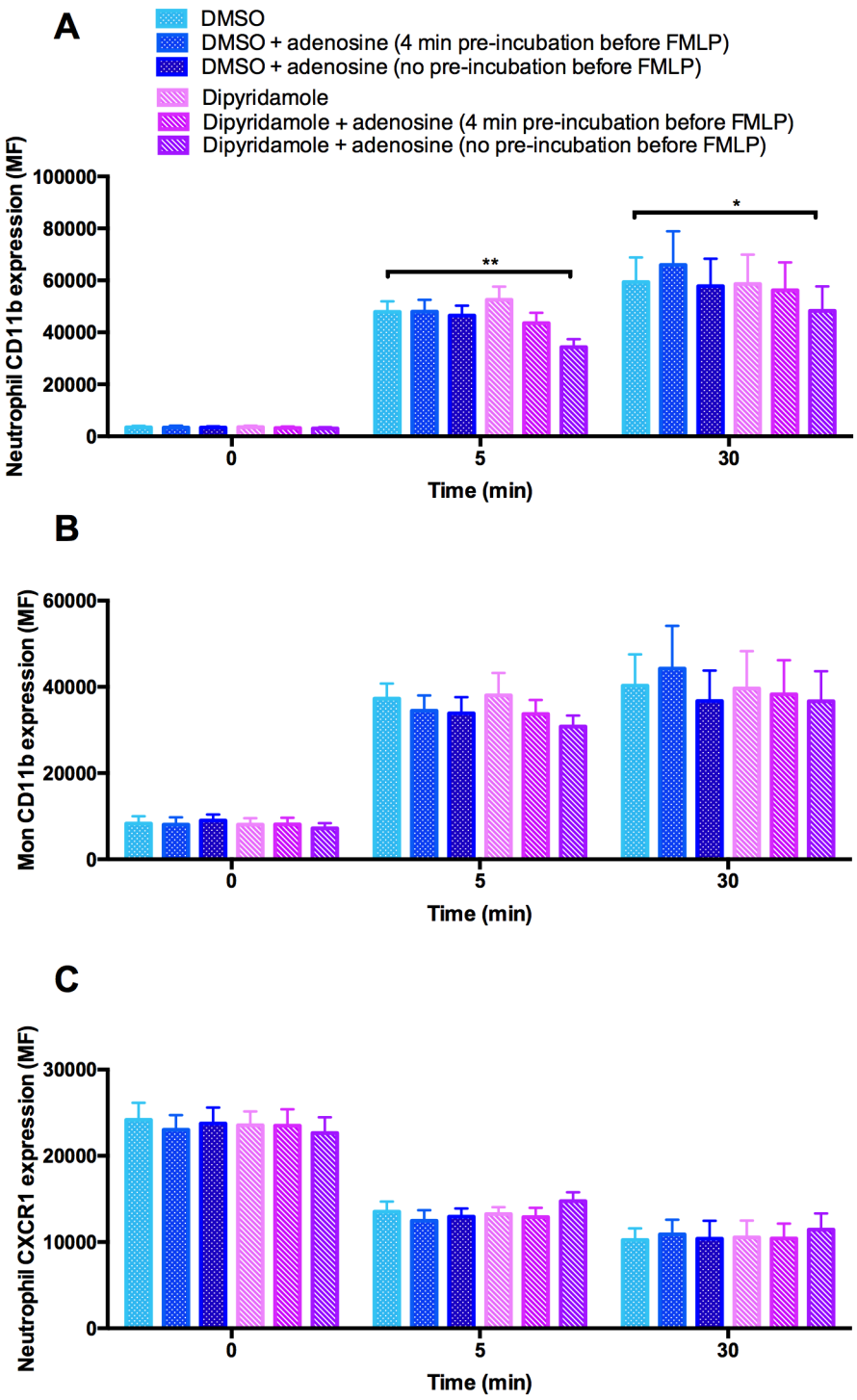

Figure 6.6 Effect of dipyridamole on neutrophil expression of CD11b (A), monocyte expression of CD11b (B) and neutrophil expression of CXCR1 (C) in response to FMLP.

Data expressed as mean ± SEM (n = 6). The effect of FMLP and the effect of adenosine and dipyridamole (each compared to their respective controls) determined using 2-way ANOVA with Dunnett's correction for multiple comparisons (*$p<0.05$, **$p<0.01$ and ***$p<0.001$).

6.7 Effect of timing of pre-incubation of adenosine on subsequent response to FMLP

In whole blood, the effects of adenosine were most prominent on FMLP-induced changes in neutrophil expression of CD11b and CXCR1 and monocyte expression of CD11b. These effects were therefore investigated in more detail in the following sections. It became apparent that the duration of pre-incubation of leukocytes with adenosine had a significant impact on the effect of adenosine, even in the presence of adenosine uptake inhibitors. Even in the presence of ticagrelor, the effect of 10^{-5} M adenosine on neutrophil expression of CD11b was most prominent when it was added at the same time as FMLP rather than pre-incubating the blood with adenosine for up to a minute (Figure 6.7A). In the presence of ticagrelor, the effects of adenosine on monocyte CD11b and neutrophil CXCR1 appeared more prolonged (Figure 6.7B and C).

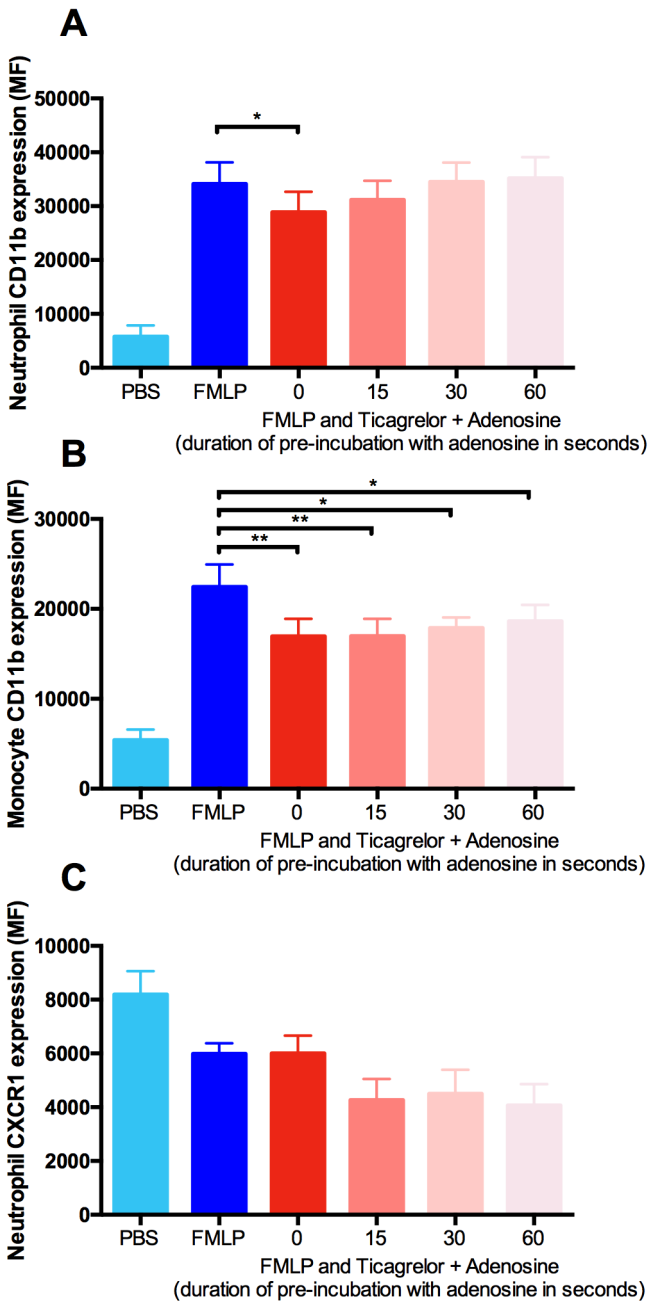

Figure 6.7 Effect of duration of pre-incubation with adenosine on FMLP induced expression of neutrophil CD11b (A), monocyte CD11b (B) and neutrophil CXCR1 (C) in the presence of ticagrelor.

Data expressed as mean ± SEM (n = 6). The effect of FMLP and the effect of ticagrelor and adenosine (each compared to their respective controls) determined using 1-way ANOVA with Dunnett's correction for multiple comparisons for the cytokines (*$p<0.05$, **$p<0.01$ and ***$p<0.001$).

6.8 Ticagrelor and dipyridamole inhibit FMLP-induced expression of CD11b in neutrophils

It was next investigated whether ticagrelor and dipyridamole preserved the effect of adenosine on FMLP-induced changes in leukocyte expression of CD11b and CXCR1 in whole blood. FMLP (10^{-7} M) significantly increased neutrophil expression of CD11b in whole blood. In the DMSO (vehicle control) samples, 10^{-8} M adenosine and 10^{-5} adenosine had no effect on neutrophil expression of CD11b. Ticagrelor alone significantly reduced FMLP-induced neutrophil CD11b expression compared to DMSO (38,800 ± 6,104 vs. 48,111; $p < 0.001$; Figure 6.8A). The combination of ticagrelor and adenosine also significantly reduced FMLP-induced neutrophil CD11b expression compared to DMSO (41,550 ± 5,215 vs. 47,558 ± 6,627; $p = 0.005$; Figure 6.8A).

Similarly, dipyridamole alone and the combination of dipyridamole and adenosine significantly reduced FMLP-induced neutrophil CD11b expression (both $p < 0.01$; Figure 6.8A). FMLP-induced monocyte CD11b expression was only significantly reduced compared to DMSO control by the combination of ticagrelor and adenosine (27,390 ± 4,278 vs. 31,017 ± 5,141; $p = 0.03$; Figure 6.8B) and the combination of dipyridamole and adenosine (26,298 ± 3,042 vs. 31,017; $p = 0.002$; Figure 6.8).

There was no significant effect of adenosine, ticagrelor or dipyridamole on neutrophil expression of CXCR1 in whole blood (Figure 6.8C).

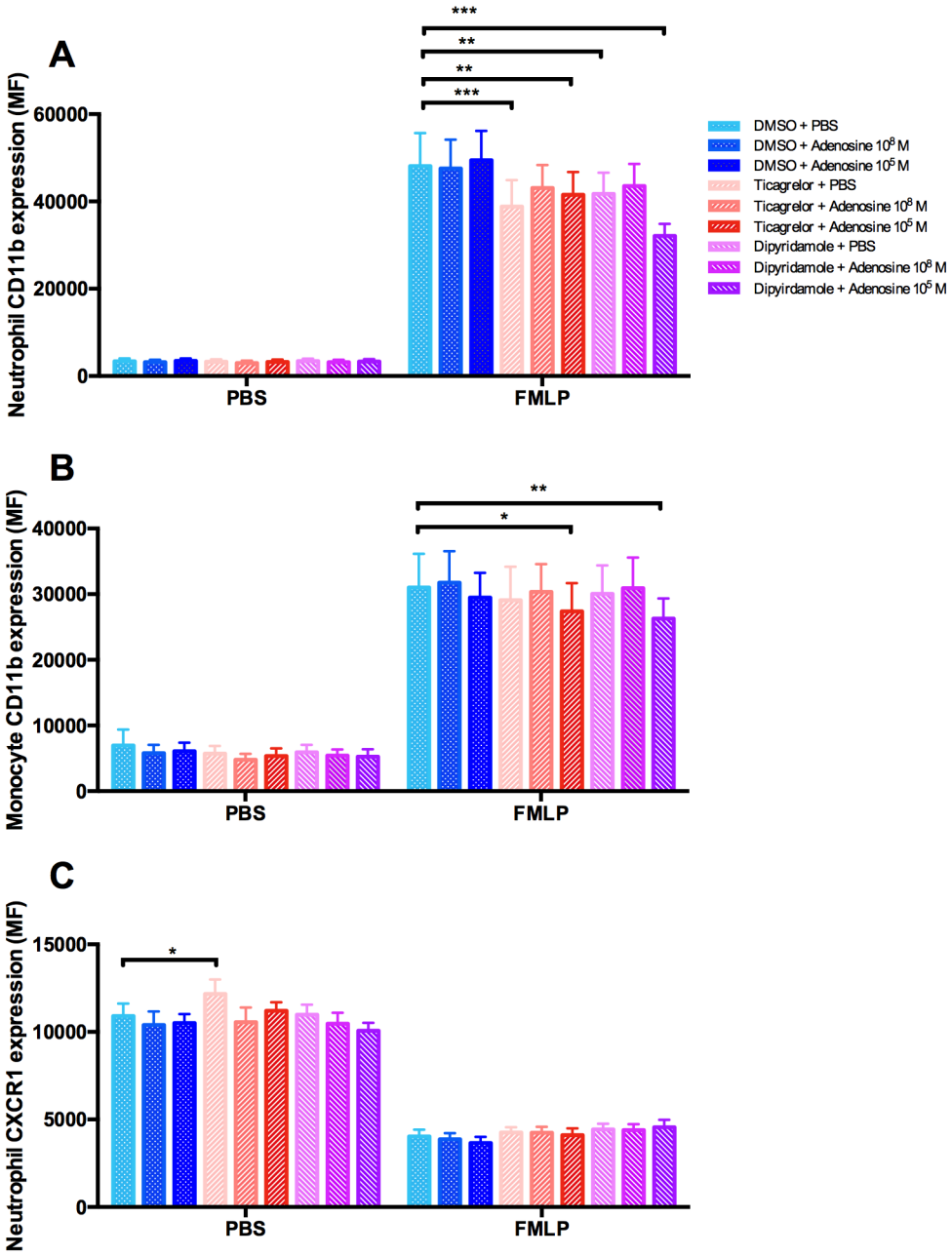

Figure 6.8 Modulatory effect of ticagrelor and dipyridamole on neutrophil expression of CD11b (A), monocyte expression of CD11b (B) and neutrophil expression of CXCR1 (C) in response to FMLP.

Data expressed as mean ± SEM (n = 8). The effect of FMLP and the effect of adenosine, ticagrelor and dipyridamole determined using 2-way ANOVA with Dunnett's correction for multiple comparisons for the cytokines (*p<0.05, **p<0.01 and ***p<0.001).

6.9 Limited additive effect of adenosine uptake inhibition and $P2Y_{12}$ inhibition

In the previous section's results, FMLP-induced neutrophil expression of CD11b was inhibited by ticagrelor even without the addition of adenosine. These effects may have been due to platelet $P2Y_{12}$ inhibition and the effect of ticagrelor was therefore next compared to the $P2Y_{12}$ inhibitor cangrelor, which does not inhibit adenosine uptake (Armstrong et al., 2014). In addition, cangrelor and dipyridamole were used in combination to determine whether the effects of $P2Y_{12}$ inhibition and adenosine uptake inhibition are additive.

In this set of experiments, FMLP-induced neutrophil CD11b expression was only significantly inhibited by the combination of ticagrelor and adenosine, dipyridamole alone, the combination of dipyridamole and adenosine, and the combination of dipyridamole, cangrelor and adenosine (all $p < 0.05$; Figure 6.9A). The combination of dipyridamole, cangrelor and adenosine did not provide any greater inhibition than the combination of dipyridamole and adenosine (Figure 6.9).

FMLP-induced monocyte CD11b expression was inhibited by adenosine alone in this set of experiments and was also inhibited the combination of ticagrelor and adenosine, the combination of dipyridamole and adenosine, and the combination of dipyridamole, cangrelor and adenosine (all $p < 0.05$; Figure 6.9).

There were no significant effects of adenosine, cangrelor, ticagrelor or dipyridamole on neutrophil CXCR1 expression (Figure 6.9).

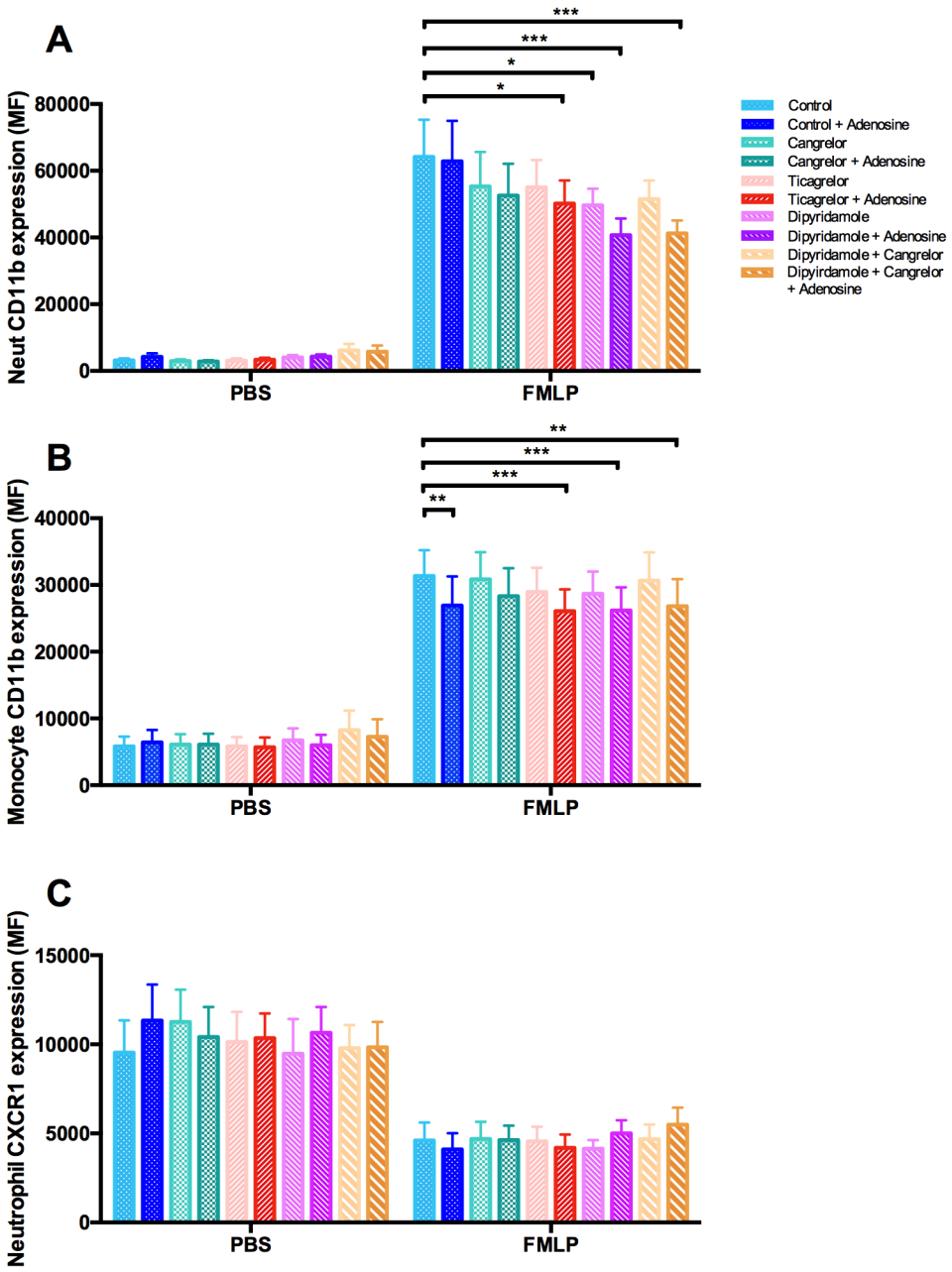

Figure 6.9 Modulatory effect of cangrelor, ticagrelor and dipyridamole on the effects of adenosine on neutrophil expression of CD11b (A), monocyte expression of CD11b (B) and neutrophil expression of CXCR1 (C) in response to FMLP.

Data expressed as mean ± SEM (n = 4). The effect of FMLP and the effect of adenosine, cangrelor, ticagrelor and dipyridamole determined using 2-way ANOVA with Dunnett's correction for multiple comparisons (*p<0.05, **p<0.01 and ***p<0.001).

6.10 Discussion

It is now well-established that ticagrelor is a weak inhibitor of cellular uptake of adenosine, in addition to its effects on platelet $P2Y_{12}$ receptors (Cattaneo et al., 2014; Bonello et al., 2014; Armstrong et al., 2014; van Giezen et al., 2012). This mechanism increases extracellular levels of adenosine, which increases plasma levels of adenosine in patients with ACS (Bonello et al., 2014). This mechanism has the potential to affect neutrophils and monocytes, which express 4 different adenosine receptors. The effects of adenosine on neutrophils and monocytes are complex due to dynamic local concentrations. Nanomolar concentrations of adenosine generally have pro-inflammatory effects, mediated by A_1 and A_3, whereas micromolar concentrations of adenosine limit excessive innate immune activation and have anti-inflammatory effects mediated by A_{2A} and A_{2B} (Barletta et al., 2012; Haskó & Pacher, 2012). It was therefore hypothesized that inhibition of adenosine uptake by ticagrelor may have a combination of pro-inflammatory and anti-inflammatory effects, dependent on the concentration of adenosine.

The results of this chapter provide novel findings relating to the effect of this mechanism on neutrophils and monocytes. Ticagrelor potentiated the stimulatory effect of nanomolar concentrations of adenosine on neutrophil phagocytosis, which was otherwise abolished by erythrocytes. The stimulatory effect of nanomolar concentrations of adenosine was mediated by neutrophil A_1 receptors. Higher concentrations of adenosine act on A_{2A} and A_{2B} receptors and micromolar concentrations of adenosine significantly inhibited FMLP-induced changes in expression of CD11b, CXCR1 and CD16 in isolated neutrophils, but only had a minimal impact on the effects of LPS. Ticagrelor in combination with adenosine inhibited FMLP-induced neutrophil and monocyte expression in whole blood, which may have been due to a combination of $P2Y_{12}$ inhibition and adenosine uptake inhibition.

These findings suggest that this adenosine-mediated mechanism of ticagrelor may cause a combination of A_1- and A_3- mediated pro-inflammatory effects and A_{2A}-and A_{2B}-mediated anti-inflammatory effects. This may serve to stimulate immune responses in the early stages of inflammation and then to limit excessive innate immune activation during the later stages of inflammation. Interestingly, adenosine

had little effect on the response to LPS, which may help to explain why the effects of ticagrelor appeared to be mostly $P2Y_{12}$ related rather than adenosine-mediated during intravenous LPS administration as described in Chapter 4.

7 General discussion

Platelet $P2Y_{12}$ inhibitors are now some of the most commonly used medications worldwide due to their established benefit in the treatment of atherothrombosis (Grove et al., 2015). Ticagrelor is a novel $P2Y_{12}$ inhibitor that has a more rapid onset of action and more potent effect than the $P2Y_{12}$ inhibitor clopidogrel (Gurbel et al., 2009). In the PLATO study, ticagrelor reduced the incidence of adverse cardiovascular events in patients with ACS compared to clopidogrel (9.8% vs. 11.7%; HR 0.84; $p < 0.001$). However, ticagrelor also reduced all-cause mortality compared to clopidogrel (4.5% vs. 5.9%; HR 0.78; $p < 0.001$), which was unexpected on the basis of previous studies.

Further analysis of the PLATO study demonstrated that ticagrelor was associated with fewer deaths following sepsis than clopidogrel (Varenhorst et al., 2014; Storey et al., 2014; Varenhorst et al., 2012). Interestingly, it has also been suggested by other observational studies that platelet $P2Y_{12}$ inhibitors may reduce mortality from sepsis (Tsai et al., 2015; Akinosoglou et al., 2014). In addition to being a more potent $P2Y_{12}$ inhibitor than clopidogrel, ticagrelor also inhibits cellular uptake of adenosine whereas clopidogrel does not (Bonello et al., 2014). Therefore it was hypothesized that both of these mechanisms could contribute to a potential beneficial effect of ticagrelor during sepsis. The impact of $P2Y_{12}$ inhibitors on pathophysiological processes that are central to sepsis responses in humans were therefore investigated using an experimental human model of systemic inflammation that involves the injection of intravenous *E.coli* endotoxin (LPS).

Intravenous injection of LPS induced the formation of platelet-monocyte aggregates, which was inhibited by ticagrelor and to a lesser extent clopidogrel. Platelet-monocyte interactions upregulate monocyte release of pro-inflammatory cytokines (Bournazos et al., 2008). Inhibition of platelet-monocyte aggregate formation therefore represents a mechanism by which ticagrelor and clopidogrel reduced the release of the major pro-inflammatory cytokines TNFα, IL-6 and CCL2. Ticagrelor, but not clopidogrel, also significantly reduced the release of G-CSF and IL-8, which may have been due to greater $P2Y_{12}$ inhibition or could have been related to adenosine-mediated effects. Sepsis is characterized by dysregulated response to

bacterial components, such as LPS, and excessive innate immune activation causes a pro-inflammatory cytokine storm, leading to collateral host tissue damage and increased mortality (Angus & van der Poll, 2013). Platelet $P2Y_{12}$ inhibitors may therefore improve mortality from sepsis by limiting excessive pro-inflammatory cytokine release.

Following sepsis, the risk of atherothrombotic events is increased by as much as 20-fold, by mechanisms that are incompletely understood (Dalager-Pedersen et al., 2014). For the first time, the results presented in this thesis demonstrate that LPS triggers alterations in fibrin clot structure, resulting in more compact clots that present greater resistance to fibrinolysis. This demonstrates a mechanism by which sepsis may increase the risk of thrombosis. Ticagrelor significantly attenuated these prothrombotic changes, which may have been secondary to its reduction in levels of pro-inflammatory cytokines, such as TNFα, which activates the extrinsic coagulation cascade (van der Poll et al., 1990).

Platelet-leukocyte interactions also upregulate leukocyte expression of adhesion molecules and facilitate adhesion of leukocytes to the endothelium (da Costa Martins et al., 2004). Ticagrelor potentiated the increase in neutrophil count that occurred following LPS administration, likely due to inhibition of non-specific sequestration of neutrophils. At 24 hours after LPS administration, there was a marked 6-fold increase in the number of intermediate monocytes, which may have been mobilized from the marginal pool. The expanded intermediate monocyte population appeared to be phenotypically similar to intermediate monocytes at baseline and expressed similar levels of CD14, CD16, CD11b, CCR2, CXCR2, TLR2 and TLR4. Both clopidogrel and ticagrelor potentiated the increase in intermediate monocyte population. Platelet $P2Y_{12}$ inhibitors may facilitate mobilization of leukocytes from the marginal pool by decreasing platelet-leukocyte interactions and thereby decreasing leukocyte adhesiveness. Emerging evidence suggests that intermediate monocytes may have a role in production of anti-inflammatory cytokines (Mukherjee et al., 2015), but further work is needed to delineate their role in ACS and sepsis.

In addition to $P2Y_{12}$-mediated effects on inflammation, ticagrelor also has adenosine-mediated effects on leukocytes. By inhibiting cellular uptake of adenosine, ticagrelor increases plasma levels of adenosine in ACS patients (Bonello et al., 2014).

Low concentrations of adenosine have predominantly pro-inflammatory effects, mediated by A_1 and A_3 receptors (Barletta et al., 2012; Haskó & Pacher, 2012). Higher concentrations of adenosine have predominantly anti-inflammatory effects, mediated by A_{2A} and A_{2B}. Ticagrelor potentiates the stimulatory effect of low concentrations of adenosine on neutrophil phagocytosis, by inhibiting adenosine uptake. In addition, ticagrelor potentiates the suppressive effect of high concentrations of adenosine on leukocyte expression of CD11b in response to FMLP.

7.1 Future work

The results of this thesis suggest that there could be potential benefits of timed administration of platelet $P2Y_{12}$ inhibitors to patients with sepsis. This is supported by observational studies, which shows that $P2Y_{12}$ inhibitors are associated with a reduction in mortality in sepsis (Tsai et al., 2015; Akinosoglou et al., 2014). In addition, $P2Y_{12}$ inhibitors have not been associated with excess bleeding during sepsis (Akinosoglou et al., 2014), which may be because they partially correct the underlying coagulopathy, which is due to excess fibrin deposition and exhaustion of the coagulation cascade (Angus & van der Poll, 2013). $P2Y_{12}$ inhibitors were administered prior to LPS administration in this study and further work is needed to demonstrate whether $P2Y_{12}$ inhibitors also reduce established systemic inflammation. In order to clarify this, the Examining the Effect of Ticagrelor on Platelet Activation, Platelet-leukocyte Aggregates and Acute Lung Injury in Pneumonia (XANTHIPPE) study (clinicaltrials.gov reference NCT01883869) and the Randomized Trial of Ticagrelor for Severe Community Acquired Pneumonia (TCAP) trial (clinicaltrials.gov reference NCT01998399) will investigate whether ticagrelor is of benefit compared to placebo in patients with pneumonia.

The results of this thesis demonstrate marked effects of platelet $P2Y_{12}$ inhibitors on mobilization of intermediate monocytes. Clarification is needed to determine the physiological roles of intermediate monocytes and to determine their role in the pathophysiology of ACS and sepsis. Further work is needed to determine whether intermediate monocytes are harmful or helpful during ACS. The ability of observational studies to determine this is limited due to multiple confounders.

The results of this thesis demonstrate that ticagrelor has adenosine-mediated effects on monocytes and neutrophils *in vitro*. However, adenosine metabolism is complex and dynamic. Further work is therefore needed to characterise the effect of ticagrelor on levels of adenosine at the local tissue level as well as at the systemic level in humans and animal models. In addition, more work is required to demonstrate whether these mechanisms improve clinical outcomes during ACS and, potentially, sepsis.

7.2 Concluding remarks

Potent inhibition of multiple inflammatory and prothrombotic mechanisms by $P2Y_{12}$ inhibitors demonstrates critical importance of platelets as central orchestrators of systemic inflammation. The findings of this thesis provide novel mechanistic insight into the lower mortality associated with $P2Y_{12}$ inhibitors in patients with sepsis in clinical studies. This suggests a promising new line of investigation for novel applications of $P2Y_{12}$ inhibitors in a syndrome that has proved elusive to almost all previous pharmacological strategies.

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9 Appendices

9.1 R code and R markdown document demonstrating the code used for Random Forest analyses of associations between intermediate monocyte count and other variables

This appendix is automatically generated by R Studio from R markdown code in order to demonstrate the code that was used to produce the Random Forest analysis for assessment of the interactions between the intermediate monocyte count at 24 hours and the other variables in the dataset. The graphs in this appendix were automatically generated using the following code and then reformatted for formal use in the PhD thesis itself.

In the first instance the following packages are loaded:

```
library(reshape2)
library(randomForest)
library(rfUtilities)
library(rfPermute)
library(gridExtra)
```

Widedat is a dataframe that includes merged values from crp.csv, cytokines1.csv, cytokines2.csv, ddimer.csv, fbc.csv and fibrin.csv in the "wide" data frame format where each variable at each timepoint is in a separate column.

```
widedat <- read.csv("./activecsv/mainprocessed.csv")
```

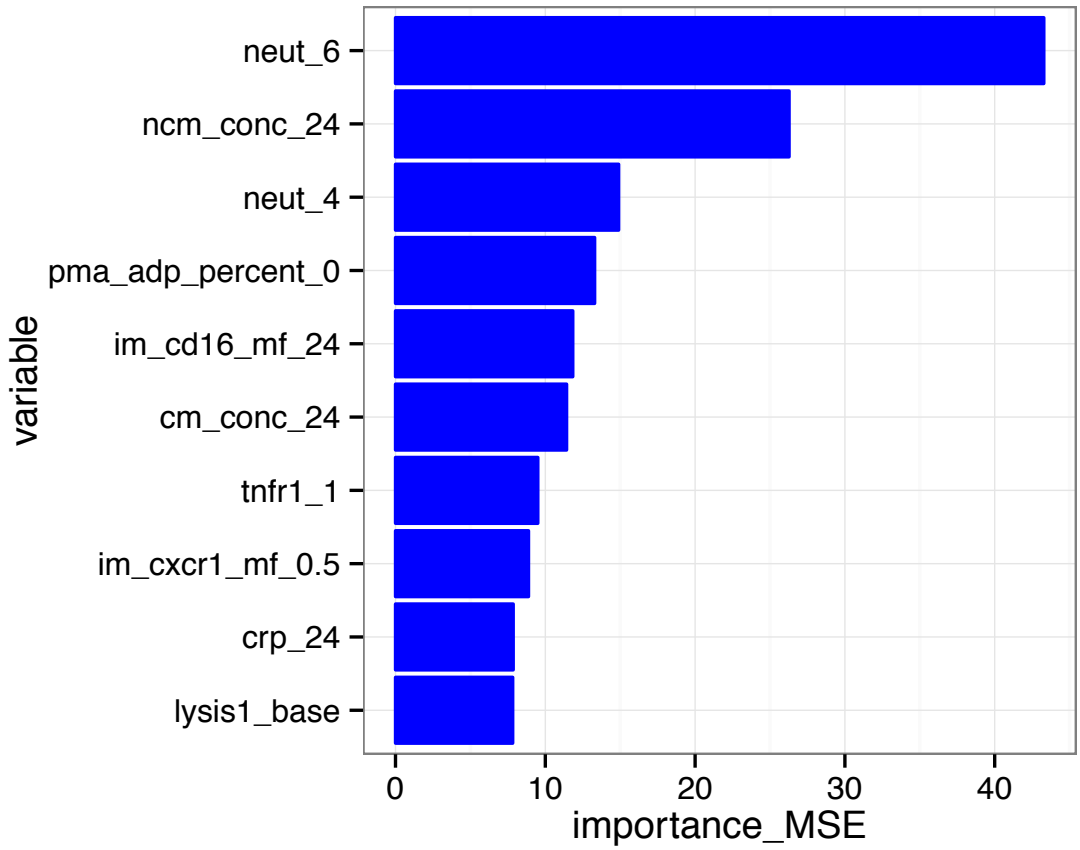
The data is divided into x explanatory variables and y, the intermediate monocyte count at 24h. Missing values are simply replaced with medians for that variable, which is required for analysis by the standard randomForest algorithm.

```
trainy <- na.roughfix(widedat[,"im_conc_24"])
trainx <- na.roughfix(widedat[,names(widedat)!="im_conc_24"])
```

Sets the random seed and runs an initial random forest on all of the variables at all of the timepoints to determine the importance of their relationship with the intermediate monocyte count at 24 hours. The 10 most important variables in the model are displayed.

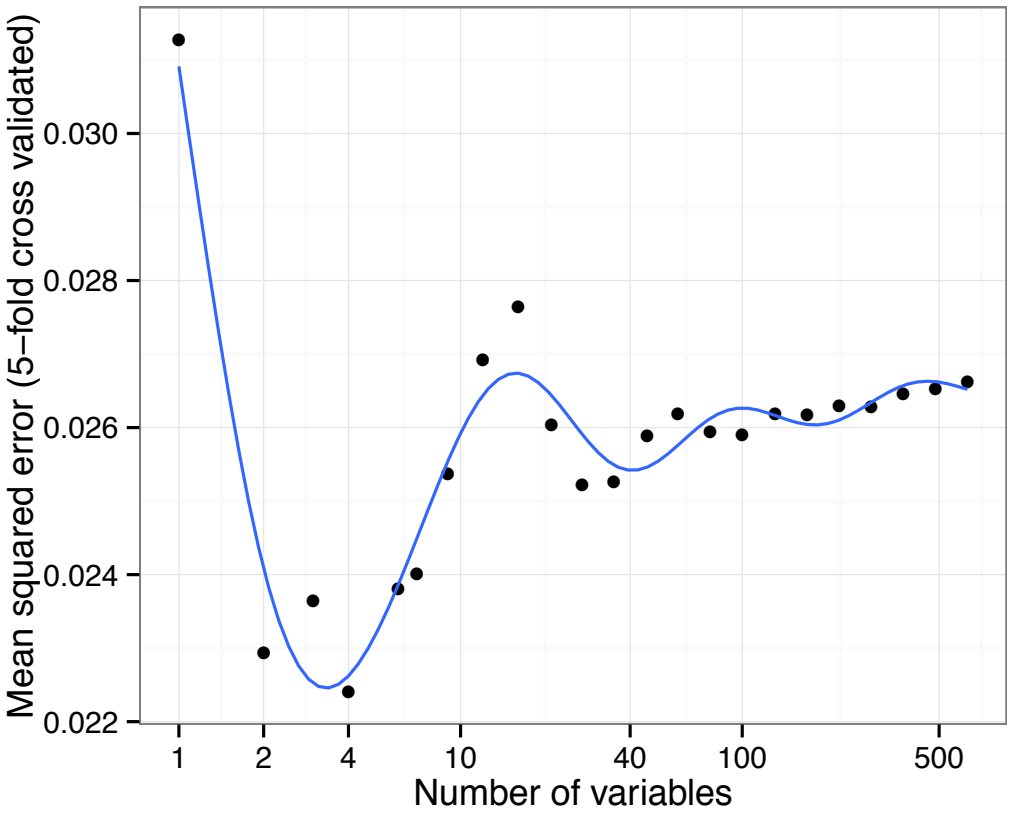
```
set.seed(100)
initialrf <- rfPermute(trainx, trainy, num.cores=8, parallel=TRUE,
                       ntree=20000,nrep=10000)
initialimp <- importance(initialrf)
initialimp <-initialimp[order(initialimp[,1], decreasing=TRUE),]
initialimp <-as.data.frame(initialimp[1:10,1, drop=FALSE])
initialimp$variable <- rownames(initialimp)
colnames(initialimp)<-c("importance_MSE","variable")
```

```
initialimp$variable <- factor(initialimp$variable, levels=rev(initialimp$va
riable))
ggplot(initialimp, aes(variable,importance_MSE))+
    geom_bar(stat="identity",color="blue",fill="blue")+coord_flip()+
    theme_bw()
```



Uses 5-fold cross validation to identify the optimal number of variables for achieving the most accurate model.

```
set.seed(100)
cvpredictors <- rfcv(trainx, trainy, ntree=100000,cv.fold=5,step=1.3)
cvpredictors <- as.data.frame(cvpredictors[1:2])
qplot(n.var, error.cv, data=cvpredictors)+geom_smooth(se=FALSE, method="gam
", formula=y~s(x))+labs(x="Number of variables", y="Mean squared error (5-f
old cross validated)")+ theme_bw()+scale_x_log10(breaks=c(1,2,4,10,40,100,5
00))
```



In this case, there is evidence of "overfitting" when all variables are included. The optimal variables to include were the 4 with the greatest importance in the initial model, which are neut_6, ncm_conc_24, neut_4 and pma_adp_percent_0.

```
namespredictors <- c("neut_6","ncm_conc_24","neut_4","pma_adp_percent_0")
```

Calculates p values for each of these variables in the initial run of random forests using all input variables. This determines the likelihood that these importance values, or more extreme importance values, could have been generated if there was no true relationship (null hypothesis).

```
set.seed(100)
pvals <- as.data.frame(initialrf$null.dist$pval)
pvals[namespredictors,1,drop=FALSE]

##                       %IncMSE
## neut_6            0.00009999
## ncm_conc_24       0.00109989
## neut_4            0.00329967
## pma_adp_percent_0 0.00999900
```

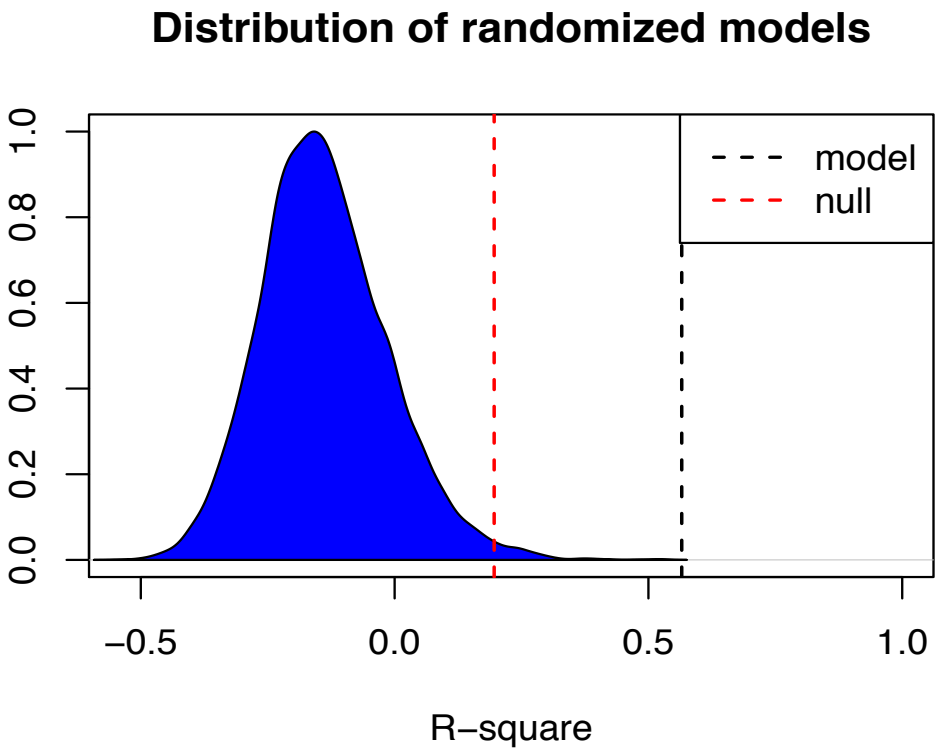
Makes a dataset using the selected variables only and runs random forests on this.

```
featuresselected<-cbind("im_conc_24"=trainy,trainx[,namespredictors])
set.seed(100)
rffit<-randomForest(im_conc_24~., data=featuresselected,ntree=20000,
                    na.action="na.roughfix")
```

Calculates the out of bag error of the model, which is a reliable estimate of the test error of the model.

Calculates a p value for the fit of the final model

```
set.seed(100)
fitsig <- rf.significance(rffit, na.roughfix(trainx), nperm=10000)
```




```
## [1] "MODEL SIGNIFICANT AT p=1e-04"

rffit

##
## Call:
##  randomForest(formula = im_conc_24 ~ ., data = featuresselected,      nt
ree = 20000, na.action = "na.roughfix")
##                Type of random forest: regression
##                      Number of trees: 20000
## No. of variables tried at each split: 1
##
##           Mean of squared residuals: 0.01146426
##                     % Var explained: 56.84
```

Creates random forests partial dependence plots for each of the selected variables.

```
featuresselected<-na.roughfix(featuresselected)
par.dep.plot <-partialPlot(rffit,featuresselected,x.var="neut_6",n.pt=30,pl
ot=FALSE)
par.dep.vals <- as.data.frame(par.dep.plot$x)
colnames(par.dep.vals)[1]<- "values"
par.dep.vals$variable <- "Neutrophil count 6h"
par.dep.vals$im_conc_24 <- par.dep.plot$y
```

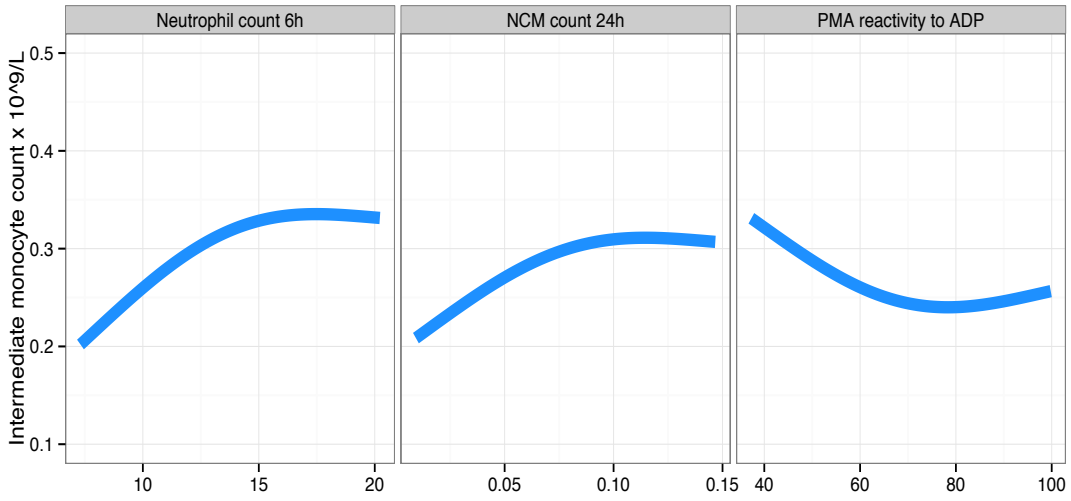
```
pardep <- par.dep.vals

par.dep.plot <-partialPlot(rffit,featuresselected,x.var="ncm_conc_24",n.pt=
30,plot=FALSE)
par.dep.vals <- as.data.frame(par.dep.plot$x)
colnames(par.dep.vals)[1]<- "values"
par.dep.vals$variable <- "NCM count 24h"
par.dep.vals$im_conc_24 <- par.dep.plot$y
pardep <- rbind(pardep,par.dep.vals)

par.dep.plot <-partialPlot(rffit,featuresselected,x.var="pma_adp_percent_0"
,n.pt=30,plot=FALSE)
par.dep.vals <- as.data.frame(par.dep.plot$x)
colnames(par.dep.vals)[1]<- "values"
par.dep.vals$variable <- "PMA reactivity to ADP"
par.dep.vals$im_conc_24 <- par.dep.plot$y
pardep <- rbind(pardep,par.dep.vals)

pardep$variable<-as.factor(pardep$variable)
pardep$variable = factor(pardep$variable,levels(pardep$variable)[c(2,1,3)])

pardepplot <- ggplot(pardep,aes(values, im_conc_24))+
    geom_smooth(se=FALSE, method="gam",formula = y ~ s(x,k=3),size=3,alpha=
0.3,color="dodgerblue")+
    facet_wrap(~variable,scales = "free_x")+theme_bw()+labs(x="",
    y="Intermediate monocyte count x 10^9/L")+expand_limits(y=c(0.1,0.5))
pardepplot
```



Creates a plot showing the linear relationship between each of the variables and the intermediate monocyte count at 24h.

```
meltfeatures <- melt(widedat, id.var=c("im_conc_24","treatment"))
meltfeatures <- meltfeatures[meltfeatures$variable%in%namespredictors,]
meltfeatures$value <- as.numeric(meltfeatures$value)
meltfeatures<-meltfeatures[meltfeatures$variable!="neut_4",]
meltfeatures$variable <- as.factor(as.character(meltfeatures$variable))


levels(meltfeatures$variable)<- c("NCM count 24h",
                                  "Neutrophil count 6h","PMA reactivity to
ADP")
```

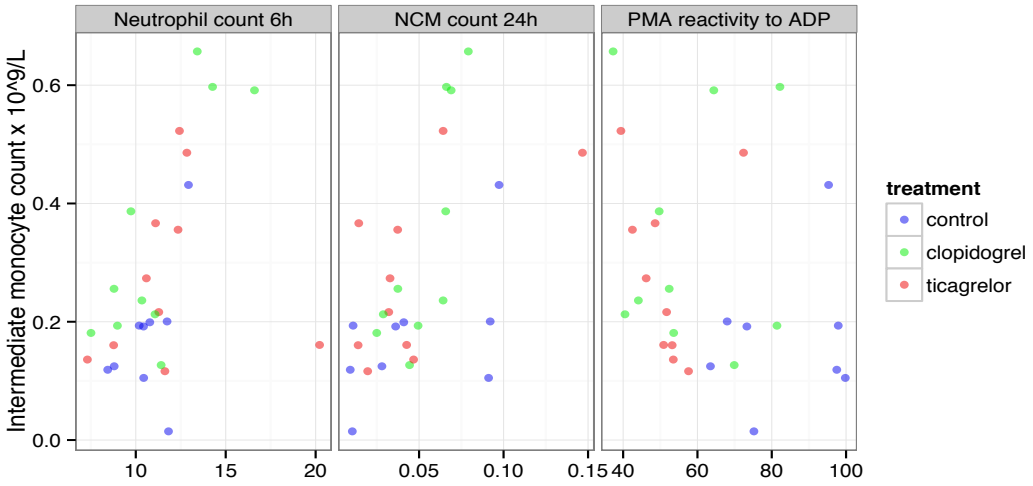
```
meltfeatures$variable = factor(meltfeatures$variable,
                               levels(meltfeatures$variable)[c(2,1,3)])
meltfeatures$treatment = factor(meltfeatures$treatment,
                                levels(meltfeatures$treatment)[c(2,1,3)])

depplot <- ggplot(meltfeatures,aes(value, im_conc_24,color=treatment))+geom
_point(alpha=0.5)+
    facet_wrap(~variable,scales = "free_x")+theme_bw()+labs(x="",
    y="Intermediate monocyte count x 10^9/L")+expand_limits(y=c(0.15,0.325)
)+
    scale_color_manual(breaks=c("control","clopidogrel","ticagrelor"),
                       values=c("blue2", "green2", "red2"))
depplot

## Warning: Removed 1 rows containing missing values (geom_point).

## Warning: Removed 1 rows containing missing values (geom_point).

## Warning: Removed 2 rows containing missing values (geom_point).
```



9.2 Ethical Approval for the Study

NHS
Health Research Authority
NRES Committee Yorkshire & The Humber - Sheffield
Yorkshire and the Humber REC Office
First Floor, Millside
Mill Pond Lane
Meanwood
Leeds
LS6 4RA

Telephone: 0113 3050128

07 February 2013

Robert Storey
University of Sheffield
Medical School, Beech Hill Road
Sheffield
S10 2RX

Dear Prof Storey

Study title:	**Study of the Effect of Ticagrelor and Clopidogrel on the Immune Response of Healthy Volunteers**
REC reference:	**13/YH/0005**
Protocol number:	**STH17062**
EudraCT number:	**2012-005514-18**
IRAS project ID:	**116021**

Thank you for your letter of 25 January 2013, responding to the Committee's request for further information on the above research and submitting revised documentation.

The further information has been considered on behalf of the Committee by the Chair.

We plan to publish your research summary wording for the above study on the NRES website, together with your contact details, unless you expressly withhold permission to do so. Publication will be no earlier than three months from the date of this favourable opinion letter. Should you wish to provide a substitute contact point, require further information, or wish to withhold permission to publish, please contact the Co-ordinator Mrs Rachel Bell, nrescommittee.yorkandhumber-sheffield@nhs.net.

Confirmation of ethical opinion

On behalf of the Committee, I am pleased to confirm a favourable ethical opinion for the above research on the basis described in the application form, protocol and supporting documentation as revised, subject to the conditions specified below.

Ethical review of research sites

NHS sites

The favourable opinion applies to all NHS sites listed in the application, subject to management permission being obtained from the NHS/HSC R&D office prior to the start of the study (see "Conditions of the favourable opinion" below).

Non-NHS sites

The Committee has not yet been notified of the outcome of any site-specific assessment (SSA) for the non-NHS research site(s) taking part in this study. The favourable opinion does not therefore apply to any non-NHS site at present. We will write to you again as soon as one Research Ethics Committee has notified the outcome of a SSA. In the meantime no study procedures should be initiated at non-NHS sites.

Conditions of the favourable opinion

The favourable opinion is subject to the following conditions being met prior to the start of the study.

Management permission or approval must be obtained from each host organisation prior to the start of the study at the site concerned.

Management permission ("R&D approval") should be sought from all NHS organisations involved in the study in accordance with NHS research governance arrangements.

Guidance on applying for NHS permission for research is available in the Integrated Research Application System or at http://www.rdforum.nhs.uk.

Where a NHS organisation's role in the study is limited to identifying and referring potential participants to research sites ("participant identification centre"), guidance should be sought from the R&D office on the information it requires to give permission for this activity.

For non-NHS sites, site management permission should be obtained in accordance with the procedures of the relevant host organisation.

Sponsors are not required to notify the Committee of approvals from host organisations

Clinical trial authorisation must be obtained from the Medicines and Healthcare products Regulatory Agency (MHRA).

The sponsor is asked to provide the Committee with a copy of the notice from the MHRA, either confirming clinical trial authorisation or giving grounds for non-acceptance, as soon as this is available.

It is the responsibility of the sponsor to ensure that all the conditions are complied with before the start of the study or its initiation at a particular site (as applicable).

Approved documents

The final list of documents reviewed and approved by the Committee is as follows:

Document	*Version*	*Date*
Advertisement	3	21 January 2013
Evidence of insurance or indemnity		07 December 2012
GP/Consultant Information Sheets	3	23 October 2012
Investigator CV		
Other: Study flow chart & study plan	2	19 September 2012
Other: Advert (email)	1	10 October 2012
Other: SMPC Clopidogrel		16 February 2012
Other: SMPC Briligue		15 November 2012
Participant Consent Form	2	25 September 2012
Participant Information Sheet	6	21 January 2013
Protocol	12.2	24 January 2013
REC application		07 December 2012
Response to Request for Further Information		25 January 2013

Statement of compliance

This Committee is recognised by the United Kingdom Ethics Committee Authority under the Medicines for Human Use (Clinical Trials) Regulations 2004, and is authorised to carry out the ethical review of clinical trials of investigational medicinal products.

The Committee is fully compliant with the Regulations as they relate to ethics committees and the conditions and principles of good clinical practice.

The Committee is constituted in accordance with the Governance Arrangements for Research Ethics Committees and complies fully with the Standard Operating Procedures for Research Ethics Committees in the UK.

After ethical review

Reporting requirements

The attached document *"After ethical review – guidance for researchers"* gives detailed guidance on reporting requirements for studies with a favourable opinion, including:

- Notifying substantial amendments
- Adding new sites and investigators
- Notification of serious breaches of the protocol
- Progress and safety reports
- Notifying the end of the study

The NRES website also provides guidance on these topics, which is updated in the light of changes in reporting requirements or procedures.

Feedback