New Players in Platelet Activation and Apoptosis

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Summary

The non-nucleated platelets are the key players in thrombosis and haemostasis and versatile mediators of inflammation and immunity. Their life, death and actions define illness and health of men. This dissertation attempted to find what events lead to the apoptotic death in platelets by nutraceutical thymoquinone, pathogen-associated peptidoglycan, the antibiotic vancomycin and finally how platelet activation and death by a recently reported chemokine CXCL16 influence platelet function. Thymoquinone triggers suicidal death of blood platelets in a Phosphoinositide 3Kinase-dependent manner, possibly through a G-protein Coupled Receptor family opioid receptor; an effect paralleled by increase of cytosolic $Ca(^{2+})$ activity, ceramide formation, mitochondrial depolarization, and caspase-3 activation. HPLC-purified fractions of peptidoglycan from *Staphylococcus aureus* 113 triggers apoptosis of platelets, characterized by annexin-V binding, increase of $[Ca^{2+}]_i$, mitochondrial depolarization, caspase-3 activation and integrin $\alpha_{IIb}\beta_3$ upregulation. The annexin-V binding was significantly blunted by anti-TLR-2 antibodies, in the absence of extracellular Ca^{2+} and by pancaspase inhibitor zVAD-FMK (1 µM). Vancomycin decreased cell volume, triggered annexin V-binding, stimulated ceramide formation and activated caspase 3. The annexin V-binding was significantly blunted by removal of extracellular Ca^{2+} but not by pan-caspase inhibition with zVAD-FMK (1µM). Vancomycin also triggers thromboxane B2 release from platelets in cyclooxygenase dependent way. The chemokine CXCL16 significantly up-regulated expression of P-selectin and activated integrin $\alpha_{IIb}\beta_3$ at the platelet surface. Consistent with these findings the effects of CXCL16 on platelet activation were inhibited by PI3K inhibitors wortmannin and LY294002 as well as by Akt inhibitor SH-6 (20 µM). The stimulation of adhesion by CXCL16 was again prevented by wortmannin, LY294002 and SH-6. Apyrase and inhibition of the platelet purinergic receptors P_2Y_1 (MRS2179, 100 µM) and especially P_2Y_{12} (Cangrelor, 10 µM) blunted CXCL16-triggered platelet activation as well as CXCL-16-induced platelet adhesion under high arterial shear stress *in vitro* indicating that CXCL16 activates platelets via ADP release-dependent paracrine activation. In conclusion the inflammatory chemokine CXCL16 triggers platelet activation and adhesion via PI3K/Akt signaling pathway and paracrine activation suggesting a decisive role for CXCL16 in the pathogenesis of atherosclerosis and thrombo-occlusive diseases.

Zusammenfassung

Thrombozyten spielen sowohl in thrombotischen und hämostatischen Prozessen eine wesentliche Rolle als auch in entzündlichen sowie Immunreaktionen. Für die Gesundheit des Menschen sind sie von essentieller Bedeutung. Im Rahmen dieser Doktorarbeit konnte herausgefunden werden, welche Ereignisse in Thrombozyten bei Kontakt mit dem Nahrungsergänzungsmittel Thymoquinone, dem Pathogen-assoziierten Peptidoglycan und dem Antibiotikum Vancomycin zu programmiertem Zellungergang führen und wodurch das Chemokin CXCL-16 Einfluss auf die Plättchenfunktion hat. Thymoquinone löst in Thrombozyten PI3-Kinase-abhängig und downstream eines G-Proteins Apoptose aus, wodurch es zu einer gesteigerten intrazellulären Ca^{2+}-Menge, zu erhöhter Ceramidbildung, zu mitochondrialen Depolarization sowie zur Aktivierung von Caspase-3 kommt. HPLC-gereinigte Fraktionen von Peptidoglycan aus Staphylococcus aureus 113 führt in Blutplättchen zur Annexin-V-Bindung, dadurch zu einer Erhöhung der $[Ca^{2+}]_i$, zu mitochondriale Depolarisation sowie zu Caspase-3-Aktivierung und Integrin $\alpha_{IIb}\beta_{33}$-Hochregulation. Die Annexin-V-Bindung wurde durch anti-TLR-2-Antikörper, die Abwesenheit von extrazellulärem Ca^{2+} sowie durch den Pan-Caspase-Inhibitor zVAD-FMK signifikant reduziert. Ebenso verringert Vancomycin das Zellvolumen, triggert die Annexin V-Bindung, stimuliert die Bildung von Cerabid sowie von aktiver Caspase-3. Die Bindung von Annexin V konnte mittels des Pan-Caspase-Inhibitors zVAD-FMK sowie durch die Entfernung von $[Ca^{2+}]_e$ erfolgreich verhindert werden. Zusätzlich triggert Vancomycin abhängig von p38 MAPK und der Cyclooxygenase die Thromboxan B2-Freisetzung aus Thrombozyten. Das Chemokin CXCL16 reguliert die Expression von P-Selektin hoch und aktiviert Integrin $\alpha_{IIb}\beta_3$ an der Plättchenoberfläche. Mit diesen Ergebnissen kohärent ist die Inhibition der beschriebenen Auswirkungen von CXCL16 sowie dessen Adhäsion durch die PI3K-Inhibitoren Wortmannin und LY294002 sowie durch den Akt Inhibitor SH-6. Die Inhibition des P_2Y_1 sowie des P_2Y_{12}-Rezeptors führte zu einer deutlichen Abnahme der CXCL16-induzierten Plättchenadhäsion bei hoher Flussrate in vitro, wodurch die Schlussfolgerung zulässig ist, dass CXCL16 Plättchen ADP-abhängig parakrin aktiviert. Zusammenfassend lässt sich sagen, dass CXCL16 Plättchen mittels PI3K-Akt sowie parakriner Prozesse aktiviert und dadurch eine maßgebliche Rolle in der Pathogenese von Atherosklerose sowie thombo-okklusiven Erkrankungen spielt.

Abbreviation

ACD : acid citrate dextrose

ADP : adenosine diphosphate

AMP : adenosine monophosphate

ANOVA : ANalysis of VAriance

ASM: acid sphingomyelinase

Apaf-1: apoptotic protease activating factor 1

BAD: B-cell follicular lymphoma-2-associated protein α

BSA : Bovine Serum Albumine

CMP: Common Myeloid Progenitor

CXCL : Cysteine-X(any amino acid)-Cysteine Ligand

cAMP: Cyclic Adenosine Mono Phosphate

cGMP: Cyclic Guanidine mono phosphate

Caspase: Cysteine-Aspertate protease

CD: Cluster for differentiation

COX : Cyclooxygenase

$DiOC_6$: 3,3′-dihexyloxacarbocyanine iodide

DNA : Deoxyribo Nucleic Acid

EDTA: Ethylene Diamine Tetraacetic Acid

EGTA : Ethylene Glycol Tetraacetic Acid

ELISA : Enzyme linked Immuno-Sorbent Assay

FACS : Fluorescence Associated Cell Sorter

FAK : Focal Adhesion Kinase

FITC : Fluoroscein Iso Thio Cyanate

Gi : Inhibitory G protein

GEF : Guanine nucleotide Exchange Factor

GP : Glycoprotein

GPCR : G-protein coupled Receptor

HEPES : 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid

HPLC: High Performance Liquid Chromatography

HRP : Horse Radish Peroxidase

HUVEC : Human Umbilical Vein Endothelial Cell

ITAM : Immunoreceptor Tyrosine-based Activation Motif
Ig : ImmunoGlobulin
IL : Interleukin
IFN : Interferone
ICAM : Intra-Cellular Adhesion Molecule
LPS : Lipo Poly Saccharide
MEP : Myeloid Erythroid progenitor
MK : Megakaryocyte
mRNA: messenger RNA
mTOR : mammalian Target of Rapamycin
MAPK: Mitogen activated protein kinase
MCP-1: Monocyte Chemoattractant Protein
MMP : Matrix Metalloprotease
MLCK : Myosine Light Chain Kinase
NF-κB : Nuclear factor κB
NO : Nitric Oxide
NOD : Nuclear binding Oligomerization Domain
PAMP : Pathogen Associated Molecular Pattern
PAR : Protease Activated Receptor
PBS : Phosphate Buffered Saline
PE : Phycoerythrin
PIP2 : Phospho Inositide diphosphate
PI3K : Phosphoinositide 3 Kinase
PG : Prostaglandin
PGN : Peptidoglycan
mPGN: monomeric PGN
PKB : Protein Kinase B
PPAR : Peroxisome Proliferator-activated Receptors
PRP : Platelet Rich Plasma
PS : Phosphatidyl serine
PSGL : P-selectin Glycoprotein Ligand
PSL : Platelet Storage Lesion
Ptx : pertusis toxin
RANTES : Regulated upon Activation, Normal T-cell Expressed, and Secreted

Rap : Ras-related protein
RBC : Red Blood Cells
ROS : Reactive Oxygen Species
SDF-1 : Stromal Derived Factor-1
STIM : Stromal Interaction Molecule 1
TBS-T : Tris Buffered Saline with Tween 20
TPO : Thrombopoitin
TF : Tissue factor
TNF Tumor Necrosis Factor
TLR : Toll-Like Receptor
Tx : Thromboxane
vWF: von Willebrand Factor
zVAD-FMK : Z-Val-Ala-Asp-FluoroMethyl Ketone

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Chapter 1

Introduction

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Introduction

Platelets were discovered in 1882 by Bizzozero who described them as blood dust (Bizzozero G. 1882). Since then platelets have been considered the key components in haemostasis (Duke 1910, Walsh 1974, Williams 1968). However with the progress of science and medicine, platelets appear to be diverse players in health and disease.

Platelets are without nucleus, which makes them atypical cells (Bizzozero G. 1882, Holmsen 1972). They originate from the megakaryocytes of bone marrow and are released into the blood stream as terminally differentiated cells (Wright 1906, Reviewed by Italiano , Shivdasani, 2003). They are discoid cells with 4-8 µM in size (White 1970) and range between $150X10^9$ – $450X10^9$/L blood in a healthy adult. Platelets are diverse players in haemostasis, thrombosis, immunity and inflammation.

1. Literature Review

1.2 Formation from Megakaryocytes

The origin of platelets from megakaryocytes was predicted by Wright (1906). The pluripotent hematopoitic stem cells produce platelets in roughly four steps (Reviewed by (Thon and Italiano, 2010):

a) Commitment to form megakaryocyte progenitors: this is accomplished through forming common myeloid progenitor (CMP), which develops into megakaryocyte-erythrocyte progenitor (MEP) and finally differentiates into megakaryocyte progenitor (MKP). This process is stimulated by thrombopoitin (TPO).
b) Proliferation of MKP to produce sufficient number of immature megakaryocytes (MK) in the bone marrow
c) Maturation of megakaryocytes (MK): The MK nucleus undergoes consecutive endomitosis to produce 64 or more nuclei per MK. The MK membrane

develops demarkation system. The MK cytoplasm increases in volume and with formation of secretory granules and mitochondria. Calcium mobilization greatly increases. The synthesis of adhesive glycoprotein complex progresses (Akkerman *et al.*, 2003).The MK moves from the osteoblastic niche to the vascular niche for platelet formation, a process regulated by CXCL-12/SDF-1 and CXCR-4 (Avecilla *et al.*, 2004), (Guerriero *et al.*, 2001). The mitochondria-rich parts of MK cytoplasm receive granules, cytoplasmic proteins and selected type and amount of mRNA and micro-RNA (miRNA) and are progressively partitioned by cytoplasmic membrane.

d) Release of proplatelets: Roughly 2000-5000 pro-platelets are released from MK.The senescent MK, which is left with the nuclei surrounded by this cytoplasmic content, exhibits a rise in caspase-3 activation and apoptotic death (Italiano *et al.*, 1999)), (reviewed by (Flaumenhaft, 2011), (Houwerzijl *et al.*, 2006). However it is important to mention that platelet formation and MK apoptosis and two tightly linked but independent pathways (Josefsson *et al.*, 2011) .

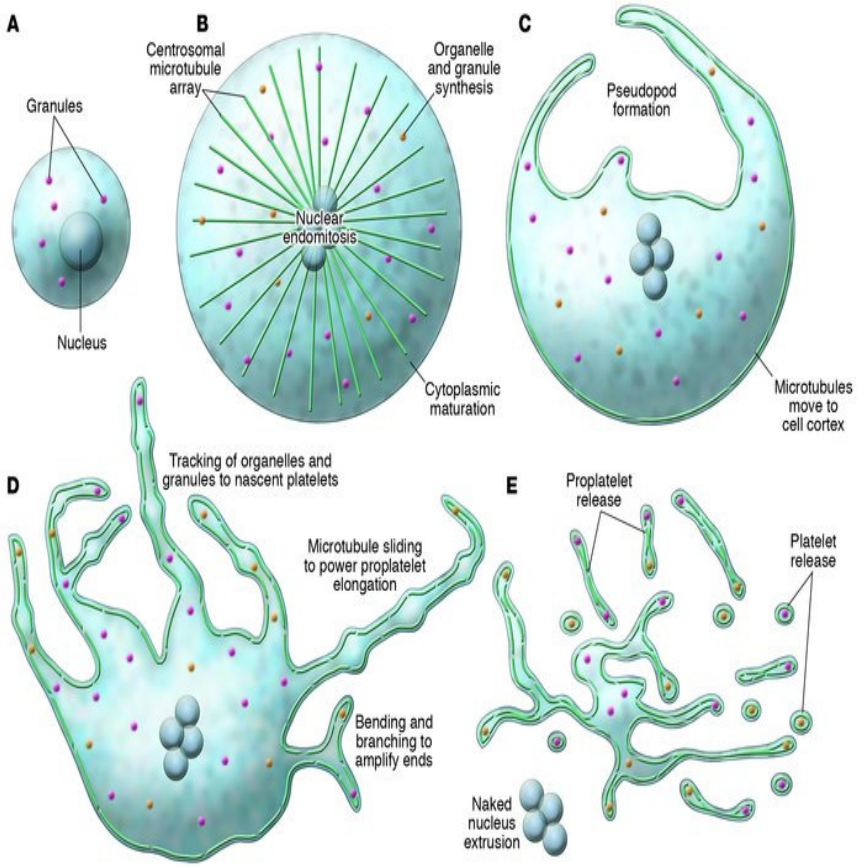


Figure 1.1 Formation of Platelets from Megakaryocytes (Patel *et al.*, 2005)

1.2 **Thrombosis and Haemostatsis by Platelets**

The most important physiologic function of plateles is to develop a haemostatic plug. This is only necessary if there is a vascular damage and blood loss. The coagulation cascade starts with release of the tissue factor (TF) and Ca^{2+} from injured vessel. Prothrombin is activated to thrombin by Factor V, Ca^{2+} and phospholipids. Thrombin is a strong inducer of platelet hyper-coagulability. Therefore platelets are activated to form a clot with the vessel wall, forming a plug that entagles erythrocytes, leukocytes and stops bleeding (William 1968, Walsh 1974, reviewed by (Velez *et al.*, 2008).

Formation of a thrombus might be looked upon in a more descriptive term. This occurs in several stages:

a. Initiation: platelets deposit on the exposed sub-endothelium and get activated. Von Willebrand factor fixed on collagen type I, III or VI are strong agonists for platelet activation. The initial slowing down of platelets on injured tissue is called tethering, which is mediated by GP Ibα. In addition, GP Ib/IX/V complex also binds to collagen, thrombospondin, alpha-thrombin, kininogen, F XI, F XII.
b. Extension: deposited and activated platelets release a number of agonists to recruit flowing platelets to the site.
c. Stabilization: the platelets accumulated in the injury site form bridges with themselves as well as with endothelilal cells and the exposed matrix to stabilize the clot under haemodynamic shear. Platelet P-selectin binds to endothelilal PSGL-1 and platelet Mac-1 binds to leukocyte to form clot (reviewed by (Rivera *et al.*, 2009b) .

1.3 **Non-haemostatic Functions of Platelets**

Platelets have indirect roles in inflammation and immunity. Platelets contain numerous granules with preformed growth factors, chemokines, inflammatory mediators and other forms of signaling molecules. RANTES, platelet factor 4, CXCL-5, IL-1β are responsible for chemoattracing leukocytes and monocytes and

stimulating activation and secretion from endothelial cells (Hawrylowicz *et al.*, 1991), (Kaplanski *et al.*, 1994).

The release of CD 40L from platelets induce endothelia to release chemokines and and to express adhesion molecules. These chemokines from endothelia recruits leukocytes and promotes the release of matrix metalloproteases (MMP). MMP destroys and remodels inflamed tissues (Sawicki *et al.*, 1997), (Henn *et al.*, 1998), (Slupsky *et al.*, 1998), (Fernandez-Patron *et al.*, 1999) .

Platelets contain pathogen-associated pattern recogntion molecules (PAMP), thereby recognize pathogens and get activated. Activated platelets can coat the pathogens, neutralizing them and at the same time trigger chemokine signal to immune cells to the site of infection (Kirschning and Bauer, 2001b), (Beutler, 2003) , (Shiraki *et al.*, 2004).

1.4 The Translational Regulation

Though anucleate and short-lived, platelets synthesize proteins *de novo* (Warshaw, 1966) as well as in activation-dependent manner (Weyrich *et al.*, 1998b) . This kind of translational regulation is debated and poorly understood. As per the current view, MK has a complex process to determine what type and amount of proteins and RNAs the platelets would have. The pre-mRNAs and mRNAs destined to be passed on to platelets bind to mRNA binding proteins, associate with the kinesin motor and are transported to platelets through microtubules, a process hypothesized to be similar in neurons (Cecchetti *et al.*, 2011). MK also pass a number of micro-RNAs (miRNA) that control post-transcriptional processes in platelets (Nagalla *et al.*, 2011). Some mRNAs are constitutively translated whereas others are translated in activation-dependent manner (eg. Bcl-3, IL-1β, tissue factors (TF) by signal-dependent pre-mRNA splicing or by mTOR mediated mechanism) (Weyrich *et al.*, 1998a). The miRNAs are also up- or down-regulated according to the stimulation (Labbaye *et al.*, 2008), (Osman and Falker, 2011) . However (Camera *et al.*, 2003) and (Lindemann *et al.*, 2001) showed proof of *de novo* mRNA synthesis in platelets. Platelets have PPAR γ and retinoid X receptor (Akbiyik *et al.*, 2004), (Ray *et al.*, 2008)).

Platelets have some novel regulatory mechanisms that inhibit of improper activation and thrombus formation in smooth healthy blood vessels. The common inhibitory mechanism is release of prostacyclin, prostaglandins, soluble gunalyl cyclases and G_s coupled signals. Nitric oxide from endothelia upegulate cyclic nucleotides cAMP and cGMP in platelets. Cyclic AMP activates PKA_c that phosphorylate and inactivate proteins of activation pathways (Gambaryan *et al.*, 2010a)) and thereby inhibit platelet aggregation, secretion and spreading. (reviewed by Smolenski, 2011, (Tan *et al.*, 2011)**.** Power (Power *et al.*, 2009) reported at least 89 peptides undergoing alternate splicing in platelets.

1.6 Calcium Regulation

Calcium influx activates myosine light chain kinase (MLCK) and calpain. Platelets have a 2-step Ca^{2+} increase in response to stimulation. It is known that primary stimulation from GP1b alpha (caused by suboptimal agonist-binding or shear stress) can lead to release of Ca^{2+} from dense granule (α/β peak), which can be stopped by raising level of cAMP, cGMP and chelators. At this stage platelets form non-permanent adhesion with other cells or matrix proetins. When integrin $\alpha_{IIb}\beta_3$ is activated, a transmebrane ion-flux is seen (γ peak) which is more intense than α/β peak, and is associated with recruitment of other platelets to the locus and formation of a stable aggregate. This second step of high Ca^{2+} influx could be stopped by AMP and PI3K inhibition ((Mazzucato *et al.*, 2002). Agonist stimulation in platelets activates inisitol 1,4,5-triphosphate mediated Ca^{2+} release from the intracellular stores. STIM-1 associated with the stores sense the Ca^{2+} depletion in the stores and trigger Ca^{2+} influx from extracellular environment through Orai-1. Serum and glucocorticoid-inducible kinase 1 (SGK-1) acts as a novel transcriptional regulator of Orai-1 in megakaryocyte and platelets partally through NF-κB (Rink and Sage, 1990), 1990, (Varga-Szabo *et al.*, 2008) , (Bergmeier and Stefanini, 2009a), (Borst *et al.*, 2012b).

1.6 Role of PI3K in Platelets

Phosphoinositide 3 Kinases (PI3K) are well-known pro-survival mediator in nucleated cells ((Tang *et al.*, 2002) , (Rojnuckarin *et al.*, 2001). PI3K comprises of a superfamily of enzymes, broadly divided into 3 types (type I, II, III) ((Wymann and Marone, 2005) , (Huang and Reichardt, 2003). Type I PI3K are the most well-studied group because of their importance in cell signalling and proliferation (Vanhaesebroeck and Waterfield, 1999). Type I PI3K have two subtypes (Subtype IA and IB), which in turn contain some isoforms, eg p110α, p110β, p110δ in subtype IA and p110γ in subtype IB (Vanhaesebroeck *et al.*, 1999). Members of the same subtype have common regulatory subunits (Maier *et al.*, 1999). Platelets express p110 β and p110γ most abundantly (Condliffe *et al.*, 2005) , where they play the common mediator of platelet adhesion, cytoskeletal remodelling and aggregation (Jackson *et al.*, 2004) . Platelets are unable to activate PLC γ2, Akt, Rap1b and signaling events involving ITAM without PI3K p110β (Martin *et al.*, 2010b). PI3K α had only been reported in GP IV mediated aggregation, secretion, Calcium mobilization (Gilio *et al.*, 2009), (Kim *et al.*, 2009a).

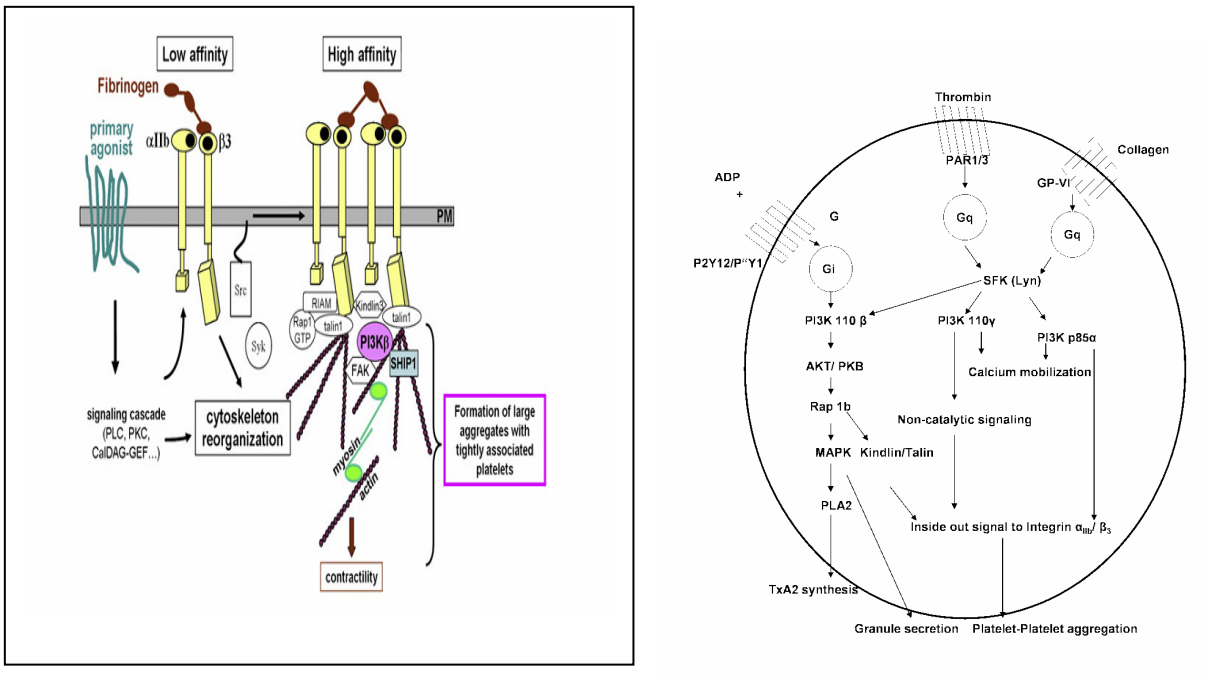


Figure 1.2: Role of PI3K Isoforms in Platelet Activation (Gratacap et. al. 2011)

Schoenwaelder and SP Jackson's group reported (Schoenwaelder *et al.*, 2007) that the different isoforms of PI3K; p110 β and p110γ, regulate platelet aggregation in two

different ways. The p110 β is activated following ADP through Gi coupled receptors and trigger Rap1b and AKT leading to Integrin $\alpha_{IIb}\beta_3$ upregulation**.** The p110γ isoform upregulates integrin following non-catalytic signaling. Later PI3K β was shown to regulate avidity of high affinity integrin receptors $\alpha_{IIb}\beta_3$ and contractile forces in clot retraction (Schoenwaelder *et al.*, 2010b). PI3K p110β is also an important downstream affector of Integrin $\alpha_{IIb}\beta_3$ (reviewed by Gratacap et al. 2011). However, PI3K $\beta^{-/-}$ mice develop smaller aggregates, therefore it is clear that PI3K p110β affects certain pathways of thrombin-mediated activation but its absence can not fully diminish thrombin-mediated platelet activation (Martin *et al.*, 2010a). In vivo experiments with a pharmacologic inhibitor of PI3K p110β was shown to reduce occlusive thrombus formation without significantly affecting the bleeding time (Jackson *et al.*, 2005) . These data led S P Jackson's team to hypothetize that selective inhibition of PI3K isoforms could be a strategy to develop anti-thrombotic drugs (Jackson and Schoenwaelder, 2006).

1.7 Delicate Balance between Activation and Quiscence

Stephan Gambaryan's group (Gambaryan *et al.*, 2009) demostrated that strong platelet agonists like thrombin and collagen initiate a negative feedback signaling through cAMP-independent but NF kB-dependent PKA_c activation so that pathologic thrombosis is controlled. The cross-talk between stimulatory and inhibitory pathways are also present in case of vWF exposure, when NO-independent soluble guanylyl cyclase and cGMP along with PKG exert an inhibitory signal (Gambaryan *et al.*, 2010b).

The regulation in short-lived platelets is too fast to be detected by conventional methods, because PIP2 accumulates to significntly higher levels within 1 minute (Holinstat *et al.*, 2009a) and Ca^{2+} signaling starts in 200 milliseconds. One proof of this regulation comes from (Crovello *et al.*, 1995) , who showed that thrombin-stimulus to human platelets is followed by a rapid and reversible histidine-phosphorylation in the cytoplasmic tail of CD62P. Kouns (Kouns *et al.*, 1992a) , (Kouns *et al.*, 1992b) had also demonstrated a reversible change in integrin $\alpha_{IIb}\beta_3$ in response to peptidomimetic inhibitors. (Dash *et al.*, 1995) showed that pp 125 FAK, pp 60 src, RAP 1b and CDC 42 Sh associate with actin in activated platelets in Ca^{2+} -

dependent but reversible manner. A D Michelson (Michelson *et al.*, 1996) reported that GP V expression is regulated reversibly by cytoskeleton.

Holinstat and group , (Holinstat *et al.*, 2009b;Holinstat *et al.*, 2009c)) came up with conclusive observation that defines irreversibility of platelet activation. PAR-1/4 mediated signal following thrombin stimulation must produce phophoinositides upto a certain concentration in order to form Rap1-GEF that results in a stable, irreversible aggregate. He also concluded that if the lipid signaling pathway can be specifically and timely intefered, conditions of infarction and stroke can, at least in theory, be avoided. Corroborating reports (Lu and Lu, 2008) suggest an intracellular phosphatidylinositol 3,4,5 triphosphate level of 20μM and more leads to irreversible platelet activation. Therefore the balance between platelets to form aggregate or to avoid being pro-coagulant are fine-tuned by complicated signaling events.

1.8 Forms of Platelet Death

1.8.1 The Natural Senescence

The current knowledge of senescent platelet death shows that intrinsic pathway of apoptosis is responsible. B T Kile (Kile, 2009e) and his group put forward a molecular clock model for senecent platelets. According to the model, the Bcl-xl pool in platelet cytoplasm (derived from megakaryocytes) weathers down with time, allowing pro-apoptotic Bak to become unrestrained. Bak and Bax complex inserts themselves into mitochondrial membranes, allwoing release of cytochrome C and oxidative stress, which ultimately leads to caspase-3 activation, PS exposure and clearence of the dead platelet from circulation by macrophages (Mason *et al.*, 2007a), (Mason *et al.*, 2007b), (Kile, 2009f).

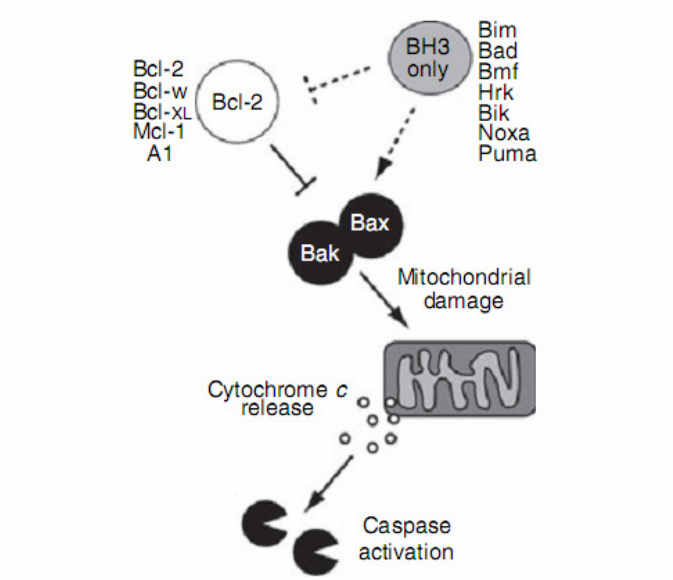


Figure 1.3 : Intrinsic pathway of platelet apoptosis (Kile, 2009a)

1.8.2 Platelet Death in Ex vivo conditions

Platelets isolated from healthy donors undergo apoptosis-like events over their storage period in the blood banks and therefore they have functional changes such as reduced adhesive function, increased pro-coagulant activity and reduced survival in transfusion recipient (Leytin *et al.*, 2003b), (Leytin and Freedman, 2003d), (reviewed by (Jackson and Schoenwaelder, 2010e) . This is called platelet storage lesion (PSL), characteized by reduction in Bcl-$_{xL}$, mitochondrial damage, increased PS, fragmentation of plasma membrane, microvesiculation, shedding of the extra-cellular domain of GPIb, caspase activation and gelsolin proteolysis (Klinger, 1996b), (Leytin and Freedman, 2003c).

1.8.3 Agonist-induced Death

Platelet agonists induce platelet activation, ie. aggregation, spreading and migration, which are the most important features for haemostatsis. Complete activation of platelets is an irreversible process ending in platelet death, where calpain plays a pivotal role (Kulkarni and Jackson, 2004a). Each agonist has its characteristic pathway of inducing activation and apoptosis simulatneously, apoptosis being independent or partially dependent on activation (Schoenwaelder *et al.*, 2009b), (Kulkarni and Jackson, 2004b), (Wolf *et al.*, 1999c)). Physiologic agonist α-thrombin

was reported to increase pro-apoptotic Bcl-2, depolarizing mitochondria, activating caspase-3 and promoting PS exposure through membrane scrambling at ≥ 1U/ml ((Leytin *et al.*, 2006b). At 0.5-1nM concentration, thrombin activates platelets but at 10nM or more concentration, upto 40% hyper-coagulant platelets are apoptotic, which shows activation is far more sensitive than apoptosis ((Leytin *et al.*, 2007c), 2007a, (Leytin *et al.*, 2006e). In case of tissue damage and injury or pathogenesis, 245nM thrombin is produced at a specific location and platelet activation by such concentrations of thrombin might be fatal. Therefore platelet apoptosis at high thrombin concentrations might be a defensive mechanism against thrombosis (Leytin *et al.*, 2007b) .

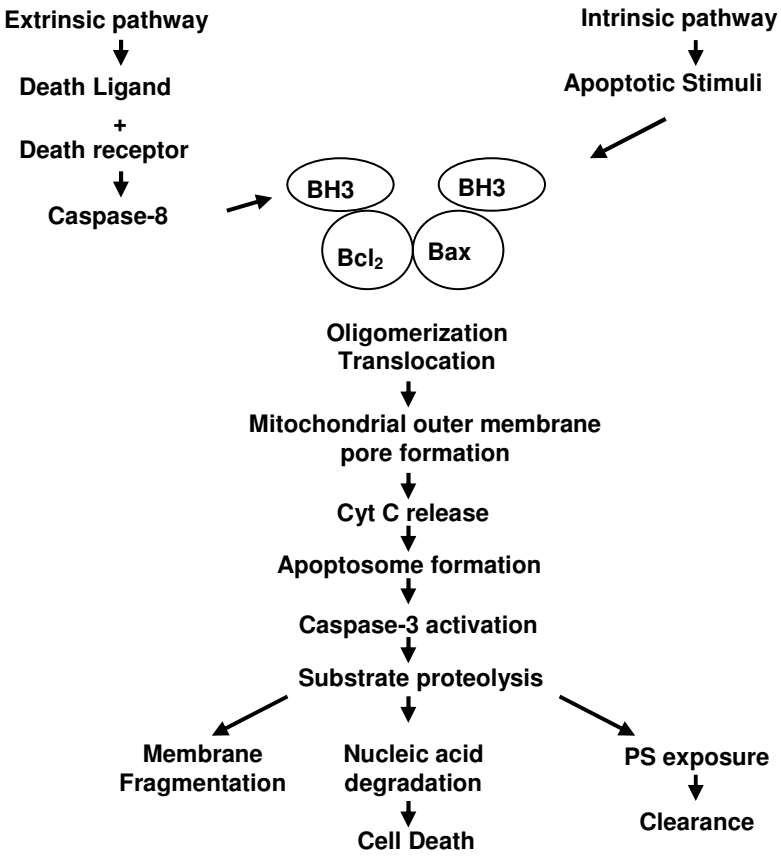


Figure 1.4 : Agonist-mediated death of Cells (Jackson and Schoenwaelder, 2010d)

The multimeric agonist von Willebrand Factor (vWF) attaches to its receptor GP1b α and activates 14-3-3 ζ, which in turn rolls Bax and Bak into pro-apoptotic action. Ultimately mitochondria is depolarized, gelsolin is cleaved and PS is exposed. Relatively higher concentration of vWF (35μM) can induce platelet apoptosis in this way (Cheng *et al.*, 2009).

1.8.4 Immune-mediated Death

In case of drug mediated immune-thrombocytopenia, IgM and IgG against platelet surface proteins coat the platelets. Coated platelets are bound by heparin and targeted by cytotoxic T cells and finally cleared away from circulation by macrophages (Kosty *et al.*, 1989) , reviewed by (Carlsson *et al.*, 2001) .

1.9 Markers Associated with Platelet Death

The Nomenclature Committee on Cell Death 2009 specified some criteria for identification of apoptotic cells by laboratory investigation (Kroemer *et al.*, 2009). According to those specifications, mitochondrial outer membrane depolarization, mitochondrial transient pore formation, ratio between pro-apoptotic and anti-apoptotic Bcl2 family proteins, cytochrome c release and Caspase 9 activation are upstream events in the intrinsic pathway. The downstream markers include Caspase 8 and Caspase-3 activation, PS exposure, platelet shrinkage, microparticle formation and cleavage of cytoskeletal proteins (reviewed by (Gyulkhandanyan *et al.*, 2012a).

1.10 Differences between apoptosis of platelet and nucleated cells

Apoptosis is the programmed death of senescent or damaged cells. Apoptosis is a mechanism of death that ensures least or no side effects. The hallmarks of apoptosis, as described for nucleated cells, are featured by activation of apoptotic Bcl-2 family proteins leading to mitochondrial depolarization, ROS production, oxidative damage of intracellular organelles and release of cytochrome C from mitochondria. This sequence of events is called the intrinsic pathway. On the other hand, the extrinsic pathway includes activation of TNF family receptors by external or internal stimuli, followed by activation of initiator caspase (caspase-8), apoptosome formation by Apaf-1, pro-caspase-9 and cytochromes from intrinsic pathway. Apoptosome releases active caspase-9 from the inactive form. Active caspase-9 activates executioner caspases (mainly caspases 3 and 7). Activation of caspase-3 is often pivotal for apoptosis because it targets nucleases and proteases and cleaves them free from their inhibitors. Active nucleases and proteases damage the nuclear

DNA, chromatin and cytoskeleton. The apoptotic cell shrinks in size, with a condensed nuclear DNA and detaches itself from neighboring cells. The expression on annexin V on the surface of dying cells signals nearby macrophages to engulf it. Thus ends the life of a nucleated cell (Chinnaiyan *et al.*, 1996) , (Waterhouse *et al.*, 1998), (Scaffidi *et al.*, 1998), (Rath and Aggarwal, 1999), (Sheridan *et al.*, 1997), (Li *et al.*, 1998), (Susin *et al.*, 1997), reviewed by (Robertson *et al.*, 2001).

Since platelets are not nucleated, their programmed death should be called apoptosis with caution. Scientists are divided in their opinion on this. While Valery Leytin (Leytin, 2012) unambiguiously terms all events of platelet death as apoptosis, Shaun P Jackson is more conservative to use the term for senescent platelet death (Jackson and Schoenwaelder, 2010b). All events of activation associated death are, according to her, necrosis; since activation-associated platelet death results in release of inflammatory mediators and aggregation that cause immune reactions and vascular occlution respectively.

Li (Li *et al.*, 2010a) described 14-3-3ζ mediated apoptosis in platelets through interaction with cytoplasmic domain of activated GP 1bα. In addition 14-3-3 activates pro-proliferative MAPK and inactivates apoptotic BAD in nucleated cells and 14-3-3 also mediates proliferative signal for megakaryocytes.

1.11 Interaction of Apoptotic Platelet with other Blood Cells

1.11.1 Erythrocytes

The role of erythrocytes in platelet function might be direct, indirect and mechanical. The knowledge of platelet-erythrocyte interaction is necessary since clinical information show a tendency of erythrocytes colocalizing with platelets in venous thrombus and erythrocyte over-production and inherent erythrocyte abnormalities are often found in cases of thrombosis. On the other hand, excessive bleeding can be treated by higher haematocrit (Leone *et al.*, 2001). Despite such importance, very little is known about platelet-erythrocyte interaction.

The direct interacton between platelet-erythrocyte might be mediated by surface receptors and ligands on these cells. The duffy antigens and Duffy antigen receptor for chemokines (DARC) on erythocytes interact with CCL and CXC subfamily of

chemokines (Apostolakis *et al.*, 2011). Platelets are known to express CXCL16 receptor, CCL and CXCR-6 (Seizer *et al.*, 2011) . Erythrocytes also express VCAM-1, ICAM-1,CD-36 in disease conditions (Swerlick *et al.*, 1993), (Cooke *et al.*, 1994), (Gee and Platt, 1995a), (Gee and Platt, 1995b), (Yang *et al.*, 2009) and ICAM-1 on endothelia bind to platelet integrin $\alpha_{IIb}\beta_3$ (Massberg *et al.*, 1999b), (Massberg *et al.*, 1999a;Massberg *et al.*, 1999c). Yang's group (2009) also postulated an important role of micromolecular depletion in erythrocyte-endothelial interaction, which is likely to be expanded for platelets. The others (Ho *et al.*, 1998b), (Lobel *et al.*, 1998;Ho *et al.*, 1998a) showed that *Plasmodium falciparum* infected erythrocytes bind to P-selectin under flow conditions and CD-36 and ICAM-1 is upregulated in these erythrocytes. A recent work reports erythrocytes possess fibrinogen receptor, which was detectable under atomic force microscopy, though the identity and mechanism of this receptor remains to be confirmed (Carvalho *et al.*, 2010) . ICAM-4 present on erythrocyte membrane can attach to high-affinity form of integrin $\alpha_{IIb}\beta_3$ on platelets under experimental condition (Hermand *et al.*, 2003). Finally, apoptotic erythrocytes express anionic aminophospholipid phosphatidylserine on surface of membrane, which provide a pro-coagulant surface for thrombin generation (Zwaal et al. 1981, (ChinYee *et al.*, 1995), (Kuypers, 1998), (Valles *et al.*, 2002).Since thrombin is the most potent trigger for platelet activation, the direct interaction between erythrocyte and platelet might be very initial event in thrombus formation under certain clinical conditions.

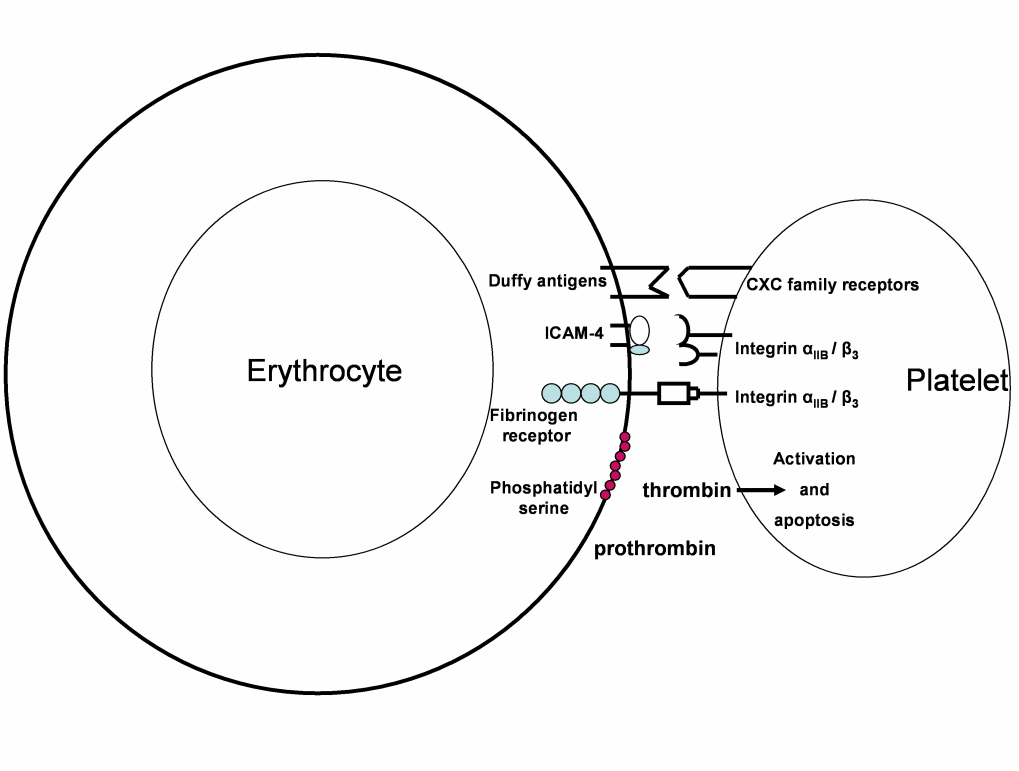


Figure 1.5: Possible Interactions between erythrocytes and platelet

(AlMomani *et al.*, 2008)) devised a micro-scale computational simulation model of RBC-platelet interaction, where platelets were found move towards the vessel wall under hydrodynamic force. The migration of platelets increased with increasing hematocrit.

1.11.2 Endothelia

Vascular injury leads to exposure of collagen, finbinogen, fibronectin, vitronectin, vWF of the tissue and release of chemoattractants which signal the platelets to the site of injury ((Gawaz *et al.*, 1996) , (Nieswandt *et al.*, 2001) , (Ruggeri, 2002a) , (Massberg *et al.*, 2003), (Nieswandt and Watson, 2003a), (Arya *et al.*, 2003). Activated endothelium expresses P-selectin, which bind to GP1bα and PSGL-1 on platelets and initiate platelet rolling (Frenette *et al.*, 1995)1995. Attachment of GP1bα to its ligand sends the inside-out signaling for integrin $\alpha_{IIb}\beta_3$ activation which stabilizes platelet-endothelial aggregate under physiologic shear stress (Bombeli *et al.*, 1998a). In addition activated platelets release a number of inflammatory mediators that stimulates endothelial NF-κB, MCP1, matrix metalloproteases and CD40L, allowing

non-haemostatic platelet-endothelial interaction to turn pro-atherogenic (Hawrylowicz et al. 1991, (Gawaz *et al.*, 1998) , (Gawaz *et al.*, 2000b), (Gawaz *et al.*, 2002) .

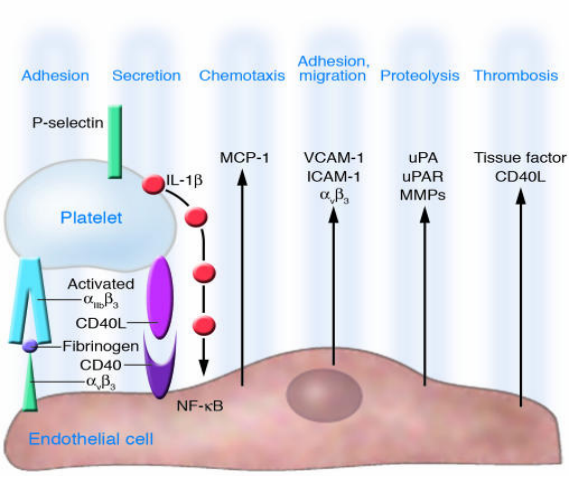


Figure 1.6: Interaction between platelet and endothelial cells (Gawaz *et al.*, 2005c)

Since integrin $\alpha_{IIb}\beta_3$ is at the heart of platelet-endothelial interaction and integrin $\alpha_{IIb}\beta_3$ is known to be upregulated by PI3K-Akt pathway (Schoenwaelder *et al.*, 2010a) 2010), interfering with platelet-specific PI3K inhibitors could be, in theory, a mechanism of reducing platelet-endothelial interaction. Such interventions might prove useful in clinical conditions such as atherosclerosis, ischemia or chronic venous insufficiency.

1.11.3 Leukocytes

Platelet interaction with monocytes are mediated by P-selectin-PSGL-1 ((Evangelista *et al.*, 1999), (Yang *et al.*, 1999). After that, CD11b and C18 bind to GP1bα (Simon et al. 2000a, (Simon *et al.*, 2000), JAM-3 (Santoso *et al.*, 2002) or ICAM-2 (Diacovo et al. 1994). Leukocyte-platelet aggregates might also form due bridging of two receptors with a common molecule. This is examplified by platelet integrin $\alpha_{IIb}\beta_3$ and leukocyte $\alpha_M\beta_2$ both bound to fibrinogen (Fitzgerald *et al.*, 1988) , reveiwed by (Gawaz *et al.*, 2005a) . Such interactions lead to selection-mediated attachment of leukocytes to endothelia, subsequent rolling, leukocyte activation and secretion and diapedesis (reveiwed by (Gawaz *et al.*, 2005b).

1.12 Chemokines in Platelet Activation and Death

Cytokines and chemokines released by inflamed endothelium or inflammatory cells have proaggregatory properties on platelets which represent an important linkage between vascular inflammation, thrombosis, and atherogenesis ((Neumann *et al.*, 1997), (May *et al.*, 2007). CXCL16 is a recently-discovered chemokine of the CXC family, CXCL16, has been proposed as a pathogenic mediator in atherosclerosis and coronary artery disease. CXCR6 is the receptor for CXCL16 (Matloubian *et al.*, 2000b;Sheikine and Sirsjo, 2008). CXCR6 promotes atherosclerosis by supporting T-cell homing and macrophage accumulation in the aortic wall as well as aortic smooth muscle cell proliferation (Chandrasekar *et al.*, 2004c;Galkina *et al.*, 2007b). Intracellular signaling followed by an activation of CXCR6 by CXCL16 was shown to involve phosphatidylinositide 3-kinase (PI3K) and its downstream effector Akt (protein kinase B) (Chandrasekar *et al.*, 2004b;Wang *et al.*, 2008a).

1.13 Practical Use of the Knowledge of Platelet Death

1.13.1 Thrombocytopenia

Thrombocytopenia refers to a condition of less than $5X10^8$ platelets/L blood. Different causes of thromocytopenia are thrombotic, idiopathic/ immune-mediated, heparin-induced, hypo-megakaryotic conditions etc. Mechanisms of platelet death are different in each case. Symptoms of thrombocytopenia include purpura, petechia, nose-bleeding, gum-bleeding, thrombosis and death if ignored (Christie *et al.*, 1990c). Heparin induced immune thrombocytopenia is a common complication, resulting from binding of serum heparin to platelet factor 4 (PF4). The complex binds to IgG, whose F(ab) domain binds to the heparin-PF4complex (Greinacher *et al.*, 1994c), (Greinacher *et al.*, 1994a;Greinacher *et al.*, 1994b) , (Amiral *et al.*, 1992) , (Kelton *et al.*, 1994) . As these immune complexes assemble on the platelet surface, the Fc domains cross link the Fc Iia receptors of nearby platelets, resulting in platelet activation (Chong *et al.*, 1989), (Warkentin and Cook, 2005) . This leads to further release of PF4, creating a positive feedback loop that propagates platelet activation and ultimately leads to removal of these platelets from the circulation, causing benign thrombocytopenia (reviewed by (Otis and Zehnder, 2010) .

One case of immune thrombocytopenia induced by drugs, high serum levels of IgG and IgM against platelet surface antigens is found in 1% of hospitalized patients undergoing long-term medication with the drug. The drug binds to F(ab) of circulating Ig and drug-Ig complex binds to integrin $\alpha_{IIb}\beta_3$ in platelet membrane. Ig bound-platelets cleared from circulation by phagocytic cells, leading to thrombocytopenic condition (Christie *et al.*, 1990b), (von Drygalski *et al.*, 2007), (Ganly *et al.*, 2011). However, there is a growing body of evidence that platelet apoptosis is responsible for paediatric thrombocytopenia (Winkler *et al.*, 2012).

1.13.2 Thrombosis

In some patients, platelet activation leads to further release of PF4, creating a positive feedback loop that propagates platelet activation and ultimately leads to removal of these platelets from the circulation (Newman and Chong, 2000) . The resultant thrombocytopenia, however, is not associated with an increased bleeding risk. Instead, ongoing platelet activation by the immune complexes results in increased thrombin production and a systemic hypercoagulable state that can culminate in venous and/or arterial thrombosis (Tardy-Poncet *et al.*, 2009) .

1.13.3 Storage and Transfusion

One limitation in blood transfusion procedure is that platelets can not be stored and supplied as required to the patients of bleeding disorders. The structural and finctional changes in stored platelets fit most closely to apoptotic changes resulting in reduced adhesion and increased pro-coagulant tendency (Klinger *et al.*, 1996), (Klinger, 1996a), (Leytin and Freedman, 2003b) , (Leytin and Freedman, 2003a;Leytin *et al.*, 2003a;Leytin *et al.*, 2003c). This is called the platelet storage lesion (PSL). S P Jackson (Jackson and Schoenwaelder, 2010a) indicated that PSL might be the result of platelet apoptosis ensuing secondary necrosis and agents preserving platelet mitochondrial function could be successfully used to improve platelet storage in the blood banks.

1.14 Insights into Pathologic and Therapeutic Agonists of Interest

1.14.1 Thymoquinone

Thymoquinone (TQ) is an active component of black cumin (*Nigella sativa)* ((Badary *et al.*, 2007), (Khader *et al.*, 2009) which is known to have anti-carcinogenic, anti-angiogenic and anti-metastatic activity (Aggarwal *et al.*, 2008), (Ali and Blunden, 2003), (Gali-Muhtasib *et al.*, 2008b) (Gali-Muhtasib *et al.*, 2008a), (Salem, 2005) . It also has anti-oxidant, anti-inflammatory and anti-toxic effects (reviewed by (Woo *et al.*, 2012). Black cumin is consumed as a spice in Asia and the Mediterranean region. Therefore blood cells are exposed to this compound. This well-known apoptosis inducing agent was applied on platelets to compare and contrast its apoptotic effect in the non-nucleated cell.

1.14.2 Peptidoglycan

The bacterial cell wall is composed of several components including peptidoglycan (PGN) (Dmitriev *et al.*, 2000;Wilk *et al.*, 2011). PGN bind to "Pattern Recognition Receptors" (PRRs) (Kirschning and Bauer, 2001a;Beutler *et al.*, 2003;Liang *et al.*, 2005;Zeytun *et al.*, 2007;Kim *et al.*, 2007;Xuan *et al.*, 2009) or “Microbe-Associated Molecular Patterns" (MAMP) (Altura *et al.*, 2011) of host cells, which are considered to include Toll-like receptor 2 (TLR2), PGN-recognition proteins (GNBP1 und PGRP-SA), and the cytoplasmic NOD1 as well as NOD2 (Chamaillard *et al.*, 2003;Carneiro *et al.*, 2004;Kaparakis *et al.*, 2010;Grubman *et al.*, 2010). Purified polymeric PGN from *S. aureus* co-localizes with NOD2 and TLR-2 in murine keratinocytes and trigger proinflammatory cytokine expression (Müller-Anstett *et al.*, 2010). The involvement of TLR2 in recognition of PGN has been a matter of debate (Dziarski *et al.*, 1996a;Takeuchi *et al.*, 1999;Schwandner *et al.*, 1999;Travassos *et al.*, 2004). PGN triggers a variety of host cell responses (Mattsson *et al.*, 1996;Dziarski *et al.*, 1996b;Rietschel *et al.*, 1998;Jin *et al.*, 1998;Schrijver *et al.*, 1999;Nakagawa and Murai, 2003;Martinon and Tschopp, 2005;Kepler and Chan, 2007) including apoptosis (Colino and Snapper, 2003;Haslinger-Loffler *et al.*, 2005;Chen *et al.*, 2005;Bluml *et al.*, 2005;Haslinger-Loffler *et al.*, 2006;Simons *et al.*, 2007).

Peptidoglycan may impact on platelet function and survival. PGN was already reported to interact with platelets (Ryc and Rotta, 1975a;Ryc and Rotta, 1978;Rotta *et al.*, 1979;Umemoto *et al.*, 1981b;Harada *et al.*, 1982b;Wannamaker,

1983b;Verhoef and Kalter, 1985;Ryc *et al.*, 1987a;Kessler *et al.*, 1991c;Polanski *et al.*, 1992b;Imegwu *et al.*, 1997a;Hoijer *et al.*, 1997b;Tydell *et al.*, 2002a;Bensing *et al.*, 2004a;Rennemeier *et al.*, 2007a;Keane *et al.*, 2010b). PGN from Gram-positive bacteria can bind to thrombospondin-1 (Rennemeier *et al.*, 2007b) and PGN-mediated platelet aggregation was observed in septicemia (Kessler *et al.*, 1991b).

TLR signaling Toll-like receptors are a group of G-protein coupled receptor that take part in innate immune response (Aderem and Ulevitch, 2000b) in mammals. TLR-2 is encoded from 4q32 gene in humans and transcripted upon stimulation by non-self antigens (Aderem and Ulevitch, 2000a). Platelets express TLR 2,4,6 and 9. TLR-2 and 4 are best studied in platelets because because they recognize components of microbial pathogens and initiate functional changes in platelets (Shiraki *et al.*, 2004), (Cognasse *et al.*, 2005). Müller and Müller-Anstett (Müller-Anstett *et al.*, 2010) reported that PGN colocalizes with TLR-2 and TLR-2 recognized bacterial pathogens in septicemia. Septicemia is often associated with thrombotic disorders (Obando *et al.*, 2011). TLR-2 forms a heterodimer with TLR-1 or TLR-6 upon activation and forms a signaling complex recruiting CD-14, MyD88 and IRAKs. The signaling event is followed by PI3K, Akt-2, p38MAPK and NFκB, finally leading to integrin $\alpha_{iib}\beta_3$ and secretion (Blair *et al.*, 2009a). In nucleated cells, PI3K transmits signals that negatively regulate TLR functions (Hazeki *et al.*, 2007).

TLR function is somewhat different in platelets. Kälvegren and group (Kalvegren *et al.*, 2010) showed that TLR-2 stimulation is mediated by purinergic receptors. P2X1 and TLR-2 together mediate the Calcium influx, cyclooxygenase, P2Y1 and P2Y12 receptor activation. Berg et al. (Berg *et al.*, 1994) showed the synthetic TLR-1/2 agonist induces physiologic platelet activation and serotonin release. Damas et al. (Damas *et al.*, 2006)) reported TLR-2 is upregulated and activated in PAR-1 agonist-treated platelets and these receptors act together in response to African tick fever agent and release sCD40L, causing inflammatory reaction. PAR-1 from epithelial cells are known to engage in complex interaction with NOD receptors, which are intracellular PGN ligand (Chung *et al.*, 2010).It is an interesting question which other receptor concert with TLR-2 in platelets upon PGN stimulation.

1.14..3 Vancomycin

Vancomycin is an antibiotic belonging to the glycopeptide group (Levine, 2006). It is specially active against Gram positive bacteria and therefore used to treat a wide range of infections (Tang *et al.*, 2011). Resistance of pathogenic bacteria to vancomycin is still low compared to other widely used antibiotics (Smith *et al.*, 1999), still vancomycin is prescribed with caution due to its side-effects. The detrimental side-effects of vancomycin include nephrotoxicity (Pritchard *et al.*, 2010a), ototoxicity (Forouzesh *et al.*, 2009;Pritchard *et al.*, 2010b) and thrombocytopenia (Mizon *et al.*, 1997), (Christie *et al.*, 1990a). The intensity of drug-mediated thrombocytopenia inspired clinical scientists to investigate the problem and gradually the mechanism of vancomycin-induced anti-platelet IgG and IgM were found out, which bind to integrin $\alpha_{IIb}\beta_3$ and possibly mediate platelet phagocytosis by macrophages (Young, 2004)), (Von Drygalski *et al.*, 2007), (Kenney and Tormey, 2008a). Suzuki and Moake in different papers (1980) also described enhanced vWF-binding after vancomycin treatment. Howard and group ((Howard *et al.*, 1997) had earlier proposed a mechanism of direct toxic effect, hapten formation and toxicity due to bystander immune response against vancomycin, yet a direct destructive effect of vancomycin on human platelets has not been experimentally shown.

Some recent groups have focused on the apoptotic events in platelets and the relation and regulation of such events in non-nucleate platelets with other events of platelet biology, such as activation, agglutination, secretion and clot retraction (Wolf *et al.*, 1999b;Schoenwaelder *et al.*, 2011),(Jobe *et al.*, 2008). Therefore the effect of the well-known anti-thrombotic antibiotic vancomycin seems to be re-investigated because alternatively, thrombocytopenia could result from excessive suicidal platelet death or apoptosis. This thesis attempted to explore if vancomycin causes direct apoptotic events in platelets and if so, what triggers the pathway.

2. Aim of the Study

The aims of this thesis was to identify the mechanisms of apoptosis in platelets and relate them to existing clinical complications. The receptors and the downstream

cascade might be subjected to pharmacological intervention to save incidents of life-threatening thrombocytopenia. The strategy can be outlined as follows:

- exposing the human platelets to a specific agent and detecting the markers of apoptosis.
- finding out a basic pathway leading to the apoptotic events.
- identifying the receptor or a key molecule that initiates the pathway.
- investigating the role of platelet activation in platelet-endothelial interaction.

Chapter 2

Materials and Methods

Chapter 2
Materials and Methods

The response of platelets to the substances of interest (Thymoquinone, peptidoglycan, vancomycin and CXCL16) were assayedthrough *in vitro* experiments.

2.1 Collection of Blood

Freshly drawn blood from healthy humans between the age of 22 to 50 years with informed consent. Platelets are sensitive to types of anticoagulants, therefore different anti-coagulants were used to study different agonists. Fresh EDTA-anticoagulated blood was obtained from the blood bank of Eberhard-Karls-Universität Tübingen, Germany, according to the Ethics Committee of the University (184/2003V) for the study of thymoquinone and peptidoglycan. To study aminoglycosides, ACD-anticoagulated blood was obtained from healthy volunteers according to the Ethics Committee of SFB Germany (273/2011 BO2). Blood was taken from a peripheral vein slowly into warm anticoagulant not to agitate the platelets.

2.2 Isolation of Platelets

In case of peptidoglycan and thymoquinone, the freshly-drawn blood was centrifuged at 160 g for 20 minutes at 20°C. The platelet rich plasma was separated and centrifuged at 2000 g at 20°C for 2 minutes and washed twice in 900 µl platelet buffer (137 mM NaCl, 2.7 mM KCl, 2 mM $MgSO_4$, 10mM HEPES, 5 mM glucose, pH 7.4) without calcium and 100 µl acid citrate dextrose solution (80 mM trisodium citrate, 52 mM citric acid, 180 mM glucose). The isolated platelets were suspended in platelet buffer with 0.1 % BSA. Care was taken not to expose the platelets to excess meachanical stress or Oxygen exposure.

To study aminoglycosides, ACD-anticoagulated blood was obtained from healthy volunteers according to the Ethics Committee of the Eberhard Karl University Tuebingen, Germany (184/2003V). The blood was centrifuged at 160 g for 20 minutes at 25° C. The platelet rich plasma was separated, added with Tyrode buffer

(137mM NaCl, 2.8mM KCL, 12mM $NaHCO_3$, 5mM glucose, 0.4mM Na_2HPO_4, 10mM HEPES, 0.1% BSA), pH 6.5 in 1:7 volumetric ratio and centrifuged at 900 g and 25°C for 10 minutes. The platelet pellet was resuspended in 250 µl of Tyrode buffer (pH 7.0).

2.3 Study of platelet apoptosis with thymoquinone

2.3.1 Stimulation of platelets with thymoquinone

The stimulation of platelets was done in platelet buffer with 5 mM $CaCl_2$ with 10^6 platelets in each reaction in a total volume of 1 ml. The platelets were added with 1 µM, 5 µM, 10 µM and 50 µM thymoquinone (Sigma-Aldrich, USA) and incubated for 1 hour at 37°C. A positive control with 1 U/ml thrombin (Calbiochem, USA) and a negative control without thymoquinone were compared with each set of experiment.

2.3.2 Inhibition of PI3K signaling

Isolated platelets were suspended in platelet buffer with 5mM $CaCl_2$ in a concentration of 10^6/ml and three sets of experiments were done. Platelets were pretreated with wortmannin (100 nM, Calbiochem, USA) or DMSO for 15 minutes and then stimulated with thymoquinone (10 µM and 50 µM) or thrombin (1 U/ml) for 30 minutes at 37°C. Samples were stained with Annexin V Fluos, Fluo 3-AM, anti-active Caspase-3-FITC or DiOC6 and measured with the Fluorescence activated cell sorter (FACS) machine from BD Biosciences (FACS Calibur). This machine detects fluorescent signal from a cell and the fluorescence is imparted by a fluorescent molecule or a fluorochrome-conjugated antibody targeted against a particlular bio-molecule of interest.

2.3.3 Inhibition of G-protein Coupled Receptor (GPCR)

10^6 platelets were suspended in platelet buffer containing 5 mM $CaCl_2$ and one set of platelets were pretreated with 1 ng/ml pertussis toxin (PTX) for 30 minutes. Both sets were then added with 1 U/ml thrombin or thymoquinone (10 µM or 50 µM). All the samples were stained with Annexin V Fluos or anti-active caspase-3-FITC and analysed with FACS.

2.4 Stimulation of isolated platelets with peptidoglycan

The platelets were stimulated in platelet buffer with 2 mM $CaCl_2$ containing 1 million platelets in each reaction so that the total volume was 1 ml. The lyophilized peptidoglycan (PGN) fraction was dissolved in MilliQ water (Millipore, USA) to produce the stock solution. Where indicated 50 ng/ml, 100 ng/ml, 250 ng/ml, 500 ng/ml and 1000 ng/ml peptidoglycan monomer preparation were added to separate sets of platelets and incubated at 37°C. Platelets were stained for Calcium influx After 5 minutes of incubation and the staining for PS exposure, integrin $\alpha_{IIb}\beta_3$ detection and caspase-3 activation were done after 30 minutes of incubation. A negative control of platelet buffer with 2 mM $CaCl_2$ without peptidoglycan (PGN) and a positive control with platelet buffer-2 mM $CaCl_2$ with 10 µM ionomycin (Calbiochem, USA) was analysed simultaneously in each set of experiments. Each blood sample was studied for primary apoptosis markers (PS exposure, Ca influx, mitochondrial depolarization and caspase-3 activation). Mitochondrial membrane depolarization was measured from platelets stimulated in phosphate buffered saline (PBS) supplemented with 1 mM $MgCl_2$, 5.6 mM glucose, 0.1% BSA and 10 mM HEPES (pH 7.4) for 30 minutes at 37°C.

2.4.1 Dependence of phosphatidylserine exposure upon pancaspase inhibitors

Isolated platelets were pre-treated for 10 min with 1 µM zVAD-FMK and then incubated with defined concentrations of peptidoglycan (50 ng/ml, 100 ng/ml, 250 ng/ml, 500 ng/ml and 1000 ng/ml) for 30 min at 37 °C. An unstimulated control and an inhibited control with 1 µM zVAD-FMK were used. All samples were stained with 1:20 dilution of Annexin V-Fluos (Immunotools, Germany), incubated for 30 min at 37 °C and subsequently subjected to FACS analysis.

2.4.2 Dependence of PS exposure on extracellular Calcium

Two sets of platelets were incubated to check the effect of Calcium on PS exposure. One set was incubated in platelet buffer with 1 mM EGTA and another set with 2 mM $CaCl_2$. Both sets were stimulated with 500 ng/ml of peptidoglycan monomer for 30 min and stained with 1:20 dilution of Annexin-V-FITC (ImmunoTools, Germany), followed by immediate FACS analysis.

2.4.3 TLR-2 dependence

Isolated platelets were suspended in platelet buffer with 2 mM $CaCl_2$ in a concentration of 10^6/ml and incubated with 2 µg/ml of rabbit anti-human anti-TLR-2 antibody (Santa Cruz, USA) for 30 minutes. The cells were centrifuged at 2000 g for 2 min and washed once in platelet buffer. Then they were stimulated with PGN monomer fraction 250 ng/ml, 500 ng/ml and 1000 ng/ml, stained with Annexin V FITC (Immuntools, Germany) and anti-caspase-3 FITC (Biovision Inc. USA). All the samples were measured by FACSCalibur (BD Biosciences, USA).

2.4.4 Effect of Lipopolysaccharide (LPS) stimulation

Isolated platelets were incubated 30 min with 5 µg/ml of LPS from *E. coli*, 0111:B4 (Sigma-Aldrich, USA) and together with 5 µg/ml of LPS and 500 ng/ml peptidoglycan, stained with Annexin V-FITC as described and measured in FACS.

2.5 Stimulation of isolated platelets with Vancomycin

The platelets were stimulated in Tyrode buffer (pH 7.4) with 2 mM $CaCl_2$ containing 10^6/ml platelets. Where indicated, vancomycin (synthesized by the Department of Microbiology and Biotechnology, Eberhard Karl University Tuebingen) was added at the indicated concentrations (1, 5, 10 and 15 µg/ml) for 30 minutes at 37° C. A negative control without vancomycin and a positive control with ionomycin 1µM was analyzed simultaneously with each set of experiment.

2.6 Stimulation of Platelets with CXCL16

Platelets were activated using 50ng/ml, 100ng/ml and 200ng/ml recombinant human CXCL16 (R&D Sytems), ADP (Sigma-Aldrich) and thrombin (Roche) in Tyrode buffer (pH 7.4, 2mM $CaCl_2$) for 30 minutes. For pharmacological inhibition of PI3K and Akt signaling pathway, the human platelets were pretreated with 100nM wortmannin, 25µM LY294002 and 20 µM SH-6 (all from Calbiochem) as described previously (Yin *et al.*, 2008b). Purinergic receptors P_2Y_1 and P_2Y_{12} were blocked with MRS2179 (Tocris Bioscience) and Cangrelor (AR-C69931MX, The Medicines Company).

2.7 Staining of stimulated platelets for flow cytometry

2.7.1 Phosphatidylserine exposure

Phosphatidylserine exposure was measured with 1:100 dilution of Annexin V Fluos (Roche, Mannheim, Germany) and incubated at 37°C for 30 minutes. Phosphatidylserine exposure was measured following stimulation with vancomycin, centrifuging the cells at 1000 g for 2 minutes followed by washing once with Tyrode buffer (pH 7.4) with 2 mM $CaCl_2$, staining with Annexin V Fluos (1:20 dilution, Immunotools, Germany) in Tyrode buffer (pH 7.4) with 2 mM $CaCl_2$ and incubating at 37° C for 30 minutes. The fluorescence was measured in FL-1 of a BD FacsCalibur (BD Biosciences, CA, USA).

2.7.2 Intracellular free Ca^{2+}

Stimulated platelets were incubated for 30 minutes with 5 µM Fluo 3-AM (Biotium, CA, USA) and measured in FL-1. 10^6/ml platelets were incubated with the defined concentrations of PGN fractions for 5 minutes in platelet buffer with 2mM $CaCl_2$ and stained with 5 µM Fluo-3AM (Biotium Inc. USA) for 30 minutes. The platelets were measured for intracellular Ca^{2+}-influx in the FL-1 of FACS.

2.7.3 Caspase-3

Formation of active caspase-3 was detected according to the manufacturer's instruction of CaspGlow Fluorescein Active Caspase-3 Staining kit from BioVision (CA, USA). Briefly, platelets were stimulated as mentioned before including one negative control with 10 minute pretreatment with 1µM Z-VAD-FMK at 37°C, 5% CO_2 and then centrifuged at 3000 rpm, 2 minutes, 25° C. The pellet was resuspended in 300 µl platelet buffer-2mM $CaCl_2$ followed by staining with 1µl FITC-DVD-FMK for 1 hour, 37°C, 5% CO_2. Stained cells were washed once with 0.5ml wash buffer from the kit and final pellet was resuspended in 0.5 ml wash buffer and measured immediately in FACS. Two sets of experiments were done to check the effect of calcium on caspase-3 formation. One set contained platelet buffer with 1 mM EGTA and another set contained platelet buffer with 2 mM $CaCl_2$ or in Tyrode buffer with 1mM EGTA or 2mM $CaCl_2$. Both contained 10^6 platelets and were stimulated with 0 ng/ml, 500 ng/ml peptidoglycan and 10 µM ionomycin . Active caspase-3 produced in the cells was measured by Caspase-3 Staining kit from BioVision (CA, USA).

2.7.4 Mitochondrial Membrane Potential

Platelets were first stimulated with thymoquinone as described before and 10^7 platelets were dissolved in Phosphate buffered saline (PBS) (Invitrogen, CA, USA) supplemented with 1 mM $MgCl_2$, 5.6 mM glucose, 0.1% BSA and 10 mM HEPES (pH 7.4) in a total volume of 1 ml and stained with 10 nM $DiOC_6$ (Invitrogen, CA, USA) for 10 minutes. The stained cells were centrifuged at 1000 g for 5 minutes at 20°C, resuspended in PBS and measured in FL-1 (Leytin *et al.*, 2006a;Leytin *et al.*, 2006d).

2.7.5 Ceramide formation

For detection of ceramide formation, 10^8/ml platelets were stimulated as described before and centrifuged at 2000 g for 2 minutes and the pellet was incubated with 50 µl of 1:5 dilution of mice antibody to human ceramide (Alexis, USA) in PBS with 1% BSA for 1 hour at 37°C and 5% CO_2. Then primary stained cells were centrifuged and the pellet was stained with 50 µl of 1:50 dilution secondary goat anti-mouse IgG (BD Pharmingen, Hamburg, Germany) for 20 minutes. The geomean of the FITC-labeled secondary Ig was measured.

2.7.6 Platelet degranulation and integrin $\alpha_{IIb}\beta_3$ activation

Platelets were stimulated as described before. Here 25 µlitre of stimulated platelets were stained with 5µl FITC-conjugated antibody to human CD62P or PAC1 antibody (BD Biosciences, USA) according to manufacturer's instruction for 30 minutes. The reaction was stopped with 75 µl PBS and analyzed by FL-1 in FACS.

2.7.7 Staining for CXCL16 Stimulation

Human platelets stimulated with CXCl16 were stained with PE-labeled mouse anti-human CXCR6 monoclonal antibody (Clone 56811, R&D Systems) (diluted 1:5) and incubated at room temperature in the dark. After incubation, the samples were added to ten times volume of PBS and analyzed on FL-2 channel of FACS-Calibur (BD Biosciences). Suitable isotype controls (mouse anti-human IgG PE) was used (BD Biosciences) along with the negative control. The platelet population was defined in the FSC/SSC dot plot and gated for further analysis. As marker for degranulation,

platelet P-selectin expression was measured using a FITC-labeled mouse anti-human P-selectin monoclonal antibody (Clone AK-4, BD Biosciences). Activated integrin $\alpha_{IIb}\beta_3$ was quantified through binding of the FITC-labeled mouse anti-human monoclonal antibody PAC-1 (BD Biosciences).

2.8 Western blot for Caspase-3 protein abundance

For western blots,10^8 platelets were stimulated with the defined concentrations of thymoquinone and washed twice in platelet buffer, lysed with 200 µl of 1x RIPA lysis buffer (Cell Signaling Technology). The cell lysate was centrifuged at 14000 rpm for 30 minutes at 4°C and the supernatant was taken for western blot.

For detection of caspase-3, 40 µg protein was loaded for electrophoresis, followed by blotting onto Protran nitrocellulose membrane (Whatman, Dassel, Germany), blocked with TBS-T (NaCl 80 g/l, Tris-HCl 24.2 g/l, Tween 20 0.1 %, pH 7.6) containing 5 % BSA and stained overnight with 1:1000 dilution of caspase-3 rabbit monoclonal antibody (Cell Signaling Technology). The blots were incubated for 1 hour with 1:5000 dilution of the HRP-conjugated anti-rabbit Ig (Cell Signaling Technology,USA). The binding of antibodies was detected by Enhanced Chemiluminescence Kit (Amersham Biosciences, Freiburg, Germany). Beta-actin (Cell Signaling, USA) was used as a loading control.

2.9 Immunofluorescence and confocal microscopy

Fresh isolated platelets were adhered to a fibrinogen surface (20 µg/ml) on chamber slides and fixed with 2 % paraformaldehyde. The platelets were washed and blocked with 2 % bovine serum albumin for 30 minutes, followed by an Triton X-100 treatment for permeabilization. For primary antibody treatment 1:100 dilution of Annexin V Fluos (Roche, Mannheim, Germany) or 1:50 Caspase-3 rabbit monoclonal antibody (Cell Signaling Technology) was incubated for 2 hours at RT. Chamber slides were washed and incubated with secondary antibody labeled with FITC (Santa Cruz) in the case of the caspase-3 staining. The actin cytoskeleton was stained with rhodamine-phalloidin (Invitrogen). Confocal microscopy was performed using a Zeiss LSM5 EXCITER Confocal Laser Scanning Microscope (Carl Zeiss Micro Imaging, Jena, Germany) with a A-Plan 63 x ocular.

2.10 Detection of Thromboxane B_2 (TxB_2) and Protaglandin E_2 (PGE_2) with ELISA

The vancomycin-treated platelets were centrifuged at 1000g for 2 minutes at 25°C and 1000 µl the supernatant was mixed with 200µl methanol (Merck, Germany) and vortexed briefly. This sample was added in 50µl volume to each of the wells of the target protein (Thromboxane B_2) coated ELISA plates (Oxford Biosciences, UK) and incubated and room temperature with shaking for 2 hours. The plate was washed three times with 300 µl wash buffer and incubated 1 hour with 50µl HRP-conjugated TxB_2 antibody. This was again washed three times with dilute wash buffer and incubated for 30 minutes with 150µl of TMB substrate for 10 minutes, blocked with 1N HCl and measured immediately at 450nm in a Biotek Power Wave XS2 ELISA reader against a standard of 0, 0.1, 0.2, 0.4, 1, 2, 4 and 10 ng/ml. The amount of TxB_2 formed in the samples were determined from a curve of concentration vs actual absorbance with Microsoft Excel 2007.

Formation of PGE_2 was determined the same way with PGE_2 standards of 0, 0.1, 0.2, 0.4, 1, 2, 4 and 10 ng/ml (Oxford Biosciences, UK).

2.11 Preparation of mouse platelets

Platelets were obtained from 10- to 12-week-old *akt1*$^{-/-}$ mice and *akt1*$^{+/+}$ mice as well as *akt2*$^{-/-}$ mice and *akt2*$^{+/+}$ of either sex. The mice were anesthetized with ether and blood was drawn from the retro-orbital plexus into four-times volume of TBS-Heparin (Tris-HCl 20mM, NaCl 137mM, Heparin 20U/ml) solution. Blood parameters were analyzed with pocH-100iv automatic hematology analyzer (Sysmex, Japan). The platelet rich plasma (PRP) was obtained by adding double volume of modified Tyrodes buffer (pH 7.4) and bringing the total volume to 3 ml with Tyrodes buffer pH 6.5, centrifuged at 120 g for 20 minutes at 25°C without brake. PRP was then carefully transfreed to another tube and centrifuged at 1200 g for 10 min at 25°C with brake to pellet the platelets. After a further washing step, the pellet of washed platelets was resuspended in modified Tyrode-HEPES buffer (pH 7.4, supplemented with 1 mM $CaCl_2$).

2.12 Flow cytometry of murine platelets

Two-color analysis of mouse platelet activation was conducted using fluorophore-labeled antibodies for P-selectin expression (Wug.E9-FITC) and the active form of $\alpha_{IIb}\beta_3$ integrin (JON/A-PE). Heparinized whole blood was diluted 1:20 in modified Tyrode buffer and washed twice. After adding 1 mM $CaCl_2$, blood samples were simultaneously mixed with antibodies in 1:10 dilution and subsequently stimulated with agonists for 15 minutes at room temperature. The reaction was stopped by addition of PBS and immediately analyzed on FL-1/FL-2 setting in FACSCalibur flow cytometer.

2.13 Investigation of platelet-Endothelial Interaction with Perfusion Chamber

The HUVEC cells from early passage were grown to confluency in complete endothelium basal medium (PAA, Austria). Sterile 100mm glass cover slips were coated for 30 minutes with 0.2% gelatin (Sigma, USA) and HUVEC cells were harvested with trypsin. 5 $X10^5$ HUVEC per ml was added to the cover slips and incubated overnight at 37^oC, 5% CO_2. Adhesion experiments under flow conditions were performed as described by HF Langer (Langer *et al.*, 2006)). Washed human platelets were suspended in PBS at 10^8/ml concentration and were perfused over the HUVEC monolayer in a flow chamber model (Havard, PHD Ultra) at high shear rates (2000^{-s}) and the cellular interaction events were recorded with a CCD camera (Carl Zeiss) with 40x magnification, followed by analysis of the number adherent platelets per high powerfield.

2.14 Statistical Analysis

The statistical significance of the results was determined by one-way ANOVA from the raw data with Graphpad Instat statistical sofware. The statistics for calcium-dependence of caspase-3, PI3K inhibition and GPCR inhibition, purinergic receptor inhibiton were determined by Student's paired t-test on the raw data of the experiments from platelet stimulation. Microsoft Excel 2007 was used for the calculation. The statistical significance between erythrocyte adhesion in treated and non-treated cells were determined with Student's paired t-test on the raw data.

Chapter 3

Results

3. Results

Programmed death in a non-nucleated cell fragment is of interest because regulation of cellular events in non-genomic level is not well-understood. Four different agonists were used to see platelet apoptotic response and major pathways involved in these signaling.

3.1 Induction of platelet apoptosis by thymoquinone

A key event of suicidal death of cells is cell membrane phospholipid scrambling with subsequent exposure of phosphatidylserine at the cell surface. The phosphatidylserine exposing cells can be identified utilizing Annexin V binding.

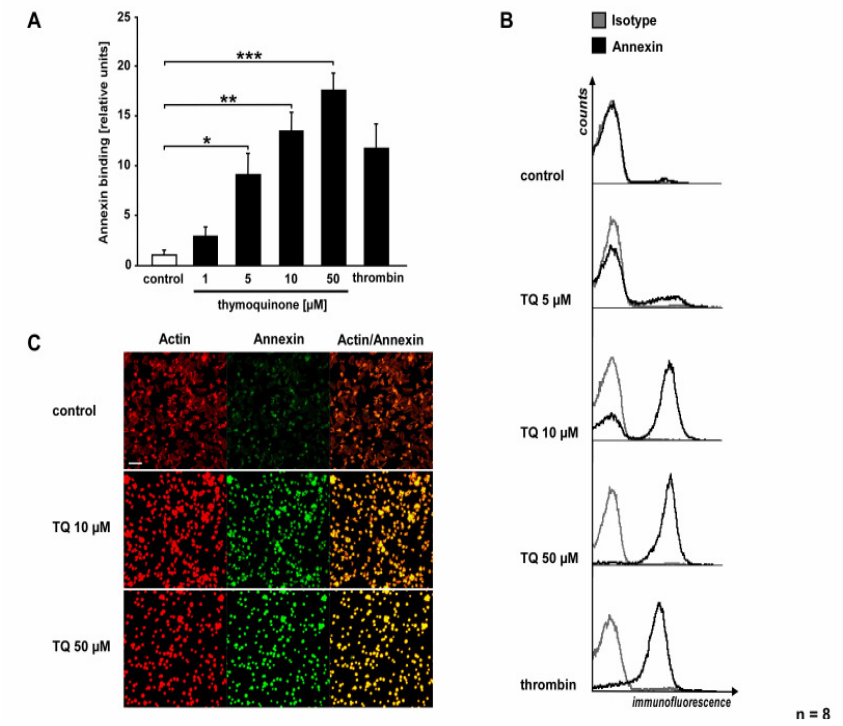


Fig. 3.1.1: PS exposure in platelets by thymoquinone

(A) Arithmetic means ± SEM (n = 8) of the relative units of human platelets binding Annexin V Fluos following a 60 minutes exposure to platelet buffer in the absence (control, open bar) and presence of thymoquinone (TQ) (1-50 µM) or of thrombin 1 U/ml (closed bars) as positive control. *p<0.05, **p<0.01 and ***p<0.001 indicate statistically significant difference to value in the absence of TQ.
(B) Representative histograms of Annexin V binding of platelets exposed for 60 minutes to (from above) 0 (control), 1-50 µM TQ or 1 U/ml thrombin.
(C) Representative immunofluorescence Annexin V staining following a 60 minutes exposure to platelet buffer in the absence (control) or presence (10 and 50 µM) of TQ. Red – rhodamine phalloidine, green – annexin V. Magnification bar represents 5 µm.

To explore, whether thymoquinone (TQ) stimulates suicidal platelet death, Annexin V binding has been determined prior to and following treatment with TQ. As illustrated in Fig.3.1, a 60 minutes exposure to TQ was indeed followed by a marked increase of the percentage Annexin V binding platelets. The effect reached statistical significance at ≥ 5 µM thymoquinone (Fig. 3.1.1).

In a wide variety of cells, cell membrane scrambling is triggered by the executor caspase-3. Thus, a second series of experiments was performed to explore whether TQ stimulates caspase-3 in platelets. As illustrated in Fig. 3.1.2, caspase-3 activity was indeed increased by a 60 minutes exposure to TQ, an effect reaching statistical significance at ≥1 µM thymoquinone. Fig. 3.2.1 A shows the dose-dependent increase of active caspase-3 in response to TQ. This caspase-3 activation is further validated by western blots, where the degradation of pro-caspase-3 reaches a statistical significance at ≥5 µM TQ (Fig. 3.1.2 C). A third technique validationg caspase-3 activation is shown with confocal microscopy, where comparable results are found (Fig. 3.1.2 D). This profound importance of caspase-3 activation in platelet death is reflected by its role is phosphatidyl serine (PS) exposure. As shown in Fig. 3.1.2.E, inhibition of caspase-3 by pan-caspase inhibitor zVAD-FMK (1µM) significantly blunts PS exposure on the surface of apoptotic platelets.

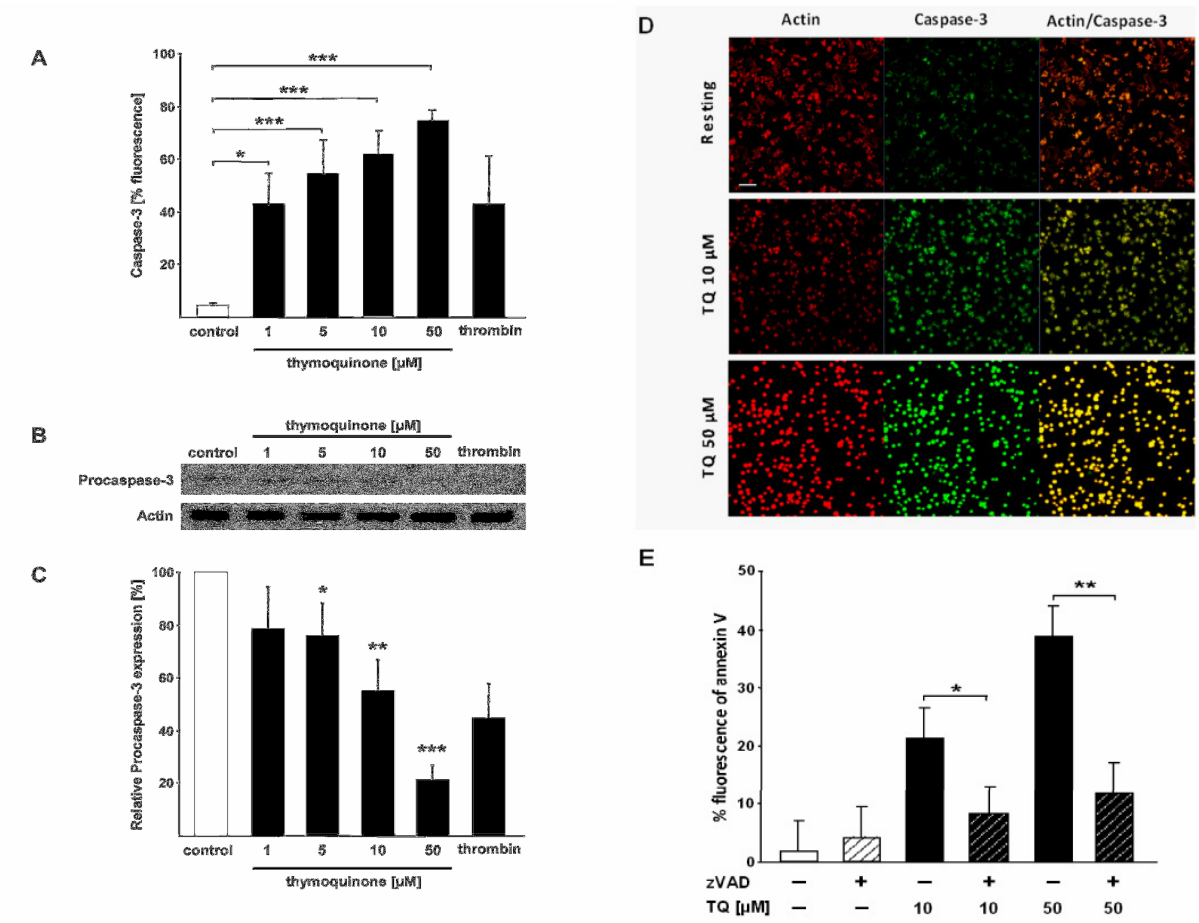


Fig 3.1.2: Caspase-3 activation in human platelets by thymoquinone.

(**A**) Arithmetic means ± SEM (n = 8) of the % human platelets expressing active caspase-3 following a 60 minutes exposure to platelet buffer 5mM $CaCl_2$ in the absence (open bar) and presence (closed bars) of thymoquinone (TQ, 1-50 µM) or of thrombin (1 U/ml) as positive control. *p<0.05, **p<0.01 and ***p<0.001 indicates statistically significant difference to value in the absence of TQ.

(B) Original Western blot of procaspase-3 abundance in human platelets following a 60 minutes exposure to platelet buffer 5 mM $CaCl_2$ in the absence (open bar) and presence (closed bars) of TQ (1-50 µM) or of thrombin (1 U/ml) as positive control. Platelet extracts were blotted with Ig against procaspase-3 (34 KDa). Protein loading was controlled by determination of β-actin.

(C) Arithmetic means ± SD (n = 4) of the ratio of procaspase-3 and β-actin following a 60 minutes exposure to platelet buffer 5mM $CaCl_2$ in the absence (control) or presence (1, 5, 10, and 50 µM) of TQ or of thrombin (1 U/ml) as positive control. *p<0.05, **p<0.01 and ***p<0.001 indicates statistically significant difference to value in the absence of TQ.

(D) Representative immunofluorescence staining of caspase-3 staining following a 60 minutes exposure to platelet buffer 5mM $CaCl_2$ in the absence (control) or presence (10 and 50 µM) TQ. Red – rhodamine phalloidine, green – caspase-3. Magnification bar represents 5 µm**.**

(E) Arithmetic means ± SEM (n = 8) of the % human platelets binding Annexin V Fluos following a 60 minutes exposure to platelet buffer without (white bar) or with (black bar) thymoquinone (TQ) in the absence (not dashed) and presence (dashed) of pancaspase inhibitor zVAD (1 µl/ml). *p<0.05 and **p<0.01 indicate statistically significant difference to value in the presence of thymoquinone but absence of zVAD.

Cell membrane scrambling can further be activated by increased cytosolic Ca^{2+} activity. Thus, an additional series of experiments explored whether TQ modifies intracellular Ca^{2+} activity. As shown in Fig. 3.1.3, a 30 minutes exposure to TQ was indeed followed by an increase of Fluo 3 fluorescence, reflecting enhanced cytosolic Ca^{2+} activity. A next series of experiments explored whether the increase of cytosolic Ca^{2+} activity was required for the stimulation of caspase-3. To this end, experiments were performed in the presence and absence of extracellular Ca^{2+}. As depicted in Fig. 3.1.3, the increase of caspase activity was significantly blunted in the absence of extracellular Ca^{2+}. However, the complete removal of extracellular Ca^{2+} did not fully prevent the thymoquinone induced stimulation of caspase-3.

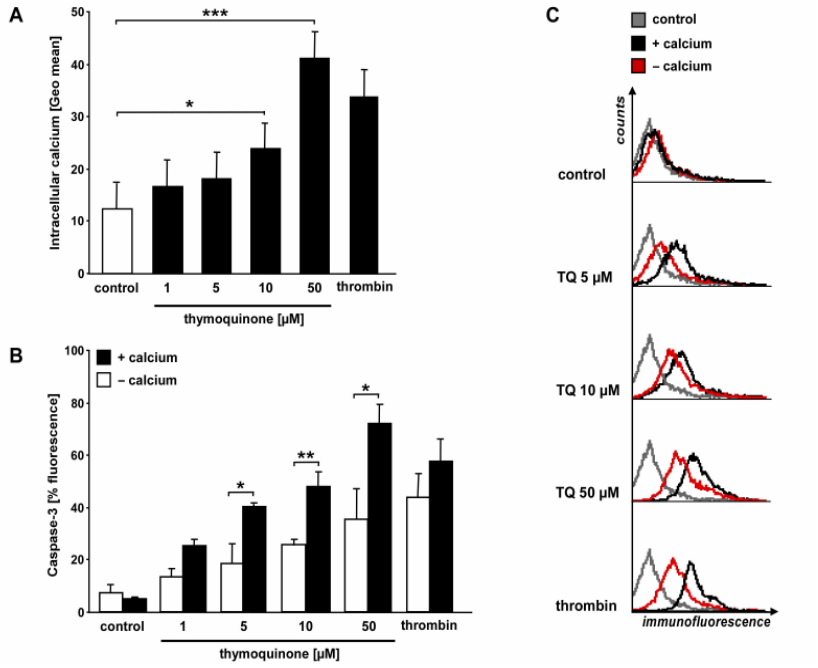
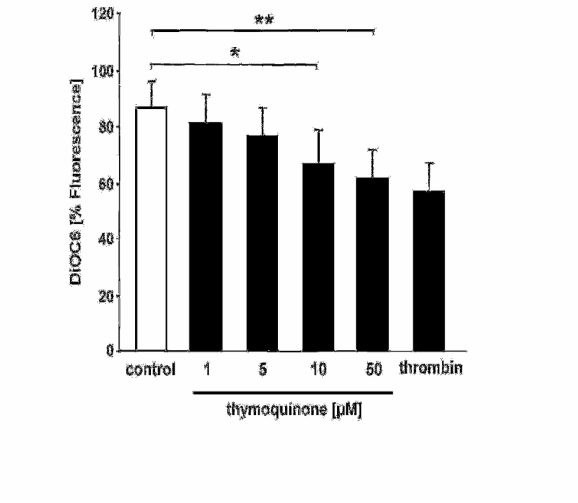


Fig 3.1.3: Calcium dependence of thymoquinone induced platelet apoptosis.
(A) Arithmetic mean ± SEM (n = 8) of Fluo 3 fluorescence in human platelets following a 60 minutes exposure to platelet buffer 5mM $CaCl_2$ in the absence (control, open bar) and presence (closed bars) of thymoquinone (TQ, 1-50 µM) or of thrombin (1 U/ml) as positive control.
(B) Arithmetic mean ± SEM (n = 8) of % of active caspase-3 (17/19 KDa) induction in TQ-treated (1-50 µM) human platelets. *p<0.05 and **p<0.01 represent significant difference between active caspase-3 induced in platelets stimulated in 0 mM $CaCl_2$ and 2 mM $CaCl_2$ by student's paired t-test.
(C) Representative histograms of caspase-3 fluorescence reflecting caspase-3 activity in platelets exposed for 60 minutes to platelet buffer 5mM $CaCl_2$ without (control, grey lines) or with (from above) 0 (control), 1-50 µM TQ or 1 U/ml thrombin as positive control in the presence (black lines) or absence (red lines) of Ca^{2+}.
In all cases**,** *p<0.05, **p<0.01 and ***p<0.001 indicates statistically significant difference to value in the absence of thymoquinone.

In search for an additional mechanism stimulating caspase activation, we explored whether TQ stimulates the formation of ceramide. As shown in Fig. 3.1.4A, a 60 minutes exposure to TQ was followed by an increase of ceramide abundance at the cell surface. The effect reached statistical significance at ≥ 10 µM thymoquinone.

Caspases are further activated following mitochondrial depolarization. Accordingly, the mitochondrial potential was determined prior to and following a 60 minutes exposure to thymoquinone. As shown in Fig. 3.1.4B, TQ treatment was followed by a decline of mitochondrial potential, an effect reaching statistical significance at ≥ 10 µM TQ.

A



B

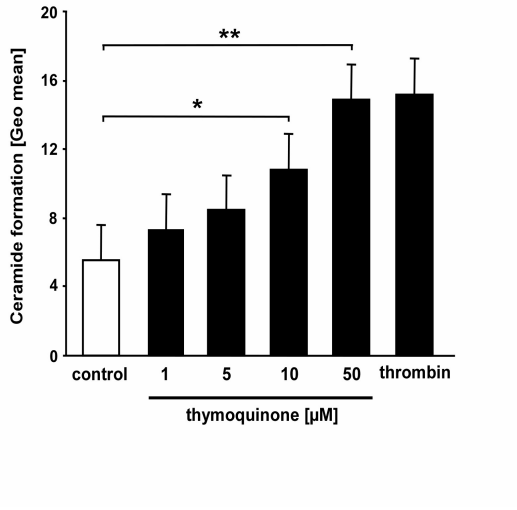


Figure 3.1.4: Depolarization of mitochondrial membrane and ceramide formation in thymoquinone (TQ)-treated human platelets.

(A) Depolarization of mitochondrial membrane after thymoquinone (TQ) treatment. Here the graph is constructed from arithmetic mean ± SEM (n = 11) with FACS analysis of TQ (1-50 µM) stimulated platelet stained with 10 nM DiOC6. *p<0.05 and **p<0.01 show significant decrease in mitochondrial membrane potential. The statistical analysis are done with one-way ANOVA.

(B) Formation of ceramide in TQ (1-50 µM) stimulated platelets. Arithmetic mean ± SEM (n = 11) for fluorescence of anti-ceramide antibodies.*p<0.05 and **p<0.01 show significant increase in ceramide formation with increasing dose of TQ. The statistical analysis are done with one-way ANOVA.

Thymoquinone has not been found to increase the activation-dependent markers of degranulation or integrin $\alpha_{IIb}\beta_3$ activation in platelets, namely CD62P and PAC-1. Additional experiments were performed to determine the signalling pathway required for the TQ induced caspase activation, The first series of experiments addressed the putative involvement of the phosphoinositide-3-kinase (PI3K)

pathway, which is known to impact on apoptosis (Zimmermann *et al.*, 2001;Duronio, 2008). As a result, the inhibition of PI3K with 100 nM wortmannin (Chakravarty, 1993d) significantly downregulated phosphatidylserine (PS) exposure, intracellular calcium influx, caspase-3 activation and abrogated the depolarization of mitochondrial membrane following TQ and thrombin treatment. Accordingly, PI3K is apparently involved in the triggering of platelet apoptosis by TQ. PI3K is acivated by G-protein coupled receptors (GPCR) (Hawkins *et al.*, 2010;Fan and Weiss, 2010). Pretreatment of of platelets with 1 ng/ml of the GPCR blocker pertussis toxin (PTX) (Chakravarty, 1993c;Carbonetti, 2010) significantly blunted the PS exposure and caspase-3 activation by TQ. Thus, TQ triggering of caspases involves PTX sensitive GPCR.

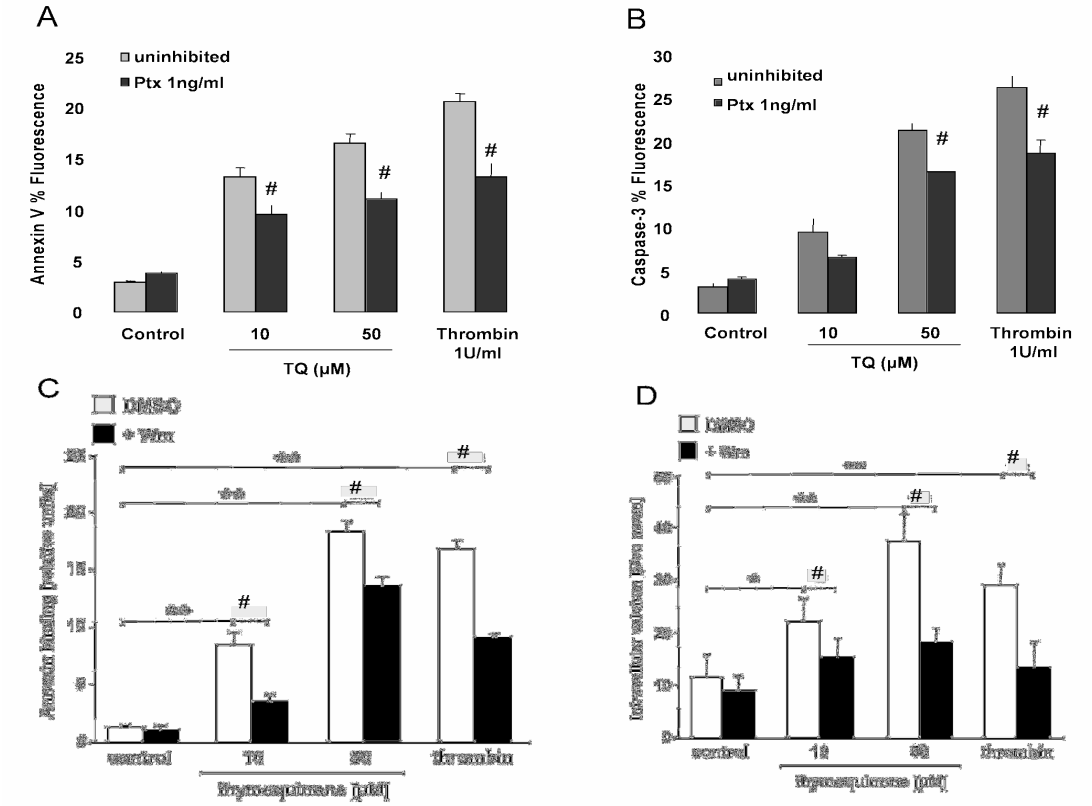


Fig. 3.1.5: Effect of the GPCR inhibitor (PTX) on TQ–stimulated platelets.
(**A)** Arithmetic mean ± SEM (n = 5) of Annexin V FITC fluorescence from human platelets preincubated with (black bar) and without (white bar) G-protein coupled receptor (GPCR) inhibitor PTX followed by stimulation with TQ (10 µM and 50 µM).
(B) Arithmetic mean ± SEM (n = 5) of anti-active caspase-3-FITC fluorescence from human platelets preincubated with (black bar) and without (white bar) PTX followed by TQ stimulation (10 µM and 50 µM).
(C) Arithmetic mean ± SEM (n = 8) of the relative units of Annexin V FITC bound to human platelets following 15 minutes preincubation with phosphoinositide-3-kinase (PI3K) inhibitor wortmannin (100 nM, black bar) and 30 minutes incubation with thymoquinone (10 µM or 50 µM). Platelets not inhibited (DMSO as solvent control) are represented as white bar.

(D) Arithmetic mean ± SEM (n = 5) from fluorescence of Fluo 3-AM in human platelets treated with (100 nM, black bar) or without wortmannin (DMSO, white bar) and 30 minutes incubation with TQ 10 µM and 50 µM (black bar).
Thrombin (1 U/ml) was used as positive control in all tests.
*p<0.05 and **p<0.01 denoted significant increase from untreated control and Student's t-test between inhibited and non-inhibited platelets exhibited significant difference with #p<0.05 and ##p<0.01.

Thymoquinone-mediated death of platelets might be termed apoptosis because the agrregation and secretion markers are not upregulated (Fig. 3.1.6).

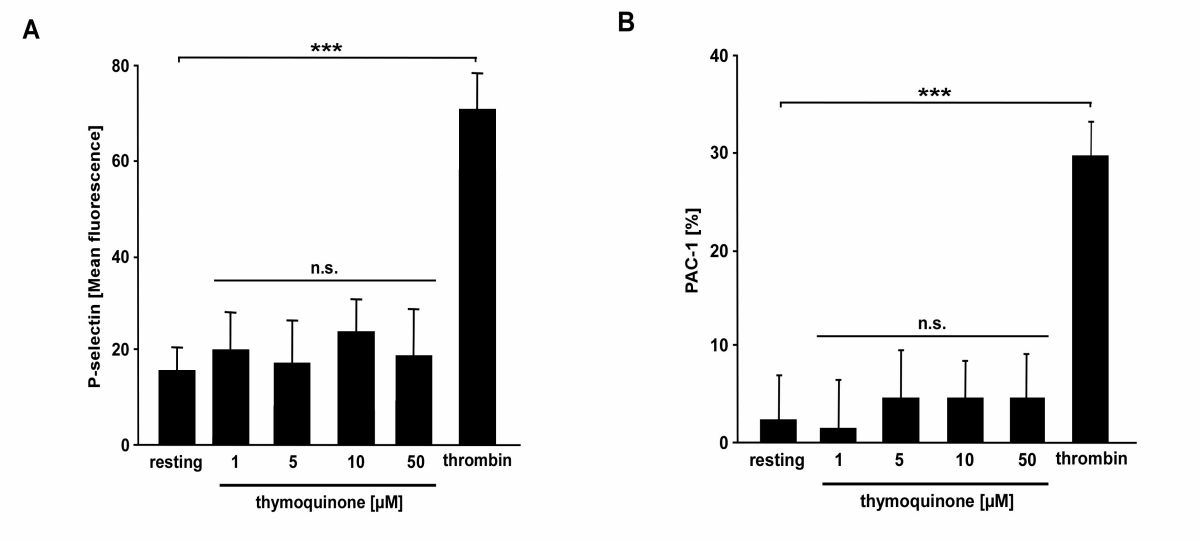


Fig. 3.1.6: Activation markers on thymoquinone (TQ) activated human platelets.
(A) Arithmetic mean ± SEM (n = 7) with FACS analysis of TQ (1 - 50 µM) stimulated platelet stained with anti-CD62P-FITC. The statistical analysis was done with one-way ANOVA. ***p<0.01 indicates statistically significant difference.
(B) Formation of Integrin IIb/IIIa in TQ (1-50 µM) stimulated human platelets. Bar diagram shows arithmetic mean ± SEM (n = 4) of fluorescence intensity from PAC1 – FITC antibodies with FACS analysis. The statistical analysis are done with one-way ANOVA. ***p<0.01 indicates statistically significant difference.

3.2 Stimulation of platelet apoptosis by peptidoglycan

The peptidoglycan (PGN) preparation was made from culture of wild type *Staphylococcus aureus* 113. The monomeric fractions containing N-acetylglucosamine and N-acetylmuramic acid with up to five amino acid residues (Fig. 3.2.1 A) was purified through HPLC. Binding of fluorescent annexin-V was taken as a measure of cell membrane phopholipid scrambling with subsequent exposure of phosphatidylserine at the cell surface. To explore whether peptidoglycan (PGN) stimulates platelet cell membrane scrambling, annexin V binding was determined prior to and following treatment with PGN. As illustrated in Fig. 3.2.1.B, a 30 min exposure to PGN increased the percentage annexin-V binding platelets, an effect reaching statistical significance at ≥250 ng/ml PGN. The highest stimulatory activity was observed with PGN monomer, whereas the dimers and oligomers were less effective (data not shown). The annexin-V binding after PGN monomer treatment was also apparent in fluorecence microscopy (Fig. 3.2.1.C). The FACS histograms are presented in Fig. 3.2.1.D.

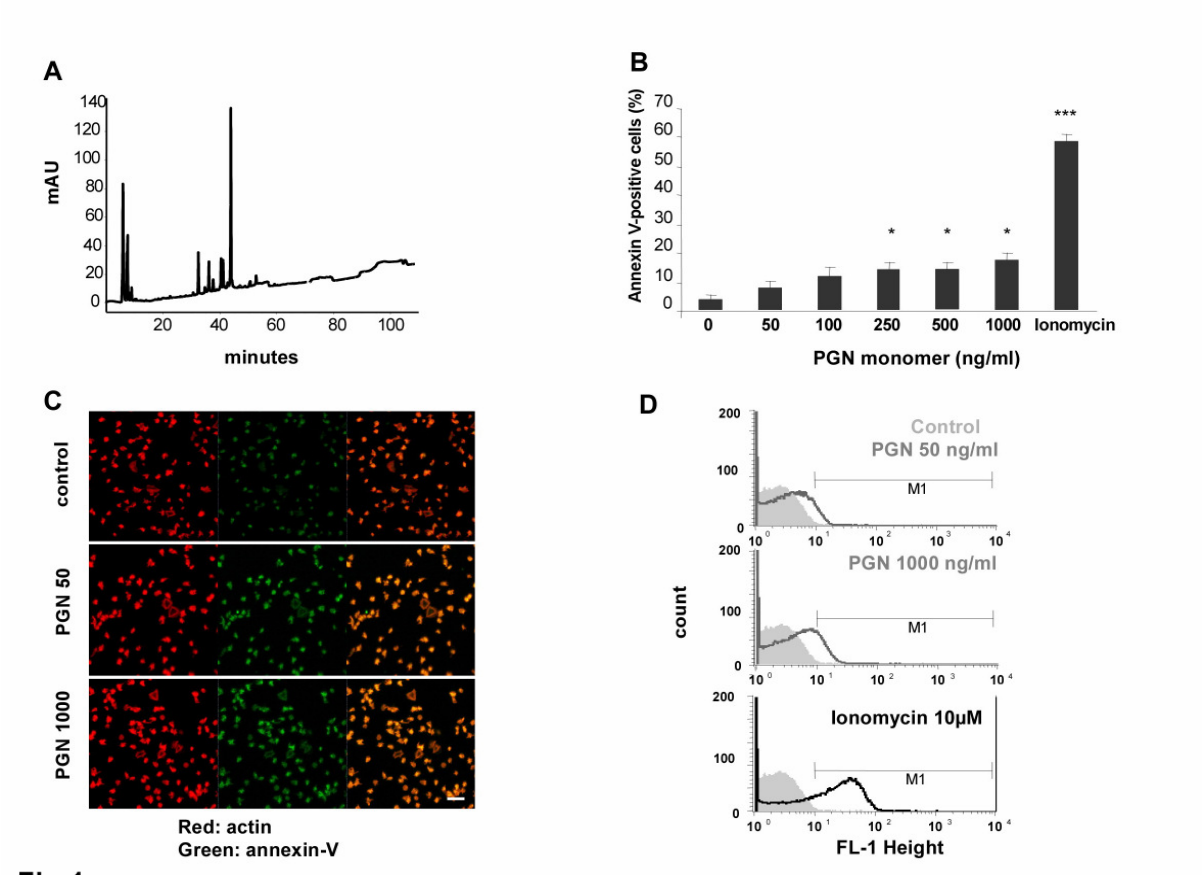


Figure 3.2.1: HPLC chromatogram of monomeric PGN and effect of PGN on phosphatidylserine exposure in human platelets.

(**A**) Monomeric PGN purified from *S. aureus* was enzymatically digested with mutanolysin possessing muramidase activity and further purified by HPLC. To ensure the identity and quality of each fraction they were reduced with sodium borohydride and analysed by RP-HPLC. Purified fractions were desalted, lyophilized and resuspended with the appropriate buffer for use in the tests.
(**B**) Arithmetic means ± SEM (n=8) of Annexin-V positive cells (%) after 30 min exposure to platelet buffer in the absence (control) and presence of 50 - 1000 ng/ml monomeric PGN. *p<0.05 indicates statistically significant difference to value in the absence of PGN (one-way ANOVA).
(C) Representative immunofluorescence staining of annexin V staining following a 30 min exposure to platelet buffer in the absence (control) or presence (50-1000 ng/ml) of PGN. Red: rhodamine-phalloidine, green: annexin-V. Magnification bar represents 10 µm.
(D) Flow cytometric histograms representing the increase in Annexin-V fluorescence in PGN 50 and 1000 ng/ml-treated platelets in comparison to negative control without PGN (light grey) and positive control with 10 µM ionomycin.

In a further series of experiments $DiOC_6$ fluorescence was employed to determine the effect of PGN on mitochondrial cell membrane potential. As illustrated

in Fig.3.2.2A, a 30 min exposure to PGN monomer was followed by a decline of the mitochondrial potential, an effect reaching statistical significance at ≥100 ng/ml of monomeric fractions. Platelets stimulated for 5 min with peptidoglycan showed a significant increase in Ca^{2+}- influx with ≥100 ng/ml of monomeric fraction (Fig. 3.2.2B). The corresponding histograms from FACS analysis are shown in Fig. 3.2.2C, D respectively.

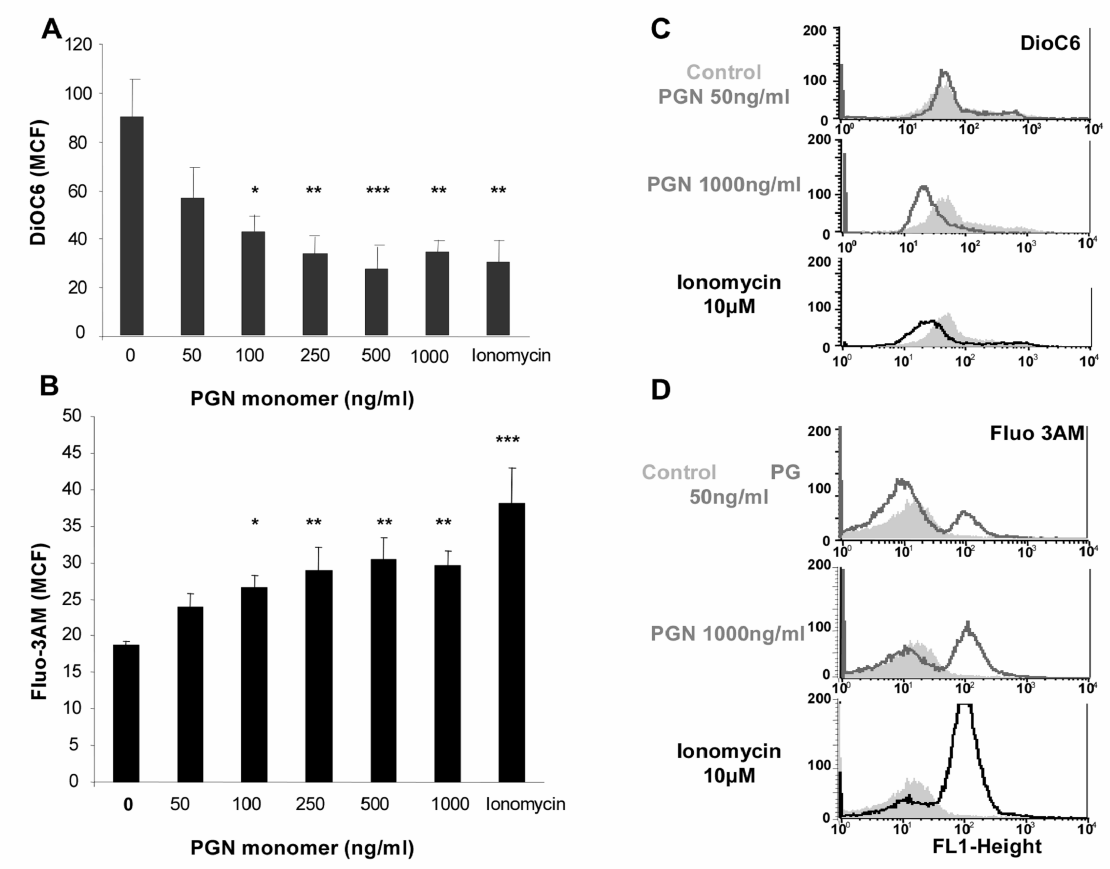


Figure 3.2.2: Mitochondrial inner membrane potential and intracellular Calcium concentration.

(**A**) Arithmetic mean ± SEM (n=14) of DiOC6 (MCF) in FACS analysis reflecting mitochondrial membrane potential of platelets following a 30 min exposure to platelet buffer in the absence (control) and presence of monomeric PGN (50-1000 ng/ml),
(**B**). Arithmetic means ± SEM (n=11) of Fluo-3AM staining for platelets stimulated 5 minutes without (control) and with monomeric PGN (50-1000 ng/ml).
(C) Flow cytometric histograms representing the shift in DiOC6 fluorescence in PGN 50 and 1000 ng/ml-treated platelets in comparison to negative control without PGN (light grey) and positive control with 10 µM ionomycin.
(D) Flow cytometric histograms representing the increase in Fluo-3AM fluorescence in PGN 50 and 1000 ng/ml-treated platelets compared to negative control without PGN (light grey) and positive control with 10 µM ionomycin.

In all cases, *p<0.05, **p<0.01 and ***p<0.001 denotes statistically significant difference from control by one-way Anova.

In addition immunofluorescence was utilized to determine the effect of PGN exposure on caspase-3 activity in blood platelets. As shown in Fig. 3.2.3, a 30 min exposure to PGN enhanced caspase-3 activity, an effect reaching statistical significance at ≥100 ng/ml monomeric PGN (Fig 3.2.3A), also showed active caspase-3 in fluorescence microscopy (Fig. 3.2.3B). However, platelets pre-treated with anti-TLR2 antibody failed to induce active caspase-3 with subsequent PGN monomer (250 ng/ml, 500 ng/ml, 1000 ng/ml) treatment, indicating the fact that caspase-3 is induced by PGN in TLR-2 dependent manner (Fig. 3.2.3C). The FACS histograms with PGN 50 and 1000 ng/ml in comparison with the negative and positive control are given (Fig. 3.2.3D).

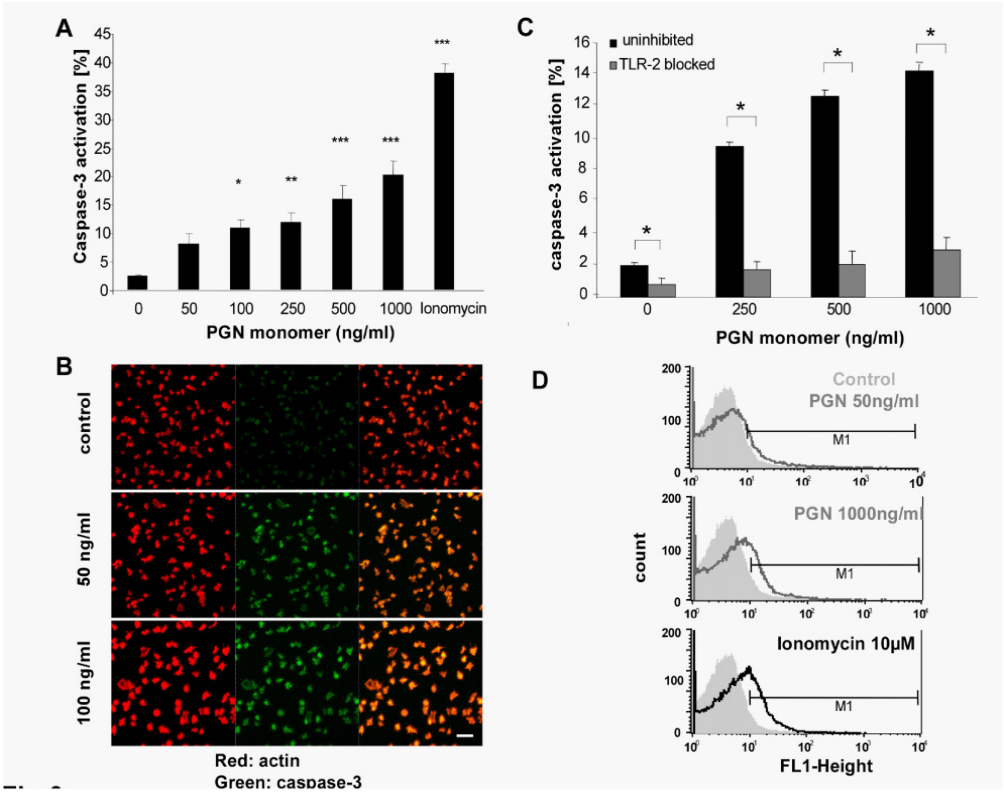


Figure 3.2.3: Effect of PGN on caspase-3 activation in human platelets.
(A). Arithmetic means ± SEM (n=7) of the % human platelets with caspase-3 activation (%) following a 30 min exposure to platelet buffer in the absence (control) and presence of monomeric PGN (50-1000 ng/ml). *p<0.05 ,**p<0.01 and ***p<0.001 indicate statistically significant difference in comparison to the value of control (one-way ANOVA)**.**
(B) Representative immunofluorescence caspase-3 staining following a 30 min exposure to platelet buffer in the absence (control) or presence (50 and 100 ng/ml) of PGN monomer. Red: rhodamine phalloidine, green: caspase-3. Magnification bar represents 10 µm.
(**C**) Arithmetic means ± SEM (n = 5) of human platelets with caspase-3 activation following 30 min treatment to PGN monomeric fraction without (black bar) and with surface TLR-2 blocking by anti-TLR-2 antibody (grey bar). *p<0.05 represents significantly reduced caspase-3 activation in TLR-2 blocked platelets in comparison to the uninhibited samples (paired t-test).
(D) Flow cytometric histograms representing the increase in active caspase-3 fluorescence in PGN 50 and 1000 ng/ml-treated platelets against negative control without PGN (light grey) and positive control with 10 µM ionomycin.

Another series of experiments tested for Ca^{2+} dependence of cell membrane scrambling. As illustrated in Fig. 4A, the stimulation of annexin V binding by PGN was virtually abolished platelets in the nominal absence of Ca^{2+} (1 mM EGTA added). Thus, PGN monomers induce PS exposure in a Ca^{2+}-dependent manner (Fig. 3.2.4A). In this experiment, ionomycin (10 µM) was used as a positive control.

Further analysis tested whether caspase-3 activity was required for the stimulation of cell membrane scrambling. To this end, platelets were exposed to PGN

in the presence and absence of the pancaspase inhibitor zVAD-FMK (1 µM). As shown in Fig. 3.2.4B, zVAD-FMK significantly blunted the increase of annexin-V binding following PGN monomer exposure.

An additional series of experiments addressed the involvement of toll-like receptors. As a result, pretreatment of platelets with 2 µg/ml anti-TLR-2 antibody significantly decreased PS exposure following stimulation with 500 ng/ml of PGN monomer (Fig. 3.2.4C).

To exclude that the observed cell membrane scrambling resulted form LPS contamination, we stimulated platelets with 5 µg/ml LPS alone or with 5 µg/ml LPS and 500 ng/ml peptidoglycan together. As shown in Fig. 3.2.4D, the stimulation with LPS alone did not produce significant PS exposure in comparison to the control and LPS and PGN monomer together produced similar PS exposure as PGN monomer alone.

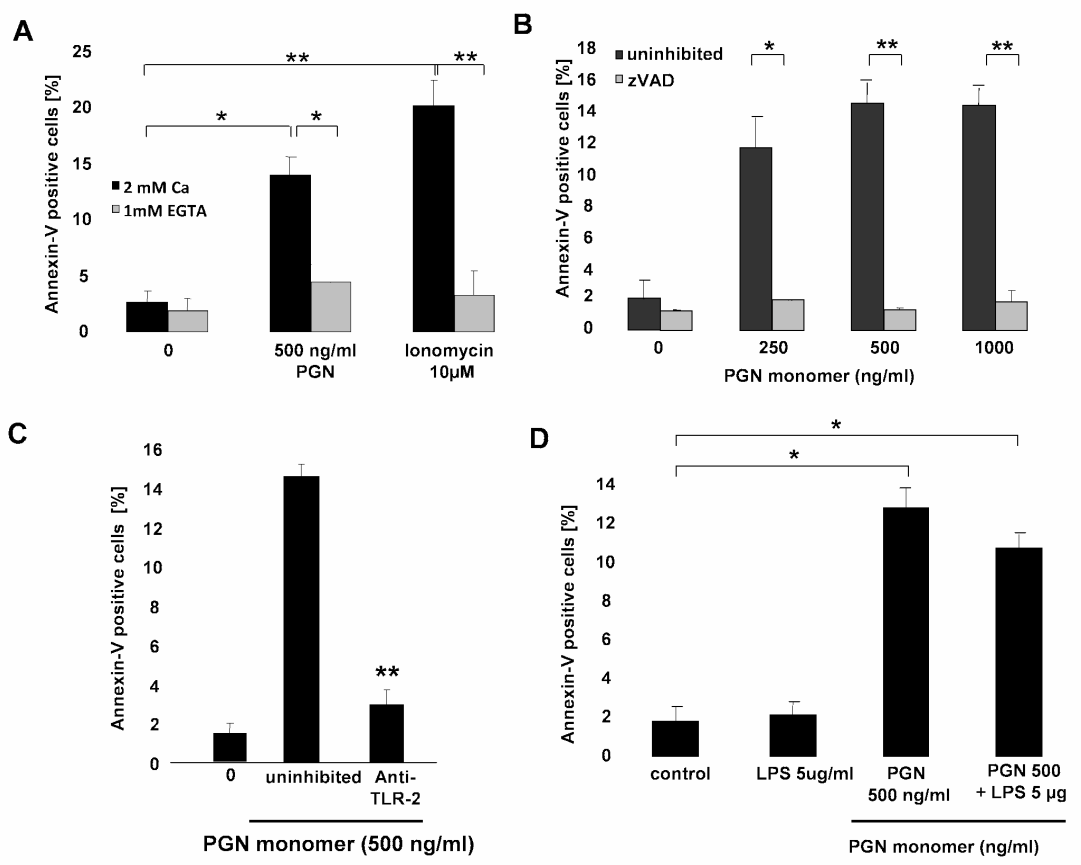


Figure 3.2.4: Mechanism of PGN-induced PS exposure.

(**A**) Dependence of PS exposure with extracellular $CaCl_2$: Arithmetic means ± SEM (n=4) of Annexin-V positive cells stimulated with 2 mM $CaCl_2$ (black bar) and with 1 mM EGTA (grey bar). *p<0.05 and **p<0.01 represents significant difference between PS exposure in presence and absence of $CaCl_2$ and in comparison to control (paired t-test).

(**B**) Arithmetic means ± SEM (n=5) of Annexin V-FITC positive platelets pre-treated with (grey bar) and without 1 µM zVAD-FMK (black bar) followed by subsequent stimulation with PGN monomers. Here *p<0.05, **p<0.01 implies significant difference between zVAD-treated and non-treated platelets (paired t-test).

(**C**) Arithmetic means ± SEM (n=8) of Annexin V-FITC fluorescence of platelets pre-treated with 2 µg/ml TLR-2 blocking antibody, and then stimulated with 500 ng/ml PGN monomeric fraction. **p<0.01 shows that PS exposure is significantly lower in platelets treated with pathway-specific inhibitors than in the uninhibited ones (paired t-test). However inhibitor-treated platelets showed almost similar quantity of PS exposure as in the uninhibited, unstimulated control.

(**D**) Arithmetic means ± SEM (n=8) of platelets treated with 5 µg/ml LPS from *E. coli* 0111:B4, PGN monomer 500 ng/ml + 5 µg/ml LPS and 500 ng/ml PGN monomer. *p<0.05 shows significantly upregulated PS in PGN monomer-treated and LPS-PGN monomer treated platelets (one-way ANOVA).

A final series of experiments showed that treatment with PGN monomers resulted in a significant upregulation of activated integrin $\alpha_{IIb}\beta_{\beta 3}$ (determined with PAC-1 antibody) at ≥250ng/ml (Fig. 3.2.5A).Double staining with PAC-1-FITC and

Annexin-V-PE revealed a significant upregulation of platelets positive for both integrin $\alpha_{II}\beta_{\beta 3}$ upregulation and phosphatidylserine exposure respectively (Fig. 3.2.5B,C) at PGN monomer ≥500 ng/ml.

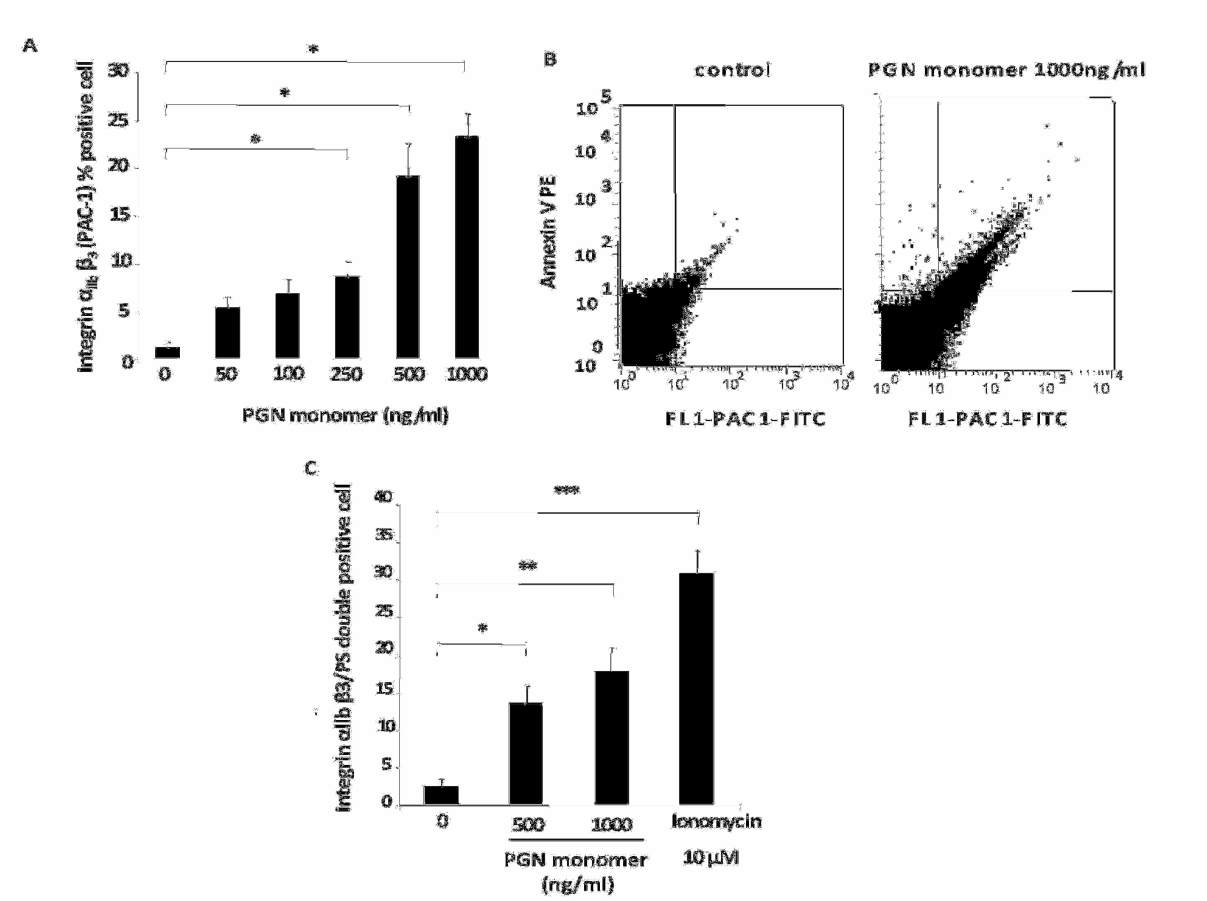


Figure 3.2.5: Simultaneous activation and apoptosis in PGN monomer-treated platelets.

(**A**) Arithmetic means ± SEM (n=6) of PAC-1 FITC positive platelets that were treated with PGN monomer. *p<0.05 represents significant upregulation of integrin $\alpha_{IIb}\beta_3$ in PGN-treated platelets in comparison to the untreated control (one-way ANOVA).
(**B**) A representative dot-plot from FACS analysis showing the increase in PAC-1 FITC and Annexin V-PE labelled platelets with PGN monomer 1000 ng/ml treatment.
(**C**) Arithmetic means ± SEM of human platelets double-positive for PAC-1 FITC and Annexin V-PE fluorescence. *p<0.05, **p<0.01 and ***p<0.001 represents significant increase in platelets expressing both integrin $\alpha_{IIb}\beta_3$ and PS, implying activation and PS exposure are simultaneous (one-way ANOVA). Ionomycin 10 µM was used as a positive control.

3.3 Vancomycin-induced platelet death

In order to determine the effect of vancomycin on platelet apoptosis, cell membrane phopholipid scrambling with subsequent exposure of phosphatidylserine at the cell surface was estimated from binding of fluorescent annexin V-FITC. As revealed by confocal microscopy, a 30 min exposure to vancomycin resulted in annexin V binding in human platelets (Fig. 3.3.1.A), an effect reaching statistical significance at $\geq$1 µg/ml vancomycin when estimated with FACS analysis (Fig. 3.3.1.B). The cell membrane scrambling was associated with an initial decrease in platelet volumes, estimated from forward scatter in FACS (Fig 3.3.1.C and 3.3.3.D).

In search for the mechanism triggering cell membrane scrambling, the effect of vancomycin on cytosolic Ca^{2+} was determined utilizing Fluo-3AM fluorescence as an indicator of cytosolic Ca^{2+} activity. As illustrated in Fig. 3.3.2.A and 3.3.2.B, a 30 minutes exposure of human platelets to vancomycin tended to increase intracellular Ca^{2+} in platelets, an effect, however, not reaching statistical significance.

Vancomycin-treated platelets also undergo mitochondrial depolarization (Fig. 3.3.2.C). Vancomycin treatment was followed by ceramide formation, an effect reaching statistical significance at 10µg/ml vancomycin as revealed with FACS analysis (Fig. 3.3.2.D).

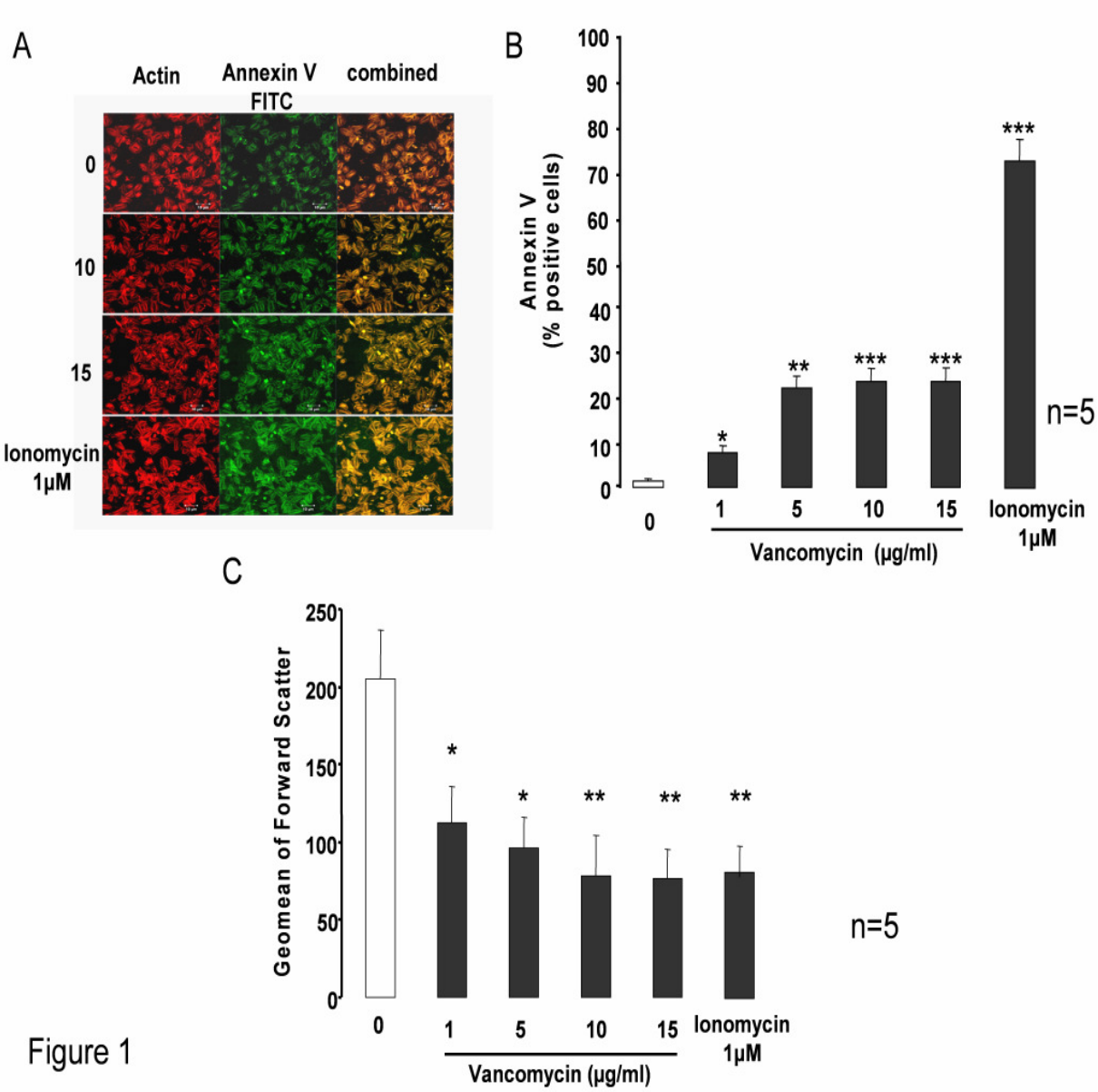


Figure 1

Figure 3.3.1: Effect of vancomycin on phosphatidylserine exposure and forward scatter of human platelets.

A. Representative immunofluorescence staining of annexin V in the absence (0) or presence (10, 50 and 100 µg/ml) of vancomycin. Red – rhodamine phalloidine, green – annexin V. Magnification bar represents 10 µm.

B. Arithmetic means ± SEM (n = 5) of the percentage of human platelets binding Annexin V-Flous following a 30 minutes exposure to Tyrode buffer 2mM $CaCl_2$ (pH 7.4) in the absence (control) and presence of 1 - 15 µg/ml vancomycin. *p<0.05, **p<0.01 and ***p<0.001 indicates statistically significant difference to value in the absence of vancomycin.

C. Arithmetic means ± SEM (n = 5) of the geomean of forward scatter of human platelets following a 30 minutes exposure to Tyrode buffer 2mM $CaCl_2$ in the

absence (control) and presence of 1 - 15 µg/ml vancomycin. *p<0.05 and **p<0.01 indicates statistically significant difference to value in the absence of vancomycin.

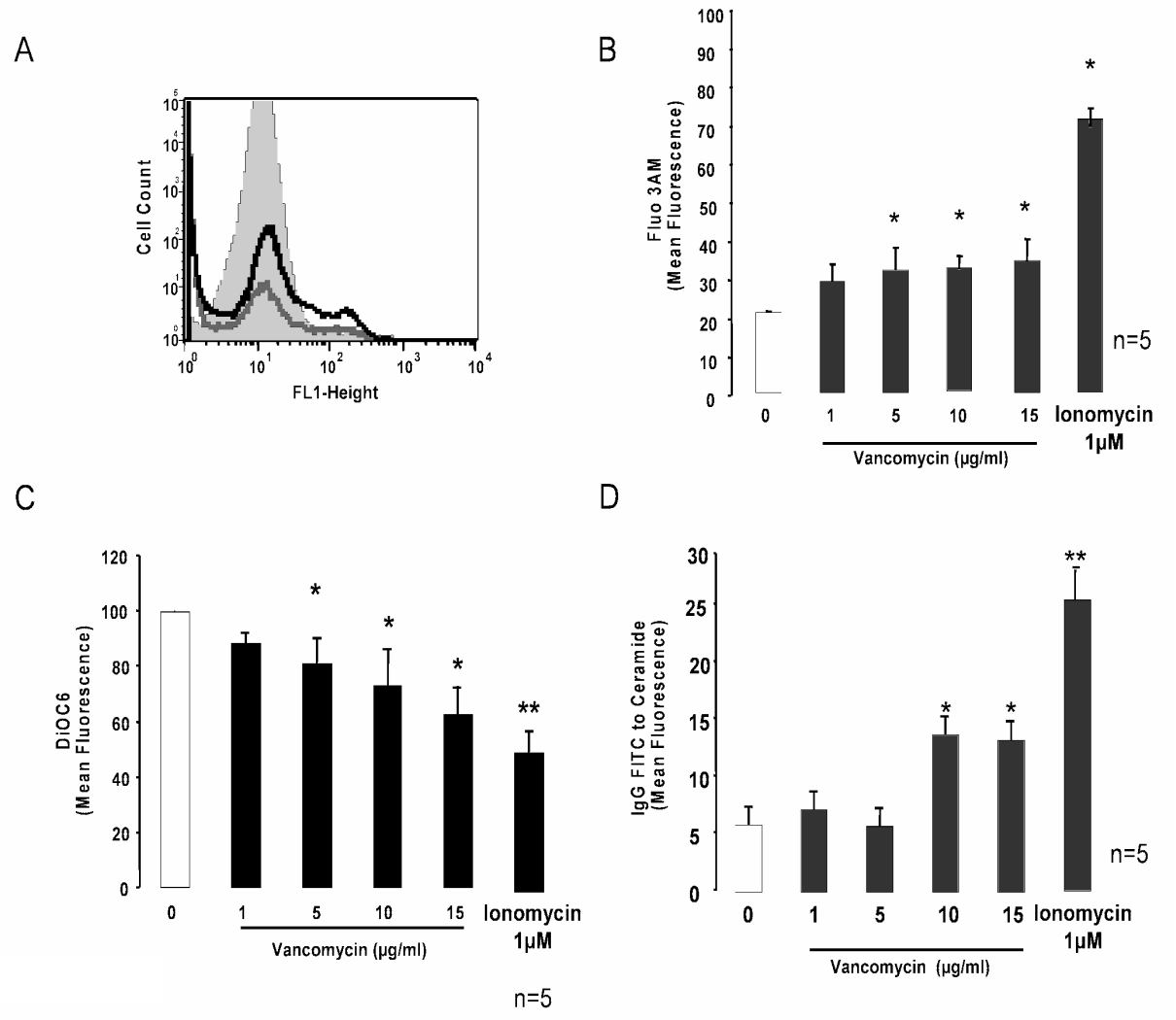


Figure 3.3.2: Effect of vancomycin on cytosolic Ca^{2+} activity and ceramide formation in human platelets

(A) Representative histogram of Fluo3 fluoresence following a 30 minutes exposure to Tyrode buffer 2mM $CaCl_2$ in the absence (control, grey shadow) or presence (15 µg/ml) of vancomycin (Vanc, grey solid line) compared to thrombin 0.1U/ml stimulated sample (black solid line).

(B) Arithmetic mean ± SEM (n = 5) of Fluo3AM fluorescence in FACS analysis reflecting Ca^{2+} mobilisation of platelets following a 30 minutes exposure to Tyrode buffer 2mM $CaCl_2$ in the absence (control, open bar) and presence (closed bars) of vancomycin (1-15 µg/ml).

(C) Arithmetic mean ± SEM (n = 5) of DiOC6 fluorescence in FACS analysis reflecting mitochondrial membrane potential of platelets following a 30 minutes exposure to platelet buffer in the absence (control) and presence of vancomycin (1-15 µg/ml). *p<0.05 shows significant decrease in mitochondrial membrane potential (one-way ANOVA).

(D) Arithmetic mean ± SEM (n = 5) of IgG-FITC attached to ceramide monoclonal Ig in FACS analysis reflecting ceramide formation in platelets following a 30 minutes

exposure to Tyrode buffer 2mM $CaCl_2$ buffer in the absence (control, open bar) and presence (closed bars) of vancomycin (1-15 µg/ml). *p<0.05 and **p<0.01 shows significant increase in ceramide formed (one-way ANOVA).

In additional experiments, caspase 3 activity was estimated utilizing FACS and immunofluorescence. As illustrated in Fig. 3.3.3.A, a 30 min exposure to vancomycin was followed by an increase of caspase 3 activity, an effect reaching statistical significance at ≥5 µg/ml vancomycin (Fig 3.3.3.B).

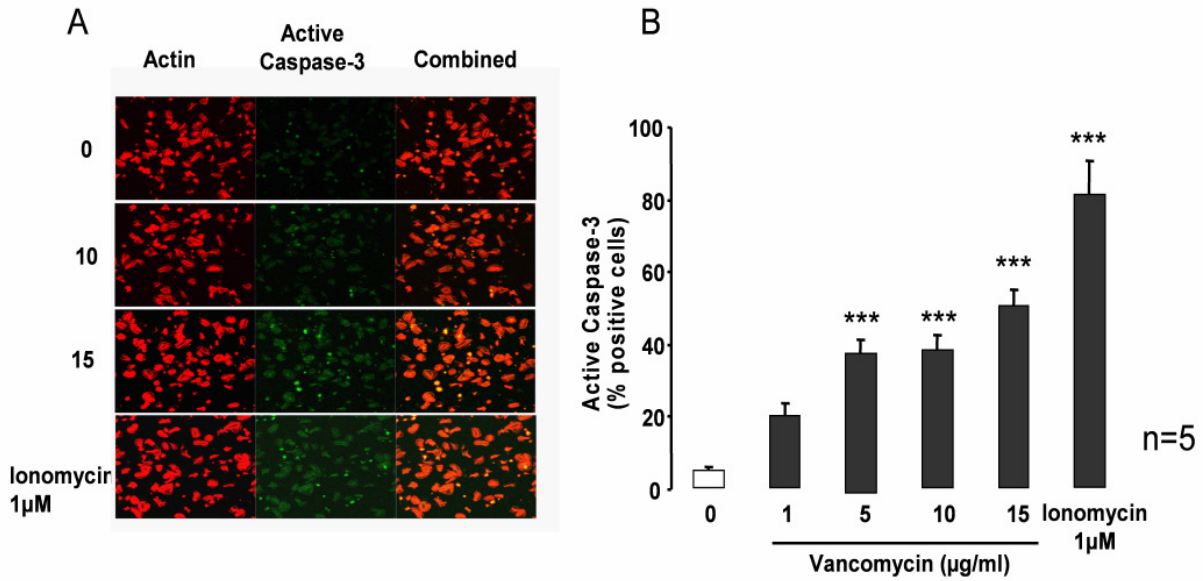


Figure 3.3.3: Effect of vancomycin on caspase activity in human platelets

(A) Representative immunofluorescence caspase-3 staining in the absence (control) or presence (10, 50 and 100 ng/ml) vancomycin. Red – rhodamine phalloidine, green – caspase-3. Magnification bar represents 10 µm.

(B) Arithmetic means ± SEM (n = 5) of the % human platelets expressing active caspase-3 following a 30 minutes exposure to Tyrode buffer 2mM $CaCl_2$ (pH 7.4) in the absence (open bar) and presence (closed bars) of vancomycin (1-15 µg/ml). ***p<0.001 indicate statistically significant difference to value in the absence of vancomycin

In order to test, whether the effect of vanomycin on cell membrane scambling depended on caspase 3 activity, platelets were exposed to vancomycin in the presence or absence of the pancaspase inhibitor zVAD-FMK (1 µM). As shown in

Fig. 3.3.4, zVAD-FMK did not significantly blunt the increase of annexin V binding following vancomycin exposure.

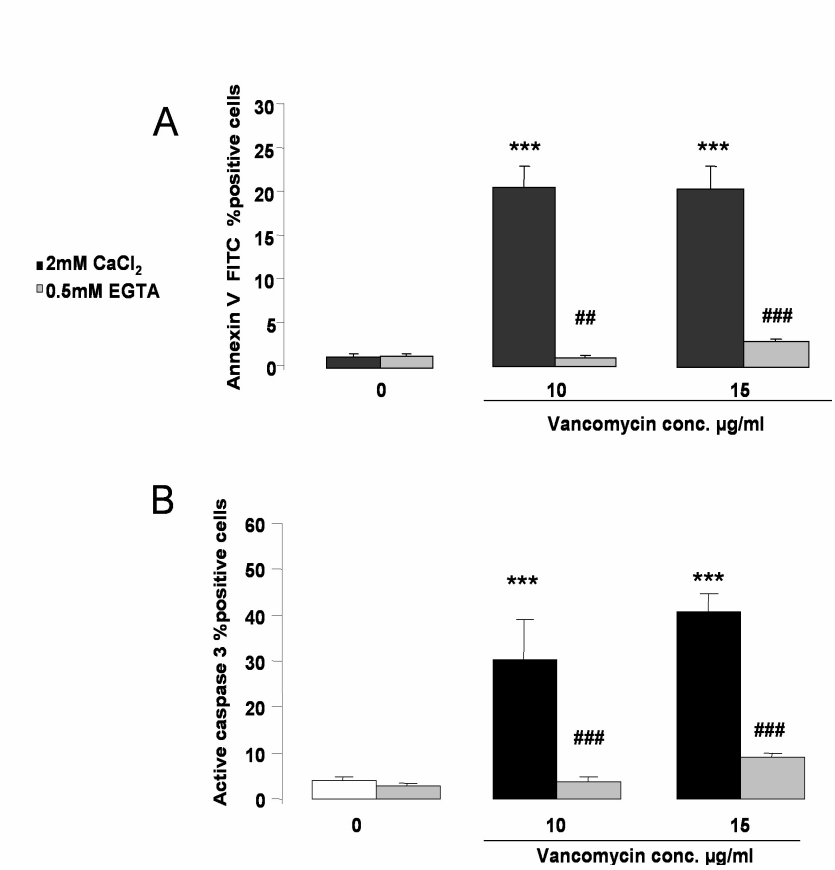


Figure 3.3.4: Influence of caspase inhibitor zVAD-FMK on vancomycin-induced phosphatidylserine exposure in human platelets

A. Arithmetic means ± SEM (n = 5) of the percentage of human platelets binding Annexin V Flous following a 30 minutes exposure to Tyrode buffer 2mM $CaCl_2$ (pH 7.4) in the absence (0, white bar) and presence of 10–15 µg/ml vancomycin in the absence (dark grey bars) and presence (light grey bars) of 1 µM zVAD-FMK. *p<0.05 and ***p<0.001 indicate statistically significant difference to value in the absence of vancomycin (one way ANOVA). ## p<0.01 indicate statistically significant difference to value in the absence of zVAD-FMK (paired T-test).

B. Arithmetic means ± SEM (n = 5) of the percentage of human platelets with active caspase-3 following a 30 minutes exposure to Tyrode buffer 2mM $CaCl_2$ (pH 7.4) in the absence (0, white bar) and presence of 15 µg/ml vancomycin in the absence (dark grey bars) and presence (light grey bars) of 1 µM zVAD-FMK. ***p<0.001 indicate statistically significant difference to value in the absence of vancomycin (one way ANOVA). ### p<0.001 indicate statistically significant difference to value in the absence of zVAD-FMK (paired T-test).

In order to explore the role of Ca^{2+} in the effect of vancomycin on cell membrane scrambling, platelets were exposed to vancomycin in the presence and absence (0.5mM EGTA) of extracellular $CaCl_2$. As illustrated in Fig. 3.3.5.A and B, the removal of Ca^{2+} (and addition of 0.5 mM EGTA) virtually abolished the effect of vancomycin (10 and 15 µg/ml) on phosphatidylserine exposure and caspase-3 activation.

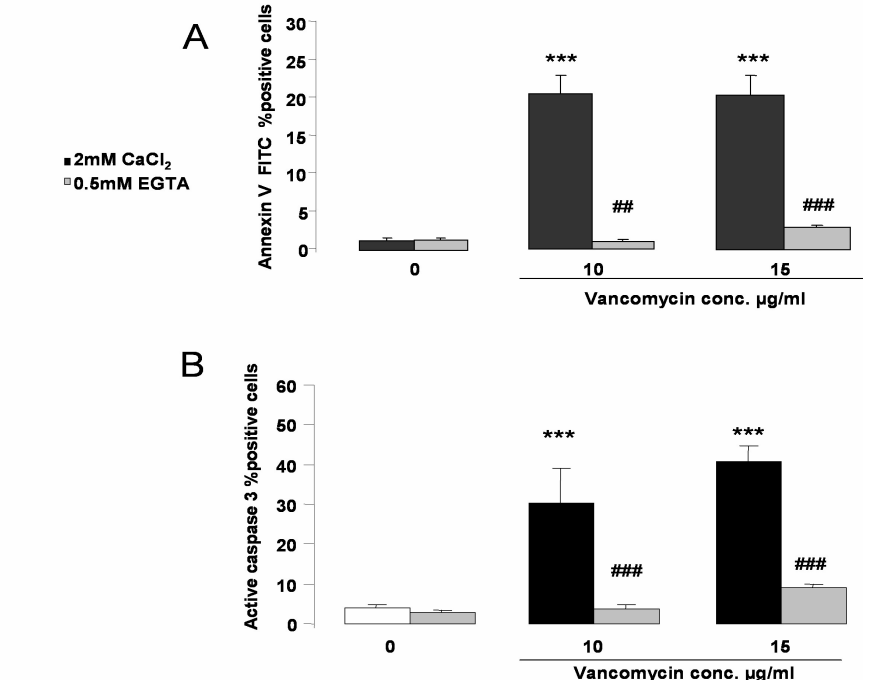


Figure 3.3.5: Effect of Ca^{2+} removal on vancomycin induced annexin V-binding in human platelets.

A. Arithmetic means ± SEM (n = 6) of percentage of human platelets binding Annexin V-Fluos following 30 minutes treatment with vancomycin in the presence (black bar) and absence (grey bar) of extracellular Ca^{2+}. ## $p<0.01$ and ###$p<0.001$ indicate statistically significant difference to value in the absence of Calcium (paired T-test) and ***$p<0.001$ indicates significant difference in comparison to non-stimulated control (one-way ANOVA).

B. Arithmetic means ± SEM (n = 5) of the percentage of human platelets with caspase-3 activation following 30 minutes treatment with vancomycin in the presence (black bar) and absence (grey bar) of extracellular Ca^{2+}.***$p<0.001$ indicate statistically significant difference from non-stimulated corresponding control (one-way ANOVA) and ###$p<0.001$ indicate statistically significant difference to value in the absence of Calcium (paired T-tes

Platelet death is associated with clinical hazards if the platelet secretes chemoattractants or upregulates adhesion molecules. These markers were also exhibited in Fig. 3.3.6. The platelet α-granule secretion marker CD62P was upregulated at ≥ 1µg/ml concentration of vancomycin (Fig. 3.3.6.A) along with aggregation marker CD41/61 (Fig. 3.3.6.B). The prostaglandin PGE2 was not elevated with vancomycin treatment (Fig. 3.3.6.C), but the platelet autocrine thromboxane B_2 (TxB_2) was secreted in response to vancomycin (Fig. 3.3.6.D). Vancomycin-dependent TxB_2 secretion was independent of p38MAPK and cyclooxygenase, as shown by SB 203580 (50nM) and Diclofenac Sodium (3µM) pre-treatment.

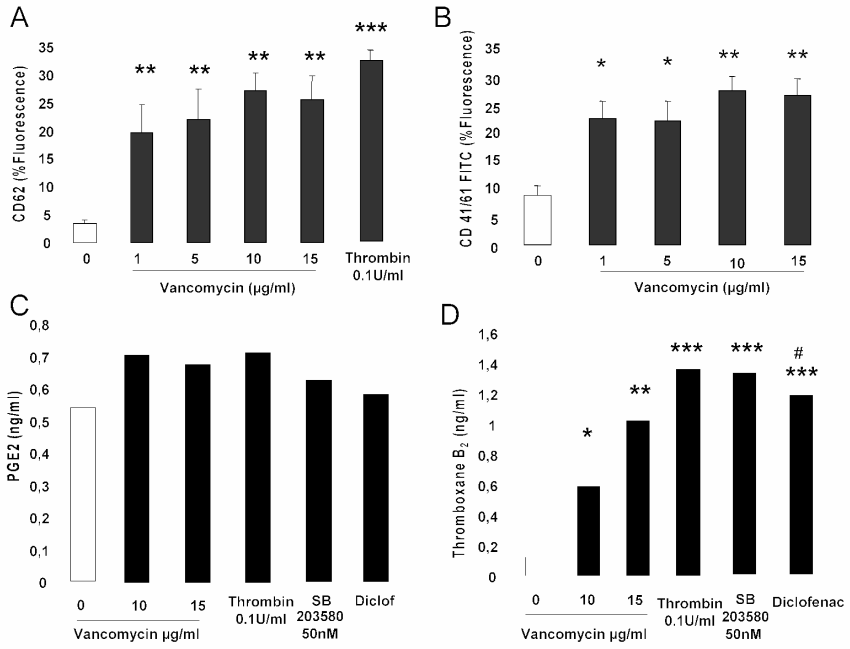


Figure 3.3.6: Activation of platelet function by vancomycin

- **A.** Arithmetic mean ± SEM (n=4) of % CD62P positive platelets after 30 minutes of vancomycin treatment compared to negative control (white bar) and positive control (thrombin 0.1U/ml).
- **B.** Arithmetic mean ± SEM (n=4) of % integrin $\alpha_{IIb}\beta_3$ positive platelets after 30 minutes of vancomycin treatment compared to negative control (white bar) and positive control (thrombin 0.1U/ml).
- **C.** Arithmetic mean ± SEM (n=3) of prostaglandin E1 (PGE-1) secretion from platelets after 30 minutes of vancomycin treatment compared to negative control (white bar) and positive control (thrombin 0.1U/ml). PGE-1 is not significantly upregulated.
- **D.** Arithmetic mean ± SEM (n=3) of Thromboxane B2 secretion from platelets after 30 minutes of vancomycin treatment compared to negative control (white bar) and positive control (thrombin 0.1U/ml).

In every case, *p<0.05, **p<0.01 and ***p<0.001 indicates statistically significant difference from negative control by one-way ANOVA. #p<0.05 stands for

statisically significant difference from vancomycin 15µ/ml-stimulated thromboxane B2 secretion.

On the whole, vancomycin induces apoptotic response in platelets in Calcium dependent manner. The response progresses through caspase-3 activation and ends in phosphati dylserine exposure, CD62P expression and integrin $\alpha_{IIb}\beta_3$ on the platelet surface.

3.4 Induction of Platelet Activation and Apoptosis by CXCL16

The CXC family chemokine CXCL16 was used to demonstrate apoptotic response together with activation in platelets. For this, 50, 100 and 200 ng/ml CXCL16 was used to stimulate 10^6/ml isolated platelets in a total volume of 1 ml Tyrode buffer (pH 7.4) 2mM $CaCl_2$.

As shown in figure 3.4.1, exposure of platelets to CXCL16 for 15 minutes triggers apoptotic response in platelets. All the classical apoptotic markers, eg. PS exposure, caspase-3 activation, depolarization of the outer membrane of mitochondria, increase of intracellular Calcium and ceramide formation are significantly higher in CXCL16 stimulated platelets. CXCL16 triggers platelet apoptosis at 100ng/ml concentration most effectively, since all the apoptotic markers are significantly up-regulated at this concentration.

Next step was to investigate what other events are associated with platelet apoptosis. Since platelet activation and cell-cell interaction are the most important clinically relevant events, attempts were taken to assess P-selectin and integrin $\alpha_{IIb}\beta_3$ exprsssion.

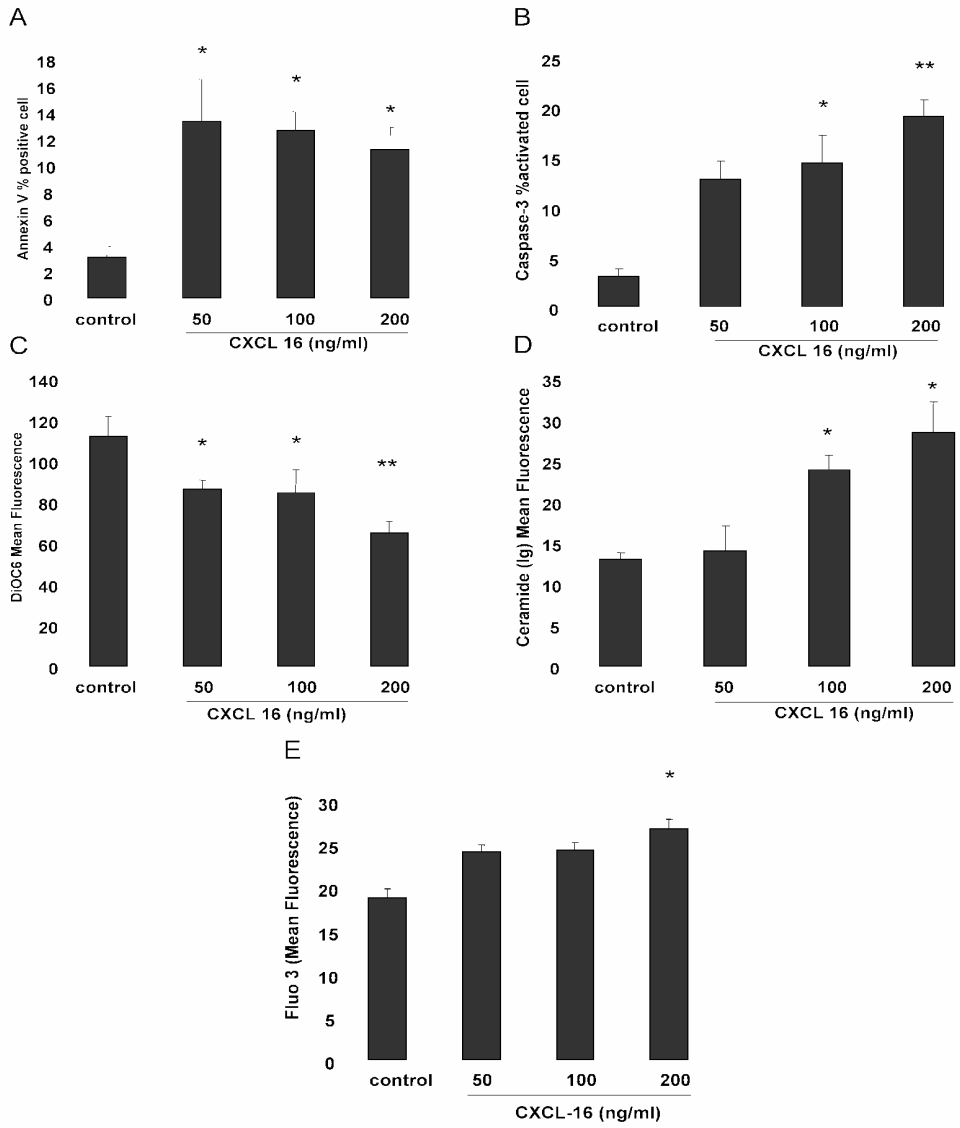


Fig. 3.4.1: CXCL16 induced apoptotic response in human platelets

A. Arithmetic mean ± SEM (n=5) of Annexin V FITC binding to human platelets stimulated with 50, 100 and 200 ng/ml CXCL16, representing PS exposure on the outer surface of membrane.
B. Arithmetic mean ± SEM (n=5) of active caspase-3 formation in human platelets stimulated with 50, 100 and 200 ng/ml CXCL-16.
C. Arithmetic mean ± SEM (n=5) of DiOC6 mean fluorescence in human platelets stimulated with 50, 100 and 200 ng/ml CXCL-16, reflecting mitochondrial outer membrane depolarization.
D. Arithmetic mean ± SEM (n=5) of ceramide formation in human platelets stimulated with 50, 100 and 200 ng/ml CXCL-16.
E. Arithmetic mean ± SEM (n=5) of Fluo-3 AM mean fluorescence in human platelets stimulated with 50, 100 and 200 ng/ml CXCL16, representing the amount of Ca^{2+} in platelets.

In each case, *p<0.05, **p<0.01 and ***p<0.001 represents statistically significant increase in parameters in comparison to platelets in absence of CXCL16.

According to flow cytometric analysis stimulation of platelets *in vitro* with CXCL16 (50, 100 and 200 ng/ml) significantly enhanced the expression of P-selectin and activated integrin $\alpha_{IIb}\beta_3$ at the platelet surface (Fig. 3.4.2 A,B). In all experiments described above stimulation with low dose ADP (5 µM) or thrombin (0.01 U/ml) was used as positive control. Thereby, the extent of platelet stimulation triggered by CXCL16 was comparable to that found after treatment with ADP (Fig. 3.4.2 A-C).

FACS analysis of human platelets revealed that the CXCL16-specific receptor CXCR6 (BONZO) is highly expressed on platelets (Fig. 3.4.2.C).

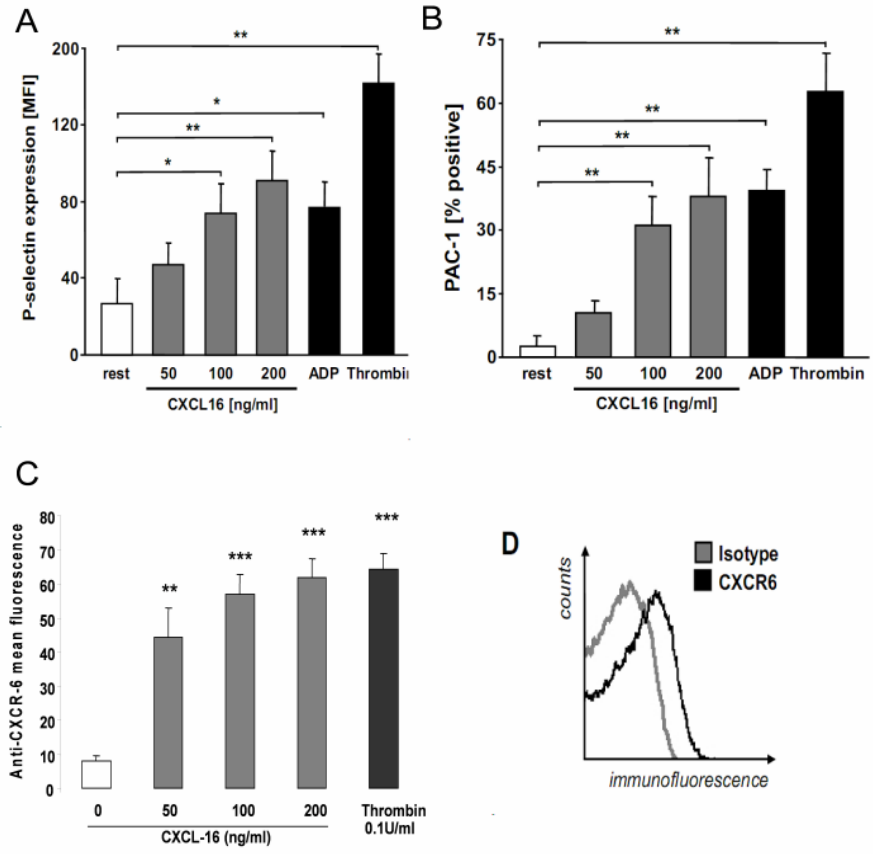


Figure 3.4.2 Activation of Platelets by CXCL16

A. Arithmetic mean ± SEM of mean fluorescence intensity of P-selectin (CD-62P) in human platelets stimulated with CXCL-16 (50, 100, 200 ng/ml). P-selectin is significantly upregulated at ≥ 100ng/ml CXCL16.
B. Arithmetic mean ± SEM of mean fluorescence intensity of integrin $\alpha_{IIb}\beta_3$ (CD41/61) in human platelets stimulated with CXCL-16 (50, 100, 200 ng/ml), determined with PAC-1 FITC binding. which is significantly upregulated at ≥ 100ng/ml CXCL16.
C. Arithmetic mean ± SEM of mean fluorescence intensity of anti-CXCR-6 antibody in human platelets stimulated with CXCL16 (50, 100, 200 ng/ml). CXCR-6 is significantly upregulated at ≥ 50ng/ml CXCL16.
D. Representative flow cytometry histogram for CXCR-6 upregulation in human platelets in comparison to isotype control.

In every case *p<0.05, **p<0.01 and ***p<0.001 represents statistically significant (ANOVA) increase in the parameter under study.

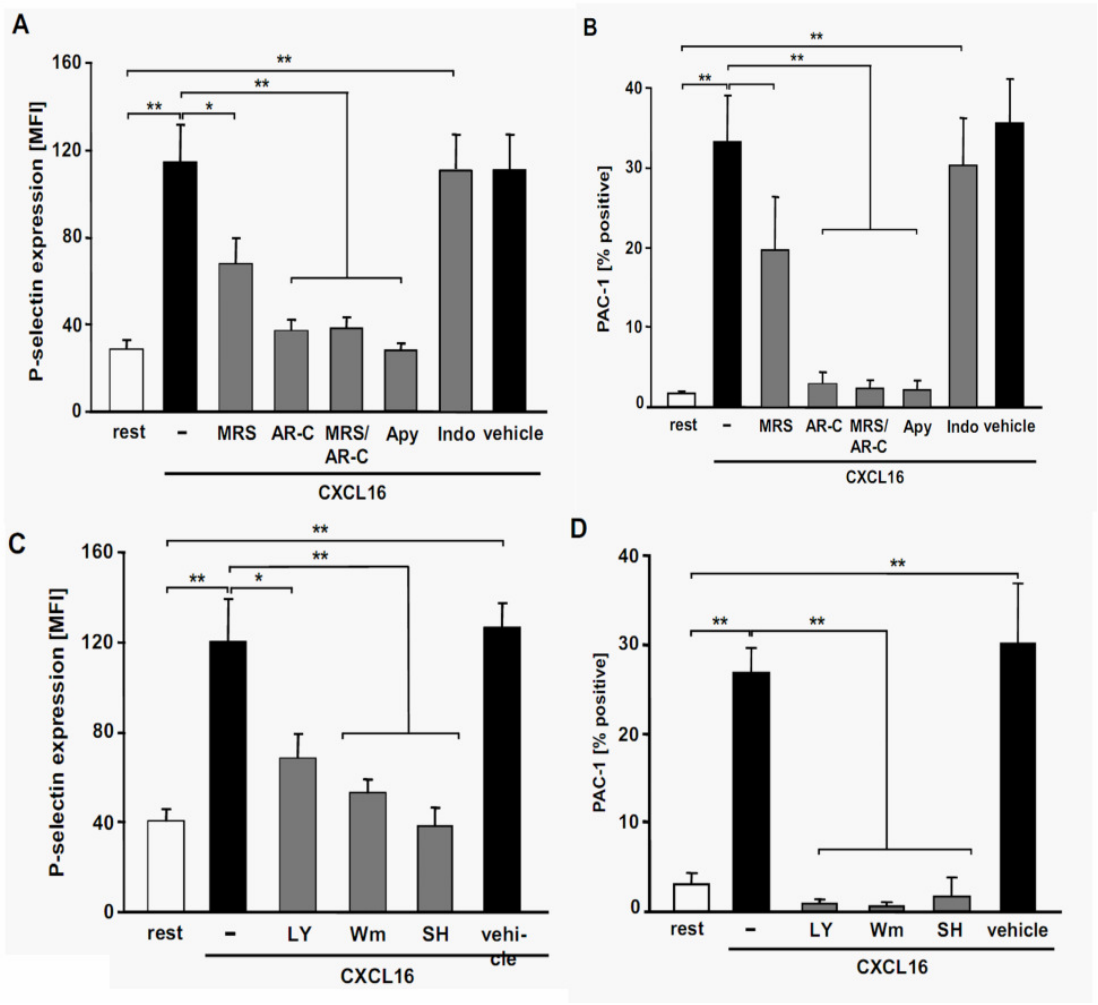


Fig 3.4.3 Role of purinergic receptors and PI3K on CXCL16 signaling

A. Flow cytometric analysis (FACS) of P-selectin expression in platelets after stimulation with CXCL16 in the presence or absence of purinergic receptors P_2Y_1 and P_2Y_{12} inhibitors MRS2179 (MRS, 100 µM), Cangrelor (ARC, 10 µM) or the combination MRS/ARC, the ADP-degrading enzyme apyrase (Apy, 1.5 U/ml) or indomethacin (Indo, 10 µM). Arithmetic means ± SEM (n = 8) are shown.

B. FACS analysis of activated glyocoprotein $\alpha_{IIb}\beta_3$ expression in platelets after stimulation with CXCL16 in the presence or absence of purinergic receptors P_2Y_1 and P_2Y_{12} inhibitors MRS2179 (MRS, 100 µM), Cangrelor (ARC, 10 µM) or the combination MRS/ARC, apyrase (Apy, 1.5 U/ml) or indomethacin (Indo, 10 µM). Arithmetic means ± SEM (n = 8) are shown.

C. Flow cytometric analysis of P-selectin expression in platelets after stimulation with CXCL16 in the presence or absence of LY (25 µM), Wm (100 nM), SH-6 (20 µM) or DMSO (vehicle) as solvent control. Arithmetic means ± SEM (n = 8).

D. Flow cytometric analysis of activated glycoprotein $\alpha_{IIb}\beta_3$ expression in platelets after stimulation with CXCL16 in the presence or absence of LY (25 µM), Wm (100 nM) SH-6 (20 µM) or DMSO (vehicle) as solvent control. Arithmetic means ± SEM (n = 8).

In every case, *p<0.05, **p<0.01, ***p<0.001 denotes statistically significant increase from untreated platelets by one-way ANOVA.

For blocking platelet thromboxane synthesis indomethacin (10 µM) was used. As shown in Fig. 3.4.3. A and B, blocking the purinergic receptors, especially P_2Y_{12}, as well as preincubation with apyrase, which completely inhibits ADP expression, significantly diminished CXCL16-triggered enhancement of platelet degranulation and integrin $\alpha_{IIb}\beta_3$ activation whereas blockage of thromboxane synthesis was without any significant effect on CXCL16-mediated platelet activation.

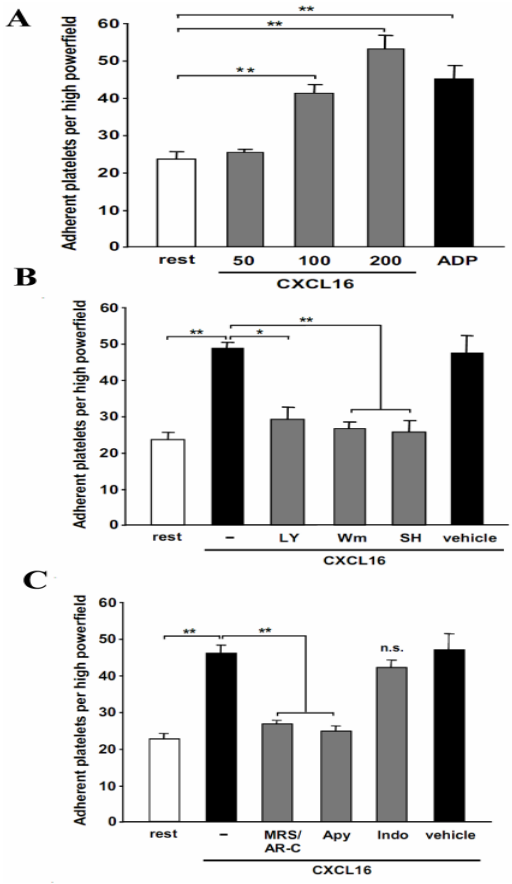


Figure 3.4.4 Role of PI3K in CXCL16 dependent interaction between platelet and endothelia

A. Arithmetic mean ± SEM (n = 17) of CXCL16 stimulated platelets adherent to human umbilical vein endothelial cells (HUVEC).
B. Arithmatic means ± SEM (n = 17) of CXCL16-stimulated platelets adherent to HUVEC cells per high powerfield under flow (high shear rate, 2000^{-s}) after preincubation with PI3K inhibitors LY (25 µM), wortmannin (100 nM) or Akt inhibitor SH-6 (20 µM).
C. Arithmetic means ± SEM (n = 19) of CXCL16-stimulated platelets adherent to HUVEC cells per high powerfield under flow (at high shear rates, 2000^{-s}) in the presence or absence of purinergic receptors P_2Y_1 and P_2Y_{12} inhibitors MRS2179 (MRS, 100 µM), Cangrelor (CGR, 10 µM) or the combination MRS/CGR, apyrase (Apy, 1.5 U/ml) or indomethacin (Indo, 10 µM). MRS, CGR or MRS/CGR as well as apyrase or indomethacin.

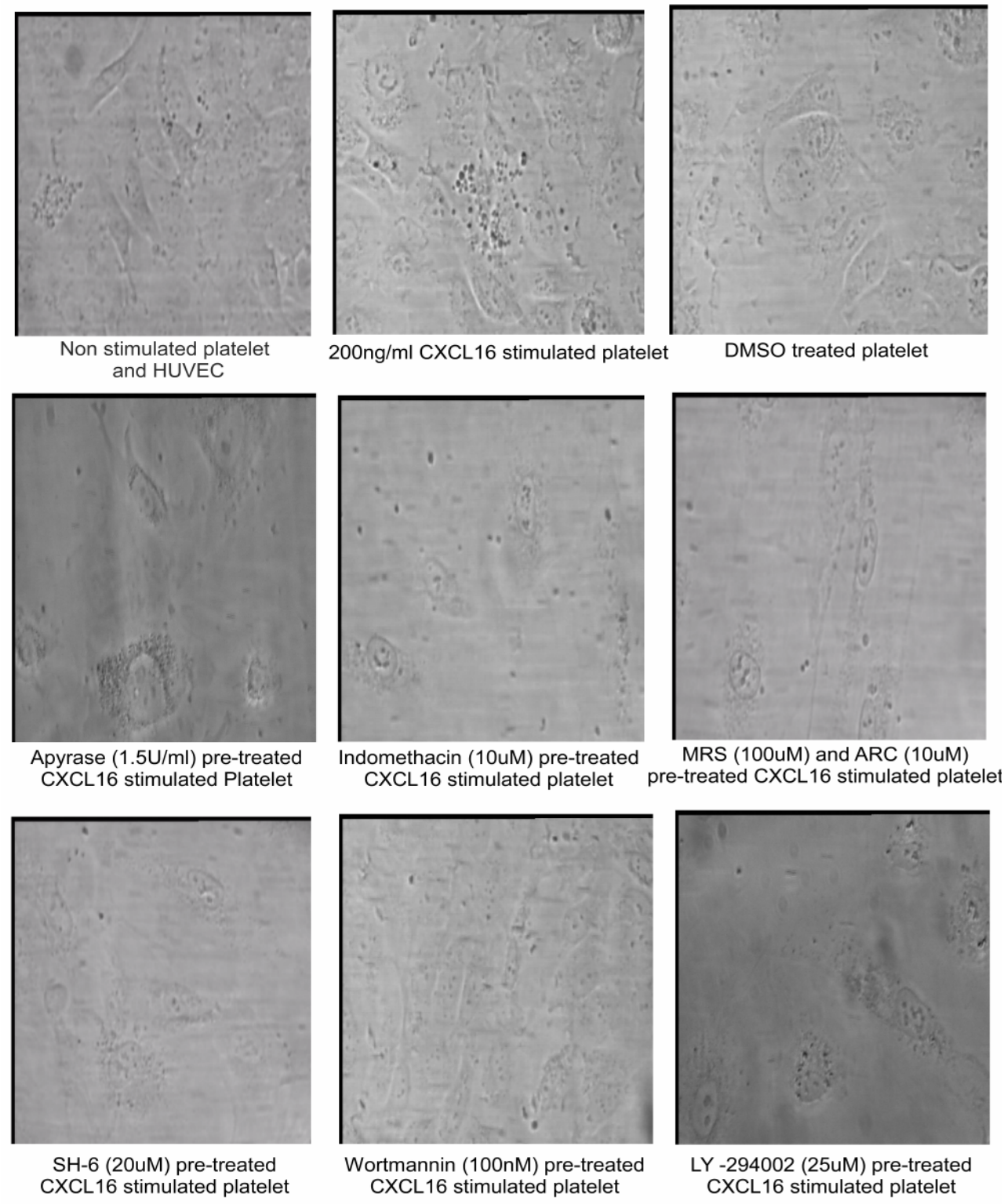


Figure 3.4.5 Representative microscopic images of platelets interacting with HUVEC under 2000s^{-1} flow rate in a single channel flow chamber. The images are taken at 20x magnification with differential interference optical settings.

As shown before in FACS analysis, blockers of purinergic receptors and apyrase potentially inhibited CXCL16-induced platelet adhesion to intact or injured vascular wall under arterial shear rates *in vitro* (Fig. 3.4.4. C). To test the functional relevance of CXCL-16-dependent platelet stimulation for platelet adhesion to the vascular wall we performed *in vitro* flow chamber experiments. In flow chamber experiments we found a significantly enhanced platelet adhesion to endothelial cells under conditions of high arterial shear rates (2000^{-s}) following treatment with increasing concentrations of CXCL16 up to 200 ng/ml.

From all the experiements conducted, CXCL16 was shown to stimulate platelet apoptosis and adhesion simulateneously. The adhesion event led to platelet-endothelial interactoin under shear stress in PI3K dependent manner. Bloking the purinergic receptor inhibited platelet adhesion to endothelia significantly. Therefore, apoptotic platelets might pose clinical risk of vascular occlusion, which appear to be due to PI3K and purinergic receptor-dependent pathways.

Chapter 4

Discussion

Chapter 4
Discussion

The impact of apoptosis on platelets and its physiological importance became evident since 2002 through the works of J Freedman and Shaun P Jackson. Their effort was initiated to investigate the molecular mechanism for platelet storage lesion and to prolong platelet life in storage conditions. They found that in *ex vivo* conditions, platelets become activated and pro-coagulant and finally dead, though death and attainment of pro-coagulant state are independent of each other (Mason *et al.*, 2007c), (Zhang and Colman, 2007), (Schoenwaelder *et al.*, 2009a), (Jackson and Schoenwaelder, 2010c), (Gyulkhandanyan *et al.*, 2012b). This study was aimed at finding different triggers of platelet apoptosis in the circulation, pathways involving the apoptotic events and possible consequence of platelet apoptosis in patho-physiology.

4.1 Stimulation of platelet apoptosis with thymoquinone

The present data demonstrates that exposure of human platelets to thymoquinone (TQ) leads to scrambling of the platelet cell membrane. The effect is paralleled by increase of cytosolic Ca^{2+} activity, depolarization of the mitochondrial potential, ceramide formation and caspase activation.

TQ has previously been shown to decrease intracellular calcium in mast cells by inhibiting its uptake and stimulating its efflux, effects mediated by inhibition of PKC (Chakravarty, 1993b). In other cells the effect of thymoquinone was shown to be inhibited by staurosporine, a substance known to inhibit protein kinase C (Tamaoki and Nakano, 1990). TQ has further been shown to inhibit PKB/AKT and extracellular signal-regulated kinase, (Yi *et al.*, 2008), glutathion depletion (Rooney and Ryan, 2005a) and/or caspase activation (El Mahdy *et al.*, 2005;Rooney and Ryan, 2005b). In this study, pretreatment with G-protein coupled receptor (GPCR) inhibitor pertussis toxin (Chakravarty, 1993f) or Phosphoinositide-3 kinase (PI3K) inhibitor Wortmannin (Chakravarty, 1993a;Chakravarty, 1993e) blunted TQ-induced phosphatidylserine exposure and active caspase-3 induction (Figure 3.1.1, 3.1.2). Thymoquinone is reported to effect μ- and κ- opioid receptors in murine models ((Abdel-Fattah *et al.*, 2000) and these receptors are present in human platelets ((Zvetkova *et al.*, 2010). Since μ- and κ- opioid receptors are $G\alpha_{i/o}$ coupled receptor ((Clark *et al.*, 2006),

(Bruchas and Chavkin, 2010), therefore this thymoquinone receptor is probably a Gαi/o associated GPCR.

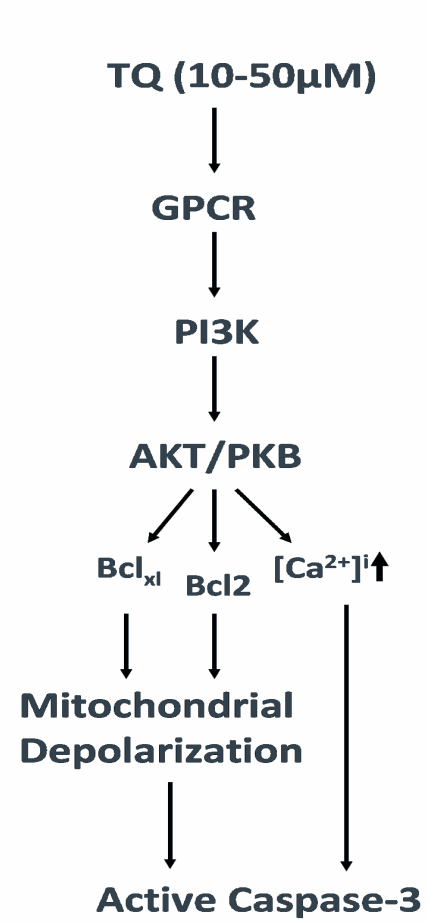


Fig 4.1 : Probable pathway of Thymoquinone-induced platelet apoptosis

The thymoquinione concentrations needed for the activation of caspases by TQ are similar to those proven effective in other cells (Mansour and Tornhamre, 2004;Kaseb *et al.*, 2007;Vaillancourt *et al.*, 2011a). *In vivo*, thymoquinone has proven effective at a daily dosage of 5 - 10 mg/kg (30 – 60 µmol/kg) (Mahgoub, 2003;Vaillancourt *et al.*, 2011b). Whether or not this dosage yields plasma concentrations affecting platelet function and/or survival, remains to be shown. Thrombocytemia may be expected to foster bleeding and inadequate platelet activation may result in thrombotic disorders (Jackson, 2007;Ombrello *et al.*, 2010). However, thymoquinone has been shown to stimulate cell membrane scrambling in erythrocytes (Qadri *et al.*, 2009a) cells devoid of nuclei and unable to modify gene expression.

Two simultaneous events might occur in the stimulated platelets: Activation and induction of apoptosis with phosphatidylserine exposure. Activation might be

characterized by degranulation-dependent P-selectin exposure on the membrane whereas apoptosis in the non-nucleate cell fragments can be assessed from phosphatidylserine exposure, caspase-3 activation, depolarization of mitochondria and release of microparticles from platelets (Qadri *et al.*, 2009b). Triggering of Ca^{2+} entry by store depletion and Ca^{2+} ionophore treatment leads in platelets to caspase 3 activation (Ben Amor *et al.*, 2006b). Thrombin-induced caspase activation and translocation was not abrogated by removal of Ca^{2+} (Ben Amor *et al.*, 2006a). Instead, the effects of thrombin on caspase translocation involves PKC and actin filament polymerization (Amor *et al.*, 2006). Thrombin is a strong stimulator of platelet activation and death because it has multiple receptors in platelet surface, PAR-1, PAR-4 and GP Ib/IX/V being the reported ones till date (Martorell *et al.*, 2008b;Rivera *et al.*, 2009a;Li *et al.*, 2010b). Contact between thrombin and its receptors trigger numerous pathways involving different types of G-proteins, intracellular protein kinases and signaling molecules such as diacyl glycerol, guanylyl cyclase (Leytin *et al.*, 2007a;Martorell *et al.*, 2008a;Stefanini *et al.*, 2009c). The ultimate effect is platelet shape change, activation of PI3K/Akt pathway, calcium dependent expression of integrin $\alpha_{IIb}\beta_3$ on the membrane and secretion of granular contents (Chakravarty, 1993g). At concentrations of 1 – 10 U/ml, which is generated during blood coagulation, thrombin activates caspase-3, depolarizes platelet mitochondria and upregulates pro-apoptotic proteins Bax and Bad (Leytin *et al.*, 2006c), at concentrations of 0.05 - 0.1 U/ml thrombin triggers only activation (Leytin *et al.*, 2007d). At this point we have a contrast of events between nucleated cells and anucleate platelets; PI3K activation is involved in pro-survival signals in nucleated cells but in our study inhibition of PI3K inhibited platelet apoptosis. PI3K is involved in platelet activation events through integrin signaling and agonist-activated platelets are dying ((Kulkarni *et al.*, 2007) because they do not have the anti-apoptotic signals needed to ovecome pro-apoptotic stimulation. In contrast thymoquinone promotes only apoptosis since CD62P and CD41/61 has not been found to be upregulated.

In conclusion, exposure of platelets to thymoquinone triggers phospholipid scrambling with cell shrinkage and phosphatidylserine exposure at the platelet surface without upregulation of the activation markers. The effects are expected to accelerate the clearance of platelets from circulating blood and thus predispose to the development of thrombocytopenia.

4.2 PGN-monomer induced platelet apoptosis

The pathogenic role of peptidoglycan is slowly becoming evident. The present study reveals a novel effect of peptidoglycan (PGN), i.e. induction of apoptosis in human blood platelets. To the best of our knowledge, PGN induced platelet death has never been shown before. The apoptotic effect of PGN is characterized by depolarization of the mitochondrial potential, increase in intracellular calcium, activation of caspase-3 and scrambling of the platelet cell membrane. The PGN induced PS exposure was blunted in the presence of the pancaspase inhibitor zVAD (1 µM). Thus, the mechanisms involved in the effect of PGN on blood platelets include caspase dependent cell membrane scrambling. Caspases have been implicated in *in vivo* studies of thrombocytopenia (James and Alperin, 1997;Piguet *et al.*, 2002). Pretreatment of platelets with TLR-2 blocking antibody virtually abolished the PGN-induced caspase-3 activation and cell membrane scrambling. In an earlier study PGN induced tissue factor expression by human monocytes and procoagulant activity (Mattsson *et al.*, 2002) and inflammatory response by triggering NF-κB, TNF-α and interleukin 8 (Wang *et al.*, 2001).

Platelets play a central role in the pathogenesis of arterial thrombosis. There are different mechanisms of platelet activation, which invariably lead to platelet apoptosis (Vanags *et al.*, 1997;Kile, 2009b). Mechanisms of platelet activation are different for specific agonists as thrombin (Coughlin, 2000;Sambrano *et al.*, 2001), collagen (Nieswandt and Watson, 2003b;Ozaki *et al.*, 2005) or vWF (De Marco *et al.*, 1985;Yin *et al.*, 2008a), resulting in triggering of distinct pathways. In activation-dependent apoptosis, the apoptotic markers are regulated by the agonist-specific pathway. PS externalization following agonist-dependent stimulation is particularly interesting because it may be involved in clearing the nucleated cells (Krysko *et al.*, 2006a) and platelets from circulation (Krysko *et al.*, 2006b) . PGN-induced cell membrane scrambling with PS externalization was abolished by inhibition of TLR-2 in the absence of extracellular calcium and following caspase inhibition. Synthetic TLR-2 ligands have previously been shown to trigger different apoptosis response of platelets, including immune-mediated platelet activation, alpha-granule release, aggregation and adhesion to collagen and regulate megakaryocyte function (Rex *et al.*, 2009;Blair *et al.*, 2009b;Beaulieu *et al.*, 2011).

In this project, the PGN monomeric fraction was isolated by HPLC and their purity were verified by mass spectrometry. Isolated human platelets were treated separately

with monomeric fraction. The PGN monomeric fraction was found to be the strongest inducer of apoptosis events in platelets, characterized by higher PS exposure, higher active caspase-3 production and sharper mitochondrial depolarization than the dimeric and polymeric fractions. *In silico* modelling of the chemical nature and molecular topology of membrane TLR2 and PGN monomeric residue predicted that a chemical bond between the NAG of PGN soluble monomer and amino acid residues 417-428 of TLR2 is thermodynamically favourable (Li *et al.*, 2011). Monomers of peptidoglycan similarly stimulate TLR-2 of dendritic cells (Volz *et al.*, 2010).

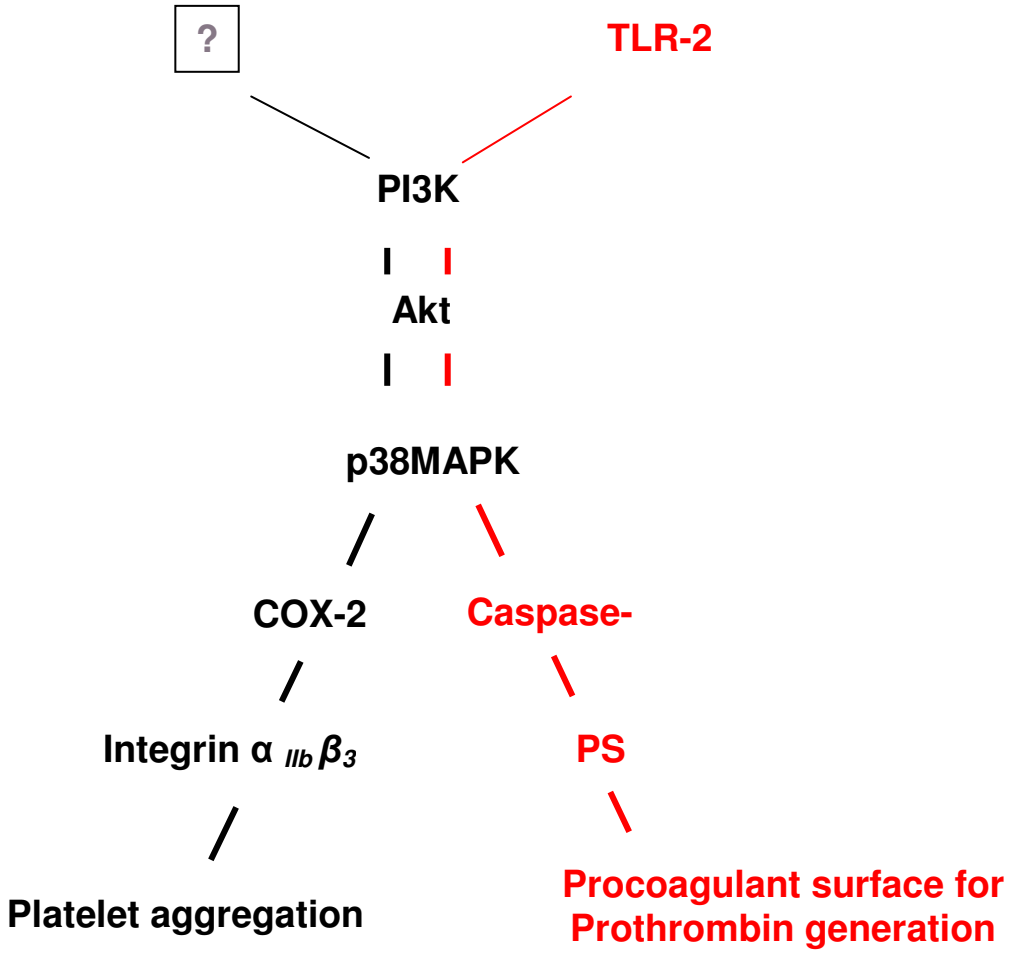


Fig. 4.2 Probable pathways of peptidoglycan-induced apoptosis in platelets

Platelet apoptosis may affect the adhesion of platelets to the vascular wall and thus play a role in hemostasis and thrombosis. PGN may indeed stimulate platelet activity (Ryc and Rotta, 1975b;Ryc and Rotta, 1978;Rotta *et al.*, 1979;Umemoto *et al.*, 1981a;Harada *et al.*, 1982a;Wannamaker, 1983a;Verhoef and Kalter, 1985;Ryc *et al.*, 1987b;Kessler *et al.*, 1991a;Polanski *et al.*, 1992a;Hoijer *et al.*, 1997a;Imegwu *et al.*, 1997b;Tydell *et al.*, 2002b;Bensing *et al.*, 2004b;Rennemeier *et al.*,

2007c;Keane *et al.*, 2010a). As shown in Fig. 3.2.5.A, 250 ng/ml peptidoglycan monomer upregulates activated integrin $\alpha_{IIb}\beta_3$, as evidenced by PAC-1 binding. Thus, stimulation of platelets by PGN could foster vascular occlusive disease. Moreover, PGN stimulates suicidal death of erythrocytes (Foller *et al.*, 2009), which similarly fosters adherence to the vascular wall and is similarly expected to compromize microcirculation. Platelet apoptosis may further result in thrombocytopenia, a known complication of *Staphylococcus aureus* bacteremia (Gafter-Gvili *et al.*, 2011). The number of platelets expressing both integrin $\alpha_{IIb}\beta_3$ and PS increased with the concentration of PGN.

In conclusion, exposure of platelets to low amounts of monomeric PGN triggers phospholipid scrambling and phosphatidylserine exposure at the platelet surface. PGN induced PS exposure is Ca^{2+}-dependent, caspase-dependent and downstream to TLR-2. Furthermore it is simultaneous with integrin $\alpha_{IIb}\beta_3$ upregulation. The effects are expected to accelerate the clearance of platelets from circulating blood and might also cause coagulopathy.

4.3 Cellular Mechanism of Vancomycin-induced thrombocytopenia

The vancomycin induced stimulation is paralleled by depolarization of the mitochondria, activation of caspase-3 and scrambling of the cell membrane evident from phosphatidylserine exposure (PS). The vancomycin induced platelet apoptosis was virtually abolished in the absence of extracellular Ca^{2+}. Thus, the presence of Ca^{2+} is apparently a prerequisite for the stimulation of cell membrane scrambling.

The current observations reveal that vancomycin itself can cause platelet death with upregulation of the markers of apoptosis and activation but phosphatidylserine (PS) exposure is independent of caspase-3. In recent past, PS exposure has been shown to be dependent on calcium and caspase-3 in platelet apoptosis (Tasneem *et al.*, 2011), Towhid et al. 2012) but calcium-dependent, caspase-3 independent in platelet activation (Schoenwaelder *et al.*, 2009c). In case of vancomycin, we find a platelet death which is calcium-dependent, caspase-3 independent and paralleled by CD62P, integrin $\alpha_{IIb}\beta_3$ upregulation and thromboxane B_2 release. Ionomycin 1µM as a positive control showed that vancomycin stimulates platelets in a manner similar to calcium ionophores, though calcium influx by vancomycin is much lower than ionomycin. Yet the total reversal of PS and caspase-3 in absence of calcium shows calcium to be a major mediator of vancomycin-induced events in platelets. The role of calcium in

platelet function takes the center stage because calcium activates myriad of kinases and effector proteins that culminate in activaion, secretion, aggregation and death (Wolf *et al.*, 1999a) (Stefanini *et al.*, 2009b),(Stefanini *et al.*, 2009a;Bergmeier and Stefanini, 2009b), (Varga-Szabo *et al.*, 2009), (Nayak *et al.*, 2011), (Borst *et al.*, 2012a). Calcium ionophore A23187 can trigger sufficient PS exposure in platelets even without mitochondrial depolarization (Arachiche *et al.*, 2009). Therefore it appears that the major trigger of vancomycin-dependent platelet stimulation includes apoptotic and activation events which are independent from previously described drug-mediated immune thrombocytopenia. Vancomycin alone could induce platelet death.

Phosphatidylserine (PS) exposing platelets are expected to be engulfed by phagocytosing cells and thus to disappear from circulating blood. Excessive PS exposure is thus expected to result in thrombocytopenia. Thus, the vancomycin induced stimulation could well contribute to or even account for the thrombocytopenia following treatment with vancomycin (Nasraway *et al.*, 2003a;Kenney and Tormey, 2008c;Anand and Chauhan, 2011). Platelet death might further be followed by adhesion of platelets to endothelial cells of the vascular wall and may thus play a role in hemostasis and thrombosis, because it has been shown that apoptotic platelets simultaneously express integrin $\alpha_{IIb}\beta_3$ (Kile, 2009c;White and Kile, 2010b).

In conclusion, vancomycin treatment of human blood platelets triggered ceramide formation, caspase-3 activation, cell shrinkage and cell membrane scrambling. The caspase-3 activation and cell membrane scrambling required the presence of Ca^{2+}. Vancomycin induced cell membrane scrambling might accelerate the clearance of platelets from circulating blood thus predisposing to the development of thrombocytopenia and foster adherence of platelets to the vascular wall thus fostering thrombosis.

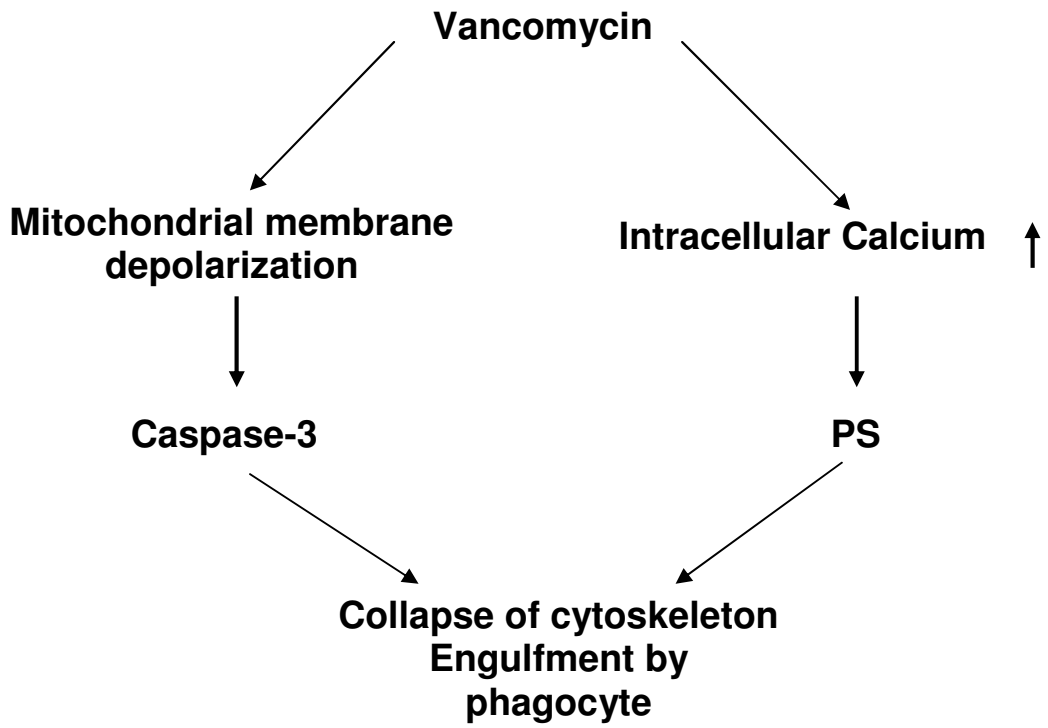


Fig. 4.3 Probable pathways of vancomycin-induced platelet apoptosis

Phosphatidylserine exposing apoptotic platelets are expected to be engulfed by phagocytosing cells and thus to disappear from circulating blood. Excessive apoptosis is thus expected to result in thrombocytopenia. Thus, the vancomycin induced apoptosis could well contribute to or even account for the thrombocytopenia following treatment with vancomycin (Nasraway *et al.*, 2003b;Kenney and Tormey, 2008b;Anand and Chauhan, 2011).

Platelet apoptosis might further be followed by adhesion of platelets to endothelial cells of the vascular wall and may thus play a role in hemostasis and thrombosis (Kile, 2009d;White and Kile, 2010a). The stimulation of platelet apoptosis by vancomycin could thus lead to vascular occlusive disease.

In conclusion, vancomycin treatment of human blood platelets triggered ceramide formation, caspase 3 activation, cell shrinkage and cell membrane scrambling. The cell membrane scrambling required the presence of Ca^{2+} and was inhibited by amitriptylin. Vancomycin induced cell membrane scrambling presumably accelerates the clearance of platelets from circulating blood thus predisposing to the

development of thrombocytopenia and foster adherence of platelets to the vascular wall thus fostering thrombosis.

4.4 CXCL16 induced platelet death and vascular adhesion

It becomes increasingly evident that platelets are important bidirectional inflammatory effectors linking inflammation, thrombosis, and atherogenesis (Weber, 2005a;Gawaz *et al.*, 2005d;May *et al.*, 2008a). Activated platelets present, secrete, and deposit chemokines and thereby exacerbate atherogenesis by inducing recruitment of mononuclear cells to inflammatory lesions of the vascular wall (Gawaz *et al.*, 2000a;Gawaz, 2004;Gleissner *et al.*, 2008b;Koenen *et al.*, 2009). Chemokines can stimulate platelet activation and adhesion amplifying the activation-dependent release of pro-atherogenic and pro-thrombotic proteins from platelet granula (Weber, 2005b;May *et al.*, 2008b). High levels of soluble CXCL16 in ACS, usually associated with acute coronary thrombosis, are associated with an increased long-term mortality (Jansson *et al.*, 2009). Nevertheless, the pathophysiological role of CXCL16 for thrombotic diseases remained unclear. In the present study we could show for the first time, that CXCL16 triggers platelet activation marker integrin $\alpha_{IIb}\beta_3$ and enhances platelet adhesion (Fig. 3.4.2.B and 3.4.3.A), major mechanisms underlying thrombotic artery occlusions (Ruggeri, 2002b).

CXCL16 binds to its only described receptor, the seven-transmembrane domain chemokine receptor CXCR-6 (BONZO) (Matloubian *et al.*, 2000a;Galkina *et al.*, 2007a), which has not been described on platelets so far.

CXCR6-dependent signaling involves the phosphatidylinositide 3-kinase (PI3K) and Akt pathway (Chandrasekar *et al.*, 2004a;Wang *et al.*, 2008b). PI3K as well as its downstream effector Akt play a decisive role in the regulation of platelet function (Stojanovic *et al.*, 2006). Two isoforms of Akt, Akt1 and Akt2, are expressed in platelets and were described to have similar overlapping functions in platelet activation (Woulfe, 2010a). Phosphorylation of both Thr^{308} and Ser^{473} are required for full enzymatic activity (Kim *et al.*, 2004b). In the present study we could show that the effect of CXCL16 dependent adhesion could be abrogated by pre-incubation with LY294002 or wortmannin (Fig. 3.4.4.B) indicating that CXCL16 activates Akt in platelets in a PI3K-dependent manner. Moreover, we found a significant reduction of CXCL16-induced platelet degranulation and integrin $\alpha_{IIb}\beta_3$

activation after preincubation with the PI3K inhibitors LY (25 µM) and Wm (100 nM) as well as with the Akt inhibitor SH-6 (20 µM) (Fig. 3.4.3 C, D).

Enhanced CXCL-16 expression has been found in atherosclerotic lesions in ApoE-deficient mice (Wuttge *et al.*, 2004). The finding that CXCL16 induces activation (P-selectin exposure and integrin $\alpha_{IIb}\beta_3$ activation) of circulating platelets, which in turn could promote release of platelet-derived inflammatory mediators resulting in enhanced leukocyte recruitment (Huo *et al.*, 2003), suggests a vicious circle that aggravates progression of atherogenesis. Mice lacking platelet P-selectin or integrin $\alpha_{IIb}\beta_3$ have been shown to be protected against development of atherosclerotic lesions indicating the predominant role of platelets in the initiation of atherogenesis (Burger and Wagner, 2003;Massberg *et al.*, 2005). Activated platelets are able to adhere to intact endothelium (Bombeli *et al.*, 1998b), a mechanism which is critically involved in the initiation of early atherosclerotic lesion formation and moreover, in promoting thrombotic processes dependent on platelet recruitment to the vascular wall (Massberg *et al.*, 2002). In the present study we could show that CXCL16 increased platelet adhesion to a human endothelial (HUVEC) cells monolayer under arterial shear stress (2000^{-s}) in a PI3K-dependent manner (Fig. 3.4.4.B).

Platelets express different chemokine receptors and some chemokines have already been demonstrated to activate platelets (Kowalska *et al.*, 2000a;Abi-Younes *et al.*, 2000a;Gear *et al.*, 2001b;Schafer *et al.*, 2004a;Gleissner *et al.*, 2008a). Partly, the activation occurs only in combination with a weak platelet agonist (Kowalska *et al.*, 2000b;Gear *et al.*, 2001a) and partly, the activation is independently from co- or prestimulation (Abi-Younes *et al.*, 2000b;Schafer *et al.*, 2004b). Although there is evolving evidence, that chemokines expressed in atherosclerotic plaques and released from mononuclear cells involved in inflammatory diseases can induce a prothrombotic state via platelet activation, only little is known about signaling pathways and mechanisms mediating these effects. Although CXCL16 is associated with occurrence and severity of thrombotic diseases as acute coronary syndromes, hitherto nothing was known about a role of CXCL16 on platelet activation.

CXCL16 shares close structural similarities with CX3CL-1 (fractalkine) (Wang *et al.*, 2008c). In recent studies fractalkine was shown to induce platelet

activation leading to enhanced platelet adhesion *in vitro*. The authors speculated that ADP or thromboxane synthesis could be involved in fractalkine-dependent platelet stimulation, an effect prevented by apyrase and aspirin (Schafer *et al.*, 2004c).

Platelet activation by ADP is mediated by its two purinergic receptors P_2Y_1 and P_2Y_{12} (Kim *et al.*, 2004a) which are furthermore described to participate in mechanisms of platelet adhesion (Leon *et al.*, 1999;Remijn *et al.*, 2002;Andre *et al.*, 2003). As shown in Fig. 3.4.3.A and B, CXCL16-triggered platelet degranulation and integrin $\alpha_{IIb}\beta_3$ activation as well as CXCL16-induced platelet adhesion to vascular wall was diminished after preincubation with the ADP-degrading enzyme apyrase or the purinergic receptor blockers MRS2179 and Cangrelor (AR-C69931MX) indicating that ADP has a potentiating effect on CXCL16-mediated platelet stimulation (Fig. 3.4.4 C). Apparently, both P_2Y_1 and P_2Y_{12} receptors are involved in CXCL16-dependent platelet activation, at which P_2Y_{12} plays the more important role (Fig. 3.3.3 C,D, Fig. 3.4.4 C), which is consistent with findings of other studies (Cosemans *et al.*, 2006). However, thromboxane synthesis was not found to be involved since CXCL16-triggered platelet activation was not affected by co-incubation with indomethacin (Fig. 3.3.3.D). ADP activates the PI3K/Akt signaling pathway in platelets through purinergic receptor signaling (Kim *et al.*, 2009b), an effect which could aggravate CXCL16-mediated platelet activation. Akt is a possible candidate for mediating further second-wave signaling by regulating platelet secretory pathways (Woulfe, 2010b). The second-wave signaling could potentiate CXCL16-induced Akt-dependent triggering of platelet activation. In this way the phosphorylation of Akt could underly synergistic effects of CXCL16 and ADP in paracrine/autocrine platelet activation.

In conclusion, the inflammatory chemokine CXCL16 triggers platelet PI3K/Akt signaling leading to degranulation and integrin $\alpha_{IIb}\beta_3$ activation. CXCL16-dependent platelet activation involves ADP-mediated paracrine/autocrine stimulation and foster platelet adhesion to intact endothelium. Thus, CXCL16 could play a decisive role in linking inflammatory diseases and thrombosis.

Concluding Remarks

1. Thymoquinone, a nutritional component in Mediterranean food with well-known apoptotic effects, cause apoptosis of human platelets without platelet activation.
2. The HPLC-purified fraction (N-acetylglucosamine covalently bound to N-acetylmuramic acid with pentapeptide side chain) of peptidoglycan from *Staphylococcus aureus 113* is a potent inducer of apoptosis in human platelets paralleled by upregulation of integrin $\alpha_{IIb}\beta_3$. The apoptotic events are downstream to TLR-2 receptor and dependent on integrin activation. P-selectin is not upregulated by purified monomeric peptidoglycan (mPGN).
3. Contrary to the long-held view of drug-induced immune thrombocytopenia, vancomycin induced clear and direct apoptotic response in platelets resembling the action of Calcium ionophores. In addition, the effect is accompanied by upregulation of $\alpha_{IIb}\beta_3$ and P-selectin and release of thromboxane B2.
4. The inflammatory chemokine CXCL16 has its receptor CXCR-6 on human platelets, which is upregulated in response to the ligand. CXCL16 induces platelet activation and apoptosis with comparable intesity and platelet activation and secretion events are dependent on PI3K pathway along with a secondary signaling from purinergic receptors. These apoptotic-activated platelets firmly adhere to endothelial cell monolayer under physiological shear stress, which is also dependent on PI3K and purinergic signaling. Therefore apoptotic platelets seem to have the ability to occlude vasculature.
5. Platelet apoptosis has a common pathway, often involving PI3K-Akt, leading to caspase-3 activation and phosphatidylserine exposure in a Calcium-dependent manner.
6. The pro-coagulatory, pro-adhesive and secretory nature of apoptotic platelets are, at least in theory, indicators of more serious pathologic events such as thrombotic occlusion or immune reaction.

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