A study of Ca^{2+} signalling during platelet-human erythroleukemia cell interactions and the heterogeneity of platelet store-operated Ca^{2+} entry in healthy donors

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Tayyaba Iftikhar - A study of Ca^{2+} signalling during platelet-human erythroleukemia cell interactions and the heterogeneity of platelet store-operated Ca^{2+} entry in healthy donors

Abstract

Growing evidence indicates a role for platelets in cancer metastasis. This study has investigated communication between human platelets and the human megakaryocytic/erythroleukemic cell line (HEL) at the level of the ubiquitous second messenger, intracellular Ca^{2+} ($[Ca^{2+}]_i$).

In mixed cell suspensions, platelets amplified $[Ca^{2+}]_i$ responses to both thrombin and Pam_3CSK_4 (TLR1/2 agonist). Both agonists evoked ATP release from platelets, but not HEL cells, over a time course similar to the enhanced $[Ca^{2+}]_i$ increase. HEL cells showed robust $[Ca^{2+}]_i$ responses to ADP and ATP and expressed multiple P2 receptor subtypes (P2X4>P2Y11>>P2X1>P2X7>P2X6> P2Y12>P2Y13> P2Y2>P2Y1). The interaction mechanism proved difficult to study with thrombin due to direct Ca^{2+} responses in both cell types. However, Pam_3CSK_4 evoked a direct response only in platelets alone. ThromboxaneA_2 dependent ATP/ADP secretion was required for the platelet-dependent HEL response to Pam_3CSK_4 as this was blocked by either apyrase or aspirin. The role of specific P2 receptors could not be confirmed due to poor antagonist specificity. At the single cell level, Pam_3CSK_4-stimulated platelets evoked HEL Ca^{2+} responses that resembled responses to ATP more than ADP. Morphological responses were also observed in HEL cells, but did not correlate with the pattern of $[Ca^{2+}]_i$ increases.

In a further set of experiments, platelet store operated Ca^{2+} entry (SOCE) in healthy individuals was assessed with a view to studying its role in cancer. Marked inter-donor heterogeneity of SOCE was observed following activation by thapsigargin that reflected variable Ca^{2+} release due in part to a cAMP-dependent pathway. Less SOCE

heterogeneity was observed with cyclopiazonic acid, which may therefore be a preferable pharmacological tool for studies of this major platelet Ca^{2+} entry pathway.

In conclusion, this study has identified a role for Ca^{2+} signalling between platelets and myeloid leukemic cells that depends upon ATP/ADP secretion and thromboxaneA_2 release and may play a role in metastasis.

Publications and Presentations arising from this thesis

Poster communications

Tayyaba Iftikhar, Catherine Vial & Martyn P. Mahaut-Smith **An investigation of calcium signalling during interactions between platelets and a cell line of megakaryocytic/ erythroleukemic origin.**
Joint British Society for Haemostasis & Thrombosis (BSHT), Anticoagulation in Practice (AiP) and UK Platelet Group meeting; Leeds, United Kingdom (November 2016).

Tayyaba Iftikhar, Catherine Vial & Martyn P. Mahaut-Smith **Intercellular signalling between platelets and a myeloid leukemic cell line.**
(XXVI Congress of the ISTH and 63rd Annual Scientific and Standardization Committee (SSC) Meeting; Berlin, Germany (July 2017)).

Papers

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Abbreviations

$[Ca^{2+}]_i$……..Intracellular Ca^{2+}

5-HT……..5-Hydroxytryptamine

ACD…….. Acid-Citrate-Dextrose

ADP…… Adenosine diphosphate

ANOVA…… Analysis of variance

ATP……..Adenosine triphosphate

BSA…… Bovine serum albumin

DAG…… Diacylglycerol

ddH_2O……Double-distilled water

Dg……Digitonin

DMSO…….. Dimethyl sulfoxide

DTS……Dense tubular system

EGF……Endothelial growth factor

EGTA……Ethylene glycol-bis (β-aminoethyl ether)-N,N,N',N'-tetraacetic acid

EMT……..Epithelial mesenchymal transition

ER…….. Endoplasmic Reticulum

FDA…….. Food and Drug Administration

FGF……Fibroblast growth factor

GAPDH…….. Glyceraldehyde 3-phosphate dehydrogenase

GP…… Glycoprotein

GPIb……Glycoprotein Ib

GPCR…….. G-protein coupled receptor

HEK-293……. Human embryonic kidney-293 cell line

HUVEC…… Human Umbilical Vein Endothelial Cells

MMP……Matrix metalloproteinases

NO……Nitric oxide

PAF……Platelet activating factor

Pam_3CSK_4…….. Pam3CysSerLys4

PECAM……Platelet endothelial cell adhesion molecule

PDGF……Platelet derived growth factors

PDGFR…….. Platelet derived growth factor receptors

PGE1……...Prostaglandin E1

PIP2……Phosphoinositide-4,5-biphosphate

PKC……..... Protein kinase C

PKA…….. Protein kinase A

PDMP……Platelet derived microparticle

PGI_2……...Prostaglandin I_2

PI3K……..Phosphoinositide 3-kinase

PLC…….. Phospholipase C

PMCA…….. Plasma membrane Ca^{2+} ATPase

PMP……Platelet microparticle

PRP……..Platelet rich plasma

PVDF……Polyvinylidene fluoride

Prb……Probenecid

PS……..Phosphatidylserine

RPM……..Rotations per minutes

RIPA…….. Radioimmunoprecipitation assay

SDS……..…Sodium dodecyl sulphate

SEM……..Standard error of the mean

SERCA…… Sarco/endoplasmic reticulum Ca^{2+}-ATPase

SOCE……..Store operated Ca^{2+} entry

TBS-T……..Tris- buffered saline and Tween-20

TEMED……..Tetramethylethylenediamine

TG……Thapsigargin

TGF……..Tissue growth factor

TLR2/1…….. Toll-like 2/1 receptors

TRPC6…….. Transient receptor potential cation channel,
subfamily C, member 6

TxA_2…… Thromboxane A_2

TP…… Thromboxane A_2 receptors

vWF……von Willebrand Factor

Chapter 1. Introduction

1.1 Functional roles of platelets

Platelets play a vital role in various physiological processes such as haemostasis and immune responses. Under normal conditions platelets are inactive, since the endothelial lining both releases inhibitors such as nitric oxide (NO) and also prevents contact with agonists such as collagen in the sub-endothelial layers (Wu, 1996, Clemetson, 2012). In addition, the intact circulation is a barrier against infection by pathogens (Clemetson, 2012, Clemetson, 2011). Following vascular damage, platelets will be activated by exposure of collagen and may also be stimulated by invading microbial pathogens via platelet immune receptors.

1.1.1 Haemostasis

Haemostasis is a complex physiological mechanism that serves to minimise the blood loss at sites of injury and maintain an intact circulation throughout the body (see Fig. 1.1). It is the first step in wound healing following tissue damage. Under normal conditions, platelets are at rest flowing in the blood circulation. The endothelial surface of the blood vessels helps to keep the platelets inactive under normal conditions. Thus, in healthy vessels there is a proper balance between inhibitory and activatory factors. NO is released from vascular endothelial cells and functions to inhibit the platelets. On injury or vascular damage, platelets are initially activated by exposure to collagen and other factors in the sub endothelial layer and by ATP and ADP released from damaged cells. These initiate the process of haemostasis leading to formation of a platelet plug to ultimately reduce blood loss.

Haemostasis is mainly divided into two parts, primary haemostasis and secondary haemostasis. The primary haemostasis includes vasoconstriction, platelet adhesion to the site of injury, platelet activation, degranulation and aggregation. The secondary phase includes clot formation via coagulation factors, particularly thrombin, and the

formation of an insoluble fibrin mesh. The fibrin mesh stabilises the platelet plug especially in large blood vessels where the platelet plug is insufficient to stop blood loss (Gale, 2011) (See the summary of role of platelets in haemostasis in Fig. 1.1).

1.1.1.1 **Primary Haemostasis**

In this phase, glycoprotein receptors on the platelet surface membrane bind to adhesion proteins exposed within the sub endothelial extracellular matrix. These adhesion proteins include collagenic proteins as type I, IV, V, VI and non-collagenic proteins such as laminin. Also they may include von Willebrand factor (vWF) and thrombospondin, the latter being mainly found in proliferative tissues (Gawaz, 2001). The first step in the adhesion phase is interaction between vWF and the platelet receptor complex GPIb-V-IX and this is called the contact phase (Jackson, 2011). The importance of the vWF:GPIb-V-IX interaction can be reflected in diseases of either the GPIb-V-IX complex (Bernard-Soulier syndrome) or vWF (von Willebrand disease) where there is an increased bleeding tendency (Kauskot and Hoylaerts, 2012).One of the earliest functional responses of platelets in haemostasis is adhesion to the subendothelial tissue. On exposure to subendothelial agonists, platelets bind to exposed collagen via vWF to GPIb-IX-V on the platelet surface. vWF is present in plasma as a soluble form while in endothelial matrix it is found in an immobilised form (Ruggeri, 1994). It is constitutively secreted by the endothelial cells and also from platelet α-granules following activation (Ruggeri, 1994, De Meyer et al., 2009). The soluble vWF in plasma does not bind to platelets with high affinity. The high affinity binding takes place under shear stress exerted by flowing blood on the immobilised vWF on the subendothelial collagen (Siedlecki et al., 1996, Gale, 2011). This assists binding of collagen to GPVI receptors which are key signalling receptors during platelet activation. In their resting state, integrins such as $\alpha_{IIb}\beta_3$, $\alpha_2\beta_1$ and $\alpha_v\beta_3$ on the platelet surface are inactive. However once platelets are activated these integrins undergo a conformational change that exposes their high affinity ligand-binding sites (Luo and Springer, 2006, Xiao et al., 2004, Shattil, 1999, Shattil et al., 2010). Among the platelet surface integrins, $\alpha_{IIb}\beta_3$ is one of the most important as it is present at the highest

density of all the platelet surface receptors. $\alpha_{IIb}\beta_3$ binds to a number of ligands and promotes platelet-to-platelet binding and aggregate formation. These ligands include fibrinogen (Fb), vWF, collagen, fibronectin and vitronectin (Varga-Szabo et al., 2008c). While $\alpha_2\beta_1$, $\alpha_V\beta_3$, $\alpha_5\beta_1$ and $\alpha_6\beta_1$ bind with collagen, vitronectin, fibronectin or laminin respectively that play a smaller role in platelet aggregation (Emsley et al., 2000, Lam et al., 1989, Varga-Szabo et al., 2008d, Sonnenberg et al., 1988). By binding with collagen, $\alpha_2\beta_1$ enables firm adhesion to collagen in addition to its role in assisting GPVI engagement to the collagen. This binding initiates the immunoreceptor tyrosine-based activation motif (ITAM) - dependent signalling downstream of GPVI (Watson and Gibbins, 1998). Activation of this pathway contributes to platelet shape change via reorganisation of actin cytoskeleton and projection of pseudopods (Aslan et al., 2012). Platelet activation via GPVI also leads to intracellular Ca^{2+} mobilisation thereby increasing the $[Ca^{2+}]_i$ level and promotes dense granule secretion (Roberts et al., 2004). A number of agonists are released from dense granules, including ADP, ATP and 5-HT. These agonists further activate the platelet via GPCR pathways (ADP, 5HT) or a Ca^{2+}-permeable ion channel (ATP). The de novo synthesis of thromboxane (TxA_2) from arachidonic acid (AA) by cycloxygenase-1 (COX-1) also takes place and supports the platelet activation by mainly binding to thromboxane receptors (TP) as well as to other platelet prostanoid receptors but to a lesser degree (Hanasaki and Arita, 1988). The agonists released from platelets throughout the primary haemostasis activate the platelets via autocrine as well as paracrine mechanisms and help recruit other platelets at the site and form a platelet aggregate (Rivera et al., 2009). This is also termed a white thrombus or platelet plug and temporarily prevents bleeding, but is delicate and can be easily disentangled from the lesion (Gawaz, 2001). For a stable clot formation, fibrinogen conversion to fibrin is required that forms a fibrin mesh to generate a stable thrombus. Fibrin generation occurs concurrently to platelet aggregation (Falati et al., 2002, Furie, 2009) and is formed in the presence of thrombin generated from the coagulation cascade.

1.1.1.2 **Secondary haemostasis**

The serine protease thrombin is produced as the end product of the coagulation pathway (Coughlin, 2000, Monagle and Massicotte, 2011). Thrombin generation involves a series of steps initiated when tissue factor (TF) in the subendothelial layer is exposed to clotting factors in plasma (also called the initiation phase of the coagulation pathway) (Coughlin, 2000). Clotting factors are the coagulation proteins and are the precursor of the proteolytic enzymes called zymogens. These factors circulate in blood as an inactive form. Each factor in activated form is depicted by suffixing letter "a" to the Roman numeral representing the respective proteins (Palta et al., 2014). TF combines with factor VIIa and this complex activates factor IX and X (Dahlback, 2000). During the amplification phase the factor Xa (activated by TF-factor VIIa complex) promotes thrombin production on a small scale (Monroe et al., 2002). This thrombin activates factor VIII, V and platelets. Thrombin acts enzymatically at platelet PAR receptors (1 and 4 in human) to reveal the tethered ligand to start receptor signalling following "intramolecular ligation" (Coughlin, 2000, Vu et al., 1991). The negative surface of platelets exposing PS serves as a site for binding procoagulant zymogens and enzymes that include factor IXa, VIIIa, X, Xa, Va and II (Tracy et al., 1979, Nesheim et al., 1988, Ahmad et al., 1989, Wolberg and Campbell, 2008). This signalling cascade at the platelet surface generates high amounts of thrombin that activates transglutaminase factor XIII which catalyses the conversion of fibrinogen to fibrin (Lasne et al., 2006). This elastic fibrin mesh forms a stable clot. In addition to the signalling events in platelets that lead to inside-out activation of $\alpha_{IIb}\beta_3$, the fibrinogen (Fb) binding results in outside-in signalling which enhances the platelet activation and granule secretion, shape change and clot formation to prevent the blood loss (Shattil and Newman, 2004). Ultimately thrombin is also involved in inhibition of the coagulation cascade to terminate haemostasis. Thrombin binds to thrombomodulin (present on endothelial cells). This complex activates protein C (APC). APC in combination with protein S cleaves procoagulant cofactors VIIIa and Va. Additionally factor V also acts as a cofactor to APC/Protein S in this reaction. These complexes are formed on the negatively charged surface of activated platelets thus this localization is

also critical in inhibition of the coagulation cascade and the crucial site (Shattil and Newman, 2004, Gale, 2011, Cramer et al., 2010, Mann et al., 1990). Serine protease inhibitors are also involved in the inhibition of the coagulation cascade. One of the major inhibitors is antithrombin that inhibits thrombin and factor Xa, IXa and XIa in the presence of heparin (Quinsey et al., 2004). Other inhibitors include heparin cofactor II (inhibits thrombin), protein Z-dependent protease inhibitor (inhibits factor Xa) and C1 inhibitor (inhibit factor XIa). Furthermore, tissue factor pathway inhibitor and alpha-2-macroglobulin are also involved significantly in terminating haemostasis by inhibition of factor IXa and thrombin respectively (Han et al., 2000, Marlar and Griffin, 1980, Suzuki et al., 1983, Tollefsen et al., 1982, Wuillemin et al., 1995, Gale, 2011, Wood et al., 2014). Eventually fibrinolysis takes place that involves lysis of the blood clot in the wound healing process and prevention of blood clots in the healthy vessels. It takes place with the help of plasmin that is generated from proenzyme plasminogen in the presence of tissue-type plasminogen activator (tPA) and urokinase-type plasminogen activator (uPA) enzymes (Rau et al., 2007, Carmeliet and Collen, 2000). Plasmin dissolves the fibrin mesh into the blood circulation that is cleared from the blood by other proteases or excreted through the kidneys or the liver (Periayah et al., 2017).

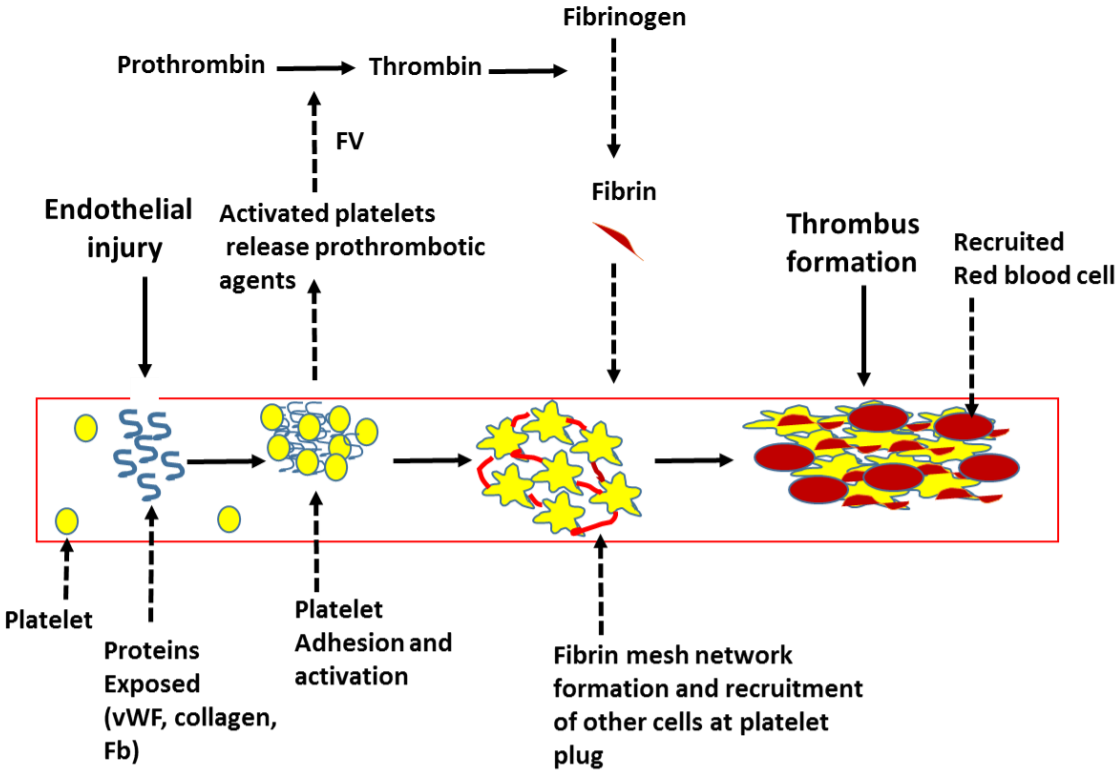

Fig 1.1 Thrombus formation

Upon endothelial injury, vWF collagen and Fb are exposed and attract platelets. The activated platelets release pro-thrombotic agents and bind activated coagulation factors that convert prothrombin to thrombin. Thrombin acts on fibrinogen converting it to fibrin. The fibrin mesh forms a network which attract other cells to the site of injury. Meanwhile an inflammatory response is also triggered by an intrinsic pathway. Activated platelets release procoagulant agents like ADP, ATP and TxA_2 that further stabilize the platelet plug. The fibrin mesh further enhances the platelet plug stabilization and recruits red blood cells and other inflammatory cells. The fibrin mesh network, along with the aggregated platelet plug, forms a thrombus on a surface.

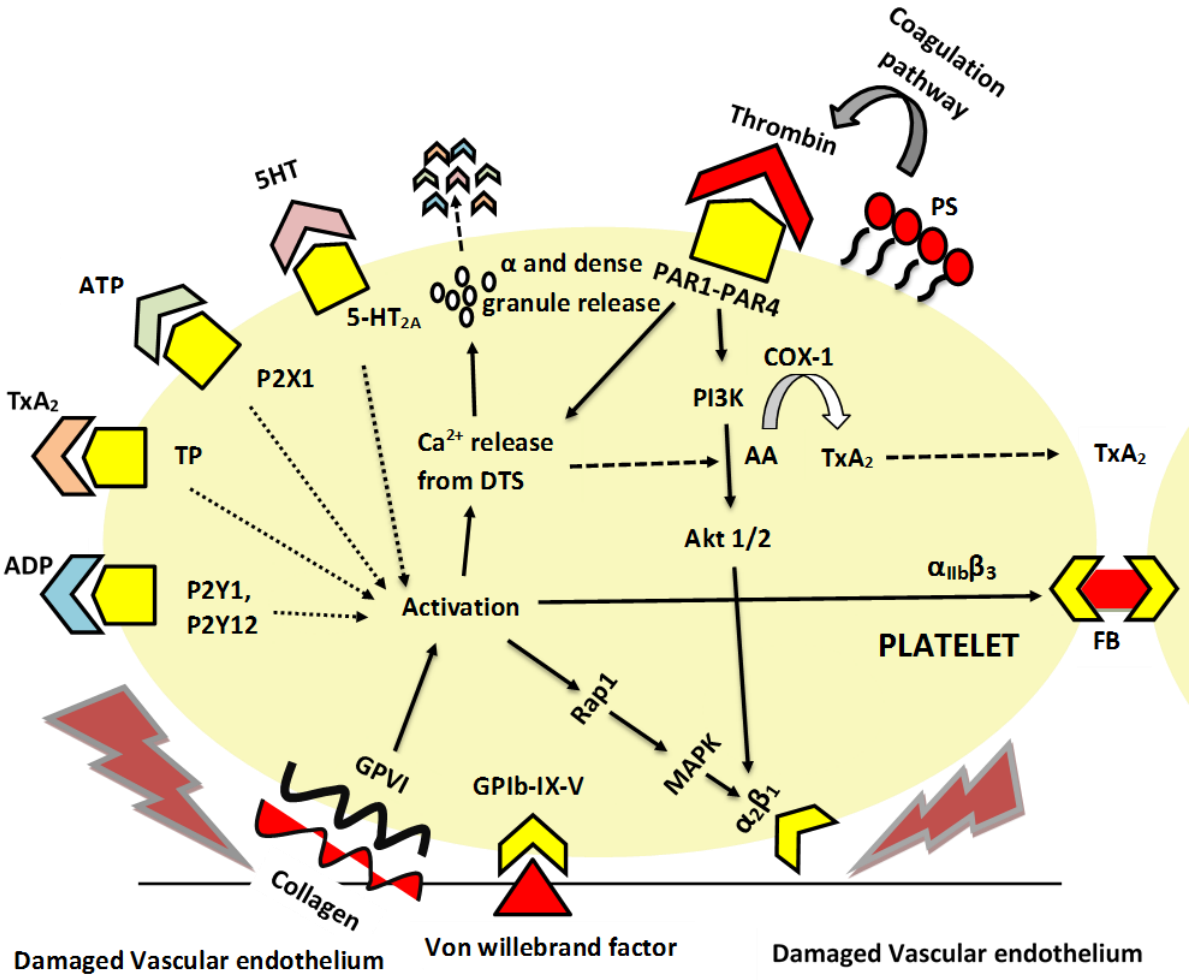

Fig. 1.2 Platelet receptors and activation pathways in haemostasis:

Platelet receptors on the platelet membrane bind to different agonists. On vascular injury, endothelial damage exposes collagen that binds to GP VI and adheres directly to $\alpha_2\beta_1$ directly and indirectly to GPIb-V-IX via von Willebrand factor. Collagen activates several signalling pathways including an elevation of intracellular Ca^{2+} level resulting in platelet and dense granule secretion and release of serotonin (5HT), ADP and ATP. These agonists activate platelets via autocrine and paracrine mechanisms by binding with 5-HT_{2A}, P2Y and P2X receptors respectively. Phosphatidylserine (PS) is expressed on the platelet surface and thrombin is generated via the coagulation pathway and binds to platelet PAR1 and PAR4. Fibrinogen (Fb) binds to $\alpha_{IIb}\beta_3$ integrins aiding platelet to platelet adhesion and fibrin mesh formation stabilising the platelet plug at the injury site. Major signalling events include Ca^{2+} increase, PI3 kinase activation and Rap1b; these lead to platelet granular secretion, shape change and integrin activation of the platelets.

1.2 Non-haemostatic physiological functions of platelets

Platelets are mainly known for their role in haemostasis but in recent years many studies have demonstrated that another major role of platelets is to contribute to the first line defence against infection and immunity (Gaertner et al., 2017), reviewed in (Semple and Freedman, 2010). Platelets are amongst the first cells that detect endothelial injury and microbial pathogens that gain access to the bloodstream via injuries (Al Dieri et al., 2012, Ali et al., 2017). Platelets also express chemokine receptors CCR1, CCR3, CCR4 and CXCR4 (Clemetson et al., 2000). These receptors detect signals from all four types of chemokines (C, CC, CXC and CX3C). These chemokines are generated at the site of infection and attract platelets. Additionally platelets express Toll like receptors (TLRs) which detect pathogen associated molecular patterns (PAMPs) (Pires et al., 2017). PAMPs detection is the first step in establishing the defence mechanism against pathogens. Human platelet TLRs recognize bacterial lipopolysacccharide (LPS), proteins, lipids and nucleic acids released from microbial pathogens. Other platelet receptors that interact with pathogens include FcγRIIa, GPIIb-IIIa (i.e. $\alpha_{IIb}\beta_3$) and GPIb (de Stoppelaar et al., 2016). Platelets interact through direct and indirect contact via these receptors. On binding with microbial agents, platelets are activated and release about 300 different biologically active agents. These agents include anti-microbial products which are collectively known as platelet microbial proteins (Moncada de la Rosa), and include kinocidins (CXCL4, CXCL7, CCL5), defensins (human B defensin 2), thymosin B4 and derivatives of platelet microbial proteins including thrombocidins and fibrinopeptide A or B (Yeaman, 2014). These agents not only impair the bacterial growth but also recruit neutrophils that form a neutrophil extracellular trap (NET) at the site of infection that reinforces the immunity against the pathogens (Ali et al., 2017). Pathogens like *S.aureus* and HIV are reportedly killed and phagocytosed by platelets (Alonso and Cox, 2015, Chakrabarti et al., 2005). Platelets help in blood clearance from these pathogens by phagocytosis. However these pathogens can also manipulate platelets by expressing the vWF-binding proteins that attach with vWF and result in prothrombin generation in the host and fibrin mesh

formation to facilitate clot formation (Claes et al., 2014). Therefore bacteria may use clots for tissue colonization and infection spreading (Flick et al., 2013). Similarly, platelet-virus interactions may in some cases alter the morphology of platelets and disturb the haemostatic balance that leads to complications like thrombocytopenia as in the case of Dengue fever (Hottz et al., 2011). As discussed above, TLRs are the pattern recognition receptors expressed on platelets and platelets contribute to phagocytosis and inflammation via these receptors. Types of TLRs include TLR1, TLR2, TLR4, TLR6, TLR8 and TLR9. One of the most important TLR ligands is LPS expressed on gram negative bacteria (McGregor et al., 2006). During sepsis, TLR4 expressed on platelets serves as a bridge between platelets and other immune cells (Yu et al., 2014). Platelet TLR4 signalling activates platelets and leads to shedding of pro-Interleukin-1β (IL-1β) rich microparticles that act as platelet agonists and amplify the LPS-induced platelet activation via an autocrine mechanism. Hence pro-Interleukin-1β is a platelet agonist that is produced by platelets and links immunity with thrombosis by activating platelets (Brown et al., 2013, Haselmayer et al., 2007). Also TLR4-activated platelets interact with neutrophils and recruit them in extracellular traps for bacteria that further supports the host innate immune system (Vasina et al., 2010). Similarly TLR2 activation by bacteria/pathogens leads to increased platelet-neutrophil adhesion and megakaryocyte stimulation that produces platelets with a higher level of expression of proinflammatory genes and proteins (Lee et al., 2014). Other TLR receptors have been shown to be activated in inflammation and thrombosis but their functions are yet to be clearly defined (Crocker et al., 2007).

In addition to haemostasis and immunity, platelets have also been reported to be involved in the physiological functions of angiogenesis and liver regeneration (Walsh et al., 2014, Lakka Klement et al., 2013, Kawasaki et al., 2010).

1.3 Platelets in pathologies

Although platelets play a vital role in haemostasis, and are involved in immunity, they are also involved in the progression of various pathological complications, including

chronic and acute inflammation, auto-immune diseases triggered by the recognition of endogenous ligands (Cognasse et al., 2015b) and cancer (Goubran et al., 2013). They are reported to be hyperactive in (or also involved In the aetiology of) a range of pathologies including diabetes mellitus (Vinik et al., 2001), hypertension (Iyú et al., 2004), hypercolesteremia (Shiraishi et al., 2010), asthma (Kornerup and Page, 2007) and atherosclerosis (Huo et al., 2003).

1.3.1 Platelets in Diabetes Mellitus

Patients with diabetes mellitus show enhanced platelet aggregation and elevated atherosclerosis resulting at least in part from decreased fibrinolytic properties due to increased PAI (plasminogen activator inhibitor) that inhibits the formation of plasmin from plasminogen (Kearney et al., 2017). In addition, there is evidence of decreased vascular synthesis of PGI_2 (Moore, 1982) and NO formation or decreased bioavailability in diabetes mellitus (Signorello et al., 2007). In health, PGI_2 is responsible for platelet inactivation by binding to inhibitory G-protein coupled receptors that stimulates adenylate cyclase, while NO causes the activation of guanylate cyclase that inhibits the phosphorylation of protein kinase leading to platelet inactivation (Vinik et al., 2001). Hyperhomocysteinaemia is a metabolic disorder mostly reported in type-2 diabetes due to reduction in vitamin B12 mediating homocysteine metabolism (Undas et al., 2005). Increased levels of homocysteine in Diabetes Mellitus activate platelets via augmented store-operated Ca^{2+} entry (SOCE) (Alexandru et al., 2008). Therefore, enhanced SOCE in diabetes may be a cause of augmented platelet aggregation/activation in diabetes mellitus partly due to hyperhomocysteinaemia. Moreover, insulin naturally antagonises platelet hyperactivity by sensitising platelets to PGI_2 and NO released by vascular endothelium (Kahn and Sinha, 1990). Therefore hyperglycaemia is closely associated with platelet hyperactivity as well as with an overall disturbance in the coagulation cascade (Vinik et al., 2001).

1.3.2 Platelets in hypercholesterolemia

Hypercholesterolemia is another example of a disease in which platelets are hyperactive, displaying augmented responses to agonists like thrombin, collagen and ADP as it is reported that elevated lipid levels induce platelet hyperactivity (Naqvi et al., 1999, Lerch et al., 1998). Platelets serve as a rich source of cholesterol that can be accumulated by smooth muscle cells and macrophages as droplets (Kruth, 1985). Platelets also enhance cholesterol ester production and accumulation in cultured peripheral mononuclear-derived macrophages (Curtiss et al., 1987). Therefore, the suppression of platelet hyperactivity is crucial in anti-atherogenesis therapy (Kahn et al., 2011). Similarly hypercholesteraemia is recognised as a cause of atherosclerosis and is associated with platelet hyperaggregability and enhanced platelet deposition as well as microvascular obstruction (Golino et al., 1987, Badimon et al., 1991). In hypercholesteraemia, platelets develop resistance to prostaglandin E_2 and I_2 thus reinforcing the platelet hyperactivity (Zucker et al., 1986). Also an in vitro study of platelets from hypercholesteraemia patients as compared to normal platelets showed fourfold reduction in response to factor Xa (Kahn et al., 2011). Shiraishi and colleagues demonstrated that in rabbit platelets, Ca^{2+} influx is more sensitive to cholesterol depletion than Ca^{2+} release from the Ca^{2+} stores involving direct activation of PKC and ADP secretion (Shiraishi et al., 2010). In their trials they treated platelets with methyl-β-cyclodextrin which is used to remove cholesterol from the membranes and found decreased platelet responses to thrombin, collagen and ADP. On the other hand, the same effect was augmented in cholesterol-rich platelets. These studies suggest that cholesterol induces hyperactivity in platelets which could lead to an involvement in the pathogenesis of atherosclerosis.

1.3.3 Platelets in hypertension

The mechanisms whereby platelet activity is altered in hypertension include endothelial dysfunction, disturbance in sympathetic and angiotensin-renin systems, decreased NO synthesis and platelet degranulation as a result of shear stress (Grassi,

1998, Gkaliagkousi et al., 2010). In hypertension platelet α2 adrenoceptors become unregulated resulting in great sensitivity to catecholamines (Grassi, 1998). Angiotensin II also affects platelet function via angiotensin receptors (AT1) on the platelet surface leading to PLC activation (Gkaliagkousi et al., 2010). Moreover angiotensin II infusion in humans elevates the P-selectin and β-thromboglobulin in the circulation that also enhance platelet-Fb binding (Larsson et al., 2000). Endothelial dysfunction in hypertension leads to underproduction of endothelial-derived NO, also results in platelet hyperactivation and enhanced platelet adhesion to endothelial cells (Radomski et al., 1987). In addition, shear stress is also a cause of platelet activation in hypertension. High blood pressure is associated with shear forces that may lead to platelet activation and degranulation (Lip et al., 2001). Furthermore platelet microparticles (PMP) production is also enhanced in hypertension and carry thrombogenic and proinflammatory agents (Labiós et al., 2006). Similarly another study demonstrated elevated levels of cytosolic Ca^{2+} in hypertensive platelets as compared to normotensive platelets that was found inversely correlated to NO levels (Camilletti et al., 2001).

This suggests that platelets play an active role in hypertension. Interestingly, some of the potent antihypertensive drugs, losartan and simvastatin, result in inhibition of platelet activation, possibly via a mechanism involving reduced sensitivity to TxA_2 (Nomura et al., 2004, Guerra-Cuesta et al., 1999). Hence some anti-hypertensive medications may aid in the prevention of cardiovascular complications in part via inhibition of platelets.

1.3.4 Platelets in renal failure

Altered activity of platelets may also contribute to both the symptoms and aetiology of renal impairment (Boccardo et al., 2004). Compromised platelet responses in renal patients are thought to be due in part to uremic toxins found in circulating blood. Impaired intrinsic platelet activation is observed as well as altered platelet interaction with the vessel wall (Kaw and Malhotra, 2006). Haemodialysis can clear the toxins

however can lead to hyper activation of platelets resulting from contact with artificial surfaces. Platelets have also been proposed to play role in glomerular pathogenesis by altering the glomerular permeability (Boccardo et al., 2004, Castillo et al., 1986).

1.3.5 Platelets in Asthma

Platelets also have a role in triggering asthma as they release various bronchoconstricting agents including histamine, serotonin, arachidonic acid metabolites and platelet activating factor (PAF) on activation (Kornerup and Page, 2007). The initial stimulus that activates platelets in asthma is unclear however, PAF is a potent activator of platelets and is released in asthmatic inflammation (Barnes, 1988). This causes recruitment of eosinophils, macrophages and platelets into the airway passages and an inflammation cascade starts (Vargaftig and Braquet, 1987). The PAF induces platelet aggregation and diapedesis in alveolar lumen (Lellouch-Tubiana et al., 1985). PAF is an important mediator of asthma by contracting bronchial smooth muscles (reviewed in (Chung and Barnes, 1991). PAF also induces asthma symptoms in baboon lungs by blocking the air passage followed by platelet aggregation after a rise in pulmonary pressure (Arnoux et al., 1988). Activated platelets induce bronchoconstriction as reported by studies of guinea pigs in which intravenously injected thrombin resulted in blockade of airway passages (Cicala et al., 1999). In another study intratracheal instillation of a bacterial endotoxin, LPS induced bronchial constriction followed by platelet activation (Vincent et al., 1993). These studies suggest an essential role of platelets in onset of asthmatic symptoms. Moreover platelets express adhesion molecules and have the ability to roll over the endothelium and bind with it. Platelets can also adhere with the leukocytes in the vasculature and act as a chaperone to help in the extravasation of the leukocytes from pulmonary microcirculation to bronchial airways thus triggering asthma (reviewed in (Chung and Barnes, 1991).

1.3.6 Platelets in Cancer

Cancer has always been found to enhance platelet activation. Platelets in cancer show marked abnormality partly due to the effect of tumour cells on platelets (called tumour cell induced platelet aggregation) (Goubran et al., 2013). In 1988, Grignani and Jamieson found that tumour cells spontaneously shed membrane vesicles in vitro that could induce strong aggregation in interacting platelets due to release or generation of pro aggregatory mediators like ADP and thrombin by the cancer cell vesicles (Grignani and Jamieson, 1988). This release of platelet agonists from cancer cells could be due to frictional cell damage by capillaries and the cytoplasmic leakage of ADP. Likewise, chemotherapy and radiotherapy also cause platelet activation by releasing agonists like ADP, ATP and TxA_2 (Grignani and Jamieson, 1988). The released ADP is extracellular and platelets have receptors, P2Y1 and P2Y12 that respond to ADP by showing aggregation (Egan et al., 2011). Also, thrombin generation is evidenced in live solid pancreatic cancer cell lines and haematological cancer cell lines, assayed by calibrated automated thrombography (CAT) (Adesanya et al., 2017). On activation, platelets express agents like P-selectin, integrins, glycoproteins, thromboxane, growth factors, organellar by-products and platelet microparticles that help in cell to cell signalling and cancer progression (Goubran et al., 2013). Some of the underlying mechanisms are discussed below.

1.4 Platelet-cancer interactions

1.4.1 Mechanisms whereby platelet activity is altered by tumours

Thrombosis is closely associated with malignancy and the major reason for this link is that cancer cells release various potent platelet stimulating agents that include TxA_2, ADP, serotonin (Hoffman et al., 2001, Donati and Falanga, 2001, Zucchella et al., 1989, Egan et al., 2011, Chiang et al., 1995, Steinert et al., 1993). Furthermore, many of the cancer types express tissue factor (TF) which promotes thrombin generation that subsequently activates surrounding platelets (Chiang et al., 1994). Similarly, cancer

cells also release chromatin protein HMGB1 (high mobility group box 1) which stimulates platelets by binding with platelet toll-like receptors especially TLR4 (Yu et al., 2014). Likewise, some of the cancer cells express podoplanin which contributes to platelet activation by binding with platelet CLEC-2 (Suzuki-Inoue et al., 2006, Lowe et al., 2012a). Cancer cells in breast, colon and prostate cancer have been shown to induce platelet dense granule secretion and release ADP, ATP and serotonin by binding with platelet FcϒRIIa receptors (Mitrugno et al., 2014). These aforementioned mechanisms lead to cancer cell induced platelet aggregation (Mitrugno et al., 2014) (see Fig. 1.3). Moreover cancer cell surface proteins co-attract platelets and granulocytes via the neutrophil-released cathepsin G that is also a platelet agonist (Shao et al., 2011, Faraday et al., 2013). Likewise, vWF-cleaving protease ADAM13 deficiency is also seen in some cancer types which causes increased vWF expression that resultantly causes excessive platelet activation (Oleksowicz et al., 1999).

In haematological cancers enhanced platelet activity is also indicated by detection of PAF-4 and β-thromboglobulin (B-TG), markers of platelet stimulation found in blood (Al-Mondhiry, 1983, Blann et al., 2001). Similarly, many cancer types, including leukaemia and colon cancer, produce elevated levels of activated blood coagulation factors such as factor V, FVIII, FIX due to expression of TF and exposure of phosphatidylserine (PS) on cell surface (Hoffman et al., 2001, Lima and Monteiro, 2013). The overall disturbance of the coagulation system and increased expression of coagulation factors leads to excessive activation of platelets (Li, 2016). Some of the main alterations of platelet surface proteins and signalling pathways in cancer patients are discussed below.

1.4.1.1 **P-selectin**

P-selectin (CD62P) is a cell adhesion receptor found on activated platelets and stimulated endothelial cells that is associated with platelet adherence onto leukocytes (Chen and Geng, 2006). It has been found to bind to various tumour cell lines including those derived from colorectal cancer, lung cancer, breast cancer, gastric cancer, malignant melanoma, neuroblastoma and adenoid cystic carcinoma. The ligand

molecules for P-selectin on tumour cells are yet to be identified but suggested as glycoprotein ligands (Degen et al., 2009). Moreover over expressed P-selectin not only facilitates platelet adhesion with leukocytes but also provides various growth factors and mitogens that promote cancer cell proliferation (Chen and Geng, 2006). An important role for P-selectin in cancer metastasis was further suggested in colon cancer when modified heparin inhibited P-selectin-mediated adhesion of cancer cells to platelets and inhibited metastasis (Wei et al., 2004). P-selectin binding with tumour cells is also mediated by PSGL-1. PSGL-1 is a P-selectin counter-receptor found in non-small cell lung cancer cells (Azab et al., 2012, Dimitroff et al., 2005, Gong et al., 2012). Similarly other P-selectin ligands CD44 and CD24 are found in colon cancer and breast cancer respectively (Dimitroff et al., 2005, Aigner et al., 1998). In mice PSGL-1/P-selectin binding resulted in platelet: cancer cells conjugates and enhanced platelet activation. Likewise, another unidentified P-selectin binding receptor, found in testicular cancer cells, was found to mediate platelet- cancer cell binding via integrins and P-selectin in platelets which subsequently enhanced platelet activation (Larrucea et al., 2007a).

1.4.1.2 **Glycoproteins and integrins**

The most influential glycoproteins in the case of adhesion are $\alpha_2\beta_1$ and $\alpha_{IIb}\beta_3$. These glycoproteins assist adhesion of platelet:cancer cell conjugates to the extracellular matrix thus facilitating tethering and migration through the matrix to new tissue (Hall et al., 2008). Anti $\alpha_{IIb}\beta_3$ and anti GPIb antibodies have shown anti-metastatic roles in animal models (Li, 2016). Platelet adhesion to cancer cells is vital in metastasis. In metastasis, tumour cells detach from the primary site and enter the blood stream where they contact other blood cells including platelets. Platelets physically interact with cancer cells via binding of ADAM-9 and fibrinogen/$\alpha_6\beta_1$ and $\alpha_{IIb}\beta_3$ with tumour cell ADAM-9 and fibrinogen/$\alpha_V\beta_3$ respectively (Lavergne et al., 2017a). Additionally, cancer cell binding to platelets via integrin-fibronectin binding leads to tumour arrest in the circulation and initiates the metastatic niche (Bendas and Borsig, 2012). Platelet activation by direct contact with cancer cells leads to tumour cell induced platelet

activation (TCIPA). This leads to platelet shape change and $\alpha_{IIb}\beta_3$ activation by binding of talin and kindlin to the β_3 chain. This further releases TxA_2, ADP, ATP, TGF-B and VEGF from platelets. These biologically active molecules consequently activate cancer cells and further promote TCIPA (Lavergne et al., 2017b).

1.4.1.3 **Thromboxane A_2**

Thromboxane synthase, which catalyses the conversion of prostaglandin H_2 to thromboxane A_2 (TxA_2), is over expressed in some cancer types such as adenocarcinoma. Interestingly, inhibition of this enzyme decreased proliferation and apoptosis in non-small cell lung cancer cells in vitro and over expression resulted in invasiveness and proliferation of cancer cells (Cathcart et al., 2011). The TxA_2 mimetic agent U46619 has been shown to induce endothelial cell migration, an effect inhibited by the TxA_2 receptor antagonist SQ29,548 (Nie et al., 2000a). This provides evidence that platelets can expedite tumour metastasis by promoting angiogenesis via production of TxA_2. The process involves the effect of fibroblast growth factor (bFGF) or vascular endothelial growth factor (VEGF) on endothelial cells that increases TxA_2 synthesis in endothelial cells three to five fold (Nie et al., 2000b). The TxA_2 receptors (TP) on platelets and endothelial cells result in IP_3-mediated Ca^{2+} mobilisation that induces a number of responses including platelet aggregation, endothelial cell migration and release of prostacyclin from endothelium to cause vasoconstriction (Nie et al., 2000a). In a study in which murine platelets were incubated with sarcoma cells (mFS6), aspirin treatment caused a significant inhibition in sarcoma-cell induced platelet aggregation, suggesting a role for TxA_2 production in TCIPA (Pacchiarini et al., 1982). Furthermore aspirin in combination with an ADPase (ATP102) synergistically reduced breast cancer and melanoma bone metastasis in mice (Bradley et al., 1997a). Although aspirin is recognised as a key platelet inhibitor, platelet aggregation induced by uterine carcinosarcoma cells was surprisingly not completely inhibited by aspirin (Bradley et al., 1997a). These results indicate a partial role for TxA_2 in platelet activation induced by cancer (Bradley et al., 1997a). Furthermore, in some of the studies aspirin failed to reduce the TCIPA that may be due to the nature of the tumour

cells which induce TxA_2 release via COX-1 independent pathways (reviewed in (Elaskalani et al., 2017a).

1.4.1.4 **Platelet microparticles**

There is emerging evidence that platelet microparticles (PMPs) are an important pathological factor in diseases such as diabetes mellitus, atherosclerosis and cancer (Burnouf et al., 2014). In cancer, PMPs are considered crucial in metastasis as they carry various bioactive molecules, including nucleic acids, growth factors, and also pro-inflammatory factors (Goubran et al., 2013). In vivo PMPs are released from platelets upon platelet activation, shear forces and apoptotic signals. In vitro they can be released during standard manipulation steps such as centrifugation and prolonged storage (Goubran et al., 2013). About 40 glycoproteins have been reported to be found on the platelet surface in their resting state and could potentially also end up on the surface of platelet-derived microparticles (Horstman and Ahn, 1999). Among these, the adhesive receptor $\alpha_{IIb}\beta_3$ is considered as a biomarker of platelet derived microparticles (Holme et al., 1998). These receptors promote cancer cell adhesion to fibrinogen and endothelial cells. Upon activation, activated platelets release certain substances from within the dense and α-granules including VEGF and fibroblast growth factors (FGF-2) that subsequently express on the platelet cell surface and act as bioactive molecules on the surface of the PMPs (Tschoepe et al., 1990). These growth factors are involved in angiogenesis. Including these proteins, some phospholipids such as sphingosine 1-phosphate (S1P) are also involved in PMP-induced angiogenesis and migration of blood vessels through EDG-1 and EDG-3 receptors on the endothelium (Kim et al., 2003). EDG-1 and EDG-3 are the endothelial cell surface receptors that bind to platelet microparticle-derived S1P and induce cell growth by DNA synthesis, cell growth and migration (Kimura et al., 2000). PMPs can also transfer $\alpha_{IIb}\beta_3$ to malignant cells leading to adhesion of the cancer cells to fibrinogen and vascular endothelium, which together with chemoattraction of cancer cells facilitates tumour proliferation as observed for lung carcinoma in mice (Janowska-Wieczorek et al., 2005).

1.4.1.5. CLEC-2/podoplanin binding in platelet-cancer interaction

Podoplanin is a transmembrane protein expressed in various metastatic cancer cell types and the native ligand for the tyrosine kinase-coupled CLEC-2 hem-ITAM receptor (Atsumi et al., 2008, Lowe et al., 2012b, Suzuki-Inoue et al., 2011). CLEC2 is expressed on the surface of platelets and its interaction with podoplanin on cancer cells is one of the main mechanisms by which platelets become activated following direct physical contact between the two cell types. These CLEC-2/podoplanin interactions lead to platelet aggregation, granular secretions, formation of platelet-tumour cell conjugates and the platelets coating the tumour cells protect them from immune attack as well as shear stress (Takagi et al., 2013, Lowe et al., 2012b). The anti-podoplanin agent MS-1 interferes with binding to CLEC-2 and was found to reduce metastasis and tumour cell proliferation in vivo (Takagi et al., 2013).

1.4.1.6 Platelet P2 receptor activation in cancer

Many pieces of evidence demonstrate that platelet P2 receptor inhibitors are effective as anti-cancer therapies. This suggests that platelet P2 receptor activation may be one of the significant factors involved in cancer progression. Significantly enhanced platelet activation via P2 receptors has been shown to occur by ADP released from several cell lines including MCF-7 where ADP induced platelet aggregation was abolished by 2-MeSAMP, a P2Y12 blocker and apyrase, a nucleotidase (Alonso-Escolano et al., 2004). Also colon carcinomas, melanoma metastasized to lymph nodes, human melanoma 1402 (Boukerche et al., 1994), neuroblastoma (Bastida et al., 1986) and human hepatoma cells all released ADP that showed a subsequent aggregation in intact platelets. These effects were more prominent in the cells derived from the metastatic stage than from the primary site (Grignani et al., 1989). The ADP released from the tumour cells activates platelets via $P2Y_1$ and $P2Y_{12}$ receptors and enhances platelet activation. This releases various angiogenic agents from platelets such as VEGF that help in cancer metastasis and progression (Bambace et al., 2010).

1.4.1.7. PAR1-PAR4 activation in platelets

Another major factor generated by tumour cells that influences platelets is thrombin (Marchetti et al., 2012). This was studied in the Hut28 cell line (mesothelioma) in which platelet aggregation was analysed in the presence of tumour cell suspensions. The Hut28 cells induced platelet aggregation that was inhibited in the presence of the thrombolytic agent, hirudin (Bastida et al., 1981, Bastida et al., 1982). Thrombin generation has been detected in many cell lines including small cell lung cancer, renal tumours, ovarian cancer and melanoma (Wojtukiewicz et al., 1990a, Zacharski et al., 1993, Wojtukiewicz et al., 1990b). Tumour-released thrombin was shown to activate TCIPA via PAR1 and PAR4 on platelets (Zucchella et al., 1989, Bambace and Holmes, 2011a). Thrombin-activated platelets release up to 300 biologically active molecules that further act on the site of injury, wound, tumour aggregates and tumour vasculature (Coppinger et al., 2004). These agents include various growth factors hence promoting tumour growth and proliferation (Coppinger et al., 2004).

	Mediators	**Effect on platelets**	**Type of cancer studied**	**References**
Diffusible agents	ADP	Direct release from cancer cells acts as a platelet agonist via P2Y1 and P2Y12 receptors.	SKNMC neuroblastoma, Small-cell lung cancer, melanoma M1Do; M2Da; M4Be, Breast carcinoma MCF7 and fibroblastoma HT-1080.	(Hoffman et al., 2001, Zucchella et al., 1989, Egan et al., 2011, Mitrugno et al., 2014, Elaskalani et al., 2017a, Jurasz et al., 2004).
	Thrombin	Induced by TF expressed by cancer cells	Human glioblastoma U87MG, Neuroblastoma and pancreatic cancer cells.	(Egan et al., 2011, Chiang et al., 1995, Elaskalani et al., 2017a, Li, 2016).
	TxA_2	Released by TCIPA and from tumour cells, induce platelet	Walker 256 carcinosarcoma cells, metastasizing squamous cell	(Li, 2016, Hoffman et al., 2001, Steinert et

		activation via autocrine and paracrine mechanism.	carcinoma of the larynx.	al., 1993, Jurasz et al., 2004, Pinto et al., 1993).
	Tumour cell-released HMGB1	Acts via platelet TLR4 activation.	murine B16F10 melanoma and Lewis lung carcinoma (LLC) tumour cells	(Li, 2016, Yu et al., 2014).
	Cathepsin B	PA4-4 cleaving leading to Platelet activation, shape change, $\alpha_{IIb}\beta_3$ activation and secretion.	Murine mucinous adenocarcinomas	(Jurasz et al., 2004, Shao et al., 2011, Faraday et al., 2013).
	Matrix metalloproteinases	Platelets activate proMMP-2 to MMP-2 by MMP-14	HT-1080, MCF-7 and metastatic prostate cancer.	(Jurasz et al., 2004, Alonso-Escolano et al., 2006).
Physical cell-cell contact	Mucin	Binds with platelets and platelets recruit granulocytes by chemoattraction and enhance platelet activation via granulocyte-released platelet activators such as cathepsin G.	neuroblastoma and small cell lung cancer	(Stone and Wagner, 1993b).
	Unknown cancer cell component	Activate platelet dense granule secretion via FcϒRIIa activation.	Prostate cancer	(Mitrugno et al., 2014, Elaskalani et al., 2017a).
	CD24, CD44, PSGL, Podocalyxin and integrins	Facilitate cancer cell- platelet adhesion particularly via platelet P-selectin.	Testicular cancer, breast cancer, colon cancer.	(Elaskalani et al., 2017a, Larrucea et al., 2007b).
	Podoplanin	Binds with platelet CLEC-2 receptors.	mouse melanoma	(Suzuki-Inoue et al., 2006, Lowe et al., 2012a).

	Enhanced GP-IX-V expression (in PLTs as well as cancer cells).	Enhances platelet adhesion	MCF7	(Jurasz et al., 2004).
	αVβ3 expression in tumour cells	Enhance platelet adhesion with tumour cells via fibrinogen binding	Breast cancer cell models (MDA-MB 435).	(Felding-Habermann et al., 2001).
Coagulant disturbances	Tissue factor expression	Induces thrombin generation.	pancreatic cancer cell line BXPC3, breast cancer cell line MCF7, leukaemia and colon cancer	(Palumbo et al., 2005a, Pucci et al., 2016a, Khorana and Fine, 2004, Lyman and Khorana, 2009, Elaskalani et al., 2017a).
	Cysteine protease and elevated FV, FVIII, FIX, FX and FXI	Activates FX independent of FVII and Enhance Thrombin generation	Acute promyeloctic leukemia, NB4 cell line	(Hoffman et al., 2001, Donati and Falanga, 2001).
Other	Decrease in vWF-cleaving proteases(ADAM13)	Increased vWF multimers in circulation and platelet activation.	HT-1080	(Hoffman et al., 2001, Donati and Falanga, 2001, Oleksowicz et al., 1999, Jurasz et al., 2004)

Table 1.1 Cancer cell-dependent factors that induce TCIPA

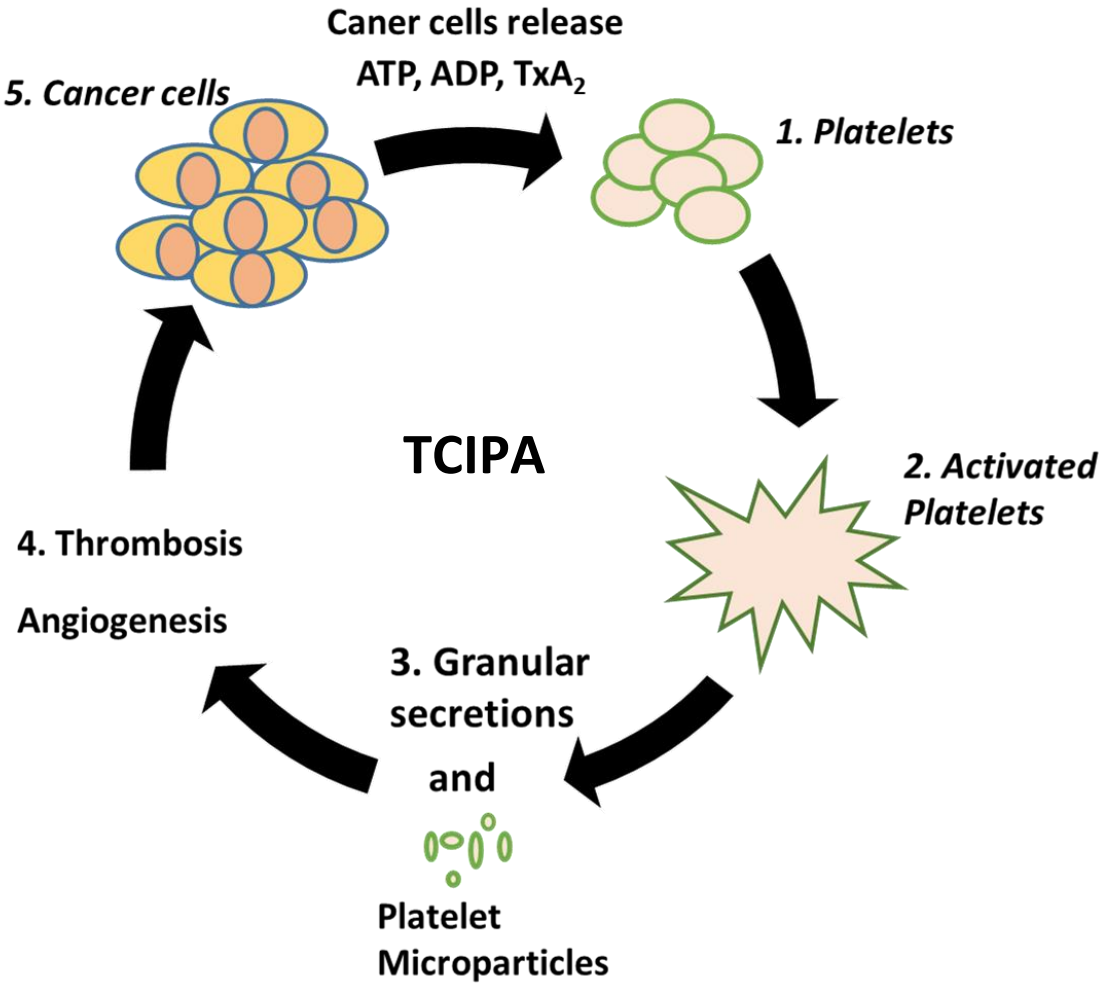

Fig 1.3 Platelet- cancer interaction:

Upon activation, platelets release granular secretions and generate microparticles that help in thrombosis and angiogenesis. The growth factors released by platelets (e.g. VEGF, PDGF) are used by cancer cells for growth. The activated platelets with adhesion receptors help in binding with cancer cells that form globules with cancer cells and protect them from the immune system. Also, the platelet-cancer cells globules help the cancer cells in protection from the shear forces in flowing blood that could damage the tumour cells. The cancer cells are leaky and release various platelet activating agents like ADP, ATP and TxA_2. These agents act on surrounding platelets and activate them and induce TCIPA (tumour cell induced platelet activation) which aids further cancer progression.

1.4.2 Effect of platelets on tumour progression

Many studies have reported an active role of platelets in cancer progression. Cancer cells not only alter the activities of platelets but are also subsequently affected by the modified platelet responses. The crosstalk between platelets and cancer cells has become a main interest in research as many studies have proposed that inhibition of platelets can be used in cancer therapy. Activated platelets affect the cancer in many ways, some of which are expanded upon in further detail below.

1.4.2.1 **Induction of metastasis by platelets**

One of the major requirements in metastasis is a change in cell phenotype to become more aggressive and invasive. The cancer cell elongates and is able to migrate more easily (Weeraratna et al., 2002, Larue and Bellacosa, 2005, Burk et al., 2008, De Craene and Berx, 2013). The metastasized tumour may reach and invade vital organs, ultimately causing organ failure and death (Kawada et al., 2008, Ikushima et al., 2009b). Circulating cancer cells may become surrounded by platelet aggregates that protect the tumour cell from the body's immune system (Palumbo et al., 2005b). Activated platelets release TGFβ1 (Assoian et al., 1983) which is known to cause metastasis in cancer cells as it alters the cell phenotype from an epithelial to mesenchymal type by acting through the P-Smad pathway (Berger, 1970, Ikushima et al., 2009a, Heldin et al., 2012, Jia et al., 2015b). These cells thus acquire the characteristic of migration and invasion of extracellular matrices (Heldin et al., 2012, Jia et al., 2015b). Colon carcinoma cells (MC38GFP isolated from Grade III cancer) and breast carcinoma cells (Ep5) that had been pre-incubated with platelets increased metastasis in mice as compared to the control tumour cells pre-incubated without platelets (Koenen, 2016, Golebiewska and Poole, 2015a, Guillem-Llobat et al., 2016, Labelle et al., 2011b, Berger, 1970). In addition to soluble platelet-released agents, direct physical contact between tumour cells and platelets also induces epithelial mesenchymal transition (EMT) and metastasis via NF-κB pathway activation in cancer

cells (Labelle et al., 2011b, Labelle et al., 2014b). In some studies, platelets have also been shown to have an inhibitory effect on cancer as platelet-derived signals can act as chemoattractants by recruiting lost immunity cells to the tumour foci. An example is C-X-C motif ligands CXCL5/7 that recruits granulocytes which have a cytotoxic effect on cancer (Labelle et al., 2014b). In general, however, platelets exhibit more means whereby they can induce metastasis that inhibit it. For example, the enzyme autotoxin (ATX) is released from platelets and promotes cancer cell growth and progression (Nam et al., 2001, Liu et al., 2009b). ATX was originally isolated as tumour motility-stimulating protein (Nam et al., 2001), it has phospholipase D activity that generates cyclic phosphatidic acid from lysophophatidylcholine (David et al., 2010, Benesch et al., 2014, Leblanc et al., 2014). Furthermore, platelets are also known to release microparticles that contain micro RNA. These micro particles are reported to enhance lung cancer (A549 cells) progression by delivering the microRNA-223 and targeting the tumour suppressing genes EPB41L3 (Liang et al., 2015). The PMPs were labelled with DiI-C_{16} and infiltration of the fluorescent vesicles was detected in A549 cells (Liang et al., 2015). The PMP-derived microRNA-223 bound to EPB41L3 mRNA transcript and inhibited the EPB41L3 translation (the tumour suppressing genes) and promoted tumour invasion (Liang et al., 2015). Unambiguously, adhesion receptors on the platelet surface also facilitate the platelet cancer interaction that further helps in adhesion of platelet-cancer cell conjugates to the endothelium and alters the vascular reactivity that helps in cancer metastasis (Varon and Shai, 2009).

1.4.2.2 **Induction of Angiogenesis by platelets**

Another important factor in metastasis is angiogenesis which takes place when endothelial cells divide to generate new blood vessels that increase oxygen and nutrient supply to the cancer cells to enhance tumour cell growth (Cross et al., 2003, Folkman, 1971). The body generates both pro-angiogenic factors and anti-angiogenic factors, the balance of which will regulate the overall process (Folkman, 1971). Platelets release both pro-angiogenic as well as anti-angiogenic factors (Walsh et al., 2015). For example, dopamine is stored in dense granules and is an anti-angiogenic

agent, in addition to its role in the central nervous system as a neurotransmitter. Intraperitoneal injections of dopamine inhibited angiogenesis as well as tumour growth (Basu et al., 2001). The same study also showed that dopamine inhibited VEGF-induced proliferation and migration of human umbilical vein endothelial cells (HUVEC) (Basu et al., 2001). Interestingly daily use of dopamine blocks stress-mediated tumour growth in vivo (Moreno-Smith et al., 2011). Dopamine exerts its inhibitory effect on angiogenesis by acting on dopamine receptors on endothelial cells to induce VEGF-R2 endocytosis that results in availability of fewer VEGF binding receptors, leading to unpaired VEGF-R2 phosphorylation (Basu et al., 2001, Elaskalani et al., 2017a). However, serotonin, which is produced in the intestine and brain, and is a pro-angiogenic agent is also stored in platelet dense granules. Serotonin is known to promote endothelial cell proliferation by activating several different kinases such as Arc, PI3K, AKT, ERK and mTOR (Liu et al., 2009a). VEGF is released from platelet granules following activation (Mohle et al., 1997) and from tumour cells to act on endothelial cells and release von Willebrand Factor (vWF) (Bauer et al., 2015). This acts on endothelial cells via tyrosine kinase-coupled receptors to cause endothelial cell proliferation and formation of new blood vessels (Cross et al., 2003). In addition to VEGF, activated platelets also release microparticles and exosomes that induce the release of angiogenic factors from cancer cells (Janowska-Wieczorek et al., 2005). Interestingly the expression of these angiogenic factors can be used as biomarkers indicative of the type and stage of cancer. For example, platelets from breast cancer patients contain a high degree of pro-angiogenic VEGF, TGFβi and PDGF (Han et al., 2014, Caine et al., 2004). Similarly, platelet-released VEGF is also associated with staging in non-Hodgkin lymphoma (Zizzo et al., 2010).

One of the most potent agonists of platelets, thrombin, also releases VEGF from α-granules via PAR-1 activation hence promoting a favourable environment for angiogenesis (Pinedo et al., 1998, Battinelli et al., 2011, Möhle et al., 1997). The pro-angiogenic activity of platelets also occurs in healthy persons and thus in cancer could represent a type of over-activity of a physiological response. For example at the site of injury, recruited platelets also induce angiogenesis (Feng et al., 2011) as well as during

embryogenesis. With this physiological role, VEGF released from platelets establishes haematopoiesis and forms new blood vessels (Ferrara et al., 1996).

1.4.2.3 **Platelet-enhancement of cancer cell adhesion**

Enhanced adhesion of cancer cells to platelets is one of the most significant mechanisms whereby these two cells interact. Platelets express a range of surface adhesion receptors, including integrins ($\alpha_{IIb}\beta_3$ and $\alpha_2\beta_1$), leucine-rich glycoproteins (GPIb/V/IX), PSGL-1 selectins (P-selectin) and immunoglobulin super family proteins such as PECAM-1 (platelet and endothelial cell adhesion molecule 1). During physiological responses, these adhesion molecules facilitate not only adhesion of platelets to themselves, but also adhesion with other cell types such as vascular endothelial or with leukocytes in host defence responses (Ruggeri, 2009). As an extension of this physiological role, platelets can form platelet-cancer cell aggregates to protect the cancer cells from immune cell-mediated lysis by natural killer cells (Karachaliou et al., 2015). The platelet-cancer cell binding takes place via fibrinogen, carbohydrate-protein binding by P-selectin and PECAM-1 binding (Stone and Wagner, 1993a). Moreover platelet-derived microparticles may also carry adhesion molecules that may bind with the cancer cells and form PMP-platelet-cancer cell conjugates (Żmigrodzka et al., 2016). These cancer cell-platelet aggregates adhere to the vessel wall, which can have a stabilizing effect on the conjugates and lead to cancer metastasis by EMT and release of growth factors from both the activated platelets and the cancer cells (Li, 2016, Janowska-Wieczorek et al., 2005). Moreover in a murine model, P-selectin deficiency in platelets and enzymatic mucin removal from cancer cells both markedly reduced the metastasis in lung cancer induced by cell injection (Radad et al., 2005). Furthermore intravenously injected metastatic B16-D5 melanoma cells in $\alpha_{IIb}\beta_3$-deficient mice showed reduced metastasis as compared to wild type mice (Echtler et al., 2017).

These cancer cell-platelet aggregates adhere to the vessel wall, which can have a stabilizing effect on the conjugates and lead to cancer metastasis by EMT and release of growth factors from both the activated platelets and the cancer cells. In addition to

the platelet-cancer cell direct contact and adhesion, cancer cell-platelet conjugates release mediators like serotonin, histamine, ATP from dense granules, TxA_2, 12-HETE and TGFβ. These agents regulate vascular permeability and mediate endothelial cell retraction that helps in exposing basal membrane, thereby facilitating cancer metastasis and invasion (Skolnik et al., 1989, Medina and Rivera, 2010, Schumacher et al., 2013b, Stanger and Kahn, 2013, Chen et al., 1992). Intravenous injected conjugates of PMP and Lewis lung carcinoma (LLC) cells resulted in increased metastatic foci to lungs and bones as compared to the LLC cells injected without PMPs into syngeneic mice (Janowska-Wieczorek et al., 2005). Platelets and PMPs also release matrix metalloproteinases (MMP)-1/2/9 which help in extracellular matrix degradation and facilitate cancer invasion to other sites (Janowska-Wieczorek et al., 2005, Alonso-Escolano et al., 2006). Lysophosphatic acid (LPA) derived from platelets has also been shown to stimulate release of two potent bone resorption stimulators, Interleukin-6 (IL-6) and Interleukin-8 (IL-8) from cancer cells that ultimately leads to osteolysis and metastasis (Boucharaba et al., 2004).

1.4.2.4 **Platelets increase cancer cell mobility**

Platelets are also known to modulate cancer cell mobility. Direct contact binding of podoplanin on the cancer cell to the platelet C-type lectin like receptor CLEC-2 leads to cycloskeletal reorganisation in the cancer cells via the ezrin/moesin-mediated pathway. This modification helps in cancer cell mobility and invasion, in part via induction of EMT (Lowe et al., 2012a). Amongst the increased mesenchymal markers that appear in EMT are fibronectin, transcription factors (Snail) and MMP-9 (Labelle et al., 2014a). Platelets conjugated with cancer cells are rapidly activated and release C-X-C motif chemokines (CXCL5 and CXCL7)(Labelle et al., 2014a). These chemokines recruit leukocytes (granulocytes and monocytes) from the blood stream to the site of platelet- cancer cell conjugates which further facilitates establishment of the metastatic niche (Labelle et al., 2014a) (Gil-Bernabé et al., 2012). This phenomenon suggests that platelets along with leukocytes contribute to promote cancer metastasis (Adinolfi et al., 2002). It has been demonstrated that in acute inflammation, P-selectin

on platelets and L-selectin on leukocytes act synergistically to enhance cancer metastasis (Borsig et al., 2002). The L-selectin on leukocytes permits adhesion to the endothelium (Imhof and Dunon, 1995). The P-selectin on the platelets bind with leukocyte PSGL-1 (P-selectin glycoprotein ligand-1). In this way, cancer cell:platelet conjugates recruit granulocytes to the endothelium. This binding enhances cytokine release from leukocytes that helps in the extravasation and mobility of cancer cells (Borsig et al., 2002). Further feedforward mechanisms that promote the metastatic niche include release of chemokines (RANTES) and MCP-2 from platelets (Popivanova et al., 2009, Läubli et al., 2009) together with release of VEGF from monocytes that increase vascular permeability and extravasation. Some studies also indicate that monocytes release cathepsin and TGFβ which reinforce the cancer cell growth and metastasis. Hence platelets in co-ordination with granulocytes as well as monocytes help in establishment of metastatic foci (Läubli et al., 2009, Popivanova et al., 2009).

1.4.2.5 **Effects of platelets on tumour cell proliferation and growth**

The effect of platelets on tumour cell growth is quite controversial. Several lines of evidence suggest tumouricidal effects of platelets, while there is much evidence that supports a tumour promoting role of platelets. The platelet-dependent effect normally depends on the tumour type (Elaskalani et al., 2017a). Cytotoxic effects of platelets on tumour cells were decreased by aspirin, indicating a role for arachidonic acid metabolites (Ibele et al., 1985, Okada et al., 1996). Similarly in another study, thrombin activated platelets showed cytotoxic effects on the myelogenous leukaemia cell line (K562) (Sagawa et al., 1993b), although the exact mechanism was unclear. It has been suggested that the extent to which platelets exert a tumouricidal effect depends on the relative sensitivity to released agents amongst the different cancer cell lines. Upon formation of aggregates around cancer cells, cytotoxic factors from platelets can form pores on the endothelium thus facilitating extravasation of aggressive cells (Sagawa et al., 1993a). Platelets also contain pro-apoptotic agents that play a role in tumour necrosis like TNF, TRAIL, TNF-α and Fas-Ligand (Fas-L) (Crist et al., 2004). Upon release from ADP or thrombin-activated platelets the Fas-ligand (FAS-L), binds with FAS-L

receptor on cancer cells that leads to caspase mediated apoptosis pathway activation (Ahmad et al., 2001). Caspases are the pro-apoptotic proteins that play an essential role in programmed cell death (Goodsell, 2000). An example of a cancer type where the malignant cells express FAS-L receptor is adult T-cell leukaemia (Ahmad et al., 2001).

The data signifying the apoptosis inducing activity of platelets is limited to certain cancer cell lines since there is much evidence suggesting platelets play a positive role in cancer cell growth and proliferation (Wang and Zhang, 2008). Platelets evoke ovarian cancer cell proliferation in vivo and in vitro by releasing TGFβ1 and promoting growth of the cancer cells (Cho et al., 2012a, Haemmerle et al., 2016). Similarly ovarian cancer tumour growth in mice was augmented by platelets via a mechanism that depended upon expression of platelet FAK (focal adhesion kinase) protein (Haemmerle et al., 2016). TGFβ1 can exert opposite effects on cancer proliferation depending upon the cell type. For example in the lung cancer cell line (A549) and the breast cancer cell line (MCF-7) proliferation was inhibited by TGFβ. In contrast, no effect of TGFβ1 was observed on colon cancer cell line (HT-29) (Roberts et al., 1985). Similarly TGFβ1 can potentiate as well as antagonise the effects of other growth factors such as PGDF and EGF (Roberts et al., 1985).

Thrombocytosis (increased platelet count) is commonly associated with malignant tumours particularly in cases of ovarian cancer (Digklia and Voutsadakis, 2014, Stone et al., 2012, Gungor et al., 2009, Lee et al., 2011). This may be the consequence of interleukin-6 (IL-6) released from tumours that facilitates thrombopoietin synthesis in liver and increases megakaryocytic development and thus platelet production from the marrow (Stone et al., 2012). Also, platelets accumulated within solid tumours via P-selectin mediated $\alpha_{IIb}\beta_3$ activation are reported in various tumour types including colorectal cancer, breast cancer hepatocarcinoma, gastric cancer, melanoma and insulinoma in mouse models that resulted in significant tumour growth as compared to the control models lacking P-selectin in platelets (Qi et al., 2015b). In a comparison of extracts from platelets, red blood cells or white blood cells on human hepatocellular

carcinoma, the platelet extract promoted the greatest level of cancer cell growth. In this analysis alpha-fetoprotein was used as a marker of growth and aggressiveness in the cell line. The possible platelet-derived growth mediators were reported as inflammatory cytokines, fibroblast growth factor (FGF), vascular endothelial growth factor (VEGF), platelet-derived growth factors (PDGF) and serotonin (Carr et al., 2014). In normal cells, PGDF acts via α- and β-tyrosine kinase receptors (PDGFRα and PDGFRβ, respectively) as a potent growth mediator (Omura et al., 1997). Furthermore PDGF also interacts with non-kinase receptors such as integrins as well as low density lipoprotein receptor-related protein (Sundberg and Rubin, 1996, Newton et al., 2005). In cancer, some receptor genes are mutated or display increased levels of expression. For example in skin tumour dermatofibrosarcoma protuberance a mutated gene, PDGF-BB is expressed that stimulates the growth of fibroblasts in an autocrine manner (Shimizu et al., 1999). Similarly in gastrointestinal stromal tumours PDGFRα gene mutations have been reported (Heinrich et al., 2003). The mutation or amplification of receptor in cancer results in an increased response to PDGF compared to normal tissues (Heldin, 2013).

Like other growth factors, ATP and ADP are also released from activated platelets (Golebiewska and Poole, 2015b, Schumacher et al., 2013a). Extracellular ATP has a role in promoting tumour growth, proliferation and migration of cells (Di Virgilio and Adinolfi, 2017, Song et al., 2015). This activation takes place via P2 receptors in cancer cells (Gendaszewska-Darmach and Kucharska, 2011, Virgilio and Solini, 2002, Ji et al., 2018). This may be an extension of the wound healing actions of ATP, which has an important role in epithelial repair (Greig et al., 2003). In lung cancer cells (A549), ATP induces a rise in $[Ca^{2+}]_i$ via activation of P2Y2 and P2Y4 receptors that resulted in Ca^{2+}-dependent release of UTP and ATP followed by proliferation of tumour cells (Schafer et al., 2003). In the cancer cell line A549, TGFβ1 augments the ATP induced Ca^{2+} signalling that accelerates migration (Miki et al., 2010). The migration induced was reversed by using suramin, a P2 receptor antagonist which indicates that ATP enhances the TGFβ1-induced cancer cell mobility and EMT (Miki et al., 2010). Additionally TGFβ1-treated cells augmented not only ATP induced Ca^{2+} transients but also store operated Ca^{2+}

influx in lung cancer cells as compared to the control cells without TGFβ1 (Miki et al., 2010). ATP also released growth factors from prostate cancer cells that stimulated cell growth and proliferation via autocrine as well as paracrine mechanisms (Sauer et al., 2001). The underlying mechanism involves ATP-induced growth stimulation by generating ROS in prostate cancer cells which was abolished by using P2 receptor antagonist, suramin (Sauer et al., 2001). In mouse melanoma cells P2X7 activation by ATP analogue benzoyl ATP (BzATP) resulted in tumour growth which was strongly inhibited by intratumoural injection of the P2X7 selective blocker, AZ10606120 (Adinolfi et al., 2012) . The pro-tumourigenic effects of ATP were also demonstrated in C6 glioma cells as stimulation of P2X7 by BzATP resulted in release of chemokines and cytokine factors from the tumour cells (Jantaratnotai et al., 2009). Moreover, the P2X7 antagonism partly reduced the release of cytokine factors and Ca^{2+} responses in rat C6 glioma cells. This led to a finding that P2Y receptor subtype is also activated by BzATP that increased the Ca^{2+} mobilisation by store depletion and subsequent SOCE (Jantaratnotai et al., 2009). Also proinflammatory factors like MCP-1, IL-8 and VEGF were released by ATP in C6 glioma cells, neuroblastoma cells and thyroid papillary carcinoma cells via activation of P2X7 and the ultimate effect on the cancer cells was accelerated migration and proliferation (Raffaghello et al., 2006). Moreover, platelet-derived ATP and ADP also induced EMT in pancreatic cancer cells via activation of P2Y12 since the P2Y12 antagonist, ticagrelor caused a reduction in cancer cell survival (Elaskalani et al., 2017c). In the same report, ATP and ADP induced epithelia mesenchymal transition (EMT) in pancreatic cancer cells (Elaskalani et al., 2017b).

Platelet based mediators	Impact on cancer cells	Cancer type studies	References
P-selectin expression	Enhance cancer cell adhesion to platelets via mucins, Podoplanin and PSGL-1.	Pancreatic islet insulinoma, squamous and germinal cancers,	(McCarty et al., 2000, Borsig et al., 2001, Mannori et al., 1995, Borsig et al., 2002, Qi et al., 2015b).
PECAM-1	Mediates platelet–cancer cell adhesion via integrins	Breast cancer cell lines MCF-7	(Timar et al., 1992).
$\alpha_{IIb}\beta_3$	Cancer cell adhesion and metastasis by bridging between platelets and cancer cells by binding to fibrinogen or von Willebrand factor.	Human umbilical vein endothelial cells (HUVEC) and melanoma cell	(McCarty et al., 2000, Trikha et al., 2002, Karpatkin et al., 1988a)
GPVI	Cancer cell arrest and metastasis via collagen mediated binding of cancer cells to vascular endothelium	Lewis lung carcinoma (D121) or melanoma (B16F10.1) cell line	(Jain et al., 2010a, Jain et al., 2009a, Jain et al., 2009b).
GPIb/IX	Cancer cell arrest and metastasis by vWF-mediated adhesion	Breast cancer and Lewis lung carcinoma.	(Jain et al., 2010a, Jain et al., 2007)
TLR4 activation	Cancer metastasis via interaction with cancer cell-released HMGB1	Murine B16F10 melanoma and Lewis lung carcinoma (LLC) tumour cells	(Yu et al., 2014, Li, 2016).
VEGF	Cancer cell proliferation, growth, mediates vascular permeability and tumour angiogenesis	Breast cancer, prostate cancer and non-Hodgkin lymphoma.	(Honn et al., 1992, Gay and Felding-Habermann, 2011b, Sabrkhany et al., 2011, Lou et al., 2015).
PDGF	Cancer cell proliferation, growth and tumour angiogenesis	Prostate cancer	(Honn et al., 1992, Gay and Felding-Habermann, 2011b, Sabrkhany et al., 2011, Heldin and Westermark,

			1999, Lou et al., 2015).
TGFβ	EMT and metastasis, cell proliferation, and tumour growth.	Ovarian cancer, mice colon and breast carcinoma cells.	(Honn et al., 1992, Sabrkhany et al., 2011, Oft et al., 1996, Oft et al., 1998, Cho et al., 2012b, Labelle et al., 2011a).
Fibrinogen	On conversion to Fibrin it is deposited around cancer cells that acts as a mask against immune surveillance and helps in formation of platelet–cancer cell conjugates and metastasis.	Haematological and breast cancer	(Palumbo et al., 2005b, Palumbo et al., 2007).
MMP1/2/9	Cancer cell invasion and metastasis via degradation of extracellular matrix	Breast cancer cells, pancreatic cancer, cervical carcinoma, colorectal cancer and melanoma	(Deryugina and Quigley, 2006, Rolli et al., 2003)
Serotonin	Activates cancer cells and facilitating invasion via increasing vascular permeability by endothelial cell retraction and exposure of basal membrane	fibrosarcoma cells	(Skolnik et al., 1989) (Elaskalani et al., 2017a)
Histamine	Activates cancer cells and facilitates invasion via increasing vascular permeability by activating cancer cell histamine H4 receptor	Melanoma, colon and breast cancer	(Medina and Rivera, 2010, Medina et al., 2011)
ATP	P2 receptor activation (P2X7). Cancer cell growth and proliferation, EMT, Promotes cancer cell extravasation via increasing vascular permeability	Prostate cancer, pancreatic cancer cells.	(Schumacher et al., 2013b, Stanger and Kahn, 2013, Verheul et al., 2007, Klement et al., 2009, Fang et al., 2010, Ko et al., 2012).
TxA_2	Enhances cancer cell invasion via increased vascular permeability	Breast cancer and melanoma bone metastasis in mice	(Chen et al., 1992, Bradley et al., 1997b).
Autotaxin	Enhances bone metastasis via generation	Metastatic breast cancer	(Palumbo et al., 2007, Li, 2016, Liu

	of LPA from phosphatidylcholine and activation of osteoclasts		et al., 2009b, David et al., 2010, Leblanc et al., 2014).
PDMP	Cancer cell adhesion, tumour angiogenesis and metastasis		(Janowska-Wieczorek et al., 2005, Mezouar et al., 2014, Brill et al., 2005).
PF4	Enhanced platelet production and accumulation in the lung, which accelerated cancer progression	Genetically modified lung cancer mouse model	(Pucci et al., 2016a).
ADP	Potent activator of platelet adhesion and aggregation, promoting platelet-cancer cell conjugates.	Pancreatic ductal adenocarcinoma	(Elaskalani et al., 2017b).

Table 1.2. Mediators released from activated platelets that have a potential role in cancer metastasis

1.5 Ca^{2+} signalling in platelet activation

As highlighted in section 1.1.1, platelets contain various receptors that respond to specific agonists leading to platelet activation. Whilst the specific agonists and receptors lead to a range of signalling events and functional responses, virtually all induce an increase in the cytosolic concentration of the second messenger, Ca^{2+} (Varga-Szabo et al., 2009). Almost all of the platelet functions including platelet activation, shape change, secretion and aggregation depend on an increase in intracellular Ca^{2+} (Feinstein et al., 1977, White et al., 1983, Wörner and Brossmer, 1975). Two major overall mechanisms are involved in agonist-evoked cytosolic Ca^{2+} increase, namely Ca^{2+} release from intracellular stores and influx from the extracellular environment through the plasma membrane (Wu, 2012). Platelets are non-excitable cells, and thus lack voltage-gated Ca^{2+} channels (MAHAUT-SMITH, 2012). Instead, a major influx pathway in the platelet is store operated Ca^{2+} entry (SOCE) via Orai1 ion

channels (Varga-Szabo et al., 2009). This pathway is activated by release of Ca^{2+} stored in the dense tubular system (DTS) in platelets (Rosado and Sage, 2000a). Platelet stimulation by agonists like collagen, ADP, thrombin, TxA_2 results in activation of phospholipase C, which has three main isoforms: PLCβ, PLCγ, PLCδ (Li et al., 2010). The PLCβ isoform is stimulated by the α-subunit of Gq heterotrimeric G proteins downstream of GPCRs for a number of platelet agonists such as ADP, serotonin, thrombin and TxA_2. In contrast, PLCγ is stimulated by GPVI receptors, integrins (α2β1, $\alpha_{IIb}\beta_3$) and GPIb-V-IX (Li et al., 2010). Upon activation, PLC leads to hydrolysis of phosphatidylinositol 4,5-bisphosphate (PIP_2) to form inositol 1,4,5-trisphosphate (IP_3) and 2-diacyl-glycerol (DAG) (Putney and Tomita, 2012). Consequently IP_3 activates Ca^{2+} permeable channels (IP_3R) on the internal stores (the DTS in platelets) causing Ca^{2+} release (Dadsetan et al., 2008). On the other hand, DAG stimulates store independent Ca^{2+} entry (Varga-Szabo et al., 2009). In platelets, two distinct Ca^{2+} stores have been identified based on the expression of sarcoplasmic/endoplasmic reticulum calcium ATPase (SERCA) isoforms (Varga-Szabo et al., 2009). The SERCA2b isoform shows a high sensitivity to thapsigargin (TG) and is expressed on the dense tubular system (DTS). In contrast, SERCA3 is expressed in the acidic stores and is sensitive to 2,5-di-(t-butyl)-1,4-hydroquinone (TBHQ) (Zbidi et al., 2011b). The resting level of Ca^{2+} in human platelets is reported to be between 50-75 nM (Vicari et al., 1994). Even minor elevations in Ca^{2+} level evoke platelet signalling that can result in shape change and activation (Rolf et al., 2001b). For this reason it is important to prevent an unnecessary rise in intracellular Ca^{2+}, thus endomembrane Ca^{2+}ATPases pump the unnecessary Ca^{2+} from the cytosol into the stores (Enyedi et al., 1986). In addition, the plasma membrane Ca^{2+}ATPase (PMCAs) pumps unwanted Ca^{2+} out of the cells across the plasma membrane. In the resting state, Ca^{2+} extrusion through PMCAs is the main regulatory mechanism (Rosado and Sage, 2000b) while in activated platelets SERCA activation precedes the PMCA-mediated Ca^{2+} extrusion (Redondo et al., 2005). Additionally Na^+/Ca^{2+} exchanger (NCX) results in very fast extrusion of excess Ca^{2+} and helps to restore the platelet resting level (Valant et al., 1992).

1.5.1 Non store operated Ca^{2+} entry in platelets

This type of Ca^{2+} entry downstream of receptor stimulation includes all pathways triggered independently of store depletion. Thus, this group includes direct ligand-gated channels and second messenger-operated ion channels (Varga-Szabo et al., 2009) which are further discussed below.

1.5.1.1 Second messenger gated Ca^{2+} entry

The main Ca^{2+}-permeable channels reported to be activated by DAG are TRPC3 and TRPC6 (Pedersen et al., 2005), although there are some controversies about the categorisation of TRPC6 as it is reported to contribute to SOCE in some studies (Soboloff et al., 2005). The degree of stimulation of TRPC6 may determine the role in the Ca^{2+} signalling pathway (Mahaut-Smith, 2012, Harper and Poole, 2011). TRP channel mRNA studies in platelets reported expression of TRPC1 and TRPC6 (Carter et al., 2006). In addition to TRPC6 other non-SOCE channels reported in platelets are TRPC3. However no significant effect of TRPC1 deficiency on Ca^{2+} level alteration was reported in mice (Varga-Szabo et al., 2008a). In contrast, studies of TRPC6-deficient mice have provided evidence for a contribution to haemostasis and thrombosis by one group (Espinosa et al., 2012). It should be noted that the relative importance of TRPC6 has been debated as another group found no evidence for a functional role of this channel in mice, although they did confirm its role in DAG-induced Ca^{2+} entry (Ramanathan et al., 2012). A further study has suggested that TRPC6 and TRPC3 channels synergise as store independent ion channels to generate PS exposure following high levels of platelet stimulation by collagen and thrombin (Harper and Poole, 2013, Hassock et al., 2002, Harper et al., 2013).

1.5.1.2 Ligand- gated Ca^{2+} entry

Ligand-gated Ca^{2+}-permeable ion channels are expressed in a range of excitable and non-excitable cells and activated by various agonist(s) including neurotransmitters such as acetylcholine, glutamate and ATP (Pankratov and Lalo, 2014). Among these

channels NMDA, nicotinic acetylcholine (Ach) and P2X channels have high Ca^{2+} permeability (Pankratov and Lalo, 2014). These channels are abundantly expressed in the central nervous system (CNS) and involved in action potential initiation. P2X receptors are ATP-gated ion channels and expressed as seven distinct isoforms (P2X1-P2X7) (Kaczmarek-Hájek et al., 2012, M Waszkielewicz et al., 2013). ATP-gated P2X1 channels are the only well-established ligand-gated Ca^{2+}-permeable channel in platelets (Jiang, 2012, Mahaut-Smith et al., 2004). Activation via ATP-gated P2X1 receptors is the fastest route for Ca^{2+}influx across the plasma membrane in the platelet as the transmembrane complex that forms the Ca^{2+} permeable pore also contains the ATP binding site and thus is activated within 10 milliseconds of ligand binding (Mahaut-Smith et al., 2011, Hechler et al., 2003, Sage and Rink, 1987, Mahaut-Smith et al., 1992). The first electrophysiological study of P2X channels in platelets was conducted by Mahaut-Smith et al in 1990 when ADP was shown to respond to directly gate ion channel opening in cell-attached membrane patches (Mahaut-Smith et al., 1990a). Later on it was demonstrated that the response generated was due to the contaminating ATP in commercial ADP (Mahaut-Smith et al., 2000). P2X1 receptors respond to many pharmacological agonists, the order being 2-meSATP = ATP > α, β-meATP (Evans and Kennedy, 1994, Kennedy and Leff, 1995) but ATP is thought to be the main physiological agonist of P2X1 (Fung et al., 2007a, Mahaut-Smith et al., 2004). Since damaged vessels and cells are a rich source of ATP release (approximately 2 μM ATP is released within 2-4 seconds of injury) the platelet P2X1 pathway is considered among the first line of activation following injury (Mahaut-Smith et al., 2004). Platelet P2X1 receptors are insensitive to elevated cytosolic cAMP and cGMP levels which have substantial inhibitory effects on IP_3 mediated Ca^{2+} mobilisation (Cavallini et al., 1996, Geiger et al., 1992). Hence P2X1 can be activated even in the presence of platelet natural antagonists NO and PGI_2 (Fung et al., 2012b). Furthermore, in addition to ATP released from the damaged vessels, activated platelets also release ATP as well as ADP (Yun et al., 2016). Hence P2X1 activation takes place via an autocrine as well as paracrine mechanism and can potentiate the functional response in synergy with P2Y and collagen receptors (Savi et al., 1998, Jin and Kunapuli, 1998b, Hechler et al., 1998, Fung et al., 2007a).

1.5.2 Store operated calcium entry

IP_3 generated following PLC activation interacts with IP_3R on the surface of the DTS leading to release of Ca^{2+} from the DTS (equivalent to the endoplasmic reticulum in other cells). The depletion of stored Ca^{2+} is sensed by STIM1 (stromal interaction molecule 1), which is a transmembrane protein situated in the ER (and thus DTS), in a way that exposes its EF hand to Ca^{2+} in the store lumen (Liou et al., 2005). When the Ca^{2+} is released from the stores the dissociation of Ca^{2+} from the DTS EF hand causes oligeromisation of STIM1 at the ER-PM (endoplasmic reticulum-plasma membrane) junction and tethering to Orai1 (store operated Ca^{2+} influx channel) on the plasma membrane (Smyth and Putney, 2012, Feske et al., 2006, Wu et al., 2007). This allows opening of Ca^{2+}-permeable Orai1 channels and thus store operated Ca^{2+} influx. SOCE is now known to be a major route for Ca^{2+} entry in platelets and contributes to adhesion, activation, aggregation and degranulation (Bergmeier et al., 2009, Braun et al., 2009a). The elevated level of Ca^{2+} in the cytoplasm activates CALDAG-GEF1 (Calcium DAG-guanine nucleotide exchange factor1) that in turn causes inside-out integrin activation in synergy with the GTPaseRap1 (Stefanini and Bergmeier, 2010). Another Ca^{2+}-dependent response is flipping of PS (phosphatidylserine) onto the outer lipid bilayer, whose negative charge on the surface provides docking sites for coagulation factors to generate thrombin (Hou et al., 2015). Since SOCE is the major Ca^{2+} influx pathway involved in platelet responses to various agonists including ADP, TxA_2, thrombin and collagen (Varga-Szabo et al., 2009), the importance of SOCE in platelet function and pathologies cannot be ignored (Egan, 2013). Various studies have demonstrated the alteration in platelet Ca^{2+} signalling and receptor expression in cancer (Egan et al., 2011, Li, 2016). Also much emerging research is found on targeting the platelet receptors in developing cancer therapy (Bambace and Holmes, 2011b). These targeting sites include platelet $P2Y_{12}$, TxA_2 receptors, COX-1 and 2 enzymes, integrin $\alpha_{IIb}\beta_3$, GPIb/IX/V or GPVI receptors, P-selectin, thrombin receptors and production, P-selectin receptors and platelet derived growth factor receptors (Wojtukiewicz et al., 2017).

However no report documents the altered levels of platelet SOCE in cancer or if the receptors in SOCE can be used as prognostic or diagnostic biomarkers in malignancy.

1.6 Altered Ca^{2+} signalling in Cancer

Increase in intracellular Ca^{2+} contributes to gene transcription, cell proliferation, migration and death. There is much evidence that Ca^{2+} homeostasis is disturbed in cancer, including altered proliferation, angiogenesis and metastasis (Cui et al., 2017). Various studies report modulation of Ca^{2+} channel expression in different cancer types, including breast cancer (Van De Vijver et al., 2002), cervical cancer (Mayr et al., 2002) and adenocarcinoma (Mayr et al., 2002). Evidence has also been found that IP_3Rs are upregulated in malignant gastric cell lines but not in cells isolated from primary tumor sites and normal gastric cells (Sakakura et al., 2003). Likewise the IP_3R2 isoform of IP_3R was dysregulated in chronic lymphocytic leukemia (Akl et al., 2013). The increased activity of IP_3R2 enhances mitochondrial activity, thereby facilitating higher metabolic activity and increased proliferation of leukemia cells (Adinolfi et al., 2002, Cui et al., 2017). Similarly, mutations in SERCA isoforms have also been reported in malignancies like colon, gastric, lung cancer, chronic plexus tumors and myeloid leukemia (Dang and Rao, 2016). Increased expression of SERCA2 resulted in increased proliferation and migration of rectal cancer (Fan et al., 2014). Ca^{2+} extrusion pathways as PMCAs are also dysregulated in cancer. PMCA2, an isoform of PMCA found on mammary epithelia is 100 fold more expressed in breast cancer cell lines as compared to normal breast. PMCA2 decreases the cytosolic Ca^{2+} level and prevents apoptosis resulting in enhanced cancer progression (Lee et al., 2005). Similarly, voltage gated Ca^{2+} channels (VGCCs) that mediate Ca^{2+} influx in response to membrane depolarization are also dysregulated in cancer. Six families of VGCCs are identified, which include L, N, P, Q, R and T type channels. L-type Ca^{2+} channels are overly expressed in colon and esophageal cancer and are correlated with the enhanced activity of cancer cells (Wang et al., 2015). TRP channels consist of more than 30 members that are divided into 7 subtypes that

include TRPC (canonical), TRPV (vanilloid), TRPA (ankyrin), TRPML (mucolipin), TRPP (polycystic), TRPM (melastatin), and TRPN (no mechanoreceptor potential C) (Wu et al., 2010). TRPC, M and V subtypes are found correlated with malignancy in breast and ovarian cancer (El Hiani et al., 2009, Yang et al., 2009, Prevarskaya et al., 2007). TRPM7, TRPM8, TRPC6 and TRPV2 are identified as overly expressed receptors in prostate carcinoma that also enhanced carcinoma progression (Gkika and Prevarskaya, 2011). Also TRPV6 expression is upregulated in esophageal squamous cell carcinoma and is suggested as a novel therapeutic target in esophageal cancer treatment (Zhang et al., 2013b). In another study TRPC1, TRPC6, TRPM7, TRPM8 and TRPV6 are found overly expressed in breast cancer and are suggested as prognostic markers of malignancy in breast cancer (Ouadid-Ahidouch et al., 2013, Dhennin-Duthille et al., 2011).

Purinergic receptors are activated by purines and pyrimidines (Ralevic and Burnstock, 1998), including the Ca^{2+}-permeable P2X ion channels and a number of GPCRs coupled to Ca^{2+} signalling via Gq heterotrimeric proteins (particularly P2Y1,2,4,6,11). P2X3 upregulation was reported in hematoma cells that enhanced the proliferation and growth of human hepatocellular carcinoma (HCC) (Maynard et al., 2015). Similarly increased P2X5 activity was reported in melanoma, breast, colorectal, brain and renal cancer and is suggested as a potential target for cancer development therapy (Kale et al., 2015). Likewise P2X7 is abundantly expressed in neuroblastoma, melanoma, leukemia, prostate, thyroid, colon and cervical cancer that enhanced cancer cell proliferation and migration of cancer cells (Kale et al., 2015). P2X7 was reported to be overexpressed in pancreatic ductal adenocarcinoma and prolonged cell survival (Kodet et al., 2015). Amongst the Ca^{2+} signalling P2Y receptors, P2Y2 is upregulated in highly metastatic breast cancer, colon cancer and hepatoma. P2Y2 overexpression in these cells increased the tumor growth via interaction with endothelial cells, increased matrix metalloproteinase-9 (MMP-9) activity and increased production of VEGF (Jin et al., 2014). In another report, P2Y2 and P2Y4 receptors were over expressed in human colon cancer (Nylund et al., 2007).

Regarding SOCE, elevated levels of STIM1 are reported in many malignancies including cervical cancer patients with observed metastasis to pelvic lymph nodes (Yang et al., 2017). Similarly in another survey, breast cancer patients with poor prognosis and failure in therapy showed elevated STIM1 levels (Van De Vijver et al., 2002). Blocking SOCE by a pharmacological inhibitor SKF96365 or by siRNA-mediated silencing of STIM1 or Orai1, impaired the focal adhesion turnover and invasive migrations of breast cancer cells (Chen et al., 2011). The underlying mechanism involves regulation of actomyosin reorganization and enhanced contractile forces that are important in tumour progression (Chen et al., 2013b, Chen et al., 2011).

1.7 Aims and Objectives

Ca^{2+} signalling is a major activation pathway in platelets, allowing these anuclear blood cells to functionally respond during haemostasis, inflammation and immunity (Rivera et al., 2009, Li et al., 2010). Less is known about the role of Ca^{2+} increases in platelet-dependent pathologies. To date various studies have documented a number of important interactions between platelets and cancer cells that may contribute to progression of the disease. One of the most common types of adult leukaemia is acute myeloid leukaemia (AML) in which precursors of haematopoietic cells are mutated resulting in abnormal production of myeloblasts (Stone et al., 2004). AML has a low survival rate (Redaelli et al., 2003). The interaction between platelets and AML cells is of interest due to a potential impact on metastasis and also changes in platelet hyperactivity. In addition, such interactions may be relevant for understanding how megakaryocytes and myeloid leukemic cells influence each other within the bone marrow environment, particularly since megakaryocytes express most if not all platelet proteins (Naeim, 2012, Thon and Italiano, 2010b). The first two results chapters of this thesis (Chapters 3 and 4) have used the HEL (human erythroleukemia) cell line to investigate the role of intracellular Ca^{2+} signalling events and intercellular signalling molecules during the interaction between platelets and AML cells. Platelet activation was studied initially with thrombin (Chapter 3), a platelet agonist shown to be elevated in cancer (Reddel et al., 2017). Secondly, since HEL cells also respond to thrombin, a

TLR ligand was used (in Chapter 4) to selectively activate platelets and understand how these two cells interact following stimulation of innate immune receptors as platelets contribute to the immune system (Ali et al., 2015). Platelet SOCE has been suggested to be modified in diabetes mellitus (see section 1.3.1), but to date not in cancer. However, changes in SOCE have been associated with cancer in other cell types (see section 1.6). At present it is not known how variable SOCE is in healthy donors or what conditions will allow the best comparison of this pathway in health and cancer. The second part of this thesis (Chapter 5) therefore aims to establish the best conditions to measure SOCE in platelets of healthy donors and to understand the reasons for any variability.

Chapter 2. Materials and Methods

2.1 Salines and reagents

2.1.1 Salines

Salines were made in ultrapure double distilled water ($UddH_2O$) delivered from an Elga Purewater® Classic system (18.2 MΩ.cm at a temperature of 24.1°C). Acid citrate dextrose anticoagulant contained 85 mM trisodium citrate, 78 mM citric acid and 111 mM glucose. Nominally calcium free saline contained 145 mM NaCl, 3 mM KCl, 1 mM $MgCl_2$, 10 mM D-glucose and 10 mM HEPES, pH adjusted to 7.35 with NaOH. 1x RIPA buffer for cell lysis prior to Western blot analysis contained 150 mM NaCl, 50 mM Trizma hydrochloride, 0.1 % SDS, 0.5 % Sodium deoxycholate, 1 % Triton X-100, pH adjusted to 8.0 with HCl. One EDTA-free protease inhibitor cocktail tablet (complete TM Mini, Roche, Switzerland) was dissolved per 10 ml of Radio-Immunoprecipitation Assay (RIPA). Buffers used for Western blotting were 1x Tris/Glycine transfer buffer (192 mM Glycine, 25 mM Tris base and 10% w/v methanol); 1x Tris-buffered saline with Tween-20 (TBS-T: 13.7 mM NaCl, 2 mM Tris base, 0.1 % w/v Tween-20, pH adjusted to 7.35 with HCl) and 1X Tris/Glycine/SDS running buffer (from National Diagnostics, Atlanta, GA, USA).

2.1.2 Reagents

Bradford Reagent was purchased from Sigma-Aldrich (Dorset, UK). Valinomycin and Bovine Serum Albumin were from Invitrogen (Paisley, UK), monoclonal Stim1 and Orai1 antibodies were purchased from Abcam (Cambridge,UK). The secondary polyclonal swine anti-rabbit antibody was purchased from Dako (Ely, UK), GAPDH (FL-335) antibodies were purchased from Santa Cruz Biotechnology (Heidelberg, Germany). Apyrase was type VII from potato (Sigma-Aldrich, Dorset, UK). PAM_3CSK_4 was purchased from Source Bioscience Lifesciences (Nottingham, UK). Chrono-Lume ATP Assay kit was purchased from Chrono-log (#395; Chrono-log Corporation, Havertown, PA, USA). NF157 was from Insight Biotechnology Ltd (Wembley, UK), MRS2179 from Cambridge Bioscience (Cambridge, UK) and NF449 from Santa Cruz

Biotechnology (Dallas, USA). Cyclopiazonic acid was from Cayman Chemical (MI, USA) and U73122 was purchased from Tocris Bioscience (Bristol, UK). For cell counting and viability testing, trypan blue stain (0.4%) was purchased from Gibco by Life Technologies (New York, USA) and the Neubauer chamber (depth 0.1mm, 1/400mm^2) was purchased from Weber Scientific International Ltd (Teddington, England). For attachment of HEL cells sterilised glass coverslips (30mm, №1.5 thickness) (Warner Instruments, MA, USA) and poly-D-lysine hydrobromide (MW>300,000) (Sigma-Aldrich, Dorset, UK) were used. Unless stated all other reagents were from Sigma-Aldrich (Dorset, UK).

2.2 HEL cell culture and cell counting

HEL cells (ECACC, Salisbury, UK) were grown in RPMI1640 supplemented with 10% foetal bovine serum (FBS) (Invitrogen, Paisley, UK) and 100 U/ml Penicillin-Streptomycin (P/S) (Invitrogen). Cells were maintained in a humidified incubator at 37^{o}C (5% CO_2) and were split after every 2 to 3 days. HEL samples for cell counting and viability were prepared by diluting 10 µl of HEL cell suspension from the cell culture flask 1:1 with the 0.4% trypan blue dye stock followed by a 1:4 dilution of this mixture with saline as recommended by a cell culture protocol from ThermoFisher Scientific (Waltham, MA 02451, USA). The suspension was incubated with dye for 5 minutes and 20 µl of the suspension was added to a Neubauer chamber. Cells were counted in 4 big squares and the total cell count was calculated by the formula:

Total cell count/ml= total no. of cells x 10^4 x dilution factor

The percentage cell viability was calculated by differentiating the non-stained viable HEL cells from the dark blue stained dead cells within the grids on the Neubauer chamber. The following formula was used for the calculation:

% Viability = (No. of viable cells/total no. of HEL cells) x 100

2.2 Phlebotomy and Platelet-rich plasma (PRP) preparation

2.2.1 Phlebotomy

Blood was collected by venepuncture from healthy donors in the age range 18-65 with their informed consent under the approval of the University Of Leicester College Of Life Sciences Human Biology Research Ethics Committee (non NHS). Blood was mixed with ACD (anti-coagulant) in a 6:1 ratio, thus for every 6 ml of whole blood 1 ml of ACD was added.

The blood and ACD mixture was collected into 15 ml Falcon tubes and centrifuged at 2500 rpm (700 x *g*) for 5 minutes to separate the platelet rich plasma (PRP) from other blood cells.

2.2.2 Preparation of Washed Platelets and Fura-2 loading

The PRP was removed from the centrifuged blood-ACD mixture and transferred to a fresh plastic tube. Aspirin and apyrase were added from 1000-fold stocks to give final concentrations of 100µM and 0.32 U/ml, respectively. The PRP was then gently stirred on a rotor until use. For spectrophotometric analysis, fura-2 AM was added to the PRP from a stock of 5 mM prepared in DMSO to make a final concentration of 2 µM, gently mixed and placed in a water bath at 37^{o}C for 50 minutes. The tube contents were protected from unnecessary exposure to light until use in experiments. The dye loading step was skipped in platelet sample preparation for aggregometry and ATP assays. The PRP was spun for 20 minutes at 1500rpm (350 x *g*) to pellet the platelets. For cuvette experiments studying HEL-Platelet interactions, the final platelet pellet obtained from the second spin was resuspended in a volume of saline 1/5th of the original volume of PRP. For all other cuvette experiments, the platelet pellet was suspended in nominally Ca^{2+}-free saline to make a volume double the volume of PRP obtained after initial centrifugation of the blood: ACD mixture.

2.3 HEL cell dye loading and attachment to glass coverslips

Approximately 28 ml of HEL cell suspension from the cell culture flask were spun at 200 x *g* for 5 minutes and resuspended in saline containing 1 mM $CaCl_2$ followed by incubation with fura-2AM (1 µM) for 45 minutes at room temperature, protected from light. The cells were then spun at 200 x *g* for 5 minutes and resuspended in nominally Ca^{2+} free saline at an average density of 15 x 10^4 cells/ml. Batches with a minimum average viability of 75 % were used in experiments. For fluorescent imaging, 8 ml of HEL suspension from the cell culture flask was spun and processed as discussed above but loaded with fluo-3 instead of fura-2 by incubation with 1 µM fluo-3AM. For attachment of HEL cells to coverslips for fluorescent imaging, glass coverslips were washed with a mixture of industrial methylated spirit and 10% HCl. After air drying, coverslips were coated with poly-D-lysine by exposure to a 0.1 mg/ml poly-D-lysine hydrobromide (MW>300,000) solution for 1 hour at room temperature. The coverslips were then washed with distilled water and allowed to dry again for 2 hours at room temperature. The prepared HEL cells were allowed to adhere to coverslips for 15-20 minutes before using for the experiment.

2.4 Spectrophotometry

2.4.1 Spectrophotometer fluorescence measurements from cell suspensions

A Cairn Spectrophotometer System (Cairn Research Ltd, Kent, UK) attached to a cuvette holder was used to make ratiometric fluorescent measurements of intracellular Ca^{2+} in suspensions of fura-2-loaded platelets and/or HEL cells. The cuvette holder was fitted with a temperature regulation system and magnetic stirrer. A 75 watt xenon arc lamp provided the excitation light which passed through a rotating filter wheel assembly containing two 380nm and four 340nm band pass excitation filters. The 340/380nm excitation light was delivered to the cuvette assembly through

a liquid light guide. The lamp was switched on about 45-50 minutes before starting experiment in order to ensure the excitation signal had fully stabilised. A Teflon magnetic stirrer bar (ScienceLab, Texas, USA) was added to each cuvette followed by 2 ml of sample which was allowed to warm to 37^{o}C for three minutes prior to recordings.

2.4.2 Background Fluorescence Measurement

Background fluorescence arising from all non-dye components of the light path, including the saline, cuvette and cells, was obtained by addition of 50 µM digitonin to the cuvette at the end of an experimental run to permeabilise the cells and release the dye, then after a further two minutes, 45 mM $MnCl_2$ was added to quench the fura-2. An increase in autoflourescence from digitonin itself was observed with an increase in gain (see Fig 2.1), therefore the final background values were adjusted by subtracting the digitonin autoflourescence values at the relevant gain.

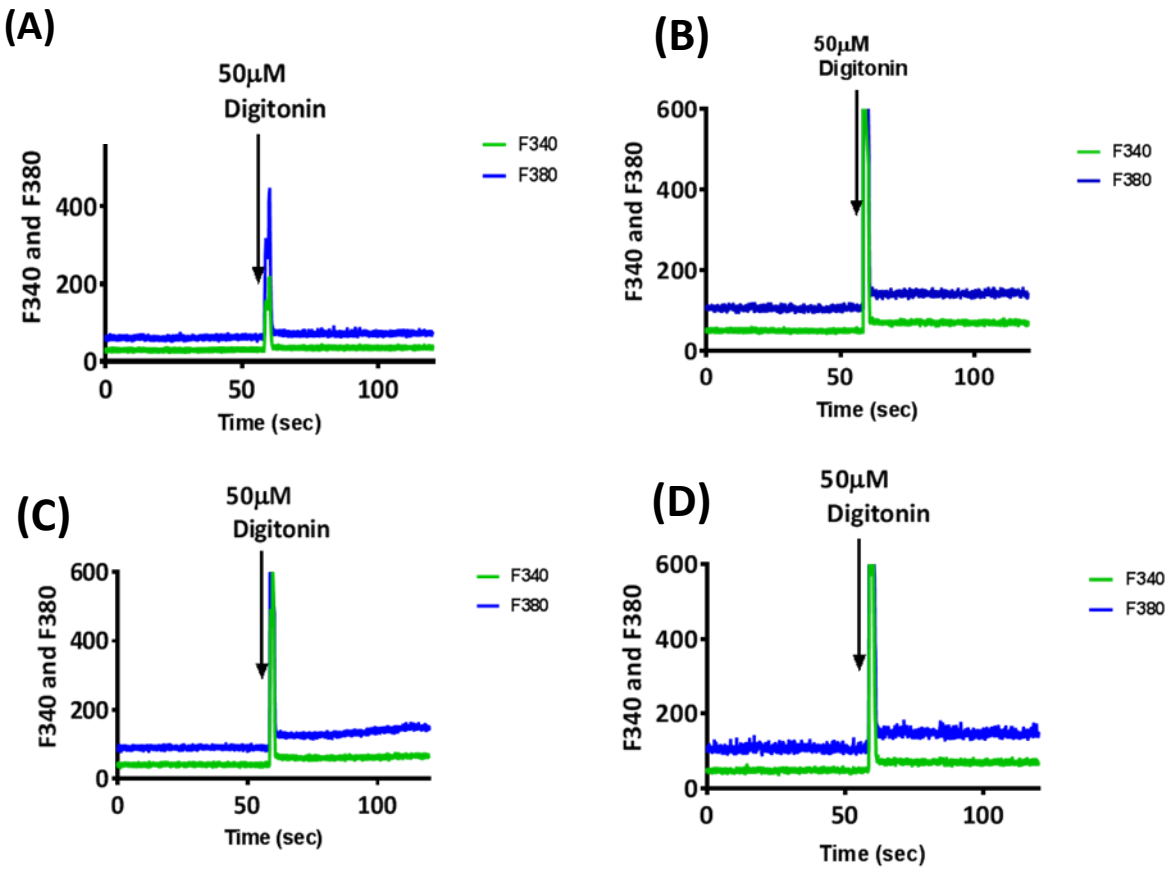

Fig 2.1 Digitonin autoflourescence at different photomultiplier gains.

Digitonin autoflourescence was measured by adding 50 µM digitonin to pre warmed nominally calcium free saline. In the traces shown, 50 µM digitonin was added at 60 s and the recording was run for two further minutes for the set photomultiplier (PMT) gain values: (A) 590 V (B) 674 V (C) 685 V (D) 700 V. The blue traces represent the fluorescence at 380 nm while the green signals indicate the fluorescence at 340 nm. As shown, the autoflourescence of digitonin increased with increase in PMT voltage.

2.4.3 Calibration of Fura-2 signals

The autofluorescence-corrected 340 and 380 nm signals were converted to intracellular Ca^{2+} ($[Ca^{2+}]_i$) using the following equation (Grynkiewicz et al., 1985).

$$[Ca^{2+}]_i = Kd \times (F380\ max / F380\ min) \times (R-R_{min})/(R_{max}-R).$$

Where Kd is the Ca^{2+} dissociation constant of fura-2 (224 nM at 37ºC and the ionic conditions used) (Grynkiewicz et al., 1985). R is the 340 nm/380 nm fluorescence ratio during an experiment. R_{min} is the minimum 340/380nm ratio obtained in Ca^{2+} free conditions by adding 15 mM EGTA followed by same concentration of TRIS base addition. Rmax is the maximum 340/380 ratio and is obtained by saturating fura-2 with Ca^{2+} by addition of 2 mM $CaCl_2$. F380 max and F380 min represent the maximum and minimum fluorescence signals respectively at 380 nm. R_{min} and R_{max} values were multiplied by a viscosity correction factor (0.85) in order to correct for the difference in functional properties of fura-2 in the cytoplasm compared to the extracellular environment (Poenie, 1990).

A lengthy set of trial experiments were performed to assess different ways of deriving the constants used for the Grynkiewicz equation. Two main sets of these validation experiments are explained below:

1. The platelet suspension was allowed to warm up for three minutes at 37°C, then 2 mM $CaCl_2$ was added to the platelets and allowed to run for two minutes. 50 µM digitonin was added in the cuvette to permeabilize the cells and obtain the R_{max} from the steady F340 and F380 signals. Afterwards, within the same cuvette, 15 mM EGTA was added and allowed to run for two minutes until the signal stabilised. This concentration of EGTA was finalised by adding a series of increasing concentrations until the signal got steady. Finally, in the same cuvette 15 mM Tris base was added to provide optimum basic (i.e; high pH) medium for EGTA to buffer Ca^{2+} to low levels and thus ensure that the R_{min} value was obtained. The measurement of autoflourescence after digitonin (see above) was conducted in a separate cuvette to avoid possible

chelation of Mn^{2+} by EGTA. However the application of this method produced a very high concentration of $[Ca^{2+}]_i$ under both resting conditions and after maximal TG-evoked responses. This could be due to the F380min value obtained in the presence of the platelets being very close to the autofluorescence level. Hence, large and very noisy ratios and $[Ca^{2+}]_i$ values were observed following elevation of intracellular Ca^{2+} with thapsigargin (TG) or during measurement of R_{max}. Therefore, the values obtained in the second method explained below were used in analysis.

2. In the second method, 2 µM K_5fura-2 (i.e. the potassium salt of fura-2) was added to >6ml of nominally calcium free saline. A 2 ml aliquot of this solution, was warmed for three minutes at 37°C and 2 mM $CaCl_2$ added to saturate the fura-2 with Ca^{2+} to provide the R_{max} value. In another cuvette a further 2 ml aliquot was warmed to 37°C the fluorescence measured after addition of 15 mM EGTA followed by 15 mM Tris base to get values for R_{min}. The R_{max} and R_{min} values were obtained after subtraction of the background signal which was assessed via two different approaches: in one, the 2 µM K_5Fura-2 in nominally Ca^{2+}-free saline was quenched with 45 mM $MnCl_2$. In the other, the fluorescence was simply measured from a 2 ml aliquot of pre-warmed nominally calcium free saline without any dye in cuvette. The values obtained with the later one were used in the calibration. Values of R_{min} and R_{max} were calculated for a range of gains used for the experiments. Using this approach, consistent $[Ca^{2+}]_i$ (nM) levels were obtained. Thus, the values from method 2 were then used in the equation to calculate the calibrated $[Ca^{2+}]_i$ (nM) concentration in all the experiments.

2.4.4 Analysis of Fura-2 signals

The parameters R_{max}, R_{min}, F380max, and F380min were measured within Cairn Research Ltd. Proprietary software (Cairn Interface v6.052). Raw data files from the Cairn spectrophotometer system were converted into ASCII files by Analyse Software Version 1.00a (Cairn Research Ltd, Faversham, Kent, UK). The data was then converted to $[Ca^{2+}]_i$ for analysis using the Grynkiewicz, Poenie & Tsien equation (see section 2.5.3) in Origin (version 9.1, Microcal Software Inc., Northampton, MA, USA) using an Origin macrofile written by Martyn Mahaut-Smith. The calibrated $[Ca^{2+}]_i$ values were

then exported to GraphPad Prism 6 software, Inc for Windows (La Jolla, California, USA) for graphical representation.

Intracellular Ca^{2+} responses were assessed from the peak increases and also from the integral of the increase for a duration of 2 minutes. To derive the integral (Area under the curve, AUC), the average baseline value was calculated and subtracted from each point after the addition of agonist or extracellular Ca^{2+}. The values throughout the 2 minutes were summed and divided by 11.45 (the data acquisition rate) to generate the $[Ca^{2+}]$i.sec value.

2.4.5 Thapsigargin-induced SOCE in platelets

2 ml of a fura-2 loaded platelet sample prepared as described in section 2.3.2, was added to a cuvette and allowed to warm at 37^{o}C for three minutes. TG was found to stimulate platelet aggregation in nominally Ca^{2+}-free medium prior to addition of 2 mM Ca^{2+} to measure SOCE, particularly following long incubations to fully deplete the Ca^{2+} stores. This aggregation was prevented by addition of 0.2 mM EGTA to chelate the extracellular Ca^{2+}. An upper estimate of free Ca^{2+} from water and salts in nominally Ca^{2+}-free saline was approximately 5 µM (Mahaut-Smith, 1990), which will be lowered to ≈2 nM by 0.2 mM EGTA (calculated using the programme Maxchelator (http://web.stanford.edu/~cpatton/webmaxcS.htm). TG was added to the cuvette followed by 2.2 mM $CaCl_2$ at varying times to set a final concentration of free Ca^{2+} at 2 mM and thus measure SOCE.

2.4.6 Ca^{2+} signalling in co-incubated suspensions of HEL cells and platelets

In addition to assessment of intracellular Ca^{2+} in suspensions of either platelets or HEL cells, Ca^{2+} responses were also measured in combined suspensions of both these cell types in which only one was loaded with fura-2 (see Fig 2.2). Extracellular $CaCl_2$ (2 mM) was added one minute after starting the recording with just the HEL cells in the cuvette. One minute after $CaCl_2$ addition, 200 µl platelets was added to the HEL cells

followed by agonist addition one minute later. The Ca^{2+} signal was recorded in for a duration of two minutes after agonist addition.

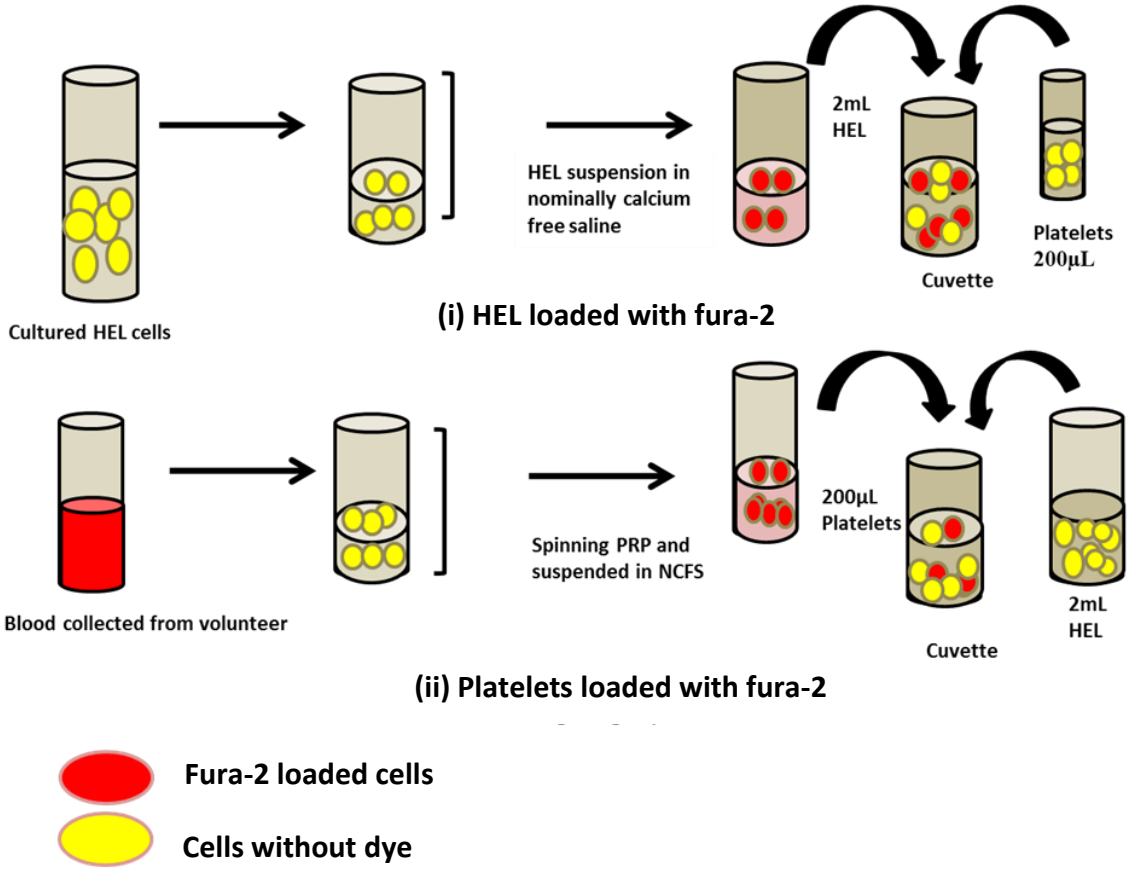

Fig 2.2. Schematic diagram for measurement of intracellular Ca^{2+} signalling in either HEL cells or platelets in mixed HEL-platelet suspension.

(i) HEL cells (2 ml) loaded with fura-2 were combined with platelets (200 µl) and the Ca^{2+} signal was analysed after agonist addition to the mixed HEL-platelet suspension (ii) Platelets (200 µl) loaded with fura-2 were incubated with HEL cells (2 ml) and the Ca^{2+} signal was analysed after thrombin addition. The red colour indicates the fura-2 loaded cells while the yellow colour represents the cells without dye.

2.5 In vitro light transmission Aggregometry

Light transmission measurements of platelet aggregation and luminescence measurements of extracellular ATP were made simultaneously with a Model 400 lumi-aggregometer (Chrono-log Corporation, Havertown, PA, USA). The luminescence and light transmission outputs were acquired to computer using a Minidigi 1A digitiser and Axoscope 9 software (Molecular Devices, Berkshire, UK). The aggregometer was allowed to warm up for 20-30 minutes after being switched on prior to starting the experiment. A cuvette with 500 µl nominally calcium free saline was placed with a Chrono-log #311 stir bar in the PPP well as the blank. A volume of platelets suspended in nominally calcium free saline was added to allow all other reagents to be added to make 500 µl final volume in cuvette in the presence of stir bar and kept in PRP well. The recording button was clicked on the screen and after 5 seconds the baseline button was pressed and released to set the baseline aggregation level. When measuring SOCE-induced aggregation, EGTA (0.2 mM) was added prior to TG addition to prevent aggregation during store depletion prior to addition of 2.2 mM Ca^{2+} (see section 2.5.5). Platelets were kept for three minutes in the cuvette well to warm up to 37^{o}C prior to recording. After recording for five minutes in the presence of 2 µM TG, 2.2 mM $CaCl_2$ was added in the cuvette containing platelets to induce SOCE in the platelets. Raw data from the Axoscope recording was imported to Origin 9.1, which allowed transfer of ASCII files to Microsoft Excel. Excel was used convert the light transmission values to percentage aggregation. Peak percentage aggregation was calculated for each run according to the following equation

$$\text{\% Light transmission (aggregation)} = \frac{\text{(Raw transmission value – Average baseline)}}{\text{(Average baseline)}} \times 100$$

Where average baseline is the mean of raw transmission values prior to stimulation. The percentage aggregation values were plotted against time using Graphpad Prism 6.

2.6 ATP secretion Assay

ATP concentrations in the extracellular saline were measured with a Chrono-Lume luciferin-luciferase assay kit in accordance with the manufacturer's instructions, in which each vial of the luciferin-luciferase reagent was dissolved in 5ml of filtered nominally Ca^{2+} free saline and 50 µl of this stock was used per 450 µl of platelets or cell suspension. Preliminary experiments showed that a significant luminescence signal was observed when 50 µl of ice-cold reagent stock was added to saline in the cuvette. Pre-warming abolished this signal, the origin of which is unclear. The time required to avoid this artefact was optimised by trials (see Fig 2.3) and shown to require at least 10 minutes warming at 37°C. ATP concentration was calculated by converting luminescence values using an ATP standard curve ranging from 4 to 1690 nM that showed a linear relationship with gradient

$$y = -0.001279x$$ **(see Fig 2.4).**

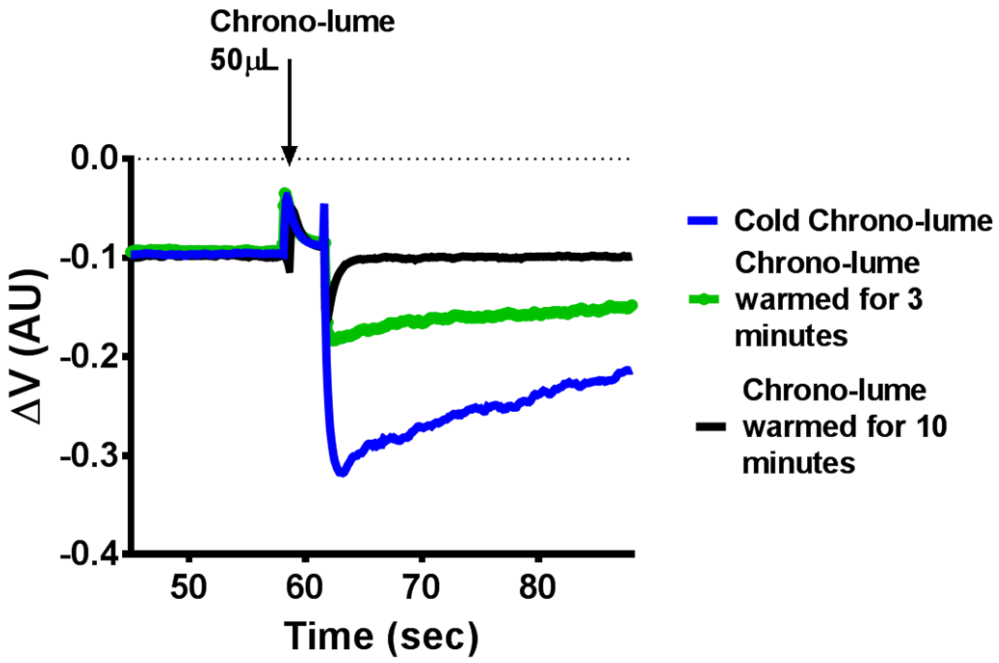

Fig 2.3 Comparison of the luminescence signal following addition of warm and cold Chronolume reagent to saline.

The traces show the luminescence signal before and after addition of 50 µl Chrono-lume reagent to a cuvette containing 450 µl saline. Prior to addition of chromolume, the saline containing 2 mM $CaCl_2$ was pre warmed for 3 minutes at 37°C. The blue trace shows the addition of cold Chrono-lume taken directly from storage on ice. The green trace shows the addition of Chrono-lume pre warmed for 3 minutes on 37°C. The black trace shows the addition of Chrono-lume pre warmed for 10 minutes at 37°C. The remaining small transients when adding the 10 minute pre-warmed condition were observed when simply opening and closing the cuvette cover, thus are artefacts within the lumi-aggregometer due to switching the light sensor path off and on.

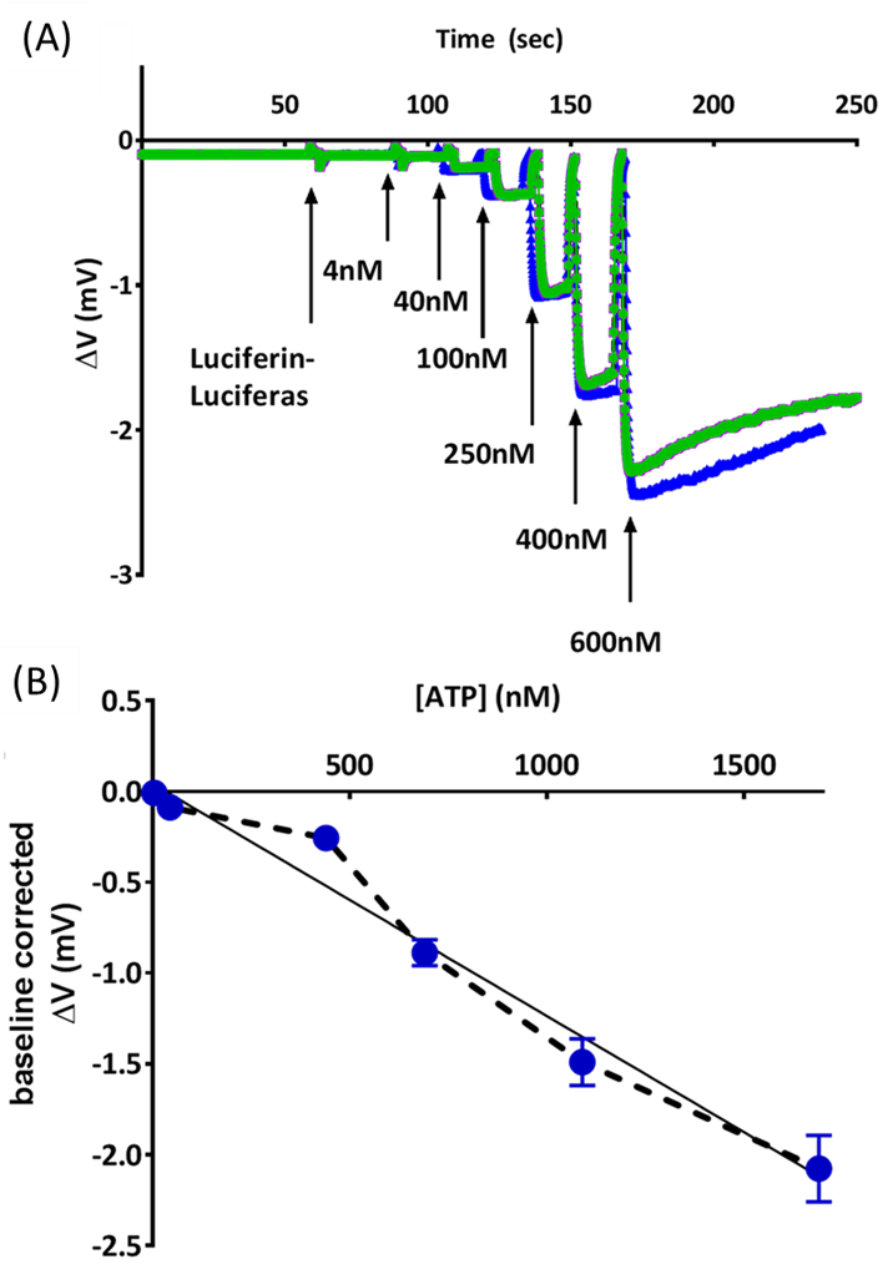

Fig 2.4 ATP concentration standard curve.

(A) Representative luminescence traces for serial addition of ATP concentrations in the range 4-600 nM. (B) Plot of luminescence signal (Y-axis) against total ATP concentration (X-axis) (points are the average of 3 separate measurements). The solid line was fit by linear regression within Graphpad Prism. The relationship of this line was used to convert luminescence signals to ATP concentrations: ΔV=-0.001279 [ATP].

2.7 Western blotting experiments

2.7.1 Preparation of Platelet Lysates

Platelet rich plasma was collected and treated with apyrase and aspirin as described in section 2.3.2. This was spun at 150 x *g* for 15 min then the platelet pellet was resuspended in nominally calcium free saline and centrifuged at 350 x *g* for 1min at room temperature. The pellet was suspended in RIPA lysis buffer containing dissolved protease inhibitor cocktail (150 mM NaCl, 0.5 % sodium deoxycholate, 50 mM Trizma® hydrochloride, 1 % Triton X-100, 0.1% SDS, 200 mM sodium orthovanadate, 2.5 mg/ml pepstatin A, 1 mg/ml leupeptin 1 mg/ml aprotinin, 100 mM PMSF) and kept on ice for 1 h to allow cell lysis. The final samples of each individual were centrifuged at 350 x *g* for 10 min at 4°C.

2.7.2 Protein Quantification and Bradford Assay

The supernatant obtained from the protocol in section 2.8.1 was used for protein quantification and analysis. Proteins were quantified using a Bradford assay with a standard curve obtained using bovine serum albumin (BSA). Multiple dilutions of (BSA) (0.625 – 10 mg/ml) were made and 5 µl of each were loaded in a 96 well plate in triplicate. Bradford reagent (250 µl) was added to each well and the absorbance at 595 nm read in an Infinite® 200 Nano Quant plate reader (Tecan, Männedorf, Switzerland). The standard curves were plotted in Microsoft Excel and protein concentrations of platelet sample were calculated (see Fig 2.5). The samples were diluted with RIPA lysis buffer to obtain the desired concentration of 2 µg/µl.

2.7.3 Western blotting

A 10 % resolving gel (30 % acrylamide, 10 % SDS, UddH_2O, 1.5 % APS, TEMED and resolving buffer at pH 8.8; National Diagnostics, USA) and 4 % stacking gel (30 % acrylamide, UddH_2O, 10 % SDS, 1.5 % APS, TEMED and stacking buffer at pH 6.8; National Diagnostics) were used to cast the gel between glass plates (with 1.0 mm spacers) assembled in a frame (BIO RAD, California, USA). Lysates (20 µl) were diluted

with 20 µl laemmli 2x sample buffer. The mixture was vortexed and heated at 70°C in a heating block for 10 min and vortexed again. 20 µl sample in laemmli buffer was loaded in each well and 10 µl pre-stained molecular weight marker (Bio-Rad Laboratories, Hertfordshire, UK; range 10-250 kDa) loaded in the first and last wells. Gels were run at 100 V for 1.5 h. Proteins were then transferred to PVDF membranes. Each gel and membrane were sandwiched between filter papers in a transfer cassette and immersed in transfer buffer (192 mM glycine, 25 mM Tris base, 10 % (v/v) methanol). Wet transfer was performed onto the PVDF transfer membrane (pore size 0.45 µM Merck Millipore, Hertfordshire, UK) in a cold room at 4°C at 100 V for one hr. The membranes were dipped into Ponceau red stain (0.1 % (w/v) in 5 % acetic acid) for 1 min and then washed 3 times with 1xTBS-T (Tris base 20 mM, NaCl 137 mM, Tween-20). Membranes were then blocked for 1 h with 5 % (w/v) skimmed milk (Premier foods, London, UK) in TBS-T in a Falcon tube on a roller at room temperature. Each membrane was then incubated with the appropriate primary antibody (see Table 2.1) in Falcon tubes overnight at 4°C on a roller. The next day membranes were washed 3-6 times for 5 min each at room temperature with TBS-T and incubated with secondary polyclonal swine anti-rabbit antibody diluted at 1:15000 in TBS-T for 1 h at room temperature on a roller. Visualisation was attained by Amersham ECL Prime Detection Kit (GE Healthcare) and the blot imaged on an ImageQuant LAS 4000 (GE Healthcare Bio-Sciences AB, Uppsala, Sweden). Protein expression was quantified by densitometric analysis using ImageJ (v1.45s, National Institutes of Health, Maryland, USA).

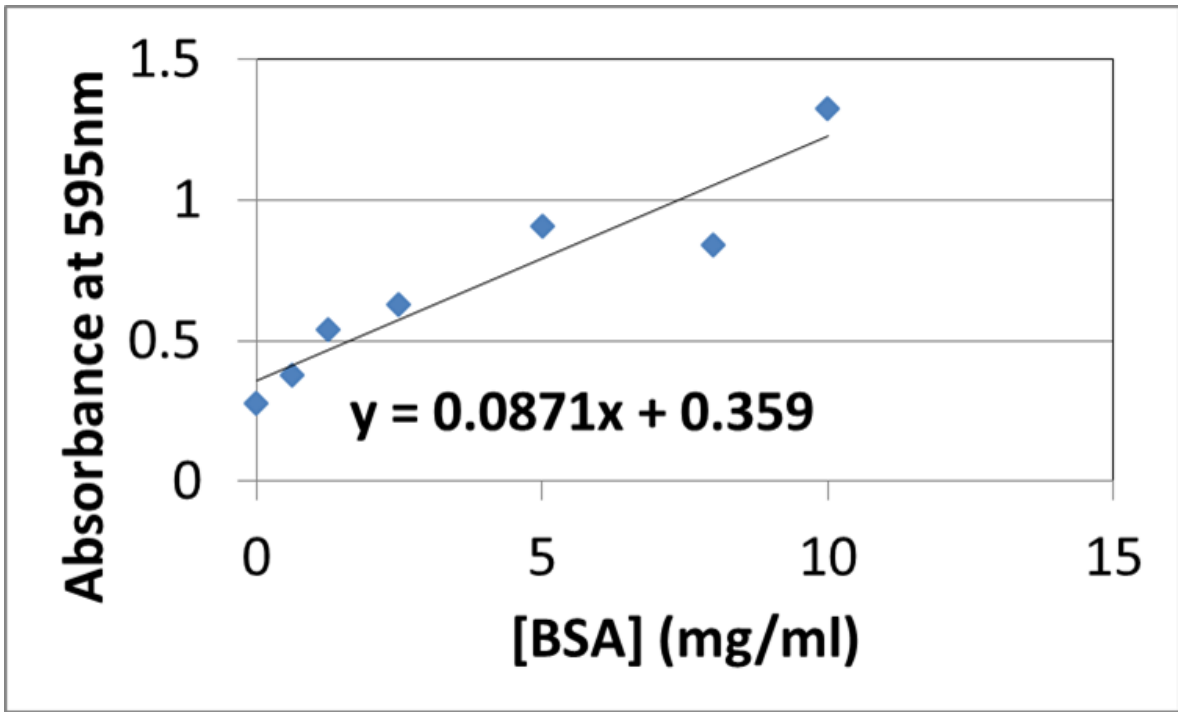

Fig 2.5 A representative Bradford assay standard curve.

Standard curve *of absorbance at 595 nm against a series of known bovine serum albumin (BSA) concentrations.*

Antibody	Species raised in	Dilution	Catalogue No and Company
Anti-Stim1	Rabbit	1:15000	(ab57834) Abcam (Cambridge, UK)
Anti-Orai1	Rabbit	1:5000	(ab59330) Abcam (Cambridge, UK)
GAPDH	Rabbit	1:500	(FL-335) Santa Cruz (Heidelberg, Germany).
Anti-Rabbit HRP	Swine	1:10000	(P0399) Dako (Ely, UK).

Table: 2.1 List of antibodies and dilutions used in Western blotting experiments.

2.8 qPCR

Total RNA was isolated from HEL cells using the QIAshredder and RNeasy kit with on-column DNase I digestion (QIAGEN, Crawley, UK.). Approximately 3 x 10^6 cells were taken from the culture, centrifuged at 1500 rpm for 5 minutes before removal of as much of the medium as possible and addition of the lysis buffer supplied within the RNeasy kit. RNA was quantified using a Nanodrop spectrophotometer (ThermoFisher Scientific) and 1 µg was reverse transcribed using a Sensiscript Reverse Transcription kit (QIAGEN). qPCR was performed using the iQSYBR Green qPCR Kit (Bio-Rad, Hemel, Hempstead, U.K) and a light cycler 480 real-time PCR system (Roche Diagnostics, Burgers Hill, U.K), with 2.5 µl of the reagent mix, 0.5 µl (0.75 µM) each of the forward and reverse primers and 1.5 µl cDNA per 20 µl reaction volume. The assay for each gene was run in triplicate for each of three separate batches of cells. qPCR expression data was normalised to the mean of β-actin (housekeeping gene) expression using the $2^{-\Delta Ct}$ method using the following equation.

$$\textbf{Relative expression (R)} = \frac{(E_{\text{gene of interest}}{}^{-Ct\ \text{gene of interest}})}{(E_{\text{housekeeping gene}}{}^{-Ct\ \text{housekeeping gene}})}$$

The human P2 receptor primer pairs are listed in the Supplementary Table S1 and were previously validated by standard end-point PCR and sequencing in the laboratory of Dr Catherine Vial (personal communication, Dr C Vial and (Wright et al., 2016a).

2.9 Fluorescence imaging of single HEL cell Ca^{2+} transients

Imaging of single HEL cell Ca^{2+} transients was carried out on an Olympus IX81 inverted microscope with a FV1000 laser scanning confocal module (Olympus, UK). All experiments used a 1.35 NA oil immersion 60X objective lens (UPLSAPO), a 1.98 µm confocal slice thickness and 488 nm excitation from a multi-line Argon laser. Fluo-3 fluorescence images were recorded at a photomultiplier gain of 715 V with the laser power set at 1.0 % (set via the Acousto-Optical Tunable Filter). Fluorescence and transmitted light images, both with a resolution of 256 x 256 pixels were collected at

an interval of 2 seconds (i.e. 0.5 Hz). Poly-D-lysine-coated coverslips were incorporated into Interchangeable Coverslip Dish (Bioptechs Inc, Butler, PA, USA) which uses an outer perspex ring and rubber washer to provide a water-tight seal. The chamber was held on the stage of the microscope using a custom Perspex mount (made in the Core Biotechnology Services Joint Biomedical Workshop, University of Leicester). 1 ml fluo-3 loaded HEL cell sample prepared as explained in section 2.4 was diluted with 1 ml saline and evenly spread over the coverslip followed by addition of 2 mM $CaCl_2$. The cells were allowed to settle for 15 minutes for attachment to the coverslip prior to recordings. Fluo-3 was excited at 488 nm and the emission was collected at 500-600 nm. After 2 minutes of recording, 200 µl platelets that had been pre-stimulated for one minute with PAM_3CSK_4 (5µg/ml), or left unstimulated as the control, were added into the chamber containing the HEL cells. The average fluorescence values from individual cells and an equivalent area in the saline were measured using a circular region of interest (ROI) within the confocal software FV1000 laser scanning confocal module (Olympus, UK) and exported to Microsoft Excel for further analysis as described below.

2.10 Data Analysis

Background corrected Fluo-3 fluorescence ratio values were calculated using the formula: $\mathbf{(F-F_{Bk})/(F_0- F_{Bk})}$, where the F represents the average fluorescence from the ROI drawn around the cell, F_0 is the average fluorescence within the same ROI before platelet addition and F_{Bk} is the average fluorescence from a ROI in the extracellular medium. The AUC of background- corrected F/F_0 was calculated by summation of values obtained after platelet addition to the end of the recording (600 s) within Prism 6. The morphological changes of HEL cells were analysed from the transmitted light images by calculating the surface area change using ImageJ 1.49 Volume Viewer 2.0 PLUGIN (National Institute of Health, USA).

2.11 Statistical Analysis

Statistical analysis in chapter 3 and 4 was performed by one-way ANOVA (followed by either Dunnett's multiple comparisons or Friedman test) or paired Student's t-tests (two-tailed), as appropriate. In chapter 5 one-way ANOVA followed by Tukey's multiple comparisons, or paired Student's t-tests (two-tailed), was used as appropriate. Results are expressed as the mean ± standard error of the mean (SEM). P values of $P<0.05$ (*), $P<0.01$ (**), $P<0.001$ (***) and $P<0.0001$ (****) were considered as statistically significant, whereas $P>0.05$ (ns) was considered not significant.

Chapter 3: An investigation of the Ca^{2+} signalling interactions between platelets and a cell line of megakaryocytic/erythroleukemic origin following thrombin stimulation

3.1 Introduction

Several pieces of evidence suggest that thrombosis is associated with a high risk of malignancy and cancer metastasis. The risk of developing venous thromboembolism (VTE), including pulmonary embolism and deep vein thrombosis, are increased up to 7 fold in cancer patients as compared to healthy individuals (Fennerty, 2006, Mandalà et al., 2011). Moreover cancer associated thrombosis is also reported as the second leading cause of death in cancer patients (Elyamany et al., 2014, Khorana, 2009). Thrombosis may be common in cancer as a consequence of tumour-induced platelet activation (see section 1.4 of the introduction for further details) (Meikle et al., 2016). In addition, the higher platelet count (thrombocytosis) may enhance the likelihood of thrombosis (see section 1.4.2.5 for further background). Finally, it is possible that the hyperactive platelets may affect signalling in the tumour cells themselves, generating a positive feedforward effect. However, at present there is limited understanding of the effect of platelets on signalling in tumour cells, with studies to date focusing on the ability of platelets to physically bind to tumour cells and protect them from the immune system (see section 1.4.1.5 for further background). An increase in intracellular Ca^{2+} is a major signalling pathway in all types of cells, including platelets, and has been implicated to have a key role in cancer development (Golebiewska and Poole, 2015b, Clapham, 2007, Capiod et al., 2007). Therefore, the analysis of interaction between platelets and cancer cells at a level of Ca^{2+} signalling could reveal further understanding of how these two cells co-operate.

A range of cell lines are available that originate from human cancer patients that could be used to study the interaction with platelets. One possibility is a myeloid leukemic cell line, human erythroleukemic cells (HEL). HEL cells grow in suspension so can be used to assess how a myeloid cell that has migrated into the blood may interact with circulating platelets. HEL cells originate within bone marrow (Naeim et al., 2013), where they will be exposed to several cell types, including megakaryocytes. Therefore, since megakaryocytes express most if not all platelet proteins (Thon and Italiano, 2010a, Shattil and Leavitt, 2001), the interactions between HEL cells and platelets may have relevance for understanding how AML cancer cells are regulated within the marrow environment.

Cancer patients are at a risk of producing 3-4 times more thrombin as compared to healthy individuals (Danckwardt et al., 2011). Therefore, this chapter has examined the interaction between platelets and HEL cells at the level of intracellular Ca^{2+} following stimulation by thrombin. This was achieved using stirred suspensions in which either platelets or HEL cells were loaded with fura-2 and comparing the responses in mixed cell populations with responses in a pure population of each cell type.

3.2 Results:

3.2.1 Platelets potentiate the thrombin-evoked response in HEL cells

The initial experiments assessed the $[Ca^{2+}]_i$ responses to thrombin in a pure population of fura-2 loaded platelets or HEL cells (Fig. 3.1 A and C, black traces). These were then compared to the responses in the platelets or HEL cells when the two cell types are mixed together. As shown in Fig. 3.1 A, the $[Ca^{2+}]_i$ response of HEL cells to thrombin was enhanced in the presence compared to the absence of platelets. On average, the 2 min integral value was increased 2.2 ± 0.23 fold; 27632 ± 789 nM.sec vs 12581 ± 3630 nM.sec, P= 0.02; n=6) by the presence of platelets (Fig. 3.1B). In contrast, HEL cells had no significant effect on the $[Ca^{2+}]_i$ response of platelets to thrombin (44022 ± 7981 nM.sec for platelets in the absence of HEL cells and 30418 ± 3776 nM.sec in a mixed population; P>0.05; n=4; see Fig 3.1 C, D).

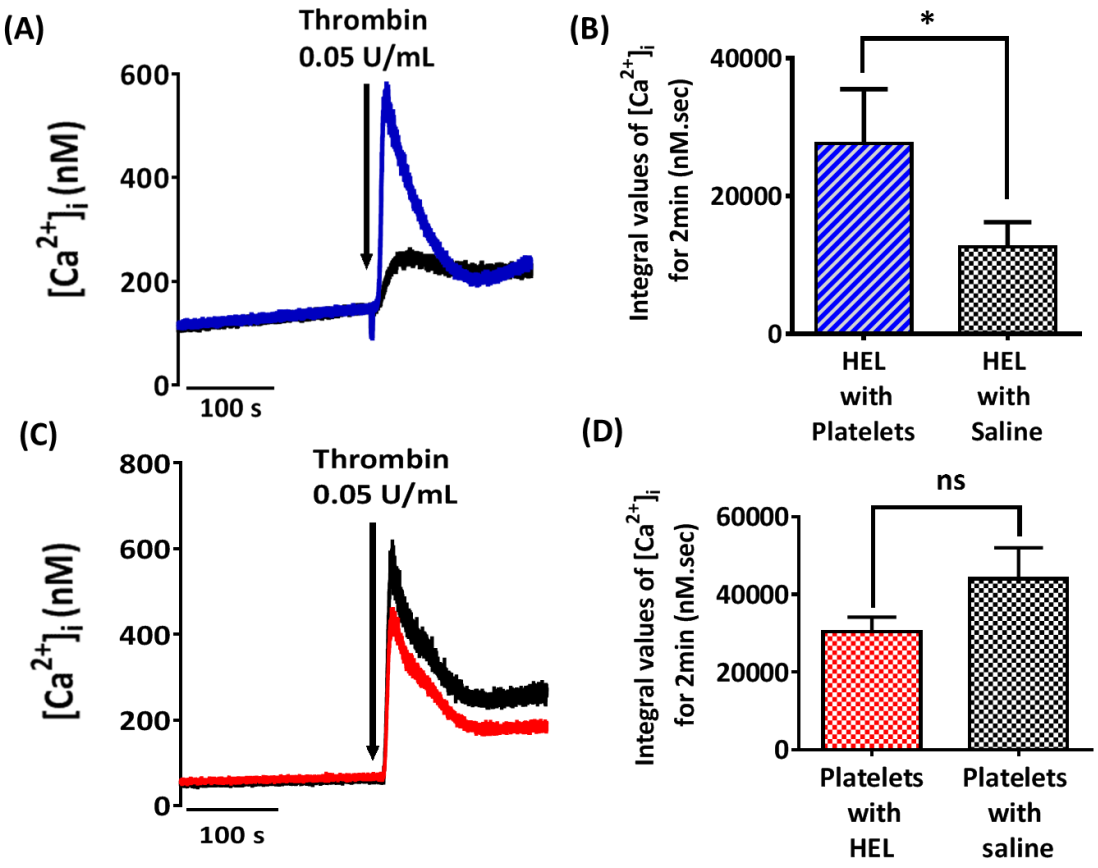

Fig 3.1 Effect of combining HEL cells and platelets in a stirred suspension on the thrombin evoked $[Ca^{2+}]_i$ responses of each of these two cell types.

*$[Ca^{2+}]_i$ responses to thrombin were recorded using fura-2 loaded into either HEL cells (A, B) or platelets (C, D). A and C show representative traces and B, D show the average integral of the increase for 2 minutes after agonist addition. Responses are from mixed suspensions of both cell types (blue, red), only HEL cells (black in A, B) or platelets (black in C, D) Responses were compared by Student's Paired t-test, n=6); *, P<0.05; ns not significant.*

3.2.2 Apyrase did not inhibit the HEL cell Ca^{2+} response in the presence of platelets, but did itself evoke a $[Ca^{2+}]_i$ increase in HEL cells

Thrombin is known to release ADP and ATP from platelets (Jiang et al., 2013) and these nucleotides stimulate Ca^{2+} responses in a variety of myeloid cells via P2X ion channels (ATP) and P2Y G-protein-coupled receptors (ADP and ATP) (Feng et al., 2015, Barbosa et al., 2011). Thus, paracrine signalling via released ADP and/or ATP could be responsible for the potentiation of HEL Ca^{2+} signals by platelets following thrombin stimulation. To assess this possibility, HEL cell responses to thrombin in mixed cell suspensions were compared in the presence and absence of the nucleotidase apyrase (He et al., 2005). Apyrase at a concentration of 0.32 U/ml did not significantly alter the platelet-induced HEL cell thrombin responses in mixed suspensions. The 2 min integral for the HEL cell response to thrombin in the presence of platelets and apyrase was 18983 ± 3743 nM.sec (see Fig 3.2 A and B), whilst in the absence of apyrase this value was 27065 ± 3594 nM.sec ($P>0.05$; n=6) (see Fig 3.2 A and B). However the shape of the response changed from a single peak followed by a plateau in the absence of apyrase to a biphasic increase with apyrase (see green traces in Fig 3.2 A). Increasing the apyrase concentration to 3.2 U/ml also did not significantly alter the integral of the thrombin-evoked Ca^{2+} response in HEL cells in a mixed platelet-HEL cell suspension (37651 ± 3659 nM.sec with 3.2 U/ml apyrase vs 29023 ± 1739 nM.sec in the absence; $P>0.05$; n=3; see Fig 3.2 C and D). However, the biphasic nature of the HEL cell response was enhanced at this higher apyrase concentration and the trend was for this high apyrase to increase the integral of the thrombin-evoked Ca^{2+} increase (Fig. 3.2 C). Moreover addition of the higher concentration of apyrase itself induced a surprising increase in Ca^{2+} signal (see Fig 3.2 C).

In the absence of platelets, a thrombin response in HEL cells was also still observed in the presence of the high apyrase concentration (3.2 U/ml) (Fig. 3.3A). The integral of this response was not significantly different in the presence of 3.2 U/ml apyrase (13314 ± 1450 nM.sec) compared to without apyrase (9236 ± 535 nM.sec; $P>0.05$, n=3) (see Fig 3.3 A and B). Addition of 3.2U/ml apyrase itself, prior to thrombin,

evoked a large transient Ca^{2+} increase in HEL cells as observed in the presence of platelets (see Fig 3.3 A and B). When fura-2-loaded platelets were studied on their own, 3.2 U/ml apyrase had no effect on either the integral or the shape of the $[Ca^{2+}]_i$ response to thrombin (see Fig 3.3 C and D). The average integral values of the thrombin mediated Ca^{2+} signal in the presence of apyrase was 44145 ± 6632 nM.sec which was not significantly varied in the absence of apyrase (46570 ± 4992 nM.sec) ($P>0.05$; n=3).

To further investigate the unexpected effect of apyrase on HEL cells, the Ca^{2+} response to a range of apyrase concentrations was studied in a pure population of HEL cells (see Fig 3.4). A clear relationship was observed between the concentration of apyrase added and the increase in $[Ca^{2+}]_i$. For example, an increase of 1090 ± 171 nM.sec integral value was observed with 0.32 U/ml apyrase while the level was raised to 9090 ± 1020 nM.sec with 3.2 U/ml apyrase ($P<0.05$; n=3) (see Fig 3.4B). One possible explanation for this effect of apyrase is that HEL cells constitutively release ATP that is converted into ADP by apyrase. ADP could then be causing autocrine stimulation of P2Y-coupled Ca^{2+} receptors in HEL cells that are more potently activated by ADP compared to ATP. This conversion can also explain the biphasic nature of the response to thrombin after addition of apyrase (see Fig. 3.2 C).

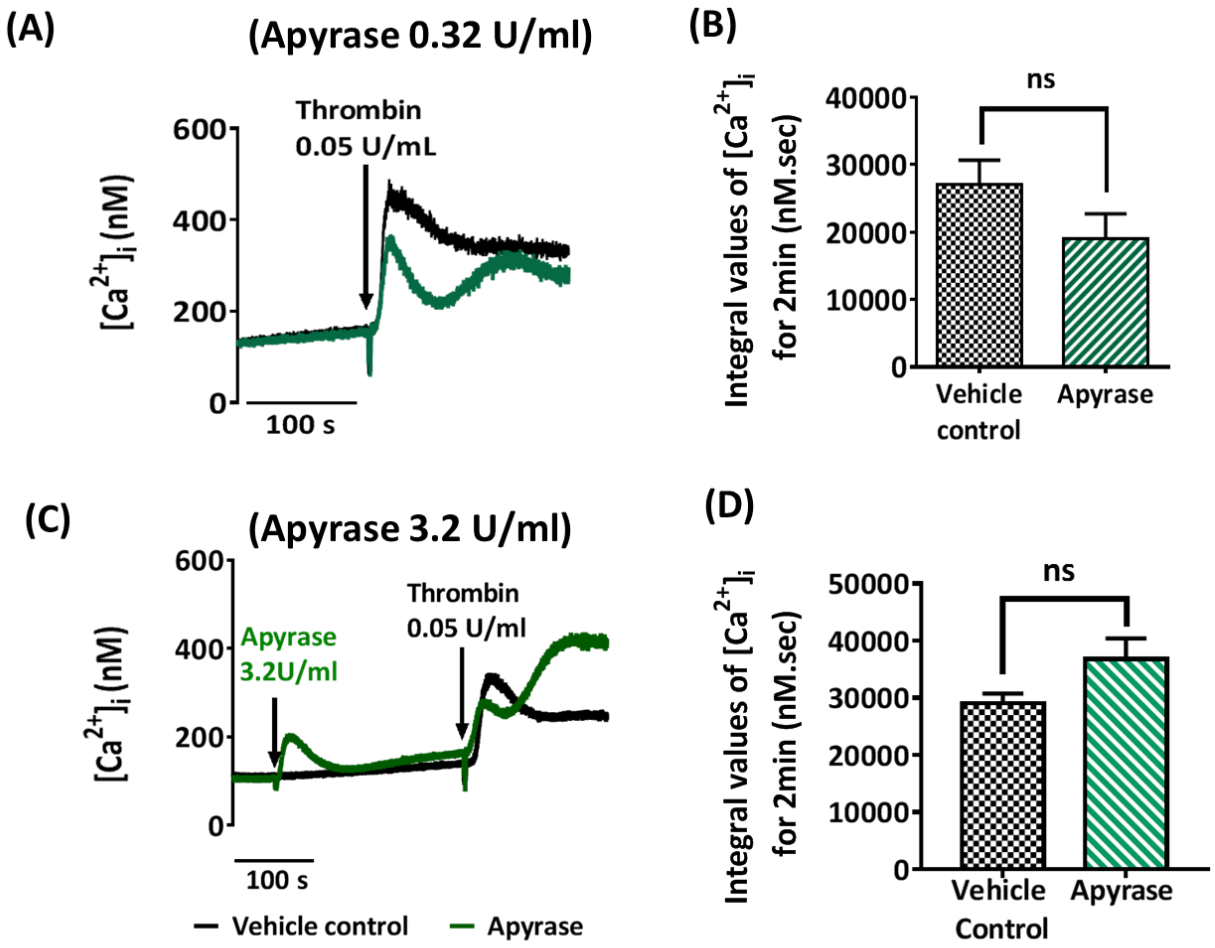

Fig 3.2 Effect of a high apyrase concentration on the HEL cell Ca^{2+} response in a mixed cell suspensions

$[Ca^{2+}]_i$ was recorded from HEL cells in the presence of platelets and in the presence or absence of either 0.32 U/ml (A,B) or 3.2 U/ml (C,D) apyrase. A,C, representative recordings; B,D, average 2 min integral of the response to thrombin. Paired t-test. Data represent the mean ± SEM, n=3.

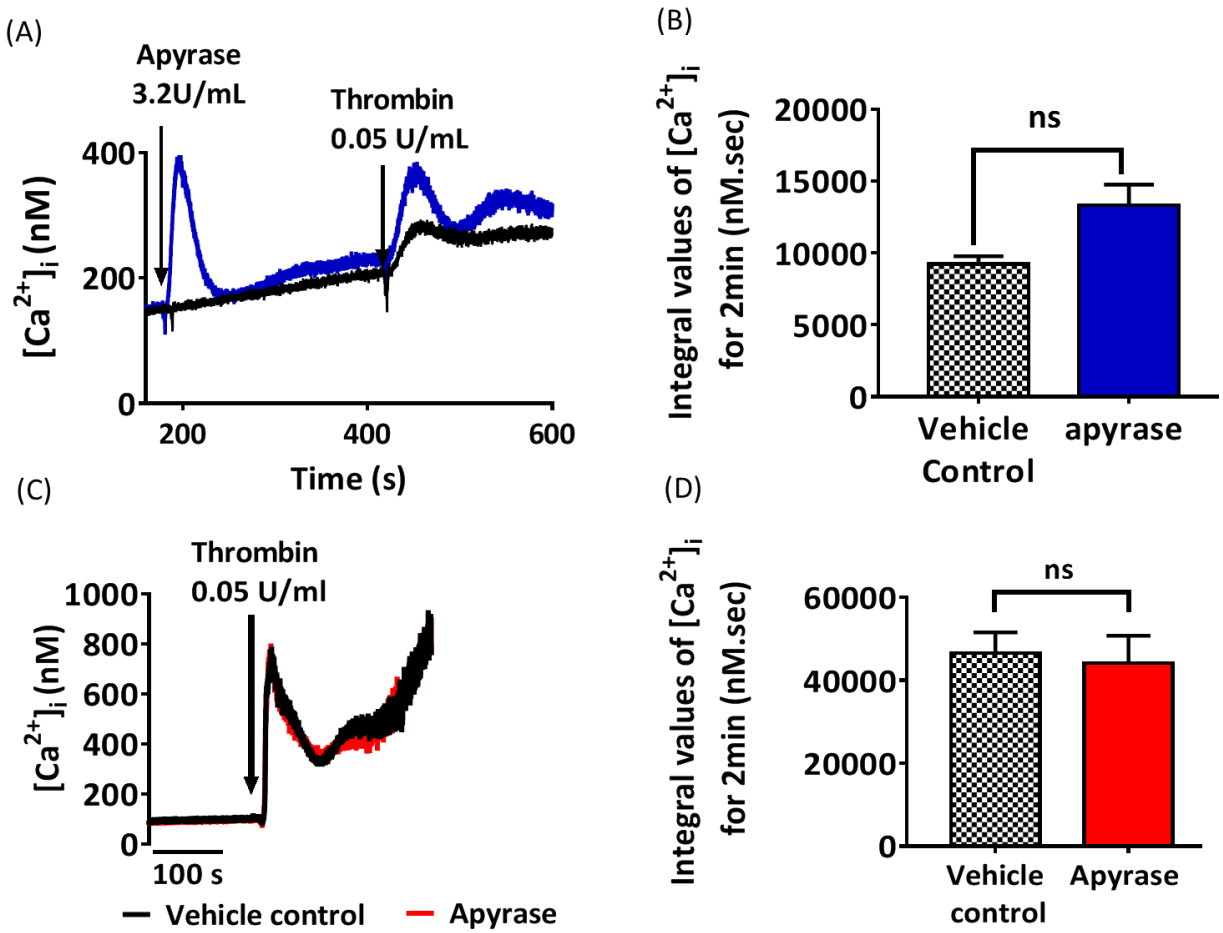

Fig 3.3 Effect of apyrase on the thrombin responses of a pure population of either HEL cells or platelets

(A) Sample recordings of responses to thrombin within a pure population of HEL cells following addition of either apyrase (blue trace) or vehicle control (black trace) 2 minutes prior to thrombin. (B) Comparison of average 2 min integrals of Ca^{2+} responses in HEL cells to thrombin in the presence (blue bar) and absence (black bar) of apyrase. No significant effect was observed on the thrombin response by apyrase (P>0.05; n=3, Paired t-test). Data represent mean ± SEM. (C) Sample recordings of responses to thrombin within a pure population of platelets following addition of apyrase (red trace) as compared to the vehicle (black trace) added prior to thrombin addition. (D) Comparison of average 2 min integral values of Ca^{2+} response to thrombin with (red bar) and without (black bar) apyrase. No significant difference observed (P>0.05; n=3). Analysis was performed with Paired t-test and the data represent the mean ± SEM.

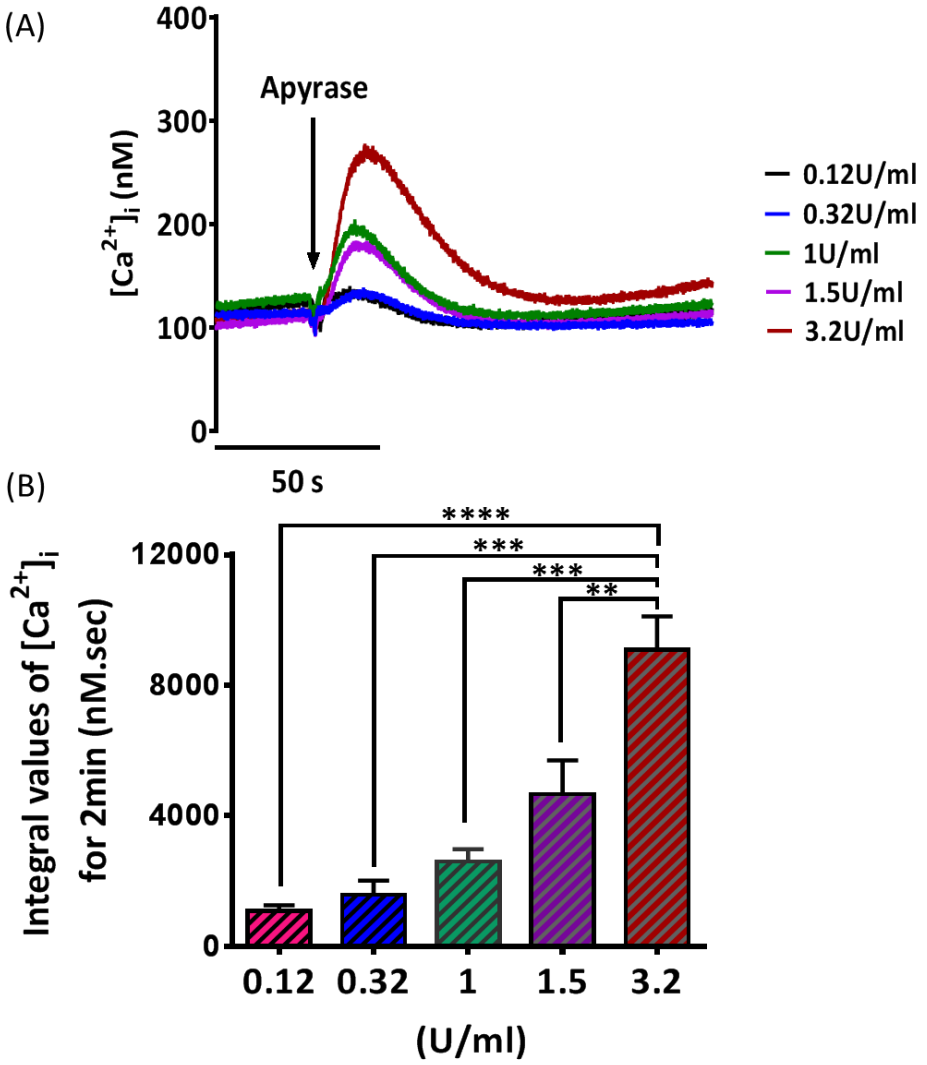

Fig 3.4 Apyrase evoked a concentration-dependent increase in intracellular Ca^{2+} in a pure population of HEL cells

*(A) Sample recordings of the $[Ca^{2+}]_i$ increase evoked by different concentrations of apyrase in a pure population of HEL cells in the presence of extracellular $CaCl_2$ (2 mM). The minimum concentration of apyrase to evoke Ca^{2+} influx in HEL cells is 0.12 U/ml (black trace) and the maximum concentration applied was 3.2 U/ml (maroon traces). (B) Comparison of average integral values of Ca^{2+} increase in HEL cells following apyrase treatment (Data represent mean ± SEM; n=3; One-way ANOVA test, multiple comparison by Tukey's test ** P<0.01; *** P<0.001, **** P<0.0001.*

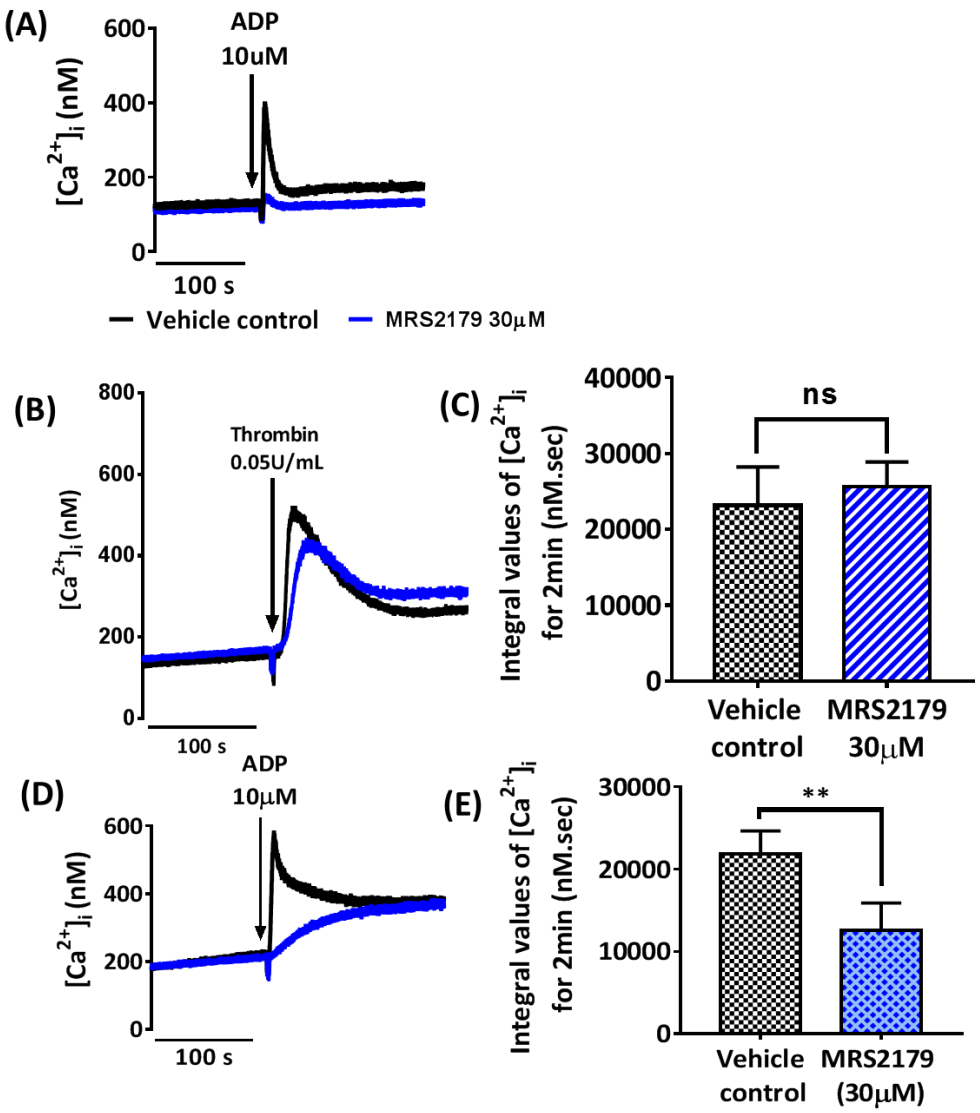

Fig 3.5 MRS2179 did not inhibit the HEL cell Ca^{2+} response to thrombin in the presence of platelets but did partially block an ADP-evoked Ca^{2+} response of HEL cells

*(A) $[Ca^{2+}]_i$ response to ADP (10 μM) in platelets under control conditions (black trace) and in the presence of the P2Y1 blocker MRS2179 (30 μM) (blue trace). (B,C) Effect of MRS2179 (30 μM for 5 minutes) on the thrombin-evoked $[Ca^{2+}]_i$ response of HEL cells in the presence of platelets; sample recordings in (B) and average 2 min integral values in (C); blue trace and bar with MRS2179 and black trace and bar in vehicle control (P>0.05; n=4). Paired t-test analysis. (D, E) Effect of MRS2179 (30 μM) on the response to ADP (10 μM) in a pure population of HEL cells. Sample traces in D and average 2 min integral in E; blue trace with MRS2179 and black trace with vehicle control. (**P<0.01; n=3) using Paired t-test. All data represent mean± SEM.*

3.2.3 Platelet-induced potentiation of thrombin-evoked Ca^{2+} response in HEL cells is not due to P2Y1 activation in HEL cells

Thrombin is known to evoke the release of ATP and ADP from platelets, and P2Y1 receptors are more potently activated by ADP than ATP (Hechler and Gachet, 2011b, Michno et al., 2007). Thus, activation of P2Y1 receptors by ADP on HEL cells could account for the enhanced responses in the presence of platelets and conversion of the thrombin-evoked HEL response to a biphasic response in the presence of apyrase. To test if the potentiation is due to an ADP-evoked response via P2Y1 in HEL cells, the selective blocker of this receptor, MRS2179 (30 μM) was used (Nandanan et al., 2000, Filippov et al., 2006, Boyer et al., 1998). The ADP response in platelets was completely blocked by MRS2179 (30 μM) (see Fig 3.5 A), confirming previous effects of this agent in platelets (Garcia et al., 2007), albeit at a higher concentration (100 μM). However, MRS2179 did not affect the thrombin response in HEL cells in the presence of platelets (see Fig. 3.5 B, C). The average 2 min integral value of the HEL response to thrombin in the presence of platelets with MRS2179 was calculated as 25661 ± 3237 nM.sec (see Fig. 3.5 C) which was not significantly different from the value in the absence of MRS2179 (21850 ± 2776 nM.sec; $P>0.05$; n=4; Fig. 3.5 C). In HEL cells (without platelets) MRS2179 significantly reduced the response to ADP although a substantial Ca^{2+} response remained (see Figs 3.5 D and E). The average 2-minute integral values were calculated as 12581 ± 3325 nM.sec in the presence of MRS2179 compared with 21850 ± 2776 nM.sec in the absence of the inhibitor ($P<0.01$; n=3; see Fig 3.5 E). Thus, although HEL cells possess a robust ADP-evoked Ca^{2+} response that is partially dependent upon P2Y1 receptors, the ability of platelets to potentiate HEL cell responses following thrombin co-stimulation does not significantly depend upon activation of P2Y1 receptors.

3.2.4 PGE1, U73122 and Aspirin did not alter the platelet-mediated potentiation of the thrombin response in HEL cells

HEL-platelet suspensions were treated with PGE1 that is known to inhibit platelets by binding to a prostanoid receptor that elevates cAMP level leading to inhibition via PKA activation (Iyú et al., 2011). PGE1 inhibition was first tested on the ADP-evoked response in platelets. The $[Ca^{2+}]_i$ response in platelets to ADP was completely abolished in the presence of PGE1 (see Fig 3.6 A). However PGE1 did not affect the platelet-induced potentiation in HEL cells following thrombin stimulation. The 2-minute integral values for the HEL thrombin response with platelets incubated in the presence of PGE1 was 31401 ± 4056 nM.sec. No significant difference was observed for the same analysis without PGE1 (33361 ± 967 nM.sec, $P>0.05$; n=3) (see Fig 3.6 C). The increase in Ca^{2+} due to PGE1 alone on HEL cells was also observed in a pure population of HEL cells (not shown). However the exact pathway of the Ca^{2+} stimulation in HEL cells by PGE1 is unknown.

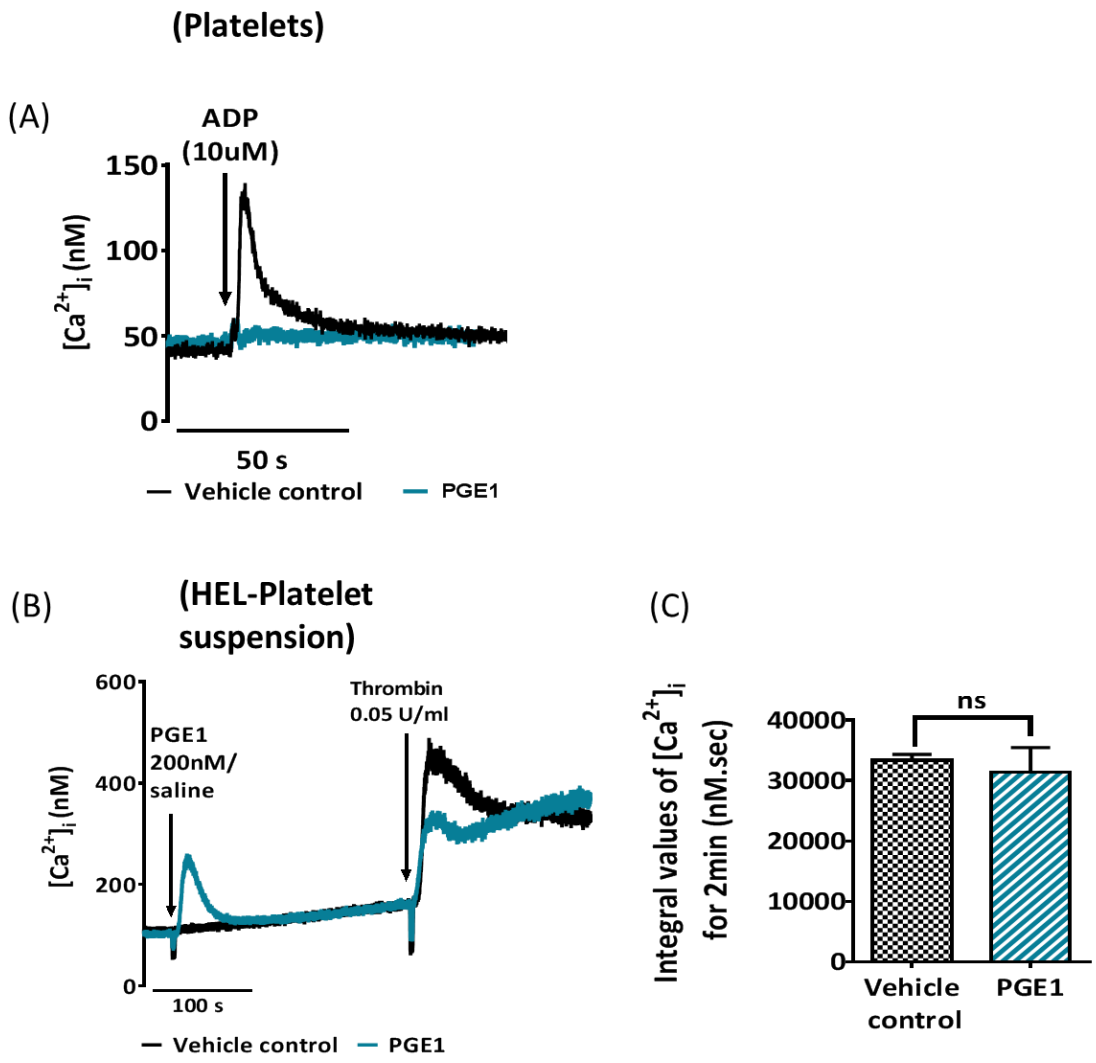

Fig 3.6 PGE1 caused no inhibition of the thrombin-evoked Ca^{2+} response in HEL cells in the presence of platelets

(A) Effect of PGE1 (200 nM, 5 minutes) on the ADP-evoked Ca^{2+} response in platelets. An inhibition in ADP-evoked Ca^{2+} signalling observed with PGE1. (B, C) Effect of 200 nM PGE1 (5 min exposure) on the thrombin-evoked Ca^{2+} response of HEL cells in a HEL-platelet suspension; B shows sample traces and C shows the average 2 min integral in the presence of PGE1 (blue trace and bar) or vehicle control (black trace and bar) No significant effect observed (P>0.05; n=3, Paired t-test). All data represent mean ± SEM.

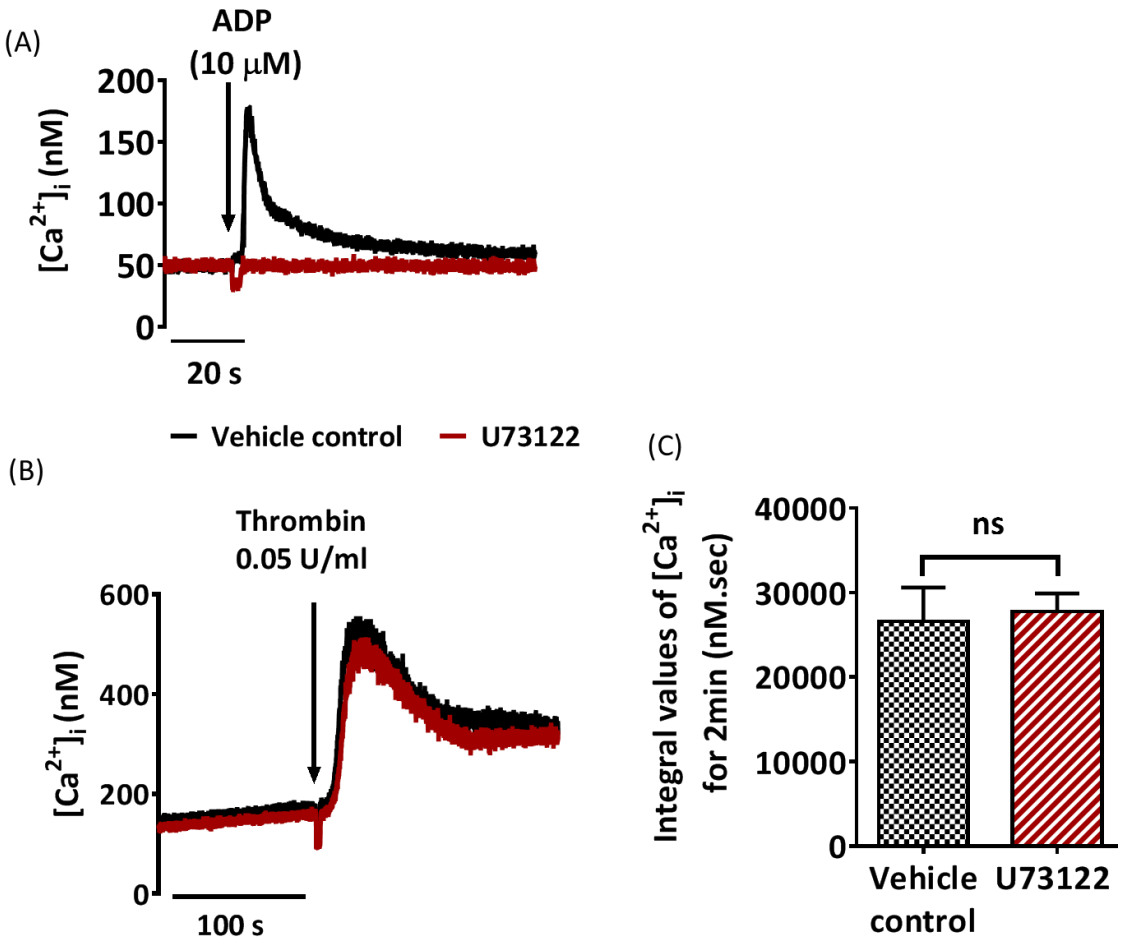

Fig 3.7 U73122 did not alter the HEL thrombin-evoked Ca^{2+} response in the presence of platelets

(A) ADP-evoked Ca^{2+} responses with 5 μM U73233 (maroon trace) as compared to the vehicle control (black trace). (B, C) Thrombin-evoked Ca^{2+} response of HEL cells in the presence of platelets with U73122 (5 μM, 5 minutes) (maroon) or vehicle control (black) Sample traces in (B) and average 2 min integrals in (C); (P>0.05; n=3 with Paired t- test analysis). Data represent mean± SEM.

Thrombin acts on PAR-1 and PAR-4 on human platelets and these receptors are coupled to PLC that mediates PIP_2 hydrolysis to IP_3 and DAG (Voss et al., 2007a). The IP_3 binds to IP_3R on DTS resulting in rise in cytosolic Ca^{2+} and together with other pathways induces platelet granular secretions (Lian et al., 2005). To test whether PLC inhibition can affect the platelet-induced potentiation in HEL cells, a PLC blocking agent, U73122 (Horowitz et al., 2005, Hollywood et al., 2010) was used. The inhibitory effect of U73122 was first confirmed on ADP-induced Ca^{2+} signalling in platelets (see Fig 3.7 A). However U73122 did not affect the potentiation induced by platelets in HEL cell response to thrombin (see Fig 3.7 B). The average 2 minutes integral values of HEL response to thrombin with platelets in the presence of U73122 was 27761 ± 2129 nM.sec and without U73122 was calculated as 26598 ± 4002 nM.sec ($P>0.05$; n=3) (see Fig 3.7C).These data imply that thrombin induced platelet secretion is not mainly via PLC activation pathway.

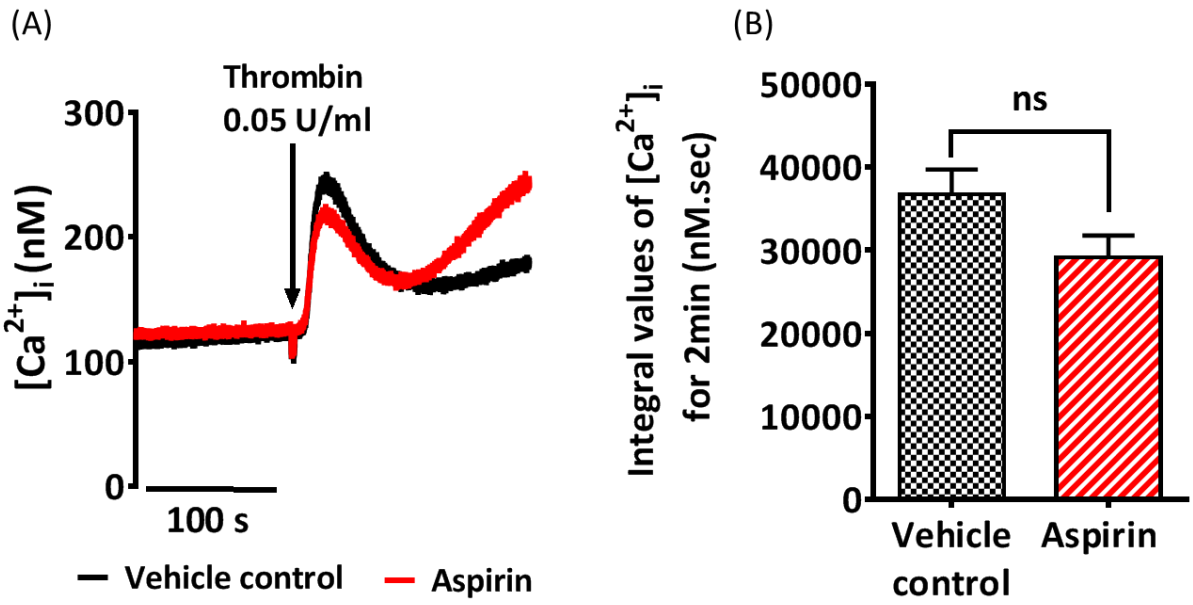

Fig 3.8 Aspirin did not alter the of the HEL cell thrombin-evoked Ca^{2+} response in the presence of platelets

HEL-platelet suspensions were treated with aspirin (100 µM) (red trace and bar) or vehicle control (black trace and bar) for 5 min prior to thrombin addition. (A) shows sample traces and (B) shows the average 2 min integral for the thrombin response No significant effect of aspirin was observed (P>0.05; n=3; Paired-t test). Data represent mean ± SEM.

Thrombin-activated platelets release TxA_2 (Garcia-Szabo et al., 1983) that is reported to activate HEL cells (Thomas et al., 1995). Therefore, another candidate that could contribute to the platelet-induced potentiation of HEL Ca^{2+} responses is TxA_2 released from thrombin stimulated platelets. Aspirin blocks the TxA_2 release from platelets via inhibition of cycloxygenase-1 (COX-1) enzyme (Warner et al., 2011). To analyse the effect of TxA_2, aspirin (100 µM) was used to block the TxA_2 release. Aspirin caused a slight reduction in the peak response of HEL cells to thrombin in the presence of platelets (see Fig 3.8 A), however there was no significant change in the average 2 min integral value; 29119 ± 2641 nM.sec in the presence and 36688 ± 3023 nM.sec in the absence of aspirin ($P>0.05$; n=3; see Fig 3.8 B).

3.2.5 P2 receptor expression of HEL cells

Based on the hypothesis that thrombin-activated platelets release ADP and ATP (Jiang et al., 2013) which act via purinergic receptors (P2 receptors) (Burnstock and Williams, 2000), the next analysis was a screening of the HEL P2 receptors that may contribute to the platelet-induced enhancement of the HEL thrombin response. The P2 receptor profile of HEL cells was determined using quantitative PCR analysis. The order of P2 receptor expression was found as P2X4>P2Y11>>P2X1> P2X7>P2X6>P2Y12>P2Y13>P2Y2>P2Y1 (see Fig 3.9). P2X4, P2Y11 and P2X1 were the most substantially expressed. The most potent agonist for these P2 receptors is ATP (Communi et al., 1999, Syed and Kennedy, 2012, Mahaut-Smith et al., 2000).

3.2.6 A high concentration of NF449 inhibits the thrombin-evoked HEL cell Ca^{2+} response in the presence of platelets

To further investigate the mechanism of platelet induced activation in HEL cells, P2 receptor blockers were used. 5-BDBD has been used to block the P2X4 receptors (Balazs et al., 2013). However, the agent was found to be autofluorescent to such an extent that the data could not be analysed as intracellular Ca^{2+} values or 340/380 nm

ratios. The P2X1-blocking effect of NF449 (Mahaut-Smith et al., 2011) was first evaluated on the platelet response to the agonist α,β-MeATP, known to selectively activate a response through this receptor in platelets (MacKenzie et al., 1996, Mahaut-Smith et al., 2000, Rolf et al., 2001a). The α,β-MeATP-evoked response was completely blocked by NF449 (1 μM) (see Fig 3.10 A). To evaluate the role of HEL P2X1, HEL-platelet suspensions were treated with 1 μM NF449. No inhibition of the HEL thrombin response in the presence of platelets was observed with NF449 (1 μM) (see Fig 3.10 B). The 2-minute integral values of Ca^{2+} influx with 1 μM NF449 was 19373 ± 4598 nM.sec, whereas without NF449 it was 20132 ± 6371 nM.sec (P>0.05; n=4) (see Fig 3.10 C). 1 μM NF449 also had no effect on the ATP-evoked Ca^{2+} increase in HEL cells, suggesting that other P2 receptors are responsible for the response to this nucleotide in the cell line (see Fig 3.10 D). Thus, P2X1 receptors are not involved in the thrombin-evoked HEL cell Ca^{2+} response in HEL-platelet suspensions. In addition, receptors other than P2X1 are responsible for the ATP-evoked Ca^{2+} increases in HEL cells.

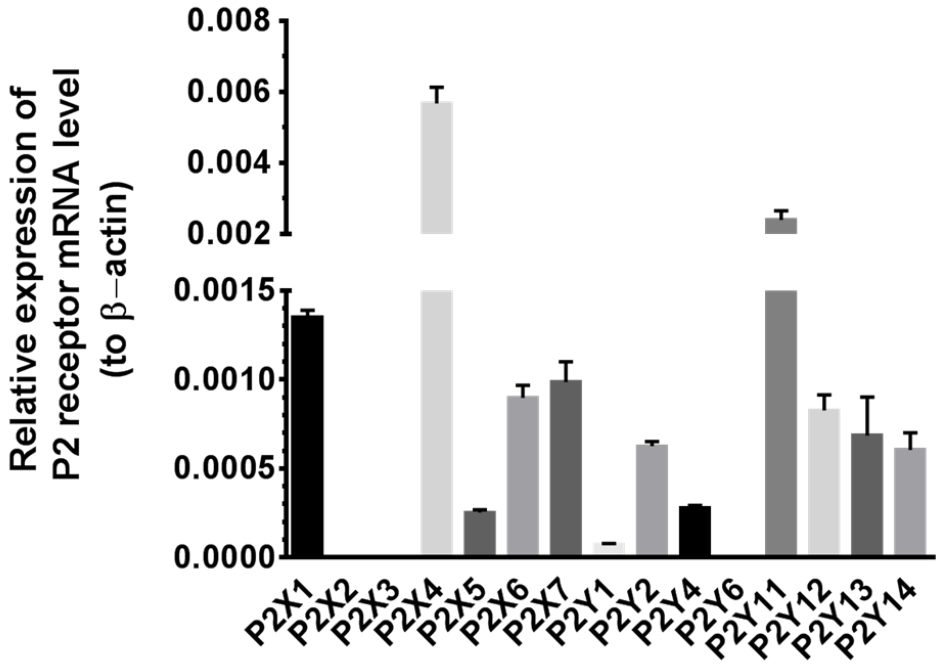

Fig 3.9 HEL cells expresses multiple P2 receptor subtypes.

P2 receptor expression in HEL cell pure population analysed with quantitative PCR. The order of P2 receptor expression is (P2X4>P2Y11>>P2X1> P2X7>P2X6>P2Y12>P2Y13> P2Y2>P2Y1) (n=3).

Interestingly, when the concentration of NF449 was increased up to 10 µM, a marked inhibition of the thrombin-evoked Ca^{2+} response of HEL cells in the mixed cell suspensions was observed (see Fig 3.11 A). The values of 2 min Ca^{2+} integral for HEL cells in the presence of 10 µM NF449 were 8449 ± 1836 nM.sec while the values without 10 µM NF449 were 27630 ± 3376 nM.sec ($P<0.05$; n=3) (See Fig 3.11 B). To further assess whether this high concentration was affecting platelets as well as HEL cells, platelets alone were treated with NF449 (10 µM) and the thrombin response assessed. An almost complete inhibition of the platelet response to thrombin was also observed with the higher concentration of this blocker (See Fig 3.11 C). This indicates that 10 µM NF449 is blocking the thrombin evoked Ca^{2+} signalling in platelets in addition to the HEL cells in the mixed cell suspensions. This action is likely a non-selective effect on either the thrombin receptors or the downstream Ca^{2+} signalling pathway.

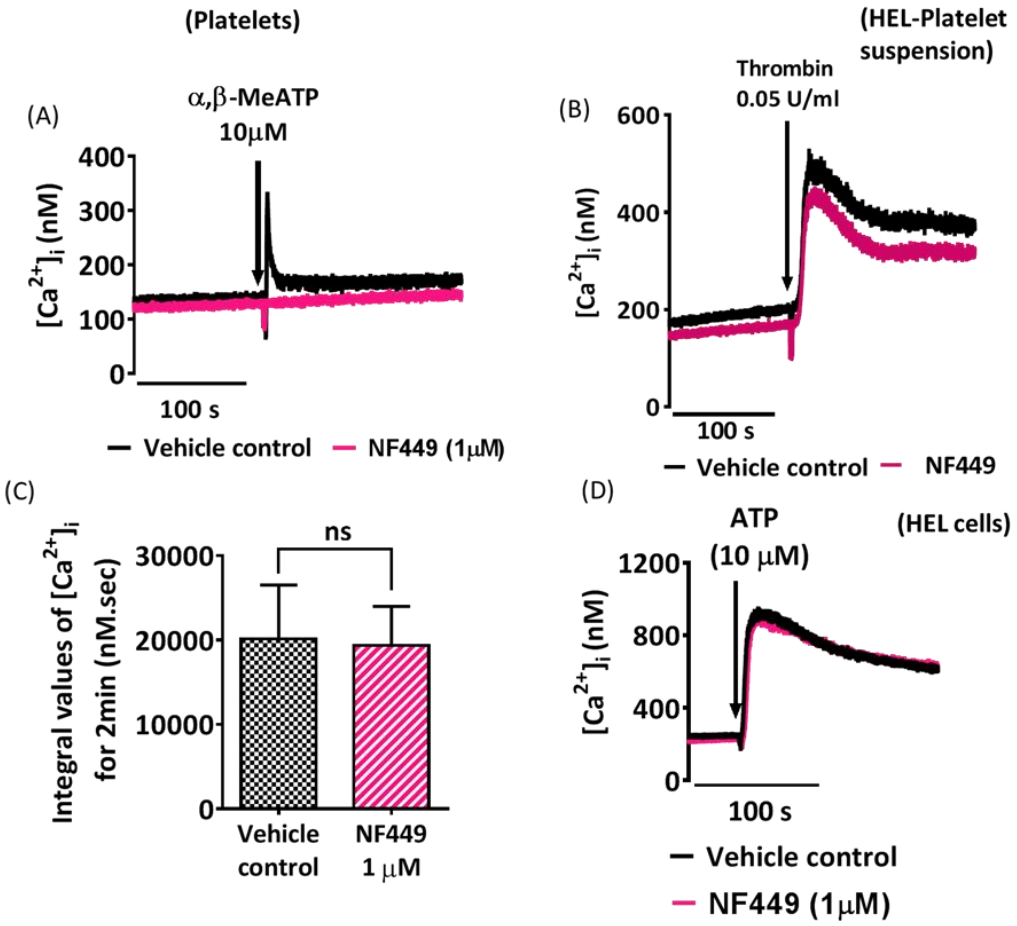

Fig 3.10 NF449 (1 μM) did not affect the HEL thrombin response in the presence of platelets

(A) Effect of NF449 (1 μM, 5 min) (pink) or vehicle control (black) on the response of a pure population of platelets to α,β-MeATP (10 μM). (B, C) Effect of NF449 (1 μM, 5 min) (pink) or vehicle control (black) on the thrombin-evoked HEL cell Ca^{2+} responses in the presence of platelets. Sample recordings in B and average 2 min integrals in C. No significant effect was observed (P>0.05; n=4; Paired t- test analysis). Data represent mean ± SEM. (D) HEL cell pure population treated with NF449 (1 μM) (pink trace) or vehicle control (black trace) prior to ATP (10 μM) addition. No reduction in ATP response observed with NF449 (1 μM).

3.2.7 NF157 inhibited the thrombin-evoked HEL cell Ca^{2+} response in the presence of platelets

Another highly expressed P2 receptor in HEL cells is P2Y11 which is reported to be selectively inhibited by NF157 (Talasila et al., 2009). NF157 caused a large reduction in the thrombin-evoked Ca^{2+} response of HEL cells in the presence of platelets (Fig. 3.12 A and B). The average 2 min Ca^{2+} integral value was reduced from 6938 ± 1520 to 3145 ± 947 nM.sec ($P<0.05$; n=4) in the presence of platelets (See Fig 3.12 A and B). The active agonists of P2Y11 are ATP>ADP (Lazarowski, 2003), which are released in similar concentrations from human platelet dense granules. Thus, it is likely that secreted ATP (and ADP) contribute to the platelet-dependent HEL Ca^{2+} response via P2Y11 receptors.

To further investigate the mechanism involved, Ca^{2+} responses to ATP and ADP were assessed in a pure HEL cell population in the presence and absence of NF157. A significant reduction in the ATP-evoked response was observed with NF157. The average 1-minute integral values of the response to ATP in the presence of NF157 was 2368 ± 243nM.sec which was significantly smaller than in the control (3625 ± 398 nM.sec, $P<0.01$; n=5) (See Fig 3.13 B). Likewise, the ADP-evoked Ca^{2+} response in HEL cells was also reduced by NF157 (see Fig. 3.13 C). The 1-minute integral value by ADP with NF157 was 989 ± 266 nM.sec and without NF157 was 3524 ± 860 nM.sec ($P<0.01$; n=5) (See Fig 3.13 D). In contrast, NF157 did not cause a significant reduction in the thrombin evoked Ca^{2+} response in a pure population of HEL cells (see Fig. 3.13 E). The average 1 minute integral values were 887 ± 93 nM.sec with thrombin in the presence of NF157, compared to 1014 ± 105 nM.sec in the absence of NF157 ($P>0.05$; n=5; Fig 3.13F). These results suggest that stimulation of platelets with thrombin releases ATP and ADP, which then act as agonist on HEL cells to potentiate the response in HEL cells when co-incubated with platelets. The use of NF157 is initial evidence that P2Y11 receptors on HEL cells are at least partially involved as platelets do not express this receptor.

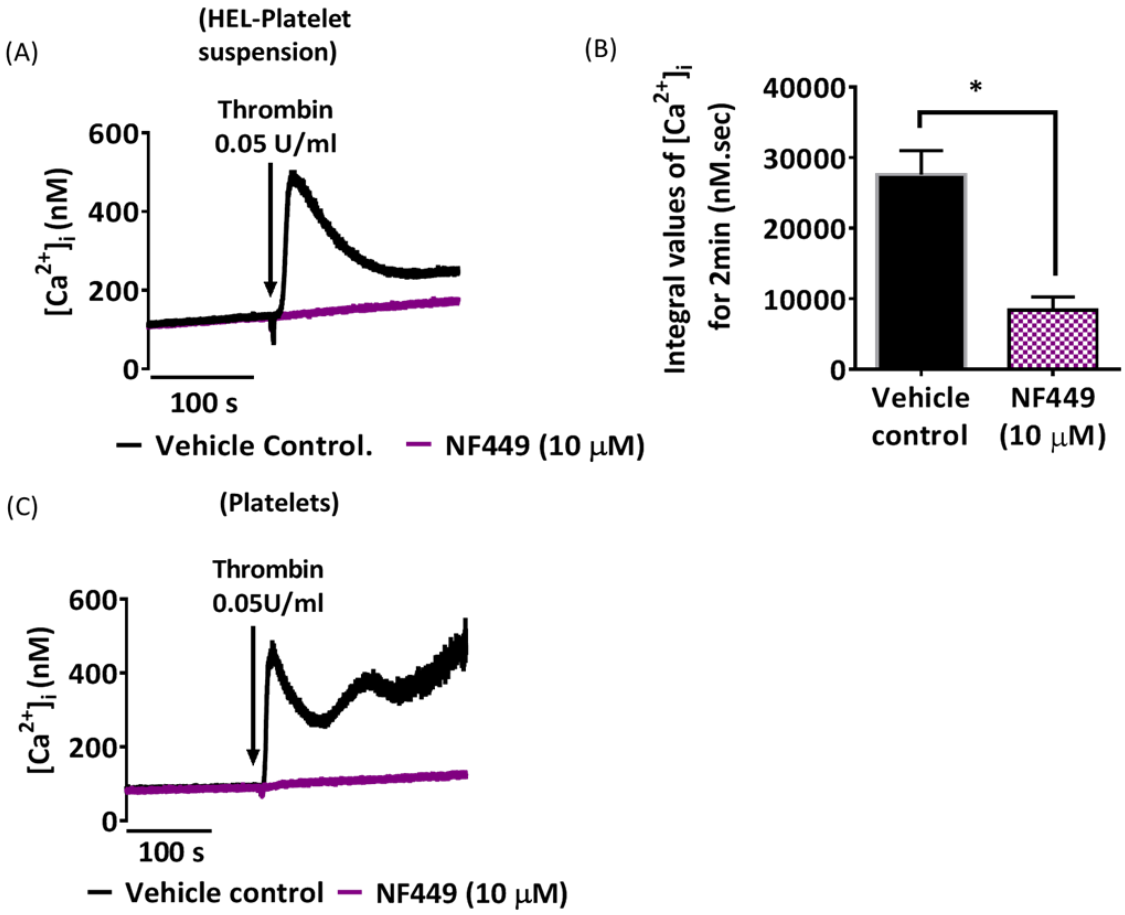

Fig 3.11 NF449 (10 μM) inhibited the thrombin-evoked Ca^{2+} response of HEL cells in the presence of platelets and a pure population of platelets

*(A, B) Thrombin-evoked Ca^{2+} responses of HEL cells in the presence of platelets after exposure to NF449 (10 μM) (purple) or vehicle control (black) for 5 minutes. Sample recordings in A and average 2 minute integrals in B. *P<0.05; n=3 with Paired t- test. Data represent mean ± SEM. (C) Effect of NF449 (10 μM) (purple) or vehicle control (black) on the thrombin response in a pure population of platelets.*

However, NF157 also significantly inhibited the thrombin-evoked Ca^{2+} response in a pure population of platelets. NF157 caused a reduction in the 1-minute integral values of the Ca^{2+} response to thrombin from 11439 ± 2232 in the control to 5389 ± 1835 nM.sec ($P<0.05$; n=4) with NF157 (See Fig 3.14 A and B). Thus, in mixed cell suspensions, the blocking action of NF157 on the HEL cell response was at least in part due to the its action at the level of the platelet. Platelets do not possess P2Y11 receptors thus this effect would appear to be due to a non-selective action on platelet thrombin receptors or their downstream Ca^{2+} signalling pathway, as observed for a high concentration of NF449.

3.2.8 Thrombin-activated platelets release ATP that can account for the potentiated HEL response via P2 receptors

As discussed above, release of ATP and ADP from thrombin-stimulated platelets can activate P2Y receptors in HEL cells. This paracrine signalling mechanism can explain at least in part the potentiation of thrombin-evoked Ca^{2+} responses in the HEL cells in the presence of platelets. To further explore this hypothesis, experiments measured extracellular ATP using a luciferin-luciferase assay. In HEL-platelet suspensions thrombin released ATP (see Fig 3.15 A) with an average maximum peak increase of 1.46 ± 0.2 µM (see Fig 3.15 A and B). However when HEL cells were stimulated with thrombin in the absence of platelets, no ATP release was detected (0.25 ± 0.05 µM) (see Fig 3.15 A and B) ($P < 0.05$). When a pure population of platelets was stimulated with thrombin a peak increase of 2.14 ± 0.5 nM ATP was observed which was not significantly different from the ATP increase in HEL-platelet suspensions (1.46 ± 0.2 nM; $P>0.05$; n=3; see Fig 3.16 B). Thus, HEL cells do not release ATP following thrombin and the increase in this nucleotide in the mixed cell suspension is entirely due to platelet secretion.

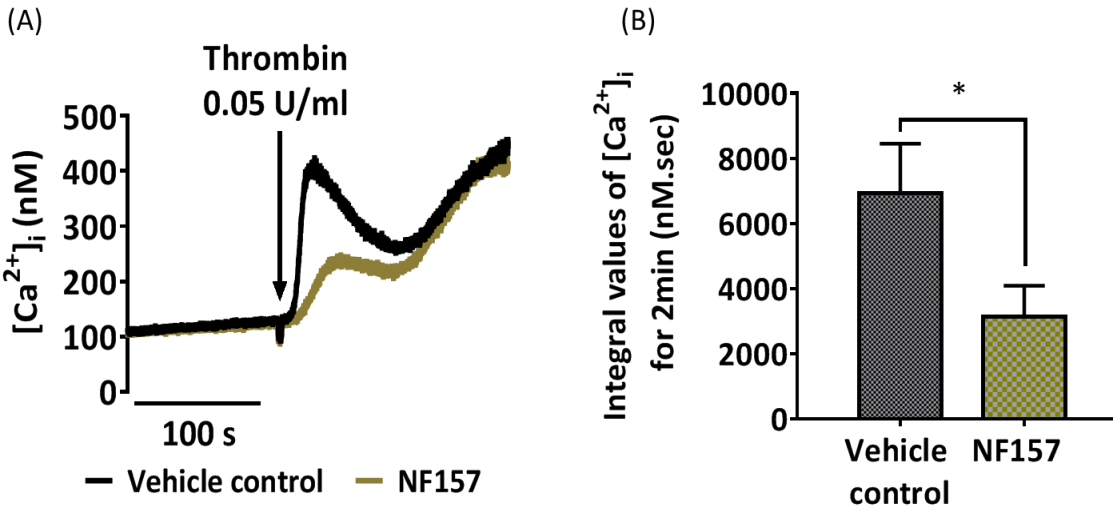

Fig 3.12 NF157 significantly reduced the HEL cell Ca^{2+} response to thrombin in the presence of platelets

*HEL-platelet suspensions were incubated with NF157 (10 µM) (brown) or vehicle control (black) for 5 minutes followed by thrombin addition. Sample traces are shown in A and the average 2-minute integrals for the thrombin response shown in B. A decrease in thrombin response was observed with NF157 (*P<0.05; n=4 with Paired t- test). Data represent mean ± SEM.*

The thrombin-evoked ATP release was also analysed in the presence of apyrase. The average ATP increase in a thrombin-stimulated HEL-platelet suspension was 2.27 ± 0.30 µM that was virtually abolished in the presence of 3.2 U/ml apyrase (0.17 ± 0.02 µM; P=0.01; n=3; see Fig 3.16 A and B). A similar effect was also observed on the ATP increase following stimulation of a pure population of platelets; without apyrase thrombin caused an increase of 1.80 ± 0.2 µM that was reduced with apyrase to 0.14 ± 0.03 µM (P<0.01; n=3; see Fig 3.16 C and D).

Interestingly, extracellular ATP was detected in HEL cells, since a signal was detected in a pure population of the cell line following addition of luciferin-luciferase (Fig. 3.16 E).

This ATP detection was reduced from 0.36 ± 0.04 to a very low level, 0.09 ± 0 nM ($P<0.05$; n=3) when HEL cells were pre-treated with apyrase (see Fig 3.16 E and F). Thus, HEL cells appear to constitutively release ATP, although do not secrete this nucleotide following thrombin stimulation.

3.2.9 NF157 and probenecid reduced the ATP release from thrombin-activated platelets

In previous experiments NF157 significantly reduced the platelet induced potentiation in HEL cells. Therefore, the ATP assay was carried out in the presence of NF157 during stimulation of a HEL-platelet suspension with thrombin. The average ATP release was significantly reduced by NF157 from 1.70 ± 0.17 µM under control conditions to 0.95 ± 0.18 µM with NF157 ($P<0.02$; n=3) (see Fig 3.17 A and B).

Several pieces of evidence state that ATP is released from platelets via pannexin channels in the cell membrane (Dahl, 2015) in addition to exocytosis. This includes thrombin-activated platelets (Taylor et al., 2014b). Therefore, the effect of the pannexin channel blocker probenecid (Silverman et al., 2008) was tested on ATP release from HEL-platelet suspensions following thrombin stimulation. Probenecid significantly reduced the ATP release from HEL-platelet suspension from 1.21 ± 0.23 to 0.4 ± 0.13 µM ($P=0.03$; n=3) (see Fig 3.18 A and B).

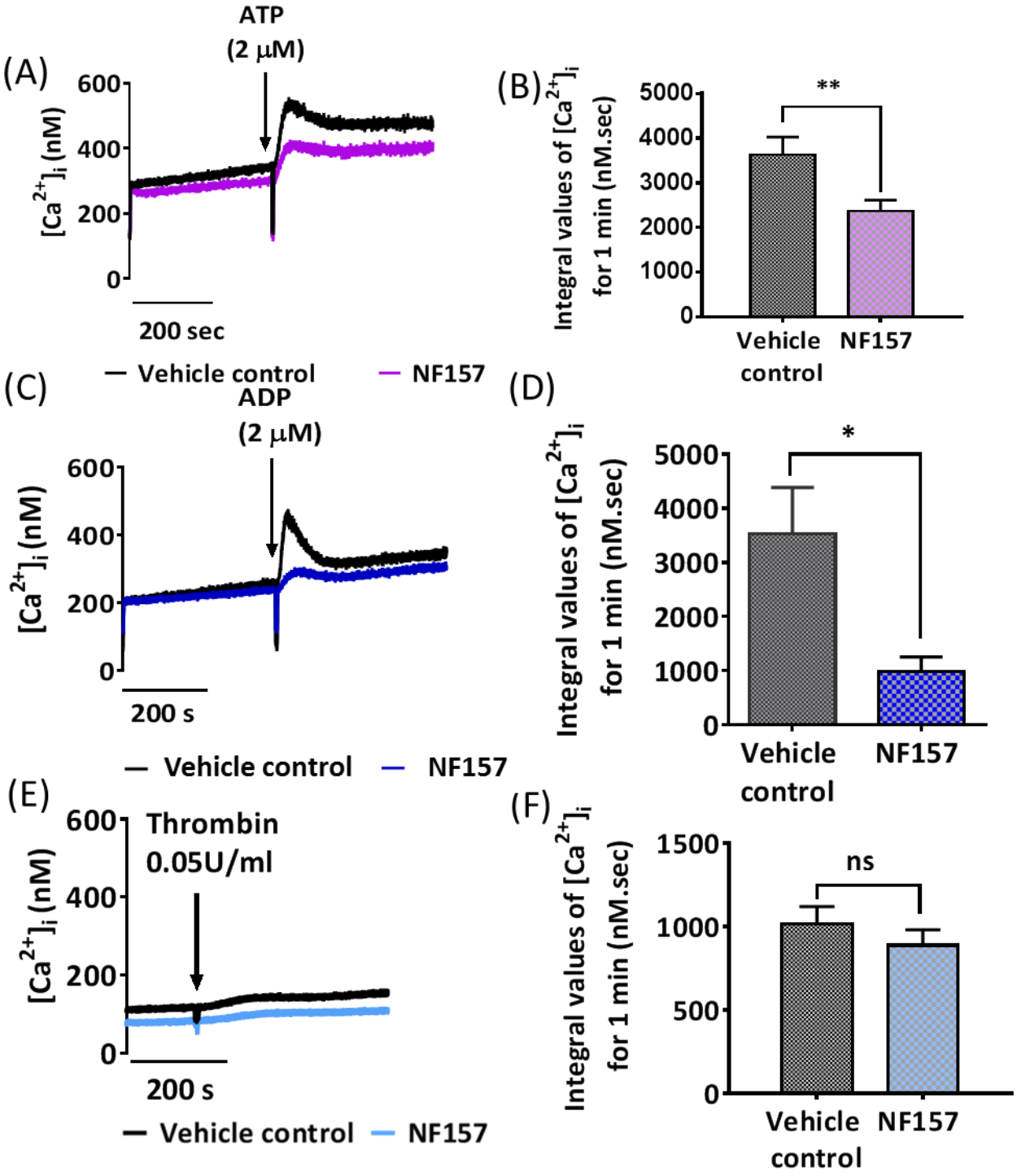

Fig 3.13 NF157 inhibited the Ca^{2+} response to ATP and ADP but not thrombin in a pure population of HEL cells

*HEL cells were loaded with fura-2 and Ca^{2+} responses to 2 μM ADP (A, B), 2 μM ATP (C, D) or 0.05 U/ml thrombin (E, F) in the presence of 10 μM NF157 (purple, deep blue or light blue) or vehicle control (black). ADP and ATP responses were significantly reduced (*P<0.05; n=5) while the response to thrombin was not significantly affected. Paired t-tests. All data represent mean ± SEM.*

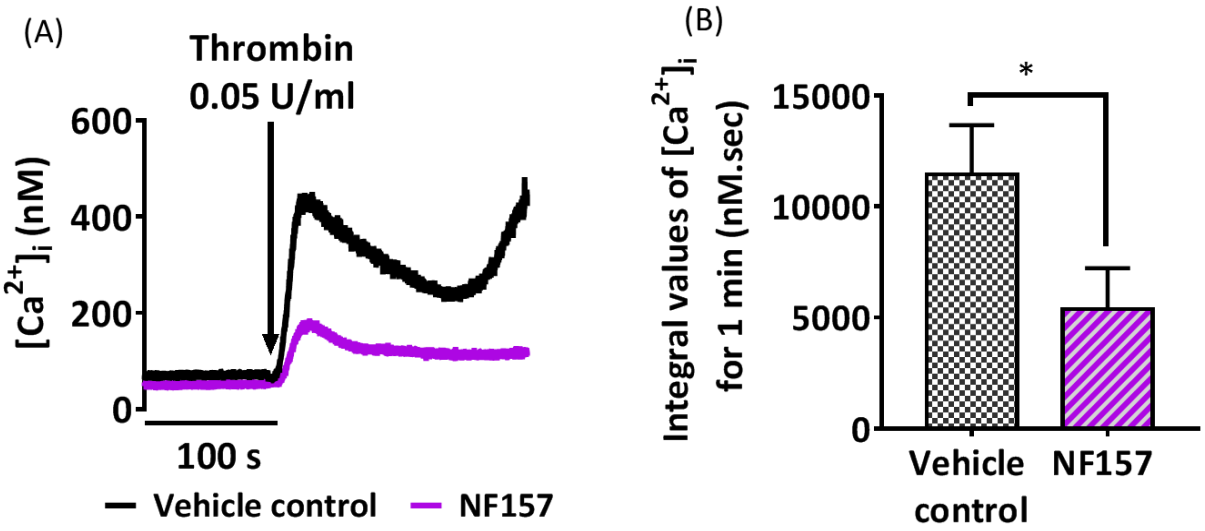

Fig 3.14 NF157 reduced thrombin evoked response in platelets

*(A) Platelets incubated with NF157 (purple traces) or with vehicle control (black traces) followed by thrombin addition. (B) A significance reduction in thrombin evoked Ca^{2+} response in platelets with NF157 (purple bar) as compared to vehicle treated (black bar) platelets (*P<0.02; n=4, Paired-t-test). Data represent mean ± SEM.*

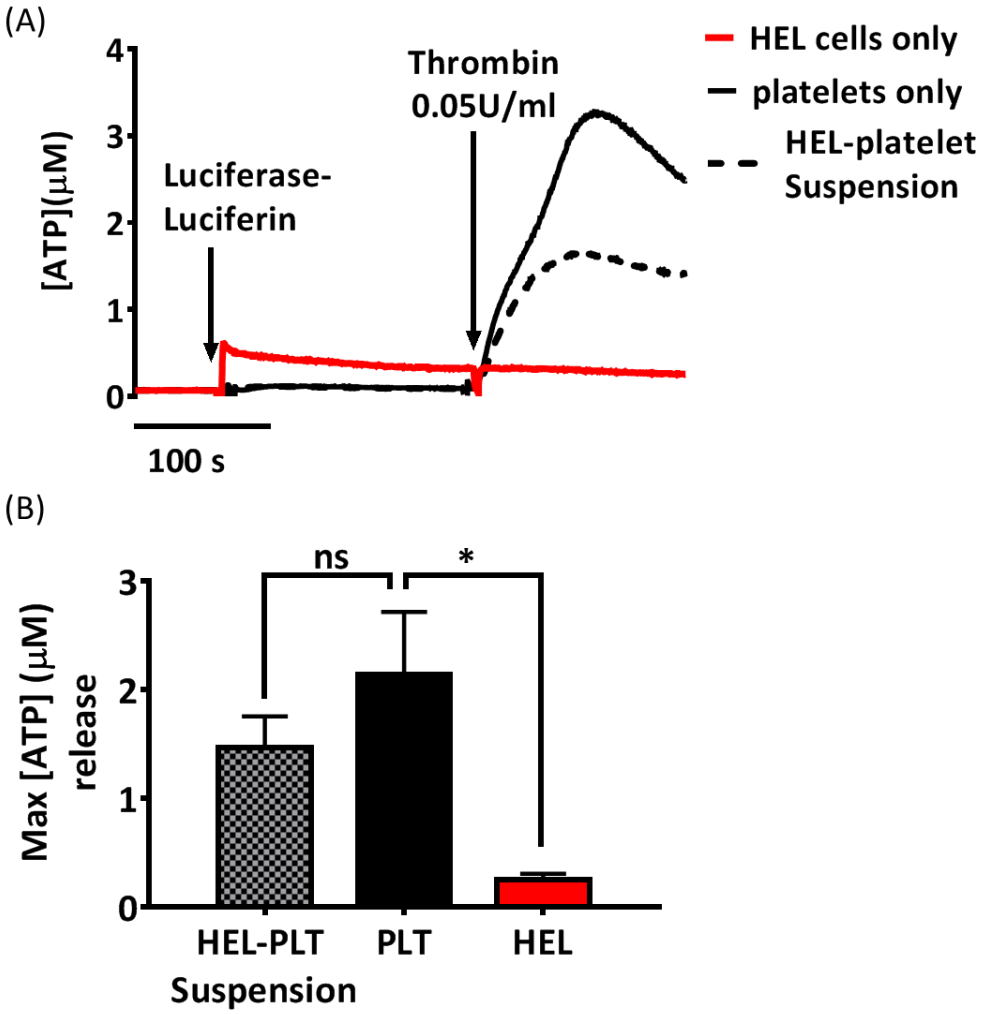

Fig 3.15 Thrombin stimulates ATP release from platelets but not HEL cells, and ATP is constitutively released from HEL cells.

(A) Sample traces and average peak increases for the extracellular ATP level of pure populations of platelets or HEL cells and both cell types combined. (B) Peak ATP levels for platelets alone and the HEL-platelet suspensions are following thrombin stimulation, whereas for HEL cells alone the peak is that detected following addition of the luciferin:luciferase mixture. (, P<0.05; ns, not significant; n=3; Paired t-test). Data represent mean ± SEM.*

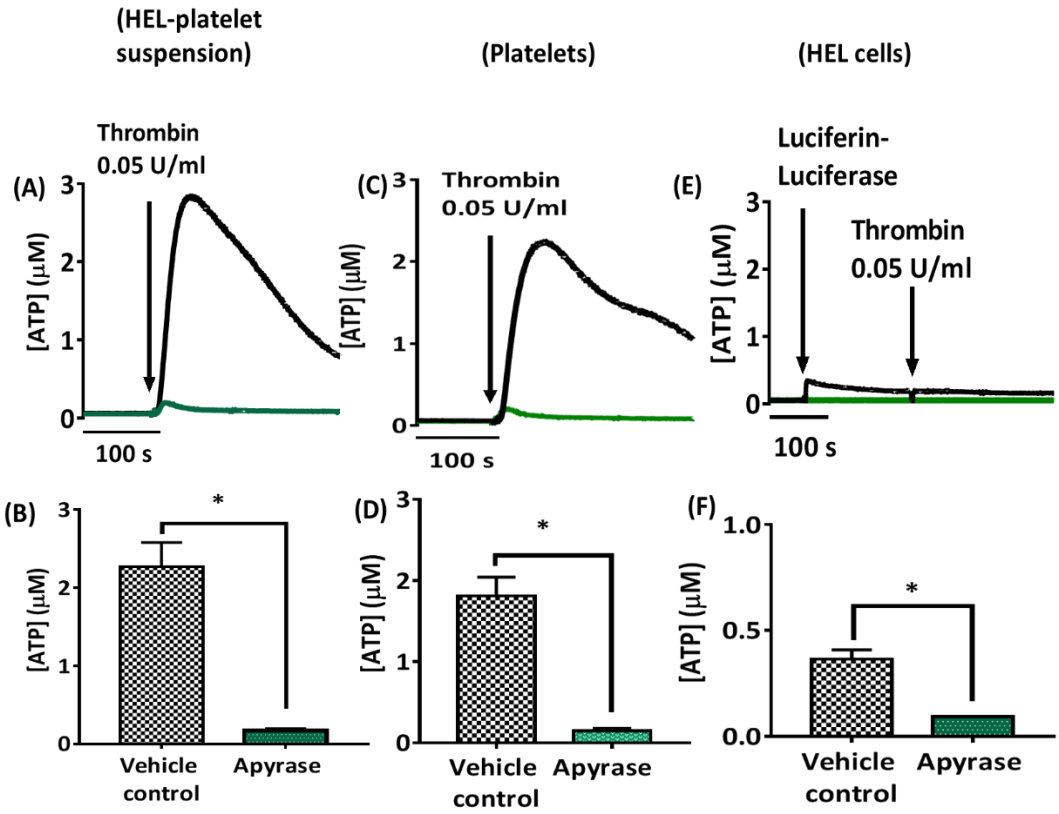

Fig 3.16 Apyrase reduced the ATP levels detected by extracellular luciferin: luciferase following thrombin-evoked secretion from platelets released constitutively from HEL cells

Sample recordings of firefly assay luminescence (A,C,E) and peak ATP increases (B,D,F) in HEL-platelet suspension (A,B), or a pure population of platelets (C,D) or HEL cells (E,F). Black traces and bars are under control conditions and green in the presence apyrase (3.2 U/ml). The peak increases in B, D, F are following thrombin for platelets and platelet:HEL cell suspensions and following addition of luciferin:luciferase for HEL cells alone. (, P<0.01; n=3; Paired t-tests). Data represent mean ± SEM.*

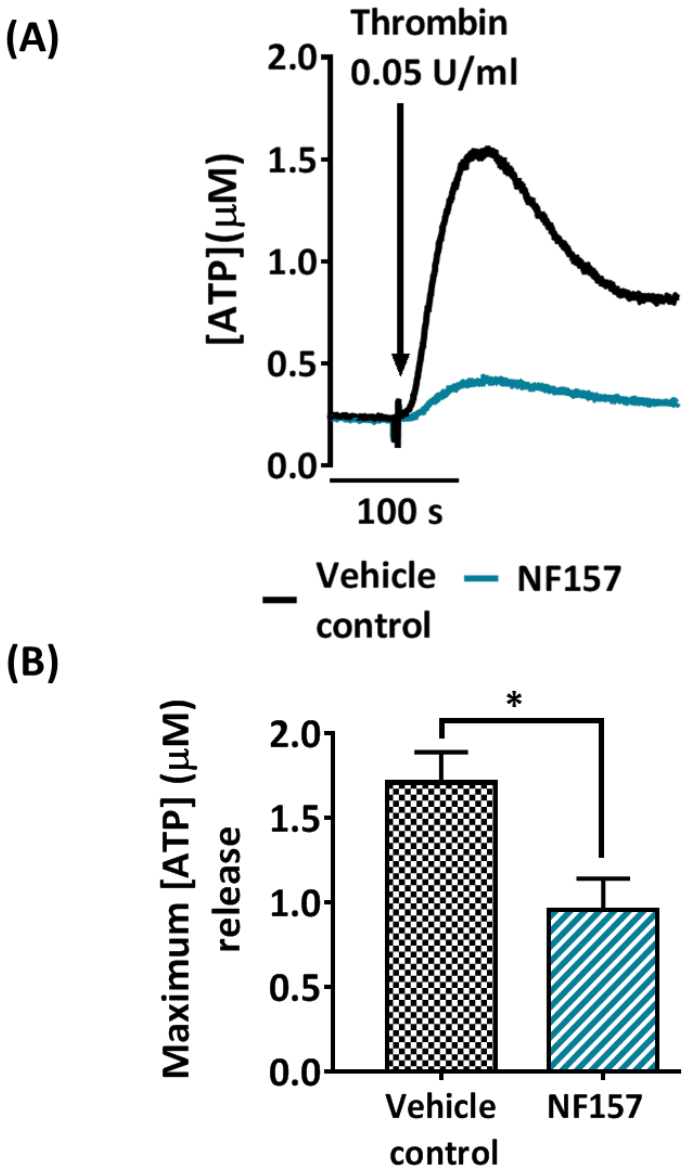

Fig 3.17 NF157 reduces the extracellular ATP level following thrombin stimulation of a HEL-platelet suspension

*HEL-platelet suspensions were incubated with NF157 (blue) or vehicle control (black) followed by thrombin addition in the presence of luciferin-luciferase. (B) NF157 reduced the ATP level detected following stimulation of a HEL-platelet suspension with thrombin (*P<0.05; n=3, Paired t-test). Data show the mean ± SEM.*

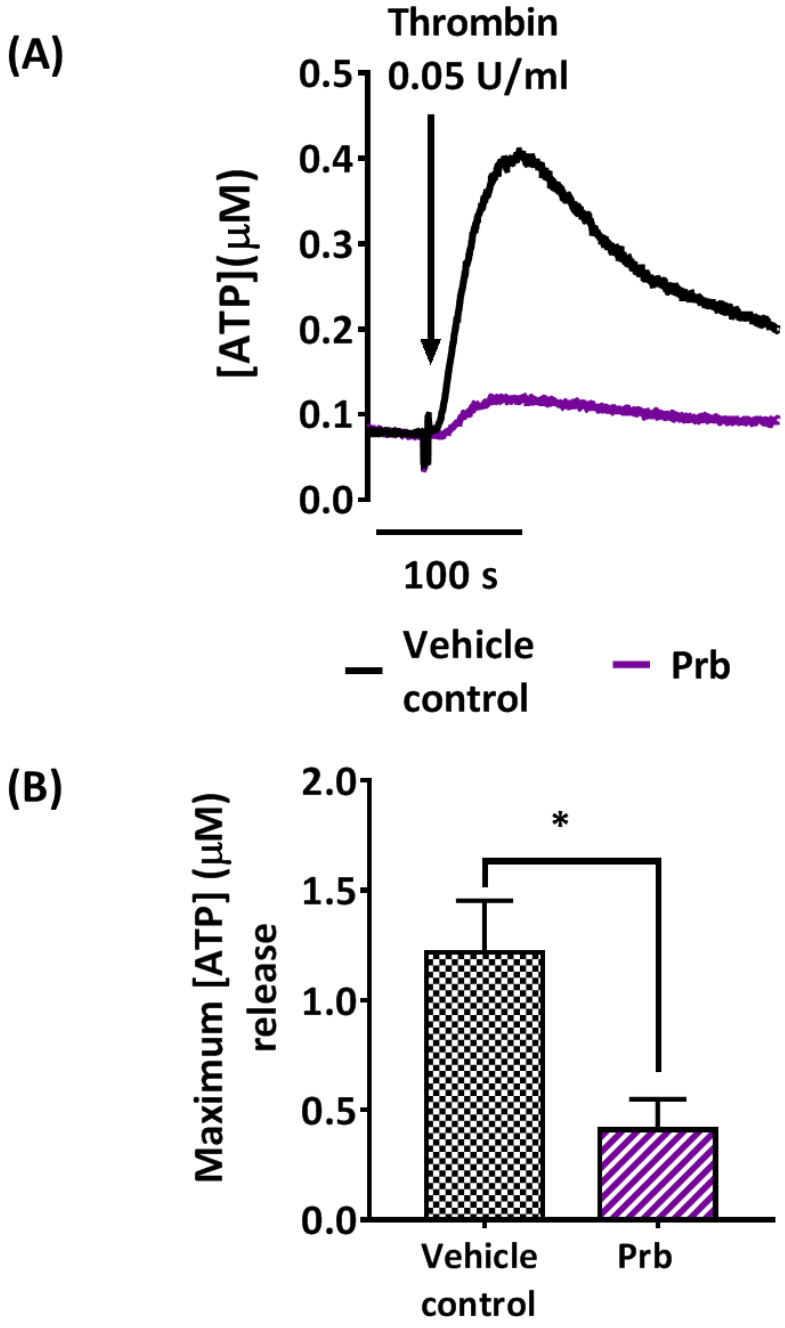

Fig 3.18 Probenecid (Prb) significantly reduced the thrombin-evoked ATP release from HEL-platelet suspensions

*HEL-platelet suspensions were incubated with probenecid (100 μM) (purple) or vehicle control (black) followed by thrombin addition. Luciferin-luciferase was present throughout. Representative recordings are shown in (A) and average peak ATP increases in B. (*P<0.05; n=3, Paired t-test). Data show the mean ± SEM.*

3.3 Discussion

This chapter mainly focuses on in vitro analysis of platelet interactions with HEL cells following stimulation by thrombin. The effect of platelets on HEL and vice versa was assessed at the level of intracellular Ca^{2+}, an important second messenger in virtually all cells. Both cell types independently responded to thrombin with a Ca^{2+} increase. Platelets induced a potentiation of the thrombin-evoked Ca^{2+} response of HEL cells, however no significant effect of HEL cells was observed on the response to thrombin of platelets. This effect is consistent with various previous findings that platelets have a stimulating effect on tumour cell activity (Borsig, 2008). Thrombin stimulated platelets release ATP, ADP and TxA_2 (Grenegård et al., 2008, Woulfe et al., 2001, Kahn et al., 1998) which are candidates for the potentiating effect since these agents are all known to be agonists of HEL cells (Chahwala and Cantley, 1984, Kalambakas et al., 1993, Mayeux et al., 1989a). The secretion is due to both release from dense granules and through pannexin-1 channels (Taylor et al., 2014a) (Fig. 3.18). However, aspirin did not reduce the thrombin-evoked response of HEL cells in the mixed suspensions, suggesting that TxA_2 release does not play a major role in the platelet-dependent HEL cell response. A previous study demonstrated that aspirin did not inhibit the α-granule secretion and P-selectin expression in platelets when treated with thrombin (Ilton et al., 1998), suggesting that thrombin evoked secretion does not depend on TxA_2 synthesis.

Consistent with an action of platelet-derived ATP and ADP in the amplification of HEL cells during thrombin stimulation, HEL cells alone showed Ca^{2+} responses to both these nucleotides. To further assess the role of ATP and ADP in the potentiated HEL release in mixed cell suspensions, apyrase type VII was used, which hydrolyses ATP to ADP and ADP to AMP (Chen and Guidotti, 2001, Ren et al., 2004). It was expected that a significant alteration in platelet-induced potentiation would be observed with apyrase due to the hydrolysing property of apyrase. Surprisingly apyrase did not significantly

reduce the potentiation at 0.32 U/ml concentration. However the pattern of Ca^{2+} signalling observed to be changed from a single peak to biphasic response with apyrase. Furthermore, this effect was pronounced at a high concentration of apyrase (3.2 U/ml) compared to at 0.32 U/ml. This could be explained by conversion of the released ATP to ADP by apyrase that then acts as an agonist of P2Y receptors on HEL cells. This idea is consistent with the ability of ADP to stimulate a Ca^{2+} increase directly in HEL cells and previous reports that ADP and ATP acts as agonists in HEL cells (Shi et al., 1995). Thus, the overall response of HEL cells to thrombin in the presence of platelets may not be reduced by apyrase because of the generation of ADP and enhanced P2Y-evoked Ca^{2+} increase. The response of HEL cells to thrombin in the presence of platelets was increased with 3.2 U/ml apyrase but the overall difference was not significant. One of the possibilities could be the conversion of released ATP into ADP that evoked the secondary response in HEL cells. Moreover, as it is well documented that tumour cells activate platelets (Lou et al., 2015), there could be a secondary response in HEL cells as a result of HEL cell induced platelet activation. Therefore, the released ATP from HEL-activated platelets may be converted to ADP by the apyrase and cause a secondary response in HEL cells. A further observation that supports this conclusion was the surprising Ca^{2+} increase of apyrase alone in HEL-platelet suspension which was also seen upon addition of apyrase to a pure population of HEL cells. Although the exact mechanism of this response was not investigated further, ATP was detected in HEL cell suspensions with the luciferin:luciferase assay and thus apyrase could be again converting this to ADP that acts on P2Y receptors on HEL cells. This could be due to the basal release of ATP from HEL cells as seen in different cancer cells including colon cancer and prostate cancer (Corriden and Insel, 2010) and acute leukemia T cells (Corriden and Insel, 2010). Indeed, the ADP-evoked response of HEL cells was partially blocked by MRS 2179. Thus, overall, there is some evidence that the platelet-dependent HEL cell thrombin response is due to release of ADP and ATP from platelets and P2 receptors in HEL cells.

Regarding the thrombin-evoked Ca^{2+} signalling pathway in HEL cells in the presence of platelets, pharmacological tools were used to assess the underlying mechanism. PGE1 is known to block platelet Ca^{2+} responses through generation of cyclic AMP, activation of protein kinase A and inhibition of IP_3 receptors (Tertyshnikova and Fein, 1998, Quinton and Dean, 1992b, Schwarz et al., 2001b). However at a concentration which abolished ADP-evoked increases in platelets, PGE1 caused slight inhibition of the thrombin-evoked HEL Ca^{2+} response in the presence of platelets but the overall effect was not significant. Furthermore, the action of PGE1 was complicated by the surprising ability of this agent to generate a rise in Ca^{2+} signal in HEL cells. This effect has been reported previously and suggested to be due to a Gi-coupled prostanoid receptor that links to Ca^{2+} release in HEL cells possibly via a βγ-induced activation of PLC (Feoktistov et al., 1997). The role of PLC was further examined, especially since thrombin is known to act through PLC activation pathway to induce platelet secretion (Murugappan et al., 2005). Surprisingly the widely used PLC inhibitor, U73122, which inhibits agonist evoked Ca^{2+}signaling in different cells including platelets (Macmillan and McCarron, 2010) did not alter the HEL cell thrombin-evoked Ca^{2+} response in the presence of platelets. At present, the lack of action of U73122 on the HEL cell response is unclear, however it could further reflect the unusual mechanism of activation of Ca^{2+} release via GPCRs in HEL cells, as shown by the unexpected effect of PGE1 via proposed Gi-coupled receptors. Alternatively, since thrombin is a very potent agonist of platelets and the positive control used to test this compound was the weak agonist ADP, the U73122 may not have been effective as an inhibitor at the level of thrombin-evoked platelet secretion.

The most highly expressed P2 receptors on HEL cells, as determined by a qPCR screen, were P2X1, P2X4 and P2Y11 which therefore represent candidates for the ATP or ADP-stimulated Ca^{2+} increases. P2X receptors are Ca^{2+}-permeable ion channels and thus will stimulate influx, whereas P2Y receptors are GPCRs and thus can cause release of Ca^{2+} from the internal stores as well as influx through second messenger or store-operated Ca^{2+} channels. P2X1, and probably all P2X subtypes, are activated by ATP but not ADP (Mahaut-Smith et al., 2000). In contrast, P2Y11 receptors are activated by ATP and ADP

and can be coupled to Ca^{2+} mobilisation (Jacobson et al., 2009, Kennedy and Leff, 1995). The overall profile of the HEL P2 receptor expression at the level of the mRNA transcripts was P2X4>P2Y11>>P2X1> P2X7>P2X6>P2Y12>P2Y13>P2Y2>P2Y1. The existence of P2Y1, P2Y2, P2Y4 and P2Y6 was also reported in a previous research carried out in megakaryocytic Dami cells (Jin et al., 1998b). These Dami cells were found contaminated with HEL cells that later on appeared to be HEL cells (Jin and Kunapuli, 1998a). Likewise in another study of HEL cells, expression of P2Y3, P2Y4, P2Y2, P2Y1 and P2Y7 receptors was also reported (Akbar et al., 1996). Similarly, expression of P2Y11 receptors has also been reported in human acute promyelocytic leukemia (APL) HL-60 cell line which shares common features with HEL cells (Van Der Weyden et al., 2000a). Furthermore presence of P2Y8, P2Y9, P2Y11, P2Y6, P2X5, P2X1 and P2X7 was also reported in HL-60 cell line which is another myeloid cell line that possibly have similar features as HEL cells (Adrian et al., 2000). One further study has reported the expression of P2Y7 in HEL cells (Robaye et al., 1997). However no specific evidence about expression of P2Y11, P2X4 and P2X1 in HEL cells has been reported prior to the present work.

The role of different HEL P2 receptors was assessed using pharmacological tools. NF449 at a concentration known to be relatively selective for P2X1 (Fung et al., 2007a, Hechler et al., 2005, Horner et al., 2005, Mahaut-Smith et al., 2011) of 1 µM did not affect the potentiation induced by platelets. Moreover 1 µM NF449 showed no inhibition of the ATP-evoked Ca^{2+} response in a pure population of HEL cells. Thus, HEL cell ATP-evoked Ca^{2+} responses do not involve P2X1 receptors. At a higher concentration (10 µM), NF449 totally abolished the HEL thrombin response in the mixed cell suspension, however this was a non-specific effect as it also abolished the platelet Ca^{2+} response to thrombin. This can be explained on the basis of the previous finding stating the non-selective inhibitory action of NF449 and other suramin derivatives on Gα proteins (Hohenegger et al., 1998). It was not possible to explore the action of P2X4 receptors as the best available selective antagonist, 5-BDBD, was too autoflourescent. A reported selective antagonist of P2Y11 receptors, NF157 abolished the thrombin-induced Ca^{2+} response in HEL cells in the presence of platelets. Although

NF157 did directly reduce the ADP and ATP-evoked responses of HEL cells, it also completely blocked the thrombin-stimulated release of ATP and Ca^{2+} increase in platelets. Platelets do not express P2Y11 receptors (Wang et al., 2003) thus, this antagonist is not acting selectively on P2 receptors and it is not possible to conclude the basis for the involvement of P2Y11 receptors in the amplified thrombin-evoked HEL cell Ca^{2+} response by platelets. It is possible that NF157 is acting non-selectively on Gα proteins as reported for NF449 (Hohenegger et al., 1998). It is still possible that P2Y11 receptors on HEL cells are involved in the paracrine effect of platelets after thrombin stimulation, however better pharmacological tools or interference RNA approaches are required in future experiments.

Although P2Y1 receptors were only expressed at a low level in HEL cells, they are a candidate for the action of ADP during apyrase treatment since they are activated by ADP more potently than ATP (Léon et al., 1997). In contrast to P2Y11, a very selective antagonist of P2Y1 has been reported, MRS2179 (Boyer et al., 1998). MRS2179 did block the platelet Ca^{2+} response, which is known to be mediated mainly via P2Y1 (Hechler and Gachet, 2011a). This agent also significantly reduced the ADP-evoked Ca^{2+} response of HEL cells, confirming that P2Y1 receptors are functional on HEL cells. However, MRS2179 did not inhibit the thrombin-induced platelet dependent potentiation of Ca^{2+} signals in HEL cells suggesting that neither platelet nor HEL cell P2Y1 receptors contribute to this effect. A slower response to ADP in HEL cells remained, suggesting that other ADP-evoked P2Y receptors are present in HEL cells. The identity of this pathway is unclear, but could be P2Y11 (van der Weyden et al., 2000b, Erb and Weisman, 2012).

3.4 Conclusion:

In this chapter the findings suggest that ATP, along with ADP, are major agents released from thrombin-activated platelets in an environment where they are mixed with a cancer cell line. These released nucleotides can act via P2 receptors to stimulate Ca^{2+} increases in the HEL cancer cell line to amplify the Ca^{2+} response beyond the

direct effect of thrombin. Consequently, ATP and ADP release and P2 receptor stimulation represent a positive feedforward mechanism whereby platelets can modulate cancer cell growth and metastasis. Therefore, targeting tumour cell P2 receptors and platelet release of ATP and/or ADP may be a useful approach in developing anti-cancer therapy. An amplification of the thrombin-evoked Ca^{2+} responses of HEL cells was observed in the presence of platelets; however it was difficult to evaluate the relative contribution of ADP and ATP to this effect due to the presence of receptors for both nucleotides. A screen of HEL cell P2 receptors and pharmacological tools suggested several candidates for the ATP/ADP-activated pathway in HEL cells. However, due to the lack of availability of suitable selective antagonists, the exact nature of the P2 receptors involved in the HEL cell line Ca^{2+} response remains unclear.

Chapter 4: Platelet-HEL cell interactions following stimulation of Toll-like receptors

4.1 Introduction

In the previous chapter, the interaction between platelets and a myeloid leukemic cell line (HEL cells) was explored at the level of Ca^{2+} signalling following stimulation by thrombin. Since thrombin directly stimulated Ca^{2+} responses in both platelets and HEL cells, the mechanisms of the underlying intercellular interactions proved complex to study. An agonist that can activate platelets without directly stimulating the cancer cell line could allow greater insight into synergy between the two cell types.

An alternative to thrombin could be an immune receptor such as the Toll-like receptors (TLRs). Indeed, since the innate immune system plays a key role in the defence against cancer development and platelets are immune competent cells, immune receptors are relevant for the study of platelet-cancer cell interactions. A summary of TLRs and their roles in cancer progression is listed in table 4.1. TLRs are expressed on the surface of the myeloid lineage cells like monocytes, macrophages, dendritic cells (DCs), mast cells as well as other immune cells such as lymphocytes and platelets (Morrell et al., 2014, Pandey et al., 2015). To date, platelet immune receptors have not been reported to play a role in cancer metastasis, however they are involved in inflammatory mechanisms and contribute to the pathophysiological process of sepsis (Morrell et al., 2014, Li et al., 2011).

Most TLRs are present on the cell surface with an exception of TLR3, TLR7 and TLR9 that are present in endosomes (Chaturvedi and Pierce, 2009). They detect foreign pathogenic molecular patterns. The different TLRs display pathogen recognition

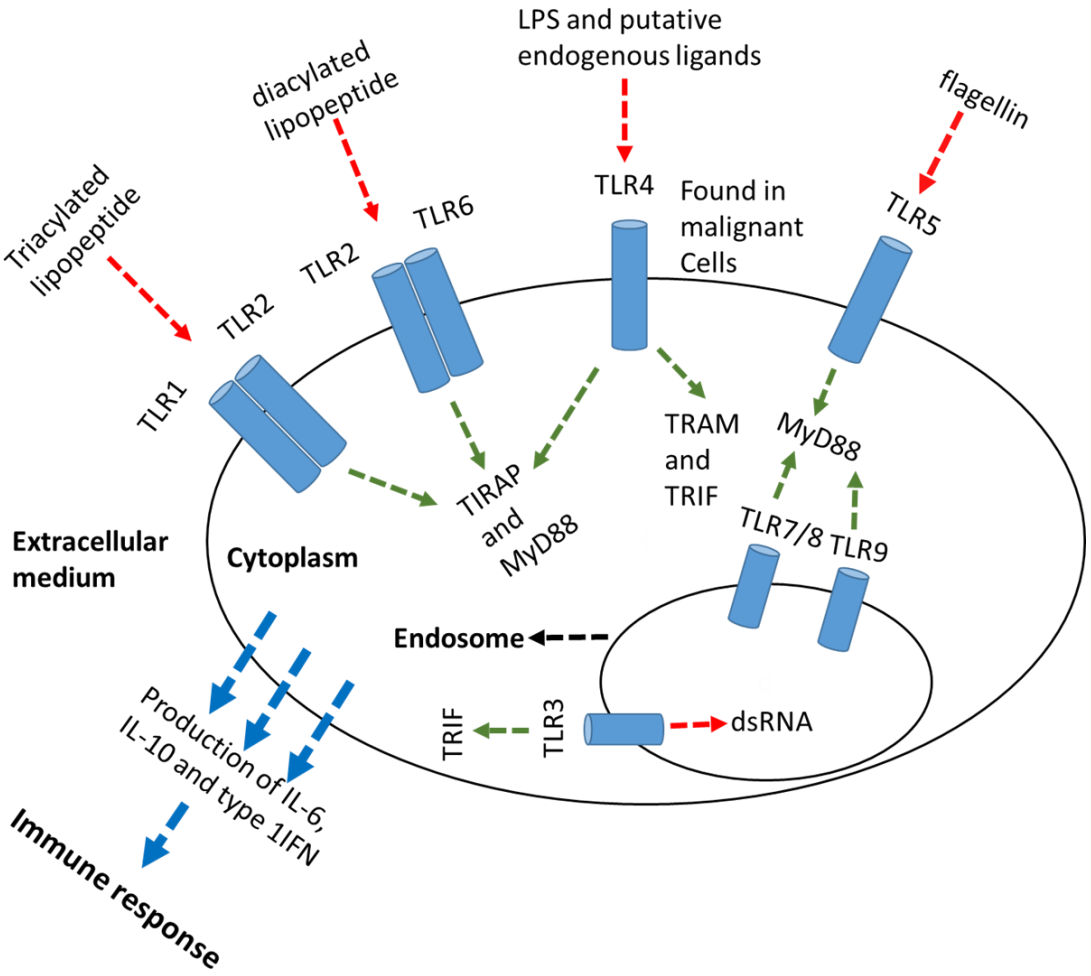

Fig 4.1 General mechanism of TLR signals involved in immune response:

TLR2 and TLR6 forms complexes that recognize diacylated lipopeptides while TLR2/6 complex identifies triaceylated lipopeptides. TLR4 binds with LPS from Gram negative bacteria, TLR5 recognizes bacterial flagellin, TLR7/8 bind with ssRNA from viruses and nucleoside analogs. TLR9 binds with CPG DNA from bacteria and viruses Ig-DNA complex and HMGB1. TLR3 recognizes virally derived dsRNA. TLR3 and 4 are expressed by malignant B cells. TLR3,7/8 and 9 are expressed in endosomes while the rest are expressed in cell membranes. The main signaling adaptors involved in TLR are MyD88, TRIF, TRAM and TIRAP. The resulting agents produced are IL-6, IL-10 and type 1 IFN.

specificity; for example TLR1/TLR2 complex binds to triacyl lipopeptides found in pathogenic bacteria (Mogensen, 2009). Similarly the TLR2/TLR6 complex present on the cell surface recognizes diacyl lipopeptides and lipoteichoic acid in Mycoplasma and Gram-positive bacteria (Mogensen, 2009, Rivadeneyra et al., 2014b) (see details in Fig. 4.1). Platelets express various types of TLRs but the most extensively studied are TLR2 and TLR4. These receptors potentially recognise pathogen associated molecular patterns in Gram-positive and Gram-negative bacteria, respectively thus platelets have a significant role in inflammation and sepsis (Aslam et al., 2006, Li et al., 2011, Kawai and Akira, 2010). TLR2 forms complexes with TLR1 or TLR6 that are crucial in recognising various pathogenic components such as lipoproteins and peptidoglycans within the microbial cell wall (Beaulieu and Freedman, 2010). It has been reported that the TLR1/2 agonist Pam_3CSK_4 activates platelet haemostatic and inflammatory responses consequently leading to fibrinogen binding, aggregation and ATP release (Rivadeneyra et al., 2014b). The exact mechanism whereby Pam_3CSK_4 causes platelet functional responses is unknown. However, it is known to stimulate PLC dependent Ca^{2+} mobilisation. This stimulation is accompanied by dense granule secretion that includes release of ATP and ADP and also the formation of TxA_2. The released ATP acts through P2X1 while ADP stimulates P2Y1 and P2Y12, further causing platelet activation and aggregation (Rivadeneyra et al., 2014b, Fung et al., 2012a, Blair et al., 2009, Kälvegren et al., 2010).

No evidence has been reported to date for the presence of TLR1/2 receptors in HEL cells. Therefore, as an alternative to thrombin, experiments in the present chapter have examined the interplay between human platelets and HEL cells at the level of intracellular Ca^{2+} following stimulation of the TLR2/1 receptor complex by Pam_3CSK_4.

TLR Type	Host cell	Cancer	References
TLR1	Monocytes, macrophages, B-lymphocytes, platelets	Prostate cancer	(Stevens et al., 2008, Panja, 2013, Koupenova et al., 2015, D'Atri and Schattner, 2017)
TLR2	Monocytes, macrophages, platelets, mast cells	Breast cancer, hepatocarcinoma	(Singh et al., 2014, Panja, 2013, Koupenova et al., 2015, D'Atri and Schattner, 2017)
TLR3	Dendritic cells, B lymphocytes, platelets	Oesophageal squamous cell carcinoma	(Farooque et al., 2009, Camerer et al., 2004, Panja, 2013, Koupenova et al., 2015)
TLR4	Monocytes, macrophages, mast cells, Dendritic cells, intestinal epithelium, platelets	Oesophageal squamous cell carcinoma, colorectal, bladder, ovarian, prostate, cervical, pancreatic, hepatocellular carcinoma, lung cancer, breast cancer	(Stevens et al., 2008, Schreibelt et al., 2010, Farooque et al., 2009, Dapito et al., 2012, Panja, 2013, Koupenova et al., 2015)
TLR6	Monocytes, macrophages, platelets, mast cells, B lymphocytes	Prostate cancer	(Stevens et al., 2008, Panja, 2013, Koupenova et al., 2015)
TLR7	Monocytes, macrophages, B-lymphocytes, platelets	Pancreatic carcinogenesis, oesophageal squamous cell carcinoma	(Farooque et al., 2009, Panja, 2013, Koupenova et al., 2015)
TLR9 TLR 10	Monocytes, macrophages, B-lymphocytes, platelets	Lung cancer, oesophageal squamous cell carcinoma Prostate cancer, nasopharyngeal	(Stevens et al., 2008, Farooque et al., 2009, Panja, 2013, Koupenova et al., 2015, D'Atri and Schattner, 2017)

Table 4.1 List of TLRs and their reported contributions to different cancer type.

4.2 Results:

4.2.1 Stimulation of platelets with a Toll-like receptor1/2 agonist evokes Ca^{2+} responses in HEL cells

In the previous chapter, thrombin-evoked -Ca^{2+}responses in suspensions of HEL cells were clearly enhanced when this cell line was co-incubated with platelets. Nevertheless, the underlying mechanism of interaction proved complicated to study as HEL cells and platelets both directly respond to thrombin with an increase in $[Ca^{2+}]_i$. I therefore looked for another agonist that may selectively stimulate platelets. One possibility is the TLR1/2 agonist, Pam_3CSK_4, known to stimulate Ca^{2+} increases and secretion in platelets (Kälvegren et al., 2010, Fung et al., 2012a). Pam_3CSK_4 caused a clear transient increase in $[Ca^{2+}]_i$ in a pure population of platelets (Fig. 4.2 A), confirming this result. In contrast there was little or no change in $[Ca^{2+}]_i$ in a pure population of HEL cells (Fig. 4.2 A, blue trace). However, in a mixed cell suspension, Pam_3CSK_4 evoked a clear response in HEL cells, indicating that the two cells are interacting to generate a Ca^{2+} increase in the cell line. This effect was analysed from multiple donors and batches of HEL cells and the average peak increases and 2 min integrals of the Ca^{2+} responses were compared (see Fig 4.2 B, C). The average 2 min Ca^{2+} integral of HEL cells in the presence of platelets was 13274 ± 1520 nM.sec which was significantly larger than in the absence of platelets (824 ± 527 nM.sec; $P<0.01$; n=4; see Fig 4.2 B). The average peak increase in the HEL cell Ca^{2+} in response to this agonist was 299.55 ± 3 nM in the presence of platelets which was also significantly larger than in the absence of platelets (9.53 ± 1 nM) ($P<0.01$; n=4). No significant difference was observed between the response to Pam_3CSK_4 of a pure population of platelets (integral 12898 ± 3463 nM.sec and peak 243.9 ± 28.8 nM) compared to the HEL cell response in the mixed cell suspension for both the integral and peak increase values ($P>0.05$; n=4) (see Fig 4.2 B and C). This suggests that HEL cell Ca^{2+} responses are induced by TLR1/2-stimulated platelets. This could be due to either a physical interaction between the two cell types or via release of agents from platelets that then diffuse to activate neighbouring HEL cells.

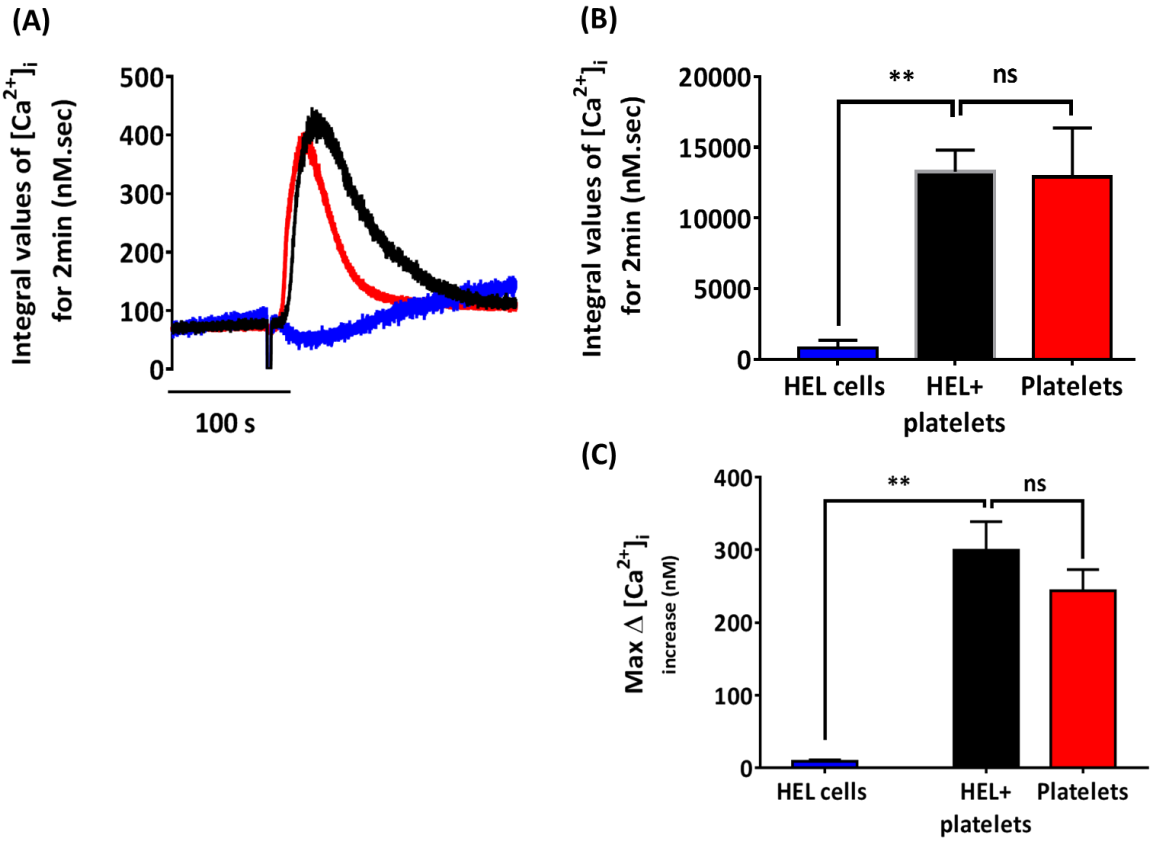

Fig. 4.2. Pam_3CSK_4, an agonist of the Toll-like receptor 1/2 complex stimulates HEL cell Ca^{2+} responses only when co-incubated with platelets.

*(A) Sample recordings of intracellular Ca^{2+} responses to Pam_3CSK_4 from either fura-2-loaded platelets (red) or fura-2-loaded HEL cells (black, blue) in a stirred suspension of only one cell type (red, blue) or both cell types combined (black). (B) Average integral values of Ca^{2+} responses increase in HEL cells when co-incubated with platelets (black bar) as compared to HEL Ca^{2+} responses to Pam_3CSK_4 without platelets (blue bar). (C) Peak average Ca^{2+} increases following addition of Pam_3CSK_4 under the three conditions described in A; n = 4; ** represents P<0.01; ; ns, not significant (One-way ANOVA; followed by Dunnett's test). Data were expressed as mean ± SEM.*

4.2.2 Evidence that Pam_3CSK_4-activated platelets release nucleotides and TXA_2.

Activated platelets are known to release the contents of their dense granules which include large amounts of ATP and ADP (Yun et al., 2016). Once released into the extracellular space, these nucleotides are known to act as agonists of P2 receptors coupled to intracellular Ca^{2+}mobilisation in a range of different cell types. Thus, such nucleotides could participate in the platelet-dependent HEL cell responses shown in Fig. 4.2. Another potential mechanism for this intercellular response is via thromboxane A_2 receptors (TxA_2-stimulated GPCRs), since TXA_2 is released from Pam_3CSK_4-stimulated platelets (Rivadeneyra et al., 2014b) and platelets (Li et al., 2003) as well as HEL cells reportedly express functional TXA_2 receptors (Mayeux et al., 1989b).

To investigate the role of nucleotide and TXA_2 release from platelets, HEL-platelet suspensions were treated with a nucleotidase (apyrase) and a cyclooxygenase inhibitor, aspirin. Platelets were treated with 3.2 U/ml apyrase for one minute prior to stimulation by Pam_3CSK_4 followed by addition to fura-2 loaded HEL cells. Interestingly this high concentration of apyrase, as compared to the saline control (which contained 0.32 U/ml apyrase) reduced the platelet-dependent peak Ca^{2+} increase in HEL cells to 30 ± 12 nM from 149.6 ± 29 nM ($P<0.001$; n=5) (see figure 4.3 C). The average integral values also showed a significant reduction with apyrase (2436 ± 1213 nM.sec) as compared to the saline control (11030 ± 2782 nM.sec) ($P<0.01$; n=5) (see Fig. 4.3 B). The apyrase used in this study (type VII from apyrase) has properties similar to human CD39 known to hydrolyse both ATP and ADP to AMP (Handa and Guidotti, 1996). Therefore, this suggests that ATP and/or ADP released from platelets and acting on P2 receptors on HEL cells is responsible for the Ca^{2+} response to Pam_3CSK_4-stimulated platelets.

Activated platelets release TxA_2 that stimulates TxA_2 receptors (TP) on the platelet surface membrane via an autocrine and paracrine mechanism. Platelet TP GPCR receptors couple to PLC activation and thus an increase intracellular calcium and also promote granule secretion and platelet aggregation via integrin $\alpha_{IIb}\beta_3$ activation (Stalker et al., 2012). TxA_2 also induces platelet shape change via the Rho kinase pathway (Smyth and Fitzgerald, 2009). HEL cells are also reported to exhibit a high density of TxA_2 receptors of the same subtype as in platelets (Mayeux et al., 1989b). Therefore, Pam_3CSK_4 stimulation of platelets could contribute to HEL cell Ca^{2+} responses via TxA_2 release both at the level of platelets and HEL cells. To investigate this possibility, aspirin was used to block platelet TxA_2 production. The Pam_3CSK_4-stimulated intracellular Ca^{2+} increase was significantly reduced by aspirin from an average peak value of 149.6 ± 29 nM in control conditions to 35 ± 7 nM in the presence of aspirin ($P<0.05$; n=5) (see Fig 4.3 C). Similarly, the average 2 min integral value was reduced from a control level of 2928 ± 971 nM.sec to 1130 ± 2782 nM.sec in the presence of aspirin ($P<0.05$; n=5) (see Fig. 4.3 B). This suggests that TXA_2 is one of the agents released from platelets on stimulation with Pam_3CSK_4 and is a major mediator of the subsequent HEL cell Ca^{2+} response. When apyrase and aspirin were added together there was also a significant decrease in the integral and peak values of the Ca^{2+} increase compared to in the absence of either inhibitor (4 ± 1 vs 149.6 ± 29 nM nM; $P<0.01$; n=5; and 2344 ± 46 vs 11030 ± 2782 nM.sec.; $P<0.01$; n=5; see Fig. 4.3 B). Although the average increase in the presence of both inhibitors was slightly lower than in the presence of either apyrase or aspirin, the difference was not significant ($P>0.05$). Taken together, these results indicate that both TxA_2 and ATP (or ADP) released from platelets are equally required for the HEL-platelet interaction following TLR1/2 stimulation.

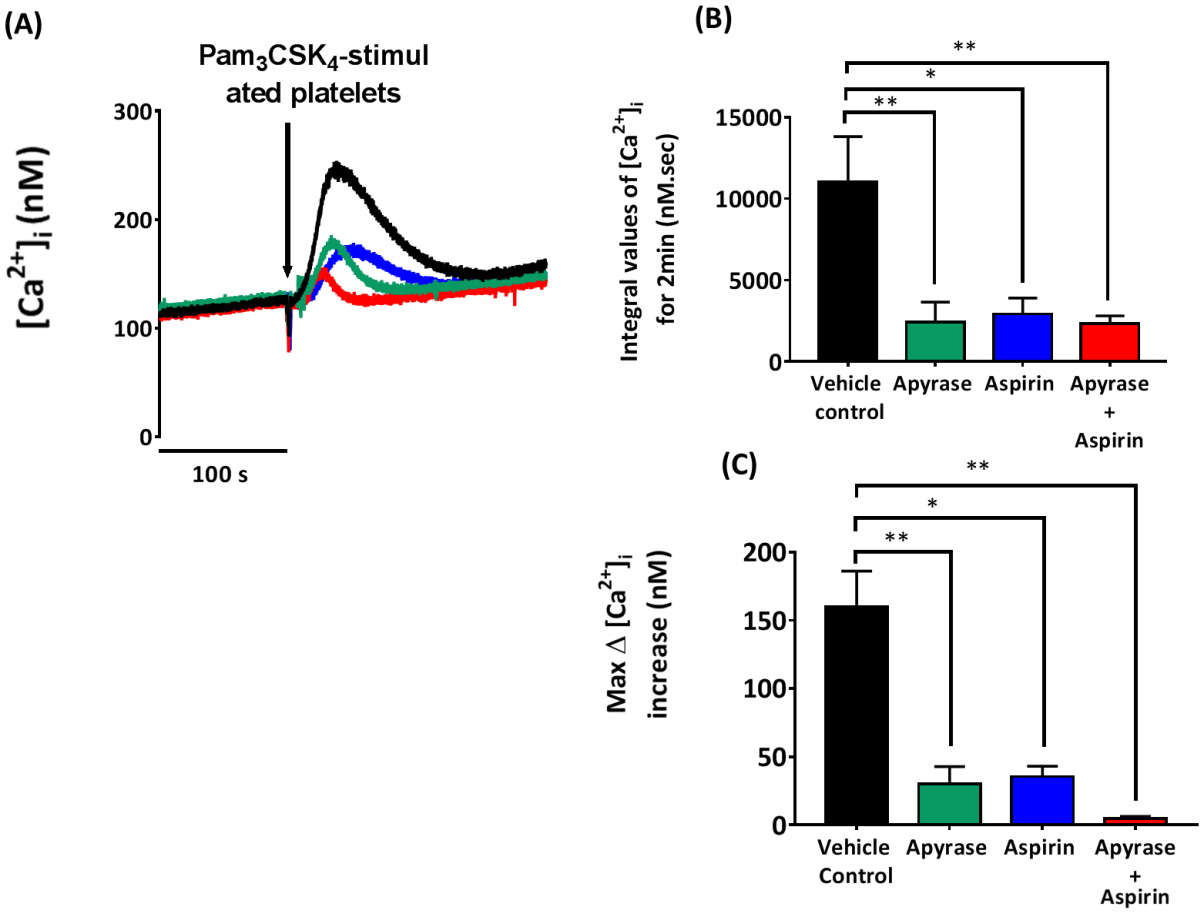

Fig. 4.3 Apyrase or aspirin reduced the platelet-dependent Pam_3CSK_4-stimulated HEL Ca^{2+} response and a combination of these two agents completely blocked the same response

*(A) Representative Ca^{2+} responses in fura-2 loaded HEL cells to Pam_3CSK_4 stimulated platelets in the presence of vehicle control (black), apyrase (green), aspirin (blue) and a combination of apyrase and aspirin (red) in stirred suspensions. (B) Average integral values of Ca^{2+} response in HEL cells under the four conditions stated in A. (C) Peak average Ca^{2+} response in HEL cells under the four conditions stated above in A. n=5;** represent P<0.01, * represent P<0.05 (One-way ANOVA followed by Dunnett's test). All data represent mean ± SEM.*

4.2.3 HEL cell purinergic receptors respond to platelet releasate

As ATP (along with ADP) is known to be secreted from platelets following Pam_3CSK_4 stimulation (Fung et al., 2012b, Kalvegren et al., 2010) and P2 receptors are able to stimulate Ca^{2+} responses in many cell types (He et al., 2003), antagonists of the most extensively expressed HEL cells-P2 receptors were used to investigate the contribution of HEL P2 receptors in the observed HEL-platelet interaction. ATP is known as an effective agonist of P2X4 and P2X1, and along with ADP is also an agonist of P2Y11 receptors (Communi et al., 1999, Syed and Kennedy, 2012, Mahaut-Smith et al., 2000) which signal through Ca^{2+} in many cells (He et al., 2003). Antagonists reported to be selective for these receptors were used to explore the Pam_3CSK_4-evoked Ca^{2+} responses following co-stimulation of platelet and HEL cell suspensions. 5-BDBD has been reported as an antagonist of P2X4 (Balázs et al., 2013, Chen et al., 2013a) and NF157 reported as a selective P2Y11 antagonist (Alkayed et al., 2012). However, 5-BDBD agent was found to be autofluorescent at fura-2 excitation wavelengths, thus preventing accurate measurement of intracellular Ca^{2+}, as already mentioned in chapter 3 (section 3.2.6). The P2X1 antagonist (NF449) was not used for these experiments since chapter 3 (see Fig. 3. 10 D) showed that ATP-evoked Ca^{2+} responses in HEL cells are not inhibited by NF449 (1 μM), indicating that the P2X1 receptors in HEL cells do not significantly contribute to this response (or that they are not functional). However, a marked reduction in HEL Ca^{2+} increases following Pam_3CSK_4 stimulation of mixed platelet:HEL cell suspensions was observed following incubation with the P2Y11 antagonist, NF157 (from control value of 253.4 ± 51 nM to 21.8 ± 4 nM) ($P<0.05$ n=4) (see Fig 4.4). The average 2 min integral values for the same analysis also showed a reduction with NF157 as compared to vehicle control (from 17617 ± 2774 to 4005 ± 2325 nM.sec; $P<0.01$ n=4). In addition to ATP, ADP is equally secreted from activated platelets (Soslau and Youngprapakorn, 1997) thus there could be a possibility that HEL cells are activated by the released ADP along with ATP. ADP is a potent agonist of P2Y1 receptors, which were also found at low levels in HEL cells (Chapter 3, section 3.2.6) and present in platelets (Jin et al., 1998a, Savi et al., 1998, Léon et al., 1999). To investigate the role of P2Y1 receptors, the selective antagonist MRS2179 (30

μM) (Fung et al., 2007b, Boyer et al., 1998) was used. The HEL cell Ca^{2+} increase observed following Pam_3CSK_4 stimulation of platelet-HEL cell suspensions, was measured as 192.5 ± 47 nM as compared to control 253.4 ± 5 nM (P>0.05 n=4) (see Fig 4.4 C), which represents a smaller average, however the difference is not significant. In addition to the change in peak response, these data were also analysed by average 2 min integral of Ca^{2+} level and no significant difference with MRS2179 was observed as compared to the control (values were changed from 17617 ± 2774 to 14428 ± 2332 nM.sec) (P>0.05; n=4) (see Fig 4.4 B). This indicates that P2Y1 receptors do not play a significant role in the platelet-induced HEL Ca^{2+} response following Pam_3CSK_4 stimulation.

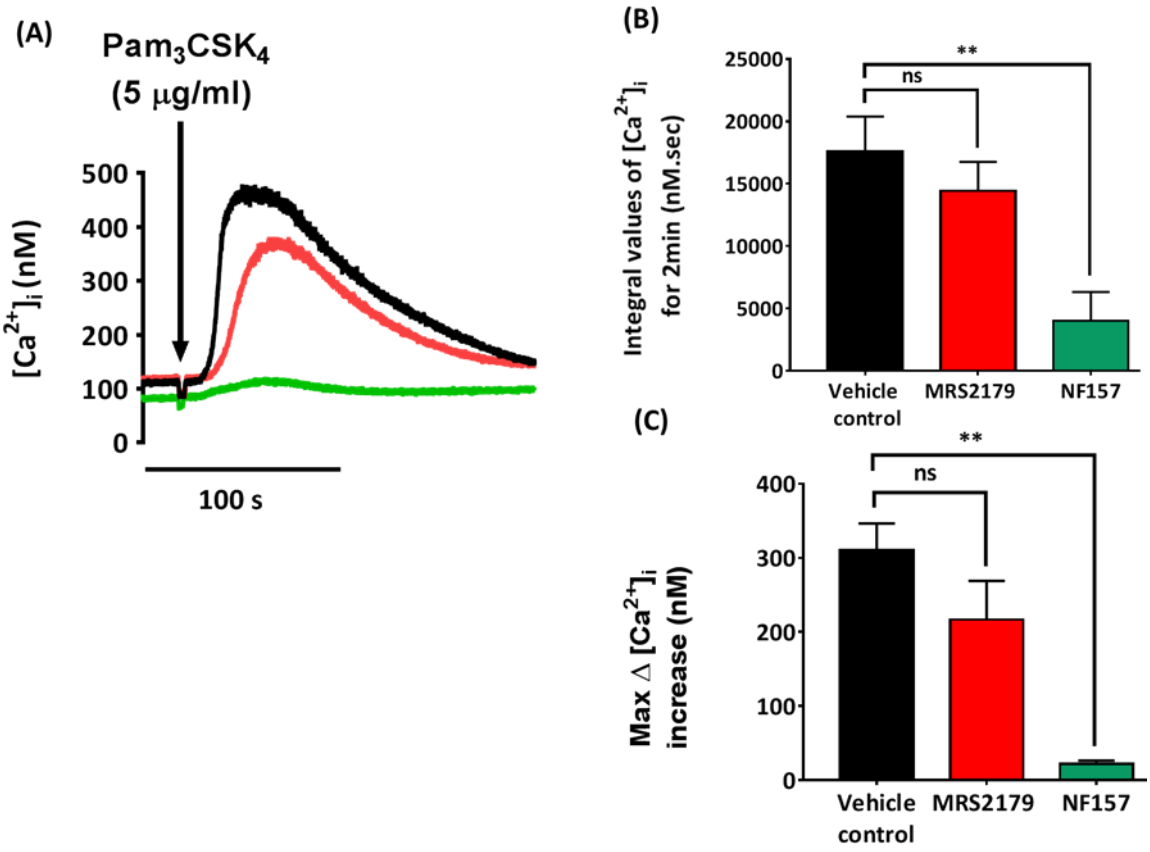

Fig. 4.4 Effect of P2 receptor blockers on platelet-dependent HEL Ca^{2+} signalling following TLR1/2 receptor stimulation

*(A) Representative traces of effect of vehicle control (black), P2Y1 blocker MRS2179 (red) and P2Y11 blocker NF157 (green) on fura-2 loaded HEL Ca^{2+} signals evoked by Pam_3CSK_4 stimulated non-dye-loaded platelets. (B) Integral values of Ca^{2+} signals induced by platelets in HEL cells under the conditions specified in A. (C) Peak average Ca^{2+}signal by under the conditions explained above in (A). n = 5; ** represents P<0.05; ns, not significant, analysed by One-way ANOVA followed by Dunnett's multiple comparison test. Data represent mean ± SEM.*

4.2.4 Pam_3CSK_4 releases ATP from platelets in HEL-platelet suspensions

To further investigate the release of ATP in HEL-platelet suspensions following stimulation by Pam_3CSK_4, bulk phase extracellular ATP measurements were carried out using a luciferin-luciferase bioluminescence assay. The effect of the apyrase concentration (3.2 U/ml) that blocked the HEL cell Ca^{2+} response in section 4.2.2 was assessed on the extracellular ATP level. Luciferin-luciferase was added to the HEL-platelet suspension two minutes prior to addition of 3.2 U/ml apyrase followed by Pam_3CSK_4 addition. An increase in ATP was detected on addition of Pam_3CSK_4 to the HEL-platelet suspension in the saline control (2.637 ± 0.5 μM) (see Fig 4.5) which was markedly reduced in the presence of apyrase to 0.223 ± 0.1 μM ($P<0.05$; n=4). As shown in chapter 3, the most extensively expressed purinergic receptor on HEL cells was P2Y11 (chapter 3 section 3.2.5). An antagonist of these receptors, NF157 significantly reduced the ATP release from HEL-platelet suspension (0.070 ± 0.003 μM) ($P<0.05$; n=5) stimulated with Pam_3CSK_4 (see Fig 4.5). However, since platelets do not express P2Y11 receptors, it is likely that this is a non-specific effect on platelet signalling or secretion, as observed for the thrombin-evoked ATP secretion in Chapter 3. Likewise, aspirin was added to assess the contribution of TXA_2 to the ATP release pathway following stimulation of HEL-platelet suspensions with Pam_3CSK_4. Aspirin was added two minutes before HEL-platelet stimulation with Pam_3CSK_4. Aspirin also showed significant reduction in ATP release by Pam_3CSK_4 as compared to the control (0.093 ± 0.02 μM compared to 2.637 ± 0.5 μM; $P<0.05$; n=4; see Fig 4.5) as compared to control (HEL response to Pam_3CSK_4-stimulated platelets without aspirin) indicating a major role for TxA_2 release in ATP secretion from platelets following stimulation by Pam_3CSK_4. Taken together, these results suggest that aspirin inhibits HEL cell Ca^{2+} responses in platelet-HEL cell mixed suspensions mainly by blocking dense granule secretion rather than preventing activation of HEL cell TXA_2 receptor activation.

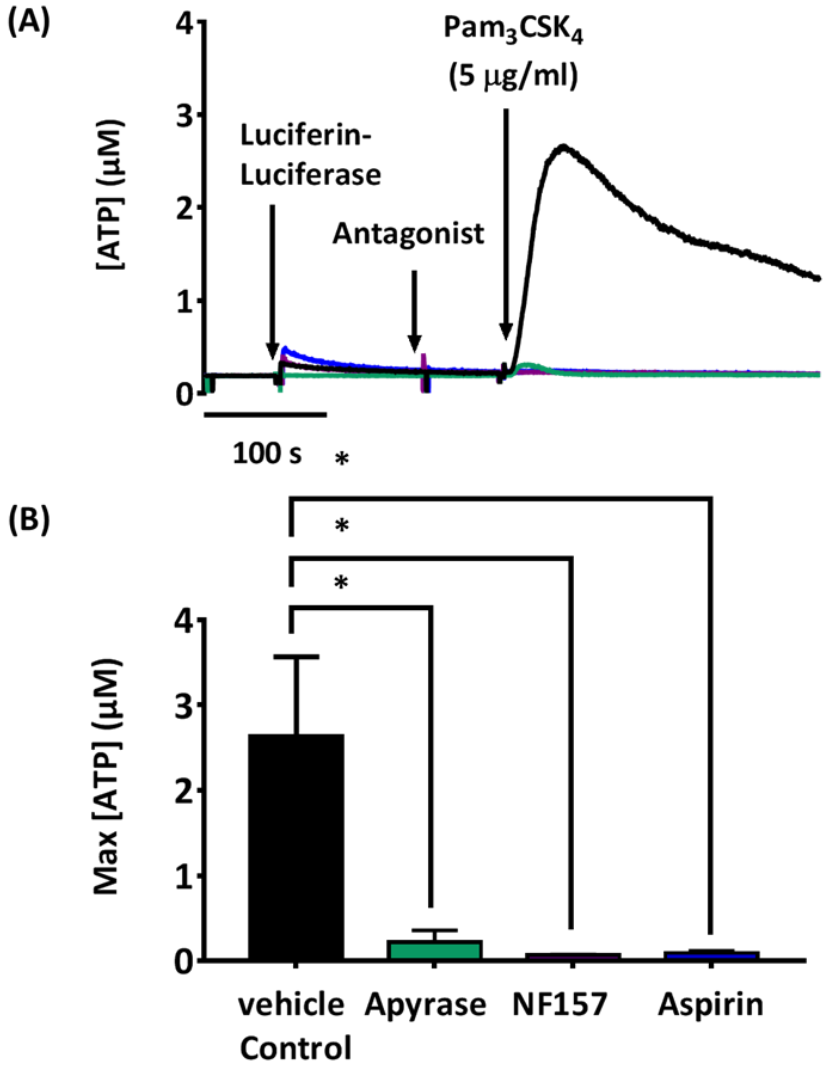

Fig. 4.5 Apyrase, Aspirin and NF157 significantly reduce the ATP release from HEL-platelet suspension on stimulation with Pam_3CSK_4

*(A) ATP release detection in HEL-platelet stirred suspension in the presence of luciferin-luciferase followed by Pam_3CSK_4 addition with prior addition of vehicle control (black), 3.2u/ml apyrase (green), 2μM NF157 (purple) and 100μM aspirin (blue). (B) The average ATP release detected by luciferin-luciferase after stimulation of platelet:HEL cells suspension with Pam_3CSK_4 in the conditions described in (A). n=4; * represents P<0.05. One-way ANOVA followed by Dunnett's multiple comparison test was performed. All data represent mean ± SEM.*

4.2.5 The P2Y11 blocker NF157 reduced Pam_3CSK_4-induced Ca^{2+} responses of platelets

To further investigate the mechanism of the action of NF157, fura-2 loaded platelets were stimulated with Pam_3CSK_4 in the absence of HEL cells with and without either MRS2179 or NF157. MRS2179 was also included as a further control since this is known to block platelet P2Y1 receptors but had no effect on the HEL Ca^{2+} response following Pam_3CSK_4 stimulation of the mixed cell suspension. NF157 completely abolished the Ca^{2+} increase evoked by Pam_3CSK_4 in platelets (from 289 ± 52 nM to 9 ± 2 nM) ($P<0.01$, n=4) (see Fig 4.6 C). A similar result was observed when comparing the 2 min integral values (11219 ± 1027 to 211 ± 4 nM.sec; $P<0.01$; n=4) (see Fig 4.6 C). In contrast, MRS2179 did not affect the Ca^{2+} increase in platelets stimulated with Pam_3CSK_4; the peak Ca^{2+} increase was 289 ± 52 nM in the control compared to 251 ± 9 nM with MRS2179 from ($P>0.05$; n=4) (see Fig 4.6 C). A similar trend but again lack of significance was observed in the 2 minute integral values, which were 11219 ± 1027 for the control compared to 10179 ± 658 nM.sec with MRS2179 ($P>0.05$; n=4) (see Fig 4.6 B). This further indicates that P2Y1 receptors have no significant role in platelet stimulation by Pam_3CSK_4.

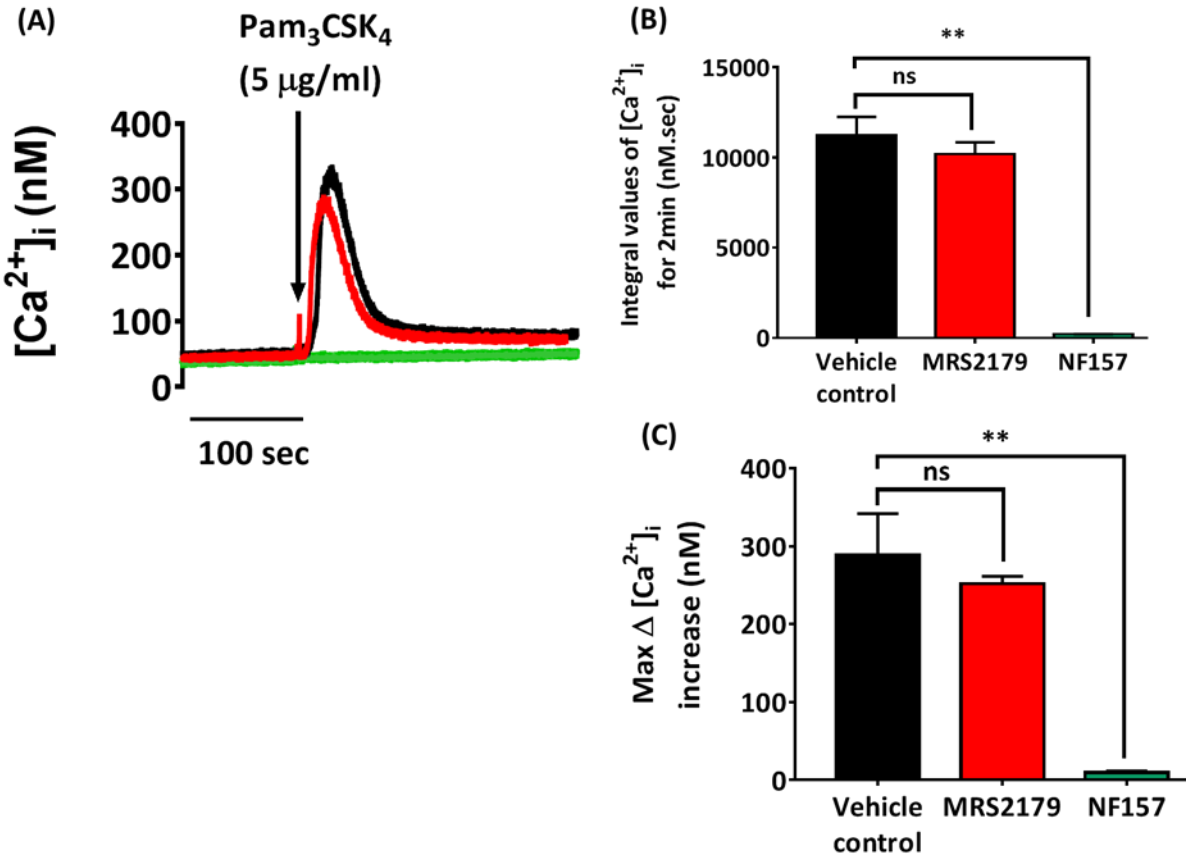

Fig. 4.6 Effect of P2Y1 and P2Y11 antagonists on Pam_3CSK_4-induced Ca^{2+} signal in platelets

*(A) Representative traces of rise in Ca^{2+} level by Pam_3CSK_4 in a pure population of fura-2 loaded platelets in the presence of vehicle control (black), MRS2179 (red) and NF157 (green). (B) Average integral values of Ca^{2+} signal by platelets under the conditions stated in A. (C) Peak average release of Ca^{2+} in above described conditions in A. n = 5; ** represents P<0.05; ns, not significant (Dunnett's multiple comparison test following One-way ANOVA). Data represent mean ± SEM values.*

Together with the ability of NF157 to block the ATP release from platelets (section 4.2.3), these data suggest that this compound is blocking platelet-dependent HEL cell Ca^{2+} responses following Pam_3CSK_4 stimulation mainly at the level of the initial platelet responses. For example, NF157 may block Pam_3CSK_4 receptors directly or downstream Ca^{2+} signalling molecules.

The effect of apyrase was also assessed on the $[Ca^{2+}]_i$ response of platelet to Pam_3CSK_4. Platelets were incubated with apyrase (3.2 U/ml) for 2 minutes prior to addition of Pam_3CSK_4 (5 µg/ml). No difference in platelet Ca^{2+} response was observed with apyrase as compared to the vehicle control (integral values changed from 10156 ± 741 to 10652 ± 479 nM.sec) (P>0.05, n=3) (see Fig. 4.7).

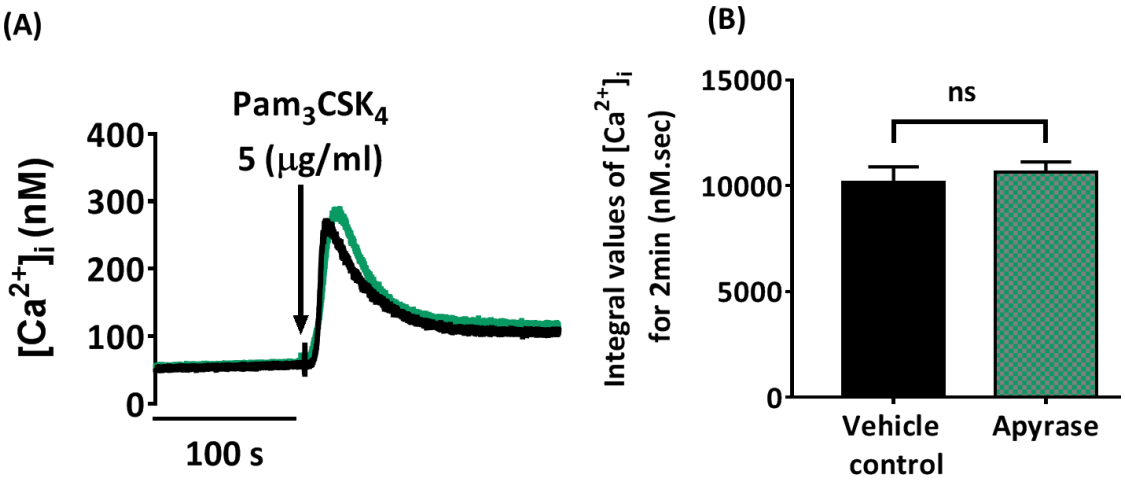

Fig. 4.7 Effect of 3.2 U/ml apyrase on Pam_3CSK_4-induced Ca^{2+} signal in platelets

(A) Representative traces of rise in Ca^{2+} level by Pam_3CSK_4 in fura-2 loaded platelets in the presence of vehicle control (black) and apyrase (green). (B) Average integral values of Ca^{2+} release in above described conditions in A. n = 3; ns represents not significant (P>0.05; n=3; Paired t-test).

4.2.6 Platelets evoke variable patterns of Ca^{2+} increases in single HEL cells

The population experiments above assess only average Ca^{2+} responses in a stirred suspension of HEL cells. To further examine Pam_3CSK_4-stimulated interactions between platelets and HEL cells at the level of intracellular Ca^{2+}, experiments were carried out using single cell fluorescence imaging. In these experiments fluo-3 loaded HEL cells were attached to a glass cover slip using poly-D-lysine followed by addition of platelets with and without Pam_3CSK_4 stimulation and Ca^{2+} responses were observed in single HEL cells. In these experiments, the transmitted light images were also acquired thereby allowing the study of any morphological response of HEL cells. Increases in HEL cell Ca^{2+} responses were observed upon addition of either unstimulated platelets or platelets pre-stimulated with Pam_3CSK_4 for 1 minute. However, a different timing and pattern of Ca^{2+} increase was observed in the HEL cells following addition of unstimulated platelets compared to addition of Pam_3CSK_4 pre-stimulated platelets (see Fig 4.8). Responses of a total of 30 cells (10 from each of 3 batches) was analysed. A rise in Ca^{2+} was observed in (93 %) 9, 9 and 8 HEL cells measured in 3 batches of 10 cells respectively upon addition of Pam_3CSK_4-stimulated platelets. However at a single cell level a heterogeneity was observed in the patterns of Ca^{2+} responses. The pattern of cells responding fell into two categories. In ≈a third of the cells (33 %), the increase occurred in the form of multiple large amplitude spikes which initiated with an average delay of 7 ± 1 s after addition of the stimulated platelets. The majority (60 %) of the cells showed an initial spike of Ca^{2+} increase followed by a sustained plateau with small spikes of Ca^{2+} increase superimposed; the delay from addition of platelets to the initial Ca^{2+} spike for this group of cells was 21 ± 1 s (see Fig 4.8 A). A delayed Ca^{2+} increase was also observed after ≈480 s in all responding cells. The remaining 7 % of cells showed no response (see Fig 4.8 A). In contrast, with non-stimulated platelets (60 %) 6, 5 and 5 HEL cells measured in 3 batches of 10 cells respectively showed a Ca^{2+} increase, although this was initiated with a greater delay (average 153 ± 11 s; range

150 to 200 s) compared to the Pam_3CSK_4-stimulated platelets (see Fig 4.8 B). About 33 % of the cells showed an initial spike followed by small spikes on top of a plateau which was followed by a second phase of Ca^{2+} increase (green traces in Fig. 4.8 B). In 27 % of the cells showed just an initial Ca^{2+} transient followed by a plateau phrase (red trace) (see Fig 4.8 B). No HEL cells responded directly to Pam_3CSK_4 (see Fig 4.8 C & D confirming that TLR1/2 receptors are not expressed on, or at least are not functionally coupled to Ca^{2+} mobilisation in HEL cells.

The earlier experiments with stirred cell suspensions suggest that Pam_3CSK_4-dependent Ca^{2+} responses in HEL cells involve release of both nucleotides and TxA_2, but that the TxA_2 works by causing further release of nucleotide from platelets rather than directly on the HEL cells. Consequently, it would be expected that the HEL Ca^{2+} responses to nucleotides mimic those induced by Pam_3CSK_4-stimulated platelets. Thus, the average concentration of ATP released from Pam_3CSK_4-stimulated platelets calculated in the above experiments (Fig 4.5) (~2 µM) was introduced to fluo-3 loaded HEL cells and the response to ATP or equal concentration of ADP was compared with the response produced by Pam_3CSK_4-stimulated platelets (see Fig 4.9 A, B & C). In this series of experiments, the Pam_3CSK_4-stimulated platelets evoked a Ca^{2+} response in 8, 9 and 7 HEL cells (measured from 3 batches each having 10 cells). The no. of HEL cells in the same batches that responded to ATP (2 µM) was 10, 9 and 10 (no. of cells= 10; from each of 3 batches), which was not significantly different from the response to Pam_3CSK_4-stimulated platelets ($P>0.05$) (see Fig 4.9 D). In contrast, a significantly lower proportion of cells (5, 4 and 6 HEL cells measured in 3 batches of 10 cells respectively) showed a Ca^{2+} response to 2 µM ADP compared to the Pam_3CSK_4 stimulated platelets ($P<0.05$) (see Fig 4.9 D).

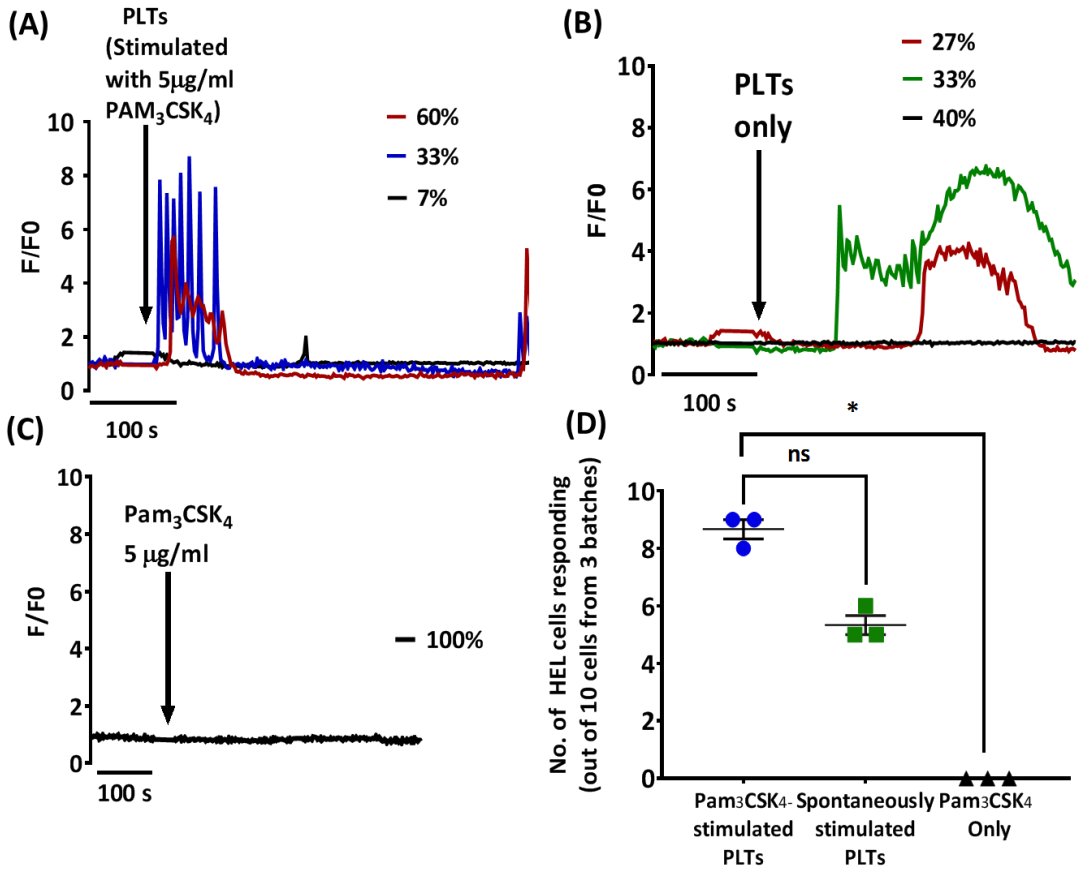

Fig. 4.8 Single HEL cell Ca^{2+} responses following addition of platelets with and without pre-stimulation by Pam_3CSK_4

*Intracellular Ca^{2+} responses of single HEL cells attached to glass coverslips recorded by imaging of fluo-3 fluorescence (F/F_0 ratio). Typical traces (A-C) and no. of cells responding (D) are shown following addition of (A) Pam_3CSK_4 stimulated platelets (PLTs) (B) unstimulated platelets (C) Pam_3CSK_4 alone (D) No. of HEL cells responding to Pam_3CSK_4-stimulated platelets (blue colour), and spontaneously-activated platelets (green colour) and to Pam_3CSK_4 in the absence of platelets (black colour), * represents $P<0.05$; ns represents not significant. Analysis is of a total of 30 HEL cells 3 different batches for each type of experiments. One-way ANOVA followed by Friedman test was performed.*

Moreover, the pattern of the Ca^{2+} response in HEL cells to Pam_3CSK_4-stimulated platelets, ATP and ADP was also compared. For these experiments, the effect of Pam_3CSK_4-stimulated platelets, ATP and ADP were compared on the same batches of HEL cells. As described above, the response after platelet addition consisted of both baseline and plateau oscillating Ca^{2+} responses followed by a delayed secondary Ca^{2+} response at about 480 s. In this set of experiments 100 % of the HEL cells responded following addition of Pam_3CSK_4-stimulated platelets (see Fig 4.9 A) (n=10 cells from a single batch). 2 µM ATP induced a Ca^{2+} response in 100% of HEL cells, which consisted of an initial peak followed by a plateau with small spikes superimposed, which is similar to the main type of pattern observed following addition of Pam_3CSK_4-stimulated platelets. However, after ATP no secondary response was observed (see Fig 4.9 C) (n=10 cells from a single batch). In contrast, 2 µM ADP evoked a Ca^{2+} response in 60% of HEL cells which consisted of a peak response followed by baseline oscillations. Of these, two thirds (i.e. 40 % of all cells) showed an early peak response and the remaining (20% of all cells) showed a delayed peak response (see Fig 4.9 B) (n=10 cells from a single batch). The no. of HEL cells (measured from 3 batches of 10 cells each) responded to Pam_3CSK_4-stimulated platelets (8, 9 and 7 HEL cells) and ATP (10, 9 and 10 HEL cells) was not significantly different ($P>0.05$). However a significant decrease in no. of HEL cells responded to ADP (5, 4 and 6 HEL cells) as compared to Pam_3CSK_4-stimulated platelets was observed ($P<0.05$). To further compare the responses of HEL to ATP and ADP with the responses of HEL to Pam_3CSK_4-stimulated platelets, the integral values of the F/F_0 increases over the baseline value were compared for a period of 8 min after addition of the stimulus. The average calculated integral of the response to Pam_3CSK_4-stimulated platelets was 636 ± 72 (no. of cells = 10, from 3 batches). The average integral response for ATP was not significantly different (652 ± 64 (F/F_0); $P>0.05$; no. of cells = 10, from 3 batches) but was significantly lower for ADP (275 ± 103; $P<0.05$; no. of cells= 10, from 3 batches) (see Fig 4.9 E). Moreover, the average peak responses for these three stimuli were also compared. The average peak F/F_0 response with Pam_3CSK_4-stimulated platelets was 8.6 ± 0.7, which was not

significantly different from that of the responses to ATP (7.2 ± 0.6; P>0.05) but was significantly different from that seen with ADP (2.6 ± 0.4; P<0.05) (see Fig 4.9 F). These data therefore suggest that either ATP or ADP can contribute to the intercellular signalling between Pam_3CSK_4-stimulated platelets and HEL cells at the level of $[Ca^{2+}]_i$. At the concentrations predicted in the bulk phase, ADP cannot replicate the effect of Pam_3CSK_4-stimulated platelets in these single cell studies. ATP appears to more closely reproduce the pattern and magnitude of the Pam_3CSK_4-stimulated platelets, but some differences were still observed. Taken together, these data suggest that ATP and ADP are either acting in combination or other factors are involved such as other secreted compounds or the physical presence of platelets.

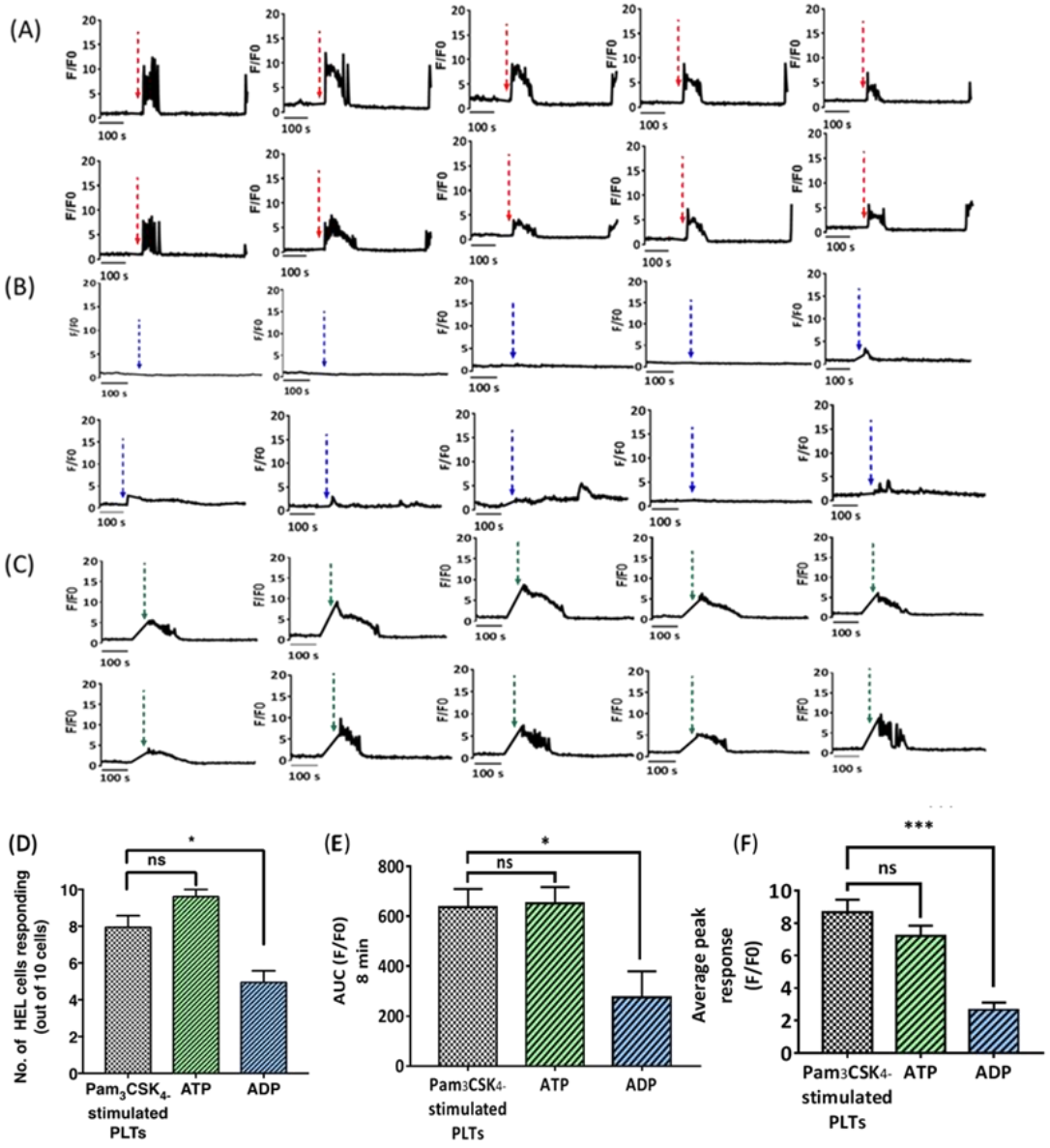

Fig. 4.9 Single cell HEL responses to (A) Pam_3CSK_4-stimulated platelets, (B) ADP and (C) ATP:

*The arrowheads indicate the time of the stimulants addition (all cells are from a single batch). (D) No. of HEL cells responding (E) Average AUC of F/F0 values by HEL cells (F) Average peak response of F/F0 by HEL cells in response to Pam_3CSK_4-stimulated platelets, ATP and ADP, * represents P<0.05, *** represents P<0.0001, ns represents non-significance (n= 10 cells, from 3 batches). Data is analysed by Dunnett's multiple comparison test following One-way ANOVA. Data represent mean ± SEM.*

4.2.7 Activated platelets induce morphological changes in single HEL cells:

Morphological studies were also carried out since cell shape change in cancer cells establishes malignancy (Friedl and Alexander, 2011). These morphological changes in cancer cells precede metastasis and are associated with modulation of Ca^{2+} pathways and cellular activation (Strobeck et al., 1999). Single HEL cells showed a significant shape change following addition of Pam_3CSK_4-stimulated platelets. The shape of the HEL cells changed from a round (see Fig 4.10 Ai) to an irregular shape with pseudopodia (see Fig 4.10 A ii). The average surface area of HEL cells before addition of Pam_3CSK_4-stimulated platelets was 162 ± 13 μm^2 and after addition was 209 ± 21 μm^2 ($P<0.05$; n=10 cells) (see Fig. 4.10 C). Pam_3CSK_4-stimulated platelets release ATP, with an average extracellular concentration of 2 μM, therefore 2 μM ATP was added directly to HEL cells to compare the shape change. A similar morphological HEL cell response was observed with ATP, changing from a round to amoeboid form. The average surface area of HEL cells increased from 153 ± 9 μm^2 to 180 ± 13 μm^2 ($P<0.05$; n=10 cells) (see figure 4.10 D). Moreover, the average percentage increase in surface area before and after addition of Pam_3CSK_4-stimulated platelets (19 ± 7 %) and ATP (13 ± 4 %) was not significantly different ($P>0.05$; n=10 cells) (see figure 4.10 E). This suggests that ATP release and P2 receptor activation can account for most of the HEL cell morphological response to Pam_3CSK_4-stimulated platelets.

The extent of the single cell shape change was also compared with the nature and magnitude of the Ca^{2+} oscillation response in HEL cells following addition of Pam_3CSK_4-stimulated platelets and ATP (see Fig. 4.11 A & B). There was no clear correlation between the two parameters. Three different HEL cells are shown in Fig. 4.11 for both these stimuli. Cells that showed no shape change (cell no 3 for addition of either ATP or Pam_3CSK_4-stimulation platelets) could display a Ca^{2+} response that was similar to that of cells that showed marked morphological responses (cells 1 and 2).

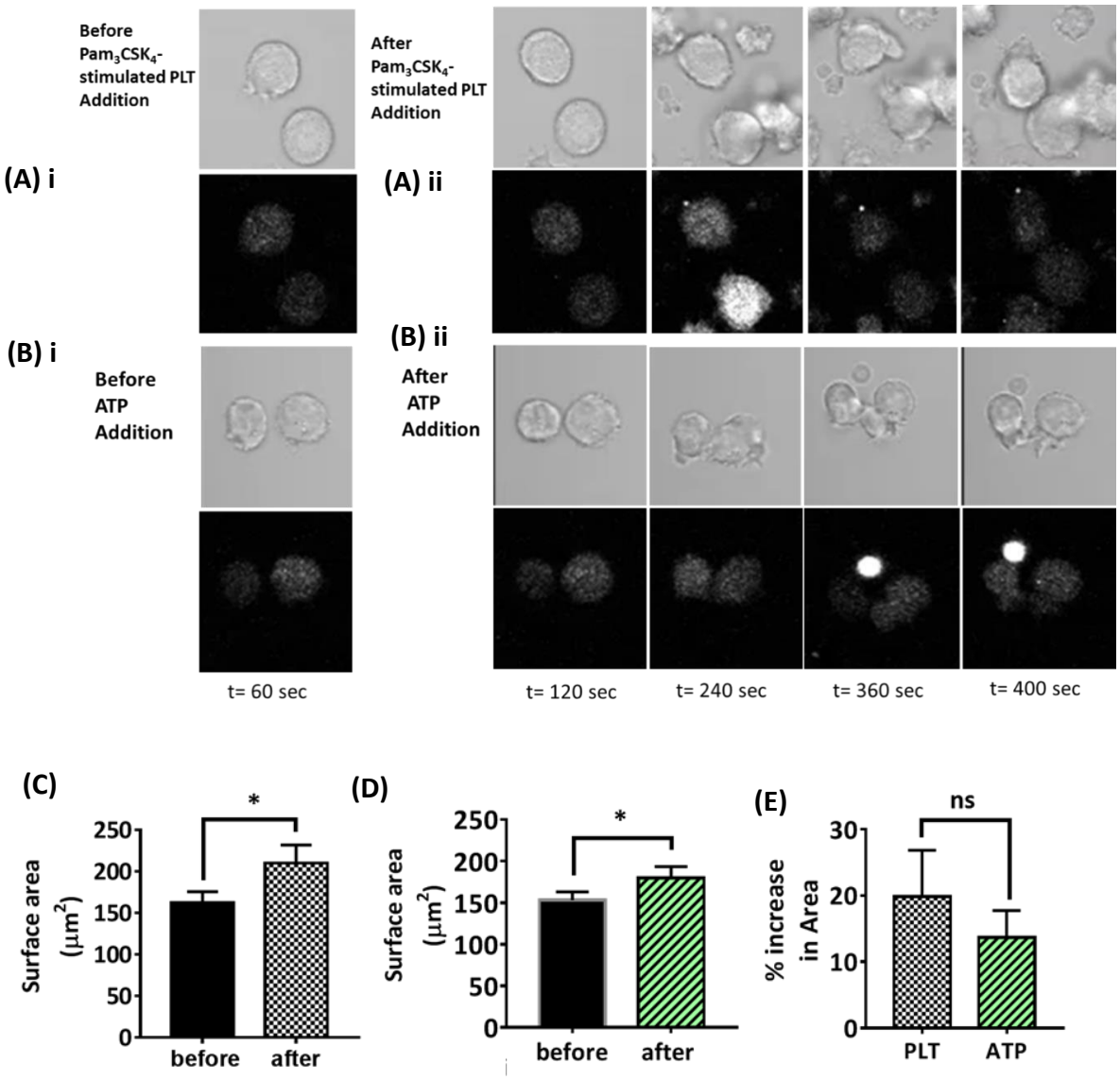

Fig. 4.10 Morphological responses of HEL cells to Pam_3CSK_4-stimulated platelets and ATP

*(A. i) HEL cell shape before addition of platelets (A. ii) after addition of platelets (B) shape of unstimulated HEL cells (B. ii) HEL cells after stimulation with ATP. (C) Surface area changes significantly with shape after HEL stimulation with Pam_3CSK_4-stimulated platelets (D) Surface area change with shape after HEL stimulation with ATP. (E) Percentage increase in surface area of HEL cells after stimulation with Pam3CSK4-stimulated platelets and ATP. n=10 cells; * represents P<0.05; ns represents non-significant, (Paired t-test is used for (C) & (D) and unpaired t-test is used for (E)). Data represent mean ± SEM.*

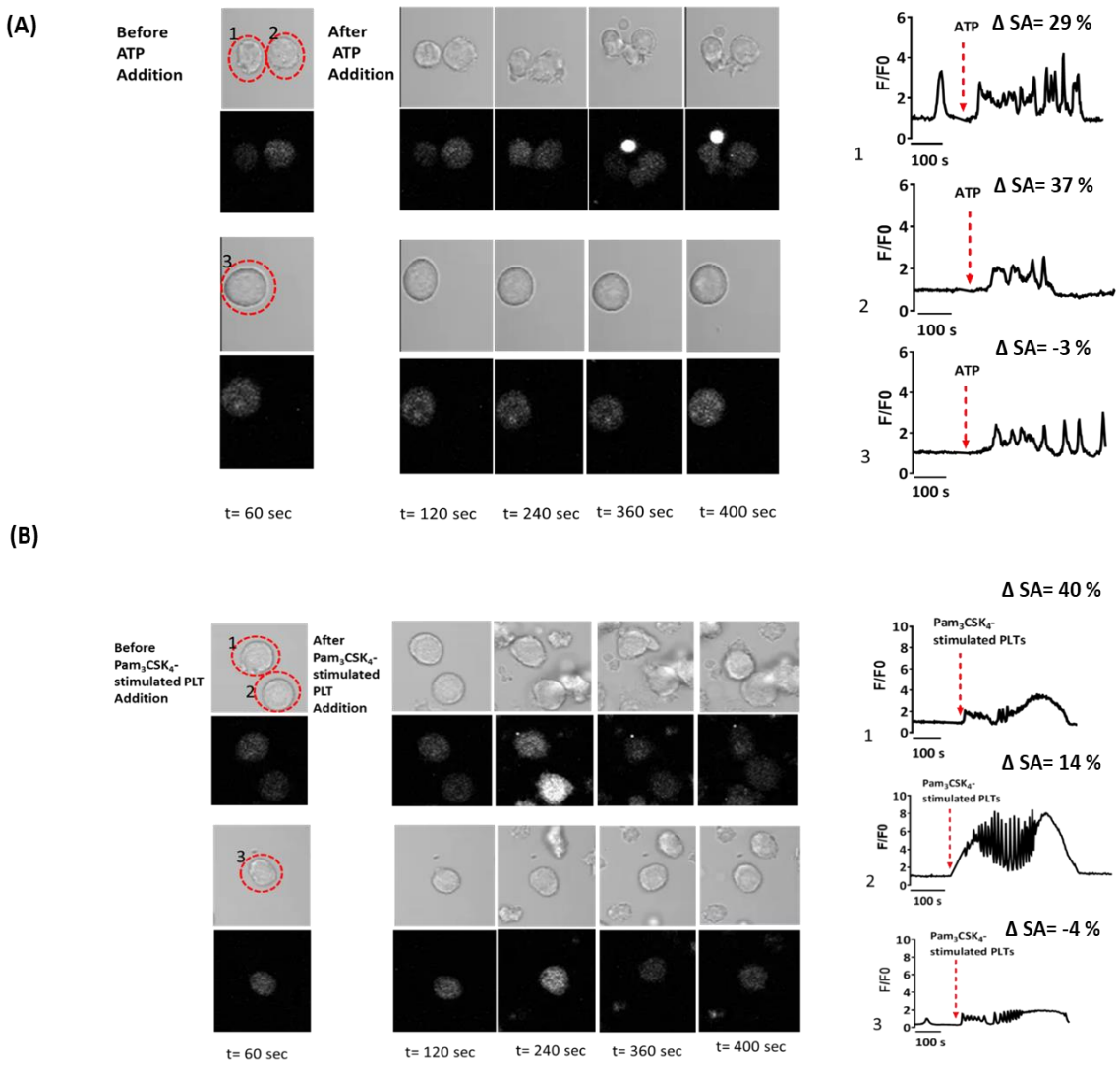

Fig. 4.11 Comparison of single cell shape change with Ca^{2+} oscillation pattern in individual HEL cells

Transmitted and fluorescence images are shown from two different experimental runs for either ATP (A) or Pam_3CSK_4-stimulated platelets (B) to compare the shape change responses (percentage change in surface area, Δ SA) of three individual HEL cells (circles 1,2,3) with the different patterns of $[Ca^{2+}]_i$ increases.

4.3 Discussion

Ca^{2+} signalling in platelets and leukemic cells may play a role during their interactions within the progression of leukemia. Therefore, the experiments reported in this chapter have examined whether platelets stimulated via their innate immune TLR1/2 receptors can communicate with HEL cells via a change in intracellular Ca^{2+} in this leukemic cell line. It is clear that Pam_3CSK_4-stimulated platelets evoke a Ca^{2+} response in HEL cells in contrast to a complete lack of Ca^{2+} response to Pam_3CSK_4 directly on the leukemic cell line. This suggests that TLR1/2 receptors are not functional in HEL cells and indeed there are no reports in the literature of TLR1/2 expression in these cells. Thus, TLR1/2-stimulated platelets must interact physically or via release of intercellular messengers to stimulate Ca^{2+} responses in HEL cells. Platelet dense and alpha granules contain >300 different components that will be released following activation (Golebiewska and Poole, 2014). In particular, platelet dense granules are a rich source of nucleotides. The stored concentration of both ATP as well as ADP is about half of a molar (Holmsen and Weiss, 1979) and is secreted with an average ATP:ADP ratio of 2:3 (Meyers et al., 1982). The average plasma concentration of ATP from fully stimulated platelets, based on the platelet density is reported as 1 to 25 µM and for ADP is 1 to 11 µM (Meyers et al., 1982). Also, Pam_3CSK_4-activated platelets have been reported to extensively secrete ATP that is comparable to collagen-mediated ATP release (Kälvegren et al., 2010, Fung et al., 2012b). Therefore, in the present experiments, apyrase was used to explore the role of the platelet-released ATP/or ADP in the generation of HEL Ca^{2+} responses following platelet activation. Apyrase virtually abolished the platelet-dependent HEL Ca^{2+} response, whereas the nucleotidase had no effect on the Pam_3CSK_4-induced Ca^{2+} response in platelets in the absence of HEL cells. Furthermore, the luciferin:luciferase firefly assay was used to show that Pam_3CSK_4 stimulated ATP release from platelets and not HEL cells, and that the extracellular ATP increase was abolished by apyrase. Together, these results indicate that nucleotides are released from activated platelets and are responsible for generating much of the

platelet-dependent Ca^{2+} signal in HEL cells. A similar inhibitory effect of apyrase has been reported on TLR1/2 induced chemokine secretion from monocytes (Ben Yebdri et al., 2009). Several studies have revealed that ATP and ADP are active agonists of Ca^{2+} mobilisation via purinergic receptors in HEL cells (Baltensperger and Porzig, 1997). Extracellular nucleotides have been reported to signal through Ca^{2+} mobilisation in a number of cancer cell types resulting in cell signalling, cell division and migration (Cui et al., 2017). These nucleotides act through P2 receptors (Mohammed Akbar et al., 1996) and the most extensively expressed P2 receptors in HEL cells detected with a qPCR screen were P2X1, P2Y11 and P2X4 (see chapter 4). ATP is an active agonist of all these receptors that can generate intracellular Ca^{2+} increases (Mahaut-Smith et al., 2000, White et al., 2003, Wilkin et al., 2001, Syed and Kennedy, 2012). It can be seen in this chapter that NF157, reported as a P2Y11 inhibitor (Ullmann et al., 2005a), significantly reduced the induced Ca^{2+} response in HEL cells. This initially suggested that P2Y11 receptors are potentially expressed in HEL cells and are actively involved in responding to ATP released from platelets. However, this P2Y11 antagonist also abolished the ATP release from Pam_3CSK_4-stimulated HEL-platelet suspension. As platelets have no P2Y11 receptors, NF157 may be acting by non-selectively blocking Pam_3CSK_4-activation in platelets. It can be possibly due to non-selective effects of NF157 in platelets that is also reported in a previous study demonstrating the effects of NF157 on P2X1 equipotent to P2Y11 (Ullmann et al., 2005a). Indeed, it was shown that NF157 also directly abolished the Ca^{2+} mobilisation response of platelets to Pam_3CSK_4. Thus, at present, the role of P2Y11 receptors in the HEL cell Ca^{2+} response to ATP or ADP secreted from Pam_3-CSK_4-stimulated platelets is unclear.

The role of other P2 receptors in the HEL cell responses to Pam_3CSK_4-stimulated platelets was also explored pharmacologically. Possibilities include P2X1, P2X4 and P2Y1. However, the P2X4 antagonist was not compatible with the current Ca^{2+} assay and the HEL cell ATP-evoked Ca^{2+} response does not involve P2X1 receptors as NF449 at 1 µM had no effect (see section 3.2.6). The P2Y1 antagonist MRS2179 has been used extensively to investigate the role of this receptor in several cell types (Lyubchenko et al., 2011, Dunne et al., 2015). Despite the presence of P2Y1 in platelets and the ability

of this reagent to block ADP-evoked Ca^{2+} responses in HEL cells (see Fig. 3.5 D & E), it failed to significantly affect the Pam_3CSK_4-induced Ca^{2+} mobilisation in platelets or HEL cells. This is in agreement with a previous study in which P2Y1 selective blocker was used to detect the effect of P2Y1 in Pam_3CSK_4 mediated nucleotide release from monocytes. Thus, although qPCR experiments have identified P2Y11 (and P2X4) as a potential candidate for the P2 receptor(s) responsible for apyrase-sensitive Ca^{2+} responses in HEL cells following TLR1/2-induced platelet dense granule secretion, the limitations of the available pharmacological agents were unable to successfully confirm this hypothesis. Future work should therefore look to interference RNA knock-down or genome editing of HEL cells to advance this work.

Experiments also demonstrated that aspirin reduced the HEL Ca^{2+} responses following Pam_3CSK_4 stimulation of platelets. HEL cells express active TxA_2 receptors (Mayeux et al., 1989b) and Pam_3CSK_4 is known to stimulate TxA_2 release (Rivadeneyra et al., 2014b, Kälvegren et al., 2010). However, it was also observed that ATP release from Pam_3CSK_4-stimulated HEL-platelet suspensions was also abolished by aspirin. One of the possible mechanisms for the action of aspirin is to block of TxA_2 release from activated platelets and thereby prevent autocrine/paracrine activation of TP receptors leading to ATP or ADP release (Cognasse et al., 2015a). This positive feedback loop of platelet activation and ATP release via $G\alpha_q$-mediated TP receptors has also been suggested in a previous study of the inhibitory effects of aspirin in platelets (Li et al., 2003). The current findings therefore suggest aspirin as an active agent against the role of platelets in cancer progression, as adjuvant use of aspirin in cancer development therapy is also suggested in many reported studies (Pasche et al., 2014, Elwood et al., 2016).

Imaging experiments within this chapter also revealed the nature of the Ca^{2+} and morphological responses in HEL cells following addition of Pam_3CSK_4-stimulated platelets. In these experiments, the Pam_3CSK_4-stimulated platelets were added and allowed to settle. In cuvette population experiments, the Ca^{2+} increase occurs as a single transient response. At the single cell level, the Ca^{2+} response was found to be

heterogeneous, but in many cells took the form of an oscillation, either from the baseline or from a plateau level. These patterns of Ca^{2+} increase have been reported in multiple cell types, including platelets and tumour cells (van Gorp et al., 2002, Rizaner et al., 2016, Pugh et al., 2017). The underlying mechanism of these oscillations was not studied here but has been the topic of many in depth reports and varies between cells. For example it can depend upon cycles of phosphorylation or dephosphorylation of the Gq-coupled GPCR which will lead to repetitive increases or decreases in IP_3. Alternatively, the oscillations may depend mainly upon the inhibition of IP_3 receptors by increasing $[Ca^{2+}]_i$ which then allows periods of refilling of the stores to generate the next round of Ca^{2+} release (Harootunian et al., 1991, Uneyama et al., 1995, Rink and Jacob, 1989). One of the reasons for the different patterns could be variability in gene expression in individual cells as reported in T cells (Arrol et al., 2008). Also in the same study the pattern of Ca^{2+} signalling was found correlated to the functional response in terms of proliferation and regulation of transcriptional activators that resulted in cytokine release in T cells (Arrol et al., 2008). Therefore, the difference in Ca^{2+} patterns can be the cause of differential transcription factors resulting in heterogeneity in Ca^{2+} signalling patterns in HEL cells.

A variable pattern of Ca^{2+} oscillations was also observed in HEL cells in response to addition of spontaneously activated platelets, although the onset was considerably longer (150-200 s) compared to the effect of Pam_3CSK_4-stimulated platelets (average 7 s). The longer delay is likely due to the time taken for platelets to become activated after settling on the glass coverslips (Hartwig, 1992) and the time for released secretions to reach the HEL cells. Alternatively, the delay of 150-200 s could be due to the time required for settling of platelets and subsequent physical interaction between platelets and HEL cells via surface receptors such as platelet integrins, particularly $\alpha_6\beta_1$ and $\alpha_{IIb}\beta_3$ (Lavergne et al., 2017a).

Following addition of ATP to the imaging chamber, Ca^{2+} oscillations were also observed, but with a very short delay of approximately 1-2 s. The shorter time

compared to that observed with Pam_3CSK_4-stimulated platelets (7 s) could be due to the time taken for the platelets to settle near the HEL cells.

Different patterns of Ca^{2+} increase was observed in HEL cells depending on the stimulus: i.e. addition of Pam_3CSK_4-stimulated platelets, ATP or ADP. The trend induced by Pam_3CSK_4-stimulated platelets was similar to the pattern induced by ATP in that both induced an initial peak of non-synchronous oscillations that was followed by a superimposed plateau for the remaining period of time. In addition, the integral of the main response was not significantly different for these two stimuli. These data provide evidence that platelet release ATP as a major component of the secretions that activate HEL Ca^{2+} signalling. However, with Pam_3CSK_4-stimulated platelets a secondary peak oscillatory response was also observed. This may be because of the secondary activation of platelets that released additional nucleotides, or TxA_2 or direct contact. Also it could be due to HEL cells induced platelet activation by direct contact with cancer cells as proposed during tumour induced platelet activation (TCIPA) (Jurasz et al., 2004). In contrast, ADP evoked a response in only 60 % of the HEL cells (compared to>80-100% of cells after addition of Pam_3CSK_4-stimulated platelets or ATP) that had a magnitude less than half the response observed with Pam_3CSK_4-stimulated platelets. These findings further support the hypothesis that ATP rather than ADP is the major secretion from Pam_3CSK_4-stimulated platelets that stimulates intact HEL cells. However, since both nucleotides are released form the dense granules at similar concentrations, there could be a synergistic contribution of ADP with ATP following platelet-dependent Ca^{2+} signalling in the cancer cell line. Several reports have documented the potential role of ATP secretion from activated platelets resulting in cancer progression (Karachaliou et al., 2015). Of the common factors that may enhance tumour progression including platelet surface receptor binding, α-granule release and dense granule release, ATP is one of the potential candidates in the paracrine activation of surrounding cancer cells. Likewise, HEL morphological changes were also observed with addition of Pam_3CSK_4-stimulated platelets. HEL shape changed from round to amoeboid with addition of Pam_3CSK_4-stimulated platelets. This may be relevant to the mechanism whereby cancer cells engage with surrounding

tissues and blood components resulting in migration through the endothelial barrier (Friedl and Alexander, 2011, Chambers et al., 2002, Sahai and Marshall, 2003). This type of morphological change was also reported in leukemic cells and suggested to involve cell-cell junctions that support single cell migration (Friedl and Alexander, 2011). Cell migration is initiated by adhesion to extracellular matrix leading to cytoskeletal contraction and shape change with altered phenotype. It involves Rho-controlled blebbing that moves the cells further ahead with membrane protrusion at the site of invasion (Lorentzen et al., 2011, Paluch et al., 2006). Correspondingly, in the case of platelets surrounding tumour cells, platelet released growth factors including PDFG, engage with tumour cell surface cadherin and enhances downstream signalling through MAPK and PI3K. This is followed by subsequent tumour cell shape change and migration via epithelial mesenchymal transition (Berx and Van Roy, 2009). This was further described by Labelle et al where MC38GFP and EP5 cancer cell lines were incubated with platelets that led to cancer cell morphological changes indicating epithelial-mesenchymal transition via TGFβ dominated pathways (Labelle et al., 2011a). HEL cell morphological changes observed on addition of Pam_3CSK_4-stimulated platelets could therefore indicate a malignant characteristic that underlies an ability to migrate and invade a peripheral tissue (Lyons et al., 2016, Labelle et al., 2011a, Gay and Felding-Habermann, 2011a, Berx and Van Roy, 2009). A similar shape change was also induced in HEL cells with ATP. These findings were in accordance with the research reported by Jelassi et al in which breast cancer cells changed shape on stimulation with ATP (Jelassi et al., 2013). The similarity between change in HEL cell shape with Pam_3CSK_4-stimulated platelets and ATP further support the conclusion based upon oscillatory patterns of Ca^{2+} increase described above that ATP is the main factor that is responsible for the platelet-dependent stimulation of HEL cells. However, ATP did not fully replicate the pattern of the Pam_3CSK_4-induced responses and thus other factors may be involved. Furthermore, the extent of the shape change did not directly correlate with the pattern or magnitude of the $[Ca^{2+}]_i$ increases, again suggesting that other signalling pathways are activated and lead to morphological events. Possible candidates include P-selectin expression on activated platelets since this is enhanced in cancer patients and has been suggested to cause platelet-cancer

interaction and metastasis (Yan and Jurasz, 2016). P-selectin appears on the platelet membrane due to alpha granule secretion and this can then bind with ligands on target cells. One such ligand reported in cancer is CD44 (Gay and Felding-Habermann, 2011a). This binding of a tumour cell ligand with platelet P-selectin not only protects tumour cells from host defence but also helps in adhesion of the tumour cell-platelet "globule" to the endothelial matrix and thus facilitates extravasation (Gay and Felding-Habermann, 2011a, Qi et al., 2015a). However, the exact mechanism of how platelets stimulate HEL cells needs additional experiments that were not possible due to time constraints during the present work. Examples are to try higher concentrations of nucleotides since the local environment of the platelet: HEL cell may see larger ATP and ADP levels than in the bulk phase. In addition, a combination of these two nucleotides could be tried along with other dense granule constituents (e.g serotonin). More sophisticated single cell imaging where the platelets are added and allowed to settle prior to stimulation could also be employed. Such experiments may require spontaneous platelet activation to be abolished, which may be achieved with an inhibitor such as PGE1.

4.4 Conclusion

TLR2/1 receptor stimulation of platelets induces Ca^{2+} responses in HEL cells via release of ATP (and possibly also ADP). P2Y1 or P2X1 receptors are not required for this response. P2Y11 receptors may be involved but improved antagonists or RNAi experiments are required to further investigate this hypothesis. TxA_2 production is also required for a large proportion of the TLR1/2-induced platelet-dependent HEL cell Ca^{2+} response which must therefore involve paracrine/autocrine activation of platelets by TXA_2 leading to ATP release. HEL cells adhered to glass cover slips show oscillatory Ca^{2+} increases as well as a shape change following exposure to Pam_3CSK_4 -stimulated platelets. However, other signalling pathways may be activated, including via direct intercellular contact.

Chapter 5. An investigation of platelet store-operated Ca^{2+} entry in healthy donors with a view to assessing its role in cancer and use as a prognostic marker of malignancy

5.1 Introduction:

A number of papers have documented an abnormal level of platelet activity in cancer that is most probably due to the effect of tumor cells on platelets (Goubran et al., 2013). Similarly, high platelet count (150,000-400,000/µl) is also associated with poor prognosis in cancer (Goubran et al., 2013). Early research indicates that cancer development could be enhanced at the site of inflammation and Rudolf Virchow first speculated the cancer-inflammation relationship (Balkwill and Mantovani, 2001, Morrison, 2012). In fact cancer is referred to as a wound that never heals; that is the natural healing system of the body is compromised in cancer (Franco et al., 2015). Given the major role of platelets in wound healing and the immune system, these small blood cells may be a major contributor in cancer pathogenesis. Therefore, in line with the hypothesis by Rudolf Virchow, the hallmarks of cancer could also be examined from the perspective of platelet activity.

In 1860, the French physician Armand Trousseau demonstrated an increased tendency of venous thrombosis in cancer (Varki, 2007). Later, a procoagulant activity was observed in his own blood which preceded his death due to pancreatic cancer (Franco et al., 2015). In the 20th century the mechanisms involved in cancer associated thrombosis were demonstrated as tumor cell induced platelet activation (TCIPA) (Gasic

et al., 1973, Karpatkin et al., 1988b).Further research has demonstrated that platelets evoke thrombus formation in cancer that initiates via platelet binding to neutrophils and production of neutrophil extracellular traps (NETs) (Gould et al., 2014). The NETs activate the intrinsic coagulation cascade that ultimately results in thrombin generation (Gould et al., 2014). Different research demonstrated the evidence of cancer biomarkers from the platelet perspective, for example CA 19-9 is a biomarker of pancreatic cancer that is found to be correlated with a high platelets/leukocyte ratio (PLR) (Miglani et al., 2013). Moreover in lung cancer, changes in the PLR is also counted as a biomarker of malignancy (Sanchez-Salcedo et al., 2016). Furthermore a high PLR in combination with a high neutrophil/lymphocyte ratio is found associated with the prognosis and stage of cancer development, and the neutrophil-platelet score is proposed as a predictor of survival in different types of cancer (Jia et al., 2015a, Emir et al., 2015, Watt et al., 2015). Also the PLR is reported as an independent (major) hallmark of venous thromboembolism in cancer (Ferroni et al., 2015).

A wide range of platelet receptors have been implicated in cancer associated thrombosis (Kim et al., 1998, Bakewell et al., 2003, Palumbo and Degen, 2007, Jain et al., 2010b). Almost all platelet receptor pathways lead to elevation in second messenger Ca^{2+} in platelets (VARGA-SZABO et al., 2009). One of the major Ca^{2+} influx pathways in platelets is SOCE that is described in detail in section 1.5.2 (see Fig.5.1 for mechanism). SOCE is a key mechanism in thrombus development (Wolf et al., 2016, Ilkan et al., 2017). STIM1 deficiency results in reduced agonist-evoked responses in platelets and impaired thrombus formation under flow in vitro (Varga-Szabo et al., 2008b). Moreover mice expressing an EF-hand mutation in STIM1 showed altered platelet activity and increased bleeding time (Grosse et al., 2007). Platelets from STIM1 knockout mice also showed impaired Ca^{2+} signals and reduced adhesion and thrombus formation compared to wild type platelets (Varga-Szabo et al., 2008b). This indicates that platelet STIM1 is a key protein in Ca^{2+} mobilization from stores and is crucial for stable thrombus formation. Similarly Orai1 (CRACM1) has been shown to be the platelet SOC channel that couples to STIM1 and essential for pathological thrombus formation (Braun et al., 2009b).

To date no study has assessed the possible role of platelet SOCE in cancer or its measurement as an indication of cancer progression. Indeed, a standardized assay for measurement of SOCE in platelets has not been fully explored, thus although tools such as thapsigargin are available to activate SOCE, it is unclear whether the length of exposure to this SERCA inhibitor or other factors can influence the quantification of SOCE. Therefore, experiments in the present chapter have conducted a careful assessment of the time-dependence of activation of SOCE in platelets from healthy donors with the aim of developing a reliable method to compare platelet SOCE between individuals in health and diseased states such as cancer.

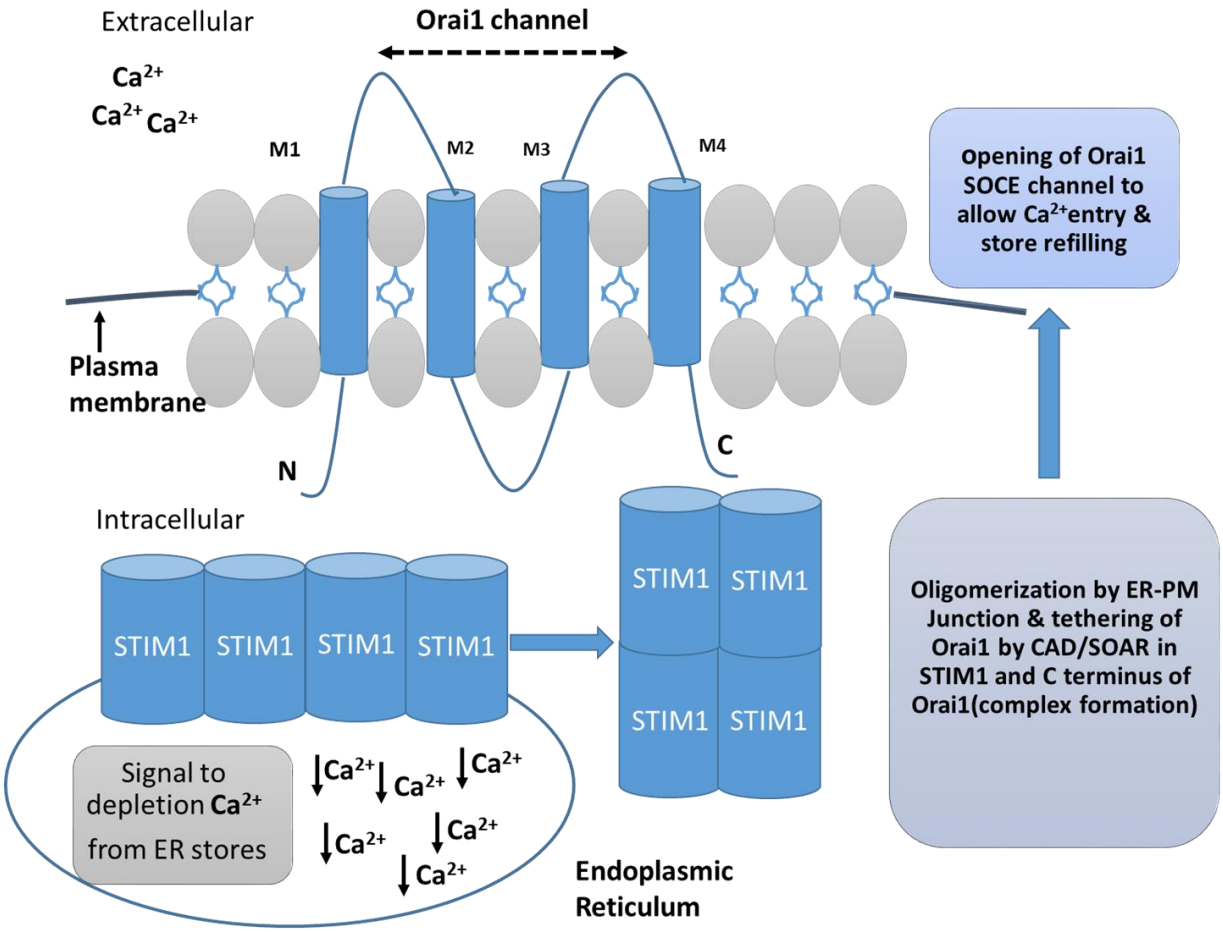

Fig 5.1 Mechanism of Sim1 and Orai1 interaction:

STIM1 is embedded in the DTS membrane. The Ca^{2+} in the DTS binds with the EF hand domain of STIM1. On depletion of Ca^{2+} from the DTS, STIM1 oligomerisation takes place. Tethering of STIM1 (CAD/SOAR) with Orai1 (C terminus) then occurs and a STIM1-Orai1 complex is formed. This opens the Orai1 in plasma membrane that allows the influx of Ca^{2+} from the extracellular environment into the cells. The Orai1 channel consists of four transmembrane proteins with a C and N terminus that interact with STIM1 on store depletion.

5.2 Results

5.2.1 Relationship between the level of SOCE and thapsigargin-incubation time

Inhibition of SERCA, for example with TG, has been used as a standard tool to activate SOCE (Lytton et al., 1991b, Sehgal et al., 2017). However, to date no study has carried out an in-depth exploration of the effect of varying the duration of exposure to the SERCA inhibitor on the activation of SOCE in platelets. Furthermore, the variability in this pathway between healthy donors has not been carefully examined. TG leads to depletion of the Ca^{2+} stores via a mechanism that is not fully understood but likely relies upon passive efflux of stored Ca^{2+} and will also depend upon the rate at which TG fully inhibits SERCAs (Watson et al., 2003). Thus, experiments initially examined the effect of increasing the duration of exposure to TG on the magnitude of SOCE. Fura-2-loaded platelets were suspended in nominally Ca^{2+}-free medium and treated with TG for a duration of 1, 2, 5, 10 and 15 minutes. SOCE was assessed by addition of 2 mM free extracellular Ca^{2+}. It was found that prolonged TG exposure caused aggregation in nominally Ca^{2+}-free saline, which could be blocked by addition of 0.2 mM EGTA at the start of the experiment. The free Ca^{2+} level was then elevated to 2 mM by addition of 2.2 mM $CaCl_2$ (for further explanation see section 2.2.5.1, chapter 2). As an overall measure of the level of SOCE, the 2 minute integral (with units of nM.sec for 2 minutes) of the Ca^{2+} increase after addition of external $CaCl_2$ was measured. The SOCE integral for 1, 2, 5, 10 and 15 minutes of TG incubation was calculated as 37323 ± 4553, 51810 ± 4582, 67948 ± 7514, 97239 ± 17243 and 82731 ± 9938 nM.sec respectively. This measurement indicates a general increase in SOCE with increased duration of TG incubation up to 10 minutes. One-way ANOVA test followed by Tukey's multiple comparison test was carried out to compare the SOCE ($P<0.05$; n=7) (see Fig 5.3). For detailed P values see supplementary table S2. The average value at 15 minutes was slightly lower than at 10 minutes, but not significantly. This suggests that

at least 10 minutes exposure to TG is required to stimulate maximal Ca^{2+} influx through SOCE for a period of 2 minutes after readdition of external Ca^{2+}.

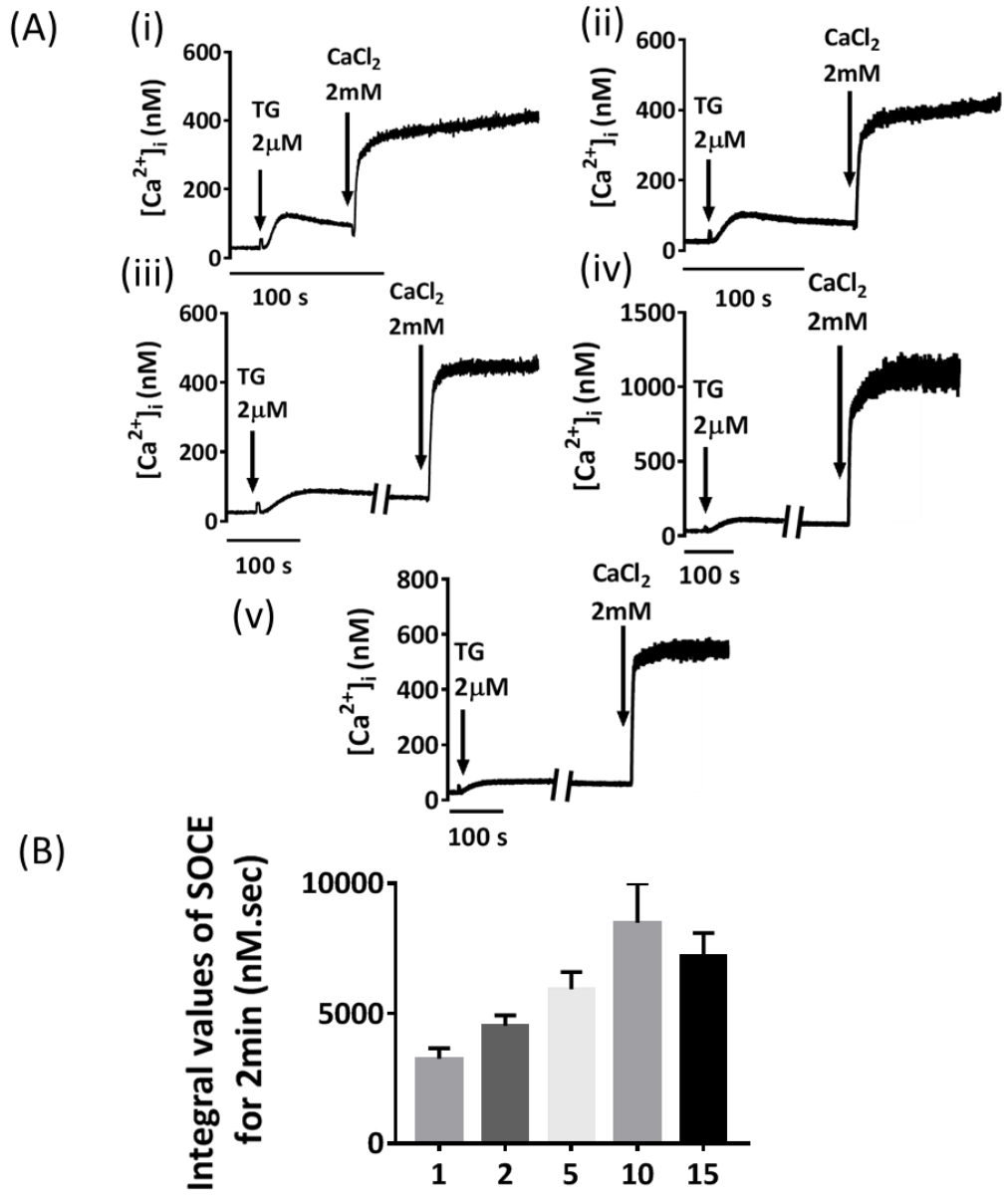

Fig 5.2 Comparison of overall increase in store operated Ca^{2+} influx with increase in TG incubation time

(A) Representative figures from one donor of SOCE following addition of 2mM Ca^{2+}after (i) 1 minute (ii) 2 minute (iii) 5 minute (iv) 10 minute and (v) 15 minutes TG incubation time. (B) Comparison of SOCE versus TG incubation time, using the 2 minute integral of the Ca^{2+} increase following $CaCl_2$ addition. One-way ANOVA followed by Tukey's multiple comparison was performed. The values at 2, 5, 10 and 15 min were significantly increased compared to 1 minute ($P<0.05$; n=7). A significant increase in the 2 minute integral was also observed between 2 minutes TG incubation time and 15 minutes ($P<0.05$; n=7) and from 5 minutes to 15 minutes ($P<0.05$; n=7) (see the summary of the statistics in supplementary Table S2).

5.2.2 SOCE increased with increase in time after addition of extracellular Ca^{2+}

The 2 minute integral is only one measurement of the level of activation of SOCE, representing the average Ca^{2+} entry over a 2 minute time period. Therefore, the intracellular Ca^{2+} increase was measured every 24 seconds throughout the period of 2 minutes after addition of 2 mM external Ca^{2+} for each of the durations of TG exposure. For a 2 minute exposure to TG, the average values for the Ca^{2+} increase at 24, 48, 72, 96 and 120 seconds after extracellular Ca^{2+} addition were calculated as 456.9 ± 62, 503.7 ± 44, 506.5 ± 48, 529.2 ± 54 and 539.5 ± 58 nM respectively. Although the general trend was upwards across these five time points, the increase was not significant ($P>0.05$; n=6) (see Fig 5.3 A) (for detail of the statistical analysis see supplementary table S3). Similarly, for a 5 minute TG incubation time, the average value of Ca^{2+} influx at 24, 48, 72, 96 and 120 seconds after extracellular Ca^{2+} addition was calculated as 619.6 ± 80, 652.8 ± 91, 678.5 ± 96, 653.2 ± 113 and 690.8 ± 99 nM respectively. A significant difference was found between 48 and 120 seconds ($P<0.05$) and 72 and 120 seconds ($P<0.05$) (n=6) (see Fig 5.3 B and supplementary table S4) for summary of the statistical analysis). Likewise, for 10 minutes TG incubation time the average value of Ca^{2+} influx at 24, 48, 72, 96 and 120 seconds after extracellular Ca^{2+} addition was calculated as 825.8 ± 175, 913.3 ± 213, 941.5 ± 217, 953.8 ± 217 and 967.3 ± 218 nM respectively.

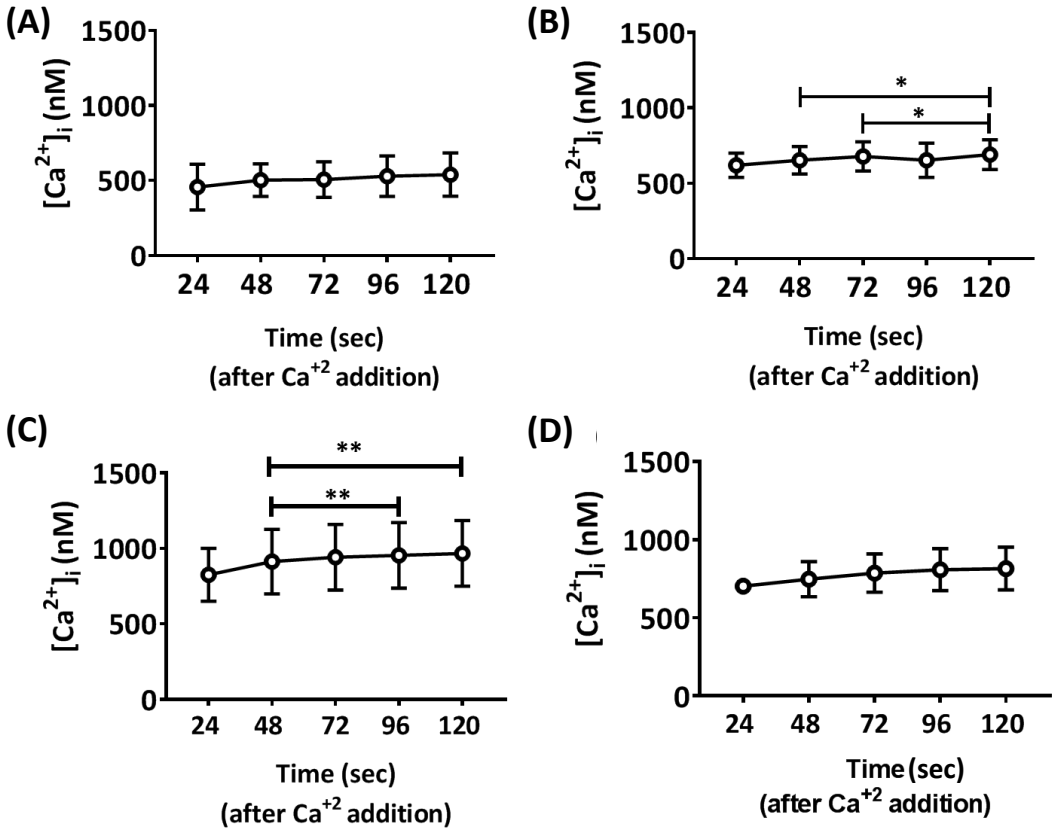

Fig 5.3 Comparison of Ca^{2+} influx at specific times following Ca^{2+} addition for different thapsigargin incubation times

Platelets were treated with 2 µM thapsigargin for different durations in the presence of 0.2 mM EGTA and extracellular Ca^{2+} increased to 2 mM by addition of 2.2 mM $CaCl_2$. (A): 2 minutes; (B): 5 minutes; (C): 10 minutes and (D): 15 minutes thapsigargin treatment before $CaCl_2$ addition. The increase in intracellular Ca^{2+} was measured at 24, 48, 96 and 120 s after Ca^{2+} addition and the average response shown from 6 donors. One-way ANOVA followed by Tukey's multiple comparison was performed. Ca^{2+} influx with 2 minute TG incubation time did not increase significantly ($P>0.05$). Ca^{2+} influx with 5 minute TG incubation time increased significantly and a significant difference was observed at 48 sec and 120 seconds ($P<0.05$) and 72 and 120 seconds ($P<0.05$) ($n=6$). Similarly Ca^{2+} influx with 10 minute TG incubation time increased significantly and a significant difference was observed at 48 sec and 96 seconds ($P<0.01$) and 48 and 120 seconds ($P<0.01$) ($n=6$). However Ca^{2+} influx with 15 minute TG incubation time did not increase significantly ($P>0.05$). All data represent mean ± SEM.

A significant difference was observed between $[Ca^{2+}]_i$ nM at 48 seconds and 96 seconds ($P<0.01$; n=6)(see Fig 5.3 C and table S5 for summary of the statistical analysis). Also, a significant difference was observed between the $[Ca^{2+}]_i$ increase at 48 and 120 seconds ($P<0.01$; n=6) (see Fig 5.3 C). However, for 15 minutes TG incubation time the Ca^{2+} influx at 24, 48, 72, 96 and 120 seconds after extracellular Ca^{2+} addition was calculated as 703.7 ± 100.9, 748.7 ± 112.5, 787 ± 122.1, 808.3 ± 134.5 and 816.2 ± 137 nM, respectively. The overall increase for 15 min TG incubation time did not increase significantly ($P>0.05$, n=6) (see Fig 5.3 D) (see supplementary table S6 for statistical summary).

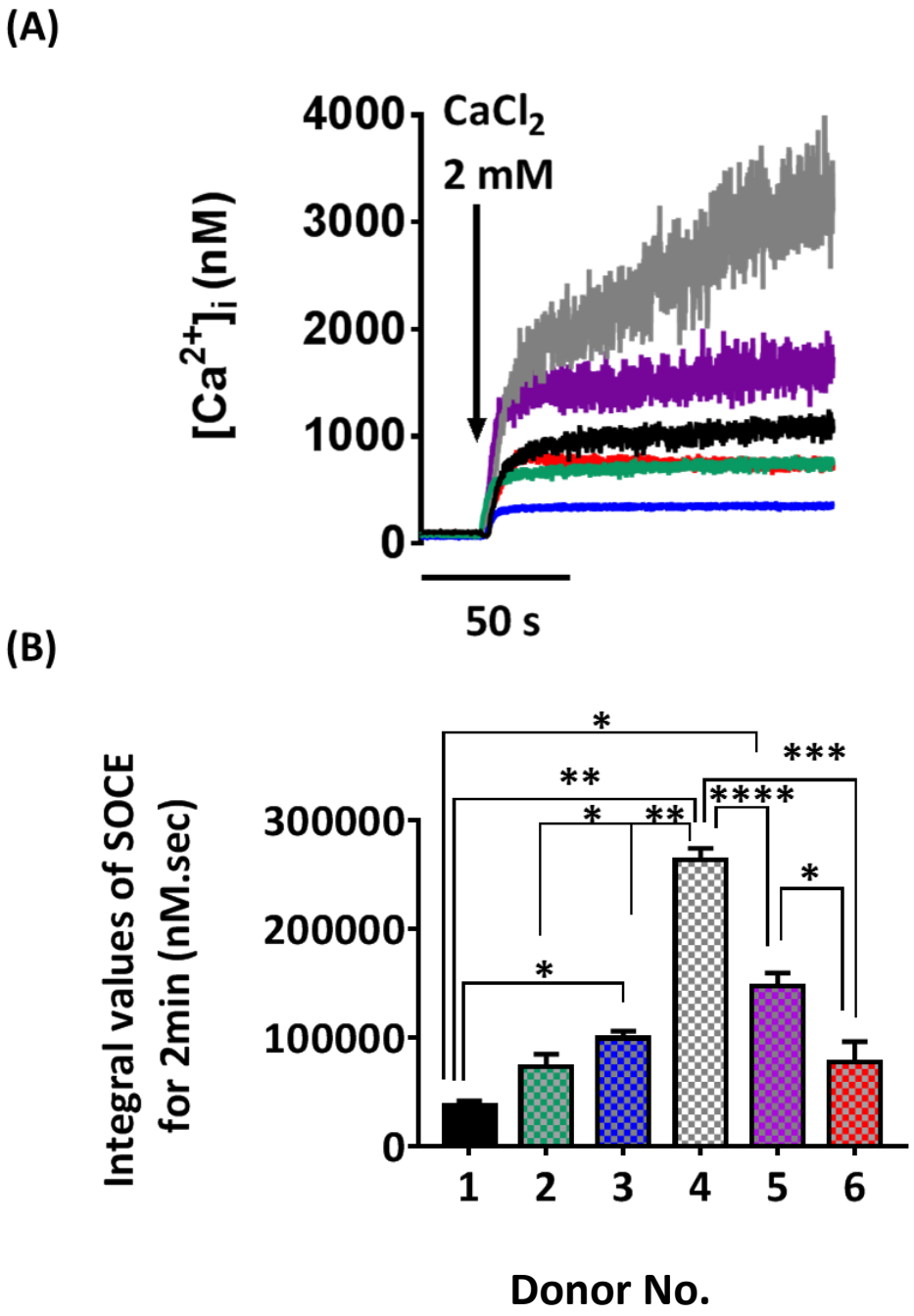

Fig 5.4 Inter-individual variability in SOCE

*(A) Representative traces of inter-individual variability in SOCE after 5 min TG-incubation time. (B) Comparison of SOCE in different individuals, using the 2 min integral of the Ca^{2+} increase immediately following $CaCl_2$ addition. Data are mean ± SEM., n=3. One-way ANOVA P<0.0001, (*p<0.05, ** P<0.01, *** P<0.001, **** P<0.0001 by Tukey's multiple range test).*

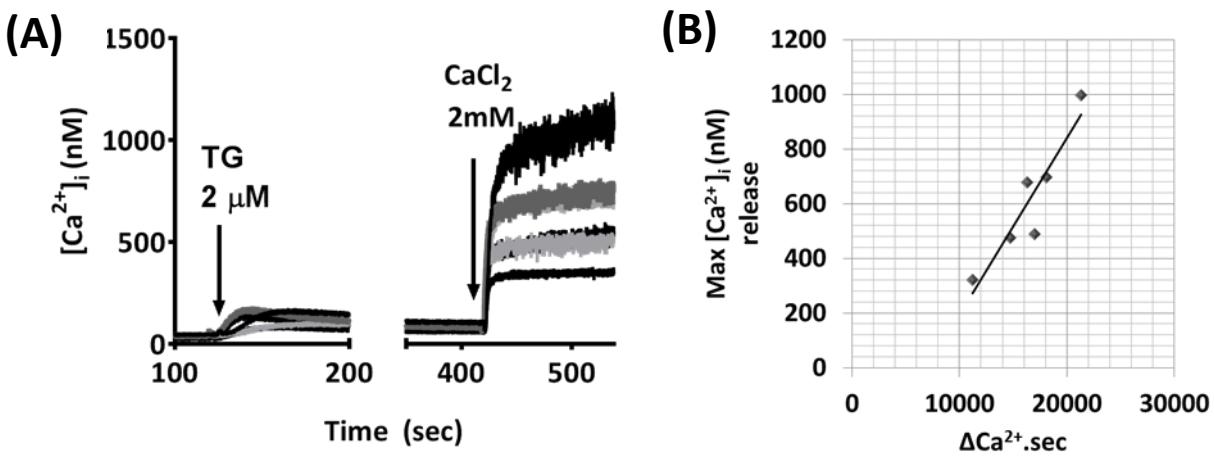

Fig 5.5 Peak of Ca^{2+} release by TG is correlated to the integral values of SOCE for 2 minutes after extracellular Ca^{2+} addition

(A) Representative recordings of SOCE in different individuals following Ca^{2+} store depletion by TG for 5 minutes. (B) Relationship between maximum Ca^{2+} release induced by TG and the 2 min integral of the subsequent SOCE. The line was fit by linear regression (R^2=0.86). Each point represents an individual donor.

5.2.3 Inter-individual variability in SOCE

The extent to which SOCE varied across a number of healthy donors was next assessed. Platelets in nominally Ca^{2+} free saline with 0.2 mM EGTA Ca^{2+} were incubated with TG and Ca^{2+} raised to 2 mM by addition of 2.2 mM $CaCl_2$ as described above. The 2 min integral of the Ca^{2+} increase following 5 min TG incubation time for different donors was calculated as 38042 ± 2181, 73615 ± 6545, 99529 ± 3782, 263351 ± 6397, 147978 ± 6749 and 77436 ± 10867 nM.sec respectively. Surprisingly the average integral values of $[Ca^{2+}]_i$ (nM) were found to be significantly different between different donors (n= 3 for each of 6 donors) (see Fig 5.4 A & B). These results suggest the level of platelet SOCE as assessed by a 5 minute exposure to TG varies significantly between healthy donors. This variation was also observed following incubation with TG for 2, 10 and 15 minutes (not shown).

5.2.4 The level of SOCE in different donors correlates with the amount of Ca^{2+} release from the DTS

The variation in SOCE between donors could simply be due to different extents to which TG releases Ca^{2+} from stores, since this clearly varied as well (Fig.5.5 A). To explore this possibility further, the peak of the TG-stimulation release was plotted against the SOCE 2 min integral for all donors (Fig.5.5 B). A strong correlation was observed for a linear relationship between these two parameters (R^2=0.86) (see Fig 5.5 B). This suggests that the most likely reason for the inter-donor variability in the magnitude of the SOCE is the amount of Ca^{2+} released from the stores by TG.

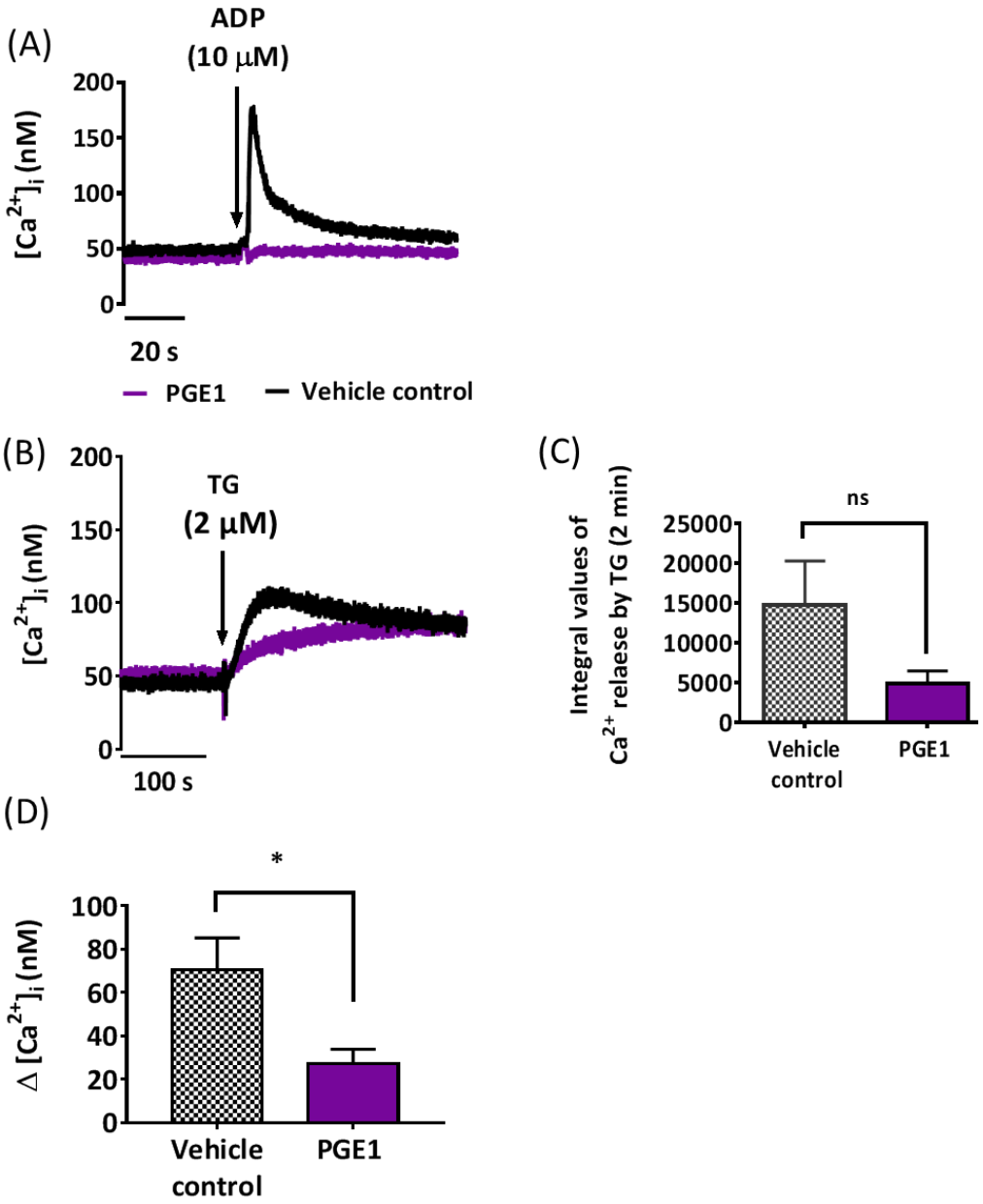

Fig 5.6 PGE1 inhibits the TG induced Ca^{2+} store release

(A) Representative recordings of the Ca^{2+} response to ADP (10 μM) in platelets in the presence of 200 nM PGE1 (purple) or vehicle control (black). (B) Representative traces of TG induced Ca^{2+} release from the platelet DTS in the presence of PGE1 (purple) or vehicle control (black). The average Ca^{2+} responses from were assessed using either the 2 min integral (C) or the peak increase (D). Using Paired t-test, the difference was significant for the peak value ($P<0.05$) but not the integral ($P>0.05$) ($n=4$). Data are the means ± SEM.

5.2.5 The TG-evoked Ca^{2+} release is partially dependent upon a pathway inhibited by cyclic AMP elevation with PGE1

It is known that TG is an inhibitor of the SERCA Ca^{2+}ATPase, and activates SOCE through depletion of the Ca^{2+} stores (Bird et al., 2008). However, the exact mechanism whereby Ca^{2+} is released from the stores following SERCA inhibition is still not clear. The main Ca^{2+}-permeable channels on the DTS store membrane are IP_3 receptors that bind cytosolic IP_3 and are responsible for Ca^{2+} release following platelet activation (VARGA-SZABO et al., 2009). Thus, variability in the background activity of IP_3 receptors could account for the inter-donor variability in the TG-induced release and subsequent SOCE. To assess this possibility, the effect of inhibiting IP_3R with PGE1-induced cyclic AMP levels (Tertyshnikova and Fein, 1998, Quinton and Dean, 1992b) was tested on the TG-induced Ca^{2+} release. To first test the inhibitory effect of PGE1 on agonist-evoked Ca^{2+} increases, ADP (10 µM)-evoked Ca^{2+} increases were measured in the presence and absence of 200nM PGE1 (200 nM for 5 minutes). 200 nM PGE1 completely abolished the ADP-evoked response in platelets (see Fig 5.6 A), an effect also reported by Fox and co-workers in which PGE1 dose dependently inhibited the Ca^{2+} responses and aggregation in platelets and a complete block of ADP response was observed with 100 nM PGE1 (Fox et al., 2004). TG induced Ca^{2+} release from platelet DTS was analysed in the presence and absence of PGE1 (see Fig 5.6 B). The Ca^{2+} release, assessed from the integral of the Ca^{2+} increase for 2 minutes after exposure of PGE1 was calculated as 4954 ± 1564 nM.sec while in the absence of PGE1 (with the vehicle control) the value was 14815 ± 5457 nM.sec, a difference which was not significant ($P>0.05$; n=4) (see Fig 5.6 C). However, PGE1 appeared to reduce the peak more than the plateau of the TG-induced response, thus the effect on the average peak value was also assessed. PGE1 did cause a significant decrease in the TG-evoked peak Ca^{2+} release (increase 70 ± 29 in control vs 27 ± 13 nM with PGE1 $P < 0.05$, n=4; Fig. 5.6 D). Thus, elevated levels of cAMP have a significant effect on the TG-induced Ca^{2+} release from the DTS, suggesting that IP_3 activity contributes to the efflux of Ca^{2+} from the stores following SERCA inhibition.

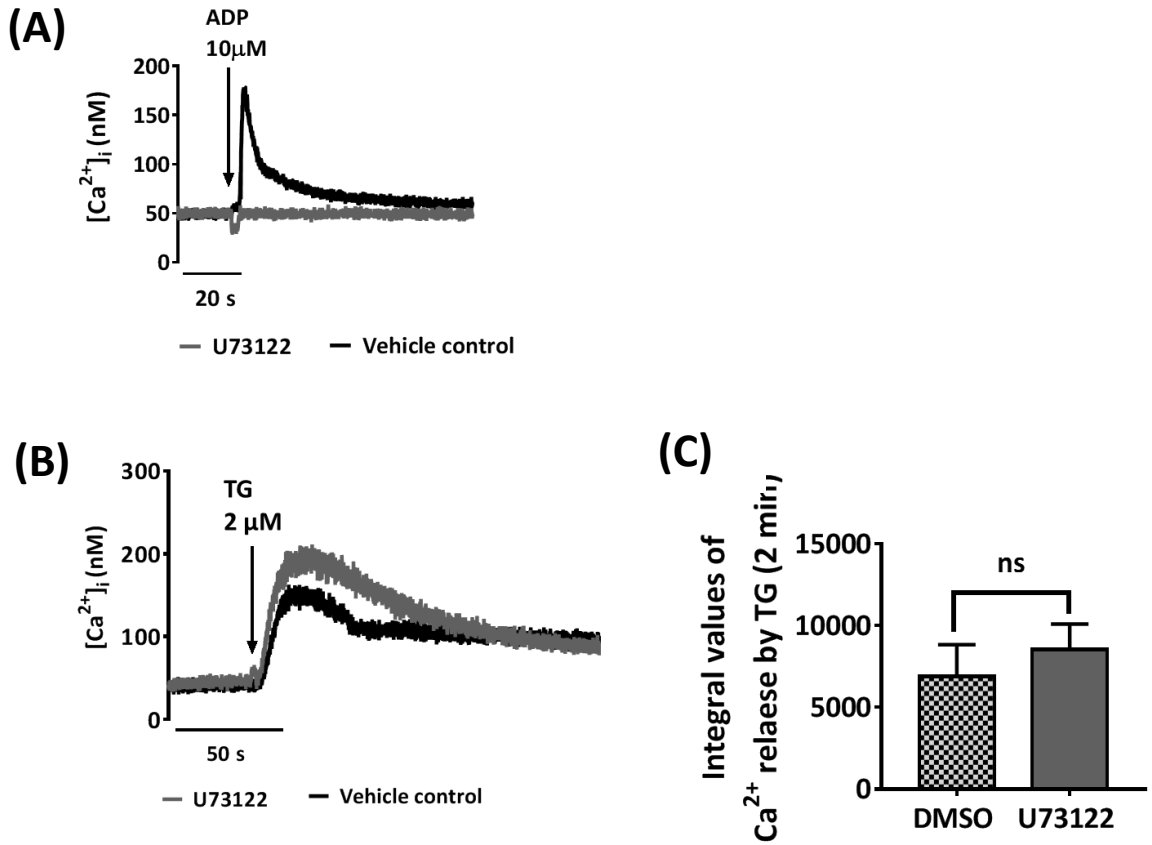

Fig 5.7 U73122 has no significant effect on the TG induced Ca^{2+} release

(A) Sample ADP (10 μM)-evoked Ca^{2+} responses in the presence of U73122 (grey) and vehicle control (black). (B) Representative traces of TG induced Ca^{2+} release from the platelet DTS in the presence of U73122 (grey) and vehicle control (black). (C) Comparison of integral values of Ca^{2+} release from stores by TG in the presence of U73122 (grey) and vehicle control (black). No significant difference was found between the two conditions (P>0.05; n=5, Paired t-test). Data represent mean ± SEM.

5.2.6 TG evoked Ca^{2+} release is independent of PLC activity

PGE1-induced cAMP formation will also inhibit PLC (Schmidt et al., 2001), thus reduced production of IP_3 and reduced IP_3 activity could account for the effect of PGE1 on TG-induced Ca^{2+} release. To assess this possibility, the PLC inhibitor U73122 (Klein et al., 2011) was tested on the TG-evoked Ca^{2+} release. The ability of this reagent to inhibit PLC was first assessed on platelets via its effect on the Ca^{2+} release stimulated by ADP, which is known to occur mainly through the generation of IP_3 (Lockhart and McNicol, 1999). 5 µM U73122 completely blocked the Ca^{2+} response evoked by 10 µM ADP (see Fig 5.7 A). TG induced Ca^{2+} release was then assessed in the presence and absence of 5 µM U73122 (see Fig 5.7 B). The integral of the Ca^{2+} increase for 2 minutes after exposure of U73122 was 8441 ± 1654 (nM.sec), which was not significantly different from the response in the vehicle control (6804 ± 1885 nM.sec; $P>0.05$; n=3) (see Fig 5.7 C). These data show that PLC activity is not significantly contributing to the TG induced Ca^{2+} release.

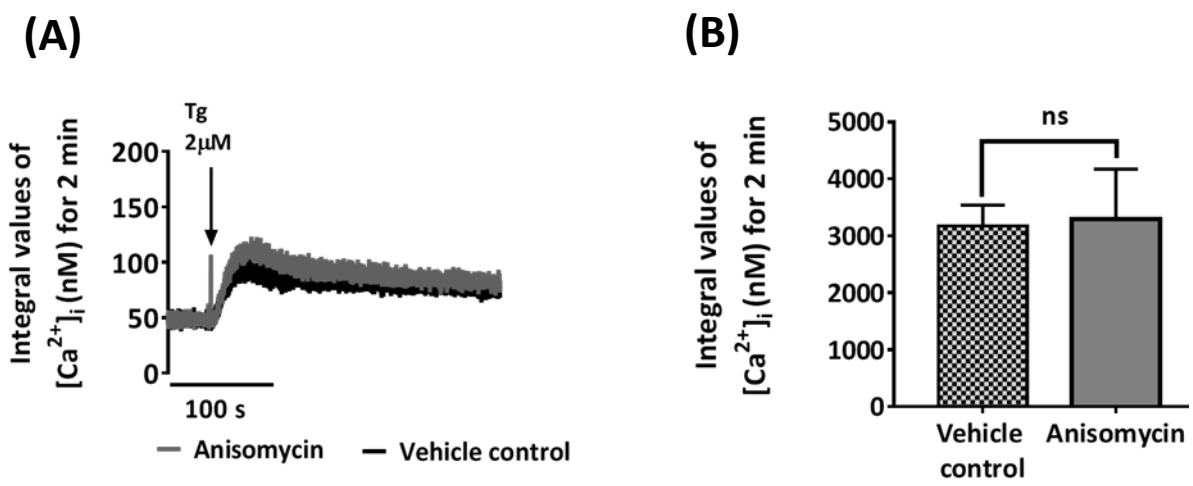

Fig 5.8 Anisomycin has no effect on TG response in platelets

(A) Representative traces of TG induced Ca^{2+} release from the platelet DTS in the presence (Grey line) and absence (black line) of anisomycin, a translocon blocker. (B) Comparison of integral values of Ca^{2+} release from stores by TG in the presence (grey bar) and absence (black pattern bar) of anisomycin. No significant difference was found between the two conditions (P>0.05; n=5, Paired t-test). Data represent mean ± SEM.

5.2.7 Anisomycin showed no effect in TG response

A pathway that has been proposed to underlie Ca^{2+} leakage from the ER stores is the translocon complex of proteins (Van Coppenolle et al., 2004). To analyse the effects of translocon on TG induced Ca^{2+} release from the DTS, experiments used anisomycin, a reported inhibitor of this pathway (Johnson et al., 2014). TG induced Ca^{2+} release was measured with and without anisomycin (see Fig 5.8). The average integral values for Ca^{2+} release for 2 minutes following TG in the presence of anisomycin was 2015 ± 3292 nM.sec, which was not significantly different from the average values obtained in the vehicle control (2563 ± 3170 nM.sec; P>0.05; n=3) (see Fig 5.8 B). Thus, the translocon

complex does not appear to be involved in the TG-induced Ca^{2+} release pathway of platelets.

5.2.8 TBHQ-activated acidic store depletion did not affect TG response in platelets

Another organellar Ca^{2+} storage site found in platelets is the acidic store (Rosado, 2011a). Ca^{2+} efflux from the acidic stores is reported to be associated with the second messenger nicotinic acid adenine dinucleotide phosphate (Rosado, 2011b). To find out if the variability in TG induced Ca^{2+} release and thus SOCE is affected by Ca^{2+} release from the acidic stores, TBHQ was used to inhibit the SERCA isoform associated with this store and thus induce acidic store depletion (Redondo et al., 2007)(see Fig 5.9 A). The average 2 minute integrals for TG-evoked Ca^{2+} release in the presence of TBHQ was 8859 ± 67 nM.sec., which was not significantly different from the response to TG in the presence of vehicle control (10432 ± 743 nM.sec; $P>0.05$; n=3) (see Fig 5.9 B). Thus, the content of the acidic stores does not influence Ca^{2+} leaks from the DTS following TG exposure.

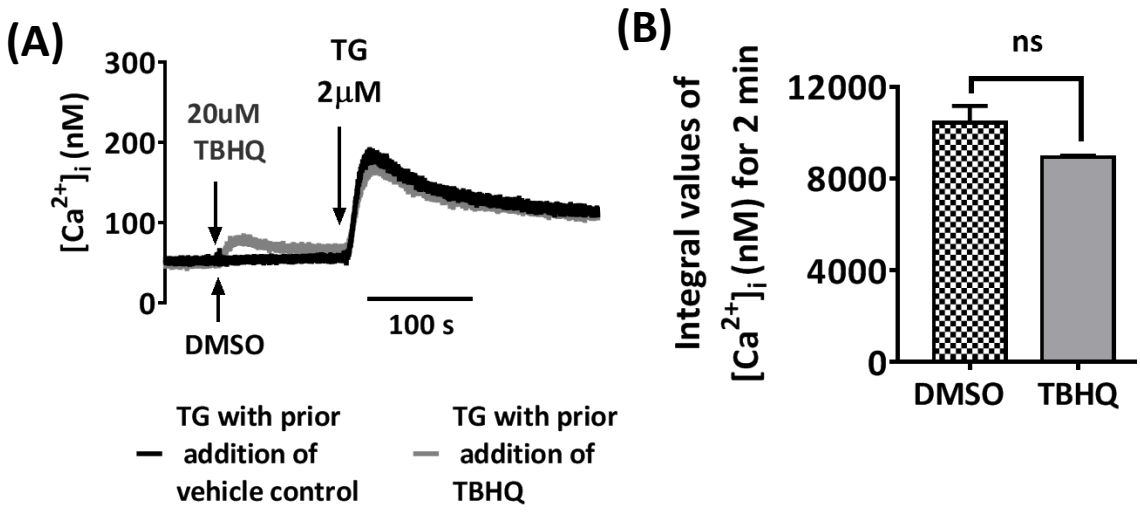

Fig 5.9 TBHQ has no significant effect on TG induced response

(A) Representative traces of TG induced Ca^{2+} release from the platelet DTS in the presence of TBHQ (grey line) or vehicle control (black line). (B) Comparison of integral values of TG-evoked Ca^{2+} release from stores in the presence of TBHQ (grey bar) or vehicle control (black pattern bar). Paired t-test was performed. No significant difference was found between the two conditions (P>0.05 by paired t-test n=5). Data represent the mean ± SEM.

5.2.9 Valinomycin showed no effect on TG-induced SOCE

Another possibility for the variation in SOCE could be due to differences in membrane potential which sets the electrical driving force for Ca^{2+} entry (Fliegert et al., 2007). To set a constant membrane potential, platelets were treated with K^+ ionophore, valinomycin. Valinomycin is known to set membrane potential at a constant level, equivalent to the K^+ equilibrium potential (Večeř et al., 1997). Platelets of every donor were treated either with valinomycin (3 µM) (grey traces in Fig 5.10 (A)) or vehicle control (black traces in Fig 5.10 (A)) and then incubated with TG for 5 minutes, followed by extracellular $CaCl_2$ addition (see Fig 5.10 A). Average 2 minute Integral values of TG-induced SOCE with vehicle control was 50880 ± 7830 which was not significantly affected by valinomycin (52392 ± 9834 nM.sec; P>0.05; n=6) (see Fig 5.10 (B)). This suggest that variation in membrane potential is not responsible for the inter-donor variability in SOCE.

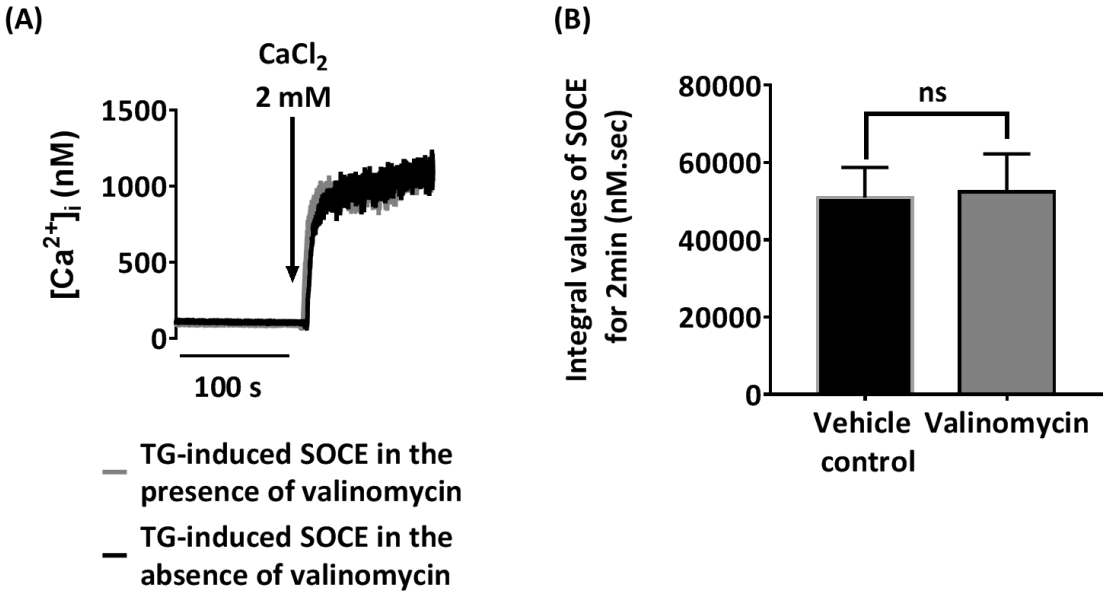

Fig 5.10 Valinomycin did not affect the TG-induced SOCE in platelets

(A) Representative traces of TG-induced SOCE in the presence of valinomycin (3 µM) (grey trace) and with vehicle control (black trace). (B) Average 2 minute integral of TG-Induced SOCE with vehicle control (black bar) and with valinomycin (grey bar). Data represent the mean ± SEM. ns=not significant; n=6 (analysed by Paired t-test).

5.2.10 Inter-donor variability of SOCE-induced platelet aggregation

A major readout of platelet function that is used clinically to assess platelet reactivity, is aggregation. However, the extent to which TG-dependent Ca^{2+} entry can induce aggregation and whether this varies between donors is unclear. Therefore, platelets were treated with TG for 5 minutes followed by addition of 2 mM Ca^{2+} (see Fig 5.11 A) during standard light transmission aggregometry measurements. Maximum percentage aggregation values were calculated for different donors and compared. The average increase in percentage aggregation observed was 55 ± 1.7, 69 ± 0.9, 31 ± 14, 47 ± 11 and 76 ± 8 % for the 5 individuals donors respectively. This was only significantly different between 2 healthy individuals (i.e. donor no. 3 & 5; n=5 donors) (see Fig 5.11 B). Thus SOCE-induced aggregation after TG shows less variability than the direct measurement of the Ca^{2+} increase itself. This may be due to the multiple steps involved in aggregation, which may themselves show some variability between healthy individuals.

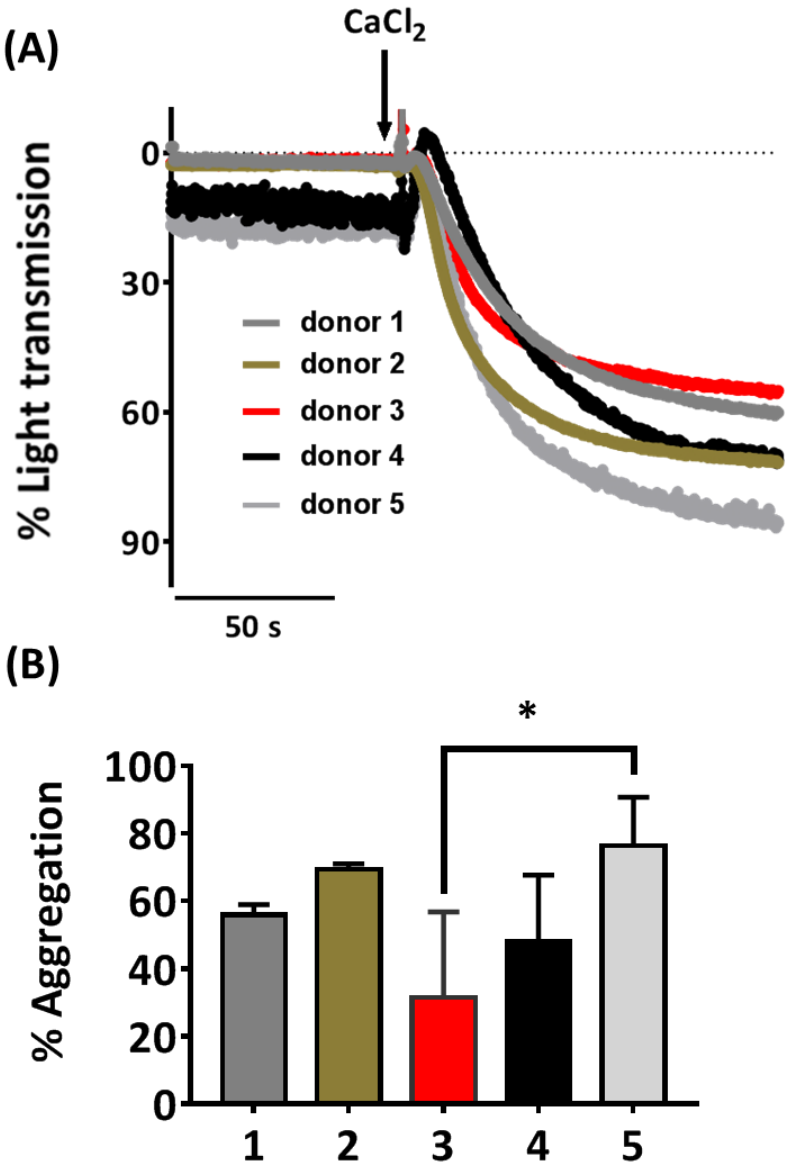

Fig 5.11 Inter-donor variability of SOCE-induced platelet aggregation

(A) Representative figures of aggregation induced by SOCE in platelets. Percentage transmission represents the percentage aggregation. (B) Statistical analysis of percentage aggregation in 5 donors induced by SOCE. A significant difference was observed in percentage aggregation of donor no. 3 and 5 (P=0.03; by Tukey's multiple comparison test following one-way ANOVA; n=3). Data represent mean ± SEM.

5.2.11 Inter-donor STIM1 and Orai1 expression

Another possible reason for the inter-individual variability in SOCE could be a difference in expression of the key proteins that take part in SOCE. These are primarily STIM1 located on the DTS and Orai1 located on the cell membrane (Rosado, 2016). The expression of these proteins in 4 donors was compared using Western blot and antibodies selective for Stim1 and Orai1, which were compared to the housekeeping gene GAPDH. Percentage band density for GAPDH was calculated as 23 ± 1, 21 ± 2, 20 ± 3 and 19 ± 1 % for each of the four individual donors (see Fig 5.12 A ii). Percentage intensity of STIM1 calculated with reference to GAPDH was 159 ± 50, 140 ± 13, 132 ± 2 and 129 ± 2 %, respectively for these four individuals (see Fig 5.12 C ii). Using one-way ANOVA and Tukey's multiple comparisons these differences were not found significant ($P>0.05$; n= 4 donors) (see Fig 5.12 C iii).

Similarly, the intensity of Orai1 calculated as a percentage relative to GAPDH was 99 ± 13, 67 ± 21, 111 ± 28 and 127 ± 13 % for each of the four individuals respectively (see Fig 5.12 B ii). One-way ANOVA and Tukey's multiple comparisons were carried out to analyse the significance in differences. No significant difference was found in Orai1 expression of individuals ($P>0.05$; n= 4 donors) (see Fig 5.12 B ii). This indicates that, using Western blot, no significant differences could be detected in the expression of the main proteins taking part in SOC influx.

5.2.12 Inter-individual variability in Ca^{2+} responses to cyclopiazonic acid

All the previous experiments suggest the main variability may occur at the level of Ca^{2+} release. Therefore, SOC influx following another inhibitor of the SERCA located on the DTS, cyclopiazonic acid (CPA), was examined for a number of donors. CPA acts via a different mechanism to TG to inhibit the SERCA (Demaurex et al., 1992, Di Marino et al., 2015). Therefore, different batches of platelets from same individuals were treated

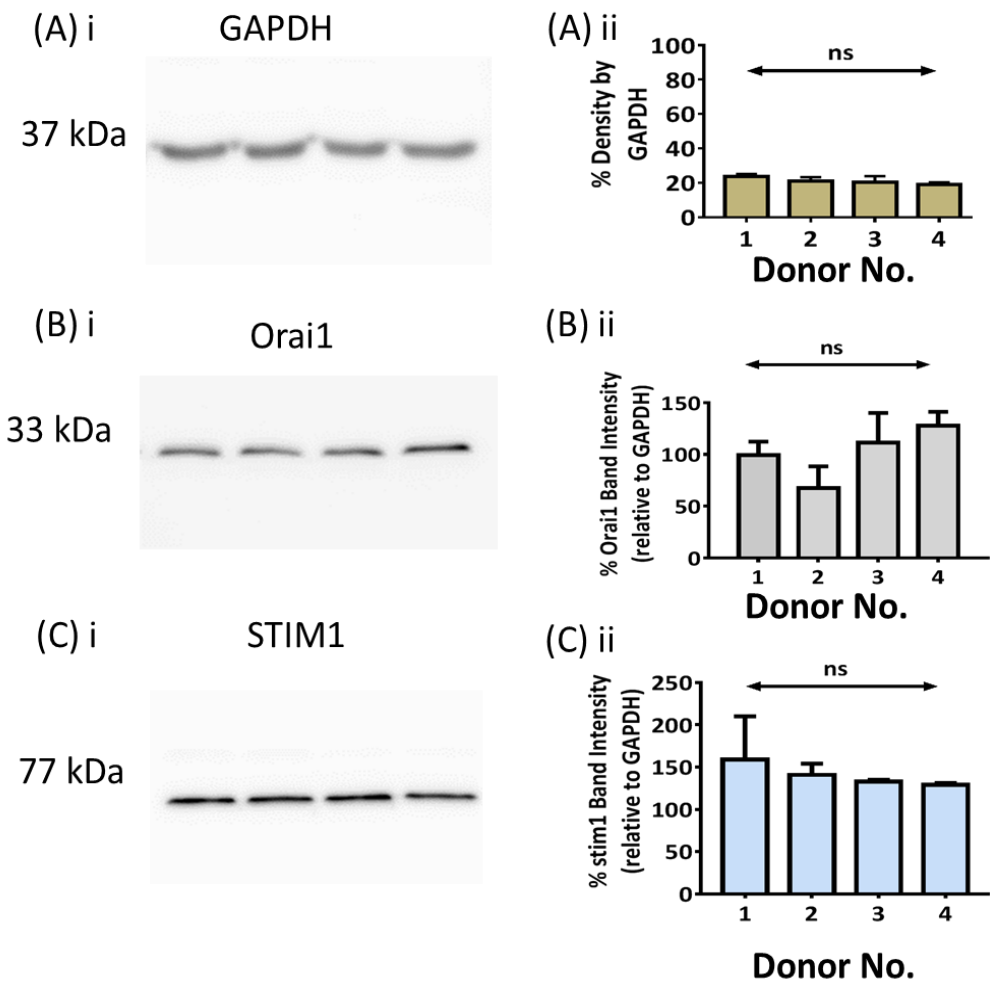

Fig 5.12 No significant inter-individual variability observed in expression of STIM1 and Orai1:

(Ai) Representative blots of GAPDH. (A ii) Comparison of percentage density of GAPDH expressed in different individuals. No significant difference was observed in percentage density of GAPDH expressed in different individuals (P>0.05; n=3). (Bi) Representative blots of Orai1. (B ii) Comparison of percentage density of Orai1 band intensity relative to GAPDH expressed in different individuals. No significant inter-individual variability was observed in percentage of Orai1 band intensity relative to GAPDH (P>0.05; n=3). (Ci) Representative blots of STIM1. (C ii) Comparison of percentage density of STIM1 band intensity relative to GAPDH expressed in different individuals. No significance difference was observed in STIM1 band intensity relative to GAPDH expressed in different individuals (P>0.05; n=3). Data represent mean ± SEM compared by one-way ANOVA test.

with CPA and TG for 5 minutes followed by addition of 2mM Ca^{2+} (see Fig 5.13 A i and B i). The average 2 minute integral values of SOCE following CPA was 65428 ± 8850, 38991 ± 2238 and 40949 ± 1563 nM.sec respectively. One-way ANOVA and Tukey's multiple comparisons were carried out to analyse the significance in differences. No significant difference was observed between all individuals (see summary of statistics in supplementary table S7) (see Fig 5.13 A ii).

In contrast, the TG-evoked SOCE within the same individuals was 263351 ± 6397, 147978 ± 6749 and 77436 ± 10867 nM.sec respectively. Using one-way ANOVA and Tukey's multiple comparisons, a significant difference was observed between all individuals ($P<0.05$; n= 3 donors) (See summary of statistics in supplementary table S3 (B)) (see Fig 5.13 B ii).

These results suggest that SOCE following Ca^{2+} store depletion with CPA- shows less variability between donors compared to thapsigargin. However, more donors would be needed to confirm whether thapsigargin and CPA behave differently in relation to the measurements made.

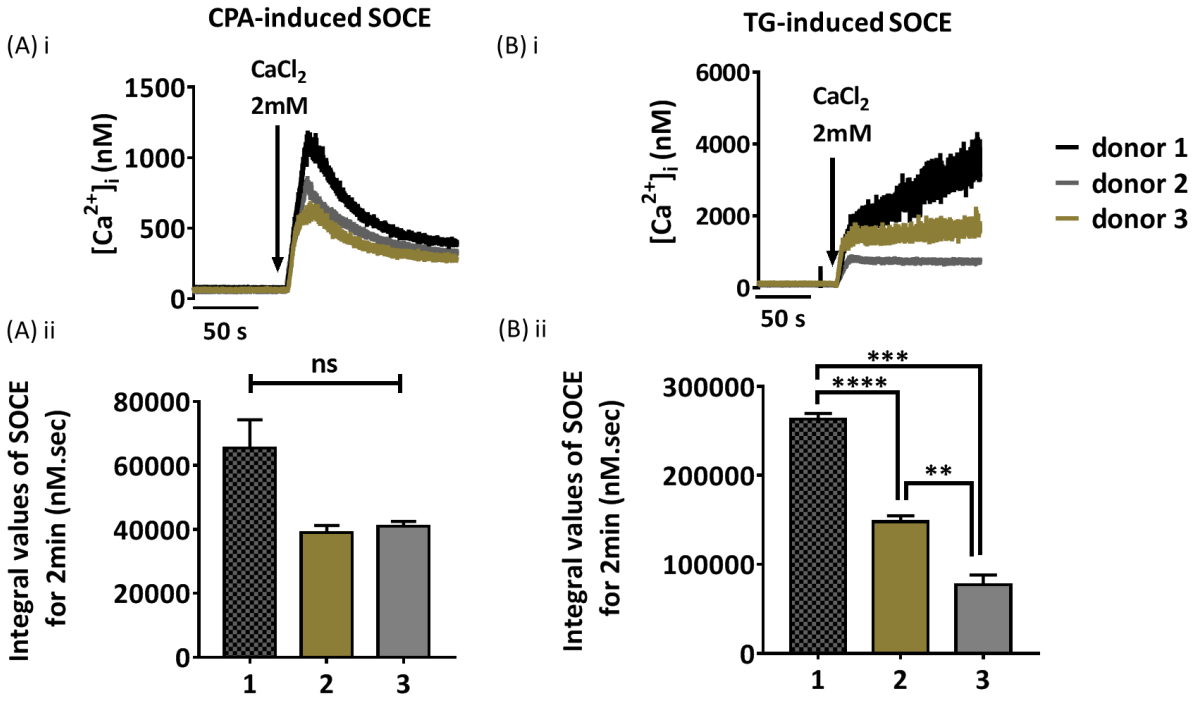

Fig 5.13 No significant inter-donor variation in SOCE following depletion of stores with cyclopiazonic acid.

*(Ai) Representative traces showing SOCE following treatment of platelets with CPA for 5 minutes in 3 different donors. (A ii) Average 2 minute integral values of CPA-induced SOCE in different donors. Data are the means ± SEM., n=3. One-way ANOVA; P>0.05. (Bi) Representative traces of TG-induced SOCE in platelets incubated with TG for 5 minutes in 3 different donors. (B ii) Average 2 minute integral values of TG-induced SOCE in different donors. Data are the means ± SEM., n=3. One-way ANOVA P<0.0006, (** P<0.01, *** P<0.001, **** P<0.0001 by Tukey's multiple range test).*

5.3 Discussion

This chapter has assessed the extent to which SOCE varies in healthy individuals and the underlying reasons for this variability. The level of SOCE with respect to an increase in TG incubation time was initially assessed. These data show that the amount of Ca^{2+} influx after TG-induced store depletion increases with the TG incubation time up to approximately 10 minutes. This indicates that the more Ca^{2+} that is depleted from stores, the greater the activation of SOCE. The amount of Ca^{2+} in intracellular stores regulates the level of SOCE Ca^{2+} influx in non-excitable cells (Parekh and Putney Jr, 2005). This conclusion was also shown by electrophysiological studies of the exceptionally Ca^{2+} selective current, called I_{CRAC}, that is now known to be carried by Orai1 channels (Hoth and Penner, 1992, Feske et al., 2006). The increase in Ca^{2+} influx with store depletion is due to increased activation of STIM1 following store depletion that promotes movement of STIM1 to a location within 10-25 nm of Orai1 in the PM (Wu et al., 2006a). Store depletion increases the ER-PM contact sites where STIM1 and Orai1 can interact by up to 62 % (Wu et al., 2006b). Studies of a T cell line show that the movement of Stim1 and its association with Orai1 occurs in proportion to the level of Ca^{2+} store depletion. Stim1 relocation and I_{CRAC} activation are minimal at a store content of more than approximately 400 nM and maximal when the level has decreased to less than 100 nM; this activation by store Ca^{2+} displayed a Kd ≈190 nM and a high level of co-operativity (≈4) (Luik et al., 2008a). This level of detail has not been studied in platelets, which are difficult to transfect. However, clearly the results in this Chapter agree with this pervious study in T cells as the more store depletion that occurs in platelets, the greater the level of SOCE. This relationship, particularly the high level of co-operativity may allow the SOCE activation mechanism to occur rapidly and strongly following agonist activation and support functional responses. In line with this notion, Stim1-/- mice clearly show reduced thrombus stability in vivo (VARGA-SZABO et al., 2009).

Comparison of platelet SOCE in healthy individuals showed a high level of variation between donors. This was unexpected since previous studies have demonstrated a non-

linear relationship between store release and I_{CRAC} studied in rat basophilic leukemic cells such that SOCE is activated over a narrow range of store Ca^{2+} depletion (Parekh et al., 1997). Although unexpected, a number of platelet receptor responses show a markedly wide range in the normal population, such as aggregation downstream of ADP or thrombin (Edelstein et al., 2014, Fontana et al., 2003). Furthermore, variable SOCE responses have been observed in other cell types, such as different clones of HEK-293 cells (Babnigg et al., 2000). A study of single HEL cells also reported a marked intercellular variability in SOCE in response to thrombin (Somasundaram et al., 1997), in that study there was no correlation found between the size of the SOCE and the amount of TG or thrombin-evoked Ca^{2+} release from the stores. The underlying basis for the variability in HEL cell SOCE was not explored in detail but a 5 fold difference in maximal TG-evoked I_{CRAC} was observed, which was taken to reflect a variability in expression of the underlying channel or activating protein (now known to be Stim1). A later study conducted on HeLa cells shows that variations in the stage of the cell cycle may contribute to the intercellular heterogeneity as the close relationship between Ca^{2+} release from stores and SOCE was broken during mitosis (Preston et al., 1991). Since platelets are derived from terminally differentiated cells (megakaryocytes), it would be expected that their SOCE would not show such inter-cellular variation due to the cell cycle. Instead, the varying amplitude of the SOCE in platelets from different individuals showed a close correlation with the peak of the TG-evoked Ca^{2+} release, suggesting that different levels of store depletion by the SERCA inhibitor were the main reason for the inter-donor variability. A range of differing factors were explored that may underlie the pathway responsible for the Ca^{2+} efflux pathway from the stores following SERCA inhibition that may then explain the variable SOCE. One possibility is background activity of the IP_3 receptor itself. TG does not stimulate an increase in IP_3 levels (Putney, 1990, Jackson et al., 1988b). The increase in cytosolic Ca^{2+} was attributed to increased expression of IP_3 in rat enterocytes that became maximal at 40 s after treatment with NAG-ST enterotoxin for cytosolic release (Hoque et al., 2001). Since the exact pathway through which the Ca^{2+} leaves the store following exposure to TG, is unknown. The main route for agonist-evoked Ca^{2+} efflux for most agonists is IP_3R and this can be totally inhibited via an increase in cAMP and PKA activation following stimulation of Gs-coupled

receptors with PGE1 (Quinton and Dean, 1992a, Tertyshnikova and Fein, 1998, Fung et al., 2012a). This property is important physiologically as a major mechanism whereby endothelial-derived inhibitors PGI_2 (and NO, via cGMP and PKG) maintain platelets in a resting state in the intact circulation (Schwarz et al., 2001a). In the present work, PGE1 reduced the TG-evoked Ca^{2+} release providing evidence that IP_3 receptor activity underlies the Ca^{2+} efflux pathway from the DTS after SERCA inhibition. Indeed, in a previous study, PGE1 was shown to reduce platelet SOCE up to 50 %, although this was interpreted as a negative modulation of SOCE by cyclic nucleotides rather than the extent of release (Rosado et al., 2001). The effect of PGE_1 indicates that IP_3Rs are a crucial route for TG-induced Ca^{2+} release from the store. Thus, variability in activity of this pathway could contribute to the inter-donor variations in SOCE. In a study using rat enterocytes, an IP_3R inhibitor (dantrolene) caused a 60 % reduction in SOCE which could be explained by altered levels of store release (Hoque et al., 2001). Thus, either variation in the background level of IP_3 or expression of the channel itself could lead to variable TG-evoked release and subsequent SOCE variable background IP_3 levels would result from altered PLC activity, however PLC inhibitors did not affect the TG induced Ca^{2+} release from the stores. This conclusion is also supported by other studies showing that Ca^{2+} stores could be depleted without activation of PLC (Jackson et al., 1988b, Thastrup et al., 1990, Putney and Tomita, 2012, Jackson et al., 1988a). Another Ca^{2+} leakage channel is the translocon that is found on the DTS and ER (Zbidi et al., 2011a, Van Coppenolle et al., 2004). However, a blocker of this pathway (anisomycin) failed to alter the TG-evoked Ca^{2+} release) in accordance with the finding that anisomycin has no effect on the calcium content of the ER (Van Coppenolle et al., 2004). Also, it was in agreement with the previous research stating that anisomycin has detectable role in platelet functional responses (Pampolina and McNicol, 1999).

Another intracellular Ca^{2+} storage organelle in platelets is the acidic store, whose regulation is poorly understood (Rosado, 2011b). However, TBHQ, an inhibitor of the SERCA3 isoform found on the acidic stores (López et al., 2005) had no influence on the TG-induced Ca^{2+} release from stores. This finding is consistent with a previous study demonstrating that prior addition of TBHQ did not abolish the TG-induced Ca^{2+} release

from the stores in bovine adrenal chromaffin cells (Robinson et al., 1992). It therefore suggests that TBHQ-sensitive acidic stores are separate from the TG-sensitive stores, at least in terms of their sensitivity to thapsigargin (Robinson et al., 1992) and that the acidic store does not contribute to the inter-donor variability in SOCE.

Differences in metabolic state may result in differences in membrane potential that could affect the activity of different drugs (Le Guennec et al., 2016). For example TG was reported to cause an extensive drop in membrane potential when added to isolated rat liver mitochondria (Vercesi et al., 1993). However, the K^+ ionophore valinomycin, which is known to set the membrane potential in platelets at the K^+ equilibrium potential (-93 mV in the salines used) (Mahaut-Smith et al., 1990b) had no effect on the TG-induced SOCE. Thus varying membrane potential does not have any effect on the inter-donor variability in TG-induced SOCE.

TG-induced SOCE also produced aggregation in platelets as reported in a previous study for both CPA and TG (Huang and Kwan, 1998). Across 5 donors, only one of 5 donors was found to have a different level of TG-induced aggregation, compared to a greater degree of variability for TG-induced SOCE measured at the level of intracellular Ca^{2+}. In previous reports, aggregation responses to a number of agonists has shown significant variation across the population which reflect heritable factors including sequence variations in GPIIb/IIIa and fibrinogen (O'donnell et al., 2001). The lower degree of variability in aggregation compared to Ca^{2+} entry may reflect the fact that multiple more steps are involved in the aggregation response, which all may show genetic variation between individuals, either in the level of expression or activity that does not necessarily correlate with that of TG-induced SOCE. Such factors include inside-out platelet activation (Malcolm and Fitzpatrick, 1992) and Fb release from the activated platelets (Kaplan et al., 1981). Using Western blotting, no difference could be detected in the expression of the main proteins involved in SOCE, namely STIM1 and Orai1. However, caution should be taken with over-interpreting these data due to the limited number of individuals studied. Also, confirmation of the antibody specificity would need to be done to confirm these results. Further work on SOCE variability should assess a large

population with a consideration of certain differentiating factors like age, gender or geographical distribution.

A final set of experiments then assessed whether the variability in SOCE was also observed with CPA, a SERCA inhibitor that also activates SOCE via activation of Orai1 channels (Luik et al., 2008a) CPA and TG both inhibit SERCA1a, but differ in their binding site (Di Marino et al., 2015). In addition, whilst the inhibition by TG is essentially irreversible (Lytton et al., 1991a), the block by CPA is reversible (Luik et al., 2008b). Interestingly, the SOCE after treatment with CPA showed less inter-donor variability compared to TG, however due to time constraints, only a small number of donors were assessed and the underlying reasons for the difference between the two SERCA inhibitors was not investigated. Nevertheless, this provides an interesting basis for future investigations for this apparent difference and whether CPA would prove to be a better tool to assess platelet SOCE.

5.4 Conclusion:

The SERCA inhibitor thapsigargin is commonly used to activate store depletion and thus store-operated Ca^{2+} entry in platelets, however a clear inter-donor heterogeneity was observed that complicates assessment of changes in this pathway during disease states such as cancer. The underlying reason for the variability is still nuclear, and may involve different levels of efflux of the store Ca^{2+} content via a pathway in part controlled by cyclic AMP and thus could be the IP_3 receptor itself. In contrast, less variation was observed following store depletion with another SERCA inhibitor, CPA. It is therefore proposed that in future studies comparison of SOCE between health and cancer should be evaluated using CPA. Further pharmacogenomic analysis with a larger cohort of donors may reveal the underlying reasons for the inter-individual variability in platelet SOCE and possible polymorphisms involved.

Chapter 6. General Discussion, Future Perspectives and Conclusions

6.1 Summary of main findings

The first two Chapters of this thesis focus on an in vitro analysis of platelet interactions with a myeloid leukemic cell line at the level of the ubiquitous second messenger intracellular Ca^{2+}. Platelets induced enhanced Ca^{2+} signalling events in co-incubated HEL cells in response to thrombin and a TLR1/2 agonist, Pam_3CSK_4. These experiments confirmed that platelets interact with surrounding leukemic cells in line with findings of previous studies on platelet-cancer cell interactions (Menter et al., 2014, Lou et al., 2015). The present work provides insight into the mechanism of crosstalk between platelets and human erythroleukemic cells at the level of intracellular Ca^{2+} responses. The findings in chapter 3 demonstrate that the platelets induce potentiation of thrombin-evoked Ca^{2+} responses of HEL cells. Whilst thrombin stimulates a direct Ca^{2+} response in HEL cells as well as platelets, the effect of this agonist on intracellular Ca^{2+} in the cell line was amplified when co-incubated with platelets. Thrombin acts on HEL cells via PAR-1 and equally expressed PAR-3 receptors (Cupit et al., 2004), whereas in human platelets this ligand stimulates both PAR1 and PAR4 receptors (Coughlin, 2000, Coughlin, 2005). A range of inhibitors together with measurement of extracellular ATP were used to investigate the intercellular signalling mechanism. Using a luciferin:luciferase assay, it was clear that thrombin-stimulated platelets release large amounts of ATP (and presumably also ADP, which is stored at similar concentrations in platelet dense granules). In contrast, HEL cells fail to release ATP in response to PAR receptor stimulation, although this nucleotide was released constitutively at lower levels from this cell line. Although HEL cells show direct Ca^{2+} responses to ATP and ADP, apyrase at a concentration of 3.2 U/ml did not reduce the overall thrombin-evoked HEL cell Ca^{2+} increase even though extracellular ATP in co-incubated

suspensions of these two cells was significantly reduced. This is in contrast to the ability of apyrase to virtually abolish platelet-induced HEL cell Ca^{2+} increases following activation of TLR1/2 receptors. This suggests that ATP is able to act as a paracrine signal between platelets and HEL cells following stimulation of TLR1/2 receptors but is less important when thrombin is the agonist. However, it was observed that after stimulation of the co-incubated platelets/HEL cells by thrombin that the HEL cell response was converted into a biphasic response by apyrase. This biphasic response could therefore be due to the secondary stimulation of HEL cells by the ADP that is converted from the ATP by the action of apyrase. Another possibility is that thrombin is able to induce secretion of paracrine agents that are not released after stimulation of TLR1/2 receptors. Certainly thrombin is recognised as one of the most powerful stimuli of platelets (Coughlin, 2000, Coughlin, 2005), whereas TLR1/2 receptor agonists are weaker relying upon amplification by secondary mediators such as TxA_2 and nucleotides (Kälvegren et al., 2010).

Thrombin acts on human platelets via PAR-1 and PAR-4 which are both coupled to Gq and G12/13 (Voss et al., 2007b). In addition, PAR1 is coupled to Gi that is downstream linked to PI3K and Akt pathways and activates $\alpha_{IIb}\beta_3$ (Kim et al., 2004). The Gq acts via PLCβ and induces Ca^{2+} mobilisation, whilst DAG is produced via PLD that activates $\alpha_{IIb}\beta_3$ via the PKC pathway and MAPK activation (Zhang et al., 2013a). The $G_{12/13}$ activates $\alpha_{IIb}\beta_3$ via Rho/Rho kinase and actin remodelling that results in shape change. All these pathways are involved in platelet secretion induced by thrombin (Voss et al., 2007b). In contrast TLR1/2 receptors are tyrosine kinase-coupled receptors leading to platelet granular secretion via PI3K/Akt pathway and ERK1/2, and p38 phosphorylation (Rivadeneyra et al., 2014a, Blair et al., 2009, Rex et al., 2009a). Thus, the signalling mechanisms through which Pam_3CSK_4 activates secretion likely differs compared to the pathways used by PAR GPCRs. It is therefore possible that the contents of the granules released by these two agonists differ. Such a difference between GPCR and tyrosine kinase receptor-linked secretion (including TLR1/2) has already been described since the secretion evoked by thrombin was shown to be abolished by PGI_2 and NO, whereas the secretion following Pam_3CSK_4 was partially resistant to these endothelial-derived

inhibitors (Fung et al., 2012a). Furthermore, in a study conducted on platelet secretion evoked by these two agonists, a significantly higher platelet factor-4 (PF-4) release was observed with thrombin as compared to Pam_3CSK_4 (Rex et al., 2009b). A number of other compounds were released following activation by both agonists; those compounds examined include thrombospondin, fibrinogen β, FXIIIa, gelsolin (α-granule protein), platelet basic protein (PBP), PF-4 (chemokines) (Rex et al., 2009b). PF-4 is known to induce platelet synthesis and increase lung cancer growth when expressed as an endocrine factor (Pucci et al., 2016b). PF-4 is a chemokine and platelet-associated PF-4 has been suggested as a biomarker of early colorectal cancer in mice (Cervi et al., 2008). Such agents released from thrombin-stimulated platelets may represent the additional paracrine mediators responsible for platelet-dependent HEL cell Ca^{2+} signals following thrombin stimulation.

A large range of compounds are stored and secreted from platelets upon stimulation (Golebiewska and Poole, 2015a, Yadav and Storrie, 2017). These include diffusible compounds other than ATP (eg. serotonin) but also surface proteins that may interact with other cells (e.g. P-selectin). For example, it is known that platelets release PDGF from α granules (Giacco et al., 2006, Witte et al., 1978, Huber et al., 2016) and in a study performed on glioblastoma cells, a biphasic Ca^{2+} response was observed which was attributed to the effect of PDGF (Vereb et al., 2005). PDGFRs are tyrosine kinase receptors that have a crucial role in tumour development (Di Rocco et al., 1998). PDGF binding to the PDGFR leads to activation of PLCϒ1 following autophosphorylation of the PDGFR (Ronnstrand et al., 1992). The PLCϒ1 results in IP_3-mediated increase in intracellular Ca^{2+} (Ronnstrand et al., 1992). Another mechanism that elevates intracellular Ca^{2+} is the production of sphingosine-1-phosphate by activation of PDGFR that depletes Ca^{2+} stores independently of the IP_3 (Claesson-Welsh, 1994). A further example is VEGF, released from α granules (Webb et al., 1998). The effect of thrombin-evoked platelet secretion was studied in breast cancer cells (MCF-7) in which PAR-1/4-stimulated platelets released VEGF and together with integrin activation induced angiogenesis and growth (Jiang et al., 2017). Hence, growth factors and hormones released from platelets can evoke a secondary Ca^{2+}response in cancer cells and may

contribute to the thrombin-evoked paracrine signalling described in Chapter 3. A number of the key secretions from the three main platelet secretory granules (α granules, lysosomes and dense granules) that are possible candidates for the thrombin-stimulated paracrine interactions with HEL cells are listed in table 6.1. Further work is required to assess the role of agents such as serotonin, PGDF, TGF and VEGF on HEL Ca^{2+} signalling in the potentiating effect of thrombin-stimulated platelets under the conditions used in this thesis.

In chapter 4, mixed platelet and HEL suspensions were treated with the TLR1/2 agonist Pam_3CSK_4, which stimulates Ca^{2+} responses in platelets but not HEL cells. This is an interesting finding particularly as TLR1/2 receptors are a key component of the innate immune system (Janeway and Medzhitov, 2002). This effect therefore demonstrates that platelets can communicate with cancer cells following stimulation of their immune receptors. Regarding the mechanism of this interaction, the response of HEL cells to Pam_3CSK_4-stimulated platelets was virtually eliminated by apyrase. This suggests that Pam_3CSK_4-activated platelets release nucleotides, which is consistent with the previous work (Fung et al., 2012a, Kälvegren et al., 2010). The present work using cell suspensions also shows that ATP (and possibly ADP) acts as a major paracrine agent between platelets and HEL cells.

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Released following dense granular secretion	**α granule secretion**	**Lysosomal secretion**
Ca^{2+} (R)	Fibrinogen (R)	Hydrolases (Cathepsin D and E) (R)
Serotonin (R)_	von Willebrand factor (R)	membrane proteins LAMP-1,-2,-3 (SE)
ATP/ADP (R)	Platelet factor-4 (R)	
Histamine (R)	β-thrombomodulin (R)	
	Growth promoting factors (R)	
	P-selectin (SE)	
	GPIb-IX-V, $\alpha_{IIb}\beta_3$ (SE)	

Table 6.1 Major components of platelet granular secretions, (R: released, SE: increased surface expression) (Adapted from (Flaumenhaft, 2013))**.**

A further key observation, which is also clinically relevant, was that aspirin reduced the Pam_3CSK_4-stimulated platelet-induced Ca^{2+} response in HEL cells to a similar extent as apyrase. Aspirin also prevented ATP secretion following TLR1/2 receptor stimulation of platelets. In contrast, the platelet-induced HEL cell Ca^{2+} response to thrombin was not affected by aspirin, consistent with the difference observed between thrombin and Pam_3CSK_4 in terms of the effect of apyrase. Being a potent agonist of platelets (Chung et al., 2002), the effect of thrombin (Gross et al., 1991) is not dependent on TxA_2 for secretions while Pam_3CSK_4 being a less potent agonist depends on TxA_2 for platelet secretions, as reported by Kalvegren and colleagues (Kälvegren et al., 2010). To further explore the relative contribution of TxA_2 in the platelet-HEL cell interactions, additional experiments should directly monitor the TxA_2 release from HEL-platelet suspension. This can be carried out using a radio- or enzyme-linked immune assays as described by (Paniccia et al., 2015).

Further evidence for a role of ATP secreted from TLR1/2-stimulated platelets in the paracrine activation of HEL cells was provided in single cell imaging experiments in Chapter 4. At the single cell level, it was seen that the HEL cells responded to the platelets via a Ca^{2+} response that was often oscillatory in nature. The results also

confirm that HEL cells do not respond directly to the TLR1/2 agonist. Single HEL cells do respond directly to ATP and ADP, with the response to TLR1/2-stimulated platelets more closely resembling ATP than ADP. However, there were still clear differences, suggesting a possible involvement of both nucleotides in combination. The Ca^{2+} oscillations induced in HEL cells may also be influenced by other platelet secretions or by physical contact between the two cell types. Hence, a further experiment with single HEL cells would be to stimulate platelets with Pam_3CSK_4 and after centrifugation, only add the supernatant to assess the effect of platelet secretions on HEL Ca^{2+} signalling. Platelets may also be interacting with HEL cells by producing microparticles that then bind to HEL cells, thus consideration should be made to the centrifugation speed to ensure that they are also sedimented. In addition to ultracentrifugation, platelet microparticles can also be removed with latex beads coated with anti-platelet antibodies (Jayachandran et al., 2012). It is also documented that platelets interact with cancer cells via increased surface expression of proteins that serve to enhance direct physical contact between the two cell types and that these have a role in metastasis (Amo et al., 2014). These proteins include P-selectin (Amo et al., 2014), $\alpha_{IIb}\beta_3$ (Karpatkin et al., 1988a), GPIbα (Jain et al., 2007) and GPVI (Farndale, 2009) which could each be selectively blocked with Inclacumab (Stahli et al., 2016), ep-tifibatide (Scarborough et al., 1993), CCP-224 (Jimenez et al., 2017), losartan and cinanserin (Taylor et al., 2014c) respectively to assess their contribution to the platelet-induced effects in the HEL Ca^{2+} response.

A summary of the interactions between platelets and HEL cells within the experiments described in Chapters 3 and 4 is given in Fig 6.1.

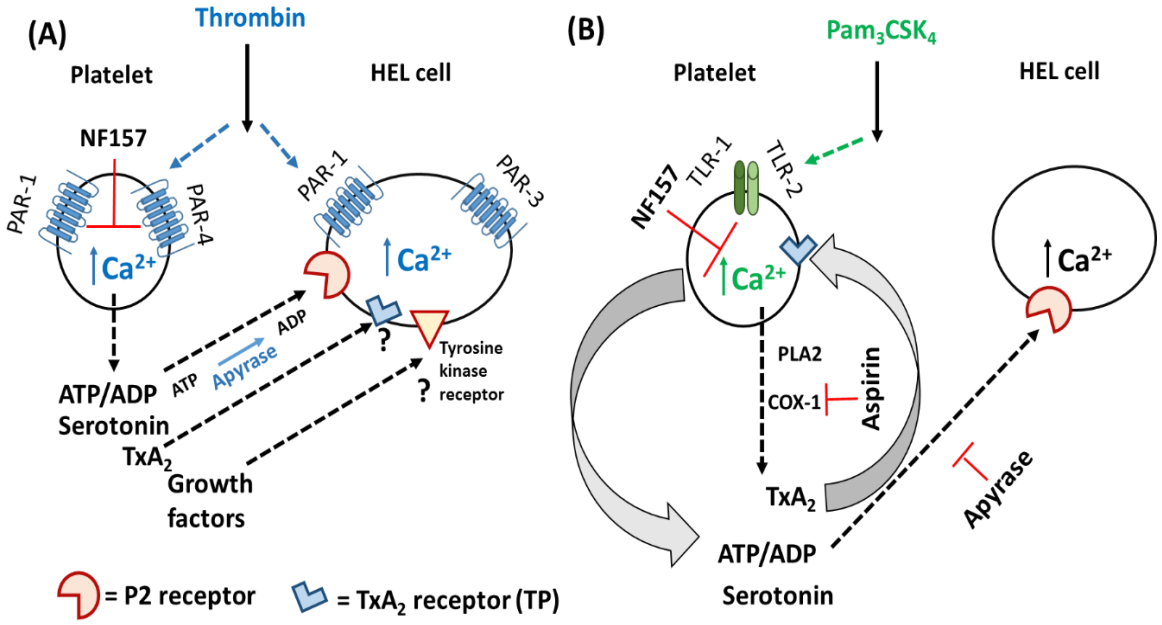

Fig. 6.1 Summary schematic showing the effect of apyrase, aspirin and NF157 on HEL cells and platelets when stimulated with thrombin and Pam_3CSK_4.

(A) Thrombin directly evokes intracellular Ca^{2+} responses in platelets by acting on PAR-1 and 4 GPCRs and in HEL cells via PAR-1 and 3 GPCRs. The stimulated platelets release ATP/ADP, TxA_2 and growth factors (VEGF, PDGF, TGF, FGF), which can all potentially stimulate HEL cells via surface receptors (P2 GPCRs for ATP/ADP; $5HT2_A$ GPCRs for serotonin; TP receptors for TxA_2 and tyrosine kinase coupled receptors for growth factors. However, apyrase and aspirin did not inhibit the platelet-induced enhanced Ca^{2+} response in HEL cells. NF157 abolished the thrombin-evoked response in platelets and platelet-induced enhanced Ca^{2+} signals in HEL response to thrombin. (B) Pam_3CSK_4 stimulated the platelets via TLR1/2 receptors but does not act directly on HEL cells. Pam_3CSK_4-stimulated platelets release ATP/ADP, serotonin and TxA_2. Aspirin and apyrase both block the Ca^{2+} signal in HEL cells and the ATP release. Thus, the platelet ATP release downstream of TLR1/2 must be dependent upon TxA_2 formation (large grey arrows). NF157 blocked the Pam_3CSK_4-evoked HEL cell response, but acts at the level of platelets, preventing both the Ca^{2+} increase and ATP release downstream of Pam_3CSK_4.

Ca^{2+} signalling and morphological changes in tumour cells are known to contribute to cell proliferation and migration (Goranov et al., 2013, Cui et al., 2017). The morphological responses observed here were based on studies of cell shape change and the relationship of this event to proliferation and metastasis is unknown. Hence in future experiments, more sophisticated measurements of HEL cell migration following platelet TLR1/2 receptor activation could be used. For example, the matrigel invasion assay (Justus et al., 2014), MTT assay (cell viability assay) (Gerlier and Thomasset, 1986) or immunohistochemical staining (Scott et al., 1991) are recommended to further confirm the metastatic and proliferative effects of platelets on co-incubated HEL cells.

A screen of HEL P2 receptors suggested candidates for surface receptors whereby ATP and/or ADP stimulate Ca^{2+} increases in HEL cells. This thesis for the first time demonstrates the expression of P2X1, P2X4 and P2Y11 in HEL cells. At a concentration selective for P2X1 receptors, the antagonist NF449 did not affect the platelet-induced potentiation of HEL cells. However at higher concentration NF449 completely inhibited the HEL response to thrombin following co-incubation with platelets. This is consistent with the non-selective effect reported for NF449 on heterotrimeric Gα subunits at a high concentration, as previously reported by Hohenegger and colleagues (Hohenegger et al., 1998). An alternative to 5-BDBD, is required to assess the contribution of P2X4 blocker due to the autoflourescence of this compound that makes it incompatible with fura-2 measurements. The P2Y11 blocker, NF157 showed a significant reduction in the thrombin response of HEL cells following co-incubation that initially indicated an involvement of this receptor in response to platelet-released nucleotides. However, NF157 also abrogated the ATP release from platelets following stimulation by thrombin as well as Pam_3CSK_4, indicating that this compound is acting at the level of the platelet not HEL cells. In other studies NF157 has been shown to exert weak antagonistic activity on P2Y1 and P2Y2 receptors and also to affect P2X1 receptors (Dreisig and Kornum, 2016, Ullmann et al., 2005b). This can be explained on the basis of the non-selective effect of NF157 as this is a structural analogue of

suramin that contains a basic phenyl carbonyl urea group and suramin is a relatively non-elective P2 receptor antagonist (Krejci et al., 2010). The only difference between NF157 and suramin is a CH_3 or F substitute (Krejci et al., 2010). However, NF157 non-selectively antagonised thrombin-evoked Ca^{2+} responses and ATP release by thrombin and Pam_3CSK_4. This may be related to a general action of suramin antagonists at higher concentrations on cell signalling pathways coupled to Ca^{2+} signals as both NF449 (Nishimura et al., 2015) and NF157 are suramin analogues (Ullmann et al., 2005b). Thus, a more selective P2Y11 antagonist is required to investigate the active role of P2Y11 receptors in HEL cells during platelet-induced potentiation. An alternative approach to using pharmacological inhibitors is via genetic modification of HEL cells to selectively delete expression of the candidate P2 receptors; for example using interference RNA or genome editing.

The second part of this thesis (Chapter 5) was to design the method of SOCE assessment in platelets and evaluate the normal level in healthy individuals. This was carried out with a view to future comparisons of this pathway in health and cancer since SOCE has been implicated in development of malignancy. These studies revealed an unexpected heterogeneity in the TG-induced platelet SOCE among healthy individuals. In contrast, another SERCA inhibitor, CPA, did not show the same level of heterogeneity of SOCE in healthy individuals. However due to time constraints and the limited available donors for repetitive blood donation, these experiments were performed on a small number of individuals. Nevertheless, they do justify extension of this work to a pharmacogenomics investigation of platelet SOCE in healthy individuals within a large group; for example as in the study by Feijge and co-workers who explored Ca^{2+} signalling variations in 51 healthy individuals (Feijge et al., 1998) . A number of studies suggest the STIM1 (Zhang et al., 2015) and Orai1 (Zui and JianJie, 2015) proteins can be used as a diagnostic tool of cancer. However a question remains whether platelet SOCE is enhanced and whether the STIM1 and Orai1 expression in platelets is altered in cancer. Once further studies in a larger population have explored the level of heterogeneity with CPA-induced SOCE in healthy donors, the feasibility of

comparing this pathway in platelets from cancer or other pathologies could be assessed.

Chapter 5 also explored the basis for the pathway responsible for store depletion and thus SOCE activation in platelets following TG exposure. The pathway through which TG-induced store depletion takes place was not fully identified, however it was shown to occur in part through a route that is cAMP-sensitive as a 50 % reduction in Ca^{2+} release was observed following stimulation of Gs-coupled receptors with PGE1. IP_3 receptors are known to be inhibited by cAMP-activated PKA (Iyú et al., 2011), thus could represent this pathway. Another unexplored channel for this study is TMEM109, a membrane protein, which may be involved in counterion Ca^{2+} transport and is found functionally active and associated with cell death in murine thymocytes (Takeshima et al., 2015). Expression of this gene has been detected in platelets (Wright et al., 2016b) and thus could represent a further candidate for the TG-induced Ca^{2+} store depletion pathway however no function has yet been reported in platelets for this protein. Thus, further studies are required to assess the pathway responsible for Ca^{2+} efflux from the DTS following SERCA inhibition, and the reason for its heterogeneity in healthy individuals.

6.2 Clinical implications:

Aspirin is a widely used anti-platelet agent, thus the demonstration that it significantly inhibits the platelet-induced potentiation of immune receptor-dependent signalling in a myeloid cancer cell may have important implications for clinical practice. Several existing pieces of evidence already suggest that aspirin is effective as a part of cancer regimen therapy (Stark et al., 2006, Holmes et al., 2010). The present work further suggests that optimized apyrase activity of human nucleoside triphosphate diphosphohydrolase-3 (CD39L3) (Moeckel et al., 2014) may also prove useful in clinical trials to minimise the chances of cancer progression as a consequence of platelet immune receptor responses and platelet hyperactivity.

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Supplementary tables

Gene Name	Primer name	qPCR eff. (%)	Sequence	Size qPCR Product
ACTB (β-actin)	HqPCR ACTB F	93.4	5’ TCCTATGTGGGCGACGAG 3’	242 bp
	HqPCR ACTB R		5’ ATGGCTGGGGTGTTGAAG 3’	

RECEPTORS

P2X1 (P2X purinoceptor 1)	HqPCR P2X1 F	98.0	5’ CTGGTGGAGGAGGTGAATG 3’	260 bp
	HqPCR P2X1 R		5’ AAGTTGAAGCCTGGGGAGAG 3’	
P2X2 (P2X purinoceptor 2)	HqPCR P2X2 F	97.2	5’ CATCGGGGTCATTATCAAC 3’	300 bp
	HqPCR P2X2 R		5’ CAGTCGCACAGGAAGGAG 3’	
P2X3 (P2X purinoceptor 3)	HqPCR P2X3 F	94.5	5’ GACCCTTTCTGCCCCATC 3’	250 bp
	HqPCR P2X3 R		5’ CACTGCCATTTTCCATTTTG 3’	
P2X4 (P2X purinoceptor 4)	HqPCR P2X4 F	95.3	5’ GGAGAACGCAGGACACAG 3’	272 bp
	HqPCR P2X4 R		5’ CCTTCCCAAACACAATGATG 3’	
P2X5 (P2X purinoceptor 5)	HqPCR P2X5 F	98.8	5’ TGCTTTCTTCTGCGACCTG 3’	235 bp
	HqPCR P2X5 R		5’ CTTCTGACTGCTGCTTCCAC 3’	
P2X6 (P2X purinoceptor 6)	HqPCR P2X6 F	96.4	5’ CCAGTGTGTGGTGTTCAATG 3’	221 bp
	HqPCR P2X6 R		5’ GTGGTTCATAGCGGCAGTG 3’	
P2X7 (P2X purinoceptor 7)	HqPCR P2X7 F	95.3	5’ GAAGAAGTGCGAGTCCATTG 3’	227 bp
	HqPCR P2X7 R		5’ GCTGTATCTCCTCTGGTTGTC 3’	

P2Y1 (P2Y purinoceptor 1)	HqPCR P2Y1 F	93.1	5’ GTCCCCTTGGTGCTGATTC 3’	232 bp
	HqPCR P2Y1 R		5’ TGGCATAAACCCTGTCATTG 3’	
P2Y2 (P2X purinoceptor 2)	HqPCR P2Y2 F	91.3	5’ GTAACCTGCCACGACACCTC 3’	222 bp
	HqPCR P2Y2 R		5’ GAAGACAGCCAGCACCAC 3’	

P2Y4 (P2X purinoceptor 4)	HqPCR P2Y4 F	90.2	5’ TAACGCCCCAACCCTATG 3’	229 bp
	HqPCR P2Y4 R		5’ GCAGGTGAGGAAAAGGACAC 3’	
P2Y6 (P2X purinoceptor 6)	HqPCR P2Y6 F	91.3	5’ AACCGCACTGTCTGCTATGAC 3’	265 bp
	HqPCR P2Y6 R		5’ CTGTCTTGGTGATGTGAAAAGG 3’	
P2Y11 (P2X purinoceptor 11)	HqPCR P2Y11 F	94.3	5’ GGTGGTTGAGTTCCTGGTG 3’	280 bp
	HqPCR P2Y11 R		5’ GTAGCGGTTGAGGCTGATG 3’	
P2Y12 (P2X purinoceptor 12)	HqPCR P2Y12 F	90.6	5’ CTGTTGTCATCTGGGCATTC 3’	247 bp
	HqPCR P2Y12 R		5’ CCTACACCCCTCGTTCTTACG 3’	
P2Y13 (P2X purinoceptor 13)	HqPCR P2Y13 F	92.6	5’ GAGCAACAAGGAAGCAACAC 3’	255 bp
	HqPCR P2Y13 R		5’ ACAAAGAAGACAGCCACGAC 3’	
P2Y14 (P2X purinoceptor 14)	HqPCR P2Y14 F	91.3	5’ TCTGCCGTGCTCTTCTACG 3’	260 bp
	HqPCR P2Y14 R		5’ CACTTCCGTCCCAGTTCAC 3’	

Table S1 list of primers used in qPCR.

TG-incubation time	**1**	**2**	**5**	**10**	**15**
1	-	ns (P=0.1038)	* (P=0.0342)	* (P=0.0496)	* (P=0.0217)
2	ns (P=0.1038)	-	ns (P=0.0749)	ns (P=0.1434)	* (P=0.0496)
5	* (P=0.0342)	ns (P=0.0749)	-	ns (P=0.3793)	* (P=0.0406)
10	* (P=0.0496)	ns (P=0.1434)	ns (P=0.3793)	-	ns (P=0.8578)
15	* (P=0.0217)	* (P=0.0496)	* (P=0.0406)	ns (P=0.8578)	-

Table S2 Summary of the comparison between different TG-incubation times (n=7).

Time after Ca^{2+} addition	24	48	72	96	120
24	-	ns (P=0.74)	ns (P=0.78)	ns (P=0.56)	ns (P=0.39)
48	ns (P=0.74)	-	ns (P=0.99)	ns (P=0.546)	ns (P=0.58)
72	ns (P=0.78)	ns (P=0.99)	-	ns (P=0.11)	ns (P=0.376)
96	ns (P=0.56)	ns (P=0.546)	ns (P=0.11)	-	ns (P=0.92)
120	ns (P=0.39)	ns (P=0.58)	ns (P=0.376)	ns (P=0.92)	-

Table S3 Comparison of increase in SOCE (with 2 min TG-incubation time) at 24, 48, 72, 96 and 120 seconds after extracellular $CaCl_2$ addition.

Time after Ca^{2+} addition	24	48	72	96	120
24	-	ns (P=0.2521)	ns (P=0.1014)	ns (P=0.8940)	ns (P=0.0856)
48	ns (P=0.2521)	-	ns (P=0.0506)	ns (P>0.9999)	* (P=0.0288)
72	ns (P=0.1014)	ns (P=0.0506)	-	ns (P=0.8918)	* (P=0.0491)
96	ns (P=0.8940)	ns (P>0.9999)	ns (P=0.8918)	-	ns (P=0.6759)
120	ns (P=0.0856)	* (P=0.0288)	* (P=0.0491)	ns (P=0.6759)	-

Table S4 Comparison of increase in SOCE (with 5 min TG-incubation time) at 24, 48, 72, 96 and 120 seconds after extracellular $CaCl_2$ addition.

Time after Ca^{2+} addition	24	48	72	96	120
24	-	ns (P=0.3098)	ns (P=0.1951)	ns (P=0.1395)	ns (P=0.1095)
48	ns (P=0.3098)	-	ns (P=0.1097)	** (P=0.0087)	** (P=0.0090)
72	ns (P=0.1951)	ns (P=0.1097)	-	ns (P=0.2028)	* (P=0.0707)
96	ns (P=0.1395)	** (P=0.0087)	ns (P=0.2028)	-	ns (P=0.0885)
120	ns (P=0.1095)	** (P=0.0090)	* (P=0.0707)	ns (P=0.0885)	-

Table S5 Comparison of increase in SOCE (with 10 min TG-incubation time) at 24, 48, 72, 96 and 120 seconds after extracellular $CaCl_2$ addition.

Time after Ca^{2+} addition	24	48	72	96	120
24	-	ns (P=0.0779)	ns (P=0.0745)	ns (P=0.1530)	ns (P=0.1569)
48	ns (P=0.3098)	-	ns (P=0.0946)	ns (P=0.2247)	ns (P=0.2227)
72	ns (P=0.0745)	ns (P=0.0946)	-	ns (P=0.6121)	ns (P=0.5117)
96	ns (P=0.1530)	ns (P=0.2247)	ns (P=0.6121)	-	ns (P=0.2637)
120	ns (P= 0.1569)	ns (P= 0.2227)	ns (P=0.5117)	ns (P=0.2637)	-

Table S6 Comparison of increase in SOCE (with 15 min TG-incubation time) at 24, 48, 72, 96 and 120 seconds after extracellular $CaCl_2$ addition.

Donor No.	1	2	3
1	-	**** (P<0.0001)	*** (P=0.0009)
2	**** (P<0.0001)	-	** (P=0.008)
3	*** (P=0.0009)	** (P=0.008)	-

Table S7 Summary of the comparison between healthy individuals average $[Ca^{2+}]_i$ (nM) with 5 minutes TG incubation time (No. of donors = 3).