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The role of formyl peptide receptors in the regulation of platelet function

A thesis submitted for the degree of Doctor of Philosophy

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Declaration

I confirm that this is my own work and the use of all material from other sources has been properly and fully acknowledged.

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Abstract

Formyl peptide receptors (FPRs) belong to the family of G protein-coupled receptors (GPCRs) and play crucial roles in the regulation of innate immunity and host defence. FPRs include three family members; FPR1, FPR2/ALX and FPR3. They bind a wide variety of structurally and chemically diverse ligands that can exert various functions. Despite a plethora of research focusing on the role of FPRs in the regulation of immunity, there is a paucity of studies on their roles on the regulation of platelet haemostatic function. Here, we demonstrate the impact of both FPR1 and FPR2/ALX on the modulation of platelet reactivity, haemostasis and thrombosis. By using selective pharmacological inhibitors for FPR1 and FPR2/ALX, and *Fpr1*- and *Fpr2/3*-deficient mice, we were able to establish instrumental roles for these receptors in the regulation of the normal platelet haemostatic function. Additionally, we report a crucial role for fMLF in the regulation of platelet function through FPR1 signalling. fMLF exerted a priming effect on platelet activation through inducing distinct functions and enhances thrombus formation under arterial flow conditions. These effects were diminished in the presence of FPR1-selective pharmacological inhibitors and in platelets obtained from *Fpr1*-deficient mice. In addition, we investigated the role of LL37 in the regulation of platelet function and its modulation on platelet reactivity under pathological conditions, such as psoriasis, via acting through FPR2/ALX. We demonstrate that LL37 activates a range of platelet functions, enhances thrombus formation, and shortens the tail-bleeding time in mice. Moreover, we report the overexpression of mCRAMP (an LL37 murine orthologue) in affected skin and plasma of a murine [imiquimod (IMQ)-induced] model of human psoriasis and its ability to enhance platelet responses via *Fpr2/3*. We also report a role for Annexin A1 and its N-terminal peptide, Ac2-26, in the regulation of platelet function through FPR2/ALX. Ac2-26 induced the activation of various platelet functions. Moreover, *AnxA1*-deficient mice demonstrate enhanced functional responses towards Ac2-26, which may be attributable to the overexpression of *Fpr2/3* in these mice. Since both FPR1 and FPR2/ALX and their ligands play critical roles in various pathological conditions, their influence on the modulation of platelet activation and thrombus formation will provide novel insights into the mechanisms that control platelet-mediated complications under various disease settings.

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Publications

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Ravishankar D, **Salamah M**, Akimbaev A, Williams HF, Albadawi DAI, Vaiyapuri R, Greco F, Osborn HMI and Vaiyapuri S. Impact of specific functional groups in flavonoids on the modulation of platelet activation. *Sci Rep.* 2018;8:9528.

Salamah M*, Ravishankar D*, Attina A, Pothi R, Vallance TM, Javed M, Williams HF, Alzahrani EMS, Kabova E, Vaiyapuri R, Shankland K, Gibbins J, Strohfeldt K, Greco F, Osborn HMI and Vaiyapuri S. Ruthenium-conjugated chrysin analogues modulate platelet activity, thrombus formation and haemostasis with enhanced efficacy. *Sci Rep*. 2017;7:5738.

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Presentations

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Abbreviations

Å – Angstrom

Ac2–26 – N–Acetyl 2–26

ACD – acid citrate dextrose

ADP – adenosine diphosphate

AGEPC – Acetyl–glyceryl–ether–phosphoryl–choline

AIDS – acquired immune deficiency syndrome

ALX – aspirin–triggered lipoxins

AMPs – antimicrobial peptides

ANOVA – analysis of variance

Anx A1– annexin A1

ApoE – apolipoprotein E

ATP – adenosine triphosphate

AU – arbitrary units

Aβ42 – β amyloid peptide

Boc–1 – t–Boc–Met–Leu–Phe

Boc–2 – t–Boc–Phe–d–Leu–Phe–d–Leu–Phe

C – control

C57BL/6 – C57 Black 6 mice

Ca^{2+} – calcium ion

$CaCl_2$ – calcium chloride

cADPR – cyclic ADP–ribose

CAMP – cathelicidin antimicrobial peptide

cAMP – cyclic adenosine monophosphate

CCL5 – C–C motif chemokine ligand 5

CCR3 – C–C motif chemokine receptor 5

CD – cluster of differentiation

CD40L – CD40 ligand

CD62P – P–selectin

CDCA – chenodeoxycholic acid

CHIPS – chemotaxis inhibitory protein of S. aureus

CLEC–2 – C–type lectinlike receptor 2

cm^2 – square centimeter

CO_2 – carbon dioxide

CRAMP – cathelicidin–related antimicrobial peptide

CRP–XL – cross–linked collagen–related peptide

CsA – cyclosporin A

CsH – cyclosporin H

CTAP–3 – connective tissue activating peptide 3

CVD – cardiovascular disease

Cy5 – cyanine 5 dye

DAG – diacylglycerol

DAMPs – damage–associated molecular patterns

DCA – deoxycholic acid

DIC – disseminated intravascular coagulation

$DiOC_6$ – 3,3'–Dihexyloxacarbocyanine iodide

DMSO – dimethyl sulfoxide

DNA – deoxyribonucleic acid

DTS – dense tubular system

E. coli – Escherichia coli

ECM – extracellular matrix

EGTA – ethylene glycol–bis(β–aminoethyl ether)–N,N,N',N'–tetraacetic acid

ELISA – enzyme–linked immunosorbent assay

eNOS – endothelial nitric oxide synthase

ERK – extracellular signal–regulated kinase

FcR – Fc receptor

FITC – fluorescein isothiocyanate

Fluo–4 AM – Fluo–4 acetoxymethyl

fMet – formyl methionine

fMLF – N–formyl–methionyl–leucyl–phenylalanine

FPA – fibrinopeptides A

FPB – fibrinopeptides B

Fpr–rs – Fpr–related sequence

FPRs – Formyl peptide receptors

FSC – forward scatter

g – g–force

g – grams

GCs – glucocorticoids

GDP – guanosine diphosphate

GP – glycoprotein

GPCR – G protein–coupled receptor

GTP – guanosine triphosphate

h – hour(s)

hCAP–18 – human cationic antibacterial protein of 18 kDa

HCl – hydrochloric acid

HDPs – host defense peptides

HEPES – 4–(2–hydroxyethyl)–1–piperazineethanesulfonic acid

HIV – human immunodeficiency virus

Hp (2–20) – a peptide derived from Helicobacter pylori

HSV–2 – Herpes simplex virus type 2

I/R – ischaemia/reperfusion

IAV – influenza A virus

IBD – inflammatory bowel disease

ICAM–1 – intercellular adhesion molecule 1

IFN – interferon

Ig – immunoglobulin

IL1β – Interleukin 1β

IMQ – imiquimod

IP receptor – prostaglandin receptor

IP3 – inositol trisphosphate

IV – intravenous

KCl – potassium chloride

kDa – kilodaltons

kg – kilogram

KO – knockout

LDH – lactate dehydrogenase

LGIC – ligand–gated ion channel

LL37 – LLGDFFRKSKEKIGKEFKRIVQRIKDFLRNLVPRTES

LPS – lipopolysaccharides

LTA – light transmission aggregometry / lipoteichoic acid

LXA4 – lipoxin A4

M – molar

Mac–1 – macrophage–1 antigen

MAPK – mitogen–activated protein kinase

mCRAMP – Mouse cathelicidin–related antimicrobial peptide

MFI – mean fluorescence intensity

mg – milligram

mg/kg – milligrams/kilogram

Mg^{2+} – magnesium ion

$MgCl_2$ – magnesium chloride

µg – microgram

µL – microlitre

µM – micromolar

Min – minute(s)

MKs – megakaryocytes

mL – millilitre

mm – millimetre(s)

MMK–1 – synthetic peptide H–LESIFRSLLFRVM–OH

mmol/L – millimolar

MMPs – matrix metalloproteinases

MMWLL – Met–Met–Trp–Leu–Leu

MW – molecular weight

n – n number

Na_2HPO_4 – disodium hydrogen phosphate

NaCl – sodium chloride

NADPH – nicotinamide adenine dinucleotide phosphate

$NaHCO_3$ – sodium bicarbonate

NETs – neutrophil extracellular traps

nm – nanometre

nM – nanomolar

NP40 – Nonidet P40

ns – not significant

OCS – open canalicular system

OH – hydroxyl group

p – probability value

PAF – platelet–activating factor

PAMPs – pathogen–associated molecular patterns

PAR – protease–activated receptors

PASI – Psoriasis Area and Severity Index scoring system

PBP – platelet basic protein

PBP10 – ten amino acid PIP2 binding peptide

PBS – phosphate buffered saline

PDB– protein data bank

PE – phycoerythrin

PECAM–1 – platelet endothelial cell adhesion molecule 1

PF4 – platelet factor 4

PFA – paraformaldehyde

PGI2 – prostacyclin

PI3K – phosphoinositide 3–kinase

PIP2 – phosphatidylinositol 4,5–bisphosphate

PIP3 – phosphatidylinositol 3,4,5–trisphosphate

PKA – cAMP–dependent protein kinase

PKB – protein kinase B

PKC – protein kinase C

PKG – cGMP–dependent protein kinase

PLAs – platelet–leukocyte aggregates

PLC – phospholipase C

PLCβ_2 – phospholipase Cβ_2

PPP – platelet–poor plasma

PRP – platelet–rich plasma

PTX – pertussin toxin

PVDF – polyvinylidene fluoride

Quin–C1 (4–butoxy–N–[2–(4–methoxy–phenyl)–4–oxo–1,4–dihydro–2H–quinazolin–3–yl]–benzamide)

RANTES (Regulated on Activation, Normal T Cell Expressed and Secreted),

RBC – red blood cell

Rho – rhodopsin

ROS – reactive oxygen species

RPM – revolutions per minute

RTKs – receptor tyrosine kinases

s / sec – second(s)

SAA – serum amyloid A

SAnxA1 – Super Annexin A1

Sc – scrambled

SDS–PAGE – sodium dodecyl sulphate polyacrylamide gel electrophoresis

SEM – standard error of the mean

SPMs – specialized pro–resolving mediators

t–Boc – tertiary butyloxycarbonyl group

TB–4 – thymosin B–4

TBS–T – Tris–buffered saline, 0.1% Tween 20

TLRs – toll–like receptors

TMB – 3,3',5,5'–Tetramethylbenzidine

TP – thromboxane prostanoid

TPO – thrombopoietin

U/mL – unit/millilitre

TRAP – thrombin–receptor activating peptide

Tris – tris(hydroxymethyl)aminomethane

TXA_2 – thromboxane A_2

U46619 – 9,11–Dideoxy–11a,9a–epoxymethanoprostaglandin

V / Veh – vehicle

VAS – Vaseline

VASP – vasodilator–stimulated phosphoprotein

VEGF – vascular endothelial growth factor

VV – vaccinia virus (VV)

vWF – von Willebrand Factor

w/v – weight/volume

WKYMVm – Trp–Lys–Tyr–Met–Val–d–Met–NH_2

WRW4 – WRWWWW; Trp–Arg–Trp–Trp–Trp–Trp–CONH(2)

WT – wildtype

5–FAM–LC – 5–carboxyfluorescein conjugated to 6–carbon linker (LC)

^{o}C – degrees centigrade

Table of Contents

Index of Figures and tables

1 – Introduction

1.1 Platelets

Platelets are small circulating blood cells that play indispensable roles in the regulation of haemostasis to prevent excessive bleeding upon vascular injury. However, their unwarranted activation under pathological conditions leads to the formation of blood clots (thrombi) within the circulation[1]. This results in reduced blood supply to vital organs including the heart and brain, which can trigger heart attacks or strokes, respectively[2]. Moreover, certain pathological conditions such as inflammation and infection results in the activation of platelets, leading to aggregation, thrombus formation in the microvasculature and in later stages, sequestration of platelets in organs such as the lungs, instigating thrombocytopenia and bleeding complications[3]. Aside from their role in haemostasis, platelets also play pivotal roles in the regulation of innate immunity, inflammatory responses and clearance of microbial infection[4-6]. In general, platelets act as sentinels[7] due to their high number in the circulation, and they play critical roles in host defence against invading pathogenic microbes such as bacteria[8]. Platelets are also known to possess direct microbicidal activities against various bacterial species[4, 7-9]. They contain different types of intracellular granules and a wide range of receptors on their surface that aid in the modulation of haemostatic responses upon vascular injury. In addition, platelets express a wide range of inflammatory receptors and mediators that enable them to recognise to a broad spectrum of molecules. Here, we describe the role of different molecules released upon inflammation and/or infection in the regulation of platelet function.

1.2 Platelet production

Platelets are small anucleate circulating blood cells that range from 1 to 3 μm in diameter and are known to play a pivotal role in haemostasis and thrombosis[10]. They are produced from the fragmentation of the cytoplasm of the megakaryocytes (MKs) in the bone marrow. This process is regulated by the cytokine thrombopoietin (TPO), a hormone produced in the kidneys and liver[11, 12], and its receptor c-Mpl[13, 14]. MKs are bone marrow cells that

originate from hematopietic stem cells and can also be found in the lungs and in the blood circulation[15, 16]. In order to produce platelets, MKs undergo endomitosis, which is DNA replication without cell division, that is stimulated by TPO and render MKs polypoid. This is followed by the maturation of MKs, wherein the nucleus is extruded, and the majority of the cytoplasm is packaged into several elongated processes known as proplatelets[17-19]. Each MK may extend 10 to 20 proplatelets, which elongates over time while thinning and branching[20]. At the tip of these proplatelets, the platelets develop and form while receiving organelles and granules transported from MKs[21]. The production of platelets can also occur in the lungs[22], where it has been demonstrated recently that MKs in the bone marrow can migrate into the lungs, and back, and produce a significant number of platelets under thrombocytopenic conditions in mice[23]. Each megakaryocyte produces around 1000-3000 platelets[24], and this process is usually completed in 5 days in human and 2-3 days in mice[25-27]. The platelet lifespan in human peripheral blood circulation is averaged between 7-10 days, while lasting 4-5 days in mouse circulation[24, 28-30]. The normal range for platelets produced by healthy individuals in the circulation is $150\text{-}400\times10^9$ per litre [31, 32].

1.3 Platelet ultrastructure

The structure of platelets aids in the implementation of its haemostatic function, and it contains the following zones: peripheral, sol-gel, organelle and membranous zone[33]. The peripheral zone of the platelets contains receptors and glycoproteins that aid in the adhesion, activation and aggregation of platelets. The Sol-gel zone allows the platelets to maintain their discoid shape via the microtubules and microfilaments and also contains the actin cytoskeleton which allows shape changes upon platelet activation. Platelets contain three distinct types of granules, which comprise the organelle zone[34-40]. Alpha granules (α) which mainly contain proteins that mediate the secondary platelet activation including fibrinogen, P-selectin and von Willebrand factor, and are the largest (200-400 nm) and most numerous (50-60 per platelet) platelet granules[35]. Conversely, dense granules (δ) contain non-proteinous molecules including serotonin, calcium, phosphate, ADP and ATP, and are smaller (~150 nm) and less numerous (3-8 per platelet) than α-granules. Moreover, platelets contain lysosomal granules (λ) which contain degrading enzymes that aid in the resolution of thrombi[37]. A diagram of the platelet structure is presented in Figure 1-1. Platelets also contain receptors that aid in their activation upon ligation with various agonists; a diagram of the different receptors expressed in platelets is presented in Figure 1-2. For example, collagen activates platelets via GPVI and the integrin α2β1, ADP activates platelets via acting through P2Y1 and P2Y12, while thromboxane A_2 (TXA_2) activates the thromboxane prostanoid (TP) receptor on platelets. Another potent platelet agonist is thrombin, which acts on protease activated receptors (PAR1 and PAR4 in humans). Finally, the membranous zone which is related to the open canalicular system (OCS) and dense tubular system (DTS), is responsible for the synthesis and release of TXA_2, which aids the aggregation of platelets[41].

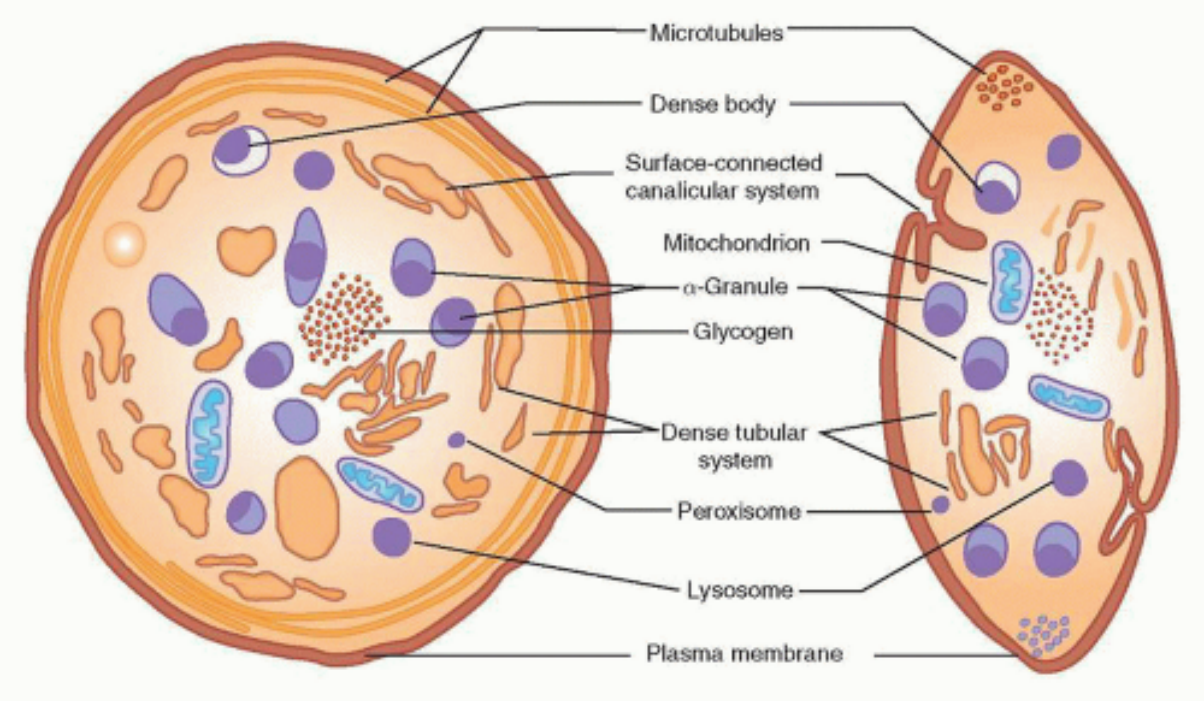

Figure 1-1: Platelet structure. Diagram of the structure of platelets demonstrating the different platelet zones including: sol-gel, organelle and membranous zone. The Sol-gel zone contains microtubules and microfilaments that allow the platelets to maintain their discoid shape, and the actin cytoskeleton that allows shape change. Additionally, platelets contain an organelle zone comprising three distinct types of granules, including α-granules, dense δ-granules λ-granules. In addition to the membranous components of the platelets (plasma membrane, canalicular system, and dense tubular system), platelets contain mitochondria, microtubules, and glycogen. Adapted from (Li *et al*., 2017)[42].

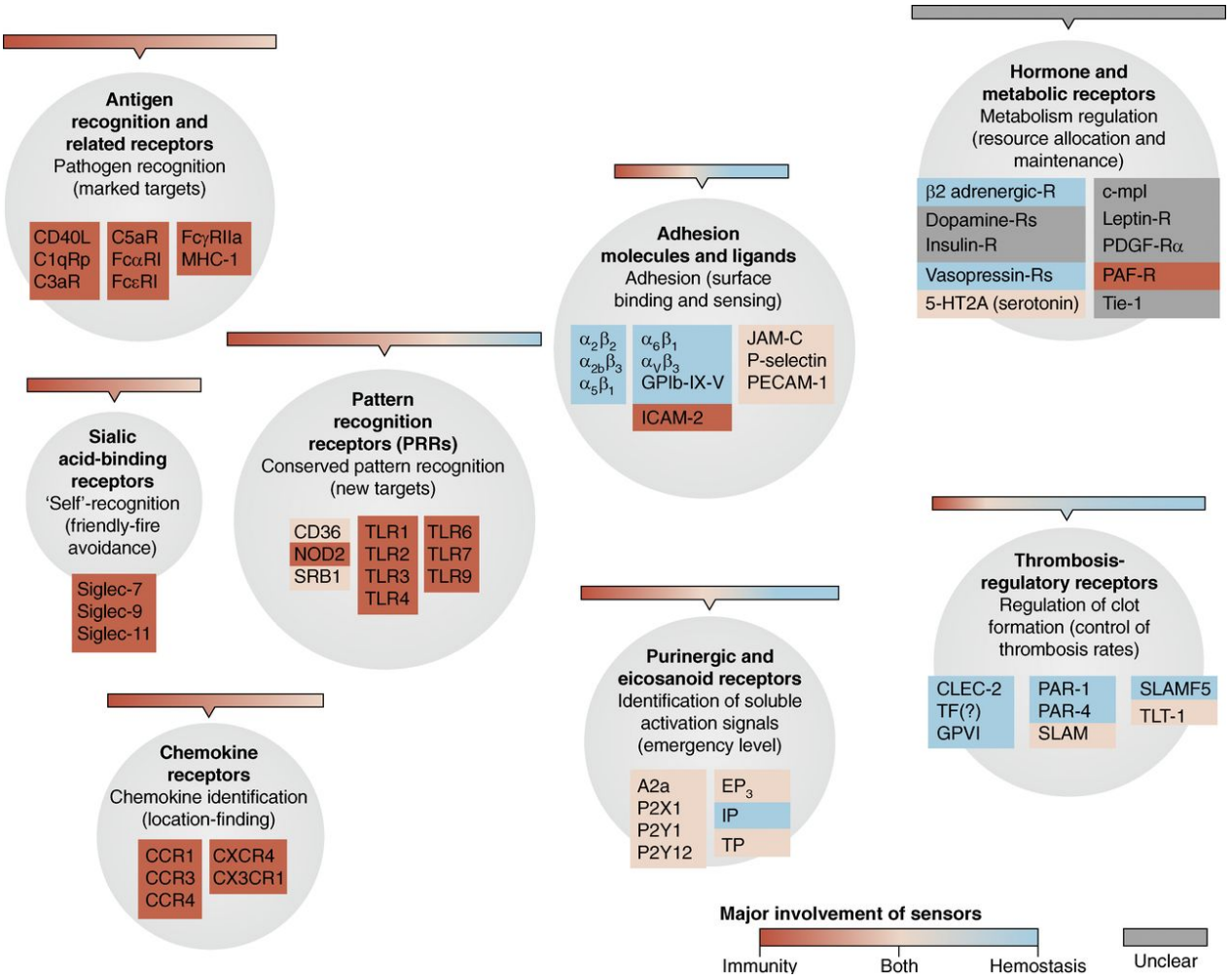

Figure 1-2: Diverse receptors expressed in platelets. Diagram of platelet receptors categorised by their major functions that are exerted in platelets. Platelets play crucial roles in the regulation of haemostasis (blue colour), immunity (red colour) or both (pink), whereas receptors of unknown functional roles are shown in grey. Adapted from (Li *et al*., 2017)[43].

1.4 Platelet function in health and disease

1.4.1 Role of platelets in haemostasis

Under normal physiological conditions, platelets circulate in a resting state. This state is regulated by molecules that inhibit the activation of platelets and are released from the intact endothelium. These molecules include nitric oxide (NO), prostacyclin (PGI2) and apyrase (which degrades ADP). The dysregulation of such molecules may lead to inappropriate platelets activation and contribute to pathological conditions[44]. Upon vascular injury however, platelets become activated and contribute to their physiological process, haemostasis, in which they are known to play a central role[45].

The haemostatic function of platelets can be categorised into the following stages: adhesion, activation, aggregation and contraction of platelets. Upon vascular injury, the subendothelial collagen is exposed, enabling the binding of von Willebrand factor (vWF), facilitating the binding to platelets and thus their adhesion to the injured vascular site. This binding is mainly facilitated by glycoprotein (GP) receptors on the surface of platelets, leading to the formation of a monolayer over the injured site to arrest the bleeding[46-48]. Notably, GPVI-mediated binding of platelets to collagen leads to a reduced production of prostacyclins and increases the production of TXA_2. Another potent platelet agonist is a synthetic peptide known as crosslinked collagen receptor peptide (CRP-XL), which is selective for GPVI only, and aids in the mechanistic investigation of signalling pathways in platelets. The binding and activation of platelets leads to initiation of various responses including, calcium mobilisation, granule secretion, tyrosine kinase signalling pathways and activation of platelet integrins. The release of platelet granules aids in the secondary platelet activation and release of chemotactic agents, attracting more platelets to the site of vascular injury. This allows the bridging of activated platelets via receptor-bound fibrinogen mediated through integrin αIIbβ3 and thus aggregation

of platelets and development of a stable platelet aggregate[49, 50]. The aforementioned responses including platelet adhesion, activation and aggregation comprise the first wave of haemostasis, and are followed by the coagulation cascade (otherwise known as the second wave of haemostasis), that leads to the development of a stable thrombus.

The activation of platelets can also lead to the exposure of phosphatidylserine on their surface, which induces a negatively charged phospholipid surface that harbours coagulation factors, rendering platelets procoagulant[51-53]. The coagulation cascade leads to the generation of thrombin, which is known as a strong platelet activator, that initiates signalling through protease activated receptor 1 (PAR1), 2 (PAR2) and GPIbα[54, 55]. In addition, thrombin facilitates the conversion of fibrinogen to fibrin, which aids in the development of a blood clot [56, 57]. In order to facilitate the repair the vascular wall damage, the platelet undergoes contraction, driving the retraction and stiffening of clots[58]. This aids in several responses, including the reinforcement of haemostasis, restoring the blood flow and promotes wound healing[59-61].

1.4.2 Function of platelets in thrombosis and cardiovascular disease

Although platelets play a central role in the regulation of haemostasis, this process, if poorly regulated, leads to inadvertent thrombus formation and the occlusion of blood vessels, a process otherwise known as thrombosis. Thrombosis is one the leading causes of death worldwide[62] and constitutes a major underlying pathology in multiple cardiovascular diseases, including strokes, ischemic heart disease and venous thromboembolism[63]. Additionally, thrombosis also occurs in atherosclerosis, wherein platelets may promote atherogenesis and augment vascular inflammation and remodelling of the arterial wall, resulting in formation of atherosclerotic plaques. The rupture of these plaques is implicated in coronary thrombosis. Various antiplatelet agents that target platelet activatory pathways have been proposed[64]. However, these agents could also target pathways that are involved in haemostasis. Therefore, their administration may lead to an increased risk of bleeding.

1.4.3 Role of platelets in innate immunity

In addition to their widely characterised role in haemostasis, platelets play an integral role in the regulation of immune and inflammatory responses and are essential in host defence, wound healing and pathogen surveillance[65-67]. A diagram of the various roles of platelets in haemostasis and immunity is presented in Figure 1-3. These roles were emphasised by the discovery of a plethora of immune receptors in platelets, including Toll-like receptors (TLRs), such as TLR2, TLR4 and TLR9, immunoglobulin receptors, Siglecs and complement receptors[68-70]. Additionally, platelets express formyl peptide receptors (FPRs), notably, FPR1 was reported to add a chemotactic function to platelets[71]. Moreover, platelet granules contain immune and inflammatory modulators that are released upon the activation of platelets and translocated to the plasma membrane. Platelets contain cytokines, such as CD40L and chemokines (CCL5), which are mainly compartmentalised in alpha-granules and aid in the recruitment and activation of other immune cells or induction of endothelial cell-mediated inflammation[72]. Platelets can also play a protective role during microbial infections. Platelets are known to contain several antimicrobial peptides including platelet factor 4 (PF4), platelet basic protein (PBP) and its derivatives, connective tissue activating peptide 3 (CTAP-3), thymosin B-4 (TB-4), CAMP and fibrinopeptides A and B (FPA and FPB), and release them upon activation in order to control microbial infection[73]. In this study we were also able to report the expression of an antimicrobial peptide (AMP), LL37, in platelets which is the sole human cathelicidin. Platelets can also directly interact with and kill pathogens. For instance, platelets may bind and wrap bacteria[74] or induce their aggregation[75], leading to degranulation. In addition to AMPs, platelets contain other inflammatory mediators, including CD40 ligands (CD40L), Interleukin 1β (IL1β) and Regulated on Activation, Normal T Cell Expressed and Secreted (RANTES)[76-80]. Additionally, platelets interact with other inflammatory leukocytes to regulate inflammation and immunological responses by releasing chemokines and cytokines

that activate and recruit them to the site of infection[81, 82]. These interactions are also crucial for the clearance of bacterial, viral, and parasitic infections[43]. For instance, platelets interact with neutrophils to facilitate the release of reactive oxygen species (ROS), proteases and myeloperoxidase[81]. Moreover, prothrombotic and inflammatory gene expression can result from the interaction of platelets with monocytes[83]. Platelets also interact with lymphocytes to increase cytotoxic T-cell activity and mediate class-switching on B-cells[84].

The activation of platelets is typically associated with eliciting pro-inflammatory responses. However, platelets have been shown to interact with anti-inflammatory and pro-resolution responses as well, which is controversial, and the underlying mechanisms are not entirely clear[85]. Notably, it has been shown that Maresin-1 interacts with FPR2/ALX on platelets and enhances their aggregation and spreading, while suppressing the release of pro-inflammatory mediators[86]. In line with this, another study has reported that the activation of platelets induces unforeseen anti-inflammatory properties[87].

Although several of these effects are beneficial to the host to aid in resolving inflammation, their dysregulation can be detrimental and may lead tissue damage and vascular injury[88]. Moreover, it may contribute to disease and exacerbation of adverse effects in various inflammatory conditions including atherosclerosis, inflammatory lung, bowel, and skin diseases, ischemic and inflammatory hepatitis, cancer, arthritis, glomerulonephritis and sepsis[89-93]. While these mediators and receptors establish a role for platelets in immunity, there is a paucity of studies on the role of these receptors and mediators in the haemostatic platelet function. Exploring such roles can lead to novel mechanisms to eliminate adverse effects in diseases associated with platelets dysfunction.

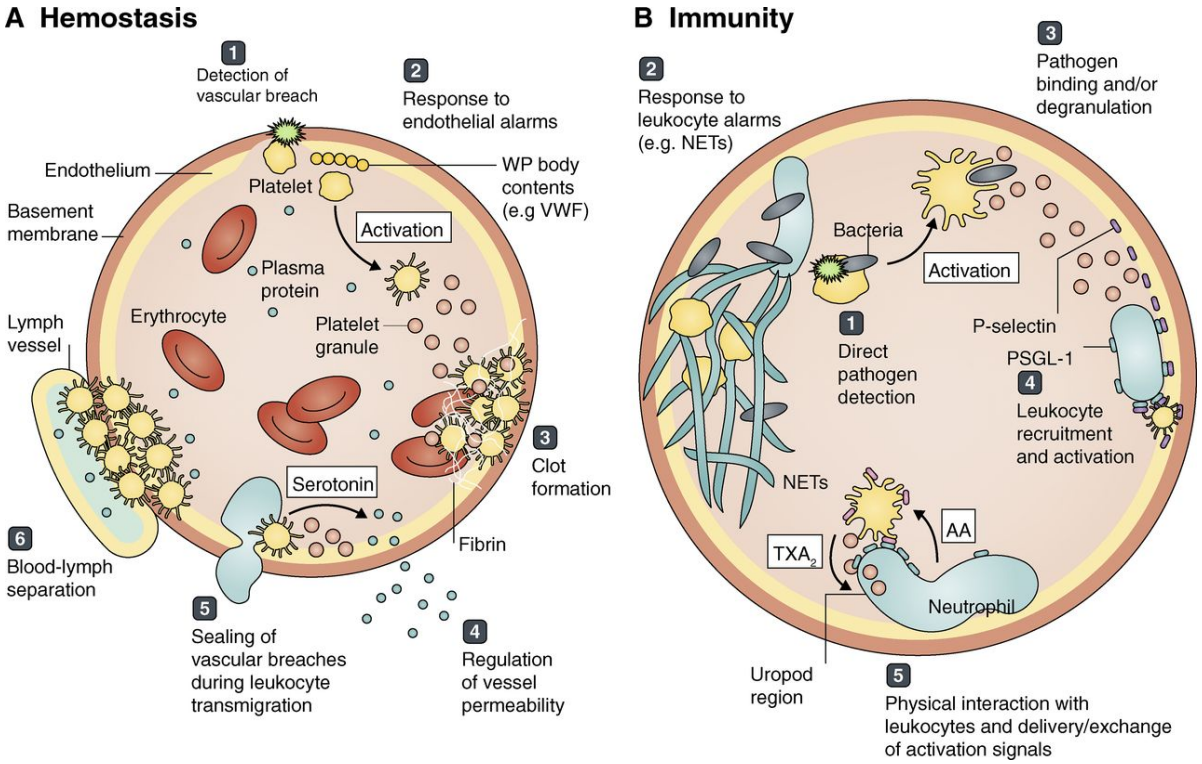

Figure 1-3: Major roles of platelets in haemostasis and immunity. Platelets are known to play pivotal roles in the regulation of haemostasis (A) and immunity (B). Upon vascular injury, platelets bind to collagen in the exposed endothelium layer (A1). Additionally, they detect molecules that are released from activated endothelium such as those released from the Weibel-Palade (WP) bodies (A2), which also contribute to the activation of platelets, and the initiation of thrombosis (A3). Meanwhile, platelets also regulate the permeability of blood vessels (A4) and prevent the loss of erythrocytes by sealing the vessels (A5). Moreover, platelets regulate the lymphovenous junction (A6). In addition to their role in haemostasis, platelets are able to detect pathogens directly (B1) or indirectly via interacting with leukocytes (B2). Platelets have the ability to directly bind to pathogens to initiate their killing (B3), or indirectly by recruiting leukocytes (B4). The interaction of platelets with leukocytes aids in the exchange of molecules, and thus initiates inflammatory responses. For example, platelets can synthesize thromboxane A_2 (TXA_2) from arachidonic acid (AA) supplemented from neutrophils (B5). Adapted from (Li et al., 2017)[43].

1.4.4 Platelet-associated inflammatory diseases

While platelets play indispensable functions, both haemostatic and immune, that are essential for haemostasis, vascular integrity and systemic homeostasis, the dysregulation of such functions can have detrimental consequences, including thrombosis and bleeding. Uncontrolled platelet function leads to the formation of blood clots within the blood vessel (thrombosis), obstructing the blood flow through the circulatory system[94]. This is considered as a key factor in several cardiovascular diseases such as myocardial infarction, strokes, atherosclerosis, and venous and arterial thrombosis[95]. Moreover, platelets have been implicated in the development of cancer, inflammatory disease such as multiple sclerosis, inflammatory bowel disease (IBD), rheumatoid arthritis and psoriasis, and in infectious disease such as sepsis, malaria and dengue, whereby they contribute to unwarranted adverse effects [88, 96-100]. The inhibition of platelet function and/or the decrease in the number of platelets (thrombocytopenia) leads to bleeding. Many treatment modalities for several chronic diseases have been associated with bleeding, including anticoagulant, antitumour, and anti-inflammatory therapy[101-103].

Since platelets interact with endothelial cells and leukocytes to induce their function, the dysregulation of such interactions can drive pathological processes. Activated platelets can interact with and adhere to leukocytes, leading to the formation of platelet-leukocyte aggregates, which can exacerbate inflammatory conditions[104]. Moreover, platelets have been shown to be implicated in the formation of neutrophil extracellular traps (NETs), which can promote the activation of platelets and trigger a procoagulant state[105, 106]. NETs can also promote thrombosis by providing a scaffold that can stimulate platelet adhesion and aggregation[107-109]. Platelets can also interact with other cell types by the production of microparticles, which can aid in cancer metastasis[110]. A diagram of the various receptors that

are present on platelets to aid the interaction with other cells types, and thus the regulation of host defence is presented in Figure 1-4.

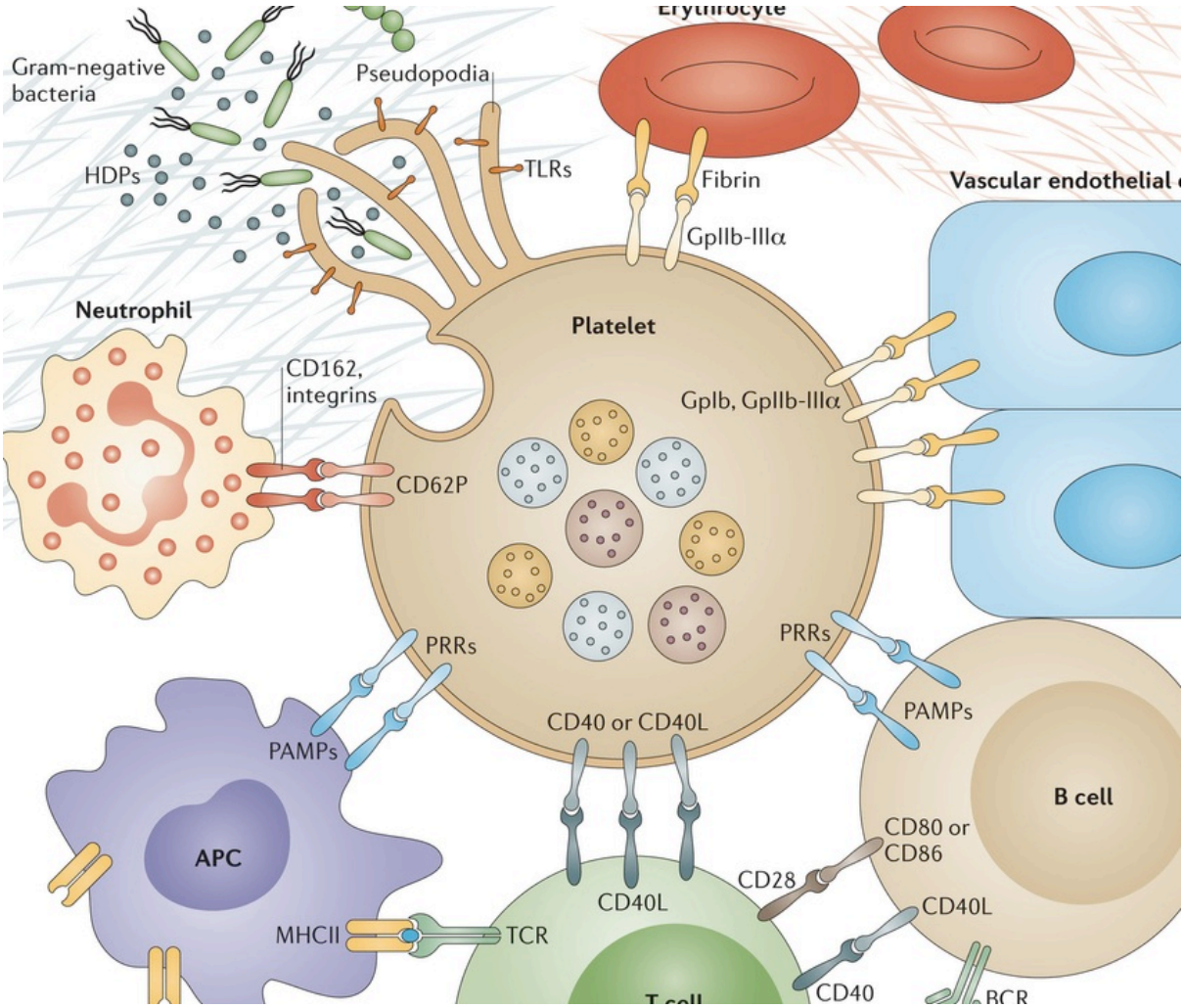

Figure 1-4: Interaction of platelets with other cell types via various receptors to initiate host defence. Platelets are able to interact with a variety of cell types in order to regulate host defence. The activation of platelets results in shape change and the formation of pseudopodia, facilitating the interaction with injured tissues and pathogens. Additionally, platelets can release host defense peptides (HDPs) that can kill pathogens. Platelets can induce the formation of neutrophil extracellular traps (NETs), which can trap pathogens. Additionally, platelets interact with antigen-presenting cells (APCs), such as monocytes and dendritic cells. Platelets also interact with T cells and B cells for the generation of cytokines and antibodies. Meanwhile, platelets also interact with the endothelium to initiate the formation of clots and regulate haemostasis. Adapted from (Yeaman, 2014)[111].

1.4.5 Platelet-associated inflammatory disease; psoriasis

Psoriasis is a chronic inflammatory cutaneous disease affecting 2-4% of the worldwide population and is characterised by hyperproliferation and abnormal differentiation of keratinocytes[112-114]. Psoriatic vulgaris (otherwise known as plaque-like psoriasis) is the most common phenotype of psoriasis and is characterised by increased redness, thickness and scaling of the skin in affected areas throughout the body, all of which are used to assess the activity and severity of psoriasis in clinical practice[115]. The most commonly used tool to assess psoriasis is the Psoriasis Area and Severity Index (PASI) scoring system, which determines therapeutic efficacy[116]. The PASI scoring system is a five-point scale (0-4) that provides a grade of the average erythema (redness), induration (thickness) and desquamation (scaling) of psoriatic plaques in four body regions including head, upper extremities, trunk and lower extremities[117].

Psoriasis fits under the umbrella of autoimmune diseases; it is triggered by an activated cellular immune system in the absence of infection or other stimuli[118]. It was previously believed that the pathomechanisms of psoriasis are limited to keratinocytes hyperplasia. However, the involvement of the immune system was later demonstrated by the accidental observation in 1979, which reported the therapeutic potential of cyclosporin A (CsA) in psoriasis, mainly by inhibiting the production of IL-2 by T-cells, which can stimulate keratinocytes[119]. CsA is an immunosuppressive agent derived from fungus that inhibits the activation and proliferation of T-cells. It acts by binding to cyclophilin, an immunosuppressant-binding protein. This binding inhibits the enzyme calcineurin, which leads to the inhibition of signal transduction pathways that are dependent on the transcription factor, NF-AT (nuclear factor of activated T cells). This ultimately inhibits the production of cytokines such as IL-2 and IFN-γ[120]. The hyperproliferation of keratinocytes in psoriasis is accompanied with the infiltration of inflammatory cells and mediators. Several effector cells were reported to

implicate psoriasis including T-helper cells, dendritic cells (DCs), neutrophils and macrophages. These cells contribute to the exacerbation of disease by releasing inflammatory mediators such as interferon (IFN)-γ, tumour necrosis factor (TNF)-α, interleukin (IL)-12, IL-17A, IL-20, IL-22 and IL-23[112]. This can in turn trigger the production of proinflammatory cytokines (IL-1, IL-6, and TNF-α) and chemokines (IL-8 [CXCL8], CXCL10, and CCL20) from keratinocytes[121].

In addition to the role of inflammatory cells and mediators, AMPs have been shown to play an integral role on the pathogenesis of psoriasis[122]. Keratinocytes are known to provide a rich source of AMPs while expressing more than 20 different types[123], including: cathelicidins, defensins, S100 proteins, lysozyme, RNase 7, elafin, neutrophil gelatinase-associated lipocalin with several others[124, 125]. Among these, the cathelicidin LL-37, human β-defensin (HBD) 2 & 3, and S100A7 protein (otherwise known as psoriasin) have been shown to be widely implicated in the development of psoriasis and highly up-regulated in psoriatic lesions[126]. Notably, LL37 has been highlighted as an immune modulatory peptide in psoriasis[127] and has been shown to be overexpressed with concentrations reaching up to 300 μM in affected skin tissues[128]. HBD2 and HBD3 were demonstrated to be highly expressed in psoriatic lesions. Moreover, high HBD copy numbers were reported in psoriasis[129-131]. This increased expression is induced by inflammatory mediators that are highly expressed in psoriatic lesions, including TNF-α, IFN-γ, IL-17A and IL-22[132, 133]. Similarly, the S100A7 protein is expressed in response to TNF-α, IL-17A, IL-22 and IL-28, and have been thought to play a chemotactic role in psoriasis[134]. Although the overexpression of HBDs has been reported by many groups, their mechanisms of action in psoriasis are not fully understood[128].

Psoriasis has been associated with complications and comorbidities that are related to systemic inflammation and cardiovascular diseases, mainly induced by the inflammatory milieu[135]. Psoriasis patients have increased risk for occlusive vascular disease, such as atherosclerosis, coronary artery disease and cerebrovascular diseases[136-139]. Psoriasis has been

suggested to be an independent risk factor for cardiovascular disease[140]. In 2006, the risk of MI and atherosclerosis has been reported in psoriasis[141-143]. This is mainly due to the activation and hypercoagulability of platelets, which has been shown to contribute to the pathogenesis of psoriasis[144]. Moreover, platelets have been shown to contribute to cutaneous inflammation and consequently atherosclerosis plaques[137, 145, 146].

Platelet indices has been demonstrated as useful indicators for the activation of platelets during psoriasis. In addition to the platelet distribution width (PDW), the mean platelet volume (MPV) have been reported to be increased in psoriasis[147, 148]. This is indicative of the presence of larger platelets, which are associated with increased reactivity, granule release, and GPIb expression. Moreover, these platelets demonstrate augmented collagen-, ADP- or thrombin-induced aggregation[149]. Additionally, the platelet mass index (PMI), formulated as MPV multiplied by platelet count, is elevated in psoriasis[141]. Unlike the MPV and PDW, the PMI not only has been shown to be a useful marker for the activation of platelets but is also indicative of the functionality of platelets; especially that associated with their capability of the formation of atherosclerotic plaques. Because the PMI was shown to be correlated with erythrocyte sedimentation rate (ESR), a marker for inflammation, it is considered as a better predictor of inflammation and atherosclerotic plaque formation in psoriasis compared to the MPV[141, 150, 151]. In addition to the platelet indices, the *in vitro* platelet aggregation was shown to be a useful predictor of platelet activation and was increased in psoriatic patients, wherein thrombin- or ADP-induced platelet aggregation was potentiated compared to the controls[152, 153].

In addition to the aforementioned markers, the elevated plasma levels of platelet inflammatory mediators have been reported in psoriasis. These mediators were shown to exacerbate the disease by mediating the activation and the recruitment of leukocytes to the site of cutaneous inflammation[154, 155]. Moreover, they mediate the leukocyte adhesion to platelets and the endothelium, all of which can contribute to the development of atherosclerosis[72]. These

mediators include soluble P-selectin (sP-selectin) and platelet-derived microparticles (PDMPs), which are also increased in psoriatic patients[96, 138, 156-159]. PDMPs were also shown to facilitate thrombus formation, endothelial dysfunction and atherosclerosis[154, 155]. Furthermore, the plasma levels of chemokines released from platelets, such as platelet factor 4 (PF4) and β-thromboglobulin (β-TG) were increased in psoriasis[160]. The elevated levels of these mediators positively correlated with the PASI scoring and thus disease severity, and their levels were reduced after treatment[96, 137, 161].

In addition to platelet indices and activation, markers of systemic inflammation were also increased, and these include: serum high sensitivity C-reactive protein (hs-CRP)[162] and IL-6[163], which has been shown to induce thrombocytosis and platelet activation[163, 164]. Moreover, IL-6 can perpetuate inflammation by inhibiting the functions of regulatory T cells[164]. These findings indicate that platelet activation and inflammation may perpetuate each other and facilitate the development of cardiovascular risks.

The use of antiplatelet agents for the treatment of psoriasis has not been explored previously. However, several biological drugs including infliximab, adalimumab and etanercept (TNF-α inhibitors), and ustekinumab (IL-12/23 inhibitor) have been associated with a reduction in PASI scoring and P-selectin expression [165]. This suggests a link between inflammation and platelet activation in psoriasis. Conversely, in a case report of cardiovascular and psoriasis comorbidity, the use of clopidogrel (an irreversible P2Y12 inhibitor) was plausibly regarded ineffective as its re-challenge in patients exacerbated and perpetuated psoriasis[166]. Moreover, re-challenge of ticlopidine or aspirin are also known to exacerbate psoriasis. However, dipyridamole, a platelet aggregation inhibitor that causes dilated blood vessels was suggested as an alternative.

1.4.6 Platelet-associated infectious disease; sepsis

Sepsis or septic shocks are characterised by life-threatening organ dysfunction stemming from dysregulated host responses to infection[167, 168]. It is the leading cause of death in intensive care units (ICUs) [169]. Cardiac arrest and organ failure are among the leading causes of death in septic shocks[170, 171]. Reported incidences of sepsis are increasing with age and as a complication of other diseases including diabetes mellitus, cancer, immunosuppression and chronic organ failure[169, 171, 172].

Platelets play a significant role in the pathophysiology of sepsis, and several modes of action have been proposed[173, 174]. Firstly, the activation of platelets[175], which may result in thrombus formation in the vasculature and hypercoagulability of blood[176], which can result in acute disseminated intravascular coagulation (DIC) and localised venous thromboembolism[177]. This consequently leads to the consumption of platelets and coagulation proteins, manifesting a bleeding diathesis in most cases of sepsis[178]. Additionally, it has been shown that systemic microvascular dysfunction due to thrombosis comprises the hallmark of organ damage in sepsis[173]. In addition to thrombosis, infection-related endothelial activation and dysfunction may also contribute to the microvascular dysfunction and leakage of blood[179, 180]. Another mode of action in which platelets may affect the clinical outcomes of sepsis is through the destabilisation of the endothelial barrier. Platelets are a major source of angiopoietin 1 (Ang-1), which stabilises the endothelium and prevents microvascular leakage. In severe cases of sepsis, the plasma and serum levels of Ang-1 are significantly declined[3, 181]. Thus, thrombocytopenia can contribute to the adverse outcomes by decreasing the delivery of Ang-1 to the endothelium.

Secondly, platelets can contribute to the pathophysiology of sepsis by interacting with other immune cells and regulating immune and inflammatory responses. As detailed

previously, platelets can elicit innate immune responses and contribute to clearance of pathogens and tissue repair[66, 68, 76, 182-184]. Additionally, platelets contribute to acute and chronic inflammatory responses, which can result in tissue and vascular injury[66, 185, 186]. All of which, can be mediated by activated platelets and exacerbated in sepsis.

Thirdly, thrombocytopenia is implicated in the pathophysiology of sepsis and can contribute to unwarranted complications and can serve as a prognostic factor for disease severity[187]. Sharma et al. reported a strong association between mortality and thrombocytopenia in 69 patients suffering from septic shock[188]. In addition, Claushuis et al. reported this association in a large cohort consisting of 931 septic patients[3]. The low platelet count in these patients upon their admission to ICUs is associated with elevated plasma levels of cytokines such as interleukin (IL)-6[189], IL-8[190] and IL-10[191], intercellular adhesion molecule 1 (ICAM-1)[192] and the chemokine fractalkine[193]. Additionally, coagulation activity is increased as evidenced by decreased antithrombin[194], and the vascular integrity is reduced as evident by the increased ration of angiopoietin Ang-2 to Ang-1[181]. It has been also shown that thrombocytopenia, independent of disease severity, is associated with increased mortality in sepsis. Thus, identifying the causes of thrombocytopenia is crucial for the management of septic patients[195]. Several causes of thrombocytopenia in sepsis, acting individually or in combination, have been previously reported[174], and include the reduced production of platelets in the bone marrow. This can be due to pre-existing conditions or the inhibition of haematopoiesis in response to drugs, inflammatory mediators or pathogenic toxins. In addition to bone marrow, the platelet count can be compromised in the periphery circulation. The increased consumption of platelets can occur by the activation of platelets and the reduction of their half-life can be triggered by pathogens or pathogenic products. Moreover, pathogen-induced apoptosis, lysis and increased phagocytic clearance of platelets can contribute to their consumption. Platelet consumption and activation can also occur by their sequestration by

leukocytes and inflammatory cells in the vascular bed. Additionally, thrombosis and coagulopathy of blood, such as in DIC, can contribute to thrombocytopenia[174, 196-200]. In addition to the aforementioned causes, the administration of drugs can induce thrombocytopenia, bleeding, hemophagocytosis and hemodilution[195], and can cause defective platelet function[201, 202].

Since platelets play an integral role in the pathophysiology of sepsis, platelet-targeted therapeutic intervention has been proposed[174]. The transfusion of platelets has been proposed to restore the platelet blood count and has been mostly used to prevent or treat bleeding in sepsis[203], whereas it was demonstrated to be ineffective in non-bleeding ICU patients[204, 205]. However, transfused platelets can present detrimental effects to the host by contributing to the inflammation, intravascular platelet activation and coagulopathy effects in sepsis[195, 204, 206]. Moreover, data collected recently from a large transfusion registry identifies platelet transfusion in sepsis as ineffectual since its association with platelet count elevation is inadequate[207]. Hence, the transfusion of platelets can be either deleterious or ineffectual, consequently, presenting a challenge in platelet-targeted therapeutic interventions in sepsis. An otherwise effective alternative strategy for restoring the blood count in sepsis that has been demonstrated recently is the clinical administration of human thrombopoietin, the main regulator of platelet production[208-211]. Conversely, TPO blockade by a chimeric (mTPOR-MBP) has been demonstrated to reduce organ damage severity in two murine experimental models; acute endotoxemia and a polymicrobial sepsis model induced by cecal ligation and puncture (CLP)[210]. Targeting platelet-associated inflammatory modulatory responses, including the inhibition of cytokines such as TNF-α and IL-1[212], platelet activating factors[213, 214], cyclooxygenase (COX)[215] and bradykinins[216] has been shown to be an ineffective therapeutic strategy in sepsis. However, activated protein C (APC) has been shown to be

effective and is approved by the Food and Drug Administration (FDA) for the treatment of human sepsis[217].

In addition to the aforementioned therapeutic strategies, the administration of antiplatelet agents has been proposed for targeting several platelet defects and deleterious pathways in sepsis[174, 218]. In mouse experimental models of acute kidney injury (AKI) and acute lung injury (ALI), the depletion of platelets or the inhibition of platelet function by administration of antiplatelet drugs has demonstrated protective roles[219]. Notably, antiplatelet therapy exhibited a reduced risk of ICU mortality, and such impact has been demonstrated over several cohorts. Notably, the blockade of P2Y12 reduced the pro-inflammatory and pro-thrombotic mechanisms in humans[220]. Additionally, the administration of acetylsalicylic acid (ASA) and GPIIb/IIIa antagonists demonstrated reduced complications and mortality in patients[221]. However, the administration of antiplatelet drugs has its drawbacks and can contribute to resistance in patients and undesirable side effects such as bleeding[222-224]. Moreover, it has been shown that antiplatelet agents demonstrate differential effects in platelets; aspirin-treated platelets can still be activated by potent platelet agonists such as thrombin[174].

1.5 Formyl peptide receptors (FPRs)

1.5.1 FPR family members

Formyl peptide receptors (FPRs) are a group of chemoattractant receptors that belong to a family of G protein-coupled receptors (GPCRs) that are mainly expressed in phagocytic leukocytes and are known to play a pivotal role in the regulation of host defence and inflammation[225]. The expression of these receptors in platelets has been demonstrated previously; the expression of FPR1 at protein level in platelets, and at transcript level in megakaryocytes[71], and the expression of FPR2/ALX in megakaryocytes, and human and mouse platelets at transcript level has been reported previously[71,226]. FPRs are so called because they were originally discovered by their ability to recognise peptides bearing *N*-formyl methionine (fMet), such as those derived from mitochondrial or bacterial proteins. Hence, these receptors are known as pattern recognition receptors with the ability to bind pathogen-associated molecular patterns (PAMPs) derived from bacteria, or damage-associated molecular patterns (DAMPs) derived from the mitochondria of damaged cells[227-229]. Nonetheless, it was later discovered that FPR ligands are not only limited to *N*-formyl peptides, but they bind numerous chemically- and structurally-diverse ligands, including non-formyl peptides, synthetic peptides, non-peptides including glycoproteins, lipids and eicosanoids such as lipoxin A4 (LXA4).

1.5.1.1 Human FPRs

The FPRs family is encoded by three genes in humans and comprises three members; FPR1, FPR2/ALX and FPR3[230]. FPR2/ALX is called so to convey its ability to interact with Lipoxin A4 and aspirin-triggered lipoxins[231]. FPR1 and FPR2/ALX share overlapping functions and significant sequence homology (69 % amino acid identity)[232], whereas FPR3 shares 58 % and 72 % identity with FPR1 and FPR2, respectively[233]. Unlike FPR1 and

FPR2/ALX which are expressed in both neutrophils and monocytes, FPR3 is only expressed in monocytes. Besides myeloid cells, FPR1 is expressed in astrocytes, microglial cells, hepatocytes and immature dendritic cells. In contrast to FPR1, FPR2/ALX displays a broader expression pattern and is found in non-myeloid cells including epithelial cells, hepatocytes, microvascular endothelial cells, astrocytoma cells, neuroblastoma cells, in addition to phagocytic leukocytes. The expression of these receptors in a variety of cells other than phagocytic cells implies their ability to activate functions beyond that of host defence.

While FPR1 binds to bacterial-derived *N*-formyl peptides with high affinity, FPR2/ALX displays low affinity to these peptides and largely binds to mitochondrial-derived formyl peptides[234, 235]. Whereas FPR3 does not bind *N*-formyl peptides[233]. Moreover, studies have shown that long amphipathic α-helical *N*-formyl peptides favour FPR2/ALX over FPR1 binding[236, 237]. The ligation of *N*-formyl peptides to FPRs in immune cells triggers a range of signalling cascades resulting in numerous biological responses including chemotaxis, degranulation, production of superoxide anions[225, 238], calcium mobilisation[239], lysosomal enzyme[240] and cytokine release, and expression of various surface markers[241, 242]. Due to their ubiquitous expression and their interaction with a wide variety of ligands, FPRs have been implicated in various pathological conditions. Notably, FPR1 is largely implicated in infectious inflammation and metastasis, while FPR2/ALX has been implicated in chronic inflammatory conditions including atherosclerosis, systemic lupus erythematous and cancer metastasis[243-246].

1.5.1.2 Mouse FPRs

Several orthologs of the human FPR1 gene (*FPR1*) have been identified in other mammalian species including mice, rabbits, horses and guinea pigs[234]. Although they share an overall sequence homology, the genes between humans and other mammalian species vary significantly in their number and sequences. Notably, the murine FPR gene family comprises at least eight members including *Fpr1*, *Fpr2* (also known as formyl peptide receptor, related

sequence 2 [*Fpr-rs2*]), *Fpr3* (also known as *Fpr-rs1*), *Fpr-rs3*, *Fpr-rs4*, *Fpr-rs5*, *Fpr-rs6*, *Fpr-rs7* and *Fpr-rs8*. The Fpr1, Fpr2 and Fpr3 genes code for receptors expressed in phagocytic leukocytes and display similarity to their human counterparts[247]. The gene product of *Fpr1* is the mouse ortholog of human FPR1. The human *FPR2/ALX* corresponds to two genes; *Fpr2* and *Fpr3* in mice, thus *Fpr2/3* is the mouse ortholog to human FPR2/ALX[234, 247-249].

While *Fpr1* displays low-affinity binding to the tri-peptide *N*-formyl-methionyl-leucyl-phenylalanine (fMLF)[248, 250], it shows higher affinity to several other *N*-formyl peptides, including bacterial fMIFL, fMIVIL and fMIVTLF, and mitochondrial fMMYALF[247, 250]. In contrast, *Fpr2* has been found to be less responsive to fMLF and fMIFL[247], but serves as a receptor for endogenous peptide agonists for FPR2/ALX, including amyloidogenic proteins SAA (serum amyloid A)[251] and $A\beta_{42}$[252], as well as F2l, which is a highly potent agonist for human FPR3[253]. Both *Fpr2* and *Fpr3* respond to Lipoxin A4 (LXA4) and aspirin-triggered lipoxin A4 (ALX)[254, 255].

The targeted deletion of *Fpr1* or *Fpr2* renders mice more susceptible to bacterial infection. Notably, neutrophils obtained from *Fpr1*-deficient mice displayed increased susceptibility to *Listeria* infection, suggesting a pivotal role for FPRs in host defence against bacterial infection[256, 257]. Moreover, *Fpr1*- and *Fpr2*-deficient mice displayed increased inflammatory responses and severe liver injury after stimulation with LPS[258, 259]. These mice were shown to behave normally under unstimulated settings. However, in several models of human diseases, both $Fpr1^{-/-}$ and $Fpr2^{-/-}$ mice respond differently compared with wildtype mice, demonstrating a regulatory role for these receptors in host defence and inflammation. The deletion of *Fpr2/3* has been associated with dysregulated responses. *Fpr2/3*-deficient mice displayed exacerbated inflammatory responses following cerebral ischaemia/reperfusion (I/R) injury, where it has been shown to mediate neutrophil-platelet aggregation[260, 261]. Furthermore,

in an experimental polymicrobial sepsis model, *Fpr2/3*-deficient mice displayed aggravated host responses, exacerbated disease severity, and myocardial dysfunction[249].

1.5.2 FPR signalling

The activation of FPRs induces a variety of responses, including chemotaxis, degranulation, and production of superoxide anion[225, 238] and lysosomal enzyme release[240]. FPRs belong to the Gi subfamily of heterotrimeric GPCRs. This was evidenced by their ability to mediate cellular responses in a pertussin toxin (PTX)-sensitive manner, where the major fMLF-stimulated functions in neutrophils were largely inhibited following the treatment of cells with PTX[262-264]. Upon agonist binding, Gi protein coupled to FPRs undergo conversion of guanosine diphosphate (GDP) to guanosine triphosphate (GTP)[65]. This induces their dissociation into α and βγ subunits, which leads to a series of signal transduction cascades[263]. This leads to the activation of the phospholipase $C\beta_2$ ($PLC\beta_2$)[265] and the phosphoinositide 3-kinase γ (PI3Kγ)[266] signalling cascades. $PLC\beta_2$ hydrolyses plasma membrane-bound phosphoinositol-4,5-bisphosphate (PIP_2) into diacylglycerol (DAG) and inositol trisphosphate (IP_3). This mediates the release of calcium from the intracellular calcium stores, mainly from the endoplasmic reticulum. The interplay of calcium and DAG subsequently leads to the activation of protein kinase C (PKC) and is crucial for the regulation of reactive oxygen species (ROS) production and NADPH oxidase.[54] Meanwhile, PI3Kγ triggers protein kinase B (PKB, also known as Akt) signalling and facilitates the conversion of PIP_2 to phosphoinositol-3,4,5-trisphosphate (PIP_3), which mediates superoxide generation[267], cytoskeletal reorganization, oxidative burst and chemotaxis in neutrophils[10, 47268, 269]. The subsequent activation of several kinases occurs, including extracellular signal-regulated kinases (ERK) and p38 mitogen-activated protein kinase (MAPK) signalling pathways.

Many cellular functions, including chemotactic responses in neutrophils, require a transient rise in intracellular calcium[270]. Recent studies indicate that chemotaxis and calcium responses in FPRs are mediated by CD38, a transmembrane glycoprotein, which catalyses the production of cyclic ADP-ribose (cADPR) from its substrate NAD^+ [271]. cADPR has been described as a regulator of calcium signalling and acts at ryanodine receptors to release calcium ions (Ca^{2+}) from intracellular stores and results in a sustained influx of extracellular Ca^{2+} required for fMLF-induced neutrophil migration[272-274]. This was evidenced by the failure of neutrophils obtained from CD38-deficient mice to migrate in response to fMLF *in vitro* and fail to accumulate at sites of *Streptococcus pneumoniae* infection *in vivo* [274]. Moreover, the ability of fMLF to induce calcium mobilisation through FPR1 in platelets has been previously reported[71]. The mobilisation of calcium in platelets is implicated with activatory responses, including the activation of integrin αIIbβ3 and granule release[275].

The activation of G_i protein-coupled receptors are known to inhibit adenylate cyclase [276] and thus, reduce the levels of cyclic adenosine monophosphate (cAMP). In addition to the aforementioned signalling pathways, the production of cAMP is involved in the regulation of chemotaxis[277]. Several studies have shown that elevation of cAMP levels inhibits chemotaxis in neutrophils[278-281]. The main target for cAMP is protein kinase A (PKA), which can regulate various signalling components, including Rho and vasodilator-stimulated phosphoprotein (VASP)[282]. cAMP signalling in platelets play a crucial role in the regulation of their function and is associated with the inhibition of platelet aggregation[283].

1.5.3 FPR ligands

FPRs are known to recognise a wide variety of ligands with diverse chemical and structural properties and can exert both pro- and anti-inflammatory responses. These ligands include formyl and non-formyl peptides, and nonpeptides such as proteins and lipids. The peptides can be categorised into three subtypes; pathogen-derived, host-derived and synthetic. The nonpeptides can either be synthetic or host derived[234].

1.5.3.1 Agonists

Bacterial-derived *N*-formyl peptides are considered as a class of pathogen-associated molecular patterns (PAMPs) as the bacterial protein synthesis is initiated with *N*-formyl methionine (fMet)[284]. These PAMPs are recognised by specialised receptors on the innate immune cells, including TLRs and FPRs[229]. The *E. coli*-derived tripeptide fMLF was one of the first characterised chemotactic formyl peptide and is the most frequently used to study neutrophil functions. It is the smallest *N*-formyl peptide that displays potent agonistic activities towards FPRs. In addition to its chemotactic responses in leukocytes, fMLF can induce intestinal epithelial cell migration and can regulate epithelial restitution and wound healing. These responses are mediated through FRP1. *N*-formyl peptides that derived from other bacterial strains have been identified, and these include pentapeptide fMIVIL from *Listeria monocytogenes* and a tetrapeptide fMIFL derived from *Staphylococcus aureus* and *Mycobacterium avium*-derived peptide fMFEDAVAWF[285]. Several other pathogen-derived nonformyl peptides have been identified to act upon FPRs. For instance, Hp (2–20), a peptide derived from *Helicobacter pylori*, has been shown to induce proliferation and the expression of vascular endothelial growth factor (VEGF) in gastric epithelial cells, suggesting that this *H. pylori* peptide may promote gastric mucosal healing[234]. Furthermore, it has been shown that HIV-1 envelope proteins can interact with FPRs[286]. Other peptides from viral strains have been

identified to interact with FPRs, including gG-2p20, which is derived from *Herpes simplex* virus type 2 (HSV-2)[234].

In addition to pathogen-derived peptides, FPRs can bind a variety of host-derived peptides. These include mitochondria-derived *N*-formyl peptides that can attract leukocytes to sites of inflammation and tissue damage. Among these, the *N*-formylated hexapeptides corresponding to the N terminus of mitochondrial NADH dehydrogenase subunits 4 (fMLKLIV) and 6 (fMMYALF), and cytochrome *c* oxidase subunit I (fMFADRW). All of which can act on both FPR1 and FPR2/ALX while fMMYALF can also act on FPR3[235]. Moreover, fMYFINILTL and its nonformylated fragment (MYFINILTL) that derived from mouse NADH dehydrogenase subunit 1 act on FPR2/ALX[287].

Another class of host-derived peptides that act on FPRs includes endogenous peptides associated with inflammatory and bacterial responses. These include the human cathelicidin antimicrobial peptide LL37 (LLGDFFRKSKEKIGKEFKRIVQRIKDFLRNLVPRTES), which is expressed in human neutrophils and derived from the enzymatic cleavage of its pro-form the human cationic antibacterial protein of 18 kDa (hCAP-18). In addition to its bactericidal activities, LL37 activates FPR2/ALX to promote chemotaxis in leukocytes and angiogenesis on endothelial cells[234]. This was evidenced by studies using CRAMP-deficient mice, wherein neovascularization was compromised during wound repair. Another antibacterial protein involved with FPR1 signalling is cathepsin G, which is a serine protease found in neutrophil granules and mediates wound healing[234]. These antimicrobial peptides are released upon cellular stimulation and aid in the recruitment of phagocytic leukocytes to the sites of infection and mediate the killing and clearance of the invading bacteria. In addition to these antimicrobial peptides, FPRs can bind peptides that play a role in the resolution of inflammation. Namely, annexin A1 (Anx A1, also called lipocortin I) and its terminal peptides

are also known to act through FPRs. Anx A1 is a glucocorticoid-regulated protein known to exert both pro- and anti-inflammatory activities through FPRs. It is abundantly expressed in neutrophils and plays a prominent role in the resolution of inflammation as it has been shown to induce neutrophil detachment and apoptosis[288].

Several other host-derived peptides have been shown to act through FPR2/ALX, some of which are associated with amyloidogenic diseases and chronic inflammation, including serum amyloid A (SAA), β amyloid peptide Aβ42, and PrP106–126. SAA is an acute-phase protein and its increase has been associated with inflammatory disease such as rheumatoid arthritis. Aβ42 is a 42-amino acid cleavage product of the amyloid precursor protein in the brain and is implicated in Alzheimer's disease. PrP106–126 is a protein fragment produced in the brain during prion disease, which can activate leukocytes through FPR2/ALX and thus contribute to inflammatory response during disease. Another endogenous host-derived peptide that can act upon FPRs is humanin (MAPRGFSCLLLLTSEIDLPVKRRA) which exerts neuroprotective activity[289] and binds to FPR2/ALX and FPR3[290]. It has been shown that the *N*-formylated humanin is more potent than the nonformylated form, demonstrating the significance of *N*-formylation in enhancing agonistic activity[291].

Several synthetic peptides that are potent chemotactic agents for leukocytes have been identified to act through FPRs. Although physiologically-irrelevant, these peptides comprise beneficial pharmacological agents for the characterization of FPRs, the investigation of their signalling pathways, and for the development of potential therapeutic agents. Among these, a hexapeptide Trp-Lys-Tyr-Met-Val-d-Met-NH_2 (WKYMVm)[292], which has been shown to stimulate lymphocytes, monocytes and neutrophils and can act on FPR1 and FPR2/ALX to a higher affinity, rendering it its most potent agonist[293]. In addition, MMK-1 (LESIFRSLLFRVM) peptide exerts potent agonistic activity towards FPR2/ALX

selectively[294, 295]. The peptide MMWLL (Met-Met-Trp-Leu-Leu) is also identified as a potent FPR1 agonist, which is 1000-fold more potent upon *N*-formylation[296]. This demonstrates the preferential recognition of *N*-formylmethionine-containing peptides by FPR1. Several synthetic nonpeptide compounds that act through FPRs have been identified. Among these, is a quinazolinone derivative known as Quin-C1 (4-butoxy-*N*-[2-(4-methoxy-phenyl)-4-oxo-1,4-dihydro-2*H*-quinazolin-3-yl]-benzamide) is a highly selective agonist for FPR2/ALX and induces chemotaxis and calcium mobilization in neutrophils[297]. Additionally, a series of compounds were shown to exert anti-inflammatory properties through FPR2/ALX, including the two pyrazolones (Compound 24 and 43)[298]. Other synthetic nonpeptide compounds that act through FPR2/ALX include Compounds 1 and 2, and arylcarboxylic acid hydrazide derivatives, which were shown to induce the production of TNFα by macrophages[299]. Nonpeptides that act through FPR1 have been also identified including AG-14, which has been shown to activate neutrophils at nanomolar concentration[300].

One of the first described lipid ligands for FPR2/ALX is the endogenous lipoxin A4 (LXA4) (5*S*,6*R*,15*S*-trihydroxy-7,9,13-*trans*-11-eicosatetraenoic acid) which is biosynthesised from arachidonic acid and is shown to exert anti-inflammatory and pro-resolving activities in various inflammatory cells[301]. LXA4 acts through FPR2/ALX to induce apoptosis and phagocytosis[302], attenuate neutrophil activity[303], inhibit production of pro-inflammatory cytokines[304], promote detachment of adherent leukocytes[305] and inhibits neutrophil infiltration [255, 306, 307]. Furthermore, LXA4 has been shown to reduce inflammation-induced pain in various pathological conditions, including ischaemia/reperfusion injury, dermal cystic fibrosis, inflammation, infections and colitis[308].

1.5.3.2 Antagonists for FPRs

The pivotal roles of FPRs in the regulation of microbial infections, host immune defence and inflammatory responses suggest that targeting such receptors may attenuate complications associated with diseases where FPRs play critical roles. Several antagonists for FPRs have been identified and include pathogen-derived, host-derived and synthetic compounds. Among these is the fungus-derived inverse agonists cyclosporin H (CsH) and A (CsA), which were shown to suppress fMLF-induced neutrophil stimulation activities through FPR1[225, 309, 310]. Additionally, FPR2/ALX-inhibitory protein (FLIPr) derived from *Staphylococcus aureus* directly binds both FPR1 and FPR2/ALX and inhibits fMLF-induced cell activation[274]. Additional pathogen-derived peptides that exert antagonistic activity for FPR1 include bacteria-derived chemotaxis inhibitory protein of *S. aureus* (CHIPS), and retrovirus-derived immunosuppressive hexapeptide LDLLDL[311]. The release of peptides by pathogens that exhibits antagonistic activities reveals mechanisms by which these pathogens may invade host defence. Host-derived endogenous antagonists for FPR1 have been identified and these include the bile acids deoxycholic acid (DCA) and chenodeoxycholic acid (CDCA) and the opioid spinorphin (LVVYPWT)[312-315].

In addition to pathogen- and host-derived antagonists for FPRs, several synthetic compounds have been identified. Previous studies have indicated that the replacement of the *N*-formyl group of fMLF with tertiary butyloxycarbonyl group (*t*-Boc) converts the peptide into an antagonist[316]. Both *t*-Boc-Met-Leu-Phe (Boc-1) and *t*-Boc-Phe-d-Leu-Phe-d-Leu-Phe (Boc-2) were originally demonstrated as selective antagonists for FPR1 when used at low concentrations[316]. However, several studies have demonstrated an inhibitory response of Boc-2 to FPR2/ALX when used at high concentrations (100 μM)[305, 317-320]. It was later demonstrated that Boc-2 is a pan-antagonist that partially inhibits FPR2/ALX at high micromolar concentrations in addition to FPR1[321]. A more selective antagonist for FPR2/ALX was later

identified, this includes the peptide WRWWWW (WRW4)[321, 322]. In addition to WRW4, the cell-permeable antagonist PBP10, which is derived from a PIP2-binding domain of the cytoskeleton protein gelsolin, demonstrates FPR2/ALX antagonistic activity by acting on the intracellular domains in the receptor. Quin-C7, which is developed through chemical modification of the FPR2/ALX agonist Quin-C1, is also identified as a highly selective FPR2/ALX antagonist[323].

1.5.4 Role of FPRs in health and disease

Given the ubiquitous expression of FPRs and their interaction with a wide variety of ligands, they have been associated in various pathological conditions, including acute and chronic inflammation, ischaemia–reperfusion injury[324], atherosclerosis[325], inflammatory bowel disease[326], neurodegenerative disease[327], diabetes and cancer[244, 328, 329].

FPRs play indispensable roles in the regulation of inflammation and resolution. This is evidenced by findings on *Fpr1*-deficient mice, wherein exacerbated infection and increased mortality were observed in these mice upon bacterial infection[256, 257]. Moreover, *Fpr2/3* deficiency caused an increase in inflammatory responses in multiple mouse inflammation models[259]. During acute inflammation, several inflammatory responses including adhesion and cellular transmigration are regulated by FPRs[330, 331]. FPR1 exerts antimicrobial responses upon binding with pathogen-derived peptides, such as the bacterial chemotactic peptide fMLF[240, 332]. Moreover, the endogenous antimicrobial peptide, LL37, is released from host cells and can interact with FPR2/ALX[333]. The dysregulated expression of these peptides has been associated with many pathological conditions, including the overexpression of LL37 in chronic skin inflammatory disease, such as psoriasis and the overexpression of fMLF in inflammatory bowel disease and metabolic diseases. Additionally, annexin A1 (ANXA1), which can exhibit both pro- and anti-inflammatory roles can interact with both FPR1 and FPR2/ALX to exert its

functions and has been associated with chronic inflammation[334, 335]. Another class of ligands that can interact with FPR2/ALX are lipoxins and resolvins, which play indispensable roles in the resolution of inflammation and anti-inflammatory responses[336]. Taken together, these demonstrate the critical role of FPRs in the regulation of inflammation and resolution, which can progress into pathophysiological responses if not regulated properly.

FPRs are also associated with neurodegenerative diseases and amyloidosis. FPR2/ALX interacts with amyloid-related peptides such as SAA, Aβ42, and PrP106–126. These peptides are elevated in inflammatory settings and can exacerbate inflammation and lead to organ damage. This may also contribute to the progression of Alzheimer's disease. Additionally, humanin has been shown to interact with FPR2/ALX to exert neuroprotective roles by competing with Aβ42 for binding to FPRs and inhibiting their effects[290, 291]. Moreover, FPR2/ALX has been associated with the progression of inflammation in prion disease[337].

FPRs were also shown to be implicated in acquired immune deficiency syndrome (AIDS), wherein the recombinant HIV-1 gp120 and gp41 have been shown to suppress the expression and function of FPRs[338, 339]. It has been shown that FPR ligands such as SAA and fMLF can significantly reduce the fusion and infection of HIV-1[340]. Thus, it has been suggested that FPR ligands may be useful for the development of anti-HIV-1 agents. In addition, FPRs have been implicated in metabolic disorders, including diabetes and obesity. It has been shown that the elevated levels of fMLF can contribute to glucose intolerance and insulin resistance via FPR1[341]. On the other hand, it has been shown that RvD1 via FPR2/ALX can enhances glucose tolerance, restore systemic insulin sensitivity and prevent macrophage accumulation in adipose tissue in obesity-induced diabetes animal model[342].

Inflammation is closely associated with cancer. Many inflammatory cells are involved in the regulation of tumour cell proliferation, metastasis and survival[343]. FPRs have been shown to play a role in the tumour growth and the progression of angiogenesis[225, 344]. In particular, ANXA1 was found to promote the growth of glioblastoma via the activation of FPR1[345]. Additionally, acute-phase reactant SAA and antimicrobial LL37[346] have been implicated in tumourigenesis[347-349]. On the contrary, LXA_4 and its analogue, BML-111, have been shown to exert anti-tumour effects[350-352]. Studies have found that these compounds can inhibit VEGF accumulation, tumour growth and angiogenesis and promoted apoptosis of tumours *in vivo*[353, 354]. Collectively, these findings uncover novel therapeutic strategies for the treatment of a broad spectrum of diseases associated with FPRs.

1.6 Bacterial peptide fMLF

1.6.1 Definition and function

N-formyl peptides are produced from the degradation of bacterial or mitochondrial proteins[355]. While FPR1 binds with high affinity to bacterial-derived *N*-formyl peptides, FPR2/ALX binds those derived from mitochondria[234, 235]. The ligation of *N*-formyl peptides to FPRs in immune cells triggers a cascade of signalling pathways, resulting in a number of biological activities. *Escherichia coli*-derived fMLF is one of the most potent chemotactic peptides that facilitates innate immune responses against bacteria via FPR1 signalling[241, 242, 356]. During infection, invading bacteria produce *N*-formyl peptides that facilitate the recruitment of immune cells to the site of bacterial infection and tissue damage, expediting rapid clearance of microbial infection and tissue damage repair[357]. fMLF is known to act mainly through FPR1 in immune cells[234]. The stimulation of neutrophils with fMLF induces a wide variety of cellular responses leading to cell migration and chemotaxis, superoxide anion generation and granule secretion[358, 359]. This triggers the release of inflammatory mediators

including the platelet-activating factor (PAF) Acetyl-glyceryl-ether-phosphoryl-choline (AGEPC)[360], proteases such as cathepsin G[361] and ROS [362], which mediate pathogen killing but can ultimately cause severe tissue injury at the site of infection[363]. Apart from its stimulatory role in neutrophils, fMLF has been shown to induce platelet chemotaxis through FPR1 to mediate innate immune responses[71].

1.6.2 Role of fMLF in disease

Despite the ability of fMLF to mediate innate immune responses, it has been associated with the pathogenesis of microbial infection and inflammatory conditions. It has been implicated in bacterial cystitis[364], pneumococcal pneumonia[365], inflammatory bowel diseases (IBDs)[366], pouchitis, colitis[367] and juvenile peridontitis[368]. Furthermore, it has been shown that the inhalation or injection of fMLF may cause bronchial inflammation[369], and induce rapid neutropenia, increasing susceptibility to infections[370]. Since the intestinal microbiota is a major source of enteric *E.coli*, and the disruption of its homeostasis has been widely implicated in the pathogenesis of IBDs, where the effect of fMLF in exacerbating the progression of this disease has been reported[356]. Moreover, fMLF (at concentrations of 1 nM to 1 μM) induced spasmogenic effects, which can contribute to abdominal cramps. The concentrations of fMLF in intestinal milieu has been reported to be at least in the micromolar range[371]. The implication of fMLF in glucose intolerance was also observed, wherein high-fat diet-fed mice displayed elevated plasma levels of circulating fMLF, sourced from enteric bacteria, which impaired glucose tolerance and insulin secretion[341]. Many of these infectious and inflammatory conditions are associated with a risk for platelet reactivity and thrombotic events that can contribute to cardiovascular complications[372-374].

1.6.3 Emerging role for fMLF in platelet function

A role for fMLF, albeit indirect, on the regulation of the haemostatic platelet function has been previously explored. fMLF has been shown to induce the upregulation of adhesion molecules[375] and severe cell aggregation in neutrophils[376]. This in turn may activate platelets and facilitates their adhesion to leukocytes and the endothelium within the blood vessel wall. The interaction between platelets and leukocytes can lead to the formation of heterotypic aggregates with leukocytes, known as platelet-leukocyte aggregates (PLAs), and may modulate their functions[81]. PLAs are mainly driven by the ligation of P-selectin on the surface of activated platelets with P-selectin glycoprotein ligand-1 (PSGL-1) on the leukocyte surface[377]. Other modes of interaction between platelets and leukocytes have been suggested and include platelet integrin αIIbβ3 binding to the neutrophil Mac-1 via fibrinogen as well as direct binding of Mac-1 on neutrophils to platelet GPIbα. These interactions consequently trigger signals that amplify prothrombotic and proinflammatory responses[378]. In line with this, fMLF has been shown to induce PLA formation[379], and several reports indicate that this is driven by linking via fibrinogen (as evidenced by the blockade of αIIbβ3[380]) and P-selectin[381]. Moreover, previous studies have reported the aggregation of platelets in response to fMLF-induced neutrophil stimulation[382-384]. These findings were attributed to the release of mediators such as platelet-activating factors (PAFs)[385] and cathepsin G[361, 386], consequential to fMLF-induced neutrophil stimulation, that can directly activate platelets and subsequently induce their aggregation[380, 384].

1.7 Antimicrobial peptide LL37

1.7.1 Definition and function

Cathelicidins are a group of endogenous cationic peptides ranging from 12-97 amino acids. They are also referred to as host defence peptides (HDPs) or AMPs, and play a crucial role in innate immune defence. They exhibit various functions including direct antimicrobial activity against bacteria, viruses, fungi, and parasites. In addition to anti-inflammatory activities, they exert immunomodulatory responses. Cathelicidins are widely distributed in both invertebrates and vertebrates, and are characterised by their pre-pro peptide sequences. All cathelicidins have a highly homologous prosequence at their N-terminus (a cathelin-like domain) followed by a highly variable C-terminal mature peptide. They are paradoxically termed so because their N-terminal signal peptide (a highly conserved prosequence) is homologous to that of cathelin, a 96-amino acid isolated from porcine neutrophils that acts as a cathepsin L protease inhibitor[387-389]. It was later shown that cathelidicins do not exert protease inhibitory roles but display anti-infective roles[390].

In order to be biologically active, cathelicidins need to undergo proteolytic processing that leads to the liberation of the mature peptide. They are initially synthetized as pre-propeptides containing the signal sequence, cathelin domain and the mature peptide. The signal sequence transports the propeptide to the granules or cell membranes. In neutrophils, the pre-propeptides are inactive and are stored at high concentrations in the azurophilic granules. Upon the activation of neutrophils, it undergoes proteolytic processing by appropriate proteases to liberate a biologically active, mature peptide[391]. This proteolytic cleavage is processed by serine proteases such as elastases, proteinase 3 and kallikreins[392]. Once the mature peptide is released, it becomes resistant to proteolytic degradation[391]. Cathelicidins are widely distributed in a variety of tissues and cells including skin, intestine, lungs, mucosa and oral cavity. They

are also largely found in the unprocessed form (pre-pro) in the granules of neutrophils as well as epithelial cells, mast cells, keratinocyte and lymphocytes. In their processed forms, they are found in various body fluids, including plasma, saliva, sweat, gastric juice, breast milk, semen and airway surface liquid[393, 394].

1.7.2 Human Cathelicidins

The only cathelicidin expressed by humans is the human cationic antimicrobial peptide of 18 kDa (hCAP18), which is encoded by the CAMP gene (chromosome 3p21.3)[388, 395]. The expression of hCAP18 is transcriptionally controlled by inflammation, Vitamin D metabolites, and endoplasmic reticulum stress via NF-κB activation[396]. Once the pre-propeptide is translated, it undergoes cleavage of the signaling sequence, resulting in the mature hCAP18 form. This form is then further processed by proteolytic enzymes (by proteinase 3 in neutrophils and kallikreins in keratinocytes) to liberate the mature, biologically active peptide LL37 (LLGDFFRKSKEKIGKEFKRIVQRIKDFLRNLVPRTES), which is named to its 37 amino acid length and two leading leucine residues[397-399]. The α-helical structure of the peptide assembles into dimers or trimers, thus avoiding peptide degradation[400]. It facilitates the penetration and destabilisation of bacterial membranes, and generation of transmembrane pores[401-404]. Moreover, LL37 neutralises endotoxin by direct binding, and exhibits chemotactic activity for human neutrophils, monocytes, and resting T cells mediated by FPR2/ALX[333].

LL37 is expressed in various immune cells, including neutrophils, macrophages, dendritic cells, NK cells and epithelial cells. It is secreted in response to infection, pro-inflammatory stimuli and/or injury in keratinocytes[126]. The dysregulated expression of LL37 has been implicated in various pathological conditions. For instance, LL37 is upregulated in chronic inflammatory diseases, including Crohn's disease[405, 406], rheumatoid arthritis[407], psoriasis[408] and eczema[409]. Its downregulation has been implicated in enteric infection[410], acute

myeloid leukaemia[411, 412], atopic dermatitis[128, 413] and chronic epithelial ulcers[414]. Additionally, patients suffering from Kostmann disease, an autosomal recessive disorder characterized by severe neutropenia and early onset of bacterial infections, report deficiency in LL37[415].

1.7.3 Mouse Cathelicidins

Similar to humans, mice solely express one cathelicidin, which is encoded by the Camp gene (chromosome 9) and shares 80% sequence homology with its human orthologue hCAP18. The mouse cathelin-related antimicrobial peptide (mCRAMP), a 34 amino acid (GLLRKGGEKIGEKLKKIGQKIKNFFQKLVPQPEQ)[415], is processed to release an active 5 kDa amphipathic peptide compromised of two α-helices that are joined by a flexible region. Unlike hCAP18, mCRAMP is not transcriptionally regulated by vitamin D metabolites. Despite differences in peptide sequence, mCRAMP exhibits significant similarities to human LL37, which renders it a useful model to investigate the function and regulation of human cathelicidins[416].

Several studies supported by CRAMP-deficient mice demonstrate the pivotal role of cathelicidins in bacterial defence[417-420]. These mice were more susceptible to bacterial and viral infection, *including P.aeruginosa, E.coli, Citobacter rodentium, Klebsiella pneumonia,* vaccinia virus (VV) and influenza (IAV), and are prone to increased disease severity[419, 421-425]. Similarly, these mice have increased colonisation and invasion of pathogenic bacteria in the colon[421] and are more susceptible to urinary tract infections[419]. Moreover, these mice display decreased vascularisation during wound repair, this indicates that cathelicidin-mediated angiogenesis is important for cutaneous wound neovascularisation *in vivo* [426].

1.7.4 Role of LL37 in disease

LL37 has been implicated in various pathological conditions, including inflammatory bowel disease, infectious and sterile inflammation, inflammatory skin disease, autoimmune disease, atherosclerosis[427]. Given the recent advances in understanding their role in multiple pathological conditions, cathelicidins can act as potential targets for the development of therapeutic strategies. The therapeutic potential of cathelicidins against infections have been explored[428]. LL37 demonstrates a promising target for the development of therapeutic agents as a substitute to systemic antibiotics[429]. Additionally, cathelicidin derivates were shown to not only provide improved therapeutic potential, but also eliminate the undesirable cytotoxic effects associated with natural cathelicidins. For instance, Omiganan, a bovine cathelicidin derivative, has been found greatly effective against pathological inflammatory conditions and infections in clinical trials[394]. In addition to infections, the utilisation of cathelicidins in wound healing has been explored. The application of cathelicidins have been shown to promote angiogenesis and re-epithelialisation and angiogenesis in gastrointestinal disorders[430]. In addition, a novel use of cathelicidin as vaccine adjuvants has been proposed[394]. LL37 fragments has also been proposed for the development of anticancer drugs since it can suppress tumour growth in gastric cancers[431]. The evolving role of LL37 in multiple autoimmune diseases, renders it a potential target for the modulation of immune responses. Given the roles of LL37 on TLR signalling, it may provide a therapeutic strategy in pathological conditions associated with such signalling pathways. In particular, the neutralisation of TLR signalling was an effective strategy in diseases such as sepsis, colitis, psoriasis and chronic pain[432]. In addition to the development of therapeutic agents, cathelicidins can act as biomarkers for disease. LL37 may serve as a biomarker for inflammatory conditions such as inflammatory skin disease and chronic pulmonary disease[409, 433].

1.7.5 Role of LL37 in psoriasis

As described previously, psoriasis is associated with the hyperproliferation of keratinocytes, inflammation of skin, increased cytokine production including type I interferons and the overexpression of AMPs including LL37[112, 434]. The upregulation of LL37 expression was first reported in 1997, wherein it was suggested that this induction is associated with a protective role in disease and that it plays a role in antimicrobial defence[435]. Contrasting to normal skin or skin from other inflammatory skin diseases, psoriatic lesions express cathelicidin in the form of LL37 exclusively[436]. Moreover, the level of LL37 in normal skin was minimal compared of that to inflamed skin from rosacea patients[437]. The normal skin mostly expresses cathelicidins (not in the form of LL37) at low levels. In inflammatory skin disease such as rosacea, the level of cathelicidin increases and is usually associated with different forms of cathelicidin[437]. Whereas in psoriasis, cathelicidins are upregulated and are expressed exclusively in the form of LL37. Another study has suggested that the increased upregulation of LL37 in psoriatic lesions is associated with low rate infection, where patients were found to be more susceptible to bacterial and viral infections due to the dysregulated expression of AMPs[128]. It was later proposed that LL37 plays an immune-modulatory role in psoriasis, where it was shown to modulate inflammation by directly activating plasmacytoid dendritic cells (pDCs) and myeloid dendritic cells (mDCs) and forming self-DNA and -RNA complexes, which leads to the activation of TLR8 in mDCs, and TLR7 and TLR9 in pDMs[127, 438] . The activation of TLR9 in turn leads to the overproduction of type I interferons (IFN-α and IFN-β)[112]. Moreover, it has been shown that LL37 reduces apoptosis in keratinocytes, which may also contribute to their hyperproliferation in psoriasis and exacerbation of inflammatory responses[439]. Serum levels of LL37 are also shown to be higher than normal in psoriatic patients and were reduced upon treatment with CsA[440].

1.7.6 Role of LL37 in atherosclerosis

In addition to psoriasis, the role of LL37 has been implicated in atherosclerosis, which is associated with innate immune responses including upregulation of type I interferons, which contribute to the development of atherosclerotic plaques and disease pathogenesis[441-444]. Similar to psoriasis, the upregulation of LL37 contributes to the development of atherosclerotic lesions in atherosclerosis[445]. It has been shown that LL37 plays a key role in the modulation of cytokines and the recruitment of inflammatory cells to atherosclerotic plaques. Notably, LL37 promotes the production of type I interferon[445]. Furthermore, atherosclerotic aortas in humans demonstrate increased transcription of LL37[446]. This was also associated with increased apoptosis of vascular smooth muscle cells in humans[447]. The association of cathelicidins with atherosclerosis has also been demonstrated in mice. In studies using pro-atherosclerotic apolipoprotein E (apoE)-deficient mice, the levels of neutrophil-associated mCRAMP were elevated in the carotid arteries of mice fed with high-fat chow. Moreover, these mice demonstrate significant protection in plaque size and recruitment of macrophages upon crossing to mice strains deficient in mCRAMP[448]. LL37 also contributes to the recruitment of inflammatory cells by activating endothelial cells and the upregulation of ICAM-1 in atherosclerosis, this in turn leads to the recruitment of inflammatory cells to the site if inflammation and may cause prothrombotic responses[426].

1.7.7 Emerging role for LL37 in platelet function

Only three studies have explored the direct role of LL37 in the regulation of platelet function. In 2006, Bucki and Janmey demonstrated the role of lipopolysaccharides (LPS) and lipoteichoic acid (LTA) on several endogenous antimicrobial peptides, including PBP 10, mellitin, magainin II and LL37[402]. In an effort to demonstrate the mechanisms by which PBP 10 triggers bacterial killing, it was compared to the aforementioned AMPs. In this study, the cytotoxic activity of such peptides on circulating blood cells including red blood cells (RBCs) and platelets was investigated. Only mellitin (5 uM) demonstrated cytotoxicity towards platelets; LL37 (5 uM) failed to exert any cytotoxicity towards platelets. Additionally, LL37 (5 uM) failed to aggregate platelets on its own and demonstrates no effects on thrombin-induced aggregation of gel-filtered platelets. LL37 (1 or 5 uM) also failed to exert any effect on calcium mobilisation using Fura-2-labelled platelets.

In 2016, Su et al. suggested an inhibitory role for LL37 in the regulation of platelet function[449]. The study shows that LL37 inhibits the aggregation of gel-filtered human platelets upon stimulation with ADP (10 μM), collagen (2 μg/mL), thrombin (0.26 U/mL) or U46619 (3 μM). Moreover, the study demonstrates that LL37 inhibits alpha-granule secretion induced by the aforementioned platelet agonists. Additionally, LL37 demonstrated antithrombotic effects in an arterio-venous shunt thrombosis model in mice. In addition, platelet spreading on immobilized fibrinogen was inhibited by pretreatment with LL37 (0.3, 0.6 and 1.2 mM). The study suggested the involvement of Src/PI3K/Akt signalling as evidenced by changes in phosphorylation upon pretreatment with LL37 (0.6 mM). The findings in this study are likely to be improbable; as the concentration of LL37 are exceptionally high and are not physiologically-relevant and even more so have been reported to exert cytotoxicity towards various eukaryotic cells[401].

On the contrary, a recently published study by Pircher et al. in 2018 demonstrated the role of LL37 in thrombo-inflammation[450]. This study demonstrates a priming role for LL37 in platelets, which was evidenced by reduced platelet activation and arterial thrombosis in CRAMP-deficient mouse models. Moreover, LL37 induced alpha-granule secretion evident by P-selectin expression. However, LL37 failed to exert a direct effect on platelet aggregation, and did not affect collagen- ADP-, or thrombin receptor activating peptide (TRAP)-induced platelet aggregation in platelet-rich plasma (PRP). In addition, LL37 failed to induce fibrinogen binding or platelet spreading on immobilized fibrinogen. In an attempt to explore the signalling mechanisms underlying such effects, the FPR2/ALX signalling was explored amongst other pathways. Notably, the inhibition of such signalling by an FPR2/ALX-selective inhibitor, WRW4, did not affect LL37 effects. It is worth noting that none of the studies mentioned above explored the effects of LL37 on FPR2/ALX signalling on platelets except for Pircher et al., wherein a very low concentration (1 uM) of WRW4 was used.

1.8 Resolving protein Annexin A1

1.8.1 Definition and function

Annexin A1 (AnxA1) is a 37 kDa pro-resolving protein that belongs to the annexin superfamily[451]. Annexins are calcium-dependent phospholipid binding proteins that share two distinctive regions; the core and the amino (N)-terminus. The core region is highly conserved between the annexin subfamilies, while the N-terminal sequence is unique to each protein[452]. It is a glucocorticoid-regulated protein that is known to play a role in the regulation of inflammation, and cell proliferation, differentiation and apoptosis[453]. It is expressed in the cytosol of various resting cells including neutrophils, monocytes, macrophages and epithelial cells[288]. Furthermore, a limited number of studies have reported its expression in platelets[454, 455]. The activation of such cells leads to the externalisation of AnxA1 on the cell membrane and/or its secretion[456]. The extracellular AnxA1 then undergoes conformational changes, exposing its active form, the N-terminal region that mediates the binding to FPR2/ALX[335, 457]. The externalisation and secretion of AnxA1 is typically accompanied by a proteolytic cleavage of its N-terminal region[458-460], such cleavage is implicated with the down-regulation of AnxA1, rather than the release of bioactive peptides. This was validated in a study whereby a cleavage-resistant AnxA1, termed Super Annexin A1 (SAnxA1), retained a prolonged function in the microvasculature[461]. While the full-length N-terminus has been shown to be inactive, a synthetic peptide containing the first 26 amino acids of its sequence displayed functionality[462]. The N-Acetyl 2–26 (Ac2-26) has been shown to act as a pharmacophore[463] and plausibly maintain the properties of the full-length protein[305, 461, 464-467]. Nonetheless, a limited number of studies reported an opposing effect[334, 468, 469]. Both full-length and cleaved forms of the protein have been found in inflammatory exudates and other extracellular biological fluids[470-473].

1.8.2 Role of Annexin A1 in disease

AnxA1 has been shown to play a protective role in various inflammatory diseases that are associated with platelet reactivity[474], including atherosclerosis[475-479], myocardial infarctions[480, 481] and strokes[260, 261, 465, 478, 482]. Furthermore, the dysregulation of AnxA1 expression has been implicated in various pathological conditions; its up-regulation is reported in cancer[483], melanoma[484], breast cancer[485-487], periods of remission in ulcerative colitis[488], and its downregulation in Crohn's disease[489], thyroid cancer[490], signifying its prognostic significance and therapeutic efficacy. Conversely, a pathogenic role of Ac2-26 has been previously described in cancer, wherein it facilitated metastasis/pro-invasiveness[491], and in rheumatoid arthritis, where it facilitated the secretion of matrix metalloproteinases (MMPs)[468].

1.9 Hypothesis

Formyl peptide receptors regulate haemostasis in platelets through the modulation of thrombus formation and haemostasis. The chemotactic bacterial peptide fMLF primes platelet activation and modulates thrombus formation through FPR1. The antimicrobial peptide LL37 exhibits prothrombotic effects and thus may implicate the pathogenesis of cutaneous inflammatory diseases such as psoriasis through interaction with platelets via FPR2/ALX. The Annexin A1 N-terminal peptide, Ac2-26, affects platelet haemostasis through FPR2/ALX.

1.10 Research objectives

The aim of this study is to determine the role of formyl peptide receptors in the regulation of platelet function. Thus, the main objectives of this study:

- Investigate the role of a range of FPR ligands in the activation of platelets, and analyse the signalling mechanisms that regulate these effects
- Determine the role of bacterial formyl peptide fMLF in the regulation of platelet function via FPR1
- Determine the role of antimicrobial peptide LL37 in the regulation of platelet function via FPR2/ALX, and its role in modulating platelet function during inflammatory disease such as psoriasis
- Determine the role of resolving protein Annexin A1 in the regulation of platelet function via FPR2/ALX

2 – The formyl peptide fMLF primes platelets and augments thrombus formation selectively via formyl peptide receptor 1

The formyl peptide fMLF primes platelets and augments thrombus formation selectively via formyl peptide receptor 1

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2.1 Abstract

Formyl peptide receptors (FPRs) are a group of G protein-coupled receptors that play pivotal roles in the regulation of innate immunity and host defence. FPRs include three family members; FPR1, FPR2/ALX and FPR3. The activation of FPR1 by its high affinity ligand, *N*-formyl-methionyl-leucyl-phenylalanine (fMLF) (a bacterial chemoattractant peptide), triggers intracellular signalling in immune cells such as neutrophils and exacerbates inflammatory responses to accelerate the clearance of microbial infection. Notably, fMLF has been demonstrated to induce intracellular calcium mobilisation and chemotaxis in platelets that are known to play significant roles in the regulation of innate immunity and inflammatory responses. Despite a plethora of research focused on the roles of FPR1 and its ligands such as fMLF on the modulation of immune responses, their impact on the regulation of haemostasis and thrombosis remains unexplored. Here, we demonstrate the effect of fMLF on the modulation of platelet reactivity, haemostasis and thrombus formation. fMLF primes platelet activation through inducing distinctive function and enhances thrombus formation under arterial flow conditions. By using selective inhibitors for FPR1 and *Fpr1*-deficient mice, we have established a fundamental role for FPR1 in the regulation of fMLF-mediated effects in platelets. Moreover, FPR1 regulates normal platelet function as its deficiency in mouse or blockade in human platelets using a pharmacological inhibitor resulted in diminished agonist-induced platelet activation. Since FPR1 plays critical roles in numerous disease conditions, its influence on the modulation of

platelet activation and thrombus formation will provide insights into the mechanisms that control platelet-mediated complications under diverse pathological settings.

2.2 Introduction

Platelets are small circulating blood cells that play indispensable roles in the regulation of haemostasis to prevent excessive bleeding upon vascular injury. However, their unwarranted activation under pathological conditions leads to the formation of blood clots (thrombi) within the circulation[1]. This results in reduced/retarded blood supply to vital organs including the heart and brain, which leads to heart attacks or strokes, respectively[2]. Moreover, platelet activation during microbial infection results in their aggregation, thrombus formation in the microvasculature and in later stages, sequestration of platelets in organs such as the lungs, instigating thrombocytopenia and bleeding complications[3]. In addition to their prominent roles in haemostasis and thrombosis, platelets play a crucial role in the regulation of innate immunity, inflammatory responses and clearance of microbial infection[4-6]. Platelets contain a broad spectrum of receptors that induce inflammatory responses during microbial infection and other pathological conditions. In addition, platelets secrete various inflammatory and immunomodulatory molecules from their granules upon activation. They also possess antimicrobial proteins including thrombocidins, cathelicidins and human β-defensins that trigger direct microbicidal activities[4]. Furthermore, platelets can directly bind and internalise invading microbes[7]. The presence of major inflammatory molecules such as formyl peptide (FPRs) and toll-like receptors (TLRs) facilitates platelets to recognise a diverse array of endogenous damage-associated molecular patterns (DAMPs) and exogenous pathogen-associated molecular patterns (PAMPs). Collectively, these properties render platelets effector and sentinel cells in primary host defence against invading pathogenic microbes[7, 8].

FPRs belong to a family of G protein-coupled receptors (GPCRs) and are predominantly expressed in immune cells, where they play a prominent role in the regulation of inflammatory responses and host defence. In humans, three FPR family members were identified; FPR1, FPR2/ALX and FPR3[9]. Although they were originally identified by their capability to recognise *N*-

formyl peptides produced from bacteria or mitochondria of damaged cells, FPRs can bind a wide variety of structurally and functionally diverse ligands. These include bacterial and mitochondrial formyl peptides, non-formylated peptides/proteins, and small lipid molecules[10]. While FPR1 binds to bacterial-derived *N*-formyl peptides with high affinity, FPR2/ALX largely binds to mitochondrial formyl peptides[9, 11]. The ligation of *N*-formyl peptides to FPRs in immune cells triggers a range of signalling cascades resulting in numerous biological activities. For example, the stimulation of FPR1 by *N*-formyl-methionyl-leucyl-phenylalanine (fMLF) in neutrophils induces degranulation, chemotaxis, production of superoxide anions, calcium mobilisation, cytokine release and expression of various surface markers[12, 13]. During microbial infection, invading bacteria release *N*-formyl peptides that facilitate the recruitment of immune cells to the site of infection and accelerate the clearance of microbial infection and repair of tissue damage[14].

Czapiga et al.[15] reported the presence of FPR1 in platelets and its ability to induce chemotactic and migratory responses upon ligation with *N*-formyl peptides, which emphasise a crucial role for FPR1 in platelet-mediated immune responses. Notably, bacterial or synthetic fMLF has been shown to act as a potent chemotactic agent through FPR1 in platelets. Despite numerous reports on the immune functions of FPR1, its impact upon ligation with fMLF on the modulation of haemostasis and thrombosis remains uncharacterised. Here, we report the ability of fMLF to prime platelets and augment thrombus formation, and the significance of FPR1 in the regulation of platelet function in the presence and absence of fMLF.

2.3 Results

2.3.1 Platelets express FPR1

The expression of FPR1 in human platelets at protein level, and in megakaryocytes at transcript level has been previously reported[15]. Furthermore, the presence of FPR1 transcripts in human and mouse platelets was demonstrated[16]. In line with these findings, here we confirmed the presence of FPR1 on the surface of human platelets by flow cytometry (Figure 2-1Ai) and in platelet lysates using immunoblot analysis (Figure 2-1Aii). Notably, the activation of platelets using 1 µg/mL cross-linked collagen-related peptide (CRP-XL) increased the level of FPR1 on the surface as determined by flow cytometry, while the level of proteins identified by immunoblots remained unchanged. These data confirm the presence of FPR1 in platelets, possibly in granules or the open canalicular system (OCS) or both, and their increase on the surface upon activation.

2.3.2 fMLF selectively binds to FPR1 on the platelet surface

A fluorescently-labelled fMLF (FITC-fMLF) was used to investigate its binding to FPR1 on the surface of platelets by flow cytometry. To ascertain the selective binding of fMLF to FPR1, platelets obtained from *Fpr1*$^{-/-}$ and *Fpr2/3*$^{-/-}$ (an orthologue of human FPR2/ALX) mice along with their controls were used in this assay. The absence of *Fpr1* in platelets obtained from *Fpr1*$^{-/-}$ mice was confirmed by immunoblot analysis (Figure 2-1Bi). The level of *Fpr1* identified in *Fpr2/3*$^{-/-}$ mouse platelets was found to be same as the controls. The binding of FITC-fMLF (5 µM) was significantly reduced in *Fpr1*$^{-/-}$ mouse platelets compared to the control and *Fpr2/3*$^{-/-}$ mice platelets (Figure 2-1Bii). These data confirm the selective binding of fMLF to *Fpr1* in mouse platelets.

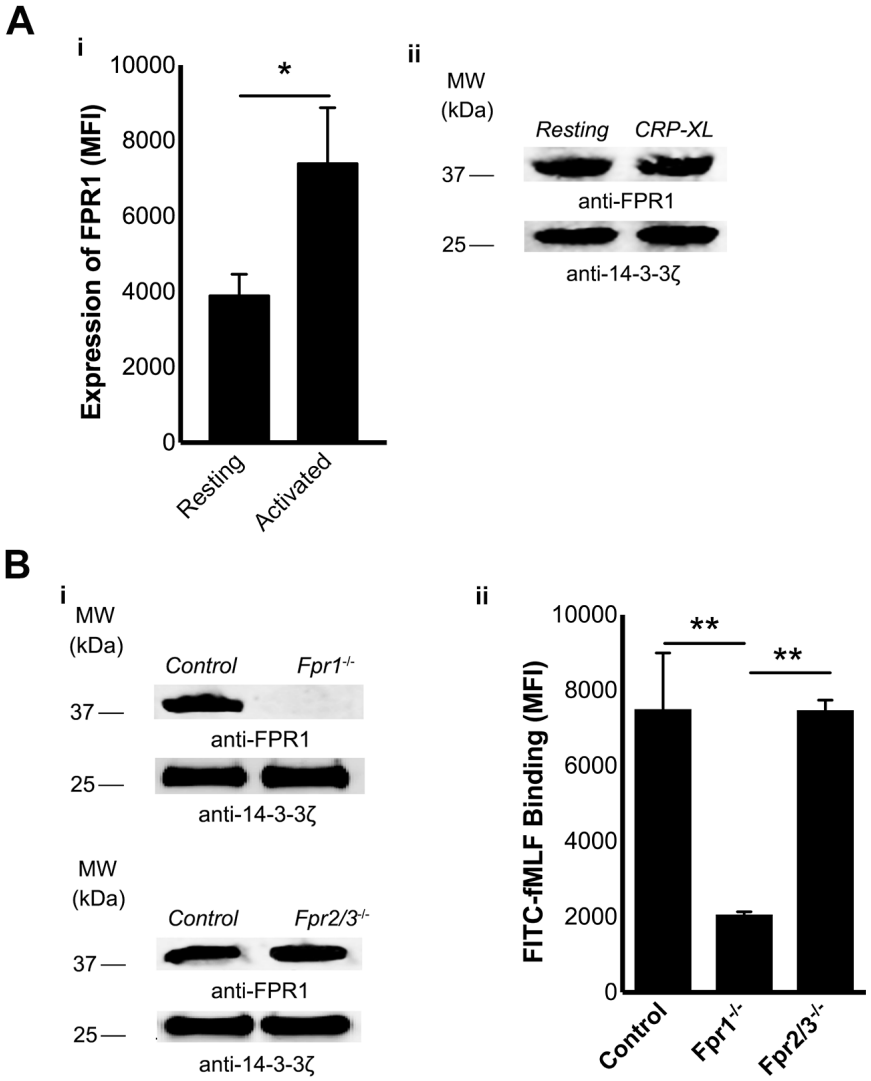


Figure 2-1: **Expression of FPR1 in platelets**. **A,** the expression of FPR1 on the surface of resting or CRP-XL(1 μg/mL)- activated human platelets was analysed using FPR1-selective and fluorescent-labelled secondary antibodies by flow cytometry (**i**). Data represent mean ± SEM (n=8). Similarly, the presence of FPR1 in human platelet lysates was confirmed by immunoblot analysis using selective antibodies (**ii**). **B**, the absence of *Fpr1* was confirmed in platelet lysates obtained from *Fpr1*$^{-/-}$ in comparison to the control and *Fpr2/3*$^{-/-}$ mice by immunoblot analysis using selective antibodies (**i**). Platelets obtained from control, *Fpr1*$^{-/-}$ and *Fpr2/3*$^{-/-}$ mice were pre-incubated with 10 μM FITC-conjugated fMLF and their level of binding to platelet surface was measured by flow cytometry (**ii**). Data represent mean ± SEM (n=5). The blots shown are representative of three separate experiments. The statistical significance was calculated by two-tailed unpaired Student's *t* test in data shown in panel **A** and by one-way ANOVA followed by Bonferroni's correction in data shown in panel **B** (*p<0.01 and **p<0.001).

2.3.3 fMLF stimulates platelet activation

To determine whether fMLF is able to stimulate platelet activation upon binding to FPR1, a range of platelet functional assays were performed. Platelet activation triggers inside-out signalling to integrin αIIbβ3 on the platelet surface and converts it to a high affinity state to allow fibrinogen binding and subsequent platelet aggregation[17]. To examine whether fMLF influences the inside-out signalling to integrin αIIbβ3 platelets, the level of fibrinogen binding on the platelet surface was measured as a marker for inside-out signalling to integrin αIIbβ3. Indeed, fMLF has increased the level of fibrinogen binding in human platelets [platelet-rich plasma (PRP)] in a concentration-dependent manner (Figure 2-2i). A minimum concentration of 1 μM fMLF has shown significant increase in fibrinogen binding compared to the resting platelets. Similarly, the level of P-selectin exposure on the platelet surface was measured as a marker for α-granule secretion by flow cytometry. The results indicate that fMLF has induced α-granule secretion in human platelets (PRP) in a concentration-dependent manner (Figure 2-2ii). Together, these data confirm the impact of fMLF on the modulation of platelet activation.

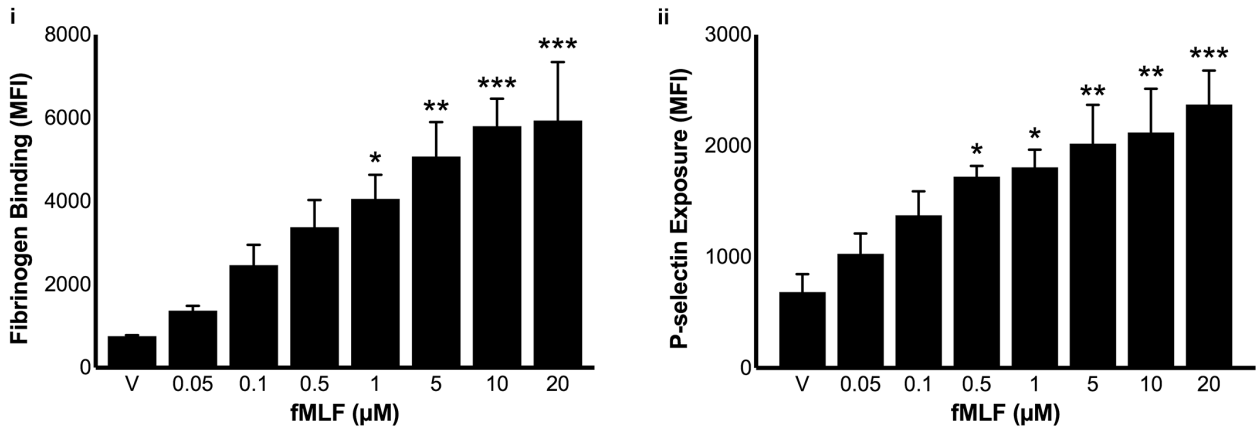


Figure 2-2: **The impact of fMLF on platelet activation**. Different concentrations of fMLF were used to determine their impact on platelet activation in human PRP by quantifying the level of fibrinogen binding (**i**) and P-selectin exposure (**ii**) using flow cytometry. Data represent mean ± SEM (n=10). The statistical significance was calculated by one-way ANOVA followed by Bonferroni's correction (*p<0.01. **p<0.001 and ***p<0.0001).

2.3.4 fMLF augments thrombus formation

Microbial infection and various inflammatory diseases including sepsis are associated with the risk of disseminated intravascular coagulation or thrombosis in the microvasculature[18]. To investigate whether fMLF has a direct impact on thrombosis, its effect on thrombus formation under arterial flow conditions was analysed. Human $DiOC_6$-labelled whole blood was pre-incubated with a control (non-formylated peptide, MLF) or fMLF (5 μM) for 10 minutes prior to perfusion over collagen-coated Vena8™ biochips. Thrombus formation was monitored for 10 minutes by acquiring fluorescent images at every 30 seconds. The images were analysed by calculating the mean fluorescence intensity. Indeed, fMLF has increased the mean fluorescence intensity of thrombi by approximately 122% compared to the controls (Figure 2-3). These data demonstrate the direct impact of fMLF on thrombus formation under arterial flow conditions in human whole blood. The effect of fMLF on other blood cells mainly leukocytes and their subsequent influence on thrombus formation cannot be ruled out under these circumstances.

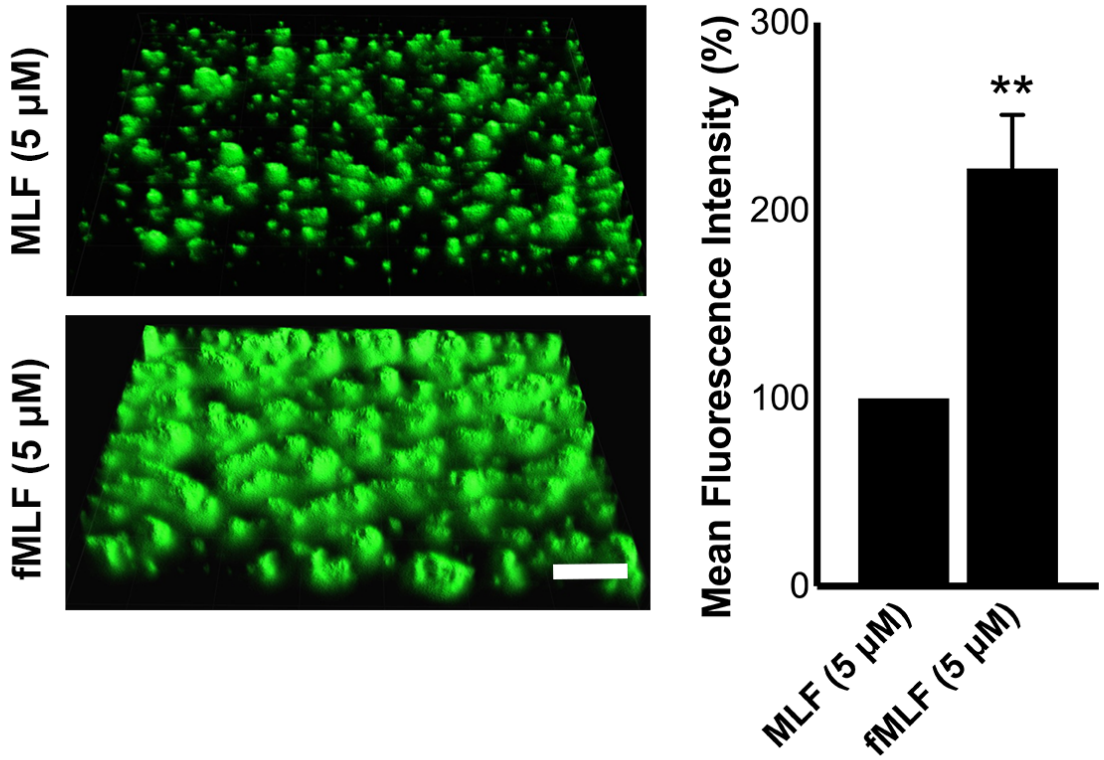
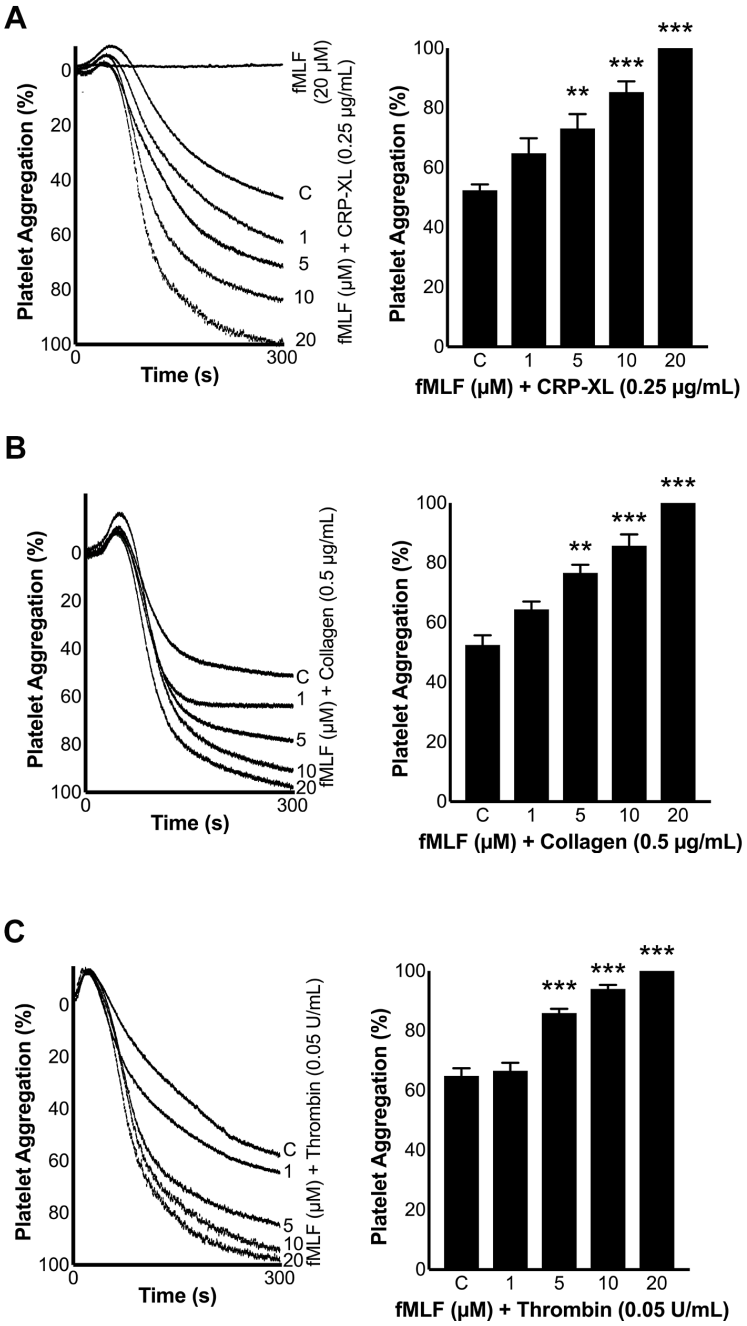


Figure 2-3: **The impact of fMLF on thrombus formation**. The effect of fMLF on the modulation of thrombus formation was analysed by using human DiOC6-labelled whole blood that pre-incubated with a vehicle control or fMLF (5 µM) for 10 minutes prior to perfusion over collagen-coated (400 µg/mL) Vena8^{TM} Biochips. Data represent mean ± SEM (n=3). Images shown are representative of three separate experiments (10x magnification; scale bar - 10 µm). The statistical significance was calculated by two-tailed unpaired Student's *t* test (**p<0.001).

2.3.5 Agonist-induced platelet aggregation is amplified by fMLF

Following the determination of the effects of fMLF on thrombus formation and platelet activation, aggregation assays were performed to establish its effects on isolated platelets. Human isolated platelets were incubated with various concentrations of fMLF (1, 5, 10 and 20 μM) prior to stimulation with different platelet agonists such as CRP-XL (0.25 μg/mL), collagen (0.5 μg/mL) and thrombin (0.05 U/mL), and the level of aggregation was monitored for 5 minutes by optical aggregometry. Notably, fMLF has failed to induce platelet aggregation on its own (Figure 2-4A) although the pre-incubation of platelets with fMLF markedly enhanced agonists-induced platelet aggregation. Maximum aggregation (100%) was obtained in human isolated platelets that treated with 20 μM fMLF and 0.25 μg/mL CRP-XL for 5 minutes (Figure 2-4A). Similar results were obtained with collagen- (Figure 2-4B) and thrombin- (Figure 2-4C) induced platelet aggregation. These data confirm the ability of fMLF to prime platelets and amplify their aggregation upon stimulation with different agonists although it was unable to aggregate platelets on its own.



<u>Figure 2-4</u>: The impact of fMLF on platelet aggregation. The effects of fMLF on CRP-XL (**A**), collagen (**B**) or thrombin (**C**)- induced platelet activation were measured using isolated human platelets by optical aggregometry. Data represent mean ± SEM (n=5). The statistical significance was calculated by one-way ANOVA followed by Bonferroni's correction ($^{**}p<0.001$ and $^{***}p<0.0001$).

2.3.6 fMLF selectively acts through FPR1 in platelets

A large number of studies indicate that fMLF binds primarily to FPR1 and exert its effects in immune cells[19-21]. The functional dependence of fMLF on FPR1 in platelets was determined using a pharmacological inhibitor for FPR1, Boc-MLF in human platelets and platelets obtained from *Fpr1*$^{-/-}$ mice through measuring the levels of fibrinogen binding and P-selectin exposure by flow cytometry. Similar to human platelets, fMLF increased the level of fibrinogen binding [Figure 2-5Ai (isolated platelets) and 2-5Aii (whole blood)] and P-selectin exposure [Figure 2-5Bi (isolated platelets) and 2-5Bii (whole blood)] on platelets obtained from control mice. However, the level of platelet activation by fMLF was significantly reduced in *Fpr1*$^{-/-}$ mouse platelets, which demonstrates its functional dependence on FPR1. Notably, the characterisation of platelets obtained from *Fpr1*$^{-/-}$ mice failed to display any defects in the size and number of platelets or the levels of major platelet receptors such as GPVI (Figure S1i), GPIbα (Figure S1ii), αIIbβ3 (Figure S1iii) and α2β1 (Figure S1iv) in comparison to the control mouse platelets. To establish the functional dependence of fMLF on FPR1 in human platelets, similar assays were performed in the presence or absence of Boc-MLF. The pre-incubation of human isolated platelets with different concentrations of Boc-MLF diminished fMLF-induced (5 μM) platelet activation as measured by the levels of fibrinogen binding (Figure 2-5Ci) and P-selectin exposure (Figure 2-5Cii). These data emphasise the involvement of FPR1 in the regulation of fMLF-mediated effects in platelets.

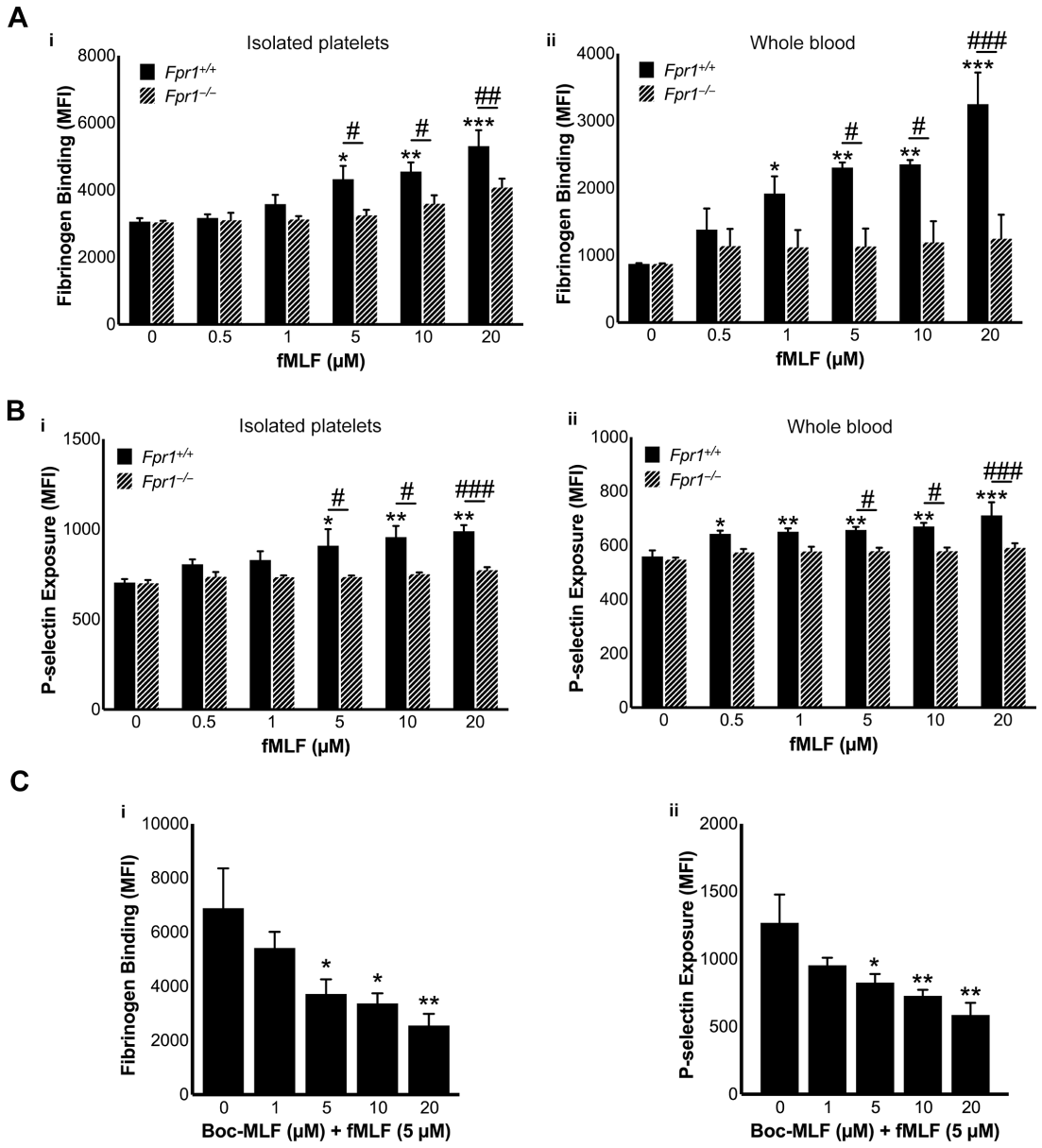
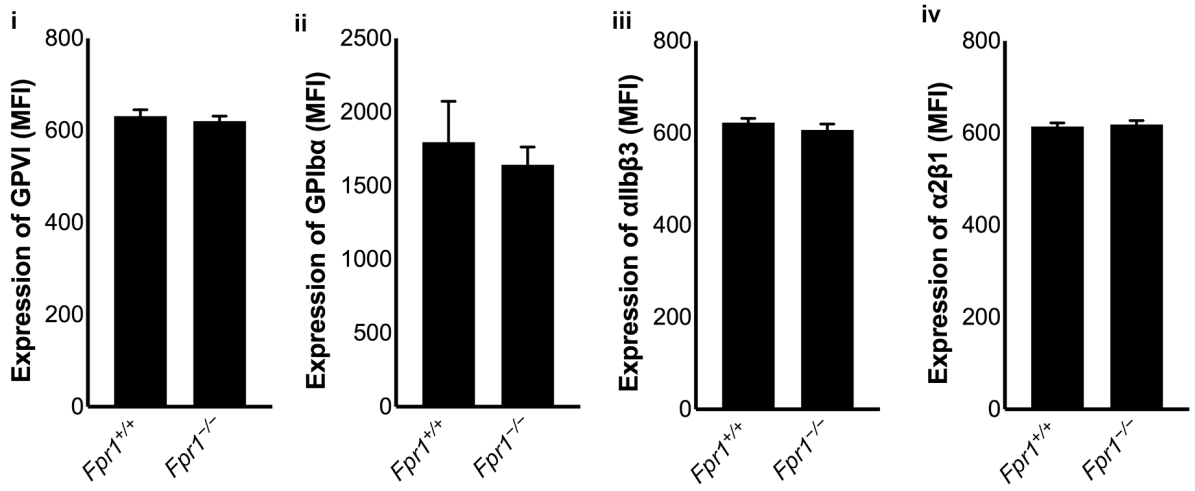


Figure 2-5: **The effects of fMLF on platelet activation are mediated through FPR1**. The platelet activation upon stimulation with various concentrations of fMLF was quantified by measuring the level of fibrinogen binding (**A**) using FITC-conjugated fibrinogen antibodies and P-selectin exposure (**B**) using PECy5-conjugated P-selectin antibodies in isolated platelets (**i**) or whole blood (**ii**) obtained from control and *Fpr1*$^{-/-}$ mice by flow cytometry. Data represent mean ± SEM (n=6 for isolated platelets & n=5 for whole blood). **C**, human isolated platelets were stimulated with fMLF (5 μM) in the presence or absence of different concentrations of Boc-MLF (1, 5, 10 and 20 μM), and the levels of fibrinogen binding (**Ci**) and P-selectin exposure (**Cii**) were analysed by flow cytometry. Data represent mean ± SEM (n=5). * Represents the significant difference between the various concentrations of fMLF within the *Fpr1*$^{+/+}$ group. # Represents the significant difference between *Fpr1*$^{+/+}$ and *Fpr1*$^{-/-}$ groups. The statistical significance was calculated by two-way ANOVA followed by Bonferroni's correction in most of the experiments except the data shown in panel **C**, which were analysed by one-way ANOVA followed by Bonferroni's correction (*p<0.01. **p<0.001 and ***p<0.0001).



Supplementary Figure 2-1: Characterisation of platelets obtained from *Fpr1*$^{-/-}$ mice. The expression levels of major platelet receptors such as GPVI (**i**), GPIbα (**ii**), αIIbβ3 (**iii**) and α2β1 (**iv**) in platelets obtained from control and *Fpr1*$^{-/-}$ mice were analysed by flow cytometry using selective fluorescent-labelled antibodies. Data represent mean ± SEM (n=8). The statistical significance was analysed by a two-tailed unpaired Student's *t* test.

2.3.7 Inhibition of FPR1 reduces the agonists-induced platelet activation

In order to study the importance of FPR1 in the regulation of normal platelet activation, further experiments were performed using human isolated platelets in the presence or absence of Boc-MLF. CRP-XL (0.25 μg/mL)-induced platelet aggregation was significantly reduced in the presence of different concentrations of Boc-MLF (1, 5, 10 and 20 μM). For example, the addition of Boc-MLF (20 μM) reduced the platelet aggregation by around 98% (Figure 2-6A). Similar results were obtained with ADP-induced platelet aggregation, wherein Boc-MLF (20 μM) reduced aggregation by approximately 70% (Figure 2-6B). Moreover, CRP-XL (0.25 μg/mL)- induced dense granule secretion as evidenced by the ATP release was significantly reduced by Boc-MLF (Figure 2-6C). Similarly, to determine if FPR1 has any influence on the outside-in signalling triggered by integrin αIIbβ3, platelet spreading assay was performed on fibrinogen-coated glass surfaces and analysed using confocal microscopy. The pre-incubation of platelets with Boc-MLF (1, 5, 10 and 20 μM) significantly decreased the number of adhered (Figure 2-7i) and spread (Figure 2-7ii) platelets, and the relative surface area of spreading on fibrinogen-coated surfaces (Figure 2-7iii) indicating a role for FPR1 in the modulation of integrin αIIbβ3-mediated outside-in signalling in platelets**.** In order to corroborate the involvement of FPR1 in the regulation of platelet function, cyclosporin H (CsH), an inverse agonist for FPR1 was employed. CsH inhibited CRP-XL (0.5 μg/ml)-induced platelet activation as measured by the levels of fibrinogen binding (Figure 2-8i) and P-selectin exposure (Figure 2-8ii). Furthermore, CsH decreased the mean fluorescence intensity of thrombi under arterial flow conditions by approximately 60% compared to the controls (Figure 2-9). These results highlight the prominent roles of FPR1 in the regulation of normal platelet function.

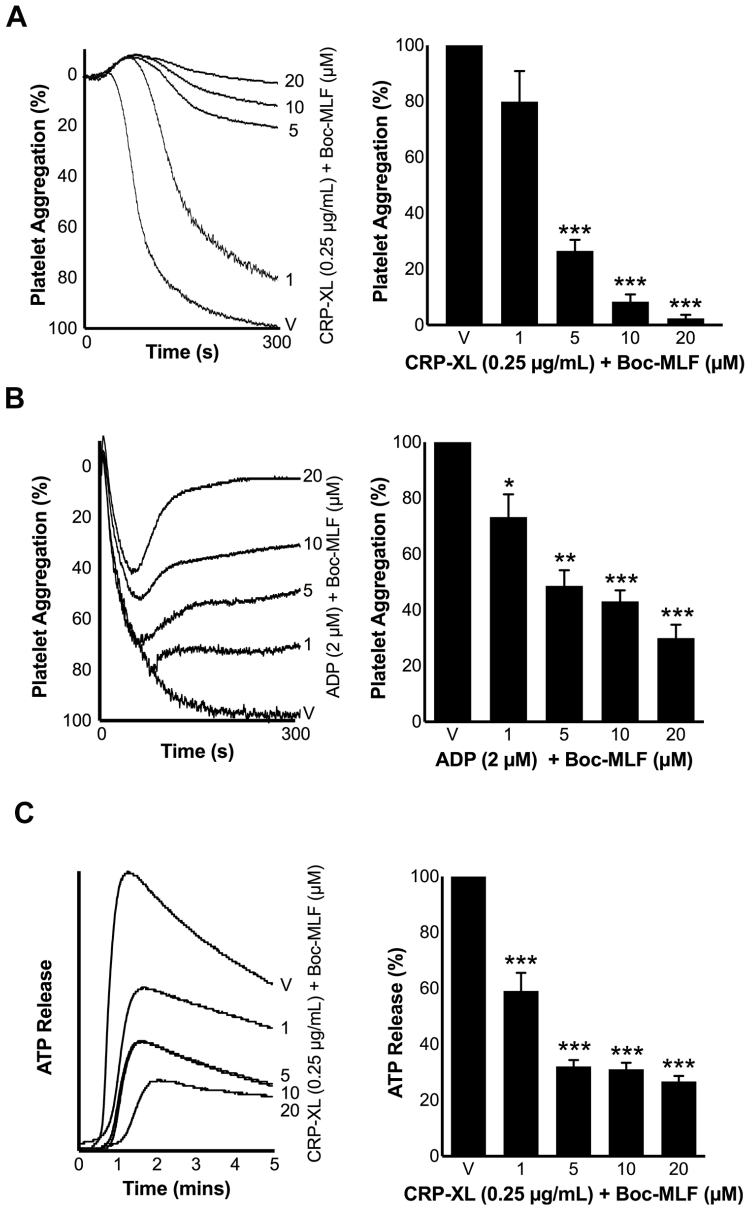


Figure 2-6: **Blockade of FPR1 using a pharmacological inhibitor reduces agonists-induced platelet activation**. The effect of different concentrations of Boc-MLF on CRP-XL (0.25 µg/mL) (**A**) or ADP (2 µM) (**B**)- induced aggregation using human isolated platelets was analysed by optical aggregometry. The level of aggregation obtained with the vehicle control was taken as 100% to calculate the extent of inhibition with Boc-MLF treated-samples. Data represent mean ± SEM (n=3). Similarly, the level of ATP secretion in human platelets upon activation with CRP-XL (0.25 µg/mL) in the presence and absence of different concentrations of Boc-MLF was measured by lumi-aggregometry (**C**). Data represent mean ± SEM (n=3). P values shown are as calculated by one-way ANOVA followed by Bonferroni's correction (*p<0.01, **p<0.001 and ***p<0.0001).

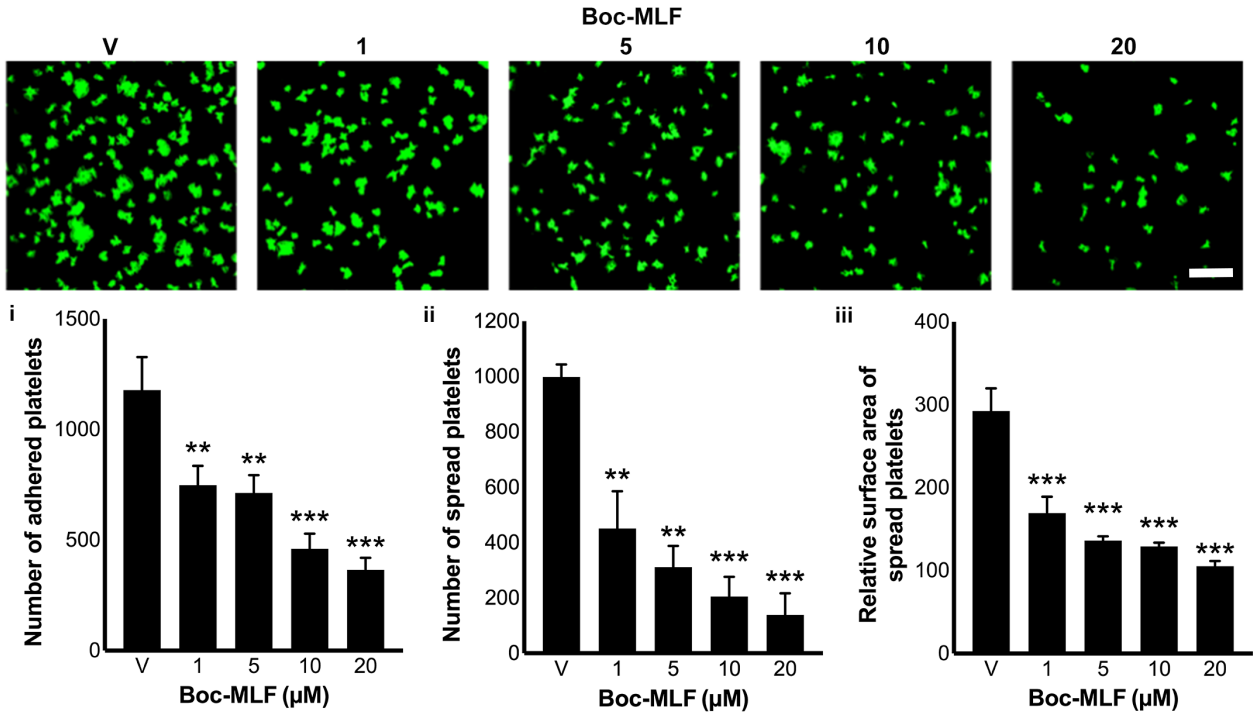


Figure 2-7: **Effect of FPR1-selective inhibitor, Boc-MLF, on platelet adhesion and spreading**. The platelet adhesion and spreading on fibrinogen-coated glass surface was analysed in the absence or presence of Boc-MLF (1, 5, 10 and 20 μM) by confocal microscopy (60x magnification; scale bar - 10 μm). The number of adhered (**i**) and spread platelets (**ii**), and the relative surface area of spread platelets (**iii**) were determined by analysing the images using ImageJ. Ten random fields of view were recorded for each sample. Data represent mean ± SEM (n=3). Data represent mean ± SEM (n=3). P values shown are as calculated by one-way ANOVA followed by Bonferroni's correction (**p<0.001 and ***p<0.0001).

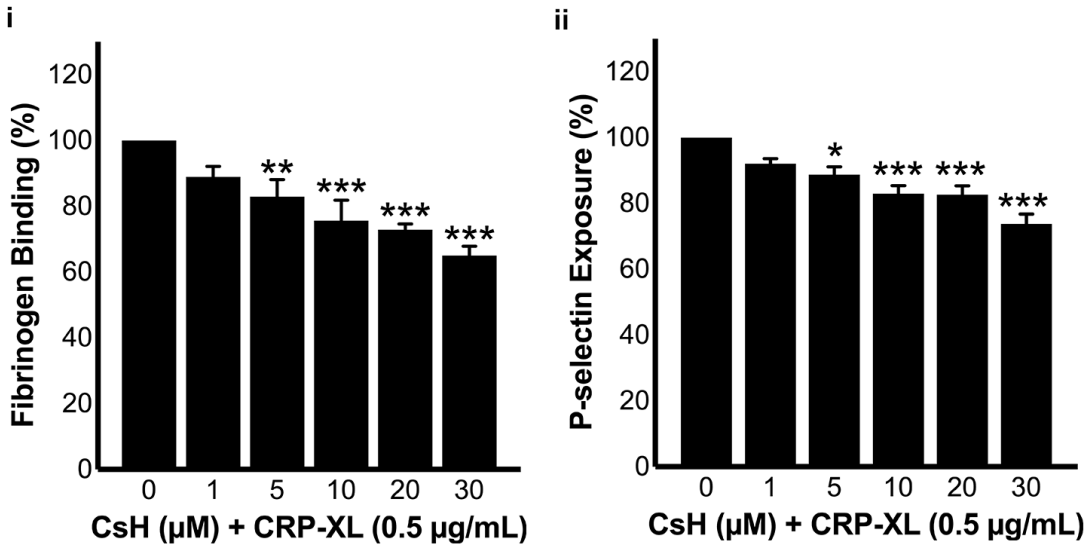


Figure 2-8: **Effect of FPR1-selective inhibitor, CsH, on platelet activation**. The levels of fibrinogen binding (**i**) and P-selectin exposure (**ii**) were analysed in human PRP by flow cytometry upon stimulation with CRP-XL (0.5 μg/mL), in the presence and absence of different concentrations of CsH (1, 5, 10, 20 and 30 μM). Data represent mean ± SEM (n=3). P values shown are as calculated by one-way ANOVA followed by Bonferroni's correction (*p<0.01, **p<0.001 and ***p<0.0001).

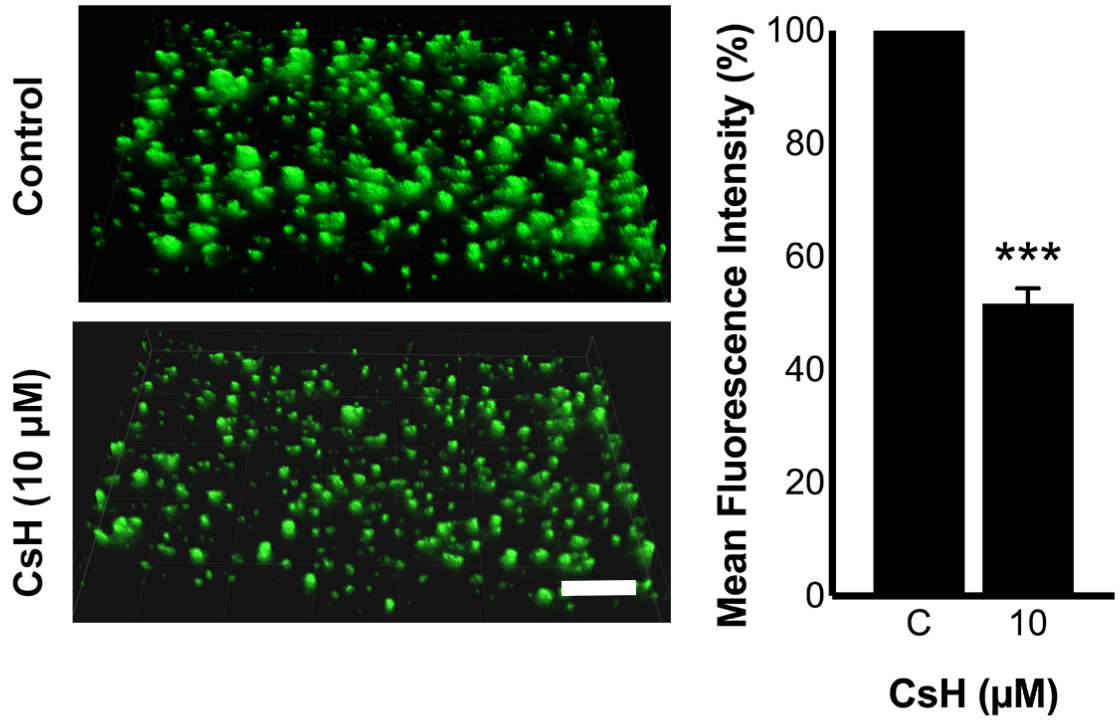


Figure 2-9: **Effect of FPR1-selective inhibitor, CsH, on thrombus formation**. The impact of CsH on the modulation of thrombus formation was analysed using human DiOC6-labelled whole blood that pre-incubated with a vehicle control or 10 µM CsH for 10 minutes prior to perfusion over collagen-coated (400 µg/mL) Vena8^{TM} Biochips. Images shown are representative of three separate experiments (10x magnification; scale bar - 10 µm). Data represent mean ± SEM (n=3). P values shown are as calculated by two-tailed unpaired Student's *t* test, respectively. (***p<0.0001).

2.3.8 Deletion of Fpr1 affects mouse platelet activation

To further examine the impact of FPR1 in platelets, the whole blood obtained from control and *Fpr1*$^{-/-}$ mice was used to assess the platelet activation upon stimulation with a range of conventional platelet agonists by measuring the levels of fibrinogen binding and P-selectin exposure using flow cytometry. Similar to the results obtained with human platelets (Figure 2-10), the level of fibrinogen binding (i) and P-selectin exposure (ii) in platelets obtained from *Fpr1*$^{-/-}$ mice upon stimulation with CRP-XL (Figure 2-10A), ADP (Figure 2-10B), AY-NH_2 (a PAR4 agonist) (Figure 2-10C) and U46619, an analogue of TXA_2 (Figure 2-10D) was largely reduced compared to their controls. To determine the influence of FPR1 on the modulation of haemostasis, tail bleeding assay was performed in control and *Fpr1*-deficient mice. A mean bleeding time of 429 seconds was observed in the control group, however *Fpr1*$^{-/-}$ mice significantly increased the bleeding time to a mean of 1128 seconds (Figure 2-11). These data indicate the importance of FPR1 in the modulation of platelet function and the maintenance of haemostasis under physiological conditions.

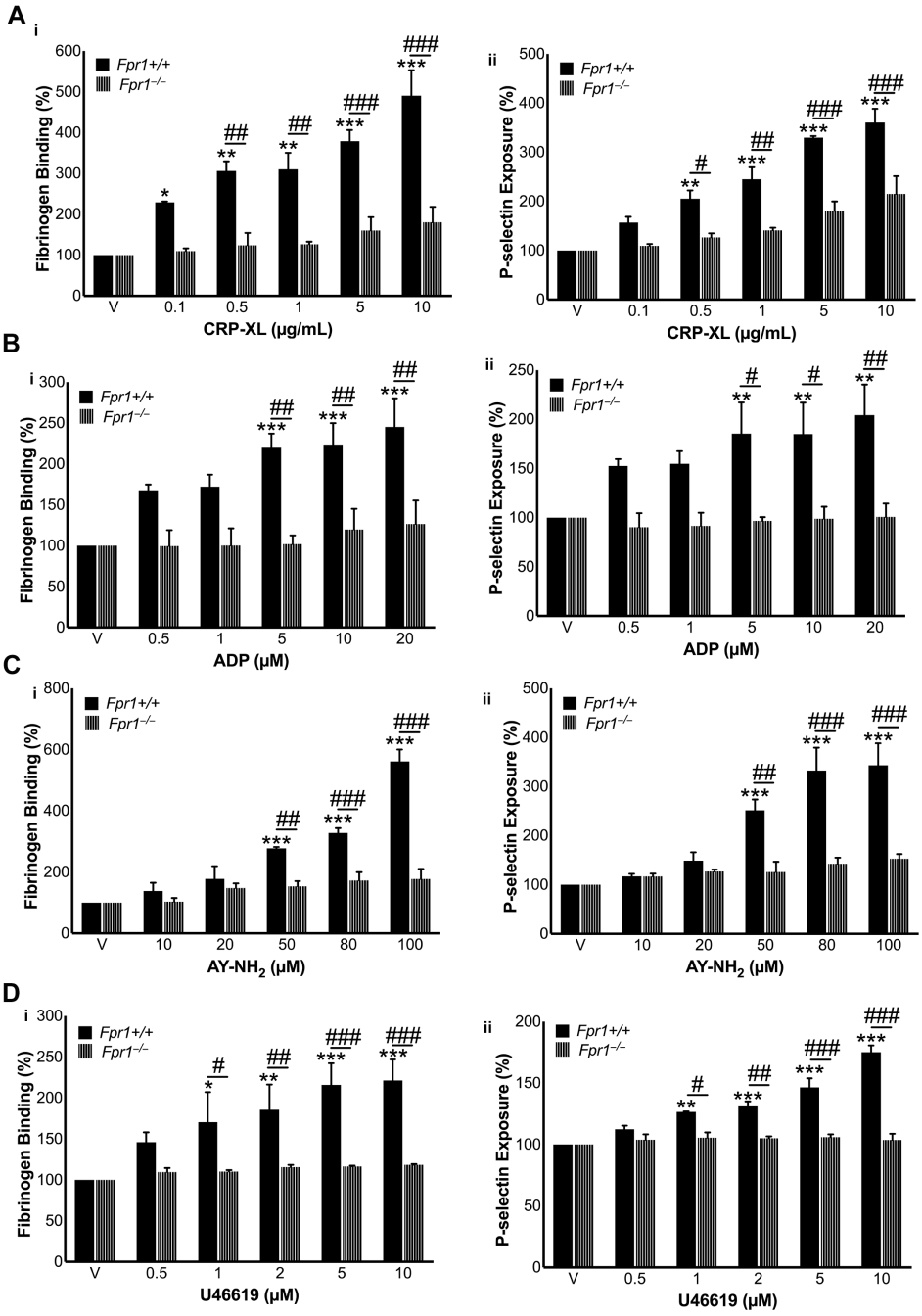


Figure 2-10: **Deletion of *Fpr1* in mice reduces the agonists-induced platelet activation.** The levels of fibrinogen binding (**i**) and P-selectin exposure (**ii**) were analysed in isolated platelets obtained from control or *Fpr1*$^{-/-}$ mice upon stimulation with various concentrations agonists such as CRP-XL (**A**), ADP (**B**), AY-NH2 (**C**) or U46691 (**D**) by flow cytometry. Data represent mean ± SEM (n=4). * Represents the significant difference between the various concentrations of agonists within the *Fpr1*$^{+/+}$ group. # Represents the significant difference between *Fpr1*$^{+/+}$ and *Fpr1*$^{-/-}$ groups. Data represent mean ± SEM (n=4). The statistical significance was calculated by two-way ANOVA followed by Bonferroni's correction (*p<0.01. **p<0.001 and ***p<0.0001).

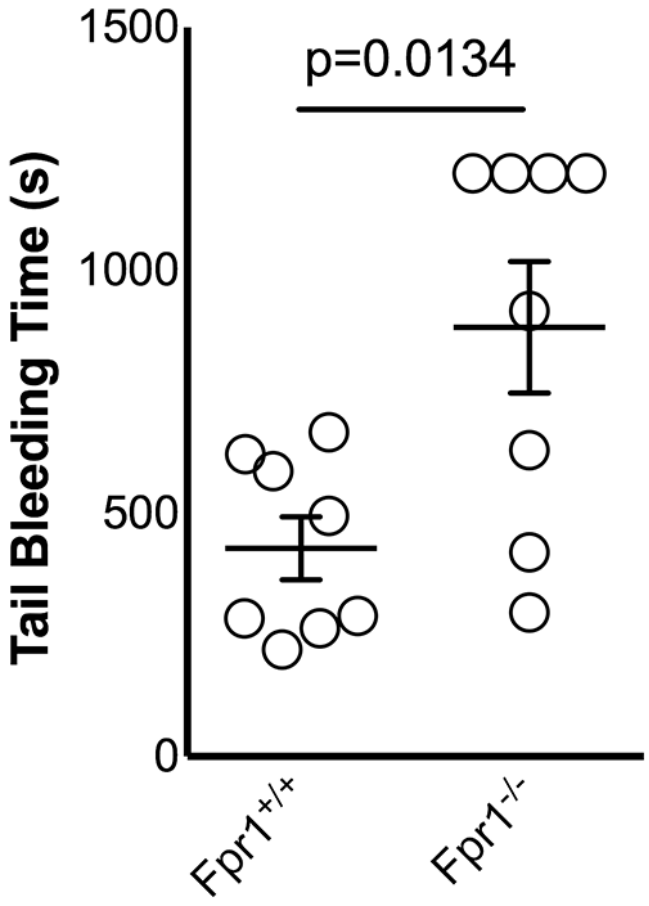


Figure 2-11: **Deletion of *Fpr1* in mice affects haemostasis**. The impact of FPR1 on the modulation of haemostasis was analysed by tail bleeding assay in control or *Fpr1*$^{-/-}$ mice. Data represent mean ± SEM (n=8). The statistical significance was calculated by non-parametric Mann-Whitney test.

2.3.9 FPR1 exerts its effects through cyclic AMP (cAMP)

cAMP is a potent inhibitor of platelet function and its level is generally reduced upon platelet activation. Stimulants of cAMP generation are known to inhibit platelet activation[22]. FPRs are G_i protein-coupled receptors[23], which are known to inhibit adenylate cyclase and thus, lead to a reduction in cAMP levels. Therefore, the deletion of genes for G_i-coupled receptors in mice generally increases the basal levels of cAMP in target cells[24, 25]. To investigate whether the effects of FPR1 in platelets are driven through cAMP-dependent signalling, the level of cAMP was quantified in platelets using a cAMP assay kit. The inhibition of FPR1 in human platelets with Boc-MLF (20μM) significantly elevated the level of cAMP compared to the controls (Figure 2-12i). Similarly, *Fpr1*$^{-/-}$ mouse platelets exhibited elevated basal levels of cAMP compared to the control mouse platelets at resting conditions (Figure 2-12ii). These data illustrate that the level of cAMP plays a key role in the regulation of FPR1-mediated function in platelets.

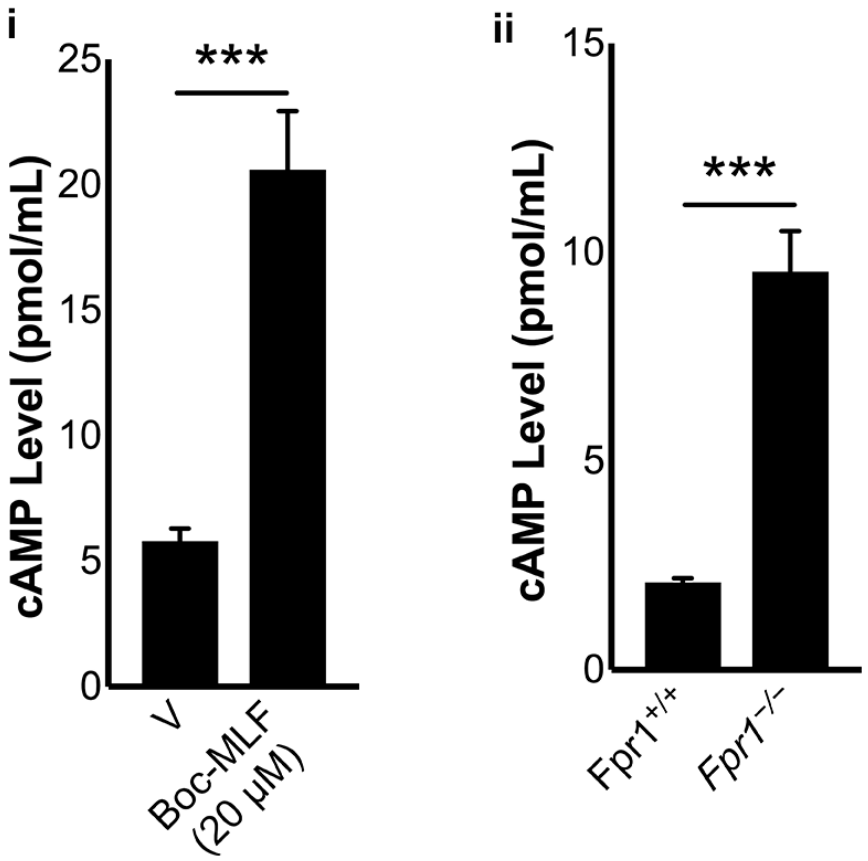


Figure 2-12: **Deletion of *Fpr1* in mice affects cAMP levels.** The level of cAMP in human isolated platelets in the presence or absence of Boc-MLF (**i**), and in control and *Fpr1*$^{-/-}$ mouse platelets (**ii**) was analysed using a cAMP assay kit. The statistical significance was calculated by two-tailed unpaired Student's *t* test (***p<0.0001).

2.4 Discussion

N-formyl peptides are released from bacteria or mitochondria of damaged cells[26, 27]. They have been demonstrated to play substantial roles in the initiation of chemotaxis and subsequent inflammatory responses in immune cells including monocytes, mast cells, eosinophils and neutrophils via FPRs[9]. Despite their ability to mediate innate immune responses, they have been associated with the pathogenesis of microbial infection and inflammatory diseases. Notably, *E. coli*-derived fMLF[28] has been implicated in bacterial cystitis[29], pneumococcal pneumonia[30], inflammatory bowel disease[31], pouchitis, colitis[32] and juvenile peridontitis[33]. It has been shown that inhalation or injection of fMLF can cause bronchial inflammation[34], and induce rapid neutropenia, thereby increasing susceptibility to infection[35]. The plasma levels of fMLF were increased in these conditions, and also in high fat diet treated mice due to altered microbiome where it impaired the glucose tolerance and insulin secretion[36]. Many of these infectious and inflammatory conditions are associated with a risk for thrombosis and other platelet-mediated complications[37-39]. The concentrations of fMLF in the intestinal milieu have been reported to be at least in micromolar ranges[40]. In this study, we demonstrate that fMLF is able to prime platelets and augment thrombus formation in micromolar concentrations. Therefore, the increased levels of fMLF under the aforementioned pathological conditions may lead to platelet activation and contribute towards thrombotic complications.

The activation of platelets facilitates their adhesion to leukocytes and leads to the formation of platelet-leukocyte aggregates (PLAs)[41]. P-selectin on the surface of activated platelets drives the formation of PLAs via binding to P-selectin glycoprotein ligand-1 (PSGL-1) on the surface of leukocytes[42]. In addition, the fibrinogen binding to integrin $\alpha_{IIb}\beta_3$ in platelets and Mac-1 in neutrophils was suggested to play an important role in the formation of PLAs[43]. PLAs are known to amplify thrombotic and proinflammatory responses in diverse inflammatory settings[44]. Indeed, fMLF has been reported to induce PLA formation[45] through fibrinogen binding in platelets and neutrophils[43]. Moreover, previous studies have reported the aggregation of platelets in response to fMLF-induced neutrophil stimulation[46-48] through release of platelet-activating factor (PAF)[49] and cathepsin G[50, 51]. In line with these, we demonstrate that fMLF has failed to induce aggregation of

isolated platelets although the pre-treatment of platelets with fMLF augmented agonist-induced aggregation. Furthermore, fMLF (but not MLF) augmented thrombus formation under arterial flow conditions in whole blood. Given that fMLF is able to upregulate the expression of adhesion molecules[52, 53] and aggregate neutrophils[43, 48], its effects on thrombus formation may be partly attributed to its interactions with leukocytes. Therefore, fMLF-induced fibrinogen binding and P-selectin exposure in platelets may directly trigger PLA formation as detailed above[54]. Although in this study, the direct impact of fMLF on platelet-mediated inflammatory responses was not analysed, the role of fMLF cannot be excluded in such responses. Together, these data demonstrate a prominent priming role for fMLF in platelets, which may augment thrombotic and proinflammatory responses through interactions with leukocytes in pathological settings.

FPR1 is a chemoattractant receptor that is widely expressed in various cell types including neutrophils, macrophages, and platelets[15]. Despite its well-characterised role in the modulation of inflammatory responses, the role of FPR1 in the regulation of platelet function is poorly studied. Here, we report a crucial role for FPR1 on the regulation of the platelet activation, haemostasis and thrombosis. In line with a previous study[15], we confirm the presence of this receptor in human platelets by immunoblot analysis, and its upregulation upon activation of platelets with an agonist. By using selective pharmacological inhibitor, Boc-MLF and an inverse agonist, cyclosporin H (CsH), the significance of FPR1 in the regulation of fMLF- and agonist -induced (such as CRP-XL and ADP) platelet activation was established. The blockade of FPR1 resulted in reduced ATP release upon activation with CRP-XL, which not only affects secondary platelet activation but may also influence the modulation of inflammatory responses[55]. Similarly, the inhibition of FPR1 impaired the ability of platelet spreading on fibrinogen, which is essential for thrombosis and subsequent wound repair[56]. The prominent role of FPR1 on the regulation of platelet activation was also corroborated using platelets obtained from *Fpr1*-deficient mice, wherein the effects of fMLF and platelet agonists were largely diminished. The selective binding of fMLF to Fpr1 was confirmed using platelets obtained from *Fpr1*-deficient mice. In addition, the haemostasis in *Fpr1*-deficient mice was affected further emphasising its significance in the regulation of platelet function. *Fpr1*-deficient mice displayed

severe inflammation, higher mortality during pneumococcal meningitis[57], increased susceptibility to *Listeria monocytogenes* infection and impaired antibacterial host defence[58, 59]. In addition, *Fpr1*-deficient mice demonstrate major roles in sterile inflammation triggered by mitochondrial *N*-formyl peptides as demonstrated by the attenuation of inflammation in response to sterile acute lung injury in these mice[60]. In line with these studies, here we demonstrate the dysfunction of platelets and affected haemostasis in *Fpr1*-deficient mice, which may also substantiate the reduced inflammatory responses due to significant contribution of platelets in inflammation and innate immunity.

As a Gi protein-coupled receptor, FPR1 triggers downstream signalling via various molecules such as phospholipase C (PLC), PI3K/AKT and MAPK, and modulates calcium mobilisation in neutrophils[13]. The ability of fMLF to induce calcium mobilisation in platelets has been previously reported[15]. Here, we report the impact of FPR1 on cAMP-mediated signalling in platelets using Boc-MLF and platelets obtained from *Fpr1*-deficient mice. Indeed, the inhibition of FPR1 in human or its deletion in mouse platelets resulted in elevated levels of cAMP, which is a potent inhibitor for platelet activation. This confirmed the involvement of cAMP-dependent signalling in the regulation of FPR1-mediated effects in platelets.

Given the significance of FPRs and their ligands under various clinical settings, the therapeutic potential of these are being largely investigated. Notably, honokiol, a plant-derived bioactive agent has been recently demonstrated to reduce the proinflammatory responses induced by fMLF in neutrophils through inhibiting FPR1[61]. In conclusion, this study demonstrates a prominent role for fMLF for priming platelet activation and augmenting thrombus formation under arterial flow conditions. Using an FPR1 selective inhibitor and *Fpr1*-deficient mice, the functional dependence of fMLF on this receptor was established. Therefore, the priming effects of fMLF on platelets may significantly contribute towards the development of thrombotic and proinflammatory diatheses during pathological settings. Hence, FPR1 may act as a potential therapeutic target and its ligands may provide a powerful platform to develop novel therapeutic agents in order to treat and prevent thrombotic and inflammatory complications during diverse pathophysiological settings.

References

1. Duhamel TA, Xu YJ, Arneja AS and Dhalla NS. Targeting platelets for prevention and treatment of cardiovascular disease. *Expert Opin Ther Targets*. 2007;11:1523-33.
2. Michelson AD. Antiplatelet therapies for the treatment of cardiovascular disease. *Nature Reviews Drug Discovery*. 2010;9:154-169.
3. Claushuis TA, van Vught LA, Scicluna BP, Wiewel MA, Klein Klouwenberg PM, Hoogendijk AJ, Ong DS, Cremer OL, Horn J, Franitza M, Toliat MR, Nurnberg P, Zwinderman AH, Bonten MJ, Schultz MJ and van der Poll T. Thrombocytopenia is associated with a dysregulated host response in critically ill sepsis patients. *Blood*. 2016.
4. Hamzeh-Cognasse H, Damien P, Chabert A, Pozzetto B, Cognasse F and Garraud O. Platelets and infections - complex interactions with bacteria. *Front Immunol*. 2015;6:82.
5. Rondina MT and Garraud O. Emerging evidence for platelets as immune and inflammatory effector cells. *Front Immunol*. 2014;5:653.
6. Nording HM, Seizer P and Langer HF. Platelets in inflammation and atherogenesis. *Front Immunol*. 2015;6.
7. Yeaman MR. Platelets in defense against bacterial pathogens. *Cell Mol Life Sci*. 2010;67:525-544.
8. Speth C, Loffler J, Krappmann S, Lass-Florl C and Rambach G. Platelets as immune cells in infectious diseases. *Future Microbiol*. 2013;8:1431-1451.
9. Ye RD, Boulay F, Wang JM, Dahlgren C, Gerard C, Parmentier M, Serhan CN and Murphy PM. International Union of Basic and Clinical Pharmacology. LXXIII. Nomenclature for the formyl peptide receptor (FPR) family. *Pharmacol Rev*. 2009;61:119-61.
10. He HQ and Ye RD. The Formyl Peptide Receptors: Diversity of Ligands and Mechanism for Recognition. *Molecules*. 2017;22.
11. Rabiet MJ, Huet E and Boulay F. Human mitochondria-derived N-formylated peptides are novel agonists equally active on FPR and FPRL1, while Listeria monocytogenes-derived peptides preferentially activate FPR. *Eur J Immunol*. 2005;35:2486-95.
12. Sklar LA, Jesaitis AJ and Painter RG. The neutrophil N-formyl peptide receptor: dynamics of ligand-receptor interactions and their relationship to cellular responses. *Contemp Top Immunobiol*. 1984;14:29-82.
13. Dorward DA, Lucas CD, Chapman GB, Haslett C, Dhaliwal K and Rossi AG. The role of formylated peptides and formyl peptide receptor 1 in governing neutrophil function during acute inflammation. *Am J Pathol*. 2015;185:1172-84.
14. Dahlgren C, Gabl M, Holdfeldt A, Winther M and Forsman H. Basic characteristics of the neutrophil receptors that recognize formylated peptides, a danger-associated molecular pattern generated by bacteria and mitochondria. *Biochem Pharmacol*. 2016;114:22-39.
15. Czapiga M, Gao JL, Kirk A and Lekstrom-Himes J. Human platelets exhibit chemotaxis using functional N-formyl peptide receptors. *Exp Hematol*. 2005;33:73-84.
16. Rowley JW, Oler AJ, Tolley ND, Hunter BN, Low EN, Nix DA, Yost CC, Zimmerman GA and Weyrich AS. Genome-wide RNA-seq analysis of human and mouse platelet transcriptomes. *Blood*. 2011;118:e101-11.
17. Yun SH, Sim EH, Goh RY, Park JI and Han JY. Platelet Activation: The Mechanisms and Potential Biomarkers. *Biomed Res Int*. 2016.
18. Semeraro N, Ammollo CT, Semeraro F and Colucci M. Sepsis-associated disseminated intravascular coagulation and thromboembolic disease. *Mediterr J Hematol Infect Dis*. 2010;2:e2010024.
19. Kirpotina LN, Schepetkin IA, Khlebnikov AI, Ruban OI, Ge Y, Ye RD, Kominsky DJ and Quinn MT. 4-Aroyl-3-hydroxy-5-phenyl-1H-pyrrol-2(5H)-ones as N-formyl peptide receptor 1 (FPR1) antagonists. *Biochem Pharmacol*. 2017;142:120-132.
20. Honda M, Takeichi T, Hashimoto S, Yoshii D, Isono K, Hayashida S, Ohya Y, Yamamoto H, Sugawara Y and Inomata Y. Intravital Imaging of Neutrophil Recruitment Reveals the Efficacy of FPR1 Blockade in Hepatic Ischaemia-Reperfusion Injury. *J Immunol*. 2017;198:1718-1728.

21. Maaty WS, Lord CI, Gripentrog JM, Riesselman M, Keren-Aviram G, Liu T, Dratz EA, Bothner B and Jesaitis AJ. Identification of C-terminal phosphorylation sites of N-formyl peptide receptor-1 (FPR1) in human blood neutrophils. *J Biol Chem*. 2013;288:27042-58.
22. Smolenski A. Novel roles of cAMP/cGMP-dependent signaling in platelets. *J Thromb Haemost*. 2012;10:167-76.
23. Wenzel-Seifert K, Arthur JM, Liu HY and Seifert R. Quantitative analysis of formyl peptide receptor coupling to g(i)alpha(1), g(i)alpha(2), and g(i)alpha(3). *J Biol Chem*. 1999;274:33259-66.
24. Yang J, Wu J, Jiang H, Mortensen R, Austin S, Manning DR, Woulfe D and Brass LF. Signaling through G(i) family members in platelets - Redundancy and specificity in the regulation of adenylyl cyclase and other effectors. *Journal of Biological Chemistry*. 2002;277:46035-46042.
25. Liu YF, Ghahremani MH, Rasenick MM, Jakobs KH and Albert PR. Stimulation of cAMP synthesis by G(i)-coupled receptors upon ablation of distinct G alpha(i) protein expression - G(i) subtype specificity of the 5-HT1A receptor. *Journal of Biological Chemistry*. 1999;274:16444-16450.
26. Schiffmann E, Corcoran BA and Wahl SM. N-Formylmethionyl Peptides as Chemoattractants for Leukocytes. *P Natl Acad Sci USA*. 1975;72:1059-1062.
27. Chadwick VS, Mellor DM, Myers DB, Selden AC, Keshavarzian A, Broom MF and Hobson CH. Production of peptides inducing chemotaxis and lysosomal enzyme release in human neutrophils by intestinal bacteria in vitro and in vivo. *Scand J Gastroenterol*. 1988;23:121-8.
28. Marasco WA, Phan SH, Krutzsch H, Showell HJ, Feltner DE, Nairn R, Becker EL and Ward PA. Purification and Identification of Formyl-Methionyl-Leucyl-Phenylalanine as the Major Peptide Neutrophil Chemotactic Factor Produced by Escherichia-Coli. *Journal of Biological Chemistry*. 1984;259:5430-5439.
29. Schwarz NT, Jung SY, Kalff JC, Chancellor M and Bauer AJ. Bacterial toxin N-formyl-methionyl-leucyl-phenylalanine acutely contracts human and rabbit detrusor through the release of eicosanoids. *J Urology*. 2002;167:2603-2612.
30. Gauthier JF, Fortin A, Bergeron Y, Dumas MC, Champagne ME and Bergeron MG. Differential contribution of bacterial N-formyl-methionyl-leucyl-phenylalanine and host-derived CXC chemokines to neutrophil infiltration into pulmonary alveoli during murine pneumococcal pneumonia. *Infection and Immunity*. 2007;75:5361-5367.
31. Anton PA, Targan SR and Shanahan F. Increased neutrophil receptors for and response to the proinflammatory bacterial peptide formyl-methionyl-leucyl-phenylalanine in Crohn's disease. *Gastroenterology*. 1989;97:20-8.
32. Leduc LE and Nast CC. Chemotactic Peptide-Induced Acute Colitis in Rabbits. *Gastroenterology*. 1990;98:929-935.
33. Perez HD, Kelly E, Elfman F, Armitage G and Winkler J. Defective polymorphonuclear leukocyte formyl peptide receptor(s) in juvenile periodontitis. *J Clin Invest*. 1991;87:971-6.
34. Peters MJ, Breslin AB, Kemp AS, Chu J and Berend N. Haematological effects of inhalation of N-formyl-methionyl-leucyl-phenylalanine in man. *Thorax*. 1992;47:284-7.
35. Yurt RW and Shires GT. Increased Susceptibility to Infection Due to Infusion of Exogenous Chemotaxin. *Arch Surg-Chicago*. 1987;122:111-116.
36. Wollam J, Johnson AM, Xu YJ, Jain M and Olefsky JM. Gut Microbiome Produced N-Formyl Peptides Act through the Receptor FPR1 to Modulate Insulin Secretion and Metabolism. *Diabetes*. 2016;65:A453-A453.
37. Danese S, de la Motte C and Fiocchi C. Platelets in inflammatory bowel disease: Clinical, pathogenic, and therapeutic implications. *Am J Gastroenterol*. 2004;99:938-945.
38. Tunjungputri RN, van de Heijden W, Urbanus RT, de Groot PG, van der Ven A and de Mast Q. Higher platelet reactivity and platelet-monocyte complex formation in Gram-positive sepsis compared to Gram-negative sepsis. *Platelets*. 2017;28:595-601.
39. Munoz-Esquerre M, Ferreiro JL, Huertas D, Marcano AL, Lopez-Sanchez M, Roura G, Gomez-Hospital JA, Dorca J, Cequier A and Santos S. Impact of acute exacerbations on platelet reactivity in chronic obstructive pulmonary disease patients. *Int J Chronic Obstr*. 2018;13:141-148.

40. Roberts EC, Hobson CH, Anderson RP and Chadwick VS. Radioimmunoassay for Formyl Methionyl Leucyl Phenylalanine .2. Demonstration of an Enterohepatic Circulation of Immunoreactive Bacterial Chemotactic Peptides in Man. *J Gastroen Hepatol*. 1990;5:38-43.
41. Zarbock A, Polanowska-Grabowska RK and Ley K. Platelet-neutrophil-interactions: Linking hemostasis and inflammation. *Blood Reviews*. 2007;21:99-111.
42. Ostrovsky L, King AJ, Bond S, Mitchell D, Lorant DE, Zimmerman GA, Larsen R, Niu XF and Kubes P. A juxtacrine mechanism for neutrophil adhesion on platelets involves platelet-activating factor and a selectin-dependent activation process. *Blood*. 1998;91:3028-3036.
43. Li NL, Hu H, Lindqvist M, Johnsson EW, Goodall AH and Hjemdahl P. Platelet-leukocyte cross-talk in whole blood: Inhibition by GPIIb/IIIa and P-selectin antagonists. *Circulation*. 2000;102:34-34.
44. Esmon CT. The interactions between inflammation and coagulation. *Brit J Haematol*. 2005;131:417-430.
45. Coeffier E, Joseph D, Prevost MC and Vargaftig BB. Platelet-Leukocyte Interaction - Activation of Rabbit Platelets by Fmlp-Stimulated Neutrophils. *Brit J Pharmacol*. 1987;92:393-406.
46. Rossaint J, Kuhne K, Skupski J, Van Aken H, Looney MR, Hidalgo A and Zarbock A. Directed transport of neutrophil-derived extracellular vesicles enables platelet-mediated innate immune response. *Nature communications*. 2016;7:13464.
47. Coeffier E, Joseph D, Prevost MC and Vargaftig BB. Platelet-leukocyte interaction: activation of rabbit platelets by FMLP-stimulated neutrophils. *Br J Pharmacol*. 1987;92:393-406.
48. Oda M, Satouchi K, Yasunaga K and Saito K. Polymorphonuclear leukocyte-platelet interactions: acetylglyceryl ether phosphocholine-induced platelet activation under stimulation with chemotactic peptide. *J Biochem*. 1986;100:1117-23.
49. Stewart AG and Harris T. Involvement of platelet-activating factor in endotoxin-induced priming of rabbit polymorphonuclear leukocytes. *J Lipid Mediat*. 1991;3:125-38.
50. Sambrano GR, Huang W, Faruqi T, Mahrus S, Craik C and Coughlin SR. Cathepsin G activates protease-activated receptor-4 in human platelets. *J Biol Chem*. 2000;275:6819-23.
51. Evangelista V, Rajtar G, de Gaetano G, White JG and Cerletti C. Platelet activation by fMLP-stimulated polymorphonuclear leukocytes: the activity of cathepsin G is not prevented by antiproteinases. *Blood*. 1991;77:2379-88.
52. Fan ST and Edgington TS. Integrin regulation of leukocyte inflammatory functions. CD11b/CD18 enhancement of the tumor necrosis factor-alpha responses of monocytes. *J Immunol*. 1993;150:2972-80.
53. Fearon DT and Collins LA. Increased expression of C3b receptors on polymorphonuclear leukocytes induced by chemotactic factors and by purification procedures. *J Immunol*. 1983;130:370-5.
54. Evangelista V, Piccardoni P, White JG, Degaetano G and Cerletti C. Cathepsin G-Dependent Platelet Stimulation by Activated Polymorphonuclear Leukocytes and Its Inhibition by Antiproteinases - Role of P-Selectin Mediated Cell-Cell Adhesion. *Blood*. 1993;81:2947-2957.
55. Dosch M, Gerber J, Jebbawi F and Beldi G. Mechanisms of ATP Release by Inflammatory Cells. *Int J Mol Sci*. 2018;19.
56. Lee D, Fong KP, King MR, Brass LF and Hammer DA. Differential Dynamics of Platelet Contact and Spreading. *Biophys J*. 2012;102:472-482.
57. Oldekamp S, Pscheidl S, Kress E, Soehnlein O, Jansen S, Pufe T, Wang JM, Tauber SC and Brandenburg LO. Lack of formyl peptide receptor 1 and 2 leads to more severe inflammation and higher mortality in mice with of pneumococcal meningitis. *Immunology*. 2014;143:447-461.
58. Liu MY, Chen KQ, Yoshimura T, Liu Y, Gong WH, Wang AM, Gao JL, Murphy PM and Wang JM. Formylpeptide receptors are critical for rapid neutrophil mobilization in host defense against Listeria monocytogenes. *Sci Rep-Uk*. 2012;2.
59. Gao JL, Lee EJ and Murphy PM. Impaired antibacterial host defense in mice lacking the N-formylpeptide receptor. *J Exp Med*. 1999;189:657-62.
60. Dorward DA, Lucas CD, Doherty MK, Chapman GB, Scholefield EJ, Morris AC, Felton JM, Kipari T, Humphries DC, Robb CT, Simpson AJ, Whitfield PD, Haslett C, Dhaliwal K and Rossi

AG. Novel role for endogenous mitochondrial formylated peptide-driven formyl peptide receptor 1 signalling in acute respiratory distress syndrome. *Thorax*. 2017;72:928-936.
61. Liu FC, Yu HP, Syu YT, Fang JY, Lin CF, Chang SH, Lee YT and Hwang TL. Honokiol suppresses formyl peptide-induced human neutrophil activation by blocking formyl peptide receptor 1. *Sci Rep-Uk*. 2017;7.

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2.5 Methods

Preparation of human platelet-rich plasma and isolated platelets

The University of Reading Research Ethics Committee approved all the experimental procedures using human blood from healthy volunteers. The blood samples were collected from healthy, aspirin-free volunteers after obtaining written informed consent. The blood was collected into VACUETTE® blood collection tubes containing 3.2% (w/v) sodium citrate. The blood samples were then centrifuged at 102*g* for 20 minutes at room temperature to obtain platelet-rich plasma (PRP). The PRP was rested at 30°C for 30 minutes prior to use. For the preparation of isolated platelets, the blood was mixed with ACD [2.5% (w/v) sodium citrate, 2% (w/v) D-glucose and 1.5% (w/v) citric acid] at 1 (ACD): 9 (blood) ratio and centrifuged at 102*g* for 20 minutes. The PRP was collected, mixed with appropriate volume of ACD, and centrifuged at 1413*g* for 10 minutes at room temperature. The resultant platelet pellet was resuspended in modified Tyrodes-HEPES buffer (134mM NaCl, 2.9mM KCl, 0.34mM Na_2HPO_4, 12mM $NaHCO_3$, 20mM HEPES and 1mM $MgCl_2$, pH 7.3) with appropriate volume of ACD, and centrifuged once again at 1413*g* for 10 minutes at room temperature. The resultant platelet pellet was resuspended to a final density of 4×10^8 cells/mL in modified Tyrode's-HEPES buffer and allowed to rest for 30 minutes at 30ºC prior to use.

Mouse blood collection and platelet preparation

The mouse strains of *Fpr1*$^{-/-1}$ and *Fpr2/3*$^{-/-2}$ on a C57BL/6 background obtained from William Harvey Research Institute, London, UK and wild type C57BL/6 mice from Envigo, UK were used in this study. The mice were sacrificed with CO_2 and the blood was directly collected by cardiac puncture into a syringe containing 3.2% (w/v) sodium citrate at 1 (citrate):9 (blood) ratio. The blood was then centrifuged at 203*g* for 8 minutes at room temperature and the PRP was collected. The remaining blood was resuspended in 500 µL of modified Tyrode's-HEPES buffer and centrifuged once again at 203*g* for 5 minutes. The resultant PRP then centrifuged at 1028*g* for 5 minutes. The

resultant platelet pellet was resuspended in modified Tyrode's-HEPES buffer at a density of $2x10^8$ cells/mL.

In vitro thrombus formation assay

In vitro thrombus formation was performed as described previously[3, 4]. In brief, human $DiOC_6$-labelled (Sigma Aldrich, UK) human whole blood was pre-incubated with a vehicle, fMLF (5 μM) or CsH (10 μM) for 10 minutes before perfusion over collagen (400μg/mL)-coated Vena8™ Biochips (Cellix Ltd, Ireland) at a shear rate of 20 dynes/cm^2. Z-stack fluorescence images of thrombi were obtained every 30 seconds for up to 10 minutes using a Nikon eclipse (TE2000-U) microscope (Nikon Instruments, UK). The fluorescence intensity was calculated by analysing the data using ImageJ software (National Institutes of Health, USA).

Tail bleeding assay

Tail bleeding assay was performed as described previously[5]. The British Home Office approved the experimental procedures. In brief, C57BL/6 (10-12 weeks old; Envigo, UK) or *Fpr1*$^{-/-}$ mice were anaesthetised using ketamine (80 mg/kg) and xylazine (5 mg/kg) administered via the intraperitoneal route and placed on a heated mat (37°C). The tail tip (1mm) was dissected, and immersed in sterile saline. The time to cessation of bleeding was measured and the assay was terminated at 20 minutes.

Platelet aggregation assay

In vitro platelet aggregation assays were performed by optical aggregometry using a two-channel platelet aggregometer (Chrono-Log Corporation, USA). Platelets obtained from human PRP (270 μL) were added into a siliconised cuvette and pre-warmed at 37°C for 90 seconds. Upon the addition of an agonist, the platelets were allowed to aggregate under continuous stirring at 1200rpm for 5 minutes at 37ºC and the level of aggregation was monitored. The platelets were pre-treated with different concentrations of fMLF (1, 5, 10 and 20 μM) for 5 minutes before the addition of CRP-XL

(0.25 µg/mL), collagen (0.5 µg/mL) or thrombin (0.05 U/mL) and the level of aggregation was monitored. Data were analysed by calculating the percentage of maximum platelet aggregation obtained at 5 minutes.

ATP secretion assay

To assess the level of dense granule secretion in platelets, ATP secretion was measured using a luciferin–luciferase luminescence substrate by lumi-aggregometry (Chrono-log, USA). The level of ATP released from platelets upon stimulation with a platelet agonist, CRP-XL (0.25 µg/mL), in the presence and absence of different concentrations of Boc-MLF was measured by observing the level of luminescence released.

Flow cytometry-based assays

In order to measure the level of fibrinogen binding and P-selectin exposure on the platelet surface, flow cytometry-based assays were performed. Five microliters of PRP or isolated platelets or whole blood were incubated with 1 µl of FITC-conjugated fibrinogen antibody (1:50) and 1 µl of $PECy^5$-conjugated anti-CD62P (P-selectin) (1:50) antibody in the presence and absence of various concentrations of fMLF or platelet agonists. The final volume was made up to 50 µl using HEPES-buffered saline (HBS) (150mM NaCl, 5mM KCl, 1mM $MgSO_4$ and 10mM HEPES, pH 7.4) and the samples were incubated for 20 minutes at room temperature. Following fixation in 0.2% formyl saline, the samples were analysed using an Accuri C6 flow cytometer (BD Biosciences, UK) by counting 5000 events within the gated population for platelets. The median fluorescence intensity was calculated using Accuri C6 software to quantify the levels of fibrinogen binding and P-selectin exposure on the surface of platelets. Similarly, for the analysis of FPR1 expression on platelets, five microliters of PRP were incubated with 1 µl of anti-FPR1 (5 µg/mL) and 2 µl of Cy5-conjugated anti-mouse IgG (80 µg/mL) with or without 1 µg/mL CRP-XL. Following 20 minutes incubation at room temperature, the platelets were fixed in 0.2% formyl saline and analysed by flow cytometry. For the

fMLF binding assay, following the incubation of isolated platelets with FITC-conjugated fMLF (5 μM) or vehicle control for 20 minutes, the platelets were fixed in 0.2% formyl saline and analysed by flow cytometry.

SDS-PAGE and immunoblotting analysis

Immunoblot analysis was performed using platelet lysates that prepared under reducing conditions. The samples were heated to 90°C for 10 minutes and subjected to SDS-PAGE using 10% resolving gels. The gels were then transferred to polyvinylidene difluoride (PVDF) membranes and blocked by incubation in 5% bovine serum albumin (BSA) in TBS-T (20mM Tris, 140mM NaCl and Tween-20, pH 7.6). Following an overnight incubation with primary anti-FPR1 antibody (1:500) (Abcam, UK), the blots were washed with TBS-T and incubated with secondary Cy5-conjugated goat anti-rabbit IgG antibodies (1:1000) (Invitrogen, UK) in TBS-T containing 5% BSA for one hour at room temperature. Following washing in TBS-T for one hour at room temperature, the blots were analysed using a Typhoon 9400 Variable Mode Imager system (GE Healthcare, UK). Equal loading of proteins in each lane was determined using anti-human 14-3-3ζ antibodies (1:1000) (Santa Cruz Biotechnology, USA).

Cyclic nucleotide assay

A cAMP ELISA kit (Cambridge Bioscience, UK) was used for the detection of the total cellular levels of cAMP in human and mouse platelets. Human isolated platelets were pre-incubated for 10 minutes with a selective inhibitor for FPR1, Boc-MLF. Similarly, platelets obtained from control or *Fpr1*$^{-/-}$ mice were also used. The platelets were incubated with 0.1M HCl and the levels of cAMP were calculated according to the manufacturer's protocol.

Platelet adhesion and spreading on fibrinogen

Isolated human platelets were treated with different concentrations of an FPR1-selective inhibitor, Boc-MLF, prior to loading onto fibrinogen (100 μg/mL)-coated coverslips and incubation

for 30 minutes. The coverslips were then washed with PBS to remove non-adhered platelets. Adhered platelets were fixed with 0.2% formyl saline for 10 minutes prior to permeabilisation with 0.2% Triton X-100 for five minutes at room temperature. Adhered platelets were stained with Alexa Fluor 488-conjugated phalloidin for 30 minutes at room temperature. The coverslips were then mounted onto slides and scanned using a Nikon A1-R confocal microscope (60x objective). Ten random fields of view were recorded for each sample. The data were analysed to quantify the number of adhered and spread platelets, and the relative area of spread platelets using ImageJ. The relative surface area of spread platelets was obtained by subtracting the surface area of resting platelets.

Statistical analysis

Data obtained in this study are represented as mean ± SEM. The statistical significance was analysed using two-tailed unpaired Student's *t* test for two-sample comparisons for the data obtained from the flow cytometric assay for FPR1 expression and platelet receptor characterisation and cAMP assay. For multiple comparisons, statistical significance was established using one-way or two-way ANOVA followed by Bonferroni's correction for data obtained from *in vitro* thrombus formation, fMLF binding, ATP release, platelet aggregation and activation. Data obtained from the tail bleeding assay were analysed using a non-parametric Mann-Whitney test. All statistical analyses were performed using Graphpad Prism 7 software (GraphPad Software Inc., USA).

References

1. Gao JL, Lee EJ and Murphy PM. Impaired antibacterial host defense in mice lacking the N-formylpeptide receptor. *J Exp Med*. 1999;189:657-62.
2. Dufton N, Hannon R, Brancaleone V, Dalli J, Patel HB, Gray M, D'Acquisto F, Buckingham JC, Perretti M and Flower RJ. Anti-inflammatory role of the murine formyl-peptide receptor 2: ligand-specific effects on leukocyte responses and experimental inflammation. *J Immunol*. 2010;184:2611-9.
3. Spyridon M, Moraes LA, Jones CI, Sage T, Sasikumar P, Bucci G and Gibbins JM. LXR as a novel antithrombotic target. *Blood*. 2011;117:5751-5761.
4. Vaiyapuri S, Jones CI, Sasikumar P, Moraes LA, Munger SJ, Wright JR, Ali MS, Sage T, Kaiser WJ, Tucker KL, Stain CJ, Bye AP, Jones S, Oviedo-Orta E, Simon AM, Mahaut-Smith MP and Gibbins JM. Gap Junctions and Connexin Hemichannels Underpin Hemostasis and Thrombosis. *Circulation*. 2012;125:2479-+.
5. Vaiyapuri S, Moraes LA, Sage T, Ali MS, Lewis KR, Mahaut-Smith MP, Oviedo-Orta E, Simon AM and Gibbins JM. Connexin40 regulates platelet function. *Nature communications*. 2013;4:2564.

3 – The endogenous antimicrobial cathelicidin LL37 underpins thrombotic complications during inflammatory diseases

The endogenous antimicrobial cathelicidin LL37 underpins thrombotic complications during inflammatory diseases

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3.1 Abstract

Platelet-associated complications including thrombosis, thrombocytopenia and haemorrhage are commonly observed during various inflammatory diseases such as psoriasis, sepsis and inflammatory bowel disease. Despite the reported evidence on numerous mechanisms/molecules that may contribute to the dysfunction of platelets, the primary mechanisms that underpin the platelet-associated complications during inflammatory diseases are yet to be determined. Here, we report the discovery of a formyl peptide receptor, FPR2/ALX in platelets, and its primary role in the development of platelet-associated complications via ligation with its ligand, LL37. LL37 acts as a powerful endogenous antimicrobial peptide but it also regulates innate immune responses. We demonstrate the impact of LL37 on the modulation of platelet reactivity, haemostasis, and thrombosis under pathophysiological conditions. LL37 activates a range of platelet functions, enhances thrombus formation, and shortens the tail-bleeding time in mice. By utilising a pharmacological inhibitor and *Fpr*2/3 (an orthologue of human FPR2/ALX)-deficient mice, the functional dependence of LL37 on

FPR2/ALX was determined. Our data demonstrate the overexpression of mCRAMP (an LL37 murine orthologue) in affected skin and plasma of a murine [imiquimod(IMQ)-induced] model of human psoriasis and its ability to enhance platelet responses via *Fpr2/3*. Since the level of LL37 is increased in numerous inflammatory diseases, these results point towards a critical role for LL37 and FPR2/ALX in the development of platelet-related complications in such diseases. Hence, a better understanding of the clinical relevance of LL37 and FPR2/ALX in diverse pathophysiological settings will pave the way for the development of improved therapeutic strategies for a range of inflammatory diseases.

3.2 Introduction

Platelets (small circulating blood cells) play pivotal roles in the regulation of haemostasis. However, their inappropriate activation under pathological conditions leads to the formation of blood clots (thrombosis) within the circulation, which is a major cause of premature death[1, 2]. Platelets also play significant roles in the regulation of innate immunity, inflammatory responses and microbial infection[3, 4]. Activation of platelets during inflammatory diseases such as psoriasis induces the formation of blood clots or disseminated intravascular coagulation in capillaries, resulting in the blockage of blood supply to tissues[5, 6]. Moreover, platelet activation results in the aggregation and sequestration of platelets, thus instigating thrombocytopenia[7, 8]. Several mechanisms have been reported to be contributing to the dysfunction of platelets under inflammatory diseases, however, the primary mechanisms that underpin the platelet activation are not yet established. In this study, we discovered the presence of FPR2/ALX in platelets and its ability to activate platelets via its ligand, the human antimicrobial cathelicidin, LL37.

Cathelicidins are a group of antimicrobial peptides produced by numerous mammalian cells in response to microbial infections[9-11]. Among these, the human cationic antimicrobial peptide 18 (hCAP18) is the only cathelicidin known to be expressed in human cells[12]. The expression of hCAP18 has been reported in cell types that remain in contact with the external environment, including epithelial cells in airways, intestine and skin, and innate immune system cells such as neutrophils, natural killer cells and monocytes[11, 13]. Normally, hCAP18 is synthesised as an inactive precursor and stored in the granules of immune cells[14, 15]; higher levels of hCAP18 have been found in neutrophils[16]. During microbial infection, the activation of immune cells releases hCAP18 to the external milieu, where it is processed by proteolytic enzymes (e.g. proteinase 3) to liberate the short, active 37 amino acid peptide LL37 (so called as it has two N-terminal leucine residues)[15, 17]. LL37 acts as a powerful antimicrobial peptide against bacteria[18], fungi[19] and viral particles[20]. Additionally, it modulates innate and adaptive immune responses by stimulating specific receptor-mediated signalling within the immune cells[21]. LL37 modulates these responses predominantly through formyl peptide receptor 2 (FPR2/ALX)[22]. Other receptors such as CXCR2[23], P2Y11[24] and P2X7[25] have also been reported to

bind LL37. The binding of LL37 activates immune cells and thus exacerbates inflammatory responses to accelerate the clearance of infection[11, 13]. Despite detailed research on the roles of LL37 in the modulation of inflammatory responses in various pathological settings, specifically psoriasis[26, 27], the effects of LL37 in the regulation of thrombosis and other platelet-related complications remained unknown for a long time. During the preparation of this manuscript, we have identified a very recent article describing a role for cathelicidins in the regulation of thrombo-inflammation, confirming some of our findings[28].

Since the level of LL37 released during inflammation is significantly higher than normal[15, 16], understanding its critical functions in the modulation of platelet reactivity will pave the way for the determination of the fundamental mechanisms underlying platelet-related complications in various inflammatory diseases and offer the potential for the development of improved therapeutic strategies for such diseases. In this study, we investigated the effects of LL37 at concentrations ($\leq$ 50 μM) that are relevant to several pathological conditions (including psoriasis[29]) on a range of platelet functional assays, and established its roles in the modulation of platelet reactivity, thrombosis and haemostasis under physiological and pathological conditions such as psoriasis using an appropriate disease model.

3.3 Results

3.3.1 Platelets store LL37 and release it upon activation

The expression of LL37 has been reported in several cell types including neutrophils where it is mainly stored in granules[30, 31]. Notably, the expression of cathelicidin antimicrobial peptide (CAMP) has been reported in human and mouse platelets at the transcript level[32]. Hence, to determine whether LL37 is present in platelets, a range of experiments were performed. The presence of LL37 in platelets was confirmed by immunofluorescence microscopy. Human isolated platelets were stained with antibodies against LL37 and phalloidin (a marker for actin), wherein LL37 appeared to be dispersed in the cytoplasm (Figure 3-1A). In order to further determine whether platelets possess LL37 and release it upon activation, an enzyme-linked immunosorbent assay (ELISA) was performed using selective antibodies for LL37. Resting and activated [by 1µg/mL cross-linked collagen-related peptide (CRP-XL)] human platelets were centrifuged, and the supernatant and platelet pellet were separated to analyse the level of LL37 by ELISA. In the resting state, the presence of LL37 was significantly higher in the lysed platelet pellet (1559±433 pM) compared to the supernatant (41±12 pM) (Figure 3-1B). However, upon activation of platelets with CRP-XL, the presence of LL37 was significantly increased in the supernatant (1490±581.7 pM) in comparison to the lysed platelet pellet (59±7.6 pM). In order to corroborate these results, the release of LL37 in human plasma (in the presence or absence of platelets) was investigated over a two-hour period by mass spectrometry. The level of LL37 was stable and significantly increased in platelet-rich plasma (PRP) over two hours compared to platelet-poor plasma (PPP), indicating its release from platelets; by contrast the level of LL37 was significantly reduced in PPP over two hours (Figure 3-1C). Together, these data confirm the presence of LL37 in platelets (between pM and nM concentrations), and its release upon agonist-induced platelet activation. Although the main source of LL37 may be neutrophils and other cells, platelets may also contribute to the elevation of LL37 upon activation within the local environment.

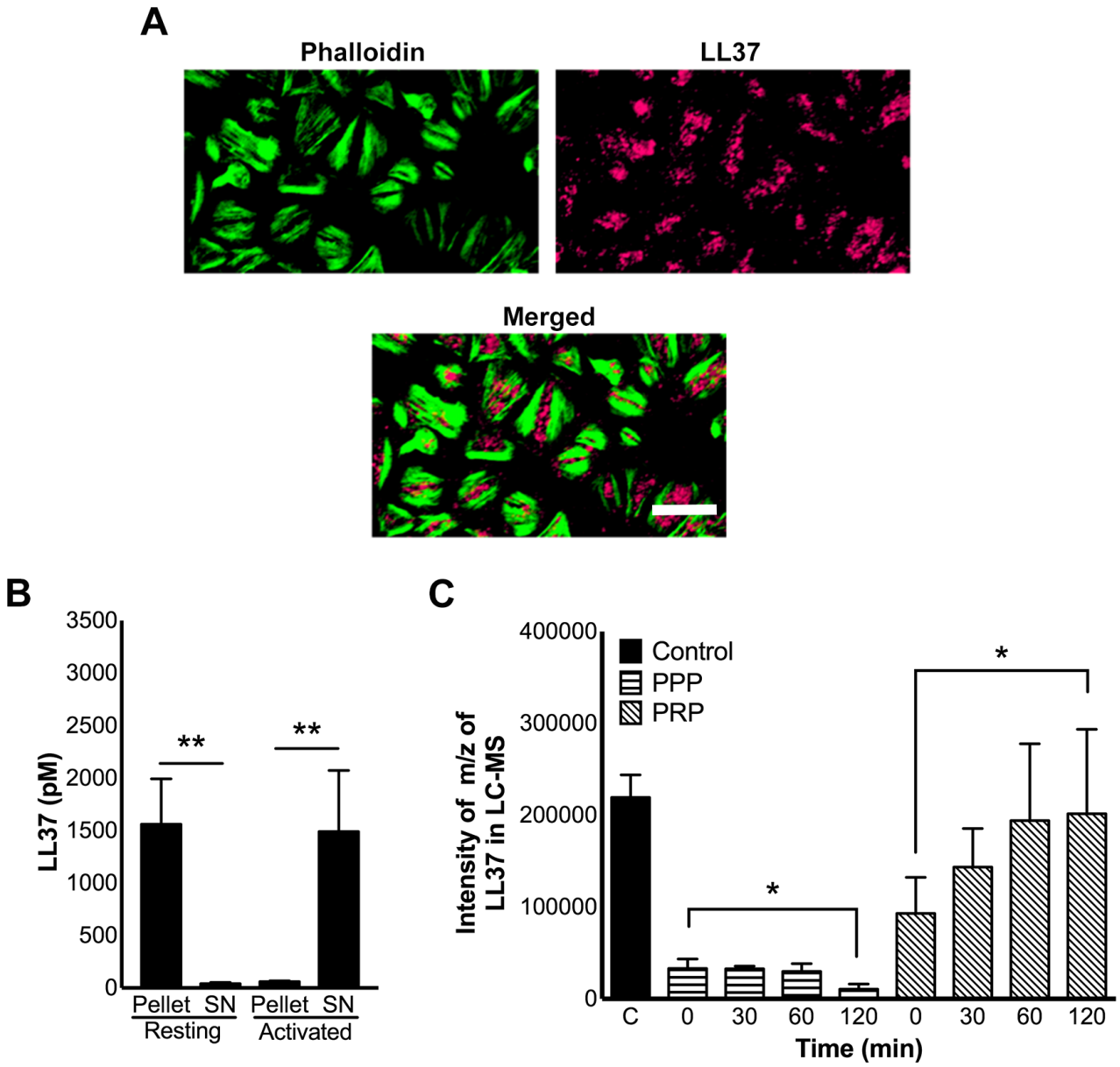

Figure 3-1: **Presence of LL37 in platelets. A**, Human platelets were stained with antibodies against LL37 (purple) and phalloidin (green) and analysed by confocal microscopy (100x magnification; scale bar - 10 µm). Images shown are representative of three independent experiments. **B**, the level of LL37 in resting and activated (1 µg/mL CRP-XL) platelet pellets and supernatants (SN) was measured by an ELISA using LL37-selective antibodies. Data represent mean ± SEM (n=5). **C**, the stability/release of LL37 in plasma was analysed by mass spectrometry (LC-MS). Graph indicates LL37 intensities (100µg/mL) spiked in platelet-rich plasma (PRP) and platelet-poor plasma (PPP) at different time points over 120 minutes. Control represents the intensity of LL37 at 100 µg/mL (unspiked). Data represents mean ± SEM (n=3). The statistical significance was established by one-way ANOVA followed by Bonferroni's correction except for the data shown in panel **B**, which was analysed by two-tailed unpaired Student's *t* test (*p<0.05 and **p<0.01).

3.3.2 LL37 augments thrombus formation under arterial flow conditions

Disseminated intravascular coagulation or thrombosis in microvasculature is a common phenomenon during inflammatory diseases such as psoriasis[33]. Therefore, to determine whether LL37 has a direct influence on thrombotic complications during inflammatory diseases, its effects on thrombus formation under arterial flow conditions were investigated using a microfluidics system by fluorescence microscopy. Human $DiOC_6$-labelled whole blood was pre-incubated with a scrambled peptide or different concentrations of LL37 (10, 20 and 50 μM) for 10 minutes prior to perfusion over collagen-coated Vena8™ biochips. Thrombus formation was monitored for 10 minutes by acquiring fluorescent images every 30 seconds (Figure 3-2i). LL37 significantly increased the mean fluorescence intensity of thrombi in a concentration dependent manner (Figure 3-2ii); the highest concentration of LL37 used (50 μM) increased the thrombus intensity by approximately 70% compared to the vehicle-treated samples (Figure 3-2iii). These data demonstrate a direct role for LL37 in the augmentation of thrombus formation under arterial flow conditions in human whole blood.

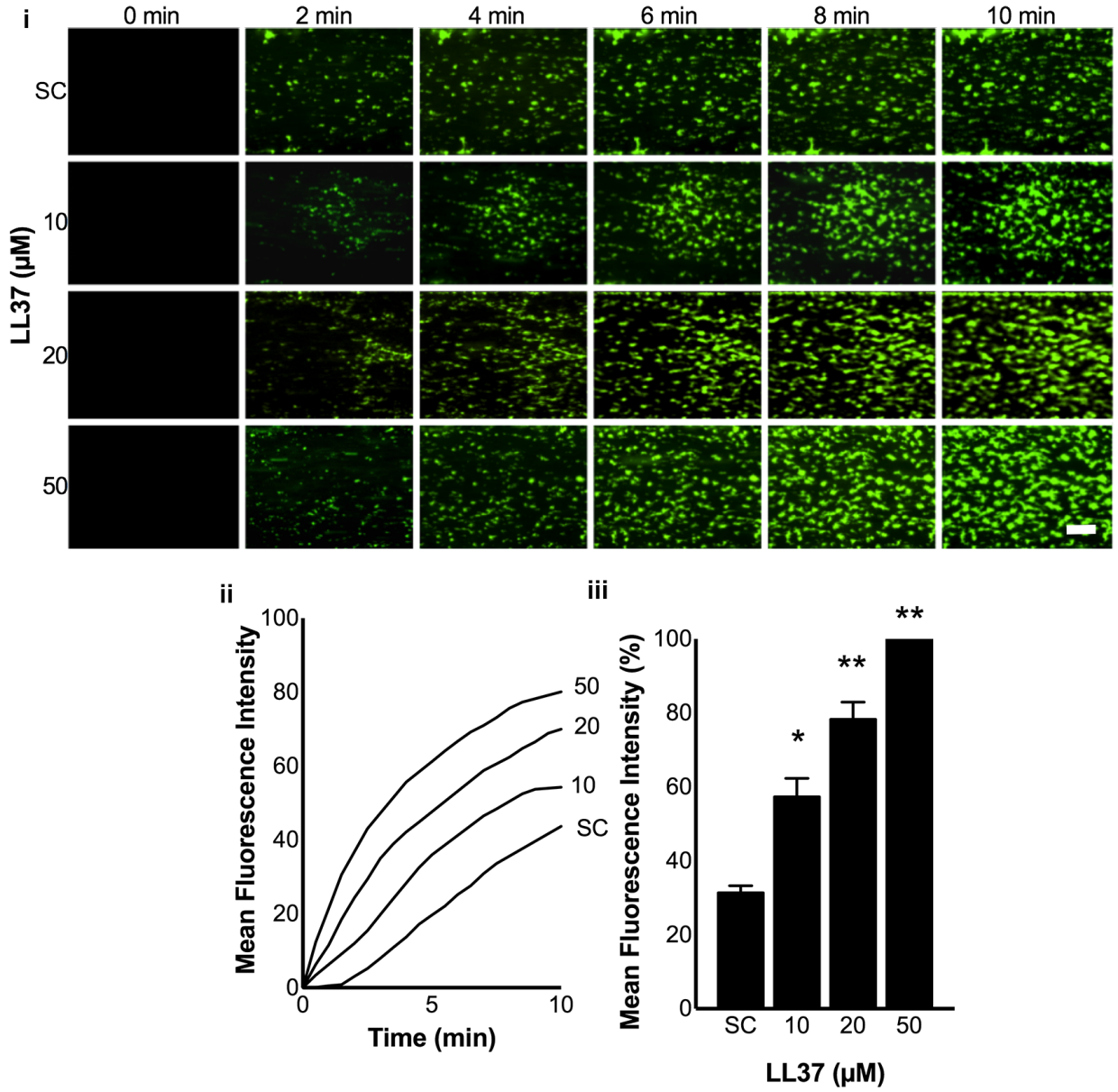

Figure 3-2: **The impact of LL37 on thrombus formation.** The effects of LL37 in the modulation of thrombus formation. Human $DiOC_6$-labelled whole blood was pre-incubated with a scrambled peptide or LL37 (10, 20 and 50 μM) for 10 minutes prior to perfusion over collagen-coated (400 μg/mL) Vena8™ Biochips. Images (**i**) (at 10 minutes) shown are representative of three separate experiments (10x magnification; scale bar - 10 μm). Data represent mean ± SEM (n=3). The statistical significance was established by one-way ANOVA followed by Bonferroni's (*p<0.05 and **p<0.01).

3.3.3 Role of LL37 in the modulation of haemostasis in mice

Since LL37 directly influenced the thrombus formation under arterial flow conditions, its effects in the modulation of haemostasis in mice under physiological conditions were determined by tail bleeding assay. Following the administration of anaesthetics to C57BL/6 mice, 20 μM of scrambled peptide or LL37 was infused via the femoral artery five minutes prior to the dissection of 1mm tail tip and the measurement of bleeding time. A mean bleeding time of 371±47 seconds was observed in the vehicle-treated group, however the infusion of LL37 significantly shortened the bleeding time in mice to a mean of 225±19 seconds (Figure 3-3). These data demonstrate the effects of LL37 in the modulation of haemostasis in mice under physiological settings, and reflect its probable function in the modification of haemostatic responses during inflammatory diseases.

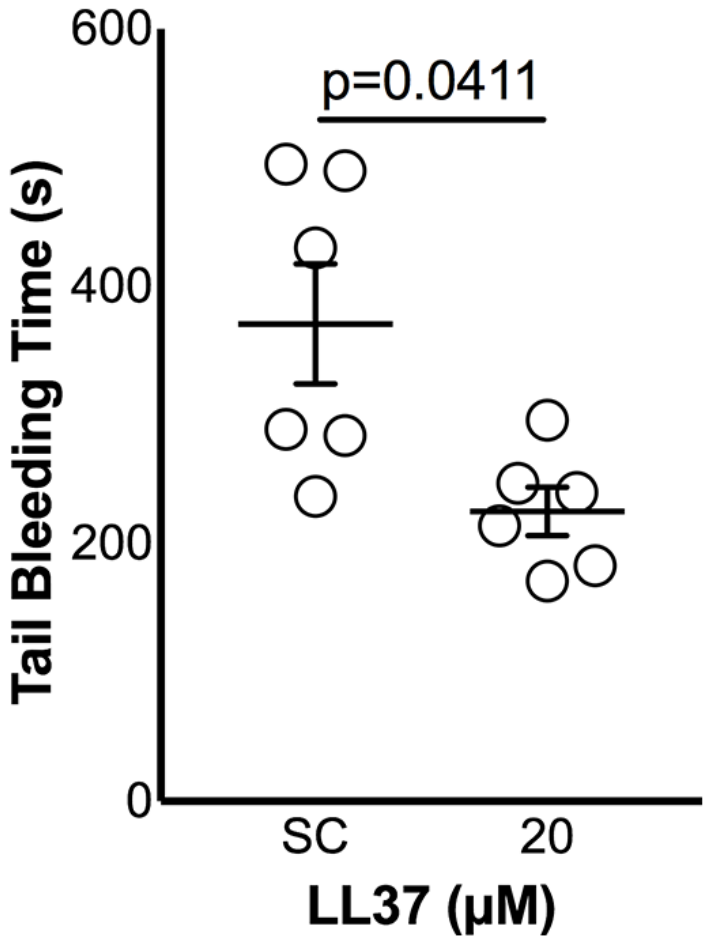

Figure 3-3: **The impact of LL37 on haemostasis.** The impact of LL37 (20 μM) on the modulation of haemostasis. C57BL/6 mice (10-12 weeks old) were anaesthetised 20 minutes before the infusion of a scrambled peptide or LL37 (20 μM) via femoral artery 5 minutes before the dissection of 1 mm of tail tip, and monitoring of time to cessation of bleeding. Data represent mean ± SEM (n=6 per group). The statistical significance was established by non-parametric Mann-Whitney test.

3.3.4 LL37 induces platelet activation

Following the confirmation of the effects of LL37 in the modulation of thrombus formation and haemostasis, a range of platelet functional assays were performed to determine the role of LL37 in the regulation of distinctive platelet functions. To examine the effects of LL37 on platelet activation, aggregation assays were performed using human isolated platelets. The platelets were treated with a vehicle control or different concentrations of LL37 (5, 10 and 20 μM) and the platelet aggregation was monitored for 5 minutes by optical aggregometry. The addition of LL37 directly induced platelet aggregation in a concentration-dependent manner. Maximum aggregation (100%) was obtained with 20 μM LL37 at 5 minutes in isolated human platelets (Figure 3-4). These data confirm the activatory effects of LL37 in platelets.

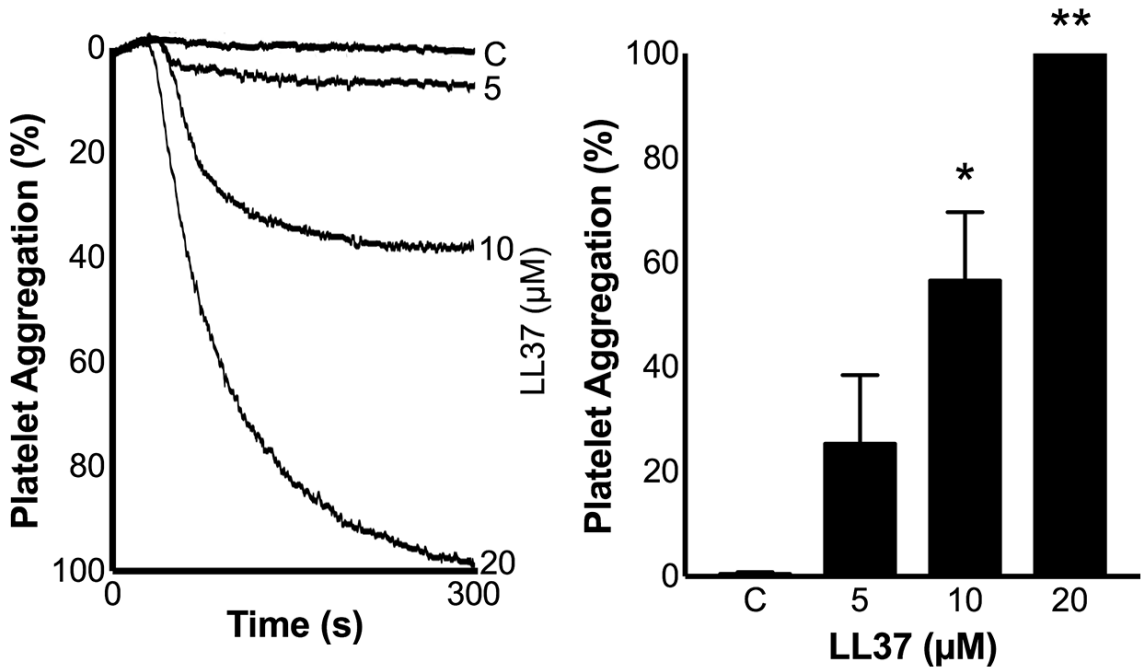

Figure 3-4: **The impact of LL37 on platelet aggregation.** The effects of LL37 on platelet activation was measured by optical aggregometry using human isolated platelets. Data represent mean ± SEM (n=6). The statistical significance was established by one-way ANOVA followed by Bonferroni's correction (*p<0.05. **p<0.01 and ***p<0.001).

3.3.5 Effect of LL37 on inside-out signalling to integrin αIIbβ3 and α-granule secretion in platelets

Platelet activation triggers inside-out signalling to integrin αIIbβ3 on the platelet surface and converts it to a high affinity state to allow fibrinogen binding and subsequent platelet aggregation[34]. To determine whether LL37 influences the inside-out signalling in platelets, the level of fibrinogen binding was measured on the platelet surface (as a marker for inside-out signalling to integrin αIIbβ3) using FITC-conjugated fibrinogen antibodies by flow cytometry. Indeed, LL37 increased the level of fibrinogen binding in a concentration-dependent manner in human isolated platelets (Figure 3-5Ai). A minimum concentration of 5 μM LL37 significantly increased fibrinogen binding compared to the resting platelets, and the highest response was obtained at 50 μM LL37. Furthermore, to determine whether the presence of plasma proteins intercedes with the response of LL37 on platelets, the effect of LL37 on platelet activation was analysed using PRP by measuring the level of fibrinogen binding. Similar to the findings observed in isolated platelets, LL37 increased the level of fibrinogen binding in a concentration-dependent manner in PRP (Figure 3-5Aii). Together, these data suggest that LL37 stimulates inside-out signalling to integrin αIIbβ3, and thus increases fibrinogen binding upon platelet activation, and its function is unaffected by the presence of plasma proteins.

Platelets contain three distinct types of granules and upon activation of platelets, the contents of such granules are released in order to enhance the secondary activation of additional platelets and recruit them to the developing thrombus. To determine the impact of LL37 on granule secretion, the level of P-selectin exposure (as a marker for α-granule secretion) was measured by flow cytometry. The results indicate that LL37 induced α-granule secretion in human isolated platelets (Figure 3-5Bi) and PRP (Figure 3-5Bii) in a concentration-dependent manner. These data confirm the impact of LL37 in the modulation of α-granule secretion.

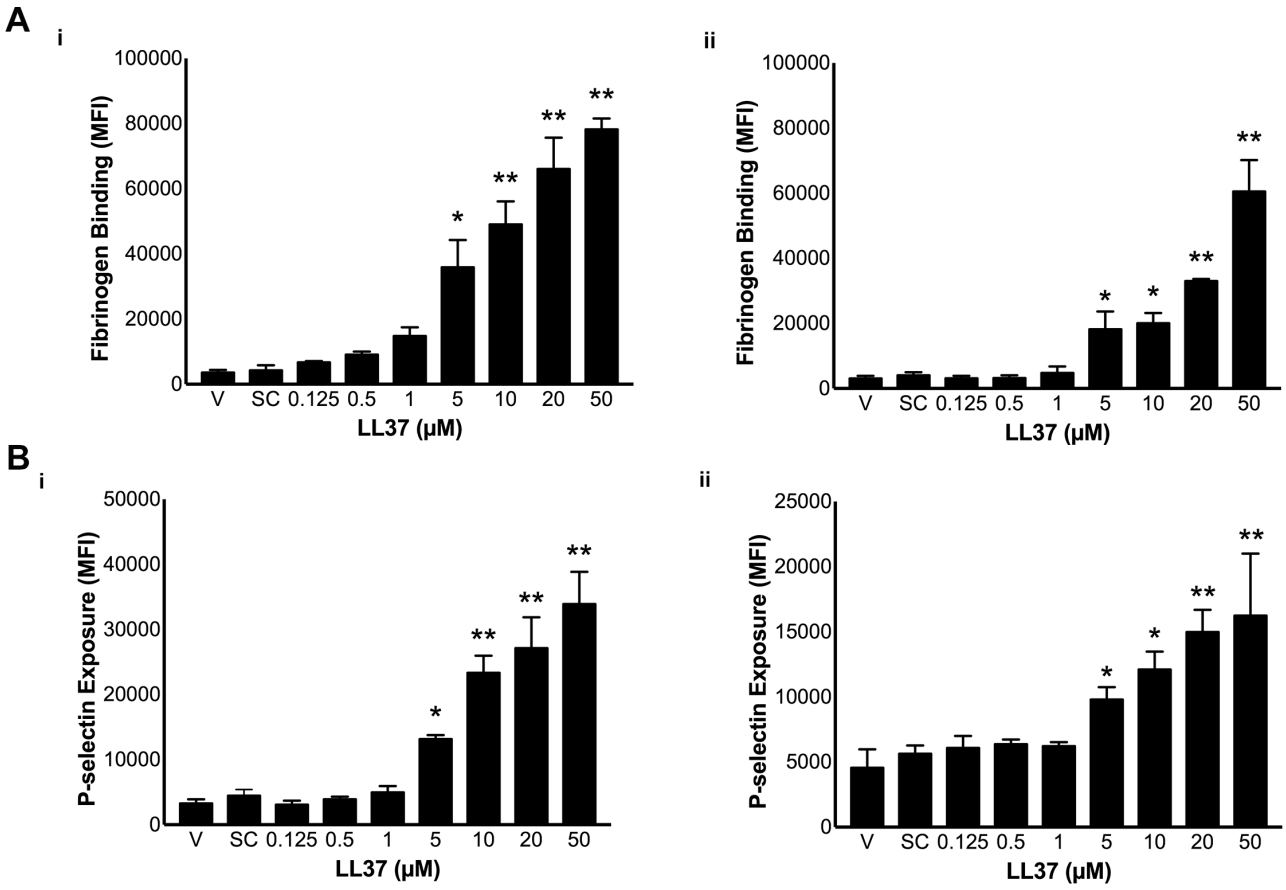

Figure 3-5: **The impact of LL37 on platelet activation. A**, the level of fibrinogen binding was analysed using FITC-conjugated fibrinogen antibodies by flow cytometry in human isolated platelets (**Ai**) or PRP (**Aii**). **B,** the level of P-selectin exposure was measured in human isolated platelets (**Bi**) or PRP (**Bii**) using PECy5-labelled P-selectin antibodies by flow cytometry. Data represent mean ± SEM (n=3). The statistical significance was established by one-way ANOVA followed by Bonferroni's correction (*p<0.05 and **p<0.01).

3.3.6 Impact of LL37 on integrin αIIbβ3-mediated outside-in signalling to platelets

Following fibrinogen binding, the integrin αIIbβ3 transduces signalling responses into platelets to allow their spreading and clot retraction. The potential of LL37 to mediate such signalling responses was measured by their ability to induce adhesion and spreading of platelets on immobilised fibrinogen under static conditions (Figure 3-6). Upon stimulation of platelets with different concentrations of LL37 (5, 10 and 20 μM), a significant increase in the number of adhered platelets to fibrinogen was observed compared to the controls (Figure 3-6i). Furthermore, LL37 displayed a marked increase in the number (Figure 3-6ii) and the relative surface area (Figure 3-6iii) of spread platelets compared to the controls. These data demonstrate the role of LL37 in the modulation of integrin αIIbβ3-mediated outside-in signalling to platelets.

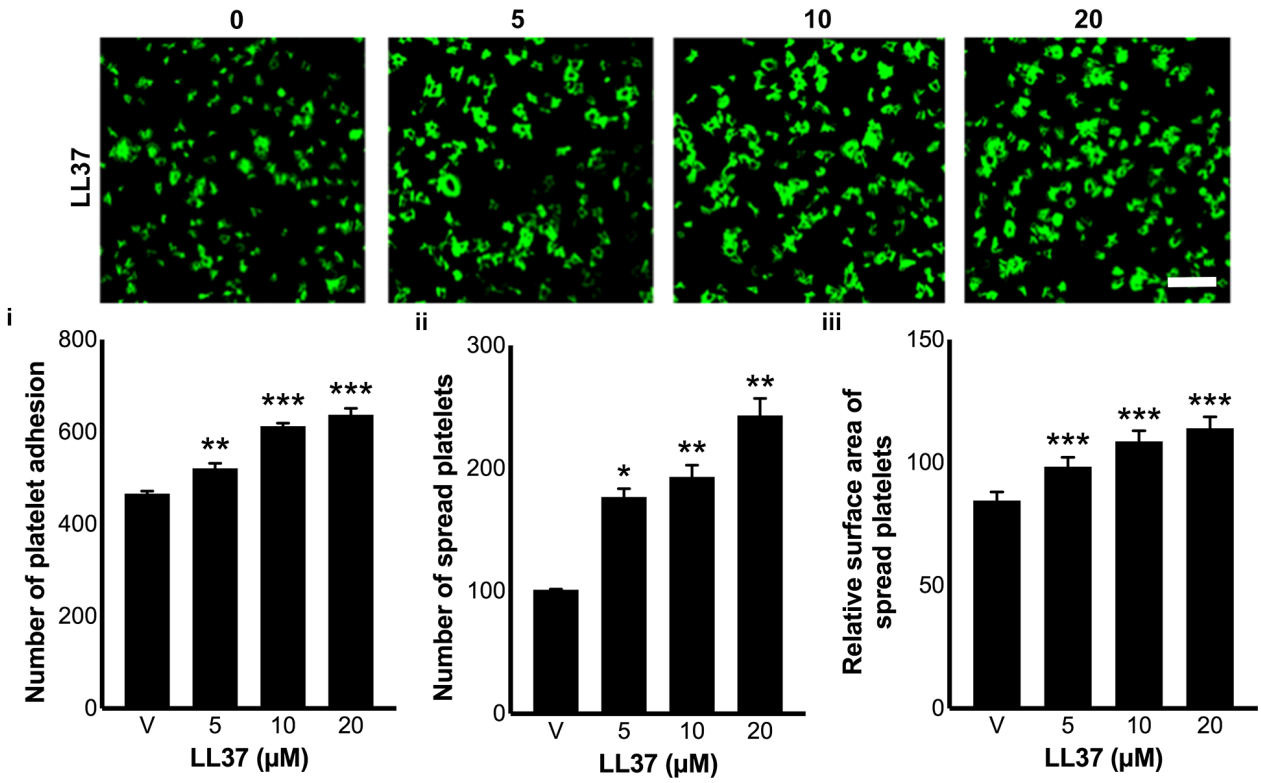

Figure 3-6: **The impact of LL37 on platelet spreading.** Platelet adhesion and spreading on immobilised fibrinogen was analysed by using platelets treated with LL37 (5, 10 and 20 µM) by confocal microscopy (60x magnification; scale bar - 10 µm). The number of adhered (**i**) and spread platelets (**ii**), and the relative surface area of spread platelets (**iii**) was determined via analysing the images using ImageJ. Ten random fields of view were recorded and analysed for each sample. Data represent mean ± SEM (n=3). The statistical significance was established by one-way ANOVA followed by Bonferroni's correction (*p<0.05, **p<0.01 and ***p<0.001.

3.3.7 Intracellular calcium levels were elevated by LL37 in human platelets

The activation of platelets results in the mobilisation of Ca^{2+} stored in the dense tubular system in platelets and the influx of extracellular Ca^{2+} across the plasma membrane[35]. Such elevation of intracellular Ca^{2+} levels plays a pivotal role in the regulation of platelet function including shape change, degranulation, aggregation and ultimately thrombus formation[36]. To assess the effects of LL37 in the modulation of calcium mobilisation, intracellular calcium levels were measured in Fluo-4 AM dye-loaded human isolated platelets. LL37 induced calcium mobilisation in a concentration-dependent manner with a maximum effect achieved with 50 μM. The level of calcium release obtained with 50 μM LL37 is similar to the level obtained with a GPVI-selective agonist, CRP-XL (1 μg/mL), although the initial kinetics of calcium release appeared to be faster for LL37 (Figure 3-7). These data suggest that LL37 is involved in the elevation of intracellular calcium levels upon platelet activation.

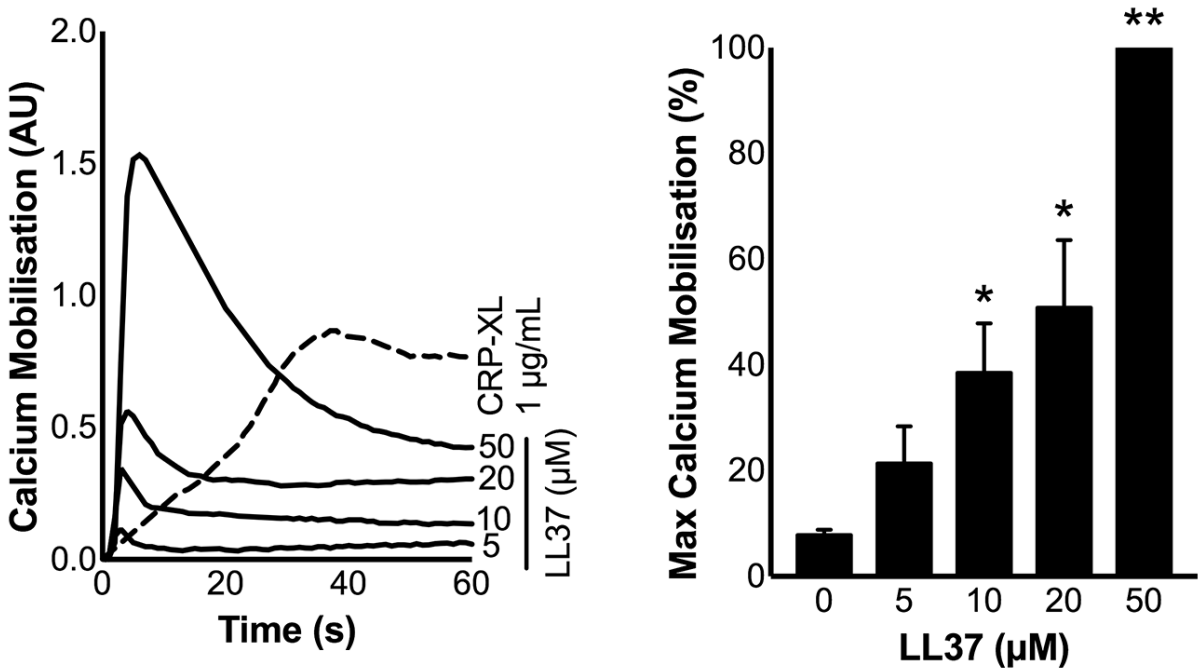

Figure 3-7: **The impact of LL37 on calcium mobilisation.** Ca^{2+} mobilisation was measured using Fluo-4 AM dye-loaded human isolated platelets upon stimulation with LL37 by spectrofluorimetry. Data represent mean ± SEM (n=4). The statistical significance was established by one-way ANOVA followed by Bonferroni's correction (*p<0.05 and **p<0.01).

3.3.8 LL37 does not exhibit cytotoxic effects in platelets at lower concentrations

A previous study[37] reported the inhibitory effects of LL37 in platelets at exceptionally high concentrations including 0.1, 0.3, 0.6 and 1.2 mM. From the literature[38], it is apparent that such concentrations are not relevant to pathological conditions except in psoriasis, where the levels of LL37 can reach up to 300 μM in affected skin tissues[39]. In addition, it has been shown previously that LL37 exhibits cytotoxic effects in eukaryotic cells, such as neutrophils and monocytes at concentrations of higher than 50 μM[40]. Such toxic effects may well be the reason behind the inhibitory effects of LL37 demonstrated in the previous study[37]. In order to determine whether the concentrations of LL37 used in the present study (≤50 μM) exhibit any cytotoxic effects in platelets, lactate dehydrogenase (LDH) cytotoxicity assay was performed. Human isolated platelets were pretreated with different concentrations of LL37. The level of LDH released was measured using a LDH cytotoxicity assay kit. The various concentrations of LL37 used in this study (1, 5, 10, 20 and 50 μM) failed to exert any significant cytotoxic effects in human isolated platelets, although 100 μM LL37 displayed significant toxicity in platelets (Figure 3-8). These results confirm that LL37 at concentrations up to 50 μM do not display any toxic effects in platelets, although higher concentrations can exhibit cytotoxic effects and thus reduction in platelet function. Such a reduction in platelet function through cytotoxicity may lead to bleeding at conditions where the level of LL37 is increased (100 μM or more).

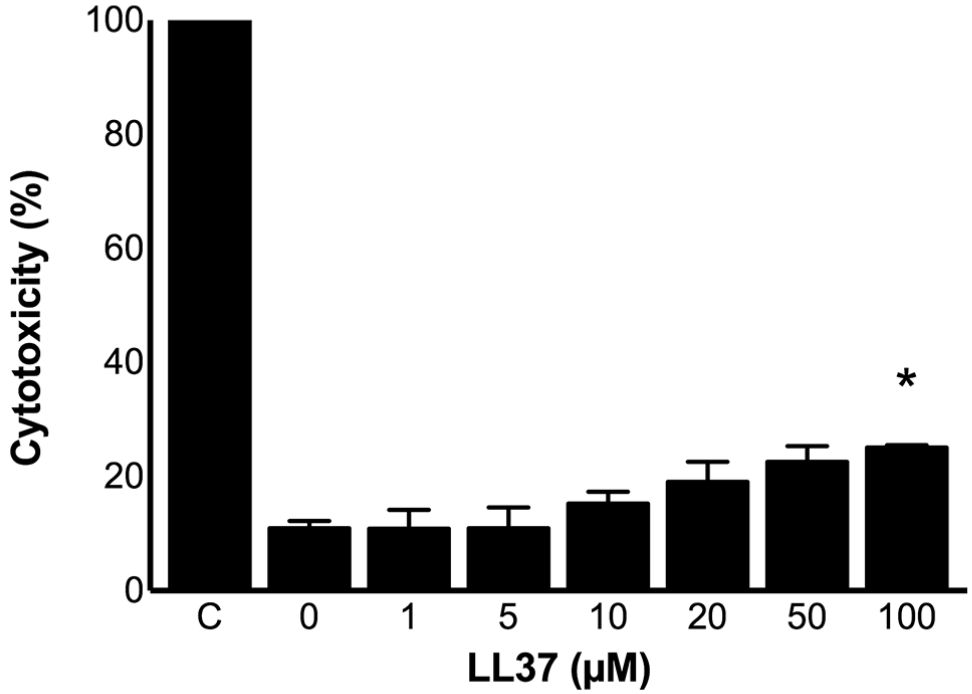

Figure 3-8: **The effect of LL37 on platelet cytotoxicity.** The cytotoxic effects of LL37 were measured in human isolated platelets using a LDH cytotoxicity assay kit. Data represent mean±SEM (n=4). The statistical significance was established by one-way ANOVA followed by Bonferroni's correction (*p<0.05).

3.3.9 Expression of FPR2/ALX in platelets

A large number of studies indicate that LL37 acts primarily through FPR2/ALX to exert its effects in immune cells[22, 41, 42]. The expression of FPR2/ALX in megakaryocytes and human and mouse platelets at transcript level has been reported previously[43,32]. Here, we confirmed the presence of FPR2/ALX in human platelet lysates using immunoblotting analysis (Figure 3-9i) and on their surface by flow cytometry (Figure 3-9ii). Notably, the activation of platelets with 1 µg/mL CRP-XL increased the level of FPR2/ALX on the platelet surface as determined by flow cytometry although the level of protein identified by immunoblots remained unchanged. Additionally, we were able to confirm the presence of FPR2/ALX in mouse platelet lysates, and its absence in *Fpr2/3*$^{-/-}$ (*Fpr2/3* is an orthologue to human FPR2/ALX) mice that were utilised in this study (Figure 3-9iii). These data confirm the presence of FPR2/ALX in platelets at protein level, and their increase on the platelet surface upon activation, which may be due to their presence in granules and/or the open canalicular system (OCS).

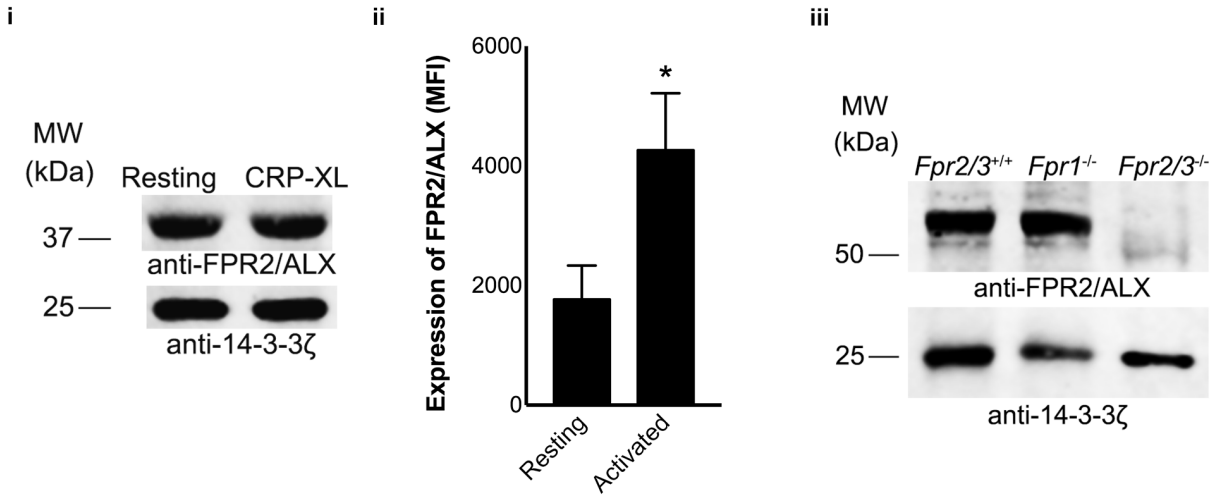

Figure 3-9: **Expression of FPR2/ALX in platelets.** The presence of FPR2/ALX was confirmed in human (**i**) and mouse (**iii**) platelet lysates by immunoblot analysis using selective antibodies. The blots are representative of three separate experiments. The expression of FPR2/ALX on the surface of resting or activated (1 μg/mL CRP-XL) human platelets was analysed using FPR2/ALX-selective and fluorescent-labelled secondary antibodies by flow cytometry (**ii**). Data represent mean ± SEM (n=4). The statistical significance was calculated using a two-tailed unpaired Student's *t* test was used (*p<0.05).

3.3.10 LL37 activates platelets through FPR2/ALX

In order to determine the functional dependence of LL37 on FPR2/ALX, the binding of LL37 to the platelet surface was confirmed using a fluorescent-labelled LL37 (5-FAM-LC-conjugated LL37) by flow cytometry. 20 μM 5-FAM-LL37 displayed marked binding to the surface of human platelets compared to a fluorescence-labelled scrambled LL37 (5-FAM-conjugated sc-LL37) (Figure 3-10i). Similarly, LL37 (20 μM) binding was analysed using platelets obtained from control, *Fpr1*- or *Fpr2/3*-deficient mouse platelets. The platelets obtained from control and *Fpr1*$^{-/-}$ mice exhibited marked binding to 5-FAM-LL37 compared to *Fpr2/3*$^{-/-}$ mouse platelets (Figure 3-10ii). Furthermore, the interactions between LL37 and FPR2/ALX was examined by molecular docking analysis using a homology model of human FPR2/ALX, which was generated based on the structural template of human delta opioid 7 transmembrane receptor (PDB code- 4N6H; 1.8 Å) using MODELLER-ModWeb server[44]. The docking analysis using PatchDock server predicted that the LL37 peptide forms prominent hydrogen bonds with key residues such as Gln-89, Ser-182, Asn-285 and Gly-275 of FPR2/ALX, that are identified to be crucial for receptor activation (Figure 3-10iii and Table 1). Together, these data confirm the binding of LL37 primarily to FPR2/ALX in human and mouse platelets.

Following the corroboration of LL37 binding to FPR2/ALX, the functional dependence of LL37 on this receptor was analysed by using a range of platelet functional assays. The activatory effects of LL37 (5, 10, 20 and 50 μM) were substantially reduced in *Fpr2/3*$^{-/-}$ mouse platelets both in isolation (Figure 3-11Ai) or whole blood (Figure 3-11Aii) compared to the controls as analysed by fibrinogen binding using flow cytometry. Similar results were obtained by analysing P-selectin exposure levels in mouse platelets in isolation (Figure 3-11Bi) or whole blood (Figure 3-11Bii). Notably, the characterisation of platelets obtained from *Fpr2/3*$^{-/-}$ mice failed to display any defects in the size and number of platelets or the levels of major platelet receptors such as GPVI (Figure 3-12i), GPIbα (Figure 3-12ii), αIIbβ3 (Figure 3-12iii) and α2β1 (Figure 3-12iv) in comparison to the control mouse platelets.

To further analyse the functional dependence of LL37 on FPR2/ALX, specific platelet functional assays were performed in the presence of a selective antagonist to FPR2/ALX (WRWWWW [WRW_4]) in human and mouse platelets. The addition of WRW_4 (5 μM) in human isolated platelets prior to activation with 20 μM LL37 inhibited platelet aggregation by approximately 40% (Figure 3-13). Similarly, the effects of LL37 (20 μM) on fibrinogen binding (Figure 3-14i) and P-selectin exposure (Figure 3-14ii) were significantly reduced in WRW_4-treated (5 μM) control platelets. These data demonstrate the involvement of FPR2/ALX in the regulation of LL37-mediated effects in platelets.

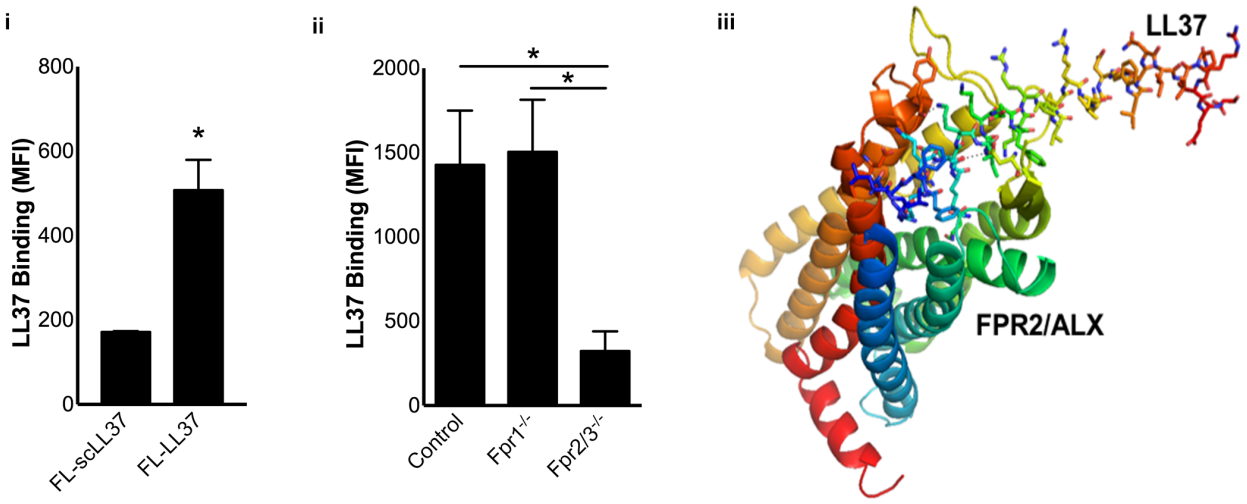

Figure 3-10: **Binding of LL37 to FPR2/ALX in platelets.** The binding of LL37 to platelets was analysed by flow cytometry. Human isolated platelets were incubated with 20μM 5-FAM-LC-conjugated LL37 or scrambled LL37, and the level of binding was analysed by flow cytometry (**i**). Data represent mean ± SEM (n=4). Similarly, platelets obtained from control, *Fpr2/3*$^{-/-}$ and *Fpr1*$^{-/-}$ mice were analysed with 20 μM 5-FAM-LC-conjugated LL37 or scrambled LL37 (**ii**). Data represent mean ± SEM (n=7). The interactions between LL37 and FPR2/ALX was analysed through structural modelling and molecular docking analysis (**iii**). The statistical significance was calculated using one-way ANOVA followed by a two-tailed unpaired Student's *t* test was used (*p<0.05).

Table 1. Summary of polar contacts between LL37 and FPR2/ALX

Hydrogen bond interactions		
Interacting LL37 residues	Interacting FPR2/ALX residues	Average distance (Å)
Gly-3 (N)	Arg-26 (NH2)	2.76
Ser-9 (OG)	Glu-89 (OE1)	3.12
Ser-9 (O)	Glu-89 (OE2)	2.61
Lys-10 (N)	Glu-89 (OE2)	2.81
Ser-9 (OG)	Asn-171 (ND2)	3.00
Gln-22 (NE2)	Ser-182 (N)	3.20
Gln-22 (OE1)	Ser-182 (OG)	2.91
Gln-22 (NE2)	Ser-182 (OG)	3.14
Gln-22 (NE2)	Ser-182 (OG)	3.18
Glu-11 (OE2)	Gly-275 (N)	3.23
Lys-8 (NZ)	Lys-276 (O)	2.53
Arg-7 (NH1)	Asn-285 (OD1)	3.08
Arg-7 (NE)	Asn-285 (OD1)	3.29

N, amide nitrogen; O, carbonyl oxygen; OG, gamma oxygen; NE2, epsilon nitrogen 2; OE1, epsilon oxygen 1; OE2, epsilon oxygen 2; NZ, zeta nitrogen; NH1, eta nitrogen 1; NE, epsilon nitrogen; NH2, eta nitrogen 2; ND2, delta nitrogen 2; OG, gamma oxygen; OD1, delta oxygen 1.

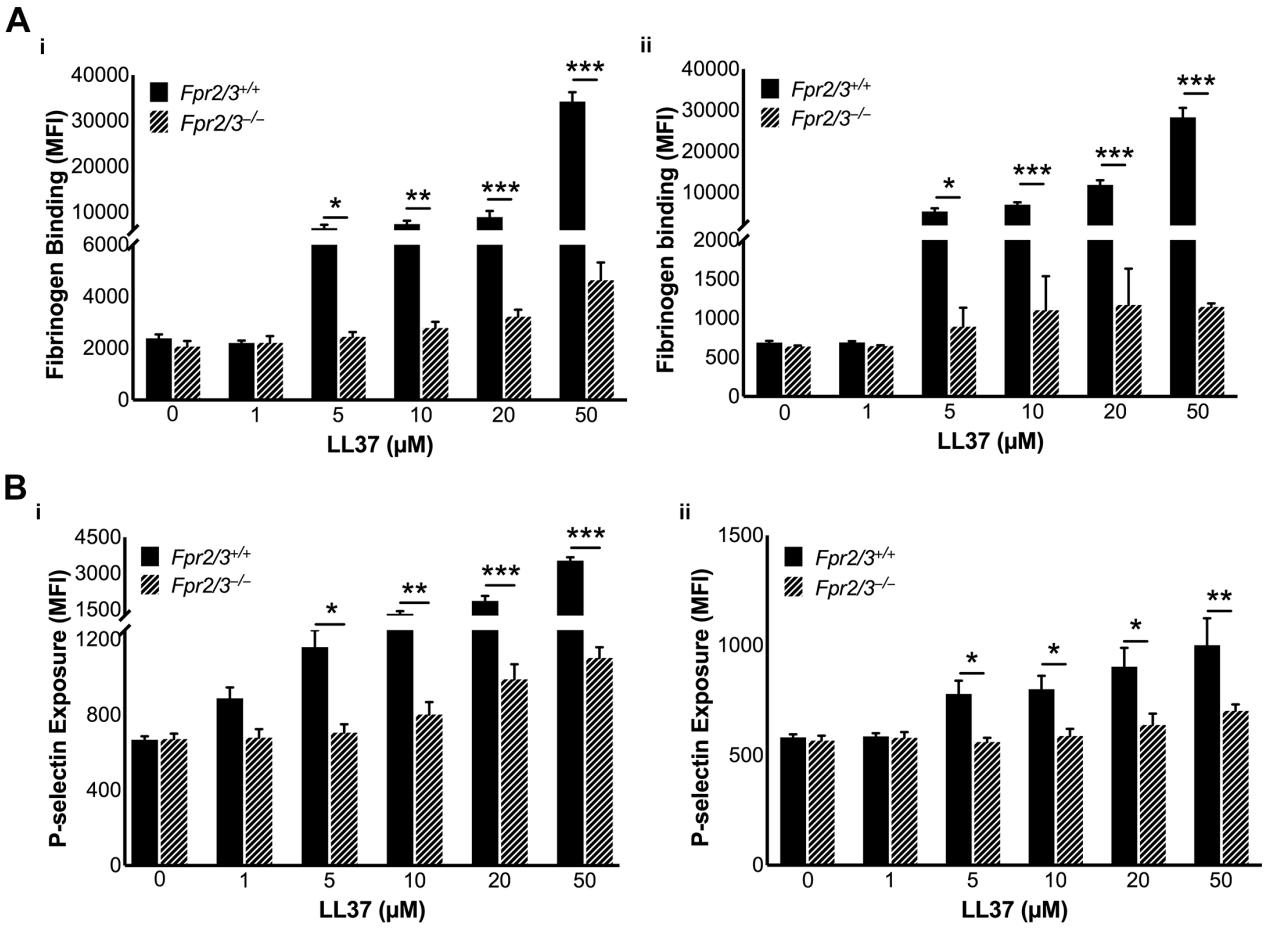

Figure 3-11: **FPR2/ALX-mediated platelet activation by LL37. A,** the level of fibrinogen binding upon stimulation with LL37 in isolated platelets (**Ai**) or whole blood (**Aii**) obtained from *Fpr2/3*$^{-/-}$ or control mice was analysed by flow cytometry. Data represent mean ± SEM (n=10 for **Ai**; n= 8 for **Aii**). **B,** Similarly, the level of P-selectin exposure was analysed using isolated platelets (**Bi**) or whole blood (**Bii**) from these mice. Data represent mean ± SEM (n=10 for **Bi**; n= 13 for **Bii**). The statistical significance was calculated using one-way ANOVA followed by Bonferroni's correction (*p<0.05, **p<0.001 and ***p<0.0001).

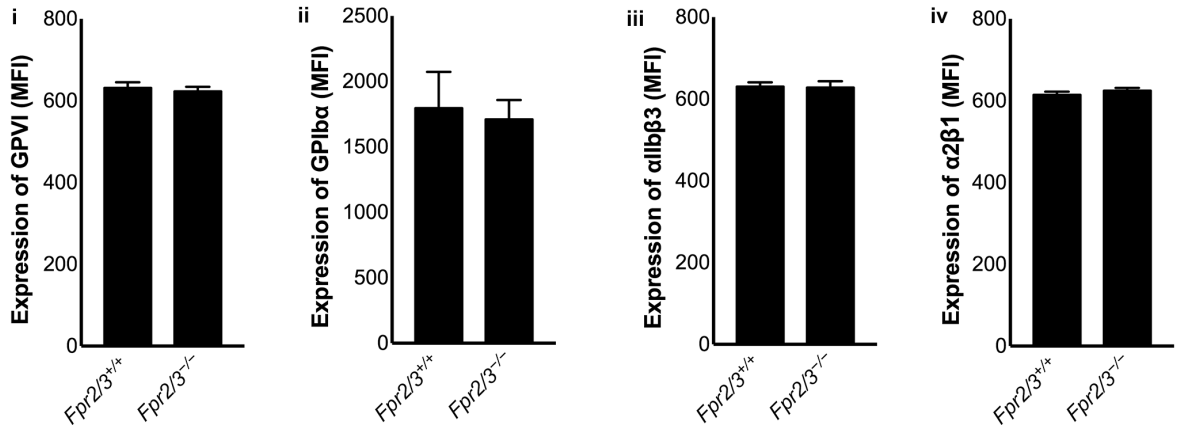

Figure 3-12: **Expression of major platelet receptors in Fpr2/3-deficient mouse platelets.** The expression levels of major platelet receptors such as GPVI (**i**), GPIbα (**ii**), αIIbβ3 (**iii**) and α2β1 (**iv**) in platelets obtained from *Fpr2/3*$^{-/-}$ and control mice were analysed by flow cytometry using selective fluorescent-labelled antibodies. Data represents mean ± SEM (n=8 per group). The statistical significance was calculated a two-tailed unpaired Student's *t* test was used.

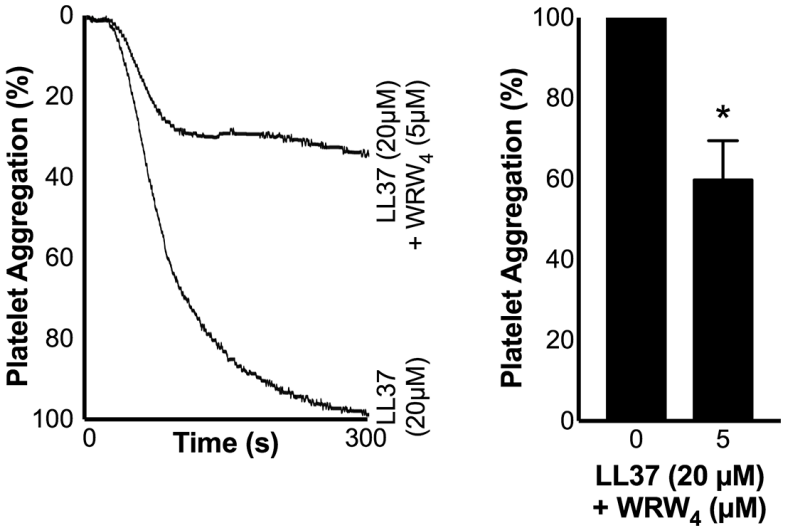

Figure 3-13: **Inhibition of LL37-mediated platelet aggregation by WRW4.** The effect of a selective inhibitor for FPR2/ALX, WRW_4 (5 μM) upon LL37-induced platelet activation was measured by optical aggregometry. Data represent mean ± SEM (n=3). The statistical significance was calculated using a two-tailed unpaired Student's *t* test was used (*p<0.05).

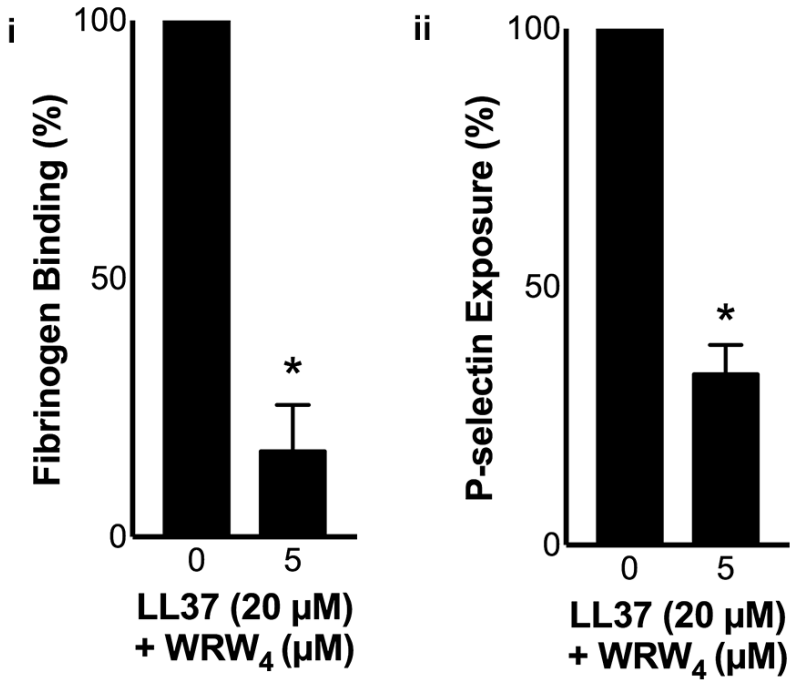

Figure 3-14: **Inhibition of LL37-mediated platelet activation by WRW4.** Mouse isolated platelets were stimulated with LL37 (20 μM) in the presence or absence WRW_4 (5 μM), and the level of fibrinogen binding (**i**) and P-selectin exposure (**ii**) were analysed by flow cytometry. Data represent mean ± SEM (n=4). The statistical significance was calculated using a two-tailed unpaired Student's *t* test was used (*p<0.05).

3.3.11 FPR2/ALX regulates platelet activation in general

In order to validate the importance of FPR2/ALX in the regulation of platelet activation in general, further experiments were performed using human isolated platelets in the presence or absence of WRW_4. CRP-XL (0.25 μg/mL)-induced platelet aggregation was significantly reduced in the presence of different concentrations of WRW_4 (2.5, 5 and 20 μM). For example, the inhibition of FPR2/ALX with WRW_4 (20 μM) reduced the platelet aggregation by around 89% (Figure 3-15A). Similar results were obtained with ADP-induced platelet aggregation, wherein WRW_4 (20 μM) reduced aggregation by approximately 82% (Figure 3-15B). Moreover, dense granule secretion (evidenced by ATP release) was significantly reduced in the presence of WRW_4 (Figure 3-15C). Furthermore, the platelet activation was also assessed using whole blood obtained from control and *Fpr2/3*$^{-/-}$ mice upon stimulation with conventional platelet agonists such as CRP-XL, ADP, AY-NH_2 (activates protease activated receptor, PAR4) and U46619, an analogue of thromboxane A_2 (TXA_2) [activates thromboxane prostanoid (TP) receptor] by measuring the level of fibrinogen binding and P-selectin exposure. Similar to human platelets, the activation of platelets obtained from *Fpr2/3*$^{-/-}$ mice upon stimulation with CRP-XL (Figure 3-16A), ADP (Figure 3-16B), AY-NH_2 (Figure 3-16C) and U46619 (Figure 3-16D) was significantly reduced compared to the controls. Additionally, pre-incubation of human platelets with WRW_4 (1.25, 2.5, 5 and 20 μM) significantly decreased the number of adhered (Figure 3-17i) and spread (Figure 3-17ii) platelets, and the relative surface area of spreading on fibrinogen-coated surfaces (Figure 3-17iii), indicating the involvement of FPR2/ALX in the regulation of integrin αIIbβ3–mediated outside-in signalling to platelets. The impact of *Fpr*2/3 on the modulation of haemostasis in mice was determined by tail bleeding assay. A mean bleeding time of 428.5±64.8 seconds was observed in the control group, however *Fpr2/3*-deficient mice significantly increased the bleeding time to a mean of 1128±71.9 seconds (Figure 3-18). These results indicate the prominence of this receptor in the maintenance of haemostasis under physiological conditions. Together, these data emphasise the impact of FPR2/ALX on the regulation of normal platelet function through a positive feedback mechanism, and thus the inhibition or deletion of this receptor results in diminished platelet function in general. This positive feedback may occur

mainly through LL37 that is released from platelets upon activation within the local environment where their concentration might be sufficient to inhibit platelet function. The presence of other FPR2/ALX ligands for platelet activation is also inevitable under these circumstances.

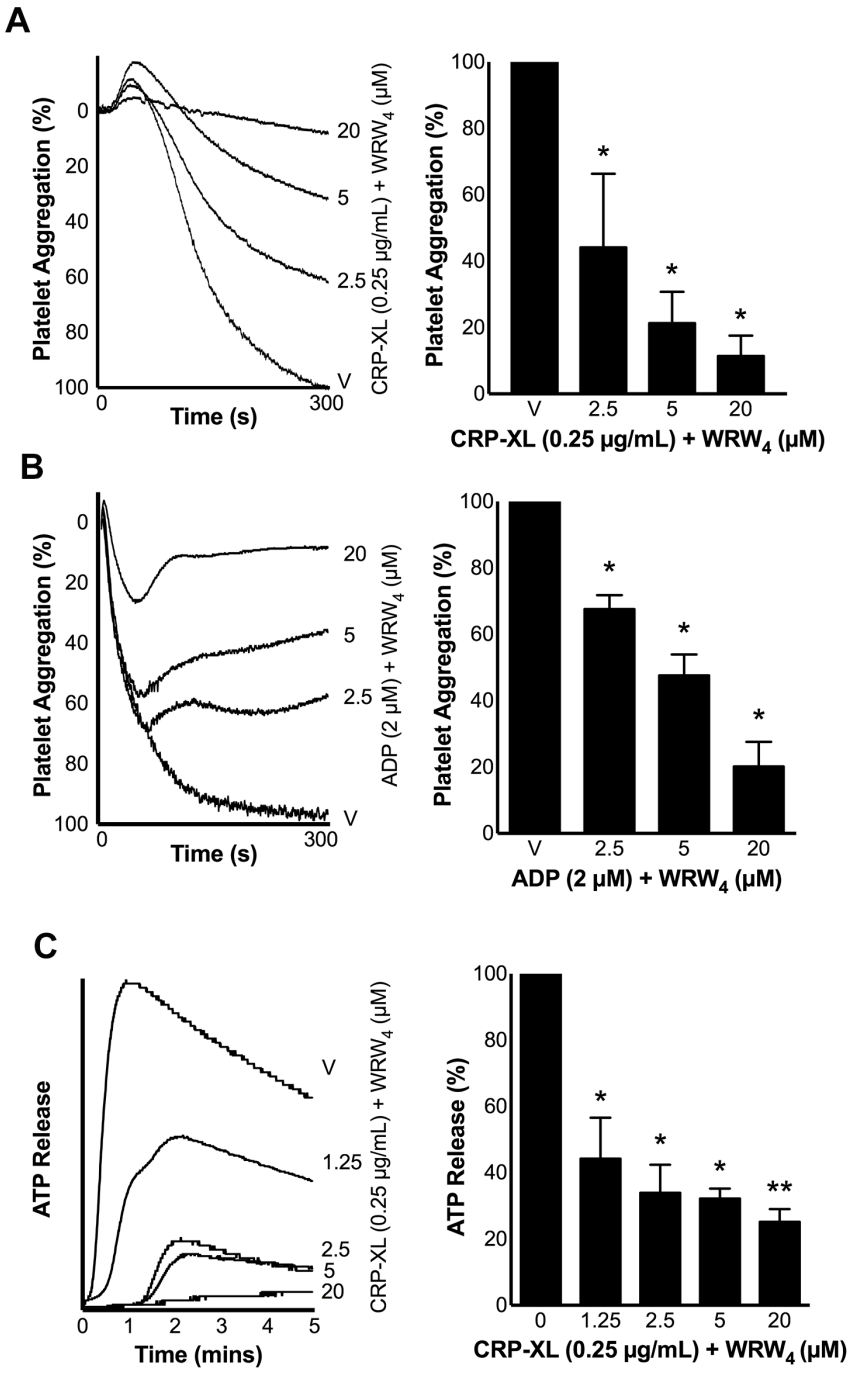

Figure 3-15: **The effect of FPR2/ALX blockade by WRW4 on platelet aggregation and ATP release.** The effects of different concentrations of WRW_4 on CRP-XL (0.25 µg/mL) (**A**) or ADP (2 µM) (**B**) -induced human isolated platelet aggregation was analysed by optical aggregometry. **C**, the level of ATP secretion in platelets treated with WRW_4 prior to activation with CRP-XL (0.25 µg/mL) was measured by lumi-aggregometry. Data represent mean ± SEM (n=3). P values shown are calculated by one-way ANOVA followed by Bonferroni's correction (*p<0.05 and **p<0.01).

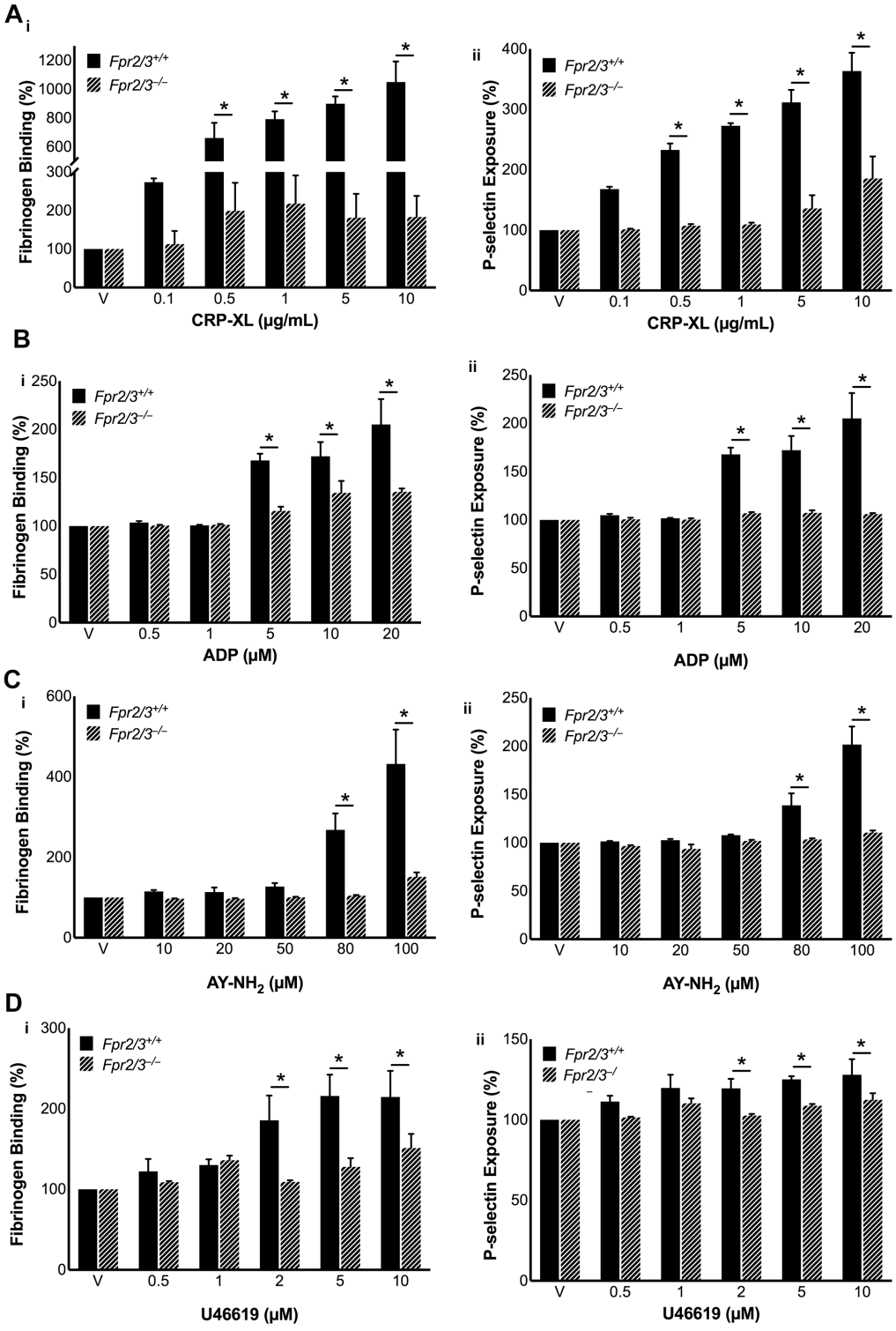

Figure 3-16: **Deletion of *Fpr2/3* in mice reduces agonist-induced platelet activation.** The levels of fibrinogen binding (**i**) and P-selectin exposure (**ii**) were analysed in platelets obtained from control or *Fpr2/3*$^{-/-}$ mice upon stimulation with various concentrations of CRP-XL (**A**), ADP (**B**), AY-NH_2 (**C**) or U46691 (**D**) by flow cytometry. Data represent mean ± SEM (n=3). P values shown are calculated by two-way ANOVA followed by Bonferroni's correction (*p<0.05).

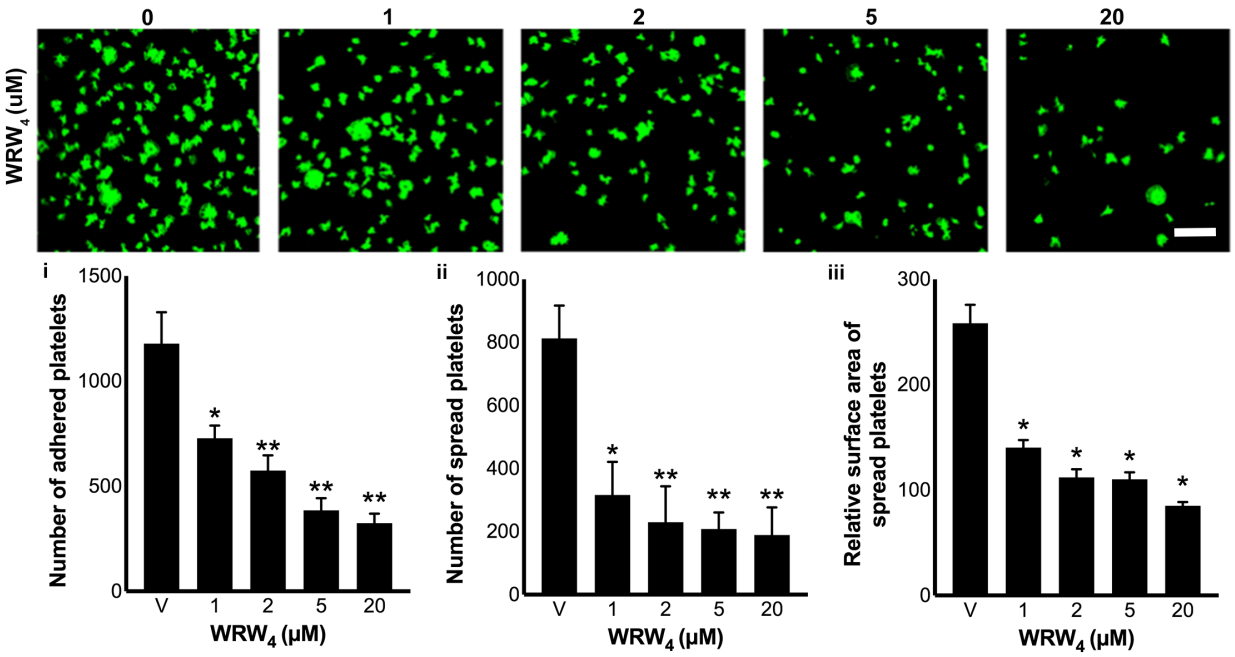

Figure 3-17: **Effect of FPR2-selective inhibitor, WRW4, on platelet adhesion and spreading.** Platelet adhesion and spreading on fibrinogen-coated glass surface was analysed in the absence and presence of WRW_4 (1.25, 2.5, 5 and 20 μM) by confocal microscopy (60x magnification; scale bar – 10 μm). The number of adhered (**i**) and spread platelets (**ii**), and the relative surface area of spread platelets (**iii**) was determined by analysing the images using ImageJ. Ten random fields of view were recorded for each sample. Data represent mean ± SEM (n=3). P values shown are calculated by one-way ANOVA followed by Bonferroni's correction (*p<0.05 and **p<0.01).

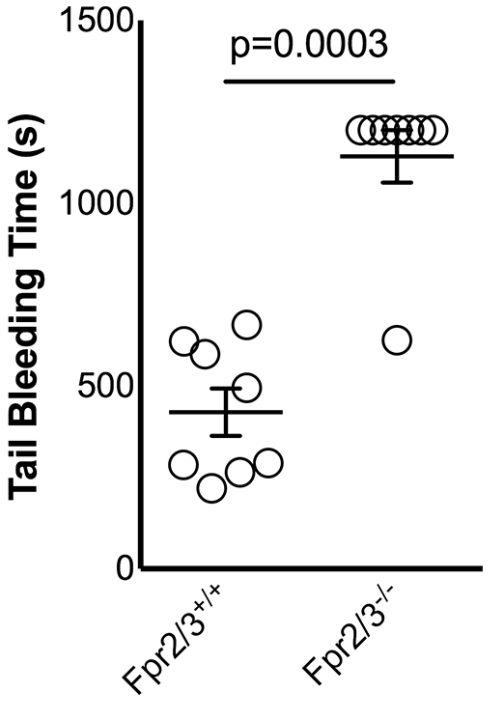

Figure 3-18: **Deletion of *Fpr2/3* in mice affects haemostasis.** The impact of FPR2/ALX in the modulation of haemostasis was analysed using tail bleeding assay in control or *Fpr2/3*$^{-/-}$ mice. Data represent mean ± SEM (n=8 per group). P values shown are calculated non-parametric Mann-Whitney test.

3.3.12 FPR2/ALX exerts its effects through cyclic AMP-dependent signalling

Cyclic AMP (cAMP) is a potent inhibitor of platelet function, and its generation is reduced upon platelet activation. Moreover, stimulants of cAMP generation are known to reverse platelet activation[45]. FPRs are G_i-coupled receptors[46], which are known to inhibit adenylate cyclase, leading to the reduction of cAMP levels. Therefore, the deletion of G_i-coupled receptor genes in mice increases the basal cAMP levels in target cells[47, 48]. In order to investigate whether the inhibition of FPR2/ALX in human or deletion of *Fpr*2/3 in mouse platelets is influenced by the cAMP-dependent signalling pathways, the level of cAMP was quantified in platelets using a cAMP assay kit. The inhibition of FPR2/ALX with WRW_4 (20 μM) significantly elevated the cAMP levels compared to the vehicle control in human platelets (Figure 3-19i). Similarly, resting *Fpr2/3*$^{-/-}$ platelets exhibited elevated levels of cAMP compared to control mouse platelets (Figure 3-19ii). These data suggest that the level of cAMP plays a key role in the regulation of FPR2/ALX-mediated functions in platelets and explain the inhibition of platelet function in general upon blocking or deletion of this receptor in platelets.

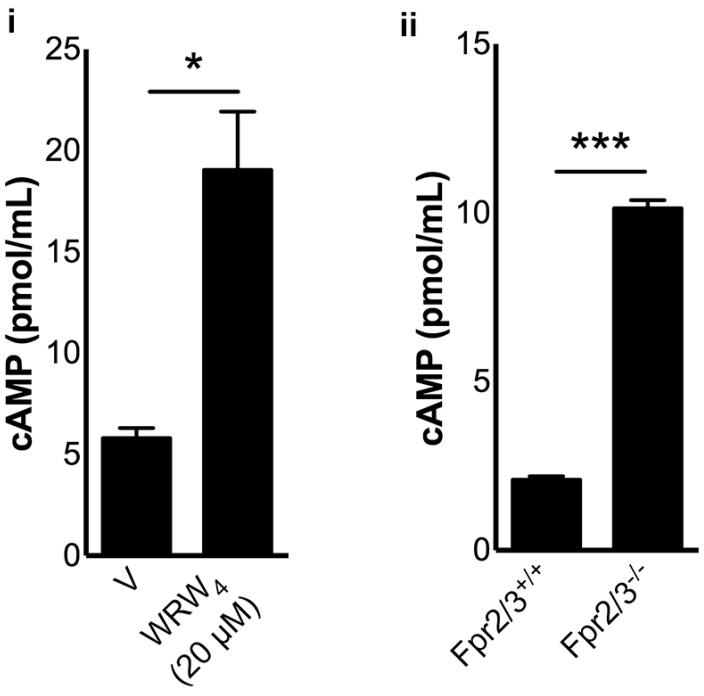

Figure 3-19: **Deletion of *Fpr2/3* in mice affects cAMP levels.** The level of cAMP in human isolated platelets in the presence or absence of WRW_4 (**i**), and control and *Fpr2/3*$^{-/-}$ mouse platelets (**ii**) was analysed using a cAMP assay kit. Data represent mean ± SEM (n=4). P values shown are calculated by two-tailed unpaired Student's *t* test (**p*<0.05 and ****p*<0.001).

3.3.13 Impact of LL37 in the modulation of platelet activation and haemostasis during psoriasis

3.3.13.1 Characterisation of a psoriasis mouse model

The level of LL37 is known to be overexpressed during psoriasis with concentrations up to 300 μM in affected skin tissues[39]. In order to determine the impact of LL37 in the modulation of platelet function and haemostasis during psoriasis, we used an animal model with psoriasis-like symptoms. Psoriasis vulgaris (plaque-type), the most common phenotype of psoriasis, is a skin inflammatory disease characterised by skin thickening along with red plaques and dry scales. In order to study the effects of LL37 under this inflammatory condition, a psoriasis mouse model was utilised.[49] To mimic human plaque-type psoriasis, mice were treated topically with a Vaseline cream containing 5% imiquimod (IMQ), an immunomodulatory agent that ligates toll-like receptor 7 (TLR7)[50]. IMQ-treated mice displayed psoriatic symptoms compared to control vaseline-treated mice. For example, IMQ-treated mice displayed significant differences in the body (Figure 3-20i) and spleen (Figure 3-20ii) weight, and skin thickness (Figure 3-20iii) compared to the control mice over five days. Moreover, based on the Psoriasis Area and Severity Index (PASI) scoring system, the clinical manifestations of psoriasis including erythema (Figure 3-20iv) and desquamation (Figure 3-20v) were significantly altered in IMQ-treated mice compared to vaseline-treated mice. Figure 3-20vi depicts the lesions on the back skin of IMQ-treated mice compared to the controls. Together, these data confirm the development of psoriasis-like symptoms in the IMQ-treated mice, rendering them an appropriate model to study human plaque-like psoriasis.

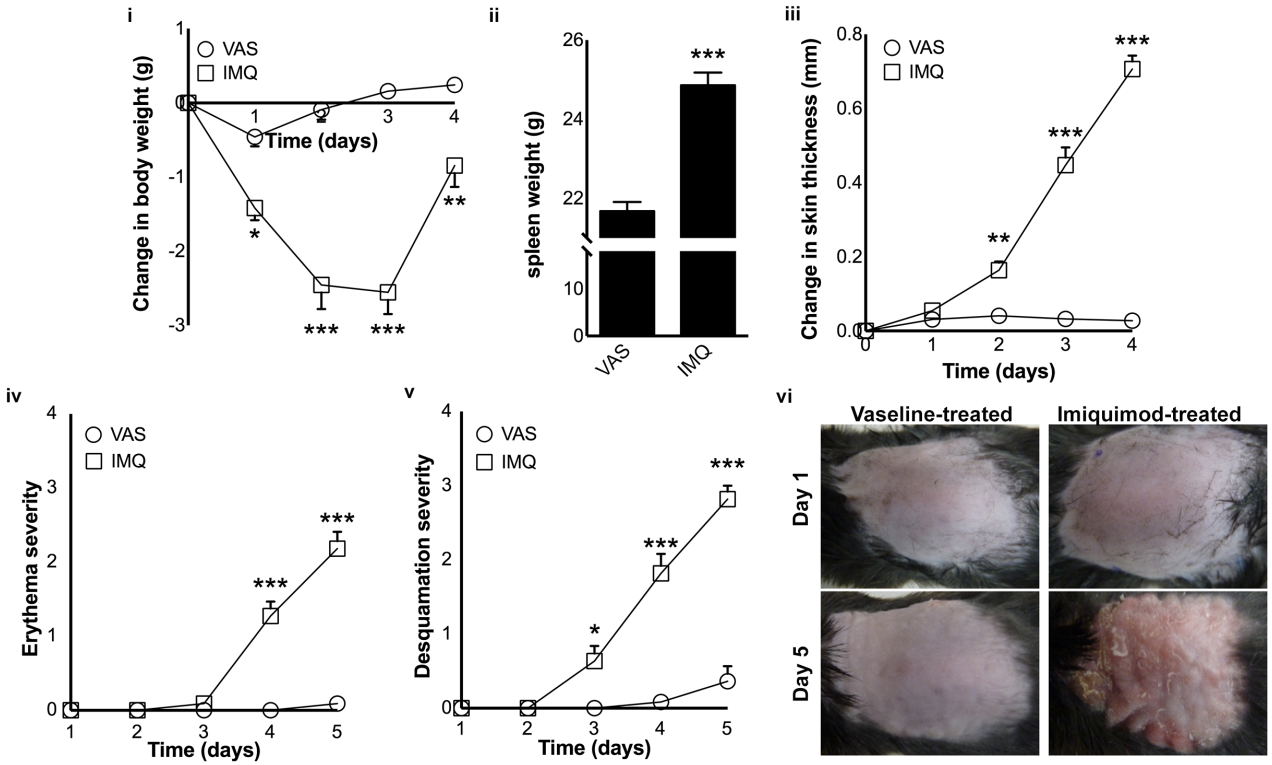

Figure 3-20: **Characterisation of a psoriasis mouse model.** Characterisation of a psoriasis mouse model was performed by measuring the body weight (**i**), spleen weight (**ii**) and skin thickness (**iii**) in IMQ-treated mice compared to the controls (Vaseline-treated mice). The PASI scoring was used to rank erythema (**iv**) and desquamation (**v**). Representative images display the lesions on the skin of IMQ-treated mice compared to the controls (**vi**). Data represent mean ± SEM (n=12 per group). P values shown are calculated by one-way ANOVA followed by Bonferroni's correction (*p<0.05, **p<0.001 and ***p<0.0001).

3.3.13.2 The effect of haemostasis in IMQ-treated mice

In order to determine whether the IMQ-treated mice exhibit a direct impact on haemostasis, a tail bleeding assay was performed. Notably, IMQ-treated mice did not exhibit significant difference in the bleeding time compared to the control group (Figure 3-21). These data demonstrate that despite the alteration of platelet function during psoriasis, the elevation of LL37 was not sufficient to impact haemostasis in these mice. Moreover, there may be a range of modulatory factors at play influencing haemostasis in these psoriatic mice.

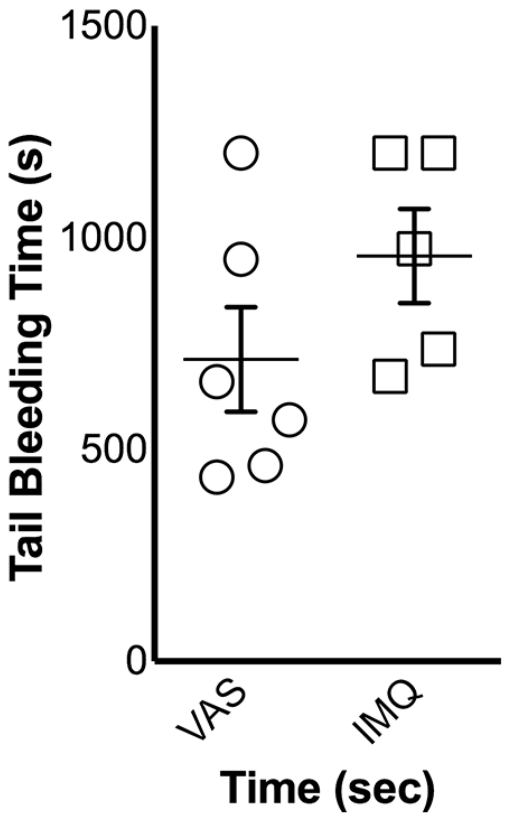

Figure 3-21: **The impact of psoriasis in the modulation of haemostasis.** The impact of psoriasis in the modulation of haemostasis was analysed in the control or IMQ-treated mice using tail bleeding assay. Data represent mean ± SEM (n=6 for IMQ-treated, and n=5 for Vaseline-treated mice). P values shown are calculated by non-parametric Mann-Whitney test.

3.3.13.3 m*CRAMP is elevated in the skin and plasma of IMQ-treated mice*

Recent studies have reported the overexpression of LL37 during psoriasis in humans mainly at local sites of inflammation and lesions[29, 51], and in the circulation[52]. In order to determine whether the LL37 murine orthologue, mCRAMP, is overexpressed both locally in the skin and plasma of IMQ-treated mice, the level of mCRAMP was measured in skin homogenates and plasma using an ELISA. The level of mCRAMP was markedly increased in the skin samples (150±2.4 pg/mL) obtained from IMQ-treated mice compared to the controls (23.1±2.7 pg/mL) (Figure 3-22i). Moreover, the level of mCRAMP was markedly increased in plasma samples (139.5±7 pg/mL) obtained from IMQ-treated mice compared to the control (35.4±1.5 pg/mL) (Figure 3-22ii). These data demonstrate the elevation of mCRAMP levels during the progression of psoriasis both locally in lesional skin and systemically in plasma.

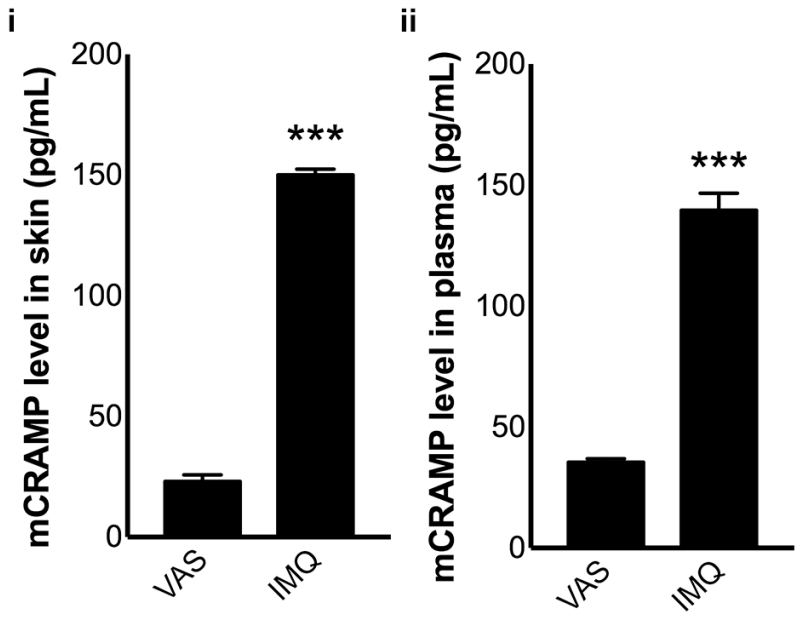

Figure 3-22: **The expression of mCRAMP in psoriatic lesions and plasma.** The level of mCRAMP in skin homogenates (**i**) or plasma (**ii**) samples obtained from IMQ-treated and control mice was analysed using mCRAMP antibodies by an ELISA. Data represent mean ± SEM (n=6 for skin; n=19 for plasma). P values shown are calculated by a two-tailed unpaired Student's *t* test (***p<0.0001).

3.3.13.4 Platelet activation is augmented during psoriasis

In order to determine whether the pathogenesis of psoriasis affects the regulation of platelet function, whole blood obtained from IMQ-treated mice was used to study platelet activation by flow cytometry. The effects of various concentrations of different platelet agonists such as, CRP-XL, ADP and U46619 were analysed in whole blood obtained from IMQ-treated and control mice. The level of fibrinogen binding and P-selectin exposure upon treatment with CRP-XL (Figure 3-23Ai and 3-23ii), ADP (Figure 3-23Bi and 3-23Bii) and U46619 (Figure 3-23Ci and 3-23Cii) was significantly increased in IMQ-treated mice compared to the controls. These data validate the impact of psoriasis pathogenesis in the modulation of platelet-mediated responses during the progression of this disease.

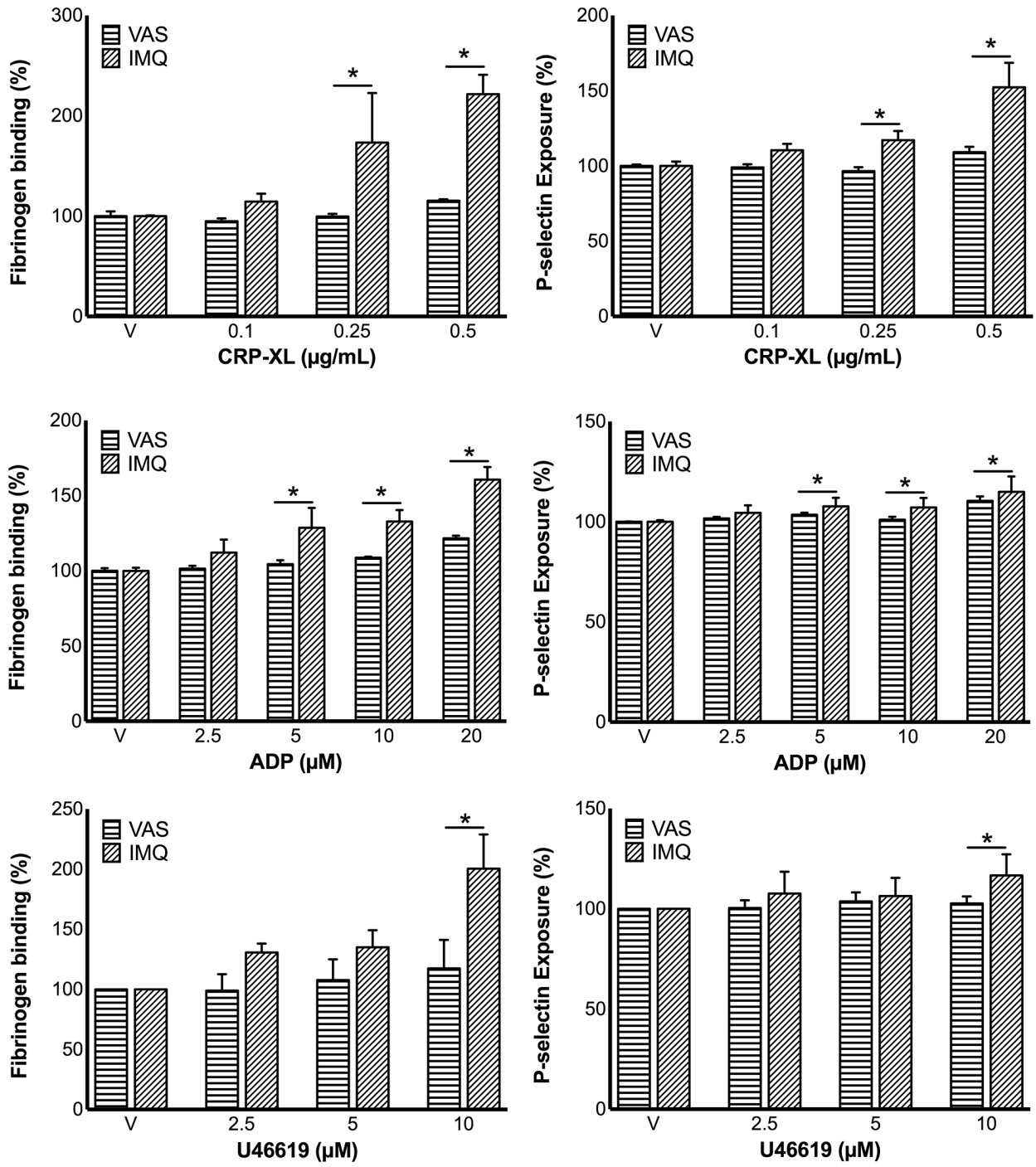

Figure 3-23: **The activation of platelets in psoriatic mice.** The activation of platelets upon stimulation with CRP-XL (n=3) (**A**), ADP (n=4) (**B**), or U46619 (n=3) (**C**) in whole blood obtained from IMQ-treated and control mice was analysed by measuring the level of fibrinogen binding (**i**) and P-selectin exposure (**ii**) by flow cytometry. P values shown are calculated by two-way ANOVA followed by Bonferroni's correction (*p<0.05, **p<0.001 and ***p<0.0001).

3.3.13.5 Effect of psoriatic plasma on healthy platelets

In order to determine the direct impact of psoriatic plasma samples on healthy platelets, the levels of fibrinogen binding and P-selectin exposure were measured using healthy mouse and human platelets in the presence and absence of IMQ-treated mouse plasma. The treatment of healthy human platelets (PRP) with psoriatic plasma markedly increased the levels of fibrinogen binding and P-selectin exposure in the absence (Figure 3-24i and 2-24ii) or presence (Figure 3-24iii and 3-24iv) of CRP-XL compared to the controls. Similar results were obtained upon the treatment of normal mouse isolated platelets, wherein psoriatic plasma markedly increased platelet activation in the absence (Figure 3-25Ai and 3-25Aii) or presence (Figure 3-25Aiii and 3-25Aiv) of a CRP-XL compared to the controls. Furthermore, psoriatic plasma also increased platelet activation in mouse PRP in the absence (Figure 3-25Bi and 3-25Bii) or presence (Figure 3-25Biii and 3-25Biv) of CRP-XL compared to the controls.

In order to investigate whether the augmentation of platelet function in IMQ-treated mice are mediated through FPR2/ALX, the effect of psoriatic plasma was investigated in the presence of WRW_4 or in *Fpr2/3*$^{-/-}$ mouse platelets. The treatment of control mouse platelets (PRP) with psoriatic plasma significantly reduced platelet activation in the presence of WRW_4 (Figure 3-26Ai and 3-26Aii). Similarly, the treatment of *Fpr2/3*$^{-/-}$ mouse platelets displayed significantly reduced platelet activation compared to the controls (Figure 3-26Bi and Figure 3-26Bii). Together, these data demonstrate the direct impact of psoriasis on platelet function and its involvement in the modulation of platelet reactivity (mainly through FPR2/ALX), which could exacerbate the pathogenesis and thrombotic complications during psoriasis at local inflammatory sites and in circulation.

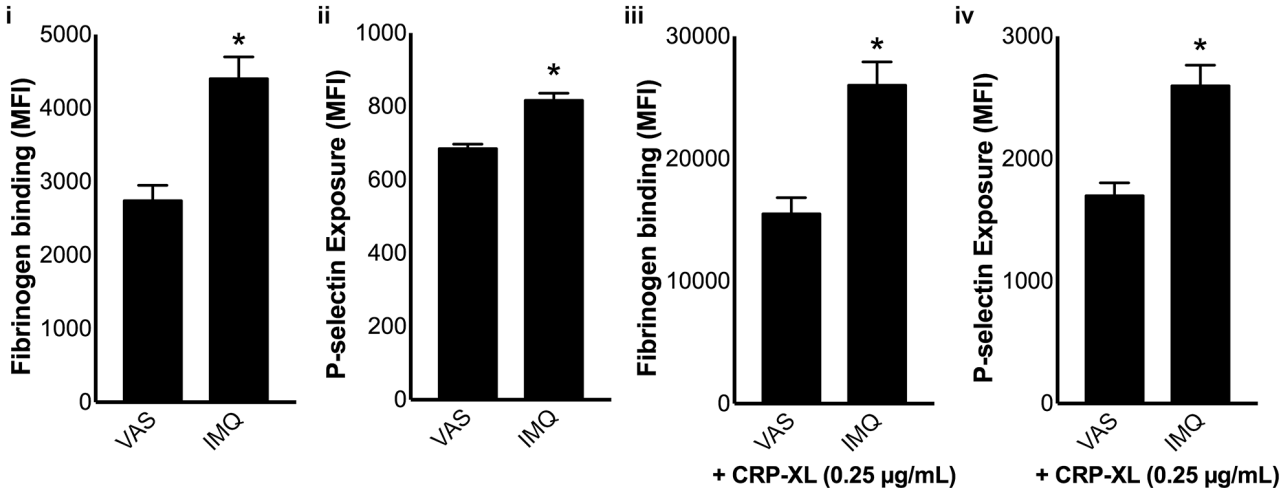

Figure 3-24: **The impact of psoriatic plasma on human platelets.** The impact of IMQ-treated mouse plasma on healthy human platelets (PRP) was analysed by measuring the level of fibrinogen binding in the absence (**i**) or presence (**iii**) of CRP-XL (0.25µg/mL) (n=18). Similarly, the level of P-selectin exposure was measured in the absence (**ii**) or presence (**iv**) of CRP-XL (0.25µg/mL) (n=18). P values shown are calculated by a two-tailed unpaired Student's *t* test (*p<0.05).

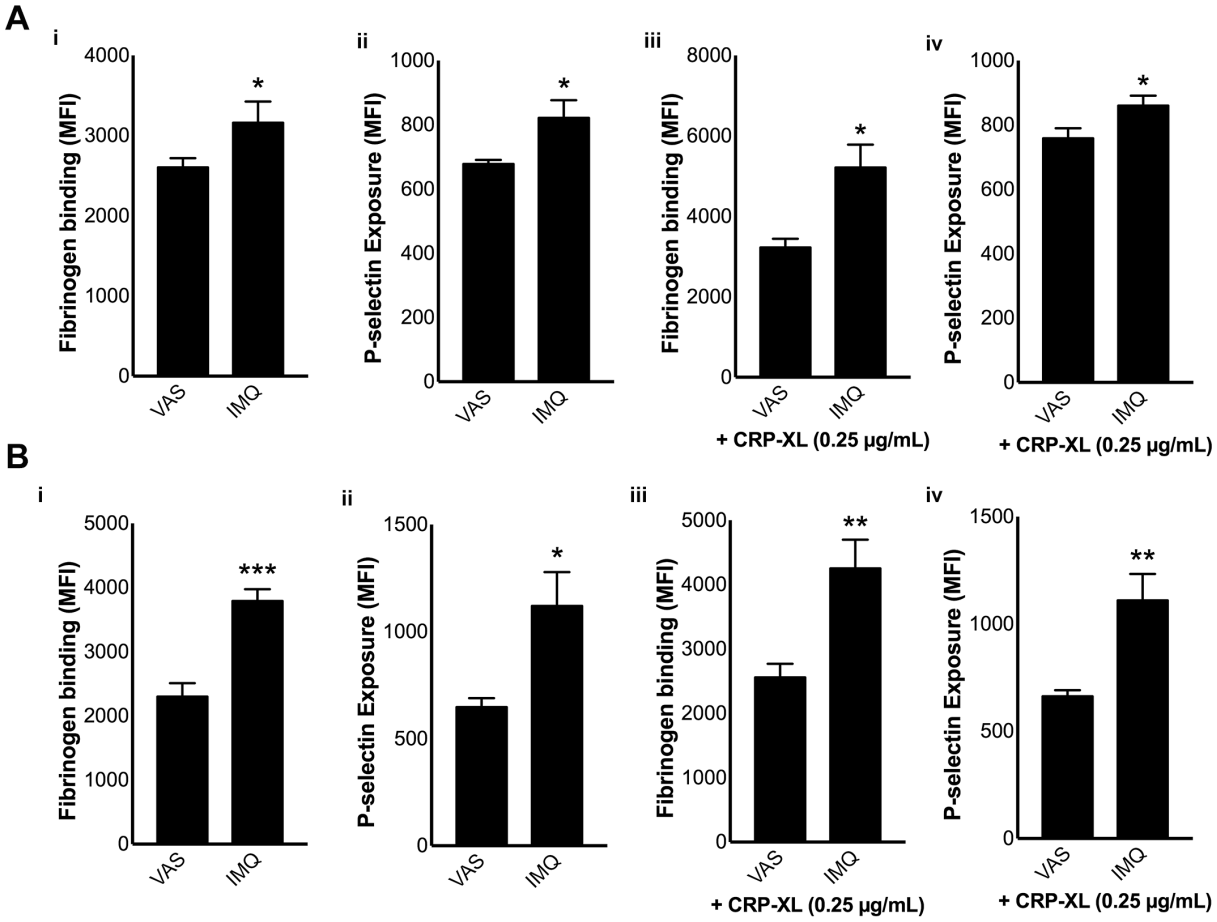

Figure 3-25: **The impact of psoriatic plasma on mouse platelets. A**, the impact of IMQ-treated mouse plasma on control mouse isolated platelets was analysed by measuring the levels of fibrinogen binding in the absence (**Ai**) or presence (**Aiii**) of CRP-XL (0.25µg/mL) (n=7). Similarly, P-selectin exposure was measured in the absence (**Aii**) or presence (**Aiv**) of CRP-XL (0.25µg/mL) (n=7). **B**, the impact of IMQ-treated plasma on control mouse PRP was analysed by measuring the levels of fibrinogen binding in the absence **(Bi)** or presence (**Biii**) of CRP-XL (0.25µg/mL) (n=6). P-selectin exposure was also measured in the absence (**Bii**) or presence (**Biv**) of CRP-XL (0.25µg/mL) (n=4). Data represent mean ± SEM. P values shown are calculated by a two-tailed unpaired Student's *t* test (**p*<0.05, ***p*<0.001 and ****p*<0.0001).

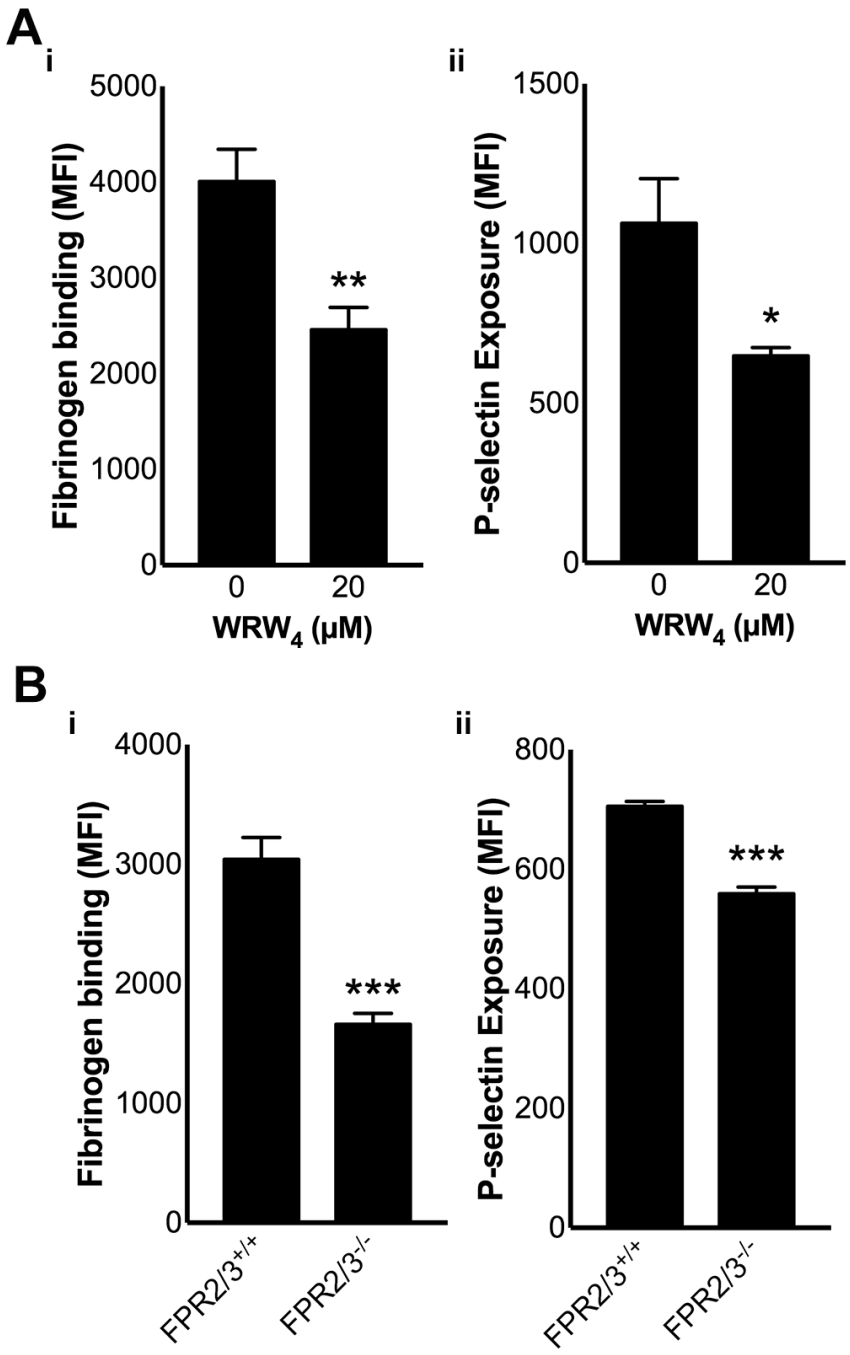

Figure 3-26: **FPR2/ALX-mediated effects of psoriatic plasma. A**, the impact of IMQ-treated plasma on healthy mouse PRP in the presence of WRW_4 was analysed by measuring the levels of fibrinogen binding **(Ai)** or P-selectin exposure (**Aii**) (n=5). **B,** the impact of IMQ-treated plasma on control and *Fpr2/3*$^{-/-}$ mouse PRP was analysed by measuring the levels of fibrinogen binding **(Bi)** or P-selectin exposure (**Bii**) (n=6). P values shown are calculated by a two-tailed unpaired Student's *t* test (*p<0.05, **p<0.001 and ***p<0.0001).

3.4 Discussion

LL37 is a powerful antimicrobial peptide that plays substantial roles in the initiation of chemotaxis and subsequent inflammatory responses in immune cells including monocytes[22], mast cells[53], eosinophils and neutrophils[54]. Moreover, the involvement of LL37 in the development of pathological conditions such as psoriasis, sepsis, inflammatory bowel disease and cystic fibrosis has been previously reported, and hence, its therapeutic potential has been analysed in detail[38]. LL37 has also been found to play a role in the pathogenesis of atherosclerosis[55, 56], wherein it leads to the development of lesions and recruitment of inflammatory cells at the site of injury[27]. In addition, LL37 has been found to play a role in wound healing[57], where the LL37 analogue, Cys-LC-LL37, has been incorporated in cotton gauzes to produce antimicrobial wound dressings[58]. However, the role of LL37 in the modulation of platelet reactivity, thrombosis and haemostasis has not been investigated previously in detail. Platelets play pivotal roles during inflammatory diseases, specifically in psoriasis where the modulation of platelet function leads to disseminated intravascular coagulation, thrombus formation in the microvasculature that culminates in thrombocytopenia. Henceforth, the impact of LL37 in the modulation of platelet reactivity, thrombosis and haemostasis under physiological and pathological conditions, such as psoriasis, was investigated in this study.

The expression of LL37 has been reported in numerous cell types including epithelial cells and immune cells such as neutrophils and monocytes[13]. Since platelets are also derived from the myeloid lineage[59], we hypothesised that platelets may possess LL37, and indeed the presence of LL37 in platelets and its release to the external milieu upon activation was confirmed. Similar to neutrophils, platelets may also contain LL37 in their granules and release it upon stimulation to enhance the secondary platelet activation. Platelets are also known to contain several other antimicrobial peptides including platelet factor 4 (PF4), platelet basic protein (PBP) and its derivatives, connective tissue activating peptide 3 (CTAP-3), thymosin B-4 (TB-4), CAMP and fibrinopeptides A and B (FPA and FPB), and release them upon activation in order to control microbial infection[60]. Similarly, they may also contribute to the elevation of LL37, the only cathelicidin in humans, upon activation to support the microbial clearance, activation of inflammatory

responses, and modulation of thrombosis and haemostasis during pathological settings. The activation of platelets during inflammatory diseases is inevitable due to the presence of several molecules that activate platelets[61]. However, here we demonstrate LL37 as a major contributor to thrombosis and other platelet-related complications during inflammatory diseases.

Thrombosis and subsequent bleeding are associated with psoriasis[62]. Similarly, disseminated intravascular coagulation, thrombosis in the microvasculature and sequestration of platelets are some of the common clinical manifestations in sepsis[63]. The level of LL37 is significantly higher in psoriasis[39] and sepsis[64] patients compared to healthy individuals. In line with thrombosis in vasculature, LL37 augmented *in vitro* thrombus formation under arterial flow conditions and shortened the bleeding time in mice. These data demonstrate a fundamental function for LL37 in the modulation of thrombosis and haemostasis under pathological settings. Similarly, LL37 induced platelet aggregation, fibrinogen binding, granule secretion, adhesion, spreading and intracellular calcium mobilisation in platelets.

The concentrations used in the present study (up to 50 μM) revealed activatory roles of LL37 in platelets. A recent study reported the inhibitory effects of LL37 in platelets at concentrations between 0.1 and 1.2 mM[37]. These concentrations are not only substantially greater than the achievable levels under a range of pathological conditions, but also exert toxic effects in several cell types[40] including platelets at 100 μM. However, in pathological conditions such as severe psoriasis, a median concentration of 304 μM LL37 in psoriatic lesions has been reported[39], which can exert cytotoxicity towards platelets at the local sites and reduce the number of functional platelets in the circulation. In other conditions such as pulmonary infections, a concentration of 5 μM LL37 has been detected[65]. Notably, the normal plasma concentration of LL37 in healthy individuals is suggested to be approximately 1.2 μM[66], which did not exert any effects on platelets. The concentrations of LL37 used in the present study are similar to those achievable during pathological conditions, such as sepsis[64], and early stages of other inflammatory diseases including psoriasis. Hence, the inhibitory effects of LL37 that were previously reported in platelets may be due to the cytotoxic effects of LL37, although additional causes cannot be excluded. Together with the previous report, our data

demonstrate that LL37 induces platelet activation at the early stages of inflammatory diseases resulting in the initiation of thrombosis and modulation of haemostasis. However, at concentrations of 100 μM and above (such as in local psoriatic lesions), LL37 may exert cytotoxic effects that can result in the reduction of platelet function and decrease the circulating platelet number, which subsequently leads to bleeding complications. We were able to demonstrate the involvement of LL37 in platelet-related complications in inflammatory diseases, psoriasis is the case in point. Furthermore, we displayed the direct impact of disease (psoriasis) pathogenesis in the dysregulation of platelet function. Together with the modulation of thrombosis and haemostasis, LL37 may also induce other platelet-related complications (e.g. thrombocytopenia and inflammation) during various inflammatory diseases where its level is elevated.

LL37 has been reported to act mainly through FPR2/ALX in other cell types[22, 30,] although additional receptors such as toll-like receptors (TLRs), receptor tyrosine kinases (RTKs), ligand-gated ion channel (LGIC), CCR3, P2Y11 and P2X7 have shown to bind this peptide[67]. To investigate the underlying molecular mechanisms through which LL37 modulates platelet function, the effects of LL37 in human platelets treated with WRW_4 and platelets obtained from *Fpr2/3*$^{-/-}$ mice were analysed. LL37-mediated activation was largely reduced by WRW_4 and in platelets obtained from *Fpr2/3*$^{-/-}$ mice compared to the controls, verifying the functional dependence of LL37 primarily on FPR2/ALX. LL37 binding assays that were performed using platelets obtained from *Fpr2/3*$^{-/-}$ mice confirmed the substantial reduction in the binding of LL37 to *Fpr2/3*$^{-/-}$ platelet surface compared to *Fpr1*$^{-/-}$ and control mouse platelets. Additionally, we were able to demonstrate that FPR2/ALX inhibition in human platelets or deletion of *Fpr2/3* gene in mice lead to the elevation of cAMP levels, which is a major inhibitory molecule for platelet activation. This indicates the involvement of cAMP-dependent signalling pathways in the regulation of FPR2/ALX in platelets. Nevertheless, the activation of platelets by LL37 through receptors other than FPRs cannot be excluded and further investigations will be needed to explore the contributions of such receptors in the modulation of platelet function upon ligation with LL37.

In conclusion, this study demonstrates that LL37 is stored in platelets and secreted upon platelet activation. Moreover, LL37 promotes thrombus formation and altered haemostasis in mice. In line with these results, LL37 induced platelet activation in a range of platelet functional assays such as platelet aggregation, inside-out signalling to integrin αIIbβ3 and outside-in signalling by the same integrin, granule secretion and intracellular calcium mobilisation. These activatory roles of LL37 were diminished in the presence of a FPR2/ALX pharmacological inhibitor, and in platelets obtained from *Fpr2/3*$^{-/-}$ mice confirming the functional dependence of LL37 primarily via this receptor in platelets. Additionally, we demonstrate an instrumental role for FPR2/ALX in the positive feedback regulation of platelet function, in which the deficiency or blockade of this receptor impaired haemostasis and the activation of platelets in general. The significant roles of LL37 and FPR2/ALX in the modulation of thrombosis and haemostasis renders them as potential candidates for the exacerbation of platelet-related complications and immune responses in numerous inflammatory diseases where platelets play essential roles. Notably, the presence of FPRs in platelets opens up new avenues to investigate the involvement of a multiplicity of FPR ligands in the modulation of thrombosis, haemostasis, and other platelet-related complications during inflammatory responses. Based on the data presented in this study, both LL37 and FPRs can act as potential therapeutic targets for cardiovascular and a range of inflammatory diseases.

References

1. Gibbins JM. Platelet adhesion signalling and the regulation of thrombus formation. *Journal of cell science*. 2004;117:3415-25.
2. Vaiyapuri S, Flora GD and Gibbins JM. Gap junctions and connexin hemichannels in the regulation of haemostasis and thrombosis. *Biochemical Society transactions*. 2015;43:489-94.
3. Hamzeh-Cognasse H, Damien P, Chabert A, Pozzetto B, Cognasse F and Garraud O. Platelets and infections - complex interactions with bacteria. *Front Immunol*. 2015;6:82.
4. Rondina MT and Garraud O. Emerging evidence for platelets as immune and inflammatory effector cells. *Front Immunol*. 2014;5:653.
5. Gisondi P and Girolomoni G. Psoriasis and atherothrombotic diseases: disease-specific and non-disease-specific risk factors. *Semin Thromb Hemost*. 2009;35:313-24.
6. Santilli S, Kast DR, Grozdev I, Cao L, Feig RL, Golden JB, Debanne SM, Gilkeson RC, Orringer CE, McCormick TS, Ward NL, Cooper KD and Korman NJ. Visualization of atherosclerosis as detected by coronary artery calcium and carotid intima-media thickness reveals significant atherosclerosis in a cross-sectional study of psoriasis patients in a tertiary care center. *J Transl Med*. 2016;14.
7. Tamagawa-Mineoka R, Katoh N and Kishimoto S. Platelet activation in patients with psoriasis: increased plasma levels of platelet-derived microparticles and soluble P-selectin. *J Am Acad Dermatol*. 2010;62:621-6.
8. Tamagawa-Mineoka R, Katoh N, Ueda E, Masuda K and Kishimoto S. Elevated platelet activation in patients with atopic dermatitis and psoriasis: increased plasma levels of beta-thromboglobulin and platelet factor 4. *Allergol Int*. 2008;57:391-6.
9. Wu WK, Wong CC, Li ZJ, Zhang L, Ren SX and Cho CH. Cathelicidins in inflammation and tissue repair: Potential therapeutic applications for gastrointestinal disorders. *Acta Pharmacol Sin*. 2010;31:1118-22.
10. Chow JY, Li ZJ, Wu WK and Cho CH. Cathelicidin a potential therapeutic peptide for gastrointestinal inflammation and cancer. *World journal of gastroenterology*. 2013;19:2731-5.
11. Vandamme D, Landuyt B, Luyten W and Schoofs L. A comprehensive summary of LL-37, the factotum human cathelicidin peptide. *Cell Immunol*. 2012;280:22-35.
12. Larrick JW, Hirata M, Balint RF, Lee J, Zhong J and Wright SC. Human CAP18: a novel antimicrobial lipopolysaccharide-binding protein. *Infect Immun*. 1995;63:1291-7.
13. Bandurska K, Berdowska A, Barczynska-Felusiak R and Krupa P. Unique features of human cathelicidin LL-37. *BioFactors (Oxford, England)*. 2015;41:289-300.
14. Wuerth K and Hancock RE. New insights into cathelicidin modulation of adaptive immunity. *Eur J Immunol*. 2011;41:2817-9.

15. Sorensen O, Arnljots K, Cowland JB, Bainton DF and Borregaard N. The human antibacterial cathelicidin, hCAP-18, is synthesized in myelocytes and metamyelocytes and localized to specific granules in neutrophils. *Blood*. 1997;90:2796-803.

16. Lowry MB, Guo C, Borregaard N and Gombart AF. Regulation of the human cathelicidin antimicrobial peptide gene by 1alpha,25-dihydroxyvitamin D3 in primary immune cells. *The Journal of steroid biochemistry and molecular biology*. 2014;143:183-91.

17. Sorensen OE, Follin P, Johnsen AH, Calafat J, Tjabringa GS, Hiemstra PS and Borregaard N. Human cathelicidin, hCAP-18, is processed to the antimicrobial peptide LL-37 by extracellular cleavage with proteinase 3. *Blood*. 2001;97:3951-9.

18. Jacobsen AS and Jenssen H. Human cathelicidin LL-37 prevents bacterial biofilm formation. *Future medicinal chemistry*. 2012;4:1587-99.

19. Wong JH, Ng TB, Legowska A, Rolka K, Hui M and Cho CH. Antifungal action of human cathelicidin fragment (LL13-37) on Candida albicans. *Peptides*. 2011;32:1996-2002.

20. Barlow PG, Svoboda P, Mackellar A, Nash AA, York IA, Pohl J, Davidson DJ and Donis RO. Antiviral activity and increased host defense against influenza infection elicited by the human cathelicidin LL-37. *PLoS One*. 2011;6:e25333.

21. Agier J, Efenberger M and Brzezinska-Blaszczyk E. Cathelicidin impact on inflammatory cells. *Central-European journal of immunology / Polish Society for Immunology and eleven other Central-European immunological societies*. 2015;40:225-35.

22. De Y, Chen Q, Schmidt AP, Anderson GM, Wang JM, Wooters J, Oppenheim JJ and Chertov O. LL-37, the neutrophil granule- and epithelial cell-derived cathelicidin, utilizes formyl peptide receptor-like 1 (FPRL1) as a receptor to chemoattract human peripheral blood neutrophils, monocytes, and T cells. *J Exp Med*. 2000;192:1069-74.

23. Zhang Z, Cherryholmes G, Chang F, Rose DM, Schraufstatter I and Shively JE. Evidence that cathelicidin peptide LL-37 may act as a functional ligand for CXCR2 on human neutrophils. *Eur J Immunol*. 2009;39:3181-94.

24. Brandenburg LO, Jansen S, Wruck CJ, Lucius R and Pufe T. Antimicrobial peptide rCRAMP induced glial cell activation through P2Y receptor signalling pathways. *Molecular immunology*. 2010;47:1905-13.

25. Tomasinsig L, Pizzirani C, Skerlavaj B, Pellegatti P, Gulinelli S, Tossi A, Di Virgilio F and Zanetti M. The human cathelicidin LL-37 modulates the activities of the P2X7 receptor in a structure-dependent manner. *J Biol Chem*. 2008;283:30471-81.

26. Hwang YJ, Jung HJ, Kim MJ, Roh NK, Jung JW, Lee YW, Choe YB and Ahn KJ. Serum levels of LL-37 and inflammatory cytokines in plaque and guttate psoriasis. *Mediators of inflammation*. 2014;2014:268257.

27. Kahlenberg JM and Kaplan MJ. Little peptide, big effects: the role of LL-37 in inflammation and autoimmune disease. *J Immunol*. 2013;191:4895-901.

28. Pircher J, Czermak T, Ehrlich A, Eberle C, Gaitzsch E, Margraf A, Grommes J, Saha P, Titova A, Ishikawa-Ankerhold H, Stark K, Petzold T, Stocker T, Weckbach LT, Novotny J, Sperandio M, Nieswandt B, Smith A, Mannell H, Walzog B, Horst D, Soehnlein O, Massberg S and Schulz C. Cathelicidins prime platelets to mediate arterial thrombosis and tissue inflammation. *Nature communications*. 2018;9:1523.

29. Lande R, Botti E, Jandus C, Dojcinovic D, Fanelli G, Conrad C, Chamilos G, Feldmeyer L, Marinari B, Chon S, Vence L, Riccieri V, Guillaume P, Navarini AA, Romero P, Costanzo A, Piccolella E, Gilliet M and Frasca L. The antimicrobial peptide LL37 is a T-cell autoantigen in psoriasis. *Nature communications*. 2014;5.

30. Gordon YJ, Huang LC, Romanowski EG, Yates KA, Proske RJ and McDermott AM. Human cathelicidin (LL-37), a multifunctional peptide, is expressed by ocular surface epithelia and has potent antibacterial and antiviral activity. *Curr Eye Res*. 2005;30:385-394.

31. Agerberth B, Charo J, Werr J, Olsson B, Idali F, Lindbom L, Kiessling R, Jornvall H, Wigzell H and Gudmundsson GH. The human antimicrobial and chemotactic peptides LL-37 and alpha-defensins are expressed by specific lymphocyte and monocyte populations. *Blood*. 2000;96:3086-3093.

32. Rowley JW, Oler AJ, Tolley ND, Hunter BN, Low EN, Nix DA, Yost CC, Zimmerman GA and Weyrich AS. Genome-wide RNA-seq analysis of human and mouse platelet transcriptomes. *Blood*. 2011;118:e101-11.

33. Chandrashekar L, Rajappa M, Revathy G, Sundar I, Munisamy M, Ananthanarayanan PH, Thappa DM and Basu D. Is enhanced platelet activation the missing link leading to increased cardiovascular risk in psoriasis? *Clin Chim Acta*. 2015;446:181-185.

34. Yun SH, Sim EH, Goh RY, Park JI and Han JY. Platelet Activation: The Mechanisms and Potential Biomarkers. *Biomed Res Int*. 2016.

35. Varga-Szabo D, Braun A and Nieswandt B. Calcium signaling in platelets. *J Thromb Haemost*. 2009;7:1057-66.

36. Bergmeier W and Stefanini L. Novel molecules in calcium signaling in platelets. *J Thromb Haemost*. 2009;7 Suppl 1:187-90.

37. Su W, Chen Y, Wang C, Ding X, Rwibasira G and Kong Y. Human cathelicidin LL-37 inhibits platelet aggregation and thrombosis via Src/PI3K/Akt signaling. *Biochem Biophys Res Commun*. 2016;473:283-9.

38. Fabisiak A, Murawska N and Fichna J. LL-37: Cathelicidin-related antimicrobial peptide with pleiotropic activity. *Pharmacol Rep*. 2016;68:802-8.

39. Ong PY, Ohtake T, Brandt C, Strickland I, Boguniewicz M, Ganz T, Gallo RL and Leung DY. Endogenous antimicrobial peptides and skin infections in atopic dermatitis. *N Engl J Med*. 2002;347:1151-60.

40. Johansson J, Gudmundsson GH, Rottenberg ME, Berndt KD and Agerberth B. Conformation-dependent antibacterial activity of the naturally occurring human peptide LL-37. *J Biol Chem*. 1998;273:3718-24.

41. Wan M, Godson C, Guiry PJ, Agerberth B and Haeggstrom JZ. Leukotriene B4/antimicrobial peptide LL-37 proinflammatory circuits are mediated by BLT1 and FPR2/ALX and are counterregulated by lipoxin A4 and resolvin E1. *Faseb J*. 2011;25:1697-705.

42. Iaccio A, Cattaneo F, Mauro M and Ammendola R. FPRL1-mediated induction of superoxide in LL-37-stimulated IMR90 human fibroblast. *Arch Biochem Biophys*. 2009;481:94-100.

43. Czapiga M, Gao JL, Kirk A and Lekstrom-Himes J. Human platelets exhibit chemotaxis using functional N-formyl peptide receptors. *Exp Hematol*. 2005;33:73-84.

44. Fenalti G, Giguere PM, Katritch V, Huang XP, Thompson AA, Cherezov V, Roth BL and Stevens RC. Molecular control of delta-opioid receptor signalling. *Nature*. 2014;506:191-6.

45. Smolenski A. Novel roles of cAMP/cGMP-dependent signaling in platelets. *J Thromb Haemost*. 2012;10:167-76.

46. Wenzel-Seifert K, Arthur JM, Liu HY and Seifert R. Quantitative analysis of formyl peptide receptor coupling to g(i)alpha(1), g(i)alpha(2), and g(i)alpha(3). *J Biol Chem*. 1999;274:33259-66.

47. Yang J, Wu J, Jiang H, Mortensen R, Austin S, Manning DR, Woulfe D and Brass LF. Signaling through G(i) family members in platelets - Redundancy and specificity in the regulation of adenylyl cyclase and other effectors. *Journal of Biological Chemistry*. 2002;277:46035-46042.

48. Liu YF, Ghahremani MH, Rasenick MM, Jakobs KH and Albert PR. Stimulation of cAMP synthesis by G(i)-coupled receptors upon ablation of distinct G alpha(i) protein expression - G(i) subtype specificity of the 5-HT1A receptor. *Journal of Biological Chemistry*. 1999;274:16444-16450.

49. van der Fits L, Mourits S, Voerman JSA, Kant M, Boon L, Laman JD, Cornelissen F, Mus AM, Florencia E, Prens EP and Lubberts E. Imiquimod-Induced Psoriasis-Like Skin Inflammation in Mice Is Mediated via the IL-23/IL-17 Axis. *Journal of Immunology*. 2009;182:5836-5845.

50. Nerurkar L, McColl A, Graham G and Cavanagh J. The Systemic Response to Topical Aldara Treatment is Mediated Through Direct TLR7 Stimulation as Imiquimod Enters the Circulation. *Sci Rep-Uk*. 2017;7.

51. Fuentes-Duculan J, Bonifacio KM, Hawkes JE, Kunjravia N, Cueto I, Li X, Gonzalez J, Garcet S and Krueger JG. Autoantigens ADAMTSL5 and LL37 are significantly upregulated in active Psoriasis and localized with keratinocytes, dendritic cells and other leukocytes. *Exp Dermatol*. 2017;26:1075-1082.

52. Pavlov SI, Ivanova II, Gerova DI, Chervenkov TG and Balev SP. Serum level of the human antimicrobial cathelicidin (hCAP18/LL-37) in patients with psoriasis vulgaris. *Scripta Scientifica Medica*. 2016;48.

53. Niyonsaba F, Iwabuchi K, Someya A, Hirata M, Matsuda H, Ogawa H and Nagaoka I. A cathelicidin family of human antibacterial peptide LL-37 induces mast cell chemotaxis. *Immunology*. 2002;106:20-26.

54. Tjabringa GS, Ninaber DK, Drijfhout JW, Rabe KF and Hiemstra PS. Human cathelicidin LL-37 is a chemoattractant for eosinophils and neutrophils that acts via formyl-peptide receptors. *Int Arch Allergy Imm*. 2006;140:103-112.

55. Edfeldt K, Agerberth B, Rottenberg ME, Gudmundsson GH, Wang XB, Mandal K, Xu QB and Yan ZQ. Involvement of the antimicrobial peptide LL-37 in human atherosclerosis. *Arterioscl Throm Vas*. 2006;26:1551-1557.

56. Doring Y, Drechsler M, Wantha S, Kemmerich K, Lievens D, Vijayan S, Gallo RL, Weber C and Soehnlein O. Lack of neutrophil-derived CRAMP reduces atherosclerosis in mice. *Circ Res*. 2012;110:1052-6.

57. Mangoni ML, McDermott AM and Zasloff M. Antimicrobial peptides and wound healing: biological and therapeutic considerations. *Exp Dermatol*. 2016;25:167-173.

58. Gomes AP, Mano JF, Queiroz JA and Gouveia IC. Incorporation of antimicrobial peptides on functionalized cotton gauzes for medical applications. *Carbohydr Polym*. 2015;127:451-61.

59. Ogawa M. Differentiation and proliferation of hematopoietic stem cells. *Blood*. 1993;81:2844-53.

60. Tang YQ, Yeaman MR and Selsted ME. Antimicrobial peptides from human platelets. *Infect Immun*. 2002;70:6524-33.

61. Nording HM, Seizer P and Langer HF. Platelets in inflammation and atherogenesis. *Front Immunol*. 2015;6.

62. Ahlehoff O, Gislason GH, Lindhardsen J, Charlot MG, Jorgensen CH, Olesen JB, Bretler DM, Skov L, Torp-Pedersen C and Hansen PR. Psoriasis carries an increased risk of venous thromboembolism: a Danish nationwide cohort study. *PLoS One*. 2011;6:e18125.

63. Semeraro N, Ammollo CT, Semeraro F and Colucci M. Sepsis-associated disseminated intravascular coagulation and thromboembolic disease. *Mediterr J Hematol Infect Dis*. 2010;2:e2010024.

64. Berkestedt I, Herwald H, Ljunggren L, Nelson A and Bodelsson M. Elevated plasma levels of antimicrobial polypeptides in patients with severe sepsis. *J Innate Immun*. 2010;2:478-82.

65. Schaller-Bals S, Schulze A and Bals R. Increased levels of antimicrobial peptides in tracheal aspirates of newborn infants during infection. *Am J Respir Crit Care Med*. 2002;165:992-5.

66. Sorensen O, Cowland JB, Askaa J and Borregaard N. An ELISA for hCAP-18, the cathelicidin present in human neutrophils and plasma. *J Immunol Methods*. 1997;206:53-9.
67. Verjans ET, Zels S, Luyten W, Landuyt B and Schoofs L. Molecular mechanisms of LL-37-induced receptor activation: An overview. *Peptides*. 2016;85:16-26.

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3.5 Methods

Preparation of human platelet-rich plasma and isolated platelets

The University of Reading Research Ethics Committee approved all the experimental procedures using human blood from healthy volunteers. The blood samples were collected from healthy, aspirin-free volunteers after obtaining written informed consent. The blood was collected into VACUETTE® blood collection tubes containing 3.2% (w/v) sodium citrate. The blood samples were then centrifuged at 102*g* for 20 minutes at room temperature to obtain platelet-rich plasma (PRP). The PRP was rested at 30°C for 30 minutes prior to use. For the preparation of isolated platelets, the blood was mixed with ACD (2.5% sodium citrate, 2% D-glucose and 1.5% citric acid) at 1 (ACD): 9 (blood) ratio and centrifuged at 102*g* for 20 minutes. The PRP was collected, mixed with appropriate volume of ACD, and centrifuged at 1413*g* for 10 minutes at room temperature. The resultant platelet pellet was resuspended in modified Tyrodes-HEPES buffer (134mM NaCl, 2.9mM KCl, 0.34mM $Na_2HPO_4.12H_2O$, 12mM $NaHCO_3$, 20mM HEPES and 1mM $MgCl_2$, pH 7.3) with appropriate volume of ACD, and centrifuged once again at 1413*g* for 10 minutes at room temperature. The resultant platelet pellet was resuspended to a final density of 4×10^8 cells/mL in modified Tyrode's-HEPES buffer and allowed to rest for 30 minutes at 30ºC prior to use.

Mouse blood collection and platelet preparation

The mouse strains of *Fpr1*$^{-/-1}$ and *Fpr2/3*$^{-/-2}$ on a C57BL/6 background obtained from William Harvey Research Institute, London, UK and wild type C57BL/6 mice from Envigo, UK were used in this study. The mice were sacrificed with CO_2 and the blood was directly collected by cardiac puncture into a syringe containing 3.2% (w/v) sodium citrate at 1 (citrate):9 (blood) ratio. The blood was then centrifuged at 203*g* for 8 minutes at room temperature and the PRP was collected. The remaining blood was resuspended in 500 µL of modified Tyrode's-HEPES buffer and centrifuged once again at 203*g* for 5 minutes. The resultant PRP then centrifuged at 1028*g* for 5 minutes. The resultant platelet pellet was resuspended in modified Tyrode's-HEPES buffer at a density of $2x10^8$ cells/mL.

Enzyme-linked immunosorbent assay (ELISA) for the detection of LL37

To investigate the presence of LL37 in platelets, a direct ELISA was performed using LL37-selective antibody (sc-166770, Santa Cruz Biotechnology, USA). Human isolated platelets were treated with a vehicle (modified Tyrode's-HEPES buffer) or CRP-XL (1 μg/mL) for five minutes under stirring conditions to obtain resting or activated platelets, respectively. The platelets were centrifuged at 1413*g* for 10 minutes at room temperature. The supernatant was collected, stored at -80°C, lyophilised, and resuspended in Nonidet P40 (NP40) buffer. The resultant pellet was also resuspended in NP40 buffer. Briefly, a 96 well plate was coated with 50 μL of various concentrations of LL37 (for the standard curve), or the resting or activated platelet samples (pellets or supernatants), and incubated at 4ºC overnight. The plate was blocked with 150 μL of assay buffer (0.5% BSA in PBS) for one hour at room temperate. Following washing three times with a wash buffer (0.1% Triton X-100 in PBS), 50 μL of anti-LL37 antibody was added and the plate was then incubated for 4 hours at room temperature. Following incubation, the plates were washed with the wash buffer before the addition of goat anti-mouse horseradish peroxidase-conjugated IgG (Life Technologies, UK) and incubation for one hour at room temperate. The plates were then washed three times, and 100 μl 3,3',5,5'-tetramethylbenzidine (TMB) substrate was added and allowed to incubate at room temperature until the development of a colour. The reaction was stopped by the addition of 100 μL stop solution (0.5M HCl). The level of absorbance was measured at 450nm using an ELISA microplate reader (EMax precision plate reader, Molecular Devices, UK).

Immunofluorescence microscopy

Human platelets were stimulated with modified Tyrode's-HEPES buffer (resting platelets) or 3 μM U46619 (activated platelets) and were left to settle on poly-L-lysine coverslips for 1 hour at 37ºC. The platelets were then fixed with 0.2% formyl saline and then permeablised with 0.2% Triton in PBS. Following washing with PBS, the coverslips were incubated with primary antibodies for LL37 (Novus Biological, UK) and phalloidin (Invitrogen, UK) overnight at 4ºC and washed with PBS prior to staining with Alexa 488 and Alexa 647-conjugated secondary antibodies for phalloidin and LL37, respectively, and incubated for one hour at room temperature in the dark. Coverslips were

extensively washed and mounted onto slides. Platelets were imaged with a magnification of 100x using a Nikon A1-R confocal microscope (Nikon Instruments, UK).

In vitro thrombus formation assay

In vitro thrombus formation was performed as described previously[3, 4]. In brief, human $DiOC_6$-labelled (Sigma Aldrich, UK) human whole blood was pre-incubated with a vehicle or different concentrations of LL37 (10, 20 and 50 μM) for 10 minutes before perfusion over collagen (400μg/mL)-coated Vena8™ Biochips (Cellix Ltd, Ireland) at a shear rate of 20 dynes/cm^2. Z-stack fluorescence images of thrombi were obtained every 30 seconds for up to 10 minutes using a Nikon eclipse (TE2000-U) microscope (Nikon Instruments, UK). The fluorescence intensity was calculated by analysing the data using ImageJ software (National Institutes of Health, USA).

Tail bleeding assay

Tail bleeding assay was performed as described previously[5]. The British Home Office approved the experimental procedures. In brief, C57BL/6 mice (10-12 weeks old; Envigo, UK) were anaesthetised using ketamine (80 mg/kg) and xylazine (5 mg/kg) administered via the intraperitoneal route for 20 minutes before the experiment and were then placed on a heated mat (37°C). Vehicle or LL37 (20 μM) was infused via femoral artery 5 minutes before the dissection of 1mm of tail tip, and the tail was immersed in sterile saline. The time to cessation of bleeding was measured and the assay was terminated at 20 minutes.

Platelet aggregation assay

In vitro platelet aggregation assays were performed by optical aggregometry using a two-channel platelet aggregometer (Chrono-Log Corporation, USA). Isolated platelets (270 μL) were added into a siliconised cuvette and pre-warmed at 37°C for 90 seconds. Upon the addition of an agonist, the platelets were allowed to aggregate under continuous stirring at 1200rpm for 5 minutes at 37ºC and the level of aggregation was monitored. To analyse the effects of FPR2/ALX on LL37-induced platelet aggregation, FPR2/ALX-selective inhibitor, WRW_4, used. The platelets were pre-treated with WRW_4 (5 μM) for 5 minutes before the addition of LL37 (20 μM) and the level of

aggregation was monitored. Data were analysed by calculating the percentage of maximum platelet aggregation obtained at 5 minutes.

ATP secretion assay

To assess the level of dense granule secretion in platelets, ATP secretion assays were performed using a luciferin–luciferase luminescence substrate by lumi-aggregometry (Chrono-log, USA). The level of ATP released from platelets upon stimulation with a platelet agonist, CRP-XL (0.25 µg/mL), in the presence and absence of different concentrations of WRW_4 was measured by observing the level of luminescence released.

Flow cytometry based assays

In order to measure the level of fibrinogen binding and P-selectin exposure on the platelet surface, flow cytometry based assays were performed. Five microliters of PRP or isolated platelets were incubated with 1 µl of FITC-conjugated fibrinogen antibody (1:50) and 1 µl of $PECy^5$-conjugated anti-CD62P (P-selectin) (1:50) antibody in the presence and absence of various concentrations of LL37 or platelet agonists. The final volume was made up to 50 µl using HEPES-buffered saline (HBS) (150mM NaCl, 5mM KCl, 1mM $MgSO_4.7H_2O$ and 10mM HEPES, pH 7.4) and the samples were incubated for 20 minutes at room temperature. Following fixation in 0.2% formyl saline, the samples were analysed using an Accuri C6 flow cytometer (BD Biosciences, UK) by counting 5000 events within the gated population for platelets. The median fluorescence intensity was calculated using Accuri C6 software to quantify the levels of fibrinogen binding and P-selectin exposure on the surface of platelets. Similarly, for the analysis of FPR2/ALX expression on platelets, five microliters of PRP were incubated with 1 µl of anti-FPR2/ALX (5µg/mL) and 2 µl of Cy5-conjugated anti-mouse IgG (80 µg/mL) with or without 1 µg/mL CRP-XL. Following 20 minutes incubation at room temperature, the platelets were fixed in 0.2% formyl saline and analysed by flow cytometry. For the LL37 binding assay, following the incubation of isolated platelets with 5-FAM-LC-conjugated LL37 or 5-FAM-conjugated scrambled LL37 (20 µM) for 20 minutes, the platelets were fixed in 0.2% formyl saline and analysed by flow cytometry.

Intracellular calcium mobilisation assay

PRP (2mL) was mixed with 2 µL of Fluo-4 AM dye (1 µM) (Life technologies, UK) and incubated for 20 minutes at 30ºC in dark. The PRP was then centrifuged at 1413*g* for 10 minutes at 20ºC. The resultant platelet pellet was suspended in 500 µL modified Tyrode's-HEPES buffer and maintained at 30ºC in dark. The platelets were stimulated with different concentrations of LL37 or platelet agonists and the level of fluorescence intensity was measured by FluoStar Optima Spectrofluorimeter (BMG Labtech, Germany) at 37ºC for 180 seconds using an excitation wavelength of 485nm and emission at 510nm. Data were analysed by calculating the percentage of calcium released at 90 seconds.

SDS-PAGE and immunoblotting analysis

Immunoblot analysis was performed using platelet lysates prepared under reducing conditions. The samples were heated to 90°C for 10 minutes and subjected to SDS-PAGE using 10% resolving gels. The gels were then transferred to polyvinylidene difluoride (PVDF) membranes and blocked by incubation in 5% milk in TBS-T (20mM Tris, 140mM NaCl and Tween-20, pH 7.6). Following an overnight incubation with primary anti-FPR2/ALX antibody (1:500) (Abcam, UK), the blots were washed with TBS-T and incubated with secondary Cy5-conjugated goat anti-rabbit IgG antibodies (1:1000) (Invitrogen, UK) in TBS-T containing 5% milk for one hour at room temperature. Following washing in TBS-T for one hour at room temperature, the blots were analysed using a Typhoon 9400 Variable Mode Imager system (GE Healthcare, UK). Equal loading of proteins in each lane was determined using anti-human 14-3-3ζ antibodies (1:1000) (Santa Cruz Biotechnology, USA).

Lactate dehydrogenase (LDH) Cytotoxicity assay

To test whether LL37 has cytotoxic effects on platelets, the level of LDH released from LL37-treated human isolated platelets was determined using LDH Cytotoxicity Assay Kit (Pierce, UK) according to the manufacturer's instructions. In brief, the platelets were incubated at 37°C under 5% CO_2 for 30 minutes. The vehicle or a range of concentrations of LL37 (1 to 100 µM) was added in

duplicates to different wells of a 96 well plate and incubated for 10 minutes. One set of wells were treated with the lysis buffer provided from the kit as a positive control for maximum LDH release, and another set was treated with modified Tyrode's-HEPES buffer for the detection of spontaneous LDH release. Results provided represent mean values from duplicate absorbance measurements, and are given as fractional LDH release compared to the positive control, which yields maximum LDH release (i.e. 100% cytotoxicity).

Cyclic nucleotide assay

A cAMP ELISA kit (Cambridge Bioscience, UK) was used for the detection of the total cellular levels of cAMP in human and mouse platelets. Human isolated platelets were pre-incubated for 10 minutes with a selective inhibitor for FPR2/ALX, WRW_4. Similarly, platelets obtained from control or *Fpr2/3*$^{-/-}$ mice were also used. The platelets were lysed with 0.1M HCl and the levels of cAMP were calculated according to the manufacturer's protocol.

Mass spectrometry analysis for LL37 stability in plasma

Plasma stability of LL37 was determined using human plasma from three individual donors. LL37 was spiked into plasma at a final concentration of 100μg/mL and the solution was subsequently incubated at 37°C for up to 2 hours. Aliquots were removed at 0, 30, 60 and 120 minutes and diluted 1:4 in ice cold methanol. Samples were centrifuged at 2500rpm for 45 min at 4ºC. The supernatant was collected and analysed by mass spectrometry (LC-MS) (Orbitrap, C8 column, Solvent system: 0.1% formic acid in water and 0.1% formic acid in acetonitrile). Stability in plasma was calculated by integrating m/z peak areas of samples using Analyst software (XCalibur, Thermofisher, UK). Similar experiments were also performed in PRP obtained from three separate donors.

Platelet adhesion and spreading on fibrinogen

Isolated human platelets were treated with different concentrations of LL37 or an FPR2/ALX-selective inhibitor, WRW_4, prior to loading onto fibrinogen (100 μg/mL)-coated coverslips and incubation for 30 minutes. The coverslips were then washed with PBS to remove non-adhered platelets. Adhered platelets were fixed with 0.2% formyl saline for 10 minutes prior to

permeabilisation with 0.2% Triton X-100 for five minutes at room temperature. Adhered platelets were stained with Alexa 488-conjugated phalloidin for 30 minutes at room temperature. The coverslips were then mounted onto slides and scanned using a Nikon A1-R confocal microscope (60x objective). Ten random fields of view were recorded for each sample. The data were analysed to quantify the number of adhered and spread platelets, and the relative area of spread platelets using ImageJ. The relative surface area of spread platelets was obtained by subtracting the surface area of resting platelets.

Structural modelling and molecular docking analysis

The X-ray crystal structures of FPR2/ALX are not available; hence, homology models of this receptor were developed to determine the plausible interactions between LL37 and this receptor. The amino acid sequence of FPR2/ALX (accession code: P25090) was retrieved from UniProt[6] and submitted to MODELLER-ModWeb server[7] for protein structural modelling. The top three structural models generated were analysed for the sequence similarity, identity and orientation of amino acids. Homology models built on the structural template of human delta opioid 7 transmembrane receptor (PDB code - 4N6H; 1.8 Å) with which FPR2/ALX was found to share 29% identity and was identified as the most appropriate model for FPR2/ALX. This homology model was validated by docking with LL37 using Sybyl-X[8]. The interactions of LL37 with this receptor was identified using PatchDock[9]. The docking results were visualised using the program, PyMOL[10] and the molecular interactions of the docked ligands were analysed by the programme, CONTACTS, as provided in the CCP4 suite of programs[11, 12]. Potential hydrogen bonds were assigned if the distance between two electronegative atoms was less than 3.3Å.

Imiquimod-induced "psoriasis-like" skin inflammation

A 4cm^2 mouse dorsal skin area was shaved and depilated (Veet, France) prior to daily topical treatment of 75mg of Aldara™ (Imiquimod, IMQ) cream (Meda Pharma, UK) or Vaseline for 4 consecutive days following a previously published protocol[13]. Sample collection and further assays were performed on day 5. Daily double fold skin thickness was measured using a micrometer (Farnell,

UK) with 0.1mm accuracy and the change in thickness was calculated against day 0 skin thickness. PASI scoring for skin scaling (desquamations) and redness (erythema) was performed by assessors blinded to the treatments. Spleen weights were normalised to body weights.

Statistical analysis

Data presented in this study are represented as mean ± SEM. The statistical significance was analysed using two-tailed unpaired Student's *t* test for two-sample comparisons for the data obtained from ELISA for LL37 and mCRAMP quantification, flow cytometric assay for FPR2/ALX expression and platelet receptor characterisation, cAMP assay, LL37 binding assay and platelet aggregometry in the presence of a FPR2/ALX-selective inhibitor. For multiple comparisons, statistical significance was established using one-way or two-way ANOVA followed by Bonferroni's correction for data obtained from *in vitro* thrombus formation, LL37 plasma stability, LL37 binding in mice, ATP release, platelet aggregation and activation, calcium mobilisation, and LDH cytotoxicity assays. Data obtained from the tail bleeding assay were analysed using a non-parametric Mann-Whitney test. All statistical analyses were performed using GraphPad Prism 7 software (GraphPad Software Inc., USA).

References

1. Gao JL, Lee EJ and Murphy PM. Impaired antibacterial host defense in mice lacking the N-formylpeptide receptor. *J Exp Med*. 1999;189:657-62.
2. Dufton N, Hannon R, Brancaleone V, Dalli J, Patel HB, Gray M, D'Acquisto F, Buckingham JC, Perretti M and Flower RJ. Anti-inflammatory role of the murine formyl-peptide receptor 2: ligand-specific effects on leukocyte responses and experimental inflammation. *J Immunol*. 2010;184:2611-9.
3. Spyridon M, Moraes LA, Jones CI, Sage T, Sasikumar P, Bucci G and Gibbins JM. LXR as a novel antithrombotic target. *Blood*. 2011;117:5751-5761.
4. Vaiyapuri S, Jones CI, Sasikumar P, Moraes LA, Munger SJ, Wright JR, Ali MS, Sage T, Kaiser WJ, Tucker KL, Stain CJ, Bye AP, Jones S, Oviedo-Orta E, Simon AM, Mahaut-Smith MP and Gibbins JM. Gap junctions and connexin hemichannels underpin hemostasis and thrombosis. *Circulation*. 2012;125:2479-91.
5. Vaiyapuri S, Moraes LA, Sage T, Ali MS, Lewis KR, Mahaut-Smith MP, Oviedo-Orta E, Simon AM and Gibbins JM. Connexin40 regulates platelet function. *Nature communications*. 2013;4:2564.
6. UniProt C. Reorganizing the protein space at the Universal Protein Resource (UniProt). *Nucleic Acids Res*. 2012;40:D71-5.
7. Webb B and Sali A. Comparative Protein Structure Modeling Using MODELLER. *Curr Protoc Bioinformatics*. 2016;54:5 6 1-5 6 37.
8. Jain AN. Surflex: Fully automatic flexible molecular docking using a molecular similarity-based search engine. *J Med Chem*. 2003;46:499-511.
9. Duhovny D, Nussinov R and Wolfson HJ. Efficient unbound docking of rigid molecules. *Lect Notes Comput Sc*. 2002;2452:185-200.
10. Grell L, Parkin C, Slatest L and Craig PA. Viz, a tool for simplifying molecular viewing in PyMOL. *Biochem Mol Biol Edu*. 2006;34:402-407.
11. Cowtan K, Emsley P and Wilson KS. From crystal to structure with CCP4 introduction. *Acta Crystallogr D*. 2011;67:233-234.
12. Winn MD, Ballard CC, Cowtan KD, Dodson EJ, Emsley P, Evans PR, Keegan RM, Krissinel EB, Leslie AGW, McCoy A, McNicholas SJ, Murshudov GN, Pannu NS, Potterton EA, Powell HR, Read RJ, Vagin A and Wilson KS. Overview of the CCP4 suite and current developments. *Acta Crystallogr D*. 2011;67:235-242.
13. Roller A, Perino A, Dapavo P, Soro E, Okkenhaug K, Hirsch E and Ji H. Blockade of phosphatidylinositol 3-kinase PI3Kdelta or PI3Kgamma reduces IL-17 and ameliorates imiquimod-induced psoriasis-like dermatitis. *J Immunol*. 2012;189:4612-20.

4 – The role of Annexin A1 in the regulation of platelet functions

The role of Annexin A1 in the regulation of platelet functions

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4.1 Abstract

Annexin A1 (AnxA1) is an endogenous protein that is known to play a role in the resolution of inflammation. It plays a pivotal role in the modulation of inflammatory responses in order to reach homeostasis. Annexin A1 is known to bind formyl peptide receptor 2 (FPR2/ALX), which are a group of G-protein coupled receptors that play a role in host defense and inflammation. Despite the detailed research in the role of Annexin A1 in the modulation of immunological response in various immune cells, including neutrophils and monocytes, its effects on the regulation of haemostasis, thrombosis and platelet-mediated inflammatory responses remain unexplored. Additionally, there is a paucity of studies to the role of platelets in the regulation of resolution of inflammation. Here, we demonstrate the role of the AnxA1 N-terminal peptide, Ac2-26, in the regulation of platelet haemostatic function, where we were able to confirm its activatory role. Ac2-26 activated platelets, evident in its ability to induce fibrinogen binding and P-selectin exposure. Moreover, it induced calcium mobilisation in a concentration-dependent manner. All of which were abrogated in the absence of Fpr2/3. Conversely, the activatory effects of Ac2-26 were exaggerated in *AnxA1*-deficient mice. This might be attributed to our findings of the overexpression of FPR2/ALX receptor in these mice. Moreover, these mice exhibited dysfunctional platelet receptor levels (GPIb), but normal haemostasis (tail bleeding assay). Understanding the function of AnxA1 and FPR2/ALX in the modulation of platelet function and platelet-associated inflammation can lead to the development of improved therapeutics that can target the resolution of inflammation without compromising the immune state of the host.

4.2 Introduction

Platelets are non-nucleated blood cells that play a primary role in haemostasis by arresting bleeding upon vascular injury. Under pathophysiological conditions, the inappropriate activation of platelets leads to the formation of blood clots (thrombus) within the circulation[1]. This can reduce or retard the blood flow to vital organs including the heart and brain, ultimately causing heart attacks and strokes, respectively[2]. Cancer, type II diabetes, and cardiovascular diseases including coronary artery disease and atherosclerosis are among diseases associated with platelet reactivity. Conversely, failure of platelets to become activated leads to excessive bleeding and coagulopathies[3]. Apart from their haemostatic function, platelets are recognised as immune cells, mediating inflammation and innate immune responses[4]. This notion was supported by the presence of known immunologic receptors on platelets, including Toll-like receptors (TLRs)[5], chemokine receptors[6] and formyl peptide receptors (FPRs)[7].

Inflammation is a protective, physiological event induced by the body to remove stimulus and facilitate tissue repair in cases of injury[8]. Under physiological conditions, inflammation is followed by a process known as 'resolution', which limits the inflammatory cell migration, induces apoptosis and clearance of activated inflammatory cells[9], regaining/restoring homeostasis. However, failure to contain such processes contributes to the chronicity of inflammatory diseases[10, 11]. A crucial step in the resolution is the production of specialized pro-resolving mediators (SPMs), including lipids (resolvins, protectins, maresins and lipoxins), and proteins such as annexin A1 (AnxA1)[12]. In particular, AnxA1 (a glucocorticoid-regulated protein) is known to play a role in the regulation of inflammation, and cell proliferation, differentiation and apoptosis[13]. It is expressed in the cytosol of various resting cells, including neutrophils, monocytes, macrophages and epithelial cells[13]. Furthermore, a limited number of studies have reported its expression in platelets[14, 15]. The activation of such immune cells leads to the externalization of AnxA1 on the cell membrane surface and/or its secretion[16]. The extracellular AnxA1 then undergoes conformational changes, exposing its active

form, the N-terminal region that mediates the binding to its *N*-formyl peptide receptor, FPR2/ALX[17]. The externalization and secretion of Anxa1 is typically accompanied by a proteolytic cleavage of its N-terminal region, such cleavage is implicated with the down-regulation of Anxa1, rather than the release of bioactive peptides[18-20]. This was validated in a study whereby a cleavage-resistant Anxa1, termed Super Annexin A1 (SAnxA1), retained a prolonged function in the microvasculature[21]. While the full-length N-terminus has been shown to be inactive, a synthetic peptide containing the first 26 amino acids of its sequence displayed functionality[22]. The *N*-Acetyl 2–26 (Ac2-26) is a peptide sequence derived from this N-terminus form and has been shown to act as a pharmacophore [23] and plausibly maintain the properties of the full-length protein[21, 24-27]. Nonetheless, a limited/small number of studies reported an opposing effect[28-30]. Both full-length and cleaved forms of the protein have been found in inflammatory exudates and other extracellular biological fluids[31-34].

AnxA1 has been shown to play a role in the regulation of inflammation, albeit an opposing one, in various cell types[13]. However, its role in the regulation of platelet function; haemostasis and thrombosis remains unexplored. AnxA1 has been linked to inflammatory diseases associated with platelet reactivity[35], including atherosclerosis[36], myocardial infarctions[37] and strokes[38]. Furthermore, the dysregulation of AnxA1 expression has been implicated in various diseases/pathologies; its up-regulation has been reported in cancer[39], melanoma[40], breast cancer[41, 42], periods of remission in ulcerative colitis[11], and its downregulation in Crohn's disease[43], thyroid cancer[44], signifying/postulating its prognostic significance and therapeutic efficacy. While numerous studies demonstrated its role in the regulation of inflammation, there is a paucity of studies on its role in the regulation of platelet function. Hence, in this study, we aimed to demonstrate the direct role of endogenous AnxA1 and its exogenous, peptidomimetic Ac2-26 in the regulation of the haemostatic function of platelets via FPR2/ALX-mediated signalling pathways using a range of platelet functional assays. We were able to confirm the activatory/haemostatic role of Ac2-26 in the regulation of platelet function. Ac2-26 activated platelets, evident in its ability to induce fibrinogen binding and P-selectin exposure. Moreover, it induced calcium mobilisation in a concentration-dependent manner. These

results were abrogated in *Fpr2/3*-deficient mice. Conversely, the activatory effects of Ac2-26 were exaggerated in *AnxA1*-deficient mice, which might be attributed to our findings of the overexpression of FPR2/ALX receptor in these mice. Moreover, these mice exhibited dysfunctional platelet receptor levels (GPIb), but normal haemostasis (tail bleeding assay). Together, these findings demonstrate a central role of FPR2/ALX in the regulation of platelet function out of the scope of haemostasis. Understanding the function of AnxA1 and FPR2/ALX in the modulation of platelet function and platelet-associated inflammation might lead to the development of improved treatment modalities.

4.3 Results

4.3.1. Characterization of platelets obtained from Anxa1−/− mice

In order to assess the effect of Annexin A1 in the regulation of platelet function in this study, we have utilized *Anxa1−/−* mice. The AnxA1 protein is encoded by the *Anxa1* gene in mice [45]. Many studies have reported biological defects associated with such deficiency, including exaggerated inflammatory responses during myocardial ischaemia and cardiovascular dysfunction (in diabetes)[46-49]. Thus, to determine whether these mice exhibit any functional defects that might influence platelets and their haemostatic function, we have investigated the levels of major adhesion platelet receptors, including GPVI, GPIbα, αIIbβ3 and α2β1, and their expression levels on the surface of platelets by using flow cytometry. Notably, the characterisation of platelets obtained from *Anxa1−/−* mice failed to display any difference in GPVI, αIIbβ3 and α2β1 surface expression, However, a significant reduction in the levels of GPIbα were observed on the surface of platelets obtained from *Anxa1−/−* mice in comparison to the control mouse platelets (Figure 4-1A). This data indicates down-regulation of GPIbα receptors on the surface of platelets obtained from *Anxa1−/−* mice.

4.3.2 Systemic haemostasis is intact in Anxa1−/− mice

Since one of the major platelet adhesion receptors was down-regulated in *Anxa1−/−* mice, we sought to investigate whether the haemostasis is compromised in these mice using a tail bleeding model. No significant difference was observed in the mean bleeding time *Anxa1−/−* mice (225.2±3.6 seconds) compared to the control group (217.5±20.4 seconds) (Figure 4-1B). These data indicate that the loss of the *ANXA1* gene does not interfere with systemic haemostasis, rendering it intact.

4.3.3 Role of Annexin A1 in platelet activation

Many studies have reported an "anti-inflammatory" role of Annexin A1 in many cell types [16, 22, 23, 26, 50, 51]. However, other studies have reported "pro-inflammatory" properties of such protein. Presumably, anti-inflammatory molecules lead to an inhibition in platelet function[52-54], whereas the activation of platelets mediates pro-inflammatory responses[4]. The activation of platelets results in inside-out signaling to the integrin αIIbβ3 on the surface of platelet, converting it to a high affinity state that allows fibrinogen binding and subsequent platelet aggregation[55]. Moreover, upon activation, platelets secrete granules that mediate in the secondary platelet activation, namely, P-selectin is released from α-granules and then exposed to the surface of platelets[56]. In order to determine the effect of the "anti-inflammatory" Annexin A1 in the regulation of platelet function, platelet activation (evident by the levels of fibrinogen binding and P-selectin exposure) induced by CRP-XL (via GPVI receptor) and ADP (acts via P2Y receptors) was measured by flow cytometry. Upon the activation of platelets by CRP-XL, the level of both fibrinogen binding (Figure 4-1Ci) and P-selectin exposure (Figure 4-1Cii) were reduced in platelets obtained from *Anxa1−/−* compared to the control platelets. Similarly, these levels were reduced upon activation of platelets by ADP (Figure 4-1Di and 4-1Dii). Together, these data indicate an activatory/protective role for the endogenous, full-length Annexin A1 in the regulation of platelet function.

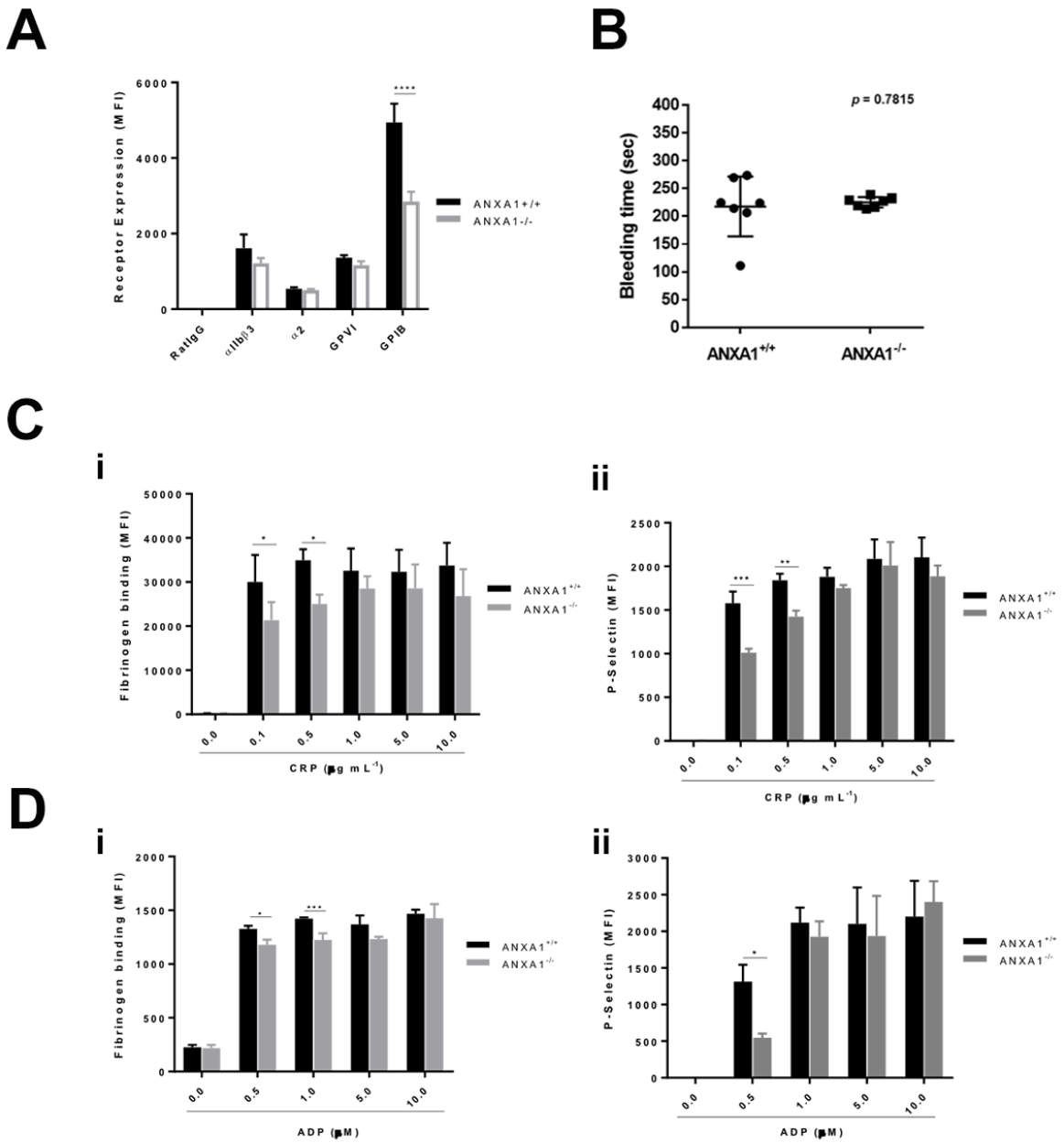

Figure 4-1: **Deletion of Anxa1 in mice affects platelet function.** The characterisation of platelets obtained from *AnxA1*$^{-/-}$ mice **(A)**. The expression levels of major platelet receptors such as αIIbβ3, α2β1, GPVI and GPIbα in platelets obtained from control and *AnxA1*$^{-/-}$ mice were analysed by flow cytometry using selective fluorescent-labelled antibodies. Data represent mean ± SEM (n=8). **B**, the impact of *AnxA1* deletion on the modulation of haemostasis was analysed by tail bleeding assay in control or *AnxA1*$^{-/-}$ mice. Data represent mean ± SEM (n=7). **C**, the levels of fibrinogen binding (**i**) and P-selectin exposure (**ii**) were analysed in whole blood obtained from control or *AnxA1*$^{-/-}$ mice upon stimulation with various concentrations of CRP-XL by flow cytometry. Data represent mean ± SEM (n=4). **D**, the levels of fibrinogen binding (**i**) and P-selectin exposure (**ii**) were analysed in whole blood obtained from control or *AnxA1*$^{-/-}$ mice upon stimulation with various concentrations of ADP by flow cytometry. Data represent mean ± SEM (n=4). The statistical significance was calculated by two-way ANOVA followed by Bonferroni's correction in most of the experiments except for the data shown in panel **A** and **B**, which were calculated by two-tailed unpaired Student's *t* test, and non-parametric Mann-Whitney test, respectively (*p<0.01. **p<0.001 and ***p<0.0001).

4.3.4 Role of Annexin A1 N-terminal cleavage peptide, Ac2-26, in platelet function

Many studies have reported "anti-inflammatory" properties of Annexin A1 in various cell types by utilization of AnxA1 recombinant proteins while others utilized its pharmacophore, the Ac2-26 N-terminal peptide cleavage molecule, to demonstrate such properties[24, 29, 57-59]. Conversely, a limited number of studies have reported opposing "pro-inflammatory" properties[29]. Hence, to investigate the role of this cleavage peptide in the regulation of platelet function, its effect on platelet activation was measured using flow cytometry. $ANXA1_{Ac2-26}$ induced platelet activation as evidenced in its ability to trigger fibrinogen binding (Figure 4-2Ai) and P-selectin exposure (Figure 4-2Aii) in a concentration-dependent manner. Moreover, these results were exaggerated/pronounced in platelets obtained from *Anxa1−/−* mice compared to control mouse platelets. Together, these data indicate an activatory role of the $ANXA1_{Ac2-26}$.

4.3.5 Ac2-26 and acts through FPR2/ALX to induce platelet activation and platelet-leukocyte aggregation

The Annexin A1 peptide is known to act through FPR2/ALX in various cell types[25, 60, 61]. Thus, following our findings on the activatory roles of ANX_{Ac2-26} on platelet function, we sought to investigate whether these effects were mediated through FPR2/ALX in platelets as well. The activatory effects of ANX_{Ac2-26} were abrogated in the presence of an FPR2/ALX-selective inhibitor, WRW_4, as evidenced in its ability to reduce the level of fibrinogen binding compared to the control (Figure 4-2Ci). Furthermore, the level of P-selectin exposure was reduced in platelets obtained from *Fpr2/3−/−* mice compared to control mouse platelets (Figure 4-2Cii). Notably, the characterization of platelets obtained from *Fpr2/3−/−* mice failed to display any defects in the size and number of platelets or the levels of major platelet receptors including GPVI, GPIbα, αIIbβ3 and α2β1 (Figure 4-2Ciii) in comparison to the control mouse platelets.

To corroborate the activatory effects of ANX_{Ac2-26} on platelet function through FPR2/ALX, its ability to induce calcium mobilization in *Fpr2/3−/−* mice was investigated. Upon the activation of platelets, mobilization of Ca^{2+} stored in the dense tubular system in platelets and influx of extracellular Ca^{2+} across the plasma membrane occurs, ultimately leading to thrombus formation[62]. To assess the ability of ANX_{Ac2-26} to induce calcium mobilization in platelets, intracellular calcium levels were measured in Fluo-4 AM dye-loaded mouse platelets using flow cytometry. Indeed, ANX_{Ac2-26} induced calcium mobilization in mouse platelets in a concentration-dependent manner. Furthermore, these effects were abrogated in platelets obtained from *Fpr2/3−/−* mice compared to the control platelets (Figure 4-2D). Together, these data confirm the role of FPR2/ALX in mediation of ANX_{Ac2-26} activatory roles in platelet function.

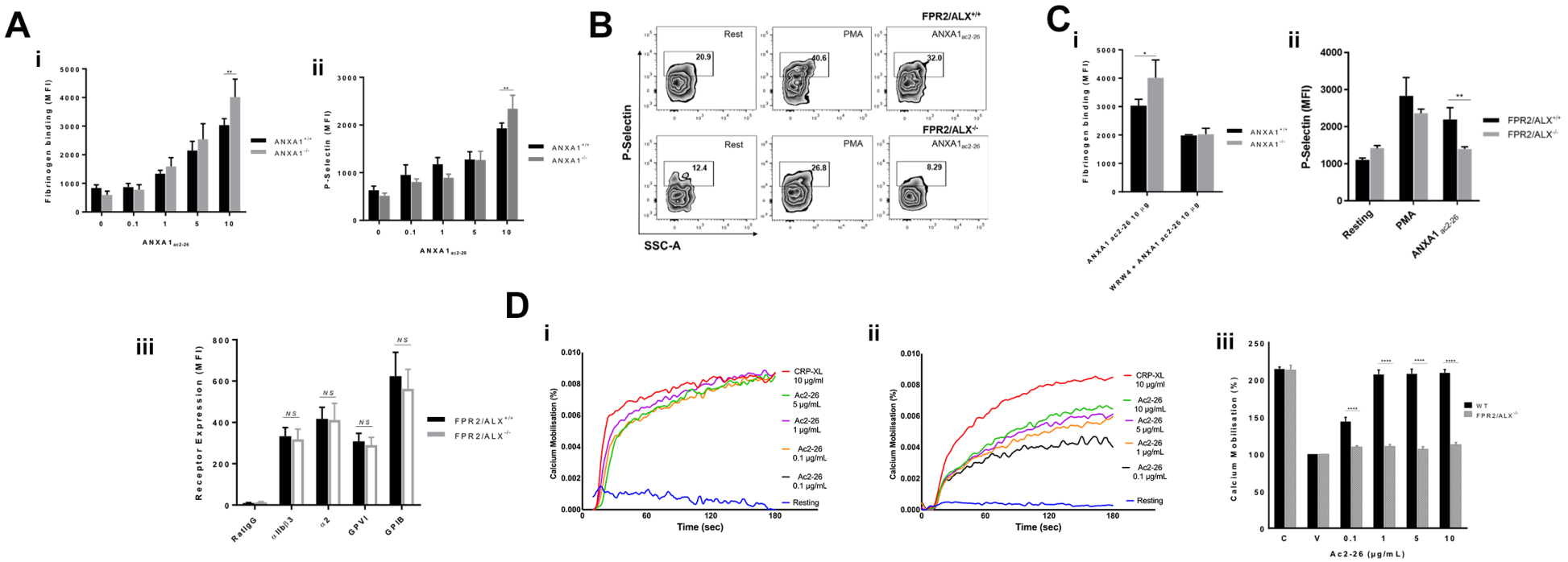

Figure 4-2: **Ac2-26 effects are mediated through FPR2/ALX in platelets.** The activation of platelets **(A).** The levels of fibrinogen binding (**Ai**) and P-selectin exposure (**Aii**) were analysed in whole blood obtained from control or *AnxA1*$^{-/-}$ mice upon stimulation with various concentrations of Ac2-26 by flow cytometry. Data represent mean ± SEM (n=4). **B**, the level of P-selectin exposure upon stimulation with Ac2-26 was measured by flow cytometry in control or *Fpr2/3*$^{-/-}$ mice. Data represent mean ± SEM (n=4). C, the level fibrinogen binding was analysed in whole blood obtained from control or *AnxA1*$^{-/-}$ mice upon stimulation with Ac2-26 in the presence or absence of WRW4 by flow cytometry (**i**), and the level of P-selectin exposure (**ii**) was analysed in whole blood obtained from control or *Fpr2/3*$^{-/-}$ mice upon stimulation with Ac2-26 by flow cytometry. Data represent mean ± SEM (n=4). **Ciii**, the characterisation of platelets obtained from *Fpr2/3*$^{-/-}$ mice; expression levels of major platelet receptors such as αIIbβ3, α2β1, GPVI and GPIbα in platelets obtained from control and *AnxA1*$^{-/-}$ mice were analysed by flow cytometry using selective fluorescent-labelled antibodies. Data represent mean ± SEM (n=8). **D**, Ca^{2+} mobilisation was measured using Fluo-4 AM dye-loaded human isolated platelets upon stimulation with Ac2-29 in platelets obtained from control or *Fpr2/3*$^{-/-}$ mice by flow cytometry. Data represent mean ± SEM (n=4). The statistical significance was calculated by two-way ANOVA followed by Bonferroni's correction in most of the experiments except for the data shown in panel **Ciii**, which were calculated by two-tailed unpaired Student's *t* test (*p<0.01. **p<0.001 and ***p<0.0001).

4.3.6 Fpr2/3 is overexpressed in Anxa1−/− mice

Following confirmation on the role of FPR2/ALX in mediating the functions of ANX_{Ac2-26} on platelets, and its exaggerated role in *Anxa1−/−* mice, we sought to investigate whether the levels of this receptor were altered in *Anxa1−/−* mice. Interestingly, the levels of *Fpr2/3* were significantly increased in *Anxa1−/−* mice compared to controls as evidenced in electron microscopy (Figure 4-3A) and flow cytometry (Figure 4-3B). These data indicate the upregulation/overexpression of *Fpr2/3* in *Anxa1−/−* mice.

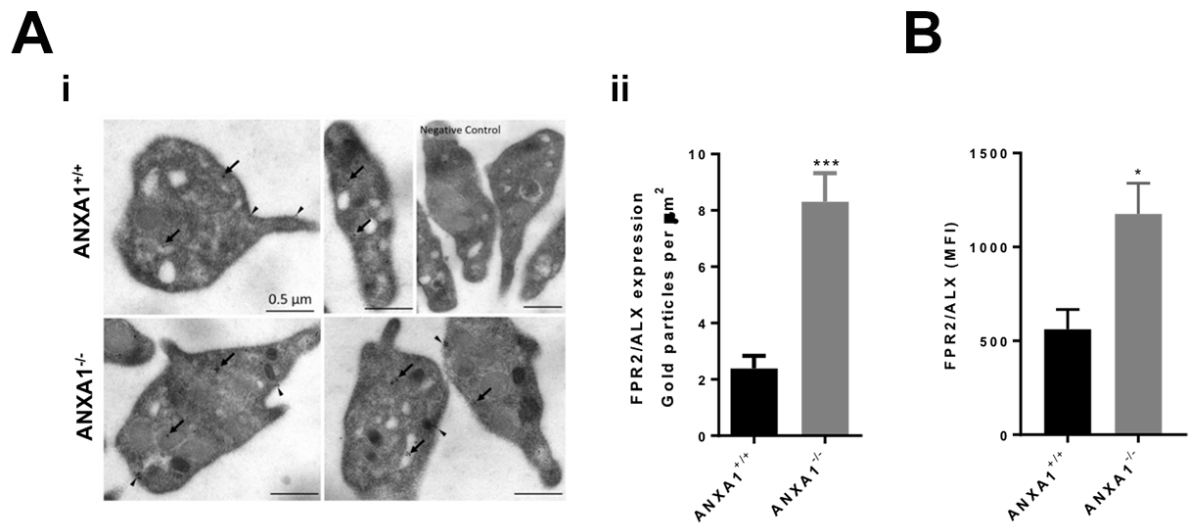

Figure 4-3: **FPR2/ALX is overexpressed in *AnxA1*$^{-/-}$. T**he presence of FPR2/ALX in overexpressed in *AnxA1*$^{-/-}$ mice as analysed by microscopy **(A)** and by flow cytometry (**B).** Data represent mean ± SEM (n=4). The statistical significance was calculated by two-tailed unpaired Student's *t* test (**p*<0.01 and ****p*<0.0001).

4.4 Discussion

AnxA1 has been shown to induce opposing physiological roles. Albeit a plethora of studies have reported its 'anti-inflammatory and pro-resolving' role in various immune cells[47], some have demonstrated its 'pro-inflammatory' capabilities[29, 30, 63, 64]. Moreover, the dysregulation of AnxA1 has been implicated in various pathological conditions, including cancer[61], infections[65], inflammatory bowel disease[43]. Furthermore, a pathogenic role of Ac2-26 has been previously described in cancer, wherein it facilitated metastasis/pro-invasiveness[66], and in rheumatoid arthritis, where it facilitated the secretion of matrix metalloproteinases (MMPs)[29]. Here, we demonstrate for the first time the role of AnxA1 and its N-terminal peptide sequence, Ac2-26, in the activation of the haemostatic platelet function through *Fpr2/3* in mice.

The *Anxa1*−/− mice utilized in this study demonstrated decreased activation of platelets compared to mice expressing the *Anxa1* gene, signifying the positive role of the full-length protein in the activation of platelet function that is induced by conventional platelet agonists such as CRP-XL (via GPIV) and ADP (via $P2Y_1$ and $P2Y_{12}$). Conversely, the activation of platelets with the N-terminal AnxA1 peptide sequence, Ac2-26, was exaggerated in *Anxa1*−/− mice compared to the control mice, this could be ascribed to a compensatory mechanism implicated with the deficiency of Anxa1 gene in these mice, buffering its deletion. Notably, the systemic haemostasis was intact and not impacted by/in the absence of AnxA1, plausibly indicating that AnxA1 may act on the site of injury, while leaving the physiological haemostasis intact. Furthermore, we observed a significant reduction in the expression of one of the major adhesion receptors on the platelets of these mice, GPIbα. Additionally, these mice displayed a marked expression/up-regulation in Fpr2/3, a receptor that mediates the signaling of both AnxA1 and its N-terminal peptide Ac2-26. This may explain the exaggerated Ac2-26 response that was observed in *Anxa1*−/− mice. The altered factors in these mice may be so in order to maintain a new physiological balance, allowing overall haemostasis. To further add to this, the dysregulation of other members of the Annexin superfamily has been previously reported in these mice[47].

Contrasting roles/mechanisms of action between ANXA1 and its N-terminal peptide Ac2-26 were previously reported[29, 59]. Here, we demonstrate that Ac2-26 evoked both an activatory platelet haemostatic function and a pro-inflammatory response, while the endogenous full-length Anxa1 limited/controlled such responses, demonstrating possible protective roles. Ac2-26 activated platelets as evident by its ability to induce calcium mobilization, integrin αIIbβ3 conformation and P-selectin exposure. All of these responses were mediated through *Fpr2/3* in platelets. A protective role of ANXA1 in many pathological conditions implicated with platelet pathogenesis has been reported previously, including atherosclerosis[36, 67-69], myocardial infarctions[37, 70] and strokes[27, 38, 71, 72]. The contrasting effects we have observed in this study could be attributed to the ability of Ac2-26 to heterodimerize FPR2/ALX with FPR1, which can result in the activation of pro-apoptotic signaling pathways and can elicit distinctive pathways[73]. Nonetheless, there is a paucity of studies addressing this phenomenon. Furthermore, Ac2-26 can active all FPRs family members[26, 28], whereas Anxa1 binds FPR2/ALX only[74]. There is a multiplicity of ligands that bind FPRs with diverse functions[75]. Notably, pro-inflammatory responses of such ligands are elicited via FPR1, while the majority of the anti-inflammatory/pro-resolving functions are mediated through FPR2/ALX[76]. Nonetheless, there are various ligands that can activate pro-inflammatory signaling mechanisms through FPR2/ALX, such as the endogenous antimicrobial peptide, LL37, that has been shown to exacerbate inflammatory responses, promoting atherosclerosis[77].

Deciphering the role of the ANXA1-FPR2/ALX axis in the regulation of platelet function could open up new avenues toward improved therapeutic strategies for the management of inflammatory responses, and particularly with those associated with bleeding or thrombotic events. Furthermore, it could provide insight into the mechanisms underlying platelet-associated complications in inflammatory diseases. Targeting ANXA1 contributes to immune dysfunction resolution in infections[65]. Since many of the pro-resolving mechanisms are mediated through FPR2/ALX, it could be utilized to suppress exacerbated inflammation without targeting the host defense[78], in contrast to anti-inflammatory mechanisms, minimizing unwarranted side effects and yielding more effective therapeutic strategies and effectual management of inflammation.

Additionally, many individuals suffering from autoimmune and inflammatory diseases are prescribed supraphysiological doses of glucocorticoids (GCs) to ameliorate signs of inflammation/inflammatory responses[79]. Nonetheless, GCs are associated with several unwarranted risks, including immunosuppression, cardiovascular disease[79, 80], Cushing's syndrome and psychiatric disorders[81]. Notably, GCs can induce the activation of platelets[82], and have been associated with hypercoagulability of blood[83]. Moreover, the positive regulation of the secretion, and the expression of AnxA1[13, 84-86] and its receptor, FPR2/ALX in monocytes[87, 88] and neutrophils[89] are implicated in response to GCs. Thus, targeting this pathway could complement current therapeutic strategies.

In conclusion, this study demonstrates a prominent role of AnxA1 and its N-terminal peptide (Ac2-26) in the regulation of platelet function, evident in the ability of Ac2-26 to induce inside-out signalling to integrin αIIbβ3 and granule secretion. The activatory roles of Ac2-26 in platelets were diminished in the presence of FPR2/ALX pharmacological inhibitors or in *Fpr2/3*-deficient mice, corroborating the functional dependence of Ac2-26 on this receptor. Moreover, we demonstrate a key role of AnxA1 in the positive regulation of platelet function. Together, the significant roles of AnxA1 and *Fpr2/3* in the modulation of thrombosis and haemostasis renders them potential attributes for the exacerbation of thrombotic complications and inflammatory responses in numerous diseases where platelet function plays essential roles. Notably, the presence of FPRs in platelets opens up new avenues to investigate the involvement of a multiplicity of FPR ligands in the modulation of thrombosis, haemostasis, and platelet-mediated inflammatory responses.

4.5 Methods

Animals

Animal experiments complied with the University of Reading Research guidelines approved by the British Home Office or Federal University of Sao Paulo Animal Care and Use Committee. Eight to ten-week-old male or female mice, wild-type (WT) C57BL/6, ANXA1$^{-/-}$ and FPR2/3$^{-/-}$ mice were used.

Mouse blood collection and platelet preparation

The mice were sacrificed with CO_2 and the blood was directly collected by cardiac puncture into a syringe containing 3.2% (w/v) sodium citrate at 1:9 ratio. The blood was then centrifuged at 203g for 8 minutes at room temperature and the PRP was collected. The remaining blood was resuspended in 500 µL of modified Tyrode's-HEPES buffer and centrifuged once again at 203g for 5 minutes. The PRP was then mixed and centrifuged at 1028g for 5 minutes. The resultant platelet pellet was then resuspended in modified Tyrode's buffer at a density of $2x10^8$ cell/mL.

Flow cytometric analysis

Five microliters of whole blood were incubated with 1 µL of FITC-conjugated fibrinogen antibody (1:50) and 1 µL of PECy5-conjugated anti-CD62P (P-selectin) (1:50) antibody in the presence and absence of increasing concentrations of ANXA1 mimetic peptide Ac2-26 ($ANXA1_{ac2-26}$: Ac-AMVSEFLKQAWFIENEEQEYVQTVK, Tocris, UK). Multiparameter acquisition was performed using a Fortessa analyser device (BD Biosciences), and the median fluorescence intensity was calculated using FlowJo software (Tree Star, Ashland, USA) to quantify the levels of fibrinogen binding and P-selectin exposure on the surface of platelets. Negative controls were set using an appropriate immunoglobulin G1 (IgG1) k-isotype–matched control for the anti-CD62P antibody, and inclusion of EGTA

(10mM) to prevent fibrinogen binding. Similarly, for the analysis of ANXA1 and FPR2/ALX expression on platelets, samples were incubated with 1 µL of anti-ANXA1 or anti-FPR2/ALX (5 µg mL^{-1}) and 2 µL of Cy5-conjugated anti-mouse IgG (80 µg mL^{-1}). Following 20 minutes of incubation at room temperature, samples were fixed in 0.2% formyl saline and analysed by flow cytometry.

Intracellular calcium mobilization assay

PRP (2 mL) derived from WT and FPR2/3$^{-/-}$ mice was mixed with 2 µL of Fluo-4 AM dye (1 µM) (Life technologies, UK) and incubated for 20 minutes at 30ºC in the dark. The PRP was then centrifuged at 1413g for 10 min at 20ºC. The resultant platelet pellet was suspended in 500 µL Tyrode's-HEPES buffer and maintained at 30ºC in the dark. The platelets were stimulated with different concentration of ANXA1 mimetic peptide Ac2-26 and the level of fluorescence intensity was measured by FluoStar Optima spectrofluorimeter (BMG Labtech, Germany) at 37ºC for 180 seconds using an excitation wavelength of 485nm and emission at 510nm. Data were analysed by calculating the percentage of calcium released at 90 seconds.

Tail bleeding assay

The experimental procedures were approved by the British Home Office. In brief, wild-type (WT) C57BL/6 and ANXA1$^{-/-}$ (8-10 weeks old male or female mice) were anaesthetised using ketamine (80 mg Kg^{-1}) and xylazine (5 mg Kg^{-1}) administered via the intraperitoneal route for 20 minutes before the experiment and were then placed on a heated mat (37ºC). 1mm of tail tip was removed using a scalpel blade and the tail tip was placed in sterile saline at 37ºC. The time to cessation of bleeding into warmed saline was measured up to 20 minutes.

SDS-PAGE and immunoblotting analysis

Immunoblot analysis was performed using platelets lysates prepared under reducing conditions. The samples were heated to 90ºC for 10 minutes and subjected to SDS-PAGE using 10% resolving gels. Equal loading of proteins in each lane was determined using anti-human 14-3-3ζ antibodies (1:1000) (Santa Cruz Biotechnology, USA). The gels were then transferred to polyvinylidene difluoride membranes and blocked by incubation in 5% milk in TBS-T (20mM Tris, 140mM NaCl, Tween-20, pH 7.6). Following overnight incubation with primary anti-FPR2/ALX antibody (1:500) (Abcam, UK), the blots were washed with TBS-T and incubated with secondary Cy^5 goat anti-rabbit IgG antibody (1:1000) (Invitrogen, UK) in TBS-T containing 5% milk for 1 hour at room temperature. Following washing in TBS-T for 1 hour at room temperature, the blots were analysed using the Typhoon 9400 variable mode imager system (GE Healthcare, UK).

Electron microscopy analysis

Platelets were fixed in a 4% paraformaldehyde, 0.5% glutaraldehyde solution (1:1) in sodium cacodylate buffer 0.1 M (pH 7.4) for 24 hours at 4 ° C. The cells were subsequently dehydrated through a methanol series and embedded in LRGold (London Resin; Reading, Berkshire, UK). To detect the localization of the FPR2, ultrathin sections (70 nm) of cells were incubated with the rabbit polyclonal anti-FPR2 (1:100; Santa Cruz Biotechnology, Santa Cruz, CA, USA) and goat anti-rabbit IgG antibody (1:50 in PBS containing 1% egg albumin) conjugated with 15 nm colloidal gold (British Biocell, Cardiff, UK). These sections were stained with uranyl acetate and lead citrate and then examined using a ZEISS EM900 electron microscope (Carl Zeiss, Jena, Germany) as described. Randomly photographed sections of cells were used for immunocytochemical analysis. The area of the cell compartment was determined with AxioVision software. The density of immunogold particles (number of gold

particles per μm^2) was calculated and expressed for each cell compartment. Values are reported as the mean ± SEM of 25-30 electron micrographs analysed per group.

References

1. Duhamel TA, Xu YJ, Arneja AS and Dhalla NS. Targeting platelets for prevention and treatment of cardiovascular disease. *Expert Opin Ther Targets*. 2007;11:1523-33.
2. Michelson AD. Antiplatelet therapies for the treatment of cardiovascular disease. *Nat Rev Drug Discov*. 2010;9:154-69.
3. Xu XR, Zhang D, Oswald BE, Carrim N, Wang X, Hou Y, Zhang Q, Lavalle C, McKeown T, Marshall AH and Ni H. Platelets are versatile cells: New discoveries in hemostasis, thrombosis, immune responses, tumor metastasis and beyond. *Crit Rev Clin Lab Sci*. 2016;53:409-30.
4. Nording HM, Seizer P and Langer HF. Platelets in inflammation and atherogenesis. *Front Immunol*. 2015;6.
5. Cognasse F, Hamzeh H, Chavarin P, Acquart S, Genin C and Garraud O. Evidence of Toll-like receptor molecules on human platelets. *Immunol Cell Biol*. 2005;83:196-8.
6. Borst O, Munzer P, Gatidis S, Schmidt EM, Schonberger T, Schmid E, Towhid ST, Stellos K, Seizer P, May AE, Lang F and Gawaz M. The inflammatory chemokine CXC motif ligand 16 triggers platelet activation and adhesion via CXC motif receptor 6-dependent phosphatidylinositide 3-kinase/Akt signaling. *Circ Res*. 2012;111:1297-307.
7. Czapiga M, Gao JL, Kirk A and Lekstrom-Himes J. Human platelets exhibit chemotaxis using functional N-formyl peptide receptors. *Exp Hematol*. 2005;33:73-84.
8. Esmon CT. The interactions between inflammation and coagulation. *Brit J Haematol*. 2005;131:417-430.
9. Maskrey BH, Megson IL, Whitfield PD and Rossi AG. Mechanisms of resolution of inflammation: a focus on cardiovascular disease. *Arterioscler Thromb Vasc Biol*. 2011;31:1001-6.
10. Sugimoto MA, Sousa LP, Pinho V, Perretti M and Teixeira MM. Resolution of inflammation: what Controls its Onset? *Front Immunol*. 2016;7.
11. Vong L, Ferraz JG, Dufton N, Panaccione R, Beck PL, Sherman PM, Perretti M and Wallace JL. Up-regulation of Annexin-A1 and lipoxin A(4) in individuals with ulcerative colitis may promote mucosal homeostasis. *PLoS One*. 2012;7:e39244.
12. Basil MC and Levy BD. Specialized pro-resolving mediators: endogenous regulators of infection and inflammation. *Nat Rev Immunol*. 2016;16:51-67.
13. Perretti M and D'Acquisto F. Annexin A1 and glucocorticoids as effectors of the resolution of inflammation. *Nature Reviews Immunology*. 2009;9:62-70.
14. Murphy CT, Peers SH, Forder RA, Flower RJ, Carey F and Westwick J. Evidence for the presence and location of annexins in human platelets. *Biochem Biophys Res Commun*. 1992;189:1739-46.
15. Eldering JA, Kocher M, Clemetson JM, Clemetson KJ, Frey FJ and Frey BM. Presence of lipocortins I and IV, but not II and VI, in human platelets. *FEBS Lett*. 1993;318:231-4.
16. Perretti M, Croxtall JD, Wheller SK, Goulding NJ, Hannon R and Flower RJ. Mobilizing lipocortin 1 in adherent human leukocytes downregulates their transmigration. *Nat Med*. 1996;2:1259-62.
17. Perretti M. The annexin 1 receptor(s): is the plot unravelling? *Trends Pharmacol Sci*. 2003;24:574-579.
18. Perretti M. Endogenous mediators that inhibit the leukocyte-endothelium interaction. *Trends Pharmacol Sci*. 1997;18:418-25.

19. Rescher U, Goebeler V, Wilbers A and Gerke V. Proteolytic cleavage of annexin 1 by human leukocyte elastase. *Biochim Biophys Acta*. 2006;1763:1320-4.
20. Vong L, D'Acquisto F, Pederzoli-Ribeil M, Lavagno L, Flower RJ, Witko-Sarsat V and Perretti M. Annexin 1 cleavage in activated neutrophils: a pivotal role for proteinase 3. *J Biol Chem*. 2007;282:29998-30004.
21. Pederzoli-Ribeil M, Maione F, Cooper D, Al-Kashi A, Dalli J, Perretti M and D'Acquisto F. Design and characterization of a cleavage-resistant Annexin A1 mutant to control inflammation in the microvasculature. *Blood*. 2010;116:4288-96.
22. Cirino G, Cicala C, Sorrentino L, Ciliberto G, Arpaia G, Perretti M and Flower RJ. Anti-inflammatory actions of an N-terminal peptide from human lipocortin 1. *Br J Pharmacol*. 1993;108:573-4.
23. Perretti M and Flower RJ. Annexin 1 and the biology of the neutrophil. *Journal of leukocyte biology*. 2004;76:25-9.
24. Bandeira-Melo C, Bonavita AG, Diaz BL, PM ES, Carvalho VF, Jose PJ, Flower RJ, Perretti M and Martins MA. A novel effect for annexin 1-derived peptide ac2-26: reduction of allergic inflammation in the rat. *J Pharmacol Exp Ther*. 2005;313:1416-22.
25. Dalli J, Montero-Melendez T, McArthur S and Perretti M. Annexin A1 N-terminal derived Peptide ac2-26 exerts chemokinetic effects on human neutrophils. *Front Pharmacol*. 2012;3:28.
26. Gavins FN, Yona S, Kamal AM, Flower RJ and Perretti M. Leukocyte antiadhesive actions of annexin 1: ALXR- and FPR-related anti-inflammatory mechanisms. *Blood*. 2003;101:4140-7.
27. Gavins FN, Dalli J, Flower RJ, Granger DN and Perretti M. Activation of the annexin 1 counter-regulatory circuit affords protection in the mouse brain microcirculation. *Faseb J*. 2007;21:1751-8.
28. Ernst S, Lange C, Wilbers A, Goebeler V, Gerke V and Rescher U. An annexin 1 N-terminal peptide activates leukocytes by triggering different members of the formyl peptide receptor family. *J Immunol*. 2004;172:7669-76.
29. Tagoe CE, Marjanovic N, Park JY, Chan ES, Abeles AM, Attur M, Abramson SB and Pillinger MH. Annexin-1 mediates TNF-alpha-stimulated matrix metalloproteinase secretion from rheumatoid arthritis synovial fibroblasts. *J Immunol*. 2008;181:2813-20.
30. Williams SL, Milne IR, Bagley CJ, Gamble JR, Vadas MA, Pitson SM and Khew-Goodall Y. A proinflammatory role for proteolytically cleaved annexin A1 in neutrophil transendothelial migration. *J Immunol*. 2010;185:3057-63.
31. Chung YW, Oh HY, Kim JY, Kim JH and Kim IY. Allergen-induced proteolytic cleavage of annexin-1 and activation of cytosolic phospholipase A2 in the lungs of a mouse model of asthma. *Proteomics*. 2004;4:3328-34.
32. Vishwanatha JK, Davis RG, Rubinstein I and Floreani A. Annexin I degradation in bronchoalveolar lavage fluids from healthy smokers: a possible mechanism of inflammation. *Clin Cancer Res*. 1998;4:2559-64.
33. Vergnolle N, Comera C and Bueno L. Annexin 1 is overexpressed and specifically secreted during experimentally induced colitis in rats. *Eur J Biochem*. 1995;232:603-10.
34. Tsao FH, Meyer KC, Chen X, Rosenthal NS and Hu J. Degradation of annexin I in bronchoalveolar lavage fluid from patients with cystic fibrosis. *Am J Respir Cell Mol Biol*. 1998;18:120-8.
35. de Jong RJ, Leoni G, Drechsler M and Soehnlein O. The advantageous role of annexin A1 in cardiovascular disease. *Cell Adh Migr*. 2016:0.
36. Kusters DH, Chatrou ML, Willems BA, De Saint-Hubert M, Bauwens M, van der Vorst E, Bena S, Biessen EA, Perretti M, Schurgers LJ and Reutelingsperger CP. Pharmacological Treatment with Annexin A1 Reduces Atherosclerotic Plaque Burden in LDLR-/- Mice on Western Type Diet. *PLoS One*. 2015;10:e0130484.

37. Qin C, Buxton KD, Pepe S, Cao AH, Venardos K, Love JE, Kaye DM, Yang YH, Morand EF and Ritchie RH. Reperfusion-induced myocardial dysfunction is prevented by endogenous annexin-A1 and its N-terminal-derived peptide Ac-ANX-A1(2-26). *Br J Pharmacol*. 2013;168:238-52.
38. Relton JK, Strijbos PJ, O'Shaughnessy CT, Carey F, Forder RA, Tilders FJ and Rothwell NJ. Lipocortin-1 is an endogenous inhibitor of ischemic damage in the rat brain. *J Exp Med*. 1991;174:305-10.
39. Sato Y, Kumamoto K, Saito K, Okayama H, Hayase S, Kofunato Y, Miyamoto K, Nakamura I, Ohki S, Koyama Y and Takenoshita S. Up-regulated Annexin A1 expression in gastrointestinal cancer is associated with cancer invasion and lymph node metastasis. *Exp Ther Med*. 2011;2:239-243.
40. Shin J, Song IS, Pak JH and Jang SW. Upregulation of annexin A1 expression by butyrate in human melanoma cells induces invasion by inhibiting E-cadherin expression. *Tumour Biol*. 2016;37:14577-14584.
41. Yom CK, Han W, Kim SW, Kim HS, Shin HC, Chang JN, Koo M, Noh DY and Moon BI. Clinical significance of annexin A1 expression in breast cancer. *J Breast Cancer*. 2011;14:262-8.
42. Sobral-Leite M, Wesseling J, Smit VT, Nevanlinna H, van Miltenburg MH, Sanders J, Hofland I, Blows FM, Coulson P, Patrycja G, Schellens JH, Fagerholm R, Heikkila P, Aittomaki K, Blomqvist C, Provenzano E, Ali HR, Figueroa J, Sherman M, Lissowska J, Mannermaa A, Kataja V, Kosma VM, Hartikainen JM, Phillips KA, kConFab AI, Couch FJ, Olson JE, Vachon C, Visscher D, Brenner H, Butterbach K, Arndt V, Holleczek B, Hooning MJ, Hollestelle A, Martens JW, van Deurzen CH, van de Water B, Broeks A, Chang-Claude J, Chenevix-Trench G, Easton DF, Pharoah PD, Garcia-Closas M, de Graauw M and Schmidt MK. Annexin A1 expression in a pooled breast cancer series: association with tumor subtypes and prognosis. *BMC Med*. 2015;13:156.
43. Sena A, Grishina I, Thai A, Goulart L, Macal M, Fenton A, Li J, Prindiville T, Oliani SM, Dandekar S, Goulart L and Sankaran-Walters S. Dysregulation of anti-inflammatory annexin A1 expression in progressive Crohns Disease. *PLoS One*. 2013;8:e76969.
44. Petrella A, Festa M, Ercolino SF, Zerilli M, Stassi G, Solito E and Parente L. Annexin-1 downregulation in thyroid cancer correlates to the degree of tumor differentiation. *Cancer Biol Ther*. 2006;5:643-7.
45. Roviezzo F, Getting SJ, Paul-Clark MJ, Yona S, Gavins FN, Perretti M, Hannon R, Croxtall JD, Buckingham JC and Flower RJ. The annexin-1 knockout mouse: what it tells us about the inflammatory response. *J Physiol Pharmacol*. 2002;53:541-53.
46. Chatterjee BE, Yona S, Rosignoli G, Young RE, Nourshargh S, Flower RJ and Perretti M. Annexin 1-deficient neutrophils exhibit enhanced transmigration in vivo and increased responsiveness in vitro. *Journal of leukocyte biology*. 2005;78:639-46.
47. Hannon R, Croxtall JD, Getting SJ, Roviezzo F, Yona S, Paul-Clark MJ, Gavins FN, Perretti M, Morris JF, Buckingham JC and Flower RJ. Aberrant inflammation and resistance to glucocorticoids in annexin 1-/- mouse. *Faseb J*. 2003;17:253-5.
48. Akasheh RT, Pini M, Pang J and Fantuzzi G. Increased adiposity in annexin A1-deficient mice. *PLoS One*. 2013;8:e82608.
49. Ng FS, Wong KY, Guan SP, Mustafa FB, Kajiji TS, Bist P, Biswas SK, Wong WS and Lim LH. Annexin-1-deficient mice exhibit spontaneous airway hyperresponsiveness and exacerbated allergen-specific antibody responses in a mouse model of asthma. *Clin Exp Allergy*. 2011;41:1793-803.
50. Perretti M, Chiang N, La M, Fierro IM, Marullo S, Getting SJ, Solito E and Serhan CN. Endogenous lipid- and peptide-derived anti-inflammatory pathways generated with glucocorticoid and aspirin treatment activate the lipoxin A4 receptor. *Nat Med*. 2002;8:1296-302.

51. Perretti M. The annexin 1 receptor(s): is the plot unravelling? *Trends Pharmacol Sci*. 2003;24:574-9.
52. Schafer AI. Effects of nonsteroidal antiinflammatory drugs on platelet function and systemic hemostasis. *J Clin Pharmacol*. 1995;35:209-19.
53. Knijff-Dutmer EA, Kalsbeek-Batenburg EM, Koerts J and van de Laar MA. Platelet function is inhibited by non-selective non-steroidal anti-inflammatory drugs but not by cyclo-oxygenase-2-selective inhibitors in patients with rheumatoid arthritis. *Rheumatology (Oxford)*. 2002;41:458-61.
54. Gladding PA, Webster MW, Farrell HB, Zeng IS, Park R and Ruijne N. The antiplatelet effect of six non-steroidal anti-inflammatory drugs and their pharmacodynamic interaction with aspirin in healthy volunteers. *Am J Cardiol*. 2008;101:1060-3.
55. Yun SH, Sim EH, Goh RY, Park JI and Han JY. Platelet Activation: The Mechanisms and Potential Biomarkers. *Biomed Res Int*. 2016;2016:9060143.
56. Rendu F and Brohard-Bohn B. The platelet release reaction: granules' constituents, secretion and functions. *Platelets*. 2001;12:261-73.
57. Trentin PG, Ferreira TP, Arantes AC, Ciambarella BT, Cordeiro RS, Flower RJ, Perretti M, Martins MA and Silva PM. Annexin A1 mimetic peptide controls the inflammatory and fibrotic effects of silica particles in mice. *Br J Pharmacol*. 2015;172:3058-71.
58. Cardin LT, Sonehara NM, Mimura KK, Ramos Dinarte Dos Santos A, da Silva WAJ, Sobral LM, Leopoldino AM, da Cunha BR, Tajara EH, Oliani SM and Rodrigues-Lisoni FC. ANXA1Ac2-26 peptide, a possible therapeutic approach in inflammatory ocular diseases. *Gene*. 2017;614:26-36.
59. Hayhoe RP, Kamal AM, Solito E, Flower RJ, Cooper D and Perretti M. Annexin 1 and its bioactive peptide inhibit neutrophil-endothelium interactions under flow: indication of distinct receptor involvement. *Blood*. 2006;107:2123-30.
60. Bena S, Brancaleone V, Wang JM, Perretti M and Flower RJ. Annexin A1 Interaction with the FPR2/ALX Receptor IDENTIFICATION OF DISTINCT DOMAINS AND DOWNSTREAM ASSOCIATED SIGNALING. *Journal of Biological Chemistry*. 2012;287:24690-24697.
61. Gastardelo TS, Cunha BR, Raposo LS, Maniglia JV, Cury PM, Lisoni FC, Tajara EH and Oliani SM. Inflammation and cancer: role of annexin A1 and FPR2/ALX in proliferation and metastasis in human laryngeal squamous cell carcinoma. *PLoS One*. 2014;9:e111317.
62. Varga-Szabo D, Braun A and Nieswandt B. Calcium signaling in platelets. *J Thromb Haemost*. 2009;7:1057-66.
63. D'Acquisto F, Piras G and Rattazzi L. Pro-inflammatory and pathogenic properties of Annexin-A1: the whole is greater than the sum of its parts. *Biochem Pharmacol*. 2013;85:1213-8.
64. Zhao B, Wang J, Liu L, Li X, Liu S, Xia Q and Shi J. Annexin A1 translocates to nucleus and promotes the expression of pro-inflammatory cytokines in a PKC-dependent manner after OGD/R. *Sci Rep*. 2016;6:27028.
65. Sena AA, Glavan T, Jiang G, Sankaran-Walters S, Grishina I, Dandekar S and Goulart LR. Divergent Annexin A1 expression in periphery and gut is associated with systemic immune activation and impaired gut immune response during SIV infection. *Sci Rep*. 2016;6:31157.
66. Boudhraa Z, Merle C, Mazzocut D, Chezal JM, Chambon C, Miot-Noirault E, Theisen M, Bouchon B and Degoul F. Characterization of pro-invasive mechanisms and N-terminal cleavage of ANXA1 in melanoma. *Arch Dermatol Res*. 2014;306:903-14.
67. Drechsler M, de Jong R, Rossaint J, Viola JR, Leoni G, Wang JM, Grommes J, Hinkel R, Kupatt C, Weber C, Doring Y, Zarbock A and Soehnlein O. Annexin A1 counteracts chemokine-induced arterial myeloid cell recruitment. *Circ Res*. 2015;116:827-35.
68. Cheuk BL and Cheng SW. Annexin A1 expression in atherosclerotic carotid plaques and its relationship with plaque characteristics. *Eur J Vasc Endovasc Surg*. 2011;41:364-71.

69. Fredman G, Kamaly N, Spolitu S, Milton J, Ghorpade D, Chiasson R, Kuriakose G, Perretti M, Farokzhad O and Tabas I. Targeted nanoparticles containing the proresolving peptide Ac2-26 protect against advanced atherosclerosis in hypercholesterolemic mice. *Sci Transl Med*. 2015;7:275ra20.
70. Ritchie RH, Gordon JM, Woodman OL, Cao AH and Dusting GJ. Annexin-1 peptide Anx-1(2-26) protects adult rat cardiac myocytes from cellular injury induced by simulated ischaemia. *Br J Pharmacol*. 2005;145:495-502.
71. Vital SA, Becker F, Holloway PM, Russell J, Perretti M, Granger DN and Gavins FN. Formyl-Peptide Receptor 2/3/Lipoxin A4 Receptor Regulates Neutrophil-Platelet Aggregation and Attenuates Cerebral Inflammation: Impact for Therapy in Cardiovascular Disease. *Circulation*. 2016;133:2169-79.
72. Smith HK, Gil CD, Oliani SM and Gavins FN. Targeting formyl peptide receptor 2 reduces leukocyte-endothelial interactions in a murine model of stroke. *Faseb J*. 2015;29:2161-71.
73. Cooray SN, Gobbetti T, Montero-Melendez T, McArthur S, Thompson D, Clark AJ, Flower RJ and Perretti M. Ligand-specific conformational change of the G-protein-coupled receptor ALX/FPR2 determines proresolving functional responses. *Proc Natl Acad Sci U S A*. 2013;110:18232-7.
74. Bena S, Brancaleone V, Wang JM, Perretti M and Flower RJ. Annexin A1 interaction with the FPR2/ALX receptor: identification of distinct domains and downstream associated signaling. *J Biol Chem*. 2012;287:24690-7.
75. He HQ and Ye RD. The Formyl Peptide Receptors: Diversity of Ligands and Mechanism for Recognition. *Molecules*. 2017;22.
76. Ye RD, Boulay F, Wang JM, Dahlgren C, Gerard C, Parmentier M, Serhan CN and Murphy PM. International Union of Basic and Clinical Pharmacology. LXXIII. Nomenclature for the formyl peptide receptor (FPR) family. *Pharmacol Rev*. 2009;61:119-61.
77. Doring Y, Drechsler M, Wantha S, Kemmerich K, Lievens D, Vijayan S, Gallo RL, Weber C and Soehnlein O. Lack of neutrophil-derived CRAMP reduces atherosclerosis in mice. *Circ Res*. 2012;110:1052-6.
78. Elajami TK, Colas RA, Dalli J, Chiang N, Serhan CN and Welty FK. Specialized proresolving lipid mediators in patients with coronary artery disease and their potential for clot remodeling. *Faseb J*. 2016;30:2792-801.
79. Liu D, Ahmet A, Ward L, Krishnamoorthy P, Mandelcorn ED, Leigh R, Brown JP, Cohen A and Kim H. A practical guide to the monitoring and management of the complications of systemic corticosteroid therapy. *Allergy Asthma Clin Immunol*. 2013;9:30.
80. Ng MK and Celermajer DS. Glucocorticoid treatment and cardiovascular disease. *Heart*. 2004;90:829-30.
81. Coutinho AE and Chapman KE. The anti-inflammatory and immunosuppressive effects of glucocorticoids, recent developments and mechanistic insights. *Mol Cell Endocrinol*. 2011;335:2-13.
82. Moraes LA, Paul-Clark MJ, Rickman A, Flower RJ, Goulding NJ and Perretti M. Ligand-specific glucocorticoid receptor activation in human platelets. *Blood*. 2005;106:4167-75.
83. Ozsoylu S, Strauss HS and Diamond LK. Effects of corticosteroids on coagulation of the blood. *Nature*. 1962;195:1214-5.
84. Mancuso F, Flower RJ and Perretti M. Leukocyte Transmigration, but Not Rolling or Adhesion, Is Selectively Inhibited by Dexamethasone in the Hamster Post-Capillary Venule - Involvement of Endogenous Lipocortin-1. *Journal of Immunology*. 1995;155:377-386.
85. Goulding NJ, Godolphin JL, Sharland PR, Peers SH, Sampson M, Maddison PJ and Flower RJ. Anti-inflammatory lipocortin 1 production by peripheral blood leucocytes in response to hydrocortisone. *Lancet*. 1990;335:1416-8.

86. Solito E, de Coupade C, Parente L, Flower RJ and Russo-Marie F. IL-6 stimulates annexin 1 expression and translocation and suggests a new biological role as class II acute phase protein. *Cytokine*. 1998;10:514-21.
87. Sawmynaden P and Perretti M. Glucocorticoid upregulation of the annexin-A1 receptor in leukocytes. *Biochem Biophys Res Commun*. 2006;349:1351-5.
88. Ehrchen J, Steinmuller L, Barczyk K, Tenbrock K, Nacken W, Eisenacher M, Nordhues U, Sorg C, Sunderkotter C and Roth J. Glucocorticoids induce differentiation of a specifically activated, anti-inflammatory subtype of human monocytes. *Blood*. 2007;109:1265-74.
89. Hashimoto A, Murakami Y, Kitasato H, Hayashi I and Endo H. Glucocorticoids co-interact with lipoxin A4 via lipoxin A4 receptor (ALX) up-regulation. *Biomed Pharmacother*. 2007;61:81-5.

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5 – General discussion

Main findings of this study:

- fMLF primes platelets and induces thrombus formation through FPR1 signalling
- LL37 triggers platelet activation through FPR2/ALX signalling
- mCRAMP implicated in pathological conditions such as psoriasis can affect platelet function via FPR2/ALX
- AnxA1 N-terminal peptide, Ac2-26, activates the platelet function through FPR2/ALX

Formyl peptide receptors are known to play a crucial role in the regulation of innate immune responses and host defence. They bind a wide variety of ligands that exhibit various functions, thus they mediate numerous biological responses through the modulation of inflammatory cells[355]. Hence, FPRs and their ligands have been implicated in the pathogenesis of various diseases, and it has been also shown that the blockade of these receptors attenuated pathophysiological responses, especially those associated with dysregulated inflammatory responses[244, 492]. Because of these observations, explorations of FPR signalling mechanisms presents an attractive approach for the development of novel therapeutic strategies as an adjunct to the treatments of inflammatory and infectious diseases, especially those associated with thromboinflammatory responses.

The hypothesis of this study was as follows; formyl peptide receptors regulate the haemostatic function of platelets through the modulation of thrombus formation and haemostasis. The chemotactic bacterial peptide fMLF primes platelet activation and modulates thrombus formation through FPR1. The antimicrobial peptide LL37 exhibits prothrombotic effects and thus may implicate the pathogenesis of cutaneous inflammatory diseases such as psoriasis through interaction with platelets via FPR2/ALX. The Annexin A1 N-terminal peptide, Ac2-26, affects platelet haemostasis through FPR2/ALX.

In order to investigate this hypothesis, the activatory roles of FPR1 and FPR2/ALX ligands, such as fMLF, LL37 and $AnxA1_{Ac2-26}$ were investigated in isolated platelets, platelet-rich plasma (PRP) and in whole blood (WB) using several platelet functional assays, as well as the mechanisms through which these FPR ligands regulate the function of platelets.

5.1 fMLF modulates platelet function through FPRl

In this study, we demonstrate the activatory role of the chemotactic bacterial peptide, fMLF, in the modulation of platelet function through FPR1. fMLF has been previously shown to induce the aggregation of platelets indirectly through neutrophils. In this study we sought to demonstrate the direct role of fMLF in the activation of platelets. Thus, the direct binding of fMLF to FPR1 in platelets was first confirmed. The ability of fMLF to induce the activation of platelets in distinct platelet functions including fibrinogen binding to integrin αIIbβ3 and Data α-granule secretion was also confirmed. These effects were abrogated in the presence of an FPR1-selective inhibitor, Boc-MLF, and in platelets obtained from *Fpr1*-deficient mice. fMLF also augmented thrombus formation under arterial flow conditions. Although this may be due in part to the effects of other mediators in whole blood. fMLF failed to aggregate platelets on its own, however, it significantly augmented CRP-XL-, collagen- and thrombin-induced aggregation in a concentration-dependent manner. These findings demonstrate the priming role of fMLF on platelets through FPR1.

5.2 LL37 induces platelet activation through FRP2/ALX and modulates disease

Following this, we were also able to demonstrate the activatory role of the endogenous antimicrobial peptide, LL37, in the modulation of platelet function through FPR2/ALX. We first sought to confirm the presence of this peptide in platelets. We demonstrate here that LL37 is present in activated platelets using confocal microscopy. Using an ELISA assay, we show that it is released from platelets (at nM concentrations) upon their activation; it release was significantly increased in activated platelets as opposed to resting platelets. In addition, we confirm the stability of LL37 in platelet-rich as opposed

to platelet-poor plasma using LC-MS. Collectively, these data demonstrate that LL37 is present in platelets and is released upon their activation.

In order to explore the role of LL37 in the regulation of platelet function, we first sought to investigate its broad role in haemostasis and thrombus formation. LL37 (20 μM) significantly shortened the bleeding time in mice using the tail bleeding assay *in vivo*. Additionally, the incubation of whole blood with LL37 significantly augmented thrombus formation under arterial flow conditions in a concentration-dependent manner (10, 20 and 50 μM). These two findings rendered LL37 an impactful modulator of platelet function. Consequently, we sought to investigate the direct role of LL37 on distinct platelet functions. Hence, using isolated platelets, we demonstrate here that LL37 (10 and 20 μM) induced immediate aggregation of platelets using light transmission aggregometry. Conversely, LL37 failed to aggregate platelets in PRP. This could be attributable to the presence of plasma protein that could bind to LL37, reducing its effect on platelet aggregation[401, 493, 494]. Nonetheless, in further distinct platelet functional assays including the binding of fibrinogen to integrin αIIbβ3 (inside-out signalling) and α-granule secretion, we demonstrate that LL37 was able to significantly increase these responses equally in platelets both in isolation and in plasma. Following to this, were also able to demonstrate that LL37 affects outside-in signalling using the platelet spreading assay, and the calcium mobilisation in platelets. These findings clearly demonstrate the ability of LL37 to directly modulate an activatory role in platelets. In contrast, a previous study on the role of LL37 concluded its negative effects on platelets[402]. The previous study revealed that 5 μM LL37 failed to aggregate gel-filtered platelets, induce calcium mobilisation and exert any cytotoxicity towards platelets. Our study also concludes that 5 μM LL37 failed to exert any of these effects although at this concentration LL37 displayed minimal and negligible effects in platelet aggregation and calcium mobilisation, and thus were interpreted as nonsignificant. Another study reported a contrasting role of LL37 in platelets; suggesting

an inhibitory role[449]. This is highly unlikely since LL37 was used at extremely high concentrations (0.3, 0.6 and 1.2 mM) that were previously reported to exert cytotoxicity towards eukaryotic cells. Indeed, we were able to confirm here that LL37 was cytotoxic towards isolated platelets at a concentration of 100 μM. Additionally, at the lower concentration (ranging from 1 to 50 μM), which were utilised in this study, LL37 failed to exert any cytotoxicity towards platelets. A recently published study confirmed some of the findings presented here, where they demonstrated that LL37 primes platelets and induces prothrombotic properties. In contrast to our findings, they report that LL37 did not induce fibrinogen binding or platelet spreading on immobilized fibrinogen, however, LL37 induced P-selectin expression, similar to our findings. This may be reflective of different experimental methods used between the two studies. They report that LL37 failed to exert aggregation in PRP, which we also reported and attributed to the presence of plasma proteins.

Following the different activatory roles of LL37, the underlying mechanisms driving these effects in platelets were investigated. Since LL37 is known to display proinflammatory effects through FPR2/ALX, we were able to confirm the dependency of this receptor on exerting the effects demonstrated here. Using a pharmacological inhibitor for FPR2/ALX, WRW4, and platelets obtained from *Fpr2/3*-deficient mice, we demonstrated that LL37 binds to this receptor and acts through it to modulate platelet function. We were the first to demonstrate this, as the recently published study reported that WRW4 (1 μM) did not affect LL37 properties in platelets; probably due to the use of a lower concentration of WRW4.

Subsequent to establishing a role of LL37 in platelets through FPR2/ALX, we sought to explore its effect in an inflammatory disease that is highly associated with LL37 and platelet-related complications using an animal model of psoriasis. The haemostasis was

not affected in mice of the psoriatic animal model used in this study. However, the level of mCRAMP in the psoriatic lesions and plasma obtained from these mice were markedly increased compared to normal controls. In addition, psoriatic platelets demonstrated prothrombotic properties as the fibrinogen binding to integrin and granule secretion upon the stimulation of platelets with CRP-XL, ADP and U46619 were increased compared to the controls. Moreover, upon the treatment of control platelets with psoriatic lesions and plasma, the fibrinogen binding and granule secretion were significantly increased in the presence or absence of CRP-XL. Upon blockade of FPR2/ALX or use of platelets obtained from *Fpr2/3*-deficient mice, the effect of psoriatic plasma on platelet activation was significantly reduced. These confirm the association of LL37 and platelet dysregulation, and the involvement of FPR2/ALX-mediated signalling in psoriasis. In this work, we demonstrate the role of psoriatic plasma obtained from mice on human blood. While the mCRAMP peptide sequence differs from that of human LL37, it was shown to be an effective model for the investigation of human cathelicidins[495]. In addition, several studies demonstrate the administration of LL37 in mice to exert its function[496-498].

5.3 AnxA1 modulates platelet activatory responses through FPR2/ALX

Succeeding the pro-thrombotic role of FPR2/ALX via ligation with LL37 in the platelets, we sought to investigate the role of AnxA1 (a pro-resolving molecule) in the regulation of platelet function. The endogenous AnxA1 is known to exert anti-inflammatory and cardioprotective properties, and thus its therapeutic potential has been previously proposed[474]. Surprisingly, we demonstrate here that the N-terminal peptide Ac2-26 exerts prothrombotic and proinflammatory properties in platelets through FPR2/ALX. In order to explore the role of AnxA1 in the regulation of platelet function, *Anxa1*-deficient mice were used in this study. Platelets obtained from *Anxa1*-deficient mice demonstrate a

normal expression of major platelet receptors including GPVI, a2 and aIIb, but a marked reduction in the expression of the GPIba receptor. Nonetheless, this finding did not impair the haemostasis as it was not affected in these mice compared to control mice. *Anxa1*-deficient mice demonstrated reduced platelet activatory properties upon stimulation with subthreshold concentrations of CRP-XL and ADP as evidenced by fibrinogen binding to αIIbβ3 and alpha-granule secretion. This affect however was overcome when higher concentrations of these two agonists were used. The exogenous AnxA1 N-terminal peptide, Ac2-26, demonstrated increased platelet activation in *Anxa1*-deficient mice, possibly due to compensatory effects. Nonetheless, we later confirmed the overexpression of FPR2/ALX in these mice, another plausible explanation for the increased platelet activation by Ac2-26 in *Anxa1*-deficient mice. Ac2-26 displayed increased fibrinogen binding to αIIbβ3, which was reversed by an FPR2/ALX-selective inhibitor, WRW4. Ac2-26 also induced increased expression of P-selectin on the surface of platelets, which was reduced in *Fpr2/3*-deficient mice. In addition, Ac2-26 induced calcium mobilisation in platelets, which was also reduced in *Fpr2/3*-deficient mice. This indicates that the effects of Ac2-26 are mediated through FPR2/ALX in platelets. Together, these findings demonstrate a role for AnxA1 in the modulation of platelet function.

5.4 FPRs regulate platelet function

In this study, we provide evidence that FPRs regulate the haemostatic platelet function by the utilisation of FPR pharmacological inhibitors and *Fpr*-deficient mice. These mice display severe inflammation in both sterile and infections inflammation in addition to increased susceptibility to infections[256, 257, 499, 500]. This supported their role in the regulation of the innate immune response and host defence. In this study, we demonstrate the consequential effect of *Fpr*-deficiency in the regulation of the platelet function. We demonstrate that mouse platelets obtained from *Fpr1*- or *Fpr2/3*-deficient mice displayed a reduced activation induced by various conventional platelet agonists, including CRP-XL, ADP, AY-NH_2, and U46619. In addition, the haemostatic function in these mice was compromised, indicated by a significantly prolonged tail bleeding time compared to control mice. The underlying mechanisms accompanying the genetic deletion of *Fpr1 or Fpr2/3* that might contribute to platelet dysfunction in these mice are beyond the scope of this study, and future studies focusing on these underlying mechanisms will provide great insight. Nonetheless, it demonstrates a crucial interplay between inflammation and thrombosis. Despite the functional abnormalities in these mice, these effects were recapitulated in human platelets by utilising FPR1 or FPR2/ALX-selective pharmacological inhibitors in several functional assays. These include reduced platelet activation (evident by fibrinogen binding and P-selectin exposure), platelet aggregation, ATP release, platelet spreading and thrombus formation. All of which were reduced in the presence of selective inhibitors for FPR1 or FPR2/ALX. FPRs belong to the family of Gi protein-coupled receptors and trigger downstream signalling pathways in neutrophils including phospholipase C (PLC), PI3K/AKT and MAPK, and can stimulate rapid calcium flux[242]. We sought to explore additional downstream signalling involved in FPR1- and FPR2/ALX-induced platelet responses. We were able to demonstrate here that the

inhibition FPR1 or FPR2/ALX, or the deletion of their respective genes in mice leads to the elevation of cAMP, which is a major inhibitor for platelet activation. This suggests the involvement of cAMP-dependent signalling pathways in the regulation of FPR-mediated effects in platelets.

In conclusion, the data presented here demonstrate the role of endogenous peptides, such as the bacterial chemotactic peptide fMLF, the antimicrobial peptide LL37 and the pro-resolving protein AnxA1 in the regulation of platelet function. These peptides are involved in the pathogenesis of various infectious and sterile inflammatory diseases. Thus, these peptides can contribute to the amplification of such diseases by the dysregulation of platelet function. Understanding the molecular mechanisms involved in such dysregulation will provide insight into the development of enhanced therapeutic strategies for various inflammatory diseases.

We provide novel findings here that demonstrate the expression of LL37 in platelets and its secretion upon their activation. Additionally, we demonstrate that the effects of LL37 in platelets are mediated through FPR2/ALX signalling pathways, which previous studies have failed to demonstrate[402, 449, 450]. Using a murine model for psoriasis, we reveal the effect of cathelicidins in platelet function through FPR2/ALX signalling during pathological conditions. In addition to LL37, we demonstrate a direct role for fMLF in distinct platelet functions, such as fibrinogen binding and P-selectin expression. Although fMLF failed to aggregate platelets on its own, it induced *in vitro* thrombus formation in whole blood, where the effect of other stimuli can contribute to the such effect. AnxA1 N-terminal peptide also exerted activatory effects towards platelet functions.

The findings of this study provide strong evidence on the role of FPRs in the regulation of the platelet haemostatic function. Moreover, it corroborates the pro-

thrombotic role of LL37, and confirms the role of FPR2/ALX signalling in mediating such responses. A priming role for fMLF and Ac2-2-6 was also demonstrated here. This supports the involvement of these peptides in mechanisms that may augment or perpetuate platelet-related complications in disease, and highlights FPRs and their ligands as potential therapeutic agents as an adjunct to the current treatments of infectious and inflammatory diseases, especially those associated with thrombo-inflammatory responses.

6 – Future direction

FPRs are traditionally known to play a role in the regulation of innate immune response and host defence. Here, we were able to demonstrate a role for these receptors and some of their ligands in the regulation of the platelet function. However, there are several questions remaining regarding their role in thrombosis and their implication in pathological conditions that are associated with platelet function. Further *in vivo* work focusing on the role of these receptors and their ligands in thrombosis will provide stronger conclusions regarding their role in thrombosis.

6.1 Role of fMLF in thromboinflammation

The role of fMLF in multiple disorders renders it an attractive target for clinical investigations. Examining the level of circulating fMLF in the plasma of patients suffering from sepsis and chronic inflammatory conditions such as inflammatory bowel disease, and its role in regulating the platelet function through FPR1 would provide insight into whether this peptide can exacerbate these inflammatory conditions.

6.2 Role of mCRAMP/ LL37 in thromboinflammation and in the pathogenesis of psoriasis

Work on human samples obtained from patients suffering from psoriasis would complement the work demonstrated here on the psoriatic mouse model. Additionally, by depleting the mCRAMP from psoriatic samples, the effect of other stimuli that can affect FPR2/ALX signalling can be excluded, and thus corroborating the involvement of this pathway. The detection of platelet markers, such as PF4, in these samples would also provide more evidence and confirm the involvement of platelets during disease progression.

6.3 Role of AnxA1 in the regulation of haemostasis and thrombosis

Additional research needs to be conducted to confirm the role of the full-length Annexin A1 protein as opposed to its N-terminal peptides, which are not found in the human body as is but are rather sequences of larger peptides. Moreover, additional experiments utilising *Fpr1*-deficient mice and FPR1-selective pharmacological inhibitors can be performed to confirm whether any of the responses observed in platelets are mediated through FPR1 signalling in platelets. Since it has been shown that Ac2-26 can elicit pro-inflammatory properties upon the heterodimerisation of FPR2/ALX with FPR1[501]. Moreover, the investigation of the heterodimerisation of FPR1 and FPR2/ALX in platelets would further provide insight into the underlying mechanisms that could contribute to the elicitation of prothrombotic effects of this peptide, since FPR2/ALX can elicit both pro-inflammatory (upon the ligation with SAA or LL37) and pro-resolving (upon ligation with AnxA1 or LXA4) properties, while FPR1 mainly triggers proinflammatory signalling[461].

References

1. Duhamel TA, Xu YJ, Arneja AS and Dhalla NS. Targeting platelets for prevention and treatment of cardiovascular disease. *Expert Opin Ther Targets*. 2007;11:1523-33.
2. Michelson AD. Antiplatelet therapies for the treatment of cardiovascular disease. *Nature Reviews Drug Discovery*. 2010;9:154-169.
3. Claushuis TA, van Vught LA, Scicluna BP, Wiewel MA, Klein Klouwenberg PM, Hoogendijk AJ, Ong DS, Cremer OL, Horn J, Franitza M, Toliat MR, Nurnberg P, Zwinderman AH, Bonten MJ, Schultz MJ and van der Poll T. Thrombocytopenia is associated with a dysregulated host response in critically ill sepsis patients. *Blood*. 2016.
4. Hamzeh-Cognasse H, Damien P, Chabert A, Pozzetto B, Cognasse F and Garraud O. Platelets and infections - complex interactions with bacteria. *Front Immunol*. 2015;6:82.
5. Rondina MT and Garraud O. Emerging evidence for platelets as immune and inflammatory effector cells. *Front Immunol*. 2014;5:653.
6. Nording HM, Seizer P and Langer HF. Platelets in inflammation and atherogenesis. *Front Immunol*. 2015;6.
7. Speth C, Loffler J, Krappmann S, Lass-Florl C and Rambach G. Platelets as immune cells in infectious diseases. *Future Microbiol*. 2013;8:1431-1451.
8. Yeaman MR. Platelets in defense against bacterial pathogens. *Cell Mol Life Sci*. 2010;67:525-544.
9. Deppermann C and Kubes P. Platelets and infection. *Semin Immunol*. 2016;28:536-545.
10. Machlus KR and Italiano JE, Jr. The incredible journey: From megakaryocyte development to platelet formation. *J Cell Biol*. 2013;201:785-96.
11. Garnock-Jones KP. Eltrombopag: a review of its use in treatment-refractory chronic primary immune thrombocytopenia. *Drugs*. 2011;71:1333-53.
12. Nugent D, McMillan R, Nichol JL and Slichter SJ. Pathogenesis of chronic immune thrombocytopenia: increased platelet destruction and/or decreased platelet production. *Br J Haematol*. 2009;146:585-96.
13. de Sauvage FJ, Hass PE, Spencer SD, Malloy BE, Gurney AL, Spencer SA, Darbonne WC, Henzel WJ, Wong SC, Kuang WJ and et al. Stimulation of megakaryocytopoiesis and thrombopoiesis by the c-Mpl ligand. *Nature*. 1994;369:533-8.
14. Kaushansky K, Lok S, Holly RD, Broudy VC, Lin N, Bailey MC, Forstrom JW, Buddle MM, Oort PJ, Hagen FS and et al. Promotion of megakaryocyte progenitor expansion and differentiation by the c-Mpl ligand thrombopoietin. *Nature*. 1994;369:568-71.
15. Levine RF, Eldor A, Shoff PK, Kirwin S, Tenza D and Cramer EM. Circulating megakaryocytes: delivery of large numbers of intact, mature megakaryocytes to the lungs. *Eur J Haematol*. 1993;51:233-46.
16. Huang N, Lou M, Liu H, Avila C and Ma Y. Identification of a potent small molecule capable of regulating polyploidization, megakaryocyte maturation, and platelet production. *J Hematol Oncol*. 2016;9:136.
17. Zimmet J and Ravid K. Polyploidy: occurrence in nature, mechanisms, and significance for the megakaryocyte-platelet system. *Exp Hematol*. 2000;28:3-16.
18. Machlus KR, Thon JN and Italiano JE, Jr. Interpreting the developmental dance of the megakaryocyte: a review of the cellular and molecular processes mediating platelet formation. *Br J Haematol*. 2014;165:227-36.
19. Schulze H, Korpal M, Hurov J, Kim SW, Zhang J, Cantley LC, Graf T and Shivdasani RA. Characterization of the megakaryocyte demarcation membrane system and its role in thrombopoiesis. *Blood*. 2006;107:3868-75.
20. Richardson JL, Shivdasani RA, Boers C, Hartwig JH and Italiano JE, Jr. Mechanisms of organelle transport and capture along proplatelets during platelet production. *Blood*. 2005;106:4066-75.

21. Italiano JE, Jr., Lecine P, Shivdasani RA and Hartwig JH. Blood platelets are assembled principally at the ends of proplatelet processes produced by differentiated megakaryocytes. *J Cell Biol*. 1999;147:1299-312.
22. Howell WH and Donahue DD. The Production of Blood Platelets in the Lungs. *J Exp Med*. 1937;65:177-203.
23. Lefrancais E, Ortiz-Munoz G, Caudrillier A, Mallavia B, Liu F, Sayah DM, Thornton EE, Headley MB, David T, Coughlin SR, Krummel MF, Leavitt AD, Passegue E and Looney MR. The lung is a site of platelet biogenesis and a reservoir for haematopoietic progenitors. *Nature*. 2017;544:105-109.
24. Schmitt A, Guichard J, Masse JM, Debili N and Cramer EM. Of mice and men: comparison of the ultrastructure of megakaryocytes and platelets. *Exp Hematol*. 2001;29:1295-302.
25. Ebbe S and Stohlman F, Jr. Megakaryocytopoiesis in the Rat. *Blood*. 1965;26:20-35.
26. Odell TT, Jr. and Jackson CW. Polyploidy and maturation of rat megakaryocytes. *Blood*. 1968;32:102-10.
27. Odell TT, Jr., Jackson CW and Friday TJ. Megakaryocytopoiesis in rats with special reference to polyploidy. *Blood*. 1970;35:775-82.
28. Aster RH. Studies of the mechanism of "hypersplenic" thrombocytopenia in rats. *J Lab Clin Med*. 1967;70:736-51.
29. Harker LA and Finch CA. Thrombokinetics in man. *J Clin Invest*. 1969;48:963-74.
30. Jackson CW and Edwards CC. Biphasic thrombopoietic response to severe hypobaric hypoxia. *Br J Haematol*. 1977;35:233-44.
31. Wong AK. Platelet biology: the role of shear. *Expert Rev Hematol*. 2013;6:205-12.
32. Walsh TG, Metharom P and Berndt MC. The functional role of platelets in the regulation of angiogenesis. *Platelets*. 2015;26:199-211.
33. White JG. A simple method for preservation of fine structure in blood cells. *Thromb Diath Haemorrh*. 1967;18:745-53.
34. Thon JN, Peters CG, Machlus KR, Aslam R, Rowley J, Macleod H, Devine MT, Fuchs TA, Weyrich AS, Semple JW, Flaumenhaft R and Italiano JE, Jr. T granules in human platelets function in TLR9 organization and signaling. *J Cell Biol*. 2012;198:561-74.
35. Maynard DM, Heijnen HF, Horne MK, White JG and Gahl WA. Proteomic analysis of platelet alpha-granules using mass spectrometry. *J Thromb Haemost*. 2007;5:1945-55.
36. Italiano JE, Jr. and Battinelli EM. Selective sorting of alpha-granule proteins. *J Thromb Haemost*. 2009;7 Suppl 1:173-6.
37. Rendu F and Brohard-Bohn B. The platelet release reaction: granules' constituents, secretion and functions. *Platelets*. 2001;12:261-73.
38. Lemons PP, Chen D, Bernstein AM, Bennett MK and Whiteheart SW. Regulated secretion in platelets: identification of elements of the platelet exocytosis machinery. *Blood*. 1997;90:1490-500.
39. Koseoglu S and Flaumenhaft R. Advances in platelet granule biology. *Curr Opin Hematol*. 2013;20:464-71.
40. Flaumenhaft R. Molecular basis of platelet granule secretion. *Arterioscler Thromb Vasc Biol*. 2003;23:1152-60.
41. Brass L. Understanding and evaluating platelet function. *Hematology Am Soc Hematol Educ Program*. 2010;2010:387-96.
42. Bentfeld-Barker ME and Bainton DF. Identification of primary lysosomes in human megakaryocytes and platelets. *Blood*. 1982;59:472-81.
43. Li JL, Zarbock A and Hidalgo A. Platelets as autonomous drones for hemostatic and immune surveillance. *J Exp Med*. 2017.
44. de Gaetano G. Historical overview of the role of platelets in hemostasis and thrombosis. *Haematologica*. 2001;86:349-56.

45. Rodvien R and Mielke CH, Jr. Role of platelets in hemostasis and thrombosis. *West J Med*. 1976;125:181-6.
46. Moroi M, Jung SM, Shinmyozu K, Tomiyama Y, Ordinas A and Diaz-Ricart M. Analysis of platelet adhesion to a collagen-coated surface under flow conditions: the involvement of glycoprotein VI in the platelet adhesion. *Blood*. 1996;88:2081-92.
47. Polanowska-Grabowska R, Simon CG, Jr. and Gear AR. Platelet adhesion to collagen type I, collagen type IV, von Willebrand factor, fibronectin, laminin and fibrinogen: rapid kinetics under shear. *Thromb Haemost*. 1999;81:118-23.
48. Nuyttens BP, Thijs T, Deckmyn H and Broos K. Platelet adhesion to collagen. *Thromb Res*. 2011;127 Suppl 2:S26-9.
49. Ma YQ, Qin J and Plow EF. Platelet integrin alpha(IIb)beta(3): activation mechanisms. *J Thromb Haemost*. 2007;5:1345-52.
50. Sanchez-Cortes J and Mrksich M. The platelet integrin alphaIIbbeta3 binds to the RGD and AGD motifs in fibrinogen. *Chem Biol*. 2009;16:990-1000.
51. Roberts HR, Hoffman M and Monroe DM. A cell-based model of thrombin generation. *Semin Thromb Hemost*. 2006;32 Suppl 1:32-8.
52. Heemskerk JW, Bevers EM and Lindhout T. Platelet activation and blood coagulation. *Thromb Haemost*. 2002;88:186-93.
53. Keuren JF, Wielders SJ, Ulrichts H, Hackeng T, Heemskerk JW, Deckmyn H, Bevers EM and Lindhout T. Synergistic effect of thrombin on collagen-induced platelet procoagulant activity is mediated through protease-activated receptor-1. *Arterioscler Thromb Vasc Biol*. 2005;25:1499-505.
54. Alberelli MA and De Candia E. Functional role of protease activated receptors in vascular biology. *Vascul Pharmacol*. 2014;62:72-81.
55. Lechtenberg BC, Freund SM and Huntington JA. GpIbalpha interacts exclusively with exosite II of thrombin. *J Mol Biol*. 2014;426:881-93.
56. Falati S, Gross P, Merrill-Skoloff G, Furie BC and Furie B. Real-time in vivo imaging of platelets, tissue factor and fibrin during arterial thrombus formation in the mouse. *Nat Med*. 2002;8:1175-81.
57. Ariens RA. Fibrin(ogen) and thrombotic disease. *J Thromb Haemost*. 2013;11 Suppl 1:294-305.
58. Lam WA, Chaudhuri O, Crow A, Webster KD, Li TD, Kita A, Huang J and Fletcher DA. Mechanics and contraction dynamics of single platelets and implications for clot stiffening. *Nat Mater*. 2011;10:61-6.
59. Carr ME, Jr. Development of platelet contractile force as a research and clinical measure of platelet function. *Cell Biochem Biophys*. 2003;38:55-78.
60. Leon C, Eckly A, Hechler B, Aleil B, Freund M, Ravanat C, Jourdain M, Nonne C, Weber J, Tiedt R, Gratacap MP, Severin S, Cazenave JP, Lanza F, Skoda R and Gachet C. Megakaryocyte-restricted MYH9 inactivation dramatically affects hemostasis while preserving platelet aggregation and secretion. *Blood*. 2007;110:3183-91.
61. Muthard RW and Diamond SL. Blood clots are rapidly assembled hemodynamic sensors: flow arrest triggers intraluminal thrombus contraction. *Arterioscler Thromb Vasc Biol*. 2012;32:2938-45.
62. The Lancet H. Thromboembolism: an under appreciated cause of death. *Lancet Haematol*. 2015;2:e393.
63. Raskob GE, Angchaisuksiri P, Blanco AN, Buller H, Gallus A, Hunt BJ, Hylek EM, Kakkar A, Konstantinides SV, McCumber M, Ozaki Y, Wendelboe A, Weitz JI and Day ISCfWT. Thrombosis: a major contributor to global disease burden. *Arterioscler Thromb Vasc Biol*. 2014;34:2363-71.
64. Franchi F and Angiolillo DJ. Novel antiplatelet agents in acute coronary syndrome. *Nat Rev Cardiol*. 2015;12:30-47.

65. Weyrich AS and Zimmerman GA. Platelets: signaling cells in the immune continuum. *Trends Immunol*. 2004;25:489-95.
66. Vieira-de-Abreu A, Campbell RA, Weyrich AS and Zimmerman GA. Platelets: versatile effector cells in hemostasis, inflammation, and the immune continuum. *Semin Immunopathol*. 2012;34:5-30.
67. Weyrich AS, Lindemann S and Zimmerman GA. The evolving role of platelets in inflammation. *J Thromb Haemost*. 2003;1:1897-905.
68. Morrell CN, Aggrey AA, Chapman LM and Modjeski KL. Emerging roles for platelets as immune and inflammatory cells. *Blood*. 2014;123:2759-67.
69. Andonegui G, Kerfoot SM, McNagny K, Ebbert KV, Patel KD and Kubes P. Platelets express functional Toll-like receptor-4. *Blood*. 2005;106:2417-23.
70. Cognasse F, Hamzeh H, Chavarin P, Acquart S, Genin C and Garraud O. Evidence of Toll-like receptor molecules on human platelets. *Immunol Cell Biol*. 2005;83:196-8.
71. Czapiga M, Gao JL, Kirk A and Lekstrom-Himes J. Human platelets exhibit chemotaxis using functional N-formyl peptide receptors. *Exp Hematol*. 2005;33:73-84.
72. Weber C. Platelets and chemokines in atherosclerosis: partners in crime. *Circ Res*. 2005;96:612-6.
73. Tang YQ, Yeaman MR and Selsted ME. Antimicrobial peptides from human platelets. *Infect Immun*. 2002;70:6524-33.
74. Youssefian T, Drouin A, Masse JM, Guichard J and Cramer EM. Host defense role of platelets: engulfment of HIV and Staphylococcus aureus occurs in a specific subcellular compartment and is enhanced by platelet activation. *Blood*. 2002;99:4021-9.
75. O'Brien L, Kerrigan SW, Kaw G, Hogan M, Penades J, Litt D, Fitzgerald DJ, Foster TJ and Cox D. Multiple mechanisms for the activation of human platelet aggregation by Staphylococcus aureus: roles for the clumping factors ClfA and ClfB, the serine-aspartate repeat protein SdrE and protein A. *Mol Microbiol*. 2002;44:1033-44.
76. Semple JW, Italiano JE, Jr. and Freedman J. Platelets and the immune continuum. *Nat Rev Immunol*. 2011;11:264-74.
77. Hartwig D, Hartel C, Hennig H, Muller-Steinhardt M, Schlenke P and Kluter H. Evidence for de novo synthesis of cytokines and chemokines in platelet concentrates. *Vox Sang*. 2002;82:182-90.
78. Henn V, Slupsky JR, Grafe M, Anagnostopoulos I, Forster R, Muller-Berghaus G and Kroczek RA. CD40 ligand on activated platelets triggers an inflammatory reaction of endothelial cells. *Nature*. 1998;391:591-4.
79. Kameyoshi Y, Dorschner A, Mallet AI, Christophers E and Schroder JM. Cytokine RANTES released by thrombin-stimulated platelets is a potent attractant for human eosinophils. *J Exp Med*. 1992;176:587-92.
80. Eisman R, Surrey S, Ramachandran B, Schwartz E and Poncz M. Structural and functional comparison of the genes for human platelet factor 4 and PF4alt. *Blood*. 1990;76:336-44.
81. Zarbock A, Polanowska-Grabowska RK and Ley K. Platelet-neutrophil-interactions: Linking hemostasis and inflammation. *Blood Reviews*. 2007;21:99-111.
82. Gawaz M, Langer H and May AE. Platelets in inflammation and atherogenesis. *J Clin Invest*. 2005;115:3378-84.
83. van Gils JM, Zwaginga JJ and Hordijk PL. Molecular and functional interactions among monocytes, platelets, and endothelial cells and their relevance for cardiovascular diseases. *Journal of leukocyte biology*. 2009;85:195-204.
84. Elzey BD, Tian J, Jensen RJ, Swanson AK, Lees JR, Lentz SR, Stein CS, Nieswandt B, Wang Y, Davidson BL and Ratliff TL. Platelet-mediated modulation of adaptive immunity. A communication link between innate and adaptive immune compartments. *Immunity*. 2003;19:9-19.

85. Slaba I, Wang J, Kolaczkowska E, McDonald B, Lee WY and Kubes P. Imaging the dynamic platelet-neutrophil response in sterile liver injury and repair in mice. *Hepatology*. 2015;62:1593-605.
86. Lannan KL, Spinelli SL, Blumberg N and Phipps RP. Maresin 1 induces a novel pro-resolving phenotype in human platelets. *J Thromb Haemost*. 2017;15:802-813.
87. Linke B, Schreiber Y, Picard-Willems B, Slattery P, Nusing RM, Harder S, Geisslinger G and Scholich K. Activated Platelets Induce an Anti-Inflammatory Response of Monocytes/Macrophages through Cross-Regulation of PGE2 and Cytokines. *Mediators Inflamm*. 2017;2017:1463216.
88. Ferrer-Acosta Y, Gonzalez M, Fernandez M and Valance WA. Emerging Roles for Platelets in Inflammation and Disease. *J Infect Dis Ther*. 2014;2.
89. Hotchkiss RS, Moldawer LL, Opal SM, Reinhart K, Turnbull IR and Vincent JL. Sepsis and septic shock. *Nat Rev Dis Primers*. 2016;2:16045.
90. Pietila MP and Thomas CF. Inflammation and infection in exacerbations of chronic obstructive pulmonary disease. *Semin Respir Infect*. 2003;18:9-16.
91. Ayala-Fontanez N, Soler DC and McCormick TS. Current knowledge on psoriasis and autoimmune diseases. *Psoriasis (Auckl)*. 2016;6:7-32.
92. Mehta JL, Saldeen TG and Rand K. Interactive role of infection, inflammation and traditional risk factors in atherosclerosis and coronary artery disease. *J Am Coll Cardiol*. 1998;31:1217-25.
93. Xavier RJ and Podolsky DK. Unravelling the pathogenesis of inflammatory bowel disease. *Nature*. 2007;448:427-34.
94. Koupenova M, Kehrel BE, Corkrey HA and Freedman JE. Thrombosis and platelets: an update. *Eur Heart J*. 2017;38:785-791.
95. Willoughby S, Holmes A and Loscalzo J. Platelets and cardiovascular disease. *Eur J Cardiovasc Nurs*. 2002;1:273-88.
96. Tamagawa-Mineoka R, Katoh N and Kishimoto S. Platelet activation in patients with psoriasis: increased plasma levels of platelet-derived microparticles and soluble P-selectin. *J Am Acad Dermatol*. 2010;62:621-6.
97. Middleton E and Rondina MT. Platelets in infectious disease. *Hematology Am Soc Hematol Educ Program*. 2016;2016:256-261.
98. Michels M, Alisjahbana B, De Groot PG, Indrati AR, Fijnheer R, Puspita M, Dewi IM, van de Wijer L, de Boer EM, Roest M, van der Ven AJ and de Mast Q. Platelet function alterations in dengue are associated with plasma leakage. *Thromb Haemost*. 2014;112:352-62.
99. Dawood BB. Study of patients with suspected platelet-based bleeding disorders [electronic resource] : a search for patients with a defect in the P2Y12 ADP receptor. 2010.
100. Ferroni P, Vazzana N, Riondino S, Cuccurullo C, Guadagni F and Davi G. Platelet function in health and disease: from molecular mechanisms, redox considerations to novel therapeutic opportunities. *Antioxid Redox Signal*. 2012;17:1447-85.
101. Bick RL. Cancer-associated thrombosis. *N Engl J Med*. 2003;349:109-11.
102. Pereira J and Phan T. Management of bleeding in patients with advanced cancer. *Oncologist*. 2004;9:561-70.
103. Tabas I and Glass CK. Anti-inflammatory therapy in chronic disease: challenges and opportunities. *Science*. 2013;339:166-72.
104. Cerletti C, Tamburrelli C, Izzi B, Gianfagna F and de Gaetano G. Platelet-leukocyte interactions in thrombosis. *Thromb Res*. 2012;129:263-6.
105. Demers M and Wagner DD. NETosis: a new factor in tumor progression and cancer-associated thrombosis. *Semin Thromb Hemost*. 2014;40:277-83.
106. Yang H, Biermann MH, Brauner JM, Liu Y, Zhao Y and Herrmann M. New Insights into Neutrophil Extracellular Traps: Mechanisms of Formation and Role in Inflammation. *Front Immunol*. 2016;7:302.

107. Xu J, Zhang X, Pelayo R, Monestier M, Ammollo CT, Semeraro F, Taylor FB, Esmon NL, Lupu F and Esmon CT. Extracellular histones are major mediators of death in sepsis. *Nat Med*. 2009;15:1318-21.
108. Semeraro F, Ammollo CT, Morrissey JH, Dale GL, Friese P, Esmon NL and Esmon CT. Extracellular histones promote thrombin generation through platelet-dependent mechanisms: involvement of platelet TLR2 and TLR4. *Blood*. 2011;118:1952-61.
109. Ammollo CT, Semeraro F, Xu J, Esmon NL and Esmon CT. Extracellular histones increase plasma thrombin generation by impairing thrombomodulin-dependent protein C activation. *J Thromb Haemost*. 2011;9:1795-803.
110. Menter DG, Tucker SC, Kopetz S, Sood AK, Crissman JD and Honn KV. Platelets and cancer: a casual or causal relationship: revisited. *Cancer Metastasis Rev*. 2014;33:231-69.
111. Yeaman MR. Platelets: at the nexus of antimicrobial defence. *Nature Reviews Microbiology*. 2014;12:426-437.
112. Nestle FO, Kaplan DH and Barker J. Psoriasis. *N Engl J Med*. 2009;361:496-509.
113. Boehncke WH and Schon MP. Psoriasis. *Lancet*. 2015;386:983-94.
114. Schon MP and Boehncke WH. Psoriasis. *N Engl J Med*. 2005;352:1899-912.
115. Ashcroft DM, Wan Po AL, Williams HC and Griffiths CE. Clinical measures of disease severity and outcome in psoriasis: a critical appraisal of their quality. *Br J Dermatol*. 1999;141:185-91.
116. Puig L. PASI90 response: the new standard in therapeutic efficacy for psoriasis. *J Eur Acad Dermatol Venereol*. 2015;29:645-8.
117. Feldman SR and Krueger GG. Psoriasis assessment tools in clinical trials. *Ann Rheum Dis*. 2005;64 Suppl 2:ii65-8; discussion ii69-73.
118. Davidson A and Diamond B. Autoimmune diseases. *N Engl J Med*. 2001;345:340-50.
119. Mueller W and Herrmann B. Cyclosporin A for psoriasis. *N Engl J Med*. 1979;301:555.
120. Lebwohl M, Ellis C, Gottlieb A, Koo J, Krueger G, Linden K, Shupack J and Weinstein G. Cyclosporine consensus conference: with emphasis on the treatment of psoriasis. *J Am Acad Dermatol*. 1998;39:464-75.
121. Nickoloff BJ. The cytokine network in psoriasis. *Arch Dermatol*. 1991;127:871-84.
122. Morizane S and Gallo RL. Antimicrobial peptides in the pathogenesis of psoriasis. *The Journal of dermatology*. 2012;39:225-30.
123. Marcinkiewicz M and Majewski S. The role of antimicrobial peptides in chronic inflammatory skin diseases. *Postepy Dermatol Alergol*. 2016;33:6-12.
124. Buchau AS and Gallo RL. Innate immunity and antimicrobial defense systems in psoriasis. *Clin Dermatol*. 2007;25:616-24.
125. Harder J and Schroder JM. Psoriatic scales: a promising source for the isolation of human skin-derived antimicrobial proteins. *Journal of leukocyte biology*. 2005;77:476-86.
126. Lai Y and Gallo RL. AMPed up immunity: how antimicrobial peptides have multiple roles in immune defense. *Trends Immunol*. 2009;30:131-41.
127. Lande R, Gregorio J, Facchinetti V, Chatterjee B, Wang YH, Homey B, Cao W, Wang YH, Su B, Nestle FO, Zal T, Mellman I, Schroder JM, Liu YJ and Gilliet M. Plasmacytoid dendritic cells sense self-DNA coupled with antimicrobial peptide. *Nature*. 2007;449:564-9.
128. Ong PY, Ohtake T, Brandt C, Strickland I, Boguniewicz M, Ganz T, Gallo RL and Leung DY. Endogenous antimicrobial peptides and skin infections in atopic dermatitis. *N Engl J Med*. 2002;347:1151-60.
129. Harder J, Bartels J, Christophers E and Schroder JM. Isolation and characterization of human beta -defensin-3, a novel human inducible peptide antibiotic. *J Biol Chem*. 2001;276:5707-13.
130. Harder J, Bartels J, Christophers E and Schroder JM. A peptide antibiotic from human skin. *Nature*. 1997;387:861.
131. Hollox EJ, Huffmeier U, Zeeuwen PL, Palla R, Lascorz J, Rodijk-Olthuis D, van de Kerkhof PC, Traupe H, de Jongh G, den Heijer M, Reis A, Armour JA and Schalkwijk J.

Psoriasis is associated with increased beta-defensin genomic copy number. *Nat Genet*. 2008;40:23-5.
132. Nomura I, Goleva E, Howell MD, Hamid QA, Ong PY, Hall CF, Darst MA, Gao B, Boguniewicz M, Travers JB and Leung DY. Cytokine milieu of atopic dermatitis, as compared to psoriasis, skin prevents induction of innate immune response genes. *J Immunol*. 2003;171:3262-9.
133. Liang SC, Tan XY, Luxenberg DP, Karim R, Dunussi-Joannopoulos K, Collins M and Fouser LA. Interleukin (IL)-22 and IL-17 are coexpressed by Th17 cells and cooperatively enhance expression of antimicrobial peptides. *J Exp Med*. 2006;203:2271-9.
134. Jinquan T, Vorum H, Larsen CG, Madsen P, Rasmussen HH, Gesser B, Etzerodt M, Honore B, Celis JE and Thestrup-Pedersen K. Psoriasin: a novel chemotactic protein. *J Invest Dermatol*. 1996;107:5-10.
135. Boehncke WH. Systemic Inflammation and Cardiovascular Comorbidity in Psoriasis Patients: Causes and Consequences. *Front Immunol*. 2018;9:579.
136. McDonald CJ and Calabresi P. Occlusive vascular disease in psoriatic patients. *N Engl J Med*. 1973;288:912.
137. Chandrashekar L, Rajappa M, Revathy G, Sundar I, Munisamy M, Ananthanarayanan PH, Thappa DM and Basu D. Is enhanced platelet activation the missing link leading to increased cardiovascular risk in psoriasis? *Clin Chim Acta*. 2015;446:181-185.
138. Ahlehoff O, Gislason GH, Lindhardsen J, Charlot MG, Jorgensen CH, Olesen JB, Bretler DM, Skov L, Torp-Pedersen C and Hansen PR. Psoriasis carries an increased risk of venous thromboembolism: a Danish nationwide cohort study. *PLoS One*. 2011;6:e18125.
139. Celik T, Unal S, Ekinci O, Ozer C, Ilhan G, Oktay G and Arica V. Mean Platelet Volume can Predict Cerebrovascular Events in Patients with Sickle Cell Anemia. *Pak J Med Sci*. 2015;31:203-8.
140. Ogdie A, Troxel AB, Mehta NN and Gelfand JM. Psoriasis and Cardiovascular Risk: Strength in Numbers Part 3. *J Invest Dermatol*. 2015;135:2148-2150.
141. Unal M. Platelet mass index is increased in psoriasis. A possible link between psoriasis and atherosclerosis. *Arch Med Sci Atheroscler Dis*. 2016;1:e145-e149.
142. Saleh HM, Attia EA, Onsy AM, Saad AA and Abd Ellah MM. Platelet activation: a link between psoriasis per se and subclinical atherosclerosis--a case-control study. *Br J Dermatol*. 2013;169:68-75.
143. Egeberg A, Thyssen JP, Jensen P, Gislason GH and Skov L. Risk of Myocardial Infarction in Patients with Psoriasis and Psoriatic Arthritis: A Nationwide Cohort Study. *Acta Derm Venereol*. 2017;97:819-824.
144. Berrettini M, Parise P, Constantini V, Grasselli S and Nenci GG. Platelet activation in psoriasis. *Thromb Haemost*. 1985;53:195-7.
145. Katoh N. Platelets as versatile regulators of cutaneous inflammation. *J Dermatol Sci*. 2009;53:89-95.
146. Tamagawa-Mineoka R. Important roles of platelets as immune cells in the skin. *J Dermatol Sci*. 2015;77:93-101.
147. Canpolat F, Akpinar H and Eskioglu F. Mean platelet volume in psoriasis and psoriatic arthritis. *Clin Rheumatol*. 2010;29:325-8.
148. Karabudak O, Ulusoy RE, Erikci AA, Solmazgul E, Dogan B and Harmanyeri Y. Inflammation and hypercoagulable state in adult psoriatic men. *Acta Derm Venereol*. 2008;88:337-40.
149. Magen E, Mishal J, Zeldin Y, Feldman V, Kidon M, Schlesinger M and Sthoeger Z. Increased mean platelet volume and C-reactive protein levels in patients with chronic urticaria with a positive autologous serum skin test. *Am J Med Sci*. 2010;339:504-8.
150. Okur N, Buyuktiryaki M, Uras N, Oncel MY, Ertekin O, Canpolat FE and Oguz SS. Platelet mass index in very preterm infants: can it be used as a parameter for neonatal morbidities? *J Matern Fetal Neonatal Med*. 2016;29:3218-22.

151. Korkmaz L, Bastug O, Ozdemir A, Korkut S, Karaca C, Akin MA, Gunes T, Kurtoglu S and Ozturk MA. The Efficacy of Propranolol in Retinopathy of Prematurity and its Correlation with the Platelet Mass Index. *Curr Eye Res*. 2017;42:88-97.
152. Hayashi S, Shimizu I, Miyauchi H and Watanabe S. Increased platelet aggregation in psoriasis. *Acta Derm Venereol*. 1985;65:258-62.
153. Kragballe K and Fallon JD. Increased aggregation and arachidonic acid transformation by psoriatic platelets: evidence that platelet-derived 12-hydroxy-eicosatetraenoic acid increases keratinocyte DNA synthesis in vitro. *Arch Dermatol Res*. 1986;278:449-53.
154. Shantsila E, Kamphuisen PW and Lip GY. Circulating microparticles in cardiovascular disease: implications for atherogenesis and atherothrombosis. *J Thromb Haemost*. 2010;8:2358-68.
155. Boulanger CM, Scoazec A, Ebrahimian T, Henry P, Mathieu E, Tedgui A and Mallat Z. Circulating microparticles from patients with myocardial infarction cause endothelial dysfunction. *Circulation*. 2001;104:2649-52.
156. Pelletier F, Garnache-Ottou F, Angelot F, Biichle S, Vidal C, Humbert P, Saas P, Seilles E and Aubin F. Increased levels of circulating endothelial-derived microparticles and small-size platelet-derived microparticles in psoriasis. *J Invest Dermatol*. 2011;131:1573-6.
157. Holme PA, Brosstad F and Solum NO. The difference between platelet and plasma FXIII used to study the mechanism of platelet microvesicle formation. *Thromb Haemost*. 1993;70:681-6.
158. Fijnheer R, Frijns CJ, Korteweg J, Rommes H, Peters JH, Sixma JJ and Nieuwenhuis HK. The origin of P-selectin as a circulating plasma protein. *Thromb Haemost*. 1997;77:1081-5.
159. Ludwig RJ, Schultz JE, Boehncke WH, Podda M, Tandi C, Krombach F, Baatz H, Kaufmann R, von Andrian UH and Zollner TM. Activated, not resting, platelets increase leukocyte rolling in murine skin utilizing a distinct set of adhesion molecules. *J Invest Dermatol*. 2004;122:830-6.
160. Tamagawa-Mineoka R, Katoh N, Ueda E, Masuda K and Kishimoto S. Elevated platelet activation in patients with atopic dermatitis and psoriasis: increased plasma levels of beta-thromboglobulin and platelet factor 4. *Allergol Int*. 2008;57:391-6.
161. Garbaraviciene J, Diehl S, Varwig D, Bylaite M, Ackermann H, Ludwig RJ and Boehncke WH. Platelet P-selectin reflects a state of cutaneous inflammation: possible application to monitor treatment efficacy in psoriasis. *Exp Dermatol*. 2010;19:736-41.
162. Coimbra S, Oliveira H, Reis F, Belo L, Rocha S, Quintanilha A, Figueiredo A, Teixeira F, Castro E, Rocha-Pereira P and Santos-Silva A. C-reactive protein and leucocyte activation in psoriasis vulgaris according to severity and therapy. *J Eur Acad Dermatol Venereol*. 2010;24:789-96.
163. Yasumoto S, Imayama S and Hori Y. Increased serum level of interleukin-6 in patients with psoriatic arthritis and thrombocytosis. *The Journal of dermatology*. 1995;22:718-22.
164. Oleksowicz L, Mrowiec Z, Zuckerman D, Isaacs R, Dutcher J and Puszkin E. Platelet activation induced by interleukin-6: evidence for a mechanism involving arachidonic acid metabolism. *Thromb Haemost*. 1994;72:302-8.
165. Kwiek B, Narbutt J, Sysa-Jedrzejowska A, Langner A and Lesiak A. Long-term treatment of chronic plaque psoriasis with biological drugs can control platelet activation: targeting the bridge between inflammation and atherothrombosis. *Postepy Dermatol Alergol*. 2017;34:131-137.
166. Mahajan VK, Khatri G, Prabha N, Abhinav C and Sharma V. Clopidogrel: a possible exacerbating factor for psoriasis. *Indian J Pharmacol*. 2014;46:123-4.
167. Lelubre C and Vincent JL. Mechanisms and treatment of organ failure in sepsis. *Nat Rev Nephrol*. 2018;14:417-427.
168. Cohen J. The immunopathogenesis of sepsis. *Nature*. 2002;420:885-91.

169. Singer M, Deutschman CS, Seymour CW, Shankar-Hari M, Annane D, Bauer M, Bellomo R, Bernard GR, Chiche JD, Coopersmith CM, Hotchkiss RS, Levy MM, Marshall JC, Martin GS, Opal SM, Rubenfeld GD, van der Poll T, Vincent JL and Angus DC. The Third International Consensus Definitions for Sepsis and Septic Shock (Sepsis-3). *JAMA*. 2016;315:801-10.
170. Leoni D and Rello J. Cardiac arrest among patients with infections: causes, clinical practice and research implications. *Clin Microbiol Infec*. 2017;23:730-735.
171. Daviaud F, Grimaldi D, Dechartres A, Charpentier J, Geri G, Marin N, Chiche JD, Cariou A, Mira JP and Pene F. Timing and causes of death in septic shock. *Ann Intensive Care*. 2015;5:16.
172. Moskowitz A, Omar Y, Chase M, Lokhandwala S, Patel P, Andersen LW, Cocchi MN and Donnino MW. Reasons for death in patients with sepsis and septic shock. *J Crit Care*. 2017;38:284-288.
173. Greco E, Lupia E, Bosco O, Vizio B and Montrucchio G. Platelets and Multi-Organ Failure in Sepsis. *Int J Mol Sci*. 2017;18.
174. Dewitte A, Lepreux S, Villeneuve J, Rigothier C, Combe C, Ouattara A and Ripoche J. Blood platelets and sepsis pathophysiology: A new therapeutic prospect in critical ill patients? *Ann Intensive Care*. 2017;7:115.
175. Yun SH, Sim EH, Goh RY, Park JI and Han JY. Platelet Activation: The Mechanisms and Potential Biomarkers. *Biomed Res Int*. 2016.
176. Levi M. The coagulant response in sepsis. *Clin Chest Med*. 2008;29:627-42, viii.
177. Semeraro N, Ammollo CT, Semeraro F and Colucci M. Sepsis-associated disseminated intravascular coagulation and thromboembolic disease. *Mediterr J Hematol Infect Dis*. 2010;2:e2010024.
178. Iba T, Gando S, Saitoh D, Wada H, Di Nisio M and Thachil J. Antithrombin supplementation and risk of bleeding in patients with sepsis-associated disseminated intravascular coagulation. *Thromb Res*. 2016;145:46-50.
179. Goldenberg NM, Steinberg BE, Slutsky AS and Lee WL. Broken barriers: a new take on sepsis pathogenesis. *Sci Transl Med*. 2011;3:88ps25.
180. Lee WL and Liles WC. Endothelial activation, dysfunction and permeability during severe infections. *Curr Opin Hematol*. 2011;18:191-6.
181. Ricciuto DR, dos Santos CC, Hawkes M, Toltl LJ, Conroy AL, Rajwans N, Lafferty EI, Cook DJ, Fox-Robichaud A, Kahnamoui K, Kain KC, Liaw PC and Liles WC. Angiopoietin-1 and angiopoietin-2 as clinically informative prognostic biomarkers of morbidity and mortality in severe sepsis. *Crit Care Med*. 2011;39:702-10.
182. Semple JW and Freedman J. Platelets and innate immunity. *Cell Mol Life Sci*. 2010;67:499-511.
183. Herter JM, Rossaint J and Zarbock A. Platelets in inflammation and immunity. *J Thromb Haemost*. 2014;12:1764-75.
184. Xu XR, Zhang D, Oswald BE, Carrim N, Wang X, Hou Y, Zhang Q, Lavalle C, McKeown T, Marshall AH and Ni H. Platelets are versatile cells: New discoveries in hemostasis, thrombosis, immune responses, tumor metastasis and beyond. *Crit Rev Clin Lab Sci*. 2016;53:409-30.
185. Dewitte A, Tanga A, Villeneuve J, Lepreux S, Ouattara A, Desmouliere A, Combe C and Ripoche J. New frontiers for platelet CD154. *Exp Hematol Oncol*. 2015;4:6.
186. Thomas MR and Storey RF. The role of platelets in inflammation. *Thromb Haemost*. 2015;114:449-58.
187. Graham SM and Liles WC. Platelets in sepsis: beyond hemostasis. *Blood*. 2016;127:2947-9.
188. Sharma B, Sharma M, Majumder M, Steier W, Sangal A and Kalawar M. Thrombocytopenia in septic shock patients--a prospective observational study of incidence, risk factors and correlation with clinical outcome. *Anaesth Intensive Care*. 2007;35:874-80.

189. Damas P, Ledoux D, Nys M, Vrindts Y, De Groote D, Franchimont P and Lamy M. Cytokine serum level during severe sepsis in human IL-6 as a marker of severity. *Ann Surg*. 1992;215:356-62.
190. Hack CE, Hart M, van Schijndel RJ, Eerenberg AJ, Nuijens JH, Thijs LG and Aarden LA. Interleukin-8 in sepsis: relation to shock and inflammatory mediators. *Infect Immun*. 1992;60:2835-42.
191. Bouchama A and Hammami M. Interleukin 10 in febrile patients and patients with sepsis. *Lancet*. 1998;351:1587-8.
192. Sessler CN, Windsor AC, Schwartz M, Watson L, Fisher BJ, Sugerman HJ and Fowler AA, 3rd. Circulating ICAM-1 is increased in septic shock. *Am J Respir Crit Care Med*. 1995;151:1420-7.
193. Hoogendijk AJ, Wiewel MA, van Vught LA, Scicluna BP, Belkasim-Bohoudi H, Horn J, Zwinderman AH, Klein Klouwenberg PM, Cremer OL, Bonten MJ, Schultz MJ, van der Poll T and consortium M. Plasma fractalkine is a sustained marker of disease severity and outcome in sepsis patients. *Crit Care*. 2015;19:412.
194. Iba T and Saitoh D. Efficacy of antithrombin in preclinical and clinical applications for sepsis-associated disseminated intravascular coagulation. *J Intensive Care*. 2014;2:66.
195. Greinacher A and Selleng S. How I evaluate and treat thrombocytopenia in the intensive care unit patient. *Blood*. 2016;128:3032-3042.
196. Stephan F, Hollande J, Richard O, Cheffi A, Maier-Redelsperger M and Flahault A. Thrombocytopenia in a surgical ICU. *Chest*. 1999;115:1363-70.
197. Claushuis TA, van Vught LA, Scicluna BP, Wiewel MA, Klein Klouwenberg PM, Hoogendijk AJ, Ong DS, Cremer OL, Horn J, Franitza M, Toliat MR, Nurnberg P, Zwinderman AH, Bonten MJ, Schultz MJ, van der Poll T, Molecular D and Risk Stratification of Sepsis C. Thrombocytopenia is associated with a dysregulated host response in critically ill sepsis patients. *Blood*. 2016;127:3062-72.
198. Thiolliere F, Serre-Sapin AF, Reignier J, Benedit M, Constantin JM, Lebert C, Guelon D, Timsit JF and Souweine B. Epidemiology and outcome of thrombocytopenic patients in the intensive care unit: results of a prospective multicenter study. *Intensive Care Med*. 2013;39:1460-8.
199. Antier N, Quenot JP, Doise JM, Noel R, Demaistre E and Devilliers H. Mechanisms and etiologies of thrombocytopenia in the intensive care unit: impact of extensive investigations. *Ann Intensive Care*. 2014;4:24.
200. Larkin CM, Santos-Martinez MJ, Ryan T and Radomski MW. Sepsis-associated thrombocytopenia. *Thromb Res*. 2016;141:11-6.
201. Levi M. Platelets at a crossroad of pathogenic pathways in sepsis. *J Thromb Haemost*. 2004;2:2094-5.
202. Yaguchi A, Lobo FL, Vincent JL and Pradier O. Platelet function in sepsis. *J Thromb Haemost*. 2004;2:2096-102.
203. Thachil J and Warkentin TE. How do we approach thrombocytopenia in critically ill patients? *Br J Haematol*. 2017;177:27-38.
204. Lieberman L, Bercovitz RS, Sholapur NS, Heddle NM, Stanworth SJ and Arnold DM. Platelet transfusions for critically ill patients with thrombocytopenia. *Blood*. 2014;123:1146-51; quiz 1280.
205. Levi M. Platelets in Critical Illness. *Semin Thromb Hemost*. 2016;42:252-7.
206. Pene F and Benoit DD. Thrombocytopenia in the critically ill: considering pathophysiology rather than looking for a magic threshold. *Intensive Care Med*. 2013;39:1656-9.
207. Ning S, Barty R, Liu Y, Heddle NM, Rochwerg B and Arnold DM. Platelet Transfusion Practices in the ICU: Data From a Large Transfusion Registry. *Chest*. 2016;150:516-23.

208. Zakynthinos SG, Papanikolaou S, Theodoridis T, Zakynthinos EG, Christopoulou-Kokkinou V, Katsaris G and Mavrommatis AC. Sepsis severity is the major determinant of circulating thrombopoietin levels in septic patients. *Crit Care Med*. 2004;32:1004-10.
209. Lupia E, Goffi A, Bosco O and Montrucchio G. Thrombopoietin as biomarker and mediator of cardiovascular damage in critical diseases. *Mediators Inflamm*. 2012;2012:390892.
210. Cuccurullo A, Greco E, Lupia E, De Giuli P, Bosco O, Martin-Conte E, Spatola T, Turco E and Montrucchio G. Blockade of Thrombopoietin Reduces Organ Damage in Experimental Endotoxemia and Polymicrobial Sepsis. *PLoS One*. 2016;11:e0151088.
211. Wu Q, Ren J, Wu X, Wang G, Gu G, Liu S, Wu Y, Hu D, Zhao Y and Li J. Recombinant human thrombopoietin improves platelet counts and reduces platelet transfusion possibility among patients with severe sepsis and thrombocytopenia: a prospective study. *J Crit Care*. 2014;29:362-6.
212. Remick DG. Cytokine therapeutics for the treatment of sepsis: why has nothing worked? *Current pharmaceutical design*. 2003;9:75-82.
213. Dhainaut JF, Tenaillon A, Le Tulzo Y, Schlemmer B, Solet JP, Wolff M, Holzapfel L, Zeni F, Dreyfuss D, Mira JP and et al. Platelet-activating factor receptor antagonist BN 52021 in the treatment of severe sepsis: a randomized, double-blind, placebo-controlled, multicenter clinical trial. BN 52021 Sepsis Study Group. *Crit Care Med*. 1994;22:1720-8.
214. Dhainaut JF, Tenaillon A, Hemmer M, Damas P, Le Tulzo Y, Radermacher P, Schaller MD, Sollet JP, Wolff M, Holzapfel L, Zeni F, Vedrinne JM, de Vathaire F, Gourlay ML, Guinot P and Mira JP. Confirmatory platelet-activating factor receptor antagonist trial in patients with severe gram-negative bacterial sepsis: a phase III, randomized, double-blind, placebo-controlled, multicenter trial. BN 52021 Sepsis Investigator Group. *Crit Care Med*. 1998;26:1963-71.
215. Bernard GR, Wheeler AP, Russell JA, Schein R, Summer WR, Steinberg KP, Fulkerson WJ, Wright PE, Christman BW, Dupont WD, Higgins SB and Swindell BB. The effects of ibuprofen on the physiology and survival of patients with sepsis. The Ibuprofen in Sepsis Study Group. *N Engl J Med*. 1997;336:912-8.
216. Fein AM, Bernard GR, Criner GJ, Fletcher EC, Good JT, Jr., Knaus WA, Levy H, Matuschak GM, Shanies HM, Taylor RW and Rodell TC. Treatment of severe systemic inflammatory response syndrome and sepsis with a novel bradykinin antagonist, deltibant (CP-0127). Results of a randomized, double-blind, placebo-controlled trial. CP-0127 SIRS and Sepsis Study Group. *JAMA*. 1997;277:482-7.
217. O'Connor PE. Recombinant human activated protein C for severe sepsis. *N Engl J Med*. 2001;345:220; author reply 220-1.
218. Akinosoglou K and Alexopoulos D. Use of antiplatelet agents in sepsis: a glimpse into the future. *Thromb Res*. 2014;133:131-8.
219. de Stoppelaar SF, van 't Veer C and van der Poll T. The role of platelets in sepsis. *Thromb Haemost*. 2014;112:666-77.
220. Thomas MR, Outteridge SN, Ajjan RA, Phoenix F, Sangha GK, Faulkner RE, Ecob R, Judge HM, Khan H, West LE, Dockrell DH, Sabroe I and Storey RF. Platelet P2Y12 Inhibitors Reduce Systemic Inflammation and Its Prothrombotic Effects in an Experimental Human Model. *Arterioscler Thromb Vasc Biol*. 2015;35:2562-70.
221. Winning J, Neumann J, Kohl M, Claus RA, Reinhart K, Bauer M and Losche W. Antiplatelet drugs and outcome in mixed admissions to an intensive care unit. *Crit Care Med*. 2010;38:32-7.
222. Gum PA, Kottke-Marchant K, Poggio ED, Gurm H, Welsh PA, Brooks L, Sapp SK and Topol EJ. Profile and prevalence of aspirin resistance in patients with cardiovascular disease. *Am J Cardiol*. 2001;88:230-5.
223. Nguyen TA, Diodati JG and Pharand C. Resistance to clopidogrel: a review of the evidence. *J Am Coll Cardiol*. 2005;45:1157-64.

224. Macchi L, Sorel N and Christiaens L. Aspirin resistance: definitions, mechanisms, prevalence, and clinical significance. *Current pharmaceutical design*. 2006;12:251-8.
225. Le Y, Murphy PM and Wang JM. Formyl-peptide receptors revisited. *Trends Immunol*. 2002;23:541-8.
226. Rowley JW, Oler AJ, Tolley ND, Hunter BN, Low EN, Nix DA, Yost CC, Zimmerman GA and Weyrich AS. Genome-wide RNA-seq analysis of human and mouse platelet transcriptomes. *Blood*. 2011;118:e101-11.
227. Wenceslau CF, McCarthy CG, Szasz T, Spitler K, Goulopoulou S, Webb RC and Dis DC. Mitochondrial damage-associated molecular patterns and vascular function. *Eur Heart J*. 2014;35:1172-U16.
228. Nakahira K, Hisata S and Choi AM. The Roles of Mitochondrial Damage-Associated Molecular Patterns in Diseases. *Antioxid Redox Signal*. 2015;23:1329-50.
229. Bloes DA, Kretschmer D and Peschel A. Enemy attraction: bacterial agonists for leukocyte chemotaxis receptors. *Nat Rev Microbiol*. 2015;13:95-104.
230. Muto Y, Guindon S, Umemura T, Kohidai L and Ueda H. Adaptive evolution of formyl peptide receptors in mammals. *J Mol Evol*. 2015;80:130-41.
231. Brink C, Dahlen SE, Drazen J, Evans JF, Hay DW, Nicosia S, Serhan CN, Shimizu T and Yokomizo T. International Union of Pharmacology XXXVII. Nomenclature for leukotriene and lipoxin receptors. *Pharmacol Rev*. 2003;55:195-227.
232. Quehenberger O, Prossnitz ER, Cavanagh SL, Cochrane CG and Ye RD. Multiple domains of the N-formyl peptide receptor are required for high-affinity ligand binding. Construction and analysis of chimeric N-formyl peptide receptors. *J Biol Chem*. 1993;268:18167-75.
233. Migeotte I, Riboldi E, Franssen JD, Gregoire F, Loison C, Wittamer V, Detheux M, Robberecht P, Costagliola S, Vassart G, Sozzani S, Parmentier M and Communi D. Identification and characterization of an endogenous chemotactic ligand specific for FPRL2. *J Exp Med*. 2005;201:83-93.
234. Ye RD, Boulay F, Wang JM, Dahlgren C, Gerard C, Parmentier M, Serhan CN and Murphy PM. International Union of Basic and Clinical Pharmacology. LXXIII. Nomenclature for the formyl peptide receptor (FPR) family. *Pharmacol Rev*. 2009;61:119-61.
235. Rabiet MJ, Huet E and Boulay F. Human mitochondria-derived N-formylated peptides are novel agonists equally active on FPR and FPRL1, while Listeria monocytogenes-derived peptides preferentially activate FPR. *Eur J Immunol*. 2005;35:2486-95.
236. Forsman H, Winther M, Gabl M, Skovbakke SL, Boulay F, Rabiet MJ and Dahlgren C. Structural changes of the ligand and of the receptor alters the receptor preference for neutrophil activating peptides starting with a formylmethionyl group. *Biochim Biophys Acta*. 2015;1853:192-200.
237. Kretschmer D, Rautenberg M, Linke D and Peschel A. Peptide length and folding state govern the capacity of staphylococcal beta-type phenol-soluble modulins to activate human formyl-peptide receptors 1 or 2. *Journal of leukocyte biology*. 2015;97:689-97.
238. Prossnitz ER and Ye RD. The N-formyl peptide receptor: a model for the study of chemoattractant receptor structure and function. *Pharmacol Ther*. 1997;74:73-102.
239. Li YS, Wu P, Zhou XY, Chen JG, Cai L, Wang F, Xu LM, Zhang XL, Chen Y, Liu SJ, Huang YP and Ye DY. Formyl-peptide receptor like 1: a potent mediator of the Ca2+ release-activated Ca2+ current ICRAC. *Arch Biochem Biophys*. 2008;478:110-8.
240. Schiffmann E, Showell HV, Corcoran BA, Ward PA, Smith E and Becker EL. The isolation and partial characterization of neutrophil chemotactic factors from Escherichia coli. *J Immunol*. 1975;114:1831-7.
241. Sklar LA, Jesaitis AJ and Painter RG. The neutrophil N-formyl peptide receptor: dynamics of ligand-receptor interactions and their relationship to cellular responses. *Contemp Top Immunobiol*. 1984;14:29-82.

242. Dorward DA, Lucas CD, Chapman GB, Haslett C, Dhaliwal K and Rossi AG. The role of formylated peptides and formyl peptide receptor 1 in governing neutrophil function during acute inflammation. *Am J Pathol*. 2015;185:1172-84.
243. Lee HY, Lee M and Bae YS. Formyl Peptide Receptors in Cellular Differentiation and Inflammatory Diseases. *J Cell Biochem*. 2017;118:1300-1307.
244. Li Y and Ye D. Molecular biology for formyl peptide receptors in human diseases. *J Mol Med (Berl)*. 2013;91:781-9.
245. Weiss E and Kretschmer D. Formyl-Peptide Receptors in Infection, Inflammation, and Cancer. *Trends Immunol*. 2018;39:815-829.
246. Yang SC and Hwang TL. The potential impacts of formyl peptide receptor 1 in inflammatory diseases. *Front Biosci (Elite Ed)*. 2016;8:436-49.
247. He HQ, Liao D, Wang ZG, Wang ZL, Zhou HC, Wang MW and Ye RD. Functional characterization of three mouse formyl peptide receptors. *Mol Pharmacol*. 2013;83:389-98.
248. Hartt JK, Barish G, Murphy PM and Gao JL. N-formylpeptides induce two distinct concentration optima for mouse neutrophil chemotaxis by differential interaction with two N-formylpeptide receptor (FPR) subtypes. Molecular characterization of FPR2, a second mouse neutrophil FPR. *J Exp Med*. 1999;190:741-7.
249. Gobbetti T, Coldewey SM, Chen J, McArthur S, le Faouder P, Cenac N, Flower RJ, Thiemermann C and Perretti M. Nonredundant protective properties of FPR2/ALX in polymicrobial murine sepsis. *Proc Natl Acad Sci U S A*. 2014;111:18685-90.
250. Southgate EL, He RL, Gao JL, Murphy PM, Nanamori M and Ye RD. Identification of formyl peptides from Listeria monocytogenes and Staphylococcus aureus as potent chemoattractants for mouse neutrophils. *Journal of Immunology*. 2008;181:1429-1437.
251. Liang TS, Wang JM, Murphy PM and Gao JL. Serum amyloid A is a chemotactic agonist at FPR2, a low-affinity N-formylpeptide receptor on mouse neutrophils. *Biochem Biophys Res Commun*. 2000;270:331-5.
252. Tiffany HL, Lavigne MC, Cui YH, Wang JM, Leto TL, Gao JL and Murphy PM. Amyloid-beta induces chemotaxis and oxidant stress by acting at formylpeptide receptor 2, a G protein-coupled receptor expressed in phagocytes and brain. *J Biol Chem*. 2001;276:23645-52.
253. Gao JL, Guillabert A, Hu J, Le Y, Urizar E, Seligman E, Fang KJ, Yuan X, Imbault V, Communi D, Wang JM, Parmentier M, Murphy PM and Migeotte I. F2L, a peptide derived from heme-binding protein, chemoattracts mouse neutrophils by specifically activating Fpr2, the low-affinity N-formylpeptide receptor. *J Immunol*. 2007;178:1450-6.
254. Vaughn MW, Proske RJ and Haviland DL. Identification, cloning, and functional characterization of a murine lipoxin A4 receptor homologue gene. *J Immunol*. 2002;169:3363-9.
255. Takano T, Fiore S, Maddox JF, Brady HR, Petasis NA and Serhan CN. Aspirin-triggered 15-epi-lipoxin A4 (LXA4) and LXA4 stable analogues are potent inhibitors of acute inflammation: evidence for anti-inflammatory receptors. *J Exp Med*. 1997;185:1693-704.
256. Gao JL, Lee EJ and Murphy PM. Impaired antibacterial host defense in mice lacking the N-formylpeptide receptor. *J Exp Med*. 1999;189:657-62.
257. Liu MY, Chen KQ, Yoshimura T, Liu Y, Gong WH, Wang AM, Gao JL, Murphy PM and Wang JM. Formylpeptide receptors are critical for rapid neutrophil mobilization in host defense against Listeria monocytogenes. *Sci Rep-Uk*. 2012;2.
258. Giebeler A, Streetz KL, Soehnlein O, Neumann U, Wang JM and Brandenburg LO. Deficiency of formyl peptide receptor 1 and 2 is associated with increased inflammation and enhanced liver injury after LPS-stimulation. *PLoS One*. 2014;9:e100522.
259. Dufton N, Hannon R, Brancaleone V, Dalli J, Patel HB, Gray M, D'Acquisto F, Buckingham JC, Perretti M and Flower RJ. Anti-inflammatory role of the murine formyl-peptide receptor 2: ligand-specific effects on leukocyte responses and experimental inflammation. *J Immunol*. 2010;184:2611-9.

260. Vital SA, Becker F, Holloway PM, Russell J, Perretti M, Granger DN and Gavins FN. Formyl-Peptide Receptor 2/3/Lipoxin A4 Receptor Regulates Neutrophil-Platelet Aggregation and Attenuates Cerebral Inflammation: Impact for Therapy in Cardiovascular Disease. *Circulation*. 2016;133:2169-79.
261. Smith HK, Gil CD, Oliani SM and Gavins FN. Targeting formyl peptide receptor 2 reduces leukocyte-endothelial interactions in a murine model of stroke. *Faseb J*. 2015;29:2161-71.
262. Bokoch GM and Gilman AG. Inhibition of receptor-mediated release of arachidonic acid by pertussis toxin. *Cell*. 1984;39:301-8.
263. Gierschik P, Sidiropoulos D and Jakobs KH. Two distinct Gi-proteins mediate formyl peptide receptor signal transduction in human leukemia (HL-60) cells. *J Biol Chem*. 1989;264:21470-3.
264. Lad PM, Olson CV and Smiley PA. Association of the N-formyl-Met-Leu-Phe receptor in human neutrophils with a GTP-binding protein sensitive to pertussis toxin. *Proc Natl Acad Sci U S A*. 1985;82:869-73.
265. Camps M, Carozzi A, Schnabel P, Scheer A, Parker PJ and Gierschik P. Isozyme-selective stimulation of phospholipase C-beta 2 by G protein beta gamma-subunits. *Nature*. 1992;360:684-6.
266. Stoyanov B, Volinia S, Hanck T, Rubio I, Loubtchenkov M, Malek D, Stoyanova S, Vanhaesebroeck B, Dhand R, Nurnberg B and et al. Cloning and characterization of a G protein-activated human phosphoinositide-3 kinase. *Science*. 1995;269:690-3.
267. Hirsch E, Katanaev VL, Garlanda C, Azzolino O, Pirola L, Silengo L, Sozzani S, Mantovani A, Altruda F and Wymann MP. Central role for G protein-coupled phosphoinositide 3-kinase gamma in inflammation. *Science*. 2000;287:1049-53.
268. Hannigan M, Zhan L, Li Z, Ai Y, Wu D and Huang CK. Neutrophils lacking phosphoinositide 3-kinase gamma show loss of directionality during N-formyl-Met-Leu-Phe-induced chemotaxis. *Proc Natl Acad Sci U S A*. 2002;99:3603-8.
269. Hannigan MO, Huang CK and Wu DQ. Roles of PI3K in neutrophil function. *Curr Top Microbiol Immunol*. 2004;282:165-75.
270. Boxio R, Bossenmeyer-Pourie C, Vanderesse R, Dournon C and Nusse O. The immunostimulatory peptide WKYMVm-NH activates bone marrow mouse neutrophils via multiple signal transduction pathways. *Scand J Immunol*. 2005;62:140-7.
271. Partida-Sanchez S, Cockayne DA, Monard S, Jacobson EL, Oppenheimer N, Garvy B, Kusser K, Goodrich S, Howard M, Harmsen A, Randall TD and Lund FE. Cyclic ADP-ribose production by CD38 regulates intracellular calcium release, extracellular calcium influx and chemotaxis in neutrophils and is required for bacterial clearance in vivo. *Nat Med*. 2001;7:1209-16.
272. Guse AH, da Silva CP, Berg I, Skapenko AL, Weber K, Heyer P, Hohenegger M, Ashamu GA, Schulze-Koops H, Potter BV and Mayr GW. Regulation of calcium signalling in T lymphocytes by the second messenger cyclic ADP-ribose. *Nature*. 1999;398:70-3.
273. Lee HC. Physiological functions of cyclic ADP-ribose and NAADP as calcium messengers. *Annu Rev Pharmacol Toxicol*. 2001;41:317-45.
274. Prat C, Bestebroer J, de Haas CJ, van Strijp JA and van Kessel KP. A new staphylococcal anti-inflammatory protein that antagonizes the formyl peptide receptor-like 1. *J Immunol*. 2006;177:8017-26.
275. Varga-Szabo D, Braun A and Nieswandt B. Calcium signaling in platelets. *J Thromb Haemost*. 2009;7:1057-66.
276. Wenzel-Seifert K, Arthur JM, Liu HY and Seifert R. Quantitative analysis of formyl peptide receptor coupling to g(i)alpha(1), g(i)alpha(2), and g(i)alpha(3). *J Biol Chem*. 1999;274:33259-66.

277. Mahadeo DC, Janka-Junttila M, Smoot RL, Roselova P and Parent CA. A chemoattractant-mediated Gi-coupled pathway activates adenylyl cyclase in human neutrophils. *Mol Biol Cell*. 2007;18:512-22.
278. Harvath L, Robbins JD, Russell AA and Seamon KB. cAMP and human neutrophil chemotaxis. Elevation of cAMP differentially affects chemotactic responsiveness. *J Immunol*. 1991;146:224-32.
279. Elferink JG and VanUffelen BE. The role of cyclic nucleotides in neutrophil migration. *Gen Pharmacol*. 1996;27:387-93.
280. VanUffelen BE, de Koster BM and Elferink JG. Interaction of cyclic GMP and cyclic AMP during neutrophil migration: involvement of phosphodiesterase type III. *Biochem Pharmacol*. 1998;56:1061-3.
281. Ariga M, Neitzert B, Nakae S, Mottin G, Bertrand C, Pruniaux MP, Jin SL and Conti M. Nonredundant function of phosphodiesterases 4D and 4B in neutrophil recruitment to the site of inflammation. *J Immunol*. 2004;173:7531-8.
282. Howe AK, Hogan BP and Juliano RL. Regulation of vasodilator-stimulated phosphoprotein phosphorylation and interaction with Abl by protein kinase A and cell adhesion. *J Biol Chem*. 2002;277:38121-6.
283. Li Z, Ajdic J, Eigenthaler M and Du X. A predominant role for cAMP-dependent protein kinase in the cGMP-induced phosphorylation of vasodilator-stimulated phosphoprotein and platelet inhibition in humans. *Blood*. 2003;101:4423-9.
284. Schiffmann E, Corcoran BA and Wahl SM. N-Formylmethionyl Peptides as Chemoattractants for Leukocytes. *P Natl Acad Sci USA*. 1975;72:1059-1062.
285. Gulden PH, Fischer P, 3rd, Sherman NE, Wang W, Engelhard VH, Shabanowitz J, Hunt DF and Pamer EG. A Listeria monocytogenes pentapeptide is presented to cytolytic T lymphocytes by the H2-M3 MHC class Ib molecule. *Immunity*. 1996;5:73-9.
286. de Paulis A, Florio G, Prevete N, Triggiani M, Fiorentino I, Genovese A and Marone G. HIV-1 envelope gp41 peptides promote migration of human Fc epsilon RI+ cells and inhibit IL-13 synthesis through interaction with formyl peptide receptors. *J Immunol*. 2002;169:4559-67.
287. Chiang N, Fierro IM, Gronert K and Serhan CN. Activation of lipoxin A(4) receptors by aspirin-triggered lipoxins and select peptides evokes ligand-specific responses in inflammation. *J Exp Med*. 2000;191:1197-208.
288. Perretti M and D'Acquisto F. Annexin A1 and glucocorticoids as effectors of the resolution of inflammation. *Nature Reviews Immunology*. 2009;9:62-70.
289. Hashimoto Y, Niikura T, Ito Y, Sudo H, Hata M, Arakawa E, Abe Y, Kita Y and Nishimoto I. Detailed characterization of neuroprotection by a rescue factor humanin against various Alzheimer's disease-relevant insults. *J Neurosci*. 2001;21:9235-45.
290. Ying G, Iribarren P, Zhou Y, Gong W, Zhang N, Yu ZX, Le Y, Cui Y and Wang JM. Humanin, a newly identified neuroprotective factor, uses the G protein-coupled formylpeptide receptor-like-1 as a functional receptor. *J Immunol*. 2004;172:7078-85.
291. Harada M, Habata Y, Hosoya M, Nishi K, Fujii R, Kobayashi M and Hinuma S. N-Formylated humanin activates both formyl peptide receptor-like 1 and 2. *Biochem Biophys Res Commun*. 2004;324:255-61.
292. Baek SH, Seo JK, Chae CB, Suh PG and Ryu SH. Identification of the peptides that stimulate the phosphoinositide hydrolysis in lymphocyte cell lines from peptide libraries. *J Biol Chem*. 1996;271:8170-5.
293. Christophe T, Karlsson A, Dugave C, Rabiet MJ, Boulay F and Dahlgren C. The synthetic peptide Trp-Lys-Tyr-Met-Val-Met-NH2 specifically activates neutrophils through FPRL1/lipoxin A4 receptors and is an agonist for the orphan monocyte-expressed chemoattractant receptor FPRL2. *J Biol Chem*. 2001;276:21585-93.

294. Klein C, Paul JI, Sauve K, Schmidt MM, Arcangeli L, Ransom J, Trueheart J, Manfredi JP, Broach JR and Murphy AJ. Identification of surrogate agonists for the human FPRL-1 receptor by autocrine selection in yeast. *Nat Biotechnol*. 1998;16:1334-7.
295. Hu JY, Le Y, Gong W, Dunlop NM, Gao JL, Murphy PM and Wang JM. Synthetic peptide MMK-1 is a highly specific chemotactic agonist for leukocyte FPRL1. *Journal of leukocyte biology*. 2001;70:155-61.
296. Chen J, Bernstein HS, Chen M, Wang L, Ishii M, Turck CW and Coughlin SR. Tethered ligand library for discovery of peptide agonists. *J Biol Chem*. 1995;270:23398-401.
297. Nanamori M, Cheng X, Mei J, Sang H, Xuan Y, Zhou C, Wang MW and Ye RD. A novel nonpeptide ligand for formyl peptide receptor-like 1. *Mol Pharmacol*. 2004;66:1213-22.
298. Burli RW, Xu H, Zou X, Muller K, Golden J, Frohn M, Adlam M, Plant MH, Wong M, McElvain M, Regal K, Viswanadhan VN, Tagari P and Hungate R. Potent hFPRL1 (ALXR) agonists as potential anti-inflammatory agents. *Bioorg Med Chem Lett*. 2006;16:3713-8.
299. Schepetkin IA, Kirpotina LN, Tian J, Khlebnikov AI, Ye RD and Quinn MT. Identification of novel formyl peptide receptor-like 1 agonists that induce macrophage tumor necrosis factor alpha production. *Mol Pharmacol*. 2008;74:392-402.
300. Schepetkin IA, Kirpotina LN, Khlebnikov AI and Quinn MT. High-throughput screening for small-molecule activators of neutrophils: identification of novel N-formyl peptide receptor agonists. *Mol Pharmacol*. 2007;71:1061-74.
301. Serhan CN. Lipoxins and aspirin-triggered 15-epi-lipoxins are the first lipid mediators of endogenous anti-inflammation and resolution. *Prostaglandins Leukot Essent Fatty Acids*. 2005;73:141-62.
302. Mitchell S, Thomas G, Harvey K, Cottell D, Reville K, Berlasconi G, Petasis NA, Erwig L, Rees AJ, Savill J, Brady HR and Godson C. Lipoxins, aspirin-triggered epi-lipoxins, lipoxin stable analogues, and the resolution of inflammation: stimulation of macrophage phagocytosis of apoptotic neutrophils in vivo. *J Am Soc Nephrol*. 2002;13:2497-507.
303. Pouliot M, Clish CB, Petasis NA, Van Dyke TE and Serhan CN. Lipoxin A(4) analogues inhibit leukocyte recruitment to Porphyromonas gingivalis: a role for cyclooxygenase-2 and lipoxins in periodontal disease. *Biochemistry*. 2000;39:4761-8.
304. Sodin-Semrl S, Taddeo B, Tseng D, Varga J and Fiore S. Lipoxin A4 inhibits IL-1 beta-induced IL-6, IL-8, and matrix metalloproteinase-3 production in human synovial fibroblasts and enhances synthesis of tissue inhibitors of metalloproteinases. *J Immunol*. 2000;164:2660-6.
305. Gavins FN, Yona S, Kamal AM, Flower RJ and Perretti M. Leukocyte antiadhesive actions of annexin 1: ALXR- and FPR-related anti-inflammatory mechanisms. *Blood*. 2003;101:4140-7.
306. Takano T, Clish CB, Gronert K, Petasis N and Serhan CN. Neutrophil-mediated changes in vascular permeability are inhibited by topical application of aspirin-triggered 15-epi-lipoxin A4 and novel lipoxin B4 stable analogues. *J Clin Invest*. 1998;101:819-26.
307. Hachicha M, Pouliot M, Petasis NA and Serhan CN. Lipoxin (LX)A4 and aspirin-triggered 15-epi-LXA4 inhibit tumor necrosis factor 1alpha-initiated neutrophil responses and trafficking: regulators of a cytokine-chemokine axis. *J Exp Med*. 1999;189:1923-30.
308. Svensson CI, Zattoni M and Serhan CN. Lipoxins and aspirin-triggered lipoxin inhibit inflammatory pain processing. *J Exp Med*. 2007;204:245-52.
309. Loor F, Tiberghien F, Wenandy T, Didier A and Traber R. Cyclosporins: structure-activity relationships for the inhibition of the human FPR1 formylpeptide receptor. *J Med Chem*. 2002;45:4613-28.
310. Wenzel-Seifert K, Hurt CM and Seifert R. High constitutive activity of the human formyl peptide receptor. *J Biol Chem*. 1998;273:24181-9.
311. Haas PJ, de Haas CJ, Kleibeuker W, Poppelier MJ, van Kessel KP, Kruijtzer JA, Liskamp RM and van Strijp JA. N-terminal residues of the chemotaxis inhibitory protein of

Staphylococcus aureus are essential for blocking formylated peptide receptor but not C5a receptor. *J Immunol*. 2004;173:5704-11.
312. Chen X, Yang D, Shen W, Dong HF, Wang JM, Oppenheim JJ and Howard MZ. Characterization of chenodeoxycholic acid as an endogenous antagonist of the G-coupled formyl peptide receptors. *Inflamm Res*. 2000;49:744-55.
313. Chen X, Mellon RD, Yang L, Dong H, Oppenheim JJ and Howard OM. Regulatory effects of deoxycholic acid, a component of the anti-inflammatory traditional Chinese medicine Niuhuang, on human leukocyte response to chemoattractants. *Biochem Pharmacol*. 2002;63:533-41.
314. Liang TS, Gao JL, Fatemi O, Lavigne M, Leto TL and Murphy PM. The endogenous opioid spinorphin blocks fMet-Leu-Phe-induced neutrophil chemotaxis by acting as a specific antagonist at the N-formylpeptide receptor subtype FPR. *J Immunol*. 2001;167:6609-14.
315. Yamamoto Y, Kanazawa T, Shimamura M, Ueki M and Hazato T. Inhibitory effects of spinorphin, a novel endogenous regulator, on chemotaxis, O2- generation, and exocytosis by N-formylmethionyl-leucyl-phenylalanine (FMLP)-stimulated neutrophils. *Biochem Pharmacol*. 1997;54:695-701.
316. Freer RJ, Day AR, Radding JA, Schiffmann E, Aswanikumar S, Showell HJ and Becker EL. Further studies on the structural requirements for synthetic peptide chemoattractants. *Biochemistry*. 1980;19:2404-10.
317. Machado FS, Johndrow JE, Esper L, Dias A, Bafica A, Serhan CN and Aliberti J. Anti-inflammatory actions of lipoxin A4 and aspirin-triggered lipoxin are SOCS-2 dependent. *Nat Med*. 2006;12:330-4.
318. von der Weid PY, Hollenberg MD, Fiorucci S and Wallace JL. Aspirin-triggered, cyclooxygenase-2-dependent lipoxin synthesis modulates vascular tone. *Circulation*. 2004;110:1320-5.
319. Nascimento-Silva V, Arruda MA, Barja-Fidalgo C, Villela CG and Fierro IM. Novel lipid mediator aspirin-triggered lipoxin A4 induces heme oxygenase-1 in endothelial cells. *Am J Physiol Cell Physiol*. 2005;289:C557-63.
320. Grumbach Y, Quynh NV, Chiron R and Urbach V. LXA4 stimulates ZO-1 expression and transepithelial electrical resistance in human airway epithelial (16HBE14o-) cells. *Am J Physiol Lung Cell Mol Physiol*. 2009;296:L101-8.
321. Stenfeldt AL, Karlsson J, Wenneras C, Bylund J, Fu H and Dahlgren C. Cyclosporin H, Boc-MLF and Boc-FLFLF are antagonists that preferentially inhibit activity triggered through the formyl peptide receptor. *Inflammation*. 2007;30:224-9.
322. Bae YS, Lee HY, Jo EJ, Kim JI, Kang HK, Ye RD, Kwak JY and Ryu SH. Identification of peptides that antagonize formyl peptide receptor-like 1-mediated signaling. *J Immunol*. 2004;173:607-14.
323. Zhou C, Zhang S, Nanamori M, Zhang Y, Liu Q, Li N, Sun M, Tian J, Ye PP, Cheng N, Ye RD and Wang MW. Pharmacological characterization of a novel nonpeptide antagonist for formyl peptide receptor-like 1. *Mol Pharmacol*. 2007;72:976-83.
324. Gavins FN. Are formyl peptide receptors novel targets for therapeutic intervention in ischaemia-reperfusion injury? *Trends Pharmacol Sci*. 2010;31:266-76.
325. Wan W and Gao JL. Leukocyte chemoattractant receptor FPR2 may accelerate atherogenesis. *Med Hypotheses*. 2012;79:101-3.
326. de Paulis A, Montuori N, Prevete N, Fiorentino I, Rossi FW, Visconte V, Rossi G, Marone G and Ragno P. Urokinase induces basophil chemotaxis through a urokinase receptor epitope that is an endogenous ligand for formyl peptide receptor-like 1 and -like 2. *J Immunol*. 2004;173:5739-48.
327. Cui Y, Le Y, Yazawa H, Gong W and Wang JM. Potential role of the formyl peptide receptor-like 1 (FPRL1) in inflammatory aspects of Alzheimer's disease. *Journal of leukocyte biology*. 2002;72:628-35.

328. Edwards BS, Bologa C, Young SM, Balakin KV, Prossnitz ER, Savchuck NP, Sklar LA and Oprea TI. Integration of virtual screening with high-throughput flow cytometry to identify novel small molecule formylpeptide receptor antagonists. *Mol Pharmacol*. 2005;68:1301-10.
329. Tsuruki T, Takahata K and Yoshikawa M. Mechanism of the protective effect of intraperitoneally administered agonists for formyl peptide receptors against chemotherapy-induced alopecia. *Biosci Biotechnol Biochem*. 2007;71:1198-202.
330. Gao JL and Murphy PM. Species and subtype variants of the N-formyl peptide chemotactic receptor reveal multiple important functional domains. *J Biol Chem*. 1993;268:25395-401.
331. Maestes DC, Potter RM and Prossnitz ER. Differential phosphorylation paradigms dictate desensitization and internalization of the N-formyl peptide receptor. *J Biol Chem*. 1999;274:29791-5.
332. Marasco WA, Phan SH, Krutzsch H, Showell HJ, Feltner DE, Nairn R, Becker EL and Ward PA. Purification and identification of formyl-methionyl-leucyl-phenylalanine as the major peptide neutrophil chemotactic factor produced by Escherichia coli. *J Biol Chem*. 1984;259:5430-9.
333. Yang D, Chen Q, Schmidt AP, Anderson GM, Wang JM, Wooters J, Oppenheim JJ and Chertov O. LL-37, the neutrophil granule- and epithelial cell-derived cathelicidin, utilizes formyl peptide receptor-like 1 (FPRL1) as a receptor to chemoattract human peripheral blood neutrophils, monocytes, and T cells. *Journal of Experimental Medicine*. 2000;192:1069-1074.
334. Ernst S, Lange C, Wilbers A, Goebeler V, Gerke V and Rescher U. An annexin 1 N-terminal peptide activates leukocytes by triggering different members of the formyl peptide receptor family. *J Immunol*. 2004;172:7669-76.
335. Perretti M. The annexin 1 receptor(s): is the plot unravelling? *Trends Pharmacol Sci*. 2003;24:574-579.
336. Krishnamoorthy S, Recchiuti A, Chiang N, Yacoubian S, Lee CH, Yang R, Petasis NA and Serhan CN. Resolvin D1 binds human phagocytes with evidence for proresolving receptors. *Proc Natl Acad Sci U S A*. 2010;107:1660-5.
337. Le Y, Yazawa H, Gong W, Yu Z, Ferrans VJ, Murphy PM and Wang JM. The neurotoxic prion peptide fragment PrP(106-126) is a chemotactic agonist for the G protein-coupled receptor formyl peptide receptor-like 1. *J Immunol*. 2001;166:1448-51.
338. Ueda H, Howard OM, Grimm MC, Su SB, Gong W, Evans G, Ruscetti FW, Oppenheim JJ and Wang JM. HIV-1 envelope gp41 is a potent inhibitor of chemoattractant receptor expression and function in monocytes. *J Clin Invest*. 1998;102:804-12.
339. Wang JM, Ueda H, Howard OM, Grimm MC, Chertov O, Gong X, Gong W, Resau JH, Broder CC, Evans G, Arthur LO, Ruscetti FW and Oppenheim JJ. HIV-1 envelope gp120 inhibits the monocyte response to chemokines through CD4 signal-dependent chemokine receptor down-regulation. *J Immunol*. 1998;161:4309-17.
340. Shen W, Li B, Wetzel MA, Rogers TJ, Henderson EE, Su SB, Gong W, Le Y, Sargeant R, Dimitrov DS, Oppenheim JJ and Wang JM. Down-regulation of the chemokine receptor CCR5 by activation of chemotactic formyl peptide receptor in human monocytes. *Blood*. 2000;96:2887-94.
341. Wollam J, Johnson AM, Xu YJ, Jain M and Olefsky JM. Gut Microbiome Produced N-Formyl Peptides Act through the Receptor FPR1 to Modulate Insulin Secretion and Metabolism. *Diabetes*. 2016;65:A453-A453.
342. Hellmann J, Tang Y, Kosuri M, Bhatnagar A and Spite M. Resolvin D1 decreases adipose tissue macrophage accumulation and improves insulin sensitivity in obese-diabetic mice. *Faseb J*. 2011;25:2399-407.
343. Balkwill F and Mantovani A. Cancer and inflammation: implications for pharmacology and therapeutics. *Clin Pharmacol Ther*. 2010;87:401-6.

344. Zhou Y, Bian X, Le Y, Gong W, Hu J, Zhang X, Wang L, Iribarren P, Salcedo R, Howard OM, Farrar W and Wang JM. Formylpeptide receptor FPR and the rapid growth of malignant human gliomas. *J Natl Cancer Inst*. 2005;97:823-35.
345. Yang Y, Liu Y, Yao X, Ping Y, Jiang T, Liu Q, Xu S, Huang J, Mou H, Gong W, Chen K, Bian X and Wang JM. Annexin 1 released by necrotic human glioblastoma cells stimulates tumor cell growth through the formyl peptide receptor 1. *Am J Pathol*. 2011;179:1504-12.
346. Coffelt SB, Marini FC, Watson K, Zwezdaryk KJ, Dembinski JL, LaMarca HL, Tomchuck SL, Honer zu Bentrup K, Danka ES, Henkle SL and Scandurro AB. The pro-inflammatory peptide LL-37 promotes ovarian tumor progression through recruitment of multipotent mesenchymal stromal cells. *Proc Natl Acad Sci U S A*. 2009;106:3806-11.
347. Malle E, Sodin-Semrl S and Kovacevic A. Serum amyloid A: an acute-phase protein involved in tumour pathogenesis. *Cell Mol Life Sci*. 2009;66:9-26.
348. Li Y, Cai L, Wang H, Wu P, Gu W, Chen Y, Hao H, Tang K, Yi P, Liu M, Miao S and Ye D. Pleiotropic regulation of macrophage polarization and tumorigenesis by formyl peptide receptor-2. *Oncogene*. 2011;30:3887-99.
349. Kim JE, Kim HJ, Choi JM, Lee KH, Kim TY, Cho BK, Jung JY, Chung KY, Cho D and Park HJ. The antimicrobial peptide human cationic antimicrobial protein-18/cathelicidin LL-37 as a putative growth factor for malignant melanoma. *Br J Dermatol*. 2010;163:959-67.
350. Zonder JA, Barlogie B, Durie BG, McCoy J, Crowley J and Hussein MA. Thrombotic complications in patients with newly diagnosed multiple myeloma treated with lenalidomide and dexamethasone: benefit of aspirin prophylaxis. *Blood*. 2006;108:403; author reply 404.
351. Perretti M, Chiang N, La M, Fierro IM, Marullo S, Getting SJ, Solito E and Serhan CN. Endogenous lipid- and peptide-derived anti-inflammatory pathways generated with glucocorticoid and aspirin treatment activate the lipoxin A4 receptor. *Nat Med*. 2002;8:1296-302.
352. Serhan CN, Takano T, Clish CB, Gronert K and Petasis N. Aspirin-triggered 15-epi-lipoxin A4 and novel lipoxin B4 stable analogs inhibit neutrophil-mediated changes in vascular permeability. *Adv Exp Med Biol*. 1999;469:287-93.
353. Chen Y, Hao H, He S, Cai L, Li Y, Hu S, Ye D, Hoidal J, Wu P and Chen X. Lipoxin A4 and its analogue suppress the tumor growth of transplanted H22 in mice: the role of antiangiogenesis. *Mol Cancer Ther*. 2010;9:2164-74.
354. Liu S, Wu P, Ye D, Huang Y, Zhou X, Li Y and Cai L. Effects of lipoxin A(4) on CoCl(2)-induced angiogenesis and its possible mechanisms in human umbilical vein endothelial cells. *Pharmacology*. 2009;84:17-23.
355. He HQ and Ye RD. The Formyl Peptide Receptors: Diversity of Ligands and Mechanism for Recognition. *Molecules*. 2017;22.
356. Marasco WA, Phan SH, Krutzsch H, Showell HJ, Feltner DE, Nairn R, Becker EL and Ward PA. Purification and Identification of Formyl-Methionyl-Leucyl-Phenylalanine as the Major Peptide Neutrophil Chemotactic Factor Produced by Escherichia-Coli. *Journal of Biological Chemistry*. 1984;259:5430-5439.
357. Dahlgren C, Gabl M, Holdfeldt A, Winther M and Forsman H. Basic characteristics of the neutrophil receptors that recognize formylated peptides, a danger-associated molecular pattern generated by bacteria and mitochondria. *Biochem Pharmacol*. 2016;114:22-39.
358. Torres M, Hall FL and O'Neill K. Stimulation of human neutrophils with formyl-methionyl-leucyl-phenylalanine induces tyrosine phosphorylation and activation of two distinct mitogen-activated protein-kinases. *J Immunol*. 1993;150:1563-77.
359. Kanaho Y, Sato T, Hongu T and Funakoshi Y. Molecular mechanisms of fMLP-induced superoxide generation and degranulation in mouse neutrophils. *Adv Biol Regul*. 2013;53:128-34.
360. Makristathis A, Stauffer F, Feistauer SM and Georgopoulos A. Bacteria induce release of platelet-activating factor (PAF) from polymorphonuclear neutrophil granulocytes: possible

role for PAF in pathogenesis of experimentally induced bacterial pneumonia. *Infect Immun*. 1993;61:1996-2002.
361. Sambrano GR, Huang W, Faruqi T, Mahrus S, Craik C and Coughlin SR. Cathepsin G activates protease-activated receptor-4 in human platelets. *J Biol Chem*. 2000;275:6819-23.
362. Follin P, Johansson A and Dahlgren C. Intracellular production of reactive oxygen species in human neutrophils following activation by the soluble stimuli FMLP, dioctanoylglycerol and ionomycin. *Cell Biochem Funct*. 1991;9:29-37.
363. Mittal M, Siddiqui MR, Tran K, Reddy SP and Malik AB. Reactive Oxygen Species in Inflammation and Tissue Injury. *Antioxid Redox Sign*. 2014;20:1126-1167.
364. Schwarz NT, Jung SY, Kalff JC, Chancellor M and Bauer AJ. Bacterial toxin N-formyl-methionyl-leucyl-phenylalanine acutely contracts human and rabbit detrusor through the release of eicosanoids. *J Urology*. 2002;167:2603-2612.
365. Gauthier JF, Fortin A, Bergeron Y, Dumas MC, Champagne ME and Bergeron MG. Differential contribution of bacterial N-formyl-methionyl-leucyl-phenylalanine and host-derived CXC chemokines to neutrophil infiltration into pulmonary alveoli during murine pneumococcal pneumonia. *Infection and Immunity*. 2007;75:5361-5367.
366. Anton PA, Targan SR and Shanahan F. Increased neutrophil receptors for and response to the proinflammatory bacterial peptide formyl-methionyl-leucyl-phenylalanine in Crohn's disease. *Gastroenterology*. 1989;97:20-8.
367. Leduc LE and Nast CC. Chemotactic Peptide-Induced Acute Colitis in Rabbits. *Gastroenterology*. 1990;98:929-935.
368. Perez HD, Kelly E, Elfman F, Armitage G and Winkler J. Defective polymorphonuclear leukocyte formyl peptide receptor(s) in juvenile periodontitis. *J Clin Invest*. 1991;87:971-6.
369. Peters MJ, Breslin AB, Kemp AS, Chu J and Berend N. Haematological effects of inhalation of N-formyl-methionyl-leucyl-phenylalanine in man. *Thorax*. 1992;47:284-7.
370. Yurt RW and Shires GT. Increased Susceptibility to Infection Due to Infusion of Exogenous Chemotaxin. *Arch Surg-Chicago*. 1987;122:111-116.
371. Roberts EC, Hobson CH, Anderson RP and Chadwick VS. Radioimmunoassay for Formyl Methionyl Leucyl Phenylalanine .2. Demonstration of an Enterohepatic Circulation of Immunoreactive Bacterial Chemotactic Peptides in Man. *J Gastroen Hepatol*. 1990;5:38-43.
372. Danese S, de la Motte C and Fiocchi C. Platelets in inflammatory bowel disease: Clinical, pathogenic, and therapeutic implications. *Am J Gastroenterol*. 2004;99:938-945.
373. Tunjungputri RN, van de Heijden W, Urbanus RT, de Groot PG, van der Ven A and de Mast Q. Higher platelet reactivity and platelet-monocyte complex formation in Gram-positive sepsis compared to Gram-negative sepsis. *Platelets*. 2017;28:595-601.
374. Munoz-Esquerre M, Ferreiro JL, Huertas D, Marcano AL, Lopez-Sanchez M, Roura G, Gomez-Hospital JA, Dorca J, Cequier A and Santos S. Impact of acute exacerbations on platelet reactivity in chronic obstructive pulmonary disease patients. *Int J Chronic Obstr*. 2018;13:141-148.
375. Fearon DT and Collins LA. Increased expression of C3b receptors on polymorphonuclear leukocytes induced by chemotactic factors and by purification procedures. *J Immunol*. 1983;130:370-5.
376. Oflaherty JT, Kreutzer DL and Ward PA. Neutrophil Aggregation and Swelling Induced by Chemotactic Agents. *Journal of Immunology*. 1977;119:232-239.
377. Ostrovsky L, King AJ, Bond S, Mitchell D, Lorant DE, Zimmerman GA, Larsen R, Niu XF and Kubes P. A juxtacrine mechanism for neutrophil adhesion on platelets involves platelet-activating factor and a selectin-dependent activation process. *Blood*. 1998;91:3028-3036.
378. Esmon CT. The interactions between inflammation and coagulation. *Brit J Haematol*. 2005;131:417-430.

379. Coeffier E, Joseph D, Prevost MC and Vargaftig BB. Platelet-Leukocyte Interaction - Activation of Rabbit Platelets by Fmlp-Stimulated Neutrophils. *Brit J Pharmacol*. 1987;92:393-406.
380. Li NL, Hu H, Lindqvist M, Johnsson EW, Goodall AH and Hjemdahl P. Platelet-leukocyte cross-talk in whole blood: Inhibition by GPIIb/IIIa and P-selectin antagonists. *Circulation*. 2000;102:34-34.
381. Evangelista V, Piccardoni P, White JG, Degaetano G and Cerletti C. Cathepsin G-Dependent Platelet Stimulation by Activated Polymorphonuclear Leukocytes and Its Inhibition by Antiproteinases - Role of P-Selectin Mediated Cell-Cell Adhesion. *Blood*. 1993;81:2947-2957.
382. Rossaint J, Kuhne K, Skupski J, Van Aken H, Looney MR, Hidalgo A and Zarbock A. Directed transport of neutrophil-derived extracellular vesicles enables platelet-mediated innate immune response. *Nature communications*. 2016;7:13464.
383. Coeffier E, Joseph D, Prevost MC and Vargaftig BB. Platelet-leukocyte interaction: activation of rabbit platelets by FMLP-stimulated neutrophils. *Br J Pharmacol*. 1987;92:393-406.
384. Oda M, Satouchi K, Yasunaga K and Saito K. Polymorphonuclear leukocyte-platelet interactions: acetylglyceryl ether phosphocholine-induced platelet activation under stimulation with chemotactic peptide. *J Biochem*. 1986;100:1117-23.
385. Stewart AG and Harris T. Involvement of platelet-activating factor in endotoxin-induced priming of rabbit polymorphonuclear leukocytes. *J Lipid Mediat*. 1991;3:125-38.
386. Evangelista V, Rajtar G, de Gaetano G, White JG and Cerletti C. Platelet activation by fMLP-stimulated polymorphonuclear leukocytes: the activity of cathepsin G is not prevented by antiproteinases. *Blood*. 1991;77:2379-88.
387. Hirata M, Zhong J, Wright SC and Larrick JW. Structure and functions of endotoxin-binding peptides derived from CAP18. *Prog Clin Biol Res*. 1995;392:317-26.
388. Larrick JW, Hirata M, Balint RF, Lee J, Zhong J and Wright SC. Human CAP18: a novel antimicrobial lipopolysaccharide-binding protein. *Infect Immun*. 1995;63:1291-7.
389. Barlow PG, Findlay EG, Currie SM and Davidson DJ. Antiviral potential of cathelicidins. *Future Microbiol*. 2014;9:55-73.
390. Zanetti M. Cathelicidins, multifunctional peptides of the innate immunity. *Journal of leukocyte biology*. 2004;75:39-48.
391. Shinnar AE, Butler KL and Park HJ. Cathelicidin family of antimicrobial peptides: proteolytic processing and protease resistance. *Bioorg Chem*. 2003;31:425-36.
392. Morizane S, Yamasaki K, Kabigting FD and Gallo RL. Kallikrein expression and cathelicidin processing are independently controlled in keratinocytes by calcium, vitamin D(3), and retinoic acid. *J Invest Dermatol*. 2010;130:1297-306.
393. Doss M, White MR, Tecle T and Hartshorn KL. Human defensins and LL-37 in mucosal immunity. *Journal of leukocyte biology*. 2010;87:79-92.
394. Nijnik A and Hancock RE. The roles of cathelicidin LL-37 in immune defences and novel clinical applications. *Curr Opin Hematol*. 2009;16:41-7.
395. Agerberth B, Gunne H, Odeberg J, Kogner P, Boman HG and Gudmundsson GH. FALL-39, a putative human peptide antibiotic, is cysteine-free and expressed in bone marrow and testis. *Proc Natl Acad Sci U S A*. 1995;92:195-9.
396. Lowry MB, Guo C, Borregaard N and Gombart AF. Regulation of the human cathelicidin antimicrobial peptide gene by 1alpha,25-dihydroxyvitamin D3 in primary immune cells. *The Journal of steroid biochemistry and molecular biology*. 2014;143:183-91.
397. Sorensen OE, Follin P, Johnsen AH, Calafat J, Tjabringa GS, Hiemstra PS and Borregaard N. Human cathelicidin, hCAP-18, is processed to the antimicrobial peptide LL-37 by extracellular cleavage with proteinase 3. *Blood*. 2001;97:3951-9.

398. Yamasaki K, Schauber J, Coda A, Lin H, Dorschner RA, Schechter NM, Bonnart C, Descargues P, Hovnanian A and Gallo RL. Kallikrein-mediated proteolysis regulates the antimicrobial effects of cathelicidins in skin. *Faseb J*. 2006;20:2068-80.
399. De Y, Chen Q, Schmidt AP, Anderson GM, Wang JM, Wooters J, Oppenheim JJ and Chertov O. LL-37, the neutrophil granule- and epithelial cell-derived cathelicidin, utilizes formyl peptide receptor-like 1 (FPRL1) as a receptor to chemoattract human peripheral blood neutrophils, monocytes, and T cells. *J Exp Med*. 2000;192:1069-74.
400. Oren Z, Lerman JC, Gudmundsson GH, Agerberth B and Shai Y. Structure and organization of the human antimicrobial peptide LL-37 in phospholipid membranes: relevance to the molecular basis for its non-cell-selective activity. *Biochem J*. 1999;341 (Pt 3):501-13.
401. Johansson J, Gudmundsson GH, Rottenberg ME, Berndt KD and Agerberth B. Conformation-dependent antibacterial activity of the naturally occurring human peptide LL-37. *J Biol Chem*. 1998;273:3718-24.
402. Bucki R and Janmey PA. Interaction of the gelsolin-derived antibacterial PBP 10 peptide with lipid bilayers and cell membranes. *Antimicrobial agents and chemotherapy*. 2006;50:2932-40.
403. Turner J, Cho Y, Dinh NN, Waring AJ and Lehrer RI. Activities of LL-37, a cathelin-associated antimicrobial peptide of human neutrophils. *Antimicrobial agents and chemotherapy*. 1998;42:2206-14.
404. Lee CC, Sun Y, Qian S and Huang HW. Transmembrane pores formed by human antimicrobial peptide LL-37. *Biophys J*. 2011;100:1688-96.
405. Kusaka S, Nishida A, Takahashi K, Bamba S, Yasui H, Kawahara M, Inatomi O, Sugimoto M and Andoh A. Expression of human cathelicidin peptide LL-37 in inflammatory bowel disease. *Clin Exp Immunol*. 2018;191:96-106.
406. Sun L, Wang W, Xiao W and Yang H. The Roles of Cathelicidin LL-37 in Inflammatory Bowel Disease. *Inflamm Bowel Dis*. 2016;22:1986-91.
407. Paulsen F, Pufe T, Conradi L, Varoga D, Tsokos M, Papendieck J and Petersen W. Antimicrobial peptides are expressed and produced in healthy and inflamed human synovial membranes. *J Pathol*. 2002;198:369-77.
408. Li D, Li J, Duan Y and Zhou X. Expression of LL-37, human beta defensin-2, and CCR6 mRNA in patients with psoriasis vulgaris. *J Huazhong Univ Sci Technolog Med Sci*. 2004;24:404-6.
409. Leung TF, Ching KW, Kong AP, Wong GW, Chan JC and Hon KL. Circulating LL-37 is a biomarker for eczema severity in children. *J Eur Acad Dermatol Venereol*. 2012;26:518-22.
410. Islam D, Bandholtz L, Nilsson J, Wigzell H, Christensson B, Agerberth B and Gudmundsson G. Downregulation of bactericidal peptides in enteric infections: a novel immune escape mechanism with bacterial DNA as a potential regulator. *Nat Med*. 2001;7:180-5.
411. An LL, Ma XT, Yang YH, Lin YM, Song YH and Wu KF. Marked reduction of LL-37/hCAP-18, an antimicrobial peptide, in patients with acute myeloid leukemia. *Int J Hematol*. 2005;81:45-7.
412. Yang YH, Zheng GG, Li G, Zhang B, Song YH and Wu KF. Expression of LL-37/hCAP-18 gene in human leukemia cells. *Leuk Res*. 2003;27:947-50.
413. Howell MD, Novak N, Bieber T, Pastore S, Girolomoni G, Boguniewicz M, Streib J, Wong C, Gallo RL and Leung DY. Interleukin-10 downregulates anti-microbial peptide expression in atopic dermatitis. *J Invest Dermatol*. 2005;125:738-45.
414. Heilborn JD, Nilsson MF, Kratz G, Weber G, Sorensen O, Borregaard N and Stahle-Backdahl M. The cathelicidin anti-microbial peptide LL-37 is involved in re-epithelialization of human skin wounds and is lacking in chronic ulcer epithelium. *J Invest Dermatol*. 2003;120:379-89.

415. Putsep K, Carlsson G, Boman HG and Andersson M. Deficiency of antibacterial peptides in patients with morbus Kostmann: an observation study. *Lancet*. 2002;360:1144-9.
416. Pestonjamasp VK, Huttner KH and Gallo RL. Processing site and gene structure for the murine antimicrobial peptide CRAMP. *Peptides*. 2001;22:1643-50.
417. Merres J, Hoss J, Albrecht LJ, Kress E, Soehnlein O, Jansen S, Pufe T, Tauber SC and Brandenburg LO. Role of the cathelicidin-related antimicrobial peptide in inflammation and mortality in a mouse model of bacterial meningitis. *J Innate Immun*. 2014;6:205-18.
418. Bergman P, Johansson L, Wan H, Jones A, Gallo RL, Gudmundsson GH, Hokfelt T, Jonsson AB and Agerberth B. Induction of the antimicrobial peptide CRAMP in the blood-brain barrier and meninges after meningococcal infection. *Infect Immun*. 2006;74:6982-91.
419. Chromek M, Slamova Z, Bergman P, Kovacs L, Podracka L, Ehren I, Hokfelt T, Gudmundsson GH, Gallo RL, Agerberth B and Brauner A. The antimicrobial peptide cathelicidin protects the urinary tract against invasive bacterial infection. *Nat Med*. 2006;12:636-41.
420. Nizet V, Ohtake T, Lauth X, Trowbridge J, Rudisill J, Dorschner RA, Pestonjamasp V, Piraino J, Huttner K and Gallo RL. Innate antimicrobial peptide protects the skin from invasive bacterial infection. *Nature*. 2001;414:454-7.
421. Iimura M, Gallo RL, Hase K, Miyamoto Y, Eckmann L and Kagnoff MF. Cathelicidin mediates innate intestinal defense against colonization with epithelial adherent bacterial pathogens. *J Immunol*. 2005;174:4901-7.
422. Kovach MA, Ballinger MN, Newstead MW, Zeng X, Bhan U, Yu FS, Moore BB, Gallo RL and Standiford TJ. Cathelicidin-related antimicrobial peptide is required for effective lung mucosal immunity in Gram-negative bacterial pneumonia. *J Immunol*. 2012;189:304-11.
423. Huang LC, Reins RY, Gallo RL and McDermott AM. Cathelicidin-deficient (Cnlp -/-) mice show increased susceptibility to Pseudomonas aeruginosa keratitis. *Invest Ophthalmol Vis Sci*. 2007;48:4498-508.
424. Howell MD, Jones JF, Kisich KO, Streib JE, Gallo RL and Leung DY. Selective killing of vaccinia virus by LL-37: implications for eczema vaccinatum. *J Immunol*. 2004;172:1763-7.
425. Barlow PG, Svoboda P, Mackellar A, Nash AA, York IA, Pohl J, Davidson DJ and Donis RO. Antiviral activity and increased host defense against influenza infection elicited by the human cathelicidin LL-37. *PLoS One*. 2011;6:e25333.
426. Koczulla R, von Degenfeld G, Kupatt C, Krotz F, Zahler S, Gloe T, Issbrucker K, Unterberger P, Zaiou M, Lebherz C, Karl A, Raake P, Pfosser A, Boekstegers P, Welsch U, Hiemstra PS, Vogelmeier C, Gallo RL, Clauss M and Bals R. An angiogenic role for the human peptide antibiotic LL-37/hCAP-18. *J Clin Invest*. 2003;111:1665-72.
427. Dombrowski Y and Schauber J. Cathelicidin LL-37: a defense molecule with a potential role in psoriasis pathogenesis. *Exp Dermatol*. 2012;21:327-30.
428. Bucki R, Leszczynska K, Namiot A and Sokolowski W. Cathelicidin LL-37: a multitask antimicrobial peptide. *Arch Immunol Ther Exp (Warsz)*. 2010;58:15-25.
429. Kruse T and Kristensen HH. Using antimicrobial host defense peptides as anti-infective and immunomodulatory agents. *Expert Rev Anti Infect Ther*. 2008;6:887-95.
430. Wu WK, Wong CC, Li ZJ, Zhang L, Ren SX and Cho CH. Cathelicidins in inflammation and tissue repair: Potential therapeutic applications for gastrointestinal disorders. *Acta Pharmacol Sin*. 2010;31:1118-22.
431. Wu WK, Wang G, Coffelt SB, Betancourt AM, Lee CW, Fan D, Wu K, Yu J, Sung JJ and Cho CH. Emerging roles of the host defense peptide LL-37 in human cancer and its potential therapeutic applications. *Int J Cancer*. 2010;127:1741-7.
432. Hennessy EJ, Parker AE and O'Neill LA. Targeting Toll-like receptors: emerging therapeutics? *Nat Rev Drug Discov*. 2010;9:293-307.

433. Jiang Y, Xiao W, Zhang Y and Xing Y. Urokinase-type plasminogen activator system and human cationic antimicrobial protein 18 in serum and induced sputum of patients with chronic obstructive pulmonary disease. *Respirology*. 2010;15:939-46.
434. Reinholz M, Ruzicka T and Schauber J. Cathelicidin LL-37: an antimicrobial peptide with a role in inflammatory skin disease. *Ann Dermatol*. 2012;24:126-35.
435. Frohm M, Agerberth B, Ahangari G, StahleBackdahl M, Liden S, Wigzell H and Gudmundsson GH. The expression of the gene coding for the antibacterial peptide LL-37 is induced in human keratinocytes during inflammatory disorders. *Journal of Biological Chemistry*. 1997;272:15258-15263.
436. Morizane S, Yamasaki K, Muhleisen B, Kotol PF, Murakami M, Aoyama Y, Iwatsuki K, Hata T and Gallo RL. Cathelicidin antimicrobial peptide LL-37 in psoriasis enables keratinocyte reactivity against TLR9 ligands. *J Invest Dermatol*. 2012;132:135-43.
437. Yamasaki K, Di Nardo A, Bardan A, Murakami M, Ohtake T, Coda A, Dorschner RA, Bonnart C, Descargues P, Hovnanian A, Morhenn VB and Gallo RL. Increased serine protease activity and cathelicidin promotes skin inflammation in rosacea. *Nat Med*. 2007;13:975-80.
438. Ganguly D, Chamilos G, Lande R, Gregorio J, Meller S, Facchinetti V, Homey B, Barrat FJ, Zal T and Gilliet M. Self-RNA-antimicrobial peptide complexes activate human dendritic cells through TLR7 and TLR8. *J Exp Med*. 2009;206:1983-94.
439. Chamorro CI, Weber G, Gronberg A, Pivarcsi A and Stahle M. The human antimicrobial peptide LL-37 suppresses apoptosis in keratinocytes. *J Invest Dermatol*. 2009;129:937-44.
440. Kanda N, Ishikawa T, Kamata M, Tada Y and Watanabe S. Increased serum leucine, leucine-37 levels in psoriasis: positive and negative feedback loops of leucine, leucine-37 and pro- or anti-inflammatory cytokines. *Hum Immunol*. 2010;71:1161-71.
441. Hansson GK. Inflammation, atherosclerosis, and coronary artery disease. *N Engl J Med*. 2005;352:1685-95.
442. Doring Y and Zernecke A. Plasmacytoid dendritic cells in atherosclerosis. *Front Physiol*. 2012;3:230.
443. Niessner A, Sato K, Chaikof EL, Colmegna I, Goronzy JJ and Weyand CM. Pathogen-sensing plasmacytoid dendritic cells stimulate cytotoxic T-cell function in the atherosclerotic plaque through interferon-alpha. *Circulation*. 2006;114:2482-9.
444. Gage J, Hasu M, Thabet M and Whitman SC. Caspase-1 deficiency decreases atherosclerosis in apolipoprotein E-null mice. *Can J Cardiol*. 2012;28:222-9.
445. Doring Y, Manthey HD, Drechsler M, Lievens D, Megens RT, Soehnlein O, Busch M, Manca M, Koenen RR, Pelisek J, Daemen MJ, Lutgens E, Zenke M, Binder CJ, Weber C and Zernecke A. Auto-antigenic protein-DNA complexes stimulate plasmacytoid dendritic cells to promote atherosclerosis. *Circulation*. 2012;125:1673-83.
446. Edfeldt K, Agerberth B, Rottenberg ME, Gudmundsson GH, Wang XB, Mandal K, Xu QB and Yan ZQ. Involvement of the antimicrobial peptide LL-37 in human atherosclerosis. *Arterioscl Throm Vas*. 2006;26:1551-1557.
447. Ciornei CD, Tapper H, Bjartell A, Sternby NH and Bodelsson M. Human antimicrobial peptide LL-37 is present in atherosclerotic plaques and induces death of vascular smooth muscle cells: a laboratory study. *BMC Cardiovasc Disord*. 2006;6:49.
448. Doring Y, Drechsler M, Wantha S, Kemmerich K, Lievens D, Vijayan S, Gallo RL, Weber C and Soehnlein O. Lack of neutrophil-derived CRAMP reduces atherosclerosis in mice. *Circ Res*. 2012;110:1052-6.
449. Su W, Chen Y, Wang C, Ding X, Rwibasira G and Kong Y. Human cathelicidin LL-37 inhibits platelet aggregation and thrombosis via Src/PI3K/Akt signaling. *Biochem Biophys Res Commun*. 2016;473:283-9.
450. Pircher J, Czermak T, Ehrlich A, Eberle C, Gaitzsch E, Margraf A, Grommes J, Saha P, Titova A, Ishikawa-Ankerhold H, Stark K, Petzold T, Stocker T, Weckbach LT, Novotny J, Sperandio M, Nieswandt B, Smith A, Mannell H, Walzog B, Horst D, Soehnlein O, Massberg

S and Schulz C. Cathelicidins prime platelets to mediate arterial thrombosis and tissue inflammation. *Nature communications*. 2018;9:1523.
451. Machado ID, Spatti M, Hastreiter A, Santin JR, Fock RA, Gil CD, Oliani SM, Perretti M and Farsky SH. Annexin A1 Is a Physiological Modulator of Neutrophil Maturation and Recirculation Acting on the CXCR4/CXCL12 Pathway. *J Cell Physiol*. 2016;231:2418-27.
452. Gerke V, Creutz CE and Moss SE. Annexins: linking Ca2+ signalling to membrane dynamics. *Nat Rev Mol Cell Biol*. 2005;6:449-61.
453. Zhao B, Wang J, Liu L, Li X, Liu S, Xia Q and Shi J. Annexin A1 translocates to nucleus and promotes the expression of pro-inflammatory cytokines in a PKC-dependent manner after OGD/R. *Sci Rep*. 2016;6:27028.
454. Murphy CT, Peers SH, Forder RA, Flower RJ, Carey F and Westwick J. Evidence for the presence and location of annexins in human platelets. *Biochem Biophys Res Commun*. 1992;189:1739-46.
455. Eldering JA, Kocher M, Clemetson JM, Clemetson KJ, Frey FJ and Frey BM. Presence of lipocortins I and IV, but not II and VI, in human platelets. *FEBS Lett*. 1993;318:231-4.
456. Perretti M, Croxtall JD, Wheller SK, Goulding NJ, Hannon R and Flower RJ. Mobilizing lipocortin 1 in adherent human leukocytes downregulates their transmigration. *Nat Med*. 1996;2:1259-62.
457. Rosengarth A and Luecke H. A calcium-driven conformational switch of the N-terminal and core domains of annexin A1. *J Mol Biol*. 2003;326:1317-25.
458. Perretti M. Endogenous mediators that inhibit the leukocyte-endothelium interaction. *Trends Pharmacol Sci*. 1997;18:418-25.
459. Rescher U, Goebeler V, Wilbers A and Gerke V. Proteolytic cleavage of annexin 1 by human leukocyte elastase. *Biochim Biophys Acta*. 2006;1763:1320-4.
460. Vong L, D'Acquisto F, Pederzoli-Ribeil M, Lavagno L, Flower RJ, Witko-Sarsat V and Perretti M. Annexin 1 cleavage in activated neutrophils: a pivotal role for proteinase 3. *J Biol Chem*. 2007;282:29998-30004.
461. Pederzoli-Ribeil M, Maione F, Cooper D, Al-Kashi A, Dalli J, Perretti M and D'Acquisto F. Design and characterization of a cleavage-resistant Annexin A1 mutant to control inflammation in the microvasculature. *Blood*. 2010;116:4288-96.
462. Cirino G, Cicala C, Sorrentino L, Ciliberto G, Arpaia G, Perretti M and Flower RJ. Anti-inflammatory actions of an N-terminal peptide from human lipocortin 1. *Br J Pharmacol*. 1993;108:573-4.
463. Perretti M and Flower RJ. Annexin 1 and the biology of the neutrophil. *Journal of leukocyte biology*. 2004;76:25-9.
464. Perretti M and Flower RJ. Modulation of IL-1-induced neutrophil migration by dexamethasone and lipocortin 1. *J Immunol*. 1993;150:992-9.
465. Gavins FN, Dalli J, Flower RJ, Granger DN and Perretti M. Activation of the annexin 1 counter-regulatory circuit affords protection in the mouse brain microcirculation. *Faseb J*. 2007;21:1751-8.
466. Bandeira-Melo C, Bonavita AG, Diaz BL, PM ES, Carvalho VF, Jose PJ, Flower RJ, Perretti M and Martins MA. A novel effect for annexin 1-derived peptide ac2-26: reduction of allergic inflammation in the rat. *J Pharmacol Exp Ther*. 2005;313:1416-22.
467. Dalli J, Montero-Melendez T, McArthur S and Perretti M. Annexin A1 N-terminal derived Peptide ac2-26 exerts chemokinetic effects on human neutrophils. *Front Pharmacol*. 2012;3:28.
468. Tagoe CE, Marjanovic N, Park JY, Chan ES, Abeles AM, Attur M, Abramson SB and Pillinger MH. Annexin-1 mediates TNF-alpha-stimulated matrix metalloproteinase secretion from rheumatoid arthritis synovial fibroblasts. *J Immunol*. 2008;181:2813-20.
469. Williams SL, Milne IR, Bagley CJ, Gamble JR, Vadas MA, Pitson SM and Khew-Goodall Y. A proinflammatory role for proteolytically cleaved annexin A1 in neutrophil transendothelial migration. *J Immunol*. 2010;185:3057-63.

470. Chung YW, Oh HY, Kim JY, Kim JH and Kim IY. Allergen-induced proteolytic cleavage of annexin-1 and activation of cytosolic phospholipase A2 in the lungs of a mouse model of asthma. *Proteomics*. 2004;4:3328-34.
471. Vishwanatha JK, Davis RG, Rubinstein I and Floreani A. Annexin I degradation in bronchoalveolar lavage fluids from healthy smokers: a possible mechanism of inflammation. *Clin Cancer Res*. 1998;4:2559-64.
472. Vergnolle N, Comera C and Bueno L. Annexin 1 is overexpressed and specifically secreted during experimentally induced colitis in rats. *Eur J Biochem*. 1995;232:603-10.
473. Tsao FH, Meyer KC, Chen X, Rosenthal NS and Hu J. Degradation of annexin I in bronchoalveolar lavage fluid from patients with cystic fibrosis. *Am J Respir Cell Mol Biol*. 1998;18:120-8.
474. de Jong RJ, Leoni G, Drechsler M and Soehnlein O. The advantageous role of annexin A1 in cardiovascular disease. *Cell Adh Migr*. 2016:0.
475. Kusters DH, Chatrou ML, Willems BA, De Saint-Hubert M, Bauwens M, van der Vorst E, Bena S, Biessen EA, Perretti M, Schurgers LJ and Reutelingsperger CP. Pharmacological Treatment with Annexin A1 Reduces Atherosclerotic Plaque Burden in LDLR-/- Mice on Western Type Diet. *PLoS One*. 2015;10:e0130484.
476. Drechsler M, de Jong R, Rossaint J, Viola JR, Leoni G, Wang JM, Grommes J, Hinkel R, Kupatt C, Weber C, Doring Y, Zarbock A and Soehnlein O. Annexin A1 counteracts chemokine-induced arterial myeloid cell recruitment. *Circ Res*. 2015;116:827-35.
477. Bagnato C, Thumar J, Mayya V, Hwang SI, Zebroski H, Claffey KP, Haudenschild C, Eng JK, Lundgren DH and Han DK. Proteomics analysis of human coronary atherosclerotic plaque: a feasibility study of direct tissue proteomics by liquid chromatography and tandem mass spectrometry. *Mol Cell Proteomics*. 2007;6:1088-102.
478. Cheuk BL and Cheng SW. Annexin A1 expression in atherosclerotic carotid plaques and its relationship with plaque characteristics. *Eur J Vasc Endovasc Surg*. 2011;41:364-71.
479. Fredman G, Kamaly N, Spolitu S, Milton J, Ghorpade D, Chiasson R, Kuriakose G, Perretti M, Farokzhad O and Tabas I. Targeted nanoparticles containing the proresolving peptide Ac2-26 protect against advanced atherosclerosis in hypercholesterolemic mice. *Sci Transl Med*. 2015;7:275ra20.
480. Qin C, Buxton KD, Pepe S, Cao AH, Venardos K, Love JE, Kaye DM, Yang YH, Morand EF and Ritchie RH. Reperfusion-induced myocardial dysfunction is prevented by endogenous annexin-A1 and its N-terminal-derived peptide Ac-ANX-A1(2-26). *Br J Pharmacol*. 2013;168:238-52.
481. Ritchie RH, Gordon JM, Woodman OL, Cao AH and Dusting GJ. Annexin-1 peptide Anx-1(2-26) protects adult rat cardiac myocytes from cellular injury induced by simulated ischaemia. *Br J Pharmacol*. 2005;145:495-502.
482. Relton JK, Strijbos PJ, O'Shaughnessy CT, Carey F, Forder RA, Tilders FJ and Rothwell NJ. Lipocortin-1 is an endogenous inhibitor of ischemic damage in the rat brain. *J Exp Med*. 1991;174:305-10.
483. Sato Y, Kumamoto K, Saito K, Okayama H, Hayase S, Kofunato Y, Miyamoto K, Nakamura I, Ohki S, Koyama Y and Takenoshita S. Up-regulated Annexin A1 expression in gastrointestinal cancer is associated with cancer invasion and lymph node metastasis. *Exp Ther Med*. 2011;2:239-243.
484. Shin J, Song IS, Pak JH and Jang SW. Upregulation of annexin A1 expression by butyrate in human melanoma cells induces invasion by inhibiting E-cadherin expression. *Tumour Biol*. 2016;37:14577-14584.
485. Yom CK, Han W, Kim SW, Kim HS, Shin HC, Chang JN, Koo M, Noh DY and Moon BI. Clinical significance of annexin A1 expression in breast cancer. *J Breast Cancer*. 2011;14:262-8.
486. Sobral-Leite M, Wesseling J, Smit VT, Nevanlinna H, van Miltenburg MH, Sanders J, Hofland I, Blows FM, Coulson P, Patrycja G, Schellens JH, Fagerholm R, Heikkila P,

Aittomaki K, Blomqvist C, Provenzano E, Ali HR, Figueroa J, Sherman M, Lissowska J, Mannermaa A, Kataja V, Kosma VM, Hartikainen JM, Phillips KA, kConFab AI, Couch FJ, Olson JE, Vachon C, Visscher D, Brenner H, Butterbach K, Arndt V, Holleczek B, Hooning MJ, Hollestelle A, Martens JW, van Deurzen CH, van de Water B, Broeks A, Chang-Claude J, Chenevix-Trench G, Easton DF, Pharoah PD, Garcia-Closas M, de Graauw M and Schmidt MK. Annexin A1 expression in a pooled breast cancer series: association with tumor subtypes and prognosis. *BMC Med*. 2015;13:156.
487. Wang LP, Bi J, Yao C, Xu XD, Li XX, Wang SM, Li ZL, Zhang DY, Wang M and Chang GQ. Annexin A1 expression and its prognostic significance in human breast cancer. *Neoplasma*. 2010;57:253-9.
488. Vong L, Ferraz JG, Dufton N, Panaccione R, Beck PL, Sherman PM, Perretti M and Wallace JL. Up-regulation of Annexin-A1 and lipoxin A(4) in individuals with ulcerative colitis may promote mucosal homeostasis. *PLoS One*. 2012;7:e39244.
489. Sena A, Grishina I, Thai A, Goulart L, Macal M, Fenton A, Li J, Prindiville T, Oliani SM, Dandekar S, Goulart L and Sankaran-Walters S. Dysregulation of anti-inflammatory annexin A1 expression in progressive Crohns Disease. *PLoS One*. 2013;8:e76969.
490. Petrella A, Festa M, Ercolino SF, Zerilli M, Stassi G, Solito E and Parente L. Annexin-1 downregulation in thyroid cancer correlates to the degree of tumor differentiation. *Cancer Biol Ther*. 2006;5:643-7.
491. Boudhraa Z, Merle C, Mazzocut D, Chezal JM, Chambon C, Miot-Noirault E, Theisen M, Bouchon B and Degoul F. Characterization of pro-invasive mechanisms and N-terminal cleavage of ANXA1 in melanoma. *Arch Dermatol Res*. 2014;306:903-14.
492. Panaro MA, Acquafredda A, Sisto M, Lisi S, Maffione AB and Mitolo V. Biological role of the N-formyl peptide receptors. *Immunopharmacol Immunotoxicol*. 2006;28:103-27.
493. Wang Y, Agerberth B, Lothgren A, Almstedt A and Johansson J. Apolipoprotein A-I binds and inhibits the human antibacterial/cytotoxic peptide LL-37. *J Biol Chem*. 1998;273:33115-8.
494. Sigurdardottir T, Andersson P, Davoudi M, Malmsten M, Schmidtchen A and Bodelsson M. In silico identification and biological evaluation of antimicrobial peptides based on human cathelicidin LL-37. *Antimicrobial agents and chemotherapy*. 2006;50:2983-9.
495. Scott MG, Davidson DJ, Gold MR, Bowdish D and Hancock RE. The human antimicrobial peptide LL-37 is a multifunctional modulator of innate immune responses. *J Immunol*. 2002;169:3883-91.
496. Kim M, Kim J, Jeong SW, Jo H and Park HJ. Long-pulsed 1064-nm Nd: YAG laser ameliorates LL-37-induced rosacea-like skin lesions through promoting collagen remodeling in BALB/c mice. *Lasers Med Sci*. 2018;33:393-397.
497. Bals R, Weiner DJ, Moscioni AD, Meegalla RL and Wilson JM. Augmentation of innate host defense by expression of a cathelicidin antimicrobial peptide. *Infect Immun*. 1999;67:6084-9.
498. Suzuki K, Murakami T, Kuwahara-Arai K, Tamura H, Hiramatsu K and Nagaoka I. Human anti-microbial cathelicidin peptide LL-37 suppresses the LPS-induced apoptosis of endothelial cells. *Int Immunol*. 2011;23:185-93.
499. Oldekamp S, Pscheidl S, Kress E, Soehnlein O, Jansen S, Pufe T, Wang JM, Tauber SC and Brandenburg LO. Lack of formyl peptide receptor 1 and 2 leads to more severe inflammation and higher mortality in mice with of pneumococcal meningitis. *Immunology*. 2014;143:447-461.
500. Dorward DA, Lucas CD, Doherty MK, Chapman GB, Scholefield EJ, Morris AC, Felton JM, Kipari T, Humphries DC, Robb CT, Simpson AJ, Whitfield PD, Haslett C, Dhaliwal K and Rossi AG. Novel role for endogenous mitochondrial formylated peptide-driven formyl peptide receptor 1 signalling in acute respiratory distress syndrome. *Thorax*. 2017;72:928-936.
501. Cooray SN, Gobbetti T, Montero-Melendez T, McArthur S, Thompson D, Clark AJ, Flower RJ and Perretti M. Ligand-specific conformational change of the G-protein-coupled

receptor ALX/FPR2 determines proresolving functional responses. *Proc Natl Acad Sci U S A*. 2013;110:18232-7.