THE ROLE OF GPVI AND CLEC-2 IN PLATELET ACTIVATION BY MISCELLANEOUS LIGANDS

BY

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Abstract

Platelets are an essential factor in wound repair and blood clotting, where exposed sub-endothelial extracellular matrix (ECM) proteins induce activation during vascular injury. However, platelets can also be activated by a diverse range of stimuli that share little-to-no resemblance in structure to each other, or to recognized ligands of platelet receptors. These stimuli include diesel exhaust particles (DEP), various peptides including 4N1-1 and Champs, lipoproteins such as PAM_3-CSK_4, and large polysaccharides for example, fucoidan, and dextran.

In this thesis, I demonstrate that this seemingly miscellaneous group of stimuli cause aggregation of human and mouse platelets through Src and Syk tyrosine kinases in association with stimulus-specific tyrosine phosphorylation of the GPVI/FcRγ-chain complex and/or CLEC-2. A critical role for GPVI and/or CLEC-2 in mediating aggregation is shown using platelets from receptor-deficient mouse platelets. Additionally, in double deficient mouse platelets these stimuli activate Src tyrosine kinases independent of GPVI and CLEC-2. DEP, fucoidan and dextran were shown to activate transfected GPVI or CLEC-2 in a cell line model. However, 4N1-1, Champs and PAM_3-CSK_4 did not activate transfected GPVI or CLEC-2 in a cell line model, nor could they bind to recombinant forms of either receptor. In addition, I demonstrate the unexpected observation that fibrin also activates GPVI revealing a new stage of haemostasis in which the generation of fibrin from fibrinogen reinforces platelet activation.

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Abbreviations

ADP	adenosine diphosphate
ATP	adenosine triphosphate
CTH	Calf thymus histones
CLEC-2	C-type lectin-like receptor 2
Coll	Collagen
CRP	Collagen-related peptide
CSK	C-terminal Src kinase
DAG	Diacylglycerol
DEP	Diesel exhaust particles
DxS	Dextran sulfate
ECM	Extracellular matrix
FcR	Fc receptor
Fp	Fibrinopeptide
GPCRs	G protein-coupled receptors
HSC	Haematopoietic stem cell
ITAM	Immunoreceptor tyrosine-based activation motif
ITIM	Immunoreceptor tyrosine-based inhibition motif
LSARLAF	Leu-Ser-Ala-Arg-Leu-Ala-Phe
NO	Nitric oxide
OCS	Open canalicular system
PAH	polyaromatic hydrocarbons
Pam_3-CSK_4	Pam3CysSerLys4
PAR	Protease activated receptor
PAS	Protein-A sepharose
PGI_2	Prostaglandin I_2
PGS	Protein-G sepharose
PKA	Protein kinase A
PLG	Plasminogen
PS	Phosphatidylserine
PTK	Protein tyrosine kinase
PTP	Protein tyrosine phosphatase
PTB	Phosphotyrosine binding
RPTP	Receptor-like PTP
Rho	Rhodocytin
SR	Scavenging receptor
SFK	Src family kinase
SH2	Src homology 2
SH3	Src homology 3
TCR	T-cell receptor
TF	Tissue factor
TLR	Toll-like receptor
TPO	Thrombopoietin
TSP	Thrombospodin-1
TxA_2	Thromboxane A_2
VASP	Vasodilator-stimulated phosphoprotein
VWF	Von Willebrand factor
WCL	Whole cell lysates

Chapter 1 GENERAL INTRODUCTION

1.1 Platelet Overview and Formation

Platelets are an essential factor in wound repair and blood clotting. When the sub-endothelial extracellular matrix (ECM) is exposed during vascular injury, activation of platelets leads to the formation of a haemostatic plug which serves to prevent excessive blood loss. Platelets are essential for haemostasis most notably in the high shear environment found in arteries and arterioles. Excessive and uncontrolled platelet activation is however detrimental to human health. One classic example is at sites of rupture of atherosclerotic plaques where uncontrolled thrombosis may lead to obstruction of blood vessels and myocardial infraction or stroke. Therefore careful regulation of platelet function is required.

The origin of all blood cells is the common haematopoetic stem cell (HSC). Blood cells are classified into two major classes that are known as myeloid (macrophages, neutrophils, basophils, eosinophils, erythrocytes, platelets) and lymphoid (T and B cells) lineages (Figure 1-1). A mutual progenitor exists for the anuclear cells platelets and erythrocytes. This progenitor produces a colony of cells from which the megakaryocyte/erythroid lineages are formed. These divide into megakaryocyte and erythroid lineages by binary fission. Platelets are produced by megakaryocytes, which are very large polyploid cells present in bone marrow. As these progenitors grow into giant cells platelets are released into the peripheral circulation by a processes called fragmentation. The hormone thrombopoietin (TPO), synthesized mainly by the liver and to a lesser extent by the kidneys, regulates platelet formation, which signals through the c-Mp1 receptor on megakaryocytes (Murone et al., 1998). Platelets survive for 7-10 days in the human circulation. In a healthy human adult, the platelet count ranges from 150–400 x 10^9 per liter.

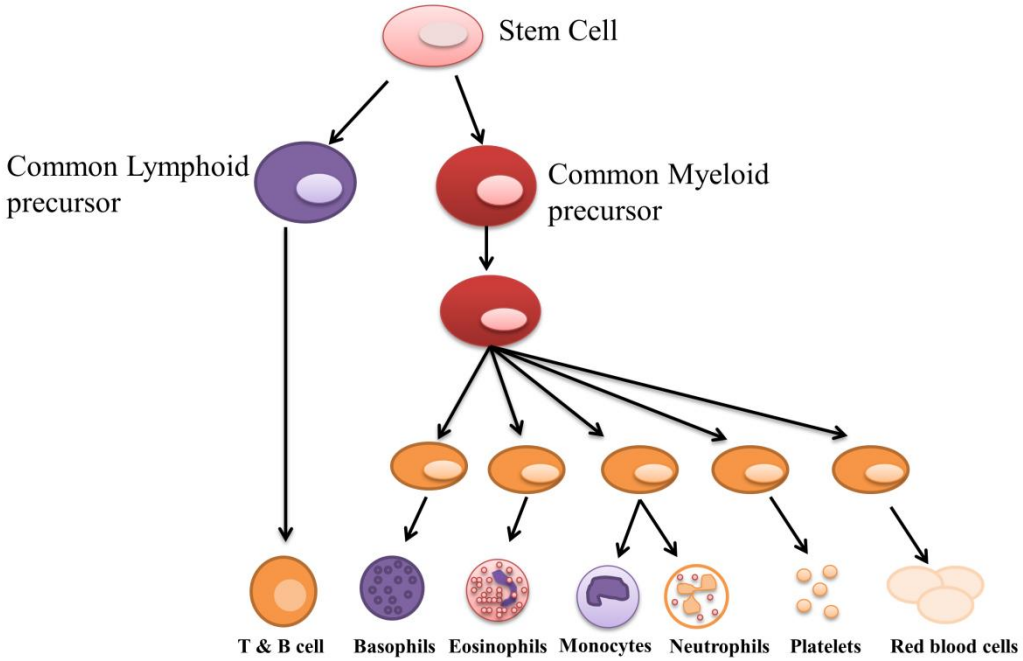

Figure 1-1 Haematopoietic stem cell (HSC). Development of white blood cells (basophils, eosinophils, monocytes, neutrophils), lymphocytes cells (T and B cells), red blood cells and platelets from a common stem cell through common myeloid and lymphoid precursors – reproduced from (Cabrita et al., 2003).

1.2 Platelet Structure

Platelets are the smallest of the blood cells, with a diameter of 2-3 μm, and a discoid shape in their inactive state. It is proposed that their small size and discoid shape helps to position platelets close to the endothelium (Ruggeri, 2009). To facilitate their function in the repair of vascular damage, red blood cells marginate platelets to the edge of blood vessels. This allows for platelets to be maintained in a position near to the endothelium, where they can respond quickly if vascular damage is detected.

When scanned under high resolution electron microscope the plasma membrane can be seen to invaginate into the cell to form a structure known as the open canicular system (OCS) (Hartwig, 2006). The function of these membrane folds is to provide additional membrane when platelets spread on a surface increasing the ratio of surface area. The OCS contains channels that facilitate the exchange of small molecules and prevents entry of larger proteins such as antibodies between the blood and the interior of the cell (Hartwig, 2006, Jin et al., 1998).

The cytoskeleton of a platelet is necessary for maintaining its discoid shape. When a platelet becomes activated, it goes through extensive structural changes which are mediated by the regulation of actin polymerisation. The platelet cytoskeleton consists of three layers:

(1) A spectrin-based skeleton that is required for attachment of the cytoplasmic surface to the plasma membrane;

(2) A microtubule network consisting of the β1 isoform of tubulin rolled into a coil and located under the cell membrane which runs along the whole circumference of platelet (Hartwig, 2006, Italiano and Shivdasani, 2003, Jin et al., 1998, Savi et al., 1998);

(3) A rigid actin filament-based network that fills the cytoplasmic space of the cell.

Actin is the most highly expressed platelet protein (Bearer et al., 2002). The filaments of actin are joined to the spectrin-based skeleton and are interlinked by two other proteins; filamin and actinin. Filamin interacts with one of the other most abundantly expressed platelet surface glycoprotein, GPIb-IX-V, which mediates tethering to sites of vessel injury through binding to von Willebrand factor (VWF) (Italiano and Shivdasani, 2003, Hartwig, 2006). Remodelling of the platelet cytoskeleton, which involves the formation of filopodia and lamellipodia, is initiated by platelet activation. In addition, platelets produce a contractile force through formation of actin stress fibres that stabilize the aggregate. Non-muscle myosin IIA and B expressed by platelets are vital for producing this contractile force (Hartwig, 2006).

As described above, platelets are formed from megakaryocytes which provide a broad range of pre-synthesized molecules required for their physiological responses. Platelets lack a nucleus and so their capability to express new proteins is much reduced (and indeed controversial). Typical cellular organelles such as mitochondria, lysosomes and endoplasmic reticulum are present in the cytoplasm of platelets. Besides these, they also contain major two types of platelet specific granules: (1) α-granules and (2) dense granules, as well as lysosomes which are of uncertain physiological significance.

About 80 α-granules are present in each human platelet having an approximate size of 0.2 - 0.4 μm in diameter (Italiano and Battinelli, 2009, Hartwig, 2006, Woulfe, 2005, Wei et al., 2009). Matrix adhesive proteins such as fibrinogen, fibronectin and VWF are stored in these granules. In addition, glycoprotein receptors are embedded in their membranes, which play a role in regulating attachment of platelets to the ECM. These include P-selectin and the major platelet integrin αIIbβ3. Coagulation factors,

including prothrombin, factors V and XIII are also present in α-granules (Harrison and Cramer, 1993).

Platelet dense granules are about 10 times less in number than α-granules and are smaller, with an approximate size of 0.15 μm in diameter (Blair and Flaumenhaft, 2009). Dense granules contain a number of small molecules that are important for cell activation including ADP, ATP, Ca^{2+} and the vasoconstrictor, serotonin (Harrison and Cramer, 1993, Kahn et al., 1999). ADP plays an essential role in 'secondary' platelet activation and platelet recruitment to the forming thrombi. Glycoproteins are also embedded in the membranes of dense and lysosomal granules that are assimilated into the plasma membrane following granule fusion, including CD63, LAMP1, LAMP2 and CD68 (Nofer et al., 2004, Kahn et al., 1998).

There are a number of other structural features which help platelets to perform their physiological functions. These include the high expression of glycoprotein receptors and signalling proteins that permit a quick response to vascular damage. In addition, expression of the negatively charged phosphatidylserine (PS) is increased in the outer lipid bilayer in activated platelets and provides a platform for the gathering of clotting factors. This helps to concentrate factors such as thrombin at the site of injury reinforcing platelet activation (Zwaal et al., 2004).

1.3 Platelet Function

Prevention of excessive blood loss by plugging the holes in damaged vessels is the fundamental physiological role of the platelets. In more recent studies, the involvement of platelets in additional processes including angiogenesis, inflammation and immunity has been reported. However, for this thesis I will concentrate on the traditional platelet actions, namely haemostasis and thrombosis.

Upon detection of exposed ECM during vascular injury, circulating platelets attach, become activated and attract other platelets from the circulation to form a haemostatic plug. At the site of injury, thrombus formation is further accelerated by thrombin generation from the coagulation cascade which also cleaves fibrinogen to fibrin. Platelet activation in haemostasis is summarised in Figure 1-2 and has been extensively reviewed (Woulfe, 2005, Wei et al., 2009, Stegner and Nieswandt, 2011, Jackson, 2011a).

1) Tethering, rolling and adhesion.

At shear conditions >500/s, as found in the small arteries, arterioles and stenotic diseased arteries, platelets accumulate by tethering and activation. The high molecular weight plasma glycoprotein VWF bridges exposed collagen and the platelet surface receptor GPIb-IX-V mediating platelet capture. The rapid 'on/off' rates of association and dissociation between GPIb-IX-V and immobilized VWF underlie the ability of the platelet to tether and roll on the VWF. A weak activation signal within the platelets is generated by this interaction and it is proposed that this helps to initiate further activation. At low shear (<500/s), conditions common in large arteries and veins, the VWF/GPIb-IX-V axis is not required. Rather, the integrins α2β1 (specific for interaction with collagen), α5β1 (specific for interaction with fibronectin), α6β1

(specific for interaction with laminin) and αIIbβ3 (which binds fibrinogen, fibronectin, VWF) are used for direct binding of the platelets to the ECM proteins indicated (Ruggeri and Mendolicchio, 2007).

2) Integrin activation and stable adhesion.

Following tethering, rolling and non-firm adhesion of platelets with the ECM, rapid, sustained activation is initiated. The low affinity collagen receptor GPVI binds to collagen, clusters, and initiates a strong and sustained activation signal. This triggers integrins αIIbβ3 and α2β1 to undergo a conformational change from a low affinity to a high affinity state and thereby bind to their immobilised ligands, namely VWF and collagen, respectively. The platelets are then strongly attached to the exposed ECM and VWF bound to collagen.

3) Spreading.

Cytoskeletal remodelling mediates the major morphological changes in the platelets induced by platelet activation. Long finger-like projections termed filopodia are extended by the now strongly attached and activated platelets. These filopodia ensure an even stronger adherence to the damaged vessel by the platelets. These projections are filled with lamellipodia, which help to increase the surface area and strengthen adhesion to the exposed subendothelial matrix and to each other. A large vascular lesion can therefore be quickly and efficiently covered by platelet spreading.

4) Secretion.

The potent platelet agonist thromboxane A_2 (TxA_2) along with the contents of α- and dense granules are rapidly released by the activated and spread platelets. A synergistic effect is produced by the combination of ADP and TxA_2 which induces maximal platelet activation. At this time the platelet plug grows with further recruitment

of new platelets. The localized generation of thrombin is accelerated by released components including Factors V, XIII and prothrombin (Wei et al., 2009).

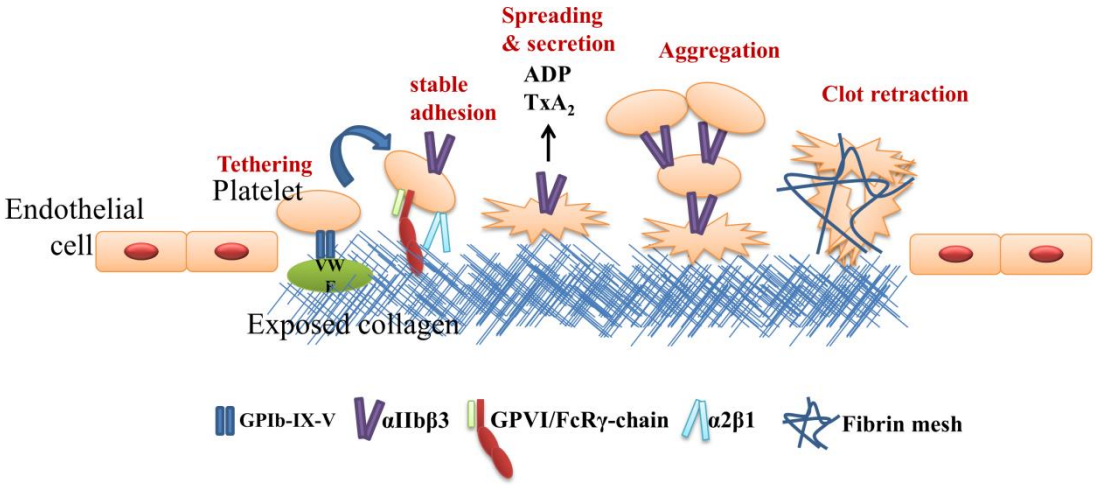

Figure1-2 Platelets tethering, adhesion, aggregation and thrombus formation. Platelet interaction with VWF leads to initiation of platelet tethering. GPVI interaction with collagen enables integrin conformational changes from low affinity to high affinity. These interactions lead to strong platelet binding to the exposed ECM and VWF. Platelets then spread and release ADP and TxA2 leading to platelets aggregation and clot formation (Jackson, 2011a, Wei et al., 2009).

5) Aggregation.

Fibrinogen derived from plasma and platelets, along with VWF, act as the facilitators of platelet aggregation. The interaction between αIIbβ3 and fibrinogen, and αIIbβ3 and VWF develop strong affinity interactions which further accelerates the growth of the thrombus (Jackson, 2007).

6) Thrombus stabilisation.

The PS exposed on the activated platelet surface, and also on the surface of activated endothelial cells, supports the activation of the coagulation cascade components (Factors V, VIII, IX and X) leading to thrombin generation (Heemskerk et al., 2002). In addition, the extrinsic coagulation pathway is initiated by the tissue factor exposed on damaged endothelial cells and smooth muscle cells in the vessel wall Figure. This pathway concludes at thrombin generation (figure 1-3). Platelet activation is increased by a high localized concentration of thrombin along with conversion of fibrinogen to fibrin and stabilisation of thrombus against embolization (Gibbins, 2004).

7) Clot retraction.

The platelet-fibrinogen/fibrin aggregate stabilises the thrombus through clot retraction via αIIbβ3. Non-muscle myosin II generates contractile forces required to pull together the interlocking cytoskeletons of platelets (Shattil and Newman, 2004).

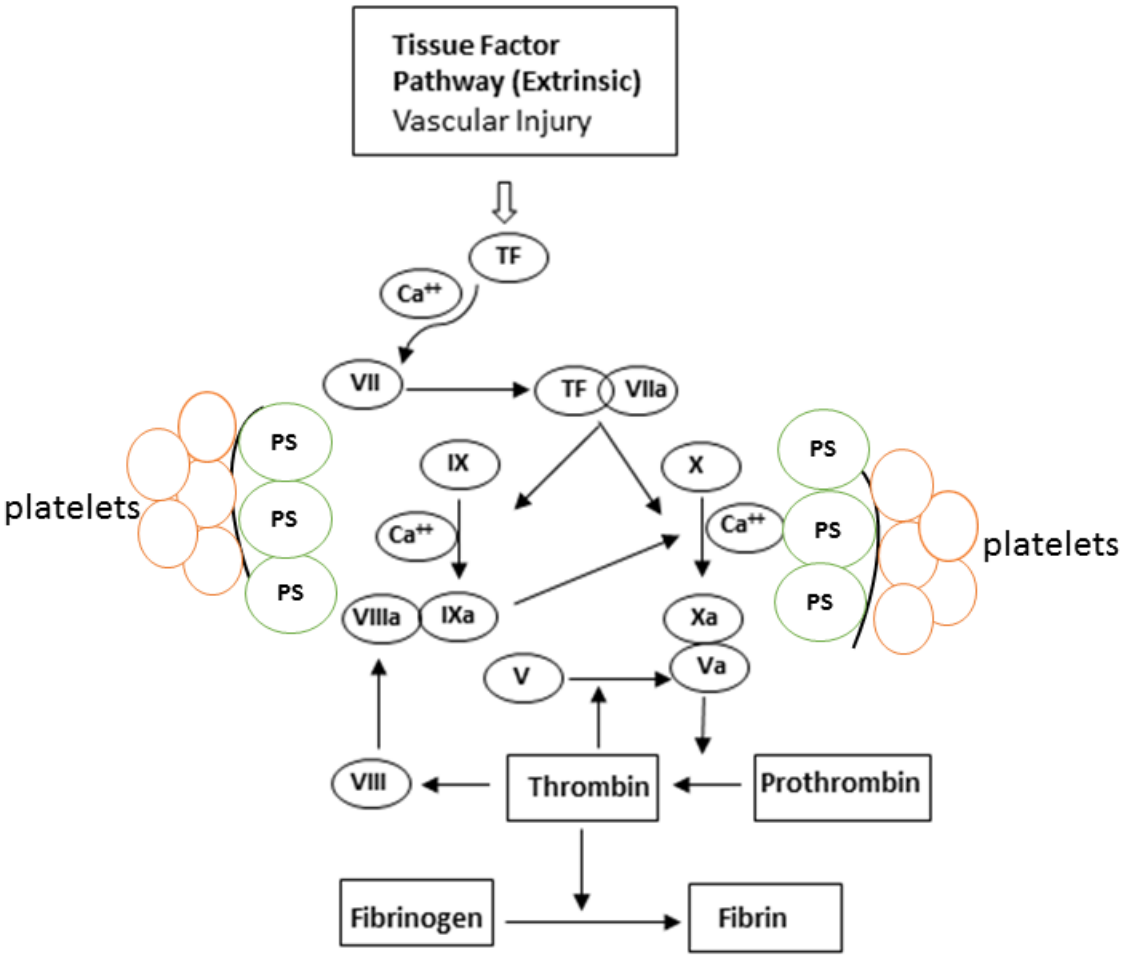

Figure 1-3 Extrinsic coagulation pathways. Tissue factor pathway includes Factors V, VIII, IX and X lead to thrombin formation and fibrinogen conversion to fibrin by the action of thrombin- reproduced from (Davie et al., 1991) .

1.4 Platelet Receptors

Receptors that control the platelet-dependent phases of haemostasis and thrombosis are expressed on the surface of platelets. They are divided into two distinct groups: tyrosine kinase-linked receptors (section 1.4.1) and G protein-coupled receptors (GPCRs) (Section 1.4.2).

1.4.1 Tyrosine Kinase-Linked Receptors

The major tyrosine kinase-linked receptors are the collagen activation receptor GPVI/FcR γ-chain complex; the collagen adhesion integrin α2β1; C-type lectin-like receptor 2 (CLEC-2); the fibrinogen/VWF integrin receptor αIIbβ3 and the VWF receptor GPIb-IX-V. The following sections describe the distinctive features of these receptors and their signalling pathways.

1.4.1.1 Collagen Receptors – GPVI and α2β1

The most abundant protein present in animals is collagen (Shoulders and Raines, 2009, Rivera et al., 2009). Vessel injury exposes blood to collagen since this fibrous structural protein is the foremost component of the ECM that lines all walls of the vessels. The most thrombogenic forms of collagen types are type I and type III present in the most luminal areas of the sub-endothelial matrix (Shoulders and Raines, 2009, Yang et al., 2000). The immunoreceptor GPVI and the integrin α2β1 are the major two collagen receptors expressed by platelets (Nieswandt and Watson, 2003, Pozgajová et al., 2006). GPVI and α2β1 bind to unrelated areas of collagen fibrils; GPVI to GPO repeat sequences, and α2β1 to GFOGER and related sequences. Affinity for their cognate motifs also differ, with GPVI binding with low affinity and activated α2β1 with high affinity. A rapid initiating signal is principally performed by GPVI

while firm adhesion is mediated by activated α2β1 (Nieswandt and Watson, 2003, Lanza et al., 1986). The two receptors work together but also have their own separate tasks in the regulation of platelet adhesion and the formation of thrombus on collagen (Sarratt et al., 2005, Massberg et al., 2003, Holtkotter et al., 2002).

1.4.1.2 GPVI/FcR γ-Chain Complex

Found in the ECM upon endothelial cell disruption, collagen and laminin are the physiological ligands of GPVI (Inoue et al. 2006). synthetic ligands include collagen-related-peptide (CRP) which is GPVI-specific (Asselin et al., 1997); and the snake toxin convulxin that bind to GPVI and GPIb-IX-V (Kanaji et al., 2003, Jandrot-Perrus et al., 1997, Canobbio et al., 2004, Wei et al., 2009) are used in *in vitro* studies to investigate downstream signalling events that are independent of integrin α2β1.

GPVI is a 62 kDa type I transmembrane receptor (Clemetson et al., 1999, Wei et al., 2009), located on chromosome 19 the human genome. Expressed solely in megakaryocytes and platelets, GPVI consists of two IgG–like domains and a stalk that is mucin-like in its extracellular region. There follows a trans-membrane region, and a small cytoplasmic tail that in humans contains 51 amino acids. This domain contains only 27 residues in the murine GPVI, suggesting that the absent amino acids may not play a critical role in GPVI function (Moroi and Jung, 2004, Jackson, 2011b).

GPVI occurs partially as a dimer on the platelets surface. Ligand-mediated clustering triggers signalling via Src family tyrosine kinases (SFKs) (Horii et al., 2009). GPVI forms a constitutive non-covalent contact with the FcR γ-chain via a positively charged arginine in its trans-membrane region (Watson et al., 2005a, Nieswandt and Watson, 2003). The interaction of GPVI with the γ-chain is essential for both expression

of GPVI on the platelet surface and for signalling, (Gibbins et al., 1996, Poole et al., 1997, Quek et al., 2000, Severin et al., 2012).

GPVI induces sequential activation of SFKs, predominantly via Lyn and Fyn, with Src also playing a minor role (Severin et al., 2012). The SFK Lyn is constitutively associated with a proline-rich area in the cytoplasmic tail of GPVI through its SH3 domains (Suzuki-Inoue et al., 2002) where it phosphorylates the conserved immunoreceptor tyrosine-based activation motif (ITAM) (YXX [L/I] X_{6-12}YXX [L/I]) in the FcR γ-chain (Gibbins et al., 1996, Poole et al., 1997). By means of its tandem SH2 domains, the tyrosine kinase Syk binds to the phosphorylated FcR γ-chain ITAM leading to auto-phosphorylation and additional phosphorylation by SFKs (Watson et al., 2005b). This highly activated protein mediates further downstream signalling events in lipid rafts, which includes formation of the 'LAT signalosome' containing the adaptors LAT, SLP-76, Gads/Grb2, Tec family kinases, The Vav family of guanine nucleotide exchange factors (GEF) and effector proteins, including PI 3-kinase and PLCγ2 (Watson et al., 2005b). Formation of the second messengers by 1 2-diacylglycerol (DAG) and inositol 1, 4, 5- trisphosphate (IP_3) is induced by PLCγ2. The two second messenger stimulate PKC and release of Ca^{2+} from intracellular stores, respectively (Figure 1-4). Integrin activation, release of α- and dense-granules, and the discharge of the secondary mediators ADP and TxA_2, which prime stimulatory signals and thrombus growth, reinforce GPVI signalling (Offermanns, 2006, Nieswandt and Watson, 2003).

Clinical cases of bleeding diathesis in patients harbouring quantitative or qualitative defects in GPVI are rare due to compensatory mechanisms of platelet activation by multiple platelet receptors. However, there has been a report of idiopathic thrombocytopenic purpura (ITP) due to reduced levels of GPVI surface expression,

where auto-antibodies directed towards GPVI were observed in the patient leading to GPVI shedding and clearance of the GPVI/FcRγ -chain complex from the platelet surface (Boylan et al., 2004). In addition, patients with genetic defects in GPVI that present with a bleeding diathesis have been reported in recent studies (Matus et al., 2013, Hermans et al., 2009). The use of GPVI-deficient mice have provided further supporting evidence for the vital role of this receptor in collagen-induced platelet activation, thrombosis and haemostasis by phenocopying GPVI deficiency in humans (Matsumoto et al., 2007, Lockyer et al., 2006, Konishi et al., 2002).

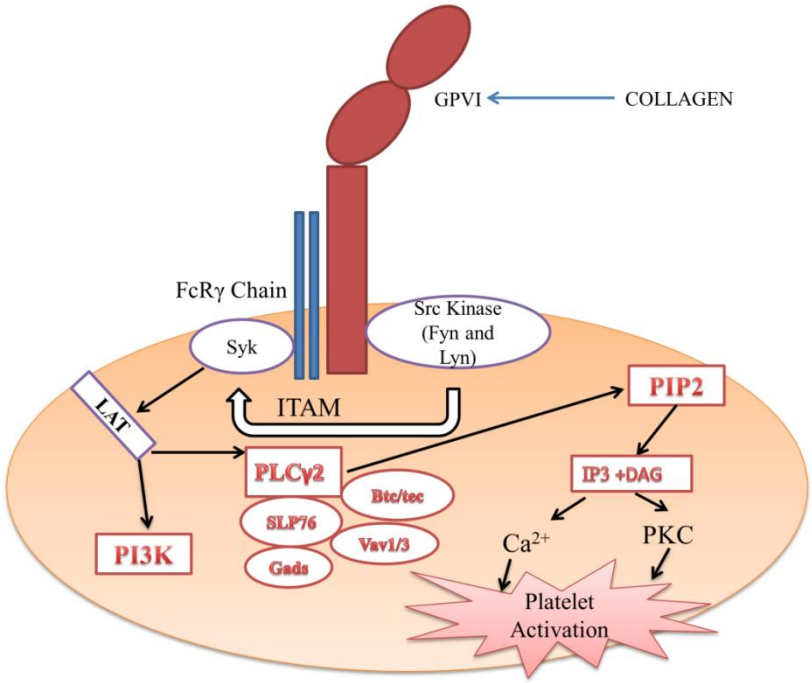

Figure 1-4 Collagen binding to GPVI leads to activation of phosphorylation of Src kinases, Syk and PLCγ2

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1.4.1.3 Integrin α2β1

The first collagen receptor identified on platelets, which functions almost predominantly as an adhesion receptor, is the integrin α2β1 (also known as GPIa/IIa) (He et al., 2003, Nieswandt and Watson, 2003). Expression levels fluctuate between individuals with approximately 2,000 – 4,000 copies per resting human platelet (Best et al. 2003; Samaha et al. 2004). It engages with high affinity to collagen types I – V (Holtkotter et al., 2002, Leitinger and Hogg, 2002). Its level of expression is unaltered upon platelet activation. The integrin is usually in a 'low-affinity' state on the surface of resting platelets. Upon interaction with collagen, 'inside-out' signalling induces a conformational change to a 'high-affinity' state (Jung and Moroi, 2000a, Jung and Moroi, 2000b). In addition to supporting platelet adhesion, binding to integrin α2β1 also increases the net binding of collagen to GPVI and therefore potentiation of signalling by the immunoglobulin receptor (Nieswandt and Watson, 2003, Jung and Moroi, 2000b). On the other hand, α2β1 signalling is much weaker than that of GPVI and possibly plays little role in thrombus formation despite the fact that the α2β1 signalling pathway utilises many of the same signalling molecules as the GPVI signalling pathway, including Src, Syk, SLP-76, and PLCγ2 (Inoue et al., 2003). It is established in genetic and pharmacological studies that α2β1 has almost no protective influence in arterial thrombosis and plays only a small part in platelet aggregate formation on collagen under flow (Holtkotter et al., 2002, Farndale et al., 2004, He et al., 2003).

1.4.1.4 CLEC-2 receptor

The C-type lectin-like (CLEC)-2 receptor is expressed on human and mouse platelets (Suzuki-Inoue et al., 2006, Severin et al., 2012). It is located on chromosome 12 in the human genome, is a type II membrane protein, and has a single trans-membrane region, an N-terminal cytoplasmic domain, and a C-terminal extracellular domain (Figure 1- 5). CLEC-2 has also been identified in inflammatory dendritic cells.

The endogenous ligand for CLEC-2 is the type I trans-membrane glycoprotein, podoplanin (Suzuki-Inoue et al., 2007a, Chaipan et al., 2006a). The expression of podoplanin on cancer cells has been shown to induce powerful platelet aggregation (Tsuruo and Fujita, 2008). In addition, CLEC-2 and podoplanin are required for lymphatic development, as mice deficient in either gene have blood-lymphatic mixing (Uhrin et al., 2010, Herzog et al., 2013).

Rhodocytin is a snake venom toxin derived from *Calloselasma rhodostoma* venom that was used in the original affinity purification of CLEC-2 receptor. It is used *in vitro* to activate CLEC-2 and induces platelet activation via Syk and Src family kinases (Suzuki-Inoue et al., 2011, Severin et al., 2011a) which subsequently leads to activation of PLCγ2 (Suzuki-Inoue et al., 2006). Platelet activation and protein tyrosine phosphorylation are blocked by SFK inhibitors such as PP2 (Severin et al., 2011a) and by the Syk inhibitors R406 or PRT-060318 (Spalton et al., 2009a, Hughes et al., 2013).

In contrast to GPVI receptors, CLEC-2 signals through a hemITAM sequence in its cytoplasmic tail, which is a single YxxL. Syk mediates phosphorylation of hemITAM with the SFKs regulating Syk (Hughes et al., 2013, Severin et al., 2011a) (Figure 1-5). Both SH2 domains of Syk are required for CLEC-2 signalling, with point mutations in either SH2 domain abolishing signalling (Fuller et al., 2007). As is the case for GPVI-FcR γ-chain, activation of Syk leads to formation of a LAT signalosome that includes the adapter SLP-76, Tec family tyrosine kinases, Vav GTP exchange factors and PLCγ2 (Fuller et al., 2007,

Suzuki-Inoue et al., 2006). In CLEC-2-deficient murine platelets, high concentrations of rhodocytin induce weak platelet aggregation in the absence of SLP-76 or combined Vav1 and Vav3, in contrast to the abolition of aggregation to CRP or convulxin (Suzuki-Inoue et al., 2006).

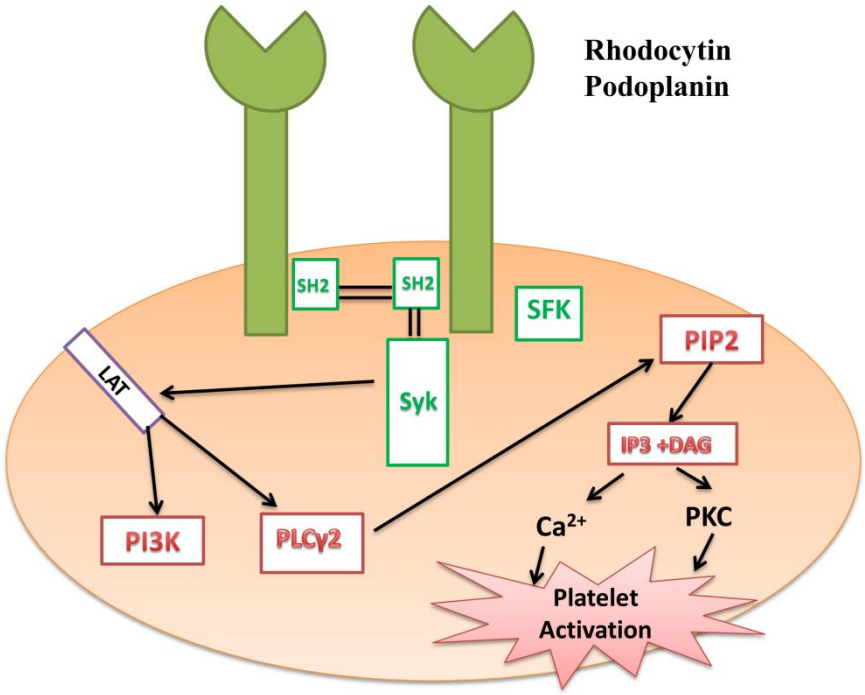

Figure 1-5 CLEC-2 by binding of Rhodocytin or podoplanin leads to activation of platelets through Src and Syk kinase.

1.4.1.5 Integrin αIIbβ3

The most abundant glycoprotein expressed on the platelet surface with approximately 60,000 – 80,000 copies per resting human platelet is the integrin αIIbβ3 (also known as GPIIb/IIIa). The GPIIb gene and the GPIIIa gene are located on chromosome 17 in the human genome (Shattil et al., 1998). αIIbβ3 is also stored in platelet α-granules, therefore upon platelet activation it translocates to the platelet surface, increasing expression by 30 – 50% (Shattil et al., 1998, Shattil and Newman, 2004). The β3 subunit is expressed in numerous cell types where it co-operates with other α subunits whereas the αIIb subunit is restricted to the megakaryocyte/platelet lineage (Shattil and Newman, 2004). Fibrinogen, fibronectin and VWF all bind to platelets via αIIbβ3.

The integrin αIIbβ3 is usually in a 'low-affinity' state on the surface of resting platelets and experiences a conformation alteration to a 'high-affinity' state when induced by 'inside-out' signalling (Shattil and Newman, 2004). Outside-in signalling, filopodia and lamellipodia formation, secretion and clot retraction are all activated by ligand mediated clustering of αIIbβ3 (Shattil et al., 1998). Adhesion and aggregate formation at sites of vascular injury require αIIbβ3 (Shattil and Newman, 2004). Outside-in signalling events of αIIbβ3 and GPVI signalling share a number of similarities (Watson et al., 2005a, Kasirer-Friede et al., 2007). The consecutive activation of SFKs, Syk, employment of adapter proteins including SLP-76 and activation of downstream effector enzymes, including PLCγ2, PI 3-kinase and Vav are all caused as a result of fibrinogen-mediated clustering of αIIbβ3 (Kasirer-Friede et al., 2007, Watson et al., 2005a).

SFKs (Src and Fyn) are successively linked with the β3 subunit and Syk becomes engaged to the integrin as a result of clustering analogous to events downstream of GPVI receptor activation. However, there are a number of key differences; firstly it remains controversial as to whether an whether an ITAM-containing protein facilitates this interaction or Syk solely interacts with the β3 subunit tail (Woodside et al., 2001b, Mocsai et al., 2006, Hughes et al., 2015); secondly, αIIbβ3 is excluded from lipid rafts: and thirdly the transmembrane adapter protein LAT, which is necessary for GPVI signalling, is not required for αIIbβ3 signalling (Wonerow et al., 2002). There is also evidence for differences between mouse and human platelets, with FcγRIIa, which is absent in the mouse genome, playing a role in human αIIbβ3 signalling (Boylan et al., 2008).

In recent years, the molecular processes that underlie outside-in integrin αIIbβ3 signalling have been studied in detail. The C-terminal Src kinase (Csk) which, as the name suggests, phosphorylates an inhibitory tyrosine in the C-terminal tail of Src, holds integrin αIIbβ3-associated Src in an inactive conformation in resting platelets (Tyr-529) (Obergfell et al., 2002, Arias-Salgado et al., 2005) Csk detaches from the αIIbβ3-Src complex and is substituted by the non-transmembrane protein tyrosine phosphatase, PTP-1B, which dephosphorylates the inhibitory tyrosine of Src and initiates the signalling pathway as integrin clustering occurs (Arias-Salgado et al., 2005). Syk is then recruited to the complex and activated via phosphorylation by Src and by autophosphorylation (Woodside et al., 2001b, Mocsai et al., 2006). Other SFKs also play a role in the initiation of outside-in integrin αIIbβ3 signalling including Fyn, Lyn and Yes (Obergfell et al., 2002, Arias-Salgado et al., 2003, Senis et al., 2009). The interaction between Syk and tyrosine phosphorylation at these sites is terminated by phosphorylation of the two conserved tyrosines in the β3-tail (Woodside et al., 2001a).

It is functionally important that phosphorylation of the two conserved tyrosine residues in the β3 subunit occurs (Law et al., 1999). A conserved NPXY motif that facilitates binding to proteins with phosphotyrosine binding (PTB) domains including the Dok family of adapter proteins is mediated by phosphorylation (Law et al., 1999, Garcia et al., 2004, Gibbins et al., 1996). A NXXY motif contains a second tyrosine and binds a separate group of PTB domain containing proteins, including Shc (Phillips et al., 2001). In a diYF knock-in mutant mouse model, increased bleeding and impairment in clot retraction is observed (Law et al., 1999). It has been proposed that this is because of loss of binding of myosin to the phosphorylated β3 tail (Phillips et al., 2001).

Glanzmann thrombasthenia, a severe bleeding disorder associated with impaired adhesion and abolished aggregation of platelets, is a result of the absence of, or reduced function of αIIbβ3 (Nurden, 2006). Mice lacking the αIIb- or β3-integrin subunits phenocopy Glanzmann thrombasthenia (Hodivala-Dilke et al., 1999). Haematopoiesis as well as platelet function have been studied in both αIIb-deficient and β3-deficient mice models (Tropel et al., 1997, Emambokus and Frampton, 2003). Extended tail bleeding times and spontaneous haemorrhage are displayed by mice that are β3-deficient. In addition, they do not form thrombi at sites of vascular damage as shown by intravital microscopy (Ni et al., 2000).

The clear involvement of αIIbβ3 in platelet adhesion and accumulation has made it a popular pharmacological target for the prevention of ischemic cardiovascular events. Antibodies including abciximab, peptides based on those isolated from venom of snakes such as eptifibitide, and analogues of an RGD-containing peptide (for example lamifiban and tirofiban) that constrain ligand binding are just some of the strategies that can be used to inhibit its function (Coller and Shattil, 2008). However,

due to excessive bleeding, these inhibitors are not used widely outside of the hospital (Quinn et al., 2003).

1.4.1.6 VWF Receptor – GPIb-IX-V Complex

Restricted to platelets and megakaryocytes, the structurally distinctive receptor complex GPIb-IX-V is expressed at a high level of 25,000 copies per platelet (Berndt et al., 2001). The genes that encode the receptor complex, GPIb α- and β-subunits, GPIX, and GPV, are all members of the leucine-rich repeat protein superfamily (Berndt et al., 2001). When first described, the complex was suggested to be made up of two GPIbα subunits, two GPIbβ subunits, two GPIX subunits and a single GPV subunit (Berndt et al., 2001). More recently, it was proposed that it exists as a molar ratio of 2:4:2:1 of GPIbα, GPIbβ, GPIX, and GPV, respectively (Luo et al., 2007).

Platelets are captured at the site of injury by reducing their velocity to allow the interaction of other receptors with the injured surface. This capturing function is carried out by GPIb-IX-V. The high shear rates observed in small arteries, arterioles, and stenosed arteries, coupled with the interaction of GPIbα and VWF restrained on collagen or on the surface of activated platelets is critical for the early tethering of platelets under flow (Savage et al., 1998). To facilitate stable adhesion, the activation of integrins is essential. Original reports suggested that the rapid on/off rate between GPIb-IX-V and VWF was not long enough to initiate signalling (Savage et al., 1996). However, more recent data reveal that weak intracellular signalling through Src family kinase and PLCγ2-related pathways is mediated by GPIb-IX-V and results in the activation of platelets and the development of αIIbβ3-mediated aggregates (Yap et al., 2002, Mangin et al., 2003). First proposed by Ozaki et al, a theoretical GPIb-IX-V signal transduction pathway was anticipated to take place, principally in lipid rafts (Ozaki et al., 2005). In agreement with this model, GPIb-IX-V is constitutively linking

with the p85 subunit of PI 3-kinase via 14-3-3ζ, (Mangin et al., 2004, Mu et al., 2008). The interaction between PI 3-kinase and Src, which produces downstream signals, including activation of PLCγ2, is induced by the interaction between GPIb-IX-V and VWF.

A condition known as Bernard-Soulier syndrome which is a congenital bleeding disorder described by mild thrombocytopenia, giant platelets and the incapability of platelets to form aggregates in response to the antibiotic ristocetin is suffered by humans missing or expressing dysfunctional GPIbα, GPIbβ or GPIX subunits (Lopez et al., 1998).

A number of coagulation factors like thrombin and Factors XI, XII and VII, which may add to the severe bleeding defect shown by Bernard-Soulier syndrome patients, also bind to GPIb-IX-V (Dumas et al., 2003, Vanhoorelbeke et al., 2007).

1.4.2 G Protein–Coupled Receptors

Various GPCRs expressed by platelets play a vital role in controlling platelet activation (Woulfe, 2005, Offermanns, 2006). The platelet activation receptors which are the most significant are: (1) the thrombin protease-activated receptors (PAR)-1 and PAR-4; (2) the ADP receptors $P2Y_1$ and $P2Y_{12}$; and (3) the TxA_2 receptor (TP) (Figure 1-6).

1.4.2.1 Thrombin receptors - Protease Activated Receptors (PARs)

Thrombin (also called Factor IIa) is a soluble plasma serine protease produced as a result of a series of enzymatic reactions, usually called the ‘coagulation cascade’ that occurs on the surface of activated platelets (Hoffman and Monroe, 2001). Active thrombin is formed by the conversion of the inactive zymogen prothrombin; this active thrombin converts the soluble fibrinogen into insoluble fibrin polymers via its serine

protease activity. Thrombin is also classified as a dominant platelet agonist (Coughlin, 2000). It activates platelets by cleaving PARs on platelet surfaces to expose peptide ligand that mediate platelet activation (Coughlin, 2005). Human platelets have two PAR receptors, PAR-1 and PAR-4 (Kahn et al., 1999). PAR-1 is the main thrombin receptor in humans but is not expressed in mice platelets, whereas PAR-4 is expressed in platelets of both humans and mice (Kahn et al., 1998). Almost 1,000 – 2,000 copies of PAR-1 are expressed by human platelets (Brass et al., 1992, Ramstrom et al., 2008). PAR-1 and PAR-4 can couple to the heterotrimeric G proteins G_q and $G_{12/13}$ (Coughlin, 2000). PAR-1 and PAR-4 activate PLCβ to produce IP3 and DAG, which subsequently release Ca^{2+} and activate PKC as described above. Both receptors are coupled to the G_q α-subunit. In addition, they activate the $G_{12/13}$ pathway resulting in activation of Rho kinase which leads to cytoskeletal rearrangements (Coughlin, 2000). Downstream of the thrombin receptors, there is a minor Src kinase-dependent component to the signalling pathway which is G protein-coupled (Coughlin, 2000, Murugappan et al., 2005). Along with PAR-4, mouse platelets also express PAR-3, however this receptor does not mediate transmembrane signalling unlike its other family members. Instead, it acts as a cofactor that binds and localizes thrombin in close vicinity of PAR-4 in mouse platelets (Coughlin, 2000).

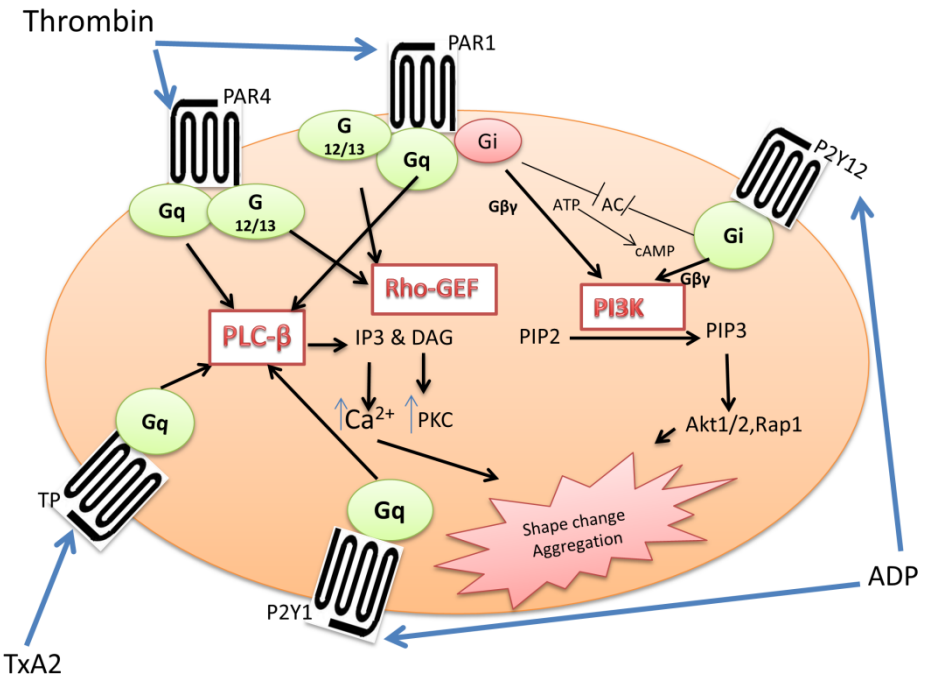

Figure 1-6 soluble agonists ADP, thrombin and TxA2 bind to their G protein coupled-receptors. G_i, G_q, and $G_{12/13}$: G proteins. DAG: 1,2-diacylglycerol, Rho-GEF: guanine nucleotide exchange factor, PLC-β: phospholipase, IP3: inositol 1,4,5-trisphosphate, PI3K: phosphatidylinositol 3-kinase, PIP2: phosphatidylinositol 4,5-bisphosphate, PIP3: phosphatidylinositol 3,4,5-triphosphate, PKC: protein kinase C. (Nieswandt et al., 2000).

1.4.2.2 ADP Receptors - $P2Y_1$ and $P2Y_{12}$

ADP is released by activated platelets, red blood cells, and damaged endothelial cells at the site of vascular injury. ADP functions to improve platelet activation through a positive feedback mechanism and is important for the recruitment of platelets to a thrombus that is developing (Offermanns, 2006). $P2Y_1$ and $P2Y_{12}$ are the two ADP receptors expressed by platelets (Gachet et al., 2006). Human platelets express a low number of copies of $P2Y_1$ (150 copies) and approximately ten-fold more of $P2Y_{12}$. Studies using selective inhibition or deletion of one or the other receptor leads to a decrease in platelet aggregation, with co-activation of $P2Y_1$ and $P2Y_{12}$ essential for ADP-induced platelet activation (Gachet et al., 2006). Therefore, by working together, activation of these two receptors results in rapid and sustained platelet activation. $P2Y_{12}$ couples to the G_i heterotrimeric G protein α-subunits while $P2Y_1$ couples to G_q (Gachet et al., 2006). Ca^{2+} mobilization, platelet shape alteration and weak, temporary aggregation in response to ADP are all initiated by the $P2Y_1$ receptor (Gachet et al., 2006). In contrast, $P2Y_{12}$ inhibits the adenylyl cyclase-dependent reduction in intracellular cAMP levels and independently acivates PI3-kinase and Rap-1B (Gachet et al., 2006). Persistent platelet aggregation and intensification of signals mediated by other agonists, including collagen, TxA_2 and the integrin αIIbβ3, are mediated by $P2Y_{12}$ activation (Gachet et al., 2006). It has also been reported that Src kinase are activated downstream of both the $P2Y_1$ and $P2Y_{12}$ receptors (Hardy et al., 2004, Shankar et al., 2006).

1.4.2.3 Thromboxane A_2 Receptor – TP

To increase and sustain activation signals and to recruit more platelets to the site of thrombus formation, activated platelets release TxA_2 which is a short-lived lipid mediator (Hamberg et al., 1975). TxA_2 also plays a role as a potent vasoconstrictor (Francois et al., 2004). This lipid mediator is produced from prostaglandin H_2 (PGH_22) using thromboxane-A synthase in activated platelets (Nakahata, 2008, Huang et al., 2004). Its lipid soluble nature allows for diffusion across the plasma membrane, thereby leaving the platelet and binding to the TxA_2 receptor (TP) on the surface of either the same or other platelets in the locality, in an auto- and paracrine way, respectively (Davi and Patrono, 2007, Suzuki-Inoue et al., 2002, Shen and Tai, 1998). Platelet activation and release are brought about by signalling through the TP receptor. Like thrombin receptors PAR-1 and PAR-4, and the $P2Y_1$ ADP receptor, the activated TP receptor in platelets couples mainly to the G_q and $G_{12/13}$ α-subunits (Djellas et al., 1999). A minor Src signalling component has also been reported (Huang et al., 2004). Patients lacking or having low TxA_2 production, exhibit a minor bleeding disorder demonstrating the significance of TxA_2 as a positive feedback agonist (Mumford et al., 2010) (Dawood et al., 2012). In addition, the TP knockout mouse model has an extended bleeding time supporting these observations (Thomas et al., 1998).

1.5 Fibrin

Fibrin is formed from soluble fibrinogen through the action of thrombin. As the final product of the coagulation cascade, fibrin plays a crucial role in the formation of stable thrombi. The interaction between thrombin and fibrinogen occurs at the central domain region of fibrinogen. The binding leads to a cleavage in fibrinogen peptides Aα and Bβ, and the release of fibrinopeptide A (FpA) and fibrinopeptide B (FpB),

respectively, a process that forms fibrin from fibrinogen (Undas and Ariens, 2011, Mosesson, 2005). In solution, FpA is released first and more promptly than FpB. However, when fibrinogen is attached to a surface, FpA cleaved slower than FpB (Weisel and Litvinov, 2013, Undas and Ariens, 2011) .

Thrombin has been reported to bind to fibrin at non-substrate high- and low-affinity binding sites. The site of high-affinity binding is at the D domain of the γ' chain, whereas that for low-affinity binding is at the E domain (Meh et al., 1996). Furthermore, the molecules released from the amino-terminal β15–42 fibrin sequence inhibit low-affinity thrombin binding. Hirudin, a peptide that inhibits thrombin activity, inhibits thrombin binding to fibrin at the high and low affinity sites (Meh et al., 1996). A low thrombin concentration leads to the formation of a thick fibrin fibre with high permeability, whereas a high thrombin concentration generates a thinner and less permeable fibrin fibre (Wolberg and Campbell, 2008). In addition, clinical studies have shown that an increase in thrombosis risk is linked to an increase in fibrin fibre density (Ariens, 2013). Thrombin also activates FXIII, a transglutaminase enzyme, which is known as a fibrin-stabilising factor that cross-links fibrin to form blood clots and support fibrinolysis resistance (Ariens, 2013, Weisel and Litvinov, 2013). Fibrin clots dissolve through the fibrinolytic system.

The fibrinolytic system is initiated by the activation of plasminogen (PLG). Plasminogen is a plasma zymogen that circulates in the inactive form of plasmin. It is activated by the tissue PLG activator (tPA), which is released from endothelial cells, and the urokinase PLG activator (uPA) produced by the kidney and tumour cells. PLG binds to tPA on a fibrin surface to generate plasmin, which then cleaves fibrin at the Lys-X and Arg-X bonds to form soluble degradation products, such as D dimer and X, Y, D and E fragments. uPA has a lower affinity than tPA for binding to fibrin, and is

able to activate PLG in the presence or absence of fibrin. However, its activity is increased up to 10-fold in the presence of fibrin (Cesarman-Maus and Hajjar, 2005).

Many studies have described the importance of fibrin in a variety of physiological processes including angiogenesis, inflammation and wound healing (van Hinsbergh et al., 2001, Mosesson, 2005, Dempfle and Mosesson, 2003, Jennewein et al., 2011). Fibrin has been shown to increase the production of cytokine and chemokines, and to regulate macrophage adhesion and leukocyte migration in inflammation (Petzelbauer et al., 2005, Szaba and Smiley, 2002).

Fibrin has been shown to bind to platelets through αIIbβ3 receptors via the sequence ATWKTRWYSMKK, which is in the γC-peptide: this region binds to the αIIb β-propeller domain. (Podolnikova et al., 2014). Polymerised fibrin has been shown to mediate adhesion and spreading of platelets (Hamaguchi et al., 1993).

1.6 Platelet Inhibition

To avoid undesirable thrombus development, platelets must be kept in a quiescent or 'resting' state in the peripheral circulation. This resting state is maintained by two major mechanisms which act via receptors for (1) prostacyclin (PGI_2) and (2) nitric oxide (NO), both of which are released from endothelial cells. Immunoreceptor tyrosine-based inhibition motif (ITIM)-containing receptors (including PECAM-, G6b-B and CEACAM1) expressed on the cell surface of platelets also inhibit platelet activation (Cicmil et al., 2002, Wong et al., 2009, Mori et al., 2008). However, they make a relatively small contribution to platelet inhibition in comparison to cAMP and cGMP elevation and so are not discussed in further detail.

1.6.1 PGI_2 Receptor

Prostaglandin I_2 (PGI_2) is produced by endothelial cells from PGH_2 by prostacyclin synthase and constitutively released into the circulation of the body, a powerful inhibitor of platelet activation, adhesion, aggregation and secretion. It has a short half-life, exerts its effects on platelets near the vessel wall and is released in low concentrations (Jin et al., 2005). G_s-coupled PGI_2 receptor initiates a signal cascade; the cAMP-dependent Ser/Thr protein kinase A (PKA) is activated by cAMP which is generated as a result of adenylyl cyclase activated by G_s. A number of the substrates for PKA which mediate platelet inhibition have been described and include vasodilator-stimulated phosphoprotein (VASP) which interacts with many cytoskeletal proteins at focal adhesions (Niebuhr et al., 1997) and in stress fibres (Sudo et al., 2003, Price and Brindle, 2000) .

1.6.2 Nitric oxide

Constitutively synthesized and release by endothelial cells, and vital in maintaing platelets in a resting state, is nitric oxide (NO). This gaseous inhibitor diffuses through the platelet plasma membrane into the cytosol where it exhibits inhibitory effects via activation of soluble guanylyl cyclase. Subsequent generation of cGMP triggers activation of the Ser/Thr protein kinase G (PKG) in concert with increasing intracellular levels of cAMP which activate PKA (Walter et al. 2009). Platelet activation is repressed when VASP is phosphorylated by both PKG and PKA (Butt et al., 1994, Li et al., 2003). Furthermore, NO also inhibits activation of PLC and IP_3-mediated mobilization of intracellular Ca^{2+} (Gambaryan and Tsikas, 2015) .

1.7 Platelet Ligands

Platelets can be activated also by a diverse range of stimuli that share little or no resemblance in structure to each other or to recognised ligands of platelet receptors, including diesel exhaust particles, various peptides (4N1-1 and Champs) and proteins (PAM3-CSK4) and large polysaccharides (e.g. fucoidan and dextran) as described below.

1.7.1 Diesel exhaust particles (DEP)

Air pollution has been shown to contribute to cardiovascular morbidity and mortality. Combustion of diesel fuel leads to the generation of gases and nanoparticulate matter with diameter <2.5 μm ($PM_{2.5}$) and <100nm (PM0.1) (D'Amato et al., 2010). It is one of the major types of nanoparticles in ambient particulate materials that cause an impact on human health (Popovicheva, 2014). DEP are made up of spherical carbon nanoparticles that contain the organic carbon, organic materials sulphated, nitrate and polyaromatic hydrocarbons (PAH) and carry an electrical surface charge (Jung and Kittelson, 2005, D'Amato et al., 2010). Visualization of DEP by using transmission electron microscope shows that DEP is spherical in appearance and present as individual particles, complex agglomerates, chained agglomerates and simple agglomerates. DEP diameter range from 40-70nM (Solomon et al., 2013). DEP have been shown in different studies to be associated with respiratory disorders and cardiovascular disease (Nemmar et al., 2007, Nemmar et al., 2003, Poss et al., 2013). DEP have been reported to cross the lung epithelium and enter the blood stream and have been proposed to cause inflammation and to be cytotoxic (Boland et al., 1999, Suzuki-Inoue et al., 2006). Several studies have shown that DEP induce platelet aggregation and activation but the mechanism remains unknown (Nemmar et al., 2003,

Forestier et al., 2012, Solomon et al., 2013, Mourao-Sa et al., 2011b, Chaipan et al., 2006a, Tang et al., 2010).

1.7.2 Fucoidan

Fucoidan is a sulphated polysaccharide. Fucoidan contains fucose, sulfate, uronic acid, galactose, glucose and xylose (Ale et al., 2011). It is composed of sulfate groups which bind to the 4-position of fucose residues (Chevolot et al., 1999). The relationship between fucoidan structure and platelets activation is not established. It is used widely for its anti-coagulant (Liu et al., 2006), anti-thrombotic (Zhu et al., 2010), and anti-oxidant effects (Wang et al., 2010). Fucoidan bioactivity, however, varies according to the algae used for extraction, due to differences in fucoidan structure and charge (Ale et al., 2011). It has been reported that inhibition of tissue factor pathway inhibitor (TFPI) by fucoidan lead to increase clotting in hemophilia A and B patients (Liu et al., 2006). Recently it is has been shown fucoidan extract from Fucus vesiculosus activate platelets (Manne et al., 2013). It is known that fucoidan binds to several receptors on macrophage membranes, including the Toll-like receptor-4 (TLR-4), CD14, CR-3, and scavenging receptor (SR) (Teruya et al., 2009).

1.7.3 Dextran

Dextran is a bacterial polysaccharide produced primarily by the *Leuconostoc* strains, with *L. mesenteroides* NRRL B512F the main strain used in industrial preparations. It is a member of the glycosaminoglycans (GAGs) family and is used as a blood plasma volume expander (Abir et al., 2004) and as an anticoagulant in biological research (Zeerleder et al., 2002). The activity of dextran sulphate on platelet function has been shown using the PFA-100 closure time (CT) test. Samples that contain dextran cause a prolongation in CT with the collagen and ADP cartridge (Zeerleder et al., 2002). Dextran sulphate has no effect on platelet aggregation in platelet rich plasma to a variety of agonists

such as ADP and collagen, but aggregation is inhibited in response to ristocetin (Zeerleder et al., 2002). Furthermore, it has also been reported that dextran sulphate can affect secondary haemostasis by prolonging activated partial thromboplastin time (aPTT) and slightly increasing prothrombin time (PT) (Zeerleder et al., 2002). Recently dextran sulphate has been shown to activate platelets through SFKs (Getz et al., 2013a) .

1.7.4 Pam_3-CSK_4

Pam3-Cys-Ser-Lys4 (Pam_3-CSK_4) is a TLR1/TLR2 complex ligand. It is an artificial triacylated lipopeptide (LP), which mimics acylated amino terminus on bacterial pathogen LPs. It binds to TLR1/TLR2 leading to stimulation of the pro-inflammatory transcription nuclear factor NF-(*k*B). Pam_3-CSK_4 has been shown to stimulate platelet aggregation and activation (Blair et al., 2009, Falker et al., 2014, Suzuki-Inoue et al., 2011, Severin et al., 2011a) through TLR2 dependent pathways leading to activation of αIIbβ3 and P selectin expression (Blair et al., 2009). Moreover, it has been shown that Pam_3-CSK_4 activates platelets through Src, Syk, LAT and PLCγ2 (Falker et al., 2014).

1.7.5 4N1-1

Thrombospodin-1 (TSP1) is a glycoprotein stored in platelet α granules. It is released upon platelet activation. A minimal sequence from the C-terminal domain of TSP1, known as 4N1-1, promotes strong platelet activation including aggregation (Tulasne et al., 2001b). 4N1-1 stimulates a similar pattern of tyrosine phosphorylation similar to that used by GPVI including tyrosine phosphorylation of the FcRγ chain, Syk, SLP-76, and PLCγ2. Platelet activation by 4N1-1 is partially dependent on the FcRγ chain, suggesting that 4N1-1 may bind to GPVI and to a second receptor (Tulasne et al., 2001b).

1.7.6 Champs peptide

Computed helical anti-membrane protein (Champs) is a method that is used to design a peptide that binds with high affinity to the helical trans-membrane of endogenous proteins. The αIIbβ3 Champs peptide, also called the integrin-activation peptide (IAP), was shown using biophysical fluorescence-based and functional assays, to bind specifically to αIIbβ3 and to induce platelet aggregation (Yin et al., 2007) . However, in human platelets, IAP has been shown to induce platelet activation through integrin αIIbβ3-dependent and independent pathways (Grygielska et al., 2009). In addition, and similar to other peptides including 4N1-1 and LSARLAF, IAP has the ability to activate platelets through the FcRγ chain, Syk and PLCγ2 (Grygielska et al., 2009).

1.8 Aims of the thesis

In previous publications it has been shown that a large number of structurally unrelated ligands have the ability to activate platelets; in most cases, the molecular basis of activation is unclear. The aim in this thesis is to investigate the signaling mechanisms of structurally diverse agonists, including DEP, fucoidan, dextran, Pam_3CSK_4, 4N1-1, and Champs peptide IAP, and specifically to test the hypothesis that they induce platelet activation through the receptors CLEC-2 and GPVI. Unexpectedly, this programme of work led to the led discovery that fibrin also activates GPVI and this has been characterized.

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Chapter 2 FUCOIDAN IS A NOVEL PLATELET AGONIST FOR THE C TYPE LECTIN-LIKE RECEPTOR 2 (CLEC-2)

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This chapter reports that sulphated polysaccharide, fucoidan is a novel platelet agonist that activates CLEC-2. In this paper I have shown that intermediate concentrations of fucoidan stimulate aggregation of mouse platelets. Platelets aggregation is blocked in mouse platelets deficient in CLEC-2. In addition, fucoidan stimulates tyrosine phosphorylation of human CLEC-2 (figure 2-6). I have prepared mouse and human platelets as shown in method of chapter 3. I have designed and performed experiments, analysed data relating to Figures 2-6. The other experiments were performed by one of the other authors in the manuscript. The manuscript was written by one of other authors. Fucoidan used in this chapter is same as the Fucoidan used in Chapter 3.

Abstract

Fucoidan, a sulfated polysaccharide from *Fucus vesiculosus*, decreases bleeding time and clotting time in hemophilia, possibly through inhibition of tissue factor pathway inhibitor. However, its effect on platelets and the receptor by which fucoidan induces cellular processes has not been elucidated. In this study, we demonstrate that fucoidan induces platelet activation in a concentration-dependent manner. Fucoidan-induced platelet activation was completely abolished by the pan-Src family kinase (SFK) inhibitor, PP2, or when Syk is inhibited. PP2 abolished phosphorylations of Syk and Phospholipase Cγ2. Fucoidan-induced platelet activation had a lag phase, which is reminiscent of platelet activation by collagen and CLEC-2 receptor agonists. Platelet activation by fucoidan was only slightly inhibited in FcRγ chain null mice, indicating that fucoidan was not acting primarily through GPVI receptor. On the other hand, fucoidan-induced platelet activation was inhibited in platelet-specific CLEC-2 knock-out murine platelets revealing CLEC-2 as a physiological target of fucoidan. Thus, our data show fucoidan as a novel CLEC-2 receptor agonist that activates platelets through a SFK dependent signaling pathway. Furthermore, the efficacy of fucoidan in hemophilia raises the possibility that decreased bleeding times could be achieved through activation of platelets.

2.1 Introduction

Platelets act as essential mediators in maintaining homeostasis of the circulatory system, and their activation is tightly regulated *in vivo* under normal physiological conditions. However, when there is vascular damage, exposure of subendothelial collagen facilitates platelet activation, mainly through the glycolprotein VI receptor (GPVI), which is essential for hemostasis (Packham, 1994, Broos et al., 2012, Clemetson, 2012). Failure of platelet activation leads to excessive bleeding. Subsequent to platelet activation, coagulation proteins are activated resulting in thrombin generation and fibrin clot formation (Shuman and Levine, 1978). Deficiencies in coagulation proteins also lead to bleeding diathesis. For example, hemophilia A is a bleeding disorder in which the patient has an increased bleeding tendency due to factor VIII absence or dysfunction (Zimmerman and Fulcher, 1985, Zimmerman et al., 1971).

Sulfated polysaccharides of natural origin, which are characterized by their biological activities, have a favorable therapeutic profile in animals and humans because most have minimal side effects. These polysaccharides are composed of a broad range of subclasses similar to heparins, glycoaminoglycans, fucoidan, dermatan, or dextran sulfates (Zimmerman et al., 1971). Notably, compounds of natural origin exhibit different biological activities depending on the concentration or dose (Liu et al., 2006, Prasad et al., 2008). Fucoidan, a non-anticoagulatory sulfated polysaccharide, is derived from the brown seaweed *Fucus vesiculosus*. When compared with other sulfated polysaccharides, fucoidan demonstrates superior procoagulant properties (Zimmerman et al., 1971, Tsuji et al., 1997, Suzuki-Inoue et al., 2006). Pharmacological activity of this compound was tested in several animal models *in vitro* and *in vivo*. In humans, *in vitro* experiments with fucoidan demonstrated several beneficial effects on ulcers and bleeding disorders (Suzuki-Inoue et al., 2006, Sobanov et al., 2001). Non-

anticoagulatory sulfated polysaccharides were originally considered as a novel approach to improve coagulation because of their inhibitory effect on physiological anticoagulation, especially fucoidan, which inhibits tissue factor pathway inhibitor and accelerates clotting in hemophilia A and B patients (Liu et al., 2006). Therefore, fucoidan is in clinical trials as a new candidate drug for hemophilia patients. In prior studies, fucoidan was shown to enhance the survival rate in murine models of hemophilia A and B (Liu et al., 2006) and a canine model of hemophilia A (Prasad et al., 2008) by decreasing bleeding times. However, the effect of fucoidan on platelet aggregation, which regulates bleeding times, has not been studied.

GPVI signaling in platelets depends on FcR γ-chain activation leading to tyrosine kinase pathways resulting in the activation of Syk and PLC γ2(Tsuji et al., 1997). C-type lectin-like receptor 2 (CLEC-2), also linked to activation of Syk and PLC γ2 (Suzuki-Inoue et al., 2006), is highly expressed in platelets and at lower levels in neutrophils and dendritic cells (Sobanov et al., 2001, Colonna et al., 2000). CLEC-2 was identified by using the snake venom protein rhodocytin, purified from *Calloselasma rhodostoma* (Suzuki-Inoue et al., 2006), as a ligand on affinity chromatography, and later shown to be an endogenous receptor for podoplanin. The crystal structure of rhodocytin shows that CLEC-2 receptors are activated through clustering by this tetrameric ligand (Watson et al., 2008, Hooley et al., 2008, Watson et al., 2009). CLEC-2 and podoplanin are implicated in tumor metastasis (Christou et al., 2008, Suzuki-Inoue, 2011) and hemostasis and thrombosis (May et al., 2009). Unlike GPVI, CLEC-2 has an immunoreceptor tyrosine-based activation motif (hemITAM) sequence that is phosphorylated by Src and Syk tyrosine kinases, whereas phosphorylation of the ITAM is mediated solely by Src kinases (Spalton et al., 2009b). Rhodocytin and podoplanin are the only two known agonists for the CLEC-2 receptor

(Christou et al., 2008, Watson and O'Callaghan, 2011, Suzuki-Inoue et al., 2007b). In the present study, we show that fucoidan causes platelet activation, leading to αIIbβ3 mediated aggregation. We show that fucoidan signals through a tyrosine kinase-dependent signaling pathway downstream of CLEC-2 receptor and propose that this contributes to a decrease in bleeding time in hemophilia animal models. These results support the notion that decreased bleeding times can be achieved in hemophilia patients through activation of platelets.

2.2 Materials and Methods

All animal experiments were performed in compliance with the Institutional Animal Care and Use Committee at Temple University (Philadelphia, PA).

2.2.1 Mice

Platelet specific CLEC-2 knock-out mice were produced as described previously Briefly, the CLEC-2 gene (*CLEC1b*) was flanked with LoxP sites, and this mouse was cross-bred with a PF4-Cre expressing mouse to drive excision of the gene in only the megakaryocyte/platelet lineage.

2.2.2 Materials

Apyrase (type VII), acetylsalicylic acid, fucoidan (80% pure, with the major contaminant being fucose), and indomethacin were obtained from Sigma. Convulxin was purified as described (Francischetti et al., 1997). YM-254890 was a gift from Yamanochi Pharmaceutical Co., Ltd (Ibaraki, Japan). FURA-2 AM was from Molecular Probes (Eugene, OR). PP3 and PP2 were purchased from Biomol Research Laboratories (Plymouth Meeting, PA). OXSI-2 was from Calbiochem (Darmstadt, Germany). Whatman protein nitrocellulose transfer membrane was obtained from Fisher Scientific (Pittsburgh, PA), LI-COR Odyssey blocking buffer

was purchased from LI-COR Biosciences (Lincoln, NE). Antibodies to Syk ($Tyr^{525/526}$), PLC γ2 (Tyr^{759}), Lat (Tyr^{191}), and f3-actin were bought from Cell Signaling Technology (Beverly, MA), Total Syk and Lat antibodies were obtained from Santa Cruz Biotechnology (Santa Cruz, CA). Monoclonal mouse anti-CLEC-2 antibody was obtained from Santa Cruz Biotechnology. FITC-labeled PAC-1and mouse IgG isotype control antibodies were obtained from BD Technologies (Franklin Lakes, NJ). P-selectin-PE antibodies were purchased from BD Biosciences.

2.2.3 Preparation of Human Platelets

Blood was collected from informed healthy volunteers in to one-sixth volume of acid/ citrate/dextrose (2.5 g of sodium citrate, 2 g of glucose, and 1.5 g of citric acid in 100 ml deionized water). Platelet-rich plasma was obtained by centrifugation at 250 X *g* for 20 min at ambient temperature and incubated with 1 mM aspirin for 30 min at 37°C. Platelets were isolated from plasma by centrifugation at 980 X *g* for 10 min at ambient temperature and resuspended in Tyrode's buffer, pH 6.5 (138 mM NaCl, 2.7 mM KCl, 2 mM $MgCl_2$, 0.42 mM NaH_2PO_4, 5 mM glucose, 10 mM PIPES (pH 6.5) containing 20 nM PGE1, 10 µM indomethacin, 500 µM EGTA, and 0.2 units/ml apyrase,). Platelets were isolated from Tyrode's buffer, pH 6.5, by centrifugation at 980 X *g* for 10 min and resuspended in Tyrode's buffer, pH 7.4 (138 mM NaCl, 2.7 mM KCl, 2 mM $MgCl_2$, 0.42 mM NaH_2PO_4,5 mM glucose, 10 mM HEPES, and 0.2 units/ml apyrase, pH 7.4). The platelet count was adjusted to 2–2.5 X 10^8/ml. Approval was obtained from the institutional review board of Temple University for these studies. Informed consent was provided prior to blood donation.

2.2.4 Preparation of Murine Platelets

All mice were maintained in a specific pathogen-free facility, and animal procedures were carried out in accordance with institutional guidelines after the Temple University Animal Care and Use Committee approved the study protocol, or they were performed under UK Home Office Project license (PPL 40/2803). Fccer1g (FcRγ knock-out) or γ-chain-deficient mice were purchased from Taconic (Germantown, NY). Age and gender-matched wild-type mice were used as controls. Blood was drawn via cardiac puncture into one-tenth volume of 3.8 % sodium citrate. Blood was spun at 100 X *g* for 10 min, and the platelet rich plasma (PRP) was separated. Red blood cells were mixed with 400 µl of 3.8 % sodium citrate and spun for a further 10 min at 100 X *g*. Resulting platelet rich plasmas (PRPs) were combined, and 1 µM PGE1 added and centrifuged for 10 min at 400 X *g*. Platelet poor plasma was removed, and the platelet pellet was resuspended in Tyrode's buffer (pH 7.4) containing 0.2 units/ml apyrase. Platelet counts were determined using a Hemavet 950FS blood cell counter (Drew Scientific Inc., Dallas, TX). For aggregation studies, a density of 2 X 10^8 platelets/ml was used.

2.2.5 Platelet Aggregation

Platelet aggregation was measured using a lumi-aggregometer (Chrono-Log, Havertown, PA) at 37°C under stirring conditions. A 0.5-ml sample of aspirin-treated washed platelets was stimulated with different agonists, and change in light transmission was measured. Platelets were preincubated with different inhibitors where noted before agonist stimulation. The chart recorder was set for 0.2 mm/s.

2.2.6 Measurement of Intracellular Ca^{2+} Mobilization

Platelet- rich plasma was incubated with 5 µM Fura-2/AM and 1 mM aspirin for 45

min at 37°C. Platelets were prepared as described above, and fluorescence was measured in a Perkin-Elmer apparatus with excitation set at 340 nm and emission set at 510 nm. Fluorescence measurements were converted to Ca^{2+} concentrations using the equation as reported (Grynkiewicz et al., 1985).

2.2.7 Flow Cytometry

Washed and aspirin-treated human platelets were used to measure agonist-dependent levels of αIIbβ3 activated receptor by PAC-1-FITC antibody and CD62P-PE antibody. Aliquots (0.5 ml) of washed platelet suspension in Tyrode's buffer (pH 7.4) containing 1 mM $CaCl_2$ were preincubated with the indicated concentration of inhibitors for 5 min at 37°C. Prior to the activation with fucoidan, an aliquot containing 10^6 platelets was gently mixed with 40 µl of anti-body mixture and incubated for 20 min at 37°C in the dark. Platelets were identified and gated according to the forward and side scatter signals. As control for immunolabeling, platelets were incubated with non-immuno-IgG isotype control antibody. To fix the platelets, 1 % parafarmaldehyde dissolved in phosphate-buffered saline was added. A total of 10,000 platelet events were acquired per sample, and the percentage of positive gated cells was analyzed. All determinations were performed on a FACS Caliber flow cytometer (BD Biosciences).

2.2.8 Immunoprecipitation

Washed human platelets (1×10^9/ ml) were stimulated with rhodocytin (50 nM) or fucoidan (50 µg/ml). Reactions were terminated by adding equal volume of 2X Nonidet P-40 lysis buffer (20 mM Tris-HCl, pH 7.6, 300 mM NaCl, 2 mM EGTA, 2 mM EDTA, 2% Nonidet P-40, 2 mM PMSF, 5 mM Na_3VO_4, 10 µg/ml leupeptin,

10 μg/ml aprotinin, 1μg/ml pepstatin). Cell debris was removed by centrifugation at 15,000 X *g* for 10 min. Antibodies against CLEC-2 (5 μg/ml) or control IgG were added to the resultant supernatant and incubated overnight with protein A Sepharose. Precipitated proteins were separated by SDS-PAGE and Western blotted with the 4G10 antibody.

2.2.9 Western Blot Analysis

Platelets were stimulated with agonists in the presence of inhibitors or vehicles for the appropriate time under stirring conditions at 37°C, and the reaction was stopped by the addition of 0.6 N $HClO_4$. The resulting acid precipitate was collected and kept on ice. The samples were centrifuged at 13,000 X *g* for 4 min followed by resuspending in 0.5 ml of deionized water. The protein was again pelleted by centrifugation at 13,000 X *g* for 4 min. The protein pellets were solubilized in sample buffer containing 0.1 M Tris, 2% SDS, 1% (v/v) glycerol, 0.1% bromphenol blue, and 100 mM DTT and then boiled for 10 min. Proteins were resolved by SDS-poly acrylamide gels and transferred to nitrocellulose membrane (Whatman Protran). Membranes were blocked with Odyssey blocking buffer for 1h at ambient temperature, incubated overnight at 4°C with the desired primary antibody, and then washed four times with TBS-T. Membranes were then incubated with appropriate secondary infrared dye-labeled antibody for 60 min at room temperature and washed four times with TBS-T. Membranes were examined with a Li-Cor Odyssey infrared imaging system.

2.2.10 Statistical Analysis

Each experiment was repeated at least three times. Results are expressed as means ± S.E. with number of observations (*n*). Data were analyzed using KaleidaGraph

software. Significant differences were determined using Student's *t* test. Differences were considered significant at $p < 0.05$.

2.3 Results

2.3.1 Fucoidan Causes Platelet Activation

As fucoidan reduced bleeding times in hemophilia animal models (Liu et al., 2006, Prasad et al., 2008), we evaluated whether fucoidan can cause platelet activation. Fucoidan caused aggregation of washed human platelets in a concentration-dependent manner (Fig. 1A). Fucoidan caused only shape change at a concentration of 10 µg/ml, whereas maximum aggregation was achieved at 50 µg/ml (Fig. 2-1A). We also evaluated the effects of fucoidan on other platelet functional responses, including integrin activation (Fig. 2-1B), dense granule secretion (Fig. 2-1C), P-selectin expression (Fig. 2-1D), and thromboxane generation (Fig. 2-1E) in human platelets. These results show that fucoidan causes platelet activation.

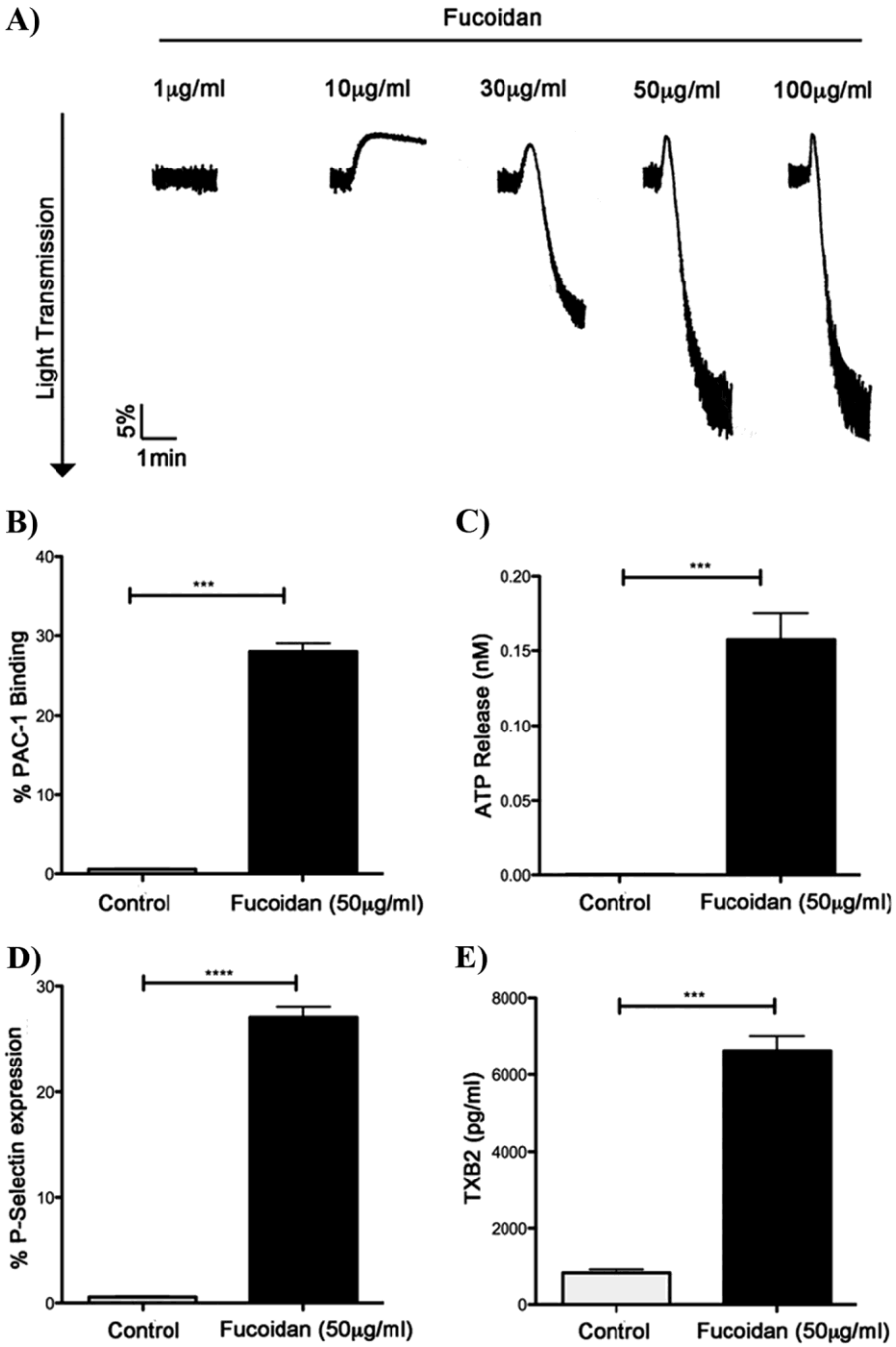

Figure 2-1 Fucoidan-induced platelet responses. (A) Washed aspirin-treated human platelets were stimulated with increasing concentrations of fucoidan at 37°C under stirring conditions. Platelet aggregation was measured using a Lumi aggregometer. The traces are representative of data from at least three independent experiments. (B) fucoidan-induced αIIbβ3 activation was measured by activating washed, aspirin-treated human platelets with fucoidan (50 µg/ml) and analyzed with FITC-PAC1 antibody by flow cytometry. (C) Dense granule secretion was assessed by measuring fucoidan-induced (50 µg/ml) ATP release from human platelets. (D) α granule expression was measured by CD62P (P-selectin) expression in response to fucoidan (50 µg/ml) and was analyzed using flow cytometry. Graphs represent mean ± S.E. of % positive cells from at least three different experiments (***, $p \leq 0.05$). (E) Washed human platelets without aspirin treatment were stimulated in a platelet aggregometer, and levels of TXB2 were determined according to established protocol (Shankar et al., 2006).

2.3.2 Role of Src Family Kinase (SFK) dependent Pathways in Fucoidan-induced Platelet Activation

Many platelet surface receptors, which mediate positive functional responses, are coupled to either $G_q$dependent or tyrosine kinase-dependent pathways (Brass, 2010) . To determine the signaling mechanism involved in fucoidan-induced platelet activation, we used the selective G_q inhibitor YM-254890 (Uemura et al., 2006, Kawasaki et al., 2006) or the pan SFK inhibitor PP2 (Li et al., 2010). Platelets pretreated with YM-254890 had only a minimum reduction in aggregation in comparison with the full inhibition of aggregation to AYPGKF (Fig. 2-2A), suggesting that the G_q pathway plays a minimal role in the activation of platelets by fucoidan, presumably through the feedback action of TxA_2. As shown in Fig. 2-2B, fucoidan induced platelet activation was completely abolished by PP2 but not by its inactive analog, PP3. These data demonstrate that SFK-dependent pathways are required for fucoidan-induced platelet activation. It is known that SFK-mediated signaling pathways involve activation and phosphorylation of Syk and Lat in platelets (Speich et al., 2008, Pasquet et al., 1999). Therefore, we evaluated the phosphorylation status of Syk and Lat following stimulation of platelets with fucoidan. As shown in Fig. 2-3A, Syk and Lat phosphorylations occurred as early as 15 s and dephosphorylated in a time-dependent manner. Fucoidan-induced Syk and Lat phosphorylations were abolished by pretreatment of platelets with PP2 (Fig. 2-3B). These data support our observation that fucoidan-mediated signaling pathways involve Syk and Lat activation downstream of SFKs. Intracellular Ca^{2+} mobilization was also measured in response to fucoidan in the presence of the pan SFK inhibitor PP2. PP2 completely blocked calcium mobilization compared with control (PP3) platelets (Fig. 2-3C). These data suggest that fucoidan activates a

SFK-dependent pathway leading to intracellular Ca^{2+} mobilization.

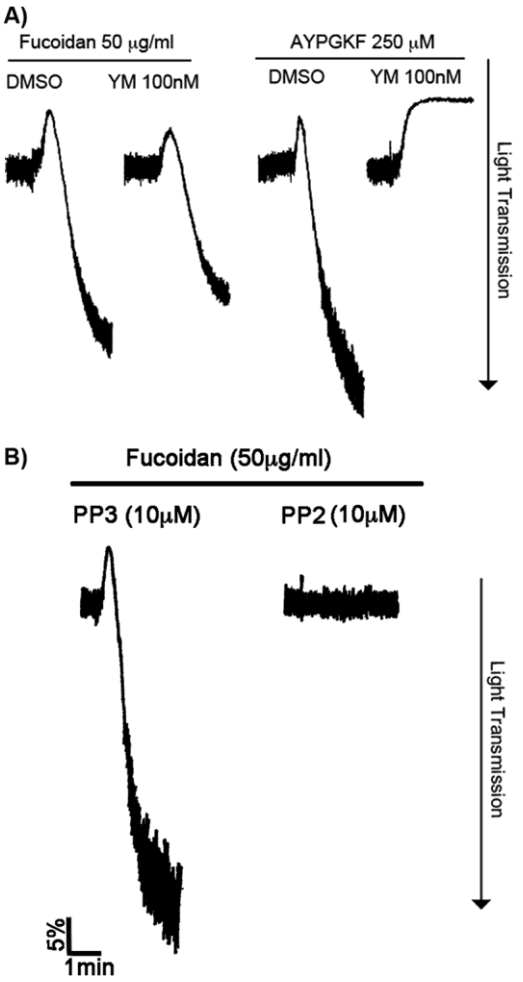

Figure 2-2 Role of Src family tyrosine kinase (SFK)-dependent pathways in fucoidan-induced platelet activation. **(**A) Washed, aspirin-treated human platelets were incubated with YM-254890 (50 nm), a selective G_q inhibitor, for 5 min and stimulated with fucoidan (50 μg/ml) for 3 min at 37°C under stirred conditions. Platelet aggregation was measured using lumi-aggregometer. The traces are representative of data from at least three independent experiments.(B) washed, aspirin-treated human platelets were pretreated with SFK inhibitor PP2 (10 μm) or PP3 (control) at 37 °C for 5 min followed by stimulation with fucoidan (50 μg/ml) for 3 min under stirred conditions. Platelet aggregation was measured by aggregometry. The tracings are representative of data from at least three independent experiments. DMSO, dimethyl sulfoxide.

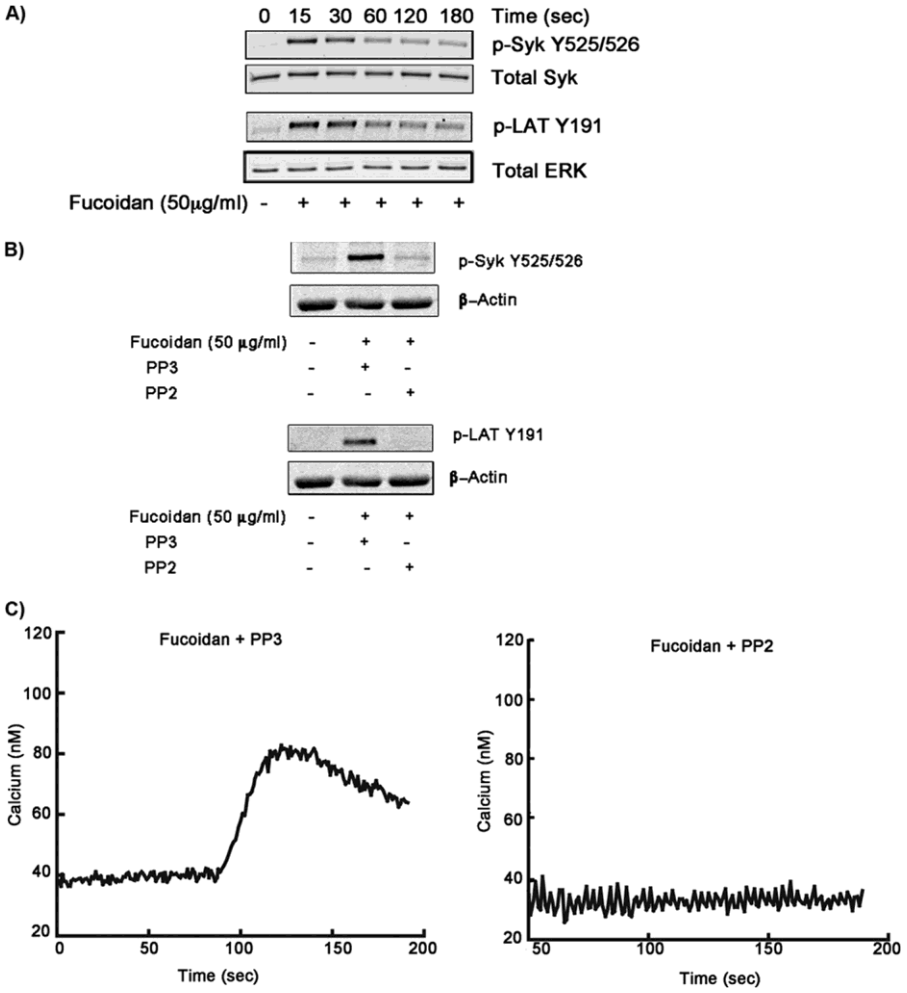

Figure 2-3 Effect of SFK inhibition on fucoidan-induced platelet activation. **(**A) fucoidan-induced phosphorylation of Syk and Lat. Washed, aspirin-treated human platelets were stimulated with fucoidan (50 μg/ml) for different time periods. Samples were subjected to SDS-PAGE and analyzed for phosphorylation of Syk and Lat. The results are representative of data from platelets at least three independent experiments. (B) Washed, aspirin-treated human platelets were stimulated with fucoidan (50 mg/ml) in the presence of the SFK inhibitor PP2 for 30 s, and the effect on Syk (Y525/26) and LAT (Y191) phosphorylation were analyzed. C, washed, aspirin-treated human platelets loaded with 5 μm FURA-2 AM were pretreated with PP3 or PP2 (10 μm) and activated with fucoidan (50 μg/ml) to measure intracellular Ca^{2+} mobilization.

2.3.3 Syk Mediates Platelet Activation by Fucoidan

The functional role of Syk in signaling by fucoidan was investigated by pretreating platelets with the Syk inhibitors OXSI-2 or Go6976 (Getz et al., 2011, Bhavaraju et al., 2008). Fucoidan-induced platelet activation was completely inhibited when treated with the Syk inhibitors, which was measured by percentage PAC1 binding using flow cytometry (Fig. 2-4A). To determine whether or not OXSI-2 was affecting the activity of SFKs, phosphorylation of Tyr^{416} (marker of SFK activation) was examined under similar experimental conditions. As demonstrated in Fig. 4B, SFK phosphorylation was not effected when a Syk inhibitor, OXSI-2 or Go6976, was used. The critical role of Syk in signaling by fucoidan was illustrated by the lack of PLCγ2 phosphorylation, which is an important downstream signaling molecule (Fig. 2-4, **C** and D). Furthermore, fucoidan-induced intracellular Ca^{2+} mobilization was completely abolished in the presence of either Syk inhibitor OXSI-2 or Go6976 (Fig. 2-4E). Inhibition of fucoidan-induced platelet activation by OXSI-2 or Go6976 suggests that Syk is an important downstream signaling molecule in fucoidan-induced platelet activation.

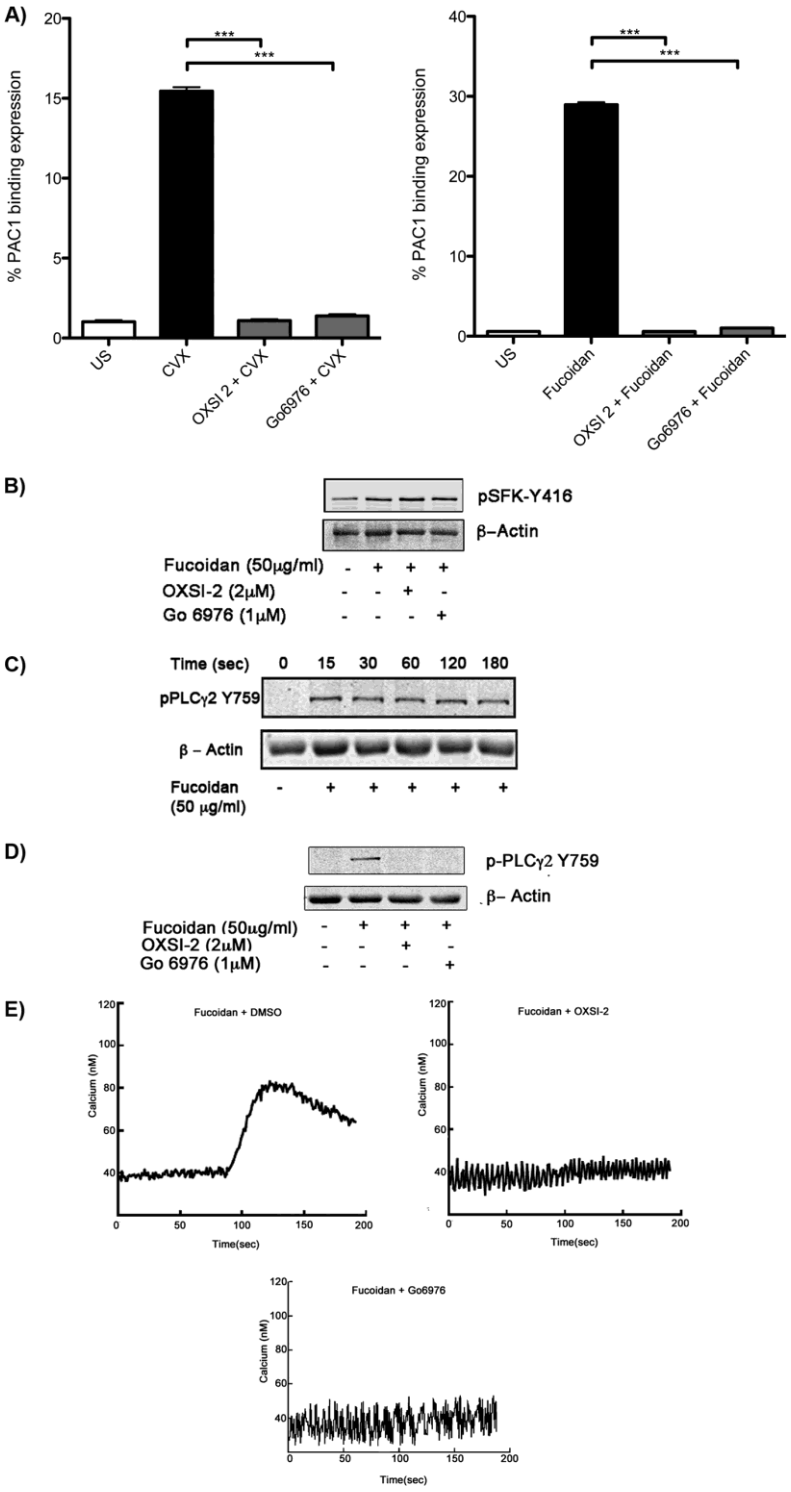

Figure 2-4 The role of Syk in fucoidan-induced aggregation, calcium mobilization, and αIIbβ3 activation. (A) Washed, aspirin-treated human platelets were pretreated with vehicle (dimethyl sulfoxide), OXSI-2 (2 μM), or Go 6976 (1 μM) for 5 min at 37°C prior to activation with convulxin (CVX; 100 ng/ml) or fucoidan (50 μg/ml) (US-unstimulated). αIIbβ3 expression was analyzed with PAC-1-FITC antibody. Graphs are represented mean ± S.E. of % positive cells from three different experiments (***, $p < 0.01$). (B) the effect of Syk inhibitors (OXSI-2 or Go 6976) on SFK phosphorylation induced by fucoidan (50 μg/ml) in human platelets was analyzed.(C) time course analysis of PLCγ2 tyrosine phosphorylation from aspirin-treated human platelets stimulated with fucoidan (50 μg/ml). (D) Platelets were stimulated with 50 μg/ml of fucoidan for 1 min. PLCγ2 tyrosine phosphorylation was measured in the presence of OXSI2, Go6976, or dimethyl sulfoxide. E, washed, aspirin-treated human platelets loaded with 5 μm FURA-2 AM were pretreated with OXSI-2 (2 μM) or Go 6976 (1 μM) and activated with fucoidan (50 μg/ml) to measure the effect of Syk inhibition in Ca^{2+} mobilization

2.3.4 Fucoidan Mediates Platelet Functional Responses through CLEC-2

The major receptors that trigger tyrosine kinase pathways in platelets are GPVI and CLEC-2 (Watson et al., 2005b, Watson et al., 2010, Hsu et al., 2001). Upon ligation of collagen to GPVI, the FcR γ-chain ITAM becomes phosphorylated by SFK members to initiate downstream signaling (Ezumi et al., 1998). Data presented in Figs. 2 and 3 suggest that the profile of activation by fucoidan is similar to that of GPVI. Previously, it was shown that FcR γ-chain-null platelets are not only unresponsive to collagen but also show no surface expression of GPVI (Jarvis et al., 2002). To evaluate whether fucoidan signals through GPVI, we made use of FcR γ-chain-null murine platelets. Wild type murine platelets aggregate in response to collagen-related peptide (10 μg/ml) (Fig.2-5A), whereas addition of collagen related peptide to FcR γ-chain null murine platelets failed to induce platelet aggregation (Fig.2-5A). Interestingly, fucoidan induced aggregation observed in FcR γ-chain null murine plate- lets was similar when compared with wild type murine platelets (Fig.2-5A). FcγRIIa is not present in murine platelets but does exist in human platelets. We therefore tested the role of this receptor by using the IV.3 monoclonal antibody that binds and blocks FcγRIIa. Blocking of FcγRIIa by IV.3 antibody did not affect fucoidan-induced platelet activation (Fig. 2-5B). These data suggest that fucoidan does not mediate its effects primarily through the GPVI or FcγRIIa receptor.CLEC-2, a C-type lectin receptor, has been shown to signal independently of the FcR γ-chain in platelets (Watson et al., 2009, Watson et al., 2010). The presence of Syk phosphorylation (Hughes et al., 2010c)and detectable levels of Lat phosphorylation in platelets activated with fucoidan (Fig.2-3) suggested that CLEC-2 (Hughes et al., 2008) may be a possible receptor candidate for fucoidan on platelets. To determine whether CLEC-2 is the

receptor that mediates fucoidan-induced platelet activation, platelet-specific (PF4-Cre) CLEC-2 receptor knock-out mice platelets were used. Wild-type murine platelets aggregated in response to fucoidan, but fucoidan failed to induce platelet activation in CLEC-2 receptor null mice (Fig.2-6A). Furthermore, tyrosine phosphorylation of the CLEC2 receptor was observed in immunoprecipitation studies when washed human platelets where activated with either rhodocytin or fucoidan (Fig. 2-6B), suggesting that fucoidan mediates platelet functional responses through the CLEC-2 receptor. We thus propose a model for the signaling events induced by fucoidan through the CLEC-2 receptor (Fig. 2-7).

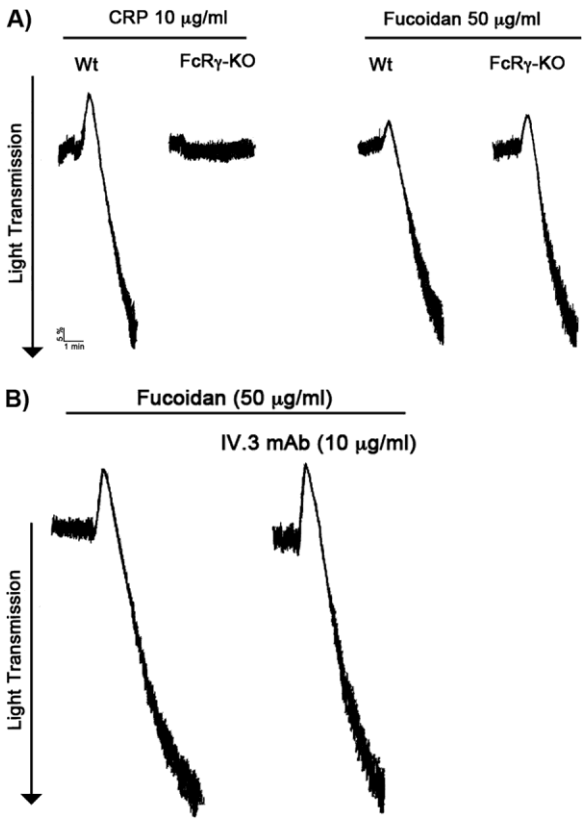

Figure 2-5 Fucoidan does not activate GPVI or FcγRIIa. (A) Fucoidan-induced platelet aggregation on FcR γ-chain-null murine platelets. Wild-type or FcR γ-chain-null murine platelets were stimulated with fucoidan (50 μg/ml) and CRP (Collagen Related Peptide) allowed to aggregate for 3 min at 37˚C under stirred conditions in a Lumi-aggregometer. The traces are representative of data from at least three independent experiments. (B) Human platelets were stimulated with fucoidan (50 μg/ml) in the absence and presence of IV.3 monoclonal antibody (10 μg/ml) for 3 min. The traces are representative of data from at least three independent experiments.

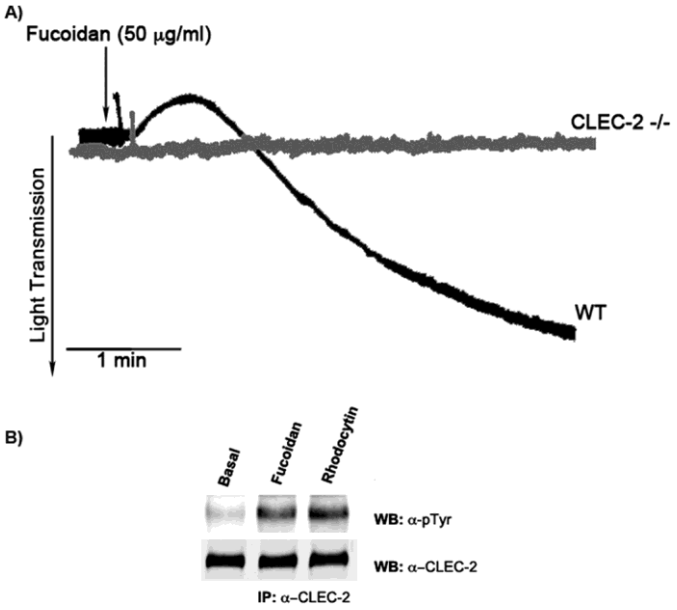

Figure 2-6 Fucoidan-induced platelet responses are mediated by CLEC-2 receptor. (A) Wild-type or CLEC-2 knock-out platelets (2×10^8/ml) were stimulated with fucoidan (50 μg/ml) and allowed to aggregate at 37°C under stirred conditions in a Lumi-aggregometer (n = 10). (B) Washed human platelets (1×10^9/ml) were stimulated with 100 nm rhodocytin or 50 μg/ml fucoidan. Reaction was terminated by addition of an equal volume of 2× lysis buffer. Platelet lysates were precleared, and detergent-insoluble debris were removed using centrifuge. Antibodies against CLEC-2 were added to the resultant supernatant and incubated overnight with protein A Sepharose. Precipitated proteins were separated by SDS-PAGE and Western blotted (WB) with the phosphotyrosine antibody. The data are a representation of three experiments.

2.4 Discussion

Fucoidan, a sulfated polysaccharide from a brown seaweed, decreases bleeding time and clotting time in hemophilia (Liu et al., 2006, Prasad et al., 2008). Decreased bleeding times in the hemophilia animal models by in *vivo* administration of fucoidan highlights the beneficial effect of fucoidan as a novel treatment (Prasad et al., 2008). Furthermore, *in vitro* studies by using platelet poor plasma from hemophilia animal models and human patients have showed that fucoidan inhibits tissue factor pathway inhibitor, thereby contributing to an increase in the extrinsic coagulation pathway activity (Liu et al., 2006). The effect of fucoidan on platelets, however, has not been studied. In the current work, we investigated whether or not fucoidan induces platelet activation and if so, what is the receptor that is mediating these signaling events. Our results suggest that fucoidan induces platelet activation (Fig.2-1) through a tyrosine kinase-dependent pathway (outlined in Fig.2-7). Using pharmacological inhibitors, we have shown that SFKs and Syk are crucial for fucoidan-induced platelet activation (Figs. 2-2 and 2-3). Our data from platelet-specific CLEC-2 receptor knock-out murine platelets suggest that CLEC-2 is the physiological receptor for fucoidan on platelets (Fig. 2-6A), thereby providing a novel CLEC-2 receptor agonist other than rhodocytin and podoplanin.

Previous studies suggest that fucoidan induced platelet activation was regulated by two important factors, molecular weight of the compound and the difference in sulfate content (Durig et al., 1997). Our results suggest that fucoidan, derived from the brown seaweed Fucus vesiculosus, with a high molecular weight of 150 –200 kDa and low sulfate content of 8 –11%, induces platelet activation

through a tyrosine kinase-dependent pathway that is mediated through the CLEC-2 receptor (Figs. 2-1 and 2-6). Previously, fucoidan has been shown to have antithrombotic effects (Millet et al., 1999, Trento et al., 2001, Durand et al., 2008). The reason for such opposite effects of fucoidan on platelets may be due to different molecular weights of fucoidan. When platelets are stimulated with fucoidan, we see a lag phase, which is reminiscent of GPVI- or CLEC-2 mediated activation. Recent studies have shown that the antibody to CLEC-2 induces dimerization of the receptor, generating a weak intracellular signal. The further clustering of CLEC-2 with a secondary antibody induces rapid and powerful activation. Similarly, tetrameric rhodocytin and polymeric podoplanin induce a much greater degree of receptor clustering (Watson et al., 2008, Hooley et al., 2008, Watson and O'Callaghan, 2011). Low molecular weight fucoidan may bind to CLEC-2 but may not be able to induce the clustering of the receptors that is necessary for platelet activation (Jarvis et al., 2002), whereas high molecular weight fucoidan induces rapid receptor clustering and platelet activation. This clustering effect is also dependent on the concentration of high molecular weight fucoidan added to platelets as shown in Fig. 2-1.

In our effort to elucidate signaling pathways by which fucoidan activates platelets, we have characterized a tyrosine kinase-dependent pathway, which leads to αIIbβ3 activation (Fig.2-1B). We also show that key signaling molecules, SFKs and Syk, are crucial for fucoidan-induced platelet activation (Figs.2-4). Downstream of the GPVI or CLEC-2 receptor, Syk kinase has been shown to play an important role in activating other effectors, which ultimately leads to the activation of αIIbβ3 (Hughes et al., 2010b, Suzuki-Inoue et al., 2004). Inhibition of Syk following GPVI stimulation shuts down all functional responses.

Consistent with the previous studies, we observed significant inhibition of PAC-1 binding in fucoidan-stimulated platelets in the presence of the Syk inhibitor OXSI-2 (32) or G06976 (Fig. 4A) (Getz et al., 2011). Platelet activation with fucoidan in the presence of the tyrosine kinase inhibitor PP2 also showed inhibition of platelet activation (Fig. 2-2). These results suggest for the first time that fucoidan-induced platelet activation depends on SFK- and Syk-dependent pathways. Fucoidan is known to activate cells such as macrophages and nerve cells (Brandenburg et al., 2010, Gao et al., 2012, Jhamandas et al., 2005), but the receptor that mediates these signaling events has not been elucidated. Fucoidan is considered as one of the agonists for scavenger receptor A in macrophages (Hsu et al., 2001) and other cell lines, but to our knowledge, scavenger receptor A has not been identified in platelets. Low molecular weight fucoidan was also been reported to bind to P-selectin (Molecular Imaging and Contrast Agent Database). In resting platelets, however, P-selectin is located on the inner wall of denes granules (Koedam et al., 1992, Mazurov et al., 1996) and is only exposed on the surface of platelets after activation. Therefore, fucoidan must first activate platelets through a receptor to expose P-selectin. Considering all our results, it is evident that fucoidan activates platelets through a tyrosine kinase-dependent receptor. The major receptors that can trigger tyrosine kinase pathways in platelets are GPVI, FcγRIIa, and CLEC-2(Watson et al., 2009, Watson et al., 2010). Of these, the GPVI receptor signaling depends on the FcR γ-chain (Jarvis et al., 2002). However, our data show that platelets lacking the FcR γ-chain are unresponsive to collagen-related peptide but still retain their ability to aggregate in response to fucoidan (Fig.2-5A). Also, fucoidan is able to induce human platelet activation in the presence of the monoclonal antibody IV.3 (Fig.2-5B),

which blocks FcγRIIa, and fucoidan also induces activation of murine platelets, which lack FcγRIIa. These results suggest that fucoidan does not mediate signaling through GPVI or FcγRIIa. CLEC-2 is a C-type lectin receptor that has been shown to signal independently of the FcR γ-chain in platelets (Watson et al., 2009, Watson et al., 2010). We observed inhibition of platelet functional responses in platelet-specific (PF4) CLEC-2receptor knock-out platelets when stimulated with fucoidan (Fig. 2-6A). To further support our conclusion that fucoidan induces platelet activation through the CLEC-2 receptor, we observed fucoidan-induced tyrosine phosphorylation of the CLEC-2 receptor (Fig. 2-6B). These results show for the first time that fucoidan mediates platelet activation through the CLEC-2 receptor. These results not only support a role for fucoidan as a novel drug for hemophilia treatment but also provide a new efficient agonist for the CLEC-2 receptor on platelets other than podoplanin and rhodocytin. In conclusion, fucoidan is a novel CLEC-2 receptor agonist that activates platelets through an SFK-dependent signaling pathway (as outlined in Fig.2-7). The stimulatory action of fucoidan on platelets strongly indicates that it may play an important role in platelet activation under diseased conditions such as hemophilia.

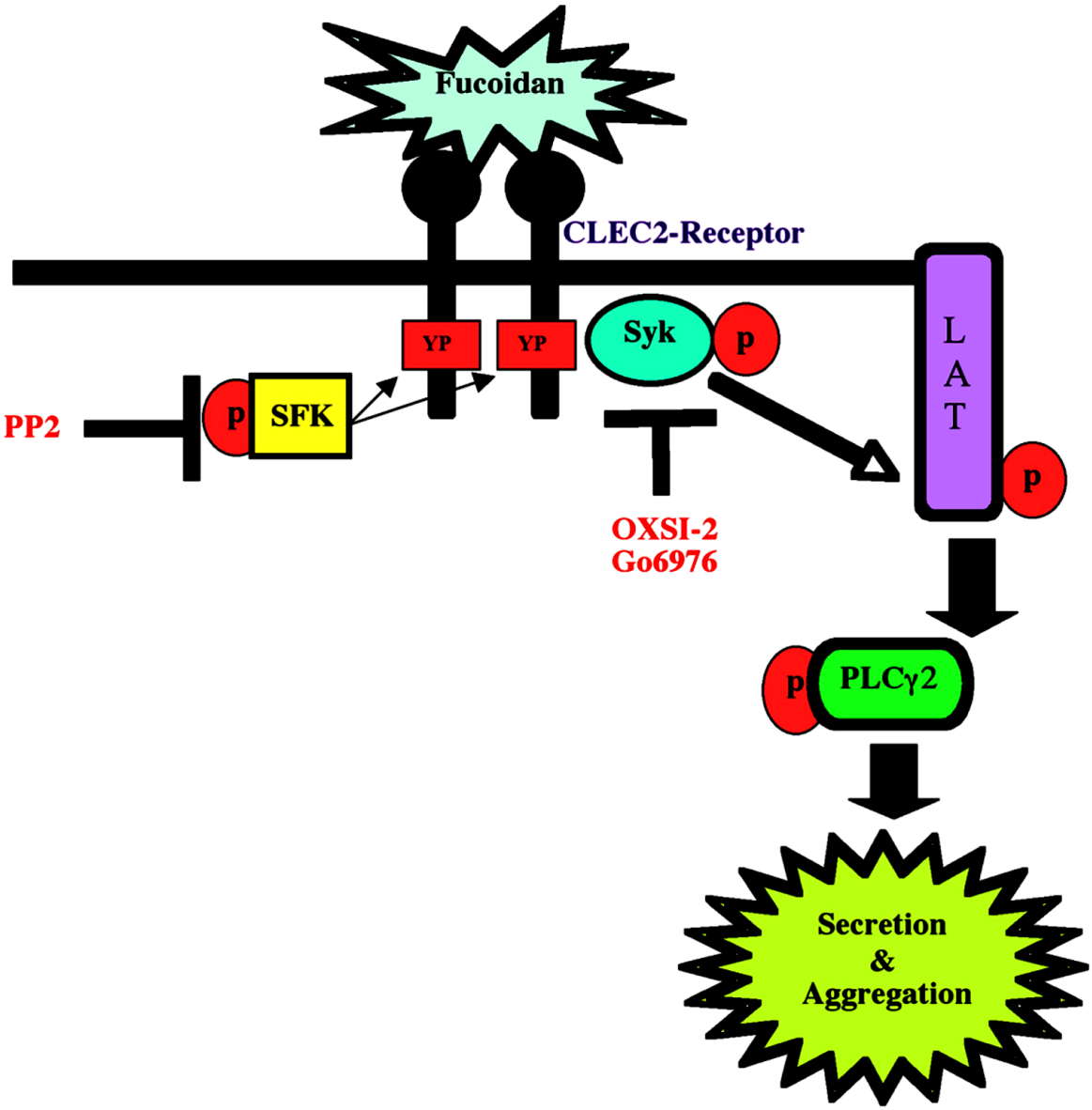

Figure 2-7 Model depicting tyrosine kinase-dependent signaling pathway in platelets activated with fucoidan. Fucoidan activates platelets through an SFK-dependent pathway through the CLEC-2 receptor. YP represents tyrosine phosphorylation sites on the cytoplasmic domain of CLEC-2 receptor**.**

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Chapter 3 ACTIVATION OF GLYCOPROTEIN VI (GPVI) AND C-TYPE LECTIN-LIKE RECEPTOR-2 (CLEC-2) UNDERLIES PLATELET ACTIVATION BY DIESEL EXHAUST PARTICLES AND OTHER CHARGED/HYDROPHOPIC LIGANDS

This research was originally published in Biochemical Journal. **Osama M. Alshehri**, Samantha Montague, Stephanie Watson, Paul Carter, Najiat Sarker, Bhanu K. Manne, Jeanette L.C. Miller, Andrew B. Herr, Alice Y. Pollitt, Chris A. O'Callaghan, Satya Kunapuli, Monica Arman, Craig E. Hughes and Steve P. Watson. Activation of glycoprotein VI (GPVI) and C-type lectin-like receptor-2 (CLEC-2) underlies platelet activation by diesel exhaust particles and other charged/hydrophobic ligands. *Biochem J,* 2015. 468,459-73 © the Biochemical Society

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This chapter reports that a miscellaneous group of agonists - including DEP, small peptides [4N1-1, Champs, and LSARLAF (Leu-Ser-Ala-Arg-Leu-Ala-Phe)], proteins (histones), large polysaccharides (fucoidan and dextran sulfate), and lipoproteins (Pam_3CSK_4) - stimulate aggregation and tyrosine phosphorylation of human and mouse platelets through the glycoprotein VI (GPVI)–FcRγ-chain complex and/or C-type lectin-like receptor-2 (CLEC-2). All of the experiments in the paper were performed by myself other than those with histones (Figures 3 and 4) and the studies on the DT40 cells which were performed with Samantha Montague and Stephanie Watson, supported by two dissertation students, Paul Carter and Najiat Sarke.

- All not shown results have been added to the Appendix except those on pages 93, 94 and 113 as these were performed by other authors in the manuscript.
- The not shown result in page 108 is shown in chapter 4 (Supplementary figure 4-2)
- I contributed experimental design, performed experiments, analysed data and wrote the manuscript.

Abstract

Platelets are activated by a range of stimuli that share little or no resemblance in structure to each other or to recognized ligands, including diesel exhaust particles (DEP), small peptides [4N1-1, Champs (computed helical anti-membrane proteins), LSARLAF (Leu-Ser-Ala-Arg-Leu-Ala-Phe)], proteins (histones) and large polysaccharides (fucoidan, dextran sulfate). This miscellaneous group stimulate aggregation of human and mouse platelets through the glycoprotein VI (GPVI)–FcRγ-chain complex and/or C-type lectin-like receptor-2 (CLEC-2) as shown using platelets from mice deficient in either or both of these receptors. In addition, all of these ligands stimulate tyrosine phosphorylation in GPVI/CLEC-2-double-deficient platelets, indicating that they bind to additional surface receptors, although only in the case of dextran sulfate does this lead to activation. DEP, fucoidan and dextran sulfate, but not the other agonists, activate GPVI and CLEC-2 in transfected cell lines as shown using a sensitive reporter assay confirming a direct interaction with the two receptors. We conclude that this miscellaneous group of ligands bind to multiple proteins on the cell surface including GPVI and/or CLEC-2, inducing activation. These results have pathophysiological significance in a variety of conditions that involve exposure to activating charged/hydrophobic agents.

3.1 Introduction

The collagen and podoplanin receptors glycoprotein VI (GPVI) and C-type lectin-like receptor-2 (CLEC-2) activate platelets through Src and spleen tyrosine kinase (Syk) tyrosine kinases via two closely related motifs, known as an immunoreceptor tyrosine-based activation motif (ITAM) and hemITAM respectively (Watson et al., 2010). An ITAM has two repeats of the amino acid sequence YXXL separated by 6–12 amino acids, whereas a hemITAM has a single YXXL motif that lies downstream of an acidic amino acid- rich region. Phosphorylation of the conserved tyrosine residues in these motifs leads to binding of Syk via its tandem SH2 (Src homology 2) domains (in the case of CLEC-2, bridging two receptors) and a signalling cascade that culminates in phospholipase C (PLC) γ 2 and platelet activation.

The GPVI–Fcε receptor 1 (FcR) γ-chain complex has been shown to mediate platelet activation to a variety of structurally unrelated peptides that have no structural resemblance to collagen or to other known platelet agonists. Activation by the peptide LSARLAF (Leu-Ser-Ala-Arg-Leu-Ala-Phe), which is from the non-coding strand of the *ITGA2B* (integrin α2B) gene in a region corresponding to the binding site for the fibrinogen γ-chain; 4N1- 1, which is derived from a sequence from the C-terminus of thrombospondin; and Champs (computed helical anti-membrane proteins), a computationally designed sequence that binds to the transmembrane of integrin subunit αIIb, is inhibited in platelets deficient in the GPVI-associated FcR γ-chain (Pearce et al., 2004b, Tulasne et al., 2001a, Grygielska et al., 2009) . Additionally, the three peptides induce platelet agglutination independently of the FcR γ-chain, indicating that they bind to other platelet surface proteins (Pearce et al., 2004b, Tulasne et al., 2001a, Grygielska et al., 2009).

CLEC-2 has been shown to mediate platelet aggregation by the sulfated polysaccharide fucoidan (Manne et al., 2013), which has no structural resemblance to the endogenous ligand, podoplanin or to the CLEC-2-specific snake venom toxin rhodocytin (also known as aggretin). Podoplanin has been shown to bind to CLEC-2 through an aspartic–glutamic acid doublet and an O-linked glycan which together form a platelet-aggregating (PLAG) domain, with the conserved sequence DEXXXS/T (Asp-Glu-Xaa-Xaa- Xaa-Ser/Thr) (Nagae et al., 2014). Rhodocytin (which is not O-glycosylated) also uses an aspartic-glutamic acid doublet to bind to CLEC-2 in association with its negatively charged C-terminus. The negatively charged fucoidan may interact with the corresponding sites on CLEC-2 to mediate activation.

Platelets are activated by other diverse stimuli that have little structural resemblance to platelet ligands including diesel exhaust particles (DEP) (Solomon et al., 2013), histones (Fuchs et al., 2011) fucoidan and dextran sulfate (Manne et al., 2013) (Getz et al., 2013a). In the present study, we have sought to investigate the role of GPVI and CLEC-2 in platelet activation by this group of agonists through measurement of protein tyrosine phosphorylation and use of transgenic mouse platelets, in combination with studies on transfected cell lines. We show that GPVI and/or CLEC-2 mediate platelet activation to this miscellaneous group of stimuli but that, in addition, these stimuli also induce tyrosine phosphorylation through binding to other surface proteins as shown using platelets double-deficient in the two (hem)ITAM receptors. We conclude that this diverse group of ligands interact with multiple proteins on the platelet surface and that they induce activation through GPVI and/or CLEC-2.

3.2 Materials and Methods

3.2.1 Reagent and antibodies

Champs (KKAYVMLLPFFIGLLLGLIFGGAFWGPARHLKK), LSARLAF and 4N1- 1 (RFYVVMWK) peptides were manufactured by Severn Biotech. DEP were purchased from the National Institute of Standards and Technology. Calf thymus histones (CTH) were purchased from Worthington Biochemical. Dextran sulfate, 50 % solution, was purchased from American Bioanalytical. The synthetic tripalmitoylated lipopeptide Pam_3-CSK_4 [tripalmitoylcysteinylseryl-$(lysyl)_4$], was from InvivoGen. All other reagents were purchased from Sigma or other named sources (Pearce et al., 2004b, Fuller et al., 2007, Mazharian et al., 2011).

3.2.2 Transgenic mice

Gp6 $^{-/-}$ mice [13] and *Clec1b* $^{fl/f;\ PF4\text{-}Cre}$ (Finney et al., 2012) have been described. These two strains were cross-bred to produce mice with platelets double-deficient in GPVI and CLEC-2 (*Gp6* $^{-/-}$; *Clec1b* $^{fl/fl;\ PF4\text{-}Cre}$). *Clec1b* $^{fl/fl}$ or wild-type mice were used as controls. All procedures were undertaken with U.K. Home Office approval (licence no. PPL 30/2721 and 30/8286).

3.2.3 Human platelets

Blood was obtained from healthy drug-free volunteers with informed consent and approval from the local ethics committee. Sodium citrate (10 %) was used as the anticoagulant. Platelet-rich plasma (PRP) and washed platelets were prepared as previously described (Grygielska et al., 2009). Washed platelets were suspended in modified Tyrode's HEPES buffer (134 mM NaCl, 0.34 mM $Na_2 HPO_4$, 2.9 mM KCl, 12 mM $NaHCO_3$, 20 mM HEPES, 5 mM glucose and 1 mM $MgCl_2$, pH 7.3) at between 2 and 10 × 10^8/ml. Aggregations and stimulations were performed in a Born (lumi)-

aggregometer (ChronoLog), at 37°C with continuous stirring at 1200 rev/min. Platelets were pre-incubated for 1 min with inhibitors or vehicle (0.2 % DMSO unless otherwise indicated) before agonist addition.

3.2.4 Mouse platelets

Blood was taken from the vena cavae of CO_2-asphyxiated mice following isofluorane anaesthesia and taken into1:10 (v/v) acid citrate/dextrose. Washed platelets were prepared as previously described (Pearce et al., 2004b) and resuspended at $2–10 \times 10^8$ /ml in Tyrode's HEPES buffer for measurement of aggregation and protein phosphorylation respectively. Platelets were pre-incubated for 1 min with inhibitors or vehicle (0.2 % DMSO unless otherwise indicated) before agonist addition.

3.2.5 Protein phosphorylation

Protein phosphorylation was measured in the presence of integrilin (9 μM), indomethacin (10 μM) and apyrase (2 units/ml), with the exception of studies in human platelets involving measurement of phosphorylation of CLEC-2 due to the positive-feedback action of secondary agonists on CLEC-2 signalling. Platelets were stimulated with agonists at 37°C with continuous stirring at 1200 rev/min in a Born (lumi)-aggregometer. Reactions were stopped and lysed with an equal volume of 2× ice cold lysis buffer containing 2 % Igepal CA-630 (Nonidet P-40 equivalent). Cell lysates were pre-cleared with Protein A/G– Sepharose or γ -Bind Plus beads (GE Healthcare) for 30–60 min at 4°C. Insoluble material was then pelleted at 13000 ***g*** for 15 min and removed. Antibodies and beads were added to platelet lysates and incubated overnight at 4°C, followed by washing with lysis buffer and elution with 2× Laemmli sample buffer. Whole cell lysates and immunoprecipitated proteins were resolved by reducing

SDS/PAGE and transferred onto PVDF membranes. Immunoblotting was performed as described previously (Poole et al., 1997).

3.2.6 NFAT–luciferase reporter assays

DT40 chicken B-cells were grown in RPMI 1640 medium supplemented with 10 % FBS, 1 % chicken serum, 100 units/ml penicillin, 100 μg/ml streptomycin, 50 μM β-mercaptoethanol and 2 mM L-glutamine. Syk-deficient Jurkat human T-cells were grown in RPMI 1640 medium supplemented with 10 % FBS, 100 units/ml penicillin, 100 μg/ml streptomycin and 2 mM L- glutamine. Cells were transfected in 0.4 ml (2×10^7 cells) of serum-free RPMI 1640 medium by electroporation using a GenePulser II (Bio-Rad Laboratories) set at 350 V and 500 μF for DT40 cells and at 250 V and 950 μF for Jurkat cells. DT40 cells were transfected with 15 μg of the nucdear factor of activated T-cells (NFAT)–luciferase reporter and either 10 μg of human CLEC-2 or 2 μg each of both human GPVI and human FcR γ-chain. Jurkat cells were transfected with 3.75 μg of the NFAT– luciferase reporter and either 2.5 μg of human CLEC-2 or 0.5 μg each of both human GPVI and human FcR γ-chain. After 20 h, live cells were counted by Trypan Blue exclusion, diluted to 2×10^6 cells/ml in serum- and antibiotic-free medium (to avoid potential binding to stimuli) and luciferase assays were carried out as described previously (Tomlinson et al., 2007) .

3.3 Data analysis

Results are shown as means ± S.E.M. for a minimum of three experiments. Statistical analyses were performed by Student's t test or using a one-way ANOVA with Bonferroni's post-hoc test. $P < 0.05$ was considered significant.

3.4 Results

3.4.1 Diesel exhaust particles activate human platelets through Src and Syk tyrosine kinases

DEP are carbon black nanoparticles coated with a variety of surface organic materials and metals that carry a net surface electrical charge. Several studies have shown that they stimulate rapid activation of platelets in Tyrode's buffer ('washed platelets') (Solomon et al., 2013). In confirmation of this, we show that DEP stimulate rapid concentration-dependent (5–50 μg/ml) aggregation (Figure 3-1Ai). In contrast, they have no effect over this concentration range in platelet-rich plasma, presumably due to binding to plasma proteins (Figure 1Ai). Aggregation is preceded by shape change and reaches a similar maximal level to that of strong platelets agonists as illustrated in Figure 1(Aii). The increase in light transmission is blocked in the presence of the αIIbβ3 antagonist integrilin, confirming that it reflects αIIbβ3-mediated aggregation rather than platelet agglutination (Figures 3-1Aii and 3-1Aiii). Shape change and aggregation are blocked by the Src kinase inhibitors PP2 and dasatinib or the Syk kinase inhibitor PRT318 (aka PRT- 060318) (Figures 3-1Aii and 3-1Aiii). In line with this, DEP stimulated tyrosine phosphorylation as demonstrated by Western blotting using the monoclonal antibody (mAb) 4G10 (Figure 3-1Bi). The increase in tyrosine phosphorylation is blocked by the Src kinase inhibitors PP2 and dasatinib and reduced by the Syk inhibitor PRT318 (Figure 3-1Bi). The greater effect of the Src inhibitors over the Syk inhibitor indicates that Src kinases lie upstream of Syk.

The presence of a prominent tyrosine-phosphorylated band at ~14 kDa (Figure 3-1Bi), corresponding to the FcR γ-chain, is indicative of GPVI activation. In line with this, DEP stimulated tyrosine phosphorylation of Syk and PLCγ2 as shown by immunoprecipitation using specific antibodies and Western blotting for

phosphotyrosine. The 14 kDa phosphorylated band is present in Syk immunoprecipitates (Figure 3-1Bi), confirming its identity as the FcR γ-chain (Gibbins et al., 1996). Phosphorylation of all three proteins is blocked in the presence of PP2 or dasatinib (Figure 3-1Bi). Phosphorylation of PLCγ2 is also blocked by PRT318, whereas phosphorylation of FcR γ-chain and Syk is retained. This is consistent with FcR γ-chain and Syk phosphorylation being mediated by Src rather than Syk tyrosine kinases as described for GPVI-activated platelets (Spalton et al., 2009a).

DEP also stimulate phosphorylation of CLEC-2, as shown by immunoprecipitation of the C-type lectin-like receptor and Western blotting for phosphotyrosine (Figure 3-1Bii). The level of tyrosine phosphorylation of CLEC-2 is weak in comparison with that induced by the CLEC-2-specific snake venom toxin rhodocytin (appendix 1). In contrast, DEP did not induce tyrosine phosphorylation of the other platelet ITAM receptor, FcγRIIa (appendix 2).

These results demonstrate that DEP activate human platelets through Src and Syk kinases in association with robust tyrosine phosphorylation of FcR γ-chain (indicative of GPVI activation) and weak tyrosine phosphorylation of CLEC-2.

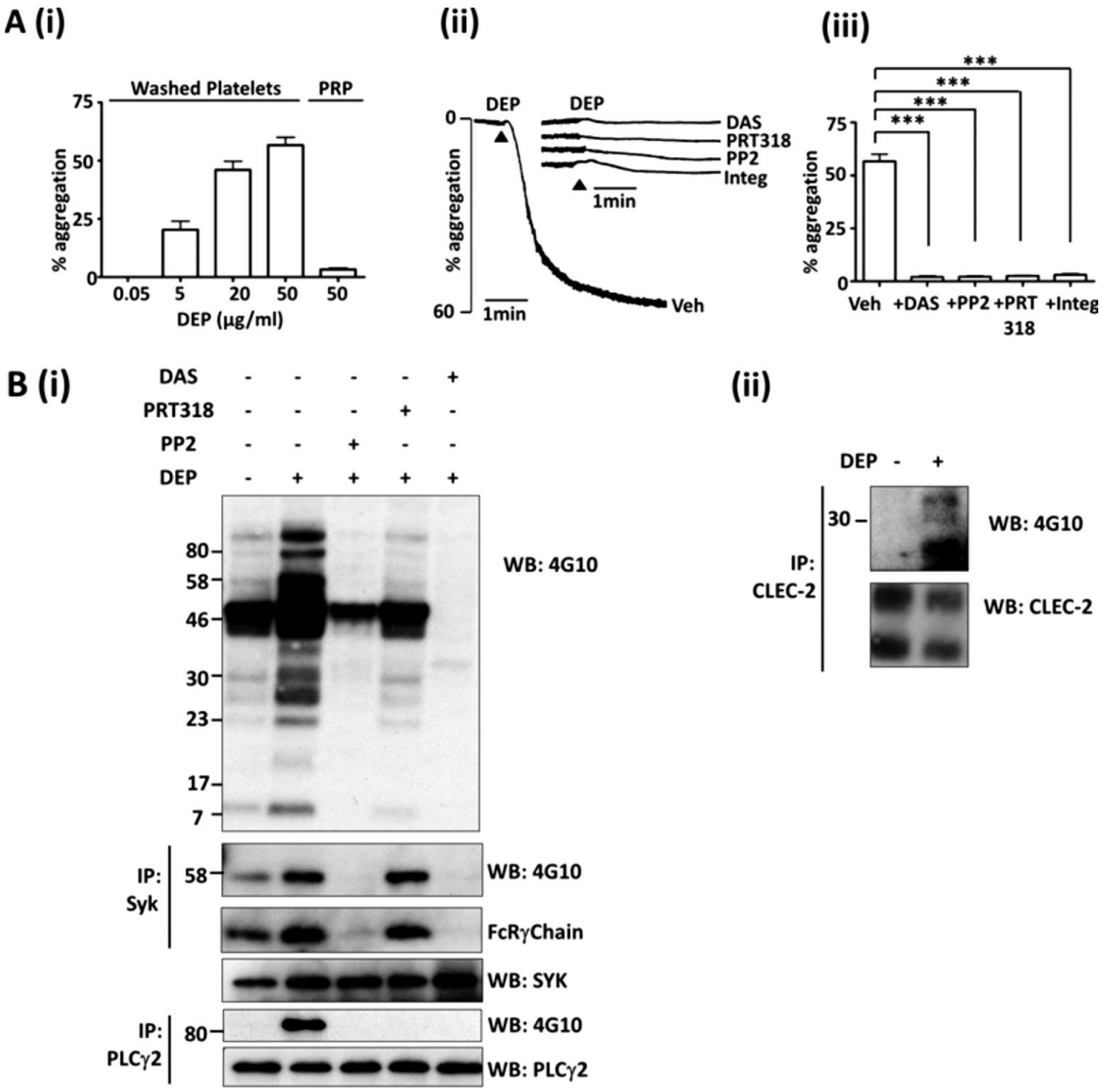

Figure 3-1 DEP activate human platelets through Src and Syk tyrosine kinases (A) (i) Dose–response curve for aggregation to DEP (0.05–50 µg/ml) in washed human platelets and platelet-rich plasma; (ii) Representative aggregation traces to DEP (50 µg/ml) following pre-incubation with dasatinib (DAS; 10 µM), PRT318 (5 µM), PP2 (10 µM), integrilin (integ; 9 µM) or DMSO (0.2 %: vehicle, Veh). (iii) Quantification of maximal aggregation is shown as means ± S.E.M., n≥3 (***P <0.001) (B). Washed platelets were pre-incubated with the inhibitors described above and stimulated with DEP (50 µg/ml) for 3 min. Experiments were performed under stirring conditions in the presence of integrilin (9 µM). (i) Whole-cell lysates or Syk and PLCγ 2 immunoprecipitates (IP) were separated by SDS/PAGE and Western blotted (WB) for phosphotyrosine using mAb 4G10 before reprobing for the precipitated protein. (ii) CLEC-2 was immunoprecipitated using a specific antibody, separated by SDS/PAGE and Western blotted for phosphotyrosine before reprobing for CLEC-2. Molecular masses are indicated in kDa. Results are representative of a minimum of three experiments.

3.4.2 Diesel exhaust particles activate mouse platelets through GPVI

DEP stimulate shape change and aggregation of washed mouse platelets, with a similar concentration–response curve and time course to that for human platelets (Figure 3-2Ai). Aggregation is again inhibited in the presence of PP2, PRT318 or integrilin, revealing a residual increase in light transmission mediated by platelet agglutination (Figures 3-2Aii and 3-2Aiii). Platelet activation is associated with tyrosine phosphorylation of the FcRγ-chain, Syk and PLCγ2, as shown by immunoprecipitation and Western blotting with mAb 4G10 (Figure 3-2B). Tyrosine phosphorylation of all three proteins was blocked by the Src kinase inhibitor PP2, whereas only phosphorylation of PLCγ 2 was blocked by the Syk inhibitor PRT318 (Figure 3-2B), consistent with human platelets.

Mice deficient in GPVI and/or CLEC-2 were used to further investigate the mechanism of platelet activation by DEP. Aggregation induced by DEP (50 μg/ml) is severely diminished in the absence of GPVI and blocked in GPVI/CLEC-2-double-deficient platelets, but unaltered in the absence of CLEC-2 (Figures 3-2Ci and 3-2Cii), with a similar result observed with a sub-maximal concentration of DEP (20 μg/ml; Supplementary Figure 3-1). The stimulation of tyrosine phosphorylation in whole- cell lysates by DEP is reduced but not blocked in the absence of GPVI, with no apparent further change in GPVI/CLEC-2- double-deficient platelets (Figure 3-2Di). Tyrosine phosphorylation was not altered in CLEC-2-deficient platelets (Figure 3-2Di). Syk phosphorylation was markedly inhibited in GPVI-deficient platelets and blocked in GPVI/CLEC-2-double-deficient platelets, but unaltered in CLEC-2-deficient platelets (Figure 3-2Dii).

These results indicate that DEP stimulate tyrosine phosphorylation of Syk predominantly through GPVI with a minor contribution from CLEC-2. In addition, DEP induce tyrosine phosphorylation independent of the two (hem) ITAM receptors.

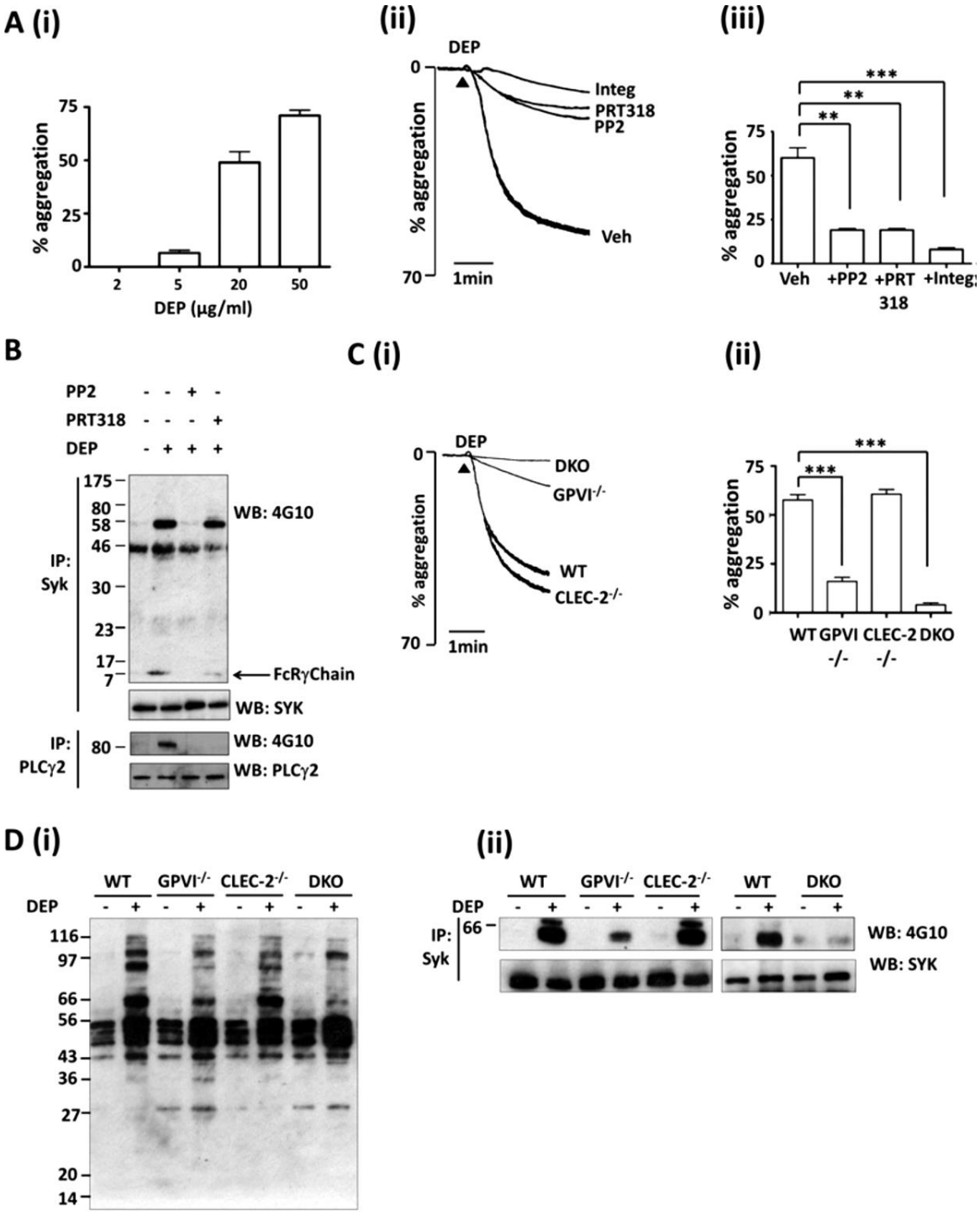

Figure 3-2 DEP activate mouse platelets through GPVI. (A) (i) Dose-response curve for aggregation to DEP (2–50 µg/ml) in washed mouse platelets. (ii) Washed platelets were pre-incubated with integrilin (integ; 9 µM), PRT318 (5 µM), PP2 (10 µM) or DMSO (0.2%: vehicle, Veh) for 3 min and then stimulated with DEP (50 µg/ml). (iii) Quantification of maximal aggregation is shown as means ± S.E.M., n=3 (**P < 0.01, ***P < 0.001). Washed platelets were stimulated with DEP (50 µg/ml) under stirring conditions for 3 min. Syk was immunoprecipitated (IP), proteins were separated by SDS/PAGE and Western blotted (WB) for phosphotyrosine. The figure is representative of three experiments. (C) Washed platelets from wild-type (WT), CLEC-2-deficient, GPVI-deficient and GPVI/CLEC-2-double-deficient (DKO) mice were stimulated with DEP (250 µg/ml): (i) representative aggregation traces; and (ii) quantification of maximal aggregation is shown as means ± S.E.M. from three experiments; (***P < 0.001).(D) Tyrosine phosphorylation in washed platelets from WT, CLEC-2-deficient, GPVI-deficient and GPVI/CLEC-2-double-deficient mice was induced by incubation with DEP (250 µg/ml) for 3 min.Tyrosine phosphorylation in (i) whole-cell lysates and (ii) Syk immunoprecipitate were analysed as described above. Molecular masses are indicated in kDa. Results are representative of a minimum of three experiments.

3.4.3 Histones activate human platelets through Src and Syk tyrosine kinases

Histones are positively charged proteins that play a key role in the organization of DNA and in gene regulation. Histones are released from damaged cells and form a central component of neutrophil extracellular traps (NETs) which are released by activated neutrophils through a process known as NETosis (Fuchs et al., 2011, Yipp and Kubes, 2013). Histones are elevated in a range of inflammatory disorders and in trauma and mediate a wide spectrum of biological effects including activation of endothelial cells and platelets (Martinod and Wagner, 2014, Abrams et al., 2013).

Calf thymus histones (CTH) are a heterogeneous mixture of all histone fractions and have been widely used to investigate histone- mediated platelet activation (Fuchs et al., 2011, Semeraro et al., 2011). CTH stimulate concentration- dependent shape change and aggregation of platelets in Tyrode's buffer and in plasma, with aggregation blocked by an αIIbβ3 receptor antagonist (Figure 3-3A; and results not shown). The CTH dose–response curve in washed platelets is approximately 10-fold lower than that in platelet-rich plasma, presumably due to reduced bioavailability as a consequence of plasma protein binding (Getz et al., 2013a). The curve is also bell-shaped, with aggregation being lost at high concentrations of CTH (Figure 3-3A; and results not shown). In contrast, the dose–response curve for ATP secretion is not bell- shaped (results not shown), indicating that loss of aggregation at high concentrations of histones is probably mediated by electrostatic repulsion of histone-coated platelets.

The stimulation of aggregation by CTH in platelet-rich plasma is blocked in the presence of the Src and Syk inhibitors, dasatinib and PRT318 respectively (Figures 3-3Bi and 3-3Bii). CTH stimulate a marked increase in tyrosine phosphorylation in platelet lysates, which is blocked in the presence of dasatinib and reduced in the presence of PRT318 (Figure 3Biii). The FcRγ -chain, Syk and PLCγ 2 were identified as

prominent tyrosine-phosphorylated proteins by immunoprecipitation and Western blotting with mAb 4G10 (Figure 3-3Biii). The increase in phosphorylation of all three proteins was blocked by the Src kinase inhibitor dasatinib. In contrast, only phosphorylation of PLCγ 2 was blocked in the presence of the Syk inhibitor PRT318 (Figure 3-3Biii), indicating that phosphorylation of the FcR γ-chain and Syk is downstream of Src kinases. CTH did not induce tyrosine phosphorylation of CLEC-2 (Figure 3-3Biii). These results indicate that histones activate human platelets through the GPVI–FcRγ -chain complex, leading to activation of Src and Syk tyrosine kinases.

3.4.4 Histones activate mouse platelets through GPVI

CTH also stimulate rapid aggregation of mouse platelets in platelet-rich plasma, which is blocked in the presence of dasatinib and markedly reduced in the presence of PRT318 (Figures 3-4Ai and 3-4Aii). Activation was associated with increased tyrosine phosphorylation in whole-cell lysates, with FcR γ-chain, Syk and PLCγ 2, but not CLEC-2, identified as phosphorylated proteins by immunoprecipitation and Western blotting (Figures 3-4Bi - 3-4Biv). Phosphorylation of FcR γ-chain, Syk and PLCγ 2 was blocked in the presence of dasatinib, whereas phosphorylation of FcR γ-chain and Syk but not PLCγ 2 was preserved (albeit reduced) in the presence of PRT318 (Figures 3-4Bi - 3-4Biv). These results demonstrate that CTH activate mouse platelets in a similar manner to human platelets.

Confirming a role for the FcR γ-chain, platelet aggregation induced by CTH was blocked in the absence of GPVI, but was unaltered in the absence of CLEC-2 (Figure 3-4C). Similarly, tyrosine phosphorylation of FcR γ-chain, Syk and PLCγ 2 induced by CTH was blocked in GPVI-deficient and GPVI/CLEC-2-double-deficient platelets (Figure 3-4D; and results not shown). Nevertheless, CTH stimulated a weak increase in tyrosine phosphorylation in whole-cell lysates of GPVI/CLEC- 2-double-

deficient platelets (Figure 3-4D) indicative of activation of additional Src kinase-linked receptors (although this does not lead to platelet aggregation). These results demonstrate that CTHs activate GPVI but not CLEC-2, but that they also bind to additional tyrosine kinase- linked surface receptors.

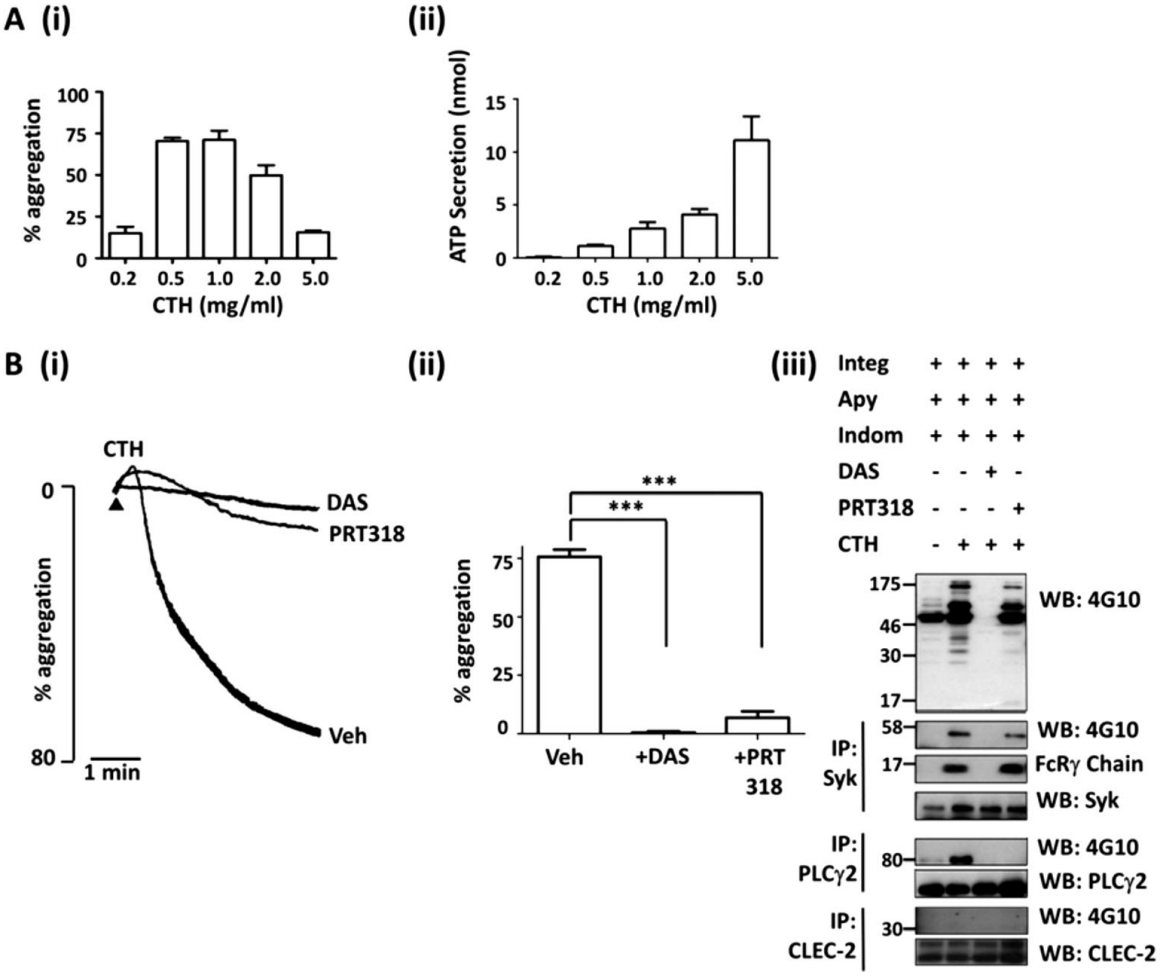

Figure 3-3 CTH activate human platelets through Src and Syk tyrosine kinases. (A) Dose response curve for aggregation to CTH (0.2–5 mg/ml) in (i) platelet-rich plasma; (ii) dose–response curve for secretion in platelet-rich plasma. (B) Plasma was pre-incubated with dasatinib (DAS; 10 μM) or PRT318 (10 μM) for 3 min before stimulation with CTH (1 mg/ml): (i) representative aggregation traces to CTH in the presence of inhibitors or vehicle (Veh; DMSO,0.2 %); (ii) quantification of maximal aggregation is shown as means ± S.E.M., n≥3 (***P < 0.001); (iii) washed platelets were stimulated for 3 min with CTH (50 μg/ml). Whole-cell lysates were immunoprecipitated (IP) for Syk, PLCγ 2 and CLEC-2, separated by SDS/PAGE and Western blotted (WB) for phosphotyrosine with mAb 4G10. Tyrosine phosphorylation of co-precipitated FcR γ-chain in the Syk immunoprecipitate, measured in the presence of integrilin (Integ), Indomethacin (indom) and apyrase (Apy) and with or without DAS and PRT318, is shown. Molecular masses are indicated in kDa. All results are representative of a minimum of three experiments.

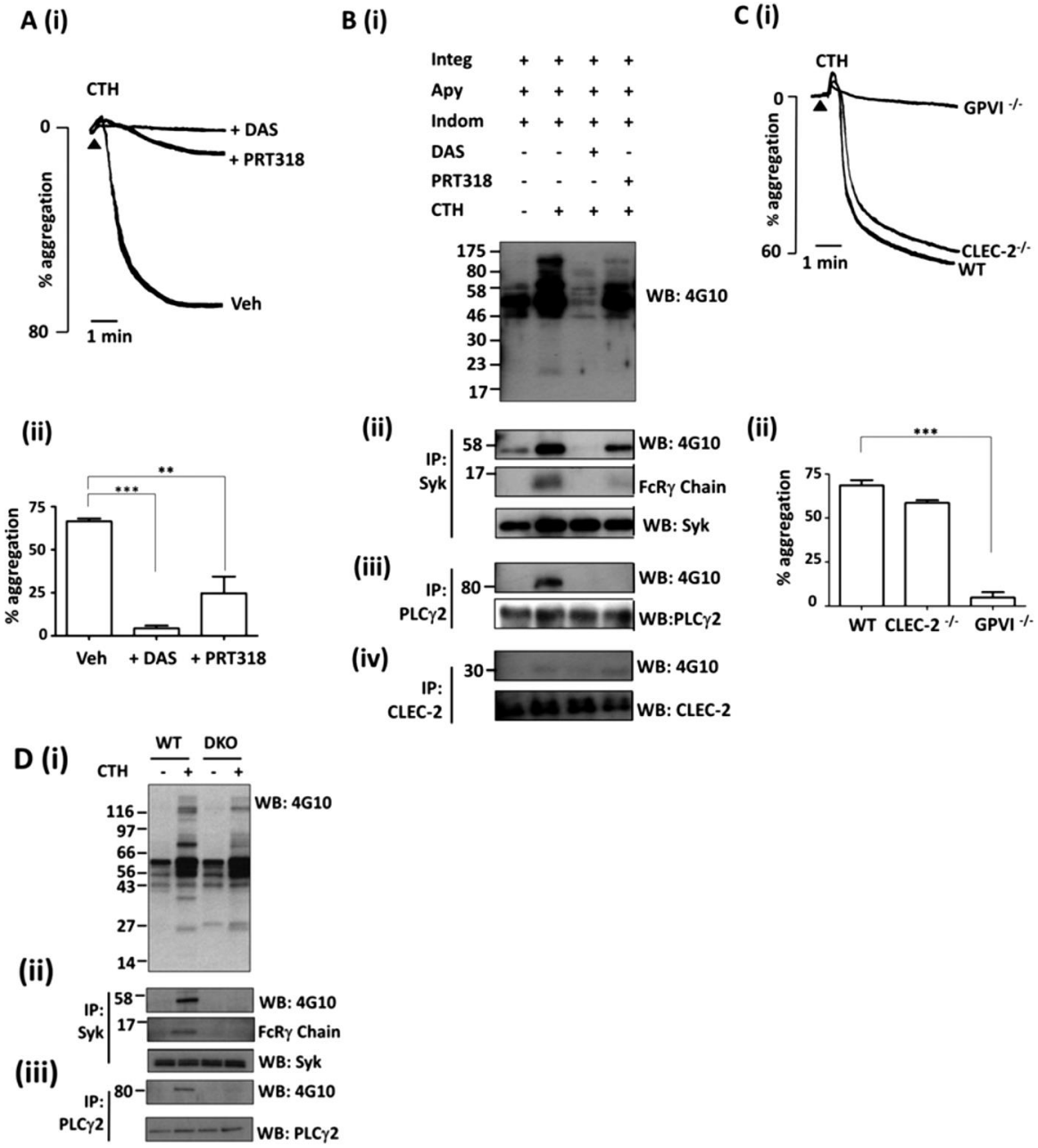

Figure 3-4 CTH activate mouse platelets through GPVI and Src and Syk tyrosine kinases.
(A) CTH (1 mg/ml) induced aggregation in platelet-rich plasma in the presence or absence of dasatinib (DAS; 10 μM) or PRT318 (10 μM). Veh, vehicle (DMSO, 0.2 %). (i) Representative aggregation traces and (ii) quantification of maximal aggregation is shown as means ± S.E.M., n ≥4 (**P < 0.01, ***P < 0.001). (B) Washed mouse platelets were incubated with CTH (50 μg/ml) for 3 min and analysed for tyrosine phosphorylation, measured in the presence of integrilin (Integ), Indomethacin (indom) and apyrase (Apy) and with or without DAS and PRT318, in (i) whole-cell lysates; (ii)Syk immunoprecipitates; (iii) PLCγ 2 immunoprecipitates; and (iv) CLEC-2 immunoprecipitates as described in Figure 2. Phosphorylation of co-precipitated FcR γ-chain (middle panel of ii) with Syk is shown. (C) Platelet-rich plasma from wild-type (WT), CLEC-2-deficient and GPVI-deficient mice was stimulated with CTH (1 mg/ml) for 3 min: (i) representative aggregation traces; and (ii) mean ± S.E.M. aggregation, n≥3 (***P < 0.001). (D) Washed platelets from WT, GPVI-deficient and GPVI/CLEC-2-double-deficient (DKO) mice were stimulated for 3 min with CTH (50 μg/ml) under stirring conditions in the presence of integrilin (9 μM). Tyrosine phosphorylation in (i) whole-cell lysates, (ii) Syk and (iii) PLCγ 2 immunoprecipitates (IP) were analysed as described above.WB, Western blot. Molecular masses are indicated in kDa. The results are from an experiment that is representative of a minimum of three experiments.

3.4.5 GPVI mediates platelet activation by 4N1-1 and Champs

We have previously reported that the structurally unrelated peptides LSARLAF, 4N1-1 and Champs stimulate aggregation of washed human and mouse platelets and that aggregation is abolished in the absence of the FcR γ-chain (Pearce et al., 2004b, Tulasne et al., 2001a, Grygielska et al., 2009). In addition, we reported that all three peptides induce αIIbβ3-independent agglutination (Pearce et al., 2004b, Tulasne et al., 2001a, Grygielska et al., 2009). The effect of these three peptides in platelets deficient in GPVI or in CLEC-2 has not been investigated. To address this, we investigated the effect of the peptides on GPVI, CLEC-2 and GPVI/CLEC-2-double-deficient platelets. We focused our studies on 4N1-1 and Champs, but also performed preliminary studies with LSARLAF (appendix 3). As anticipated from the studies on FcR γ-chain-deficient mice (Tulasne et al., 2001a, Grygielska et al., 2009) aggregation of mouse platelets by 4N1-1 and Champs was inhibited in GPVI-deficient platelets and in the presence of inhibitors of Src and Syk tyrosine kinases (Figures 3-5A and 3-5B). In all cases, a residual increase in light transmission was observed which is due to αIIbβ3- independent agglutination, as it was not blocked by integrilin (appendix 4). Similar observations were previously reported in platelets from mice deficient in the FcR γ-chain (Tulasne et al., 2001a, Grygielska et al., 2009) . The reduction in aggregation was accompanied by a decrease but not abolition of tyrosine phosphorylation to 4N1-1 and Champs in platelet lysates from GPVI-deficient mice (Figures 3-5C and 3-5D). In contrast, aggregation was not significantly altered to any of the three peptides in the absence of CLEC-2 (appendix 5).These results demonstrate a critical role for GPVI but not CLEC-2 in mediating platelet activation by 4N1-1, Champs and LSARLAF. In addition, the peptides bind to one or more other surface receptors that induce tyrosine phosphorylation and platelet agglutination.

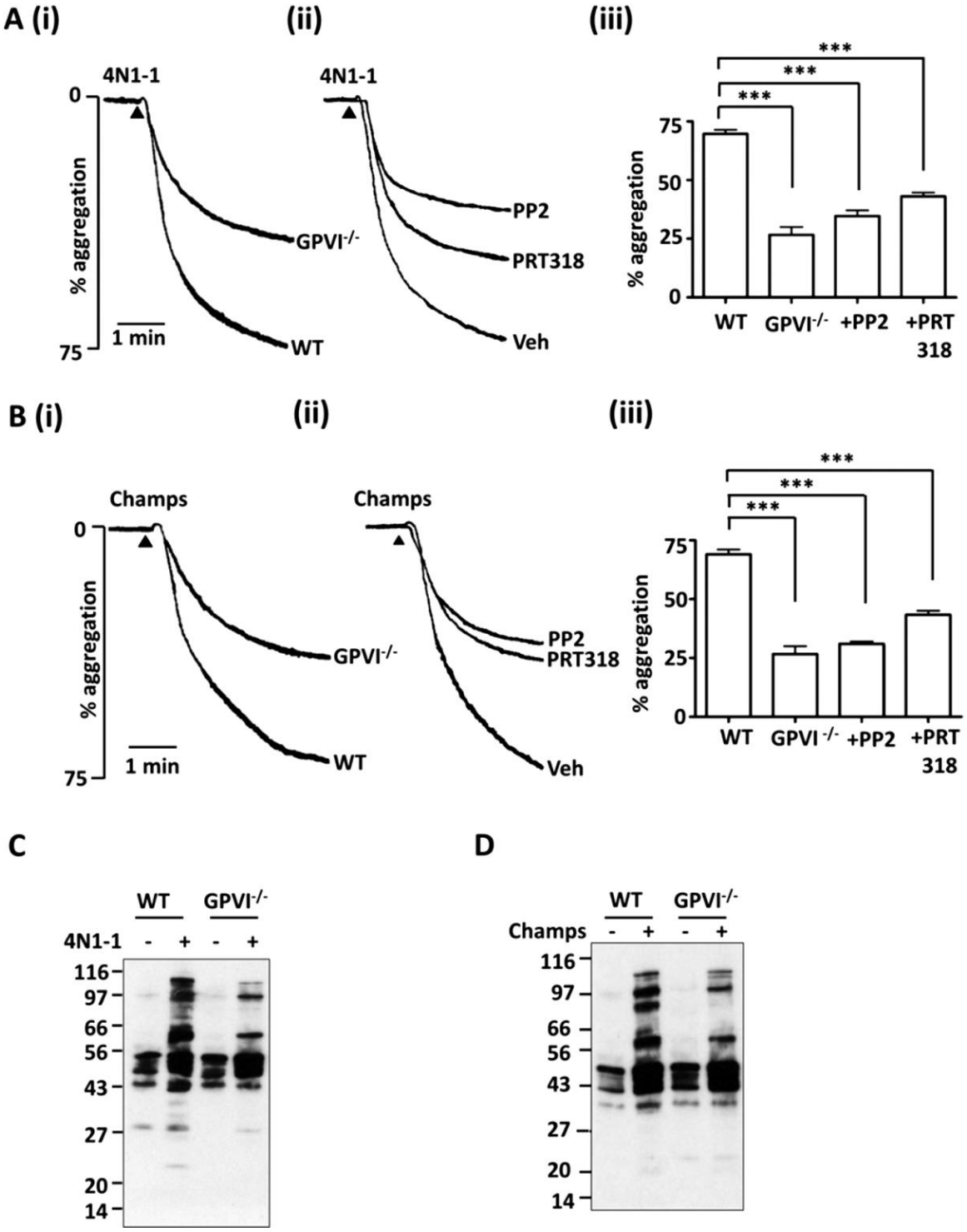

Figure 3-5 4N1-1 and Champs activate mouse platelets through GPVI. Washed platelets from wild-type (WT) and GPVI-deficient mice were stimulated with (A) 4N1-1 (100 μM) and (B) Champs (30 μg/ml). (i) Representative aggregation traces. (ii) Platelets were pre-incubated with PP2 (10 μM) and PRT318 (5 μM) for 3 min prior to 4N1-1 or Champs stimulation. (iii) Maximum aggregation results from three experiments are shown as means ± S.E.M. (*P < 0.05, **P < 0.01, ***P < 0.001). Washed platelets from WT and GPVI-deficient mice were stimulated with (C) 4N1-1 (100 μM) or (D) Champs (30 μg/ml) for 3 min. Protein phosphorylation was measured as described in Figure 3-2. Molecular masses are indicated in kDa. Results are representative of three experiments.

3.4.6 CLEC-2 and GPVI mediate activation of mouse platelets by fucoidan

We have previously reported that the sulfated polysaccharide fucoidan activates human and mouse platelets through Src and Syk tyrosine kinases and that activation is lost in mouse platelets deficient in CLEC-2 (Manne et al., 2013). In the present study, we show that higher concentrations of fucoidan induce partial aggregation of CLEC-2-deficient platelets and that this is blocked in mouse platelets double-deficient in GPVI and CLEC-2 (Figures 3-6Ai and 3-6Aii). Shape change is observed in some but not all experiments (Figure 3-6Ai). In contrast, aggregation is not altered in GPVI- deficient mouse platelets in response to maximal or sub-maximal concentrations of fucoidan (Figures 3-6Ai and 3-6Aii; Supplementary Figure 3-2), consistent with results in FcR γ-chain-deficient mouse platelets (Manne et al., 2013).

The stimulation of whole-cell tyrosine phosphorylation by fucoidan was inhibited but not abolished in CLEC-2-deficient platelets (Figure 3-6Bi), with no apparent change in GPVI-deficient platelets (appendix 6). A further reduction in tyrosine phosphorylation is seen in GPVI/CLEC-2-double-deficient platelets, although a residual increase in phosphorylation, including a band of 90 kDa, is still present (Figure 3-6Bii). This residual phosphorylation is blocked by the Src kinase inhibitors PP2 or dasatinib (appendix 7). Tyrosine phosphorylation of Syk by fucoidan is markedly inhibited in the absence of CLEC-2 and blocked in GPVI/CLEC-2-double-deficient platelets, whereas Syk phosphorylation is not altered in the absence of GPVI (Figure 3-6Biii). A weak band corresponding to the FcR γ-chain is present in Syk immunoprecipitates from control platelets, which is lost in platelets deficient in GPVI, GPVI/CLEC-2- double-deficient and, surprisingly, CLEC-2-deficient platelets (Figure 6Biii). These results confirm that fucoidan activates both GPVI and CLEC-2 in platelets, but suggest that it also binds to one or more additional platelet surface

receptors which leads to an increase in tyrosine phosphorylation but not platelet activation.

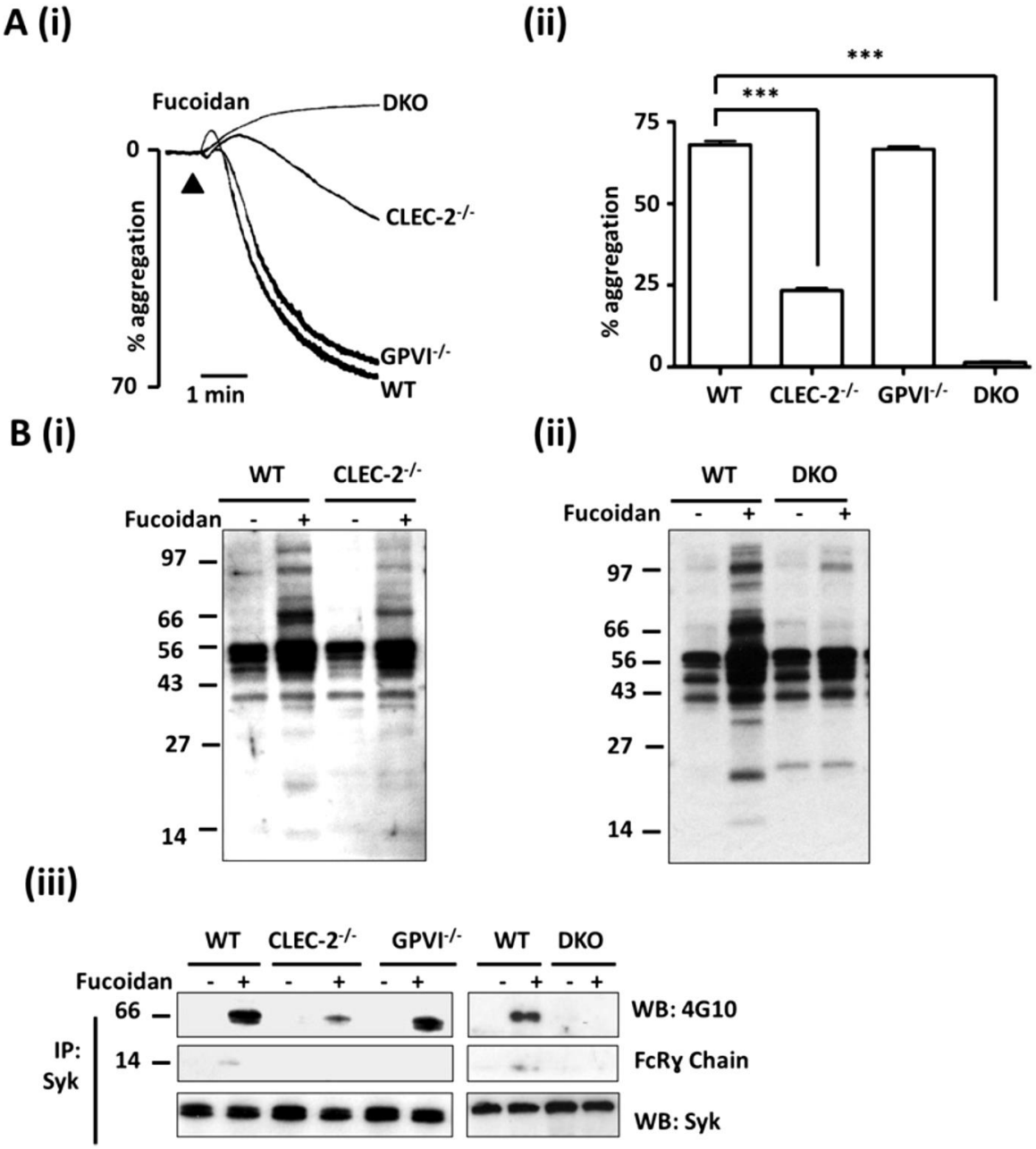

Figure 3-6 Fucoidan activates mouse platelets through GPVI and CLEC-2. (A) (i) Washed platelets from wild-type (WT), CLEC-2-deficient, GPVI-deficient and GPVI/CLEC-2-double-deficient (DKO) mice were stimulated with fucoidan (100 μg/ml) and monitored for aggregation. (ii) Quantification of maximal aggregation, shown as means ± S.E.M., n>3 (***P < 0.001). (B) Washed platelets from WT and (i) CLEC-2-deficient (ii) GPVI/CLEC-2-double-deficient mice were stimulated with fucoidan (100 μg/ml) for 3 min. Protein phosphorylation was measured as described in Figure 3-2. (iii) Syk was immunoprecipitated as described in Figure 3-2 and phosphotyrosine of co-precipitated FcR γ-chain in the Syk immunoprecipitate is shown. WB, Western blot. Molecular masses are indicated in kDa. Results are representative of three experiments.

3.4.7 CLEC-2 and GPVI mediate activation of mouse platelets by dextran sulfate

Dextran sulfate has been reported to induce aggregation of washed human platelets through a Src kinase-dependent but Syk-independent pathway (Getz et al., 2013a). We have confirmed this result using two further inhibitors of Src and Syk tyrosine kinases, PP2 and PRT318, which block or slow the onset of aggregation respectively (Figures 3-7Ai and 3-7Aii). In addition, we show that dextran sulfate stimulates tyrosine phosphorylation of CLEC-2 in human platelets (Figure 3-7Aiii), which may account for the delay in aggregation observed in the presence of PRT318 (Figure 3-7Ai).

Dextran sulfate stimulates weak aggregation of mouse platelets, which, in contrast with human platelets, is inhibited in the presence of PP2 or PRT318 (Figure 3-7B). A small increase in light transmission is observed in the presence of either inhibitor (Figure 3-7B) and in the presence of integrilin (appendix 8), which is likely to be due to platelet agglutination. A similar reduction in aggregation is seen in CLEC-2-deficient and in GPVI/CLEC-2-double-deficient platelets, whereas the response is not altered in the absence of GPVI alone (Figures 3-7Ci - 3-7Ciii). Dextran sulfate stimulated tyrosine phosphorylation of CLEC-2 and Syk in mouse platelets (Figures 3-7Di and 3-7Dii). Phosphorylation of Syk was markedly inhibited in the absence of CLEC-2 and blocked in GPVI/CLEC-2-double-deficient platelets, but unaltered in the absence of GPVI (Figure 3-7Di; and appendix 9). These results demonstrate that dextran sulfate induces activation of mouse platelets through a CLEC-2-driven pathway, with a minor role for GPVI. In contrast, dextran sulfate activates human platelets through a Syk-independent pathway, making it likely that activation is independent of CLEC-2 and/or GPVI.

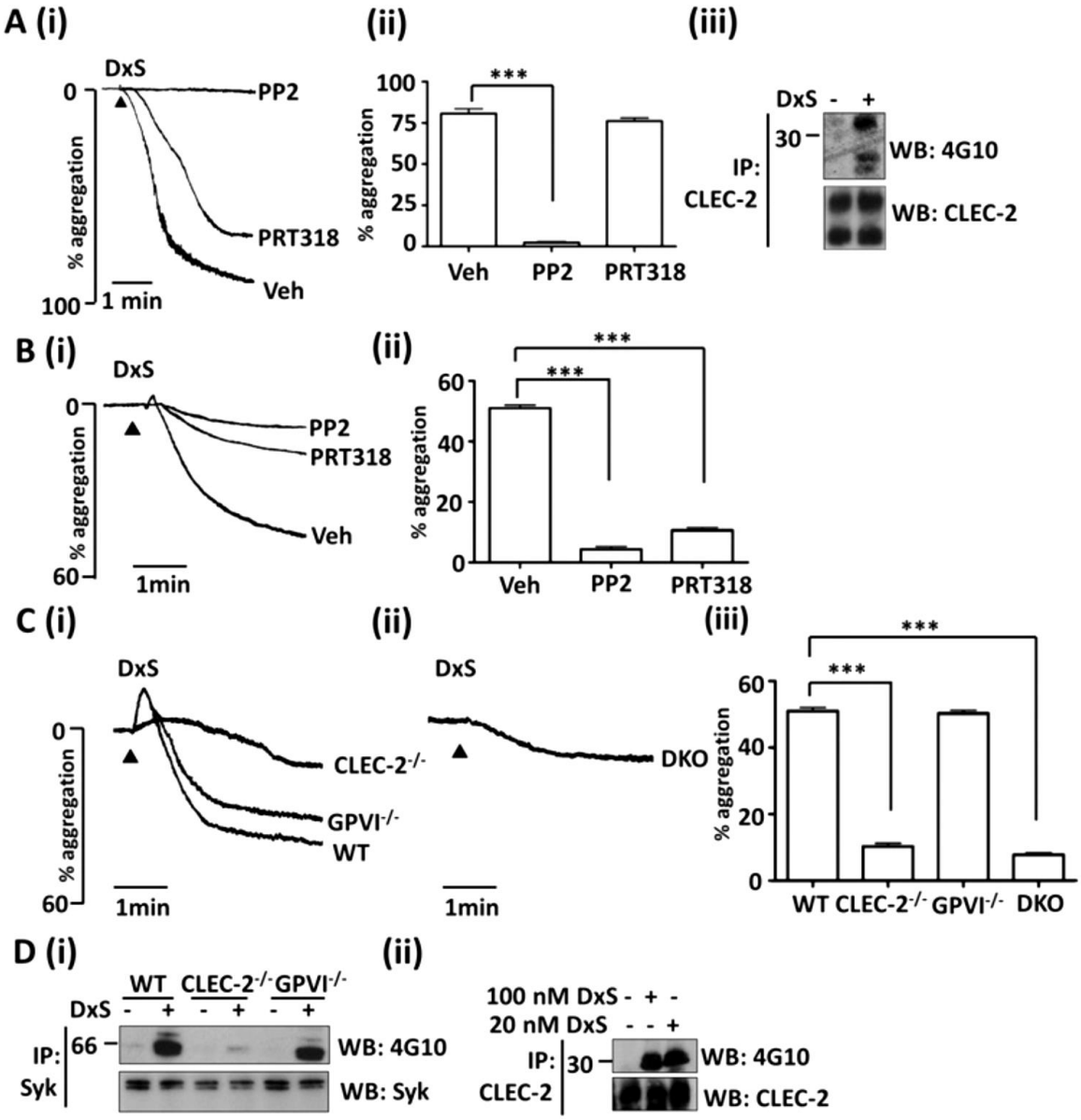

Figure 3-7 Dextran sulfate activates human and mouse platelets through a Src kinase activation. (i) Washed human platelets were pre-incubated with PP2 (10 μM) and PRT318 (5 μM) before stimulation with dextran sulfate (DxS; 20 nM). Veh, vehicle (DMSO, 0.2 %). (ii) Quantification of maximal aggregation is shown as means ± S.E.M., n>3 (***P < 0.001). (iii) CLEC-2 was immunoprecipitated (IP) as described in Figure 1. Results are representative of three experiments. Washed mouse platelets were pre-incubated for 3 min with PP2 (10 μM) and PRT318 (5 μM) before stimulation with dextran sulfate (20 nM). (i) Representative aggregation traces. (ii) Quantification of maximal aggregation shown as means ± S.E.M. from three experiments (***P < 0.001). (C) (i) Washed platelets from wild-type, CLEC-2-deficient, GPVI-deficient, and (ii) GPVI/CLEC-2-double-deficient (DKO) mice were incubated with dextran sulfate (20 nM) and monitored for aggregation. Results are representative of three experiments. (iii) Mean ± S.E.M. aggregation from three experiments (***P < 0.001). (D) (i and ii) Protein phosphorylation of Syk and CLEC-2 was measured after dextran sulfate stimulation of washed mouse platelets as described in Figure 3-2. WB, Western blot. Molecular masses are indicated in kDa. Results are representative of three experiments.

3.4.8 Pam_3-CSK_4 stimulates phosphorylation of the FcR γ–chain

The toll-like receptor (TLR)-1/2 agonist Pam_3-CSK_4 stimulates aggregation of human platelets in association with phosphorylation of a number of proteins that are regulated by platelet ITAM receptors, including Syk and PLCγ 2 (Falker et al., 2014). In the present study, we show that Pam_3-CSK_4 induces transient tyrosine phosphorylation of the FcR γ-chain in human platelets, which can be detected after 10 s but not after 300 s (Figures 3-8Ai and 3-8Aii). Syk phosphorylation occurs in parallel with phosphorylation of the FcR γ-chain (Figures 3-8Ai and 3-8Aii). In contrast, CLEC-2 is not phosphorylated by Pam_3-CSK_4 in human platelets (appendix 10). Pam_3-CSK_4 stimulates sustained phosphorylation of FcR γ-chain and Syk in mouse platelets, but again has no effect on CLEC-2 phosphorylation (Figure 3-8B and appendix 11). This increase in phosphorylation of the FcR γ-chain and Syk is blocked in GPVI deficient platelets (Figure 3-8B). Surprisingly, however, Pam_3-CSK_4 induced aggregation is not altered in GPVI-deficient mouse platelets (Figures 3-8Ci and 3-8Cii) or the presence of inhibitors of Src and Syk tyrosine kinases (appendix 12).These results demonstrate that Pam_3-CSK_4 induces platelet aggregation through a tyrosine kinase-independent pathway despite robust activation of GPVI (as shown by FcR γ-chain and Syk phosphorylation) suggesting that it activates platelets through multiple receptors.

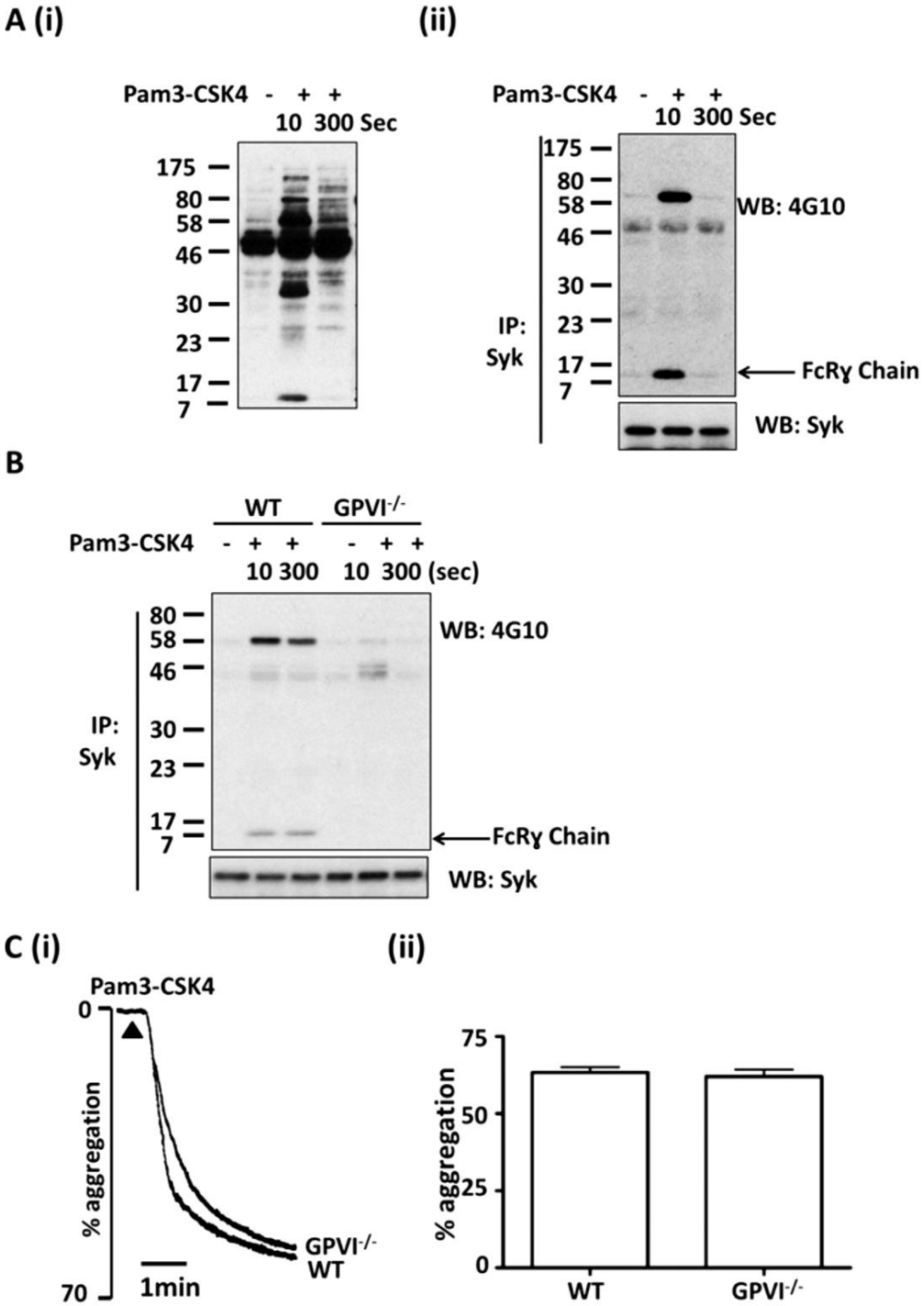

Figure 3-8 Pam_3-CSK_4 activates Syk in human and mouse platelets. (A) (i and ii) Washed human platelets were pre-incubated with integrilin (9 μM) and stimulated under stirring conditions with Pam3-CSK4 (30 μg/ml) for 10 and 300 s before lysis. Protein phosphorylation was measured as described in Figure 3-1. WB, Western blot. Molecular masses are indicated in kDa. (ii) Syk was immunoprecipitated and phosphotyrosine of co-precipitated FcR γ-chain in the Syk immunoprecipitate (IP) is also shown. Results are representative of three experiments. (B) (i and ii) Washed platelets from wild-type (WT) and GPVI-deficient mice were stimulated with Pam3-CSK4 (30 μg/ml): (i) representative aggregation traces; and (ii) results shown as mean ± S.E.M. aggregation from three experiments. (C) Protein phosphorylation in WT and GPVI deficient mouse platelets in response to Pam_3-CSK_4 (30 μg/ml) for 10 and 300 s was measured as described in Figure 3-2. Results are representative of three experiments.

3.4.9 The effect of the structurally diverse stimuli GPVI and CLEC-2-transfected cell lines

To seek confirmatory evidence that the above agonists directly activate GPVI and/or CLEC-2, we investigated their activation of transfected cell lines. We used human GPVI and human CLEC- 2 in these studies. We investigated the action of the above agonists on GPVI- transfected chicken DT40 B-cells and human Jurkat-T cells using an NFAT–luciferase reporter as a readout for activation of PLCγ 2 (Gibbins et al., 1996). We performed these studies in the absence of serum or antibiotics to prevent a possible interaction with one or more of this structurally diverse group of ligands. We used two cell lines in case of possible species differences in activation. We observed a similar level of activation of GPVI by DEP, fucoidan and dextran sulfate in transfected Jurkat T-cells (Figure 3-9A) and DT40 B-cells (appendix 13) to that induced by collagen, but not by the other agonists used above, namely CTH, LSARLAF and Pam_3-CSK_4 (Figure 3-9B). We were, however, unable to investigate 4N1-1 and Champs as both peptides stimulated a marked increase in luciferase activity in mock- transfected cells (appendix 14). We also investigated whether these stimuli were able to activate CLEC-2 in transfected DT40 cells. We were unable to perform similar studies on human Jurkat cells as CLEC-2 is unable to signal through the Syk-related kinase ζ-chain-associated protein of 70 kDa (Zap-70), which is expressed in these cells (Hughes et al., 2013). DEP, fucoidan and dextran sulfate induced activation of CLEC-2, whereas the other agonists had no effect (Figures 3-9B and 3-9C). These results therefore confirm that DEP, fucoidan and dextran sulfate are able to activate GPVI and CLEC-2. The inability of the other stimuli to activate the two platelet (hem)ITAM receptors could reflect a loss of avidity due to the absence of other key surface receptors or even binding to inhibitory ITIM receptors in these cell lines (see the discussion).

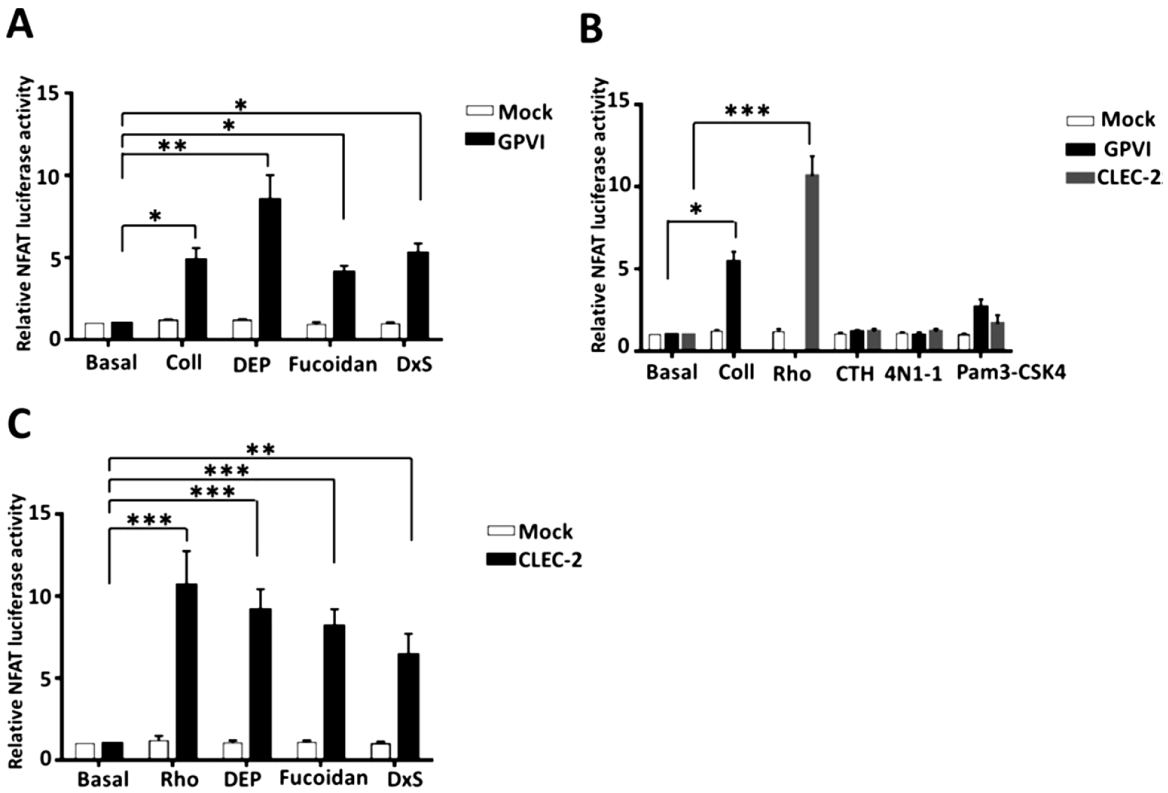

Figure 3-9 Miscellaneous platelet stimuli activate GPVI and CLEC-2 in transfected cell lines. (A) The effect of DEP (50 μg/ml), fucoidan (150 μg/ml) and dextran sulfate (DxS; 100 nM) on GPVI-transfected Jurkat T-cells was monitored using an NFAT–luciferase reporter assay. Cells were transfected with either an empty vector (mock) or a GPVI–FcRγ -chain and an NFAT–luciferase reporter as described in the Experimental section and stimulated for 6 h. Luciferase activity was measured as previously described (Tomlinson et al., 2007). Results are representative of three experiments, and are means ± S.E.M, n-3 (*P <0.05, **P <0.01). (B) The effects of CTH (100 μg/ml), 4N1-1(100 μM) and Pam_3-CSK_4 (50 μg/ml) on GPVI- or CLEC-2-transfected DT40 B-cells was monitored using the NFAT–luciferase reporter assay described above. Results are means ±S.D. n=3 (**P <0.01). (C) The effect of DEP, fucoidan and dextran sulfate on CLEC-2-transfected DT40 cells was monitored using an NFAT–luciferase reporter assay as described in the Experimental section and stimulated for 3 h. Results are representative of three experiments, and are means ± S.E.M., n-3 (**P <0.01, ***P <0.001). Coll, collagen; Rho rhodocytin

Table 3-1 Summary of platelet activation by miscellaneous ligands. The Table summarizes the mechanism of platelet activation by the ligands that have been used in the present study. The columns represent the following: Receptor phosphorylation: phosphorylation of FcR γ-chain or CLEC-2; Aggregation: dependency on Src and Syk tyrosine kinases; Agglutination: whether agglutination (αIIbβ3-independent aggregation) is present.

Ligand	Receptor phosphorylation	Aggregation block by Src	Aggregation block by Syk	Agglutination
DEP	FcR γ-chain, CLEC-2 (weak)	Yes	Yes	No
CTH	FcR γ-chain	Yes	Yes	No
4N1-1	FcR γ-chain	Yes	Yes	Yes
Champs	FcR γ -chain	Yes	Yes	Yes
Fucoidan	FcR γ-chain (weak), CLEC-2	Yes	Yes	No
Dextran sulfate	CLEC-2	Yes	Yes	Yes
Pam_3 - CSK_4	FcR γ-chain (transient)	No	No	No

3.5 Discussion

The present study has investigated the mechanism of platelet activation by a diverse group of ligands that were selected on their known ability to activate platelets but their lack of structural resemblance to known platelet agonists. The results imply that all of these stimuli induce activation through GPVI and/or CLEC-2 but that they also bind to additional Src kinase-coupled surface receptors. However, with the exception of dextran sulfate, it appears that this additional binding only induces weak signalling, insufficient to cause full platelet activation and aggregation. The three structurally unrelated small peptides, Pam_3-CSK_4 and histones all activate GPVI, whereas DEP, fucoidan and dextran sulfate activate both GPVI and CLEC-2, with the former showing a preference for the collagen receptor over CLEC-2 and vice versa for fucoidan and dextran. This marked heterogeneity in terms of receptor specificity within this group is summarized in Table 3-1.

The mechanism of activation of GPVI and CLEC-2 by DEP, fucoidan and dextran sulfate appears to be through direct binding as shown in transfected cell lines. In contrast, we did not observe activation of GPVI and/or CLEC-2 in transfected DT40 or Jurkat cells using the other ligands described above, namely histones, 4N1-1 and Pam_3-CSK_4 (with the caveat that this could not be investigated for Champs and LSARLAF because of activation of mock-transfected cells). While we cannot rule out that this is due to an alternative conformation of these two proteins relative to that on a platelet surface, a more likely explanation is that of too low an affinity as discussed above, with binding on platelets being influenced by avidity. The ability of these ligands to activate GPVI and/or CLEC-2 on platelets may therefore be the result of enhanced

avidity mediated by binding to multiple surface proteins including multiple copies of GPVI/CLEC-2.

Evidence of binding to other surface proteins by the above ligands is provided by the observation that all of these stimuli also induce tyrosine phosphorylation independently of GPVI and CLEC-2 as shown using GPVI/CLEC-2-double-deficient platelets. However, only in the case of dextran sulfate does this lead to activation. It is therefore likely that binding to these other receptors facilitates binding to GPVI and CLEC-2 through increased avidity. In addition, this could also influence activation of one or both of the (hem)ITAM receptors through binding to other surface proteins such as tyrosine phosphatase-binding proteins, e.g. cluster of differentiation (CD) 148 (Senis et al., 2009, Severin et al., 2011b) and the constitutively phosphorylated ITIM receptor G6b-B (Mori et al., 2008). Altering the distribution of GPVI or CLEC-2 relative to these proteins could lead to receptor activation. The critical role of GPVI and CLEC-2 in mediating platelet activation by this diverse group of agonists, relative to other receptors, may reflect their ability to induce powerful activation of Syk through ITAM and hemITAM-based pathways respectively. In contrast, other surface glycoproteins that activate Syk, such as the GPIb-IX-V complex and integrin αIIbβ3, induce relatively weak activation of Syk most probably because of the absence of a cytosolic (hem) ITAM.

We have also shown that aggregation induced by low concentrations of the ligands that have been used in the present study is partially inhibited by agents that prevent the formation (indomethacin) or action (apyrase) of the feedback agonists, thromboxane A_2 and ADP (appendix 15). This is similar to platelet activation by low concentrations of most platelet agonists, including both GPVI and CLEC-2 ligands.

The stimuli that form the focus of the present study differ markedly in size, charge and hydrophobicity, and include large polysaccharides, charged/hydrophobic peptides, highly basic proteins and DEP, which are carbon black nanoparticles with surface electrical charge. Such diversity in terms of structure, along with the differential roles of GPVI and CLEC-2, indicates that they are unlikely to induce activation through a single mechanism, consistent with the differential roles of the two (hem)ITAM receptors in mediating activation to these ligands, as described above. Furthermore, this structural diversity indicates that they are unlikely to mediate activation through a 'close-fitting' binding interface with a high degree of surface complementarity, but via hydrophobic and charge interactions that lead to receptor clustering or altered interactions with surrounding proteins. The binding of fucoidan to CLEC-2 is a possible example of this and we speculate that this may occur at the binding sites in CLEC-2 for the podoplanin aspartic–glutamic acid doublet and *O*-glycan or the negatively charged C-terminus of rhodocytin as predicted from co-crystallization (Jung et al., 2012). The binding of podoplanin and rhodocytin to CLEC-2 is abrogated on mutation of just one of the four conserved arginines in the C-type lectin-like receptor, emphasizing the importance of a charged interaction (Jung et al., 2012). The negatively charged nature of fucoidan and dextran sulfate, and the multiple charges on DEP, would be consistent with such a mechanism.

The possibility that this diverse group of agonists could exert their effects on platelets through the interaction with a single membrane protein or with the membrane bilayer however seems unlikely. The observation that the majority of the stimuli are unable to activate GPVI or CLEC-2 in transfected DT40 cells argues against an interaction with the phospholipid membrane being the sole cause of activation. The diversity of structures also argues against an effect on a single surface receptor.

Moreover, we have shown that representative stimuli from this group (i.e. DEP, histones, fucoidan and 4N1-1) stimulate granule secretion and tyrosine phosphorylation in platelets from patients deficient in the two major platelet glycoprotein receptors, GPIbα and integrin αIIbβ3 (not shown and appendix 16). Furthermore, we have also shown the stimulation of ATP secretion by this diverse group of ligands is blocked in the presence of a Src kinase inhibitor (appendix 17) demonstrating that it is not mediated by lysis. Together, these data indicate that this group of ligands bind to multiple proteins including GPVI, CLEC-2 and other Src kinase-coupled receptors.

The differential roles of GPVI over CLEC-2 in platelet activation by the various ligands could reflect differences in their proximal signalling events. For example, platelet activation by GPVI but not by CLEC-2 is inhibited in the absence of the membrane tyrosine phosphatases CD148 (Severin et al., 2011b, Senis et al., 2009). Additionally, whereas Src kinases mediate phosphorylation of the ITAM in the GPVI–FcR*γ*-chain complex, phosphorylation of the hemITAM in CLEC-2 is mediated by Syk (Hughes et al., 2013, Spalton et al., 2009a, Severin et al., 2011b). Moreover, GPVI is predominantly monomeric on resting platelets and undergoes dimerization upon activation to a high-affinity state that enables binding to collagen (Jung et al., 2012). In contrast, CLEC-2 is expressed as a dimer in resting platelets (Hughes et al., 2010b). Measurement of GPVI and CLEC-2 dimerization/oligomerization using techniques such as FRET and super-resolution microscopy in the presence of this miscellaneous group of ligands will provide important insights into the mechanism of regulation.

Interestingly, GPVI-deficient platelets also have a 50 % reduction in expression of the FcR γ-chain (supplementary 4-2). This demonstrates that platelet activation is mediated by the GPVI–FcR*γ*-chain complex rather than through the indirect cross-linking of the FcR γ-chain.

Several of the stimuli that have been investigated in the present study, namely the three charged peptides, DEP and dextran sulfate, also induce platelet agglutination, possibly through negation of surface membrane charge or promotion of low-affinity interactions between homophilic ligands such as platelet-endothelial cell adhesion molecule (PECAM)-1 receptors or ligand pairs such as ephrins and Eph kinases (aka ephrin receptors). Many of these low-affinity interactions are thought to play a critical role in late-stage platelet activation(Brass et al., 2011).

The present results are important to a number of physiological and pathological processes. Histones are implicated in a variety of pathological conditions, including trauma and thrombo- inflammatory disorders such as deep vein thrombosis (Abrams et al., 2013, Brill et al., 2012). Histones exert a multitude of effects in the vasculature including endothelial cell damage and platelet activation. These effects are likely to be mediated by multiple mechanisms including activation of ion channels, TLRs and, as shown in the present study, through (hem) ITAM receptors. The significance of platelet activation by DEP is less clear as they were unable to induce activation in the presence of plasma presumably due to plasma protein binding. On the other hand, they have been shown to potentiate aggregation *in vivo* in the presence of other agonists (Solomon et al., 2013) although it is not known whether this involves activation of GPVI and CLEC-2. Furthermore, other types of nanoparticles may have increased bioavailability *in vivo* and may mediate activation by similar mechanisms to those described above.

Platelets are activated by several other structurally unrelated stimuli where uncertainty exists with regard to the underlying mechanism. For example, the TLR1/2 ligand Pam_3-CSK_4 has been shown to stimulate tyrosine phosphorylation of several proteins in the (hem)ITAM signalling cascade in human platelets, including Src kinase family, Syk, linker of activated T-cells (LAT) and PLCγ 2 (Falker et al., 2014) In the

present study, we have shown that Pam3 -CSK4 stimulates transient phosphorylation of the FcRγ-chain and Syk but not CLEC-2, and this is lost in GPVI- deficient mice even though platelet aggregation is not altered.

This is in contrast with human platelets where phosphorylation of Syk and the downstream LAT signalosome is independent of GPVI (Falker et al., 2014) , although it is possible that this may reflect a difference in the methodologies used to block GPVI function. Examples of other ligands that induce activation and for whom uncertainty exists in regard to the underlying mechanism include oxidised-low density lipoprotein (ox-LDL), the ionic sclerosing agent sodium tetradecyl sulfate, the charged inositol phospholipid phosphatidylinositol 3, 4, 5-trisphosphate (PIP3) and various amyloid proteins. It will be of interest to establish whether mechanisms similar to those described in the present study underlie activation by one or more of these ligands.

In conclusion, the present study demonstrates that a diverse range of ligands activate platelets through GPVI and/or CLEC-2. In addition, these ligands activate other unidentified Src kinase- coupled surface proteins, although only in the case of dextran sulfate does this lead to platelet aggregation. The mechanisms described in the present study may have relevance to a variety of physiological and pathological situations ranging from the elevation of histones through to exposure to nanoparticles and contact with exogenous surfaces.

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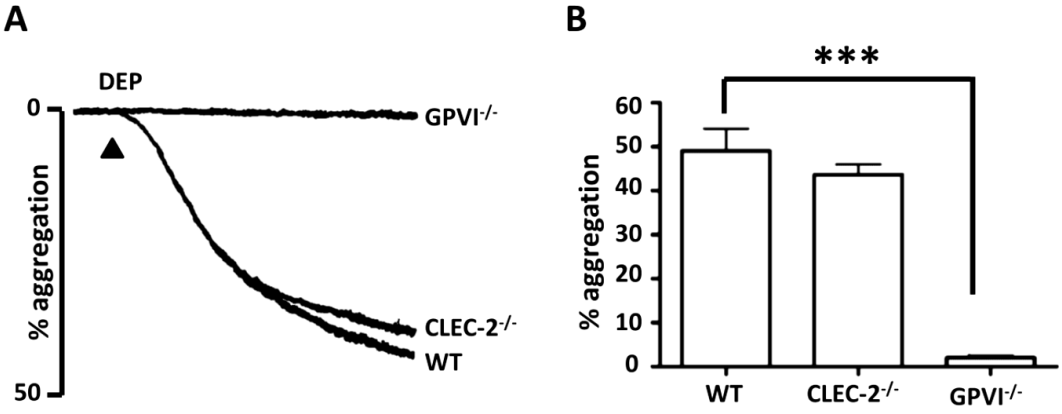
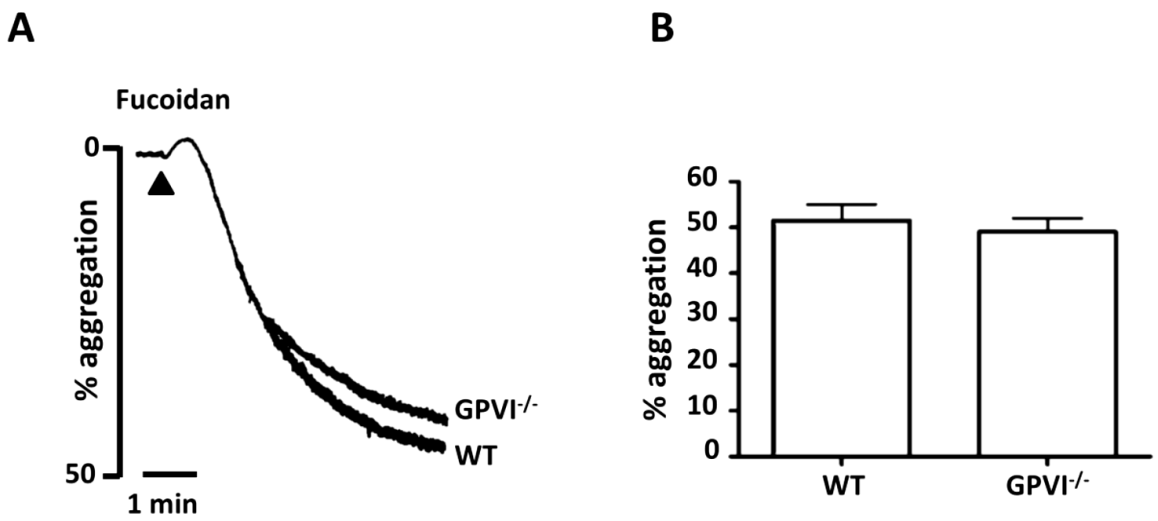
3.7 Supplementary



Supplementary figure 3-1 A submaximal dose of diesel exhaust particles (DEP) activates mouse platelets through GPVI. (A) Washed platelets from wild type (WT), CLEC-2-deficient, and GPVI-deficient mice were stimulated with DEP (20 μg/ml) and monitored for aggregation. (B) Quantitation of maximal aggregation, shown as mean ± SEM, from 3 experiments; (*** P<0.001).



Supplementary figure 3-2 A sub-maximal dose of fucoidan activates mouse platelets through CLEC-2 alone. (A) Washed platelets from wild type (WT) and GPVI-deficient mice were stimulated with fucoidan (50 μg/ml) and monitored for aggregation. (B) Quantitation of maximal aggregation, shown as mean ± SEM, from 3 experiments**.**

Chapter 4 FIBRIN ACTIVATES GPVI IN HUMAN AND MOUSE PLATELETS

This research was originally published in *Blood*. **Osama M. Alshehri**, Craig E. Hughes, Samantha Montague, Stephanie K. Watson, Jon Frampton, Markus Bender, and Steve P. Watson. Fibrin activates GPVI in human and mouse platelets. *Blood*. 2015; 126:1601-8.

Word Count: 4,464

This chapter demonstrates that fibrin binds to GPVI leading to the platelet activation and thrombus growth and stability. I contributed to this study by performing all of the *in vitro* experiments other than the GPVI ELISA which was performed by Samantha Montague. The in *vivo* experiments were undertaken by Markus Bender. Stephanie Watson performed studies on GPVI-transfected cell lines that were not included in the paper. The studies were designed with Craig Hughes.

- All not shown results have been added to the Appendix except those in page 130 and 136 were performed by one of the other authors in the manuscript

Abstract

The glycoprotein VI (GPVI)-Fc receptor γ (FcRγ) chain is the major platelet signaling receptor for collagen. Paradoxically, in a $FeCl_3$ injury model, occlusion, but not initiation of thrombus formation, is delayed in GPVI-deficient and GPVI-depleted mice. In this study, we demonstrate that GPVI is a receptor for fibrin and speculate that this contributes to development of an occlusive thrombus. We observed a marked increase in tyrosine phosphorylation, including the FcR γ-chain and Syk, in human and mouse platelets induced by thrombin in the presence of fibrinogen and the αIIbβ3 blocker eptifibatide. This was not seen in platelets stimulated by a protease activated receptor (PAR)-4 peptide, which is unable to generate fibrin from fibrinogen. The pattern of tyrosine phosphorylation was similar to that induced by activation of GPVI. Consistent with this, thrombin did not induce tyrosine phosphorylation of Syk and the FcR γ-chain in GPVI-deficient mouse platelets. Mouse platelets underwent full spreading on fibrin but not fibrinogen, which was blocked in the presence of a Src kinase inhibitor or in the absence of GPVI. Spreading on fibrin was associated with phosphatidylserine exposure (procoagulant activity), and this too was blocked in GPVI-deficient platelets. The ectodomain of GPVI was shown to bind to immobilized monomeric and polymerized fibrin. A marked increase in embolization was seen following $FeCl_3$ injury in GPVI-deficient mice, likely contributing to the delay in occlusion in this model. These results demonstrate that GPVI is a receptor for fibrin and provide evidence that this interaction contributes to thrombus growth and stability.

4.1 Introduction

Glycoprotein VI is an immunoglobulin superfamily receptor expressed on the surface of platelets in association with the Fc receptor γ chain (FcR γ-chain). Ligand binding of GPVI results in receptor clustering and Src kinase-dependent tyrosine phosphorylation of the FcR γ-chain immunoreceptor tyrosine-based activation motif (ITAM). This initiates a signalling pathway involving Src and Syk tyrosine kinases, various adapter proteins, and results in activation of phospholipase-C (PLC) $\gamma 2$ (for review see) (Watson et al., 2010).

GPVI is the major signalling receptor for the extracellular matrix protein, collagen (Ryo et al., 1992, Moroi et al., 1989, Arai et al., 1995), and is also activated by other endogenous ligands including laminin (Inoue et al., 2006); adiponectin (Riba et al., 2008) and the Ig superfamily protein EMMPRIN (CD147/Basigin) (Seizer et al., 2009). GPVI is also activated by a variety of exogenous and synthetic ligands including collagen-related peptide (CRP), which consists of glycine-proline-hydroxyproline (GPO) repeats(Knight et al., 1999), the snake venom toxin, convulxin (Polgar et al., 1997, Jandrot-Perrus et al., 1997), various artificial peptides including 4N1-1 and LSARLAF, antisense nucleotides (Flierl et al., 2015) and diesel exhaust particles (Alshehri et al., 2015).

GPVI plays a critical role in haemostasis and thrombosis through integrin activation, supporting adhesion and the initial stages of platelet aggregation. Despite this, patients and mice deficient in GPVI exhibit mild or no apparent impairment in haemostasis due to compensatory pathways of initiation of platelet activation including tissue factor-driven thrombin generation (Bynagari-Settipalli et al., 2014) and ADP release from damaged cells. GPVI also plays a critical role in the maintenance of a healthy endothelial cell layer, a process known as vascular integrity

(Boulaftali et al., 2013). The contribution of GPVI to thrombosis varies according to the experimental model. This is illustrated by its role in $FeCl_3$ and laser injury models, which is dependent on the level of injury to the vessel wall and the degree of exposure of the subendothelial matrix (Bender et al., 2011, Dubois et al., 2006, Konstantinides et al., 2006, Massberg et al., 2003). In the absence of a breach in the endothelium, thrombus formation is mediated by endothelial cell activation leading to tissue factor exposure and thrombin generation (Dubois et al., 2006, Konstantinides et al., 2006, Massberg et al., 2003, Eckly et al., 2011)

The focus of research on GPVI in haemostasis and thrombosis has been on its interaction with collagen. Paradoxically, however, the time to occlusion rather than initiation of thrombus formation has been shown to be prolonged in GPVI-deficient and GPVI-depleted mice following $FeCl_3$ injury, suggesting a role for GPVI in thrombus growth (Bender et al., 2011) even though collagen is only thought to play a role at the site of lesion. In the present study, we provide an explanation for this paradox by demonstrating that GPVI is a receptor for fibrin, which is generated from fibrinogen by the action of thrombin. Thus, GPVI is a receptor for both collagen and fibrin, and plays a critical role in thrombus growth as well as in initiating thrombus formation.

4.2 Materials and Methods

4.2.1 Reagents

Human fibrinogen was obtained from Enzyme Research Laboratories (Swansea, UK). The α- phospho-tyrosine (4G10) monoclonal antibody was from Millipore (Abingdon, UK). Alexa-488 fibrinogen, Alexa-488 phalloidin, and Alexa-568 phalloidin were from Molecular Probes (Life Technologies, Paisley, UK). The α-Syk antibody (SC-573) was from Insight Biotechnology (Wembley, UK).The α-CLEC-2

antibodies (AYP1 and AYP2) have been described previously (Gitz et al., 2014) .FITC-Annexin V was from BD Bioscience (Oxford, UK). FITC-conjugated α-mouse αIIbβ3, α2, CLEC-2, GPIbα, GPV, GPVI, GPIX, and IgG antibodies were from Emfret Analytics (Würzburg, Germany). Dasatinib was from LC Laboratories (Woburn, MA, USA). PAR-4 peptide (Ala-Tyr-Pro-Gly-Lys-Phe: AYPGKF) was produced by Alta Bioscience (Birmingham, UK). HRP-conjugated secondary antibodies and ECL reagent was from Amersham Biosciences (GE Healthcare, Bucks, UK). HRP-conjugated secondary antibody for ELISA was from Dako (Ely, UK). The α-GPVI antibody (1A12) was provided by Dr Elizabeth Gardiner (Melbourne, Australia). SuperSignal ELISA Pico chemiluminescent substrate was from Pierce (Rockford, IL, USA). All other reagents were purchased from Sigma Aldrich (Poole, UK).

4.2.2 Mice

$Gp6^{-/-}$ mice (GPVI KO) were provided by Dr Jerry Ware (Kato et al., 2003). $Itga2b^{-/-}$ mice (αIIb-deficient mice) have been previously described (Emambokus and Frampton, 2003).Wild-type mice (WT) were generated from breeding of heterozygotes or purchased from Harlan Laboratories (Hillcrest, UK). All procedures were undertaken with UK Home Office approval under PPL30/2721 and PPL30/8286.

4.2.3 Washed platelet preparation

Blood was drawn from CO_2-asphyxiated mice or from consenting, healthy, drug free volunteers into 10% acid citrate dextrose or sodium citrate, respectively. Ethical approval for the donation of blood by volunteers was granted by Birmingham University Internal Ethical Review (ERN_11-0175). Washed platelets were obtained by centrifugation using prostacyclin and resuspended in modified Tyrode's buffer

(134 mM NaCl, 0.34 mM Na_2HPO_4, 2.9 mM KCl, 12 mM $NaHCO_3$, 20 mM HEPES (4-(2-hydroxyethyl)-1 piperazineethanesulfonic acid), 5 mM glucose, and 1 mM $MgCl_2$; pH 7.3) as previously described (Hughes et al., 2015). Platelets were used at 2 x 10^7/mL for static adhesion or 5 x10^8/mL for other studies.

4.2.4 Protein phosphorylation

Washed platelets were pre-treated with 9μM eptifibatide (where stated), 10μM indomethacin and 2 U/ml apyrase or solvent controls. Platelets were stimulated with thrombin (1 U/ml) or PAR-4 peptide (AYPGKF; 150 μM) at 37°C with stirring (1200rpm) in an aggregometer for 60 sec. Where stated, stimulations were performed in the presence of fibrinogen (200 μg/ml) and GPRP (Gly-Pro-Arg-Pro; 5 μM). Activation was terminated with 2×ice-cold lysis buffer (300 mM NaCl, 20 mM Tris, 2 mM EGTA, 2 mM EDTA, 2% IGEPAL CA-630 (NP-40 equivalent); pH 7.4 plus 2.5 mM Na3VO4, 100 μg/ml AEBSF, 5 μg/ml leupeptin, 5 μg/ml aprotinin, 0.5 μg/ml pepstatin). Whole cell lysates were prepared by boiling a sample of lysate with SDS sample buffer. Syk was immunoprecipitated with α-Syk antibody (SC-573) and protein A-Sepharose beads for 2 hrs. The beads were then washed and proteins eluted by boiling in SDS sample buffer. IPs and WCLs were then separated by SDS-PAGE, electro-transferred, and Western blotted. Western blots were imaged with a Licor Odyssey-FC (Cambridge, UK) or autoradiographic film.

4.2.5 Platelet spreading

Coverslips were coated in the presence of 200 μg/ml fibrinogen (± 5 μM GPRP) for 60 min followed by washing with phosphate-buffered saline and then blocking with 5 mg/ml heat-inactivated bovine serum albumin for 60 min. Fibrin-coated coverslips were generated by the addition of 1 U/ml thrombin for 15 min to coverslips that had

been incubated with 200 μg/ml fibrinogen for 30 min. Thrombin was neutralised by addition of 5 U/ml hirudin for 30 min. Following coating, coverslips were washed and blocked with 5 mg/ml heat inactivated bovine serum albumin for 60 min. Platelets were spread on fibrinogen or fibrin for 45 min at 37°C before washing with Tyrode's buffer followed by fixation with paraformaldehyde (3.7%). Where stated, platelets were pre-incubated with dasatinib (10 μM) or hirudin (5 U/ml). For actin staining, platelets were permeablised with 0.1% Triton X-100 and stained with Alexa-488 phalloidin for 45 min. In some studies, Alexa-488-fibrinogen was used for fluorescent imaging to monitor conversion to fibrin, in which case, platelets were counterstained with Alexa-568 phalloidin for 45 min. For phosphatidylserine (PS) staining, platelets were incubated with FITC-Annexin V in Tyrode's buffer plus 2 mM $CaCl_2$ for a further 15 min, followed fixation, and counter-staining for actin using Alexa-568 phalloidin for 45 min. Platelets were imaged on a Zeiss Axiovert 200 M microscope. Platelet surface area was analysed using ImageJ (NIH, Bethesda, USA).

4.2.6 Flow cytometry

Surface glycoprotein expression was measured in wild type and GPVI-deficient mouse platelets by staining whole blood with selected FITC-conjugated antibodies and analyzing with a BD Accuri C6 flow cytometer (BD Biosciences, Oxford, UK).

4.2.7 GPVI ELISA

Binding of GPVI to fibrin/fibrinogen was measured using an adapted version of a soluble GPVI ELISA (Al-Tamimi et al., 2009b). Wells of a Nunc Maxisorp micro-titre plate (Thermo Scientific, Paisley, UK) were coated with fibrin/fibrinogen using the same protocol as above. Wells were washed six times with 100 μl of PBS-T (0.2%), blocked with 1% (w/v) BSA for 1hr at RT, and then washed six times with

PBS-T. GPVI ectodomain (prepared from NEM treated plasma) was added to the wells for 1 hr at RT, followed by washing six times with PBS-T. A standard curve was generated by a serial dilution of GPVI ectodomain into GPVI-depleted plasma. Primary antibody, (1A12; 1 μg/ml) was then incubated for 1hr followed by six washes. Secondary, α- mouse HRP-conjugated antibody (2.6 μg/ml) was then incubated for 1 hr, followed by six washes, followed by 100μl of SuperSignal substrate for 1 min. Light emission was measured using a Wallac-Victor2 luminescence plate reader (PerkinElmer, Waltham, MA, USA) for 10 sec/well. Amounts of bound GPVI were calculated from the standard curve.

4.2.8 Intravital microscopy

4-5 weeks old WT or GPVI KO mice (Bender et al., 2013) were anesthetised, and the mesentery was exteriorised through a midline abdominal incision. Arterioles (40-60 μm diameter) were visualised with a Zeiss Axiovert 200 inverted microscope (x10) equipped with a 100-W HBO fluorescent lamp source, and a CoolSNAP-EZ camera (Visitron Systems, Puchheim, Germany). Digital images were recorded and analysed offline using Metavue software. Injury was induced by topical application of a droplet of $FeCl_3$ from 3-mm2 filter paper saturated with 20% $FeCl_3$. The droplet rapidly dissipated through the vessel. Adhesion and aggregation of fluorescently labelled platelets (Dylight-488 conjugated α-GPIX Ig derivative) in arterioles were monitored for 40min or until complete occlusion occurred (blood flow stopped for longer than 1min).

4.3 Statistical analysis

Statistical analysis was by ANOVA with a Bonferroni post-test, or for the ELISA, a one-tailed t-test. For in vivo experiments, results are shown as the mean ± SD of a minimum of six mice. Statistical analysis was by t –test.

4.4 Results

4.4.1 Fibrin stimulates tyrosine phosphorylation in human and mouse platelets through GPVI.

The binding of fibrinogen to integrin αIIbβ3 induces outside-in signalling leading to activation of Src and Syk tyrosine kinases and a signalling cascade that culminates in activation of PLCγ2 (Wonerow et al., 2003, Clark et al., 1994). In addition, αIIbβ3 is a receptor for fibrin, which binds to multiple sites in the αIIb β-propeller (Podolnikova et al., 2014). It is not known however whether binding of fibrin to the integrin also induces outside-in signalling. To address this, we compared tyrosine phosphorylation in human platelets stimulated by thrombin, which stimulates fibrin formation, and by a PAR-4 peptide. The contribution of fibrin polymers to tyrosine phosphorylation in this assay can be established using GPRP, which blocks polymerization of fibrin monomers.

Thrombin stimulates tyrosine phosphorylation in human platelets under aggregating conditions, which is slightly increased in the presence of added fibrinogen (Suppl. Fig. 4-1). A similar increase in tyrosine phosphorylation is induced by the PAR-4 peptide. For both agonists, tyrosine phosphorylation is marginally reduced or unaltered in the presence of GPRP. These results indicate that fibrin does not play a major role in mediating tyrosine phosphorylation under aggregating conditions, which is predominantly mediated through binding of fibrinogen to integrin αIIbβ3 as previously reported (Watson et al., 2005b, Huang et al., 1993, Haimovich et al., 1993)

A role for fibrin in mediating tyrosine phosphorylation may however have been masked by outside-in signalling by fibrinogen. To address this, we performed studies in the presence of eptifibatide, which blocks the binding of fibrinogen to αIIbβ3. Under these conditions, thrombin stimulates a much smaller increase in

tyrosine phosphorylation in platelet lysates, which is increased significantly in the presence of fibrinogen, including a band of 72 kDa, which was identified as Syk by immunoprecipitation and western blotting (Fig. 4-1Ai) (NB a decrease in Syk signal in the Syk reprobes corresponds with the increase in phosphorylated Syk. This is due to the blotting antibody not detecting phospho-Syk). In addition, a tyrosine phosphorylated band of 14 kDa was present in Syk immunoprecipitates, which corresponds to the FcR γ-chain. The marked increase in tyrosine phosphorylation, including Syk and the FcR γ-chain, is blocked in the presence of GPRP indicating that it is mediated fibrin polymerisation. In contrast, tyrosine phosphorylation induced by a PAR-4 peptide (which is unable to generate fibrin) is not altered in the presence of added fibrinogen or GPRP. A similar set of results are seen in mouse platelets stimulated by thrombin and by the PAR-4 peptide in the presence of eptifibatide (Fig. 4-1Aii and appendix 18). These results show that fibrin increases tyrosine phosphorylation in human and mouse platelets, including Syk and FcR γ-chain.

We used αIIb-deficient mouse platelets to investigate whether integrin αIIbβ3 is required for the increase tyrosine phosphorylation induced by fibrin in the presence of eptifibatide (in view of the possibility that the increase was mediated by binding of fibrin to separate site on the integrin). As shown in (Fig.4-1Bi), tyrosine phosphorylation, including Syk and the FcR γ-chain, was again induced by thrombin in the presence of fibrinogen despite the absence of αIIb-integrin subunit, and this was blocked in the presence of GPRP. These results demonstrate that fibrin stimulates tyrosine phosphorylation of Syk and FcR γ-chain in mouse platelets independent of the major platelet integrin, αIIbβ3. The pattern of tyrosine phosphorylation was similar to that induced by activation of GPVI, including phosphorylation of the FcR γ-chain. To investigate a possible role for GPVI, we studied the effect of thrombin in

mouse platelets deficient in GPVI in the presence of eptifibatide to block binding of fibrinogen to integrin αIIbβ3. Platelets from these mice have approximately 50% of the wild type level of the GPVI-binding partner, the FcR γ-chain, while the level of other major platelet glycoprotein receptors was similar to that in wild type platelets (Suppl. Fig. 4-2). Further, activation of platelets by thrombin is not altered in GPVI-deficient mice (not shown) suggesting that the levels of PAR-4 are unchanged. Fibrin stimulated only a minor increase in tyrosine phosphorylation in GPVI-deficient platelets, and failed to induce phosphorylation of Syk or the FcR γ-chain (Fig. 4-1Bii). The minor increase in phosphorylation in platelet lysates was not altered in the presence of GPRP (Fig. 4-1Bii). These results demonstrate that the marked increase in tyrosine phosphorylation, including the FcR γ-chain and Syk, by fibrin in platelets is mediated by GPVI. The podoplanin receptor CLEC-2, which signals through a similar pathway to that of GPVI, was not phosphorylated (Suppl. Fig. 4-3) ruling out a role for the C-type lectin-like receptor in the response to fibrin.

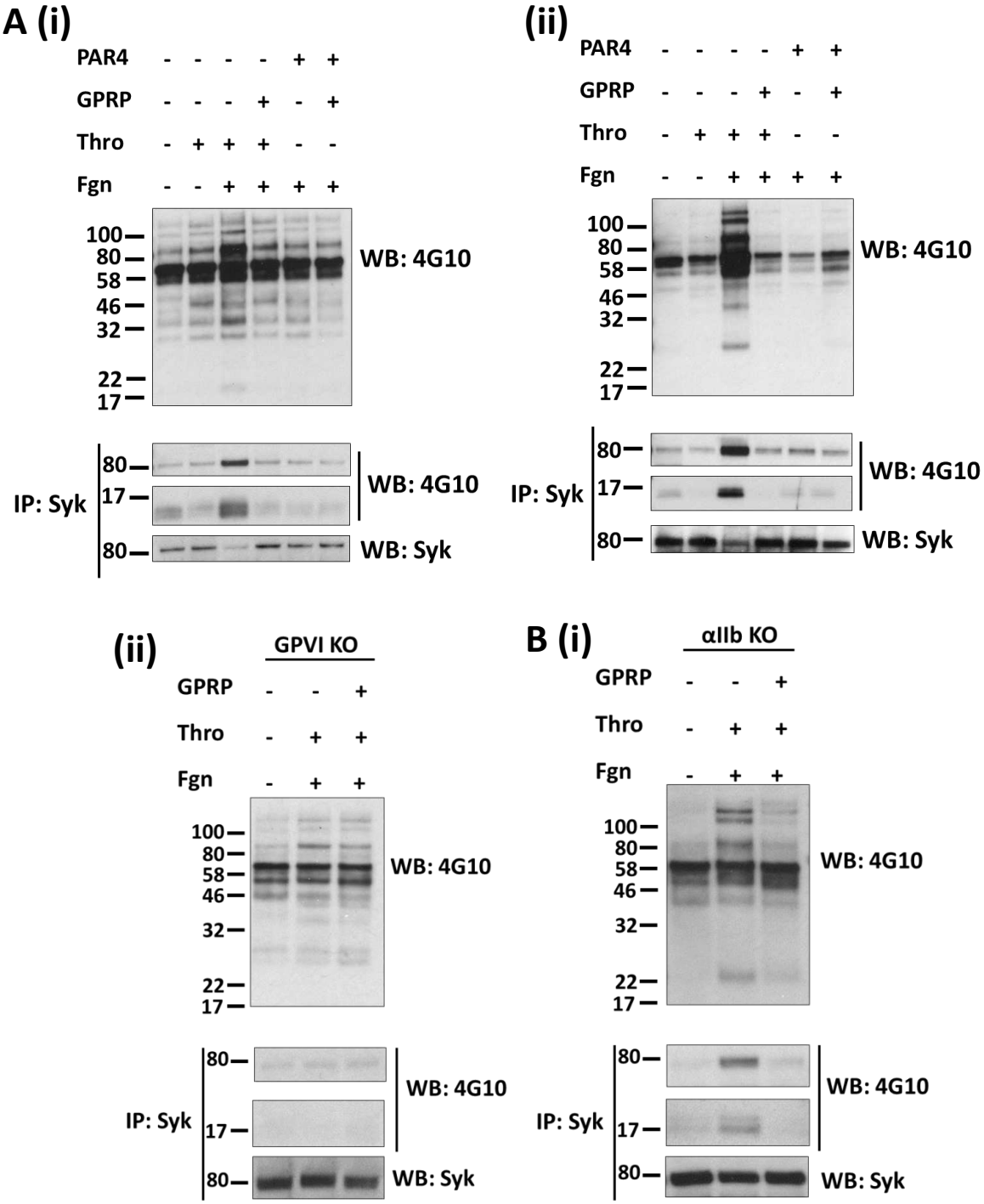


Figure 4-1 Fibrin stimulates tyrosine phosphorylation in a GPVI-dependent manner. Platelets from (Ai) human, (Aii) mice, (Bi) αIIb-deficient mice, and (Bii) GPVI-deficient mice were stimulated with thrombin (1 U/mL) or PAR-4 peptide (150 mM) in the presence of eptifibatide and, where shown, fibrinogen (200 mg/mL) and GPRP (5 mM). Stimulations were stopped after (A) 1 or (B) 3 minutes with the addition of 23 lysis buffer. A sample of the WCL was removed, and the remaining lysate was used to IP Syk. WCLs and IPs were separated by SDS-polyacrylamide gel electrophoresis and western blotted for pTyr, Syk, and the FcR γ-chain, which coprecipitates with Syk. The results are shown as representative of 3 experiments**.**

4.4.2 Fibrin stimulates platelet spreading and pro-coagulant activity via GPVI

We next asked whether activation of GPVI by fibrin could induce platelet activation. To address this, we monitored spreading of mouse platelets on a fibrin surface and compared this to spreading on fibrinogen. To ensure a similar level of coating of fibrin and fibrinogen, coverslips were coated with fibrinogen prior to treatment with thrombin (hirudin was later added to block thrombin as described in the methods). We used Alexa-488 fibrinogen to visualise fibrin and fibrinogen. Fibrinogen forms a homogenous surface on the coverslip, which was converted to a heterogeneous network of fibrin following treatment with thrombin. Large areas of fibrin are easily distinguishable (Suppl. Fig. 4-4), but following longer exposures, smaller areas of fibrin can be seen. Both large and smaller coatings of fibrin are associated with platelet adhesion and spreading (Fig. 4-2A and appendix 19).

Mouse platelets form actin stress fibres and lamellipodia sheets on polymerised fibrin but not on fibrinogen, which induces formation of filopodia and partial lamellipodia (Fig. 4-2B).Actin nodules (Calaminus et al., 2008) and filopodia can be seen in platelets that have undergone partial spreading, which is likely to reflect an earlier stage of spreading on fibrin (appendix 20). Spreading on fibrin is blocked by the Src kinase inhibitor dasatinib but unaltered in the presence of hirudin, demonstrating that it is not mediated by residual thrombin (Fig. 4-2B). Spreading of platelets on fibrin was abolished in the absence of GPVI (Fig. 4-2C) demonstrating that fibrin induces spreading through the collagen receptor. Fibrin also supports full spreading of human platelets (Suppl. Fig. 4-5). Interestingly, mouse platelets underwent full spreading on fibrin in the presence of GPRP, which prevents polymerisation of fibrin (Fig. 4-3A). This demonstrates both fibrin

monomers and fibrin polymers are able to induce full spreading of platelets indicating that they share the epitope for binding to, and activation of GPVI. The inhibitory effect of GPRP on GPVI activation in suspension studies as shown in (Fig. 4-1) is likely to be due to loss of avidity (and therefore the ability to crosslink GPVI) rather than loss of binding to fibrin. This is overcome by presentation of fibrin monomers on a 2-dimensional surface. We modified a previously described ELISA assay (Al-Tamimi et al., 2009b) to verify that monomeric and polymerised fibrin bind to GPVI. Using this assay, we observed a similar level of binding of the ectodomain of GPVI to fibrin monomers and fibrin polymers (Fig. 4-3B). In contrast, GPVI did not bind to immobilised fibrinogen (Fig. 4-3B).

During the latter stages of platelet activation, phosphatidylserine (PS) is exposed on the outer leaflet of the plasma membrane and provides a negatively charged, pro-coagulant surface for the generation of thrombin. This process is important for thrombus formation *in vivo* as it generates thrombin on the platelet surface, which serves both as a positive feedback step in platelet activation and mediates conversion of fibrinogen to fibrin, thereby stabilising the thrombus. As fibrin generation is an integral part of this positive feedback loop, and activation of GPVI is associated with PS exposure, we determined whether fibrin induces PS exposure in platelets by measurement of binding of FITC-conjugated Annexin V (Munnix et al., 2005). Fibrin induced a 3-fold increase in number of PS-exposing platelets compared to fibrinogen, which was inhibited in GPVI-deficient platelets (Fig. 4-4A). Thus, fibrin stimulates PS exposure through activation of GPVI.

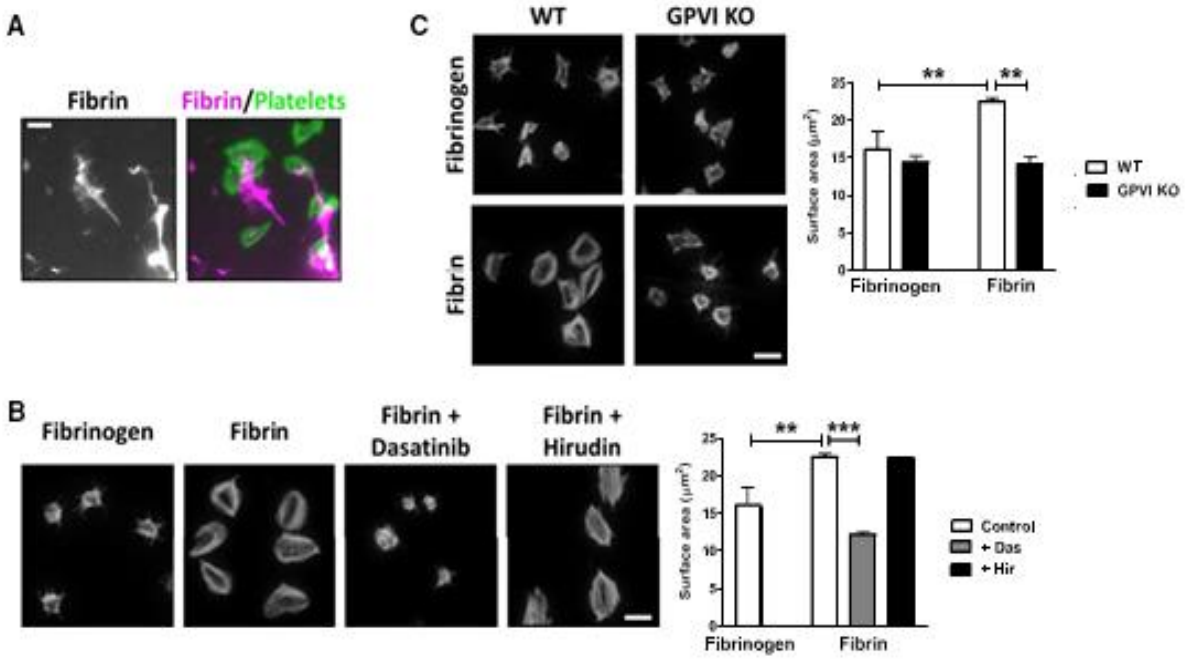


Figure 4-2 Fibrin stimulates spreading in a GPVI-dependent manner. Fibrinogen was coated onto glass coverslips, and converted into fibrin by treatment with thrombin. Hirudin was used to neutralise any residual thrombin before washing and blocking. (A) Alexa-488 fibrinogen was used to visualise fibrin formation. Platelets (2×10^7/ml) were allowed to spread on coated coverslips, followed by actin staining with Alexa-568 phalloidin. Scale bar = 5 μm. (B) Platelets (2×10^7/ml) were allowed to spread on non-fluorescent fibrinogen or fibrin-coated coverslips, followed by actin staining with Alexa-488 phalloidin. Where shown, platelets were pre-incubated with dasatinib (10 μM) or hirudin (5 U/ml). Scale bar = 5 μm. (C) WT or GPVI KO platelets were allowed to spread on fibrinogen or fibrin-coated coverslips, followed by actin staining with Alexa-488 phalloidin. Scale bar = 5 μm. The results are shown as mean ± SEM of three experiments. *P*<0.01; ***P<0.001.

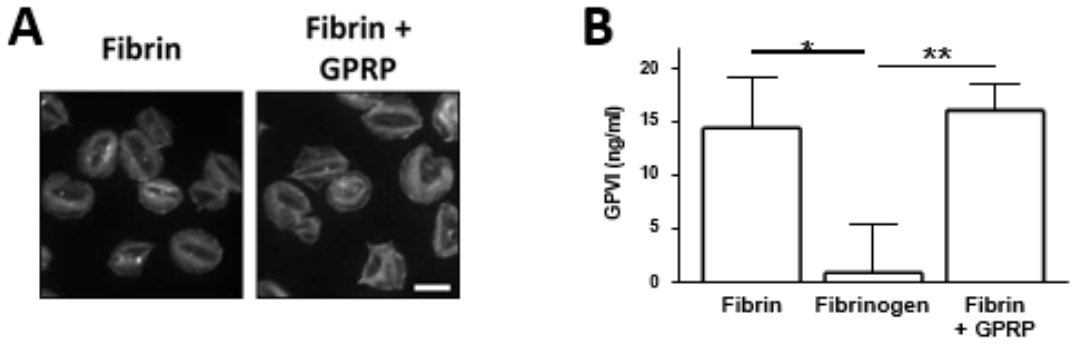


Figure 4-3 Fibrin polymerisation is not essential for platelet spreading or GPVI binding. (A) Fibrinogen was coated onto glass coverslips, and converted into fibrin by treatment with thrombin. GPRP (5 μM) was used to inhibit polymerisation of fibrin monomers. Hirudin was used to neutralise any residual thrombin before washing and blocking. Platelets (2×10^7 /ml) were allowed to spread, followed by actin staining with Alexa-488 phalloidin. Scale bar = 5μm. The results are representative of three experiments. (B) Fibrinogen was coated onto the wells of Nunc Maxisorp plates, and converted into fibrin by treatment with thrombin. GPRP (5 μM) was used to inhibit polymerisation of fibrin monomers. The ectodomain of GPVI was incubated, and following washing, adherent GPVI was detected with a HRP-conjugated antibody. The results are shown as mean ± SEM of three experiments performed in duplicate. *P<0.05; **P<0.01.

4.4.3 Time to occlusion but not initiation of thrombus formation following $FeCl_3$ injury is diminished in GPVI-deficient platelets

The observation that GPVI is a receptor for fibrin raises the question as to whether the delayed occlusion in a $FeCl_3$ injury model (Bender et al., 2011)is due to loss of activation of GPVI by fibrin rather than by collagen, which is not present in the growing thrombus. To address this, we studied thrombus formation by intravital fluorescence microscopy following $FeCl_3$-injured mesenteric arterioles in GPVI-deficient mice. The onset of aggregation occurred at similar times after injury in wild type and GPVI-deficient mice indicating that it was likely to be mediated by endothelial cell activation rather than by exposure to collagen (Fig. 4B and supplementary video). Complete vessel occlusion was observed within 20 min in wild type vessels ((Bender et al., 2011) and not shown). In contrast, vessel occlusion was markedly delayed or absent in GPVI-deficient mice and constant embolisation was seen (Fig. 4-4B and supplementary video). The rate of embolisation from thrombi of more than 10 µm was increased by 6.6 fold in GPVI-deficient mice. Consequently, most vessels remained open or occlusion was significantly delayed. These results demonstrate a critical role for GPVI in thrombus stabilisation relative to thrombus initiation in this model of $FeCl_3$-injury.

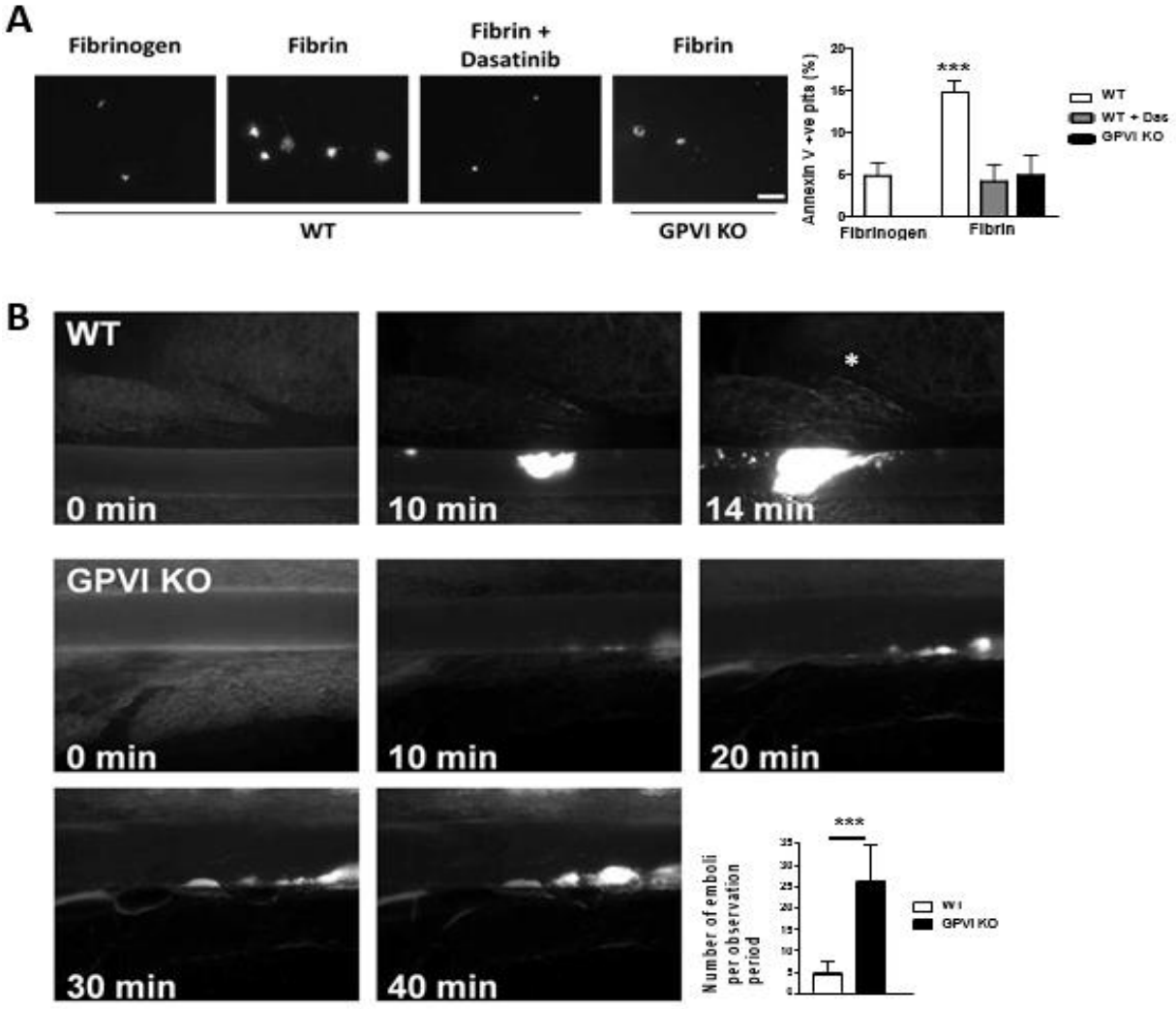


Figure 4-4 GPVI KO platelets have reduced PS exposure and reduced thrombus stability. (A) WT or GPVI-deficient platelets were allowed to spread on fibrinogen or fibrin- coated coverslips, followed by incubation with FITC-Annexin V. Actin was counterstained with Alexa-568 phalloidin to count total number of adherent platelets (appendix 21). Scale bar, 20μm. The results are shown as mean ± SEM of 3 experiments. (B) Representative fluorescent images before and after injury. Asterisk indicates vessel occlusion. Embolisation rate was determined by counting embolised thrombus fragments (size >10μm) after an initial thrombus had formed (thrombus size at least 10μm). Observation period: 40 minutes or until vessel occlusion. The results are shown as mean ± SD of 6 or more mice. ***P <0.001.

4.5 Discussion

Until now, the focus on the role of GPVI in haemostasis and thrombosis has been on the onset of response because of expression of the ligands, collagen and laminin, in the sub-endothelial matrix. Strikingly however, a marked delay in vessel occlusion rather than in initiation of thrombus formation is seen in mice-deficient in GPVI in a $FeCl_3$ injury model indicating a role for GPVI in thrombus growth(Bender et al., 2011) . In the present study, we identify fibrin as a novel ligand for GPVI. We speculate that loss of this interaction mediates the delay in vessel occlusion, and the increase in embolisation in GPVI-deficient mice following topical $FeCl_3$ injury.

The interaction of fibrinogen with platelets is dependent on sustained activation of the integrin through the platelet ADP receptor, $P2Y_{12}$ (Stefanini et al., 2015) but becomes irreversible over time, as a result of conversion to fibrin. Fibrin is formed from fibrinogen by the action of the protease thrombin. Thrombin exposes a site of interaction allowing the fibrin monomers to polymerise. These are then cross-linked by Factor XIIIa forming covalently linked polymers.

Fibrin polymers stabilise the growing thrombus through crosslinking of surface receptors, including integrin αIIbβ3, and by clot retraction, which links the integrin to the platelet cytoskeleton. The interaction of fibrin and fibrinogen with αIIbβ3 is mediated through distinct epitopes. Fibrinogen binds to αIIbβ3 via the carboxy-terminal peptide sequence of the γC-peptide (GAKQAGDV). In contrast, fibrin binds to the integrin through a unique sequence in the γC-peptide, ATWKTRWYSMKK, which binds to the αIIb β-propeller (Podolnikova et al., 2014, Podolnikova et al., 2005). The distinct roles of these epitopes are illustrated by the differential effects of mutation of these sequences

on adhesion and clot retraction (Rooney et al., 1998, Cohen et al., 1989). In the present study, we show that fibrin but not fibrinogen activates GPVI thereby indicating the presence of additional functional differences between the closely related matrix proteins. Further work is required to establish the structural basis of this. In our initial experiments, we asked whether fibrin stimulates tyrosine phosphorylation in human and mouse platelets through integrin αIIbβ3. Under aggregating conditions, fibrin polymerisation is associated with a relatively minor increase in tyrosine phosphorylation, most likely because this is masked by fibrinogen-dependent αIIbβ3-outside-in signalling. Consistent with this, tyrosine phosphorylation is dramatically reduced by the αIIbβ3-blocker eptifibatide, which blocks binding of fibrinogen but not fibrin to the integrin. Tyrosine phosphorylation is markedly increased however in the presence of added fibrinogen in the presence of eptifibatide following stimulation by thrombin but not a PAR-4 peptide, with the FcR γ-chain and Syk being identified as major phosphorylated proteins. Studies on mouse platelets deficient in the integrin subunit, αIIb, confirmed that phosphorylation is independent of integrin αIIbβ3.

An increase in FcR γ-chain and Syk phosphorylation is indicative of GPVI activation. Consistent with this, tyrosine phosphorylation of FcR γ-chain and Syk by fibrin was abolished in GPVI-deficient platelets, which express 50% of the wild type level of FcR γ-chain and normal levels of the other major platelet glycoprotein receptors. The increase in phosphorylation was blocked by GPRP demonstrating that it is mediated by fibrin polymers and not by monomeric fibrin. However, both immobilised monomeric and polymerised fibrin induce spreading of platelets demonstrating that polymerisation is not essential for binding and activation of the glycoprotein receptor. Further, activation

of GPVI by immobilised fibrin leads to PS exposure, which is also abolished in GPVI-deficient platelets. This suggests that on a monolayer, the avidity of the fibrin surface is able to support GPVI activation in the absence of cross-linking of fibrin. In solution, fibrin requires polymerisation to induce clustering and activation of GPVI. Confirmation of binding of GPVI to both monomeric and polymerised fibrin was shown using a modified ELISA.

This study therefore adds to the growing number of ligands of the collagen receptor on platelets, which includes the endogenous proteins, laminin, adiponectin, and EMMPRIN, as well as a range of miscellaneous exogenous ligands including small charged peptides, sulphated sugars, antisense nucleotides and diesel particles (Flierl et al., 2015, Alshehri et al., 2015).

This work is of significance with regard to the delay in occlusion and increased embolisation that is seen following $FeCl_3$ injury in GPVI-deficient mouse platelets, which we speculate is due to loss of platelet activation by fibrin and a corresponding reduction in aggregation and PS exposure and therefore reduced thrombin generation. Following submission of this manuscript, Mammadova-Bach et al demonstrated that fibrin supports thrombin generation in human platelets through activation of GPVI (Mammadova-Bach et al., 2015). We therefore propose that activation of GPVI by fibrin plays a key feedback role in promoting coagulation and thrombin formation during haemostasis and thrombosis.

These findings add to the potential significance of GPVI as an anti-thrombotic target (Zahid et al., 2012, Dutting et al., 2012) by extending its role to the propagation of an occlusive thrombus. This unexpected role, coupled with its relatively minor role in

haemostasis as shown by the mild bleeding seen in GPVI-deficient human and mouse (Kato et al., 2003, Hermans et al., 2009, Matus et al., 2013) platelets, indicates that targeting the binding of fibrin to GPVI may prevent vessel occlusion at sites of arterial thrombosis, without causing a major bleeding diathesis.

4.6 References

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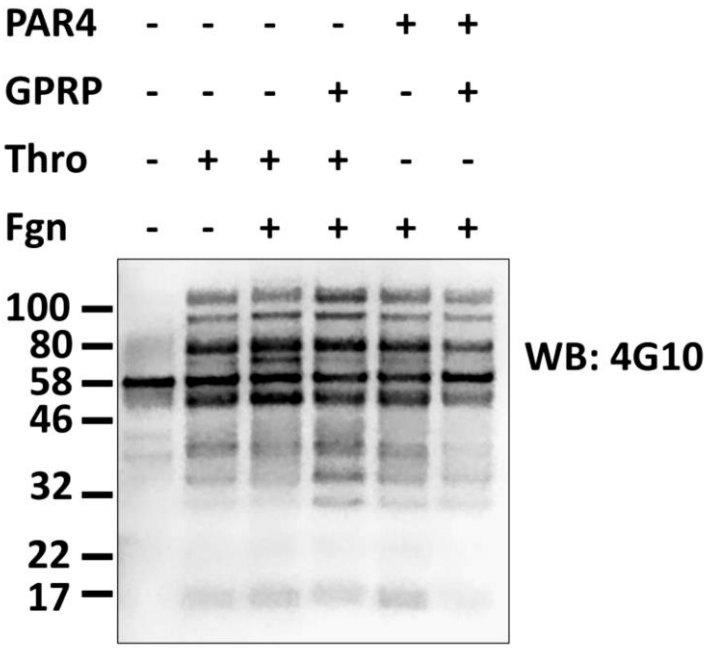
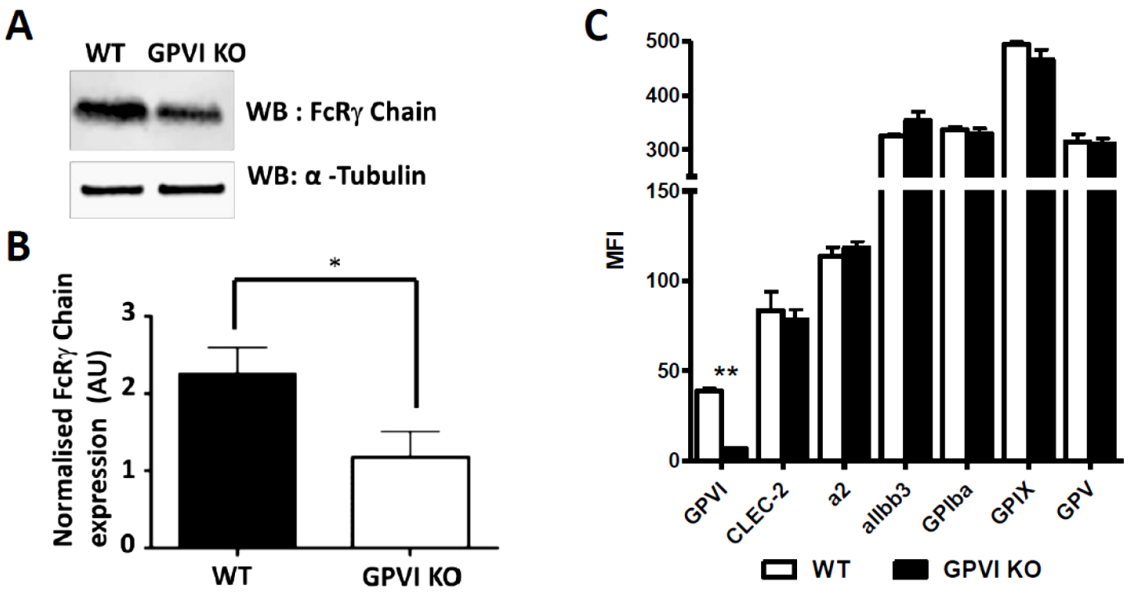
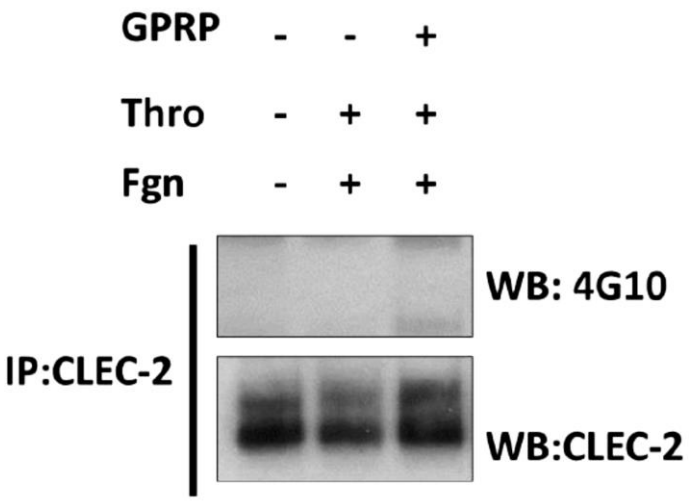
4.7 Supplementary



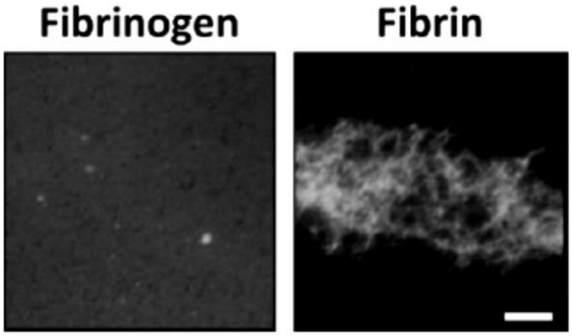
Supplementary figure 4-1 Tyrosine phosphorylation in human platelets under aggregation conditions. Human platelets (5×10^8/ml) were stimulated with thrombin (1 U/ml) or PAR-4 peptide (150 μM) under aggregating conditions and, where indicated, in the presence of fibrinogen (200 μg/ml) and GPRP (5 μM). Aggregations were stopped after 1min with the addition of 2× lysis buffer. A sample of the whole cell lysate (WCL) was removed and the remaining lysate used to immunoprecipitate (IP) Syk. WCLs and IPs were separated by SDS-PAGE and Western blotted for pTyr and Syk (phospho-FcR γ–chain co-precipitates with Syk. The results are representative of three experiments.



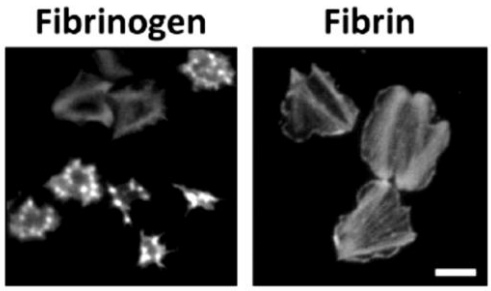
Supplementary figure 4-2 GPVI KO platelets express lower levels of FcRγ -chain, but normal levels of surface glycoproteins. Platelet lysates were prepared from WT and GPVI-deficient platelets (5×10^8/ml), and proteins separated by SDS-PAGE and western blotted for FcRγ –chain (A) and quantified (B). The results are shown as mean ± SEM of three experiments. (C) Platelets from WT and GPVI-deficient platelets were analysed by flow cytometry for the stated proteins. The results are shown as mean ± SD of four experiments. *P<0.05, ** P<0.01.



Supplementary figure 4-3 Fibrin does not stimulate tyrosine phosphorylation of CLEC-2. Human platelets (5×10^8/ml) were stimulated with thrombin (1 U/ml) in the presence of eptifibatide (9 μM), fibrinogen (200 μg/ml), and where shown, GPRP (5 μM). Aggregations were stopped after 1 min with the addition of 2× lysis buffer and the lysate used to immunoprecipitate (IP) CLEC-2. IPs were separated by SDS-PAGE and western blotted for tyrosine phosphorylation and CLEC-2. The results are representative of three experiments.



Supplementary figure 4-4 Thrombin treatment converts fibrinogen into polymerised fibrin networks. Fibrinogen was coated onto glass coverslips, and converted into fibrin by treatment with thrombin. Alexa-488 fibrinogen was used to visualise fibrin formation. Scale bar = 5μm.



Supplementary figure 4-5 Fibrin stimulates spreading in human platelets. Fibrinogen was coated onto glass coverslips, and converted into fibrin by treatment with thrombin. Hirudin was used to neutralise any residual thrombin before washing and blocking. Human platelets (2×10^7/ml) were allowed to spread on fibrinogen or fibrin-coated coverslips, followed by actin staining with Alexa-488 phalloidin. Scale bar = 5μm.

Chapter 5 CLEC-2 EXPRESSION IS MAINTAINED ON ACTIVATED PLATELETS AND ON PLATELET MICROPARTICLES

This research was originally published in *Blood*. Eelo Gitz, Alice Y. Pollitt, Jerney J. Gitz-Francois, **Osama Alshehri**, Jun Mori, Samantha Montague, Gerard B. Nash, Michael R. Douglas, Elizabeth E. Gardiner, Robert K. Andrews, Christopher D. Buckley, Paul Harrison and Steve P. Watson. CLEC-2 expression is maintained on activated platelets and on platelet microparticles. *Blood*. 2014; 124:2262-70.

Word Count: 4,109

This chapter reports that CLEC-2 activation induces proteolytic cleavage of GPVI and FcγRIIa but not of itself and that CLEC-2 but not GPVI is detected on platelet derived microparticles. I contributed in this paper by characterisation of new mouse monoclonal antibodies against human CLEC-2 namely AYP1 and AYP2 (figure 5-1 A). I have designed and performed experiments, and analysed the data that presented in Figure 5-1A. The manuscript was written by one of other authors. I read the manuscript prior to submission and made comments.

Abstract

The C-type lectin-like receptor CLEC-2 mediates platelet activation through a hem-immunoreceptor tyrosine-based activation motif (hemITAM). CLEC-2 initiates a Src- and Syk-dependent signaling cascade that is closely related to that of the 2 platelet ITAM receptors: glycoprotein (GP) VI and FcγRIIa. Activation of either of the ITAM receptors induces shedding of GPVI and proteolysis of the ITAM domain in FcγRIIa. In the present study, we generated monoclonal antibodies against human CLEC-2 and used these to measure CLEC-2 expression on resting and stimulated platelets and on other hematopoietic cells. We show that CLEC-2 is restricted to platelets with an average copy number of ~2000per cell and that activation of CLEC-2 induces proteolytic cleavage of GPVI and FcγRIIa but no to fit self. We further show that CLEC-2 and GPVI are expressed on $CD41^{+}$ microparticles in megakaryocyte cultures and in platelet-rich plasma, which are predominantly derived from megakaryocytes in healthy donors, whereas microparticles derived from activated platelets only express CLEC-2. Patients with rheumatoid arthritis, an inflammatory disease associated with increased microparticle production, had raised plasma levels of microparticles that expressed CLEC-2 but not GPVI. Thus, CLEC-2, unlike platelet ITAM receptors, is not regulated by proteolysis and can be used to monitor platelet-derived microparticles

5.1 Introduction

Platelets play an essential role in limiting blood loss from the damaged vasculature, but can also block blood flow in diseased vessels through formation of an occluding thrombus.(Jackson, 2011a). Platelet adhesion and activation are ordinarily triggered by exposure to subendothelial extracellular matrix proteins. Initial adhesion to the damaged vessel wall is regulated through the interaction of the glycoprotein (GP)Ib-V-IX complex with von Willebrand factor immobilized on exposed collagen fibers. Platelet activation and recruitment to a growing thrombus is regulated by the collagen receptor GPVI and the platelet-secreted agonists adenosine diphosphate and thromboxane (TX)A_2 (Broos et al., 2011). Thrombin, generated through the coagulation cascade, further activates platelets and strengthens clot formation by converting fibrinogen into fibrin. Adenosine diphosphate, TxA_2, and thrombin signal via heterotrimeric G protein-coupled receptors. The collagen receptor GPVI signals through an immunoreceptor tyrosine-based activation motif (ITAM) via its associated subunit, the FcR γ-chain. The signals from G protein-coupled receptors and GPVI synergize to mediate activation of integrin αIIbβ3-dependent platelet aggregation (Broos et al., 2011).

Human platelets express 2 other receptors that signal through a closely related pathway to that of GPVI: the low affinity immunoglobulin ITAM receptor FcγRIIa and the podoplanin hemITAM receptor C-type lectin-like receptor 2 (CLEC-2). FcγRIIa signals through a single ITAM in its cytosolic domain and is a critical mediator of platelet activation in immune thrombocytopenia (McKenzie et al., 1999, Stolla et al., 2011, Urbanus et al., 2013), heparin-induced thrombocytopenia (Warkentin et al., 2011), bacterial infection

(Tilley et al., 2013, Arman et al., 2014), and cancer (Mitrugno et al., 2014). CLEC-2, a type II transmembrane protein, signals via a single YxxL sequence known as a hemITAM and is the receptor for the type I transmembrane GP podoplanin, which is widely expressed outside of the vasculature, including lymphatic endothelial cells, type 1 lung alveolar cells, lymph node stromal cells, and the choroid plexus epithelium. Podoplanin is also present on inflammatory macrophages (Hou et al., 2010, Kerrigan et al., 2012) on a subset of activated T-helper (Th)17 cells (Peters et al., 2011, Miyamoto et al., 2013).The function of CLEC-2 in hemostasis is, however, unclear, with reports indicating that it either plays a minor role (May et al., 2009, Suzuki-Inoue, 2011) or that it plays no role (Hughes et al., 2010a). More recently, CLEC-2 has been shown to play a vital collaborative role with GPVI in thrombosis (Bender et al., 2013).

There is increasing recognition that platelet (hem)ITAM receptors play a pivotal role in processes beyond hemostasis. Platelet-specific deletion of CLEC-2, or deletion of one of its downstream signaling proteins, Syk, SLP-76, or PLCγ2, leads to a number of developmental problems including blood-lymphatic mixing in midgestation (Suzuki-Inoue, 2011, Bertozzi et al., 2010, Finney et al., 2012).

GPVI and CLEC-2 are also required for the maintenance of vascular integrity at sites of inflammation. Mice with reduced platelet counts and deficiency in GPVI and CLEC-2 exhibit severe bleeding following inflammatory challenge, and this is independent of the major platelet receptors involved in hemostasis including integrin aIIbb3 (Boulaftali et al., 2013). In patients with rheumatoid arthritis, GPVI signaling amplifies inflammation through collagen-dependent platelet microparticle production (Boilard et al., 2010).

(hem)ITAM receptors signal through Src and Syk tyrosine kinases. Src family

kinases and/or Syk phosphorylate the conserved YxxL motifs, which allow Syk to bind to 2 phosphorylated tyrosines via its tandem SH2 domains. Activation of Syk in this way gives rise to a signaling cascade that triggers PLCγ2 and Ca21 mobilization, generation of TxA_2, integrin activation, and granule secretion (Bergmeier and Stefanini, 2013). Activation of GPVI or FcγRIIa is also associated with extracellular metalloproteinase-mediated ectodomain shedding of GPVI (Gardiner et al., 2007) and intracellular calpain mediated cleavage of FcγRIIa, resulting in deletion of the ITAM domain (Gardiner et al., 2008). Significantly, activation of either receptor results in proteolysis of the other, and in both cases, this is dependent on Src and Syk activation (Gardiner et al., 2008, Gardiner et al., 2004).

In the present study, we generated monoclonal antibodies (mAbs) to human CLEC-2 and used one of these, AYP1, to measure the level of CLEC-2 in resting and stimulated human platelets and on microparticles. We show that in contrast to GPVI and FcγRIIa, CLEC-2 is not regulated by proteolysis on activation or in response to stimulation of GPVI and FcγRIIa and that CLEC-2 but not GPVI is expressed on microparticles derived from activated platelets. The physiological significance of these findings is discussed

5.2 Materials and Methods

5.2.1 Antibodies and other materials

A recombinant extracellular fragment of human CLEC-2 (aa 68-229) was produced as described (Christou et al., 2008) used to raise mouse α-human CLEC-2 mAbs AYP1 and AYP2 (IgG1). A detailed description of other antibodies and materials used can be found in the Supplemental Methods.

5.2.2 Patients

Citrated blood samples from healthy individuals and from patients with rheumatoid arthritis were obtained with informed consent approved by the local ethics committee (ERN_11-0175 and 071Q270612, respectively). All patients with rheumatoid arthritis satisfied the 1987 American College of Rheumatology (ACR) criteria for rheumatoid arthritis(Arnett et al., 1988).

5.2.3 Cell isolation and culture, protein deglycosylation, Western blots and measuring sGPVI

A detailed description can be found in the Supplemental Methods.

5.2.4 Microparticle preparation

For analysis of microparticles, platelet-free plasma (PFP) was collected from citrated venous blood by double centrifugation at 2,500g for 15 minutes (Lacroix et al., 2013) Microparticles within PFP were used for subsequent studies. Microparticles from activated platelets were collected by incubating washed platelets with indicated agonists for one hour at 37°C, followed by double centrifugation at 2,500g for 15 minutes. Microparticles from megakaryocyte cultures (day 12) or from platelet concentrates (day 5) were collected using the same centrifugation protocol. Freshly prepared microparticles were analysed by flow cytometry.

5.2.5 Flow cytometric analysis

Platelets and microparticles were analysed by flow cytometry using an AccuriTM C6 flow cytometer (BD Biosciences). Platelets were characterized by forward scatter (FSC) and side scatter (SSC). Appropriate antibodies, as indicated in the figure legends, were added and incubated for 15 minutes at 37°C. A total of 10,000 events were analysed for each sample. Microparticles were characterized using an APC-conjugated antibody against integrin αIIb (CD41a; BD Biosciences) against an isotype- matched control. The threshold was set on fluorescence (FL4) to exclude background noise. $CD41^+$ microparticles were then analysed for surface expression of CLEC-2 and GPVI using Alexa Fluor 488-conjugated AYP1 and 1G5, respectively, and appropriate isotype-matched controls. Antibodies were centrifuged at 18,000g for 5 minutes to remove protein aggregates prior to assay. All other reagents were filtered with a 0.22 μ M filter.

Leukocytes were analysed using a FACSverse flow cytometer (BD Biosciences). Antibody incubations were on ice for 20 minutes. Peripheral blood mononuclear cells (PBMCs) and polymorphonuclear cells (PMNs) were first discriminated by their FSC and SSC characteristics. Live cells were selected based on negative staining for Sytox Red followed by selection for negative staining of CD41. An antibody against CD45 was used as a general marker for leukocytes. The following markers were used to distinguish leukocyte subsets: CD3 (T-cell), CD11c (dendritic cell), CD14 (monocyte), CD16 (neutrophil) and CD19 (B-cell). The leukocyte subsets were analysed for surface expression of CLEC-2 by Alexa Fluor 488-conjugated AYP1 relative to isotype-matched controls.

5.2.6 Statistical analysis

Data are means ± SEM, unless stated otherwise. Statistical analysis was performed

using GraphPad Prism 5 (San Diego, CA, USA) using Mann-Whitney test. P-values less than 0.05 were considered significant.

5.3 Results

5.3.1 Characterisation of mouse monoclonal antibodies against human CLEC-2

The extracellular domain of human CLEC-2 was used to generate mAbs to the C-type lectin receptor. One of these antibodies, AYP1 (IgG1), immunoprecipitated a 32 and 40 kDa doublet from lysates of control and rhodocytin-stimulated human platelets (Figure 5-1A). A second mouse α-human CLEC-2 mAb, AYP2 (IgG1), recognized the two bands by Western blot, which correspond by molecular mass to differential glycosylated forms of human CLEC-2 described previously (Suzuki-Inoue et al., 2006) (Figure 1A,B). AYP1 did not detect CLEC-2 by Western blot, indicating that the antibody binds to a conformational epitope that is lost during the denaturing conditions of SDS-PAGE (not shown). Probing for tyrosine phosphorylation demonstrated that AYP1 immunoprecipated both non phosphorylated and phosphorylated forms of CLEC-2 (Figure 5-1A). AYP1 but not AYP2 recognised surface expressed CLEC-2 when analysed by flow cytometry as shown using doxycycline-treated 293T Rex cells (Figure 5-1C) (Chaipan et al., 2006b). AYP1 also detected CLEC-2 on human platelets by flow cytometry but did not detect expression on monocytes, neutrophils, T- and B-cells or dendritic cells in whole blood (Figure 5-1D; gating strategy described in Supplemental Figure 5-1A). Analysis of leukocytes with a commercially available polyclonal antibody against CLEC-2 confirmed these findings (Supplemental Figure 5-1B). This restricted distribution of CLEC-2 agrees with the BioGPS database (Supplementary Figure 5-2) and shows that the C-type lectin receptor is selectively expressed on platelets in human blood. We used an established flow cytometry assay (Best et al., 2003) to

determine the level of expression of CLEC-2 in healthy individuals. The receptor was present at 2016 ± 239 (mean ± SD) copies per platelet (Figure 2A-D). This is within the same order of magnitude as the other platelet ITAM receptors GPVI (1250-9600) (Best et al., 2003, Chen et al., 2002, Burkhart et al., 2012) and FcγRIIa (1000-4000) (Kelton, 2005).

The full characterization of antibodies recognizing human platelets is complicated by expression of FcγRIIa, which can bind the Fc portion of antibodies. To address this, we generated Fab fragments of AYP1 and demonstrated that these antibody fragments also bound to human platelets. Furthermore, AYP1 Fab fragments were able to block platelet activation triggered by rhodocytin and Podoplanin suggesting that these ligands have binding sites on CLEC-2 that overlap with the AYP1 epitope (Figure 5-3A and 5-3B). Crosslinking of the AYP1 Fab fragments with a secondary antibody induced powerful platelet aggregation (Figure 5-3C) and enhanced fibrinogen binding and P-selectin expression (Figure 5-3D), which was blocked by inhibition of Src and Syk tyrosine kinases using PP2 and PRT 060318, respectively. This is consistent with CLEC-2-mediated platelet activation requiring receptor clustering and activation of Src and Syk tyrosine kinases.

AYP1 thus recognizes CLEC-2 in its native conformation and can be used to inhibit Podoplanin and rhodocytin-mediated platelet activation. A second mouse mAb, AYP2, can be used to recognize the denatured form of CLEC-2 following SDS-PAGE.

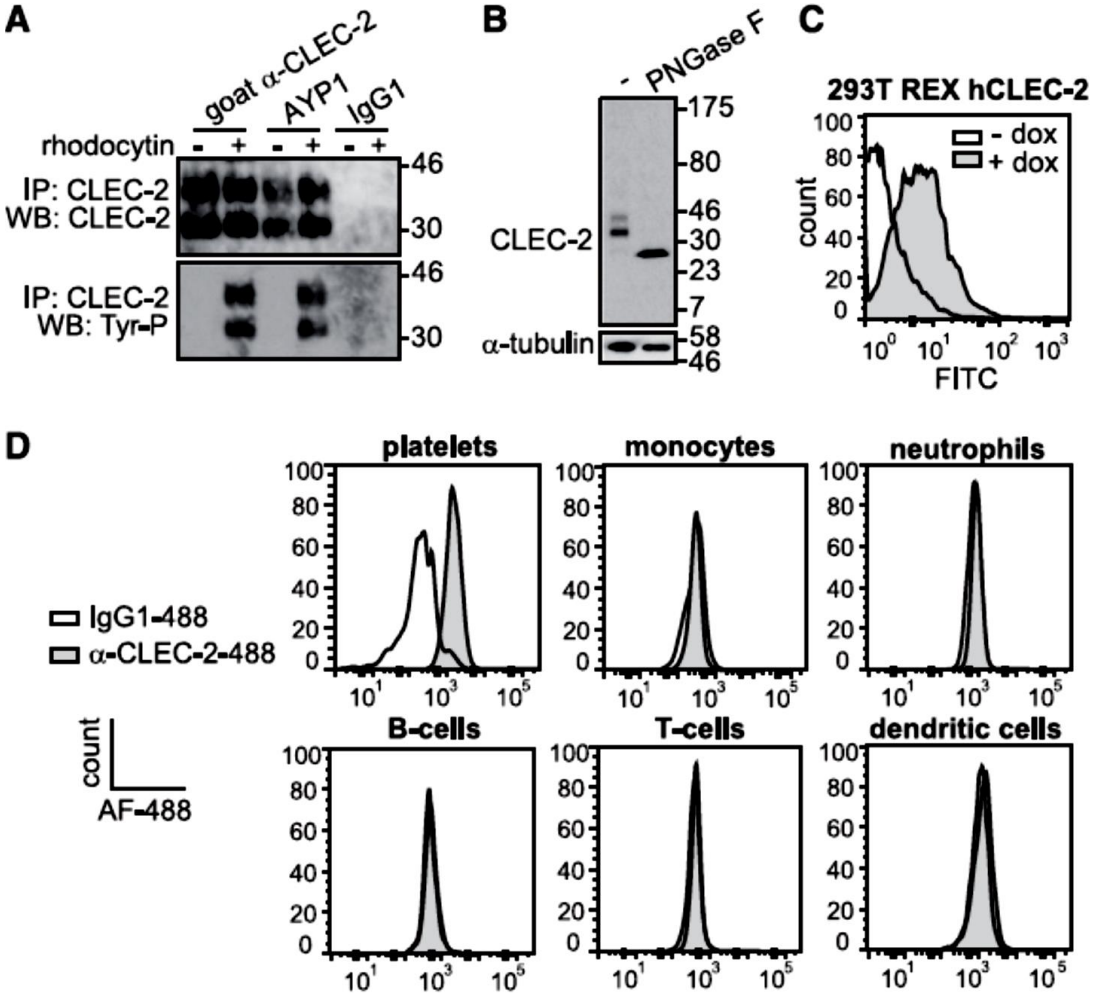


Figure 5-1 AYP1 recognizes CLEC-2 on human platelets. (A) Washed platelets were incubated for 5 minutes at 37°C under stirring conditions in the absence and presence of 100 nM rhodocytin, followed by lysis and immunoprecipitation with 2 µg/mL of goat α-human CLEC-2 (previously characterized antibody), AYP1 or IgG1 coupled to protein-G sepharose. Proteins were separated on a 12% SDS- PAGE gel under reducing conditions, transferred onto a PVDF membrane and probed with AYP2. Membranes were subsequently stripped and reprobed with α-phosphotyrosine antibody 4G10. (B) Deglycosylation reduces CLEC-2 to a single band. Lysates of resting platelets were incubated in the absence or presence of Peptide-N-Glycosidase F (PNGase F), probed with AYP2 and stripped and reprobed with an antibody against α-tubulin. (C) Analysis of AYP1 binding to 293T Rex cells with doxycycline-inducible protein expression of CLEC-2. Cells were incubated in the absence or presence of 1 µ g/mL of doxycycline for 24 hours and AYP1 binding was determined by flow cytometry using a FITC-conjugated sheep α-mouse secondary antibody. (D) Flow cytometric analysis of CLEC-2 on platelets and leukocytes. Platelets and leukocytes were isolated and incubated with saturating concentrations of either Alexa Fluor-488 (AF-488) conjugated α-CLEC-2 antibody AYP1 or isotype-matched control for 30 minutes at room temperature (platelets) or on ice (leukocytes) and analysed immediately. Leukocyte subset discrimination is described in Supplementary Methods and gating strategy is shown in Supplemental Figure 5-1A. Data are representative of at least 3 independent experiments.

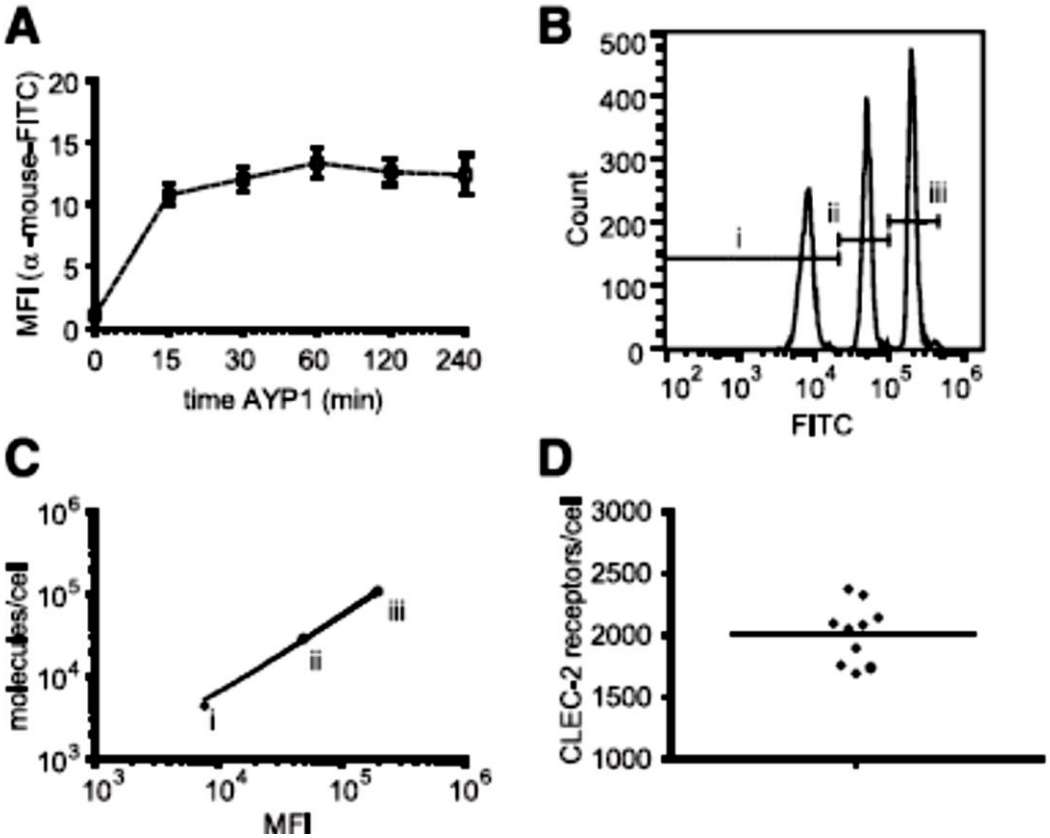


Figure 5-2 Quantification of platelet CLEC-2 expression. The number of surface copies of CLEC-2 per platelet was determined using the mouse α-human CLEC-2 antibody AYP1 and the Platelet Calibrator Kit from Biocytex. (A) Washed platelets were pre-incubated with 2.5 µg/mL AYP1 (saturating concentration) at room temperature for indicated times and subsequently incubated with a FITC-conjugated α-mouse antibody for another 15 minutes. Flow cytometric analysis shows that binding of AYP1 remains stable over the tested time period. (B) Calibrator beads, coated with batch- defined increasing concentrations of mouse IgG1 antibody molecules (i: 4400, ii: 29000 and iii: 10800), were stained with FITC-conjugated α mouse antibody and analysed by flow cytometry. (C) The geometric mean fluorescence intensity (MFI) of the three bead populations were plotted against the corresponding number of mouse IgG1 molecules. Linear regression revealed an R^2 of 0.996. (D) Washed platelets were pre-incubated with AYP1 for 30 minutes at room temperature and incubated FITC-conjugated α-mouse antibody. The MFI was determined by flow cytometry and used to quantify surface expression of CLEC-2 by extrapolation from the linear regression line of (C). CLEC- 2 copy number was determined for ten donors of diverse ethnic backgrounds (aged 23 to 55 years) with a mean of 2016 ± 239 (mean ± SD).

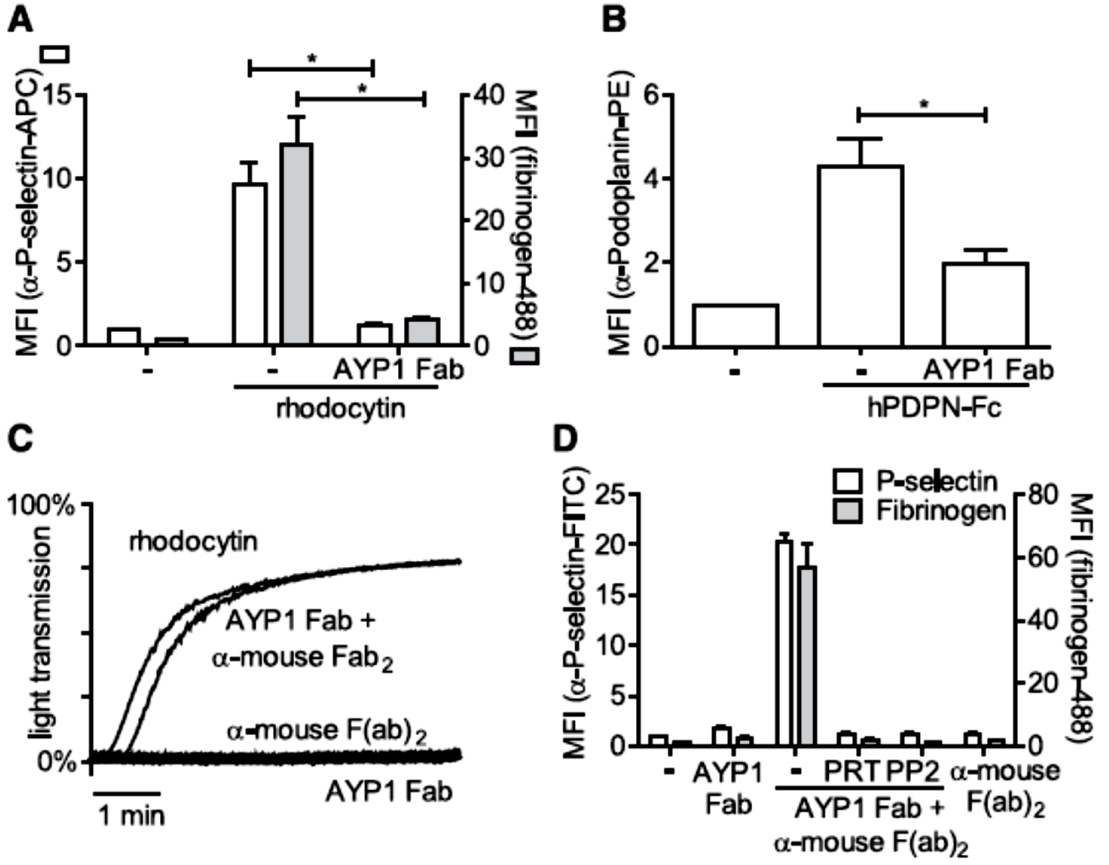


Figure 5-3 The effect of AYP1 on CLEC-2 signalling. (A,B) Washed platelets were pre-incubated with 2.5 µg/mL AYP1 Fab fragments for 15 minutes at room temperature and stimulated with (A) 100 nM rhodocytin or (B) 10 µg/mL hPDPN-Fc for 15 minutes at 37°C. (A) Platelet activation by rhodocytin was determined by flow cytometric analysis of P-selectin expression and fibrinogen binding and (B) Podoplanin binding using a PE-conjugated α-human Podoplanin antibody. (C) Aggregation of washed platelets induced by 100 nM rhodocytin or AYP1 Fab (2.5 µg/mL) crosslinked with 20 µg/mL α-mouse Fab-specific $F(ab)_2$ fragments. Single incubation with either AYP1 Fab or α-mouse $F(ab)_2$ fragments did not trigger aggregation. The traces are representative of three independent experiments. (D) Flow cytometric analysis of P-selectin expression and fibrinogen binding induced by crosslinking AYP1 Fab. Washed platelets were incubated with AYP1 Fab, α-mouse $F(ab)_2$, or both for 15 minutes at 37°C and immediately analysed. Pre-incubation with either the Syk inhibitor PRT-060318 (PRT; 5 µM) or the Src family kinase inhibitor PP2 (20 µM) for 10 minutes at room temperature prevented platelet activation induced by crosslinking AYP1 Fab. Data are presented as the ratio of MFI of treated over control platelets (n=4).

5.3.2 CLEC-2 is not regulated by shedding or internalisation upon platelet activation

The newly generated antibodies were used to investigate whether CLEC-2 is regulated by proteolytic cleavage as is the case for GPVI and FcγRIIa. Washed platelets were incubated under a variety of conditions that cause down-regulation of GPVI and FcγRIIa, and in addition, were subject to CLEC-2 stimulation by rhodocytin or crosslinked AYP1. Incubation with N-ethylmaleimide (NEM), a reagent which reacts with free sulfhydryl groups, or the calmodulin antagonist W7, which both induce metalloproteinase-mediated shedding of GPVI (Gardiner et al., 2004, Arthur et al., 2005), had no effect on surface expression of CLEC-2 as measured by flow cytometry or cleavage of CLEC-2 as measured by western blot (Figure 5-4A and 5-4B).Binding of GPVI by CRP, or FcγRIIa by crosslinked IV.3 Fab fragments, or CLEC-2 by crosslinked AYP1 Fab, also failed to alter surface expression or induce cleavage of CLEC-2. A reduced level of CLEC-2 expression was observed by flow cytometry but not by western blotting when platelets were stimulated by rhodocytin, which is likely to reflect competition between rhodocytin and AYP1 for binding to the C-type lectin-like receptor. To investigate this further, we monitored the time course of rhodocytin binding to platelets by flow cytometry using a previously described polyclonal Ab.30 Figure 5-4C shows that binding of rhodocytin is stable for up to 120 minutes. Similarly, crosslinked AYP1 Fab remained stably bound to CLEC-2 over the tested incubation time. In line with these findings, incubation with NEM, W7 or a rat α-mouse CLEC-2 mAb also failed to induce proteolytic cleavage of murine CLEC-2 (Supplemental Figure 5-3). Together, these findings indicate that CLEC-2 is not shed or internalized from the platelet surface upon auto-activation or in response to activation of GPVI and FcγRIIa, or agents which induce cleavage of the two ITAM receptors.

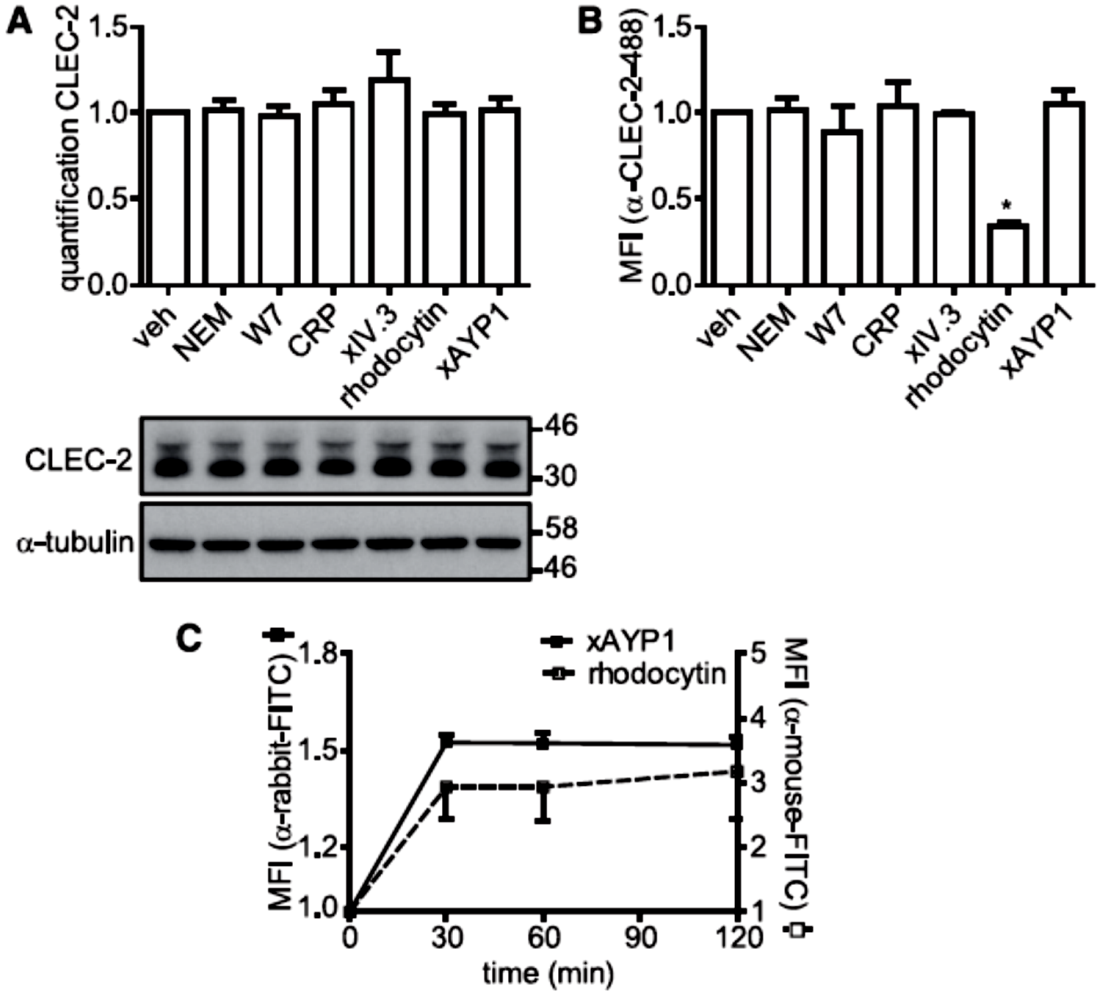


Figure 5-4 CLEC-2 is not shed or internalized following activation. (A, B) Washed platelets were incubated with vehicle control (veh; DMSO), NEM (2 mM), W7 (150 µ M), CRP (10 µg/mL), IV.3 Fab (5 µg/mL) crosslinked with 20 µg/mL α-mouse Fab-specific F(ab)2 fragments (xIV.3), rhodocytin (100 nM) or AYP1 Fab (2.5 µg/mL) crosslinked with 20 µg/mL α-mouse Fab-specific F(ab)2 fragments (xAYP1) for 1 hour at 37°C. (A) Platelet lysates were blotted and probed with AYP2. Membranes were subsequently stripped and reprobed with an antibody against α-tubulin. Quantification is presented as the mean ratio of treated over control platelets (n=3). (B) Flow cytometric analysis of the surface expression of CLEC-2. After treatment, platelets were incubated with Alexa Fluor 488-conjugated AYP1 for 15 minutes at 37°C and immediately analysed. Alexa Fluor 488-conjugated AYP1 Fab was used to assess CLEC-2 expression after AYP1 Fab crosslinking. (C) Analysis of surface-bound rhodocytin and crosslinked AYP1 Fab. Washed platelets were incubated with rhodocytin or xAYP1 for indicated times at 37°C. Surface-bound rhodocytin was determined by incubation with rabbit α-rhodocytin antibody, followed by incubation with $F(ab)_2$ fragments of FITC-conjugated swine α-rabbit IgG. Surface-bound xAYP1 was assessed by incubation with $F(ab)_2$ fragments of polyclonal FITC-conjugated sheep α-mouse IgG. Data are presented as the ratio of MFI of treated over control platelets (n=5).

5.3.3 Activation of CLEC-2 induces proteolytic cleavage of GPVI and FcγRIIa

GPVI and FcγRIIa are regulated by metalloproteinase-mediated ectodomain shedding (Gardiner et al., 2007) and by intracellular calpain-mediated cleavage (Gardiner et al., 2008, Nazi et al., 2011), respectively. We therefore investigated whether CLEC-2 ligation also affects expression of GPVI by probing platelet lysates with an α-human GPVI cytoplasmic tail antibody which detects full-length (~62 kDa) and the proteolytically cleaved remnant (~10 kDa) of platelet-associated GPVI and of FcγRIIa by probing with a western blotting antibody against the low affinity immune receptor (Figure 5). In agreement with previous reports (Gardiner et al., 2008, Gardiner et al., 2004), incubation with NEM, W7, CRP or crosslinked IV.3 Fab induced shedding of GPVI (Figure 5-5A). Similarly, we show that CLEC-2 activation by rhodocytin or by AYP1 Fab crosslinking induces proteolysis of GPVI and concomitant generation of the remnant GPVI fragment. CLEC-2-mediated shedding of GPVI was confirmed by flow cytometry (Figure 5-5B). Further, proteolysis of GPVI in response to CLEC-2 was inhibited by the broad-range metalloproteinase inhibitor, GM6001, and by selective inhibition of Src (PP2) and Syk (PRT-060318) kinases (Figure 5-5B). Pre-incubation with the membrane-permeable calpain inhibitor E64d did not prevent CLEC-2-induced shedding of GPVI (Figure 5-5B). Western blot analysis revealed that activation of CLEC-2 also resulted in proteolytic cleavage of FcγRIIa, which was inhibited by E64d, PRT-060318 and PP2, but not by GM6001 (Figure 5-5C). No loss of FcγRIIa was detected by flow cytometry (Figure 5-5D), confirming earlier findings that the extracellular domain remains platelet-associated after proteolysis (Gardiner et al., 2008). These results collectively demonstrate that CLEC-2 signalling induces metalloproteinase-dependent shedding of GPVI and calpain-dependent proteolytic cleavage of FcγRIIa and that this proteolysis requires activation of Src and Syk tyrosine kinases.

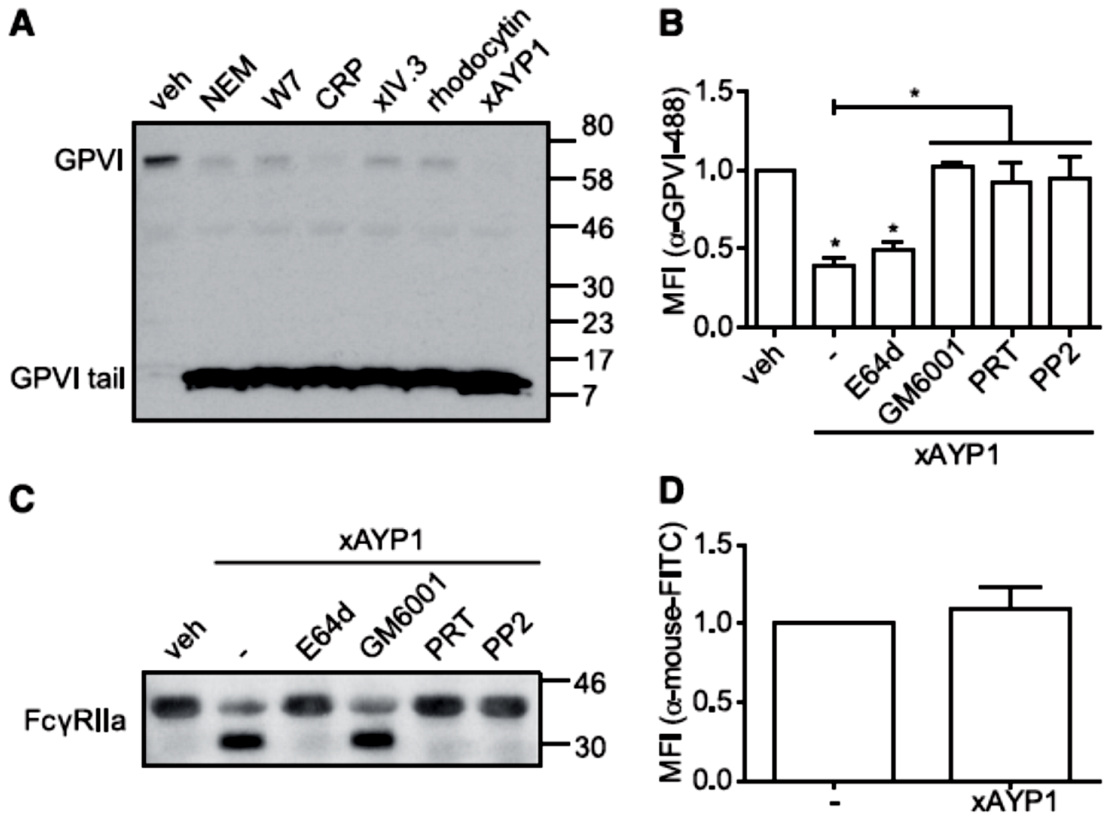


Figure 5-5 Activation of CLEC-2 leads to proteolytic cleavage of GPVI and FcγRIIa. (A) Washed platelets were incubated with vehicle control (veh; dimethylsulfoxide), NEM (2 mM),W7 (150 mM), CRP (10 mg/mL), IV.3 Fab (5 mg/mL) cross-linked with 20 mg/mL a-mouse Fab-specific $F(ab)_2$ fragments (xIV.3), rhodocytin (100 nM), or AYP1 Fab (2.5 mg/mL) cross-linked with 20 mg/mL a-mouse Fab-specific F(ab)2 fragments (xAYP1) for 1 hour at 37°C. Platelet lysates were blotted and probed with an antibody against the cytoplasmic tail of GPVI, which detects both full-length and the cytoplasmic tail remnant of GPVI. Blot is representative for at least three independent experiments. (B) Flow cytometric analysis of the surface expression of GPVI. Washed platelets were preincubated with the calpain inhibitor E64d (100 mM), the metalloproteinase inhibitor GM6001 (100 mM), the Syk inhibitor PRT-060318 (PRT; 5 mM), or the Src family kinase inhibitor PP2 (20 mM) for 10 minutes at room temperature, followed by incubation with AYP1 Fab (2.5 mg/mL) cross-linked with 20 mg/mL a-mouse Fab-specific $F(ab)_2$ fragments (xAYP1) for 15 minutes at 37°C. After treatment, platelets were incubated with Alexa Fluor 488-conjugated a-GPVI antibody 1G5 for 15 minutes at 37°C and immediately analysed. (C) Washed platelets incubated under the conditions of B were blotted and probed with mouse a-human FcγRIIa-biotin. The blot is representative for at least three independent experiments (D) Flow cytometric analysis of FcγRIIa surface expression on platelets after AYP1 Fab cross-linking. Data are presented as the ratio of MFI of treated over control platelets (n =3).

5.3.4 Microparticles from activated platelets retain CLEC-2 but lose GPVI expression

Megakaryocytes and activated platelets shed small submicron fragments into the circulation, accounting for between 70% and 90% of microparticles in the blood stream (Horstman and Ahn, 1999, Joop et al., 2001, Berckmans et al., 2001) as shown by positive staining for the megakaryocyte/platelet integrin subunit αIIb (CD41). In healthy individuals, the majority of circulating $CD41^+$ microparticles originates from megakaryocytes rather than from activated platelets (Flaumenhaft et al., 2009, Rank et al., 2010).We analyzed $CD41^+$ microparticles isolated from plasma, primary human megakaryocytes, and activated platelets for surface expression of CLEC-2 and GPVI. $CD41^+$ microparticles isolated from plasma of healthy volunteers expressed CLEC-2 and GPVI (Figure 5-6A). These microparticles stained negative for CD45, indicating that they were not fused with microparticles of leukocyte origin (supplemental Figure 5A). A similar level of CLEC-2 and GPVI expression was found on $CD41^+$ microparticles isolated from megakaryocytes cultured for 12 days (Figure 5-6B 5-6C). In contrast, microparticles derived from platelets activated with either CRP or AYP1 Fab cross-linking contained CLEC-2 but not GPVI, suggesting that treatment of microparticle formation in platelets results in proteolytic cleavage of the collagen receptor. Incubation of $CD41^+$microparticles isolated from plasma with CRP or cross-linked AYP1 Fab failed to induce loss of either receptor, indicating that microparticles are not capable of GPVI proteolysis (supplemental Figure 5-5B). Microparticles formed by incubating platelets with concentrations of CRP or AYP1 Fab that do not induce full activation, or microparticles formed during storage of platelet concentrates, also retained CLEC-2 but lost GPVI expression (supplemental Figure 5-5C - 5-5E). Together, these findings indicate that the presence of GPVI can be used to distinguish

microparticles derived from either megakaryocytes or activated platelets.

Elevated platelet-derived microparticle levels are associated with a number of cardiovascular and inflammatory diseases, including arterial thrombosis(Mallat et al., 2000, Lee et al., 1993) 4344 heparin-induced thrombocytopenia(Hughes et al., 2000) 45, immune thrombocytopenia (Jy et al., 1992), malaria infection (Couper et al., 2010), AIDS48 and rheumatoid arthritis (Boilard et al., 2010, Knijff-Dutmer et al., 2002). To verify whether the presence of GPVI on microparticles allows discrimination of megakaryocyte- from platelet-derived microparticles, we isolated microparticles from peripheral blood plasma samples from patients with rheumatoid arthritis and analyzed CLEC-2 and GPVI levels on $CD41^+$ microparticles. As expected, the total number of $CD41^+$ microparticles was increased in the patient group (Table 5-1). Levels of CLEC-2 were similar to levels on microparticles from healthy controls, but GPVI levels were markedly decreased (Figure 5-6D - 5-6E; Table 5-1). This reduction agrees with an increase in levels of plasma soluble GPVI (Table 5-1). These results suggest that measurement of GPVI levels on $CD41^+$/CLEC-2^+ microparticles in plasma can be used to determine whether patients have increased levels of microparticles derived from activated platelets.

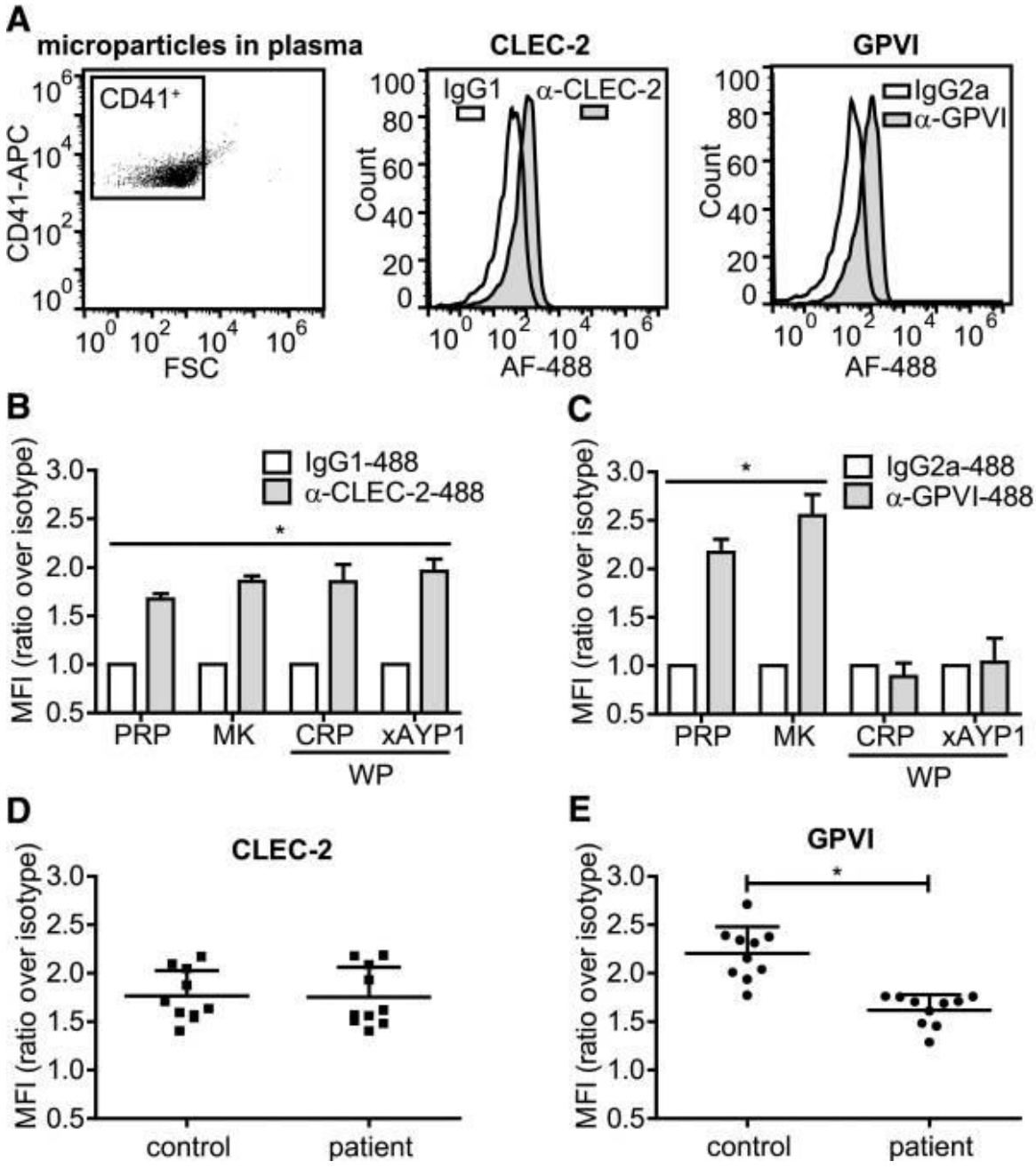


Figure 5-6 Microparticles from activated platelets maintain CLEC-2, but lose GPVI expression. (A) Microparticles were isolated from fresh PRP as described in the methods section and (left) $CD41^+$ microparticles were analyzed for (center) CLEC-2 and (right) GPVI expression using Alexa Fluor 488-conjugated α-CLEC-2 antibody AYP1 and α-GPVI antibody 1G5, respectively. True positivity was determined using isotype-matched controls. (B-C) $CD41^+$ microparticles isolated from PRP from a 12-day megakaryocyte (MK) culture or from washed platelets (WP) that were incubated for 1 hour at 37°C with CRP (10 µg/mL) or AYP1 Fab (2.5 µg/mL) cross-linked with 20 µg/mL α-mouse Fab-specific F(ab)2 fragments (xAYP1) were analyzed for (B) CLEC-2 and (C) GPVI expression. Data are presented as the ratio of MFI of CLEC-2 or GPVI over isotype controls (n = 4). (D-E) Analysis of microparticles from healthy controls and patients with rheumatoid arthritis. $CD41^+$ microparticles isolated from fresh PRP were analyzed for (D) CLEC-2 and (E) GPVI expression. Data are presented as the ratio of MFI over isotype controls (mean ± standard deviation; n = 10).

Table 5-1 $CD41^+$ microparticles and sGPVI levels in rheumatoid arthritis patients.

Parameters	**Rheumatoid arthritis (n = 10)**	**Healthy controls (n = 10)**	***P***
Platelet count ($\times 10^6$/L)			
Median	250	297	NS
Range	178-306	143-350	
$CD41^+$ microparticles ($\times 10^6$/L)			
Median	1372	164	<.01
Range	619-7325	33-295	
CLEC-2 on $CD41^+$ microparticles (ratio MFI over isotype)			
Median	1.6	1.7	NS
Range	1.4-2.2	1.4-2.2	
GPVI on $CD41^+$ microparticles (ratio MFI over isotype)			
Median	1.6	2.2	<.01
Range	1.3-1.8	1.8-2.7	
sGPVI (ng/mL)			
Median	27	8	<.01
Range	14-50	6-17	

NS, not significant

5.4 Discussion

In the present study, we generated human mAbs to CLEC-2 and demonstrated the following: (1) in peripheral blood of healthy individuals, CLEC-2 is restricted to platelets with a copy number of 2016 ± 76 per cell; (2) CLEC-2 is not regulated by proteolytic cleavage or internalization following autoactivation or in response to activation of the ITAM receptors GPVI and FcγRIIa; (3) activation of CLEC-2 leads to Src and Syk kinase-mediated proteolytic cleavage of GPVI and FcγRIIa; (4) megakaryocyte-derived microparticles express CLEC-2 and GPVI, whereas microparticles derived from activated platelets lose GPVI; and (5) the greater expression of CLEC-2 relative to GPVI on microparticles and increased soluble GPVI levels in patients with rheumatoid arthritis provides evidence of activation of platelets in the circulation during this inflammatory disorder.

CLEC-2 was first identified during a bioinformatic screen of human myeloid cells for C-type lectin-like receptors, with mRNA transcripts reported in monocytes, granulocytes, and dendritic cells (Colonna et al., 2000).A later study of the human transcriptome found high levels of CLEC-2 mRNA in bone marrow, liver, and whole blood, but not in leukocytes (supplemental Figure 2) (Su et al., 2004b). In agreement with this study, we observed expression of CLEC-2 on platelets but not on myeloid and lymphocyte populations isolated from peripheral blood. Del Rey and colleagues were also unable to detect CLEC-2 expression on cells other than platelets in synovial fluid of rheumatoid arthritis patients and failed to detect the receptor on human immature and mature dendritic cells (Del Rey et al., 2014). Expression of CLEC-2 on human cells other than platelets has thus far only been identified on the monocytic leukemia THP-1 cell line (Chang et al., 2010). On the other hand, studies in mice using a selective mAb, 17D9, have reported expression of CLEC-2 on a broad range of hematopoietic

cells, including neutrophils, natural killer cells, tissue-resident dendritic cells, and macrophages, along with enhanced expression during inflammatory insults (Kerrigan et al., 2009, Mourao-Sa et al., 2011a, Acton et al., 2012). The functional significance of this differential distribution is not known.

Of considerable interest in this study is the observation that, in contrast to the related ITAM receptors GPVI and FcγRIIa, CLEC-2 is not regulated by proteolytic cleavage on activation or in response to activation of either of the ITAM receptors. Shedding of GPVI is mediated by a disintegrin and metalloproteinase family pathway (Bender et al., 2010). In contrast, proteolysis of FcγRIIa occurs via activation of calpain. Both receptors are also cleaved after treatment with the calmodulin inhibitor W7 to dissociate calmodulin from a binding site within the cytoplasmic tails of GPVI and FcγRIIa (Gardiner et al., 2008). There is no identifiable calmodulin binding motif within the cytoplasmic tail of CLEC-2.

These observations give rise to the question as to why the levels of GPVI and FcγRIIa are controlled by proteolytic cleavage but not those of CLEC-2. Down-regulation of GPVI and FcγRIIa by proteolysis may help to limit uncontrolled platelet activation (and thereby thrombus formation) in the circulation either in response to weak constitutive signaling by either receptor (Tomlinson et al., 2007, Mori et al., 2008), or in the case of FcγRIIa, in response to contact with immune complexes. It could also be important to limit the time and extent of platelet activation, for example, in relation to the role of GPVI in the maintenance of vascular integrity, although this would also apply to CLEC-2 (Boulaftali et al., 2013). Chronic ligand engagement of GPVI could contribute to persistent platelet activation and inflammation in the vessel wall, as observed in rheumatoid arthritis, deep vein thrombosis, and atherosclerosis, and this would be countered by shedding.

One potential explanation as to why CLEC-2 is not subject to the same regulatory pathways could be related to the absence of podoplanin in the vasculature or in the immediate vicinity of damage to the vessel wall. Furthermore, functional engagement of CLEC-2 and podoplanin may require a sustained interaction. In this context, it may be important to recognize CLEC-2 as both a signaling receptor and a ligand that regulates podoplanin signaling. The role of CLEC-2 and podoplanin in prevention of blood-lymphatic mixing (Suzuki-Inoue et al., 2010, Bertozzi et al., 2010, Finney et al., 2012) and in maintenance of integrity in high endothelial venules (Herzog et al., 2013) may require sustained activation of podoplanin to permit changes such as gene regulation to occur. At the same time, it is possible that the shedding of GPVI by CLEC-2 may represent an important mechanism to limit thrombus growth during contact between the 2 vasculatures. Although the present results demonstrate that CLEC-2 is not down-regulated on human platelets *in vitro*, it is noteworthy that down-regulation of CLEC-2 has been described in mouse platelets in *vivo* on exposure to a rat a-mouse CLEC-2 mAb, INU1(May et al., 2009). The molecular basis of this down-regulation and its physiological significance are unclear. We showed that the binding of CLEC-2 induces proteolysis of GPVI and FcγRIIa. In the same context, GPVI is capable of inducing proteolysis of FcγRIIa and vice versa (Gardiner et al., 2008). The physiological implication of this is unclear, as this could reflect the shared signaling mechanisms by all 3 receptors. Shedding of GPVI by CLEC-2 during lymphatic development may represent an important mechanism to limit thrombus growth during this important physiological process. Alternatively, CLEC-2–induced proteolysis of GPVI and FcγRIIa could regulate a novel pathway of regulation of platelet function.

Platelet-derived microparticles are generated in response to a range of pathologic processes, and their determination and characterization may provide insights into the molecular mechanism of disease (Boilard et al., 2010, Mallat et al., 2000, Knijff-Dutmer et al., 2002). They may also play physiological roles in processes ascribed to platelet function. For example, microparticles could potentially mediate some of the functions ascribed to CLEC-2 on platelets in processes such as development of the lymphatics and lymph nodes (Bénézech et al., 2014). Using flow cytometry, we demonstrate that megakaryocyte-derived microparticles in plasma of healthy individuals express CLEC-2 and GPVI, whereas GPVI is absent from microparticles derived from activated platelets. It has recently been shown that megakaryocyte-derived microparticles can be distinguished from platelet-derived micro- particles by the absence of surface-expressed P-selectin and lysosome-associated membrane protein-1 and by the presence of full-length filamin A (Flaumenhaft et al., 2009). In line with our study, microvesicles derived from mouse megakaryocytes also stained positive for GPVI (Flaumenhaft et al., 2009).As P-selectin and lysosome-associated membrane protein-1 are also expressed by other cells (McEver et al., 1989, Sarafian et al., 1998) and determination of filamin A requires isolation of microparticles and western blotting, screening for $CD41^{+}$/$CLEC\text{-}2^{+}$/ $GPVI2^{-}$ microparticles by flow cytometry may provide an attractive alternative to quantitate platelet-derived microparticles in circulation. This is illustrated in the present study by analysis of plasma samples from patients with the chronic inflammatory disease rheumatoid arthritis, which is associated with increased microparticle production (Boilard et al., 2010, Knijff-Dutmer et al., 2002). Increased microparticle production in rheumatoid arthritis patients has been attributed to

collagen-mediated platelet activation around the vasculature of the joint, which is in close contact with fibroblast-like synoviocytes and extracellular matrix (Boilard et al., 2010). Importantly, in the present study, we show that the majority of the circulating micro- particles lack GPVI but retain CLEC-2 and CD41 expression in this inflammatory disorder, suggesting that they are partly derived from megakaryocytes and part from platelet activation. The reduced levels of GPVI expression on microparticles and elevated levels of soluble GPVI in patients with rheumatoid arthritis support the role of these measurements as biomarkers of disease.

In summary, we describe a novel mouse mAb against human CLEC-2, AYP1, and use this to demonstrate the restricted distribution of CLEC-2 to platelets, the resistance of the C-type lectin receptor to proteolysis, and the CLEC-2–induced cleavage of GPVI and FcγRIIa. The observation that microparticles derived from activated platelets retain CLEC-2 but lose GPVI highlights the potential use of measurement of surface expression of platelet receptors to screen for platelet activation in a wide variety of cardiovascular and inflammatory diseases.

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5.6 Supplementary

5.6.1 Supplemental methods

5.6.1.1 Materials and antibodies

We used the following products (with sources): human serum albumin fraction V (First Link Ltd., Wolverhampton, UK), calpain inhibitor E64d (L-3-carboxy-trans-2,3-epoxypropionyl-L-leucylamido-(3-methyl)butane),broad-range matrix metalloproteinase inhibitor GM6001, *N*-ethylmaleimide (NEM) and calmodulin inhibitor W7 (Calbiochem, La Jolla, CA), enhanced chemiluminescence reagent (Thermo Fisher Scientific, Waltham, MA), PLATELET calibrator kit (Biocytex, Marseille, France), Calcium ionophore (A23187), Histopaque-1119 and -1077, paraformaldehyde and Src family kinase inhibitor PP2 (Sigma-Aldrich, Dorset, UK), Lymphoprep (Axis-Shield, Oslo, Norway), MiniMACS CD34 MicroBead kit, human thrombopoietin (TPO), human stem cell factor (SCF), interleukin (IL)-3, IL-6 and Flt3 ligand (Myltenyi Biotec, Surrey, UK), prostacyclin (PGI2) (Cayman Chemical, Ann Arbor, MI), PRT-060318 (Portola Pharmaceuticals Inc., San Francisco, CA) and Sytox Red live/dead cell staining solution and StemPro-34 medium (Life Technologies, Paisley, UK). Rhodocytin was purified from *Calloselasma rhodostoma* venom as previously described (Eble et al., 2001). Crosslinked collagen-related-peptide (CRP) was purchased from Dr. Richard Farndale (Cambridge University, UK). Human IgG1 Fc-tagged recombinant human podoplanin (hPDPN-Fc) was generated as described (Schacht et al., 2005).

The following antibodies were used: goat IgG, polyclonal goat human CLEC-2, goat human FcγRIIa-biotin (R&D Systems, Minneapolis, MN), donkey goat IgG-FITC (Jackson Immunoresearch, Suffolk, UK), goat mouse IgG (Fab specific), FITC-conjugated sheep mouse IgG (whole molecule) $F(ab')_2$ fragments and mouse human tubulin (Sigma-Aldrich), APC conjugated mouse human P-selectin (Biolegend, San

Diego, CA), PE-Cy7-conjugated mouse human CD3, APC-eFluor® 780 conjugated mouse human CD14 and PE-conjugated rat human podoplanin clone NZ-1.3 (eBioscience, San Diego, CA), PE-Cy7-conjugated mouse human CD11c and CD16, PE-Cy5-conjugated mouse human CD19, PE- and APC-conjugated mouse human integrin IIb (CD41), PE-conjugated mouse IgG1 and mouse human CD45 (BD Biosciences, Oxford, UK), FITC-conjugated mouse human CD34 (Myltenyi Biotec), HRP-conjugated sheep mouse, donkey rabbit IgG and streptavidin (GE Healthcare, Little Chalfont, UK), FITC-conjugated swine rabbit F(ab)2 (Dako, Glostrup, Denmark), mouse phosphotyrosine 4G10 (Merck Millipore, Billerica, MA) and Alexa Fluor-488 conjugated goat rat IgG and fibrinogen (Life Technologies). Mouse human GPVI clone 1G5 (Gardiner et al., 2007), rabbit human GPVI cytoplasmic tail (Gardiner et al., 2007) and polyclonal rabbit rhodocytin antibody (Suzuki-Inoue et al., 2006) were generated as described in the corresponding references. Mouse human FcγRIIa monoclonal antibody IV.3 was produced and purified in-house from a hybridoma cell line purchased from ATCC (Middlesex, UK). Conjugation of antibodies to Alexa Fluor-488 (LifeTechnologies) was performed according to the manufacture's protocol with an average fluorophoreto-protein ratio of 8. Fab fragments of the mouse human CLEC-2 antibody AYP1 and FcγRIIa2 antibody IV.3 were made using a kit from Thermo Fisher Scientific. Rat mouse CLEC-2 antibody 17D9 was from AbD Serotec (Oxford, United Kingdom) and dialyzed using a Slide-A-Lyzer Dialysis Cassette (Thermo Fisher Scientific) to remove azide. Antibodies were matched with isotype controls from the same company or, in the case of in-house produced antibodies, from R&D Systems.

5.6.1.2 Platelet preparation

Venous blood from healthy drug-free volunteers was taken into sodium citrate (f.c. 0.32%). Washed platelets were obtained by centrifugation using prostacyclin (PGI_2; 1

μg/mL) to prevent activation during the isolation procedure (Suzuki-Inoue et al., 2003). Platelets were resuspended in modified-Tyrode's buffer (134 mM NaCl, 0.34 mM Na_2HPO_4, 2.9 mM KCl, 12 mM $NaHCO_3$, 20 mM N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid (HEPES), 5 mM glucose, 1 mM $MgCl_2$, 1 mM $CaCl_2$, 2.5 μM $ZnCl_2$; pH 7.3). Platelets were used at a concentration of $2x10^8$/mL or as indicated. Platelet concentrates were prepared by the buffy coat method and stored according to the UK blood transfusion services guidelines (Services, 2005)

For preparation of mouse platelets, blood was collected from terminally CO2-narcosed mice by cardiac puncture into 1:10 (vol:vol) acid citrate dextrose (120mM sodium citrate, 110mM glucose, 80mM citric acid). Washed platelets were prepared as previously described (Pearce et al., 2004a) and resuspended in modified-Tyrode's buffer (134 mM NaCl, 0.34 mM Na_2HPO_4, 2.9 mM KCl, 12 mM $NaHCO_3$, 20 mM N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid (HEPES), 5 mM glucose, 1 mM $MgCl_2$, 1mM $CaCl_2$, 2.5 μM $ZnCl_2$; pH 7.3). Platelets were used at a concentration of $2x10^8$/mL.

5.6.1.3 *In vitro* culture of human megakaryocytes

$CD34^+$ cells were isolated from human umbilical cord blood by Lymphoprep density gradient centrifugation followed by magnetic cell sorting using MiniMACS CD34 MicroBead kit according to the manufacturer's instructions. The purity of the isolated population was based on CD34 expression determined by flow cytometry and always exceeded 90%. $CD34^+$ cells were cultured for 5 days in StemPro-34 medium supplemented with 20ng/ml human thrombopoietin (TPO), 50 ng/ml human stem cell factor (SCF), 10 ng/ml interleukin (IL)-3, 10 ng/ml IL-6 and 50 ng/ml Flt3 ligand. To induce megakaryocyte development, 1 ng/mL SCF, 30 ng/ml TPO, 7.5 ng/ml IL-6 and

13.5 ng/ml IL-9 were added to the culture media on day 5 of subculture and incubated for an additional 12 days. Microparticles were isolated at the end of the culture.

5.6.1.4 Leukocyte isolation

Human peripheral blood mononuclear cells (PBMCs) and polymorphonuclear cells (PMNs) were isolated from EDTA-anticoagulated blood by gradient separation using Histopaque according to the manufacturer's protocol. Freshly prepared PBMCs and PMNs were resuspended in PBS at $1x10^6$ cells/mL and analysed by flow cytometry.

5.6.1.5 Cell culture

293T Rex cells with doxycycline-inducible expression of CLEC-2 (Chaipan et al., 2006b) were cultured in Dulbecco's modified Eagle's medium supplemented with 10% foetal calf serum and antibiotics. Protein expression of CLEC-2 was induced by treating the cells with 1µg/mL of doxycycline for 24 hours.

5.6.1.6 Protein deglycosylation

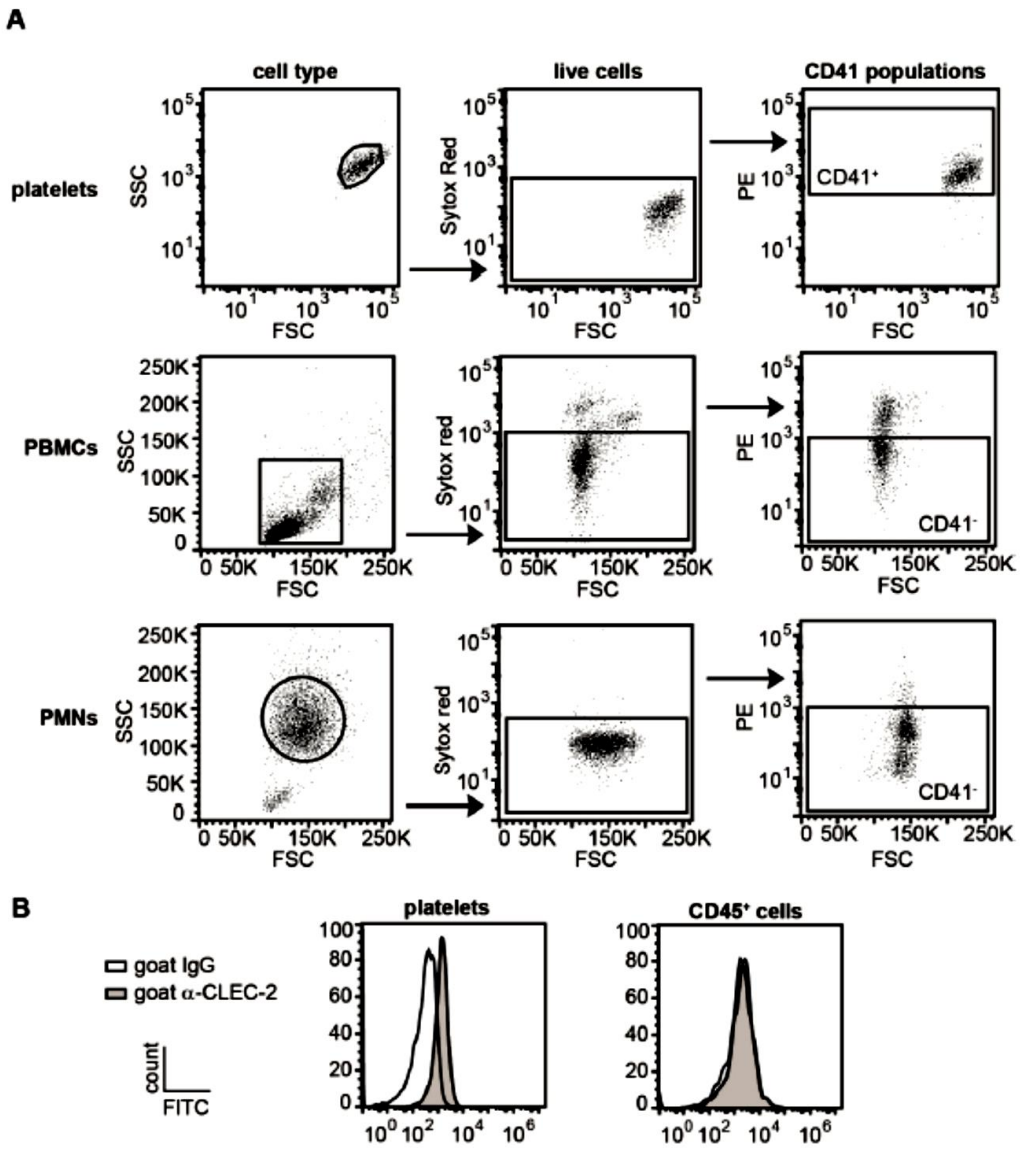
Proteins in platelet lysates were deglycosylated using Peptide-*N*-Glycosidase F (PNGase F) from New England Biolabs (Hitchin, UK) according to the manufacturer's instructions. In short, lysates were denatured for 10 minutes at 100^{o}C and incubated without or with PNGase F for 2 hours at 37^{o}C.

5.6.1.7 Measurement of sGPVI

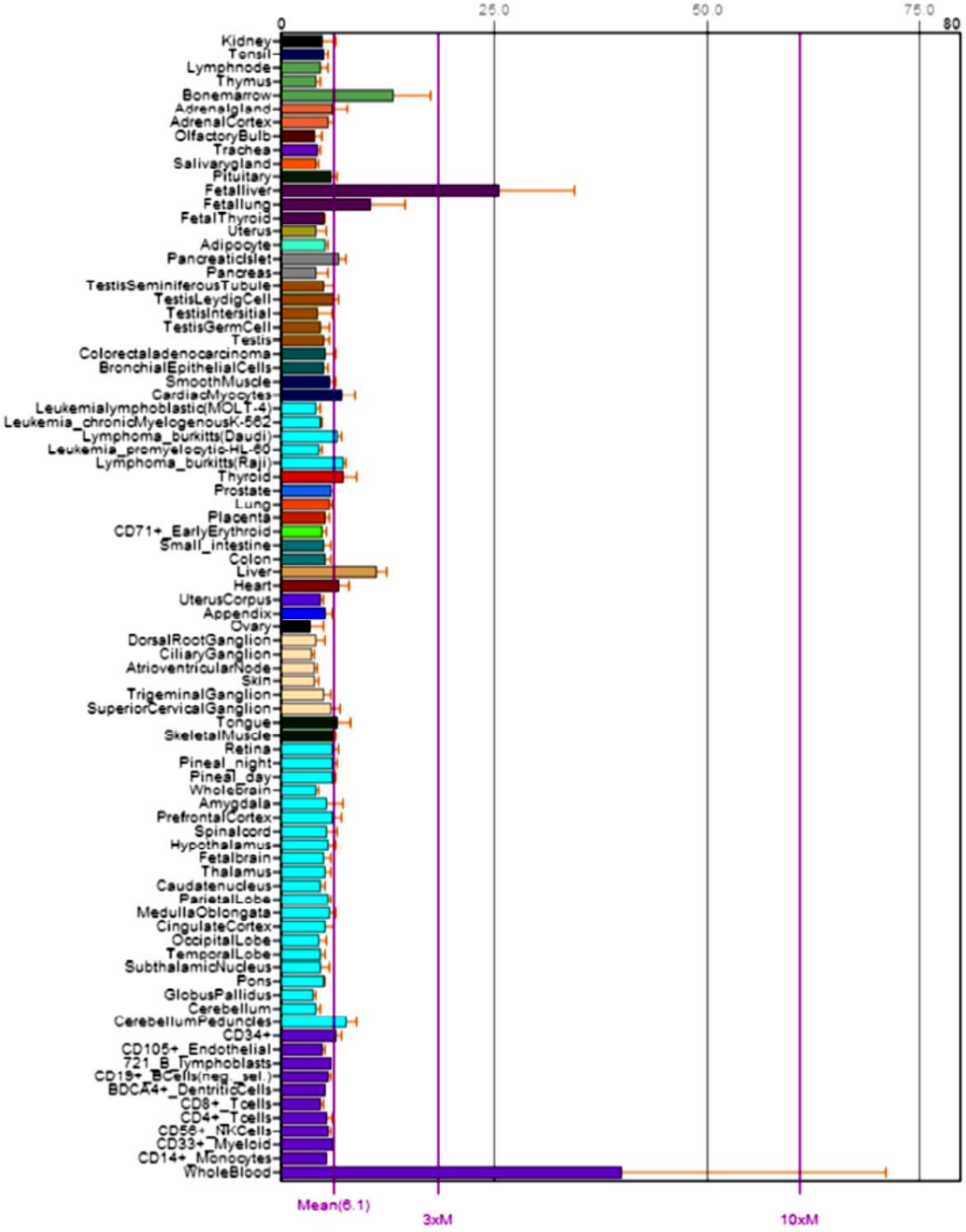
Levels of sGPVI were determined in plasma from healthy controls and patients with rheumatoid arthritis in triplicate by enzyme-linked immunosorbent assay (ELISA) as previously described (Al-Tamimi et al., 2009a)

5.6.1.8 Immunoprecipitations and western blots

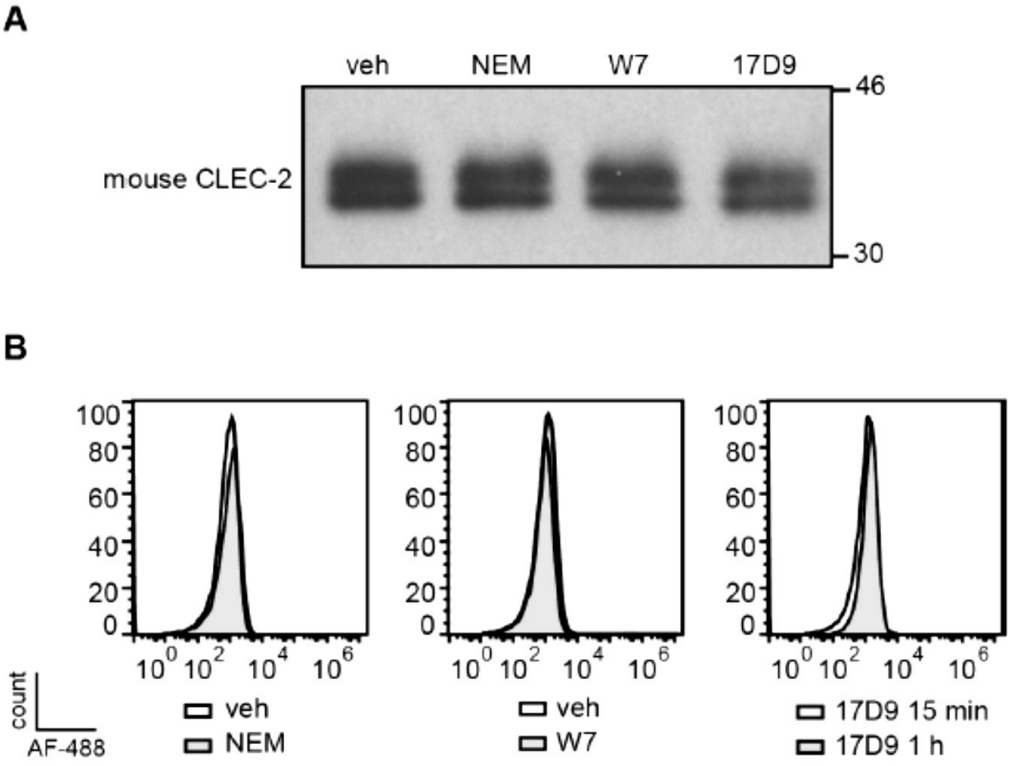
For immunoprecipitations, washed platelets ($5x10^8$/mL) were lysed with an equal volume of 2x lysis buffer (20 mM Tris/HCL (pH 7.5), 300 mM NaCl, 2 mM EGTA, 2 mM EDTA, 2% NP-40, 2 mM PMSF, 5 mM Na_3VO_4, 10 μg/mL leupeptin, 10 μg/mL aprotinin, and 1 μg/mL pepstatin). Proteins were captured by antibodies bound to protein G-Sepharose. For lysate analysis, platelet suspensions were fixed for 15 minutes at 4°C with 1% paraformaldehyde, centrifuged (15,000*g*, 2 minutes) and dissolved in 1x lysis buffer. Sample buffer (5x) was subsequently added under reducing conditions. Samples were separated by SDS-PAGE alongside a molecular weight marker and transferred onto a polyvinylidene difluoride (PVDF) membrane. After blocking, the membranes were incubated with primary antibody overnight, washed, and then incubated with horseradish peroxidase-conjugated secondary antibody for 1 hour. Protein bands were visualized by enhanced chemiluminescence. Quantification was performed with ImageJ software.



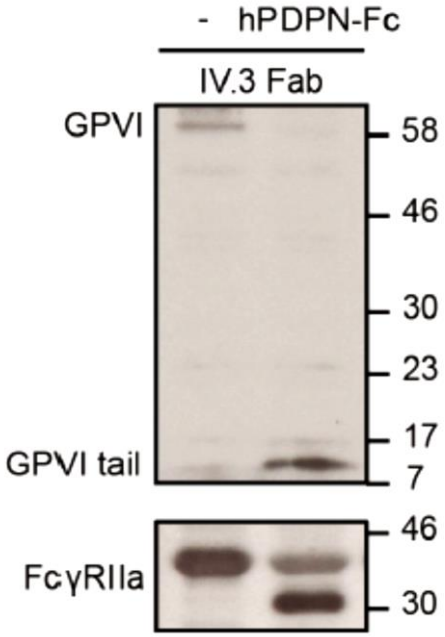
Supplemental Figure 5-1 Flow cytometric analysis of CLEC-2 expression on platelets and leukocytes. (A) Gating strategy. Freshly prepared platelets, peripheral blood mononuclear cells (PBMCs) and polymorphonuclear cells (PMNs) were identified by forward scatter (FSC) and side scatter (SSC). Sytox Red- cell populations (live cells) were gated and analysed for CD41 expression.Surface expression of CLEC-2 on platelets was performed within the $CD41^+$ gate and on PBMCs and PMNs in the CD41- gate. (B) Platelets and $CD45^+$ cells were analysed for CLEC-2 expression using another, commercially available polyclonal goat human CLEC-2 antibody (R&D Systems). Antibody binding was assessed with a secondary donkey goat IgG-FITC antibody**.**



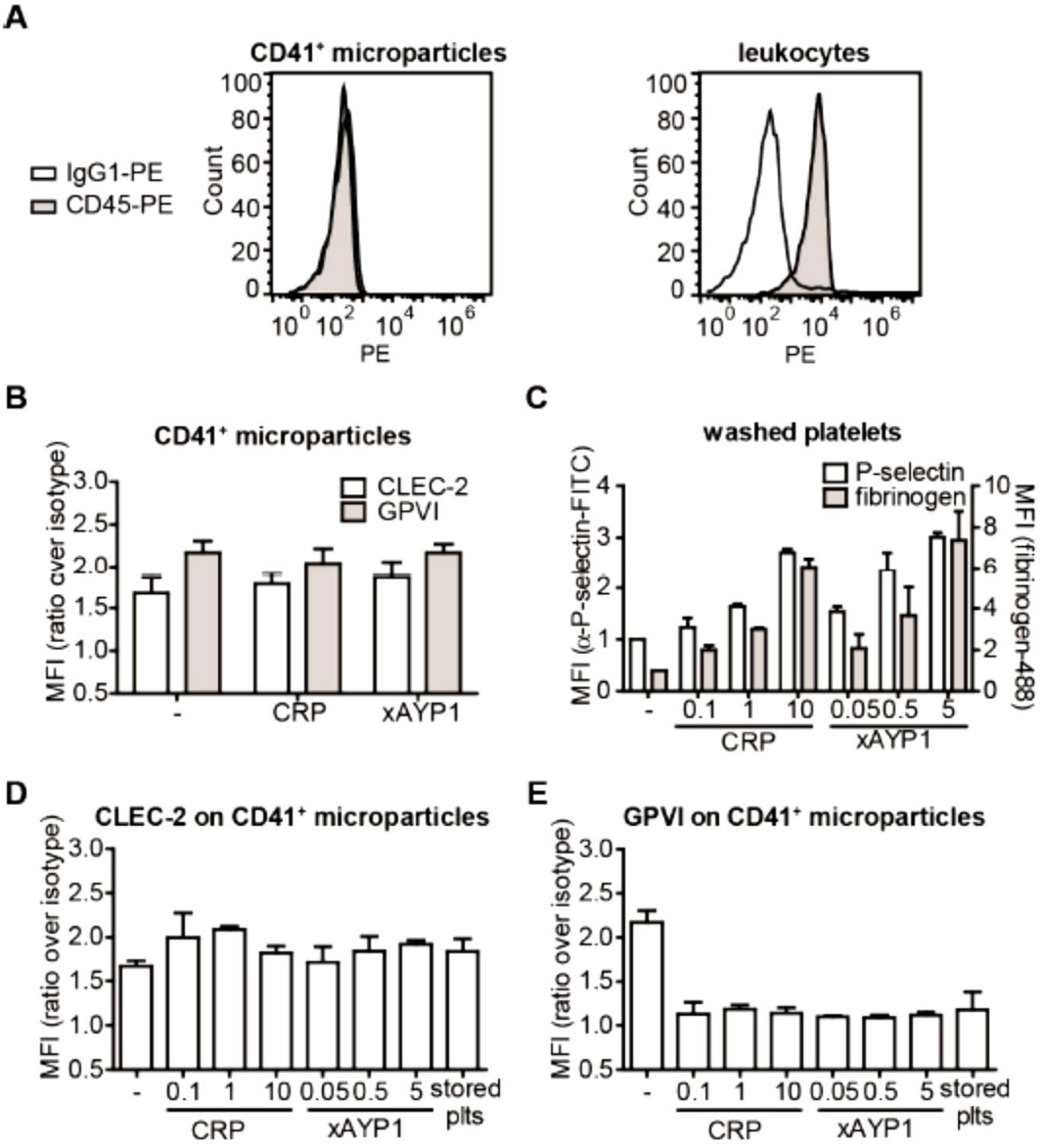
Supplemental Figure 5-2 Tissue- and cell-specific analysis of CLEC-2 mRNA expression in humans. This dataset has been generated and described elsewhere (Su et al., 2004a) and the graphic representation was created using the gene portal BioGPS.org.(Wu et al., 2009)



Supplemental Figure 5-3 Murine CLEC-2 is not susceptible to proteolytic cleavage. (A) Washedmouse platelets were incubated with vehicle control (veh; DMSO), NEM (2 mM), W7 (150 μM) or with rat mouse CLEC-2 mAb 17D9 (10 μg/mL) for 1 hour at 37ºC. Platelet lysates were blotted and probed with 17D9 for detection of CLEC-2. (B) Flow cytometric analysis of the surface expression of murine CLEC-2. Washed mouse platelets were incubated with vehicle control (veh; DMSO), NEM (2 mM) or W7 (150 μM) for 1 hour or with rat mouse CLEC-2 mAb 17D9 (10 μg/mL) for 15 minutes and 1 hour at 37ºC. DMSO, NEM and W7 treated platelets were pre-incubated with 17D9 for 15 minutes, followed by incubation with Alexa Fluor 488-conjugated goat rat IgG for 15 minutes at room temperature. 17D9 treated platelets were also incubated with Alexa Fluor 488-conjugated goat rat IgG for 15 minutes at room temperature. All samples were immediately analysed. Data are representative of three independent experiments.



Supplemental Figure 5-4 Podoplanin induces proteolytic cleavage of GPVI and FcγRIIa.Washed platelets were incubated with IV.3 Fab (5 μg/mL) to block FcγRIIa and either incubated on a petri dish coated with BSA (20 μg/mL) or hPDPN-Fc (20 μg/mL) for 1 hour at 37ºC. Non-adhered platelets on the BSA-coated petri dish were removed and lysed. Adhered platelets on the hPDPN-Fc coated petri dish were lysed and protein concentrations in both lysates were equalized before loading onto a gel. Following blotting, the membrane was probed with an antibody against the cytoplasmic tail of GPVI, which detects both full-length and the cytoplasmic tail remnant of GPVI, and with mouse human FcγRIIa-biotin. The blots are representative for three independent experiments.



Supplemental Figure 5-5 Analysis of CD41⁺ microparticles. **(**A) CD41⁺ microparticles isolated from PRP or isolated leukocytes (positive control) were analysed for CD45 expression by flow cytometry. Data are representative of three independent experiments. (B) Microparticles isolated from PRP were incubated for 1 hour at 37ºC with CRP (10 µg/mL) or AYP1 Fab (2.5 µg/mL) crosslinked with 20 µg/mL mouse Fab-specific $F(ab)_2$ fragments (xAYP1) and analysed for CLEC-2 and GPVI expression. Data are presented as the ratio of MFI of CLEC-2 or GPVI over isotype controls. (C-E) Analysis of CLEC-2 and GPVI expression on microparticles formed during suboptimal platelet activation or platelet storage. (C) Washed platelets were incubated for 1 hour at 37ºC with indicated concentrations (µg/mL) of CRP or AYP1 Fab crosslinked with 20 µg/mL mouse Fab-specific $F(ab)_2$ fragments (xAYP1) and analysed for P-selectin expression and fibrinogen binding. Data are presented as the ratio of MFI of treated over control platelets. (D, E) CD41⁺ microparticles formed during these incubations or after platelet storage for five days were analysed for (D) CLEC-2 and (E) GPVI expression. Data are presented as the ratio of MFI of CLEC-2 or GPVI over isotype controls (n=3).

5.6.2 Supplementary References

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Chapter 6 General Discussion

6.1 Summary

The major two finding of this thesis are (i) that DEP nanoparticles and several structurally unrelated charged/hydrophobic agonists, fucoidan, dextran sulphate, peptides 4N1-1 and Champs, and the synthetic lipoprotein Pam_3-CSK_4, activate platelets through the (hem)ITAM receptors GPVI and CLEC-2; and (ii) the identification of fibrin as a novel ligand for GPVI.

I have determined that platelet activation by the diverse group of ligands named above is mediated by Src and Syk tyrosine kinases, and is dependent on GPVI and/or CLEC-2 receptors. DEP, fucoidan and dextran sulphate induce activation through direct binding to GPVI and/or CLEC-2, while it remains unclear whether activation of GPVI by 4N1-1, Champs and Pam_3-CSK_4 is mediated by direct binding or by an indirect action (that presumably leads to clustering of GPVI). In addition, I have shown that fibrin binds directly to GPVI.

In the thesis, I demonstrate that miscellaneous charged/hydrophobic agents activate GPVI and CLEC-2 in platelets, including PAM3-CSK4. This observation indicates the need for caution in ascribing the mechanism of platelet activation to PAM_3-CSK_4 to TLR2, especially since the signalling pathway through which its specific receptor, TLR2, activates platelets is not clear. In light of the present observations, further studies on the mechanism of platelet activation by PAM_3-CSK_4, including the use of blocking antibodies to TLR2, are warranted.

These observations have important implications for haemostasis and life-threatening arterial thrombosis. The observation that fibrin binds to and activates GPVI advances the ‘textbook’ model of platelet activation, with fibrin mediating both clot

consolidation and further platelet activation (including PS exposure and thrombin generation). The discovery that structurally diverse ligands such as DEP activate platelets through Src and Syk tyrosine kinases raises the possibility that inhibition of either class of kinase could be used to reduce the risk of arterial thrombosis in areas of increased exposure to car exhaust fumes and other nanoparticles, albeit that there remains a need for further experimental evidence to confirm a causal link.

6.2 The mechanism of platelet activation by structurally unrelated charged/hydrophobic ligands

I have shown that GPVI and CLEC-2 can be activated *in vitro* by structurally diverse ligands. In moderate concentrations, several of the ligands are selective to one or other of the two ITAM receptors, although at higher concentrations, they activate both receptors. This raises the question as to how these diverse stimuli induce activation of multiple receptors over a similar concentration range, including whether this involves direct binding to the receptor or to associated membrane proteins.

6.2.1 Do DEP and other hydrophobic/charged ligands activate platelets by clustering of GPVI and CLEC-2 in lipid rafts?

GPVI and CLEC-2 signal in cholesterol-rich domains of the surface membrane known as lipids (Wonerow et al., 2002, Locke et al., 2002, Pollitt et al., 2010, Manne et al., 2015). These domains are rich in signaling proteins including the Src family kinases (SFKs) Fyn and Lyn, although Src itself is excluded from these domains (Watson et al., 2010). Receptor activation is mediated by translocation and clustering of the two receptors in the cholesterol-rich lipids rafts, which brings them into contact with Src and Syk family kinases. Depletion of membrane cholesterol with β-methylcyclodextrin, thus leading to membrane raft disruption, inhibits signaling by GPVI and CLEC-2

(Wonerow et al., 2002, Pollitt et al., 2010). It seems likely that this diverse group of stimuli mediate activation by inducing clustering of GPVI and CLEC-2 in lipid rafts.

DEP are made up of spherical carbon nanoparticles that contain the organic materials sulphate and nitrate. The surface of DEP carries an electrical charge which may underlie binding to GPVI and CLEC-2, with the multimeric nature of DEPs leading to crosslinking of both receptors. A similar mechanism may mediate platelet activation by other charged and hydrophobic ligands. In this way clustering of GPVI and CLEC-2 in lipid rafts could lead to receptor activation.

It is also possible that one or more of this group of ligands could bind to other membrane proteins and indirectly promote clustering of the two receptors, although this may not necessarily occur in lipid rafts. While direct evidence of this is not available, it is noteworthy that several of the ligands used in this study induce tyrosine phosphorylation at similar concentrations that mediate binding to GPVI and CLEC-2.

6.2.2 Do other receptors contribute to platelet activation?

In Chapter 2, I demonstrated that low and intermediate concentrations of fucoidan induce platelet aggregation and phosphorylation through CLEC-2 but that at higher concentrations it also activates GPVI and other tyrosine kinase-linked membrane proteins. The significance of the latter to platelet activation is not known. Fucoidan has been reported to bind to several receptors on macrophages such as Toll-like receptor-4 (TLR-4) and scavenging receptors (SRs) (Teruya et al., 2009). Platelets express both TLR4 receptor and the SRs CD36, SRBI, LOX-1 and CD68 (Valiyaveettil and Podrez, 2009), and both TLR4 and CD36 are coupled to Src kinases (Gong et al., 2008, Magwenzi et al., 2015). Several of the other ligands used in this study may also bind to

several surface receptors through charge/hydrophobic interactions, and it is possible that these contribute to platelet activation.

6.2.3 Does dextran sulphate activate human platelets through a novel signaling pathway?

Dextran sulphate has been shown to activate human platelets through SFKs independent of Syk as discussed in Chapter 3. This demonstrates that dextran sulphate signals in human platelets through a novel pathway that may be independent of GPVI and CLEC-2. In contrast, as shown in Chapter 3 using transgenic mice, activation of mouse platelets by dextran sulphate is mediated by CLEC-2 and GPVI, indicating an important species difference. PI3K-Akt offers a possible pathway through which dextran sulphate can induce platelet aggregation and secretion, as dextran sulphate has been reported to activate PI3K and Akt independently of the $P2Y_{12}$ receptor (Getz et al., 2013b), although this pathway alone is likely to be insufficient to mediate platelet activation. Thus, the mechanism underlying activation by dextran sulphate remains to be determined and also why this is induced by dextran sulphate in human but not in mouse platelets.

6.2.4 Differential features between human and mouse platelets activation by Dextran sulfate

Mouse platelets are the gold-standard animal for platelet studies, primarily because of the availability of a large number of transgenic mice strains. Nevertheless, there are a number of significant differences between mouse and human platelets including the absence of FcγRIIA and PAR-1 in mouse platelets. While these differences do not invalidate the use of mouse platelets, they indicate the need for caution and the importance of confirming observations between human and mouse platelets as far as possible. The observation of the differential dependence on Syk activation by Dextran

sulphate in this study further indicates an important difference between platelets from the two species. The explanation for this difference is not known. However, the present results also show that dextran sulfate activates CLEC-2 in both human and mouse platelets.

6.2.5 Mechanism of platelet activation and platelet agglutination by 4N1-1 and Champs peptides

It has previously been reported that the peptide 4N1-1 stimulates aggregation of washed human and mouse platelets, and that aggregation is abolished in the absence of the FcR γ-chain, suggesting that it is mediated through GPVI. A residual increase in light transmission is seen in FcR γ-chain-deficient or integrin αIIb-subunit deficient mouse platelets in response to high concentrations of 4N1-1, reflecting agglutination. Similarly, Champs peptide induces agglutination in mice deficient in the integrin subunit αIIb or the FcR γ-chain. The observation that both peptides induce platelet agglutination at similar concentrations to those at which activate the GPVI/FcR γ-chain complex suggests that multiple interactions take place with surface proteins over a similar concentration range, with agglutination possibly being mediated by neutalisation of surface platelet charge.

6.2.6 Conclusions on the mechanism of platelet activation by charged/hydrophobic ligands

There appear to be multiple interactions with surface proteins that underlie platelet activation by the series of charged/hydrophobic ligands used in this study. At the centre of these is activation of GPVI and CLEC-2, the major two tyrosine kinase signalling receptors which induce powerful activation of platelets through Src and Syk tyrosine kinases. In addition, the observation of increased protein tyrosine phosphorylation and/or agglutination in platelets from mice deficient in both receptors demonstrates that

this group of ligands bind to other surface proteins or possibly to the phospholipid themselves, and that in some cases this can lead to functional responses. The similar concentrations at which these events are seen suggest a major role for charged/hydrophobic interactions in mediating these interactions. The multimeric nature of many of these ligands is likely to lead to clustering of membrane proteins, including GPVI and CLEC-2. Whether activation occurs in membrane-specific domains such as lipid rafts or tetraspanin domains remains to be determined.

6.2.7 The clinical significance of GPVI and CLEC-2 as receptors for DEPs

There is considerable interest in the possible role of DEP in platelet activation and thrombus formation through exposure in the environment but little direct evidence that this occurs because of the challenge in designing a random controlled trial (RCT). Polycyclic aromatic hydrocarbons are one of the toxic compounds of DEPs, along with other chemical compounds, such as carbon monoxide and dioxide (Zielinska et al., 2004). In addition, DEP induce a localised inflammatory response (Siegel et al., 2004), which could also contribute to platelet activation and pathogenic thrombosis. Exposure to pollutants is linked to series health issues, notably in the frail and elderly, but the underlying mechanisms remain to be determined. DEP exposure is linked to a variety of health conditions, including cardiovascular and lung disease, and cancer (Krivoshto et al., 2008), with platelet activation foremost as the underlying mechanism.

One way to address the potential role of DEPs in mediating thrombus formation is determination of the mechanism of platelet activation, and to develop or identify pharmaceutical agents that block activation. Even then, it will be difficult to design a RCT that specifically addresses the potential for DEPs to induce thrombus formation through this pathway due to the long term nature over which the pathology occurs and

the present of other compounding factors. Nevertheless, the work in this thesis is important in that it establishes the mechanism of platelet activation by DEPs.

6.2.8 GPVI and CLEC-2 as targets for development of novel antiplatelet drugs

Patients at risk of arterial thrombosis are treated long term with antiplatelet agents including aspirin and $P2Y_{12}$ receptor antagonists such as clopidogrel. These antiplatelet agent target the two feedback agonists, thromboxane A_2 and ADP which activate platelets through GPCRs. However, both sets of drugs induce bleeding which in some cases can be life-threatening (Swieringa et al., 2014). It is therefore important to develop new forms of antiplatelet drugs that prevent thrombosis in the absence of a bleeding diathesis.

GPVI/FcRγ complex and CLEC-2, along with FcγRIIA in human platelets, are the only three receptors that activate platelets through a hem(ITAM)-driven pathway that involves Src and Syk tyrosine kinases. A small number of patients have been identified with function-disrupting mutations in GPVI in association with mild bleeding e.g. (Hermans et al., 2009). In addition, GPVI knockout mouse also exhibit a mild increase in tail bleeding times in some but not all studies(Dutting et al., 2012). In contrast, the general consensus is that neither CLEC-2 nor FcγRIIA play a role in haemostasis due to the absence of an identified ligand at the site of vascular damage and normal bleeding in mice deficient in CLEC-2 (FcγRIIA is absent in the rodent genome). Thus, CLEC-2 and FcγRIIA could represent novel targets as antiplatelet agents for thrombotic conditions in which they play a role, such as the up-regulation of podoplanin that is seen in liver in Salmonella infected mice (Hitchcock et al., 2015)

It is also possible that activation of (hem)ITAM receptors could be beneficial to patient with bleeding disorders. It has been reported that fucoidan decreases the

bleeding time in murine models of haemophilia A and B and that this leads to an increase in survival (Liu et al., 2006). It may also be possible to use fucoidan in combination with other antiplatelet drugs to reduce the side effects of the bleeding.

6.3 The binding of fibrin to the GPVI receptor

As discussed in Chapter 4, fibrin was identified as a novel ligand for GPVI. The delay in vessel occlusion and the increase in embolisation in GPVI-deficient mice, following topical $FeCl_3$ injury may be explained by the loss of this interaction. Fibrin activates platelets through the GPVI signalling pathway, as summarised in Figure 6-1. The stabilisation of the platelet aggregate by the conversion of fibrinogen to fibrin is the last stage in thrombus formation, but our results now suggest that this can lead to further platelet activation. Thus, GPVI may play an essential role in stabilisation of the aggregate through its interaction with fibrin as well as in the initiation of aggregation through the interaction with collagen.

GPVI collagen-binding domain residues have been mapped (Horii et al., 2006). It will be important to establish if fibrin binds to the same residues as it may be possible to interfere with the binding of one but not the other ligand. In this way, it may be possible to block the interaction of GPVI with fibrin or collagen, and potentially develop a novel antithrombotic drug that does not give rise to bleeding (at the moment, we do not know if the bleeding diathesis is due to loss of interaction with collagen and/or fibrin). Thus, if collagen and fibrin are shown to bind to separate sites, then the development of inhibitors that block just one of these interactions may lead to a new form of treatment of cardiovascular disease without excessive bleeding. Further, with increasing recognition of the importance of personalised medicine, and that thrombosis can have many causes, it may be that selective inhibition of the interaction with fibrin

or collagen is beneficial under different conditions such as the increase in thrombosis observed in diabetes, ischemic attack and stroke which are associated with elevated fibrinogen levels and, in some cases GPVI/FcR expression (Vinik et al., 2001, Cabeza et al., 2004, Di Napoli and Singh, 2009, Bigalke et al., 2010). In addition, anti-GPVI agents may help us investigate whether there is a link between fibrin or collagen and GPVI in patients who suffer from atherosclerosis and cardiovascular diseases.

6.4 Final thoughts

The results obtained from this thesis demonstrate the primary role of GPVI and the CLEC-2 in the activation of platelets by a miscellaneous group of ligands including fibrin. This may lead to new ways to target platelet activation in pathological diseases, such as exposure to DEP, or by selective inhibition of GPVI activation by fibrin. Further the absence of a recognised role for CLEC-2 in haemostasis, and the absence of information on the significance of the binding of fibrin to GPVI in haemostasis, indicates that it may be possible to develop inhibitors of these two receptors that target thrombosis in the absence of excessive bleeding.

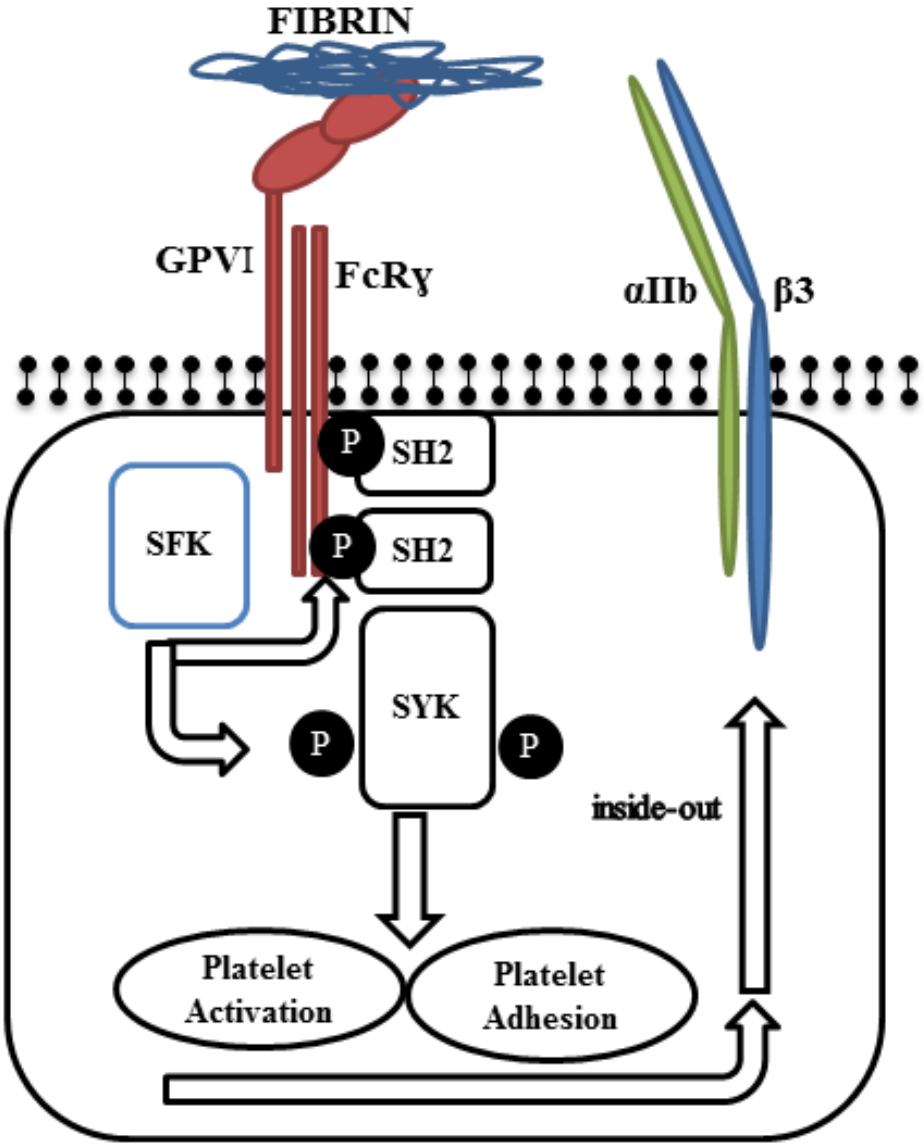

Figure 6-1 Signalling pathway of GPVI receptor, following the activation of platelets by fibrin. Fibrin binds directly to GPVI, leading to the phosphorylation of SFK and Syk and platelet activation, which induces inside-out signalling and the activation of integrin αIIbβ3.

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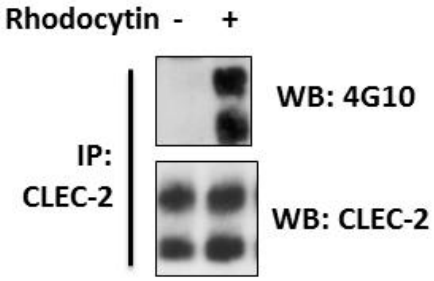
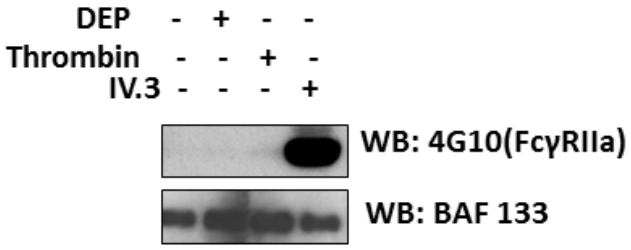
Appendices

Appendix 1.



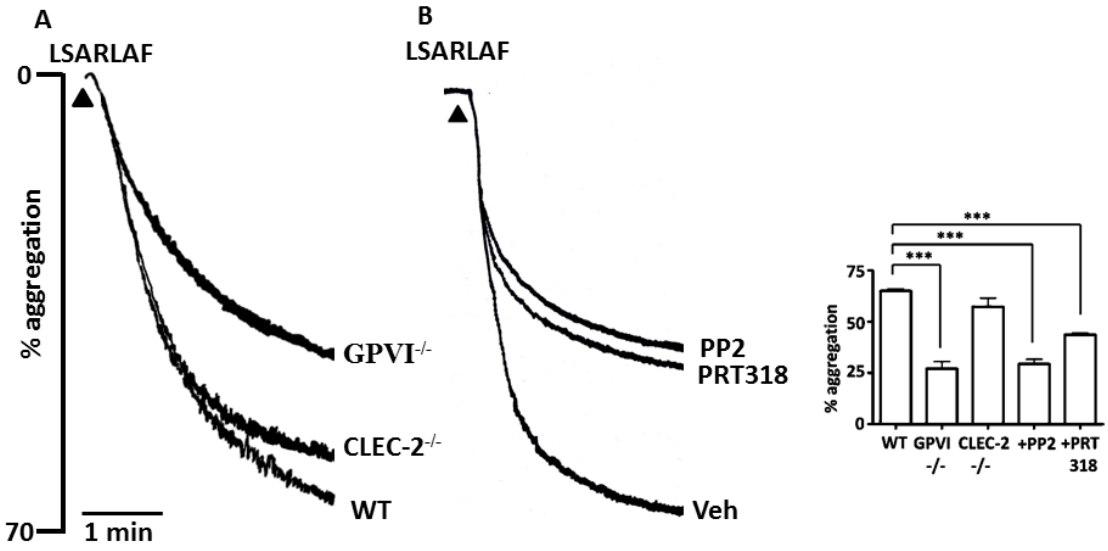
Rhodocytin stimulates tyrosine phosphorylation of CLEC-2. Washed human platelets were stimulated with Rhodocytin (100 nM) for 3 min. Experiments were performed under stirring conditions in the presence of Integrilin (9 μM). CLEC-2 was immunoprecipitated using a specific antibody, separated by SDS PAGE and Western blotted for pTyr before reprobing for CLEC-2. Results are representative of a minimum of three experiments.

Appendix 2.



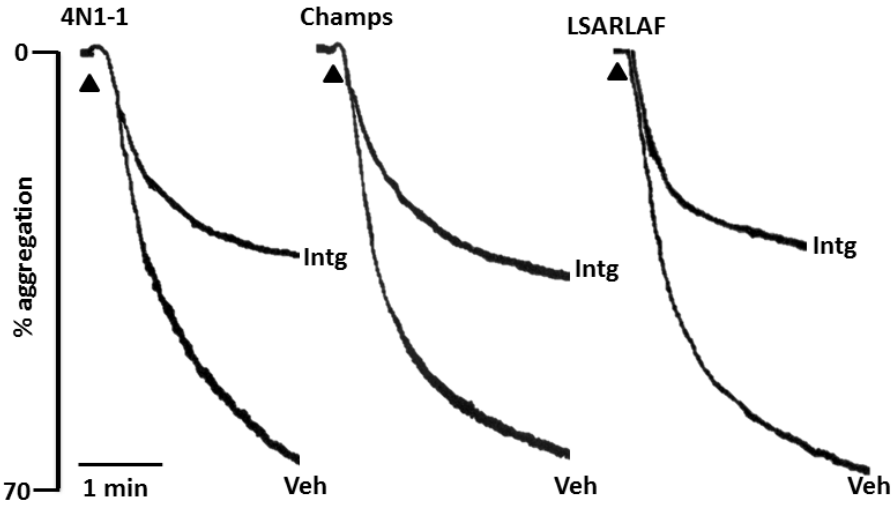
Diesel exhaust particles (DEP) do not induce tyrosine phosphorylation of FcγRIIa. Washed human platelets were stimulated with DEP (50 μg/ml), thrombin (1 U/ml) and mAb IV.3 (2 μg/ml), crosslinked with a secondary cross-linker sheep α-mouse $F(ab)_2$ (15 μg/ml) for 3 min. The latter was used as a positive control for FcγRIIa phosphorylation. FcγRIIa was immunoprecipitated using mAb BAF 133, separated by SDS PAGE and western blotted for pTyr before reprobing with for the ip'd protein. Results are representative of a minimum of three experiments.

Appendix 3.



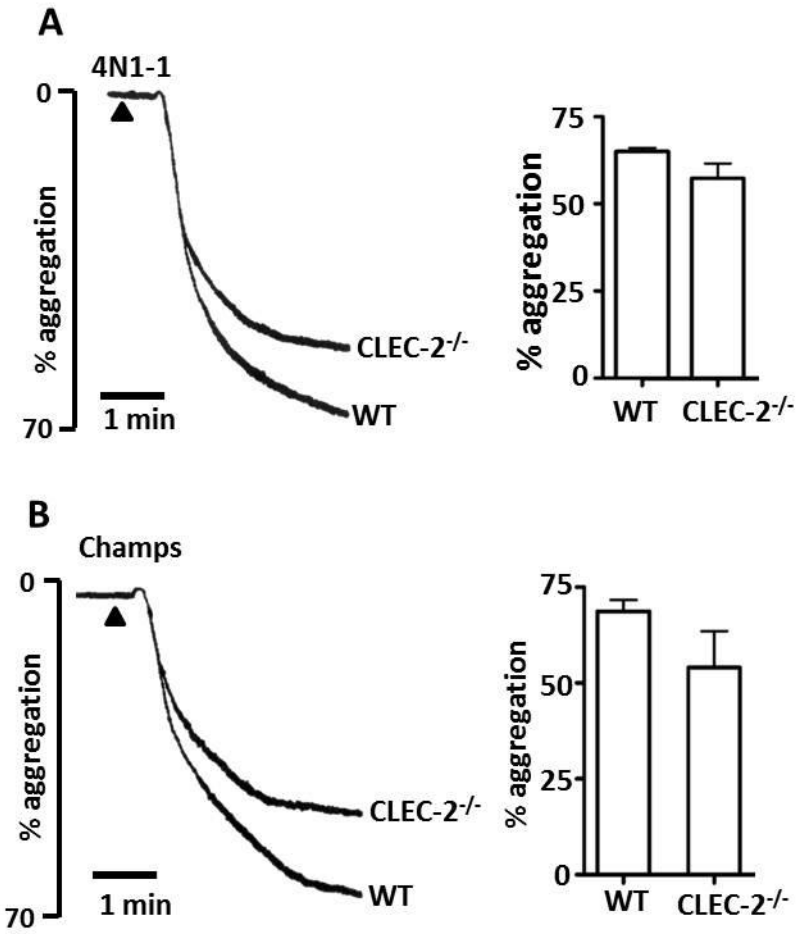
LSARLAF activates mouse platelets through GPVI. Washed platelets from (A) wild type (WT), GPVI-deficient mice and CLEC-2-deficient mice were stimulated with LSARLAF (1 mM), and (B) Platelets were pre-incubated with PP2 (10 µM) and PRT318 (5 µM) for 3 min prior to stimulation; Representative aggregation results from 3 experiments are shown as mean ±SEM (*** P<0.001). Results are representative of three experiments

Appendix 4.



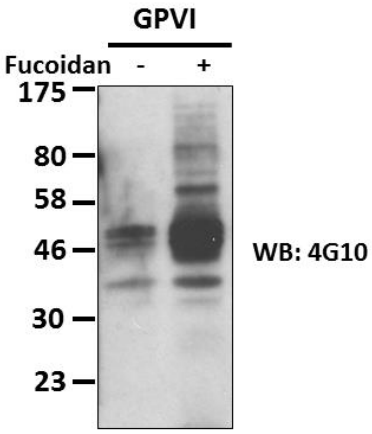
4N1-1, Champs and LSARLAF induce platelets agglutination. Platelets were pre-incubated with integrillin (9µM) for 3 min prior to 4N1-1, Champs or LSARLAF stimulation. Results are representative of three experiments.

Appendix 5.



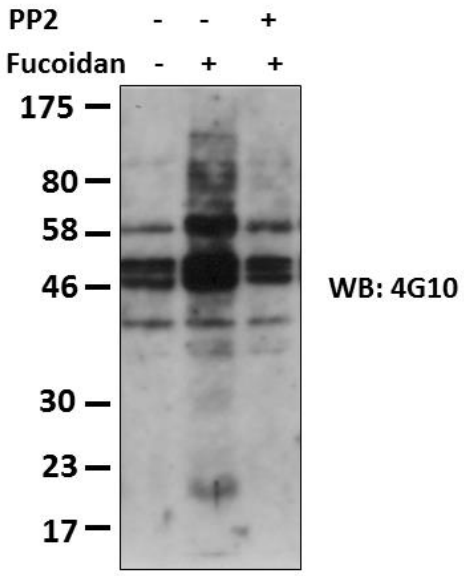
4N1-1 and Champs do not activate mouse platelets through CLEC-2. Washed platelets from wild type (WT) and CLEC-2-deficient mice were stimulated with (A) 4N1-1 (100 μM) and (B) Champs (30 μg/ml). Representative aggregation traces shown with mean results from three experiments ± SEM.

Appendix 6.



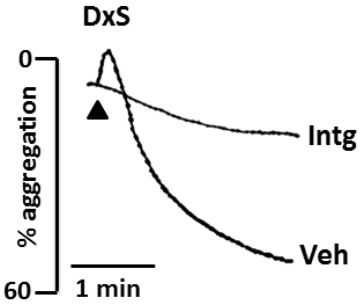
Fucoidan stimulates tyrosine phosphorylation in GPVI-deficient platelets. Washed platelets prepared from GPVI-deficient mice were stimulated with fucoidan (100 μg/ml) for 3 min. Tyrosine phosphorylation of whole cell lysates were separated by SDS-PAGE and western blotted for pTyr using mAb 4G10. Results are representative of three experiments

Appendix 7.



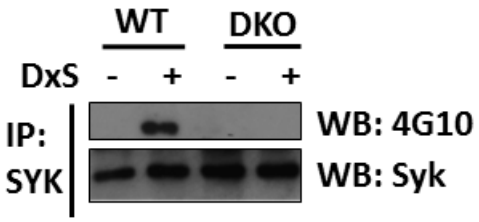
Stimulation of tyrosine phosphorylation by fucoidan is blocked by PP2. Mouse platelets were pre-incubated with PP2 (10 μM) for 3 min prior to stimulation with Fucoidan (100 μg/ml). Protein separated by SDS-PAGE and Western blotted for pTyr. The figure is representative of three experiments.

Appendix 8.



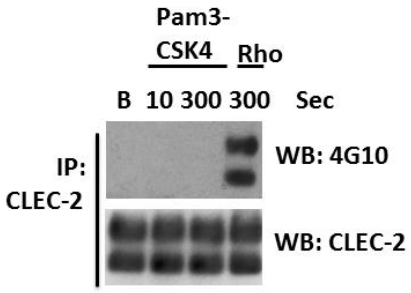
Dextran sulphate induces platelet agglutination. Washed human platelets were pre-incubated with integrilin (9 μM) for 3 min before stimulation with dextran sulphate (20 nM). Results are representative of three experiments

Appendix 9.



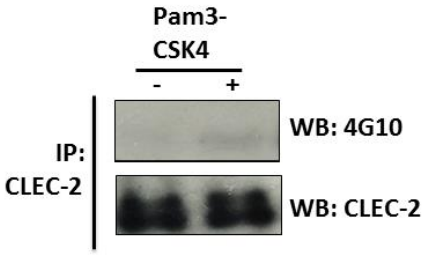
Inhibition of Syk phosphorylation in GPVI/CLEC-2-double-deficient by dextran sulphate. Protein phosphorylation of Syk was measured after dextran sulphate (20 nM) stimulation of washed platelets from wild-type (WT) and GPVI/CLEC-2-deficient mice. Results are representative of three experiments.

Appendix 10.



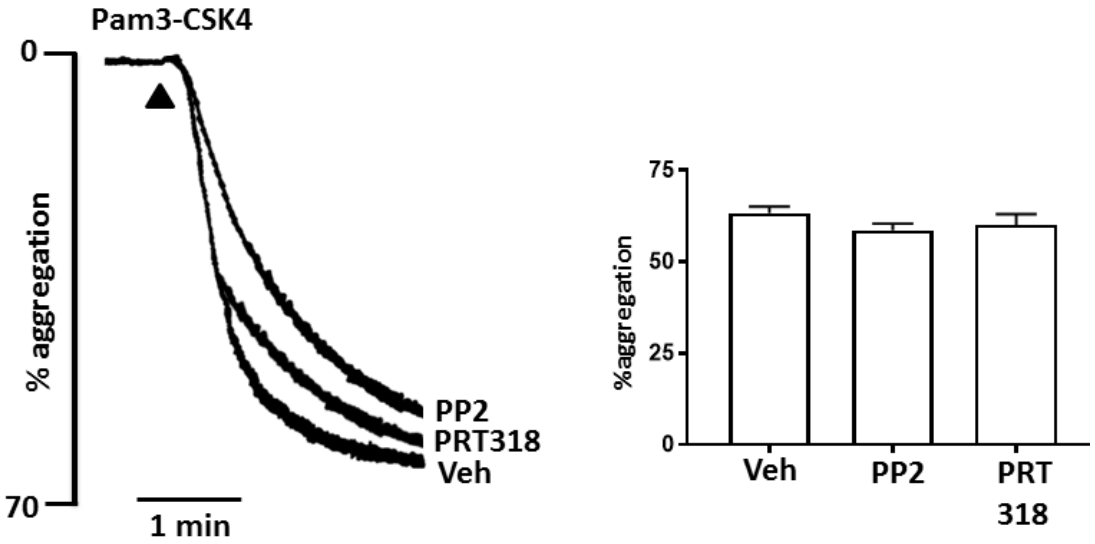
CLEC-2 is not phosphorylated by Pam_3-CSK_4 in human platelets. Washed human platelets were preincubated with integrilin (9 μM) and stimulated under stirring conditions with Pam_3-CSK_4 (30 μg/ml) for 10 and 300 sec or Rhodocytin (100 nM) before lysis. CLEC-2 was immunoprecipitated using a specific antibody, separated by SDS PAGE and Western blotted for pTyr before reprobing for CLEC-2. Results are representative of three experiments.

Appendix 11.



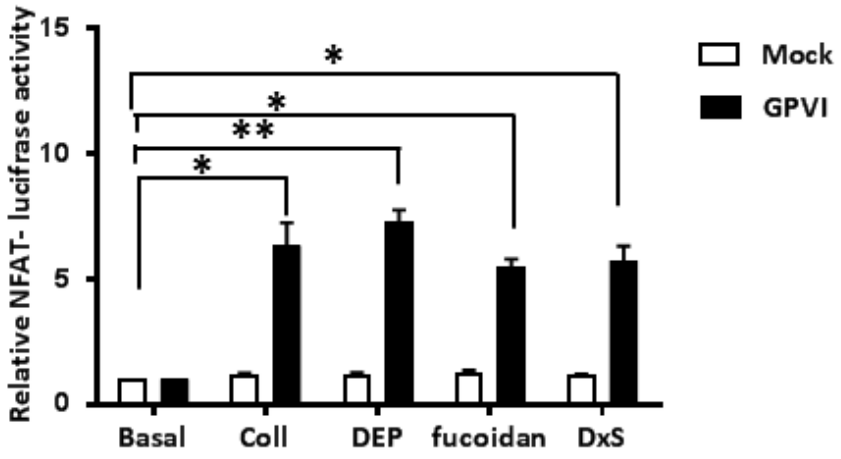
CLEC-2 is not phosphorylated by Pam_3-CSK_4 in mouse platelets. Washed mouse platelets were preincubated with integrilin (9 μM) and stimulated under stirring conditions with Pam_3-CSK_4 (30 μg/ml) before lysis. CLEC-2 was immunoprecipitated using a specific antibody, separated by SDS PAGE and Western blotted for pTyr before reprobing for CLEC-2. Results are representative of three experiments.

Appendix 12.



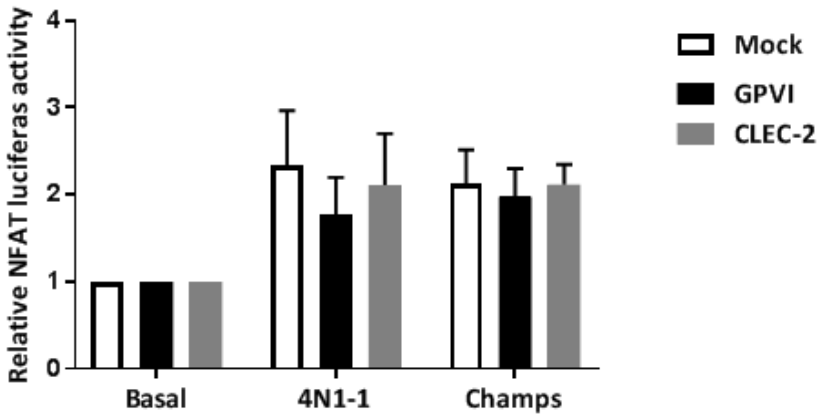
Pam$_3$-CSK$_4$ induce platelet aggregation in the presence of PP2 and PRT318. Platelets were pre-incubated with PP2 (10 μM) and PRT318 (5 μM) for 3 min prior to stimulation with Pam$_3$-CSK$_4$ (30 μg/ml). Results are representative of three experiments

Appendix 13.



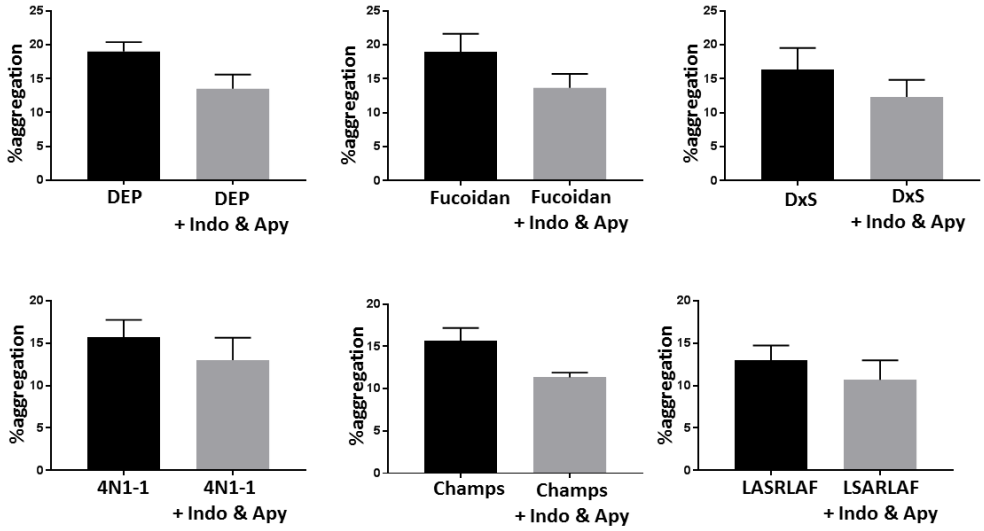
Miscellaneous platelet stimuli activate GPVI in transfected cell lines. The effect of diesel exhaust particles (DEP), fucoidan and dextran sulphate on GPVI- transfected DT40 cells was monitored using an NFAT-luciferase reporter assay. Cells were transfected with either an empty vector (mock) or GPVI/FcR γ-chain and an NFAT-luciferase reporter and stimulated for 6 hours. Luciferase activity was measured as previously described in chapter 3. Results are representative of three experiments, mean ± SEM, n ≥3 (* $P<0.05$, ** $P<0.01$).

Appendix 14.



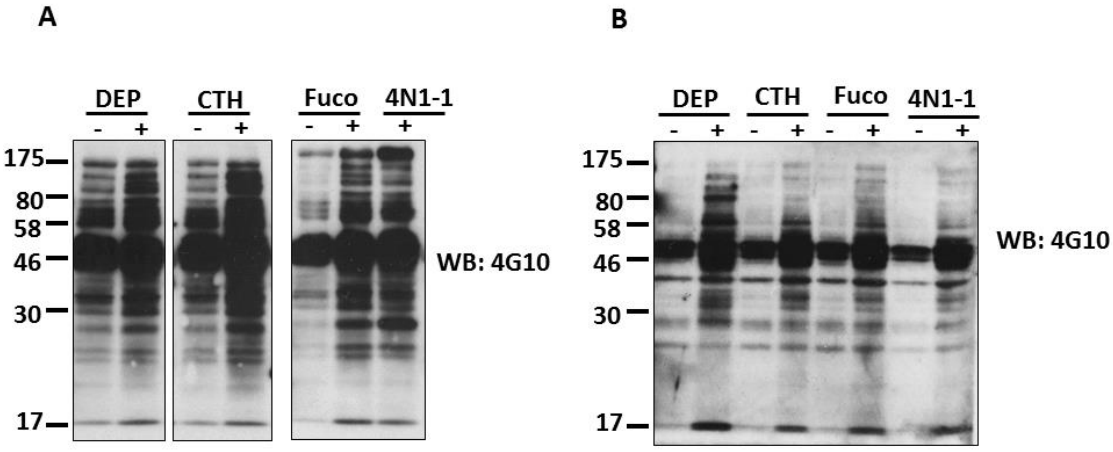
4N1- and Champs activate Mock, GPVI and CLEC-2 in transfected cell lines. The effect of 4N1-1 and Champs on Mock, GPVI and CLEC-2 transfected DT40 cells was monitored using an NFAT-luciferase reporter assay. Cells were transfected with either an empty vector (mock) or GPVI/FcR γ-chain and an NFAT-luciferase reporter and stimulated for 6 hours. Luciferase activity was measured as previously described in chapter 3. Results are representative of three experiments. There was no significant difference.

Appendix 15.



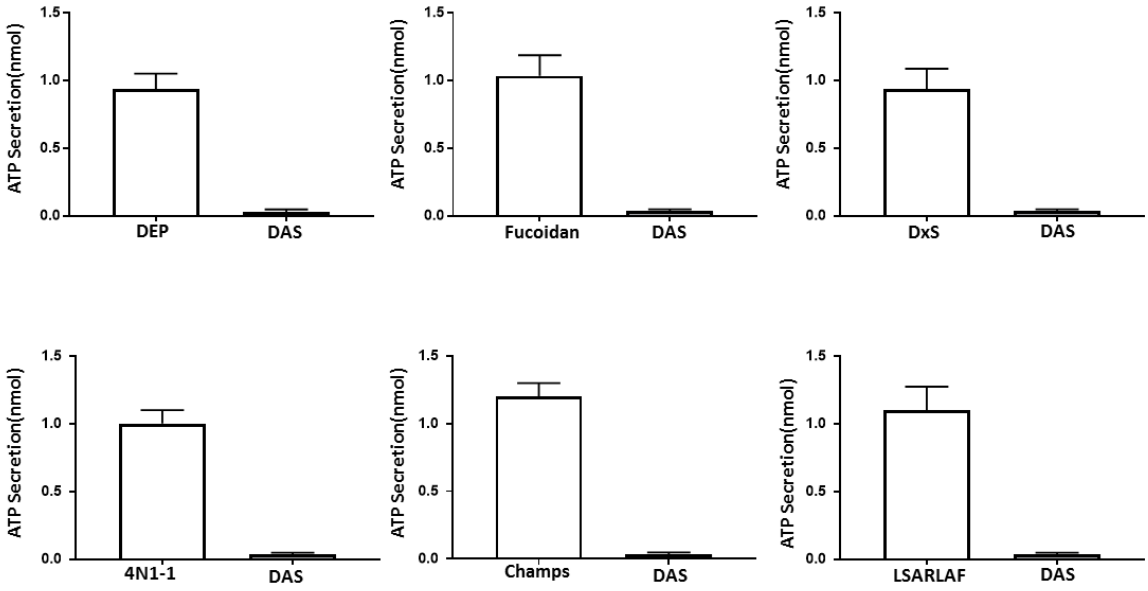
Platelets aggregation induced by low concentrations of the ligands in the presence of indomethacin and apyrase. Washed platelets were pre-incubated with the inhibitors (10 μM) indomethacin and (2 U/ml) apyrase and stimulated with DEP (5 μg/ml), fucoidan (30 μg/ml), DxS (5 nM), 4N1-1 (20 μM), Champs (2.5 μg/ml) and LASERLAF (0.25 mM). Results are representative of three experiments. There was no significant difference.

Appendix 16.



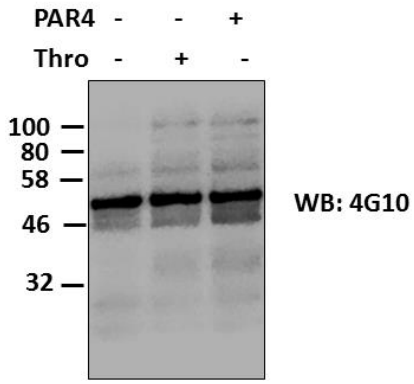
DEP, CTH, 4N1-1 and fucoidan induce platelets tyrosine phosphorylation from patients deficient in integrin αIIbβ3 and GPIbα .Washed human platelets from patients deficient in (A) integrin αIIbβ3 and (B) GPIbα were stimulated with DEP (50 µg/ml), CTH (50 µg/ml) 4N1-1 (100 µM) or Fucoidan (100 µg/ml) for 3 min. Protein phosphorylation was measured as described in Figure 3-2. Molecular masses are indicated in kDa. Results are shown from a single experiment.

Appendix 17.



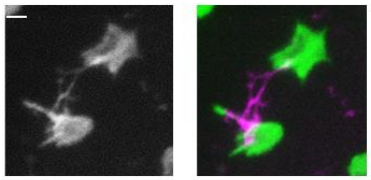
Stimulation of ATP secretion by diverse group of ligands is blocked in the presence of a Src kinase inhibitor. ATP was measured using luciferin-luciferase.

Appendix 18.



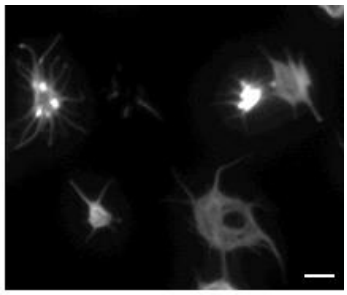
Platelets stimulated by thrombin and PAR-4 peptide. Mouse platelets from WT mice were stimulated with thrombin (1 U/mL) or PAR-4 peptide (150 mM) in the presence of eptifibatide. WCLs were separated by SDS-polyacrylamide gel electrophoresis and western blotted for pTyr. The results are shown as representative of 3 experiments.

Appendix 19



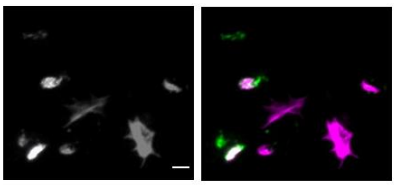
Smaller coatings of fibrin are associated with platelet adhesion and spreading. Alexa-488-fibrinogen was used to visualise fibrin formation. Platelets (2×10^7/ml) were allowed to spread on fibrin coated coverslips, followed by actin staining with Alexa-568-phalloidin. Scale bar = 5μm. Results are representative of three experiments.

Appendix 20.



Actin nodules and filopodia in platelets that have undergone partial spreading. Mouse platelets (2×10^7 /ml) were allowed to spread on fibrin coated coverslips, followed by actin staining with Alexa-488-phalloidin. Scale bar = 5μm. Actin nodules can be seen as bright spots in the upper land part of the figure. The figure is representative of three experiments.

Appendix 21.



Using Alexa-568-phalloidin to counterstain actin: Mouse platelets (2×10^7 /ml) were allowed to spread on fibrin coated coverslips, followed by incubation with FITC-Annexin V (green). Actin was counterstained with Alexa-568-phalloidin (magenta) to count total number of adherent platelets. Scale bar =10μm. The result is representative of three experiments.