University of Reading

Role of Bacterial Lipopolysaccharides in the Modulation of Platelet and Megakaryocyte Function

Thesis submitted for the Degree of Doctor of Philosophy

School of Chemistry, Food, and Pharmacy

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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.

Thomas Michael Vallance

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PUBLICATIONS & PRESENTATIONS

Review Articles

1. **Toll-like Receptor 4 Signalling and Its Impact on Platelet Function, Thrombosis, and Haemostasis.**
   Vallance TM, Zeuner MT, Williams HF, Widera D, Vaiyapuri S
   Mediators of Inflammation. 2017; 2017: 9605894.

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Posters

9. Vallance TM, Widera D, Vaiyapuri S. (2017) Impact of Toll-like Receptor 4-Mediated Signalling in the Modulation of Platelet Activation, Haemostasis, and Thrombosis. World Congress on Inflammation
10. Vallance TM, Widera D, Vaiyapuri S. (2017) Impact of Toll-like Receptor 4 Signalling on Platelet Function. Reading School of Pharmacy PhD Showcase
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12. Vallance TM, Ravishankar D, Albadawi DAI, Layfield H, Sheard J, Vaiyapuri R, Dash P, Patel K, Widera D, Vaiyapuri S. (2018) Effect of Ultrapure LPS on Platelet Activation. British Pharmacological Society

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ABSTRACT

Cardiovascular disease is a group of inflammatory diseases that is responsible for the plurality of deaths in the world. In ischaemic diseases, aberrant platelet activation leads to thrombus formation and occlusion of key arteries. Furthermore, platelets are critically involved in sepsis, where thrombocytopaenia is correlated with worse patient outcomes. Therefore, immune receptors, such as Toll-like receptor 4 (TLR4), have been identified as potential targets for reducing platelet activation by decoupling immune functions from haemostasis. Multiple experimental techniques were used to determine the impact of ultrapure lipopolysaccharide (LPS) chemotypes on platelet and megakaryocyte function as LPS is a specific ligand for TLR4.

To determine whether LPS modulates platelet activity, LPS and platelets were co-incubated under different conditions. We determined that ultrapure LPS derived from various species of Gram-negative bacteria cannot significantly modulate platelet activity, as measured by aggregation, fibrinogen binding, and P-selectin exposure, except under specific conditions. This is potentially due to activation dependent cell-surface expression of TLR4. Subsequently, to investigate whether LPS induces inflammatory signalling in megakaryocytes, a novel megakaryocyte reporter cell line, Meg-01R, was developed and used to determine that ultrapure LPS is not sufficient on its own to modulate megakaryocyte function. Characterisation of this cell line suggests that MyD88-dependent signalling pathways are active in Meg-01R cells but TLR4 is not present in sufficient quantities at the cell surface. As LL37 directly binds to LPS and is also a strong platelet agonist, we investigated the impact of LPS on LL37-induced platelet activation. Here, we discovered a TLR4-independent cell-independent formation of LL37-LPS micelles that prevents LL37-induced platelet activation.

Based on these results, LPS and TLR4 are not sufficient, *in vitro*, to decouple the immune function of platelets from haemostasis or induce changes in megakaryocyte function however it may still play an important role in conjunction with other immune receptors.

ABBREVIATIONS

5-FAM-LL37 – 5-carboxyfluorescein-LL37

Ac2-26 – Acetyl-AMVSEFLKQAWFIENEEQEYVQTVK

ACD – Acid citrate dextrose

ADP – Adenosine diphosphate

AMP – Antimicrobial peptide

ANOVA – Analysis of variance

AP-1 – Activator protein-1

ATP – Adenosine triphosphate

AUC – Area under the curve

BML-111 – (5*S*,6*R*)-methyl 5,6,7-trihydroxyheptanoate

BSA – Bovine serum albumin

C87 – 3-phenyl-1-(4-phenyl-2-thiazolyl)-1*H*-pyrazole-4,5-dione 4-[2-(4-chloro-3-nitrophenyl)hydrazine]

CD14 – Cluster of differentiation 14

$CD34^+$ – Cluster of differentiation 34-positive

CD42b – Cluster of differentiation 42b; platelet glycoprotein Ib

CD61 – Cluster of differentiation 61; integrin β_3

CD62P – Cluster of differentiation 62P; P-selectin

CD63 – Cluster of differentiation 63

CD284 – Cluster of differentiation 284; Toll-like receptor-4

cGKI – cGMP-dependent protein kinase I

cGMP – Cyclic guanosine monophosphate

cIAP – Cellular inhibitor of apoptosis

CMAC – 7-amino-4-chloromethylcoumarin

CMC – Critical micelle concentration

c-MPL – Thrombopoietin receptor

CRAMP – Cathelicidin-related antimicrobial peptide

CRP-XL – Cross-linked collagen-related peptide

CryoTEM – Transmission electron cryomicroscopy

CtxB – Cholera toxin B-subunit

CVD – Cardiovascular disease

CXCR2 – C-X-C motif chemokine receptor-2

Cy5 – Cyanine5

DAG – Diacylglycerol

DAMPs – Damage-associated molecular pattern

DAPI – 4',6-diamidino-2-phenylindole

DD – Death domain

DIC – Disseminated intravascular coagulation

DMEM – Dulbecco's modified Eagle's medium

DNA – Deoxyribonucleic acid

ECL – Enhanced chemiluminescence

EDTA – Ethylenediaminetetraacetic acid

EGF – Epidermal growth factor

EGFR – Epidermal growth factor receptor

ERK1/2 – Extracellular signal-related kinase-1/2

FcR – Fc receptor

FCS – Foetal calf serum

FITC – Fluorescein isothiocyanate

FPR2/ALX – Formyl peptide receptor 2/Lipoxin A4 receptor

GFP – Green fluorescent protein

GPIb – Glycoprotein Ib

GPVI – Glycoprotein VI

gp96 – Heat shock protein 90kDa β member-1

GPI – Glycosylphosphatidylinositol

HBS – HEPES-buffered saline

hCAP-18 – Human cationic microbial peptide-18

HEK293 – Human embryonic kidney 293

HEPES – 4-(2-hydroxyethyl)-1-piperazineethanesulphonic acid

HMGB1 – High-mobility group box-1

HSP – Heat shock protein

HSP60 – Heat shock protein 60

ICAM-1 – Intracellular adhesion molecule-1

ICC – Immunocytochemistry

IgG – Immunoglobulin G

IκB – Inhibitor of NF-κB

IκBα – Inhibitor of NF-κB α

IKK – IκB kinase

IKKα – IκB kinase-α

IKKβ – IκB kinase-β

IKKγ – IκB kinase-γ; also known as NEMO

IKKε – IκB kinase-ε

IL-1β – Interleukin-1β

IL-6 – Interleukin-6

IL-8 – Interleukin-8

IMD0354 – *N*-[3,5-bis(trifluoromethyl)phenyl]-5-chloro-2-hydroxybenzamide

$Ins(1,4,5)P_3$ – Inositol 1,4,5-trisphosphate

IRAK – IL-1 receptor-associated kinase

IRAK1/2 – IL-1 receptor-associated kinase-1/2

IRAK4 – IL-1 receptor-associated kinase-4

IRF3 – Interferon regulatory factor-3

JNK1/2 – c-Jun N-terminal kinase-1/2

LAMP-1 – Lysosomal associated membrane protein-1

LBP – Lipopolysaccharide binding protein

LDH – Lactate dehydrogenase

LL37 – LLGDFFRKSKEKIGKEFKRIVQRIKDFLRNLVPRTES

LPS – Lipopolysaccharide

LPS_{EC} – *Escherichia coli*-derived lipopolysaccharide

LPS_{RS} – *Rhodobacter sphaeroides*-derived lipopolysaccharide

LPS_{SM} – *Salmonella enterica minnesota*-derived lipopolysaccharide

LRR – Leucine-rich repeat

Luc – Luciferase

Mal – MyD88 adaptor-like

MAPK – Mitogen-activated protein kinases

MCP-1 – Monocyte chemoattractant protein-1

MD-2 – Lymphocyte antigen-96

Meg-01R – Meg-01-NF-κB-GFP-Luc

MK – Megakaryocyte

MyD88 – Myeloid differentiation primary response-88

NEMO – NF-κB essential modulator; also known as IKKγ

NET – Neutrophil extracellular trap

NF-κB – Nuclear factor of κ-light-polypeptide-gene enhancer in B cells

NMR – Nuclear magnetic resonance

P2X7R – P2X7 receptor

PAF4 – Platelet-activating factor-4

PAMP – Pathogen-associated molecular pattern

Pam3CSK4 – Pam3CSKKKK

PAR – Protease-activated receptor

PBMC – Peripheral blood mononuclear cell

PBS – Phosphate-buffered saline

PBS-T – Phosphate-buffered saline with Tween-20

PBT – Phosphate-buffered saline with Triton X-100

PDGF-AB – Platelet-derived growth factor-AB

PE – Phycoerythrin

PE-Cy5 – Phycoerythrin-cyanine5

PEB – PBS-EDTA-BSA

PFA – Paraformaldehyde

PGI_2 – Prostaglandin I_2

Phenol-TEA-DOC – Phenol-triethylamine-deoxycholate

PI3K – Phosphoinositide 3-kinase

$PI(4,5)P_2$ – Phosphatidylinositol-4,5-bisphosphate

PKB – Protein kinase B; also known as Akt

PKC – Protein kinase C

PLCγ2 – Phospholipase Cγ2

Poly(A:U) – Polyadenylic-polyuridylic acid

Poly(I:C) – Polyinosine-polycytidylic acid

PRAT4A – Protein associated with TLR4

PRP – Platelet-rich plasma

PRR – Pattern-recognition receptor

PTX – Pertussis toxin

PVDF – Polyvinylidene fluoride

qPCR – Quantitative polymerase chain reaction

RAGE – Receptor for advanced glycation end-products

RANTES – Regulated upon activation, normally T-cell expressed, and presumably secreted

RPMI-1640 – Roswell Park Memorial Institute-1640

RSTB – Reducing sample treatment buffer

RT – Room temperature

SAA – Serum amyloid A

sCD14 – Soluble cluster of differentiation 14

sCD40L – Soluble cluster of differentiation 40 ligand

S.D. – Standard deviation

SDS-PAGE – Sodium dodecyl sulphate – polyacrylamide gel electrophoresis

SNAP23 – Synaptosomal-associated protein-23

SNARE – Soluble N-ethylamide-sensitive factor attachment protein receptor

Syk – Spleen-associated tyrosine kinase

TAK1 – TRAF-activated kinase-1

TANK – TRAF family member-associated NF-κB activator

TBK1/NAK – TANK-binding kinase-1/NF-κB activating kinase

TGFβ – Transforming growth factor β

TIR – Toll/IL-1 receptor

TIRAP – TIR domain-containing adaptor protein

TLR – Toll-like receptor

TLR1/2 – Toll-like receptor-1/2

TLR2 – Toll-like receptor-2

TLR3 – Toll-like receptor-3

TLR4 – Toll-like receptor-4

TNFα – Tumour necrosis factor-α

TNFRI/II – TNF receptor I/II

TPO – Thrombopoietin

TRAF3 – TNF receptor-associated factor-3

TRAF6 – TNF receptor-associated factor-6

TRAM – TRIF-related adaptor molecule

TRAP6 – Thrombin receptor activator peptide-6

TRIF – TIR domain-containing adaptor-inducing interferon-β

TxA_2 – Thromboxane A_2

$uLPS_{EC}$ – Ultrapure *Escherichia coli*-derived lipopolysaccharide

$uLPS_{SM}$ – Ultrapure *Salmonella enterica minnesota*-derived lipopolysaccharide

vWF	– von Willebrand factor
WB	– Whole blood
WP	– Washed platelets
WRW4	– WRWWWW
XTT	– Sodium 3’-[1-(phenylaminocarbonyl)- 3,4- tetrazolium]-bis (4-methoxy 6-nitro) benzene sulphonic acid hydrate

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1.1 GENERAL INTRODUCTION

Cardiovascular diseases (CVDs) contribute to the plurality of deaths in the world and adversely affect the quality of life for millions of individuals (particularly those >75 years old) as well as having a substantial economic impact[1,2]. The most common forms of CVDs are driven by a complex, multifactor process that involves deposition of lipids underneath the arterial endothelium, extravasation and activation of immune cells, and formation of a fibrous cap[3]. This atherosclerotic plaque can expand via multiple mechanisms including endothelial desquamation or fibrous cap rupture leading to the exposure of the subendothelial matrix and lipid core, triggering thrombus formation which can occlude blood flow and induce a myocardial infarction or an ischaemic stroke[3].

A key cell type in CVDs is the platelet. Platelets are responsible for maintaining haemostasis under normal conditions but they also act as sentinels that can alert nearby immune cells to infections and damage[4]. The immune role of platelets is further emphasised by their ability to interact with leukocytes to promote additional functions (e.g. neutrophil extracellular trap formation) during systemic infections (sepsis) and their ability to secrete antimicrobial compounds[5–7]. During myocardial infarction and ischaemic stroke, platelets become activated by the exposure of subendothelial collagen and drive the formation of the pathological thrombus via aggregation with other platelets[8]. It is this pathological thrombus which is responsible for inducing ischaemia in key tissues. Therefore, platelets are a key target for preventing CVDs. Current antiplatelet therapies, aspirin and clopidogrel, have been used for decades but both have problems with low efficacy (only decreasing the prevalence of thrombotic events by <25%) and increased adverse bleeding events[9,10]. Despite the pressing need and the decades of research that has been conducted, an improved antiplatelet therapy has yet to been discovered and brought to the clinic.

Recently, a hypothesis has arisen to explain why CVDs are more prevalent in “older” individuals than “younger” individuals[11,12]. Indeed, platelet reactivity increases with age and this has been suggested to be due to the increased systemic inflammation seen during aging[11,13]. This hypothesis also links to the suggestion that antiplatelet therapies should target immune receptors [e.g. Toll-like receptors (TLRs), formyl

peptide receptors (FPRs)] rather that haemostatic receptors [e.g. glycoprotein VI (GPVI), protease-activated receptor 1/4 (PAR1/4), purinergic receptor ($P2Y_{12}$)]. This would decouple the immune function of platelets from the haemostatic function of platelets and therefore preserve haemostasis whilst preventing aberrant platelet activation.

Lipopolysaccharide (LPS) is a component of the cell wall of Gram-negative bacteria that ligates to TLR4[14]. This receptor has been implicated in platelet activation, priming, and promoting interactions with leukocytes such as neutrophils[6,15]. Administration of LPS can induce sepsis and be fatal[16]. TLR4 has also been suggested to respond to lots of endogenous damage-associated molecular patterns that could be released into the blood stream during atherosclerotic lesion rupture and therefore potentiate platelet function during these conditions and exacerbate arterial occlusion[17–19]. Furthermore, TLR4 has been implicated in the generation of platelets from megakaryocytes[20]. Moreover, bacterial infection and LPS has been implicated in atherosclerosis as they can increase intracellular adhesion molecule-1 (ICAM-1) expression on human endothelial cells[21]. Taken together, TLR4 is an interesting target for novel therapeutic agents in the prevention of CVDs, as well as sepsis, and LPS is a useful tool for examining these functions[6].

LL37 is an endogenous antimicrobial peptide and the only cathelicidin produced by humans[22,23]. In addition to this function, LL37 can act as a chemoattractant and a powerful platelet agonist and has been detected in arterial thrombi and atherosclerotic plaques[7,24–26]. Furthermore, studies in $Apoe^{-/-}$ $Cramp^{-/-}$ mice suggest that murine cathelicidin-related antimicrobial peptide (mCRAMP; the mouse homologue of LL37) is important for determining plaque size as removal of mCRAMP led to a reduction in lesion size[27]. Therefore, LL37 may also be critically involved in CVDs due to its ability to both potentiate atherosclerotic lesion formation and directly activate platelets when the atherosclerotic lesion ruptures. Intriguingly, LL37 is also known for its direct interactions with LPS during which they can form complex superstructures and either potentiate or inhibit inflammation[28–31]. However, how this function may associate with CVDs is currently unclear.

In this thesis, we will examine the influence of LPS on platelet and megakaryocyte function to determine the role of TLR4 in these cell types. Moreover,

this influence will be tested in the presence of multiple platelet agonists, including LL37 as these two molecules are known to closely associate with each other, to evaluate platelet/megakaryocyte TLR4 as a potential therapeutic target for CVDs.

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1.2

Review Article

Toll-Like Receptor 4 Signalling and Its Impact on Platelet Function, Thrombosis, and Haemostasis

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Platelets are anucleated blood cells that participate in a wide range of physiological and pathological functions. Their major role is mediating haemostasis and thrombosis. In addition to these classic functions, platelets have emerged as important players in the innate immune system. In particular, they interact with leukocytes, secrete pro- and anti-inflammatory factors, and express a wide range of inflammatory receptors including Toll-like receptors (TLRs), for example, Toll-like receptor 4 (TLR4). TLR4, which is the most extensively studied TLR in nucleated cells, recognises lipopolysaccharides (LPS) that are compounds of the outer surface of Gram-negative bacteria. Unlike other TLRs, TLR4 is able to signal through both the MyD88-dependent and MyD88-independent signalling pathways. Notably, despite both pathways culminating in the activation of transcription factors, TLR4 has a prominent functional impact on platelet activity, haemostasis, and thrombosis. In this review, we summarise the current knowledge on TLR4 signalling in platelets, critically discuss its impact on platelet function, and highlight the open questions in this area.

1. Introduction

Platelets are small, anucleated, and short-lived blood cells with a range of important functions beyond their classical roles in haemostasis [1–3]. The function of platelets in haemostasis has been well documented and is linked to their capacity to respond to the damaged endothelium [4–6]. Following vessel damage and initial activation, platelets secrete a wide variety of small molecules and proteins from intracellular granules in order to activate and recruit more circulating platelets and immune cells, such as leukocytes [4]. In addition to these secretion events, platelets undergo dramatic shape changes that enable them to cover the site of injury and prevent bleeding [4]. Thrombosis (blood clot formation) mediated by platelets occurs in the arteries under pathological conditions and significantly obstructs the blood flow to major organs such as the heart and brain resulting in heart attacks and strokes, respectively [7]. In addition to their physiological functions, platelets can be involved in different pathological conditions, for example, in atherosclerosis [8, 9]. If the atherosclerotic plaque ruptures, the exposure of the subendothelial matrix and release of procoagulatory matrix proteins, such as collagen, are sufficient to initiate the formation of a thrombus (blood clot) at this site [4, 10]. Thrombus poses a significant systemic risk because it is formed in a narrowed blood vessel and so has the potential to completely occlude the vessel and trigger a myocardial infarction or ischaemic stroke [10].

Platelets also have pivotal roles in the innate immune system, which includes cells that combat general infections (e.g., neutrophils), and is responsible for the eradication of pathogens to protect the body from infection [11, 12]. During the immune response, platelets have been shown to interact with and respond to many species of Gram-positive and Gram-negative bacteria through different receptors [13, 14]. Moreover, platelets are capable of internalising specific types of bacteria and viruses although the function of this phenomenon is poorly understood [15, 16]. The ability of platelets to

participate in such a wide range of functions and their ability to synthesise certain new proteins despite lacking a nucleus have generated significant scientific interest [2, 3, 17].

In addition, platelets play a role in the development of disseminated intravascular coagulation (DIC), a common complication observed in patients with sepsis [18–20]. During DIC, platelets are activated and form smaller thrombi in the microvasculature or aggregates that are sequestered in organs such as the lungs. Together, this leads to thrombocytopenia, a reduction in the number of circulating platelets. Mild thrombocytopenia is defined as less than 1.5×10^{11} platelets per litre of blood compared to between 1.5 and 4.0×10^{11} in healthy individuals, but more severe thrombocytopenia is defined as less than 0.5×10^{11} platelets per litre [6, 20, 21]. Furthermore, it has been discovered that platelets can promote the formation of neutrophil extracellular traps (NETs) which have cytotoxic actions on host cells beyond their beneficial antibacterial effects [22].

Notably, conditions such as sepsis and DIC have been suggested to be linked to several platelet receptors, especially Toll-like receptor (TLR) 4 [8, 18, 22, 23]. In human nucleated cells, especially in professional antigen-presenting cells, the binding of a ligand to TLR1, 2, 4, 5, 6, 7, 8, 9, and 10 results in the activation of the so called myeloid differentiation factor-88- (MyD88-) dependent pathway, whereas TLR3 activates the MyD88-independent pathway [12, 23, 24]. In contrast to most TLRs which signal exclusively through one of the two pathways, TLR4 is able to activate both MyD88-dependent and MyD88-independent signalling [12, 24].

Platelets contain all of the proteins (e.g., MyD88 and interferon regulatory factor 3 (IRF3)) that are required for signal transduction through TLR4 and so at first glance it would appear that platelets utilise the same mechanisms as in nucleated cells [2, 25]. However, as we will explain in more detail in the subsequent sections, this cannot be the case as both the MyD88-dependent and the MyD88-independent pathways culminate in the activation and nuclear translocation of transcription factors, and this step would not be applicable in anucleated cells like platelets [2, 12, 26]. Before examining the evidence for the TLR4 signalling pathways in platelets, it is worth reviewing the pathways in nucleated cells for use as a benchmark.

2. TLR4 Signalling in Nucleated Cells

2.1. TLR4 Ligands. Lipopolysaccharide (LPS) is a component of Gram-negative bacterial cell membranes and a powerful ligand for TLR4 [27, 28]. LPS is composed of a lipid A moiety (responsible for the molecule's interactions with TLR4), the core oligosaccharide, and the O-antigen polysaccharide [27, 29, 30]. The lipid A moiety is localised in the outer cell membrane and is formed from a 1,4-bis-phosphorylated diglucosamine molecule linked to variable acyl chains (e.g., six chains in *Escherichia coli* LPS) [27, 29]. The phosphate groups and acyl chains of LPS are important for interactions with TLR4, and alterations in these can shift the molecule from being an agonist to an antagonist [27, 31]. LPS may not be the only ligand for TLR4 as damage-associated molecular patterns (DAMPs), such as high-mobility group box 1 (HMGB1) and heat shock proteins (HSPs), have also been suggested to be capable of inducing activation through this receptor [12, 32].

Although the immunogenic region of LPS is inside the bacterial cell membrane, it is capable of eliciting an immune response due to the presence of lipopolysaccharide-binding protein (LBP) [27, 29, 33]. LBP is a soluble protein that is synthesised by hepatocytes and found in the blood [28, 33]. It is capable of binding to areas rich in LPS (e.g., LPS aggregates and Gram-negative bacterial membranes) and promotes the exposure of the molecule's hydrophobic regions [34]. Subsequent to this, LPS monomers, via a process facilitated by albumin, can associate with CD14 (cluster of differentiation 14), a high affinity, horse shoe-shaped, glycosylphosphatidylinositol- (GPI-) anchored membrane protein [28, 31, 33–35]. CD14 forms a dimer with the dimerisation interface at the C-terminal end and LPS-binding pockets at the N-terminal end [33]. The transfer of LPS to TLR4 and the breakdown of LPS aggregates (micelles) into monomers are mediated by CD14 [28, 31, 33, 36]. Albumin can bind LPS, and other hydrophobic molecules, via hydrophobic interactions between domain III (on albumin) and the fatty acid chains of LPS [34]. Furthermore, albumin is capable of transferring LPS to TLR4 on its own although this requires approximately 10-fold higher concentrations of LPS compared to CD14 [34].

2.2. TLR4 Receptor. Similarly to CD14 (the molecule responsible for transferring LPS to TLR4), the ectodomains of TLR4 are horse shoe-shaped due to the presence of several leucine-rich repeats (LRRs) [33, 37]. Like other type I membrane-spanning proteins, the membrane-spanning domain of TLR4 is comprised of a single helix that serves to link the intracellular and extracellular domains [31]. The intracellular domain of TLRs contains a Toll/IL-1 receptor (TIR) domain common to all of the adaptor protein molecules involved at this stage of signalling [1, 31].

For signalling via TLR4 to occur, TLR4 requires heteromeric association with myeloid differentiation factor 2 (MD-2) [38, 39] (Figure 1). MD-2 is required because TLR4 does not bind LPS directly [27]. This is exemplified by the ability of human MD-2 to bind LPS in the absence of TLR4 [31]. MD-2 is constitutively associated with TLR4 through an interaction in the central region of TLR4 and may be responsible for the recognition of different LPS chemotypes [33, 39].

TLR4 has been detected on the plasma membrane and in intracellular compartments (such as the early endosome) of both nucleated cells and platelets [24, 40, 41]. In addition, TLR4 is capable of internalisation, as has been shown following prolonged exposure to LPS [24, 40–42]. The mechanisms behind the internalisation of TLR4 differ between cell types and may be required for MyD88-independent signalling [40, 43]. The intracellular forms of TLRs are not inactive as may be expected for an internalised extracellular receptor but are capable of recognising ligands (such as LPS) in endosomes, lysosomes, and endolysosomes [12, 43, 44]. Notably, plasma membrane localisation of TLR4 requires HSP

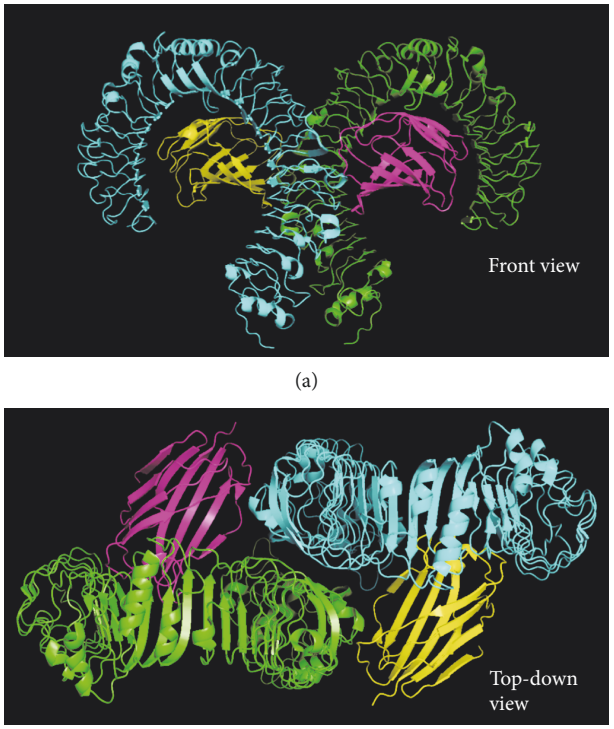

Figure 1: Structure of TLR4/MD-2 ectodomains, in a heterotetrameric form, as seen from (a) or (b). The TLR4 molecule (green) is constitutively bound to MD-2 (magenta), and the TLR4* (cyan) molecule is constitutively bound to MD-2* (yellow). Dimerisation interfaces form between TLR4 and MD-2* and vice versa. Images were created by adapting the structure of TLR4 (PDB code: 3FXI) using PyMOL [37].

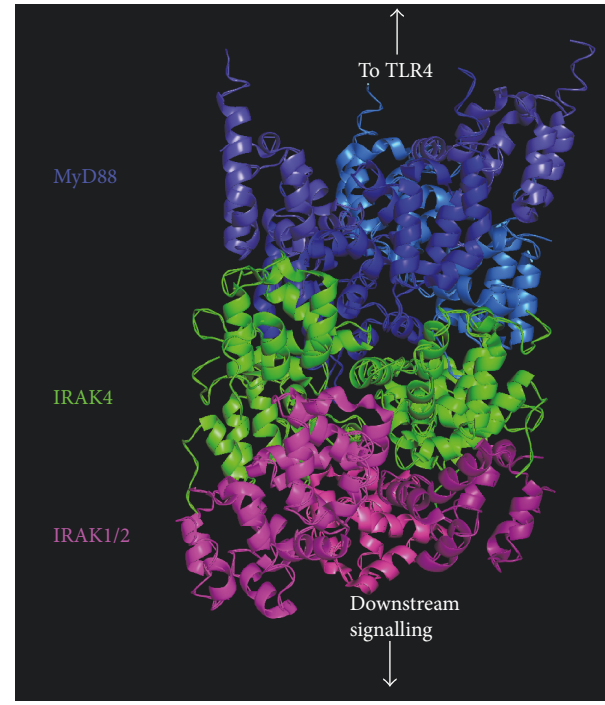

Figure 2: Structure of the Myddosome showing the protein death domains (DD). The Myddosome is formed of six MyD88 molecules, four IRAK4 molecules, and four IRAK1/2 molecules arranged in a single-stranded helix. MyD88 occupies the two layers closest to the plasma membrane whereas IRAK4 and IRAK1/2 form the two subsequent layers. The image was created by adapting the structure of the Myddosome (PDB code: 3MOP) using PyMOL [47].

90 kDa β member 1 (gp96) and protein associated with TLR4 (PRAT4A) acting as chaperones [44, 45]. Moreover, MD-2 has been reported to play a role in TLR4 localisation at the plasma membrane as its absence traps TLR4 in the Golgi apparatus [38].

2.3. TLR4 Activation. In order to activate the TLR4 signalling pathway, two receptor complexes need to dimerise to bring together the intracellular TIR domains (Figure 1) [31]. LPS and MD-2 (constitutively bound) binding to TLR4 is required for the TLR4 complex dimerisation to take place [27]. This dimerisation occurs due to the formation of a dimerisation domain that incorporates a hydrophobic patch on TLR4 and one of the acyl chains of LPS [33]. The remaining acyl chains are hidden in the hydrophobic cavity of MD-2 [27]. Ectodomain dimerisation leads to an interaction between the two intracellular domains of the TLR4 monomers [31]. This builds a platform onto which the intracellular signalling complexes can be formed [33]. At this stage, the two pathways diverge but there is still disagreement about what happens during this step [31].

2.4. The MyD88-Dependent Pathway. For the MyD88-dependent pathway, the TIR domain-containing adaptor protein (TIRAP), also known as MyD88 adaptor-like (Mal) protein, interacts with the TIR domain of the receptor enabling it to recruit MyD88 to the plasma membrane [31]. TIRAP presence at the plasma membrane is mediated by its phosphatidylinositol-4,5-bisphosphate- ($PI(4,5)P_2$-) binding domain [43, 46]. It has been suggested that TIRAP may bind to TLR4's TIR domain using complementary charge distributions because of the observation that charges differ between TLR3 (cannot bind TIRAP) and TLR4 and TLR2 (can or only bind TIRAP, resp.). However, the exact details and structures involved during this binding have not been determined, partially due to the lack of a crystal structure for the TIR domain of TLR4 [31]. Once TIRAP has bound to the receptor, it recruits MyD88 via an interaction between their respective TIR domains [43, 46, 47].

MyD88 contains a death domain (DD) at its N-terminal end, which is crucial for the subsequent signalling cascade as it enables the construction of a large multimeric complex called the Myddosome (Figure 2) [47]. The Myddosome is formed of six MyD88, four interleukin- (IL-) 1 receptor-associated kinase 4 (IRAK4), and four IRAK1/2 molecules, all of which contain DDs, arranged in a single-stranded left-handed helix [24, 47]. As shown in Figure 2, this helix has multiple levels with the first two levels comprised solely of MyD88, IRAK4 is found in the third level, and IRAK1/2 is found in the fourth level [47]. Following assembly, IRAK4 undergoes an activating autophosphorylation process thereby enabling it to phosphorylate, and activate, IRAK1/2 [47]. Phosphorylation of IRAK1/2 stimulates disassociation

from the Myddosome and triggers polyubiquitination of tumour necrosis factor (TNF) receptor-associated factor (TRAF) 6 [47, 48]. TRAF6 interacts with TRAF-activated kinase 1 (TAK) and IRAK1/2, and this complex in turn interacts with NF-κB essential modulator (NEMO) to stimulate the activating phosphorylation of IκB kinase- (IKK-) β and the degradation of IκB [24, 49–51]. Degradation of IκB and the release of inhibition on NF-κB permit it to translocate into the nucleus and enhance expression of proinflammatory cytokines including TNFα and IL-1β [44, 50, 51]. The MyD88-dependent signalling downstream of LPS stimulation is dependent on TLR4 remaining at the plasma membrane as inhibition of internalisation increases NF-κB activity [52].

Activation of mitogen-activated protein kinases (MAPKs) downstream of MyD88 and TAK1 is also involved in TLR4-mediated responses in nucleated cells [44, 48]. MAPKs include a range of proteins including extracellular signal-regulated kinase (ERK) 1 and 2, c-Jun N-terminal kinase (JNK) 1 and 2, and p38 [48]. These kinases are capable of activating the transcription factor, activator protein 1 (AP-1) [48]. This part of the MyD88-dependent pathway is dependent on the downregulation of TRAF3, via ubiquitination by cellular inhibitor of apoptosis (cIAP), near the plasma membrane where it has a negative regulatory role [48]. A summary of all the signalling pathways in nucleated cells is shown in Figure 3.

2.5. The MyD88-Independent Pathway. TRIF-related adaptor molecule (TRAM) is responsible for recruiting TIR domain-containing adaptor-inducing interferon-β (TRIF) in the MyD88-independent pathway [31]. Signalling through this pathway occurs following specific internalisation of the TLR4-MD-2 heterotetramer, its bound ligand, and CD14 [53–55]. The protein responsible for the internalisation (clathrin or caveolin) of TLR4 varies between cell types and with time although dynamin and CD14 are always necessary [40, 43, 52, 55]. Whereas CD14 is only required at low concentrations of LPS for MyD88-dependent pathway signalling (with other proteins such as albumin capable of transferring LPS to MD-2), CD14 is always necessary for MyD88-independent signalling [34, 55, 56]. As internalisation of TLR4 occurs, the decrease in $PI(4,5)P_2$ in the local area leads to a weakening of the interaction between TLR4 and TIRAP and thus propagates the breakdown of the Myddosome [24, 43]. Interestingly, endocytosis of TLR4 does not appear to be dependent on TLR4-mediated signalling, with cells lacking TIRAP, MyD88, TRAM, or TRIF retaining the capacity to internalise the receptor [55]. This has been suggested to be a result of phospholipase Cγ2 (PLCγ2) and spleen-associated tyrosine kinase (Syk) activation in a CD14-dependent and TLR4-independent manner [55].

Upon internalisation, TLR4 enters the endosome, a region of the cell where TRAM and TRAF3 are present and from where MyD88-independent signalling can begin [24, 43, 48, 55]. When recruited to the TLR4-TRAM-TRIF complex by TRIF, TRAF3 is polyubiquitinated thus stimulating the activation of TRAF family member-associated NF-κB activator- (TANK-) binding kinase- (TBK-) 1 and IKKε [48]. TBK1 and IKKε are then free to phosphorylate IRF3, which is activated upon phosphorylation and dimerisation and stimulates the production of type I interferons [48, 56].

3. TLR4 Signalling in Platelets

3.1. Platelet Activation upon Vascular Damage. The response of platelets to "classical" agonists and the subsequent activation in haemostasis have been well defined [4–6]. During vascular injury, there is exposure of the subendothelial matrix and proaggregatory proteins, such as von Willebrand factor (vWF) and collagen, to the flow of blood. vWF is immobilised on collagen, and its association with GPIb-V-IX, a large glycoprotein (GP) complex, represents the initial interaction between platelets and the damaged vessel. This interaction slows down the platelets enabling them to interact with the exposed collagen via GPVI and platelet activation to ensue [57–59]. Binding of collagen to GPVI promotes an intracellular signalling cascade involving tyrosine kinase-mediated (e.g., Syk) activation of PLCγ2. The degradation of $PI(4,5)P_2$ by PLCγ2 into diacylglycerol (DAG) and inositol 1,4,5-trisphosphate ($Ins(1,4,5)P_3$, also known as IP_3) induces indirect activation of protein kinase C (PKC) [59].

Platelet activation induces shape change and modulation of integrin $\alpha_{IIb}\beta_3$ affinity to allow the formation of a platelet plug with fibrinogen used as a bridging molecule to surrounding platelets [5, 6]. Integrin activation is critical for a successful aggregation response. In resting platelets, integrin $\alpha_{IIb}\beta_3$ is in a low affinity state but a conformational change during platelet activation enables high-affinity binding of ligands. PKC activation has a key role in modulating integrin $\alpha_{IIb}\beta_3$ affinity [59, 60].

Furthermore, activation of platelets leads to degranulation and the secretion of adenosine diphosphate (ADP) and the synthesis and release of thromboxane A_2 (TxA_2), resulting in the activation of more platelets and recruitment of them to the thrombus [57–59]. Moreover, prothrombin is cleaved into thrombin following interactions involving tissue factor, factor VIIa, and factor Xa on the activated platelet surface. Thrombin is able to activate platelets through a cleavage of a region in the extracellular domains of protease-activated receptors (PARs) 1 and 4. Together, these agonists activate more circulating platelets and thus stimulate the formation of a platelet plug to seal the damaged region [5–7].

3.2. TLR4 Expression in Platelets. The presence of TLR4 on platelets is not disputed, and it was first identified on mouse and human platelets using flow cytometry by Andonegui et al. [36]. In addition, the same research group demonstrated that TLR4 displays functional effects in platelets. Furthermore, the discovery was backed up independently by Cognasse et al. in the same year, also through flow cytometry-based experiments [41]. Other research groups have also confirmed the presence of TLR4 on platelets through immunoblot analysis [42, 61, 62]. The amount of TLR4 expressed on the surface of platelets is variable, and an intracellular pool has also been identified [30, 41, 42].

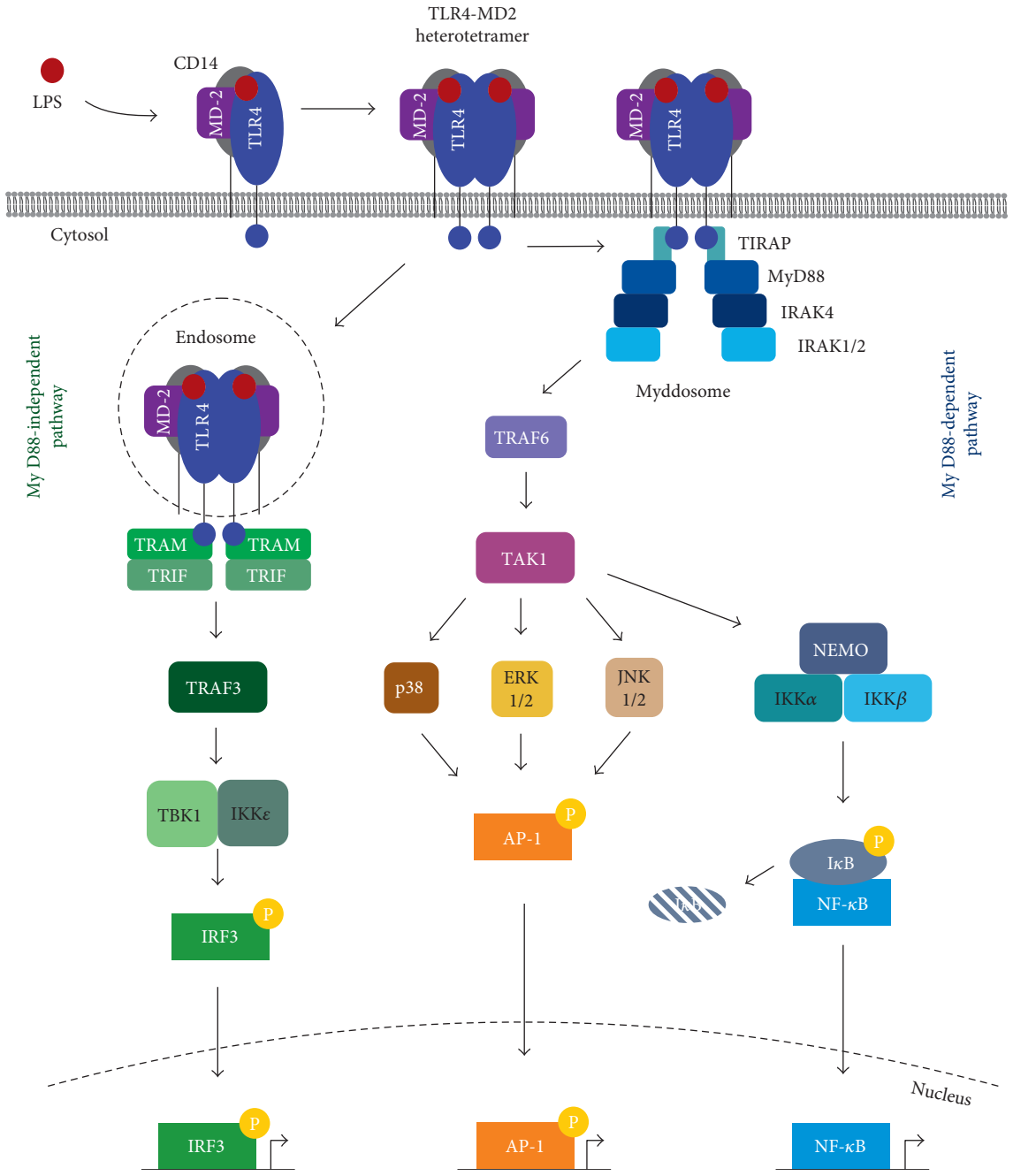

Figure 3: Summary of intracellular TLR4 signalling pathways in nucleated cells. LPS is transferred to CD14 (or albumin), via a process involving LBP and albumin, which transfers LPS to TLR4:MD-2 to complete the heterotetramerisation. There are two major signalling pathways, namely, the MyD88-dependent and -independent pathways, for TLR4 signalling. In the MyD88-dependent pathway, TIRAP (or Mal) enables MyD88 binding to TLR4 and formation of the Myddosome, which contains MyD88, IRAK4, and IRAK1/2. The kinases found at the base of the Myddosome activate TRAF6 and TAK1 followed by the activation of NEMO and its associated kinases. IKKβ stimulates the degradation of inhibitory IκB, which leads to nuclear translocation of NF-κB and transcription of proinflammatory genes. In addition, TAK1 activates JNK1/2, ERK1/2, and p38, which can then stimulate the transcription factor AP-1. In the MyD88-independent pathway, following CD14-dependent internalisation into the endosomes, TRAM and TRIF are recruited to TLR4 before activating TRAF3. Activation of TRAF3 activates TBK1 and IKKε, which phosphorylate and activate the transcription factor IRF3 that stimulates the transcription of anti-inflammatory cytokines.

A big difference in TLR4 signalling between platelets and nucleated cells is that although platelets contain the intracellular signalling proteins required for TLR signalling (Figure 4), they do not have all of the necessary extracellular components (e.g., CD14) [2, 63, 64]. Membrane-bound CD14 is absent in platelets; however, this problem is overcome by high levels of soluble CD14 in the plasma [14, 30, 63, 65]. This may prevent "priming" of platelets

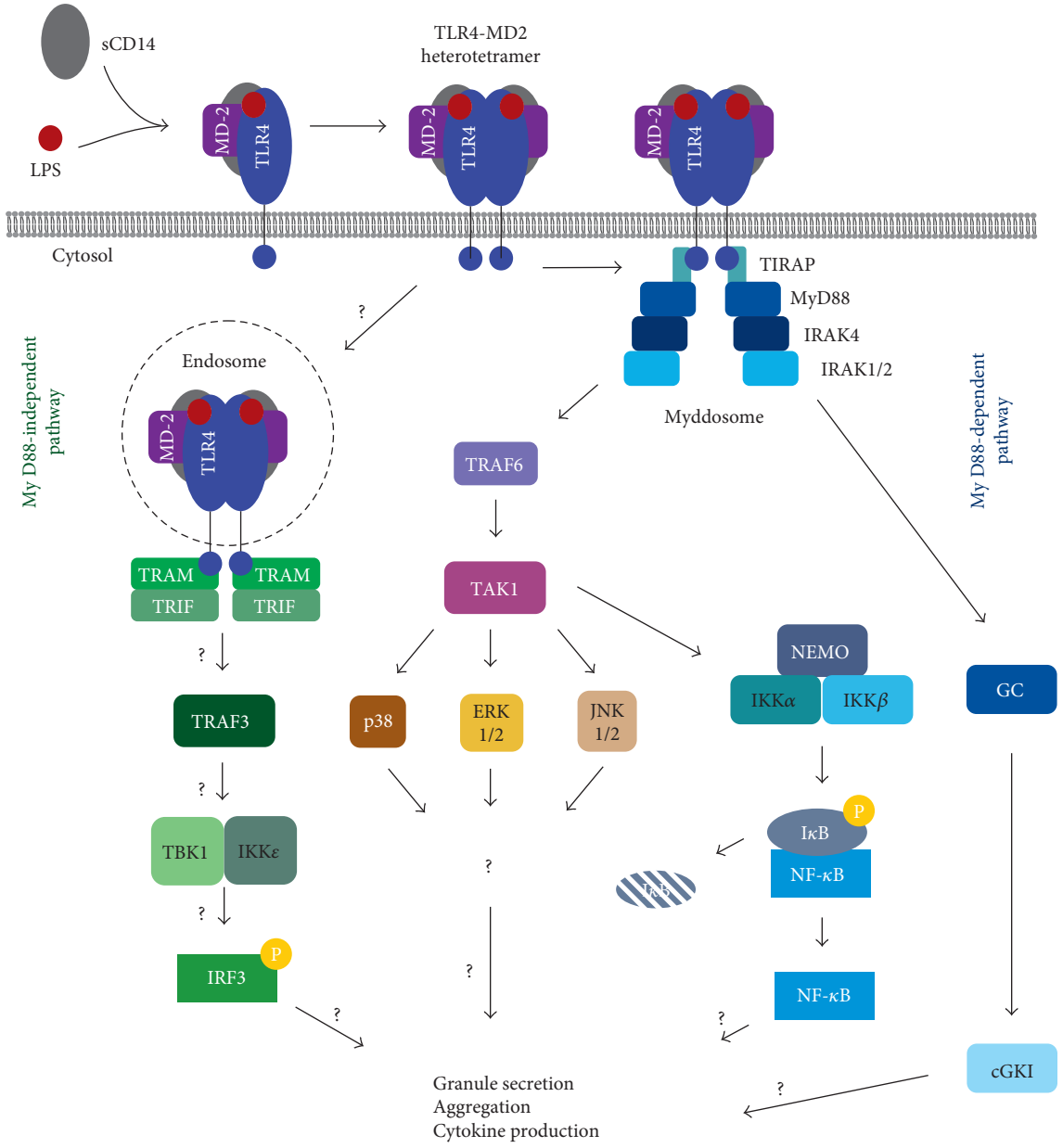

Figure 4: Summary of intracellular TLR4 signalling in platelets. Although the individual steps of the MyD88-dependent pathway have been observed, signalling in its entirety downstream of TLR4 has not been confirmed. Similar to nucleated cells, the proteins required for signalling through MyD88-dependent and -independent pathways are present in platelets but it is currently unclear how they mediate their effects. Signalling downstream of MyD88 can also be mediated by cGKI. The presences of TLR4, MyD88, IRAK1, TRAF6, TAK1, JNK, MAPK, IκBα, NF-κB, TRIF, TRAF3, TBK1, IKKε, and IRF3 have all been confirmed by immunoblot analysis [2, 25]. Question marks (?) denote aspects of the pathway that have not been confirmed.

at low concentrations of LPS whilst responses at higher concentrations are not affected. Moreover, the requirement for higher concentrations of LPS could prevent NET formation in response to minor bacterial infections, thus protecting against unwarranted endothelial damage [22]. Furthermore, the absence of membrane-bound CD14 may also have an impact on MyD88-independent signalling which requires CD14 for the endocytosis of TLR4 and LPS [55]. The loss of CD14 caused by "washing" platelets appears to reduce the magnitude of the response to LPS although a response is still present [63, 66, 67].

3.3. TLR4 Activity in Platelets. A strong piece of evidence for TLR4 activity in platelets comes from experiments conducted by Clark et al. They demonstrated that high concentrations of LPS led to an interaction between platelets and neutrophils that stimulated the formation of NETs [22]. The researchers also linked this activity to sepsis, a disease that is commonly associated with platelet TLR4 [19]. This was achieved by determining the production of NETs in the blood samples of sepsis patients [22]. It is unclear whether it was the LPS in the blood or another substance that stimulated this response as certain proteins that may stimulate platelets in

a TLR4-dependent manner are also released into the blood during sepsis, for example, HMGB1 [22, 32]. Similarly, further evidence for the role of platelet TLR4 is provided by the observation that the levels of soluble cluster of differentiation 40 ligand (sCD40L) are raised following treatment of platelets with LPS [63, 66–69]. This is important because platelet α-granules are the largest source of sCD40L, and CD40L is involved in inflammatory responses elucidated by the endothelium and immune cells [1, 68–70]. Increases in sCD40L levels have been suggested to directly involve TLR4 [69].

Many attempts have been made to characterise the responses of platelets to LPS and other TLR4 agonists although there have been conflicting results. Evidence from different studies agree that exposure of platelets to LPS stimulates the release of tumour necrosis factor- (TNF-) α, a molecule that is produced downstream of the MyD88-dependent pathway in nucleated cells [42, 50, 71]. Although platelets lack genomic DNA, they still contain mRNA transcripts that can be processed and spliced following stimulation of platelets by LPS or thrombin [63, 72]. Transcripts that are affected include IL-1β (a proinflammatory cytokine) and cyclooxygenase-2 (produces a platelet agonist, TxA_2) [63]. In addition, IL-1β mRNA has been shown to be spliced in platelets in a TLR4-dependent manner with JNK and protein kinase B (PKB) (found downstream of the MyD88-dependent pathway) activity increasing during splicing [65]. Furthermore, splicing of IL-1β was diminished in the presence of JNK or PKB inhibitors. However, the mechanism of action has not yet been elucidated [65]. Platelet shape change as a result of actin filament formation has also been suggested [63]. A comprehensive examination of cytokine release from platelets after treatment with LPS was conducted by Cognasse et al. [30]. They demonstrated that the expression of CD63 and release of sCD40L and platelet-activating factor 4 (PAF4) were increased; release of regulated upon activation, normally T-expressed, and presumably secreted (RANTES), angiogenin and platelet-derived growth factor- (PDGF-) AB were decreased (along with TLR4 expression); meanwhile, there was no change in the expression of soluble P-selectin, epidermal growth factor (EGF), transforming growth factor β (TGFβ), or IL-8 [30]. Upregulation of P-selectin following LPS exposure is controversial with evidence both for [32, 61, 63] and against [26, 30, 66].

3.4. The Role of MyD88 in Platelets. It is unclear whether the traditional TLR4 pathways are responsible for all the effects mediated by TLR4 ligands on platelet function. MyD88$^{-/-}$ mouse platelets have been used to demonstrate that this protein is necessary for the effects of LPS in enhancing aggregation and granule secretion in platelets. Some effects downstream of MyD88 have also been shown to be mediated by the cyclic guanosine monophosphate- (cGMP-) mediated signalling pathway [61].

In contrast, one research study demonstrates that there is virtually no role for MyD88 in modulating platelet function during Gram-negative (*Klebsiella pneumoniae*) bacterial infection [71]. Differences in responses were observed in systemic MyD88$^{-/-}$mice compared to the controls; however, these differences could not be recovered by transfusing wild-type platelets into the MyD88$^{-/-}$ mice. Furthermore, some changes in the secretion of TNFα and monocyte chemoattractant protein-1 (MCP-1) were observed that could be the result of deletion of platelet MyD88, thus suggesting that signalling to NF-κB is still intact and functioning [71].

The results of this study are somewhat limited for several reasons. For example, the observed effects were not shown to be mediated by TLR4 as competitive antagonists, blocking antibodies for TLR4, and platelets derived from TLR4-deficient mice were not used in their experimental settings. Furthermore, this study did not use pure LPS (or other potential TLR4 ligands), but rather whole *Klebsiella pneumoniae* bacteria, which means that other bacterial components or exotoxins may have been able to influence cellular activities. More specifically, there was no investigation into the success of the platelet transfusions as the recipient mice were not depleted of their platelets and transfused platelets may have been sequestered in organs such as the lungs and spleen. The possibility of adaptive mechanisms in the MyD88-deficient mice was not investigated either; nor was an alternative signalling pathway suggested. Nevertheless, this study highlights the necessity for further research in order to confirm the significance of MyD88 in TLR4-mediated signalling in platelets.

3.5. Priming Platelets. There is evidence suggesting that LPS (and therefore TLR4-mediated signalling) has a "priming" role in platelets. LPS on its own is unable to induce aggregation in washed platelets, but it can potentiate agonist-induced aggregation responses. This was elucidated through the use of classical agonists such as collagen and thrombin [14, 26, 61]. Despite washed platelets being used, sCD14 was still detectable on platelets via flow cytometry [61]. Similar results have been obtained with platelet-rich plasma (PRP) using agonists such as adenosine diphosphate (ADP) [63]. The response was mediated by TLR4 as demonstrated through the use of TLR4$^{-/-}$ mouse platelets [61]. An intriguing observation from this was that the different bacterial strains of LPS tested had different potencies [61]. This has also been observed with the LPS from *Rhodobacter sphaeroides* demonstrating its ability to act as a competitive TLR4 antagonist [63]. This priming behaviour in platelets is also supported by studies using NF-κB and IKKβ inhibitors [26, 73, 74].

The identification of TLR4:MyD88 coupling to the cGMP-dependent pathway is important as this pathway stimulates platelet aggregation from a subthreshold concentration of an agonist (0.02 U/mL of thrombin) [75]. Thus, there is a precedent for TLR4 to have a priming role in platelet aggregation. The response to cGMP-analogues was biphasic with an initial stimulatory response followed by an inhibitory response [75]. An interesting point to consider is that whilst cGMP-dependent kinase I (cGKI) inhibition affected aggregation and secretion to low agonist concentrations (excluding ADP), there was no effect on calcium mobilisation [76] and TLR4 is also incapable of modulating calcium mobilisation [77]. cGKI has been proposed to

be involved positively in the G_i-pathway, and so activation of cGKI could help amplify platelet responses in a similar manner to the $P2Y_{12}$ receptor [76].

3.6. NF-κB in Platelets. Given that platelets lack a nucleus, it may appear that the presence of a signal transduction pathway that culminates in transcription factor activation would have no role in platelet function. This initially prompted some researchers to claim that TLR4 and its downstream signalling proteins in platelets were relics left over from their formation by megakaryocytes. Furthermore, certain experiments concluded that there were no responses mediated by TLR4 with specific bacterial species, lending support to this argument [78]. Other concerns arose from different LPS chemotypes derived from diverse bacterial species having diverse potencies when it comes to elucidating a response [61, 63, 66, 79]. NF-κB, however, appears to have a role in platelet function, suggesting a nongenomic role, especially when the ability of NF-κB inhibitors to reduce the proaggregatory effects of TLR4 is considered [26, 80].

Notably, IKKβ is involved in the phosphorylation of soluble N-ethylmaleimide-sensitive factor attachment protein receptors (SNAREs), particularly synaptosomal-associated protein 23 (SNAP23), and thus, IKKβ has an important role in granular secretion [73]. Phosphorylation of SNAREs is known to occur downstream of PKC when thrombin is used as an agonist [73]. This is relevant because IKKβ is found downstream of both this classical agonist pathway and the MyD88-dependent pathway, suggesting a mechanism by which TLR4 activation could lead to the secretion of granules that has been shown in some studies [32, 51, 73]. Further investigations have revealed that IKKβ activity occurs downstream of TAK1, found in the MyD88-dependent pathway [25]. This evidence points towards the ability of the MyD88-dependent pathway to promote SNARE complex formation and may explain some of the "priming" activity induced by TLR4 ligands. However, it is unclear whether IKKβ directly phosphorylates SNAP23 or whether it occurs due to the activation of NF-κB. It has been shown that NF-κB activity is involved in modulating dense and α-granule secretion upon activation with low agonist doses by using inhibitors of IκBα phosphorylation and ubiquitination (to indirectly inhibit NF-κB activity) [74, 80]. Moreover, NF-κB inhibition decreases binding of platelets to fibrinogen [80]. This suggests that NF-κB is responsible for modulating secretion in this case although one of the inhibitors used is likely to directly inhibit IKKβ. Inhibition of aggregation has also been seen to be mediated by NF-κB inhibitors downstream of TLR4, suggesting that TLR4 and NF-κB activity is connected in platelets [26].

3.7. Other Ligands. Although LPS has been the predominant ligand mentioned in this review, other ligands have also been suggested to bind to TLR4; however, this area is highly controversial [9, 67, 81, 82]. HMGB1 is one such possible ligand and has been shown to have effects in platelets in an autocrine and paracrine manner [32]. With a presence in the plasma and on NETs, the DNA-binding protein released from dead/dying cells or activated immune cells has opportunities to interact with platelets in many conditions, for example, sepsis [22, 32, 83, 84]. HMGB1 has been reported to elicit similar responses in platelets compared to LPS, including the priming effects. These effects were also shown to involve TLR4, MyD88, and cGKI although there is not yet clear evidence indicating exactly how these proteins relate. ERK was another protein that had a change in its activity as a result of treatment with HMGB1 dependent on the presence of TLR4 [32]. HMGB1 has also been shown to have a role in tumour metastasis in a mechanism involving TLR4 [84]. Platelets are known to aid in cancer metastasis by forming protective thrombi around metastasising cells [85], and subsequent experiments by Yu et al. demonstrated that deletion of TLR4 in mice led to fewer metastatic tumours [84]. However, evidence from nucleated cells exists implying that HMGB1 acts solely as a TLR ligand-binding protein (e.g., LPS) and potentiates signalling through TLRs (alarmin effect) [86, 87]. Thus, the effects observed in the studies might be due to the binding of HMGB1-LPS colligation to TLR4 [87]. Moreover, recent studies have shown that, instead of direct binding to TLR4, HMGB1 directly exerts effects (such as activation of NF-κB and MAPKs) on cells through binding to the receptor for advanced glycosylation end-products (RAGE) [88].

Another ligand that has been suggested to alter platelet activity in a TLR4-dependent manner is cellular fibronectin [9]. It has been shown that cellular fibronectin can modify platelet activity in a similar manner to LPS by potentiating aggregation induced by low doses of thrombin and increasing phosphorylation of NF-κB and IKKα/β [9]. Furthermore, it was shown that the presence of TLR4 in mouse platelets significantly increased thrombus growth when treated with cellular fibronectin [9]. These findings suggest a possible effect of cellular fibronectin that may be mediated in a TLR4-dependent manner.

Histones have also been proposed to be ligands for TLR4 and are found in the blood during sepsis following release from neutrophils or necrotic cells [89–92]. They are important for the organisation of DNA in nucleated cells and, like HMGB1, appear in NETs [93]. Histones (especially H4) have interactions in the blood, and they have been reported to have a role in chemokine production in whole blood, platelet aggregation, and also thrombocytopenia in mice [89, 93]. However, these studies concluded that it was monocytes, and not platelets, that were responsible for the TLR4-dependent production of cytokines (even though histone H4 did associate with platelets) whereas the impact of TLR4 on histone-induced aggregation and thrombocytopenia was not examined [89, 93]. In contrast, it has been shown that histones can stimulate P-selectin exposure and thrombin generation on platelets in a TLR2- and TLR4-dependent manner [81].

Additionally, HSP60, a cell-stress marker, has been proposed to trigger TLR4-mediated signalling in a vascular smooth muscle cell line, with implications in atherosclerosis. However, the effects of this protein have not been tested on platelets despite an increased expression of HSP60 on endothelial cells in sheer stress environments [94, 95]. Serum amyloid A (SAA) is a potential ligand for TLR4 that is

released, primarily from the liver, during an inflammatory response [96, 97]. Platelets have been shown to adhere to SAA in an integrin $\alpha_{IIb}\beta_3$-dependent manner; however, it has not been determined whether or not this integrin is solely responsible for this behaviour as the research was conducted before the discovery of TLR4 on platelets [97]. Further research is required to determine whether these proposed ligands are having an effect due to direct binding to TLR4 or if it is the result of a more complex interaction, as has been suggested for HMGB1 [87].

4. TLR4 Signalling in Platelets: What Is Still to Discover?

Although many studies have linked TLR4 activity in platelets to immune responses, there have not been many studies to explore the signalling pathways downstream of TLR4 or MyD88 [26, 32, 61]. This is of particular interest as this receptor, with so many potential ligands and possible functions, operates through a pathway that classically results in gene transcription, but this end result is not achievable due to the lack of a nucleus in platelets. Moreover, the presence of all the signalling proteins in the pathways has been confirmed [2, 25] but whether the entirety of each pathway is functional, in platelets, has not been elucidated. Currently, individual steps of the MyD88-dependent pathway have been seen but not tied together downstream of TLR4. The MyD88-independent pathway in platelets also lacks considerable amounts of detail, including study of its activity. Furthermore, platelet TLR4 expression levels have been linked to more severe disease states in inflammatory responses [8, 9, 81, 84, 98–101]. This obviously makes TLR4 an interesting receptor to target for the prevention and/or treatment of cardiovascular diseases. However, it is challenging due to the important contribution of TLR4 to innate immunity. Determination of the effector proteins involved and their responses may lead to the discovery of novel pathways downstream of TLRs and present TLR4 as a novel therapeutic target for the treatment of cardiovascular diseases and other pathological settings such as inflammatory disease.

Abbreviations

ADP: Adenosine diphosphate
AP-1: Activator protein 1
CD63: Cluster of differentiation 63
cGKI: cGMP-dependent kinase I
cGMP: Cyclic guanosine monophosphate
cIAP: Cellular inhibitor of apoptosis
DAG: Diacylglycerol
DAMPs: Damage-associated molecular patterns
DD: Death domain
DIC: Disseminated intravascular coagulation
EGF: Epidermal growth factor
ERK: Extracellular signal-regulated kinase
GP: Glycoprotein
gp96: Heat shock protein 90 kDa β member 1
GPI: Glycosylphosphatidylinositol
HSP60: Heat shock protein 60
HMGB1: High-mobility group box 1
HSP: Heat shock protein
IκB: Inhibitor of NF-κB
IKK: IκB kinase
IL: Interleukin
$Ins(1,4,5)P_3$: Inositol 1,4,5-trisphosphate
IRAK: IL-1 receptor-associated kinase
IRF3: Interferon regulatory factor 3
JNK: c-Jun N-terminal kinase
LBP: Lipopolysaccharide-binding protein
LPS: Lipopolysaccharide
LRR: Leucine-rich repeat
Mal: MyD88 adaptor-like
MAPK: Mitogen-activated protein kinases
MCP-1: Monocyte chemoattractant protein-1
MD-2: Myeloid differentiation factor-2
MyD88: Myeloid differentiation factor 88
NEMO: NF-κB essential modulator
NET: Neutrophil extracellular trap
NF-κB: Nuclear factor of κ-light-polypeptide-gene-enhancer in B cells
PAF4: Platelet-activating factor 4
PAR: Protease-activated receptor
PDGF-AB: Platelet-derived growth factor-AB
$PI(4,5)P_2$: Phosphatidylinositol-4,5-bisphosphate
PKB: Protein kinase B
PKC: Protein kinase C
PLCγ2: Phospholipase Cγ2
PRAT4A: Protein associated with TLR4
PRP: Platelet-rich plasma
RAGE: Receptor for advanced glycation end-products
RANTES: Regulated upon activation, normally T-cell expressed, and presumably secreted
SAA: Serum amyloid A
(s)CD14: (Soluble) cluster of differentiation 14
sCD40L: Soluble cluster of differentiation 40 ligand
SNAP23: Synaptosomal-associated protein 23
SNARE: Soluble N-ethylmaleimide-sensitive factor attachment protein receptor
Syk: Spleen-associated tyrosine kinase
TAK1: TRAF-activated kinase 1
TBK1: TRAF family member-associated NF-κB activator- (TANK-) binding kinase 1
TGFβ: Transforming growth factor β
TIR: Toll/IL-1 receptor
TIRAP: TIR domain-containing adaptor protein
TLR: Toll-like receptor
TNF: Tumour necrosis factor
TRAF: TNF receptor-associated factor 3
TRAM: TRIF-related adaptor molecule
TRIF: TIR domain-containing adaptor-inducing interferon-β
TxA_2: Thromboxane A_2
vWF: von Willebrand factor.

Conflicts of Interest

The authors declare that no conflicts of interest exist.

Authors' Contributions

The manuscript was written by Thomas M. Vallance, Darius Widera, and Sakthivel Vaiyapuri. The figures were created by Marie-Theres Zeuner and Thomas M. Vallance. Further contributions for the preparation of this manuscript were provided by Marie-Theres Zeuner and Harry F. Williams. Darius Widera and Sakthivel Vaiyapuri contributed equally to this work.

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1.3 LL37: ITS IMPACT ON THE IMMUNE SYSTEM, PLATELETS, AND ASSOCIATION WITH LIPOPOLYSACCHARIDES

Introduction

LL37 is a cathelicidin that is already known to interact with the immune system as a chemoattractant and inducer of neutrophil extracellular trap formation[1–3]. Moreover, LL37 has been detected in atherosclerotic plaques and arterial thrombi whilst mice lacking LL37 develop smaller atherosclerotic plaques[2,4,5]. More recently, LL37 was determined to be a strong platelet agonist[2,6]. Therefore, LL37 presents itself as an important protein involved in the progression and acceleration of cardiovascular disease with roles in both lesion and thrombus growth. Furthermore, LL37 is known as an antimicrobial peptide and interacts with lipopolysaccharides (LPS), and other small molecules, on a molecular level, leading to complex functions and structures[7–9]. Despite these two important functions, no research has been conducted to determine the influence of LPS and LL37, in combination, on cardiovascular disease. Here, we investigate the current knowledge pertaining to LL37's role as a bioactive molecule.

Cathelicidins

Cathelicidins are a group of cationic antimicrobial peptides (AMPs) that are expressed in a wide range of vertebrates, including humans, and invertebrates[10–13]. Interestingly, primates and rodents appear to express only one cathelicidin each whereas other mammals, birds, and fish express several[10]. With their multiple roles, cathelicidins are an important component of the immune system[11,13,14]. Specifically, cathelicidins can kill fungi, bacteria (including the disruption and destruction of bacterial biofilms), and are antiviral and antiparasitic, a function theorised to occur via pore-formation in the membrane/surface of the pathogen[11]. Additionally, cathelicidins can eliminate pathogens via activation of immune cells. Cathelicidins across all species share a highly conserved N-terminal region whereas the C-terminal region is more variable with large structural differences present between species, for example, some cathelicidins have α-helices whereas others contain β-hairpins[10].

Direct anti-microbial activity

During neutrophil degranulation, inactive human cationic microbial peptide-18 (hCAP-18) is released and processed extracellularly to release active LL37[15]. The protein responsible for the extracellular processing depends on the host cell type e.g. by proteinase-3 from human neutrophils and kallikrein-5 and -7 from human keratinocytes[11,14–17]. LL37 is a 4.5 kDa cathelicidin with 37 amino acids that was first detected, in humans, in neutrophils but it has since been found to be expressed in multiple cell types including epithelial cells in the gastrointestinal and respiratory tract, the skin, and also in platelets[6,10,18].

LL37 can kill bacteria directly via permeabilising bacterial cell membranes[19]. Mammalian cells are protected from this permeabilisation by the presence of cholesterol and neutral phospholipids in the phospholipid bilayer[14]. Initially, LL37 molecules are drawn to the negative charge of bacterial membranes, specifically the phosphate groups in the LPS of Gram-negative bacteria. Multiple models have been proposed to be responsible for the membrane permeabilisation including: the “carpet” model, in which the AMP covers the membrane and induces membrane curvature and micelle formation; the “barrel-stave” model, in which AMP molecules insert themselves across the bilayer with a central hydrophilic core in a manner similar to ion channels; and finally the “toroidal” model, which combines elements of the previous two models. AMP molecules induce curvature of the membrane leading to pore formation however the hydrophilic lipid heads remain incorporated into the pore[18,19]. For LL37, the toroidal model is the prevailing theory for its mechanism of action as experiments conducted utilising nuclear magnetic resonance (NMR) determined that the peptide is parallel to the lipid bilayer, there is a change in the orientation of lipid head groups, and there is an absence of membrane fragments[18].

Role of LL37 in the innate immune system

As well as having direct antibacterial effects, LL37 can also stimulate innate and adaptive immune cells[13,14]. As evidence for their role in the immune system, LL37 is protective against Gram-negative sepsis-induced mortality in rat and mouse models (including cecal ligation and puncture)[3,20]. Specifically, LL37 promotes calcium mobilisation and migration of neutrophils and can induce release of neutrophil

extracellular traps (NETosis)[1,3,13,14,21]. LL37 has also been reported to potentiate interleukin (IL)-1β release from primary human monocytes that have been primed with 1ng/mL *Escherichia coli* LPS (LPS_{EC})[22]. Meanwhile, LL37 also has anti-inflammatory effects as it can inhibit LPS-induced (10ng/mL-100ng/mL) TNFα production in THP-1 cells and peripheral blood mononuclear cells (PBMCs) in a concentration-dependent manner[9,20]. LL37 also inhibits LPS-induced production of IL-8 and IL-6 in human gingival fibroblasts whilst preventing nuclear factor of κ light polypeptide gene enhancer in B-cells inhibitor α (IκBα) degradation and p38 phosphorylation[23]. Furthermore, it was suggested that the inhibitory effect of LL37 in these experiments was due to an interaction between LL37 and LPS as the greatest effect was seen when they were added simultaneously[9].

LL37 has been suggested to induce its effects via a multitude of different receptors. These receptors include formyl peptide receptor 2 (FPR2/ALX)[6,13], C-X-C motif chemokine receptor 2 (CXCR2)[13,24], epidermal growth factor receptor (EGFR)[7], P2X7 receptor (P2X7R)[22], and glycoprotein VI (GPVI)[2]. Migration of neutrophils driven by LL37 is G_i-mediated as migration was significantly inhibited by pertussis toxin (PTX; inhibits G_i-mediated signalling) treatment, which suggests that it is FPR2/ALX- or CXCR2-mediated as they both couple to G_i[1,25]. Additionally, human embryonic kidney (HEK293) cells stably transfected with FPR2/ALX migrate in response to LL37 and mobilise calcium unlike their parental cell[1]. Moreover, LL37-induced calcium mobilisation is desensitised following treatment with Su peptide (a specific FPR2/ALX agonist) and vice-versa[1]. Furthermore, proteoliposomes specifically expressing reported receptors demonstrated binding to FPR2/ALX only[26]. Conversely, in neutrophils, CXCR2 is internalised following LL37 stimulation whilst the selective CXCR2 inhibitor SB225002 inhibits calcium mobilisation induced by LL37[24]. In these experiments, no intracellular calcium mobilisation was observed when neutrophils were activated in calcium/magnesium-free buffer[24]. Treatment of NCI-H292 cells (a human mucoepidermoid pulmonary carcinoma cell line) with AG1478 (an EGFR tyrosine kinase inhibitor) prevents LL37-mediated LPS-internalisation[7]. In monocytes, P2X7R inhibitors partially inhibited LL37-induced IL-1β release whilst PTX had no significant effect when administered 30 minutes or 6 hours prior to LL37 stimulation[22].

Interaction of LL37 with platelets

In addition to its beneficial effects, LL37 has been detected in arterial thrombi, in atherosclerotic plaques, and overexpression can lead to psoriasis and rosacea[2,4,5,11,14]. In relation to platelets, Su *et al.*[27] observed that LL37 has inhibitory effects on platelet activation however the concentrations used in this study ranged from 0.1mM to 1.2mM, levels which were determined to be cytotoxic to platelets by experiments conducted in our laboratory[6,27]. In contrast, Salamah *et al.*[6] and Pircher *et al.*[2] independently demonstrated that LL37 can induce platelet activation as measured by aggregation, fibrinogen binding (as a marker for integrin $\alpha_{IIb}\beta_3$ activation[28]), P-selectin exposure (as a marker of α-granule secretion[29]), and calcium mobilisation[2,6]. However, both groups propose different mechanisms of action responsible for driving platelet activation[2,6]. GPVI has been proposed to be a possible receptor for LL37 in platelets as it was determined that LL37-induced P-selectin exposure was unaffected by incubation with G_i (PTX) or G_q (cholera toxin) inhibitors however Src (Dasatinib) and Syk (R406) inhibitors and GPVI blocking antibodies partially inhibited P-selectin exposure[2]. No inhibition of P-selectin exposure was seen when platelets were incubated with WRW4 (a FPR2/ALX antagonist) or A438079 (a P2X7R inhibitor)[2]. Moreover, Syk$^{-/-}$ murine platelets retained normal thrombin-induced P-selectin exposure although this response was inhibited when cathelicidin-related antimicrobial peptide (CRAMP) was tested as the agonist[2]. However, results from our group demonstrate that Fpr2/3$^{-/-}$ platelet responses to LL37 are almost completely ablated and that FPR2/ALX antagonism by WRW4 inhibits LL37-mediated platelet activation (as measured by aggregation, fibrinogen binding, and P-selectin exposure), therefore suggesting a role for FPR2/ALX[6]. Interestingly, responses of Fpr2/3$^{-/-}$ mouse platelets to cross-linked collagen-related peptide (CRP-XL), adenosine diphosphate (ADP), and AY-NH_2 were also abrogated[6].

Interaction of LL37 with LPS

LL37 is a cationic peptide with a net charge of +6 and an α-helical structure (when in the presence of lipid A or millimolar concentrations of anions)[10,18,19]. Thus, it easily binds to negatively-charged compounds such as extracellular deoxyribonucleic acid (DNA) and LPS[10]. HEK293 cells stably transfected with cluster of differentiation

14 (CD14) and Toll-like receptor 4 (TLR4) produce IL-8 in response to 1.5ng/mL LPS_{EC} and this can be inhibited by the presence of 5.5μM LL37[30]. Following establishment of this protocol, LL37 was truncated into multiple different forms which were then tested at the same concentration to determine the important sequences for inhibition of LPS-induced effects. These experiments revealed both that a 19 amino acid central sequence (IGKEFKRIVQRIKDFLRNL) is key for LL37's inhibitory effect and that this is dependent on the cationicity (explains 62.5% of the inhibitory effect) and the hydrophobicity (explains 75.5% of the inhibitory effect) of the truncated peptide[30]. This finding is supported by another study that identified an overlapping 18-amino acid long sequence (KEFKRIVQRIKDFLRNLV) which retained the ability to bind LPS and inhibit cytokine production from RAW 264.7 cells[31]. Furthermore, loss of all or part of this sequence reduced the binding of LPS to LPS-binding protein (LBP)[30]. However, increasing the cationicity of LL37 by substituting acidic residues for neutral or basic residues led to a decrease in LL37-LPS binding as IL-6 production from BEAS-2B cells increased 4-fold[32]. Furthermore, LL37 binding to LPS is pH-dependent with LL37-LPS interactions and LL37 oligomers decreasing as pH decreases[32]. Moreover, the presence of a truncated form of LL37, LL29, can decrease the interaction of LL37 and LPS, leading to an increase in IL-6 production from BEAS-2B cells[32].

LPS forms aggregates under physiological conditions and these aggregates are important for their function[8,33]. However, transmission electron cryomicroscopy (CryoTEM) images reveal that LL37 disrupts LPS aggregate structures, but that this is dependent on the length of the O-antigen[8]. LPS is composed of three regions: lipid A, core oligosaccharides, and the O-antigen polysaccharide[8,34]. The length of the O-antigen determines whether LPS is classed as "rough" (with a short or non-existent O-antigen) or "smooth" (with a long O-antigen)[35]. CryoTEM images reveal that smooth LPS (LPS_{EC} O111:B4) forms elongated, branching micelles that become thinner, shorter, and with fewer branches following addition of LL37. However, rough LPS chemotypes (LPS_{EC} D21 or LPS_{EC} E7) form lamellae that are converted into elongated structures with irregular toroids in the presence of LL37. It is suggested that LL37 interaction with phosphate groups on lipid A and core oligosaccharides induces curvature in LPS micelles, driving their shape change, but a larger proportion of LL37 interacts with the O-antigen in smooth LPS hence the lack of an aggregate shape change[8,36]. However, these experiments were performed with very high concentrations

of LPS (6mg/mL) and therefore may not be representative of physiological concentrations of LPS (300pg/mL)[8,37].

Conclusion

LL37 is an important molecule in innate immunity but can also be responsible for pathological conditions. LL37 is a multi-factor molecule that exerts its effects via multiple mechanisms (direct receptor binding and scavenging pathogen-associated molecular patterns and damage-associated molecular patterns) and, possibly, different receptors. Although lots of work has been done to investigate the role of LL37, its role in platelet activation has been lacking until very recently and, to-date, no work has been conducted to elucidate its role in combination with platelets in non-sterile inflammation.

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1.4 RATIONALE

Cardiovascular disease is the largest cause of mortality in the world. Atherosclerosis, the root cause of both myocardial infarction and ischaemic stroke, has been identified to involve both classic thrombotic mechanisms as well as chronic inflammation. Therefore, numerous platelet inflammatory receptors have been investigated for their potential to upregulate platelet activity during thrombotic conditions. One particular receptor, Toll-like receptor 4 (TLR4), which will be the focus of this thesis, has drawn lots of attention due to its potential to signal through two different pathways with different activities. However, the role of TLR4 in platelet activation is controversial due to differences in experimental techniques and preparation of its ligand, lipopolysaccharide (LPS). In this thesis, we examine the role of TLR4 in platelet activation using ultrapure LPS derived from various bacterial species.

Platelets are anucleate descendants from haematopoietic stem cells and derive most of their proteins from their parental cell, megakaryocytes (MKs). Therefore, platelet TLR4 may be a relic from MKs. Investigations into MK biology have been limited due to the difficulties associated with acquiring large numbers of MKs and also by differences in human and murine physiology. Megakaryoblastic cell lines exist for use in signalling studies however they lack easily detectable readouts. In this thesis, we develop a Meg-01 (megakaryoblastic cell line) reporter cell line (Meg-01R) for the investigation of NF-κB activity and incubate it with multiple pro- and anti-inflammatory molecules and inhibitors, including ultrapure and non-ultrapure LPS chemotypes. Furthermore, we investigate the presence and localisation of TLR4 in this cell type.

LL37 is a platelet agonist that can have both pro- and anti-inflammatory effects on other cell types and its overexpression has been linked to atherosclerosis and psoriasis. LL37 is known to interact with LPS and suppress LPS-induced pro-inflammatory cytokine release. Therefore, in this thesis, we investigated the impact of different LPS chemotypes and TLR4 antagonists on LL37-induced platelet activation to investigate a role for TLR4 in platelet activation induced by this inflammatory molecule.

Overall this study has further established the detailed roles of bacterial LPS and its receptor, TLR4, in the modulation of platelet and MK function.

1.5 AIMS AND OBJECTIVES OF EXPERIMENTAL CHAPTERS

2.1 Effect of ultrapure lipopolysaccharides derived from diverse bacterial species on the modulation of platelet activation

Hypothesis: Ultrapure LPS chemotypes will modulate platelet reactivity in response to Gram-negative bacterial infection

Aims: To elucidate whether different ultrapure LPS chemotypes can potentiate or inhibit platelet activation

Experimental system: Luciferase assay, immunoblotting, immunocytochemistry, flow cytometry, aggregometry

Chapter connection: Need to determine whether the effects previously observed to occur with LPS chemotypes are due to ligation of LPS to TLR4 or are due to other factors

2.2 Development and characterisation of a novel, megakaryocyte NF-κB reporter cell line for investigating inflammatory responses

Hypothesis: Development of a reporter megakaryocyte cell line will enable the investigation of NF-κB-dependent inflammatory responses in this cell type

Aims: To develop and characterise a Meg-01R cell line and elucidate whether ultrapure LPS can induce NF-κB activity

Experimental system: Immunoblotting, XTT assay, immunocytochemistry, flow cytometry, luciferase assay

Chapter connection: TLR4 is also expressed on megakaryocytes but it is unknown whether this is solely for packaging into platelets or can modulate megakaryocyte behaviour to alter platelet phenotype

2.3 Ultrapure lipopolysaccharide derived from *Rhodobacter sphaeroides* counteracts LL37-induced platelet activation

Hypothesis: LPS_{RS} counteracts the LL37-induced platelet activation under pathophysiological conditions

Aims: To determine the mechanism of action for LPS_{RS}-mediated inhibition of LL37-induced platelet activation

Experimental system: Aggregation, platelet spreading on immobilised fibrinogen, immunoblotting, flow cytometry

Chapter connection: LPS_{RS} is a TLR4 antagonist yet can inhibit LL37-mediated platelet activation which suggests a potential role for TLR4 in mediating platelet activation induced by inflammatory compounds

EXPERIMENTAL CHAPTERS

2.1 Effect of ultrapure lipopolysaccharides derived from diverse bacterial species on the modulation of platelet activation

2.2 Development and characterisation of a novel, megakaryocyte NF-κB reporter cell line for investigating inflammatory responses

2.3 Ultrapure lipopolysaccharide derived from *Rhodobacter sphaeroides* counteracts LL37-induced platelet activation

2.1 Effect of ultrapure lipopolysaccharides derived from diverse bacterial species on the modulation of platelet activation

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Rationale of this chapter

There is lots of controversy in the literature about whether TLR4 on platelets alters platelet activation due to differences in techniques and lipopolysaccharide (LPS) preparations. To determine the effect of Toll-like receptor 4 (TLR4), and its ligands, LPS (derived from various bacterial species), on platelet activation, thrombosis, and haemostasis, we performed a range of experiments in this chapter using ultrapure LPS. Three different ultrapure LPS chemotypes were chosen to investigate different aspects of TLR4 activity. LPS derived from *Escherichia coli* O111:B4 (LPS_{EC}) is a commonly used chemotype for stimulating pro-inflammatory signalling in platelets and other cell types. LPS derived from *Salmonella enterica minnesota* R595 (LPS_{SM}) has been reported to be a biased agonist towards the MyD88-independent pathway in a glioblastoma cell line, a pathway which has been suggested to have anti-inflammatory effects, and was tested to determine whether it could decrease platelet activity and suggest potential new anti-platelet therapeutics. Finally, *Rhodobacter sphaeroides* LPS (LPS_{RS}) was tested as a TLR4 antagonist to determine if there is any constitutive TLR4 activity in platelets. We detected TLR4 on the surface of platelets and found that its surface expression level increased during platelet activation. However, the different ultrapure LPS chemotypes did not influence platelet activity except under specific circumstances. This suggests that platelet TLR4 does not play a major role on its own in cardiovascular diseases such as atherosclerosis.

Contribution to this chapter

As the first author, I contributed nearly 85% to this chapter by designing and performing experiments, analysing the data, and preparing the manuscript and figures.

Experimental contribution

All experiments were performed by me apart from the experiments in Figure S2E and S5 (performed by DR). DAIA and HJL assisted with Figures S2, S3, and S4. JS assisted with the confocal microscopy in Figure 2C.

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Effect of ultrapure lipopolysaccharides derived from diverse bacterial species on the modulation of platelet activation

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Platelets are small circulating blood cells that play essential roles in the maintenance of haemostasis via blood clotting. However, they also play critical roles in the regulation of innate immune responses. Inflammatory receptors, specifically Toll-like receptor (TLR)-4, have been reported to modify platelet reactivity. A plethora of studies have reported controversial functions of TLR4 in the modulation of platelet function using various chemotypes and preparations of its ligand, lipopolysaccharide (LPS). The method of preparation of LPS may explain these discrepancies however this is not fully understood. Hence, to determine the impact of LPS on platelet activation, we used ultrapure preparations of LPS from *Escherichia coli* (LPS_{EC}), *Salmonella minnesota* (LPS_{SM}), and *Rhodobacter sphaeroides* (LPS_{RS}) and examined their actions under diverse experimental conditions in human platelets. LPS_{EC} did not affect platelet activation markers such as inside-out signalling to integrin $\alpha_{IIb}\beta_3$ or P-selectin exposure upon agonist-induced activation in platelet-rich plasma or whole blood whereas LPS_{SM} and LPS_{RS} inhibited platelet activation under specific conditions at supraphysiological concentrations. Overall, our data demonstrate that platelet activation is not largely influenced by any of the ultrapure LPS chemotypes used in this study on their own except under certain conditions.

Platelets (small, circulating blood cells) are responsible for blood coagulation upon vascular injury although their unwarranted activation leads to thrombosis. Platelets also play critical roles in the regulation of innate immune responses through diverse molecular mechanisms[1–4]. Toll-like receptors (TLRs) are a group of immune receptors that recognise pathogen- (PAMP) and damage-associated molecular patterns (DAMP)[5]. In humans, TLR4 is expressed in various immune cells and it plays critical roles in the regulation of inflammatory responses. The high affinity ligand for TLR4 is lipopolysaccharide (LPS), a molecule that is found in the outer membrane of Gram-negative bacteria[1,2]. TLR4 may activate two different signalling pathways (the MyD88-dependent or -independent pathway) depending on the ligand involved, as demonstrated in a glioblastoma cell line[6].

It has been widely reported that TLR4 is functional in platelets[3,7]. Moreover, various signalling molecules involved in the MyD88-dependent and -independent signalling pathways downstream of TLR4 have been reported to be present in platelets[8,9] which also emphasise the notion that TLR4 is functional in platelets. The presence of signalling molecules involved in both pathways suggests the potential binding of TLR4 with different LPS chemotypes to trigger either MyD88-dependent or -independent signalling[10]. Binding of *Escherichia coli* LPS (LPS_{EC}) to TLR4 has been reported to increase the level of fibrinogen binding on the surface of platelets under arterial flow conditions[7]. Furthermore, circulating platelets have been reported to respond differently to diverse LPS chemotypes[11]. Moreover, it has been demonstrated that "rough (as referenced in the previous report – 'without the O antigen')" LPS [obtained from *Salmonella minnesota* (LPS_{SM})] is capable of enhancing platelet aggregation in PRP whereas "smooth (as referenced in the previous report - 'with the O antigen')" LPS (obtained from LPS_{EC})

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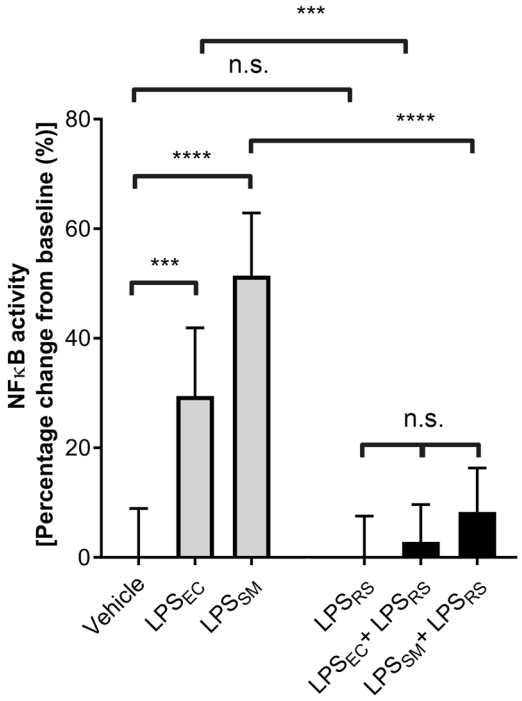

Figure 1. Effect of LPS chemotypes on the U251-NF-κB-GFP-Luc reporter cell line. After 3.5 hours of starvation, the cells were incubated with or without 10 μg/mL LPS_{RS} for 30 minutes. Then, U251-NF-κB-GFP-Luc cells were treated with 1 μg/mL LPS_{EC} or 1 μg/mL LPS_{SM} for 24 hours before lysis and measurement of luciferase activity by spectrophotometry. The results were normalised to the mean of vehicle control treated cells or the mean of the LPS_{RS} control. The data represent percentage change from control ± S.D. (n = 6). The *p* values shown are as calculated by a one-way ANOVA with Bonferroni's post-hoc test (***$p < 0.001$, and ****$p < 0.0001$).

has no effect[12]. This is also reflected in "rough" LPS significantly inducing the release of platelet-derived microparticles on its own and with agonist whilst "smooth" LPS had no significant effect[12]. Diverse platelet responses such as platelet-neutrophil interactions[3], fibrinogen binding[13], and sCD40L secretion[14] have also been observed following stimulation by LPS_{EC} although there is still no overall consensus on the LPS-induced effects in platelets[2]. For example, Claushuis *et al.*[15] suggested that LPS_{EC} is only capable of influencing mitochondrial respiration in platelets although they have reported a significant increase in P-selectin exposure on platelets obtained from septic patients[15]. Furthermore, Koessler *et al.*[16] recently suggested that the preparation of platelets is a factor in the response to LPS as it could only potentiate platelet responses in washed platelets and not in platelet-rich plasma (PRP)[16]. The reasons for the discrepancies of the results reported in the previous studies[7,13,15,17–19] are unclear although the inadequate purification of LPS may result in the presence of bacterial contaminants, such as cell wall components that act as TLR2 ligands, and might be responsible for the controversial results[20,21].

In order to determine the impact of LPS chemotypes obtained from various bacterial species on the modulation of platelet activation under different experimental settings, we used ultrapure LPS_{EC}, LPS_{SM}, and *Rhodobacter sphaeroides* (LPS_{RS}, which is a TLR4 antagonist[13,22,23]) and analysed their effects in platelets. Here, we demonstrate the inability of ultrapure LPS chemotypes from various bacterial species to directly modulate platelet reactivity under diverse settings at physiological concentrations in contrast to conventionally prepared LPS.

Results

LPS_{EC} and LPS_{SM} induce NF-κB activity in a reporter cell line. In order to confirm whether the ultrapure LPS chemotypes used in this study are functionally active and selective to TLR4, they were individually tested in U251-NF-κB-GFP-Luc cells, a reporter cell line for NF-κB signalling selectively via TLR4 (as they do not express TLR2)[24]. The results (Fig. 1) demonstrate that both LPS_{EC} and LPS_{SM} are able to significantly increase NF-κB activity whereas LPS_{RS} did not affect this activity (as expected for a TLR4 antagonist) in comparison to the controls. Notably, no significant increase in NF-κB activity induced by LPS_{EC} or LPS_{SM} was seen in the presence of LPS_{RS}. These data suggest that the LPS chemotypes used in this study are capable of ligating to TLR4 and induce its downstream signalling, and that LPS_{RS} acts as an antagonist for TLR4.

TLR4 is prominently detected in activated platelets. The presence of TLR4 on the platelet surface has been described previously and its level has been reported to increase following platelet activation[7,11,15,25,26]. To corroborate the presence of TLR4 in platelets, immunoblotting was performed using human isolated platelets that were stimulated with 0.5 μg/mL CRP-XL [a potent agonist acting via platelet glycoprotein VI (GPVI)][27]. The presence of TLR4 was predominantly detectable in activated platelets compared to the resting cells (Fig. 2A,B).

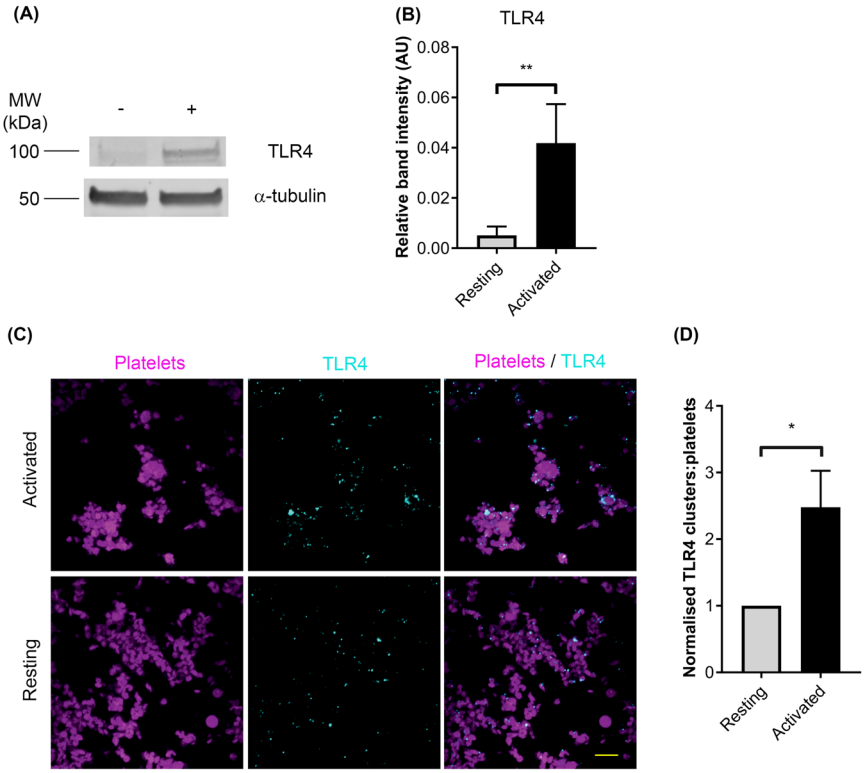

Figure 2. Expression of TLR4 in platelets. (**A**) Resting (−) and CRP-XL (0.5 μg/mL) activated (+) platelets were examined for the presence of TLR4 via immunoblotting. The level of α-tubulin was detected as a loading control. The blot shown is representative of five separate experiments using platelets obtained from five separate donors. (**B**) Quantification of the presence of TLR4 in platelet lysates compared to the expression of α-tubulin using immunoblots. Data represent mean ± S.D. and the *p* value was calculated using Student's t-test (n = 5, $^{**}p < 0.01$). (**C**) The level of TLR4 expression on resting and 0.5 μg/mL CRP-XL activated platelets was analysed by confocal microscopy using a 100x objective. The platelets are shown in magenta and the TLR4 is displayed in cyan. The images shown are representative of data obtained from three separate individuals. The scale bar represents 10 μm. (**D**) Quantification of the level of TLR4 in confocal microscopy images obtained from different donors. Three different regions of three images for each donor were analysed for the presence of TLR4 clusters and presented as mean ± S.D. *P* value ($^{*}p < 0.05$) was calculated using Student's t-test.

The selectivity of TLR4 antibody used was confirmed using U251-NF-κB-GFP-Luc cell lysates as a positive control and HEK293 lysates as a negative control (Fig. S1A). The increased level of TLR4 was also detected in platelets that were stimulated with 0.5 μg/mL collagen (Fig. S1B). Furthermore, the presence of TLR4 was confirmed through immunostaining of platelets (Fig. 2C) where a significant increase on the surface of platelets was observed upon activation with 0.5 μg/mL CRP-XL (Fig. 2D).

LPS_{EC} does not affect platelet activation. To investigate the effect of different ultrapure LPS chemotypes on platelet activation, flow cytometry-based assays were performed to measure the levels of fibrinogen binding (a marker for inside-out signalling to $\alpha_{IIb}\beta_3$[28]) and P-selectin exposure (a marker for α-granule secretion[1,29]). As TLR4 signalling in platelets was generally considered as pro-inflammatory, we hypothesised that LPS_{EC} may directly activate platelets. Therefore, LPS_{EC} was initially tested in platelets in the absence of an agonist[7,13,18]. The effect of diverse concentrations of (0.125 μg/mL–2 μg/mL) ultrapure LPS_{EC} on platelet activation was determined using human PRP or whole blood under different conditions such as various temperature and incubation times. The conditions tested were: incubation of PRP with LPS_{EC} at room temperature for 20 (Fig. 3A,B) or 25 minutes (Fig. S2A,B), and incubation of PRP with LPS_{EC} at 37 °C for 25 (Fig. 3C,D) or 50 minutes (Fig. S2C,D). These results demonstrate that LPS_{EC} does not induce platelet activation in PRP as measured by the levels of fibrinogen binding and P-selectin exposure at these conditions. To determine whether LPS_{EC} has priming roles on platelets as reported previously[30], its effect on CRP-XL-activated platelets (Fig. 3E,F) was analysed through preincubating it for 5 minutes with PRP prior to stimulation with 0.5 μg/mL CRP-XL for 20 minutes at room temperature. Again, the LPS_{EC} failed to increase the level of CRP-XL-induced platelet activation. Furthermore, whole human blood was used to investigate the effect of LPS_{EC} on platelet activation in the presence of other blood cells but no significant change was observed (Fig. 3G,H). Together, these data demonstrate that ultrapure LPS_{EC} was unable to significantly increase either the level of fibrinogen binding or P-selectin exposure on human platelets under any of the conditions tested in this study. However, the non-ultrapure version of LPS_{EC} was able to significantly increase fibrinogen binding in platelets under similar conditions without the presence of a platelet agonist (Fig. S2E). In contrast, PRP from the same donors did not respond to ultrapure LPS_{EC} (Fig. S2E).

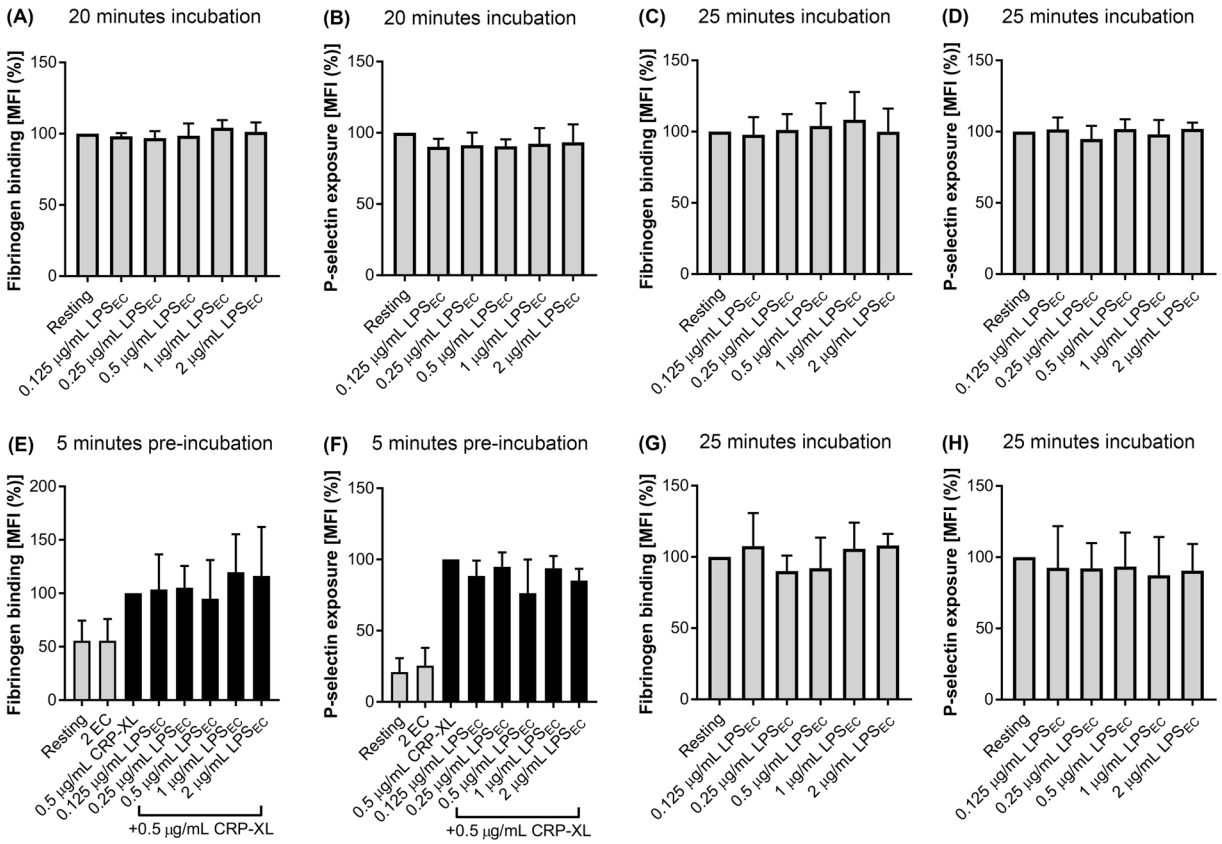

Figure 3. Effect of LPS_{EC} on platelet activation. The level of fibrinogen binding (as a marker for inside-out signalling to integrin $\alpha_{IIb}\beta_3$) (**A**) and the level of P-selectin exposure (as a marker for α-granule secretion) (**B**) was measured in PRP upon incubation with LPS_{EC} for 20 minutes at room temperature (n = 4). In order to determine the impact of temperature on LPS-induced effects in platelets, the level of fibrinogen binding (**C**) and P-selectin exposure (**D**) in PRP was measured by incubating PRP with LPS_{EC} for 25 minutes at 37 °C (n = 5). To determine if the LPS chemotypes possess priming effects in platelets, the level of fibrinogen binding (**E**) and P-selectin exposure (**F**) was measured in PRP upon preincubation with LPS_{EC} for 5 minutes followed by stimulation with a vehicle control or 0.5 µg/mL CRP-XL for 20 minutes at 37 °C (n = 3). Similarly, the level of fibrinogen binding (**G**) and P-selectin exposure (**H**) in human whole blood was measured upon incubation with LPS_{EC} for 25 minutes at 37 °C (n = 3). The data were normalised to either their resting control (100%: **A**–**D** and **G**,**H**) or their 0.5 µg/mL CRP-XL control (100%; **E**,**F**) and analysed using one-way ANOVA and Dunnett's post-hoc test. Data represent mean ± S.D.

LPS_{SM} modulates platelet activation at specific conditions. A previous report using U251-NFκB-A1 cells has suggested that ultrapure LPS_{SM} possesses the capacity to bias signalling mediated by TLR4 towards the MyD88-independent pathway[6]. In order to determine the effect of LPS_{SM} on platelets, various concentrations of this LPS were tested on the modulation of platelet activation upon stimulation with CRP-XL. Similar to above experiments with LPS_{EC}, the levels of fibrinogen binding and P-selectin exposure were measured in platelets upon treatment with LPS_{SM} in the presence and absence of CRP-XL.

To determine the effect of LPS_{SM} on platelets in the absence of an agonist, PRP that was incubated with 2 µg/mL LPS_{SM} was analysed along with CRP-XL-activated platelets as a control in different experimental conditions. The results (Fig. 4A–H) demonstrate that LPS_{SM} does not instigate platelet activation in isolation.

To investigate whether LPS_{SM} has any modulatory effects on the agonist-induced platelet activation, platelets were treated with LPS_{SM} and 0.5 µg/mL CRP-XL simultaneously at room temperature for 20 minutes. These results (Fig. 4A,B) suggest that LPS_{SM} does not affect CRP-XL-induced platelet activation when they were treated simultaneously at room temperature. Subsequently, the platelets were stimulated with a low (0.25 µg/mL) concentration of CRP-XL (Fig. S3A,B) and LPS_{SM}. Again, LPS_{SM} did not affect platelet activation induced by a low agonist concentration. Similarly, to investigate whether preincubation of platelets with LPS_{SM} affects agonist-induced platelet responses, PRP was preincubated with LPS_{SM} for 5 minutes at room temperature prior to activation with 0.5 µg/mL CRP-XL but again this did not affect platelet function (Fig. S3C,D).

Many previous studies reporting the ability of LPS to modulate platelet function were conducted at room temperature[13,15,18,29]. To make the experiments more physiologically relevant, the platelets were incubated at 37 °C. This enabled the investigation of whether temperature was a factor conferring the modulation of platelet activation by LPS_{SM}. The data obtained with platelets that were preincubated at 37 °C for 5 minutes with LPS_{SM} followed by 20 minutes of stimulation with 0.5 µg/mL CRP-XL at 37 °C suggest that LPS_{SM} did not alter platelet activity

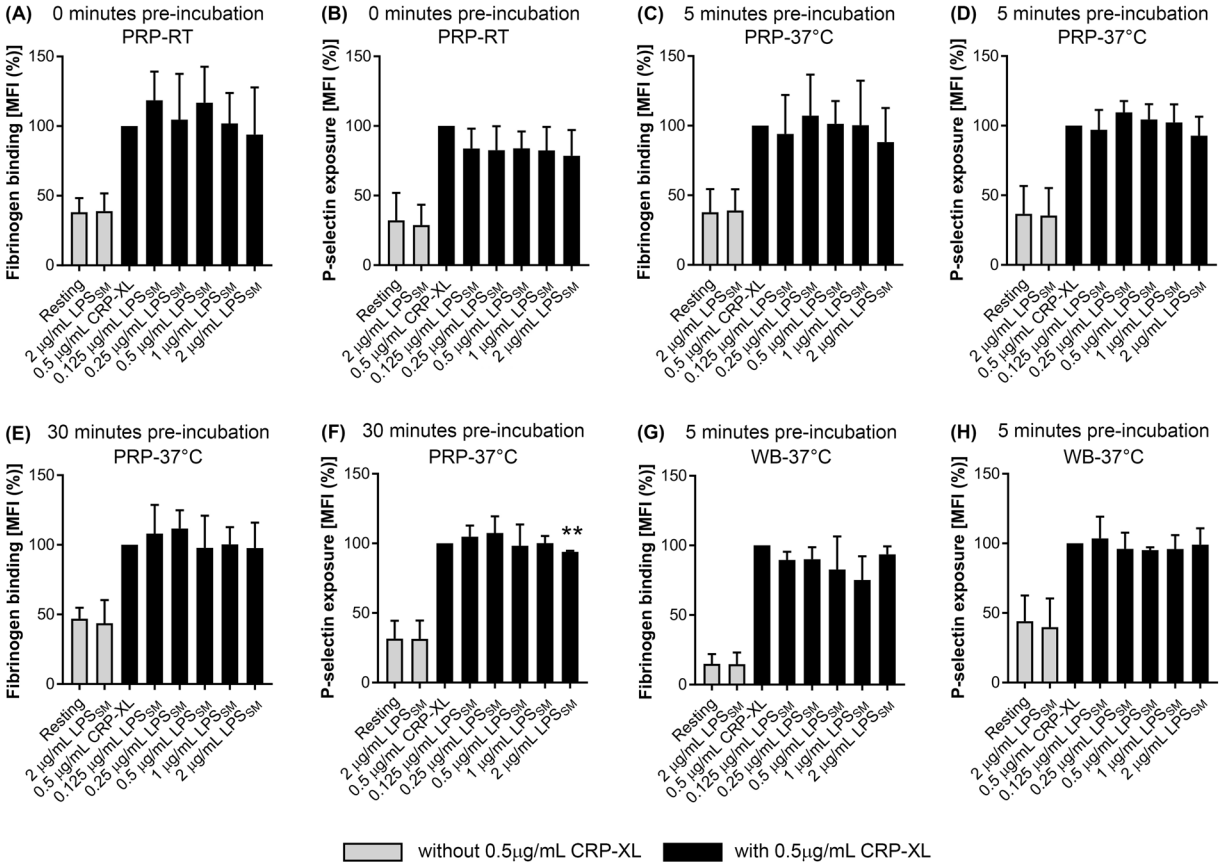

Figure 4. Effect of LPS_{SM} on platelet activation. To determine the impact of LPS_{SM} on the modulation of platelet activation, the level of fibrinogen binding (**A**) and P-selectin exposure (**B**) was analysed in PRP in the presence of different concentrations of LPS_{SM} and 0.5 µg/mL CRP-XL after simultaneous incubation for 20 minutes at room temperature (n = 5). To determine the impact of temperature on LPS-mediated effects in platelets, the level of fibrinogen binding (**C**) and P-selectin exposure (**D**) in PRP was measured upon preincubation with LPS_{SM} for 5 minutes followed by stimulation with 0.5 µg/mL CRP-XL for 20 minutes at 37 °C (n = 6). Similarly, the preincubation of PRP with LPS_{SM} was increased to 30 minutes prior to stimulation with 0.5 µg/mL CRP-XL for 20 minutes at 37 °C, and the level of fibrinogen binding (**E**) and P-selectin exposure (**F**) was analysed (n = 4). Furthermore, the level of fibrinogen binding (**G**) and P-selectin exposure (**H**) was analysed in human whole blood upon preincubation with LPS_{SM} for 5 minutes followed by stimulation with 0.5 µg/mL CRP-XL for 20 minutes at 37 °C (n = 3). The data were normalised to their 0.5 µg/mL CRP-XL control, and the *p* value (**$p < 0.01$) shown was calculated using one-way ANOVA and Dunnett's post-hoc test. Data represent mean ± S.D. The samples treated in the absence of 0.5 µg/mL CRP-XL are represented with empty bars.

(Fig. 4C,D) under these conditions. To determine the impact of incubation time on LPS_{SM}-induced effects, the pre-incubation times were increased from 5 minutes to 30 minutes. The increase in preincubation time did not enable LPS_{SM} to significantly affect fibrinogen binding (Fig. 4E) although, P-selectin exposure stimulated by 0.5 µg/mL CRP-XL was significantly reduced (by approximately 5%) only at a concentration of 2 µg/mL LPS_{SM} (Fig. 4F). At higher concentrations of LPS_{SM}, with 10 minutes of pre-incubation at 37 °C, LPS_{SM} was unable to influence fibrinogen binding or P-selectin exposure induced by CRP-XL (Fig. S3E,F). However, under these conditions, 5 µg/mL LPS_{SM} was able to significantly potentiate (by around 30%) the P-selectin exposure induced by 10 µM TRAP-6 but fibrinogen binding was unaffected (Fig. S3G,H). Moreover, ADP-induced fibrinogen binding and P-selectin exposure were unaffected by LPS_{SM} (Fig. S3I,J). Furthermore, whole human blood was used to investigate the effect of LPS_{SM} on platelet activation. The whole human blood was incubated with LPS_{SM} for 5 minutes at 37 °C prior to platelet activation by 0.5 µg/mL CRP-XL for 20 minutes at 37 °C but it did not affect platelet activation in whole blood (Fig. 4G,H). In conclusion, while 2 µg/mL LPS_{SM} is capable of significantly inhibiting (only by around 5%) CRP-XL-induced P-selectin exposure on the platelet surface under specific conditions (PRP; 30 minutes of preincubation at 37 °C), in general, LPS_{SM} did not affect platelet function under any of the conditions tested in this study.

LPS_{RS} inhibits platelet activation at specific conditions. LPS_{RS} has been suggested to be an antagonist for TLR4[13,22,23]. Here, the effect of ultrapure LPS_{RS} was investigated upon activation of platelets with CRP-XL by measuring the level of fibrinogen binding and P-selectin exposure using flow cytometry. LPS_{RS} was tested in the absence of a TLR4 agonist to investigate potential endogenous activity of the receptor as previous work has suggested that some TLR4 molecules are in an active conformation in unstimulated cells[23]. Furthermore, endogenous

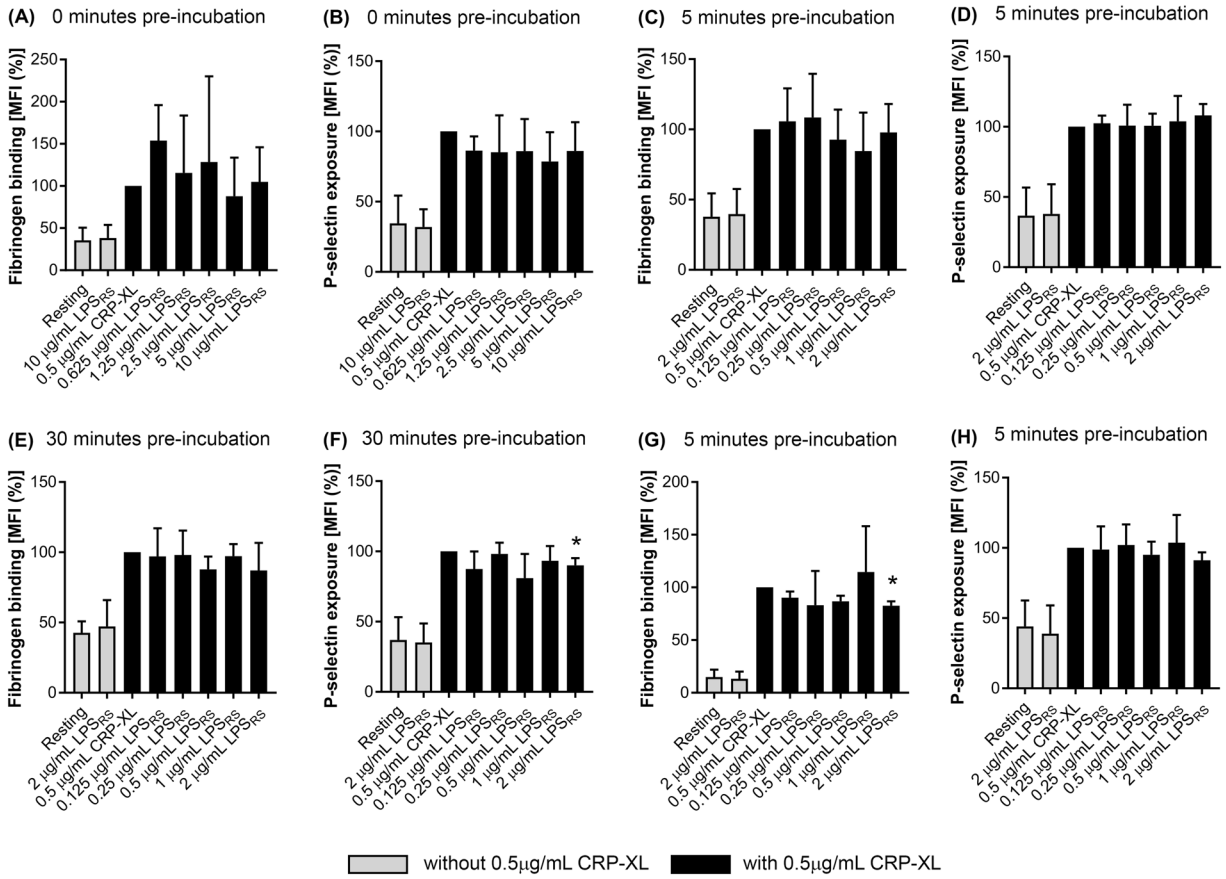

Figure 5. Effect of LPS_{RS} on platelet activation. To determine the impact of LPS_{RS} on the modulation of platelet activation, the level of fibrinogen binding (**A**) and P-selectin exposure (**B**) was analysed in PRP in the presence of different concentrations of LPS_{RS} and 0.5 µg/mL CRP-XL after incubation for 20 minutes at room temperature (n = 4). To determine the impact of temperature on LPS-mediated effects in platelets, the level of fibrinogen binding (**C**) and P-selectin exposure (**D**) in PRP was measured upon preincubation with LPS_{RS} for 5 minutes followed by stimulation with 0.5 µg/mL CRP-XL for 20 minutes at 37 °C (n = 6). Similarly, the preincubation of PRP with LPS_{RS} was increased to 30 minutes prior to stimulation with 0.5 µg/mL CRP-XL for 20 minutes at 37 °C, and the level of fibrinogen binding (**E**) and P-selectin exposure (**F**) was analysed (n = 5). Furthermore, the level of fibrinogen binding (**G**) and P-selectin exposure (**H**) was analysed in human whole blood upon preincubation with LPS_{RS} for 5 minutes followed by stimulation with 0.5 µg/mL CRP-XL for 20 minutes at 37 °C (n = 3). The data were normalised to their 0.5 µg/mL CRP-XL control, and the *p* value (*$p \leq 0.05$) shown is as calculated using one-way ANOVA and Dunnett's post-hoc test. Data represent mean ± S.D. The samples treated in the absence of 0.5 µg/mL CRP-XL are represented with empty bars.

ligands, such as high mobility group box 1 (HMGB1), have been suggested to activate TLR4[31,32]. Similar to LPS_{SM}, LPS_{RS} was tested in resting platelets in all the conditions used in the above experiments (Fig. 5A–H) and the results demonstrate that LPS_{RS} is unable to significantly alter platelet activation in resting platelets.

To determine the impact of LPS_{RS} on agonist-induced platelet activation, platelets were incubated simultaneously with LPS_{RS} and 0.5 µg/mL CRP-XL for 20 minutes at room temperature (Fig. 5A,B) and the levels of fibrinogen binding and P-selectin exposure were measured. The results show that LPS_{RS} did not significantly inhibit P-selectin exposure or fibrinogen binding under these conditions. LPS_{RS} was also tested in conjunction with the lower concentration of CRP-XL (0.25 µg/mL) under the same conditions (Fig. S4A,B). However, it was unable to significantly inhibit platelet activation at this concentration of CRP-XL. Platelets were also preincubated with LPS_{RS} at room temperature for 5 minutes (Fig. S4C,D) before stimulation with CRP-XL (0.5 µg/mL) but it did not alter platelet activation under these conditions. The influence of LPS_{RS} was also tested at 37 °C (Fig. 5C,D) with 5 minutes of preincubation with LPS_{RS} followed by 20 minutes stimulation with 0.5 µg/mL CRP-XL and it did not affect platelet activation. When the preincubation time was increased to 30 minutes, although the level of fibrinogen binding was unaffected under these conditions (Fig. 5E), a significant decrease (approximately 10%) in P-selectin exposure induced by 0.5 µg/mL CRP-XL (Fig. 5F) was observed. The effect of LPS_{RS} on platelet activation in whole blood was examined. Although a decrease in fibrinogen binding was observed (approximately 20%, Fig. 5G), no significant inhibition of P-selectin exposure (Fig. 5H) was observed following 5 minutes of preincubation with LPS_{RS} and 20 minutes of stimulation with CRP-XL at 37 °C. Together, these data demonstrate that ultrapure LPS_{RS} is able to significantly inhibit P-selectin exposure (α-granule secretion in PRP) following

30 minutes preincubation, and inside-out signalling to integrin $\alpha_{IIb}\beta_3$ in whole blood, but in other conditions, LPS_{RS} does not alter platelet activation either directly or upon activation with a platelet agonist.

Moreover, following 10 minutes of preincubation with ultrapure LPS_{RS}, platelet fibrinogen binding and P-selectin exposure induced by 0.5 μg/mL CRP-XL (S4E-F), 10 μM TRAP-6 (S4G-H), and 10 μM ADP (S4I-J) were unaffected by the presence of ultrapure LPS_{RS}. Interestingly, when these experiments were repeated using a non-ultrapure version of $LPS_{RS,}$ fibrinogen binding (but not P-selectin exposure) induced by 0.5 μg/mL CRP-XL was potentiated by 5 μg/mL LPS_{RS} (Fig. S4K,L). Furthermore, no significant change in responses evoked by 10 μM TRAP-6 was observed in fibrinogen binding and P-selectin exposure (Fig. S4M,N). However, a significant increase was observed in P-selectin exposure (but not fibrinogen binding) in platelets induced by 10 μM ADP in the presence of 10 μg/mL LPS_{RS} (Fig. S4O,P). This provides further evidence that there may be a contaminant present in these non-ultrapure versions of LPS that may be responsible for stimulating platelet activity.

LPS does not affect aggregation of pre-activated platelets. As the availability of TLR4 on the platelet surface has been suggested to increase upon platelet activation[11,26], platelets were pre-activated with a low concentration of ADP (0.5 μM) prior to treatment with LPS_{EC}, LPS_{SM}, or LPS_{RS} and further activation with a higher dose of ADP (4 μM) (Fig. 6A). The results (Fig. 6B,C) suggest that none of the three ultrapure LPS chemotypes are capable of significantly modifying the aggregation of platelets under these conditions. Similar results were obtained when the experiment was conducted, using the same procedure, with 0.1 μg/mL collagen (low dose) and 0.25 μg/mL collagen (high dose; Fig. 6D,E) or 1 μM TRAP-6 (low dose) and 10 μM TRAP-6 (high dose; Fig. 6F,G). These data suggest that priming platelets did not affect the ability of LPS chemotypes to modulate platelet function.

LPS does not exert cytotoxic effects in platelets. To determine if the ultrapure LPS chemotypes used in this study exert toxic effects in platelets, various concentrations of LPS chemotypes were analysed in platelets using a lactate dehydrogenase (LDH) assay. The results suggest that the LPS chemotypes [LPS_{EC} (Fig. S5A), LPS_{SM} (Fig. S5B), and LPS_{RS} (Fig. S5C)] at the concentrations used in this study were unable to induce cytotoxicity in platelets. The results demonstrate that ultrapure LPS chemotypes do not induce any direct cytotoxic effects on platelets.

Discussion

Several studies have reported controversial results on the effects of LPS (specifically LPS_{EC}) on platelet function. A key source of criticism revolves around the potential contamination of LPS preparations, possibly by cell wall components that may stimulate TLR2, and the interference of these contaminants on platelet reactivity[20]. The structure of LPS varies between chemotypes and these structural differences have been proposed to be responsible for conferring different downstream activities[10,11,33]. In order to address some of these previous concerns and to determine the precise actions of LPS on platelets, we determined the impact of ultrapure LPS chemotypes from various bacterial species on the modulation of platelet activation.

Initially, the activity of LPS chemotypes was analysed using an NF-κB reporter cell line (which selectively express TLR4 and lack TLR2) to confirm their ability to selectivity bind TLR4 and induce downstream signalling. Our results demonstrate that both ultrapure LPS_{EC} and LPS_{SM} are capable of increasing luciferase activity in U251-NF-κB-GFP-Luc cells compared to the controls whereas LPS_{RS} did not affect the activity as it is an antagonist for TLR4[23]. Moreover, LPS_{EC} and LPS_{SM} were incapable of significantly inducing signalling to NFκB in the presence of LPS_{RS}. Similar results were reported in a previous study to demonstrate the impact of ultrapure LPS chemotypes in the modulation of luciferase activity in the same cell type[24].

The expression of TLR4 on the platelet surface has been widely reported and indeed, in some studies this has been reported to increase upon activation of platelets by a diverse range of agonists including thrombin, convulxin, TRAP-6, ADP, and adrenaline[11,16,26]. Here, we also demonstrate that platelets express low levels of TLR4 on the surface at resting conditions, however its level increases following activation with agonists such as CRP-XL and collagen. Moreover, Tsai *et al.*[26] suggested that in resting platelets, TLR4 is associated with myosin-9 in the intracellular α-granules, and during activation by thrombin, calpain (a protease) is activated and it cleaves the interactions between these two proteins and liberates TLR4 enabling its transport to the platelet surface. Consistent with this previous study, we demonstrate that TLR4 (at 94 kDa) was only prominently detectable in activated platelets. The resting platelets may contain TLR4, however, if it is associated with myosin-9 in α-granules, it may possess a greater molecular mass. Thus, it is not expected to appear at 94 kDa. Furthermore, this would suggest that the preincubation of TLR ligands is superfluous as there may not be a substantial number of TLR4 receptors present on the platelet surface. Moreover, the level of platelet activation by agonists could be a key factor for TLR4 exposure on the cell surface. However, we demonstrate that LPS_{RS} and LPS_{SM} treatments required 30 minutes of preincubation at 37 °C before a significant (~10%) decrease in P-selectin exposure was observed at a supraphysiological concentration under specific conditions.

Previously, it has been reported that LPS_{EC} can enhance platelet aggregation[13,18] and alter the release of different cytokines[11,29]. Furthermore, NF-κB (the transcription factor activated downstream of the MyD88-dependent pathway) is involved in platelet activation induced by classical platelet agonists[2,6,34]. Notably, platelets obtained from TLR4-deficient mice have been shown to possess similar aggregation behaviour compared to control mouse platelets[35]. Currently, there is no consensus regarding platelet response to LPS_{EC} as some reports suggest that it can induce P-selectin exposure and fibrinogen binding[7,13,18,30] whereas others suggest that it does not[11,15,17]. Ultrapure LPS_{EC} has been used by Berthet *et al.*[11] and Claushuis *et al.*[15] where there was no significant increase in P-selectin exposure observed. In line with these previous studies, here we also report that LPS_{EC} does not modulate platelet activation under the diverse settings used in this study. However, a non-ultrapure version of LPS_{EC} did induce a significant increase in the binding of fibrinogen to platelets which supports the hypothesis that the

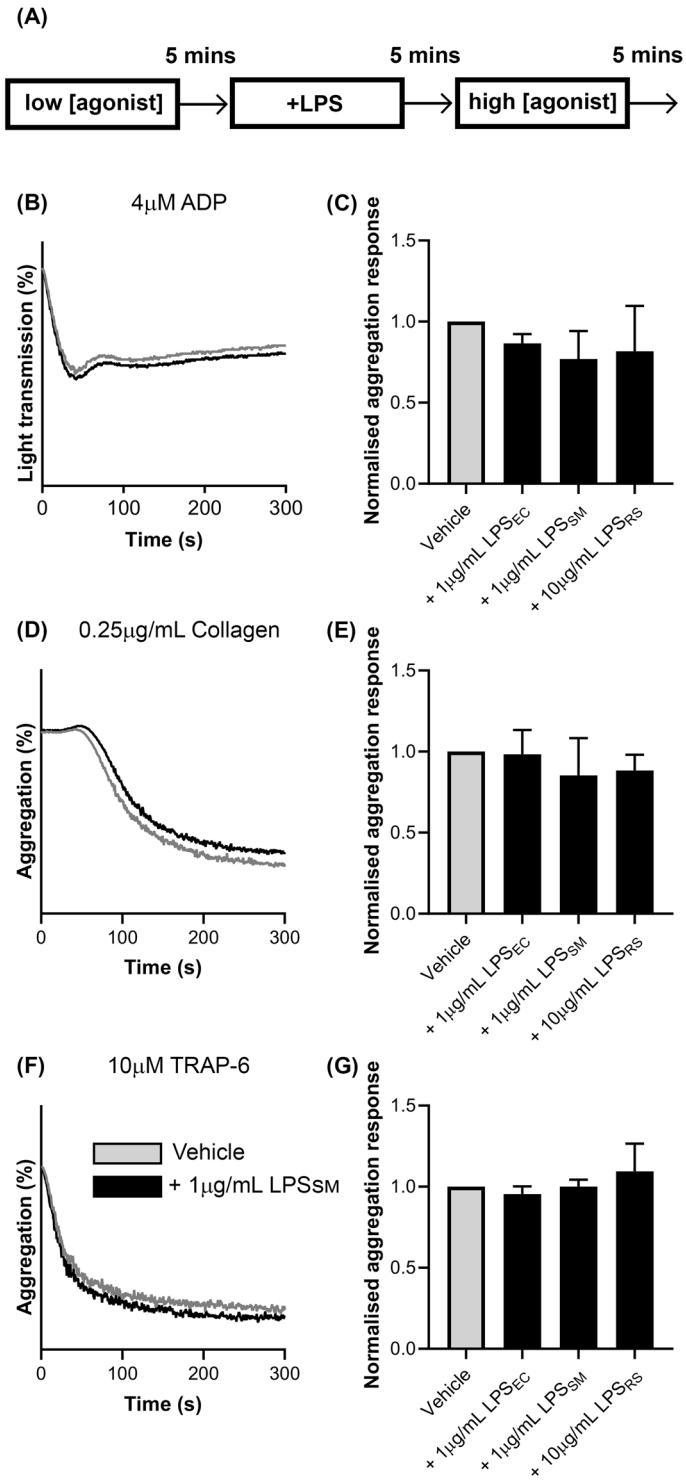

Figure 6. Effect of pre-activation of platelets on the LPS-mediated actions. Aggregometry was used to determine the effect of mild pre-activation by a low dose of different platelet agonists on LPS-modulated actions in PRP. (**A**) demonstrates the schematic protocol for this experiment. A representative trace demonstrating the aggregation induced by ADP in the presence of a vehicle control or 1 μg/mL LPS_{SM} is shown in (**B**). The extent of activation by 4 μM ADP following pre-activation with 0.5 μM ADP and treatment with vehicle, 1 μg/mL LPS_{EC}, 1 μg/mL LPS_{SM}, or 10 μg/mL LPS_{RS} is shown in (**C**). (**D**) A representative aggregation curve induced by 0.25 μg/mL collagen in PRP following pre-activation by 0.1 μg/mL and incubation with a vehicle control or 1 μg/mL LPS_{SM}. (**E**) Shows the normalised results for this experiment. (**F**) A representative trace of aggregation induced by 10 μM TRAP-6 following pre-activation with 1 μM TRAP-6 and incubation with a vehicle control or 1 μg/mL LPS_{SM}. The normalised aggregation response for this experiment is shown in (**G**). In all traces, the LPS-treated trace is represented in black whilst the vehicle is represented in grey. Data represent mean ± S.D. (n = 3) and were analysed using one-way ANOVA.

contaminants found in LPS preparation may be responsible for inducing the observed effects in platelets. The incubation times and concentrations of LPS_{EC} (0.125 μg/mL and 2 μg/mL) used in this study are comparable to other studies where they have used concentrations ranging from 100 ng/mL[15] to 500 ng/mL[29], 1 μg/mL[13,18,36] to 3 μg/mL[11], and 5 μg/mL[3,7] to 10 μg/mL[30].

Moreover, LPS_{SM} did not affect fibrinogen binding and P-selectin exposure in the absence of a platelet agonist. The preincubation (30 minutes) of LPS_{SM} with platelets was able to significantly reduce agonist-induced P-selectin exposure (but only by around 5%) suggesting that α-granule secretion may be inhibited at supraphysiological concentrations of LPS_{SM}. Moreover, the level of fibrinogen binding was unaffected by LPS_{SM} under any of the conditions tested.

LPS_{RS} was also tested for its effects on platelet activity under similar conditions to LPS_{SM} but it did not display any significant effects in the absence of a platelet agonist. However, it affected α-granule secretion but only at supraphysiological concentrations after 30 minutes of preincubation in the PRP. Conversely, the non-ultrapure LPS_{RS} significantly increased fibrinogen binding to platelets upon activation with CRP-XL and P-selectin exposure on platelets upon activation with ADP, which suggests that another factor may be present in the non-ultrapure version that induces the effects reported here and in other studies. To investigate whether pre-activation of platelets would augment TLR4-mediated effects, a low dose of agonist (ADP, TRAP-6, or collagen) was used to prime platelets prior to LPS treatment and further stimulation with a greater concentration of the same agonist. These experiments did not lead to an increase in the effect induced by any of the LPS chemotypes used.

Under pathological conditions such as in sepsis, a plethora of factors are present in the bloodstream and they may be responsible for the observed *in vivo* effects of LPS on platelets. This could be explained by synergistic effects that were not tested in this study although this was suggested by other studies[15,19,37]. In addition to platelets, many other immune cells present in the blood may also respond to LPS and interact with platelets thereby augmenting their activity[1,3,38]. Despite the presence of immune cells in the whole blood, the results from this study suggest that the LPS_{EC} and LPS_{SM} treatment is unable to alter fibrinogen binding or P-selectin exposure on platelets in whole blood. However, LPS_{RS} was capable of modulating inside-out signalling to integrin $\alpha_{IIb}\beta_3$, but not α-granule secretion following stimulation with an agonist at specific concentrations.

Overall, we conclude that the actions of LPS chemotypes on platelets may not be direct and, during pathological conditions, this may be driven or augmented through other molecules that are released under those circumstances. Moreover, the purity of LPS must be ensured prior to testing them in platelets either through a reporter cell line or other experiments to confirm their selectivity to TLR4 and the absence of potential impurities for other molecules. It is important that physiological effects mediated via other receptors, such as TLR2, are not misinterpreted as TLR4-specific effects. This will ensure that solid foundations are available for clinical research, as TLR4 remains an attractive target for certain immunological diseases. The results presented in this study will form a strong basis for future studies to investigate the impact of different LPS chemotypes on the modulation of platelet function, haemostasis, and thrombosis under diverse pathophysiological settings.

Methods

Materials. Ultrapure lipopolysaccharides (LPS) from *Escherichia coli* O111:B4, *Salmonella minnesota* R595, and *Rhodobacter sphaeroides* and their non-ultrapure alternatives (where available) were purchased from InvivoGen, France and used throughout all the experiments. Ultrapure versions of LPS were purified by an additional phenol-TEA-DOC step compared to the non-ultrapure versions that underwent extraction via a phenol-water mixture[21].

Human blood collection and platelet preparation. The blood was obtained from healthy human volunteers with informed consent in accordance to the procedures approved by the University of Reading Research Ethics Committee (UREC: 17/17), and the platelets were prepared as described previously[39–42]. All methods were performed in accordance with the relevant institutional and national guidelines and regulations. Briefly, the blood was drawn via venepuncture into vacutainers containing 3.2% (w/v) citrate and used in assays where whole blood was required. For the preparation of platelet-rich plasma (PRP), the blood was centrifuged at 102 *g* for 20 minutes at 20 °C and the PRP was carefully collected for further experiments. For the preparation of isolated platelets, the blood was mixed with 15% (v/v) acid citrate dextrose [ACD: 2.5% (w/v) sodium citrate, 2% (w/v) glucose and 1.5% (w/v) citric acid] prior to centrifugation at 102 *g* for 20 minutes at 20 °C. The PRP was then collected and centrifuged at 1413 *g* for 10 minutes at 20 °C in the presence of 50 ng/mL prostaglandin I_2 (PGI_2) before the plasma was removed and the platelet pellet was resuspended in modified Tyrode's-HEPES buffer (134 mM NaCl, 2.9 mM KCl, 0.34 mM $Na_2HPO_4.12H_2O$, 12 mM $NaHCO_3$, 20 mM HEPES, 1 mM $MgCl_2$, and 5 mM D-glucose, pH 7.3) with 12% (v/v) ACD. Following another centrifugation at 1413 *g* for 10 minutes in the presence of 50 ng/mL PGI_2, the platelet pellet was resuspended in fresh modified Tyrode's-HEPES buffer and left to rest for 30 minutes at 30 °C prior to use.

Cell culture and luciferase assay. The U251-NF-κB-GFP-Luc cells[24] grown in high glucose DMEM supplemented with 10% (v/v) foetal calf serum (FCS) and 2 mM L-glutamine (Sigma Aldrich, UK) were seeded on a 24-well plate at 1×10^5 cells/well and left in a 37 °C humidified incubator with 5% CO_2 until >80% confluency was achieved. The cells were then starved in serum-free high glucose DMEM supplemented with 2 mM L-glutamine for 3.5 hours prior to addition of vehicle (endotoxin-free water) or 10 μg/mL LPS_{RS} for 30 minutes. Subsequently, cells were treated with a vehicle control, 1 μg/mL LPS_{EC}, 1 μg/mL LPS_{SM}, or 10 μg/mL LPS_{RS} (or their combinations). After 24 hours, the cells were washed with sterile phosphate-buffered saline (PBS) (Sigma Aldrich, UK) before being lysed on a rocker in cell culture lysis buffer (Promega, UK) for one hour at room temperature. The cell lysates were centrifuged for 5 minutes at 5000 *g* at room temperature. The level of luciferase activity of each lysate after the addition of luciferase assay substrate (Promega, UK) was measured by a SpectraMax iD3 multi-mode microplate reader (Molecular Devices, USA).

Immunocytochemistry. The isolated human platelets were suspended in modified Tyrode's-HEPES buffer containing 10 μM Cell Tracker, CMAC (Thermo Fisher Scientific, UK) for one hour. The platelets were mixed on a rotational plate shaker at 330 rpm for 10 minutes at room temperature in the presence of 0.5 μg/mL cross-linked collagen-related peptide (CRP-XL) (obtained from Professor Richard Farndale, University of Cambridge, UK) or modified Tyrode's buffer as a control before they were fixed by the addition of 0.2% (v/v) formyl saline. The platelets were centrifuged at 2500 *g* for 5 minutes, washed in PBS, then resuspended in 5% goat serum in PBS for blocking for 30 minutes. After blocking, the platelets were washed with PBS and resuspended in 1/100 anti-TLR4 [76B357.1] antibody (Abcam, UK) in PBS and incubated for one hour prior to washing and incubating with 1/300 goat anti-mouse Alexa Fluor 647-conjugated secondary antibody for one hour. Subsequently, the platelets were washed, resuspended in Mowiol [containing 0.1% (v/v) 1,4-phenylenediamine dihydrochloride], and mounted on a microscope slide. A Nikon A1-R Confocal Microscope was used for image acquisition using a 100x objective. The level of expression of TLR4 was quantified using ImageJ.

Immunoblotting. The isolated human platelets were treated with either modified Tyrode's-HEPES buffer (resting control) or 0.5 μg/mL CRP-XL for five minutes in an aggregometer prior to lysis in reducing sample treatment buffer [RSTB; 69 mM sodium dodecyl sulphate, 5% (v/v) 2-mercaptoethanol, 10% (v/v) glycerol, and 25 mM Tris-HCl]. Subsequently, the platelet lysates were boiled at 90 °C for 10 minutes and analysed by SDS-PAGE in 4–15% pre-cast gels (Bio-Rad, UK) and then transferred to a PVDF membrane (GE Healthcare, UK) using a Semi-Dry Transfer System (Bio-Rad, UK). The membrane was blocked with 5% (w/v) bovine serum albumin (BSA) in PBS with 0.1% (v/v) Tween-20 (PBS-T) for 1 hour at room temperature. Then it was incubated overnight at 4 °C with anti-TLR4 antibody (1/250 dilution) and for 1 hour at room temperature with anti-α-tubulin [B-7] or anti-14-3-3ζ antibody (1/2000 dilution) (Santa Cruz Biotechnology, USA). 30 μg of HEK-293 or U251-NF-κB-GFP-Luc cell lysates were used as a TLR4-negative or -positive control respectively. The primary antibodies were detected by using Cy5-conjugated goat anti-mouse IgG (1/2500 dilution) (Thermo Fisher Scientific, UK) in a Typhoon 9400 variable mode imager (GE Healthcare, UK) (488 V) and images were analysed using ImageJ.

Flow cytometry-based assays. Rabbit polyclonal FITC-conjugated anti-human fibrinogen antibodies (Dako, UK) were used to measure the level of fibrinogen binding as a marker for inside-out signalling to integrin $\alpha_{IIb}\beta_3$[28] and PE-Cy5-conjugated mouse anti-human CD62P antibodies (BD Biosciences, UK) were used to measure the level of P-selectin exposure as a marker for α-granule secretion from platelets[1,29]. The human PRP was incubated with both the antibodies in HEPES-buffered saline (HBS: 150 mM NaCl, 5 mM KCl, 2 mM $MgSO_4.7H_2O$, and 10 mM HEPES, pH 7.4) for various time periods (0, 5, 20, 25, 30 and 50 minutes) with and without different concentrations of LPS chemotypes (LPS_{EC}, LPS_{SM}, and LPS_{RS}). After preincubation, the platelets were exposed to modified Tyrode's-HEPES buffer (vehicle) or 0.5 μg/mL CRP-XL, 0.25 μg/mL CRP-XL, 10 μM TRAP-6 (Abcam, UK), or 10 μM ADP (Sigma, UK) for various time points at room temperature or 37 °C. The platelets were then fixed using 0.2% (v/v) formyl saline and the level of fluorescence was measured using an Accuri C6 Flow cytometer (BD Biosciences, UK).

Platelet aggregation. The PRP was pre-activated with 0.5 μM ADP, 1 μM TRAP-6, or 0.1 μg/mL collagen (ChronoLog, UK) for five minutes prior to the addition of different ultrapure LPS chemotypes and incubation for another five minutes before initiating aggregation with 2 μM ADP, 10 μM TRAP-6, or 0.25 μg/mL collagen respectively. The aggregation was monitored for five minutes using a Chrono-Log (Model 700) aggregometer (USA) under constant stirring conditions at 37 °C.

Lactate dehydrogenase (LDH) assay. In order to determine if the LPS chemotypes have direct cytotoxic effects, a LDH cytotoxicity assay was performed using a LDH Cytotoxicity Assay Kit (Thermo Fisher Scientific, UK) according to the manufacturer's instructions. Briefly, human PRP was incubated at 37 °C for 30 minutes prior to incubation with different concentrations of LPS chemotypes or a vehicle control (endotoxin free water) for 5 minutes. Subsequently, the reaction mixture provided in the kit was added to platelets and incubated for 30 minutes and the reaction was stopped using a stop solution (provided in the kit). The positive control referred to in the results was supplied in the kit. The absorbance of the samples was read at 490 nm and 650 nm using a Fluostar Optima (BMG Labtech, Germany) spectrofluorometer.

Statistical analysis. All the data obtained in this study were analysed using GraphPad Prism 8. The data obtained from cell culture experiments were analysed using one-way ANOVA with the differences between treatments investigated using a Bonferroni's post-hoc test. Comparisons for relative band intensity in immunoblot images and TLR4 cluster:platelet ratios were analysed via Student's t-test. Flow cytometry experiments were analysed using one-way ANOVA. The differences between the vehicle control (for experiments involving LPS_{EC}) or the positive control (0.25 μg/mL, 0.5 μg/mL CRP-XL, 10 μM TRAP-6, or 10 μM ADP) and the experimental mean were tested for statistical significance through the use of a Dunnett's post-hoc test. The normality of distribution was examined for all the datasets and non-parametric tests were used where appropriate (Friedman's test with Dunn's post-hoc test).

Data availability

The datasets generated and analysed in this study are available from the corresponding author on reasonable request.

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Author contributions
T.M.V., D.W. and S.V. designed the experiments, analysed the data and wrote the manuscript. T.M.V., D.R., D.A.I.A., H.L., J.S. and R.V. performed the experiments and analysed data. P.D. and K.P. have provided advice and support for the design of experiments.

Competing interests
The authors declare no competing interests.

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Effect of ultrapure lipopolysaccharides derived from diverse bacterial species on the modulation of platelet activation

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Supplementary Information

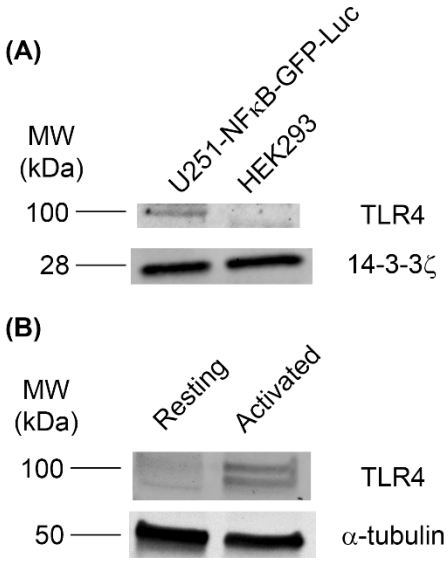

Figure S1: **TLR4 expression in platelets and control cells. (A)** U251-NF-κB-GFP-Luc cell lysates (left) and HEK293 cell lysates (right) were used as positive and negative controls respectively to validate the anti-TLR4 antibody used. **(B)** Platelet lysates stimulated with 1µg/mL collagen and their resting equivalents were also probed

for their TLR4 expression using the anti-TLR4 antibody. The blot shown is representative of three separate experiments.

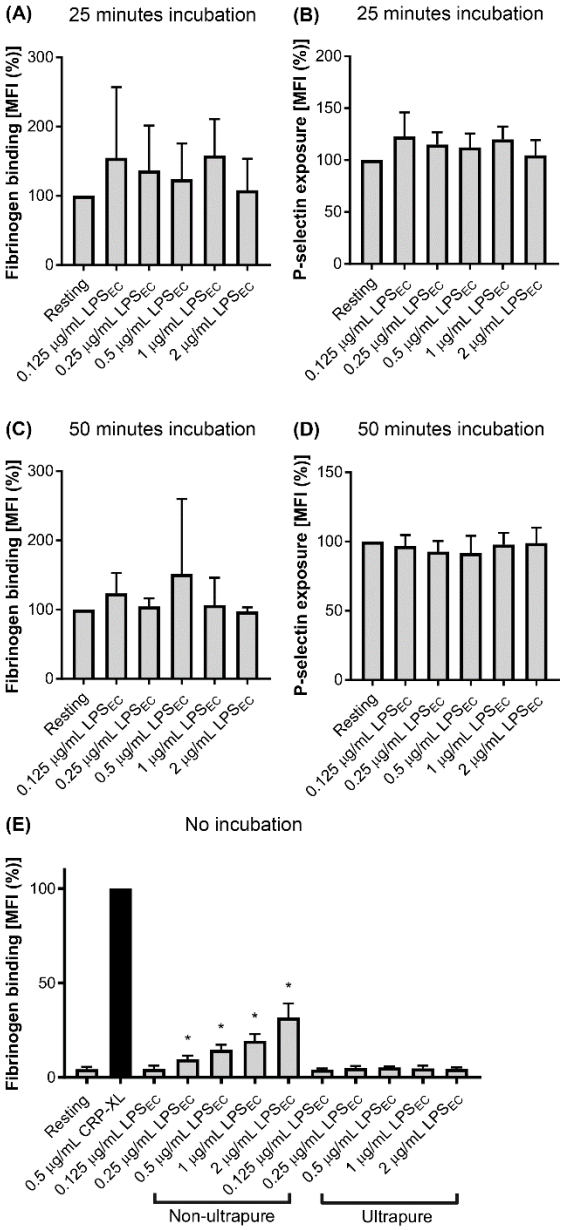

Figure S2: **Effect of LPS_{EC} on platelet activation.** The level of fibrinogen binding and P-selectin exposure in PRP was measured upon incubation with ultrapure LPS_{EC} for 25 minutes at room temperature (**A**, **B**) or 50 minutes at 37°C (n=5) (**C**, **D**). Furthermore, the level of fibrinogen binding to platelets treated with a non-ultrapure version of LPS_{EC} following 20 minutes of incubation was determined (n=3) (**E**). All data were normalised to their resting control and analysed using one-way ANOVA and Dunnett's post-hoc test (*p<0.05). Data represent mean ± S.D.

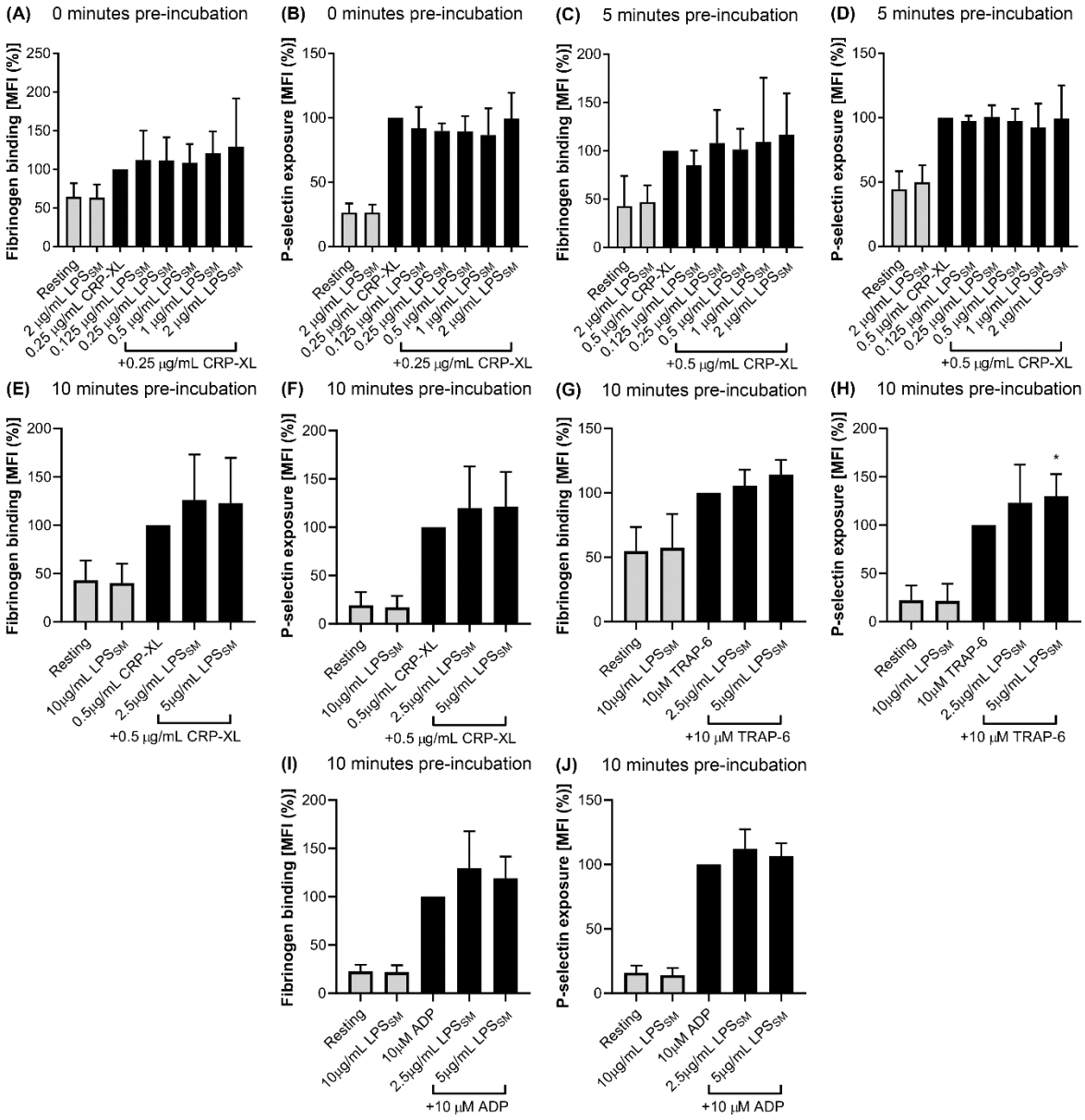

Figure S3: ***Effect of LPS$_{SM}$ on platelet activation***. The level of fibrinogen binding and P-selectin exposure was measured in PRP in the presence of different concentrations of LPS$_{SM}$ and 0.25µg/mL CRP-XL after simultaneous incubation for 20 minutes at room temperature (**A**, **B**) (n=3) or preincubation with LPS$_{SM}$ for 5 minutes followed by stimulation with 0.5µg/mL CRP-XL for 20 minutes at room temperature (n=5) (**C**, **D**). Similarly, higher LPS$_{SM}$ concentrations were tested with 0.5µg/mL CRP-XL following 10 minutes of pre-incubation and the level of fibrinogen binding (**E**) and P-selectin exposure (**F**) was measured (n=7). This experimental set-up was repeated with 10µM TRAP-6 (**G** and **H**; n=7) and 10µM ADP as platelet agonists (**I** and **J**; n=7). The data were normalised to their 0.5µg/mL CRP-XL, 10µM TRAP-6, or 10µM ADP controls and analysed using one-way ANOVA and Dunnett's post-hoc test (*p<0.05). Data represent mean ± S.D.

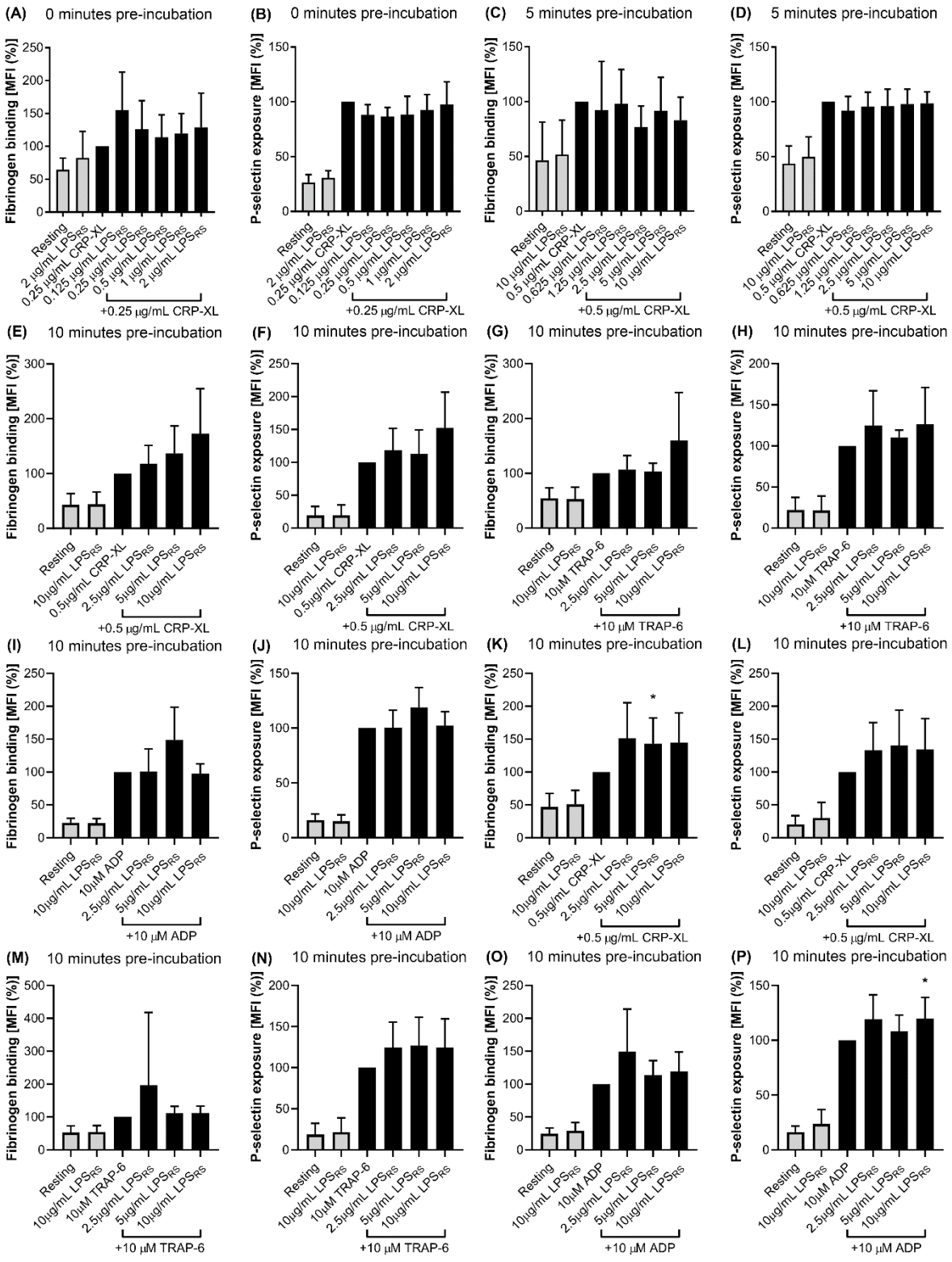

Figure S4: ***Effect of LPS_{RS} on platelet activation.*** The level of fibrinogen binding and P-selectin exposure was measured in PRP in the presence of different concentrations of LPS_{RS} and 0.25µg/mL CRP-XL after incubation for 20 minutes at room temperature (**A**, **B**) (n=4) or preincubation with LPS_{RS} for 5 minutes followed by stimulation with 0.5µg/mL CRP-XL for 20 minutes at room temperature (n=4) (**C**, **D**). Similarly, ultrapure LPS_{RS} concentrations were tested with 0.5µg/mL CRP-XL following 10 minutes of pre-

incubation and the level of fibrinogen binding (**E**) and P-selectin exposure (**F**) was measured (n=7). This experimental set-up was repeated with 10µM TRAP-6 (**G** and **H**; n=7) and 10µM ADP as platelet agonists (**I** and **J**; n=7). Furthermore, non-ultrapure LPS_{SM} was also tested for its effect on fibrinogen binding and P-selectin exposure in response to 0.5µg/mL CRP-XL (**K** and **L**; n=9), 10µM TRAP-6 (**M** and **N**; n=10), and 10µM ADP (**O** and **P**; n=10) following 10 minutes of pre-incubation. The data were normalised to their 0.5µg/mL CRP-XL, 10µM TRAP-6, or 10µM ADP controls and were analysed using a one-way ANOVA and Dunnett's post-hoc test (*p<0.05). Data represent mean ± S.D. The samples treated in the absence of 0.5µg/mL CRP-XL are represented with empty bars.

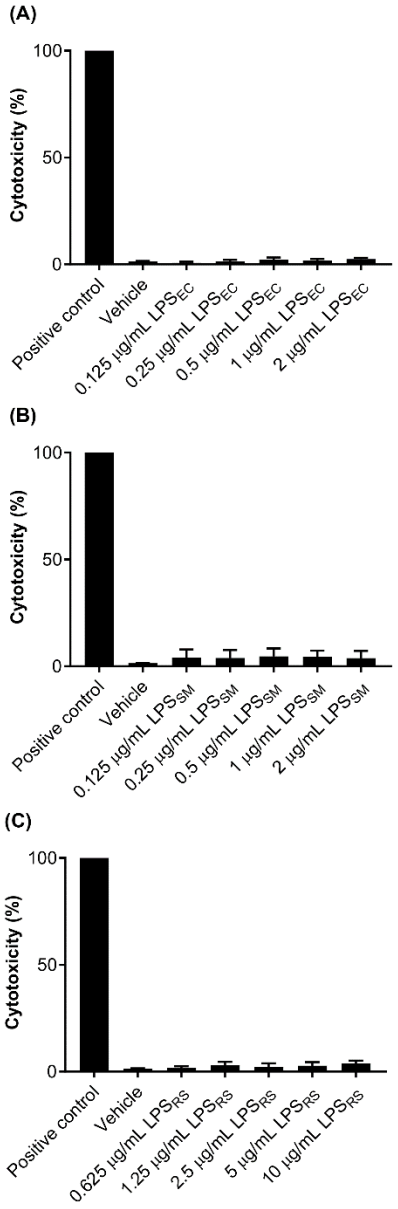

Figure S5: ***Cytotoxic effects of LPS chemotypes in platelets.*** The cytotoxic effects of LPS_{EC} (**A**), LPS_{SM} (**B**), and LPS_{RS} (**C**) in platelets were investigated using a lactate

dehydrogenase (LDH) cytotoxicity assay. All the results were normalised to the positive control. Data represent mean ± S.D. (n=3) and analysed with one-way ANOVA with Dunnett's post-hoc test.

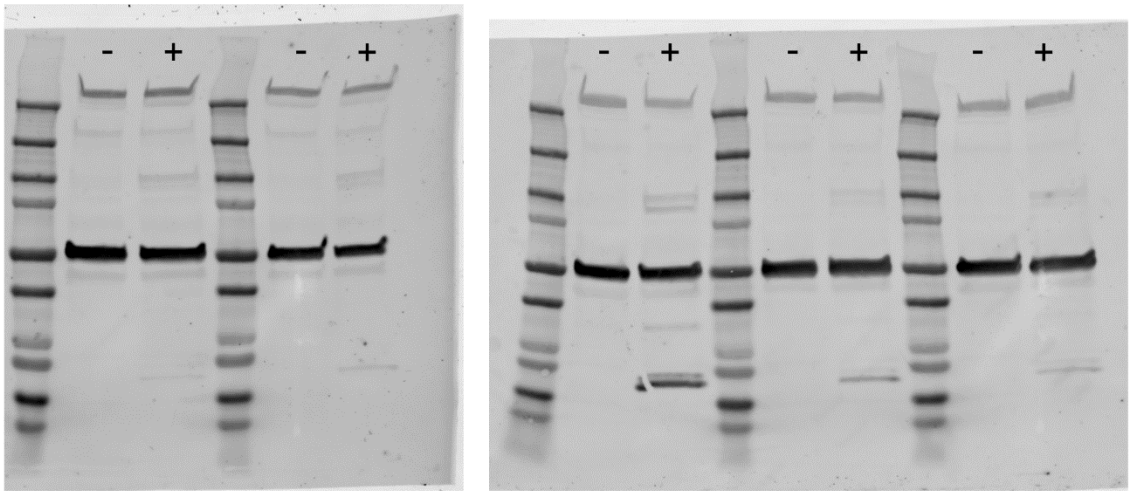

Figure S6: **Full length immunoblots of TLR4 and α-tubulin in whole platelet lysates shown in Figure 2.** Resting (-) and CRP-XL (0.5µg/mL) activated (+) platelets were examined for the presence of TLR4 via immunoblotting. The level of α-tubulin was detected as a loading control.

2.2 Development and characterisation of a novel, megakaryocyte NF-κB reporter cell line for investigating inflammatory responses

Vallance TM, Sheard JJ, Meng Y, Torre EC, Widera D, and Vaiyapuri S

Rationale of this chapter

Following the conclusions of the previous chapter, we aimed to determine whether TLR4 and its ligands, ultrapure (without any impurities) LPS, may influence megakaryocyte (MK) function and thereby impact the phenotype of the platelets produced under pathological scenarios. Due to the difficulties in obtaining MKs in large numbers, relevant readouts, and differences in murine and human morphology, research into the inflammatory responses of MKs has been largely restricted. Hence, here for the first time, we have developed a reporter cell line for NF-κB signalling to study the functions of MKs in response to various (specifically pro- and anti-inflammatory) molecules that are able to affect NF-κB signalling. We have successfully transduced the megakaryoblastic Meg-01 cell line with a reporter construct to stably express luciferase in response to NF-κB activity. We demonstrate that the transduced (Meg-01R) cells responded to a range of pro- and anti-inflammatory molecules as determined by the level of luciferase activity. However, the ultrapure LPS molecules were unable to alter NF-κB activity although the non-ultrapure version of LPS was able to increase its activity. The level of TLR4 on the surface of MKs was also found to be minimal compared to the level found inside the cell. In conclusion, although the LPS molecules were unable to alter the NF-κB activity of MKs, this reporter cell line can be used as a novel tool to measure inflammatory signalling using various molecules that are able to alter NF-κB signalling in MKs.

Contribution to this chapter

As the first author, I contributed nearly 85% to this chapter by designing and performing experiments, analysing the data, and preparing the manuscript and figures.

Experimental contribution

All the experiments were performed by me in their entirety apart from Figure 7A and 7B which were conducted by YM. JJS transduced the Meg-01 cells to produce the Meg-01R cell line and assisted with confocal microscopy.

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Development and characterisation of a novel, megakaryocyte NF-κB reporter cell line for investigating inflammatory responses

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ABSTRACT

Due to the difficulties in acquiring large numbers of megakaryocytes, the impact of inflammatory responses on these cells and their ability to produce fully functional platelets under various pathological conditions has not been investigated in detail. The primary objective of this study is to develop and functionally characterise a novel megakaryocyte NF-κB reporter cell line in order to determine the effects of various inflammatory molecules on megakaryocytes and their signalling pathways.

A Meg-01-NF-κB-GFP-Luc (Meg-01R) cell line was developed by inserting a reporter NF-κB-GFP-Luc cassette into normal Meg-01 cells to produce luciferase following activation of NF-κB to enable easy detection of pro- and anti-inflammatory signalling. Meg-01 and Meg-01R cells were shown to have comparable characteristics including the expression of both GPIb and integrin β_3. Meg-01R cells responded to various inflammatory molecules as measured by the level of luciferase activity. For example, pro-inflammatory molecules such as TNFα and Pam3CSK4 increased NF-κB activity, whereas an anti-inflammatory molecule, LL37 reduced its activity. Meg-01R cells were also found to be sensitive to inhibitors (IMD0354 and C87) of pro-inflammatory pathways. Notably, Meg-01R cells were able to respond to LPS (non-ultrapure) although it was not able to react to ultrapure LPS due to the lack of sufficient TLR4 molecules on their surface.

For the first time, we report the development and characterisation of a novel megakaryocyte NF-κB reporter cell line (Meg-01R) as a robust tool to study the inflammatory responses/signalling of megakaryocytes upon stimulation with a range of pro- and anti-inflammatory molecules.

INTRODUCTION

Platelets (small circulating blood cells) are known for their ability to regulate haemostatic, innate immune, and inflammatory responses [1,2]. A large number of studies have focussed on elucidating the roles of platelets in the regulation of innate immune/inflammatory responses and the molecular mechanisms responsible for these activities [1,3–5]. However, only a relatively small number of studies have focussed on determining the impact on megakaryocytes (MKs), the precursors of platelets, during immune/inflammatory responses [6,7]. This is an under-researched area although the reactivity and proteome of platelets produced during inflammation are modified in order to accustom the pathological conditions [8–10].

MKs differentiate from haematopoietic stem cells (the same lineage from which innate immune cells such as neutrophils and monocytes are derived), produce large quantities of proteins, and possess an extended invaginated membrane system for packaging into numerous platelets [7]. MKs in the bone marrow produce platelets by extending proplatelets into sinusoids where the shear flow of blood leads to budding of platelets into the bloodstream [7,11]. Platelets are also produced in the mouse lungs, where MKs may interact with pathogen-associated molecular patterns (PAMPs) and damage-associated molecular patterns (DAMPs), however whether this function translates into humans remains unclear [12]. The environment surrounding MKs may alter the functions of platelets, for example, producing platelets that are more pro-aggregatory or aggressive towards pathogens, although, the molecular mechanisms behind these actions are still poorly understood [8–10]. Studies have shown that in mice, lipopolysaccharide (LPS) from Gram-negative bacteria causes an increase in circulating platelet count [8]. Mice lacking functional Toll-like receptor (TLR)-4 (the receptor for LPS) have a significant reduction in the number of circulating platelets [8]. Furthermore, some studies suggest that LPS treatment increases platelet production from MKs, as well as MK ploidy [13,14], although this may be due to the release of

several factors from macrophages [15]. Hence, determining the impact of MKs during inflammatory responses and subsequent platelet production will aid in better understanding of the significance of MKs and platelets in the regulation of thromboinflammation in various pathophysiological scenarios.

Meg-01 is a megakaryoblastic cell line which is frequently used as a surrogate for elucidating signalling pathways and functions in MKs due to the difficulties associated with acquiring large numbers of primary MKs [16–19]. Meg-01 was chosen as the cell line to be transduced as it shares similar features with MKs whilst not expressing markers for lymphocytes or erythrocytes [19,20]. Furthermore, although TLR2-induced cytokine release and the presence of TLRs has been demonstrated in other MK-like cell lines, Meg-01 cells have been proven to degrade IκB downstream of TLR2 activation, which confirms that this cell line is a valid choice for elucidating inflammatory signalling pathways [16,21–23]. TLR2 is closely related to TLR4 and it signals through the same MyD88-dependent pathway. Treatment of Meg-01 cells with a TLR2 agonist, Pam3CSK4 resulted in increased MK ploidy, activated NF-κB, and altered protein expression [16], and these results suggest that the MyD88-dependent pathway is active in MKs. Due to the lack of a reliable system to determine the role of MKs in the regulation of inflammatory responses, in this study we developed a robust, Meg-01-NFκB-Luc-GFP (Meg-01R) reporter cell line and functionally characterised them using a range of inflammatory molecules.

MATERIALS & METHODS

The data that support the findings of this study are available upon reasonable request.

Cell culture

Meg-01 cells were grown in RPMI-1640 media supplemented with 10% (v/v) foetal calf serum (FCS) and 2mM L-glutamine (Sigma-Aldrich, UK). Cells were kept at 37°C in a humidified 5% CO_2 incubator. Every 2-3 days, the cells were removed by scraping, counted, and resuspended at a concentration of $2.5x10^5$cells/mL in a vented suspension flask (Sarstedt, Germany). The media for Meg-01R cells was supplemented with 1µg/mL puromycin for selection (Apollo Scientific, UK). All experiments were conducted in the absence of antibiotics and antimycotics.

THP-1 cells were grown in RPMI-1640 media supplemented with 10% (v/v) FCS, 0.2% (v/v) penicillin/streptomycin mix, and 0.4% (v/v) amphotericin B (Sigma-Aldrich). These cells were kept in a humidified 5% CO_2 incubator at 37°C.

HEK-293 cells were grown in high glucose DMEM containing 10% (v/v) FCS and 2mM L-glutamine in a humidified 5% CO_2 incubator at 37°C.

Doubling time was calculated using Meg-01 and Meg-01R cells cultured in parallel under normal growth conditions in normal growth media using the formula:

$$\text{Doubling time} = \frac{\text{Duration (h)} \times \log_{10}(2)}{\log_{10}([\text{final}]) - \log_{10}([\text{initial}])}$$

Immunoblotting

Meg-01 and THP-1 cells were lysed in cell lysis buffer (Promega, UK) at a concentration of $10x10^6$ cells/mL. Protein concentration was quantified using Coomassie protein assay reagent (ThermoFisher Scientific, UK) and lysates were boiled in the presence of reducing sample treatment buffer [RSTB; 2% (w/v) sodium dodecyl sulphate, 5% (v/v) 2-mercaptoethanol, 10% (v/v) glycerol, and 0.3% (w/v) Tris] at 90°C for 10 minutes. 20µg of protein was run through a pre-cast 10% polyacrylamide gel (Bio-Rad, UK) at 200V. After gel electrophoresis, protein was transferred to a methanol-activated 0.2µm polyvinylidene difluoride (PVDF) membrane (GE healthcare) using a Semi-Dry Transfer System (Bio-Rad). PVDF membranes were blocked in 5% (w/v) bovine serum albumin (BSA) in phosphate-buffered saline (PBS)-Tween-20 [PBS-T; 0.1% (v/v) Tween-20] for 1 hour at room temperature.

The concentration of antibodies used when blocking overnight depended of the protein being detected but primary and secondary antibodies were always diluted in 5% BSA in PBS-T. Antibody details are found in Supplementary Table 1.

Proteins were visualised through either fluorescence or enhanced chemiluminescence (ECL). For the fluorescence method, proteins were incubated with goat Cy5-conjugated anti-mouse/rabbit antibodies (Thermofisher Scientific) at a final concentration of 1/2500 and imaged using a Typhoon 9400 variable mode imager (GE healthcare). For ECL, goat horseradish peroxidase-conjugated anti-mouse/rabbit antibodies (ThermoFisher Scientific) were used at 1/2500. The membranes were then

imaged using an ImageQuant LAS 4000 mini (GE Healthcare). Images were processed using ImageJ.

XTT assay

Meg-01 cells ($2.5x10^4$) were seeded in a 96-well low adhesion plate (Sarstedt, Germany) for 24 hours prior to treatment with vehicle or 3ng/mL-30µg/mL puromycin. Meg-01 cells were incubated with puromycin for 24 hours or 48 hours and sodium 3'-[1-(phenylaminocarbonyl)- 3,4- tetrazolium]-bis (4-methoxy 6-nitro) benzene sulphonic acid hydrate (XTT) reagent (Sigma-Aldrich, UK) was added for 8 hours before the absorbance of the sample was measured at 490nm and 650nm.

For assessment of changes in metabolic activity, Meg-01 and Meg-01R cells were cultivated at increasing concentrations on a suspension 96-well plate prior to addition of XTT reagent. The absorbance of the sample was measured following 6 hours of incubation.

Lentiviral transduction

Meg-01R cells were generated as described previously [24,25]. Briefly, HEK-293 cells were transfected with pGreenFire-NFκB-Puro vector (System Biosciences, USA) for viral production prior to transduction of Meg-01 cells. Viral production proceeded for 48 hours. Viral particles were isolated from HEK-293 cells by removing the cells with centrifugation for 10 mins at 1000*g* (4°C). The supernatant was filtered through a 0.45µm diameter filter and then centrifuged at 100,000*g* for 1 hour at 4°C. The viral pellet was resuspended in PBS and left to precipitate overnight at 4°C. To aid transduction of Meg-01 cells, 5µg/mL of polybrene was added to the PBS-virus suspension and incubated with $3.75x10^6$ Meg-01 cells for 30 mins at 4°C. Following transduction, normal cell culture medium (RPMI-1640 with 10% FCS and 2mM L-glutamine) was added, and the cells were left for 96 hours prior to the addition of 1µg/mL puromycin for the selection of transduced cells. Dead cells were removed via an extra centrifugation step (300*g* for 10 minutes) in PBS.

Flow cytometry

Meg-01R cells were washed in PBS, centrifuged at 300*g* for 5 minutes and resuspended in sterile-filtered 0.2% formyl saline [0.9% (w/v) NaCl and 0.2% (v/v) formaldehyde] for 10 minutes at room temperature. Following fixation, cells were blocked with 10% (v/v) FcR blocking reagent (Miltenyi Biotec, Germany) in PBS (for

non-permeabilised samples) or 0.02% (v/v) PBT (for permeabilised samples) for 30 minutes at room temperature.

For GPIb [cluster of differentiation 42b (CD42b)] and integrin β_3 (CD61) staining, cells were incubated with 1/50 dilution of anti-CD42b [SP219] rabbit antibody or 1/50 dilution of anti-CD61 [CRC54] mouse antibody (Abcam) in PEB buffer [PBS with 2mM ethylenediaminetetraacetric acid (EDTA) and 0.5% (w/v) BSA] for 20 minutes at room temperature. Cells were then washed in PEB and incubated with anti-rabbit Alexa Fluor 647® or anti-mouse Alexa Fluor 647® (ThermoFisher Scientific, UK) respectively for 20 minutes in the dark. Finally, cells were diluted 1/10 in PEB and analysed in an Accuri C6 Sampler Plus.

For TLR4 staining, cells were incubated with 1/10 dilution of anti-TLR4 CD284-PE [HTA125] antibody or its isotype IgG2a-PE control (Miltenyi Biotec) in PEB buffer in the dark at room temperature. Samples were then washed twice with PEB buffer prior to analysis of the samples in an Accuri C6 Sampler Plus (BD, Belgium). Each step was followed by spinning down the cells at 300*g* for 5 minutes at room temperature.

Luciferase assay

The luciferase assay performed in this study was modified from previous experiments [24]. Briefly, $3.75x10^5$ Meg-01R cells were seeded into a 24-well suspension plate in starvation media (RPMI-1640 with 2mM L-glutamine) and left in a 37°C 5% incubator for 4 hours. Treatments were introduced to cells in RPMI-1640 containing 20% FCS and 2mM L-glutamine. For experiments involving inhibitors, Meg-01R cells were incubated with the inhibitor during and after the starvation period. Following induction, cells were incubated for 24 hours. Meg-01R cells were removed and any remaining adherent cells were rinsed off with media and collected. Meg-01R cells were washed prior to resuspension in cell lysis buffer (Promega, UK). Each sample was agitated for 2 hours and then centrifuged at 5000*g* for 5 minutes at room temperature. Luciferase activity was measured by addition of luciferase substrate (Promega) and the luminescence at all wavelengths was recorded using a SpectraMax iD3 multi-mode microplate reader (Molecular Devices, UK).

Confocal microscopy

For each condition, $1x10^6$ Meg-01 cells were stained in suspension. Cells were fixed in 4% (w/v) paraformaldehyde for 10 minutes prior to blocking with FcR blocking reagent in PBS (non-permeabilised) or 0.02% PBT (permeabilised). 1/100 dilution of mouse anti-TLR4 antibody (Abcam) was used to detect TLR4 after incubation with cells for 1 hour at room temperature. 1/300 dilution of Alexa Fluor 555®-conjugated goat anti-mouse IgG antibody (ThermoFisher Scientific) was used to visualise binding of the anti-TLR4 antibody to its target. Finally, the nuclei of Meg-01 cells were stained with 1/2000 4',6-diamidino-2-phenylindole (DAPI) before resuspension in Mowiol 4-88 (Sigma-Aldrich) and mounting on a microscope slide. Slides were then imaged using a Nikon A1R confocal microscope and images were processed using Nikon NIS-Elements software and ImageJ.

Statistical analysis

Logarithmic dose-response curves for puromycin toxicity were generated using a four-parameter curve with variable slope. For cell doubling time, an unpaired Student's t-test was used to compare the mean values. For grouped data, analysis was performed using multiple t-tests with the type I error rate controlled for using the Holm-Sidak method. For experiments containing multiple comparisons, data were analysed using a one-way ANOVA with Bonferroni's post-hoc test. All statistical analysis was conducted using GraphPad Prism 8 (GraphPad, USA). Data are represented as mean ± SD.

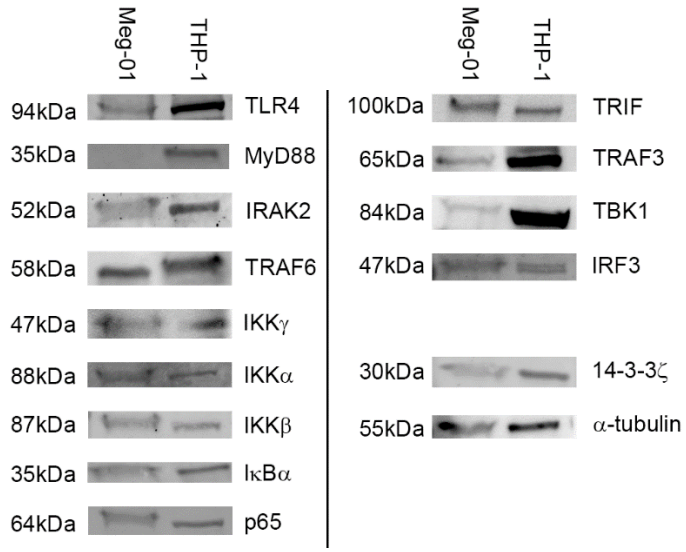

Figure 1: **Presence of proteins involved in Toll-like receptor 4 signalling pathways in Meg-01 cells.** Whole cell Meg-01 and THP-1 lysates were investigated via immunoblotting for the expression of TLR4, MyD88, IRAK2, TRAF6, IKKγ, IKKα, IKKβ, IκBα, p65, TRIF, TRAF3, TBK1, and IRF3, 14-3-3ζ and α-tubulin were used as loading controls. Proteins were detected via either fluorescence or enhanced chemiluminescence.

RESULTS

Meg-01 cells express various signalling proteins involved in TLR4 pathways

To determine whether Meg-01 cells are capable of inducing MyD88-dependent and -independent signalling upon ligation of inflammatory molecules such as LPS with TLR4, immunoblotting analysis was performed using Meg-01 cell lysates to confirm the presence of various signalling proteins. Meg-01 cell lysate was tested concurrently with an equivalent mass of THP-1 cell (a monocyte cell line capable of responding to LPS [26]) lysate as a positive control. As expected, THP-1 cells express notable levels of TLR4, MyD88, IRAK2, TRAF6, IKKγ, IKKα, IKKβ, IκBα, p65, TRIF, TRAF3, TBK1, IRF3, and control proteins, 14-3-3ζ and α-tubulin (Figure 1). MyD88 was not detectable by this assay however this is likely to be due to a low expression level of this protein.

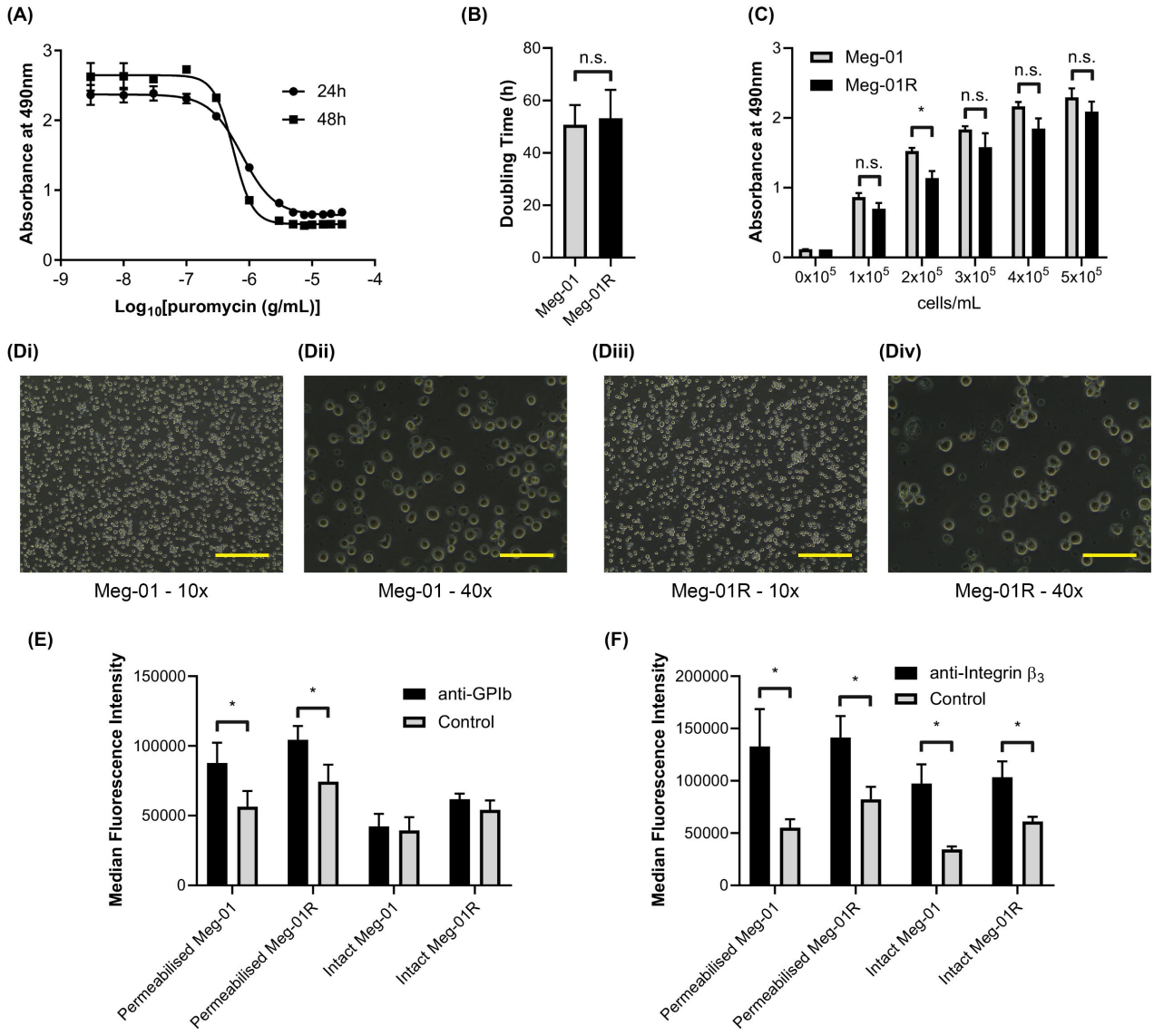

Figure 2: **Characterisation of Meg-01 and Meg-01-NFκB-Luc-GFP reporter (Meg-01R) cells.** (A) Meg-01 cells were treated with puromycin and their viability was tested by an XTT assay to determine the susceptibility of naïve cells. Data represent mean ± SD after 24 hours or 48 hours incubation of $5x10^4$ cells with puromycin (n=3). (B) The growth rate of Meg-01 and Meg-01R cells was compared to determine if the transduction process hindered growth. Data was analysed using a Student's t-test. (C) An XTT assay using different cell concentrations was performed to determine if the transduction process altered cell metabolism (n=3). Data were analysed using multiple t-tests corrected using the Holm-Sidak method. (D) The morphology of Meg-01 at 10x (i) and 40x (ii) and Meg-01R cells at 10x (iii) and 40x (iv) were compared using light microscopy. Scale bar represents 400µm at 10x and 100µm at 40x. Images shown are representative of 5 regions of interest taken from 3 separate flasks. Expression of GPIb

(E) and integrin β_3 (F) intracellularly and on the surface of Meg-01 and Meg-01R cells was compared to determine if the transduction process had modified the expression of megakaryocytic lineage cell markers (n=4 and n=3 respectively). Data were analysed using multiple t-tests corrected via the Holm-Sidak method. *p<0.05.

Development of a Meg-01R (NF-κB) reporter cell line

Viral particles containing the NF-κB reporter sequence were generated from HEK-293 cells in order to develop an NF-κB reporter Meg-01 cell line, Meg-01R, to aid in determination of the role of megakaryocyte-lineage cells during inflammation. The commercially-available luciferase gene was used to produce a non-endogenous protein whose activity is easily detectable [27]. Successful transduction was confirmed by stimulating Meg-01R cells with tumour necrosis factor-α (TNFα) and treating cell lysates with luciferin.

The optimal concentration of puromycin required to eradicate ~80% of non-transfected Meg-01 cells was determined using an XTT assay (Figure 2A). The results demonstrate that puromycin was capable of inducing cell death both over 24 hour and 48 hour time scales with an EC_{50} of 760ng/mL and 560ng/mL, respectively. Based on these results, a concentration of 1µg/mL puromycin was used to select transduced cells, which contain a puromycin-resistance gene, in subsequent experiments.

To elucidate differences or similarities between the transduced Meg-01R and standard Meg-01 cells, the doubling time of these cells were compared. The growth rate of Meg-01 (50.7 hours ± 4.35) and Meg-01R (53.2 hours ± 6.28) cells cultured parallelly was not significantly different (Figure 2B). Moreover, their viability was determined using an XTT assay and it did not show any significant differences after 6 hours except when cells were seeded at $2x10^5$ cells/mL (Figure 2C). This slight reduction may be explained by the presence of 1µg/mL puromycin throughout the experiment to prevent non-transfected cells from interfering with the results. To investigate whether the transduction altered the cellular morphology, light microscopy was used to analyse Meg-01 and Meg-01R cells. Meg-01 cells at both 10x (Figure 2Di) and 40x (Figure 2Dii) exhibited a spherical morphology when cultivated in suspension, and the same morphology was observed in Meg-01R cells (Figure 2Diii-2Div). Furthermore, the expression of specific cell markers was investigated using flow

cytometry. GPIb was detectable in permeabilised cells of both Meg-01 and Meg-01R at similar levels (Figure 2E) although it was not detected on intact cells. Integrin β_3 was detectable in both permeabilised and intact Meg-01 and Meg-01R cells (Figure 2F) [19]. Together, these data suggest that the transduction in Meg-01R cells did not affect the proliferation, growth, and major characteristics of these cells compared to Meg-01.

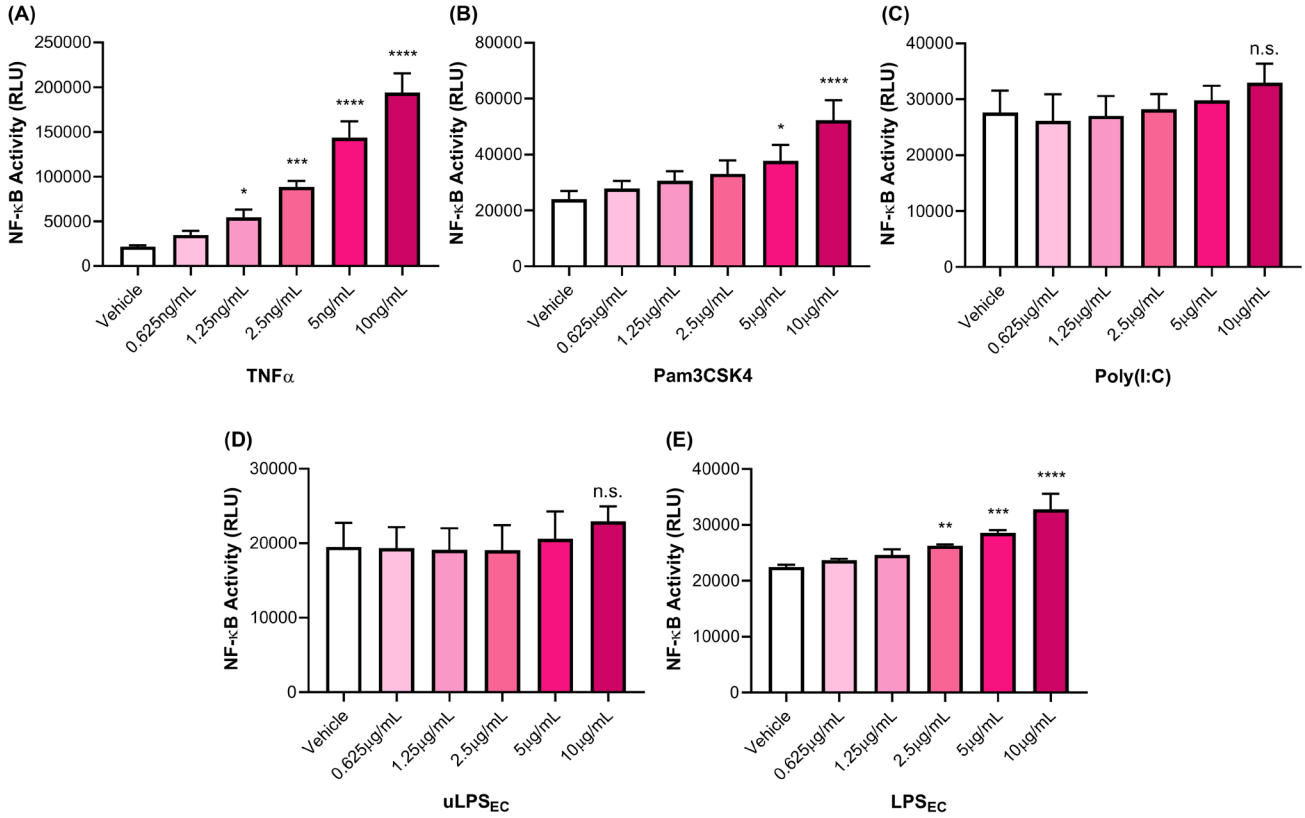

Figure 3: **NF-κB activity is altered by different pro-inflammatory molecules.** Meg-01R cells were incubated with TNFα (A) or a TLR1/2 agonist Pam3CSK4 (B) for 24 hours and the level of NF-κB activity was recorded. Furthermore, NF-κB activity was recorded in Meg-01R cells challenged with various concentrations of a TLR3 agonist, Poly(I:C) (C), or ultrapure (D) or non-ultrapure (E) versions of the TLR4 agonist LPS_{EC} for 24 hours. The level of NF-κB activity was measured by quantifying the level of luminescence. Data represent mean ± SD (n=3) and statistical significance (*p<0.05, **p<0.01, ***p<0.001, and ****p<0.0001) was calculated using a one-way ANOVA with Bonferroni post-hoc test.

Inflammatory molecules stimulate NF-κB activity in Meg-01R cells

To determine whether Meg-01R cells respond to various inflammatory molecules, luciferase assays were performed as a measure for NF-κB activity. Meg-01R cells were incubated with increasing concentrations of TNFα [Figure 3A; a ligand for tumour necrosis factor receptor I and II (TNFRI/II), both of which are expressed on Meg-01 cells [18]], Pam3CSK4 (Figure 3B; a ligand for TLR1/2 [16]), Poly(I:C) (Figure 3C; a ligand for TLR3 [28]), or ultrapure and non-ultrapure preparations of LPS from *Escherichia coli* [$uLPS_{EC}$ (a ligand for TLR4 [29]; Figure 3D) and LPS_{EC} (Figure 3E) respectively] 24 hours prior to lysis, and addition of luciferin to measure the level of luminescence. The results demonstrate that TNFα induced significant luciferase/NF-κB activity in a concentration-dependent manner (from 1.25ng/mL to 10ng/mL). Similarly, Pam3CSK4 increased NF-κB activity at 5μg/mL and 10μg/mL. In contrast, Poly(I:C) did not significantly increase NF-κB activity at the concentrations tested. Interestingly, whilst $uLPS_{EC}$ was incapable of inducing NF-κB activity from Meg-01R cells, the non-ultrapure preparation tested was able to stimulate significant NF-κB activity. These results demonstrate that Meg-01R cells produce functional luciferase as a marker for NF-κB activity and respond to various inflammatory molecules.

FPR2/ALX ligands do not modulate NF-κB activity in Meg-01R cells

Formyl peptide receptor 2 (FPR2/ALX), a seven-transmembrane receptor that couples to G_i proteins, is involved in the regulation of the innate immune system and has been found to be expressed on platelets [30–33]. Since the ligands of FPR2/ALX perform both pro- and anti-inflammatory activities, their impact on modulating NF-κB activity was analysed in Meg-01R cells. LL37 (an FPR2/ALX agonist that is known to activate platelets [32]), Ac2-26 (an annexin-1 mimetic peptide that acts as a pro-resolution mediator to control inflammation [34]), and amyloid-β1-42 (reported to act via TLR2/4, FPR2/ALX, and RAGE [24,35]) were tested in unstimulated Meg-01R cells. Based on its activity in other cell types, LL37 was hypothesised to increase NF-κB activity but instead significantly inhibited NF-κB activity when used at a concentration of 10μM (Figure 4A) whereas Ac2-26 (expected to be anti-inflammatory; Figure 4B) and amyloid-β (expected to be pro-inflammatory; Figure 4C) were unable to modulate NF-κB activity at any of the concentrations tested. Furthermore, BML-111 (a synthetic lipoxin A4 analogue and ligand for FPR2/ALX that is known as a pro-resolution mediator [36]) was tested at a range of concentrations in both the presence and

absence of 2.5ng/mL TNFα. In these experiments, TNFα was able to significantly induce the NF-κB activity over 24 hours whereas BML-111 (Figure 4D) was unable to significantly modulate the NF-κB activity in either stimulated or unstimulated Meg-01R cells. Further experiments would be necessary to determine the mechanisms that regulate the anti-inflammatory effects of LL37 in Meg-01R cells.

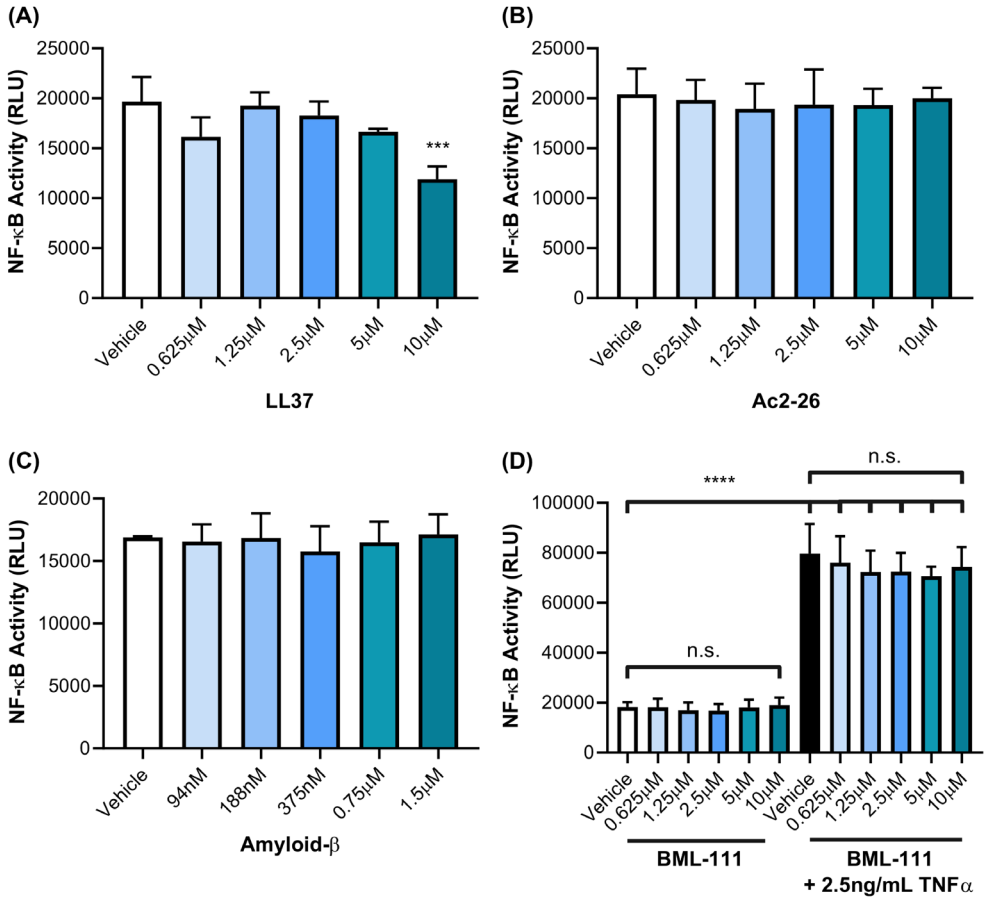

Figure 4: **Effect of FPR2/ALX ligands on NF-κB activity of Meg-01R cells.** Various ligands for FPR2/ALX were tested at different concentrations to determine their impact on NF-κB activity in Meg-01R cells over a 24-hour period. The FPR2/ALX agonist LL37 (A), the annexin A1-mimetic Ac2-26 (B), and amyloid-β (C) were tested in the absence of an inflammatory stimulus. An FPR2/ALX agonist, BML-111 (D) was tested in both the presence and absence of 2.5ng/mL TNFα. Data represent mean ± SD (n=3) and statistical significance (***p<0.001 and ****p<0.0001) was calculated using a one-way ANOVA with Bonferroni post-hoc test.

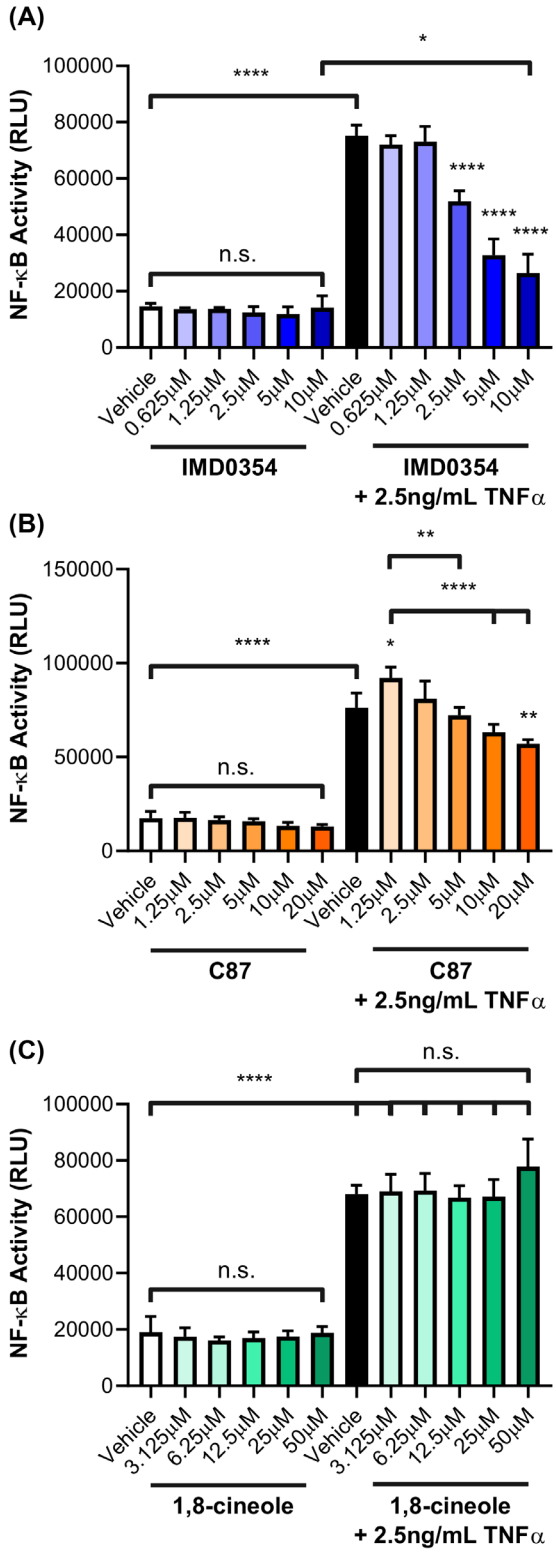

Figure 5: **Impact of TNFα-pathway inhibitors on luciferase activity in Meg-01R cells.** Different concentrations of an IKKβ inhibitor, IMD0354 (A), a TNFα antagonist C87 (B), and 1,8-cineole (C) were tested in both the presence and absence of 2.5ng/mL TNFα over a 24-hour period. Data represent mean ± SD (n=3) and statistical significance (*p<0.05, **p<0.01, and ****p<0.0001) was calculated using a one-way ANOVA with Bonferroni post-hoc test.

Small molecule inhibitors of TNFα signalling pathways impair NF-κB activity

IMD0354, C87, and 1,8-cineole were reported as small molecule inhibitors that are able to affect TNFα-induced NF-κB activity in other cell types. IMD0354 acts as an inhibitor of IKKβ to prevent the phosphorylation and subsequent degradation of IκB [24,37]. C87 acts as an antagonist to TNFα by directly binding to TNFα and thus has been proposed to disrupt the TNFα-TNFRI/II complex [38,39]. 1,8-cineole is a plant-derived compound that has been shown to inhibit nuclear translocation of p65 and degradation of IκBα [25,40]. In the absence of

TNFα, IMD0354 (Figure 5A), C87 (Figure 5B), and 1,8-cineole (Figure 5C) do not alter the NF-κB activity in Meg-01R cells. However, when cells were stimulated with 2.5ng/mL TNFα, IMD0354 significantly inhibited NF-κB in a dose-dependent manner. C87 was also capable of inhibiting NF-κB activity in Meg-01R cells at 20μM. Interestingly, C87 mildly potentiated TNFα-induced NF-κB activity at 1.25μM, however this increase was not observed at higher concentrations. In this cell type, 1,8-cineole was unable to inhibit TNFα-induced NF-κB activity. These results demonstrate that the inhibitory potential of various small molecule inhibitors can be examined using Meg-01R cells.

TLR4 is predominantly present inside Meg-01 cells

To further scrutinise the lack of Meg-01R response to the ultrapure preparation of LPS, the level of TLR4 expression on the surface and inside of Meg-01 cells was examined. The results obtained using confocal microscopy demonstrate that TLR4 is largely detectable inside Meg-01R cells as only the permeabilised cells show strong binding to anti-TLR4 antibodies (Figure 6A and B). The z-stack image in Figure 6C further demonstrates that TLR4 is expressed ubiquitously within the cytoplasm.

Additionally, the absence of TLR4 on the surface of Meg-01R cells was corroborated using a flow cytometry-based assay. Here, the binding of an anti-TLR4 antibody to the surface of Meg-01R cells was not significantly different from the isotype controls (Figure 6D). Conversely, the binding of the anti-TLR4 antibody was significantly greater in permeabilised cells compared to their isotype control. Further experiments are required to determine whether activating Meg-01R cells with specific molecules (for example valproic acid) might elevate the levels of TLR4 on the surface which would then enable ligation with LPS molecules in order to exert inflammatory effects (e.g. NF-κB activity).

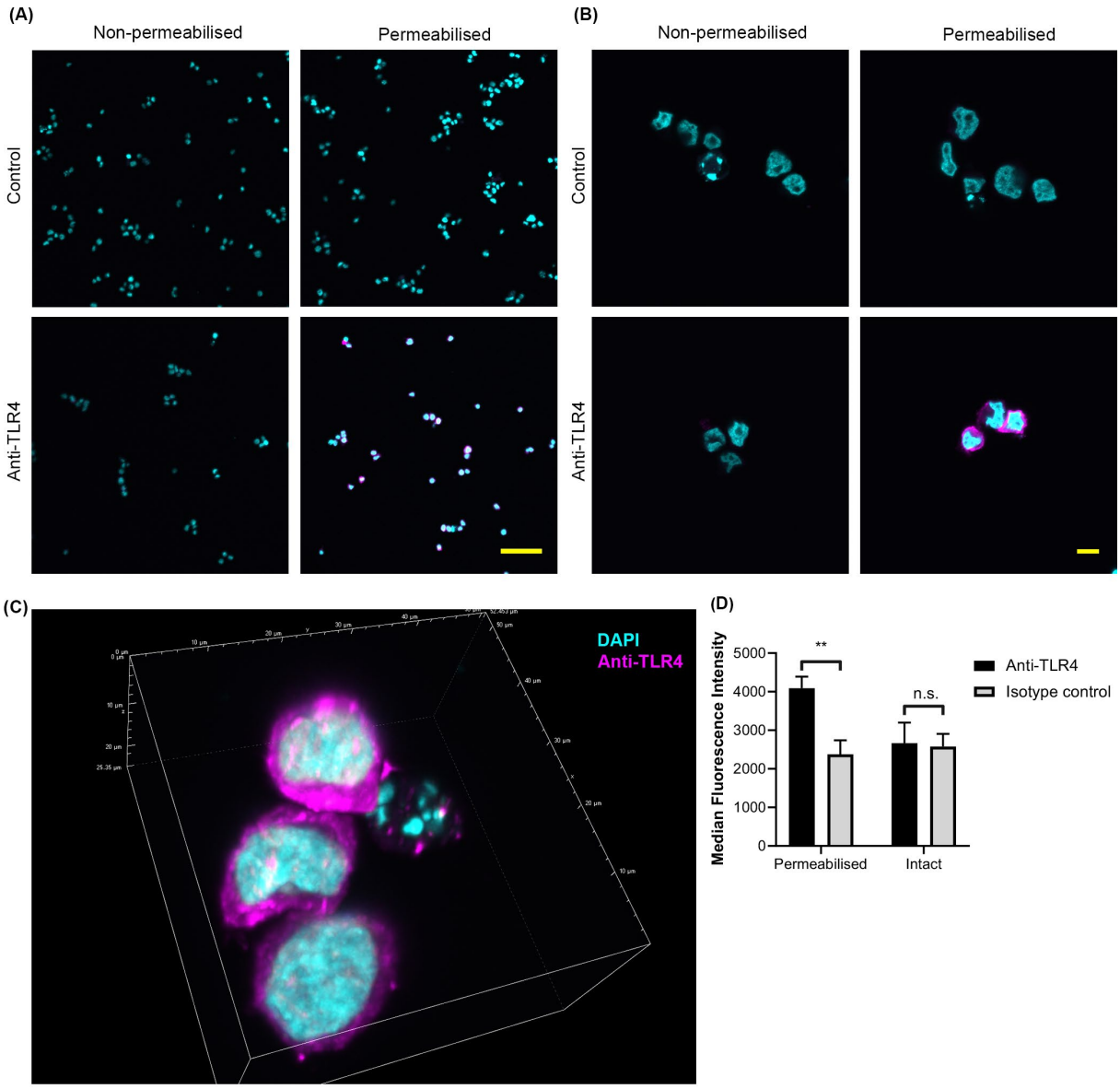

Figure 6: **TLR4 is detectable mainly inside Meg-01 cells.** (A) 20x magnification images of fluorescently labelled Meg-01 cells acquired via confocal microscopy. Images represent non-permeabilised negative control, permeabilised negative control, permeabilised cells with the anti-TLR4 antibody present, and non-permeabilised cells with an anti-TLR4 antibody present (clockwise from the top left). Scale bar shows 100µm. (B) Same as A but images were acquired with 100x magnification. Scale bar denotes 10µm. (C) 3D reconstruction of Meg-01 cells taken with a 100x objective showing the distribution of TLR4 in the cytoplasm. In all images, nuclei have been stained with DAPI (cyan) and the anti-TLR4 antibody is shown in magenta. (D) Flow cytometry-based assays were used to quantify the location of TLR4 by quantifying antibody binding to both permeabilised and non-permeabilised cells. Median

fluorescence intensity of TLR4 expression in non-permeabilised and permeabilised Meg-01R cells (n=3) was calculated. Data represent mean ± SD and were analysed using multiple t-tests with the type I error rate corrected for using the Holm-Sidak method (**p<0.01).

CD14 and LL37 do not enable LPS to stimulate NF-κB activity in Meg-01R cells

To determine if TLR4-induced NF-κB activity could be promoted by CD14 (a TLR4 co-receptor [5,41,42]) or LL37 (a molecule capable of binding LPS [43–45]), the assays were performed in the presence and absence of these molecules along with LPS chemotypes. LL37 has been shown to be capable of transmitting LPS across the cell membrane of human epithelial cells [44].

Meg-01R cells were co-incubated with $uLPS_{EC}$ or ultrapure *Salmonella minnesota* LPS ($uLPS_{SM}$) for 24 hours with/without CD14 or LL37. A physiologically relevant concentration of 2μg/mL was chosen for CD14 [46]. The results show that CD14 did not significantly alter NF-κB activity in Meg-01R cells on its own, and it was not capable of promoting $uLPS_{EC}$ or $uLPS_{SM}$ to stimulate NF-κB activity (Figure 7A). Furthermore, LPS chemotypes did not induce any activity on their own. When LL37 and $uLPS_{EC}$ or $uLPS_{SM}$ were added simultaneously (Figure 7B) or following 15 minutes pre-incubation with LL37 (Figure 7C), there was no change in NF-κB activity compared to LL37 alone. These results demonstrate that CD14 and LL37 do not modulate LPS-induced NF-κB activity.

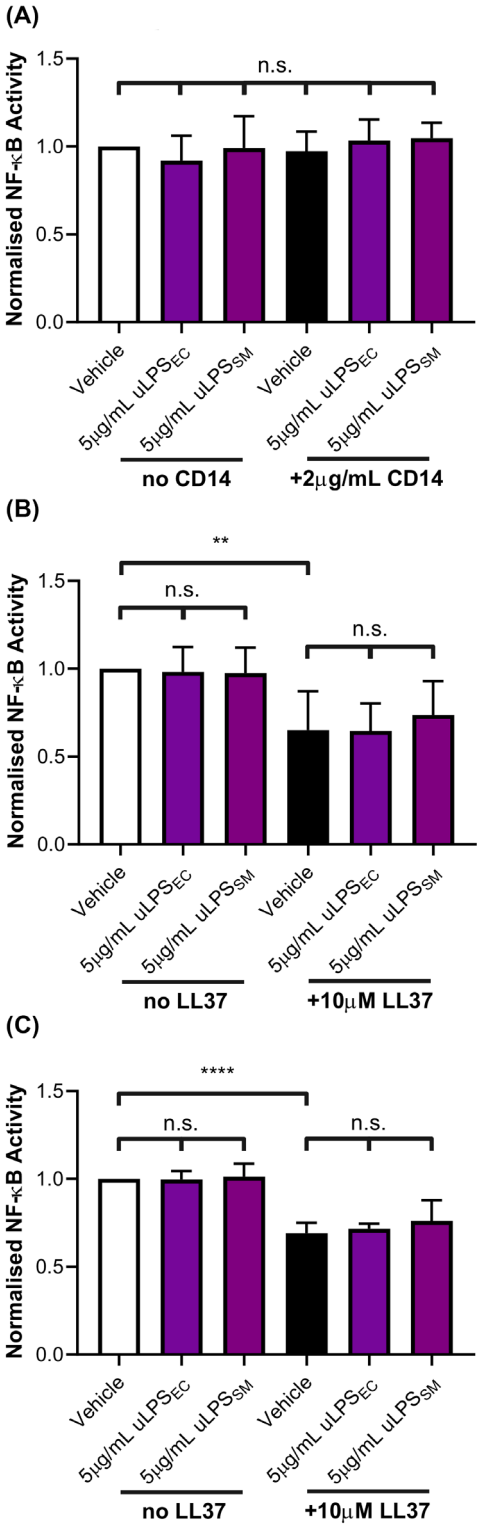

Figure 7: **The LPS-binding molecules CD14 and LL37 are unable to induce LPS to activate NF-κB.** NF-κB activity was measured in lysates from Meg-01R cells stimulated with $uLPS_{EC}$ or $uLPS_{SM}$ in the presence of 2μg/mL CD14 (A; n=3), or 10μM LL37 following no co-incubation prior to addition (B; n=6) or 15 minutes co-incubation (C; n=5). Data represent mean ± SD and statistical significance (**p<0.01 and ****p<0.0001) was calculated using a one-way ANOVA with Bonferroni post-hoc test.

DISCUSSION

The role of MKs during inflammation is not fully understood. Thrombopoietin (TPO) is known to stimulate the growth and development of MKs and it is removed from the plasma by platelets which means that, during thrombocytopaenia (which can be induced by inflammatory diseases such as sepsis [5]), high levels of TPO are detected in the plasma [7,47]. Very few studies have been conducted to examine the effect of PAMPs using MK cell lines which demonstrate that NF-κB can be activated [16,28]. Mice lacking TLR4 have significantly lower platelet counts than their wild-type counterparts but intravenous injection of a sublethal dose of LPS (0.2mg/kg) leads to a significant increase in platelet count a week after administration regardless of whether TLR4 was present or not [8]. Moreover, TLR2 and TLR4 have been shown to induce the production of IL-6, via NF-κB, in $CD34^+$ cells which leads to increased MK maturation and platelet production [14]. Furthermore, circulating platelets have

been shown to have higher expression of TLR2 and TLR4 in particular disease states which suggests that MKs can respond to inflammatory states to tailor the platelet phenotype to the altered pathological situation [8,10,48]. Moreover, as platelets are anucleate cells and TLRs lead to activation of transcription factors, it has been suggested that platelet TLRs are relics left over from MKs or haemopoietic stem cells [6].

TLR4 is unique amongst the TLR family of receptors in that it can signal via two distinct pathways [5]. These two pathways are known as the MyD88-dependent pathway and the MyD88-independent pathway [49–51]. TLR1, 2, 4, 5, 6, 7, 8, 9, and 10 can all activate the MyD88-dependent pathway whereas TLR3 and TLR4 activate the MyD88-independent pathway [5,49,52]. Here, we demonstrate that a plethora of proteins required for both MyD88-dependent signalling (TLR4, IRAK2, TRAF6, IKKγ, IKKα, IKKβ, IκBα, and p65) and MyD88-independent signalling (TLR4, TRIF, TRAF3, TBK1/NAK, and IRF3) are present in Meg-01 cells. Interestingly, MyD88 itself was detectable in the THP-1 cells used as a positive control but was not detectable in Meg-01. This is likely due to a low expression level of MyD88 compared to a specialised immune cell as MyD88 has been detected in platelets [53] and MyD88-dependent signalling has been reported in Meg-01 cells [16]. Further research, utilising quantitative polymerase chain reaction (qPCR) methods or increased lysate concentrations, is required to confirm the presence of MyD88 in Meg-01 cells. One complication with identifying proteins in Meg-01 cells and MKs however is their ability to produce platelet-like particles or platelets respectively as it can be difficult to ascertain whether the proteins are present for signalling in the progenitor cell or merely produced for packaging into the progeny.

IκB prevents binding of p65 (a member of the NF-κB family of transcription factors) to its specific DNA promoter sequence but is degraded following phosphorylation by IKKβ and ubiquitinated prior to its degradation [5,54,55]. p65 is a transcriptional activator that enables transcription of inflammatory cytokines in response to PAMPs and DAMPs, such as ligands of TLRs and TNFα [5,55,56]. The MyD88-dependent pathway directly leads to NF-κB activation brought about by IκBα degradation however cross-talk between the two pathways mean that the MyD88-independent pathway can also induce NF-κB activation [5,28].

Meg-01 cells and MKs are reported to express TLR1, 2, 3, 4, and 6 and are frequently used as a surrogate for elucidating signalling pathways in MKs [16–19,28,57–59]. As these receptors can all induce NF-κB activation, we endeavoured to develop a reporter cell line to determine the role of MKs in regulating inflammation. Therefore, we transfected HEK-293 cells with a previously published and commercially-available reporter plasmid (pGreenFire-NFκB-Puro) [24] to produce lentiviral particles that can stably transfect other cell types with this reporter construct. Successfully transduced cells were selected for with puromycin, a compound which is cytotoxic to mammalian cells. A concentration capable of killing ~80% of non-transduced Meg-01 cells was used to ensure that a pure population of Meg-01-GFP-Luc-NFκB-Puro (Meg-01R) cells developed.

Initially, the Meg-01R cell line was compared with Meg-01 cells to determine whether the transduction process adversely affected the cells or changed their phenotype. Growth rate and morphology remained constant throughout the two cell types and only a minor defect in viability was detected which may be due to the presence of puromycin. Furthermore, Meg-01R cells express MK lineage markers in the same compartments as Meg-01 cells. The Meg-01R cells were then characterised by stimulating them with a range of PAMPs and DAMPs. Firstly, a DAMP, TNFα was tested for its effects as Meg-01 cells have been reported to express both TNFRI and TNFRII, two receptors that stimulate NF-κB activation [18,56]. TNFα was able to significantly activate NF-κB to transcribe luciferase in a concentration-dependent manner.

Pam3CSK4 is a synthetic ligand for the TLR1/2 heteroreceptor and it has previously been shown to induce phosphorylation of NF-κB's p65 subunit and degradation of IκB over the period of one hour [16]. Phosphorylation of p65 was also shown to occur in murine MKs following 30 minutes of treatment with Pam3CSK4 [16]. In accordance with this previous study, Pam3CSK4 was able to induce luciferase production downstream of NF-κB albeit not as strongly as the lowest concentration of TNFα tested.

Poly(I:C) is a synthetic ligand for TLR3 and, although TLR3 couples to the MyD88-independent pathway, signalling downstream of TLR3 can lead to NF-κB activation [28]. Poly(I:C) and Poly(A:U) (another synthetic TLR3 ligand) have been shown to induce IκB degradation and phosphorylation of p65 in human CD34+ cells

derived from umbilical blood [28]. In this study, Poly(I:C) was incapable of stimulating the NF-κB activity at any of the concentrations tested. This discrepancy may be due to changing expression levels and characteristics observed during MK maturation.

The preparation of LPS_{EC} can alter the characteristics of the responses induced by this ligand therefore an ultrapure and a non-ultrapure version were both tested. This has been hypothesised to be due to the presence of bacterial contaminants in non-ultrapure preparations that can activate other pro-inflammatory receptors such as TLR2 [29,52]. The ultrapure version of LPS_{EC} was unable to activate NF-κB however the non-ultrapure preparation was capable of inducing activity at a range of concentrations. This finding lends more weight to the hypothesis that many of the effects observed when using LPS_{EC} may be induced by contaminants in the preparations.

Moreover, FPR2/ALX ligands were tested on Meg-01R cells to determine if they could affect NF-κB activity in this cell type. The agonist, LL37 [32], was able to significantly inhibit NF-κB activity on its own over a period of 24 hours in contrast to the annexin A1-mimetic, Ac2-26 [34]. Amyloid-β (1-42) is a neuropeptide involved in the progression of Alzheimer's disease. Indeed, it has been related to inflammation in the brain and is capable of inducing NF-κB activity in a glioblastoma cell line [24,35]. However, amyloid-β was incapable of activating NF-κB at any of the tested concentrations in this study. This may be due to the concentrations tested being subthreshold. Interestingly, the synthetic lipoxin A4-analogue, BML-111 was incapable of modulating NF-κB activity in the absence or presence of TNFα [36]. However, this may be due to signalling via a different downstream pathway.

A potential application of this novel reporter cell line is for the identification of compounds that affect inflammatory responses in MKs during disease states [15]. To evaluate this, Meg-01R cells were treated with an IKKβ inhibitor, IMD0354 [37], a TNFα antagonist, C87 [38], or 1,8-cineole for 24 hours in the presence or absence of TNFα. Neither IMD0354 nor C87 had a significant effect on unstimulated Meg-01R cells however they could both significantly inhibit the increase in NF-κB activity induced by TNFα. 1,8-cineole had no effect in either stimulated or unstimulated cells. Interestingly, the lowest concentration of C87 tested (1.25μM) potentiated the NF-κB activity induced by TNFα although the mechanism behind this is currently unknown.

Meg-01 cells have previously been described to express TLR4 when the cells were fixed with methanol, a fixative known to be capable of permeabilising cells [58]. To confirm the distribution of TLR4 in Meg-01 cells, they were fixed with 4% PFA (for immunocytochemistry) or 0.2% formyl saline (for flow cytometry) and then cells were left intact or permeabilised with PBS containing Triton-X100. Moreover, two different anti-TLR4 antibodies were used to confirm its presence depending on the detection method. With both methods, TLR4 was not detectable on the surface of Meg-01 cells however it was detectable intracellularly. Previous studies have suggested that megakaryocytes express TLR4 on their surface but this increases during megakaryocyte maturation [59]. Future work should investigate the effect of TLR4 ligands on NF-κB activity in differentiated Meg-01R cells (created following incubation with valproic acid [60]) in combination with measuring the change in surface receptor expression levels to determine if this reporter cell line can also be useful for signalling induced by this receptor.

Although TLR3 is predominantly detectable intracellularly, poly(I:C) is capable of being internalised leading to its activation [28]. In contrast, LPS is not capable of crossing the cell membrane on its own [44]. LPS is internalised after binding to CD14-TLR4 to enable it to enter endosomes and induce MyD88-independent signalling [41,42]. Furthermore, LL37 is known to be capable of binding LPS and other negatively-charged molecules, usually in an anti-inflammatory capacity [43–45,61,62]. Shaykhiev *et al.* [44] demonstrated, through the use of primary human bronchial epithelial cells and human pulmonary mucoepidermoid carcinoma NCI-H292 cell line, that 20µg/mL LL37 can induce the intake of LPS in epithelial cells over the course of 24 hours [44]. Importantly, this internalisation was shown to be independent of TLR4 and dependent of caveolae and functional epidermal growth factor receptor [44]. Three treatments – 2µg/mL CD14, simultaneous addition of 10µM LL37 (45µg/mL) and LPS, addition of 10µM LL37 and LPS after 15 minutes of co-incubation – were tested to determine if these proteins could deliver LPS to its target receptor. 2µg/mL CD14 had no effect on NF-κB activity in Meg-01R cells and was unable to promote LPS to induce luciferase synthesis. Furthermore, in both conditions, LL37 significantly inhibited luciferase synthesis however this was not modulated by the simultaneous addition of LPS, nor the co-incubation of LL37 and LPS. This may suggest that TLR4 in Meg-01R

cells is not found in the endosome and therefore it may not stimulate MyD88-independent signalling nor interact with internalised LL37/LPS complexes.

In conclusion, here we developed a megakaryocyte reporter cell line and demonstrated that megakaryocytes are responsive to a range of PAMPs and DAMPs and their pathway inhibitors. This reporter cell line is particularly useful for investigating TNFR-mediated and TLR2-mediated inflammation due to the robust increase in NF-κB activity that could be detected and modulated by small molecule inhibitors. However, this model is not suitable for investigating TLR4 signalling in MKs as TLR4 is expressed intracellularly in this cell type. More work is required to determine if this cell line is capable of investigating signalling by FPR2/ALX ligands. Furthermore, this tool can be applied to screen both pro-inflammatory and anti-inflammatory molecules.

ADDENDUM

T.M. Vallance, D. Widera, and S. Vaiyapuri designed the experiments, analysed the data, and wrote the manuscript. T.M. Vallance, J.J. Sheard, Y. Meng, and E.C. Torre performed the experiments and analysed the data. K. Patel has provided advice and support for the design of experiments.

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DISCLOSURES

The authors declare no competing interests.

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2.2 Supplementary information

Antibody	Clone	Concentration	Manufacturer
TLR4	76B357.1	1/250	Abcam (UK)
IκBα	E130	1/500	Abcam (UK)
MyD88	D80F5	1/500	Cell Signalling Technologies (UK)
IRAK2	Polyclonal	1/500	Cell Signalling Technologies (UK)
TRAF6	D21G3	1/500	Cell Signalling Technologies (UK)
IKKγ	DA10-12	1/500	Cell Signalling Technologies (UK)
IKKα	3G12	1/500	Cell Signalling Technologies (UK)
IKKβ	D30C6	1/1000	Cell Signalling Technologies (UK)
TRIF	Polyclonal	1/500	Cell Signalling Technologies (UK)
TRAF3	Polyclonal	1/500	Cell Signalling Technologies (UK)
TBK1/NAK	D1B4	1/500	Cell Signalling Technologies (UK)
p65	F-6	1/500	Santa Cruz Biotechnology (USA)
IRF3	FL-425	1/500	Santa Cruz Biotechnology (USA)
14-3-3ζ	1B3	1/2000	Santa Cruz Biotechnology (USA)
α-Tubulin	B-7	1/2000	Santa Cruz Biotechnology (USA)

Supplementary Table 1: **Antibody clones and concentrations used for detection of proteins in Meg-01 and THP-1 cells.**

2.3 Ultrapure lipopolysaccharide derived from *Rhodobacter sphaeroides* counteracts LL37-induced platelet activation

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Rationale of this chapter

After establishing that ultrapure LPS molecules are unable to modulate platelet and MK function on their own, we hypothesised that they might interact with other inflammatory molecules released under pathological conditions. Hence, we sought to determine the impact of ultrapure LPS on LL37-induced actions in platelets. LL37 is an antimicrobial peptide and a powerful inflammatory molecule that is known to strongly activate platelets. Here, we investigated the influence of LPS on platelets stimulated with LL37. While LPS_{EC} or LPS_{SM} did not show any effects on LL37-induced platelet function, we found that LPS_{RS} was capable of significantly inhibiting LL37-induced platelet activation. Further experiments confirmed that LL37-LPS_{RS} micelles are formed and counteract the effects of LL37 on platelets. Furthermore, we demonstrate a role of TLR4 in modulating outside-in signalling via integrin $\alpha_{IIb}\beta_3$. These data suggest that LPS_{RS} can be used as a therapeutic agent to reduce the increased levels of LL37 under inflammatory diseases such as atherosclerosis and psoriasis. Although we have planned to complete more experiments, particularly to determine the impact of LPS_{RS} under *in vivo* settings in the presence and absence of LL37, we were unable to perform these experiments due to the current restrictions. However, these experiments will be performed in the near future.

Contribution to this chapter

As the first author, I contributed nearly 85% to this chapter by designing and performing experiments, analysing the data, and preparing the manuscript and figures.

Experimental contribution

Aggregation experiments (Figure 1) were performed by me or with the help of CYL (Figure 1B and 1E). Platelet spreading experiments (Figure 2) were conducted by myself or with the help of MSNT. Platelet counting and morphological analysis was performed by myself. Figure 3A and B were performed by me and 3C was conducted in collaboration with AG. Experiments in Figure 4, S1, and S2 were performed by me.

Publication status: Manuscript in preparation

Ultrapure lipopolysaccharide derived from *Rhodobacter sphaeroides* counteracts LL37-induced platelet activation

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ABSTRACT

Objective

LL37 is a powerful platelet stimulant in pathological conditions where it acts as an immune modulator. We examine the influence of lipopolysaccharides (LPS) on LL37-induced platelet activation and determine its mechanism of action.

Approach and results

LL37-induced platelet aggregation was inhibited by 10µg/mL *Rhodobacter sphaeroides LPS* (LPS_{RS}) and 5µM FP12 [both known as Toll-like receptor 4 antagonists (TLR4)] whereas aggregation was unaffected by LPS derived from *Escherichia coli* or *Salmonella minnesota* (LPS_{SM}). Furthermore, LPS_{RS} was capable of inhibiting LL37-induced platelet adherence to immobilised fibrinogen as well as outside-in signalling through integrin $\alpha_{IIb}\beta_3$.

Immunoblotting for phosphoproteins and 5-FAM-LL37 binding studies reveal that inhibition of platelet activation was driven by an extracellular process as serine 473 phosphorylation of Akt and 5-FAM-LL37 binding to platelets was significantly reduced in the presence of LPS_{RS}.

Finally, it was determined that LPS_{RS} was able to form detectable micelles with LL37, in a concentration-dependent manner, at all concentrations tested. Moreover,

LPS_{SM} was also able to form detectable micelles with LL37 at specific concentrations, albeit at lower numbers that LPS_{RS} at an equivalent concentration.

Conclusion

In conclusion, LL37-induced platelet activation is inhibited by LPS_{RS} due to the formation of LL37-LPS micelles that prevent LL37 from binding to and activating platelets. Therefore, this discovery could potentially lead to the development of novel treatments for diseases caused by overexpression of LL37, such as atherosclerosis or psoriasis.

INTRODUCTION

Cardiovascular disease is the largest cause of mortality in both the developing and the developed word[1,2]. Both myocardial infarction and ischaemic stroke can trace their origins back to atherosclerotic lesions, that rupture and induce thrombus formation in the heart or brain respectively[3,4]. LL37 is a platelet agonist that has been detected in atherosclerotic lesions and arterial thrombi and therefore presents itself as a potential drug target for mitigating thrombus size and reducing arterial occlusion[5–7]. Furthermore, LL37 overexpression has been strongly linked to the pathogenesis of psoriasis, a disease that has a very high impact on quality of life[8].

Cathelicidins are polypeptides with anti-microbial properties that can interact with the adaptive and innate immune systems[9]. LL37, derived from human cationic antimicrobial protein-18 (hCAP18), is the only cathelicidin produced in humans[9,10]. LL37 is released by immune cells, epithelial cells, and platelets and has been reported to signal through multiple receptors including formyl peptide receptor 2 (FPR2/ALX), glycoprotein VI (GPVI), P2X7 receptor, C-X-C motif chemokine receptor 2 (CXCR2), and the epidermal growth factor receptor (EGFR)[6,7,9–14]. LL37 acts as a chemotactic agent for neutrophils, monocytes, and T-cells, in addition to its role as a platelet agonist[13–15]. Platelets are responsible for haemostasis and also possess a sentinel role in the innate immune system[16–18]. Moreover, LL37-stimulated platelets drive the production of neutrophil extracellular traps (NETs)[6]. The mechanism of action behind LL37-induced platelet activation has yet to be fully elucidated as both FPR2/ALX and GPVI (with Syk) have been proposed to be the target receptors in platelets[6,7].

The effect of lipopolysaccharides (LPS) derived from Gram-negative bacteria, and the role of their receptor, Toll-like receptor 4 (TLR4), on platelets remains controversial[19–25]. LPS is comprised of three regions: the hydrophobic lipid A domain containing multiple acyl chains ranging from 12-16 carbon atoms in length, an oligosaccharide core, and an O-antigen polysaccharide[26,27]. LPS is negatively-charged, due to a 1,4-bis-phosphorylated diglucosamine backbone and numerous oligosaccharides, and commonly binds to positively-charged LL37[9,16]. The binding of LPS to LL37 has been reported to be anti-inflammatory as it reduces tumour necrosis factor (TNF)-α production from THP-1 cells[28] and also pro-inflammatory as it increases interleukin (IL)-1β production and adenosine triphosphate (ATP) release from LPS-primed monocytes[29].

Furthermore, LL37 reduces IL-8 and IL-6 secretion (induced by LPS) in primary human gingival fibroblasts, BEAS-2B cells (a bronchial epithelium cell line), and peripheral blood mononuclear cells whilst also preventing inhibitor of NF-κB α (IκBα) degradation and interferon regulatory factor (IRF)-3 phosphorylation[30–32]. Experiments by Molhoek *et al.*[32] reveal that the cationic and the hydrophobic nature of LL37 are largely responsible for the peptide's inhibition of LPS-mediated cellular activity[32].

Further characterisation of LL37-LPS complexes demonstrated that the structure of these complexes is partially dependent on the length of the O-antigen polysaccharide in LPS[33,34]. Smooth LPS (with a longer O-antigen region) in the presence of LL37 forms short, unbranching micelles whereas rough LPS (containing no O-antigen or a very short O-antigen region) forms elongated structures with irregular toroids (doughnut-shaped structures)[33]. LL37 was determined to interact with rough LPS chemotypes via the core oligosaccharides and the 1,4-bis-phosphorylated glucosamine phosphate groups of the lipid A moiety[33,34]. Furthermore, LL37 altered the structure of rough LPS aggregates (from lamellae to toroids), possibly by increasing the curvature of the LPS bilayer[33].

As LL37 and LPS are known to interact and modulate their properties and activities, we examined the impact of ultrapure *Rhodobacter sphaeroides* LPS (LPS_{RS}) on LL37-induced platelet activation, to determine its potential as a novel therapeutic for treatment of conditions caused by an overexpression of LL37. Subsequently, the mechanism of action of LPS_{RS}-mediated platelet inhibition was determined. Here, we

demonstrate that LPS_{RS} inhibits LL37-mediated platelet activation and outside-in signalling via integrin $\alpha_{IIb}\beta_3$, potentially by quenching the LL37 present in solution to prevent it from binding to its target receptor.

METHODS

Materials

Ultrapure lipopolysaccharide from *Rhodobacter sphaeroides* (LPS_{RS}), *Escherichia coli* O111:B4 (LPS_{EC}), and *Salmonella enterica minnesota* R595 (LPS_{SM}) were purchased from Invivogen (France) and resuspended in endotoxin-free water (Sigma-Aldrich, UK). LL37 was purchased from Tocris (UK). All other chemicals were purchased from Sigma-Aldrich unless otherwise stated.

Preparation of human isolated platelets

The isolated platelets used in these experiments were obtained as described previously[35–37]. Briefly, human whole blood was drawn from healthy, aspirin-free donors who gave informed consent. Whole blood was collected in vacutainers containing 3.2% (v/v) citrate and further mixed with 12% (v/v) acid citrate dextrose [ACD; 2.5% (w/v) sodium citrate, 2% (w/v) glucose, 1.5% (w/v) citric acid] before being centrifuged at 102*g* for 20 minutes. Platelet-rich plasma (PRP) was extracted and centrifuged in the presence of 12% (v/v) ACD and 50ng/mL prostacyclin (PGI_2) for 10 minutes at 1413*g*. Following removal of the supernatant and resuspension in modified Tyrode's-HEPES buffer (134mM NaCl, 2.9mM KCl, 0.34mM $Na_2HPO_4.12H_2O$, 12mM $NaHCO_3$, 20mM HEPES, 1mM $MgCl_2$, pH 7.3) containing 5mM D-glucose, 12% (v/v) ACD, and 50ng/mL PGI_2, the platelet suspension was further centrifuged for 10 minutes at 1413*g*. The platelet suspension was resuspended in modified Tyrode's buffer containing D-glucose, to produce isolated platelets, and allowed to rest for 60 minutes at 30°C prior to the beginning of experiments.

Platelet aggregation

Human isolated platelets were stimulated with multiple concentrations of LL37 in the presence or absence of varying concentrations of different ultrapure LPS chemotypes. To determine the extent of aggregation, a Chrono-Log Model 700

aggregometer (USA) or a Chrono-Log Model 490 4+4 aggregometer (USA) was used to measure light transmission through the sample under constant stirring conditions at 37°C. Both the maximum aggregation and the area under the curve (AUC) were recorded to account for complex trace patterns. Lysates for immunoblotting were obtained from these experiments by mixing aggregated platelets with reducing sample treatment buffer [RSTB; 69mM sodium dodecyl sulphate, 5% (v/v) 2-mercaptoethanol, 10% (v/v) glycerol and 25mM Tris-HCl].

Platelet spreading

Coverslips (VWR, UK) were coated with 100µg/mL fibrinogen (in modified Tyrode's-HEPES buffer) for 30 minutes and blocked with 1% (w/v) BSA in modified Tyrode's-HEPES buffer for 1 hour. The coverslips were then washed three times with phosphate-buffered saline (PBS). Following incubation of isolated platelets ($2x10^7$cells/mL) with either 10µg/mL LPS_{RS} or vehicle for 10 minutes, they were treated with 10µM LL37. Platelets were then allowed to adhere to the fibrinogen-treated coverslips for 0, 5, 10, 15, 20, or 30 minutes prior to fixation in 0.2% formyl saline [0.9% (w/v) NaCl and 0.2% (v/v) formaldehyde] for 10 minutes. Following washing with PBS, platelets were permeabilised with PBS containing 0.2% (v/v) Triton X-100 (PBT) for 5 minutes and the actin cytoskeleton of the adhered platelets was stained using Alexa Fluor 488®-conjugated phalloidin (Fisher Scientific, UK) before another wash step. Finally, one drop of Mowiol 4-88 was added to the coverslip as a mounting medium and left overnight at 4°C to polymerise. Images of platelets on coverslips were acquired by a Nikon A1-R Confocal Microscope using a 60x oil-immersion objective and analysed using ImageJ.

Immunoblotting

Whole human platelet lysates lysed by RSTB were boiled for 10 minutes at 90°C to aid protein denaturation before 30µL of sample was loaded into a 4-15% pre-cast gel (Bio-Rad, UK). After lysates were run through the gel under 200V constant voltage, they were transferred to a polyvinylidene difluoride membrane (GE Healthcare, USA) using a Semi-Dry Transfer System (Bio-Rad). Following transfer, these membranes were blocked in 5% (w/v) bovine serum albumin (BSA; Fisher Scientific) in PBS with 0.1% (v/v) Tween-20 (PBS-T) for 1 hour at room temperature. Membranes were then incubated overnight at 4°C with phospho-Akt (Ser473) [D9E] rabbit monoclonal

antibodies (Abcam, UK). Cy5-conjugated goat anti-rabbit IgG antibodies (1/2500 dilution) (Thermo Fisher Scientific, UK) and a Typhoon 9400 variable mode imager (GE Healthcare) (488V) were used to detect the binding of the p-Akt antibody, and 14-3-3ζ [1B3] (Santa Cruz Biotechnology, USA) was used as a loading control. Images were quantified using ImageJ and intensity presented as a ratio of pS473-Akt band intensity to 14-3-3ζ band intensity.

Binding of LL37 to platelets

Human isolated platelets were incubated, at room temperature, with 10µg/mL LPS_{RS} or vehicle for 10 minutes. Subsequently, the platelets were stimulated with 10µM 5-FAM-LL37 for 5 minutes and the entire sample was diluted 1 in 6 in HEPES-buffered saline (150mM NaCl, 5mM KCl, 2mM $MgSO_4.7H_2O$, and 10mM HEPES, pH 7.4) that had previously been filtered using a 0.2µm diameter filter. Fluorescence on platelets was detected using an Accuri C6 CSampler flow cytometer (Becton-Dickinson Biosciences, USA).

Flow cytometry

LL37 and LPS were added to 0.2µm-filtered Tyrode's-HEPES buffer at the appropriate concentrations and incubated together for 5 minutes on a rotational plate shaker at room temperature. 50µL of each sample was run through a BD Accuri C6 CSampler flow cytometer and the total number of events was recorded. Blank samples were run following every range of LPS concentrations to minimise background events.

Statistical analysis

All data analysis was performed using GraphPad Prism 8. Differences in maximal platelet aggregation and AUC were calculated using a paired t-test or a repeated-measures ANOVA with Bonferroni or Dunnett's post-hoc test where appropriate. Total count of spread platelets was normalised to each donor's final count following 30 minutes of 10µM LL37 and compared with the treated group using multiple t-tests corrected for using the Holm-Sidak method. Similarly, differences in platelet morphology between treatment groups were tested for using multiple t-tests corrected for using the Holm-Sidak method. Immunoblotting values were compared using a repeated measures ANOVA with Bonferroni's post-hoc test. Differences in 5-FAM-LL37 binding were determined using a paired t-test. All flow cytometry data was tested

with a one-way ANOVA followed by a Bonferroni post-hoc test if the F-value was statistically significant. $p<0.05$ was considered statistically significant.

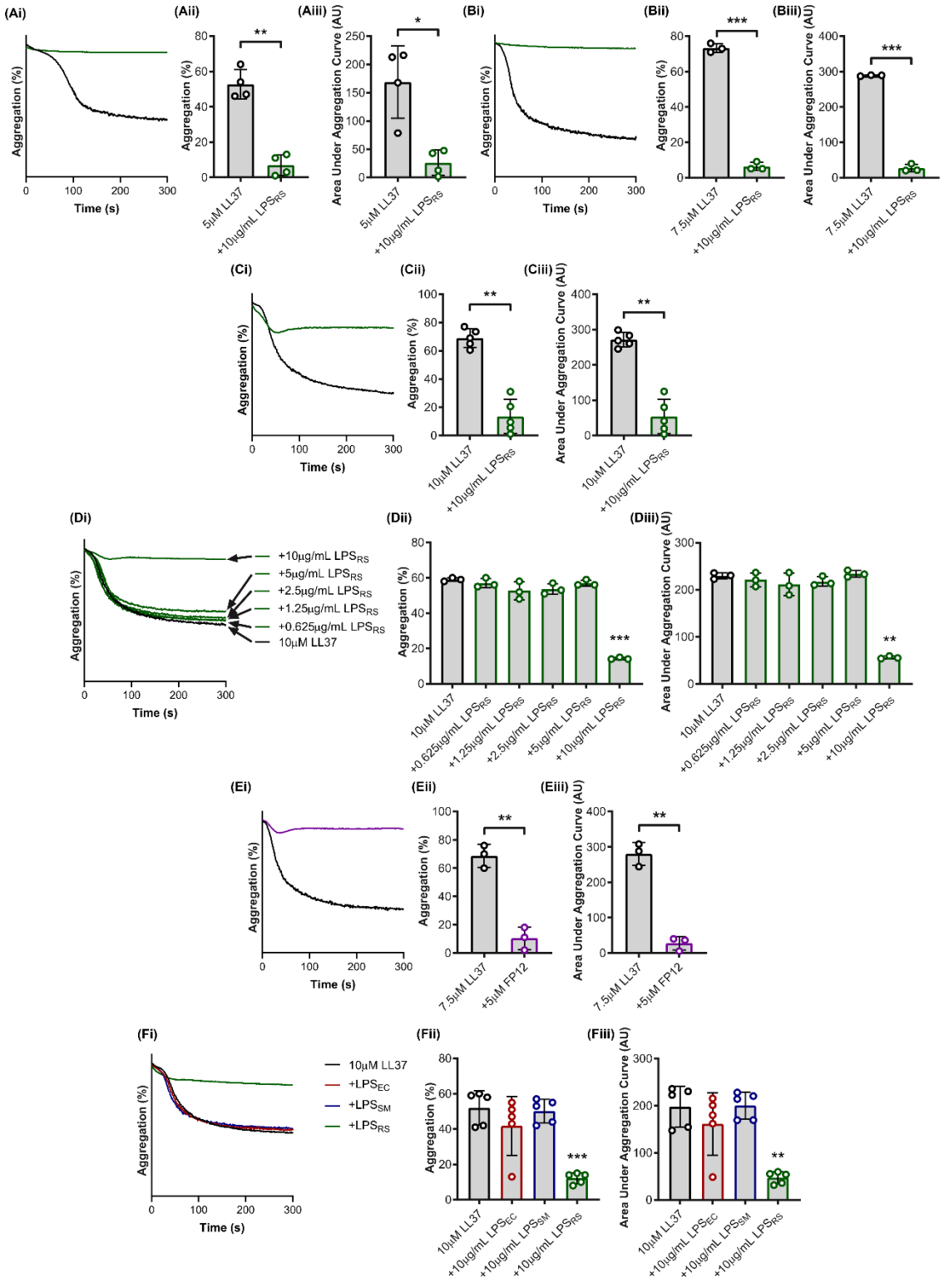

Figure 1: ***Effect of LPS chemotypes on isolated platelet aggregation induced by LL37.*** The effect of 10μg/mL LPS_{RS} on platelet aggregation (i) with maximum platelet aggregation (ii) and area under the aggregation curve (AUC; iii) quantified. Aggregation was induced by 5μM LL37 (A; n=4), 7.5μM LL37 (B; n=3), or 10μM LL37 (C; n=5) following 10 minutes of pre-incubation. Data represent mean ± SD and were analysed via a paired t-test. (D) Concentration-response curve (i) for maximum aggregation (ii) and AUC (iii) for 10μM LL37-stimulated platelets following incubation with 0.625μg/mL-10μg/mL LPS_{RS}. Data represent mean ± SD (n=3) and analysed via a repeated measures ANOVA with Bonferroni post-hoc test. (E) The effect of 10 minutes pre-incubation with 5μM FP12 on platelet aggregation (i) with maximum aggregation (ii) and AUC (iii) induced by 7.5μM LL37 quantified. Data represent mean ± SD and were analysed via a paired t-test (n=3). (F) Platelet aggregation for 10μM LL37-stimulated platelets incubated with vehicle or 10μg/mL LPS_{EC}, LPS_{SM}, or LPS_{RS} with maximum aggregation (ii) and AUC (iii) quantified. Data represent mean ± SD (n=5) and analysed via repeated measures ANOVA and compared to 10μM LL37 with Dunnett's post-hoc test. P-values shown are as calculated (*p<0.05, **p<0.01, and ***p<0.001) by GraphPad Prism.

RESULTS

LL37-induced platelet aggregation is reduced by TLR4 antagonists

When platelets are exposed to agonists, they become activated and form aggregates. As previously reported[6], LL37 is capable of inducing aggregation in isolated platelets (Figure 1) but not PRP (data not shown). To determine the impact of LPS_{RS} (a known TLR4 antagonist[38,39]), isolated platelets were incubated with LPS_{RS} for 10 minutes at 37°C prior to addition of LL37 for 5 minutes. Under these conditions, pre-incubation with 10μg/mL LPS_{RS} inhibited the LL37-induced aggregation (Figure 1Ai and 1Aii) and the area under the curve (AUC) (Figure 1Aiii). This inhibitory effect was also observed for the maximum aggregation (Figure 1Bi and 1Bii) and AUC (Figure 1Biii) induced by 7.5μM LL37 and by 10μM (Figure 1Ci, 1Cii, and 1Ciii respectively). Interestingly, the significant decrease in maximum aggregation (Figure 1Di and 1Dii) or AUC (Figure 1Diii) induced by LPS_{RS} on 10μM LL37-induced platelet activation was only achieved with 10μg/mL LPS_{RS} as there was no inhibitory effect

observed following pre-incubation of platelets with 0.625µg/mL, 1.25µg/mL, 2.5µg/mL, or 5µg/mL LPS_{RS}. Based on these results, concentrations of 10µM LL37 and 10µg/mL LPS_{RS} were chosen for use in subsequent experiments.

To corroborate the above results, a small molecule inhibitor of TLR4, FP12, was used and the same reduction to maximum aggregation (Figure 1 Ei and 1Eii) and AUC (Figure 1Eiii) was observed[40]. These results demonstrate that pre-incubation of platelets with TLR4 antagonists (LPS_{RS} and FP12) significantly reduce LL37-induced platelet activation.

LL37-induced platelet aggregation is unaffected by TLR4 agonists

To determine whether the inhibitory property was common to all TLR4 ligands (LPS chemotypes from different species) or solely its antagonists, 10µg/mL LPS_{EC} and 10µg/mL LPS_{SM} were also tested for their effect on LL37-induced platelet aggregation. However, the inhibitory effect on maximal aggregation (Figure 1Fi and 1Fii) and AUC (Figure 1Fiii) induced by LPS_{RS} and FP12 was not seen when platelets were treated with an equivalent concentration of LPS_{EC} or LPS_{SM}. These results suggest that TLR4 antagonists, but not agonists, are able to inhibit LL37-induced platelet activation.

LPS_{RS} inhibits platelet spreading on fibrinogen and outside-in signalling through integrin $\alpha_{IIb}\beta_3$

Platelets change shape and spread during activation to increase their surface area and reduce the volume of blood lost through the site of injury. Platelet spreading on fibrinogen involves actin remodelling driven via outside-in signalling through integrin $\alpha_{IIb}\beta_3$ and hence can be used to investigate defects in these pathways[41].

LL37 is known to induce platelets to spread on fibrinogen-coated surfaces over a period of 20 minutes[7]. Here, platelets were treated with 10µM LL37 in the presence or absence of 10µg/mL LPS_{RS} and exposed to fibrinogen for different periods of time (0, 5, 10, 15, 20, or 30 minutes) (Figure 2A). As expected, the number of platelets bound to fibrinogen increased over time. At all time points however, pre-treatment with LPS_{RS} significantly inhibited platelet adherence to fibrinogen (Figure 2B). At 30 minutes, there was an ~80% decrease in the number of bound platelets to fibrinogen following pre-treatment with LPS_{RS}.

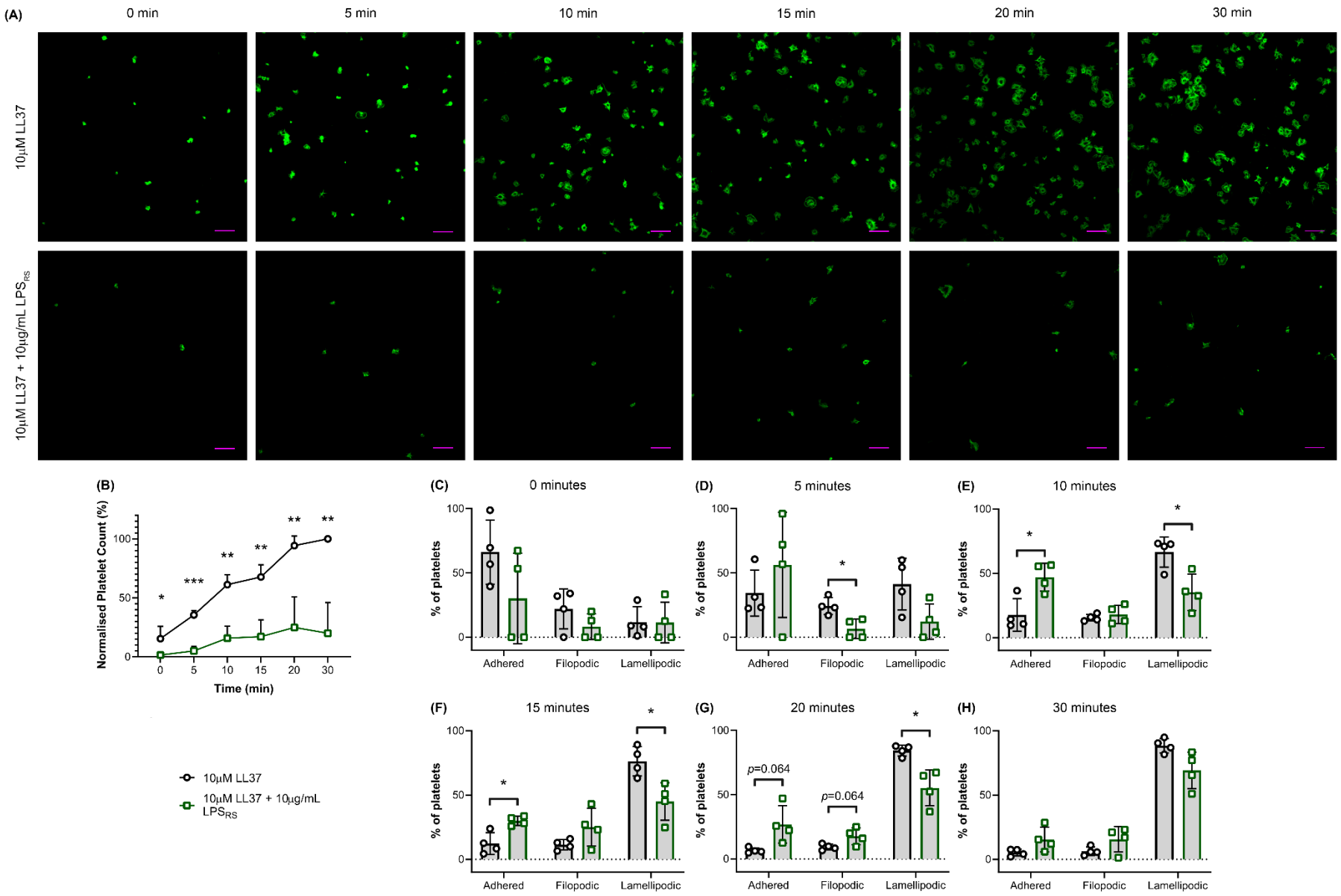
(A)
0 min
5 min
10 min
15 min
20 min
30 min
10μM LL37
10μM LL37 + 10μg/mL LPS$_{RS}$
(B)
Normalised Platelet Count (%)
Time (min)
10μM LL37
10μM LL37 + 10μg/mL LPS$_{RS}$
(C) 0 minutes
(D) 5 minutes
(E) 10 minutes
(F) 15 minutes
(G) 20 minutes
(H) 30 minutes
% of platelets
Adhered
Filopodic
Lamellipodic
p=0.064

Figure 2: ***Effect of LPS_{RS} on LL37-induced platelet spreading on fibrinogen.*** (A) Representative images of isolated platelets that were stimulated with 10μM LL37 in the presence or absence of 10μg/mL LPS_{RS} and allowed to adhere to fibrinogen-coated coverslips for different time points. (B) Total platelet count was recorded in LPS_{RS}/LL37-treated samples and normalised to total number of adhered platelets treated with 10μM LL37 after 30 minutes. Distribution of platelets between adhered, forming filopodia, and forming lamellipodia (including completely spread) was calculated for 0 minutes (C), 5 minutes (D), 10 minutes (E), 15 minutes (F), 20 minutes (G), and 30 minutes (H). Data represent mean ± S.D. (n=4). The mean for each donor (5 regions of interest) is shown in the bars. The *p* values were calculated using multiple t-tests corrected for using the Holm-Sidak method (*p<0.05, **p<0.01, and ***p<0.001).

Furthermore, platelets were manually examined to determine the number of adhered (present with a circular morphology), filopodic (adhered and producing finger-like projections), or lamellipodic (adhered and producing flattened extensions, including fully spread) platelets. At 0 minutes (Figure 2C), there were no significant differences in platelet morphology between control and LPS_{RS}-treated platelets. However, at 5 minutes (Figure 2D), there was a significant decrease in the percentage of filopodic platelets treated with LPS_{RS}. At later time points [10 min (Figure 2E), 15 min (Figure 2F), and 20 min (Figure 2G)], there was a significant increase in the proportion of LPS_{RS}-treated platelets with a circular morphology (adhered to fibrinogen; 10 and 15 min) and a significant decrease in the proportion of lamellipodic LPS_{RS}-treated platelets (10, 15, and 20 min). At 30 minutes (Figure 2H), there were no significant changes in adhered, filopodic, or lamellipodic platelets between the untreated and LPS-treated groups.

These results demonstrate that the pre-incubation of platelets with 10μg/mL LPS_{RS} decreases total platelet binding at all time points. The impact of LPS_{RS} on the spreading of adhered platelets is unclear due to the low number of adhered platelets.

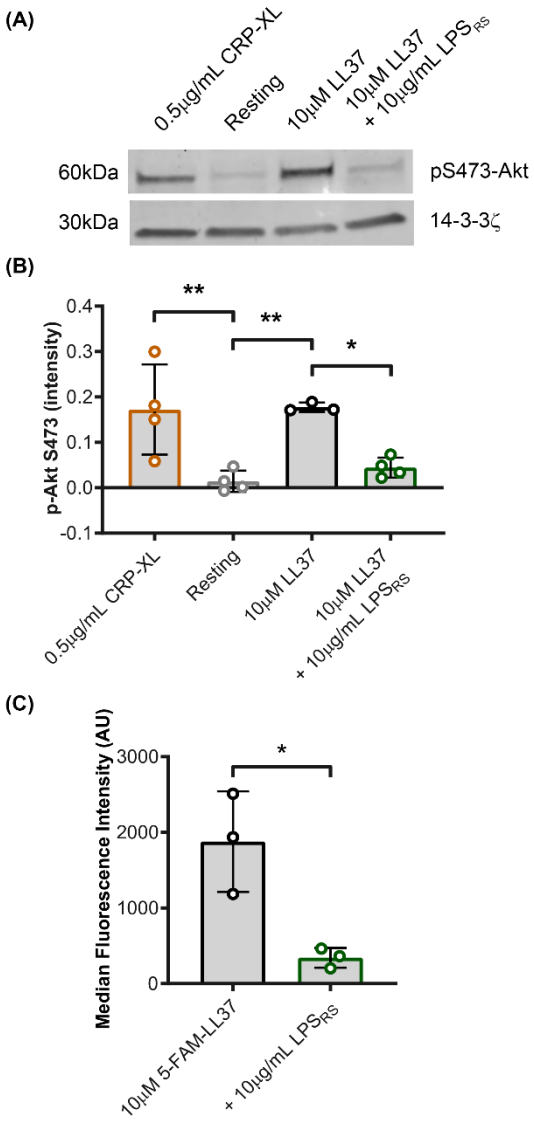

Figure 3: ***Effect of LPS_{RS} on LL37-induced Akt phosphorylation and binding of LL37 to platelets.*** (A) Phosphorylation of Ser473 on Akt in whole platelet lysates was determine via immunoblotting in resting, CRP-XL-induced, and LL37-induced platelets in the presence or absence of LPS_{RS} (10µg/mL). 14-3-3ζ was used as a loading control. (B) Quantification of the intensity of the bands observed in the immunoblotting detection of Akt Ser473 phosphorylation. Data represent mean ± SD (n=4) and were analysed using a one-way ANOVA with Bonferroni's post-hoc test. (C) Binding of fluorescent 5-FAM-LL37 to human isolated platelets in the presence or absence of 10µg/mL LPS_{RS}. Data represent mean ± SD and were analysed using a paired t-test (n=3) (*p<0.05 and **p<0.01).

LPS_{RS} inhibits LL37-induced phosphorylation of Akt

LL37 has been proposed to bind to FPR2/ALX in platelets[7] which couples to and signals through G_i proteins[42]. The βγ-subunit of G_i activates phosphoinositide 3-kinase (PI3K) which in turn induces Akt activation[43]. PI3K and Akt are also activated downstream of GPVI[44]. To determine the stage at which this signalling pathway may be interrupted, we utilised immunoblotting to examine the phosphorylation of a key protein (Akt) downstream of G_i (Figure 3A and B). Ser473 phosphorylation was investigated because it was the earliest measurable point in the signalling cascade plus this phosphorylation site is required for full activation of Akt[44,45]. Activation of platelets with 0.5µg/mL cross-linked collagen-related peptide (CRP-XL), prior to lysis,

significantly increased the phosphorylation of Ser473 on Akt compared to resting platelet lysates. Similarly, 10μM LL37 significantly increased phosphorylation of Akt at this site, however it was significantly reduced when LL37-induced platelets were pre-incubated with 10μg/mL LPS_{RS}. In conclusion, LL37-induced phosphorylation on Akt was inhibited by LPS_{RS} thereby suggesting an upstream point of inhibition, possibly extracellular.

LPS_{RS} significantly reduces the binding of LL37 to platelets

As the inhibition induced by LPS_{RS} occurred upstream of Akt, we investigated whether LL37-binding to platelets was affected. LL37 binds to FPR2/ALX which is expressed on platelets[7] whereas LPS binds to TLR4 which is also expressed in platelets[22]. To investigate whether LPS_{RS} was inhibiting binding of LL37 to its receptor, 5-FAM-LL37 (LL37 conjugated to a fluorophore) was used to determine whether 10μg/mL LPS_{RS} significantly decreased binding of LL37 to platelets (Figure 3C). As previously demonstrated[7], 10μM 5-FAM-LL37 was capable of binding to platelets however the level of fluorescence detected on platelets was significantly reduced following pre-treatment with 10μg/mL LPS_{RS}. This suggests the ability of LPS_{RS} to inhibit LL37 binding to platelets.

LPS_{RS} and LPS_{SM} form micelles with LL37

Closer examination of flow cytometry data containing platelets treated with LL37 and LPS_{RS} revealed the presence of a new population of events outside of the platelet gate. To determine whether this new population originated from platelets, vehicle or 10μM LL37 was combined with 1.25μg/mL (Figure 4A), 2.5μg/mL (Figure 4B), 5μg/mL (Figure 4C), 10μg/mL (Figure 4D), or 20μg/mL (Figure 4E) of LPS_{EC}, LPS_{SM}, and LPS_{RS} in the absence of platelets. On their own, none of the LPS chemotypes formed detectable micelles. Furthermore, incubation of LPS_{EC} or LPS_{SM} with LPS_{RS} did not lead to formation of detectable micelles (Supplementary Figure 1).

In the presence of LL37, LPS_{EC} did not form detectable micelles at any of the concentrations tested. However, both LPS_{SM} and LPS_{RS} formed significant numbers of detectable micelles. LPS_{SM} only formed significant numbers of micelles at a concentration of 20μg/mL with 10μM LL37. Meanwhile, LPS_{RS} formed significant numbers of micelles at all concentrations tested and, additionally, significantly more micelles than LPS_{SM} at all concentrations. Furthermore, LPS_{RS}-LL37 micelles formed

in a concentration-dependent manner (Figure 4F). Representative forward scatter (FSC) and side scatter (SSC) plots can be found in Supplementary Figure 2. These results demonstrate that LPS_{RS} and LL37 form detectable micelles when incubated together whereas LPS_{EC} and LPS_{SM} do not except at very high concentrations of LPS_{SM}.

These results demonstrate that LPS_{RS} and LL37 form micelles that are not present when each compound is tested individually. These micelles may prevent activation of platelets induced by LL37 by sequestering LL37 away from its receptor.

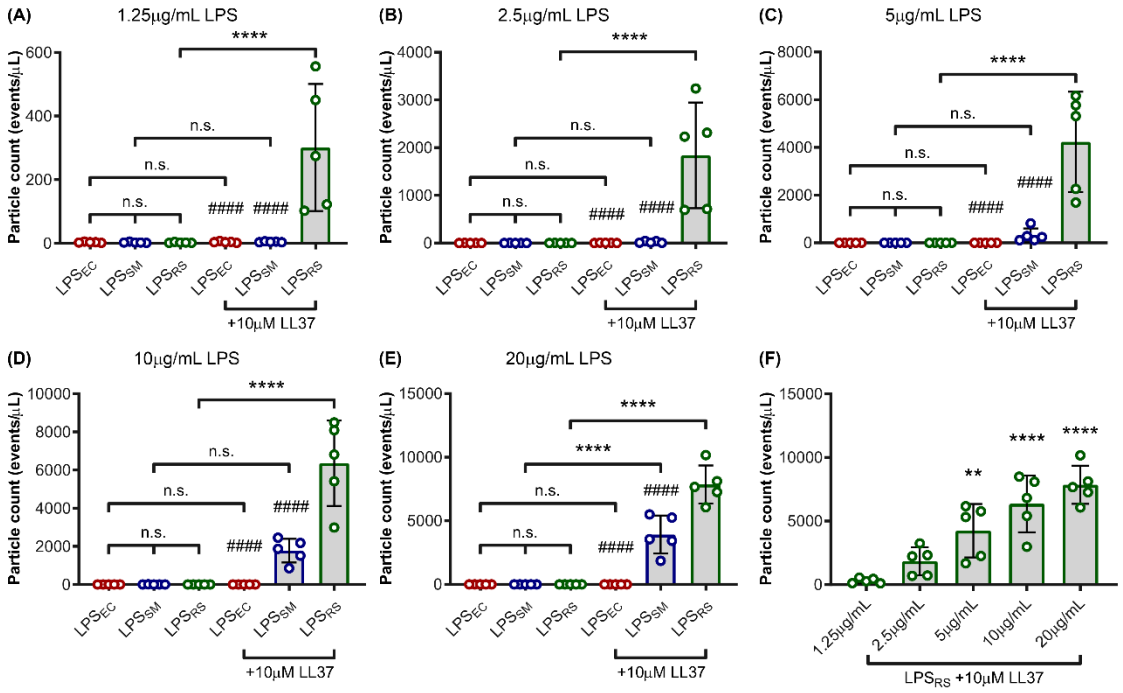

Figure 4: ***Analysis of LL37-LPS micelles by flow cytometry.*** (A) Vehicle and increasing concentrations of LPS_{EC}, LPS_{SM} or LPS_{RS} were incubated with or without 10µM LL37 together for 5 minutes prior to being processed by flow cytometry. The LPS concentrations chosen were 1.25µg/mL, 2.5µg/mL (B), 5µg/mL (C), 10µg/mL (D), or 20µg/mL (E). An increasing concentration of LPS_{RS} was incubated with 10µM LL37 to determine how the number of LL37-LPS_{RS} micelles was affected by LPS_{RS} concentration (F). Data represent mean ± SD (n=5) with differences analysed using a one-way ANOVA and Bonferroni's post-hoc test (**p<0.01 and ****p<0.0001 compared to its vehicle control, ####p<0.0001 compared to 10µM LL37 with LPS_{RS}).

DISCUSSION

LL37 has been strongly implicated in cardiovascular diseases due to its presence in atherosclerotic lesions and arterial thrombi[5,6]. LL37 also acts as a chemoattractant for immune cells and therefore increases the inflammatory response in the sites where it is present[13,15]. Moreover, mice lacking cathelicidin-related antimicrobial peptide (CRAMP; the murine analogue of LL37) generate smaller atherosclerotic plaques than their control counterparts[14]. Furthermore, overexpression of LL37 is important for the development of psoriasis and individuals with psoriasis face a reduced life-expectancy primarily due to an increase in cardiovascular disease[8]. Therefore, LL37-induced inflammatory responses represent an important target for drug development.

Previously, we provided further evidence for the growing argument that TLR4 does not impact platelet activity[19,20,23,25,46] although this was in response to “classical” platelet agonists such as CRP-XL, adenosine diphosphate (ADP), and thrombin[19]. As platelets have roles in the immune system, we sought to investigate the potential of TLR4 to modulate platelet activation induced by a molecule directly involved in the innate immune system. LL37 is an immune peptide known to be able to activate platelets despite its potential to be an anti-inflammatory molecule[6,7]. The mechanism of action for LL37 stimulation of platelets is not completely established as multiple mechanisms have been proposed in platelets[6,7] and in other cell types[12]. In this study, we investigate the impact of LPS (specifically the chemotype derived from *Rhodobacter sphaeroides*) on LL37-induced platelet activation and establish the mechanism behind its actions.

As previously reported[6,7], aggregation of isolated platelets was induced following incubation with LL37. However, aggregation was inhibited by pre-incubation of isolated platelets with LPS_{RS}. Interestingly, only 10µg/mL LPS_{RS} was able to inhibit 10µM LL37-induced platelet aggregation with no concentration-dependent effect. Furthermore, LPS_{EC} and LPS_{SM} were unable to inhibit LL37-mediated platelet aggregation when tested at the same concentration. This suggested that this effect may be due to LPS_{RS}’s properties as a TLR4 antagonist[39]. This supposition was also supported by the discovery that a synthetic TLR4 antagonist, FP12, was also capable of inhibiting LL37-induced platelet activation at concentrations similar to those used to

inhibit LPS-induced TLR4 activation in human embryonic kidney (HEK)-Blue hTLR4 cells[40].

Platelets stimulated with LPS_{EC} have been reported to adhere to and spread on fibrinogen-coated surfaces[22,47]. Moreover, platelets stimulated with LL37 have also been reported to adhere to and spread on fibrinogen, a process driven by outside-in signalling[7,41,48]. Therefore, we determined the impact of LPS_{RS} on LL37-induced platelet spreading. Outside-in signalling through integrin $\alpha_{IIb}\beta_3$ also appeared to be inhibited as lamellipodia formation after 10, 15, and 20 minutes was significantly reduced compared to LL37 alone. However, this defect was recovered at 30 minutes of incubation. The effect of LPS_{RS} on outside-in signalling is complicated by the overall lack of platelet activation in the LPS_{RS}/LL37-treated platelets as significantly lower numbers of platelets adhered to fibrinogen to begin with. This decreased number of adhered platelets could influence platelet spreading. Conversely, it may suggest a role for TLR4 during outside-in signalling and suggest a physiologically relevant function for the movement of TLR4 to the cell surface during platelet activation[19,49]. Future work should investigate the impact of LPS_{RS} (or TLR4-neutralising antibodies) on platelet spreading induced by an agonist whose activity is not mitigated by the presence of LPS_{RS}, for example CRP-XL[19].

FPR2/ALX is a 7-transmembrane receptor that couples to different isoforms of G_i[42]. Here we demonstrate that LL37 can induce phosphorylation of serine 473 in Akt, a signalling protein found downstream of the Gβγ-subunit of G_i and also downstream of GPVI[43,44]. Furthermore, as this phosphorylation is inhibited by the presence of LPS_{RS}, inhibition of LL37-induced signalling is most likely to occur upstream in this signalling pathway or extracellularly. Another key piece of evidence for the mechanism of action was obtained using fluorescently-conjugated LL37 (5-FAM-LL37), which is known to bind to platelets[7]. We determined, via flow cytometry, that the presence of 10μg/mL LPS_{RS} significantly inhibited 5-FAM-LL37 binding to platelets. Therefore, suggesting that LPS_{RS} abrogates LL37-induced platelet activation by preventing binding of LL37 to its receptor.

At this stage, our foremost hypothesis was that TLR4 heterotetramers interacted with the receptor for LL37 in platelets (FPR2/ALX in our model) to aid LL37 binding. Therefore, the loss of the heterotetramer, induced by TLR4 antagonists[39,40], would

prevent ligand binding. However, experiments combining high concentrations of LPS_{RS} with LL37 in the presence of platelets revealed a novel particle population with similar side-scatter to platelets but smaller forward-scatter that appeared in a concentration-dependent manner. These novel particles also appeared in the absence of cells which suggests a non-platelet origin.

LPS is well known for its ability to form aggregates which is essential for LPS activity[50]. Furthermore, LL37 has been reported to bind to LPS in multiple settings, usually with the result of decreasing inflammatory activity induced by LPS, such as inflammatory cytokine release[28,30,31]. Conversely, LL37 has been proposed to aid translocation of LPS across the cell membrane to induce inflammation[11]. As far as we are aware, this is the first time that LPS has been reported to be an inhibitor of LL37-induced effects, in any cell type. Although LPS forms aggregates on its own[33,51,52], the presence of LL37 alters the morphology of aggregates by inducing greater membrane curvature, with the final structure depending on whether the LPS chemotype involved is rough (lacking the O-antigen) or smooth (O-antigen present)[33].

Both LPS_{SM} and LPS_{RS} could form micelles in the presence of LL37 however LPS_{SM} only formed significant numbers at a concentration of 20µg/mL whereas LPS_{RS} formed micelles at all concentrations tested and, additionally, formed significantly more micelles than LPS_{SM} did at the same concentration. This is likely to be due to differences between their critical micelle concentrations (CMC), the concentration at which LPS molecules form micelles or aggregates[51,52]. The CMC for LPS_{RS} is unknown however the CMC for LPS_{SM} R595 is predicted to be 10µg/mL and the CMC for LPS_{EC} O111:B4 is 22µg/mL in 20mM Tris and 0.15M NaCl at pH 7.5[51]. FP12 also shares a lipidic structure similar to LPS but with fewer acyl chains, compared to LPS_{RS}, due to the absence of a second glucosamine moiety[38,40]. No pure LPS micelles were visible in our system at any concentration tested however the LL37-LPS_{SM} micelles were only detectable above the CMC which suggests that the micelles become detectable due to a structural change induced by an interaction with LL37[51]. Given the structural similarities to LPS_{RS}, FP12 is likely to inhibit LL37-induced platelet activation via the same mechanism however this will need to be confirmed using the same techniques.

As the number of detectable micelles increase with increasing concentrations of LPS_{RS}, the lack of a concentration-response curve found in aggregation experiments

may be due to high concentrations of free LL37 in solution able to interact with platelet receptors, at lower LPS_{RS} concentrations. Once enough LL37-LPS micelles have been formed, sufficient LL37 is removed from solution to prevent a full aggregation/activation response from platelets. This explains why some minor platelet activation remains and why LPS_{SM}, although it can form LL37-LPS micelles, cannot inhibit platelet aggregation at the concentrations tested due to low micelle numbers of 1773±277.7 (mean ± S.E.M.) compared to 6359±1001 for LPS_{RS}. Therefore, we propose that LPS (specifically LPS_{RS}) acts as a scavenger to prevent LL37-induced platelet activation although the direct interaction of TLR4/FPR2 cannot be ruled out.

In conclusion, TLR4 antagonists, such as LPS_{RS} and FP12, are capable of inhibiting LL-37 induced platelet activation via extracellular micelle formation incorporating both molecules. This could lead to a new line of treatments for certain inflammatory diseases, including atherosclerosis and psoriasis[9], where excess LL37 leads to pathological outcomes. However, future work needs to be conducted to determine whether the scavenging mechanism of action occurs *in vivo*.

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AUTHOR CONTRIBUTIONS

T.M.V., A.G., M.S.N.T., C.Y.L., H. J. L., and D.A.I.A performed the experiments and analysed the data. T.M.V., D.W., and S.V. designed the study. T.M.V., D.W., and S.V. wrote the manuscript.

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CONFLICTS OF INTEREST

The authors report no conflicts of interest.

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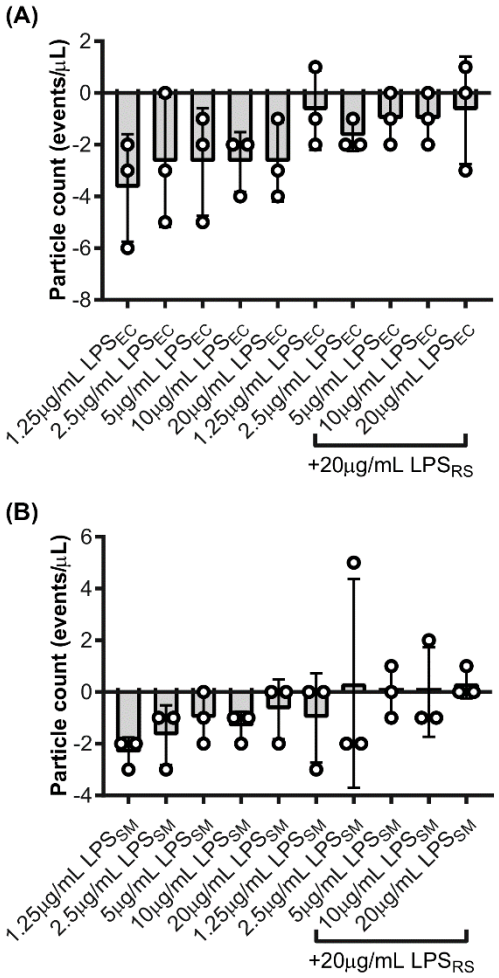
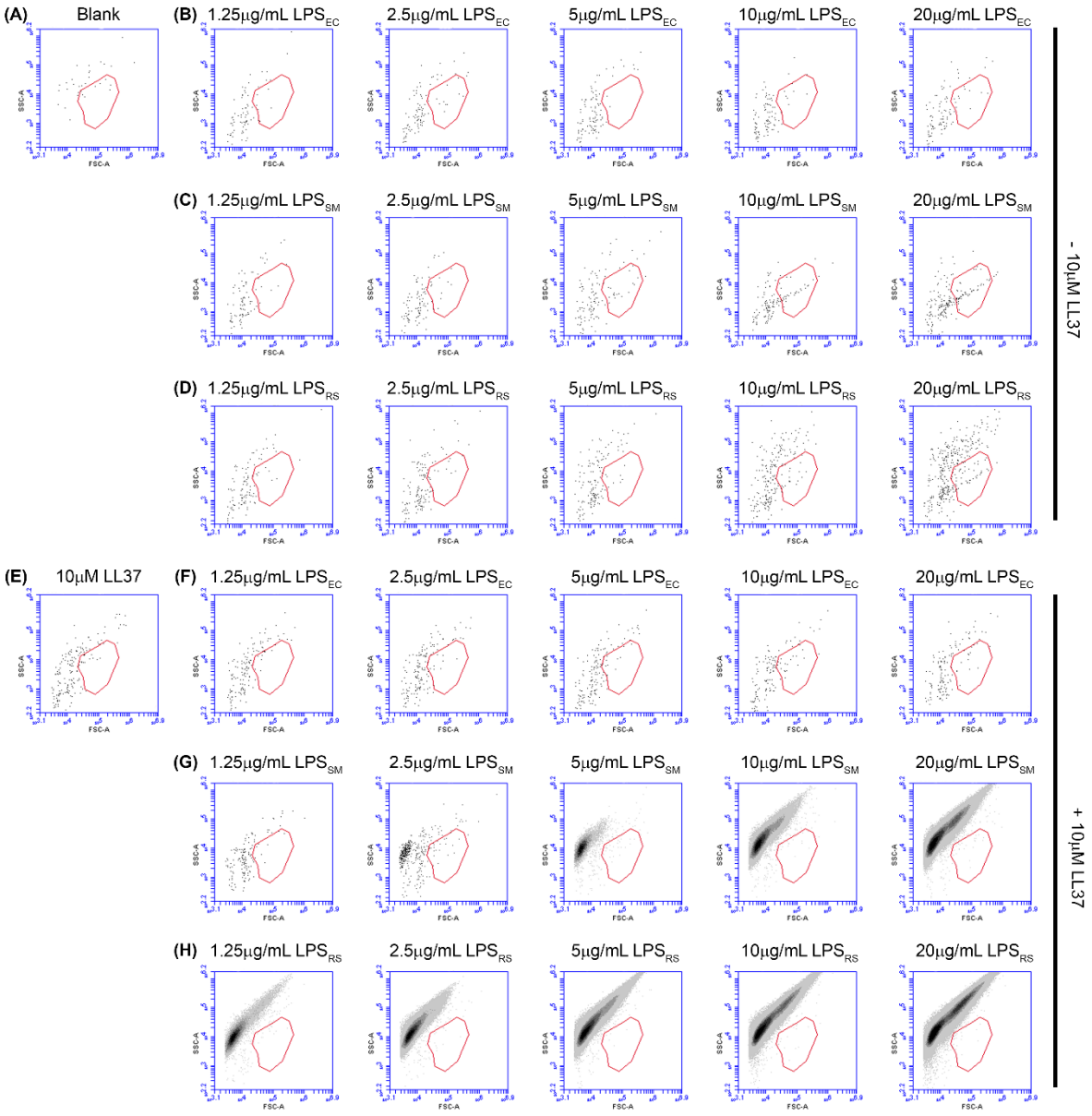
2.3 Supplementary information



Supplementary Figure 1: ***Micelle formation between different LPS chemotypes.*** Vehicle or 20µg/mL LPS_{RS} was incubated with increasing concentrations of LPS_{EC} (A) or LPS_{SM} (B) and the concentrations of detectable particles was measured using a flow cytometer. Data represent mean ± SD (n=3) with statistical differences determined via a one-way ANOVA.



Supplementary Figure 2: ***Representative forward and side scatter plots of LL37-LPS micelles***. (A) Representative scatter graph of forward scatter (FSC-A) and side scatter (SSC-A) in 50μL of 0.2μm-filtered Tyrode's buffer. (B) Representative scatter graphs of increasing concentrations of LPS_{EC} in the absence of 10μM LL37. (C) Representative scatter graphs of increasing concentrations of LPS_{SM} in the absence of 10μM LL37. (D) Representative scatter graphs of increasing concentrations of LPS_{RS} in the absence of 10μM LL37. (E) Representative scatter graph of 10μM LL37 in 0.2μm-filtered Tyrode's buffer. (F) Representative scatter graphs of increasing concentrations of LPS_{EC} in the presence of 10μM LL37. (G) Representative scatter

graphs of increasing concentrations of LPS_{SM} in the presence of 10μM LL37. (H) Representative scatter graphs of increasing concentrations of LPS_{RS} in the presence of 10μM LL37. The red polygon highlights the area where platelets would be detected.

GENERAL DISCUSSION

3. GENERAL DISCUSSION

Myocardial infarctions and ischaemic strokes, together representing thrombotic diseases, are the leading cause of human death in the entire world[1,2]. Both of these diseases are induced by aberrant platelet activity for which there is currently no ideal treatment. For example, current treatments for cardiovascular diseases (CVDs) include non-platelet targeting drugs, such as warfarin and heparin, and anti-platelet drugs, such as aspirin and clopidogrel[3,4]. Whilst effective, these treatments do not eliminate the risk of myocardial infarction or ischaemic stroke entirely and have adverse side effects, most notably, increased bleeding events. Therefore improved treatments are being desperately sought after[3–5]. These new treatments would require normal haemostatic and immune functions to be maintained whilst inhibiting the potentiation and platelet activation induced by sterile inflammation in important arteries in the heart and brain.

In recent years, inflammation has come to the forefront of the battle against numerous diseases, including CVDs[6,7]. Indeed, chronic inflammation seen during aging has been proposed to be responsible for increasing platelet activation in older mice and humans[8]. Furthermore, platelets have been proposed to act as sentinels in the innate immune system as they express inflammatory receptors and fluid dynamics means that platelets exist in the periphery of the blood flow, allowing them to scan the endothelial layer for damage[3,9]. Moreover, platelets have been reported to be capable of engulfing bacteria and viruses however, as they cannot destroy the engulfed material, the purpose is currently unknown[10,11]. Therefore, the inflammatory role of platelets has attracted a great deal of interest from scientists developing new antiplatelet treatments.

TLR4 in platelets

Main Findings

Specifically, Toll-like receptor 4 (TLR4) has been a focus of study for numerous research groups as it is expressed on platelets[12,13], and its surface expression level is increased during platelet activation[14] and in humans with cardiovascular disease[15,16].

The influence of lipopolysaccharides (LPS) on platelet function and the role of its receptor, TLR4, has remained controversial since the discovery of TLR4 on platelets in 2005[12,13,16–23]. However, many studies that reported an effect induced by LPS used non-ultrapure versions[21,22,24–27] or whole bacteria[28–31] whereas groups that utilise ultrapure LPS[18,20,23,32,33] (LPS that is purified using a phenol-water mix and an additional phenol-TEA-DOC step[34]) observed much less profound effects. Therefore, other contaminants may be present in LPS which can activate immune receptors e.g. TLR2 ligands [Vallance et al. *in preparation* (a)][17,35,36].

In an attempt to fully elucidate the role of TLR4 on platelet function, three different ultrapure LPS chemotypes [derived from *Escherichia coli* (LPS_{EC}), *Salmonella minnesota* (LPS_{SM}), or *Rhodobacter sphaeroides* (LPS_{RS})] were tested under a range of different conditions (temperature and pre-incubation time) to account for the variations in experimental methodology seen in numerous studies. Furthermore, these three different LPS chemotypes are proposed to induce different effects due to their different structures[23,37]. LPS_{EC} is commonly used as a pro-inflammatory molecule that signals via TLR4 and the pro-inflammatory MyD88-dependent pathway[12,17,38]. LPS_{SM} is another TLR4 ligand that can bias TLR4 signalling down the anti-viral and anti-inflammatory MyD88-independent pathway in a glioma cell line, but has also been suggested to induce soluble cluster of differentiation 40 ligand (sCD40L) release from platelets[23,38]. LPS_{RS} is a TLR4 antagonist that prevents formation of the TLR4 heterotetramer and therefore prevents endogenous signalling via this receptor[39]. Ultrapure LPS is specific for TLR4 and lacks any TLR2 cross-activity and therefore prevents confusion between the two receptors that both signal via the MyD88-dependent pathway[17,34].

This study provides a comprehensive examination of the influence of different LPS chemotypes on integrin $\alpha_{IIb}\beta_3$ activation (as measured by fibrinogen binding[40]) and α-granule secretion (as measured by P-selectin exposure, a protein that is important for platelet-leukocyte interactions[32,41]) in platelets in a range of conditions with variations in temperature and pre-incubation time, in response to a range of platelet agonists. Furthermore, platelet aggregation in response to these agonists was likewise not significantly altered by the presence of LPS chemotypes. Moreover, it describes how ultrapure LPS chemotypes have no impact on these measures of platelet activation, except under specific conditions, whereas non-ultrapure versions do. This

is important for comparison of the different experimental setups used in the literature to clarify the precise role of TLR4 in platelets.

Moreover, this lack of activity may be partially explainable by changes in surface TLR4 expression on platelets during activation by CRP-XL or collagen. Under resting conditions in healthy humans, there may not be sufficient numbers of TLR4 on the cell surface that are required to alter platelet function. However, when platelets are activated, there is processing of TLR4 and translocation to the cell membrane[14]. The data gathered using LPS_{RS} was particularly enlightening as it suggests that there is no endogenous TLR4 activity induced by resting TLR4 heterotetramers[39]. These data suggest that TLR4 is not a good target for pharmacological therapies aiming to decouple platelet immune functions from haemostasis.

Limitations

When platelets become activated, multiple events occur. As well as integrin activation (which enables fibrinogen and collagen binding) and degranulation, platelets also undergo shape changes, interact with immune cells, produce surfaces for thrombin generation, undergo clot retraction and spreading, *de novo* synthesis of proteins, and a host of other activities[5,41–45]. Therefore, the effect of LPS on platelets could not be completely elucidated in this thesis solely using platelets. Furthermore, the experiments involving the effect of LPS of platelets is limited as other bacterial compounds (such as TLR2 agonists) were not tested to elucidate the compound responsible for the potentiator effects seen in other studies[21,22,24–27].

Secondly, a major limitation shared by almost all platelet research arises from donor selection. As this research was conducted in a university environment, most of the volunteers who donated blood were healthy people between the ages of 20 and 35. However, the majority of deaths induced by ischaemic stokes and myocardial infarctions occur in individuals >65 years old[2]. In the age group tested here, platelets are less potentiated and the atherosclerotic plaques that are present inside their arteries are likely to be smaller and less developed, and therefore less likely to rupture and narrow the lumen sufficiently to occlude blood flow to a major organ[6,8]. Aged platelets are more reactive to common platelet agonists due to higher levels of circulating tumour necrosis factor α (TNFα) which provokes megakaryocytes (MKs) into producing primed platelets[8]. As the aim of all platelet research is to develop new

treatments with the potential of replacing current treatments (aspirin and clopidogrel), it severely hampers research when the platelets tested in basic research are phenotypically and behaviourally different to the platelets that will be treated in the clinic. Conversely, sepsis can affect people of all ages and therefore the limitation described above is mitigated[46,47]. This issue can be addressed by recruiting more donors from older age groups however this is associated with challenges related to controlling for different medications prescribed to older individuals (polypharmacology).

Additionally, based on the data provided in this chapter, we cannot conclusively state that we were not trying to modulate a maximal response in the stimulated platelets. This could have been addressed by using an independent platelet agonist to induce full activation of platelets as a reference. High concentrations of cross-linked collagen-related peptide (CRP-XL) were used however this synthetic agonist can have variable activity and the flow cytometry histograms suggested that these concentrations were not sufficient to fully activate all of the platelets present. However, as we cannot demonstrate a higher value for fibrinogen binding or P-selectin exposure that CRP-XL alone, we cannot state that we used a sub-maximal concentration and therefore any influence by LPS may be lost because the platelets were fully activated.

Future work

Furthermore, future work investigating the effect of LPS on platelet function via TLR4 should be conducted using ultrapure variants to investigate cytokine release. Of particular note, sCD40L and regulated upon activation, normally T-cell expressed, and presumably secreted (RANTES) release from platelets is modulated by the presence of ultrapure LPS and so further experiments should be conducted to determine the signalling pathways involved in these events[23,29,32,48]. Specifically, the pathways responsible for these effects could be elucidated through the use of pharmacological inhibitors of proteins such as IKKβ (IMD0354[49,50]; to inhibit MyD88-dependent signalling) and TBK1/NAK (BX795[51]; to inhibit MyD88-independent signalling). Furthermore, as the role of TLR4 is likely to tie in to the immune role of platelets, such as interaction with immune cells[22,41], the role of ultrapure LPS-stimulated platelets on neutrophil extracellular trap formation (NETosis) and platelet-leukocyte interactions should be investigated in detail.

Role of LPS in the modulation of megakaryocyte function

Main Findings

Inflammation of MKs is an important area of research because the platelet phenotype is altered under septic conditions due to an altered MK transcriptome. For example, during sepsis, murine platelets become cytotoxic in the spleen and lungs due to the introduction of granzyme B into packaged platelets[52]. Furthermore, platelet numbers in TLR4$^{-/-}$ mice are reduced compared to wild-type controls[53]. Current MK research is stymied by difficulties in obtaining large numbers of primary MKs, limits in the functional assays that can be performed, and their non-human origin[54,55]. Therefore, we developed and characterised a Meg-01 reporter cell line (Meg-01R), derived from human cells[54], that produces luciferase in response to NF-κB activation. We believe that this novel tool can be used to investigate inflammation induced by numerous factors and therefore be of great use to the scientific community. We discovered that normal Meg-01 cells express a plurality of the proteins involved in the MyD88-dependent and MyD88-independent pathways, as well as TLR4 itself [Vallance et al. *in preparation* (a)]. Meg-01 cells were then transduced to incorporate an NF-κB reporter sequence into their genome and selected for using puromycin. The NF-κB reporter sequence contains NF-κB binding domains and a gene for the transcription and translation of stable luciferase. Therefore luciferase activity can be used as a measure of NF-κB activity[50,56].

NF-κB activity in these Meg-01R cells increased significantly in response to TNFα and a TLR2 agonist, Pam3CSK4. Thereby providing the first evidence for TNFα-induced activity in Meg-01 cells[57]. Interestingly, increased NF-κB activity was not detected in Meg-01R cells treated with increasing concentrations of ultrapure LPS_{EC} but produced significantly greater amounts of luciferase (hence greater NF-κB activity) when the same concentration of non-ultrapure LPS_{EC} was used. This provides further evidence that non-ultrapure LPS_{EC} includes contaminants that can induce NF-κB activity. It is necessary to consider, however, that TLR agonists may have synergistic effects together and thus the TLR4-dependent effects (determined using anti-TLR4 antibodies or TLR4 antagonists, such as eritoran and LPS_{RS}) observed by others may still have merit[22,25,28,30,58]. Furthermore, the lack of NF-κB activity observed is not due to the absence of pro-inflammatory activity in ultrapure LPS chemotypes as we have

previously shown that ultrapure LPS is capable of inducing NF-κB activity in another reporter cell line, U251-NF-κB-GFP-Luc[36,50]. Using the Meg-01R reporter cell line, we were able to provide further evidence that the purity of LPS is important for preventing generation of false results.

Moreover, we show via both immunocytochemistry and flow cytometry that TLR4 is not detectable on the surface of Meg-01 or Meg-01R cells but were detectable intracellularly when cells were permeabilised with Triton-X100. Whether these intracellular TLR4 receptors are functional is unclear as attempts to introduce ultrapure LPS intracellularly failed. CD14 was used initially to supplement the soluble CD14 present in the foetal calf serum and to drive internalisation of LPS as CD14 is necessary for TLR4 internalisation[59]. However, NF-κB activity in Meg-01R cells incubated for 24 hours with CD14 and LPS_{EC} or LPS_{SM} was not significantly altered. We hypothesise that this occurred because the lack of surface TLR4 hindered the internalisation of LPS. The only human cathelicidin, LL37, was also used to induce internalisation of LPS into Meg-01R cells and activation of intracellular TLR4[60]. A previous study by Shaykhiev *et al.*[61] proposed that LL37 could transmit LPS across the cell membrane to endosomes, whereupon LPS disassociates following a pH change and ligates to endosomal TLR4 to induce pro-inflammatory signalling. This internalisation was dependent upon epidermal growth factor receptor (EGFR) which is expressed on the surface on Meg-01 cells[62]. Again, no significant increase in NF-κB activity was induced by LPS compared to LL37 alone. There are several potential reasons for this lack of an effect: the intracellular TLR4 may not be present in endosomes in Meg-01R cells (they could be pre-packaged into platelet-like particles); LL37 and LPS may not be interacting optimally and therefore LPS may not be internalised; or sufficient EGFR is not present at the cell surface to enable internalisation [Vallance et al. *in preparation* (a)].

Further characterisation of the Meg-01R cell line demonstrated that they are unresponsive to FPR2/ALX agonists (apart from LL37) under these experimental conditions. Specifically, BML-111, a synthetic lipoxin A4 analogue[63], did not alter resting or TNFα-stimulated Meg-01R cell NF-κB activity. However, NF-κB activity induced by TNFα was significantly reduced by the presence of an IKKβ inhibitor, IMD0354[49,50], and a TNFα antagonist, C87[64,65]. These data therefore demonstrate that Meg-01R cells can be applied in elucidating the effect of pro- and anti-inflammatory

molecules on NF-κB activity in MKs in a manner that avoids the conventional methods, such as immunoblotting for phosphorylated proteins, which are dependent on the quality of the antibodies available[55,66]. This reporter cell line enables quicker and reliable screening of inflammatory compounds and their inhibitors and so provide the building blocks for a prophylactic anti-platelet drug that acts by keeping platelets in a suppressed, non-inflammatory, naïve state.

Limitations

Meg-01 cells are a megakaryoblastic cell line, not a megakaryocytic cell line, as the differentiation from megakaryoblast into MK is terminal and MKs do not divide[54,67]. This means that Meg-01 cells are not an entirely accurate reflection of MKs as receptor levels on MKs, particularly TLR4 expression levels, have been reported to change during MK maturation[12,54,68,69]. Moreover, some proteins that may be detected in MKs appear to have no role as they are produced for packaging into platelets. Indeed, another limitation of Meg-01 cells is the lack of detectable readouts. We have addressed the issue of the lack of readouts by incorporating an NF-κB-dependent luciferase gene into the genome of the cells to generate a reporter cell line [Vallance et al. *in preparation* (a)].

Interestingly, MKs primarily reside in bone marrow and therefore do not have much exposure to the circulating environment[67,68,70]. It has already been proposed that other cell types act as intermediaries between MKs and the outside environment to encourage them to grow and release platelets therefore they would lack the need for pattern recognition receptors (PRRs)[71]. MKs would only need to express c-MPL to detect circulating thrombopoietin levels and receptors for damage-associated molecular patterns (DAMPs) released by the intermediary cell type. Currently, the physiological and anatomical milieu cannot be replicated *in vitro*.

Future work

Treatment of Meg-01 cells with valproic acid (a histone deacetylase inhibitor) has been proposed to induce megakaryopoiesis and increase formation of proplatelets and platelet-like particles[72]. Whether TLR4 surface expression on Meg-01 increases during valproic acid treatment is not known however these differentiated cells could be examined for modulation of NF-κB activity by ultrapure LPS. Localisation of TLR4 in Meg-01 cells could be improved via investigation of co-expression with markers of the

endosome or Golgi apparatus, such as cholera-toxin B-subunit (CtxB) or lysosomal associated membrane protein 1 (LAMP-1)[61]. Furthermore, if these differentiated cells yield similar results to those obtained using human umbilical cord-derived $CD34^+$ cells[73] then this reporter cell line could mitigate the need for costly and time consuming experiments involving primary cells, whilst also avoiding ethical issues.

Additionally, FPR2/ALX is likely to be expressed in Meg-01 cells and MKs as it is expressed in both haematopoietic stem cells and platelets[74,75]. However, the surface expression of FPR2/ALX on Meg-01 cells and MKs is currently unknown. Whether FPR2/ALX is expressed at the cell surface or not raises different and interesting questions:

- Cell surface expression would invite investigation into the absence of NF-κB activity modulation induced by the alternative ligands examined
- The absence of FPR2/ALX cell surface expression would suggest an alternative FPR2/ALX-independent mechanism of action, potentially via LL37 internalisation involving cell surface-expressed EGFR[61,62]
- As FPR2/ALX is a seven-transmembrane receptor[76], the potential role of biased signalling differentially induced by peptide and lipidic ligands cannot be ignored

To investigate these questions, and to determine whether LPS can be internalised into MK lineage cells without surface TLR4, fluorescent LL37 (5-FAM-LL37) could be utilised both in the presence and absence of LPS to examine its location. Furthermore, FPR2/ALX cell surface expression could be investigated using the differentiated reporter cell line to determine whether its cellular localisation changes during maturation as TLR4's does[12].

Impact of lipopolysaccharide in the modulation of LL37-induced platelet reactivity

Main Findings

Most of the work investigating the effect of LPS, and therefore the impact of TLR4 signalling on platelets, has been conducted using classical platelet agonists to stimulate a response to be potentiated[21,25,36]. The current model contends that a wound occurs, leading to exposure of collagen from the subendothelial matrix and therefore

platelet activation[17,41,43]. Any Gram-negative bacteria entering the blood vessel at this point would inadvertently release LPS, which potentiates platelet function to prevent the spread of infection[17,41]. Uncontrolled systemic inflammation caused by the spread of the pathogen through the vasculature leads to sepsis, a disease with a very high mortality rate that is characterised by thrombocytopaenia and can also involve disseminated intravascular coagulation (DIC)[77–79]. Platelets are critically involved in sepsis as platelet count is strongly correlated with patient outcomes[80]. Furthermore, this model posits that platelets also interact with immune cells, particularly neutrophils, and increase/alter their function (e.g. stimulate them to produce NETs)[41]. However, pro-inflammatory molecules have been suggested to activate platelets[41] and therefore we investigated whether LPS could modulate platelet activation in response to these agonists to study the interactions between inflammatory molecules and their receptors in the modulation of platelet function.

LL37 was recently discovered to be able to activate platelets[75,81] although Su *et al.* suggested that concentrations in the millimolar range lead to inhibition of platelet activation[82]. The concentrations of LL37 used by Su *et al.* have since been determined to be cytotoxic, thus explaining the observed inhibitory effect[75,82]. Incubation of isolated platelets with LL37 led to platelet activation however this was inhibited by pre-incubation with a specific concentration of LPS_{RS}, a TLR4 antagonist [Vallance et al. *in preparation* (b)][37,39]. Moreover, a synthetic TLR4 antagonist, FP12[83], inhibited LL37-induced platelet activation whilst other TLR4 ligands, namely LPS_{EC} and LPS_{SM}, had no effect on platelet function. This strongly suggested a role for TLR4 in mediating LL37-induced platelet activation. Especially as the mechanism of LL37-induced platelet activation is still unclear with multiple receptors proposed, including FPR2/ALX and GPVI, however inhibition of these receptors individually does not completely abrogate platelet activation[75,81].

Furthermore, LPS_{RS} inhibited LL37-induced platelet adhesion to fibrinogen and also its spreading. LL37-induced platelet spreading in TLR4-inhibited platelets was slower than platelet spreading in vehicle-treated cells which suggests that TLR4 (possibly through endogenous signalling) may have a role in modulating outside-in signalling via integrin $\alpha_{IIb}\beta_3$. However, the end point at 30 minutes was the same for both treatments [Vallance et al. *in preparation* (b)]. If this is a physiological function, this could partly explain why TLR4 surface expression increases during platelet

activation. Furthermore, this corresponds with the currently proposed mechanism by which TLR4 and other PRRs potentiate platelet function when pathogens are present. Faster wound closure means that fewer pathogens would have the opportunity to enter the circulation, which therefore reduces the risk of sepsis.

The absence of activated Akt phosphorylation and decreased binding of LL37 to platelets induced following pre-treatment with LPS_{RS} suggests that the inhibition of LL37-induced platelet activation occurs due to an extracellular event. This extracellular event was determined to be the formation of LL37-LPS_{RS} micelles, which LPS_{EC} was incapable of, and LPS_{SM} could only do at the highest concentration tested in significantly fewer numbers than LPS_{RS} did at the same concentration. These micelles appear to remove LL37 from the solution to prevent it binding to its receptor and therefore platelets are not stimulated. This could be very important for atherosclerosis and psoriasis, where LL37 is suggested to have pathological roles[81,84–88]. Conversely, this would not be beneficial during sepsis as, although LL37 can induce NETosis during these conditions[89], it has also been shown to be protective in murine models of sepsis[89,90].

Limitations

The lack of *in vivo* work is a major limitation throughout this thesis however was necessary in some circumstances. For example, the precise role of TLR4 on platelet and MK function is difficult to ascertain due to the complex interactions that occur between components of the innate immune system[22,41,71]. LL37-LPS micelles have not yet been characterised for their impact *in vivo* or in plasma[91]. Further work is needed to properly characterise these micelles, especially in a disease model of atherosclerosis or psoriasis where these treatments could have the greatest impact[75,85].

The role of TLR4 in platelet spreading has not previously been examined. Therefore, the reduction in platelet spreading observed here represents a potential novel role for TLR4. However, as only LL37 and LL37+LPS_{RS} were tested during the platelet spreading experiments it is unclear whether the observed effects were due to a global inhibition of platelet activity or if TLR4 antagonism was having a significant effect. The decrease in platelet spreading observed with LPS_{RS} treatment could be related to the platelets not spreading due to a lack of neighbouring cells. The influence

of TLR4 antagonism on platelet spreading should be investigated by stimulating the platelets with an agonist (such as CRP-XL) whose responses TLR4 has been suggested to not modulate.

The platelet spreading results are further complicated by the potential of human misinterpretation. Currently, identification of platelet morphology must be conducted manually which can lead to differences of interpretation by different individuals and is also time consuming. This limitation was addressed however by using a single individual to quantify the gathered images to prevent misidentification based on different definitions of adhered, filopodic, and lamellipodic platelets.

Future Work

Here, we demonstrated that LL37-induced platelet activation could be attenuated by pre-incubation of platelets with LPS_{RS}. This is the first time that LPS has been shown to inhibit LL37-induced effects. Furthermore, this discovery suggests the possibility of a scavenger class of drugs for use in the treatment of psoriasis and rosacea as modern treatments currently include the use of topical glucocorticosteroids and vitamin D_3 analogues, which are inconvenient and can lead to skin irritation[87,92]. Before non-bioactive LPS or similar compound can be considered for use as a potential therapeutic for atherosclerosis or psoriasis, complete characterisation of LL37-LPS micelles is required to determine their size and their pharmacokinetic profile. Furthermore, these structures have only been confirmed in the absence of plasma proteins and therefore micelle formation must be observed in the presence of other lipids and plasma proteins and in whole blood. Moreover, although LL37-LPS micelles have no apparent effect on platelets, they may still be capable of interacting with other cell types, especially those more sensitive to LL37 than platelets. Furthermore, even in the absence of lipid A region ligation to TLR4, LPS is a large, complex structure with an extended O-antigen polysaccharide with the potential to interact with local cell types, potentially via the dectin-1 receptor[93]. However, it is currently unknown whether LPS_{RS} has an O-antigen or if this structure is important for LL37-LPS micelle formation. Further work needs to be conducted to determine if other regions of LPS molecules can interact with and stimulate immune cells, and therefore lead to a pathological inflammatory response[78].

In addition, the inhibition in platelet spreading following activation across twenty minutes suggests a novel role for TLR4 during platelet activation and outside-in signalling via integrin $\alpha_{IIb}\beta_3$ which is another potential avenue for future work to deduce the role of TLR4 in platelet activity. Furthermore it would imply that the influence of TLR4 occurs as a long-term effect, as would be supported by the results gathered by other groups which require prolonged incubation of LPS with platelets before significant changes in behaviour are observed[18,23,25,36]. This may suggest that inflammatory signalling via TLR4 potentiates outside-in signalling via integrin $\alpha_{IIb}\beta_3$ and increases platelet spreading during bacterial infection to aid in confinement of pathogens[78].

Wider context and implications

The results reported in this thesis have important implications for prior work that was conducted using non-ultrapure forms of LPS. The platelet responses reported by these studies cannot be definitively described as being solely TLR4-mediated effects. Even studies that utilised TLR4 antagonists and anti-TLR4 antibodies must be interpreted carefully as there is potential synergism between TLR2 and TLR4 signalling[21,22,24,94,95]. Studies involving ultrapure LPS, or LPS that has been demonstrated to have no TLR2 activity, should be considered the gold standard in this field. Furthermore, recent studies are more predisposed to use ultrapure LPS than before which suggests a shift in understanding in the platelet-TLR4 community[18,20,36].

Platelet phenotype is subject to alteration during chronic inflammation conditions. For example, platelet reactivity is increased in older individuals (the exact morphological changes are unclear)[8], novel mouse platelets can be modified to produce granzyme B during septic conditions that are cytotoxic to host cells[52], and integrin α_{IIb} mRNA expression is increased in human and mouse platelets and MKs during sepsis[96]. Despite the clear evidence for altered platelet phenotype and behaviour, very little is known about the mechanisms behind this change due to the lack of suitable models. Our reporter cell line addresses some of these issues and can be used to easily determine which compounds can increase or decrease inflammatory signalling in MKs thereby allowing determination of some of the mechanisms involved. Furthermore, depending on the responses of the differentiated reporter cell, this could

provide valuable information on the responsiveness of platelet-producing MKs and the potential for direct PAMP and DAMP modulation of the platelet phenotype which could explain the change in platelet and MK protein expression exhibited in less than 24 hours[52,96].

Formation of LL37-LPS micelles has the potential to be a novel therapeutic. LL37 is already known to be a strong platelet agonist[75,81], a chemotactic agent[84], and to be present in atherosclerotic lesions[81,85,86]. Therefore, LL37 presents itself as an important mediator of CVD. A model can be proposed in which: 1) neutrophils extravasate into the developing lesion (neutrophil-dependent monocyte adhesion to the endothelium is mediated *in vivo* by CRAMP in mice in an FPR2/ALX-dependent manner)[6,97] where they 2) deposit LL37 in the growing lesion (this LL37 can also be transported to the apical membrane of the endothelium)[85,97]; 3) LL37 attracts more neutrophils and monocytes and increases intracellular adhesion molecule-1 (ICAM-1) expression on endothelial cells[97,98], contributing to the growth of the lesion; 4) the 'vulnerable' plaque erupts and releases the plaque contents, including LL37, into the blood stream[6]; 5) LL37 and other plaque contents interact with platelets and LL37 causes direct platelet activation[75,81]; 6) a thrombus forms and occludes a blood vessel either at the lesion site or downstream, thereby inducing ischaemia, necrosis of the tissue, and potentially death[6,45]. Ideally, a treatment could be found that removes LL37 from all of these situations to prevent influx of immune cells (step 3), which leads to plaque instability, and prevent pathological LL37-induced platelet activation (step 5). LPS_{RS} and FP12 would therefore be useful in this model as it would prevent the additional LL37-induced platelet activation during plaque rupture seen in step 5. This would mitigate the thrombus size and therefore reduce the risk of arterial occlusion. Furthermore, LPS_{RS} may potentially interact with the immobilised LL37 presented on the apical membrane of endothelial cells and therefore inhibit the neutrophil-dependent adherence of monocytes that is required for extravasation[97].

This novel treatment would represent the "Holy Grail" of antiplatelet therapies, as arterial thrombosis could be inhibited (although not removed entirely) whereas primary haemostasis would be unaffected. Therefore, there would be no increased risk of bleeding. Additionally, although the final compound used in the clinic would ideally not react with any other cell types, the failure of a phase III clinical trial for eritoran (E5564; another TLR4 antagonist) to exhibit protective effects in sepsis[99] and the lack

of a significant effect in a phase II clinical trial for coronary artery bypass surgery[100] suggest that administration of a drug that also inhibits TLR4 may not adversely affect normal biological functions[101].

Although the complete structure of LPS_{RS} is currently unknown and LPS is heterogenous even within the same bacterium, we can infer that the LPS-like molecule should not have an O-antigen polysaccharide because LL37 binds to the two phosphate groups on the diglucosamine backbone of lipid A and the core region[102]. Furthermore, more work needs to be conducted to determine the optimal number of acyl chains. At the same concentration, LPS_{EC} (seven acyl chains) was unable to form detectable micelles whereas LPS_{SM} (six acyl chains) could and LPS_{RS} (five acyl chains) formed more[37].

Conclusion

In conclusion, the role of TLR4 in platelets and MKs is still not fully understood. Here, we have shown that ultrapure LPS chemotypes cannot significantly modulate platelet integrin $\alpha_{IIb}\beta_3$ activation or α-granule secretion except under specific conditions. This may be due to low levels of TLR4 on the surface of resting platelets, which increases when platelets are activated. Moreover, experiments involving the novel Meg-01 reporter cell line revealed that LPS cannot induce signalling via TLR4 in this cell type because the TLR4 receptors are expressed intracellularly where LPS cannot ligate to them. Furthermore, different responses were seen between non-ultrapure and ultrapure LPS preparations in both platelets and Meg-01R cells which reinforces the hypothesis that other contaminants are present in the non-ultrapure preparations that can evoke inflammatory responses, possibly via TLR2. Therefore, great care must be taken when interpreting results using LPS. However, TLR4 may have a role in outside-in signalling via integrin $\alpha_{IIb}\beta_3$ as platelet spreading was inhibited over 20 minutes although TLR4 inhibition was not sufficient on its own to prevent spreading on fibrinogen.

Additionally, LPS_{RS} was shown to inhibit LL37-induced platelet function however this occurred in a TLR4-independent, cell-independent manner involving formation of LL37-LPS_{RS} micelles to prevent LL37 binding to its native receptor. Therefore, LL37

quenching is a potential novel therapeutic, for use in LL37-mediated pathological conditions, such as atherosclerosis and psoriasis, that requires further research.

Together, these data show that the precise role of TLR4 in platelet and MK function is still unclear and has been confused by studies conducted using non-ultrapure preparations of LPS. However, it is unlikely that TLR4 is just a relic left over from haematopoietic stem cells. The findings of this thesis suggest that, currently, platelet TLR4 is not sufficient to abrogate inflammatory responses and therefore not a suitable target for the development of novel treatments aiming to separate inflammatory responses from normal haemostasis in atherosclerosis, psoriasis, and sepsis. However, the further research proposed in this discussion may yet still yield new discoveries and novel therapeutic agents that can improve the quality of life for many individuals as well as reduce mortality worldwide.

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