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Abbreviations

ACD:	Acid citrate dextrose
ADP:	Adenosine diphosphate
ALK:	Anaplastic lymphoma tyrosine kinase
BSA:	Bovine serum albumin
Caspase:	Cysteine-aspartic proteases
CD62:	P-selectin
CRAC:	Ca^{2+} release-activated Ca^{2+}
CRP:	Collagen-related peptide
CytC:	Cytochrome c
DAG:	Diacylglycerol
DCFDA:	2',7'- dichlorodihydrofluorescein diacetate
ER:	Endoplasmic reticulum
FBG:	Fibrinogen
FITC:	Fluorescein isothiocyanate
FSC:	Forward scatter
GIF:	Glycosylation-inhibiting factor
GP:	Glycoprotein
G protein:	Heterotrimeric guanine nucleotide-binding protein
GPCR:	G-protein-coupled receptor
$IC_{50:}$	Half maximal inhibitory concentration
IP_3:	Inositol 1,4,5-trisphosphate
ITAM:	Immunoreceptor tyrosine-based activation motif

MK:	Megakaryocyte
MLCK:	Myosin light-chain kinase
MP:	Platelet microparticle
MPTP:	Mitochondria permeability transition pore
MPV:	Mean platelet volume
mRNA:	Messenger RNA
NCKX:	Na^+/Ca^{2+}-K^+ exchanger
NCX:	Na^+/Ca^{2+} exchanger
NSCLC:	Non-small cell lung cancer
Orai:	Calcium release-activated calcium channel protein
PAR:	Protease-activated receptor
PDGF:	Platelet-derived growth factor
PE:	Phycoerythrin
PF4:	Platelet factor 4
PI3K:	Phosphoinositide 3-kinase
PIP_2:	Phosphatidylinositol 4,5-trisphosphate
PKC:	Protein kinase C
PLC:	Phospholipase C
PM:	Plasma membrane
PMCA:	Plasma membrane Ca^{2+} ATPase
PRP:	Platelet-rich plasma
PS:	Phosphatidylserine
ROS:	Reactive oxygen species
RT:	Room temperature

RyR:	Ryanodine receptor
SD:	Standard deviation
SERCA:	Sarco/endoplasmic reticulum Ca^{2+} ATPase
SOCE:	Store-operated Ca^{2+} entry
SOC:	Store-operated Ca^{2+} channel
STIM:	Stromal interaction molecule
TF:	Tissue factor
TGFβ:	Transforming growth factor-beta
TRPC:	Transient receptor potential cation channel
TXA_2:	Thromboxane A_2
VEGF:	Vascular endothelial growth factor
vWF:	von Willebrand factor
Δψm:	Mitochondrial membrane potential

1. Introduction

Platelets play vital roles in the physiological process of haemostasis and further regulate vascular inflammation, immunity, and atherogenesis (Gawaz, 2004; Sonmez and Sonmez, 2017; Borst et al., 2012a). Platelets are distinct from most mammalian cells because they do not have a nucleus. Accordingly, they were previously called “cellular fragments”. However, this outdated definition has been abandoned since platelets are proposed to participate in the pathophysiology of type II diabetes, atherosclerosis, cancer cell metastasis, and immune responses (Morrell et al., 2014; Garraud et al., 2013; Lannan et al., 2015)

1.1 Platelet generation

Platelets do not have genomic DNA because they are non-nucleated. Megakaryocytes (MKs) are the precursors of platelets and can produce a total of 1000 to 3000 platelets (George, 2000). Approximately 100×10^9 platelets are produced by MKs every day. In human cells, the proportion of MKs is approximately 0.05–0.1% of all nucleated cells residing in the bone marrow. The platelet number changes depending on the need for different platelet functions (Lebois and Josefsson, 2016). Although platelets are anucleated, they possess many cytoplasmic messenger RNAs inherited from MKs, which maintain survival and physiological platelet functions (Rowley et al., 2012; Harrison and Goodall, 2008). The mechanism by which platelets are formed has become increasingly apparent (Yamada, 1957).

Two hypotheses have been proposed to explain platelet formation, and these hypotheses have been incorporated into a complete model of platelet formation.

The first and primary model for platelet formation has been described as follows: MKs produce pseudopodia in the bone marrow by a demarcation membrane system that is highly redundant (Radley and Haller, 1982; Behnke, 1968) The sinusoids of the bone marrow have thin endothelial cells, which bind firmly to others and might also overlap (Becker and De Bruyn, 1976). Platelets, as well as proplatelets (which are elongated chains of MK cytoplasm that are larger than normal platelets and subsequently divided into many platelets) (Becker and De Bruyn, 1976; Tong et al., 1987), later germinate due to the localized activation of caspases (De Botton et al., 2002). The MK pseudopodia infiltrate the endothelial cells, probably due to their role in regulatory processes (Becker and De Bruyn, 1976).

The second model claims that the production site of platelets is located in the lung but not in the bone marrow, which further contributes to the model described above. MKs or proplatelets are first released from the marrow and then enter the lung, where they are converted into platelets (Lefrançais et al., 2017; Pedersen, 1978; Trowbridge et al., 1982). MKs or large fragments of MKs are cleaved into platelets in the circulation. However, the extent of pulmonary platelet production is difficult to measure with current methods (Trowbridge et al., 1982; Pedersen, 1978; Levine et al., 1993). Evidence for this mechanism includes the finding that MKs can pass through the bone marrow of the endotheliocyte barrier and analyses of MKs or their nuclei in pulmonary vessels and circulation (Poujol et al., 1998; Kaufman et al., 1965; Pedersen, 1978). Initially, MKs that escaped from the bone marrow and were trapped in the lung were thought to be aberrant. However, this process is now considered a significant route of cell trafficking (Kuter, 2016).

1.2 Specific platelet granules

Granules of platelets contain many agonists, such as serotonin, ADP and perhaps tachykinins, which mediate the function of platelets, such as forming aggregates (Graham et al., 2004). Another critical factor discovered in the granules of platelets is called insulin-like growth factor-1 (IGF-1) (Chan and Spencer, 1998), which is a growth factor related to cell differentiation and proliferation (Delafontaine et al., 2004).

Secretory granules of platelets that are important for the physiological functions of these non-nucleated cells have been classified into three types: 1) α-granules, 2) dense granules and 3) lysosomes. Among these granules, α-granules and dense granules occupy a large proportion and have critical functions (George, 2000). The membranes of the α-granules and dense granules are composed of MKs (Rendu and Brohard-Bohn, 2001). α-granules of platelets contain various factors and proteins (Rendu and Brohard-Bohn, 2001). The granule body forms in the early development of MKs. Some of the granule contents, such as platelet factor 4 (PF4), transforming growth factor-β and von Willebrand factor (vWF), are transferred into the α-granules and assembled in the MKs (Greenberg et al., 1987; Nurden, 2011). PF4 is abundant in the α-granules of platelets (Kowalska et al., 2010), and the concentration was estimated to be approximately 7–17 μg/10^9 platelets (Peterson et al., 2010). A PF4 variant, which differs in only three amino acids and is encoded by a second non-allelic gene, inhibits vascular generation (Hagedorn et al., 2002; Vandercappellen et al., 2011). PF4 inhibits the binding between different growth factors and cells (Bikfalvi, 2004a; Bikfalvi, 2004b; Perollet et al., 1998). Other proteins are transported from the plasma to the α-granules of both MKs and platelets (Handagama et al., 1989; Handagama et al., 1995).

1.3 Primary haemostasis

Haemostasis is a highly conserved process that has an intricate physiological function. After vascular injury, the underlying subendothelial matrix is exposed during endothelial layer disruption. Then, the haemostatic mechanism is triggered to form a thrombus (Baz and Mekhail., 2013).

In short, coagulation involves two major processes: primary haemostasis (characterized by three overlapping steps: platelet adhesion, activation and aggregation) (Hoffman and Monroe 2001; Monroe 2002) and secondary haemostasis (followed by fibrin network deposition and maturation) (Palta et al., 2014; Xu et al., 2016). Figure 1 illustrates a toolkit of primary haemostasis.

During primary haemostasis, the matrix of the procoagulant subendothelium, which includes vWF, collagen, and fibrinogen (FBG), is exposed. In addition, subendothelial matrix proteins bind to their receptors, such as glycoprotein (GP) receptors (e.g., collagen-GPVI, vWF-GPIb/IX/V complex and collagen-α2β1), and immediately trigger primary haemostasis (Figure 1). Following the stimulation of injured vessels, rapid increases in intracellular Ca^{2+} induce the release of dense granule contents from platelets (Jones et al., 2011). Platelets are activated in parallel by the combination of agonists such as thromboxane A_2 (TXA_2, Reed, 2004), thrombin (Ivanciu and Stalker, 2015) and ADP (Morimoto et al., 1990) to their G-protein-coupled receptors (GPCRs). Polyphosphates from the dense granules can promote the cofactor activity of factor V and facilitate the formation of dense fibrin and fibrils to prevent fibrinolysis. Platelet clots and fibrin further form as a result. The α-granules play a crucial role during platelet activation. For example, vWF and the coagulation factors FXIII, FV and FBG are all produced from platelet α-granules (Repetto and De Re, 2017).

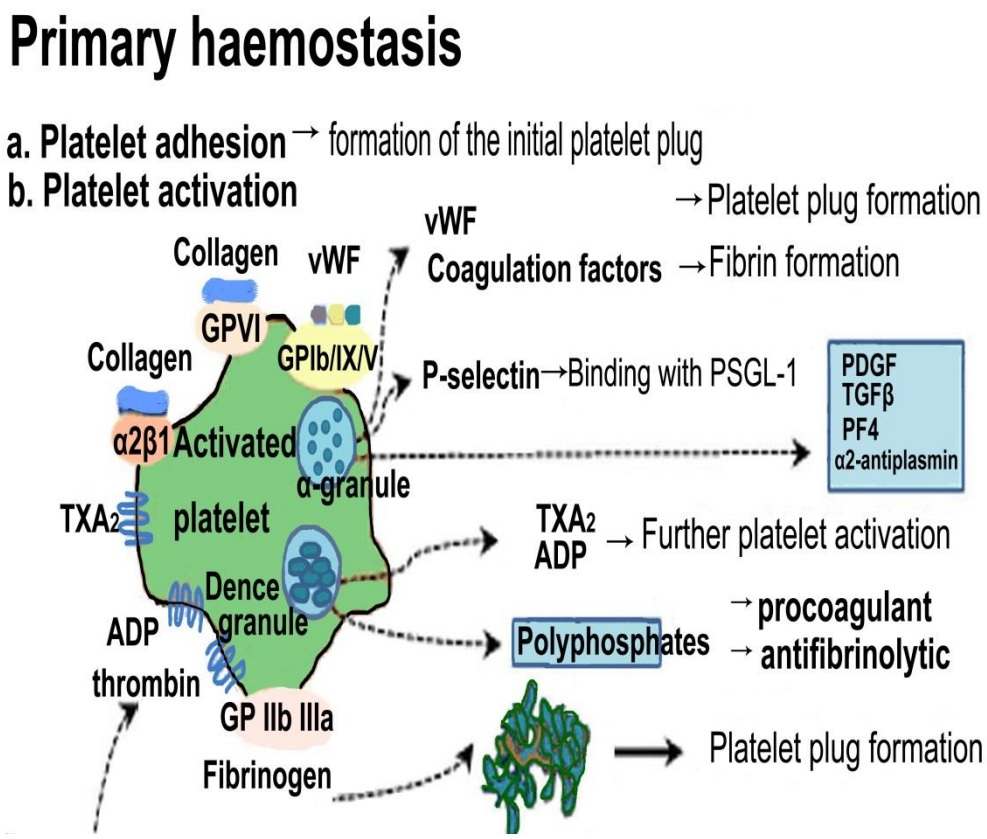

Figure 1: Mechanism of primary haemostasis (from Repetto and De Re, 2017)

Figure shows the formation of primary haemostasis which consists of platelet (a) adhesion, (b) activation, and (c) aggregation, is triggered by the exposure of procoagulant subendothelial matrix (consisting of proteins including fibronectin, collagen, vWF, laminin, and FBG), which afterwards binds to GP receptors. GP: glycoprotein; vWF: von Willebrand factor; TXA_2: thromboxane A_2; PSGL-1: P-selectin glycoprotein ligand 1; PDGF: platelet-derived growth factor; TGF-β: transforming growth factor β; PF4: platelet factor 4.

In addition, adhesion molecules, such as P-selectin (Orkin et al., 2008; Coleman and Tsongalis, 2009), which are located at the platelet surface, can bind to their ligands *in vivo* on leukocytes (offering scaffolds for the formation of fibrin) and endothelial cells. Furthermore, platelet aggregation can be activated by platelet integrin αIIbβ3 *via* the FBG/vWF complex (Repetto and De Re, 2017).

The process of haemostasis can be summarized as a spatiotemporal regulation of platelet adhesion, activation, and aggregation, followed by the generation of coagulation cascades and thrombin (Golebiewska and Poole, 2015; Ivanciu and Stalker, 2015; Xu et al., 2016).

1.4 Calcium regulation

1.4.1 Platelet Ca^{2+} store and Ca^{2+} release

The physiological function of platelets depends on the increase in intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$), which can be induced *via* endogenous release from Ca^{2+} stores and extracellular Ca^{2+} influx (Figure 2). Two types of independent intracellular stores have been identified in the cytoplasm of platelets. The first one, which is termed the dense tubular system (DTS), is a membrane system of the endoplasmic reticulum (ER) harbouring several kinds of enzymes that are essential for proper protein folding (Gresele et al., 2017) and is one of the major internal Ca^{2+} stores. Following a stimulus, inositol 1,4,5-trisphosphate (IP_3) moves to the surface of the DTS to bind to IP_3R, which subsequently triggers the release of Ca^{2+} through the DTS and ultimately activates the related Ca^{2+} channels to accomplish these reactions (Jardin et al., 2008).

Another store involving secretory granules and mitochondria is lysosome-related acidic organelles (Jardin et al., 2008). The ER plays pivotal roles in intracellular calcium storage, as well as these organelles, and is responsible for protein assembly and modification (Soboloff and Berger, 2002). Recent discoveries have shown that ER stress at sublethal levels can contribute to protecting cells. However, sustained ER stress will lead to prolonged inhibition of protein synthesis, which eventually results in cell death (Isaacs, 2005; Zhang and Berger, 2004). Figure 2 shows platelet activation *via* Ca^{2+} influx.

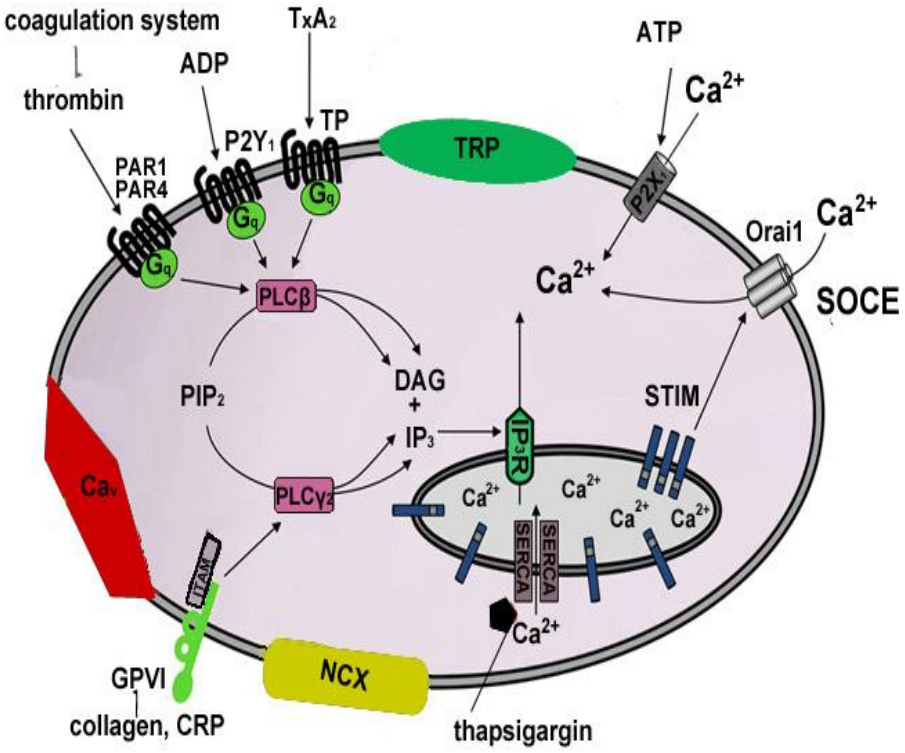

Figure 2: Ca^{2+}-mediated platelet activation (from Braun et al., 2011)

The release of Ca^{2+} is mainly dependent on two signaling pathways. PLC (β and γ_2) hydrolyze PIP_2 into IP_3 and DAG following agonists activation. 1) Soluble ligands such as thrombin and TXA_2 translocate to their receptors GPCRs, initiating PLCβ. 2) Agonists such as collagen or CRP bind with their receptor GPVI, activating PLCγ_2. Intracellular Ca^{2+} pools are discharged by the binding between IP_3 and its receptor IP_3R, in turn triggering SOCE which depends on STIM1/2 and Orai1. SERCA (SR Ca^{2+}-ATPase) pumps Ca^{2+} from ER to cytosol, which further induces Ca^{2+} influx through SOCs. TRP, Ca_v and NCX indicate Ca^{2+} channels of transient receptor potential, voltage-dependent Ca^{2+} channels and Na^+/Ca^{2+} exchanger, consecutively. Thapsigargin: SERCA inhibitor. DAG: diacylglycerol.

The inhibition of sarco-ER Ca^{2+}-ATPase (SERCA) usually results in ER Ca^{2+} depletion, thus inducing Ca^{2+} entry through store-operated channels (SOCs), further resulting in platelet apoptotic events. Several SERCA inhibitors, such as thapsigargin, have been extensively used to determine the role of Ca^{2+} stores in Ca^{2+} signalling (Hakii et al., 1986). SERCA, which can carry cytosolic Ca^{2+} into

the DTS *via* ATP consumption, has been shown to be an important Ca^{2+} pump family member in platelets (Flaumenhaft, 2016). Moreover, various inhibitors have been developed as potential anticancer agents (Michelangeli and East, 2011).

1.4.2. Ca^{2+} pathways

Intracellular Ca^{2+} release plays a vital role in the initial period of platelet reactions. Moreover, steady and continuous platelet function depends on the entry of high extracellular Ca^{2+} levels, which could immediately fill the depleted store again. Intracellular Ca^{2+} activity is modulated *via* Ca^{2+} entry and Ca^{2+} discharge mechanisms. A range of Ca^{2+} signaling pathways have been identified, including ryanodine receptor (RyR); transient receptor potential (TRP); Na^{+}/Ca^{2+}-K^{+} exchanger (NCKX); plasma membrane Ca^{2+} ATPases (PMCAs); Ca^{2+} release-activated Ca^{2+} current (CRAC); voltage-dependent Ca^{2+} channels (Ca_V); SR Ca^{2+}-ATPase (SERCA) and Na^{+}/Ca^{2+} exchanger (NCX) (Mahaut-Smith et al., 2011; Sage et al., 2002; Shumilina et al., 2011; Harper and Sage, 2007).

1.5 Platelet activation

In non-excitable cells such as platelets, receptor-agonist reactions result in the stimulation of phospholipase C (PLC) through the tyrosine kinase signalling pathway or G-protein-coupled signalling pathway. IP_3 generation is followed by PIP_2 hydrolysis *via* PLC, which can bind with the DTS receptors and thus finally deplete Ca^{2+} stores. This mechanism was originally detected in neutrophils in 1986 (Putney, 1986) and suggested that the Ca^{2+} entry is store-regulated (Sargeant et al., 1992), which has recently been termed store-operated calcium entry (SOCE) (Lang et al., 2013a; Parekh et al., 2005).

1.5.1 Platelet agonists and receptor pathways

Physiological platelet agonists induce distinct signalling pathways. The binding between agonists and receptors could trigger platelet activation as well as the downstream cascades of “inside-outside” signalling (Goggs and Poole, 2012). The elevated $[Ca^{2+}]_i$, which is widely related to cellular interactions, e.g., shape change and adhesion receptor activation, plays many roles during the activation of platelets (Hathaway et al., 1979; Offermanns et al., 1997; Rink et al., 1982).

Two types of signalling pathways have been extensively investigated in platelets. One is called the tyrosine kinase pathway; platelet activation depends on tyrosine phosphorylation (Stegner and Nieswandt, 2011; Bergmeier and Stefanini, 2009).

Agonists like collagen, convulxin and collagen-related peptide (CRP) mainly activate platelets through a mechanism dependent on the glycoprotein (GP) VI and downstream signalling pathways, such as the Fc-receptor γ-chain (FcRγ) and diverse tyrosine kinases (Varga-Szabo et al., 2008; Nieswandt and Watson, 2003). The phosphorylation of the immunoreceptor tyrosine-based activation motif (ITAM) and Syk may trigger the activation of these cascades. Downstream phosphoinositide 3-kinase (PI3K) is further activated upon phospholipase $Cγ_2$ ($PLCγ_2$) activation, which ultimately triggers platelet Ca^{2+} mobilization and aggregation (Stegner and Nieswandt, 2011). In addition, integrin αIIbβ3 (which binds to FBG) and GPIb-V-IX (which binds to vWF) also rely on the tyrosine kinase pathway to activate platelets (Watson et al., 2005; Jackson et al., 2003).

The other pathway is called the GPCR signalling pathway. PLCβ isoforms and numerous enzymes are activated following the binding of agonists to their respective receptors. The reactions of ligand-receptors trigger various

downstream cascades through various G proteins and therefore modulate shape change, Ca^{2+} mobilization, and aggregation of platelets (Offermanns, 2006; Gachet, 2001; Devanathan et al., 2015).

1.5.2 Orai/STIM-mediated SOCE

Recent studies upon the interactions of stromal interaction molecule (STIM) and Orai have uncovered the mechanisms of activation and regulation of store-operated Ca^{2+} channels (SOCE). The pore-forming subunits are called Orai (mammalian cells express three isoforms termed Orai1, Orai2 and Orai3) and STIM (mammalian cells express two isoforms termed STIM1 and STIM2) (Oh-hora and Rao, 2008). Orai proteins contribute to Ca^{2+}-selective channels that are located on the plasma membrane (PM; Lewis, 2007; Motiani et al., 2013). Every Orai protein consists of one cytosolic C-terminus, four-transmembrane (TM) sections linked through three rings (intracellular 1 and extracellular 2), and one cytosolic N-terminus (Prakriya, 2013).

Two types of molecules, namely, STIM1 and Orai1, have attracted the attention of researchers in the last two decades (Lang et al., 2013a). STIM1, which is localized in the ER, has been proven to be a Ca^{2+} sensor (Hyser et al., 2013; Maschalidi et al., 2017). Orai1, which is found in the PM, has been suggested to be the major SOCs transmembrane protein (Ong et al., 2007). Orai1 is the major subtype among the three subtypes. STIM1 translocates to the PM, where it activates Orai1, the pore-forming unit of Ca^{2+} release-activated Ca^{2+} (CRAC) channels, during ER Ca^{2+} depletion (Smyth et al., 2006; Berna-Erro et al., 2012).

SOCE, which is essential for the regulation of numerous physiological reactions in almost all kinds of cells (Parekh and Putney, 2005), has been proposed as the most important Ca^{2+} influx mechanism in platelets (Alonso et al., 1991; Sage et

al., 1992). However, the precise mechanism and the role of the involved molecules have not been fully elucidated.

1.6 Platelet apoptosis

Platelets have a brief life in the blood circulation before they are cleared by the reticuloendothelial system. The platelet lifespan ranges from 8 to 10 days in humans (Leeksma and Cohen, 1955) and approximately 4 to 5 days in mice (Ault and Knowles, 1995). The short life span of platelets is not due to the lack of nuclei, because the lifespan of circulating erythrocytes is up to 3 months in humans (Shemin and Rittenberg, 1946; McArthur et al., 2018). Platelets may enter apoptosis, which is also known as programmed cell death. The normal platelet count ranges from 150×10^3 to 450×10^3 platelets/μl in the bloodstream in human and typically remains within a narrow range for individuals (Johnson et al., 2016; George, 2000). In addition, mitochondria might play essential roles in regulating apoptosis of platelets (Leytin et al., 2009). Elevated platelet apoptosis has been discovered in various processes, such as malaria, bacterial infection, type II diabetes, and uraemia (Leytin, 2012).

1.6.1 Apoptosis pathways

Various factors trigger platelet apoptosis through multiple mechanisms. Apoptotic platelet pathways can also be defined as the intrinsic pathway and extrinsic pathway, similar to nucleated cells (Kile, 2014). Furthermore, inhibitors of actin polymerization, PKC and caspase activation in platelets can lead to downregulated apoptosis as well as treatment with cyclosporin A (CsA) and αIIbβ3 integrin (Leytin, 2012).

Intrinsic pathway

In general, the apoptosis of ageing platelets is mediated through the intrinsic pathway. A molecular model has been established based on previous studies (Kile, 2009; McArthur et al., 2018). In brief, prosurvival proteins, e.g., Bcl-2 family proteins, maintain platelet survival by changing the Bak/Bax ratio according to the apoptotic signalling. For instance, BH3-only proteins can be activated by apoptotic signalling factors, such as DNA injury, hypoxia and radiation. The Bak/Bax proteins can be released by activating BH3-only protein through binding to prosurvival proteins. The Bak/Bax complex produces a pore at the surface of the mitochondrial membrane, and then, cytochrome c is released. This complicated process finally induces caspase 9 activation, as well as the rest of the apoptotic caspase cascades (Green and Reed, 1998), phosphatidylserine (PS) externalization and platelet decomposition (Vogler et al., 2011; Zhu et al., 2018).

Extrinsic pathway

The process of platelet apoptosis, which depends on the intrinsic pathway, has been intensively studied. However, whether apoptosis in platelets can be triggered *via* the extrinsic pathway remains controversial. Although evidence has indicated the existence of an extrinsic apoptotic pathway in platelets, this pathway has not been definitively confirmed. Some researchers believe that mitochondria act as the hub in platelet apoptosis induced *via* external stimulation (Lien et al., 2013). Besides, circulating apoptotic platelets can be eventually cleared through the exposure of circulating platelets to high pathological shear pressure from apoptogenic stimulation (Gyulkhandanyan et al., 2017).

In addition to triggering the intrinsic apoptosis pathway, mechanical stimuli and chemical forces decrease extra-mitochondrial apoptotic responses, such as

segmentation of gelsolin and other cytoskeleton proteins, caspase-3 activation, membrane blebbing, expansion of filopodia and platelet microparticle (MP) shedding, PS exposure and platelet shrinkage (Lang et al., 2013b; Gyulkhandanyan et al., 2017).

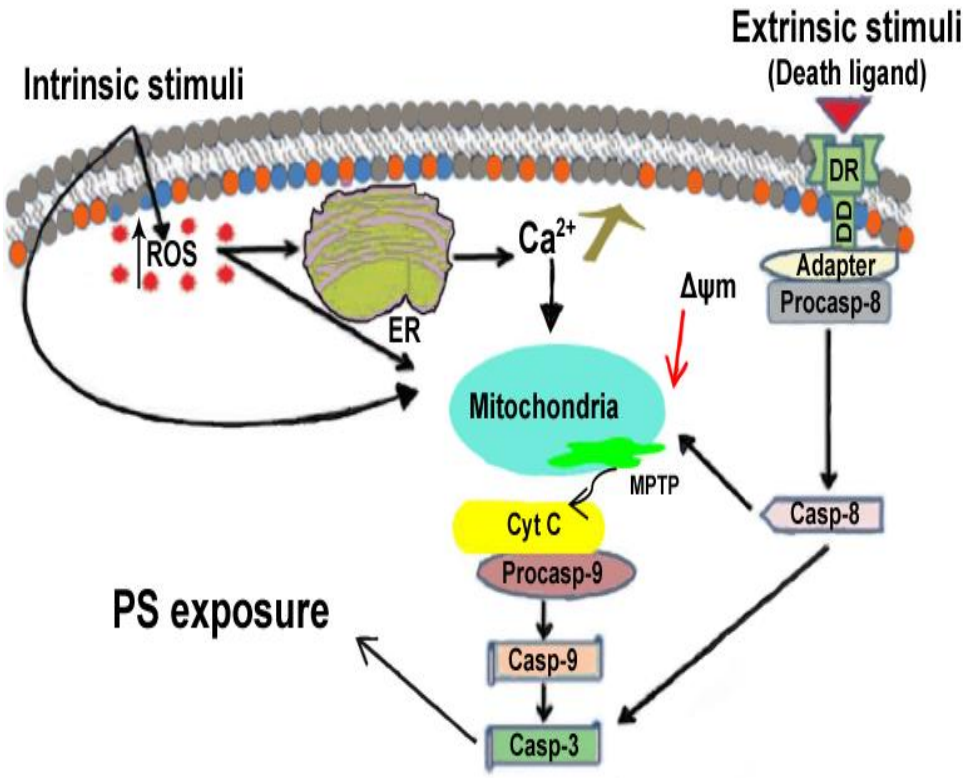

Figure 3: The mechanism of platelet apoptosis (from Thushara et al., 2013)

Figure represents general signalling pathways of platelet apoptosis *via* intrinsic stimuli and extrinsic stimuli. The extrinsic pathway (triggered by the death ligand), and the intrinsic pathway (induced by intracellular signals) come together in the execution period, resulting in a series of reactions, such as the opening of the MPTP (mitochondrial permeability transition pore), the increase of $[Ca^{2+}]_i$ and ROS, the discharge of Cyt C (cytochrome c), a decrease of Δψm (mitochondrial inner membrane potential), and the activation of caspase cascades, etc. These changes finally lead to apoptotic events in platelets.

Thushara's report (Figure 3) showed that depolarization of the mitochondrial inner membrane (Δψm) could activate caspase cascades and induce apoptosis of human platelets (Thushara et al., 2013). A high level of $[Ca^{2+}]_i$ can directly lead to loss of Δψm by activating the mitochondrial permeability transition pore (MPTP) (Towhid et al., 2011; Sveshinkova et al., 2015). In addition, the potential mediator calpain binding with a high level of $[Ca^{2+}]_i$ can elicit Δψm loss, which can induce platelet apoptosis in a Ca^{2+}-mediated manner (Zhang et al., 2011). Reactive oxygen species (ROS) can regulate the release of cytochrome c, SOCE and $[Ca^{2+}]$ (Rosado et al., 2004), as well as caspase-3 activation (Lopez et al., 2007). Moreover, ROS treatment triggers activation of Bid and Bax, which was proven by previous experiments, and accordingly mediates apoptosis of platelets (Lopez et al., 2008). Furthermore, caspase-8 can be activated by TPEN (Lopez et al., 2009) and resveratrol (Lin et al., 2009b). Figure 3 depicts platelet apoptosis depending on different signalling pathways.

1.6.2 Thrombin-induced platelet apoptosis

Agonist stimulation can induce platelet activation as well as platelet apoptotic events (Leytin, 2012). Thrombin, a natural platelet agonist, has been identified as a coagulation factor that can convert FBG to fibrin to generate blood plugs (Thushara et al., 2013) and is dependent on G-protein-coupled protease-activated receptors as a potent inducer of platelet activation (Coughlin, 2005; Lundblad and White, 2005).

Platelet apoptosis triggered by thrombin is not as sensitive as platelet activation. Thrombin at low concentrations can evoke high-level expression of P-selectin and integrin. The loss of Δψm, as well as caspase-3 activation and PS externalization, *via* a thrombin-mediated mechanism, however, is induced in a dose-dependent manner (Leytin et al., 2007). Thrombin activates PAR-1 at the

nucleated cell surface and further modulates apoptosis (Flynn and Buret, 2004). Similarly, PAR-1 signalling pathways are involved in thrombin-initiated stimulating responses and proapoptotic reactions in platelets, which consist of the activation of protein kinase C (PKC), intracellular calcium mobilization, PLC and stimulation of Rho/RhoA kinase, leading to a reorganization of actin cytoskeleton and myosin light-chain kinase activation (Coughlin, 2005; Flynn and Buret, 2004).

1.6.3 Platelet PS exposure

In many mammalian cells, PS is one of the phospholipids in the PM. In intact platelets, PS is situated in the inner leaflet of the phospholipid bilayer and forms asymmetrical distributions with other phospholipids, such as sphingomyelin and phosphatidylcholine (Zwaal, 1978). The asymmetry of membrane phospholipids is regulated by scramblases, floppases and flippases (Hankins et al., 2015). Platelet activation *via* tissue damage or other factors leads to the externalization of PS *via* transport from the inner membrane layer to the cell membrane surface (Perrotta et al., 2003). Then, exposed PS binds to its receptor Tim 4 at the surface of macrophages, which can clear the senescent platelets by a combination with several proteins (Dransfield et al., 2015; Toda et al., 2012). In nucleated cells, PS exposure is one of the essential apoptotic events. The level of PS exposure can reflect the proportion of apoptosis in total stored platelets. The percentage of PS externalization increases in apoptosis, which is driven by different mechanisms (Kile, 2014; Arachiche et al., 2009). Furthermore, platelet function can be directly affected by the proportion of apoptotic platelets (Rinder and Smith, 2003; Shapira et al., 2000)

The platelet PS externalization, which appears after the decrease or loss of Δψm, is a contributor to blood coagulation (Lentz, 2003). PS exposure is an indicator of mid-stage apoptosis (Mourdjeva et al., 2005).

1.7 STIM1 and STIM2 (STIM1/2)

The store-operated Ca^{2+} entry across the platelet plasma membrane (SOCE) (Prakriya and Lewis, 2015) has been converted into a real mechanical model by the discovery of STIM1. The highly homologous protein STIM was detected in many species, including *Caenorhabditis* and *Drosophila* (Roos et al., 2005; Strange et al., 2007; Liou et al., 2005). The STIM family members contain STIM1 and STIM2, which are both expressed in human platelet ER (Berna-Erro et al., 2012). Except for the end of the C-termini and N-termini, the molecular structures of STIM1 and STIM2 are similar. In lymphocytes, STIM1 is a significant activator in antigen-receptor-regulated Orai1 reactions. In contrast, STIM2 manages the level of cytoplasmic Ca^{2+} in resting conditions (Feske et al., 2006; Vig et al., 2006).

STIM protein domain structures

Activation of STIM typically begins with Ca^{2+} discharging from the EF-hand domain, which is located at the ER lumen (Manji et al., 2000), (Soboloff et al., 2006). Usually, STIM1 is distributed extensively throughout the ER (Wu et al., 2006), (Liou et al., 2007) but transfers to PM junctions within a few seconds after store depletion (Liou et al., 2005). Although STIM1 is also present in the PM (Hewavitharana et al., 2008; Spassova et al., 2006), it functions mainly in the ER to stimulate SOCs by moving to the ER-PM junctional region (Lewis, 2007). A decrease in the stimuli initiated by ER Ca^{2+} then improves its discharge from STIM to initiate conformational changes. The resulting physical reactions with

Orai channels trigger Ca^{2+} influx as well as their activation through the PM (Berry et al., 2018). The N-terminal Ca^{2+}-sensing region of STIM1 consists of the EF-hand and SAM domains. The EF-SAM domain in the presence of Ca^{2+} shows high α-helical structures, whereas EF-SAM without Ca^{2+} loading shows a much looser structure (Stathopulos et al., 2006). The C-terminal region in the cytoplasm includes broad helical regions that bind with PM Orai channels, which can cover most ER-PM junctional gaps (Luik et al., 2008). The depletion of stores is proposed to enhance STIM1 in the PM (Zhang et al., 2005; Hauser and Tsien, 2007); however, the activation of SOCs does not require the PM to embed STIM1 (Hewavitharana et al., 2008; Baba et al., 2006).

STIM1-deficient platelets and STIM2-deficient platelets were documented very well in previous studies. However, to the best of our knowledge, the effect of STIM1/2 double conditional deletion on platelet function remained elusive.

1.8 Ceritinib

Ceritinib (Figure 4), an ATP-competitive inhibitor of anaplastic lymphoma tyrosine kinase (ALK) and IGF-1R (Gabay et al., 2015), has been used to treat advanced cancer patients such as non-small cell lung cancer (NSCLC), particularly those who harbor ALK genetic alterations (Shaw et al., 2013; Seto et al., 2013; Solomon et al., 2014).

Further kinases and other targets reported to be sensitive to ceritinib include IGF1R, FAK1, RSK1/2, CAMKK2, FER and ERK1/2 (Kuenzi et al., 2017). The drug is approved for the following indications: ROS1/ALK overexpressed advanced carcinoma, ALK-positive crizotinib naive metastatic NSCLC, ALK-positive NSCLC along with central nervous system metastasis, etc (El-Osta and Shackelford, 2015). Published side effects of ceritinib treatment include

diarrhea, vomiting, nausea, and decreased appetite (Califano et al., 2017). Pharmacokinetic studies revealed that ceritinib is taken up by cytochrome P450 (CYP) 3A (Mok et al., 2017), and eliminated by the CYP3A enzyme (Morcos et al., 2017). The IC_{50} of ceritinib is 0.2 nM (Selleck Chemicals, USA) and around 97% of the drug is bound to plasma proteins (Mok et al., 2017).

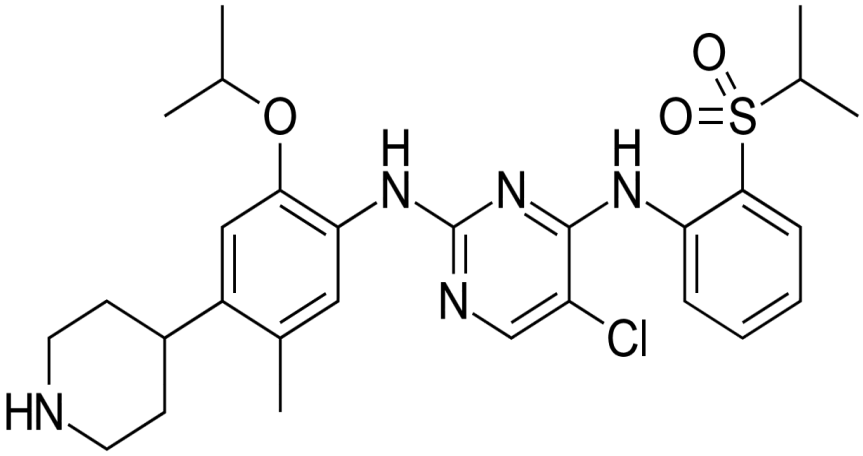

Figure 4: Chemical structure of ceritinib (from Bedi et al., 2018)

Changes of platelet activation biomarkers such as platelet count and the mean platelet volume (MPV) have been reported in many studies on NSCLC (Aoe et al., 2004; Kumagai et al., 2015; Maráz et al., 2013). Ceritinib has previously been shown to trigger apoptosis of tumour cells (Van Erp et al., 2017) and suicidal death or eryptosis of anucleated erythrocytes (Al Mamun Bhuyan et al., 2016). Whether ceritinib treatment affects platelet apoptosis, has not been determined.

1.9 Aim of the study

To the best of our knowledge, there is no report about the effect of combined STIM1/2 deletion on platelet activation and apoptosis. Moreover, nothing is known about the effect of ceritinib on platelets. Thus the current investigation addresses the following objectives:

To explore the impact of STIM1/2 on platelet activation and apoptosis and to investigate whether ceritinib modifies platelet Ca^{2+} signalling, activation and apoptosis, measurements were made without and with exposure of the platelets to collagen-related peptide, a stimulator of platelets mimicking *in vivo* activation by collagen (Nieswandt and Watson, 2003) and being effective by stimulation of STIM/Orai (Zhang and Trebak, 2011). Moreover, some experiments were performed without and with exposure of the platelets to thrombin, which is effective by stimulation of STIM/Orai (Zhang and Trebak, 2011).

2. Materials and Methods

2.1 Materials

2.1.1 Chemicals or antibodies

The used concentrations and manufacturers of chemicals or antibodies in the present studies are listed as follows:

Name	concentration	Manufacturer
Thrombin	0.01 U/ml	Roche, Switzerland
CRP	2-5 μg/ml	R. Farndale, University of Cambridge
Orai1 rabbit anti-mouse antibody	1:200 diluted	Abcam, Germany
Anti-rabbit secondary antibody	1:250 diluted	CF^{TM} 488A; Sigma, USA
Fluo-3	3 μM	Biotinium, USA
Thapsigargin	1 μM	Invitrogen, Germany
P-selectin antibody	1:10 diluted	Emfret, Germany
Integrin αIIbβ3 antibody	1:10 diluted	Emfret, Germany
Annexin-V FITC	1:20 diluted	Mabtag, Germany
Caspase-3 Staining Kit	3:100 diluted	BioVision, CA, USA
CD9-APC antibody	1:100 diluted	Abcam, Germany

CD9-PE antibody	1:100 diluted	Abcam, Germany
DCFDA	10 µM	Sigma-Aldrich, Germany
Praformaldehyde	0.5%	Germany Carl Roth

2.1.2 Mice

Mice with double conditional deficiency of STIM1/2 and mice of C57BL/6 of either gender were used in the present studies. The mice with STIM1 conditional deficiency in PF4 expressing platelets have been described previously (Ahmad et al., 2011). The STIM1/2 double deficient mice of *Stim1/2*$^{fl/fl}$ *Pf4-Cre*$^{+}$ (*Stim1/2*$^{fl/fl}$ *Pf4-Cre*) and the corresponding control mice of *Stim1/2*$^{fl/fl}$ *Pf4-Cre*$^{-}$ (*Stim1/2*$^{fl/fl}$) were generated and genotyped in the laboratory of Prof. Harald Langer (previously: University of Tübingen; current affiliation: Medical Clinic II, University of Lübeck). Briefly, *Pf4-Cre* mice were hybridized with *Stim1/2*$^{fl/fl}$ mice to remove the gene of STIM1/2 in platelets. The generation of *Pf4-Cre* mice (Tiedt et al., 2007) and *Stim1/2*$^{fl/fl}$ mice (Oh-hora et al., 2008; Mancarella et al., 2013) have been explained in detail previously. The C57BL/6 wild-type mice (from Physiology Institute I, University of Tübingen) were used for the ceritinib-related experiments. All assays complied with German animal welfare law. Free access to water and control chow was provided before taking blood (Ssniff, Soest, Germany).

2.2 Preparation of platelets

Platelets were isolated from STIM1/2 mice (including *Stim1/2*$^{fl/fl}$ mice and *Stim1/2*$^{fl/fl}$ *Pf4-Cre* mice, 6-8 weeks old, n = 4-6 pairs) and C57BL/6 mice (2-3 months old, n = 4-5) of the whole blood. Before isolation of platelets, the mice

were killed by cervical dislocation (Carbone et al., 2012), approximately 700 µl of blood was taken from the retro-orbital plexus after the mouse was anaesthetized properly using diethylether (Roth, Germany) (Pelzl et al., 2013). All operations (including anaesthetization and sacrifice) on mice before platelet isolation were kindly done by my colleagues (Anja Umbach and Hong Chen, University of Tübingen). Then, the blood was mixed with 200 µl acid-citrate-dextrose (ACD) buffer in a 2 ml tube (tube 1) (Honisch et al., 2015). Modified Tyrode buffer (200 µl) was gently added into ACD-mixed blood in tube 1. Afterwards, the tube was centrifuged at 260 × g for 5 min, and the supernatant was transferred to tube 2 to obtain platelet-rich plasma (PRP). Then, platelets were pelleted after 640 × g for 5 min centrifugation and moved into tube 3 (Cao et al., 2018a).

Platelets isolated from C57BL/6 mice were partially preincubated with ceritinib (Medchem Express, Germany) diluted with Dimethyl sulfoxide (Sigma, Germany) for 0.5 h at 37°C before stimulation.

Tube 1	700 µl blood+ 200 µl ACD+ 200 µl Modified Tyrode buffer
Tube 2	PRP
Tube 3	Platelets

Experiments were performed with ACD buffer consisting of (in mM), D-glucose 180, citrate 80, citric acid 50 (pH 4.6) and Modified Tyrode buffer consisting of (in mM): NaCl 133, Ca^{2+} 1, D-glucose 5, HEPES 10, $NaHCO_3$ 12, KCl 2.8 and BSA 0.1% (pH 7.4).

Automated hematology analyzer (KX21-N, Sysmex, Germany) was used to measure the concentration of washed platelets. Besides, fresh heparin-anticoagulated whole blood 70 µl was obtained from *Stim1/2$^{fl/fl}$ Pf4-Cre* mice and corresponding littermates *Stim1/2$^{fl/fl}$* mice to measure blood parameters such as platelet count and MPV.

2.3 Ceritinib concentrations used

According to Kuenzi's dose-response curves data, ceritinib inhibited the viability of the most sensitive NSCLC cells (including H650, H23, H1155 and A549 cells) with the IC_{50} ranging from 1 to 2 µM (Kuenzi et al., 2017). Another report showed that ceritinib higher than 1.3 µM could trigger erypotosis (Al Mamun Bhuyan et al., 2016). In the present research, ceritinib has been used at concentrations of 0.9, 1.8 and 2.6 µM, values similar to total plasma concentration in patients under treatment (Nishio et al., 2015).

2.4 FACS analysis

In our work, FACS-Calibur (BD Biosciences, USA) which consists of 4 LASER channels (FL-1, FL-2, FL-3, and FL-4) was utilized for data analysis. All the channels are wavelength-specific:

Name of the LASER channel	wavelength (nm)
FL-1	533/530
FL-2	585/540
FL-3	670
FL-4	675/625

The platelets were stimulated with thrombin of 1×10^{-2} U/ml (5×10^{-3} U/ml in aggregation) or CRP of 2 µg/ml in all detections (except for PS exposure and Caspase-3 activation where 5 µg/ml of CRP was employed) (Liu et al., 2015).

Detection of PS exposure and forward scatter (FSC)

Quantification of apoptotic cells was determined by a FACS Calibur using the Annexin-V-FITC apoptosis kit. Briefly, the indicated agonists (thrombin or CRP) were used to treat platelets (1×10^8/ml) with modified Tyrode buffer at 37°C for 10 min. Afterwards, the samples were stained utilizing an Annexin-V FITC kit containing 1 mM extracellular Ca^{2+} followed by a one-time wash. After a 20 min incubation at 37°C, the samples were immediately analysed at excitation/emission wavelengths of 488/530 nm (FL-1 channel). Then, platelet volume was estimated from forward scatter (Gilio et al., 2010).

Caspase-3 activity

To evaluate the effect of STIM1/2 on platelet caspase-3 activity, we treated platelets (1×10^7/ml) with the indicated agonists in modified Tyrode buffer at 37°C for 10 min. Afterwards, the samples were incubated with a Caspase-3 Staining Kit at 37°C for half an hour. After incubation, all samples were measured with a FACS Calibur in the FL-1 channel (Rukoyatkina et al., 2017).

Ca^{2+} response

Fluo-3 was utilized to detect the Ca^{2+} flux in FACS measurements. The platelets (1×10^6/ml) were first labelled with Fluo-3 dye in a 37°C incubator for 0.5 h. The loaded calcium molecule is a fluorescence-enhanced indicator bound to Ca^{2+}. Then, the samples were stimulated with thrombin for 100 seconds or CRP for 150 seconds and analyzed with the FL-1 channel (Liu, 2018).

Platelet Orai1 protein expression

Platelets (1 × 10^8/ml) were treated with the indicated agonists for 15 min. Afterwards, Tyrode buffer was used to stop the reaction, followed by a 660 × g centrifugation for 5 min. After the samples were washed, they were treated with the Orai1 rabbit anti-mouse antibody for 1.5 h. After removal of the antibody and another wash, the anti-rabbit secondary antibody was employed to incubate platelets for 60 min. During the process, 1% paraformaldehyde was used to fix the platelets within 10 min before the samples were treated with the anti-rabbit secondary antibody. After incubation, platelet samples were analyzed in the FL-1 channel (Liu, 2018).

SOCE

SOCE detection was based on the extracellular Ca^{2+} discharge, and Ca^{2+} was then added in the presence of thapsigargin (1 μM). Platelets were resuspended in 150 μl Ca^{2+}-free buffer to measure this parameter. Subsequently, Fluo-3 was used to stain the platelets for 0.5 h at 37°C. After the samples were rewashed, thapsigargin was utilized to treat the loaded platelets in Ca^{2+}-free Tyrode buffer for 10 min. Finally, 1 mM $CaCl_2$ was added to the treated samples containing thapsigargin. The final concentration of platelets was adjusted to 1 × 10^6/ml. The sample fluorescence was determined by utilizing FACS after 5 min in the FL-1 channel (Liu, 2018).

ROS abundance

DCFDA fluorescence was utilized to evaluate ROS abundance. Platelets (1 × 10^8/ml) were preincubated with DCFDA for 0.5 h at 37°C. Afterwards, CRP was employed to treat the DCFDA-labelled platelets for 10 min. The intensity of DCFDA fluorescence was measured with the FL-1 channel (Liu, 2018).

P-selectin and integrin abundance

Platelets (1 × 10^6/ml) were suspended in the mixture, which consisted of the indicated agonists and fluorophore-loaded antibodies (integrin αIIbβ3 conjugated PE antibody and P-selectin conjugated FITC antibody) at RT for 15 min. Before measurements, the reaction was stopped by supplementing the volume to 200 μl. The two-colour loaded samples were measured by FACS (FL-1 channel and FL-2 channel) (Liu et al., 2016).

Aggregation

Platelet aggregation induced by agonists was investigated according to a previous report (De Cuyper et al., 2013). Briefly, aggregation was evaluated from the proportion of double-labelled cells to total-coloured events. The events in the Q2 region symbolized the double-labelled cells with treatment of the indicated agonists (thrombin or CRP), and the total-coloured events were counted as the sum for the three regions Q1, Q2 and Q4. CD9 antibodies were utilized to stain cells (5 × 10^7/ml) at RT for 15 min. Subsequently, 200 μl of modified Tyrode buffer was used to suspend the platelet pellet after discharging the redundant antibodies. The CD9 APC-labelled platelets were mixed with CD9 PE-loaded cells in a 1:1 ratio, followed by shaking at 600 rpm for 10 min in a 37°C incubator. Next, platelets were activated with agonists upon shaking at 1000 rpm for 4 min. During activation, stained cells were fixed by paraformaldehyde. After incubation, the samples were measured in the FL-1 channel and FL-4 channel, respectively.

2.5 Statistical analysis

GraphPad Prism 5.1 software was employed to analyse the statistical difference between the collected data by unpaired t-test or ANOVA (one-way) with a Tukey test as appropriate. The results are presented as arithmetic means ± SD, n represents the number of independent experiments and $p < 0.05$ denotes statistical significance.

3. Results

3.1 The role of STIM1/2 in platelet function

The present study examined whether STIM1/2 affects blood platelet physiological parameters. To achieve this objective, platelets were isolated from the indicated *Stim1/2$^{fl/fl}$ Pf4-Cre* mice and the corresponding *Stim1/2$^{fl/fl}$* littermates. The platelet count in *Stim1/2$^{fl/fl}$ Pf4-Cre* mice was considerably higher (p-value < 0.05) than that of *Stim1/2$^{fl/fl}$* mice. However, other platelet parameters, including MPV, erythrocyte number and platelet distribution width, showed no significant difference between *Stim1/2$^{fl/fl}$ Pf4-Cre* mice (n = 5, 3 females) and *Stim1/2$^{fl/fl}$* mice (n = 5, 3 females) (Table1).

Table 1: Blood parameters of *Stim1/2$^{fl/fl}$ Pf4-Cre* mice and corresponding *Stim1/2$^{fl/fl}$* mice.

Arithmetic means ± SD are shown (unpaired t-test), (n = 5), * (p < 0.05) denotes statistical difference.

	Stim1/2$^{fl/fl}$	***Stim1/2$^{fl/fl}$ Pf4-Cre***
Platelet number (10^3/µl)	*1018 ± 78*	*1293 ± 116 **
Mean platelet volume (MPV) (fl)	*6.3 ± 0.5*	*6.2 ± 0.6*
Erythrocyte number (10^6/µl)	*9.3 ± 0.3*	*9.4 ± 0.7*
Platelet Distribution Width (PDW) (fl)	*7.0 ± 0.8*	*7.1 ± 0.9*
Mean erythrocyte volume (MCV) (fl)	*51.5 ± 1.2*	*49.2 ± 1.5*

Hemoglobin (g/dl)	*14.6 ± 0.7*	*14.8 ± 1.2*
Erythrocyte hemoglobin concentration (MCHC) (g/dl)	*30.7 ± 0.6*	*30.2 ± 1.7*
Hematocrit (%)	*48.0 ± 4.5*	*46.2 ± 4.2*
Hemoglobin/erythrocyte (MCH) (pg)	*15.7 ± 1.0*	*15.8 ± 1.2*

The role of STIM1/2 in platelet apoptosis

To investigate the impact of the transmembrane molecule STIM1/2 on platelet apoptosis, we used annexin-V-binding FITC analysis to quantify platelet PS abundance by flow cytometry. FSC was utilized to analyse platelet size. As shown in Figure 5, the percentage of annexin-V binding, which is shown by the marked area, was similarly low between *Stim1/2*$^{fl/fl}$ and *Stim1/2*$^{fl/fl}$ *Pf4-Cre* platelets in resting groups. After treatment with thrombin or CRP, the proportion of annexin-V-positive cells was markedly enhanced in both genotypes. However, the impact of the agonists was significantly attenuated in *Stim1/2*$^{fl/fl}$ *Pf4-Cre* platelets compared to *Stim1/2*$^{fl/fl}$ platelets.

The volume of the two types of untreated platelets was similarly high *in vitro*. Treatment with thrombin was followed by marked cell shrinkage, which was determined from FSC in both groups. However, the impact of thrombin was significantly blunted in STIM1/2-deficient platelets. Moreover, FSC markedly decreased in the presence of CRP in both genotypes. Again, defective STIM1/2 considerably reduced the impact of CRP in platelets (Figure 6).

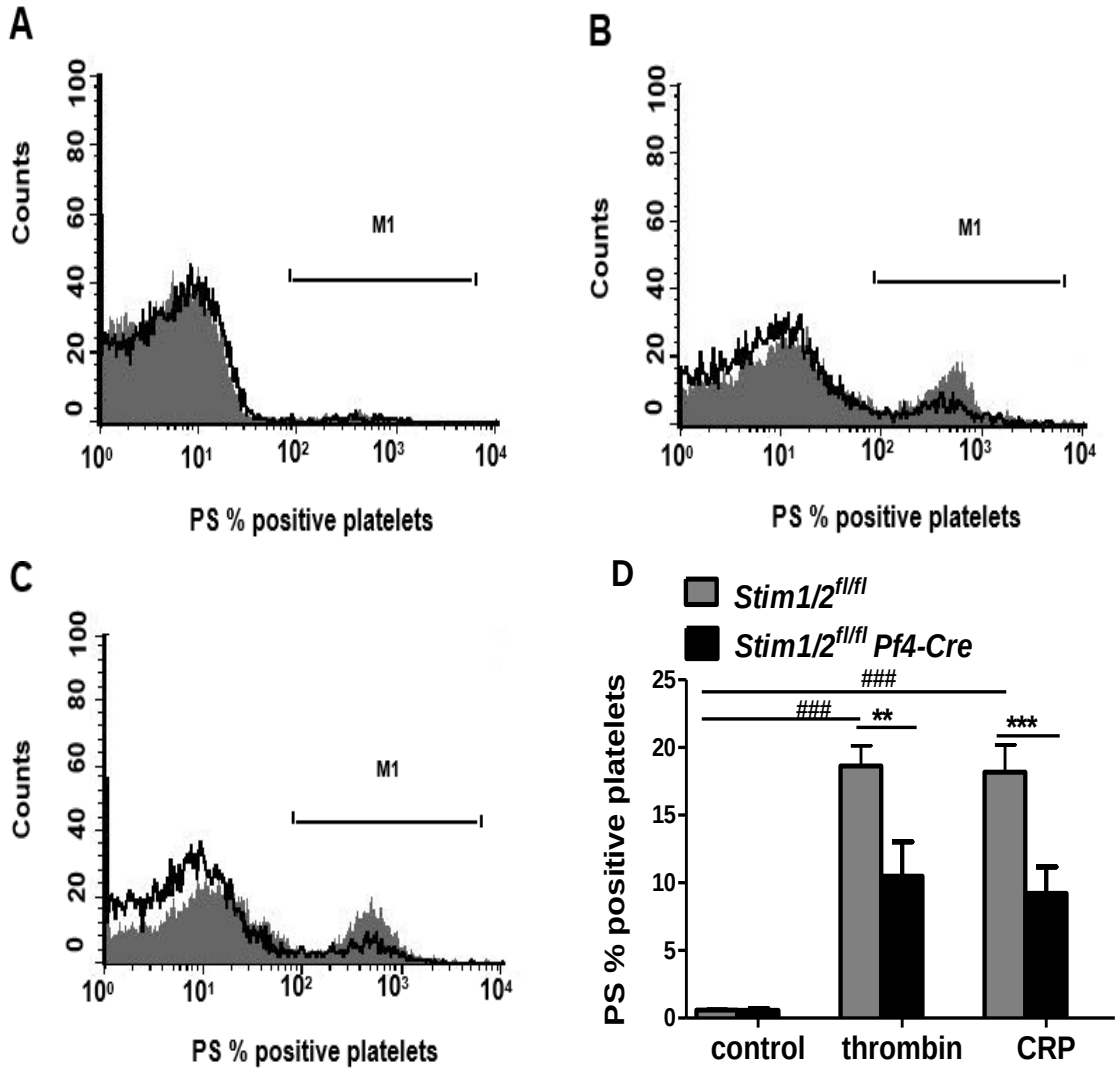

Figure 5: STIM1/2 sensitivity of thrombin- or CRP-induced PS exposure in platelets

A-C. Representative histograms (M1 areas, annexin-V fluos) showing PS exposure at the platelet surface in the absence (A) and presence of (B) thrombin or (C) CRP for 10 min. Black patterns show *Stim1/2$^{fl/fl}$ Pf4-Cre* platelets and grey parts represent *Stim1/2$^{fl/fl}$* platelets. Thrombin: 1×10^{-2} U/ml, CRP: 5 µg/ml.
D. Bar charts indicate *Stim1/2$^{fl/fl}$ platelets* (grey-filled bars) and *Stim1/2$^{fl/fl}$ Pf4-Cre* platelets (black-filled areas) binding to annexin-V-Fluos without and with a 10 min stimulation in the presence of CRP or thrombin. Arithmetic means ± SD are shown, n = 4 independent experiments. ** ($p < 0.01$) and *** ($p < 0.001$) represent statistically significant differences from double conditionally defective STIM1/2, ### ($p < 0.001$) denotes statistically significant differences in the presence of CRP or thrombin (ANOVA).

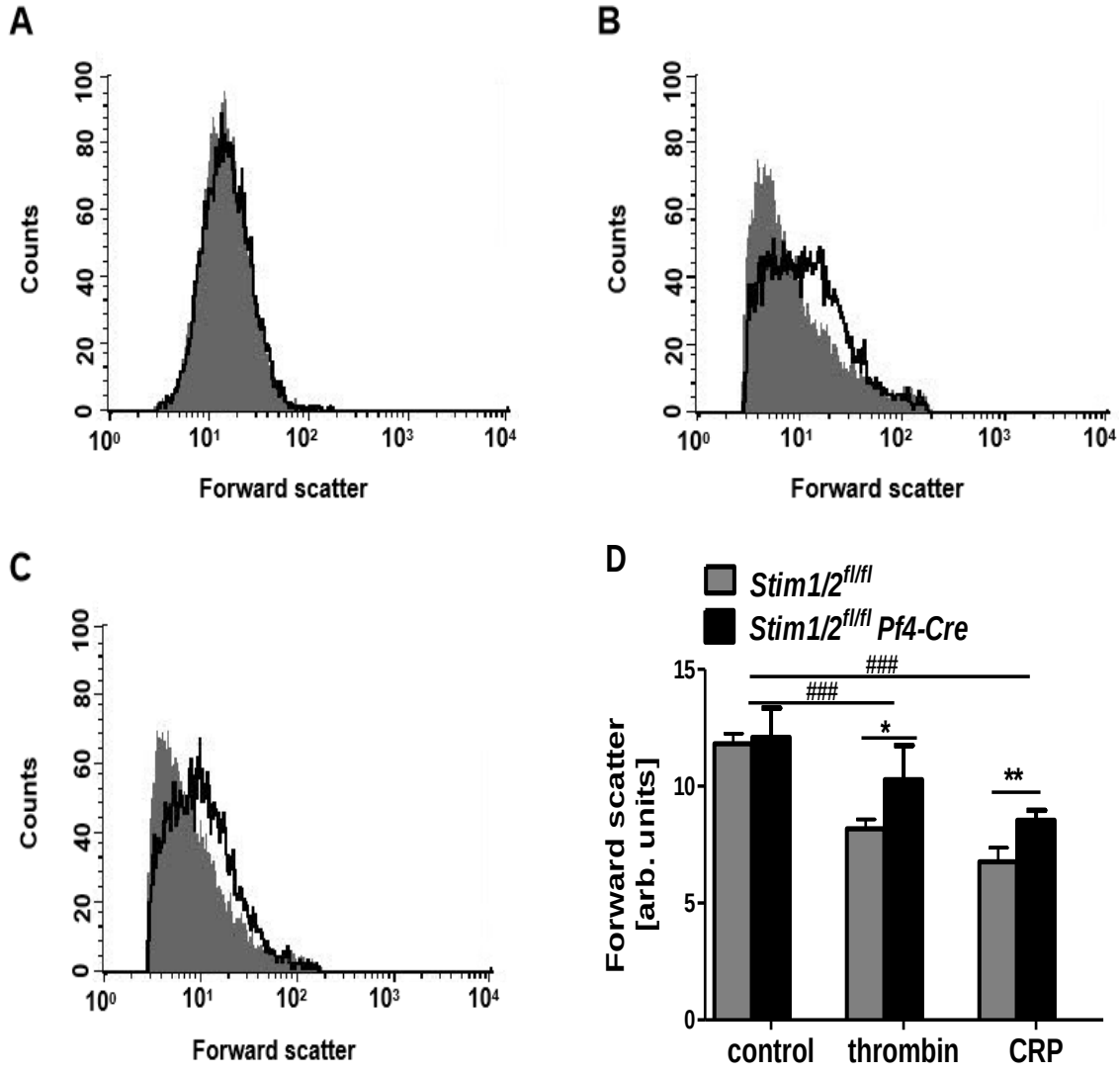

Figure 6: STIM1/2 sensitivity of thrombin- or CRP-induced platelet shrinkage

A-C. Typical histograms of cell shrinkage volume represented from FSC without (A) and with (B) thrombin or (C) CRP in *Stim1/2*$^{fl/fl}$ platelets and *Stim1/2*$^{fl/fl}$ *Pf4-Cre* platelets for 10 min. Black patterns show *Stim1/2*$^{fl/fl}$ *Pf4-Cre* platelets and grey parts represent *Stim1/2*$^{fl/fl}$ platelets. Thrombin: 1×10^{-2} U/ml, CRP: 5 µg/ml.

D. Bar charts representative of FSC from the light intensity units after stimulation for 10 min with thrombin or CRP between *Stim1/2*$^{fl/fl}$ (grey-filled bars) and *Stim1/2*$^{fl/fl}$ *Pf4-Cre* (black-filled areas) platelets. Arithmetic means ± SD are shown, n = 4. ### ($p < 0.001$) represents statistically significant differences with the treatment of CRP or thrombin, * ($p < 0.05$), ** ($p < 0.01$) show statistically significant differences from double conditionally deficient STIM1/2 (ANOVA).

Further experiments examined whether double conditional deficiency of STIM1/2 could affect caspase activity, which was quantified with CaspGlow Fluorescein. As depicted in Figure 7, the proportion of caspase-3-positive platelets (M1 area) was similarly low in the resting groups (Figure 7A, D). With the stimulation of thrombin or CRP, caspase-3-positive platelets significantly increased in both groups. The effect of the agonists, however, was blunted considerably in *Stim1/2$^{fl/fl}$ Pf4-Cre* platelets compared with *Stim1/2$^{fl/fl}$* platelets (Figure 7B-D).

The role of STIM1/2 in the platelet Ca^{2+} response

To further determine whether the decrease in platelet apoptotic events induced by STIM1/2 is related to $[Ca^{2+}]_i$, we used Fluo-3 to investigate cytosolic Ca^{2+} activity (Figure 8). Resting *Stim1/2$^{fl/fl}$* and *Stim1/2$^{fl/fl}$ Pf4-Cre* platelets showed similar $[Ca^{2+}]_i$ values (Figure 8A, D). In the presence of thrombin, $[Ca^{2+}]_i$ was sharply enhanced in both *Stim1/2$^{fl/fl}$* and *Stim1/2$^{fl/fl}$ Pf4-Cre* platelets (Figure 8B, D). The increased $[Ca^{2+}]_i$, however, was substantially less pronounced in double conditionally deficient STIM1/2 platelets than *Stim1/2$^{fl/fl}$* platelets. After CRP treatment for 150 s, $[Ca^{2+}]_i$ was strongly enhanced in both the *Stim1/2$^{fl/fl}$ Pf4-Cre* and *Stim1/2$^{fl/fl}$* groups. Similar to apoptotic events, the $[Ca^{2+}]_i$ was again significantly decreased in double conditionally deficient STIM1/2 platelets (Figure 8C, D).

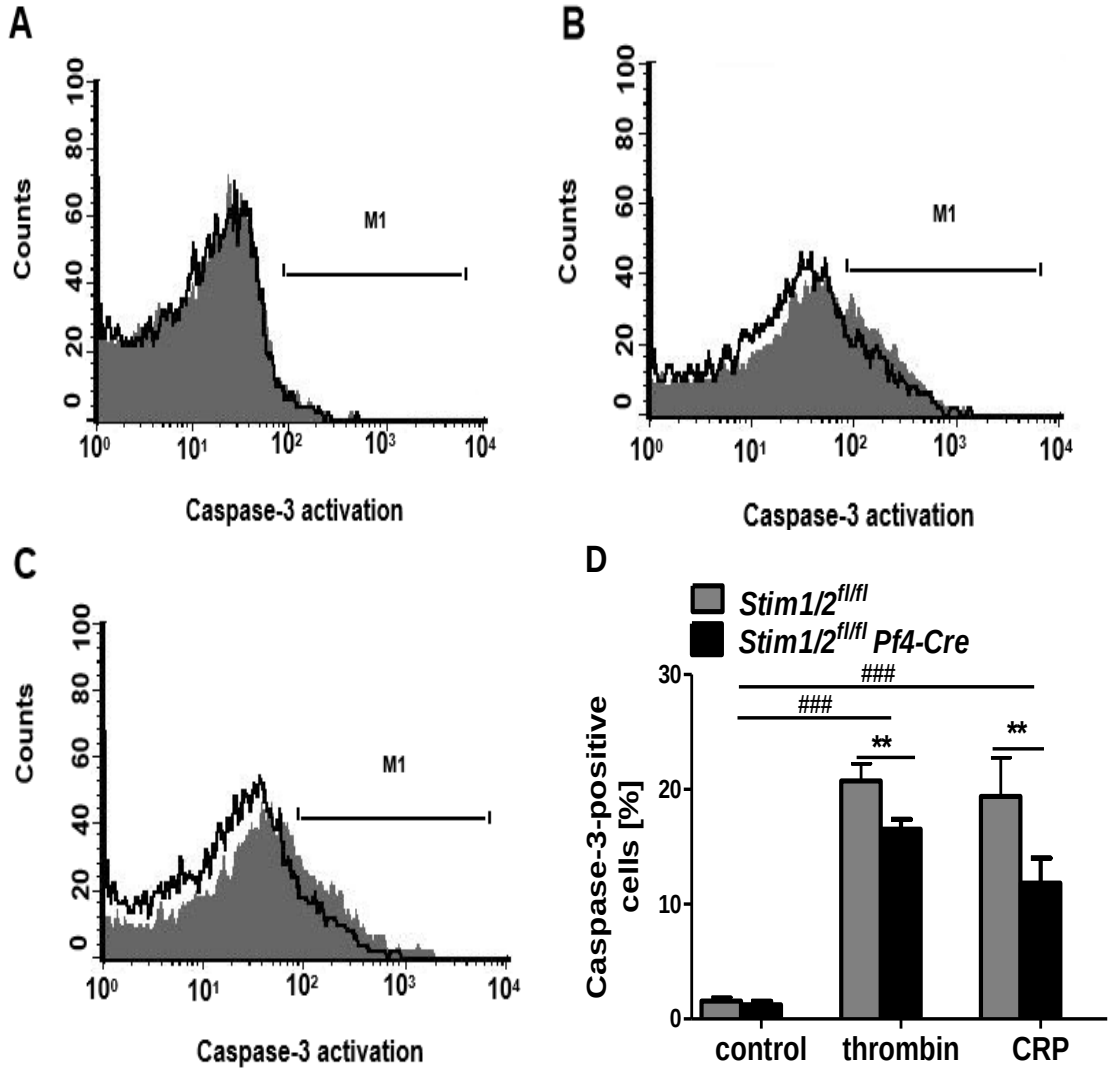

Figure 7: STIM1/2 sensitivity of thrombin- or CRP-induced platelet caspase-3 activity

A-C. Representative histograms (M1 areas) indicate caspase-3 activity in the absence (A) and presence of (B) thrombin or (C) CRP in platelets for 10 min. Black patterns show *Stim1/2$^{fl/fl}$ Pf4-Cre* platelets and grey parts represent *Stim1/2$^{fl/fl}$* platelets. Thrombin: 1 × 10^{-2} U/ml, CRP: 5 µg/ml.

D. Bars representative of the proportional activity of caspase-3 in *Stim1/2$^{fl/fl}$* platelets (grey-filled bars) and *Stim1/2$^{fl/fl}$ Pf4-Cre* platelets (black-filled areas). Arithmetic means ± SD are shown, n = 4. ** ($p < 0.01$) denotes significant differences from double conditionally defective STIM1/2, ### ($p < 0.001$) denotes significant differences with the stimulation of thrombin or CRP (ANOVA).

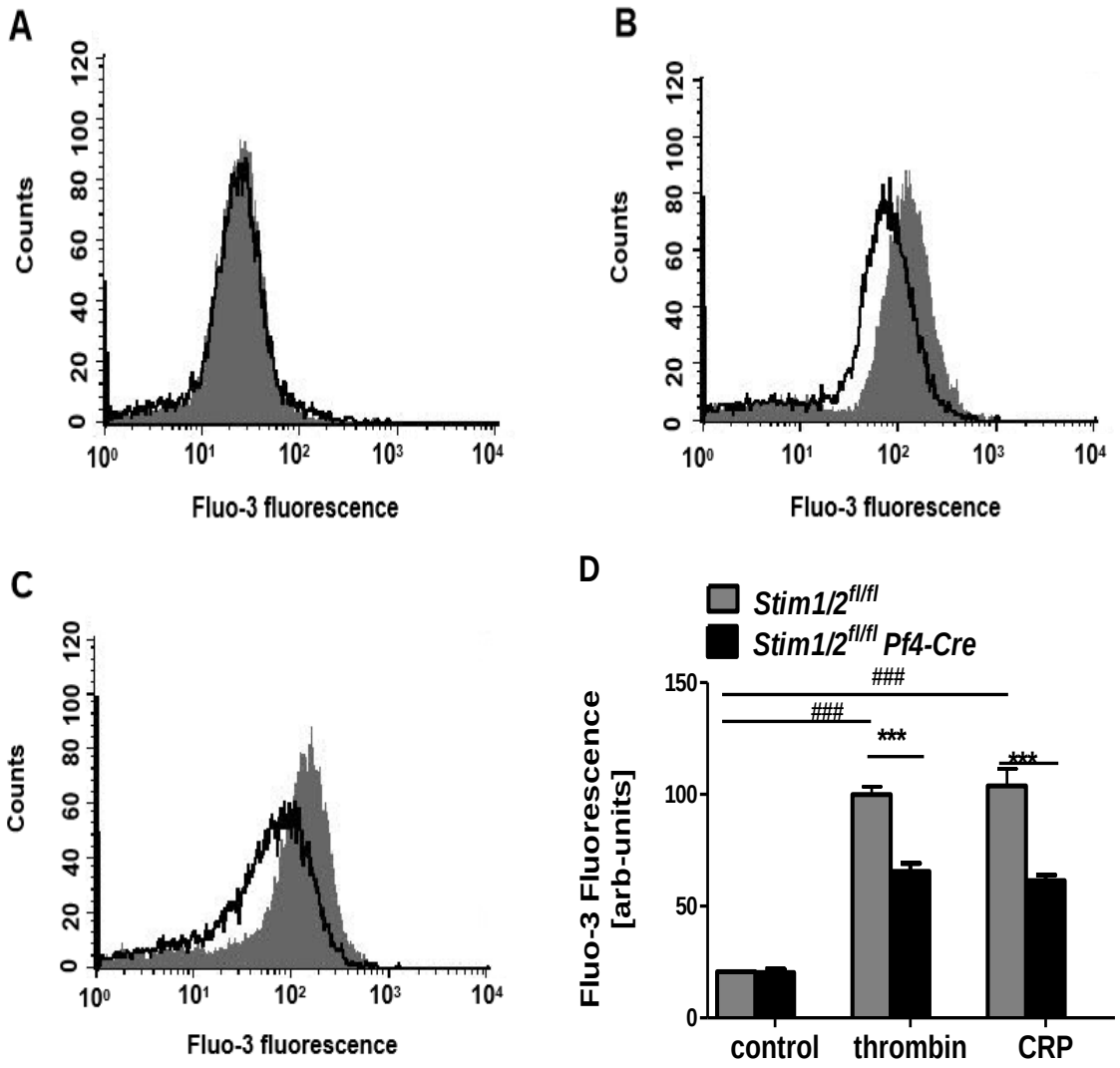

Figure 8: STIM1/2 sensitivity of thrombin- or CRP-induced increase of platelet intracellular Ca^{2+} release

A-C. Original histograms represent cytosolic Ca^{2+} influx utilizing Fluo-3 abundance without (A) and with stimulation by (B) thrombin for 100 s or (C) CRP for 150 s. Grey parts denote *Stim1/2$^{fl/fl}$* platelets, black lines: *Stim1/2$^{fl/fl}$ Pf4-Cre* platelets. Thrombin: 1 × 10^{-2} U/ml, CRP: 2 µg/ml.

D. Bar charts show the Fluo-3 fluorescence in *Stim1/2$^{fl/fl}$* samples *(*grey-filled bars*)* and *Stim1/2$^{fl/fl}$ Pf4-Cre* samples *(*black-filled areas*).* Arithmetic means ± SD are shown, n = 4. *** ($p < 0.001$) shows significant differences from double conditionally defective STIM1/2, ### ($p < 0.001$) indicates statistical differences from the treatment of CRP or thrombin (ANOVA).

The alterations in $[Ca^{2+}]_i$ were quantified from Fluo-3 abundance. The SOCE was evident from the increase in $[Ca^{2+}]_i$ following an extracellular Ca^{2+} re-addition after the Ca^{2+} pool was depleted in the presence of the sarco/endoplasmic reticulum Ca^{2+} ATPase inhibitor thapsigargin.

As shown in *Stim1/2*$^{fl/fl}$ platelets in Figure 9, $[Ca^{2+}]_i$ determined from Fluo-3 fluorescence moderately increased after a 10 min treatment in the presence of 1 µM thapsigargin and markedly rose following the subsequent addition of 1 mM extracellular Ca^{2+} (5 min). In contrast, $[Ca^{2+}]_i$ was almost abolished in *Stim1/2*$^{fl/fl}$ *Pf4-Cre* platelets compared to *Stim1/2*$^{fl/fl}$ platelets after the treatment of both thapsigargin and re-addition of 1 mM extracellular calcium (Figure 9).

For further determination of whether the Orai1 channel is involved in the process, the alterations of Orai1 protein in defective STIM1/2 platelets were measured utilizing FACS analysis. As shown in Figure 10, Orai1 protein abundance at the platelet surface was not significantly altered in resting platelets but was markedly enhanced in the presence of thrombin or CRP for 15 min. Again, Orai1 abundance was significantly decreased in *Stim1/2*$^{fl/fl}$ *Pf4-Cre* platelets compared to the corresponding control *Stim1/2*$^{fl/fl}$ platelets in the presence of both agonists (Figure 10).

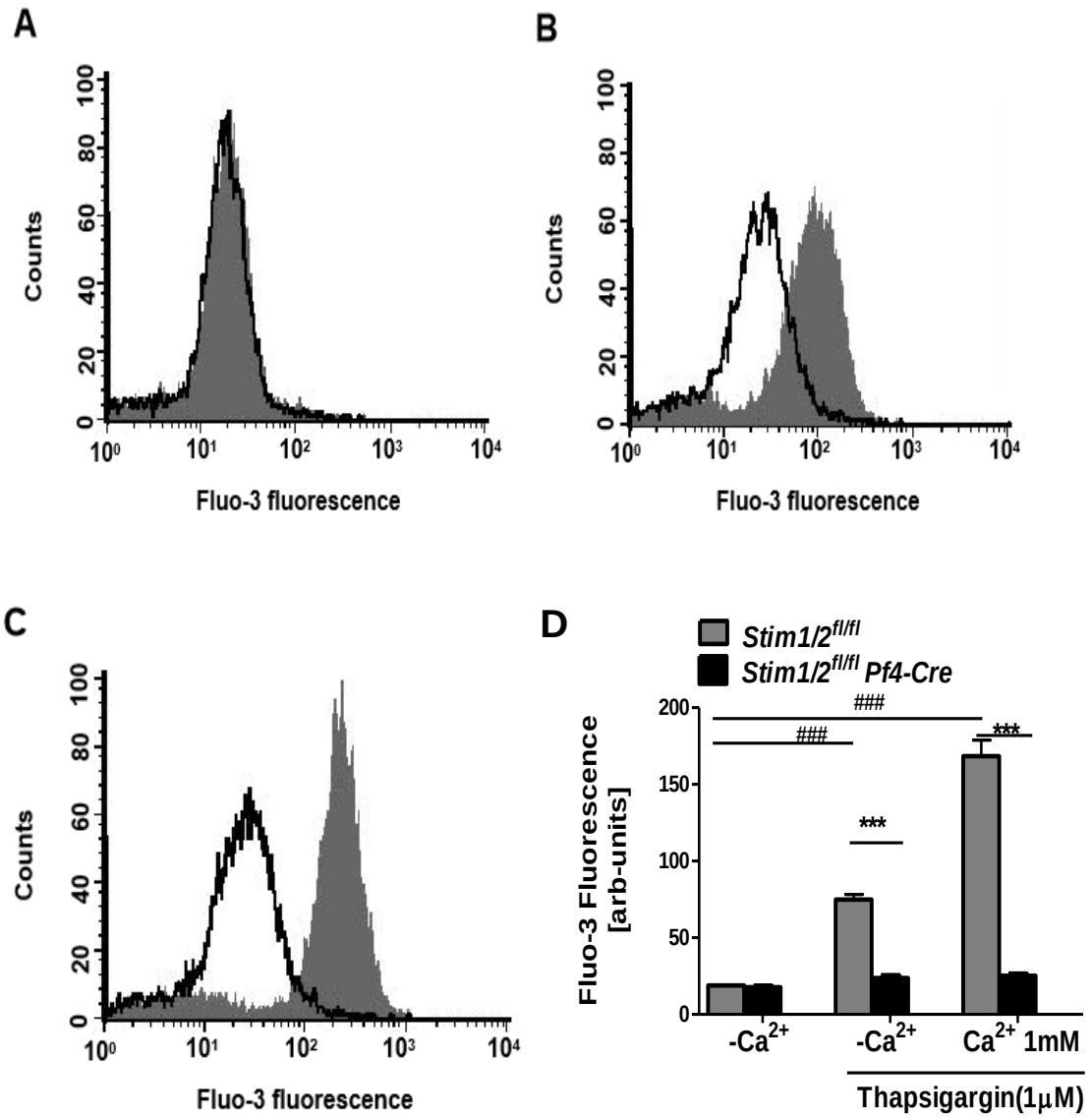

Figure 9: STIM1/2 sensitivity of thapsigargin-induced platelet SOCE

A-C. Representative histograms of Fluo-3 in fluorescence spectrometry before (A), during and after the depletion of Ca^{2+} through the addition of 1 µM thapsigargin in the absence (B, 10 min) and presence (C, 5 min) of 1 mM $CaCl_2$ in control (grey parts) and *Stim1/2$^{fl/fl}$ Pf4-Cre* (black patterns)

D. Bar charts show Fluo-3 fluorescence in control (grey-filled bars) and *Stim1/2$^{fl/fl}$ Pf4-Cre* platelets (black-filled areas). Arithmetic means ± SD are shown, n = 4. ### ($p < 0.001$) denotes significant differences from the exposure of CRP or thrombin, *** ($p < 0.001$) denotes statistical differences from double conditionally defective STIM1/2 (ANOVA).

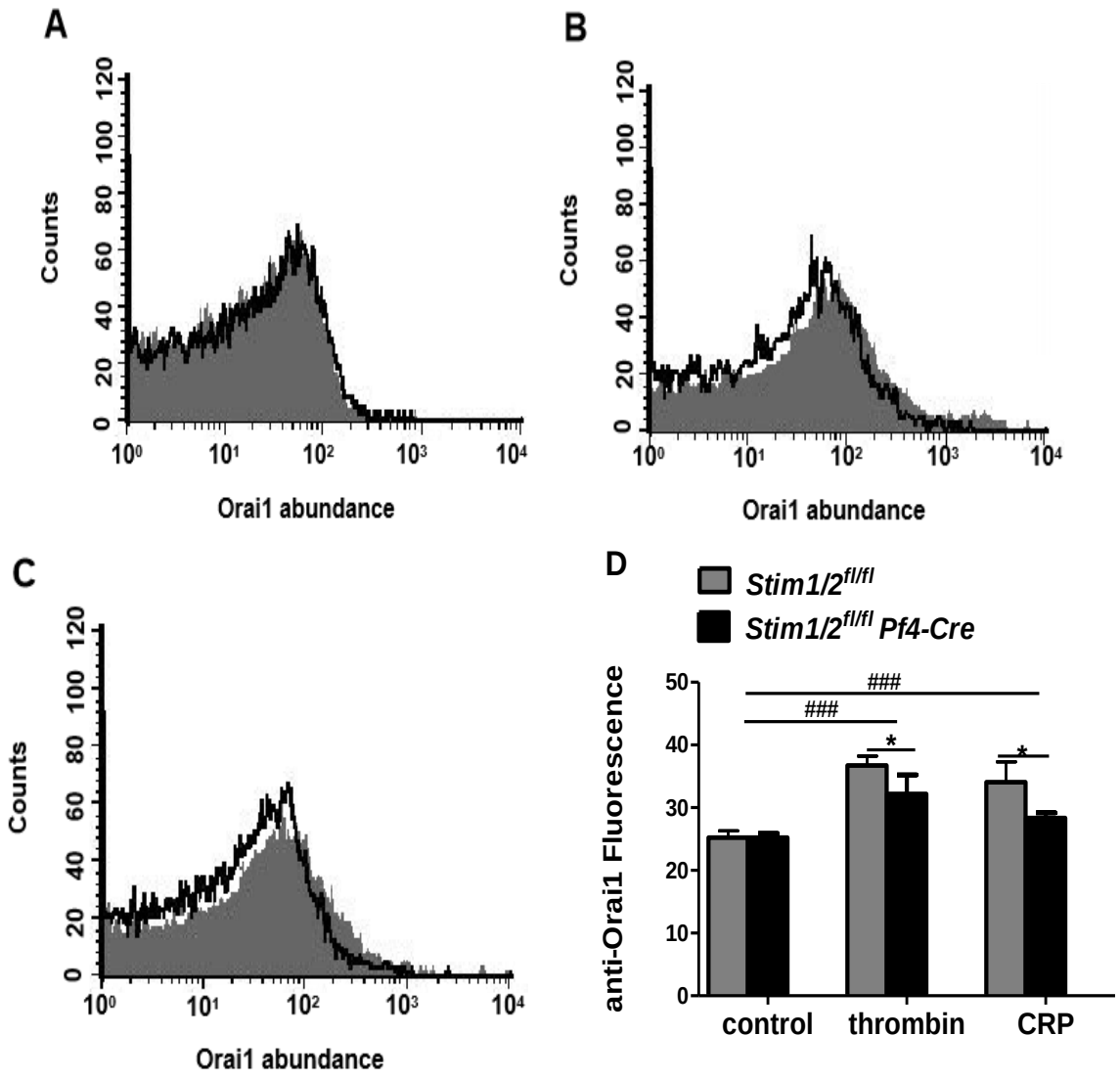

Figure 10: STIM1/2 sensitivity of thrombin- or CRP-induced increase of platelet Orai1 expression

A-C. Original histograms of anti-Orai1 abundance determined by FACS in the absence (A) and presence of (B) thrombin or (C) CRP in platelets for a 15 min treatment. Black patterns show *Stim1/2$^{fl/fl}$ Pf4-Cre* platelets and grey parts represent *Stim1/2$^{fl/fl}$* platelets. Thrombin: 1 × 10^{-2} U/ml, CRP: 2 µg/ml.

D. Charts showing anti-Orai1 fluorescence in *Stim1/2$^{fl/fl}$* platelets (grey-filled bars) and *Stim1/2$^{fl/fl}$ Pf4-Cre* samples (black-filled areas) in response to thrombin or CRP. Arithmetic means ± SD are shown, n = 4. ### ($p < 0.001$) indicates statistical differences from the treatment of CRP or thrombin, * ($p < 0.05$) shows statistically significant differences from double conditionally defective STIM1/2 (ANOVA).

The role of STIM1/2 in platelet activation

The role of STIM1/2 in platelet degranulation was explored in further experiments and was assessed using FACS analysis of platelet P-selectin. The degranulation was negligible at the surface in control platelets that were obtained from *Stim1/2*$^{fl/fl}$ and *Stim1/2*$^{fl/fl}$ *Pf4-Cre* mice, respectively (Figure 11A, D). Following exposure to thrombin or CRP, P-selectin abundance was significantly enhanced in both control and STIM1/2-deficient platelets, and the increase was markedly decreased in *Stim1/2*$^{fl/fl}$ *Pf4-Cre* groups (Figure 11C, D).

To test the effect of STIM1/2 on integrin αIIbβ3, we treated platelets with the fluorescent-labelled specific antibody. Again, the activated integrin αIIbβ3 fluorescence at the platelet surface was negligible in resting cells of both the control and *Stim1/2*$^{fl/fl}$ *Pf4-Cre* groups (Figure 12A, D). The expression of integrin αIIbβ3 was markedly enhanced following thrombin and CRP treatment for 15 min for both genotypes. The impact of thrombin and CRP was again significantly attenuated in *Stim1/2*$^{fl/fl}$ *Pf4-Cre* platelets compared to *Stim1/2*$^{fl/fl}$ platelets.

The role of STIM1/2 on platelet aggregation

For analysis of the impact of STIM1/2 on the aggregation of platelets in the presence of agonists, CD9 PE- and CD9 APC-specific antibodies were employed to treat the samples for 15 min at RT. Double-colour detection was performed with a FACS machine. As depicted in Figure 13, the proportion of two-coloured events indicating aggregation was negligible in both genotypes of resting platelets and was strongly enhanced after 4 min of stimulation with thrombin or CRP. However, the effect of the indicated agonists on platelet aggregation was not affected by the STIM1/2 deficiency.

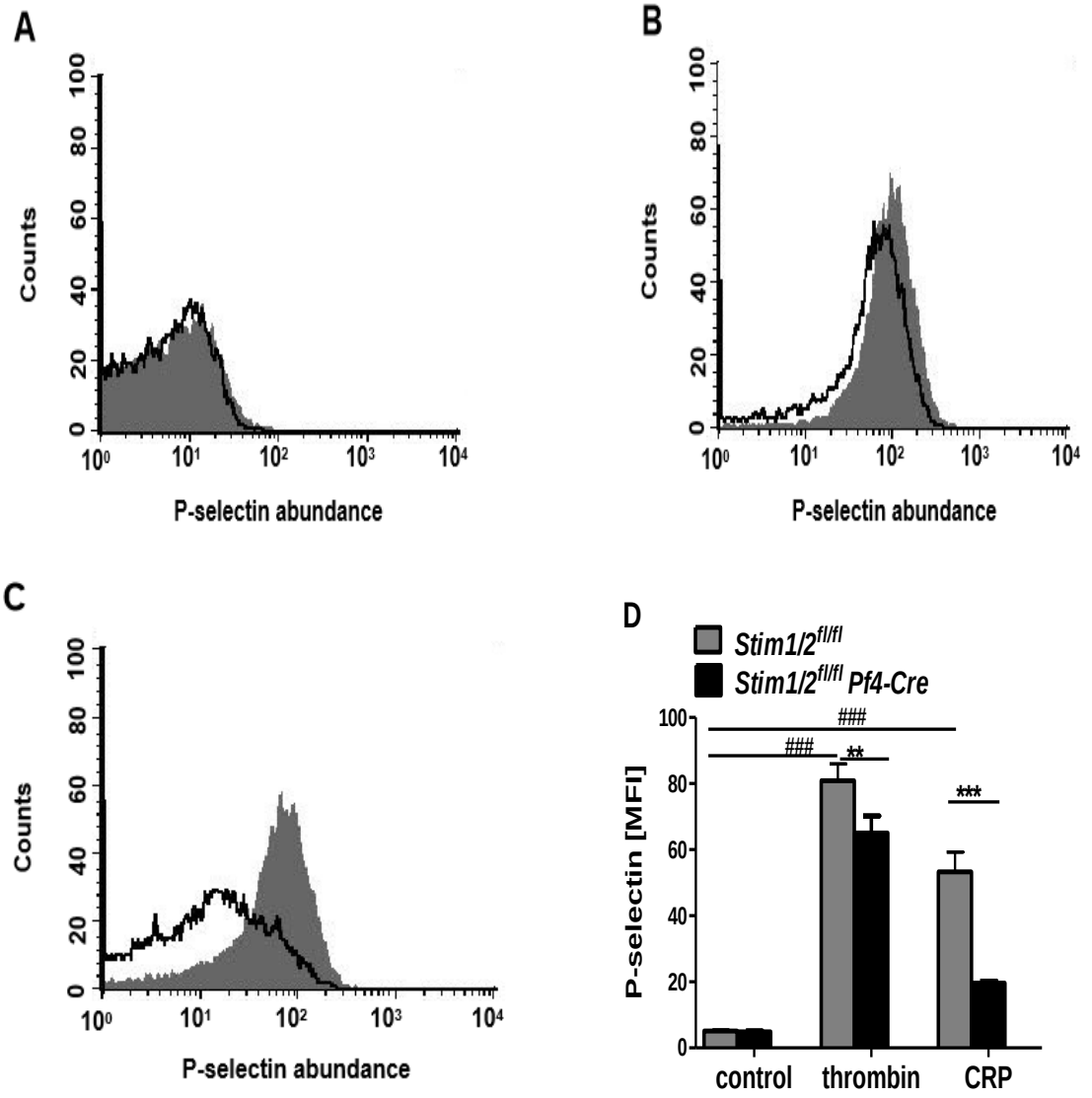

Figure 11: STIM1/2 sensitivity of thrombin- or CRP-induced platelet degranulation

A-C. Typical histograms showed platelet degranulation determined from P-selectin in the absence (A) and presence of (B) thrombin or (C) CRP for a 15 min treatment. Black patterns show *Stim1/2*$^{fl/fl}$ *Pf4-Cre* cells and grey parts represent *Stim1/2*$^{fl/fl}$ samples. Thrombin: 1 × 10^{-2} U/ml, CRP: 2 µg/ml.

D. Bars represent P-selectin expression in the control (grey-filled bars) and *Stim1/2*$^{fl/fl}$ *Pf4-Cre* (black-filled areas) cells. Arithmetic means ± SD are shown, n = 4. ** ($p < 0.01$) and *** ($p < 0.001$) show statistically significant differences from double conditionally deficient STIM1/2, ### ($p < 0.001$) denotes statistically significant differences from the exposure of CRP or thrombin (ANOVA).

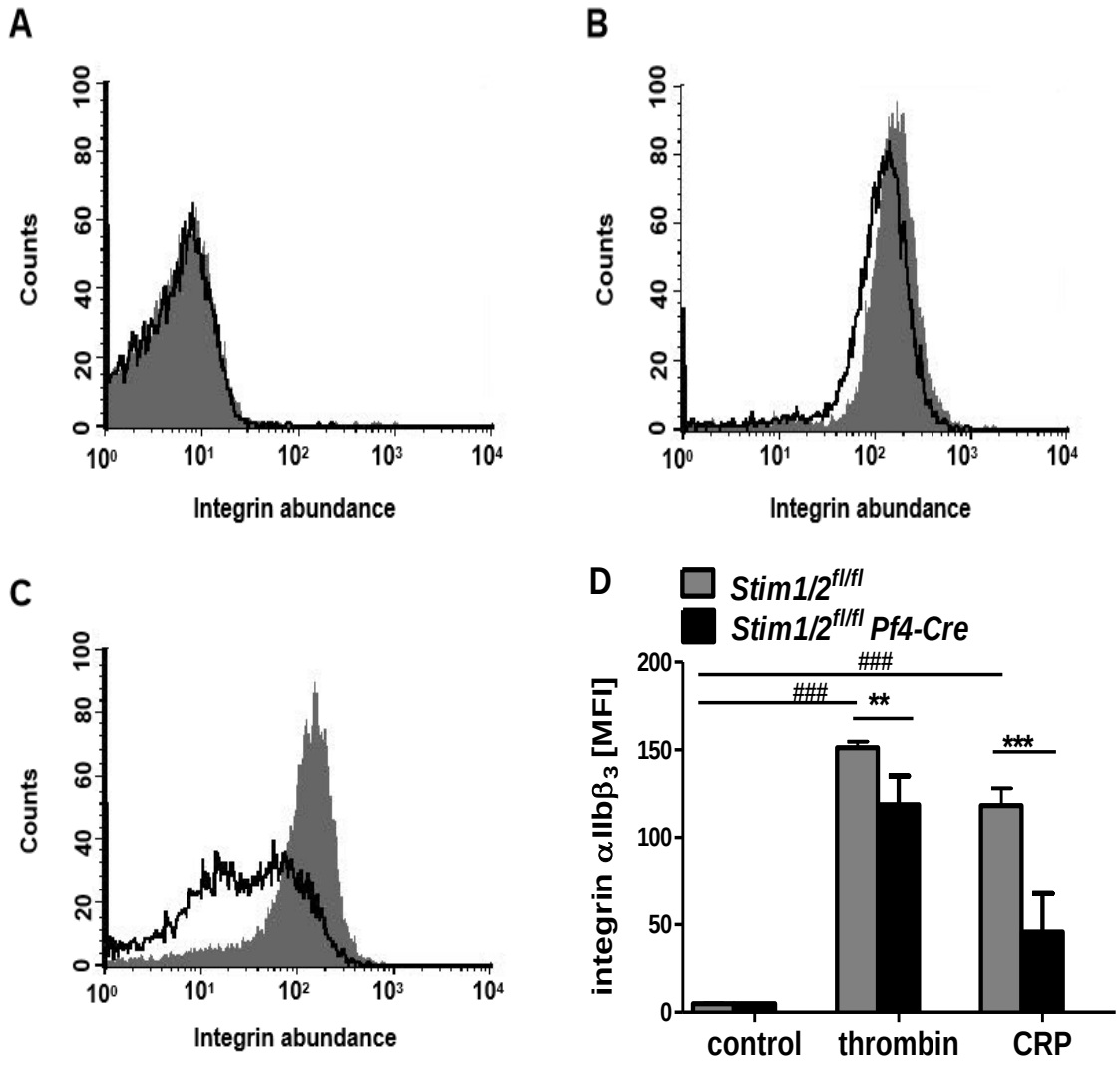

Figure 12: STIM1/2 sensitivity of thrombin- or CRP-induced platelet integrin αIIbβ3 activation

A-C. Typical histograms indicate the αIIbβ3 abundance of platelet αIIbβ3 activation before (A) and after treatment with (B) thrombin or (C) CRP for 15 min. Black patterns show *Stim1/2$^{fl/fl}$ Pf4-Cre* platelets and grey parts represent *Stim1/2$^{fl/fl}$* platelets. Thrombin: 1 × 10^{-2} U/ml, CRP: 2 µg/ml.

D. Bar charts represent the abundance of activated integrin between *Stim1/2$^{fl/fl}$* (grey-filled bars) and *Stim1/2$^{fl/fl}$ Pf4-Cre* (black-filled areas) cells after 15 min of treatment at RT. Arithmetic means ± SD are shown, n = 4. ### ($p < 0.001$) shows statistically significant differences from the treatment of CRP or thrombin, ** ($p < 0.01$) and *** ($p < 0.001$) represent statistically significant differences from double conditionally defective STIM1/2 (ANOVA).

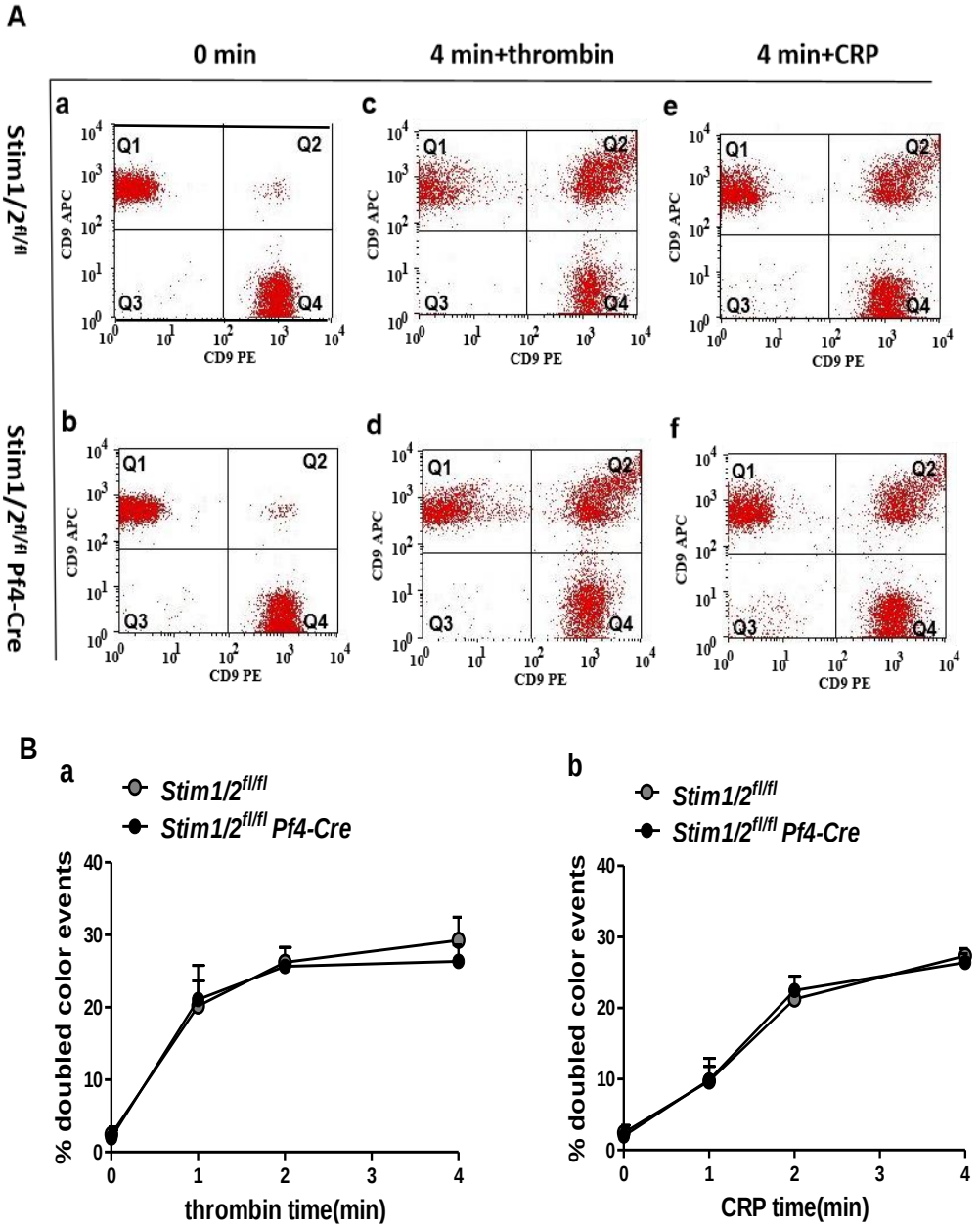

Figure 13: STIM1/2 insensitivity of thrombin- or CRP-induced platelet aggregation

A. Original dot traces show platelet aggregation in *Stim1/2*$^{fl/fl}$ (a, c, e) and *Stim1/2*$^{fl/fl}$ *Pf4-Cre* samples (b, d, f) in the presence of thrombin (c, d) or CRP (c, d) from 0 min (a, b) to 4 min of treatment. Thrombin: 5×10^{-3} U/ml, CRP: 2 µg/ml.
B. Two-coloured dot areas (Q2) denote aggregation of platelets in the presence of (a) thrombin and (b) CRP at the indicated time points (0, 1, 2, 4 min). Grey-filled circles: *Stim1/2*$^{fl/fl}$ platelets, black-filled circles: *Stim1/2*$^{fl/fl}$ *Pf4-Cre* platelets. Arithmetic means ± SD are shown, n = 4. No significant difference was found from double conditionally defective STIM1/2 (ANOVA).

3.2 Ceritinib-sensitive platelet activation and apoptosis

Ceritinib, an ATP-competitive inhibitor of ALK and IGF-1R (Gabay et al., 2015), has been reported to be useful for patients who harbour ALK genetic alterations (Seto et al., 2013; Solomon et al., 2014). ALK mutations have been documented in various malignancies (Shackelford et al., 2014). The present work focused on the effects of ceritinib on platelet function, including apoptosis activation and Ca^{2+} signalling.

CRP-induced effects of ceritinib on platelet apoptosis

Ca^{2+}-regulated phospholipid scrambling also contributes to platelet apoptosis. Hence, further experiments were designed to explore the effect of ceritinib on membrane scrambling of platelets and cell size using FACS analysis. As depicted in Figure 14A and C, the proportion of annexin-V (M1 area) was similarly low in vehicle platelets, regardless of treatment with (0-2.6 μM) ceritinib. Following treatment with CRP, platelet PS exposure was significantly enhanced. There was no statistical difference for ceritinib treatment at 0.9 μM, which was consistent with the previous report of annexin-V-binding in erythrocyte (Al Mamun Bhuyan et al., 2016). However, ceritinib (1.8 and 2.6 μM) treatment significantly blunted annexin-V-binding FITC (Figure 14B, C).

The volume of platelets was examined from FSC. As indicated in Figure 14D and F, platelet FSC was similar before and after ceritinib treatment. After a 10 min exposure to CRP, platelet volume was significantly reduced, and again, the decrease in platelet volume was markedly attenuated by ceritinib at 2.6 rather than 0.9 or 1.8 μM (Figure 14E, F).

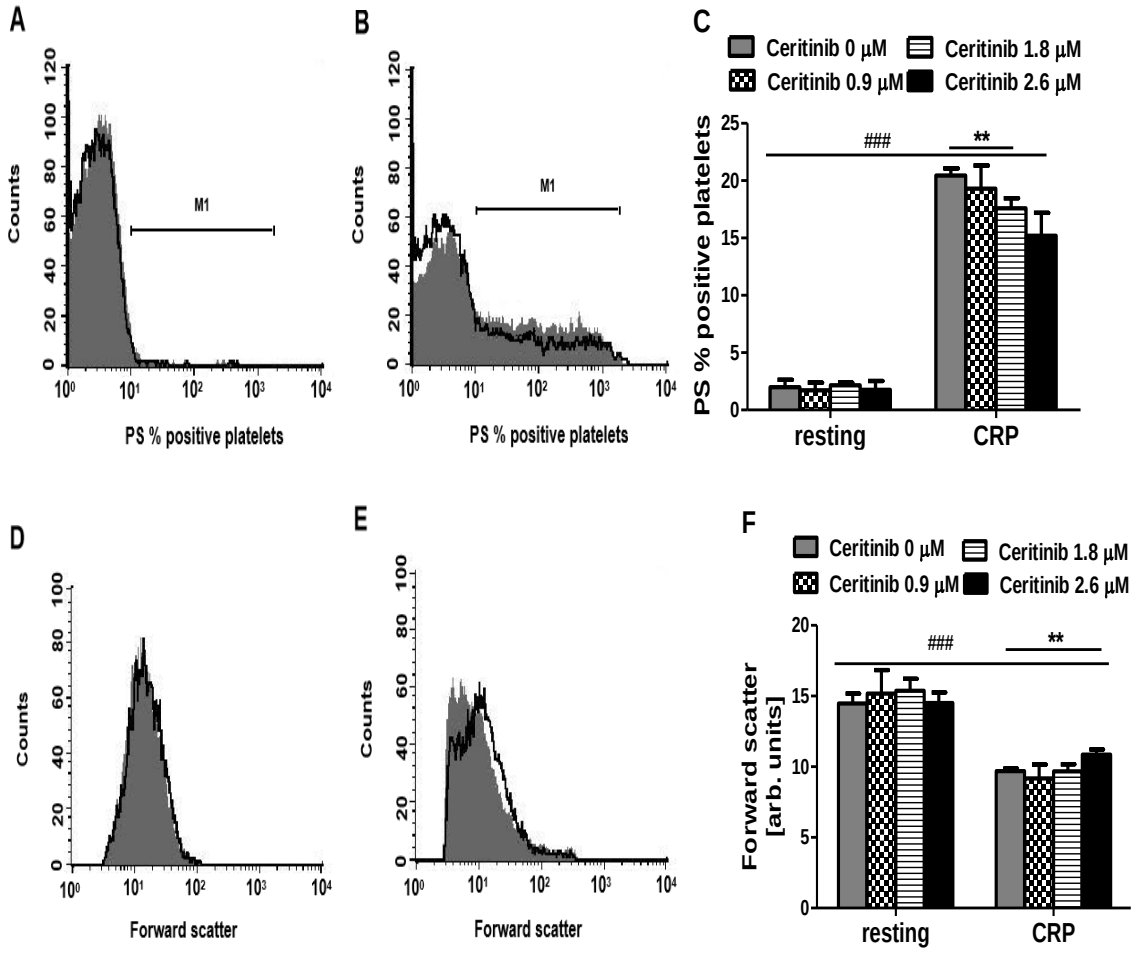

Figure 14: Ceritinib-sensitive of CRP-induced PS exposure and shrinkage in platelets

A, B. Representative histograms (M1 areas) indicate the annexin-V abundance of platelets after a 10 min exposure in the absence (A) and presence (B) of CRP. Grey parts denote vehicle platelets and black patterns represent 2.6 µM ceritinib incubated cells, CRP: 5 µg/ml.
C. Bar charts denote annexin-V staining of platelets treated with vehicle (grey-filled bars) and (0-2.6 µM) ceritinib after 10 min of stimulation in the absence (resting bars) and presence (CRP bars) of the indicated agonist. Arithmetic means ± SD are shown, n = 4.
D, E. Typical histograms showed platelet size without (D) and with (E) a 10 min CRP treatment. Grey parts denote vehicle cells and black patterns represent 2.6 µM ceritinib incubated platelets, CRP: 5 µg/ml.
F. Bars reflect platelet FSC with (right bars) and without (left bars) a 10 min CRP treatment in the vehicle (grey-filled bars) and (0-2.6 µM) ceritinib-incubated cells. Arithmetic means ± SD are shown, n = 4. ** ($p < 0.01$) denotes statistical differences from ceritinib treatment, ### ($p < 0.001$) denotes statistical distinctions from agonist treatment (ANOVA) (from Cao et al., 2018a).

Platelet caspase-3 was detected using a staining kit. As depicted in Figure 15A and C, the proportion of activated caspase-3 platelets was low prior to CRP treatment, regardless of the presence or absence of ceritinib. Moreover, caspase-3-positive cells strongly increased in both groups after treatment with the indicated agonist; again, ceritinib treatment strongly interfered with this increase (Fig. 15B, C).

The effect of ceritinib on the platelet Ca^{2+} response

Further experiments were performed using Fluo-3 abundance and FACS analysis to explore whether ceritinib affects CRP-induced platelet Ca^{2+} signalling (Figure 16). As shown in Figure 16A and C, ceritinib substantially decreased $[Ca^{2+}]_I$ prior to CRP treatment. Following exposure to the indicated agonist, platelet $[Ca^{2+}]_i$ was markedly enhanced. Again, the stimulatory effect of CRP was attenuated by ceritinib (Figure 16B, C).

Orai1 protein alterations in platelets were measured with a fluorescence-related antibody by FACS analysis. As depicted in Figure 17A and D, no alteration in surface Orai1 abundance was detected before and after the ceritinib treatment. After CRP treatment, Orai1 abundance significantly increased in both groups. However, the increased Orai1 surface abundance was markedly blunted in ceritinib-incubated samples (Figure 17C, D).

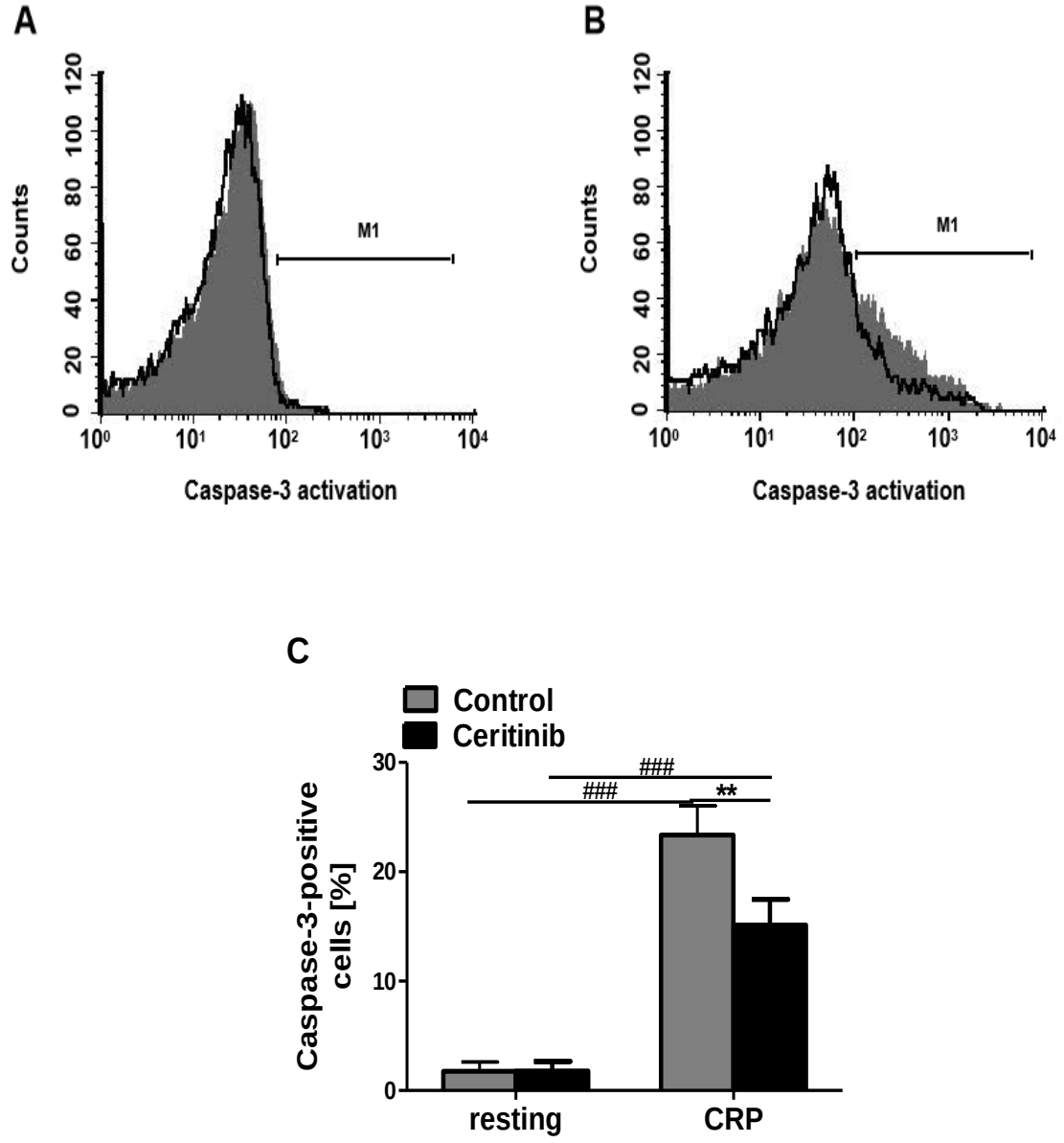

Figure 15: Ceritinib-sensitive of CRP-induced caspase-3 activity

A, B. Typical histograms (M1 areas) reflect caspase-3 fluorescence in platelets in the absence (A) and presence (B) of a CRP exposure for 10 min. Grey parts denote control platelets and black patterns represent 2.6 µM ceritinib treated platelets, CRP 5 µg/ml.
C. Bar charts indicate caspase-3-positive cells of the vehicle and ceritinib-treated samples before and after exposure to the indicated agonist for 10 min. Arithmetic means ± SD are shown, n = 4. ### ($p < 0.001$) points out significant distinctions from the lack of CRP, ** ($p < 0.01$) denotes statistical differences from ceritinib treatment (ANOVA) (from Cao et al., 2018a).

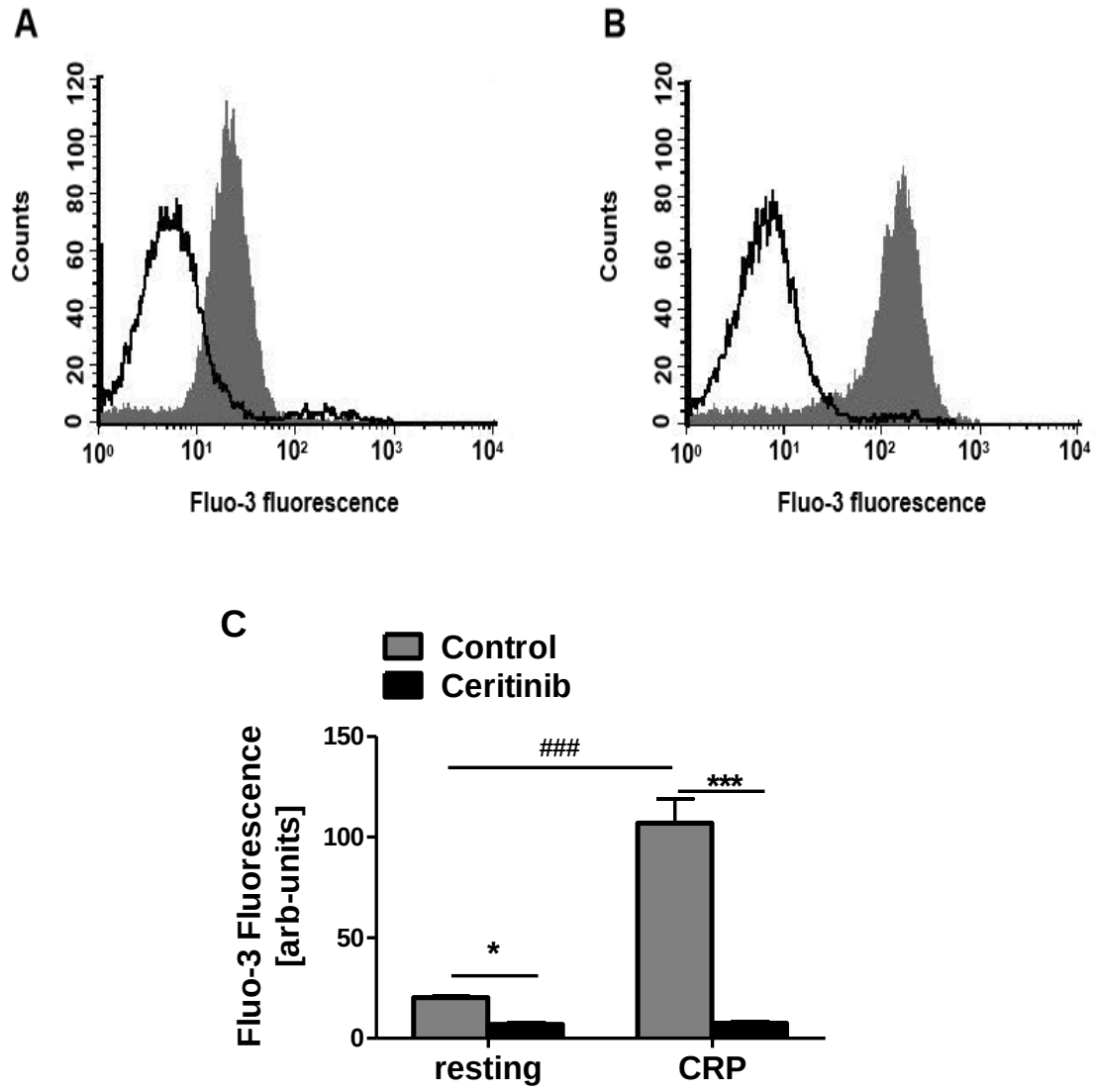

Figure 16: Ceritinib sensitivity of CRP-induced increase of platelet Ca^{2+} concentration

A, B. Typical histograms represent intracellular Ca^{2+} activity from Fluo-3 abundance in platelets in the absence (A) and presence (B) of a 150 s exposure to CRP. Grey parts denote vehicle-treated cells and black patterns represent 2.6 µM ceritinib treated platelets, CRP: 2 µg/ml.
C. Representative bar charts of Fluo-3 abundance in platelets. Grey-filled bars: control cells, black-filled areas: ceritinib treated platelets. Arithmetic means ± SD are shown, n = 4. ### ($p < 0.001$) denotes statistical differences from agonist stimulation, * ($p < 0.05$) and *** ($p < 0.001$) denote significant differences from the lack of ceritinib (ANOVA) (from Cao et al., 2018a).

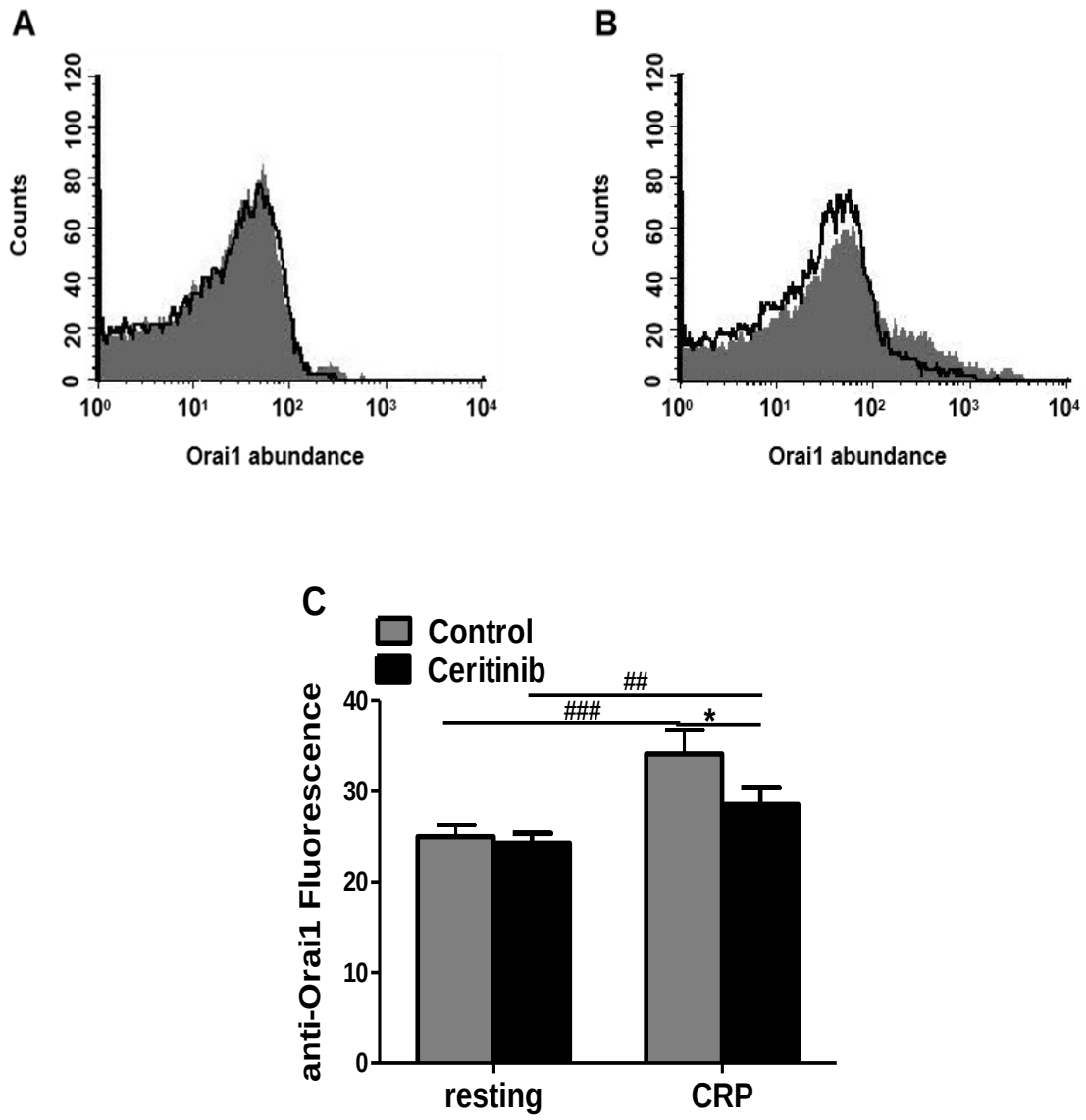

Figure 17: Ceritinib-sensitive of CRP-induced increase of Orai1 protein abundance at the platelet surface

A, B. Representative histograms reflecting Orai1 fluorescence in platelets in the absence (A) and presence (B) of CRP for 15 min exposure. Grey parts denote vehicle cells and black patterns represent 2.6 µM ceritinib incubated platelets, CRP: 2 µg/ml.
C., Bar charts represent Orai1 fluorescence without and with CRP treatment for 15 min. Grey-filled bars: vehicle samples, black-filled areas: ceritinib treated platelets. Arithmetic means ± SD are shown, n = 4. ## ($p < 0.01$) and ### ($p < 0.001$) indicate statistical differences from indicated agonist stimulation, * ($p < 0.05$) represents statistical differences from ceritinib treatment (ANOVA) (from Cao et al., 2018a).

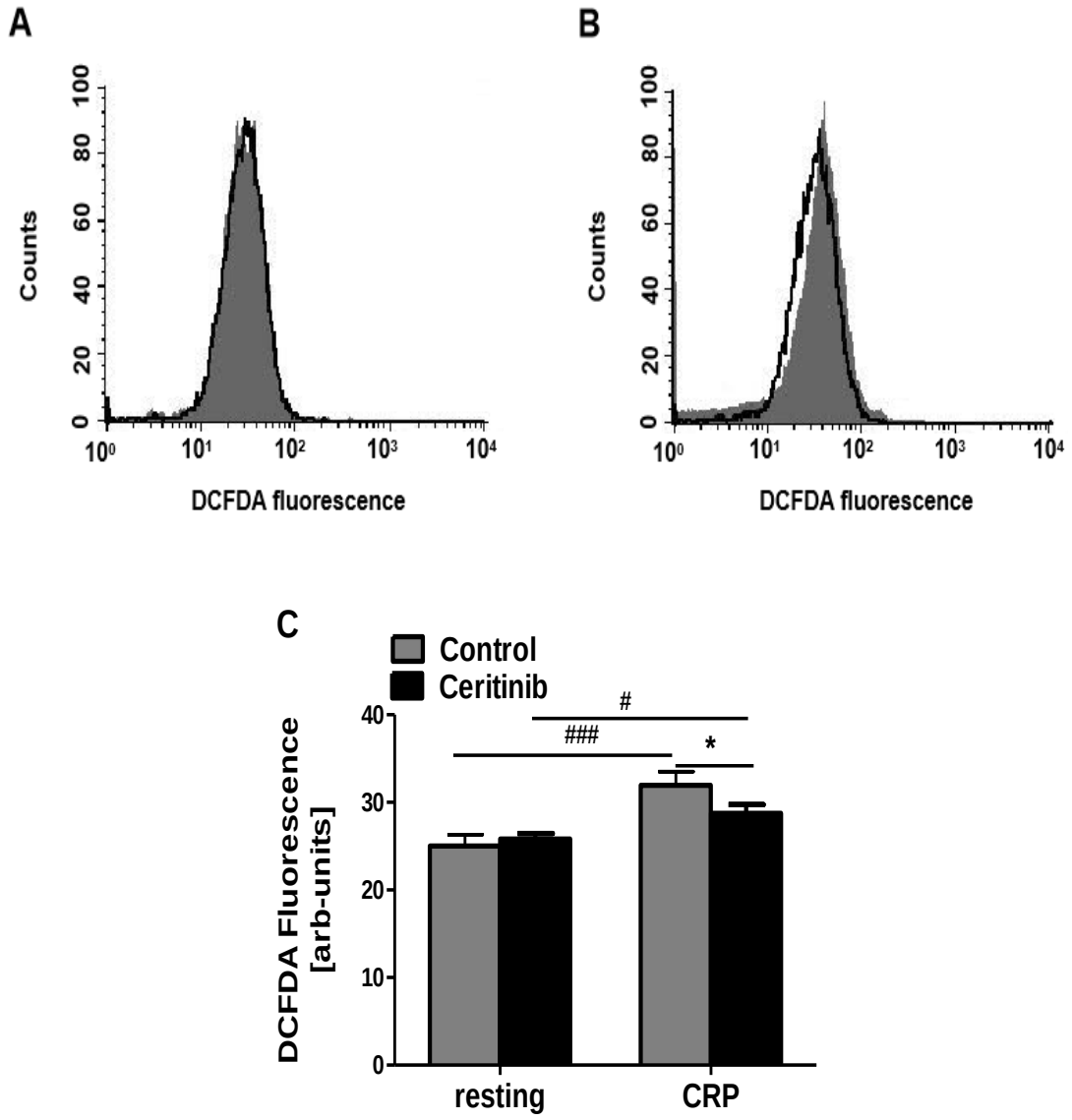

Figure 18: Ceritinib sensitivity of CRP-induced ROS formation

A, B. Typical histograms indicate ROS from DCFDA abundance in the absence (A) and presence (B) of a 10 min exposure to CRP in murine platelets. Grey parts denote control platelets and black patterns represent 2.6 µM ceritinib treated platelets, CRP: 2 µg/ml.
C. DCFDA abundance-related ROS in the control (grey-filled bars) and ceritinib (black-filled areas)-treated platelets in the presence and absence of a 10 min exposure to CRP. Arithmetic means ± SD are shown, n = 4. # ($p < 0.05$) and ### ($p < 0.001$) indicate significant differences from agonist stimulation, * ($p < 0.05$) represents significant differences from ceritinib treatment (ANOVA) (from Cao et al., 2018a).

CRP-induced effects of ceritinib on platelet oxidative stress

DCFDA fluorescence was used to explore the impact of ceritinib on platelet ROS abundance in further experiments. As shown in Figure 18, the DCFDA fluorescence was similarly high in resting platelets, regardless of treatment with or without 2.6 µM ceritinib. Platelet ROS abundance was significantly enhanced by exposure to CRP. However, ceritinib considerably blunted DCFDA fluorescence (Figure 18B, C).

Effect of ceritinib on CRP-induced platelet activation

As shown in Figure 19A and C, platelet P-selectin abundance was negligible without or with exposure to ceritinib. P-selectin abundance was sharply and markedly enhanced in both types of platelets in the presence of CRP. However, the impact of CRP markedly decreased with ceritinib treatment (Figure 19B, C). In addition, the impact of ceritinib on platelet activation was determined from the integrin αIIbβ3 antibody. Integrin αIIbβ3 abundance was the same as that of P-selectin (Figure 19D-F).

CRP-induced effects of ceritinib on platelet aggregation

The experiment was designed to further explore the effect of ceritinib on platelet aggregation. For this goal, CD9-PE and CD9-APC were employed to label the resuspended samples. As shown in Figure 20, CRP quickly enhanced platelet aggregation. However, ceritinib treatment significantly blunted the double-coloured events in the presence of the agonist.

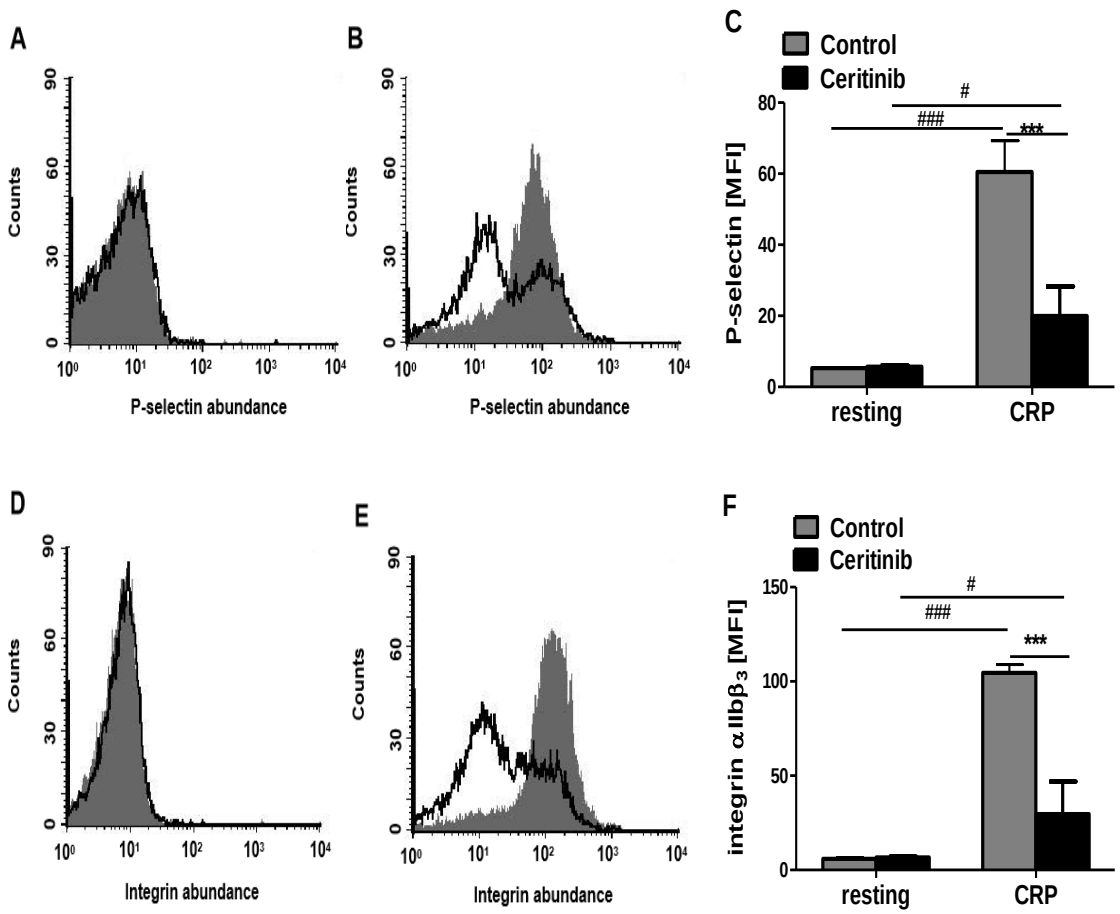

Figure 19: Ceritinib-sensitive of CRP-induced platelet degranulation and integrin activation

A, B. Typical histograms indicating P-selectin fluorescence prior to (A) and after (B) CRP stimulation for 15 min in platelets. Grey parts denote control platelets and black patterns represent 2.6 µM ceritinib incubated samples, CRP: 2 µg/ml.
C. Bar charts reflect P-selectin abundance from the FITC antibody at the platelet surface. Grey-filled bars: vehicle platelets, black-filled areas: 2.6 µM ceritinib incubated platelets. Arithmetic means ± SD are shown, n = 4.
D, E. Representative histograms indicating the activated αIIbβ3 cells in the absence (D) and presence (E) of a 15 min exposure to CRP. Grey parts denote vehicle cells and black patterns represent 2.6 µM ceritinib treated samples, CRP: 2 µg/ml
F. Bars denote integrin αIIbβ3 activation at the platelet surface. Grey-filled bars: vehicle cells, black-filled areas: ceritinib treated platelets. Arithmetic means ± SD are shown, n = 4. # ($p < 0.05$) and ### ($p < 0.001$) point out significant differences from agonist stimulation, *** ($p < 0.001$) represents statistical differences from ceritinib treatment (ANOVA) (from Cao et al., 2018a).

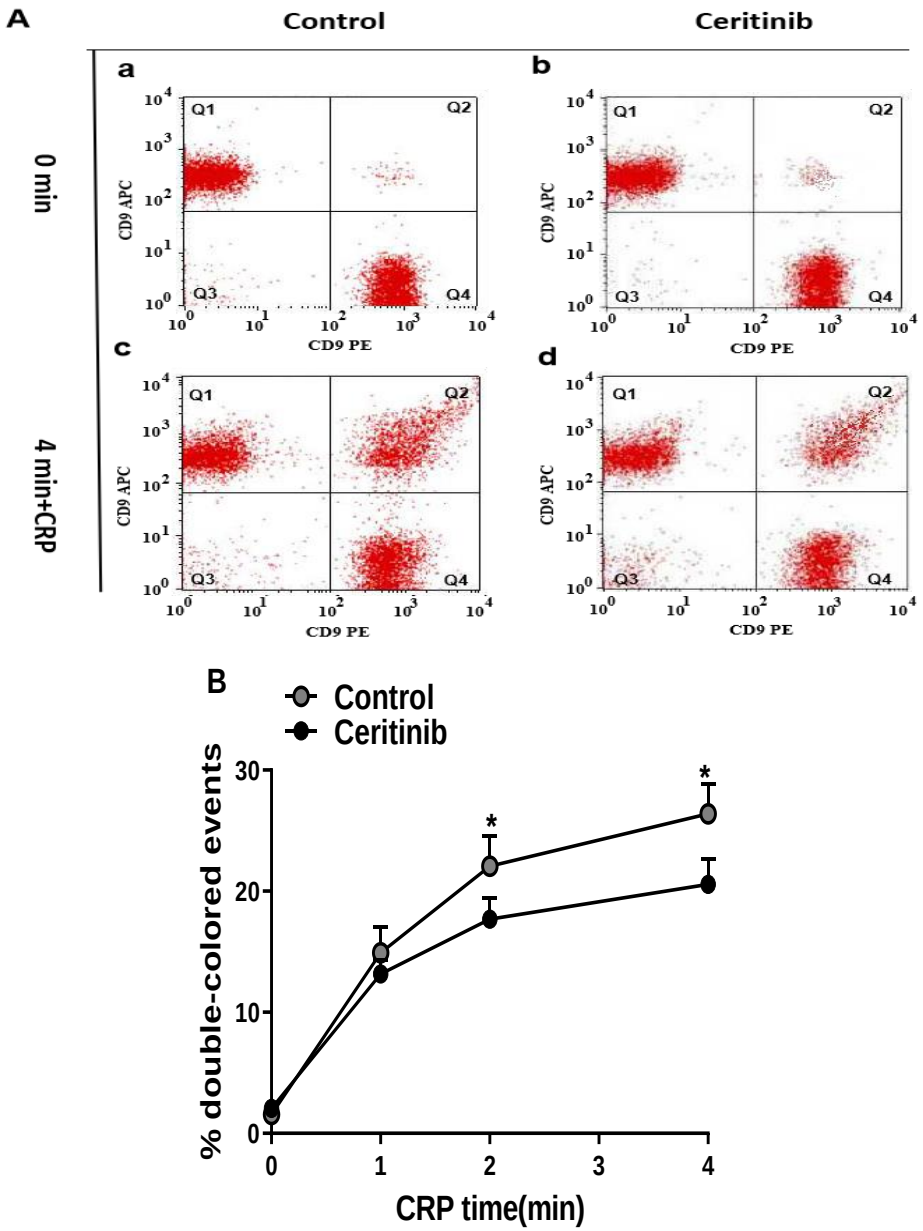

Figure 20: Ceritinib sensitivity of CRP-induced platelet aggregation

A. Typical dot blots denote aggregation from double-coloured samples in the vehicle platelets (a, c) and ceritinib-treated (2.6 µM) platelets (b, d) before (a, b) and after a 4 min (c, d) stimulation with 2 µg/ml CRP.

B. Two-coloured dot areas (Q2) represent platelet aggregation in the absence and presence of agonist treatment at the indicated time points. Grey circles: vehicle cells, black circles: ceritinib incubated samples. Arithmetic means ± SD are shown, n = 4. * ($p < 0.05$) denotes statistically significant differences from the lack of ceritinib (ANOVA) (from Cao et al., 2018a).

4. Discussion

Platelets play significant roles in haemostasis and are involved in various pathophysiologic processes, such as vascular occlusion and inflammation (De Gaetano, 2001; Harrison, 2005). In platelets, Ca^{2+} influx is in large part accomplished by SOCE, which is mediated *via* Orai/STIM (Lang et al., 2013a). Intracellular Ca^{2+} concentration has a strong influence during the complex activation process in platelets, which involves platelet degranulation, aggregation, integrin activation, platelet oxidative stress (Rakesh et al., 2014) and PS exposure (Bergmeier and Stefanini, 2009). Currently, platelets are proposed to support cancer cell metastasis, atherosclerosis, and type II diabetes (Lannan et al., 2015).

The present study uncovered the roles of STIM1/2 and the influence of ceritinib in the regulation of platelet Ca^{2+} signalling and activation as well as apoptosis, which were realized using conditional deletion of STIM1/2 in a platelet model and analysis of the ceritinib effects.

4.1 The effect of double conditionally deficient STIM1/2 on platelet function

STIM1 and STIM2 belong to the STIM family and are both found in human platelet ER (Berna-Erro et al., 2012). STIM2 has been suggested as a weaker activator than STIM1 during the interaction with Orai1 (Bird et al., 2009), partially due to the different time courses of STIM2 activation (Zhou et al., 2009; Parvez et al., 2008). Moreover, STIM1 exhibits higher Ca^{2+} sensitivity than STIM2 (Brandman et al., 2007), leading to a constitutive Ca^{2+} influx in several cell types, probably owing to high resting Ca^{2+} levels in the ER (Parvez et al., 2008;

Soboloff et al., 2006). In particular, STIM1 is considered to be the major isoform in SOCE *via* STIM induction (Berna-Erro et al., 2012). However, the roles that STIM1/2 play in platelets have not been fully elucidated.

Either STIM1-deficient cells or STIM2-deficient cells were used in previous studies. STIM1 KO in C57/BL6 mice was perinatally lethal, while STIM2 KO mice survived several weeks after delivery (Oh-Hora et al., 2008). The double STIM1/2 KO mice have not been described, probably due to early embryonic lethality (Varga-Szabo et al., 2008). Because of these potential limitations, the conditional knockout of both STIM1 and STIM2 in platelets has become one of the most effective approaches to understand the effect of STIM on platelet function. Double conditional knockout of STIM1 and STIM2 was reported before in mouse thymocytes (Cheng et al., 2012; Oh-Hora et al., 2008), in which the T-cell function was pronouncedly declined, and the mice showed severe self-immune-like symptoms (Mancarella et al., 2013).

Prior to activation, PS exposure, caspase-3 activation, $[Ca^{2+}]_i$, Orai1, P-selectin, αIIbβ3 integrin activation, and FSC were similar in platelets isolated from mice with conditional deletion of STIM1/2 and their corresponding littermates. Treatment with thrombin or CRP significantly enhanced platelet PS exposure, caspase-3 activation, $[Ca^{2+}]_i$, surface Orai1 abundance, degranulation, and integrin αIIbβ3 activation and markedly decreased cell volume. The effect of the indicated agonists was significantly less in *Stim1/2*$^{fl/fl}$ *Pf4-Cre* platelets than in *Stim1/2*$^{fl/fl}$ platelets. However, thrombin or CRP still significantly enhanced platelet PS exposure, caspase-3 activation, $[Ca^{2+}]_i$, surface Orai1 abundance, degranulation, and integrin αIIbβ3 activation and still decreased cell volume in *Stim1/2*$^{fl/fl}$ platelets. In contrast, thapsigargin-induced SOCE was virtually abolished in double conditionally deficient STIM1/2 platelets. Thus, it appears

that thrombin- or CRP-induced platelet activation and apoptosis only partially depends on SOCE and STIM1/2.

STIM has been proposed to participate in platelet SOCE (Prakriya and Lewis, 2015). Our studies demonstrated that STIM1/2 plays an essential role in platelet activation and apoptosis, especially in platelet SOCE. However, STIM1/2 is not critical for the aggregation of platelets under flow conditions.

STIM1/2 is an important molecule in the SOCE of platelets based on the present results. When comparing the platelet activation and apoptosis of STIM-deficient platelets, we detected both differences and similarities to prior work. In accordance with a previous report (Varga-Szabo et al., 2008), the most important impairments were platelet activation of P-selectin and integrin αIIbβ3 in STIM1 KO platelets by GPVI induction. As shown by our data, GPVI-dependent P-selectin and αIIbβ3 activation in *Stim1/2*$^{fl/fl}$ *Pf4-Cre* platelets were impaired by nearly half compared to those of *Stim1/2*$^{fl/fl}$ platelets. However, platelet aggregation had no noticeable difference in *Stim1/2*$^{fl/fl}$ *Pf4-Cre* platelets and control platelets. This impairment in activation, however, did not affect platelet aggregation *in vitro*. A similar conclusion was reached in Ahmad's research (Ahmad et al., 2011).

Contrary to recent findings (Gilio et al., 2010; Varga-Szabo et al., 2008) (Table 2), our data suggested that STIM1/2 is not necessary for platelet aggregation. One explanation for these results is methodological diversity. Another more critical difference among these investigations comes from the use of different kinds of platelet models. Notably, both Gilio and Varga-Szabo performed studies utilizing chimeric mice with a total STIM1 KO in hematopoietic cells and the present work used a conditional deletion of STIM1/2 in PF4 mice, which may be the reason why different findings were achieved. As mentioned above, the

STIM1/2 double knockout of whole cells can lead to early embryonic lethality. Also, deficient STIM1 in every cell results in possible restrictions in experiments assessing thrombosis of whole blood in the chimeric mice (Ahmad et al., 2011). Various inflammatory factors are systemically released due to lethal radiotherapy, which is required to generate chimeric mice (Van der Meeren et al., 2001). The inflammatory factors might have a negative effect on circulating platelets (Oleksowicz et al., 1994). Thus, chimeric mice should recover for some time before they are used for experiments. In the present study, the aforementioned complications were minimized in STIM1/2 conditional KO platelets. STIM1/2 double conditionally deficient models were also generated and analysed as in T cells (*Stim1*$^{fl/fl}$*Stim2*$^{fl/fl}$ *CD4-Cre*) (Oh-Hora et al., 2008) and smooth muscle cells (*SM22α-CreKI*$^{+/-}$*/Stim1/2*$^{loxP/loxP}$) (Mancarella et al., 2013) before.

Different results were obtained using different types of agonists and varying concentrations, which uncovered the complex mechanism of platelet aggregation. These discoveries suggest that platelet aggregation is not influenced by SOCE directly *via* STIM1/2. Ahmad suggested that intracellular Ca^{2+} stores might activate $P2Y_{12}$ signalling, further triggering the Rap1/integrin reaction (Ahmad et al., 2011). According to his results, platelet P-selectin and integrin activation were mediated *via* a dose-dependent mechanism in the presence of thrombin receptor PAR4-activating peptide. The present data indicated that thrombin could trigger significant and stable activation at the platelet surface. One likely explanation is that STIM2 plays a compensatory role in the absence of STIM1, driven by thrombin at specific concentrations. In STIM1/2 double conditional knockout platelets, the feasible effect of this compensation is eliminated. The compensatory effect between STIM1 and STIM2 may have led to different results in different cells when the function of a

single gene was inhibited. Further efforts need to be made to explore the interaction between STIM1/2-mediated SOCE and agonist-induced platelet aggregation.

PS exposure regulates the structure and physiological function of platelets in several ways. For example, PS located at the platelet surface first combines with macrophages and is subsequently cleared by engulfment (Badlou et al., 2006), triggering thrombin release and platelet pro-coagulant activity (Wolfs et al., 2005; Harper and Poole, 2011; Mahaut-Smith, 2013). Moreover, PS exposure can activate blood coagulation and consequently contribute to haemostasis (Lhermusier et al., 2011).

A similarity was investigated in platelets with PS exposure that were isolated from mice with a mutated, inactive form of Orai mediated *via* STIM1 (Bergmeier et al., 2009), and this finding was stated by a previous report in *Stim1*$^{-/-}$ platelets (Gilio et al., 2010). The data showed that in the presence of the GP (VI)-dependent agonist convulxin, PS exposure was considerably enhanced. With the cotreatment of thrombin, PS exposure was regularly observed in platelets of *Stim1*$^{-/-}$ chimeric mice (Table 2).

Accordingly, it was argued that SOCE of platelets might be useless for thrombi formation at injured vessels where thrombin is expressed as a coagonist (Gilio et al., 2010). There are reasons to doubt this speculation. As shown in our data, platelet PS exposure was substantially attenuated by thrombin in the *Stim1/2*$^{fl/fl}$ *Pf4-Cre* model, which was consistent with previous research by Ahmad (Ahmad et al., 2011). In addition, in *Stim1/2*$^{fl/fl}$ *Pf4-Cre* platelets, caspase-3 activation was significantly decreased in the presence of thrombin or CRP. Thus, we suggest that STIM1/2-mediated SOCE is essential in regulating platelet apoptosis.

Table 2: The effect of STIM1 or STIM2 on platelet $[Ca^{2+}]_i$, activation and apoptosis (Modified from Gilio et al., 2010 and Varga-Szabo et al., 2008)

The arrows or ns indicate statistical decrease ($p < 0.05$) or no statistical difference from WT mice respectively

deficient mice	measurements	GPVI dependent	G protein dependent
$Stim1^{-/-}$	$[Ca^{2+}]$	collagen/CRP ↓	thrombin/ADP ↓
	P-selectin, αIIbβ3	CRP/Convulxin ↓	thrombin/ADP ns
	PS exposure	Convulxin ↓	thrombin ns
		(Convulxin + thrombin) ns	
$Stim2^{-/-}$	$[Ca^{2+}]$	CRP ns	
	PS exposure	collagen ns	

These data suggest that thrombin and CRP trigger Ca^{2+} entry into blood platelets by at least two mechanisms, e.g., STIM1/2-dependent SOCE and a second mechanism that does not require the Ca^{2+} sensor STIM1/2. In conclusion, our data reveal that a lack of STIM1/2 in blood platelets virtually abolishes SOCE but only moderately attenuates thrombin/CRP-induced Ca^{2+} entry, integrin activation, degranulation and apoptosis.

4.2 Ceritinib may modify platelet activation and apoptosis

Platelet dysfunction and thrombotic disorders have been shown to be important manifestations of cancer progression (Li, 2016). Therefore, patients who are diagnosed with cancers have an increased risk of suffering hyper-reactions (Blann et al., 2001) and thrombotic events (Schulman and Lindmarker, 2000) in platelets.

Ceritinib is an ATP-competitive inhibitor of ALK and IGF-1R (Gabay et al., 2015) that has been widely used to treat patients who harbour ALK genetic alterations (Seto et al., 2013; Solomon et al., 2014). The present research revealed that ceritinib inhibits activity and apoptosis in platelets. Notably, ceritinib strongly attenuated the effect of CRP on FSC, PS, $[Ca^{2+}]_i$, Orai1 protein abundance, ROS, integrin activation, platelet degranulation, caspase-3 activity and aggregation.

The narrow range of ceritinib concentrations tested precludes safe conclusions as to the target responsible for the drug effect on platelets. Ceritinib concentrations required for significant inhibition of apoptotic cell membrane scrambling (≥ 1.8 μM) and apoptotic cell shrinkage (≥ 2.6 μM) are similar to concentrations previously used *in vitro* to trigger apoptosis of tumour cells (Hu et al., 2015; Wang et al., 2018), but several orders of magnitude higher than the IC_{50} of ALK (0.2 nM, Selleck Chemicals, USA) or the IGF-1 receptor (8 nM, Sullivan and Planchard, 2016) and the inhibitory effect of ceritinib on platelet apoptosis is thus unlikely due to inhibition of ALK or IGF-1R. The effective ceritinib concentrations are in the range of concentrations observed in the total plasma of ceritinib-treated patients (Nishio et al., 2015). However, as 97% ceritinib is bound to plasma proteins (Mok et al., 2017), the free ceritinib concentration in plasma of ceritinib-treated patients is substantially lower. Thus,

the presently observed inhibition of platelet activation and apoptosis may occur *in vivo* follow ceritinib intoxication. Whatever the primary target, the inhibition of enhanced $[Ca^{2+}]_i$ is expected to be an important reason for ceritinib-mediated inhibition of platelet activation and apoptosis. CRP triggers Ca^{2+} influx, which is mainly dependent on Orai1/STIM-mediated SOCE in platelets (Tanwar and Motiani, 2018; Zhu et al., 2011).

The decline of CRP-induced apoptosis was caused by the decreased Ca^{2+} influx in ceritinib-treated platelets. PS exposure of platelets promotes the activation of coagulation and consequently accelerates the haemostasis reaction (Lhermusier et al., 2011). However, the impact of ceritinib on platelets contrasted that on erythrocytes, in which it increased $[Ca^{2+}]_i$, an effect explaining the activation of eryptosis (Al Mamun Bhuyan et al., 2016). The contrary influences of ceritinib in platelets and erythrocytes are reminiscent of that of cholestasis, bilirubin or bile acids on platelet activation and eryptosis (Kile, 2009; Gowert et al., 2017; Lang et al., 2015; Shiao et al., 1993). The mechanism of Ca^{2+} entry into blood platelets and of that in erythrocytes remains unclear.

In view of the inhibitory effect of ceritinib on platelet activation and apoptosis, one possibility is that this substance may countervail thrombosis. The different effects of ceritinib on apoptosis may be due to the preferential selection of different signalling pathways in different cell types. Regardless, the hypothesis of ceritinib-sensitive signalling pathways requires additional experimental evidence. Despite the preliminary findings, ceritinib could, at least in theory, further prolong the life of platelets, which is related to apoptosis (Cao et al., 2018b; Lang et al., 2016).

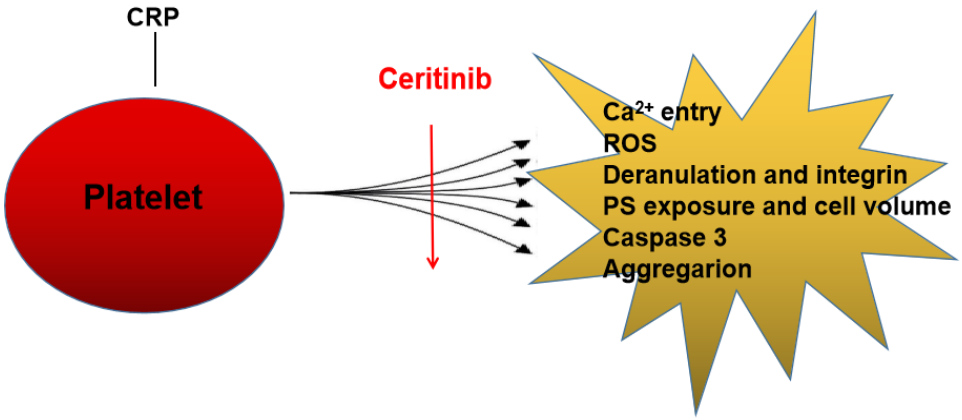

Figure 21: Ceritinib-sensitive CRP-induced platelet activation and apoptosis

Figure indicates the effect of ceritinib on platelet function *via* CRP stimulation. Ceritinib significantly inhibits CRP-triggered platelet activation and apoptosis which are related to PS exposure, cell volume, $[Ca^{2+}]_i$, P-selectin abundance, ROS abundance, integrin αIIbβ3 activity, caspase-3 activity, and aggregation.

In conclusion, ceritinib treatment blunted the effect of CRP on cell membrane scrambling, cell shrinkage, $[Ca^{2+}]_i$, P-selectin abundance, ROS abundance, integrin αIIbβ3 activity, caspase-3 activity, and aggregation in platelets (Figure 21). Those effects require, however, ceritinib concentrations by far higher than those required for ALK and IGF-1R inhibition and higher than the free drug concentration in patient blood.

5. Summary

Change of intracellular Ca^{2+} concentration is involved in many physiological processes, including platelet activation and apoptosis. The majority of Ca^{2+} entry into activated platelets, which probably comes from Orai/STIM-regulated SOCE. The present work investigated the impacts of STIM1-STIM2 and of ceritinib on platelet Ca^{2+} signalling, activation and apoptosis.

STIM1/2 is described as a Ca^{2+} sensor that plays decisive roles in Orai/STIM-mediated SOCE. The current work showed that the number of platelets from the blood was significantly lower in *Stim1/2*$^{fl/fl}$ mice than *Stim1/2*$^{fl/fl}$ *Pf4-Cre* mice. No significant difference was found in platelet aggregation between the two genotypes. Besides, without thrombin and CRP stimulation, platelet degranulation, Orai1, PS exposure, integrin αIIbβ3 activation, SOCE and $[Ca^{2+}]_i$ were similar in *Stim1/2*$^{fl/fl}$ mice and *Stim1/2*$^{fl/fl}$ *Pf4-Cre* mice. CRP or thrombin treatment led to a sharp increase in degranulation, Orai1, PS exposure, integrin αIIbβ3 activation, SOCE and $[Ca^{2+}]_i$ in both genotypes. All effects, however, were markedly attenuated in *Stim1/2*$^{fl/fl}$ *Pf4-Cre* platelets compared to *Stim1/2*$^{fl/fl}$ platelets. These data suggest that thrombin and CRP trigger Ca^{2+} entry into blood platelets by at least two mechanisms, e.g., STIM1/2-dependent SOCE and a second mechanism that does not require the Ca^{2+} sensor STIM1/2. In conclusion, our data revealed that the lack of STIM1/2 in blood platelets virtually abolishes SOCE but only moderately blunts thrombin- and CRP-induced Ca^{2+} entry, integrin activation, degranulation and apoptosis.

Ceritinib is effective in ALK-positive non-small cell lung carcinoma treatment and triggers apoptosis of tumour cells. The present work indicated that platelet $[Ca^{2+}]_i$ was strongly decreased by treatment with very high concentrations of ceritinib.

Furthermore, cell shrinkage, annexin-V-binding, $[Ca^{2+}]_i$, Orai1 abundance, integrin activation, platelet degranulation, ROS formation and aggregation were remarkedly increased following stimulation with CRP. At very high concentrations ceritinib blunted those effects of CRP. In conclusion, at excessive concentrations ceritinib counteracted CRP-induced platelet activation, apoptosis and aggregation.

Zusammenfassung

Die Veränderung der intrazellulären Ca^{2+}-Konzentration ist an vielen physiologischen Prozessen wie an der Thrombozytenaktivierung und Apoptose beteiligt. Der überwiegende Anteil am Ca^{2+} Einstrom in aktivierte Thrombozyten beruht wahrscheinlich auf dem von Orai/STIM regulierten, speicherabhängigen Ca^{2+}-Einstrom (SOCE). Die Auswirkungen von STIM1-STIM2 (STIM1/2) sowie von Ceritinib auf den Thrombozyten-Ca^{2+}-Signalweg, Aktivierung und Apoptose wurden in dieser Studie untersucht.

STIM1/2 wurde als Ca^{2+}-Sensor beschrieben, der in Orai/STIM-vermittelter SOCE eine entscheidende Rolle spielte. Die vorliegende Studie ergab, dass die Anzahl der Thrombozyten von *Stim1/2*$^{fl/fl}$-Mäusen auffallend niedriger als die von *Stim1/2*$^{fl/fl}$ *Pf4-Cre*-Mäusen war. Ohne Aktivierung mit Thrombin oder CRP war kein statistisch signifikanter Unterschied der Thrombozytenaggregation zwischen beiden Genotypen erkennbar. Außerdem waren vor der Thrombin- und CRP-Stimulation die Thrombozyten-Degranulation, Orai1, Phosphatidylserin (PS)-Exposition, Integrin-αIIbβ3-Aktivierung, SOCE und intrazelluläre Ca^{2+}-Konzentration ($[Ca^{2+}]_i$) in Thrombozyten von *Stim1/2*$^{fl/fl}$-Mäusen und *Stim1/2*$^{fl/fl}$ *Pf4-Cre*-Mäusen ähnlich. Die Behandlung von CRP oder Thrombin führte zu einem signifikanten Anstieg der Degranulation, Orai1, PS-Exposition, Integrin-αIIbβ3-Aktivierung, SOCE und $[Ca^{2+}]$ in beiden Genotypen. Alle Effekte waren in *Stim1/2*$^{fl/fl}$ *Pf4-Cre*-Plättchen deutlich schwächer als in *Stim1/2*$^{fl/fl}$-Plättchen. Diese Daten weisen darauf hin, dass der durch CRP oder Thrombin induzierte Einstrom von Ca^{2+} in Blutplättchen von mindestens zwei Mechanismen reguliert wird, d.h. STIM1/2-abhängige SOCE und durch einen zweiten Mechanismus, der den Ca^{2+}-Sensor STIM1/2 nicht erfordert. Unsere Daten zeigen, dass das Fehlen von STIM1/2 in Blutplättchen

den SOCE tatsächlich aufhebt, aber Thrombin- und CRP-induzierten Ca^{2+}-Einstrom, die Integrinaktivierung, -degranulation und -apoptose nur mäßig mindert.

Ceritinib ist bei der Behandlung des ALK-positiven nicht-kleinzelligen Lungenkarzinoms wirksam. Die Substanz löst Apoptose von Tumorzellen aus. Die vorliegende Arbeit hat ergeben, dass thrombozytäres $[Ca^{2+}]_i$ in Anwesenheit von sehr hohen Ceritinib-Konzentrationen eine signifikante Abnahme zur Folge hat. Außerdem führte die Exposition der Thrombozyten mit CRP zu einem beachtlichen Anstieg von $[Ca^{2+}]_i$, Orai1-Abundanz, Integrin-αIIbβ3-Aktivierung, Thrombozytengranulation, Zellschrumpfung, Annexin-V-Bindung und ROS-Bildung und –aggregation. Diese Wirkungen von CRP wurden durch Ceritinib gehemmt. Zusammenfassend wirken hohe Konzentrationen an Ceritinib der CRP-induzierten Thrombozytenaktivierung, -apoptose und -aggression entgegen.

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7. Declaration of Contributions

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8. Publications

Cao H, Al Mamun Bhuyan A, Umbach AT, Ma K, Borst O, Gawaz M, Zhang S, Nürnberg B, Lang F. Garcinol A Novel Inhibitor of Platelet Activation and Apoptosis. Toxins (Basel). 2019 Jul 1;11(7). pii: E382.

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