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SCHOOL OF LIFE SCIENCES

MODULATION OF TLR4 SIGNALLING BY NOVEL SYNTHETIC GLYCOLIPIDS

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I. Abbreviations

ANOVA	Analysis of variance
Blk AB	Blocking Antibody
Cas1	Caspase 1
CD14	Cluster of differentiation 14
CD30	Cluster of differentiation 30
CD36	Cluster of differentiation 36
CD86	Cluster of differentiation 86
CEP	2-(ω-carboxyethyl) pyrrole
CpG	5'- Cytosine, phosphate, Guanine -3' Sequence
CRP	C-reactive protein
DAMP	Damage associated molecular pattern
DNA	Deoxyribonucleic acid
DTT	Dithiothreitol
dsRNA	Double Stranded Ribonucleic Acid
ECL	Enhanced chemiluminescence
ELISA	Enzyme linked immunosorbent assay
EOTAXIN-1	Eosinophil chemotactic protein 1
EOTAXIN-2	Eosinophil chemotactic protein 2
ERK	Extracellular signal-related kinase
FP (FP7, FP12…)	Francesco Peri (Francesco Peri 7, Francesco Peri 12…) small molecule series naming
GCSF	Granulocyte colony-stimulating factor
GM-CSF	Granulocyte-macrophage colony-stimulating factor
HDL	High-density lipoprotein
HRP	Horseradish peroxidase
I-309	Small-inducible cytokine A1
iCAM-1	Intercellular adhesion molecule 1
IFNAR	Interferon alpha/beta receptor
IFNα	Interferon alpha
IFNβ	Interferon beta
IFNγ	Interferon gamma
IgG	Immunoglobulin G
IKK	Inhibitor of nuclear factor kappa-B kinase
IL-10	Interleukin 10
IL-11	Interleukin 11
IL-12p40	Interleukin 12 p40 subunit
IL-12p70	Interleukin 12 p70 subunit
IL-13	Interleukin 13
IL-15	Interleukin 15
IL-16	Interleukin 16
IL-17A	Interleukin 17
IL-1R	Interleukin 1 receptor
IL-1α	Interleukin 1 alpha
IL-1β	Interleukin 1 beta
IL-2	Interleukin 2

IL-6R	Interleukin 6 receptor
IL-8	Interleukin 8
IP10	Interferon gamma induced protein 10
IRAK1	Interleukin-1 receptor-associated kinase 1
IRAK4	Interleukin-1 receptor-associated kinase 4
IRF-3	Interferon regulatory factor 3
I-TAC	Interferon-inducible T-cell alpha chemoattractant
JNK	c-Jun N-terminal kinase
KC	Keratinocyte chemoattractant
KO	Knockout
LBP	Lipopolysaccharide binding protein
LDL	Low-density lipoprotein
LIX	C-X-C motif chemokine 5
LPS	Lipopolysaccharide
LRR	Leucine rich repeat
M1	Classically activated macrophage
M2	Alternately activated macrophage
mAB	Monoclonal antibody
Mal	Myelin and lymphocyte protein
MAPK	Mitogen activated protein kinase
MCP-1	Monocyte chemoattractant protein 1
MCP-2	Monocyte chemoattractant protein 2
M-CSF	Macrophage colony-stimulating factor 1
MD-2	Myeloid differentiation factor 2, lymphocyte antigen 96
MDA	Malondialdehyde
MIG	Monokine induced by gamma-INF
MIP-1α	Macrophage inflammatory protein 1 alpha
MIP-1α	Macrophage inflammatory protein 1 alpha
MIP-1β	Macrophage inflammatory protein 1-beta
MIP-1γ	Macrophage inflammatory protein 1 gamma
MIP-1δ	Macrophage inflammatory protein 1-delta
MKK	Mitogen Activated Protein Kinase Kinase
mmLDL	Minimally modified low-density lipoprotein
MMP	Matrix Metalloproteinase
MPLA	Monophosphoryl lipid A
MyD88	Myeloid differentiation factor primary response 88
NF-κB	Nuclear Factor kappa-light-chain-enhancer of activated B cells
nLDL	Native low-density lipoprotein
NLRP3	NACHT, LRR and PYD domains-containing protein 3
NS	Non-significant
oxHDL	Oxidised high-density lipoprotein
oxLDL	Oxidised low-density lipoprotein
oxPC	Oxidised phosphocholine
oxPE	Oxidised phosphatidylethanolamine
PAMP	Pathogen associated molecular pattern
PDGF-BB	Platelet-derived growth factor BB
PMA	Phorbol 12-myristate 13-acetate
RANTES	Regulated upon Activation Normal T Cell Expressed and Secreted
RAW264	Immortalised mouse macrophage cell line

R-Form (Ra, Re…)	Rough Form
RNA	Ribonucleic acid
ROS	Reactive oxygen species
SAPK	Stress-activated protein kinase
SD	Standard deviation
SDF-1α	Stromal cell-derived factor 1 alpha
S-Form	Smooth Form
SMC	Smooth Muscle Cell
SR-Form	Semi-rough Form
ssRNA	Single Stranded Ribonucleic Acid
STAT-1	Signal transducer and activator of transcription 1-alpha/beta
TAB1	TAK1-binding protein 1
TAB2	TAK1-binding protein 2
TAK1	Transforming growth factor beta-activated kinase 1
TBK1	TANK-binding kinase 1
TBS-T	Tris buffered saline with Tween 20
TECK	Thymus-expressed chemokine
TGFβ	Transforming growth factor beta
THP-1	human monocyte cell line
TIMP-1	Tissue inhibitor of metalloproteinases 1
TIMP-2	Tissue inhibitor of metalloproteinases 2
TLR (TLR1,TLR2…)	Toll-like receptor (Toll-like receptor 1, Toll-like receptor 2, etc…)
TMB	3,3',5,5'-Tetramethylbenzidine
TNF RI	Tumour necrosis factor receptor 1
TNF RII	Tumour necrosis factor receptor 2
TNFα	Tumour Necrosis Factor alpha
TRAF6	TNF receptor-associated factor 6
TRAM	Translocating chain-associated membrane protein
TRIF	TIR domain containing adaptor inducing interferon beta
VCAM-1	Vascular cell adhesion protein 1
VEGF	Vascular endothelial growth factor
WT	Wildtype
XCL1	X-C Motif Chemokine Ligand 1, Lymphotaxin
TYK (TYK1..)	Tyrosine kinase (Tyrosine Kinase 1 etc)
JAK1	Janus activated kinase 1
MS	Multiple sclerosis
HMEC	Human mammary epithelial cell

II. Abstract

MODULATION OF TLR4 SIGNALLING BY NOVEL SYNTHETIC GLYCOLIPIDS - Charys Palmer (2021)

Background: Vascular diseases represent a significant burden on healthcare systems in the developed world, heavily contributing to mortality and morbidity. TLR4 is an innate immune receptor integral to host defence against pathogens. However, overactivation of this response can be detrimental and is associated with development and progression of chronic inflammatory based conditions such as vascular diseases. Modulation of TLR4 activity therefore represents a potential avenue for development of pharmacological interventions.

Objective: Professor Peri (University of Milano) has developed a series of synthetic glycolipid small molecule modulators of TLR4. The current body of work aimed to screen and assess the potential of several predicted agonist and antagonists to affect TLR4 signalling in a cellular system, investigate the mechanism of action of these compounds and to determine their validity as modulators of haematopoietic TLR4 signalling in human and mouse cell lines.

Methods: Human and mouse monocyte and macrophages were used as a model. MTT was used to determine cytotoxic effects of ligands and small molecule antagonists and agonists. TLR4 activation was stimulated by exposure of cells to bacterial endotoxin or sterile oxidised lipids and effects. FP compounds were tested in the presence or absence of TLR4 ligands. Two readouts were used: activation of signalling mediators and endpoint proinflammatory protein production. These were measured using Western blot, ELISA, and a proinflammatory protein antibody array.

Results: Screening revealed two antagonists and one agonist which were selected for further mechanistic studies. FP7 and FP12 were shown to negatively regulate both human and mouse MyD88-dependent signalling and human TRIF-dependent pathways. FP11 and FP18 were more

effective at inducing MyD88-dependent signalling than TRIF-dependent but may still activate signalling via IFNAR.

Conclusions: Results from the current body of work provide novel evidence towards the effects and mechanism of action of FP antagonists and agonists as modulators of TLR4-dependent proinflammatory signalling. Together, this helps to validate two antagonist and two agonist molecules as potential novel therapeutics for further preclinical investigations for treatment of inflammatory based disorders.

LAY SUMMARY

Heart disease is a major contributor to death and long-term disability in the developed world. There is evidence to suggest that some abnormal signalling in cells may contribute to disease progression. In response to the presence of bacterial threat, an immune system is activated which initiates a defensive reaction. This response is broad and can cause damage to both the threat and the host organism. In some cases, the immune system can also be activated without a threat present and has been implicated in a range of diseases, including heart disease. As such, specific control of the immune system may provide a means of treatment. Further to this, increasing activity of specific molecules such as Toll like receptor 4 (TLR4) may also be of value in initiating immune response. To determine if a series of potential drug candidates were capable to affect TLR4, immune cells were used to test the activation and suppression capability of several candidates. Two of these were capable of reducing activation of TLR4 caused by bacterial products, and two others caused activation of the system. These were selected as candidates of interest and investigated further to determine the means by which they could interact with signalling. This provided evidence of the effects of these drug candidates and their value as items of interest for further clinical investigation.

III. Acknowledgements

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IV. Contents

V. Figures

VI. Publications

There are two articles currently published in relation to the following body of work and two further articles which are currently either under review or in progress prior to submission for this year.

1. Facchini FA, Minotti A, Luraghi A, Romerio A, Gotri N, Matamoros-Recio A, Iannucci A, **Palmer C**, Wang G, Ingram R, Martin-Santamaria S, Pirianov G, De Andrea M, Valvano MA, Peri F. Synthetic Glycolipids as Molecular Vaccine Adjuvants: Mechanism of Action in Human Cells and In Vivo Activity J Med Chem. 2021 Aug 26;64(16):12261-12272.

2. **Palmer C**, Facchini F, Peri F, Neumann F, Pirianov G. The synthetic glycolipid-based TLR4 antagonist FP7 negatively regulates TRIF-dependent TLR4 signalling in macrophages Innate Immun. 2021 Apr;27(3):275-284.

3. **Palmer C**, Neumann F, Ahmad F, Leake DS, Peri F, Pirianov G. The synthetic glycolipid-based TLR4 antagonist FP7 negatively regulates in vitro and in vivo haematopoietic and non-haematopoietic vascular TLR4 signalling. Innate Immun. 2018, 24(7):411-421.

4. Facchini FA, Zaffaroni L, Minotti A, Rapisarda S, Calabrese V, Forcella M, Fusi P, Airoldi C, Ciaramelli C, Billod JM, Schromm AB, Braun H, **Palmer C**, Beyaert R, Lapenta F, Jerala R, Pirianov G, Peri F. Structure-Activity Relationship in Monosaccharide-Based Toll-Like Receptor 4 (TLR4) Antagonists. J Med Chem.12;61(7):2895-2909, 2018.

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1. Introduction

TLRs are a family of innate immune receptors referred to as pattern recognition receptors. The Innate immune system is part of a predefined encoded response which provide a fast, generalised reaction to pathogens. TLRs provide a protective effect against invading pathogens and are involved in initialising the inflammatory response. However, aberrant TLR activity has been associated with sepsis, diabetes, metabolic syndrome, cardiovascular diseases, neurodegenerative diseases, and stroke (Higashimori *et al.*, 2011; Hernanz *et al.*, 2015). Among these, cardiovascular diseases represent a significant impact on global healthcare, being the leading cause of mortality and morbidity in the developed world (Wilkins *et al.*, 2017). The absence of TLR4 has been shown to protect against experimental models of cardiovascular diseases in animal-based studies (Michelsen *et al.*, 2004; Owens *et al.*, 2011). This suggests that molecules capable of reducing TLR activity may be of clinical interest.

TLR4 is known as the receptor for gram-negative bacteria. The receptor detects the presence of gram-negative bacteria through binding to LPS, a component of the bacterial outer membrane. LPS is not bound directly to TLR4, but rather delivered via LBP and CD14 to a hydrophobic pocket on the co-receptor (MD-2) which is attached in complex with the TLR4 ectodomain. This interaction causes a conformational change in the F126 loop of MD-2 which re-positions several residues important for initiating dimerisation of TLR4. A remaining free acyl chain of Lipid A interacts with a hydrophobic patch on the second receptor, forming the TLR4/MD2 signalling complex (Park *et al.*, 2009). Dimerisation of TLR4 causes the intracellular signalling domains of these receptors to come together, creating a dimer which acts as a scaffold for Mal/MyD88 and TRAM/TRIF. Signalling via MyD88 or TRIF dependent pathways and results in endpoint production of pro-inflammatory cytokines and interferons. While this is an important defence mechanism, this response is broad and nonspecific,

causing damage both to invading pathogens as well as to host tissues, which may be detrimental to heath if sustained.

It is thought that sterile inflammation rather than bacterial induced inflammation is linked to many chronic inflammatory based diseases (Kolodgie *et al.*, 2002; Chai *et al.*, 2005). Aside from LPS, TLR4 has been shown to be activated by a variety of self-derived molecules including fibronectin, heatshock proteins, small fragments of hyaluronan and oxidised low-density lipoproteins (LDL) (Taylor *et al.*, 2004; Miller *et al.*, 2005). Absence of TLR4 has been shown to provide protective effects against cardiovascular disease progression in mice, implicating TLR4 in disease pathology (Michelsen *et al.*, 2004; Owens *et al.*, 2011). The presence of molecules such as oxLDL in atherosclerotic lesions may be linked to the sustained inflammatory response associated with the condition and may drive TLR4 involvement in atherosclerotic progression. TLR4 activation can occur in response to both LPS and oxLDL, despite structural differences between these molecules. Recognition of LPS by TLR4 relies on interaction of LPS with TLR4 and MD-2 to form dimers, aided in by a delivery system involving LBP and CD14. TLR4 activity in response to OxLDL is also associated with involvement of other receptors including TLR2, TLR6 and CD36, the absence of which may diminish responsiveness to oxLDL (Stewart *et al.*, 2010). These sterile ligands are thought to interact with TLRs in a different manner to LPS.

Despite the obvious involvement of TLR4 in disease progression complete removal or blocking of TLR4 activity may not be desirable due to the important role of TLR4 in host-defence. Suppression of TNFα may result in the re-emergence of dormant infections (Lin *et al.*, 2010). A more nuanced approach in modulation of TLR4 receptor activity that allows for a reduction in activation, while retaining immune functionality, may therefore be more appropriate.

A series of small molecule agonists and antagonists of TLR4 were designed and developed by Professor Peri (University of Milano). These molecules shared structural similarities to the active Lipid A moiety of LPS, responsible for binding to and triggering TLR4 signalling, and were designated

as ‘FP’ followed by an identifying number. Some of these compounds have previously been shown to downregulate LPS induced TLR4-dependent signalling and are thought to interact with the MD-2 co-receptor of TLR4 in a similar way to LPS (Cighetti *et al.*, 2014). They are thought to be highly specific to TLR4, and studies have shown that FP7 does not affect several other TLRs, which are stimulated by different ligands (Perrin-Cocon *et al.*, 2017). Further investigation of these molecules is required to understand the more specific effects and differences between each of the structural variants. In this regard, FP7 and its derivative series were evaluated here in both human and mouse macrophage cell lines.

1.1. Toll-like receptors

Toll-like receptors are a class of transmembrane glycoproteins found in both invertebrate and vertebrate species. Originally discovered in fruit flies, drosophila toll was associated with dorsoventral patterning (Belvin and Anderson, 1996), and later in the response to fungal and bacterial infections (Lemaitre *et al.*, 1996; Michel *et al.*, 2001). A human homologue of toll was later identified and described, being similar to drosophila toll but exhibiting one LRR region rather than two separated regions of LRRs (Medzhitov, Preston-Hurlburt and Janeway, 1997).

The number of different TLRs found in species can vary greatly, ranging between one and hundreds in some invertebrates. However, in some cases, this high variability in number of TLRs may be the result of repeat coding of the same receptor (Pujol *et al.*, 2001; Davidson *et al.*, 2008; Buckley and Rast, 2012). In vertebrates the number of TLRs is typically more consistent, with most species having a similar array of TLRs which is much more conserved between species and is likely due to their immunological importance. Conversely, the high amount of variation in number of TLRs in invertebrates may be related to their developmental role.

Vertebrate TLRs are an important part of the innate immune response. TLRs recognise and bind to broad classes of molecules, known as PAMPs (Pathogen associated molecular patterns), associated with the presence of pathogens and regulate the inflammatory response. Following recognition of PAMPs the TLR ectodomain forms dimers. This causes subsequent intracellular dimerisation of TIR signalling domains which triggers an intracellular signalling cascade and facilitates transcription and production of proinflammatory proteins. While there is variation between TLRs between species, much of the functional activity of these receptors is conserved indicating the importance of TLRs in species survival. While this provides a necessary defensive response to pathogens, sustained activation of the TLRs can also be damaging.

There are six main families of vertebrate TLRs. The TLR1, TLR7 and TLR11 families all contain several TLRs within the group, whereas TLR3, TLR4 and TLR5 families consist of only one TLR in each case.

These families are thought to have been present at the point of divergence between all vertebrate species, though TLR15 from the TLR1 group similarities may be the result of some convergent evolution of a paralogue within birds and reptiles (Temperley *et al.*, 2008; Boyd *et al.*, 2012). Humans do not express a representative of the TLR11 family, though a pseudogene for this receptor has been reported, suggesting an ancestral presence of TLR11. This could have been selected against due to redundancy of this receptor in humans, or perhaps self-initiated responses that made TLR11 presence less viable as a protection strategy. In most cases, Vertebrate species exhibit one representative TLR from each receptor family (Roach *et al.*, 2005). These typically have somewhat conserved structure and function between vertebrate species, indicative of their importance in host-defence and survival.

1.1.1. **Structure and function of toll-like receptors**

TLRs are transmembrane glycoproteins made up of an extracellular domain, responsible for ligand recognition, connected via an alpha helix to an intracellular signalling domain similar in structure to that of IL-1R (Botos, Segal and Davies, 2011). The extracellular domain of the TLRs is made up of 6-28 LRR regions with 2-5 cystines at the C-terminus (Matsushima *et al.*, 2007). This gives TLRs a typical solenoid or horseshoe shaped structure which may allow for greater flexibility (Figure 1).

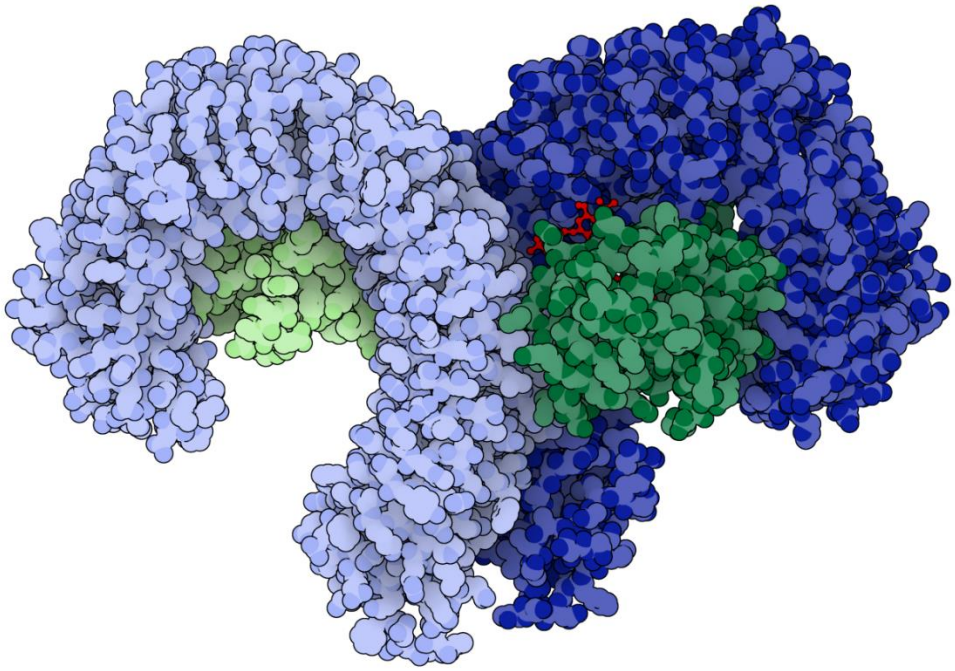

Figure 1. Structure of extracellular TLR4/MD-2 signalling complex. Blue = TLR4 (light/dark - dimer), Green = MD-2 (light/dark – associated with corresponding TLR4), Red = Lipid IV A ligand bound to MD-2 pocket, PDB ID: 3VQ1 (Ohto et al.*, 2012). Images produced using protein imager* (Tomasello, Armenia and Molla, 2020).

LRR structures are a common among receptors and signalling molecules, and are regions which are typically associated functional activity, facilitating protein interactions. These structures have been reported in a host of organisms, from bacteria to humans. In addition to TLR4, CD14 also have LRR regions which are associated with ligand interaction (Kim *et al.*, 2005). NLRs, another group of innate immune receptor, are known to have a similar LRR ectodomain. NLRP3, important for IL-1β production, has been shown to contain the LRR motif which aids in interactions with NEK7 and subsequent inflammasome activation (Sharif *et al.*, 2019). TLRs are typically quite similar in structure to one another, but the LRR regions can vary considerably in length, from around 22-30 residues (Mi and Lee, 2008). Most of the variation between TLR ectodomains occurs in the middle of the LRR region, where ligand binding is thought to occur.

Although the structure of TLRs are relatively conserved between vertebrates, owing to functional significance, the structure of invertebrate TLRs is not the same. The most obvious difference between vertebrate and invertebrate TLRs is the breaking up of this LRR region by a cysteine cluster in invertebrates, which in turn creates a long and short chain LRR region in such species TLRs (Rock *et al.*, 1998)This likely corresponds to the functional difference of TLRs between such organisms.

TLRs are heavily glycosylated, though this glycosylation is not thought to be associated with ligand recognition. While removal of glycosylation sites may impair TLR activity, it does not necessarily inhibit binding or recognition of ligands (Liu *et al.*, 2008). Despite this, the glycosylation sites of TLRs are highly conserved across species, suggesting these are functionally important. TLR2 secretion is shown to be impaired by the removal of two specific glycosylation sites (Weber, Morse and Gay, 2004), and TLR4 is also dependent on some of these glycosylation sites for transport to the cell surface(da Silva Correia and Ulevitch, 2002). Asparagine residue mutations in TLRs can affect glycosylation sites, but the reduction in TLR activity seen following such mutations is thought to be the result of structural or conformational changes rather than the loss of a glycosylation site itself (Weber, Morse and Gay, 2004; Liu *et al.*, 2008). Glycosylation of TLRs is therefore important in

receptor processing and secretion but may not be directly involved in the binding efficiency of the receptor.

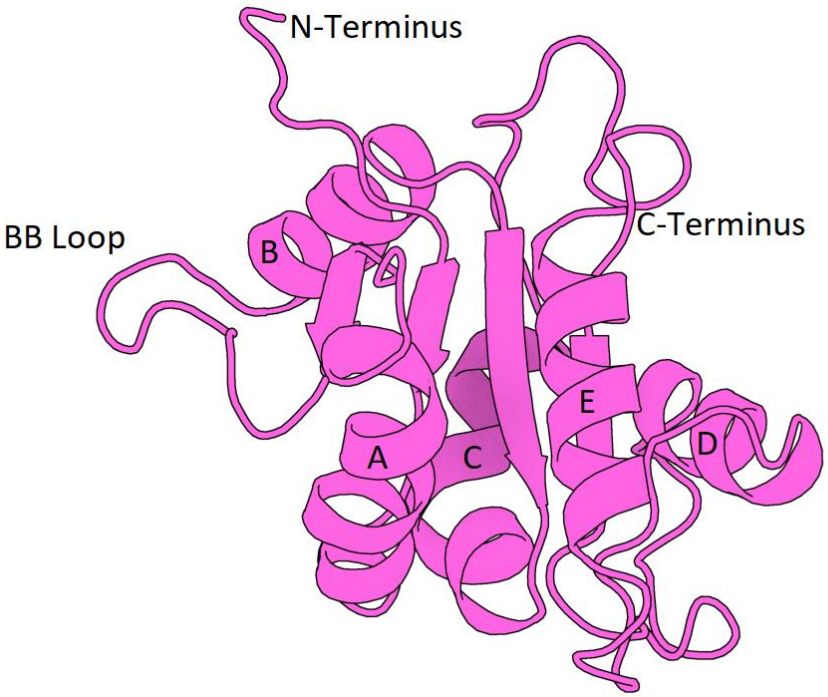

Figure 2. Crystal structure of TIR domain of TLR2. Alpha Helices are designated as A-E from N- to C-terminus. PDB ID: 1FYW (Xu et al*., 2000). Images produced using protein imager* (Tomasello, Armenia and Molla, 2020)

TLR ectodomains are known to form dimers on recognition of PAMPS. These dimers may be in the form of heterodimer or homodimers. TLR2 forms heterodimers with TLR1 or TLR6 in a ligand specific manner which may allow for more nuanced threat detection of triacylated and diacylated lipopeptides respectively (Mi and Lee, 2008). TLR4 typically forms a homodimer when bound to LPS, but it is thought that a TLR4/TLR6 heterodimer may be involved in activity relating to amyloid-β and oxLDL (Stewart *et al.*, 2010). Heterodimers may allow for recognition of a greater variety of ligand. Following ectodomain dimerisation, the intracellular regions of TLRs are also brought together.

The intracellular signalling, TIR domain containing region of TLRs is structurally similar to in signalling domain of IL-1R(Botos, Segal and Davies, 2011). The crystal structure analysis of TLR cytoplasmic TIR domains determines these to be made up of a β-sheet core and five α-helices surrounding, designated A-E (Figure 2). While the signalling domains of TLRs are relatively comparable to one another, there are some conformational differences seen between TLRs (Xu *et al.*, 2000). There is a highly conserved region between TLRs which is located just before α-helix B referred to as the BB loop (Figure 2). The BB loop area of the TIR domain is thought to be important in interactions with adaptor molecules such as MyD88 (Xu *et al.*, 2000). Mutations in this region are associated with subsequent loss of receptor function, suggesting the integral role of the BB-loop in signalling (Slack *et al.*, 2000). The TIR domain of TLR4 interacts with TIR domains on MyD88 and TRIF to facilitate signal transduction.

1.1.2. **Ligands of toll-like receptors**

TLRs detect the presence of pathogens through binding to and recognising molecules associated with their presence. These ligands can vary dependent on the specific receptor involved, but usually consist of pathogen associated nucleic acids or shed components. The location of each TLR is often associated with the type of molecule that it is detecting. TLRs which detect genetic material such as dsRNA or CpG DNA are typically located in endosomal compartments, whereas those associated with external components such as LPS or flagellin are found on the surface membrane of the cell (Lee, Avalos and Ploegh, 2012). These molecules bind to the receptors and cause dimerisation to occur, which activates the subsequent signalling chain through intracellular MyD88 or TRIF recruitment.

Humans are known to possess 10 TLRs, TLR1-TLR10, which are associated with different classes of ligands. TLR1, TLR2, TLR4, TLR6, and TLR5 are found on the outer membrane of the cell. TLR2 is known to detect triacylated and diacylated lipopeptides, forming heterodimers in conjunction with TLR2 and TLR6 to recognise these respectively (Mi and Lee, 2008). TLR4 is known as the receptor for gram-negative bacteria via associated endotoxin (LPS) and TLR5 is responsible for detection of flagellin (O'Neill, Golenbock and Bowie, 2013). In endosomal compartments, TLR3, TLR7, TLR8 and TLR9 detect the presence of pathogen associated nucleic acids. TLR3 detects dsRNA (Liu et al., 2008), ssRNA is detected by TLR7 and TLR8, and CpG DNA by TLR9 (O'Neill, Golenbock and Bowie, 2013). Together, these provide an automatic detection system for a broad range of invading pathogens and produce an initial non-specific response to threats before more specialised processes come into action. While TLR1-9 all have recognised ligands, the role of TLR10 in immune function is less understood.

As yet, TLR10 has no known associated ligands. An apparent regulatory effect of TLR10 on other TLRs has been observed, as blocking of the receptor can lead to upregulation of TLR2. Mice transfected with human TLR10 show suppressed cytokine release via MyD88 and TRIF-dependent inhibition

(Oosting *et al.*, 2014; Jiang *et al.*, 2016). This may suggest an immunomodulatory function of the receptor but is yet to be confirmed under normal conditions.

1.1.3. **Ligands of sterile inflammation**

Aside from the classic ligands associated directly with pathogenic invasion, TLRs have also been seen to react to some endogenous host-derived molecules, known as DAMPs (Damage Associated Molecular Patterns). These DAMPs may be indicative of an otherwise undetected infection, whereby pathogens inflict damage to surrounding cells and tissue causing the release of these molecules which stimulate receptors as indirect signs of a threat. These DAMPs include molecules such as heatshock proteins, fibronectin, small fragments of hyaluronan and oxidised lipids, among others. In some cases, DAMPs are thought to trigger TLR response in the absence of pathogens and may be associated with the aberrant receptor activity seen in many disease pathologies.

Sterile ligands of TLRs may be responsible for the sustained activation of inflammatory response in inflammatory based conditions. OxLDL has been implicated in cardiovascular disease and may contribute to disease progression. Activation of TLR4 response by oxLDL is thought to involve several alternate receptors to canonical TLR4 activation by LPS, including TLR2, TLR6 and CD36 (Stewart *et al.*, 2010). High levels of circulating oxLDL and CD36 have been associated with high-risk groups (Ramos-Arellano *et al.*, 2014) and are associated with TLR4 activation of macrophages as well as the formation of foam cells, an important part of atherosclerotic plaque progression (Chávez-Sánchez *et al.*, 2014). Further to this, self-nucleic acids have also been shown to cause aberrant activation of TLR9. TLR9 can be activated by mitochondrial DNA which has been implicated as a contributing factor to sustained inflammation seen in patients post severe trauma (Zhang *et al.*, 2010) and has also been associated with IL-1β production and inflammasome activation (Shimada *et al.*, 2012). TLR9 may also be activated by IgG bound chromatin, which has been implicated in lupus (Hoque *et al.*, 2011). Therefore, sterile ligands of TLRs are of clinical interest in development of strategies for combatting inflammatory based diseases.

Sterile ligands may interact with receptors differently than PAMPs. Some are thought to cause formation of unique heterodimer combinations in response to activation. TLR4 and TLR6 are thought

to form a heterodimer in response to oxLDL and amyloid-β stimulation and TLR2 and TLR4 are both important in hyaluronan driven inflammation (Jiang *et al.*, 2005). Other receptors may also be associated with ligand recognition processes. CD36 has been shown to be indispensable in oxLDL induced inflammation. A lack of CD36 was associated with downregulation of oxLDL stimulated production of MIP-2 and RANTES in mice. A reduction was also seen in NF-κB activation in response to oxLDL and amyloid-β in absence of CD36, whereas the presence or absence of CD14 does not appear to be important (Stewart *et al.*, 2010). Due to the different mechanisms that pathogen and host originating ligands are recognised through, antagonists of TLRs in response may interact with DAMP-dependent signalling differently to canonical PAMPs.

1.1.4. **Activation of toll-like receptor signalling in human and murine models**

Mice a regularly used as a model for investigating cardiovascular disease through angiotensin stimulated atherosclerotic development. Translation of findings into an animal model is an important bridging step towards proving the clinical relevance of a new molecule in a living system. However, fundamental differences between humans and mice can sometimes lead to differences in response to such interventions. The immune response of mice is geared towards tolerance to repeated stimuli in order to prevent tissue damage. This is in contrast to humans which utilise a strategy of resistance whereby threats are eliminated quickly. This difference in immune strategies may be due to the difference in the environments in which humans and mice find themselves in. The difference between human and mouse immune system response may result in difficulties in translating findings from studies in mice to humans.

While human and mouse TLRs share some similarities, there are distinct differences in the expression, activity, and distribution of receptors between humans and mice. Humans have 10 known TLRs, whereas 12 TLRs have been identified in mice so far. Humans are known to possess TLRs 1-10, while mice possess TLRs 1-7 and 9-13. TLR8 is absent in mice and TLR12 and TLR13 are absent in humans, with TLR11 only being present in the form of a pseudogene. In mice, TLR2 is expressed in the T-cells, but not in humans where circulating leukocyte TLR2 expression is more pronounced than in mice. Poly I:C can result in TLR3-dependent NF-κB and MAPK activation, resulting in downstream TNFα and IL-6 production in mice, but not humans (Lundberg *et al.*, 2007). Mouse susceptibility to LPS cytotoxic effects is markedly reduced as compared to humans which require lower doses for cytotoxicity due to resistance mechanisms in mice (Matsuguchi *et al.*, 2000) Conversely, mice are more sensitive to TLR5 based recognition of flagellin. Variations in TLR responses such as this could potentially affect the comparability of human and mouse *in vivo* models.

While both humans and mice are known to possess TLR4, mouse TLR4 may react to ligands slightly differently than human TLR4. In this regard, Tetracyclated lipid IVa was shown act as an agonist of mouse receptor activity, while functioning as an antagonist in humans (Ohto *et al.*, 2012). The MD-2 co-receptor is attached to TLR4 and facilitates ligand interaction through binding of acyl chains from Lipid A into a hydrophobic pocket on the co-receptor, alongside interactions with a second patch on the second recruited TLR4 receptor (Park *et al.*, 2009)Antagonism may occur by binding of mimetic molecules to the hydrophobic pocket of MD-2, which are fully accommodated in the hydrophobic pocket, preventing subsequent binding by LPS. It has been suggested that short chain lipid A molecules exhibit antagonistic effects in mice, whereas long chains are more effective in human models. This may be due to differences in the MD-2 receptor between species (Chebrolu *et al.*, 2015). The means of antagonist action by these molecules is thought to be one of competitive exclusion, thus a reduction in binding efficiency caused by structural differences of MD-2 could result in the lower antagonistic potential. It is therefore important to investigate the activity of TLR4 modulators in both humans and mouse model systems. The verification of findings in mouse cells, as well as human cell lines will help to inform future *in vivo* studies.

1.2. Toll-like receptor 4

Toll-Like receptor 4 (TLR4) is expressed in both hematopoietic and non-hematopoietic cells within the body. Deletion of TLR4 in mice is associated with a subsequent lack of LPS response and increased susceptibility to gram-negative infection (Poltorak *et al.*, 1998). TLR4 is therefore known as the receptor for gram-negative bacteria through this capacity to bind to and initiate an inflammatory response in the presence of gram-negative associated LPS.

TLR4 does not recognise the presence of LPS directly. Instead, the ligand is delivered by CD14 and LBP to the hydrophobic pocket of the MD-2 co-receptor (Figure 3) (Oblak and Jerala, 2015). Agonistic activity is driven by accommodation of acyl chains within the hydrophobic pocket of MD-2 alongside an additional chain outside of this pocket which interacts with a hydrophobic region on the surface of a second recruited TLR4, serving to stabilise the receptor complex (Oblak and Jerala, 2015) This triggers a conformational change which initiates homodimerisation of the receptor complex. Intracellular signalling is then initialised by the adaptor proteins MyD88 and Mal or TRIF and TRAM which initiate activation of two separate pathways (Kenny and O'Neill, 2008). These result in downstream phosphorylation and activation of signalling mediators, NF-κB and MAPKs, and subsequent production of proinflammatory proteins.

Following recognition of ligands, dimerisation of TLR4 ectodomain occurs. Subsequent dimerisation of intracellular signalling domains occurs and initiates recruitment of MyD88 or TRIF, facilitated by bridging adaptors Mal and TRAM. This initiates a signalling cascade that results in the release of NF-κB and MAPKs; JNK (c-JUN N-terminal kinase), ERK (extracellular signal-regulated kinase) and P38. This causes subsequent production of proinflammatory proteins or interferons.

TLR4 has been associated with a wide variety of inflammatory based diseases including sepsis. TLR4 response to LPS has also been associated with conditions such as sepsis, where aberrant activation of the receptor results in a sustained inflammatory state that is damaging and potentially life-threatening (Kuzmich *et al.*, 2017). This contributed to around 11 million deaths worldwide in 2017,

though survival rates have improved over the last three decades (Rudd *et al.*, 2020). However, sepsis can also have long lasting aftereffects and may contribute to impaired immune functionality in survivors (Ammer-Herrmenau *et al.*, 2019). Further to this, cardiovascular and neurodegenerative diseases have been associated with TLR4 activation, but the involvement may not be linked to canonical activation of the receptor by LPS (Higashimori *et al.*, 2011; Hernanz *et al.*, 2015). There is therefore a need to modulate TLR4 in specific conditions.

Cardiovascular disease represents a major contributor to mortality and morbidity in the developed world. TLR4 is also known be activated by endogenous molecules such as small fragments of hyaluronan and oxLDL which are often referred to as DAMPs or ligands of sterile inflammation and may be involved in progression of cardiovascular disease (Xu *et al.*, 2001; Taylor *et al.*, 2004). As such, therapeutic strategies involving modulation of TLR4 response, and more specifically TLR4 response to the presence of sterile ligands, could be beneficial in treatment in these conditions.

1.2.1. **The role LBP, CD14 and MD-2 in TLR4 signalling**

LBP and CD14 facilitate ligand recognition through binding and presenting LPS to MD-2. LBP is a long acute phase protein which is produced in the liver and exists in a soluble state which enters the blood stream. Circulating LBP has a high affinity for LPS and will bind to this in the bloodstream (Palsson-McDermott and O'Neill, 2004). LBP attaches to LPS and subsequently can present the molecule to CD14.

CD14 can exist either as a membrane bound protein or may be soluble in nature. LBP is thought to aid in the transfer of LPS to CD14 but may not always be necessary for LPS recognition. CD14/LPS complex formation has also been reported to occur in the absence of LBP (Hailman *et al.*, 1994). Where LBP does facilitate LPS binding, the acute phase protein interacts with the inner curve of CD14, forming a complex to deliver LPS. This interaction only occurs when LBP is bound to LPS and not between LBP and CD14 alone (Kim and Kim, 2017). Following the interaction resulting in LPS/LBP/CD14 complex formation, LPS is transferred to CD14 through a series of brief electrostatic interactions between a basic region of LBP and the acidic inner arch of CD14 which are only initiated when LBP is bound, after which LBP dissociates from LPS/CD14, which was determined using Single-molecule fluorescence microscopy (Kim and Kim, 2017). CD14 then serves to deliver LPS to the MD-2 co-receptor, which may have a higher binding affinity for LPS than does CD14 (Koraha *et al.*, 2005). However, CD14 appears to be capable of binding to a broader range of LPS molecules than MD-2, being less specific in binding to molecules with different levels of acylation perhaps due to its role in other TLR signalling pathways (Koraha *et al.*, 2005). CD14 does not appear to be associated with TLR4/MD-2 in the active signalling complex and may only have a temporary association; depositing LPS before dissociating once LPS is transferred (Akashi *et al.*, 2003). TLR4 signalling in response to LPS can still be achieved in absence of CD14, but absence of MD-2 abolishes receptor functionality (Haziot *et al.*, 1998; Nagai *et al.*, 2002). CD14 and LBP are important external proteins which aid in

the recognition of LPS by the receptor by functioning as a delivery system but are not always necessary for LPS associated TLR4 signalling.

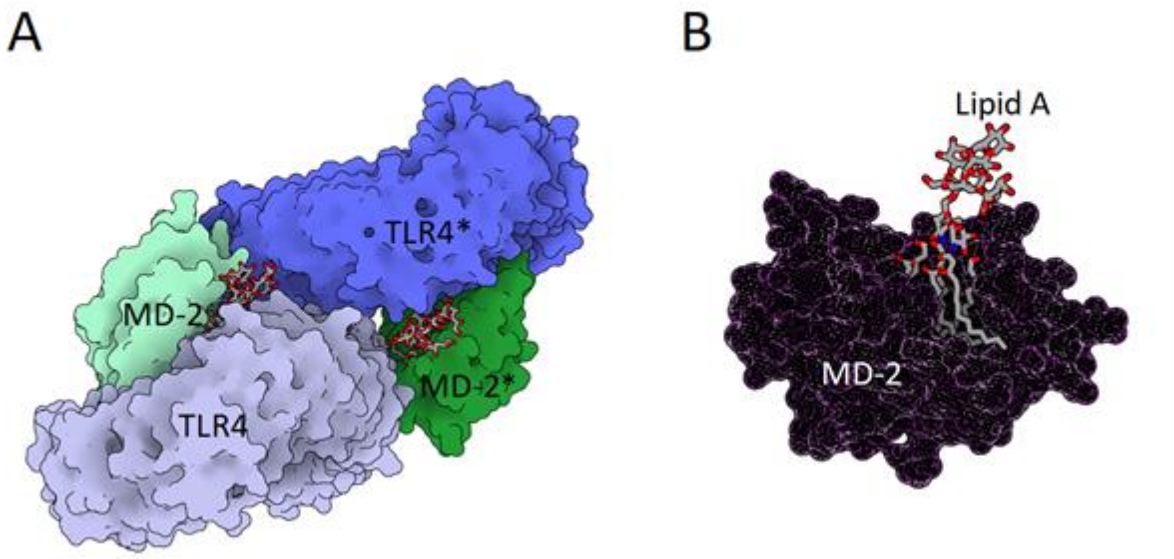

Figure 3. Binding of LPS to TLR4/MD-2 A) The Lipid A domain of E.coli LPS bound to the hydrophobic pocket of MD-2, viewed from above, forms a dimerisation interface, interacting with the recruited TLR ectodomain. B) 5 lipid chains are bound within the pocket of MD-2, while one extends outside, maing contact a patch on the second recruited TLR4 receptor. The phosphate groups bind to the receptor complex through interaction with positively charged residues on each of the TLR4 receptors. PBD ID: 3FFXI (Park et al*., 2009). Images produced using protein imager* (Tomasello, Armenia and Molla, 2020)

TLR4 differs from other TLRs in that it is the only receptor which relies on a co-receptor molecule in order to detect its associated ligand. MD-2 is bound to TLR4 and has been shown to be crucial in ligand recognition. Absence of MD-2 results in failure of TLR4 response to LPS (Nagai *et al.*, 2002). The MD-2 co-receptor is formed in such a way that a hydrophobic pocket is present between two antiparallel beta sheets. This hydrophobic pocket has a high binding affinity to the acyl chains of LPS.

When LPS is delivered to the MD-2 co-receptor, this causes a conformational change in MD-2 which is associated with recruitment of the second TLR4 receptor, facilitates receptor dimerisation and subsequent intracellular signal domain associations (Figure 3 A) (Ohto *et al.*, 2012; Kim and Kim, 2017). The essential role of MD-2 in ligand recognition makes this a good choice to target for modulators of TLR4.

It was originally thought that all acyl chains of LPS were located within the hydrophobic pocket upon LPS binding to MD-2 and the conformational change in the co-receptor was related to accommodation of these chains. However, crystal structure analysis revealed that 6 chain *Escherichia coli* LPS has acyl chains do not all fit within the pocket (Figure 3 B). One of these chains remains projecting outside and interacts with a hydrophobic patch on the second recruited TLR4 receptor (Park *et al.*, 2009)A synthetic TLR4 agonist, Neoseptin-3, is also known to interact with the hydrophobic region of the receptor and can induce TLR4 signalling, exhibiting similar conformational changes as seen by LPS despite not being structurally similar (Wang *et al.*, 2016). Though it is not clear at present, interaction with this interface is thought to drive dimerisation in response to LPS binding.

1.3. Activation of TLR4 signalling pathways

TLR4 differs from other TLRs in that it has been associate with both MyD88 and TRIF-dependent signalling pathways. Dimerisation of the ectodomains of TLR4 results in a corresponding dimerisation of TIR domains which recruit either MyD88 or TRIF signalling molecules. The intracellular region of TLR4 does not allow for direct interaction and requires the aid of bridging adaptor proteins Mal and TRAM to facilitate interactions with MyD88 and TRIF respectively (Kenny and O'Neill, 2008). Mal and TRAM contain TIR domains which interface with the TIR domain of TLR4, as well as those of MyD88 and TRIF respectively (Ve *et al.*, 2012, 2017). Mal is proposed to have a phosphatidylinositol 4,5-bisphosphate region which is important for membrane recruitment (Kagan and Medzhitov, 2006) while TRAM is bound to the membrane and relocates upon activation of TRIF signalling (Rowe *et al.*, 2006; Tanimura *et al.*, 2008) Various binding sites have been proposed for TIR-TIR interactions, but as yet are these have not been confirmed. However, dimerisation of TLR4 ectodomains, which brings together the TIR domains of TLR4 may causes an increase in affinity for TIR-TIR interactions through formation of a cooperative assembly (Nimma *et al.*, 2017).

MyD88 signalling occurs through formation of the Myddosome, whereby recruitment of MyD88 and IRAKs forms a platform signal transduction (Lin, Lo and Wu, 2010). Downstream activation of NF-κB and MAPKs lead to endpoint production of proinflammatory cytokines. Absence of MyD88 may diminish, but not abolish response to LPS leading to the proposal of an MyD88 dependent signalling pathway. This alternate pathway was attributed to TRIF (Yamamoto et al., 2003). TRIF dependent signalling occurs following endosomal relocation of TLR4, TBK1 and IRF activation. These pathways further interact, with MyD88 signalling impaired by the absence of TRIF (Yamamoto *et al.*, 2003). These intracellular signalling cascades results in downstream production of proinflammatory proteins and interferons (Figure 4).

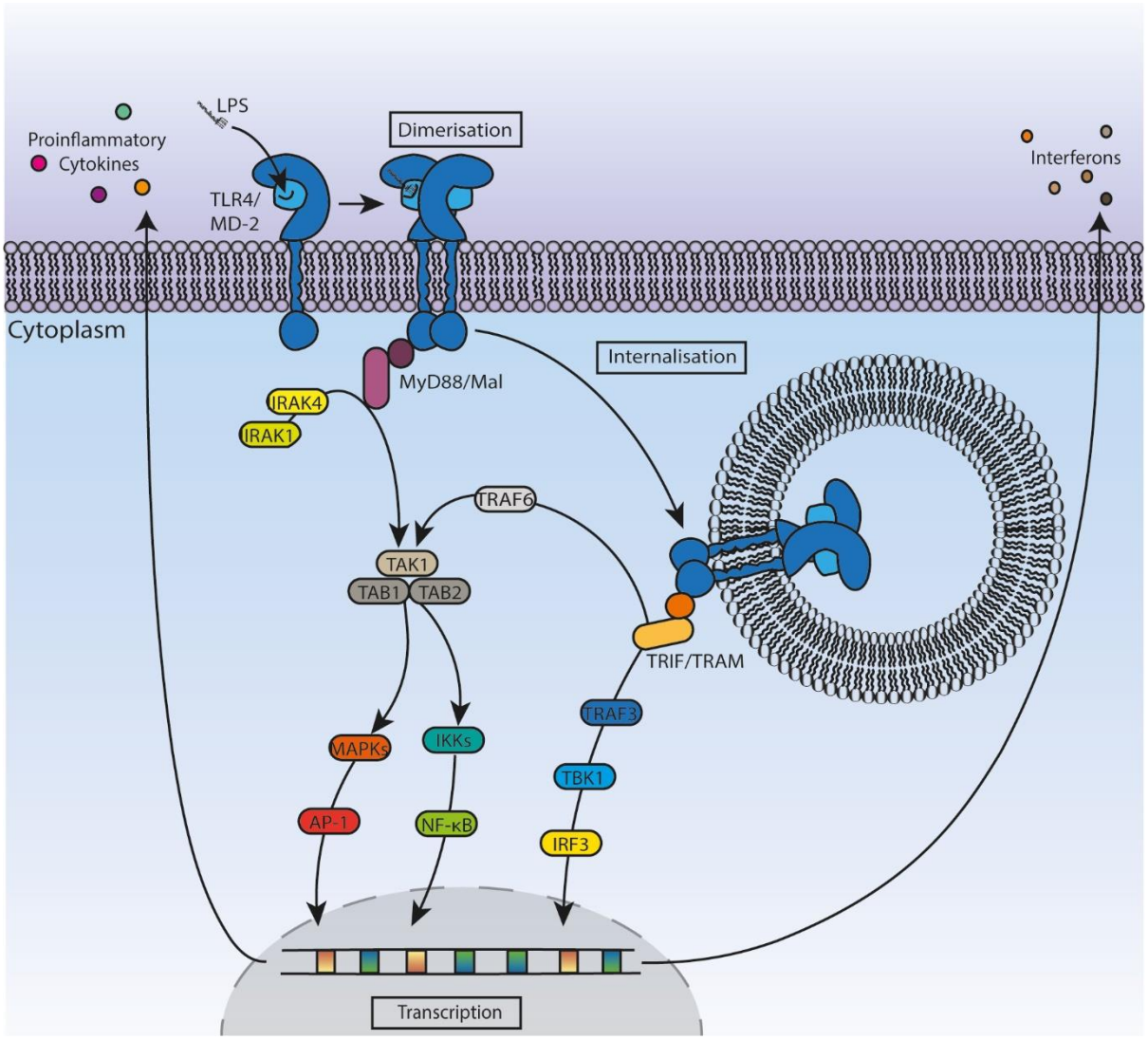

Figure 4. TLR4 signalling via MyD88-dependent and TRIF-dependent signalling pathways. LBP and CD14 serve as a delivery system for LPS to the MD-2 co-receptor. These form a complex which initiates dimerisation of the ectodomains, recruitment of adaptor molecules and initiates intracellular MyD88-depedent signalling leading to downstream MAPK and NF-κB activation and subsequent proinflammatory protein production, or TRIF-dependent signalling via TRAF3 and TBK1 which results in IRF activation and endpoint production of interferons (Wang et al., 2001; Lu, Yeh and Ohashi, 2008)

1.3.1. **MyD88-dependent TLR4 signalling pathway**

MyD88-dependent signalling is activated following stimulation of TLR4 with LPS. For signal transduction to occur, a signalling complex is formed with Mal, MyD88 and the intracellular region of TLR4. Cleavage of Mal by Cas1 initiates the signalling cascade for MyD88-dependent signalling. Inhibition of Cas1 prevents cleavage of Mal and can result in subsequent inhibition of MyD88-dependent TLR4 signalling (Miggin *et al.*, 2007). Cas1 is therefore an integral accessory to MyD88-dependent signalling, following which IRAKs 1 and 4 are recruited to the process.

IRAK1 and IRAK4 were shown to play an important role in LPS induced signalling. IRAK deficiency produces a slight reduction in downstream NF-κB and MAPKs activation (Swantek *et al.*, 2000). IRAK4 deficiency can prevent IL-6, TNFα and IL-1 production in response to LPS challenge in KO mice, which showed almost complete inhibition compared to WT animals (Suzuki *et al.*, 2002). Survivability of animals following *Staphylococcus aureus* infection was severely impaired, with 0% survival rate after 10 days, compared to 80% in WT mice. However, KO mice were protected from LPS induced septic shock (Suzuki *et al.*, 2002). This showed that IRAKs, and more specifically IRAK4, are in important part of LPS induced TLR4 signalling.

Beyond IRAK recruitment, TRAF 6 has been shown to be an important part of MyD88-dependent signalling. Following LPS stimulation, TRAF6 deficient macrophages showed a delay in IKK and MAPK activation and a substantial reduction of TNFα and IL-6 was seen after at 4 and 12 hours (Gohda, Matsumura and Inoue, 2004). This was proposed to be a result of a secondary late phase activation of NF-κB and may be the result of synergistic activation of IKKs via TRIF (Yamamoto *et al.*, 2003). Phosphorylation of IKK leads to release of NF-κB, whereas MKK6 is associated with JNK and P38 MAPK pathways.

1.3.2. **TRIF-dependent TLR4 signalling pathway**

TLR4 signalling can still occur in the absence of MyD88. While it was observed that responsiveness to LPS is diminished in absence of MyD88, there remained some activation of NF-κB, MAPKS, IRF-3 and IP-10 release (Kawai *et al.*, 1999, 2001). This responsiveness to LPS in absence of MyD88 led to the proposal of an alternate MyD88-independent pathway, which was later attributed to a TRIF-dependent signalling cascade (Yamamoto et al., 2003).

TLR4 is the only TLR known to utilise both MyD88 and TRIF-dependent signalling, where others are usually associated with one or the other. TLR4 activation was shown to be impaired in absence of TRIF and capacity for NF-κB activation was lost in MyD88 and TRIF deficient mice (Yamamoto *et al.*, 2003). As with MyD88-dependent signalling, an adaptor protein is required to facilitate the interaction with the signalling domain. Electronegative TRAM bridges TRIF and the signalling domain of TLR4 to allow for interaction between the two, which are generally positively charged and would otherwise not be interact with one another. Absence of TRAM in peritoneal macrophages and lung fibroblasts were both associated with an absence of downstream phosphorylation of STAT-1 compared to controls, while Poly I:C stimulated cells showed no difference in WT and KO cells (Yamamoto *et al.*, 2003).

While TRIF-dependent signalling can still occur in the absence of MyD88, the same is not the case for MyD88-dependent signalling. Absence of TRIF can result in a lack of response to stimuli and TRAM deficient mice also showed diminished response to LPS as compared to wild-type mice, where negligible levels of TNFα, IL-6 and IL-12 production in 1-100ng/mL treatments versus control animals, suggesting an important involvement of TRIF/TRAM associated signalling in MyD88-dependent proinflammatory cytokine production (Yamamoto *et al.*, 2003). TRIF has been proposed to contain binding sites for TRAF6, and therefore TRAF6 was thought to be involved in TRIF signalling (Sato *et al.*, 2003). However, TRAF6 deficiency had no apparent impact on TRIF-dependent IP10 and CD86 expression. Conversely, TRAF6 was found to be essential for MyD88-dependent signalling (Gohda,

Matsumura and Inoue, 2004). TRAF6 may facilitate cross-pathway interactions as TRAF6 facilitates phosphorylation of IKK and MKK6 via a complex consisting of TAK1, TAB1 and TAB2 (Wang *et al.*, 2001). TRIF may be associated with these TRAF6 interactions and thus play an essential role in MyD88-dependent signalling. This could explain the necessity of TRIF for MyD88-dependent cytokine production.

Following LPS stimulation, endocytosis of the TLR4 receptor complex occurs to initiate TRIF-dependent signalling. Receptor internalisation can occur within 60-120 minutes of LPS stimulation of the receptor, and perhaps prior(Kobayashi *et al.*, 2006). TLR4 endocytosis is reliant on dynamin and as such, dynasore inhibition of dynamin can prevent endocytosis of the receptor complex. The use of dynasore could inhibit LPS induced TRIF-dependent signalling, while still allowing for some diminished NF-κB activity (Kagan *et al.*, 2008). Receptor internalisation is thus thought to be integral to TRIF-dependent signalling. Further to this, TRAF3 is indispensable for TRIF mediated production of IFN and IP10 and has been shown to colocalise with TRIF/TRAM within cells following relocation to the endosome (Tanimura *et al.*, 2008). LBP and CD14 are thought to play a much more significant role in TRIF-dependent signalling than MyD88-dependent signalling and are important for receptor internalisation (Tsukamoto *et al.*, 2018). TBK1 and IRF-3 are phosphorylated within 30-120 minutes of LPS exposure. However, without LBP, IRF-3 appears to be absent and TBK1 phosphorylation is somewhat diminished. This is further reflected in IFNβ production (Tsukamoto *et al.*, 2018). MyD88-dependent signalling is therefore thought to be initiated from the plasma membrane, while TRIF-dependent signalling occurs from endosomes. Endocytosis of the receptor complex is therefore an important part in TRIF-dependent signalling and vital for downstream IFN production.

1.3.3. **Proinflammatory protein production via TLR4 signalling pathways**

TLR4 activation is associated with downstream production of numerous proinflammatory cytokines and chemokines. These may then go on to interact with further cytokine associated receptors to produce a cascading chain of cytokine/receptor interactions and production. LPS stimulation has been shown to induce various interleukins and other proinflammatory proteins including IL-1β, IL-6, IL-8, TNFα, MMPs and Interferons among others (Suzuki *et al.*, 2002; Gohda, Matsumura and Inoue, 2004). These proinflammatory proteins represent an endpoint for TLR4 signalling and can have biological and clinical significance, thus representing reasonable targets to assess the effectiveness and validity of TLR4 modulators. The role of these molecules in both normal immune function and pathogenesis is thus important to consider.

Monocytes and macrophages are known to produce IL-1β which is subsequently released from cells. IL-1β can further upregulate various chemokines and cytokines, and may stimulate SMC proliferation (Nathe et al., 2002). NLRP3 Inflammasome activation is involved in the cleavage of the IL-1β precursor form by caspase-1. An increased level of inflammasome activation have been reported in human carotid plaques and elevated levels of IL-1β and IL-18 were also reportedly present (Kirii *et al.*, 2003). IL-1β deficiency has also been proposed to provide protective effects against atherosclerotic plaque development, potentially granting a 33% reduction. Lack of IL-1β was associated with subsequent downregulation of VCAM-1 and MCP-1 and may bring about a reduction in further monocyte recruitment into atherosclerotic plaque sites (Paramel Varghese *et al.*, 2016). IL-1β may also be produced in the presence of cholesterol crystals, in absence of LPS (Rajamäki *et al.*, 2010). IL-1β is therefore an important target to consider when investigating the value of pharmacological interventions intended for treatment of cardiovascular diseases.

IL-6 plays both positive and negative roles in immune regulation and disease pathology. IL-6 is responsible for a variety of processes in immune function and haematopoiesis. These include functions such as stimulating production of variety of acute phase proteins; CRP, Fibrinogen, Serum

amyloid A and Hepcidin (Heinrich, Castellt and Andust, 1990; Gillmore *et al.*, 2001) and involvement in some aspects of T-cell differentiation (Bettelli *et al.*, 2006; Yuk *et al.*, 2017). IL-6 is also responsible for producing pyrogenic effects (Chai *et al.*, 1996). It may be released in response to sterile stimuli following damage and can be elevated in patients following surgical procedures in an age-dependent association (Giannoudis et al., 2010; Howell et al., 2016). Elevated levels of IL-6 have also been associated with various inflammatory based disease. IL-6 induced upregulation of VEGF has been associated with rheumatoid arthritis (Nakahara *et al.*, 2003). In this regard, IL-6 was found to produce a synergistic increase in VEGF in conjunction with IL-1β or TNFα, which was not observed between IL-1β and TNFα in absence of IL-6. In blocking IL-6R with an anti-IL-6R mAB, this synergistic effect was also diminished. This effect may be responsible for improved conditions of rheumatoid arthritis patients which has been associated with anti-IL-6R mAB therapy (Nakahara *et al.*, 2003). A large-scale meta-analysis of cardiovascular disease development over several years found elevated IL-6 levels to be a significant predictor of cardiovascular risk (Zhang *et al.*, 2018). This could be associated with the role that IL-6 and TNFα are thought to play in endothelial dysfunction, as has been seen in diabetic mice (Lee et al., 2017). IL-6 is therefore another important target for modulation of TLR4 signalling.

TNFα was originally discovered and its noticed for its role in cell death and potential as an anti-tumour agent. TNFα production is associated with further cytokine and chemokine production via TNFR1/TNFR2 and release of MMPs (Ramesh and Reeves, 2002; Hosokawa *et al.*, 2019). TNFα is released early following TLR4 activation by LPS at sufficient quantities to be detectible within 3 hours (Huber *et al.*, 2006). TNFα may further activate NF-κB via its associated receptor and produce a secondary, later activation after initial TLR4 activation (Han *et al.*, 2002). Elevated TNFα is also associated with an increase in ROS and subsequently implicated in endothelial dysfunction (Zhang *et al.*, 2009; Lee *et al.*, 2017). In addition, TNFα has been implicated in Parkinson's and may exacerbate neurodegeneration (Boka et al., 1994). However, suppression of TNFα may allow for the resurgence

of dormant infections (Lin *et al.*, 2010). As such, complete suppression of TNFα may not always be a desirable outcome for treatment strategies.

1.3.4. **Interferon beta and Interferon alpha/beta receptors**

Interferons are separated into two main groups, type I interferons, and type II interferons. Type I interferons consist of many structurally similar molecules, while type II interferons have only 1 representative in IFNγ. IFNAR signalling is the associated with type 1 interferons including IFNβ. IFNβ-dependent IFNAR signalling occurs via JAK/STAT pathways. The type I interferon receptor is comprised of two units; IFNAR1 and IFNAR 2 which are associate with TYK2 and JAK1, respectively. Phosphorylation of TYK2/JAK1 following activation of the receptor facilitates further phosphorylation of STATs. IFNβ is associated with further production of IP10 and NO release from cells. Type-1 interferon neutralising antibodies are capable of causing a significant reduction in nitrate levels following stimulation (Zughaier *et al.*, 2005).

Interferon therapies have been investigated for treatment of a range of conditions. IFNβ therapies have been shown to have some positive effect in MS patients (Maimaitijiang *et al.*, 2019). IFNβ may also show some anti-cancer potential, with the potential bring about a reduction in processes associated with recurrence of triple-negative breast cancer in a HMEC model system (Doherty *et al.*, 2017). However, not all effects of IFNβ are universally positive. IFNβ may prevent attachment and differentiation of monocytic cells, causing detachment though not apoptosis. This is thought to be through an impairment in VLA-5 integrin functionality by calpain-1 (Yıldırım *et al.*, 2015). Anti-angiogenic effects of IFNβ may contribute to ischemia. Blocking of IFNβ can stimulate proliferation of VSMCs and may aid in angiogenesis, allowing for the creation of collateral arteries which may aid in bypassing arterial occlusions (Schirmer, Bot and …, 2010). Modulation of IFNβ may be beneficial or detrimental in disease pathology depending on the situation.

1.4. LPS as a master TLR4 ligand

LPS is a glycolipid form part of the outer membrane of most gram-negative bacteria. This serves as a protective barrier to the organism and provides a layer of resistance to external threats such as bacteriophages, immune elements, or antibiotics. It is possible for some gram-negative bacteria to remain viable despite blockade of Lipid A synthesis, though growth may be slower in modified strains (Steeghs *et al.*, 1998). However, LPS deficient variants are likely to be more susceptible to antibiotics and other threats which would otherwise be resisted (Steeghs *et al.*, 2001). LPS provides a barrier against large hydrophobic molecules entering bacterial cells, which can be lost in its absence.

When gram-negative bacteria are present in an organism, these outer membrane components such as LPS are shed and picked up by immune system. LPS it is recognised and can stimulate an inflammatory response via TLR4 which helps to deal with the associated bacterial threat. Pathogens must employ various strategies to avoid detection by the immune system and may make modifications to the LPS structure (Al-Qutab et al., 2006). Structural variations in LPS may lead to a different immune response to unique to these LPS variants as pathogens attempt to circumvent immune detection.

Different species of LPS may differentially activate TLR4 based MyD88 or TRIF -dependent signalling, activating one or the other to a lesser or greater extent. Following exposure to several species of LPS, *Salmonella* LPS was found to be more effective at inducing MyD88-independent IFNβ inducible NO production, while *E.coli* LPS was more effective at inducing MyD88 associated TNFα than either *Salmonella minnesota* or *Salmonella typhimurium* (Zughaier *et al.*, 2005). The difference in MyD88-dependent or TRIF-dependent signalling responses to different LPS species could relate CD14 and LBP interactions, which are thought to be of greater importance for TRIF-dependent signalling(Tsukamoto *et al.*, 2018)

1.4.1. **LPS structure and variations**

The structure of LPS can vary between species, though it is typically comprised of three distinct regions. These three regions are referred to as the O-antigen, polysaccharide core and Lipid A domain (Figure 5). Of these, the Lipid A domain is the active moiety that interacts with the hydrophobic pocket of MD-2. While the different regions of LPS can vary between bacterial species, the Lipid A domain remains relatively conserved across strains and is thus the region that is recognised by TLR4/MD-2 (Mi and Lee, 2008; Oblak and Jerala, 2015).

Structural modifications can occur in the Lipid A domain of LPS. Highly acylated forms of LPS are more potent activators of immune response than less acylated forms. In this regard, hexa-acylated Lipid A structures have been shown to be more effective at inducing TLR4 signalling. An optimal acyl chain length of 10 carbons has been proposed, though 8 is considered sufficient to induce a response (Stöver *et al.*, 2004). *Porphyromonas gingivalis* may present with either tetra-acylated or penta-acylated variants of the Lipid A region in the presence of different environmental stimuli. In medium containing hemin, *P.gingivalis* Lipid A converts to the tetra acylated form which does not stimulate immune response as effectively (Al-Qutub *et al.*, 2006; Ding *et al.*, 2013). Modifications to the Lipid A domain of LPS may therefore provide a mechanism for gram-negative bacteria to circumvent immune detection.

LPS can be separated into further chemotypes dependent on the structure of the O-antigen and Polysaccharide core regions. These LPS chemotypes are separated into three main categories: smooth, semi-rough and rough type LPS. Smooth, S-form LPS has a full polysaccharide chain and usually represents the wild-type composition and semi-rough or SR-Form may have a reduction in the number of repeating units in the O antigen region, while rough, or R-form LPS show a further reduction in residues within the polysaccharide core (Huber et al., 2006). These are further broken down into categories that identify the number of residues present. Incomplete forms are named in

sequence Ra, Rb, Rc and so on, based on the number of residues absent from the polysaccharide chain (Figure 5).

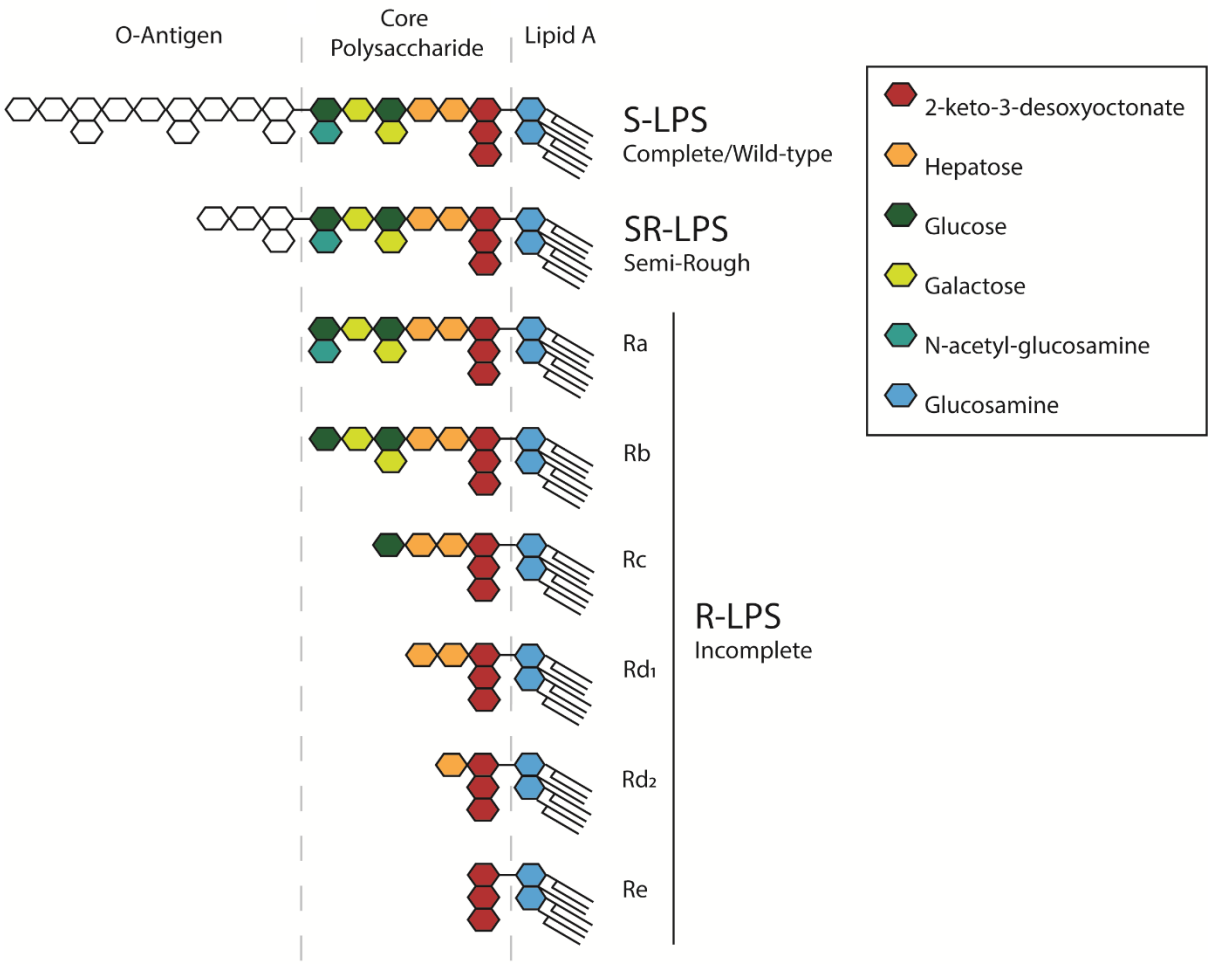

Figure 5. Structural diagram representing different LPS chemotypes. S-form, SR-form, and R-Form LPS are defined by the number of sugar residues present, whereby R-Form LPS are subdivided into further categories associated with a decrease in chain length (Huber et al.*, 2006).*

Modifications to the O-antigen and Polysaccharide core region of LPS may also result in different biologically significant interactions and may exist within a species (Huber *et al.*, 2006). S-form and R-form LPS may differentially activate TLR4 MyD88-dependent and TRIF-dependent signalling

pathways. R-form LPS are capable of inducing higher levels of TNFα than S-Form (Huber *et al.*, 2006). Further to this, S-form LPS may be more dependent extracellular delivery components CD14 to be recognised by TLR4. IL-6 production following S-LPS treatment appears to be more dependent on LBP and CD14 than R-form LPS for which high concentrations alone are sufficient to produce a similar increase (Huber *et al.*, 2006)It is interesting to note that LBP and CD14 are also more important for TRIF-dependent signalling (Tsukamoto *et al.*, 2018). As the O-antigen region of LPS may play an important role in protection of gram-negative bacteria from Macrophage uptake, reductions in this region could represent a means to reduce defence in exchange for stealth (Murray, Attridge and Morona, 2006) Modifications to LPS structure may therefore be a trade-off between protective effects of the LPS barrier and means to effectively evade of immune detection.

1.5. Cardiovascular diseases: risk factors and pathology

Cardiovascular diseases are one of the leading causes of mortality and morbidity in the developed world. Around 45% of deaths in Europe are attributed to cardiovascular disease, including ischemic heart disease and stroke, and represents a significant burden on healthcare system around the world (Wilkins et al., 2017). Cardiovascular disease is therefore an important target for development of novel pharmacological intervention strategies.

The relationship between developed countries and higher prevalence of cardiovascular disease points to the potential of social and lifestyle-based risk factors which may be possible to modify through behavioural interventions in areas. The main risk factors for cardiovascular disease consist of smoking, diabetes, hyperlipidaemia, and elevated blood pressure (Khot *et al.*, 2003). Risk factors are largely avoidable through behavioural alterations and adoption of healthy lifestyle choices. Smoking is a major risk factor which can lead to endothelial dysfunction (Widlansky et al., 2003) and is further associated in both men and women with an earlier onset of cardiovascular events (Khot *et al.*, 2003).

The relationship with alcohol consumption and cardiovascular risk is thought to be more complex. While high levels of alcohol consumption are linked to hypertension and cardiovascular risk (Xu *et al.*, 2001), moderate alcohol consumption appears to be beneficial in reducing cardiovascular risk as compared to no alcohol consumption at all(Ronksley, Brien and Turner, 2011). Moderate alcohol consumption has been linked to an increase in HDL which may contribute to the protective effects seen in this regard (Brien et al., 2011). It is possible that these effects may also be associated with the type of alcohol being consumed, whereby beer and wine may be associated with greater protective effects than consumption of spirits (Chiva-Blanch *et al.*, 2013). This suggests a more dose-dependent relationship between alcohol and cardiovascular disease.

Atherosclerosis is an inflammatory based condition, characterised by the accumulation of lipids in the intima following damage to the endothelium (Figure 6). Initial damage occurs as the result of

stress factors such as hypertension and can lead to endothelial dysfunction. Endothelial dysfunction following damage leads to production of chemoattractant molecules and the recruitment of monocytes, and other leukocytes (Wang and Bennett, 2012). Macrophages in the endothelium consume oxLDL which fails to break down efficiently, resulting in the formation of senescent foam cells. Release of inflammatory proteins causes further production of chemoattractant molecules and migration of smooth muscle cells into plaques (Wang and Bennett, 2012). These combined factors contribute to the development of atherosclerotic plaque and sustained inflammatory response therein.

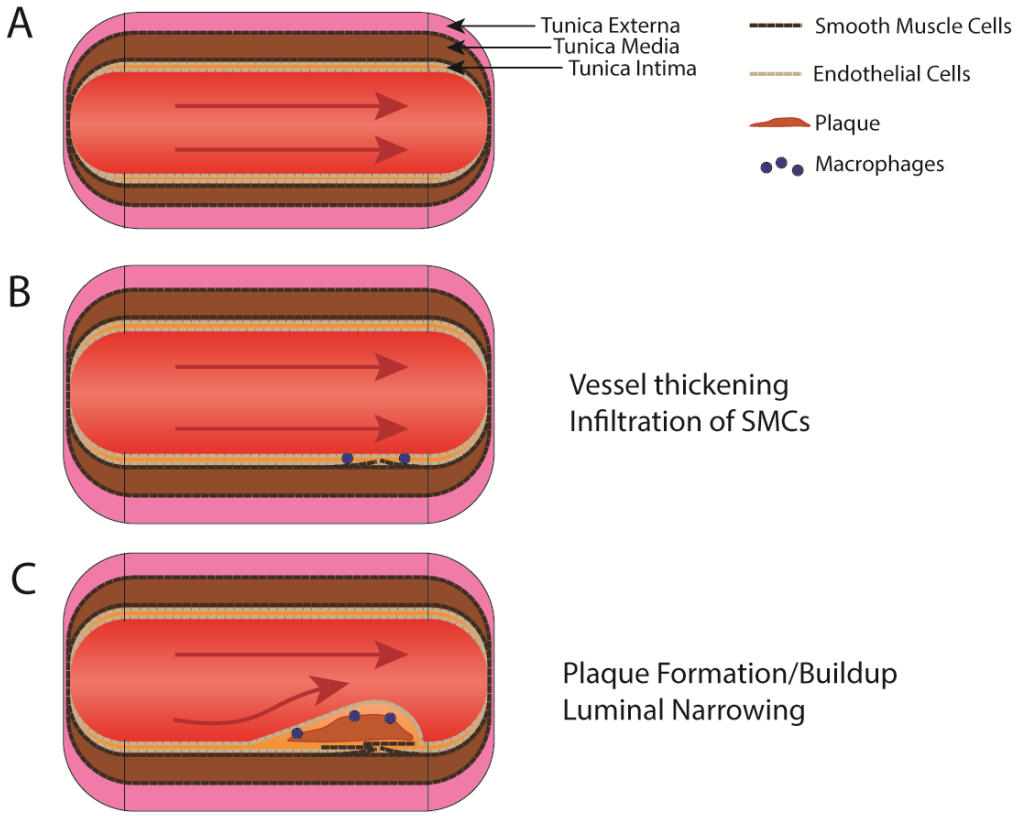

Figure 6. Progression or normal vessel to formation of atherosclerotic plaque. A = normal vessel, B = thickening of the vessel wall and subendothelial layer, migration of vascular smooth muscle, C = Atherosclerosis, plaque formation and narrowing of lumen (Xu et al.*, 2017).*

1.5.1. **Oxidised Low-Density Lipoprotein and the role of sterile inflammation in atherosclerosis**

OxLDL is comprised of a large variety of lipid molecules including cholesterol, phospholipids, and others (Table 1). Of these, it is the cholesterol and polyunsaturated fatty acids which are susceptible to oxidation. Oxidation of these complex molecules can result in hundreds of structurally different products (Miller and Shyy, 2017). While the main association with oxLDL is that of disease, oxidised lipids in the body are known to be involved in both positive and negative processes.

Table 1. The composition of oxLDL (Miller and Shyy, 2017b)

	Approx. number of molecules
Unesterified Cholesterol	600
Cholesterol Esters	1600
Phospholipids	700
Triglycerides	185
Apolipoprotein B-100	1

There are a variety of oxidised lipids present in the body which have a plethora of roles within the body. Among these are oxPC (oxidised phosphocholine), oxPE (oxidised phosphatidylethanolamine), oxCE (Oxidised Cholesterol Esters), MDA (malondialdehyde) and CEP (2-(ω-carboxyethyl)pyrrole). These serve roles in wound healing and homeostasis, antioxidant, and protective anti-microbial functions. On the other hand, oxidised lipids are thought to contribute to a range of adverse effect such as inflammation and inflammasome activation, foam cell formation, platelet activation and may also exhibit a synergistic effect with LPS that overstimulates immune response (Miller and Shyy, 2017). The role of oxidised lipids in the body is complex and may be both beneficial and harmful dependent on the specific context.

TLRs are known to be involved in progression of atherosclerosis. However, the method of activation in conditions such as atherosclerosis is through to be the result of non-bacterial ligands. OxLDL is prevalent in atherosclerotic plaques and is known to play an important part in foam cell formation. In this regard, TLR4 has been shown to be involved in internalisation of oxLDL by which absence or blocking of the receptor has prevented this event (Howell *et al.*, 2011; Chávez-Sánchez *et al.*, 2014). However, oxLDL is involved in activation of the receptor via different mechanics to that seen in LPS activation.

OxLDL produced by different methods in the lab may have different effects on cell response and signalling. NF-κB activation has been demonstrated following oxLDL stimulation in some cases(Chávez-Sánchez *et al.*, 2014). However, minimally oxLDL (mmLDL) may activate ERK and JNK signalling pathways, but not NF-κB (Choi *et al.*, 2012). Further, mmLDL produced through using the *in vitro* 15-lipooxygenase modification method is not thought to bind to CD36 (Miller and Shyy, 2017). This suggests that the method by which oxLDL is produced can have an impact on the final product and its receptor interactions.

Activation of TLR4-dependent signalling by oxLDL involves a cluster of receptors, including TLR4. Upregulation IL-6 and IL-1β following oxLDL stimulation has been shown to be dependent on TLR4, but also TLR2 and CD36. Blocking of any of these receptors is associated with a partial reduction in cytokine production, although TLR2 is not seen as important in foam cell formation(Chávez-Sánchez *et al.*, 2014). CD36 and oxLDL have been reported at higher levels among obese subjects which represent a high-risk factor for cardiovascular disease pathologies(Ramos-Arellano *et al.*, 2014). CD36 is associated with TLR4/TLR6-dependent signalling induced by oxLDL and is indispensable in activation of oxLDL induced inflammation (Stewart *et al.*, 2010). Oxidised phospholipids are partially involved in CD36 binding and may form attachments to apoprotein (Boullier *et al.*, 2000). More recently a carboxyl group present in 7-ketocholesteryl-9-carboxynonanoate has been shown to be critical for oxLDL binding to CD36 and activation of downstream signalling mediators (Li *et al.*, 2017).

It has also been suggested that urokinase receptor may be involved in oxLDL induced production and can affect IL-6 and IL-8 production, as well as colony stimulating factors G-CSF (granulocyte colony stimulating factor) and GM-CSF (granulocyte-macrophage colony stimulating factor) in vascular smooth muscle cells following activation (Kiyan *et al.*, 2014). As such, molecules only interacting with TLR4 could be less effective at preventing activation by sterile ligands as compared to LPS.

1.5.2. **Pharmacological treatment of cardiovascular diseases**

The typical method of intervention for cardiovascular disease in high-risk groups is the use of statins. These help to manage the risk factors such as inflammation, thrombogenicity reduction, and improved endothelial function (Riccioni and Sblendorio, 2012). Statins can indirectly affect TLR4 through disruptive lipid raft modifications (Chansrichavala *et al.*, 2010). These modifications to the lipid raft may also affect a number of other receptors which are shuttled to this region of the cell membrane following activation. Statin therapy is widely considered safe and effective form of management. These can be used in combination with other strategies such as anticoagulants and ACE inhibitors which are more effective in reducing mortality in synergy with one another (Kashani *et al.*, 2006).

HDL has the potential to de-oxidise LDL, becoming oxidised itself. Higher hydrophilic attraction of HDL may spontaneously transfer oxPC and oxidised cholesterol from the surface of LDL (Parthasarathy, Barnett and Fong, 1990; Rasmiena *et al.*, 2016). This oxidation can be removed from oxidised HDL in the liver, but not from oxLDL . Thus, HDL plays an important role in the clearance of lipid peroxides (Linna *et al.*, 2013). Due to the role of oxLDL in atherosclerosis, HDL levels have been of interest as a means of intervention. However, treatment strategies that artificially increase HDL have been somewhat ineffectual (Linna *et al.*, 2013; Kingwell *et al.*, 2014).

Cytokines play a part in development of atherosclerosis and cardiovascular disease. As such, therapies targeting cytokines and their associated receptors have been investigated in the past. Recently an IL-1β targeting antibody, Canakinumab, was shown to reduce rates of cardiovascular events in patients when associated with reduced IL-6 levels following treatment (Ridker *et al.*, 2018). However, one potential issue with cytokine targeted therapies is that the function of many cytokines may overlap and therefore target one may not result in an overall improvement.

A complication that can arise in some cases of cardiovascular disease is abdominal aneurysm formation. Atherosclerosis has been linked to aneurysm formation as luminal narrowing may result

in a compensatory effect whereby the blood vessel widens to prevent blockage (Sakalihasan, Limet and Defawe, 2005). Levels of TIMP-2 and PAI-1 have been linked to formation of aneurisms, or alternative athero-occlusive disease whereby remodelling does not occur and luminal narrowing reduces blood flow (Defawe *et al.*, 2003). While luminal widening may improve blood flow, there is a risk of rupture of the associated with this countermeasure. Current treatment for aneurysms requires surgical intervention by means of a high-risk procedure. In the case of rupture, there is a very small timeframe within which surgical intervention may be successful. Screening methods allow for early detection in high-risk individuals. However, many asymptomatic cases go undiagnosed (Kent et al., 2010). This can pose a serious risk to health and survival.

MMPs are responsible for breaking down collagen and elastin and are associated with plaque destabilisation and arterial remodelling. MMP9 has been seen at elevated levels in aneurisms. This involvement of MMPs has made them of interest in the control of aneurism formation through the use of protease inhibitors (Defawe *et al.*, 2003; Sakalihasan, Limet and Defawe, 2005). Doxycycline has shown positive effects in reducing MMP9 and subsequently reducing the formation of aneurisms, though does not appear to have a broader effect on atherosclerosis as a whole (Manning, Cassis and Daugherty, 2003). TLR4 is overexpressed in human aneurismal aortas and TRIF knockout animals show protection from aneurism formation (Vorkapic *et al.*, 2015). Thus, TLR4 and specifically TRIF-dependent signalling may be important targets for risk reduction.

1.5.3. **Modulation of TLR4 signalling as a strategy of pharmacological intervention of cardiovascular diseases**

TLR4 is known to play an important part in the progression of atherosclerotic disease. This has been demonstrated in TLR4 deficient mice, which are shown to be protected from development of experimentally induced atherosclerosis (Michelsen *et al.*, 2004; Owens *et al.*, 2011). TLR4 is known to be involved in the sustained inflammatory response as seen in atherosclerotic plaques. IP10, IL-6 and IL-8 have been linked to the proliferation and migration of SMCs into atherosclerotic lesions (Vink *et al.*, 2002) and chemoattractant molecules such as MCP-1 and MIP-1 and RANTES which facilitate macrophage infiltration may be induced through TLR4 (Vink *et al.*, 2002). There is also evidence to suggest TLR4 plays an important part in foam cell formation (Hansson and Libby, 2006). Therefore, Modulators of TLR4 activity may provide new avenues of treatment for inflammatory based cardiovascular diseases.

Several strategies exist that have been tested as a means to prevent TLR4 activation in response to LPS. Pharmacological intervention has involved the targeting of various parts of the activation process. These may attempt to sequester LPS, to inhibit delivery molecules such as LBP and CD14, to prevent binding to MD-2/TLR4, or to interact with TLR4 directly (Peri and Piazza, 2012). A good recent example is the CANTOS trial, which showed that limiting the bioavailability of IL-1β (produced mainly via TLR4 pathways) reduced clinical cardiovascular events by 15% in a trial with over 10000 patients who had recently had a heart attack (Ridker *et al.*, 2017). The findings of this report strongly support the use of TLR4 regulation as a potential therapeutic target for control or management of aneurism and atherosclerosis.

Several compounds have been found to negatively regulate TLR4-dependent inflammation following oxLDL stimulation. Lycopene (Figure 7 A) has recently been shown to reduce oxysterol induced cytokine production in THP-1 macrophages via inhibition of TLR4-dependent NF-κB and MAPKs, possibly through disrupting receptor complex formation as a result of lipophilic interaction with cell

membranes rather than directly binding to TLR4/MD-2 (Palozza *et al.*, 2011). Similarly, Puerarin (Figure 7 B), a plant derived isoflavone, has been shown to decrease the oxLDL induced proinflammatory response in macrophages via a TLR4/NF-κB and downregulate CD36 (Zhang *et al.*, 2015). However, the exact means by which these molecules affect oxLDL induced TLR4 signalling remains to be elucidated. The results suggest that TLR4 modulators may be effective in reducing oxLDL induced signalling.

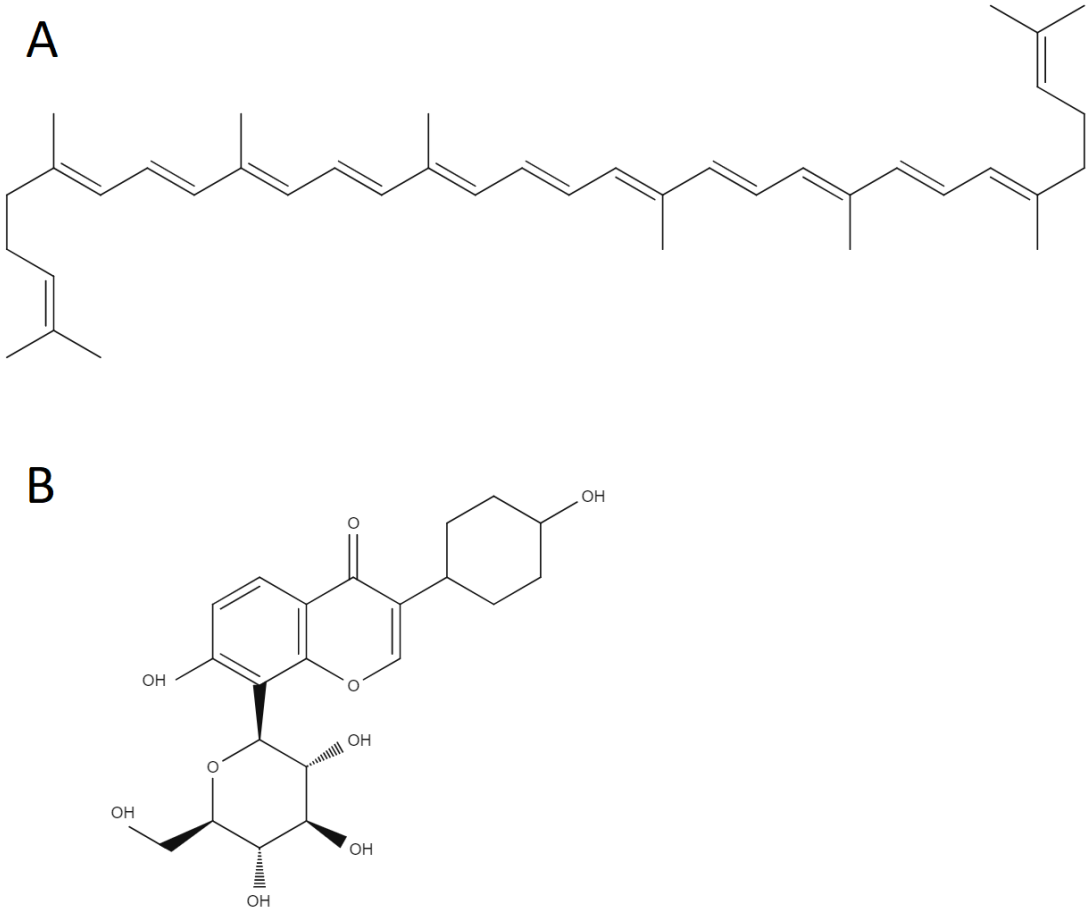

Figure 7. Structure two proposed modulators of oxLDL associated activation of proinflammatory signalling. A = Lycopene, B= Puerarin (Palozza et al.*, 2011; Zhang* et al.*, 2015)*

1.6. The role of immune system in physiological and pathological inflammation: monocytes/macrophages

Monocytes and macrophages are important regulators of immunity and homeostasis throughout the body. Monocytes are mobile and circulate in the blood, make up around 10% of nucleated cells present in human blood. Macrophages are located in specific tissues where they reside as resident macrophages or are formed from monocytes at sites of inflammation. Resident macrophage populations have been found to self-maintain populations within tissue and may not require additions in the form of monocyte derived macrophages (Hashimoto *et al.*, 2013).

Monocytes may be separated into several subsets, associated with different levels of surface receptor expression, usually of CD14 and CD16. The two main subsets are CD14+ CD16- and CD14+ CD16+, although further low CD14 variants and other less defined versions exist which can be identified via flow cytometry (Ziegler-Heitbrock and Hofer, 2013). These different subsets may contribute to functionally significant physiological effects. For example, subsets of monocytes may in part be associated with a predisposition to develop into macrophages or dendritic cells (Randolph et al., 2002). The ratio of different monocyte subsets may vary throughout infection, though the exact role of these population shifts is not entirely clear (Ziegler-Heitbrock and Hofer, 2013). Monocytes populations are therefore considered to be heterogeneous and may change in respond to environmental variations.

Monocytes are recruited by chemoattractant molecules and may differentiate into macrophages which can subsequently be divided in two main phenotypic forms. M1 macrophages are activate by LPS or IFNγ and associated with an aggressive response via pro-inflammatory cytokine production and iNOS. Alternately IL-4 activated M2 macrophages serve the functions of repair and homeostasis and is associated with expression of arginase-1 and CD206 (de Waal Malefyt *et al.*, 1993; Orecchioni *et al.*, 2019). These phenotypes are not necessarily fixed and M1 and M2 macrophages may be subdivided or exist in intermediate states between the two (Murray *et al.*, 2014). M1 and M2

macrophages are therefore considered to be proinflammatory or anti-inflammatory forms respectively though may exist in more specific specialised states to respond to localised situations.

1.6.1. **The role of TLR4, monocytes and macrophage polarisation in atherosclerosis**

Adhesion molecules facilitate cellular attachment to the extracellular matrix. VEGF and MCP can regulate the attachment of monocytes and facilitate transport of cells through the endothelial barrier and into the arterial wall (Stadler et al., 2007). These play an important role in damage and repair mechanisms in blood vessels. Absence of VEGF-2 can result in impaired capacity for vascular remodelling and thrombus repair (Alias *et al.*, 2014). Alternatively, adhesion molecules have also been implicated in atherosclerosis, allowing monocytes to enter into plaques and contribute to the sustained inflammatory condition (Potteaux et al., 2011).

In atherosclerosis, monocytes circulating in the blood and are recruited into plaques where they differentiate into macrophages (Figure 8). These macrophages accumulated in the arterial wall can become polarised into proinflammatory (M1) or anti-inflammatory (M2) subtypes. Higher levels of M1 macrophages in atherosclerotic plaques are associated with greater plaque instability (Cho *et al.*, 2013) These are associated with elevated levels of IL-6, MCP-1 and MMPs and can become foam cells through endocytosing LDL within the plaques, which may be oxidised externally or in lysosomes within the cell (Ahmad and Leake, 2019). These then contribute to production of a range of inflammatory cytokines, chemoattractant molecules and MMPs which cause further recruitment of leucocytes, smooth muscle cell migration, matrix degradation and necrosis of cells within plaques which are associated with a reduction in plaque stability (Vink et al., 2002; den Dekker et al., 2010). As monocytes and macrophages play an important part in disease progression and can be cultured, these have been selected as an *in vitro* model for the basis of this work.

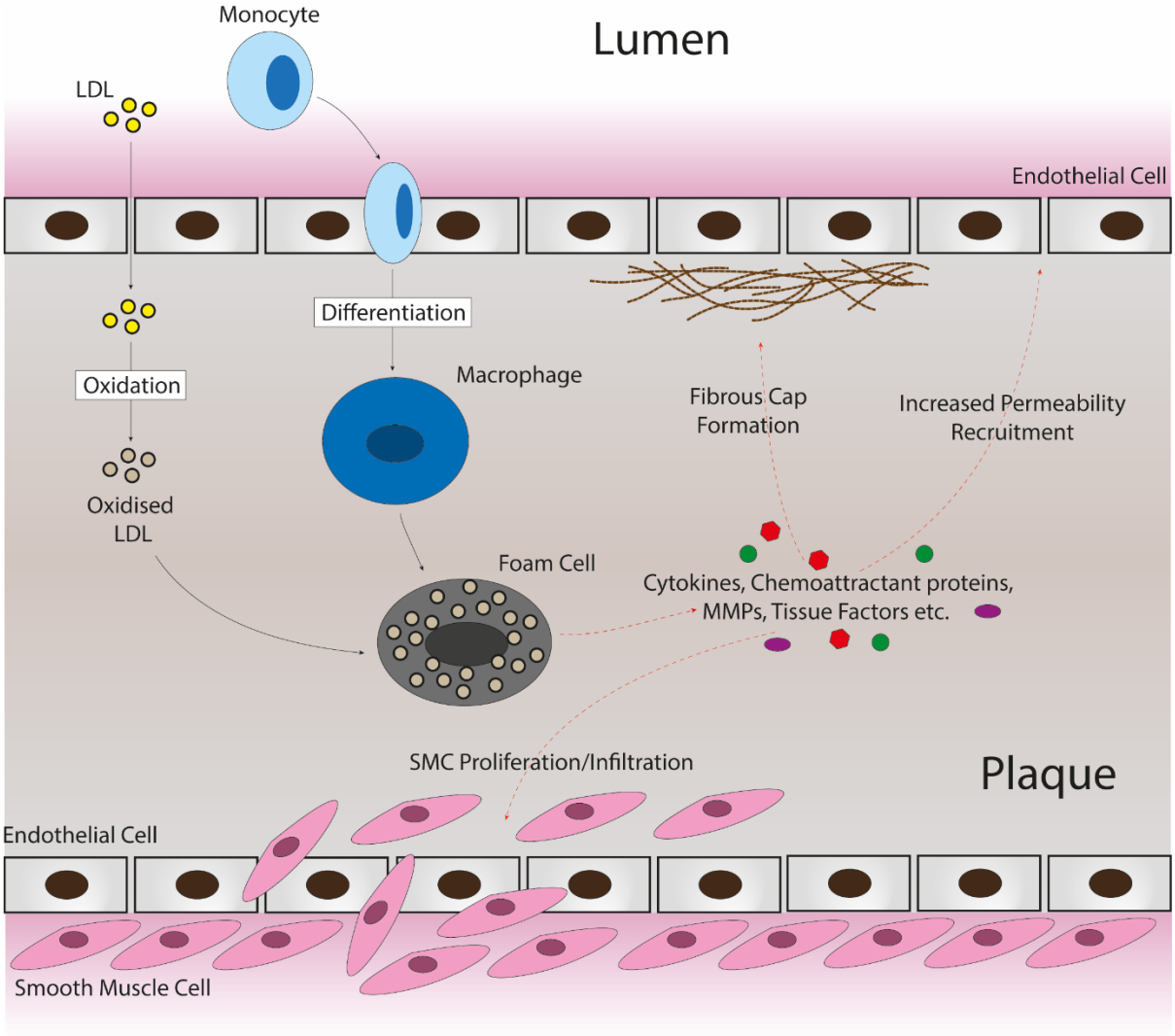

Figure 8. Recruitment to cells to atherosclerotic plaques. The formation of atherosclerotic plaques is associated with accumulation of lipids in the intima, recruitment of leukocytes, foam cell formation and infiltration of SMCs. Macrophages form foam cells through endocytosis of LDL within plaques and contribute to the sustained inflammatory response which facilitates further recruitment of leukocytes, SMC proliferation and the formation of fibrous cap and necrotic core (Hansson and Libby, 2006)

1.6.2. **Primary versus THP-1 responses of monocytes and macrophages**

THP-1 cells are an immortalised monocytic cell line derived from leukemic human monocytes. These are a commonly used representative of human monocyte and macrophage responses, although there is some difference between THP-1 cell activity and primary cell response. THP-1 monocytes and macrophage have previously been shown to express IL-1β, IL-6, IL-8 and TNFα in response to LPS stimulation, though may not produce iNOS (Chanput *et al.*, 2010). This lack of iNOS production appears to be paralleled in primary cells also (Skorokhod *et al.*, 2007). However, further studies have reported iNOS production in macrophages (Zughaier *et al.*, 2005). This disparity may be the result of experimental difference such as use of LPS of different origins. Similarities in timed effects are typically observed in cytokine production, whereby the release of TNFα is comparable between THP-1 cells and PBMCs (Bruckmeier *et al.*, 2012). Despite this, following stimulation LPS can elicit a much stronger response in PBMCs than in THP-1 cells through expression of higher concentrations of TLR4 associated cytokines (Bruckmeier *et al.*, 2012; Schildberger *et al.*, 2013). Further to this, PMA differentiated THP-1 macrophages may not show the same expression profiles as primary cells in association with M1/M2 phenotypes. For example, CD206 expression does not appear to associate with M2 phenotype in THP-1 cells despite being present in PBMCs. Additionally, CD163 appears to be absent (Tedesco *et al.*, 2018). In most cases, studies carried out in THP-1 cells can provide a decent representation of normal monocyte and macrophage function and predict response in PBMC, but it is necessary to verify results in primary and full system *in vivo* models where possible.

1.7. Cancer immunotherapy and TLRs

Immunotherapeutic strategies take advantage of the natural systems by which the immune system removes potential malignancies from the body. Natural defence mechanisms are capable of recognising and potentially suppressing the development of aberrant host-derived cells within the body. Cancer immunotherapy encompasses methods of stimulation of immune response through strategies such as vaccination using cancer associated antigens, external development to specific antibodies, innate immune stimulation, and cytokine treatments.

Despite the promise of immunotherapeutic strategies as a means to combat cancers, many early immunotherapy strategies did not yield much success. This may be in part due to the ability of many cancers to suppress natural immune response in order to circumvent detection. More recently, combination therapies have shown more success. Pre-treatment of patients with lymphodepleting chemotherapy was shown to improve the effectiveness of T-cell and IL-2-dependent therapies in treatment of metastatic melanoma (Rosenberg and Dudley, 2009; Sharma *et al.*, 2011). Combination therapies thereby represent an interesting avenue for development of novel cancer treatment strategies.

TLR agonists have the potential to improve the effectiveness of cancer immunotherapies through priming immune response. TLR agonists have previously show potential to be used as adjuvants for anticancer vaccines. Activation of antigen producing cells, such as macrophages and dendritic cells, can also be used to prime T-cells in cancer immunotherapies. A synthetic glycolipid, CCL-34, has recently been shown stimulate autophagy via TLR4 and upregulate T-cell proliferation, which contributed to antigen specific immune activity in mice (Chou *et al.*, 2020). TLR2 upregulation can help to counteract the immunosuppressive effect of Treg cells and lead to tumour regression (Zhang *et al.*, 2011) Further to this, co-delivery of multiple TLR activators may also act as potent adjuvants may provide stronger synergistic effects. Combined MPLA/CpG administration using lipid nanocarriers can facilitate concurrent activation of TLR4 and TLR9 and has been shown to exhibit

stronger anti-tumorigenic effects and T-cell stimulatory responses than alone or in conjunction without nanocarrier delivery (Kuai *et al.*, 2018) Despite positive effects seen in some TLR therapies, there have also been negative effects seen in response to TLR agonists. This may depend in part on the exact mechanism by which agonists are affecting TLR signalling. While some TLR2 agonists may downregulate the Treg suppressive effects, TLR4 upregulation by HMGB1 has been shown to increase immunosuppressive effects in Treg cells which may facilitate cancer progression (Zhu *et al.*, 2011) Novel antagonists which provide differential activation of TLR signalling may be of interest in TLR agonists used in conjunction with cancer immunotherapies.

1.8. Modulation of TLR4 by novel lipid A mimetic molecules

Previously two TLR4 antagonists have reached clinical trials for the treatment of sepsis. Eritoran functioned as a competitive antagonist which prevented LPS binding to accessory molecules such as CD14 and LBP and subsequently the MD-2 pocket (Kim *et al.*, 2007), while TAK242 is thought to interact with TLR4 signalling through binding to the intracellular signalling domain (Takashima *et al.*, 2009). TAK242 appeared to function as a specific MyD88-dependent antagonist (Kawamoto *et al.*, 2008). However, both molecules were discontinued in various phases of clinical evaluation due to lack of efficacy on upscaling. TAK242 failed to suppress sepsis related cytokine production, while Eritoran was discontinued after phase 3 large scale studies failed to replicate earlier success (Rice *et al.*, 2010; Opal *et al.*, 2013). Alternative methods of sequestering LPS using Polymyxin B have also proved ineffectual in sepsis treatments. Polymyxin B can bind to LPS and may be used to remove LPS from samples. However, Polymyxin B could not bring about a reduction in the level of circulating LPS in patients suffering from sepsis (Vincent *et al.*, 2005). A further meta-analysis of Polymyxin B in sepsis trials showed no significant effect on 28-day mortality from 6 separate trials in 857 participants (Fujii et al., 2018). As such, there is a clinical interest in identifying new TLR4 modulators.

Lipid A mimetics are structurally analogous of the lipid A domain of LPS (Figure 9). These molecules may interact with TLR4/MD-2 in a comparable way to LPS, but not necessarily cause activation of the receptor. Synthetic glycolipids can be formed through modification of existing molecules such as Lipid IVa or Lipid X. Deacylation, glucosamine backbone modification and phosphate group removal can be achieved within bacterial strains through the use of enzymatic reactions. These strategies can be employed to develop unique synthetic molecules with controlled structures, designed for specific activity (Peri and Calabrese, 2014). In previous studies, these molecules have been shown to function in both an agonistic and antagonistic manner on TLR4 induced signalling, as do LPS lipid A variants (Peri and Piazza, 2012; Cighetti *et al.*, 2014).

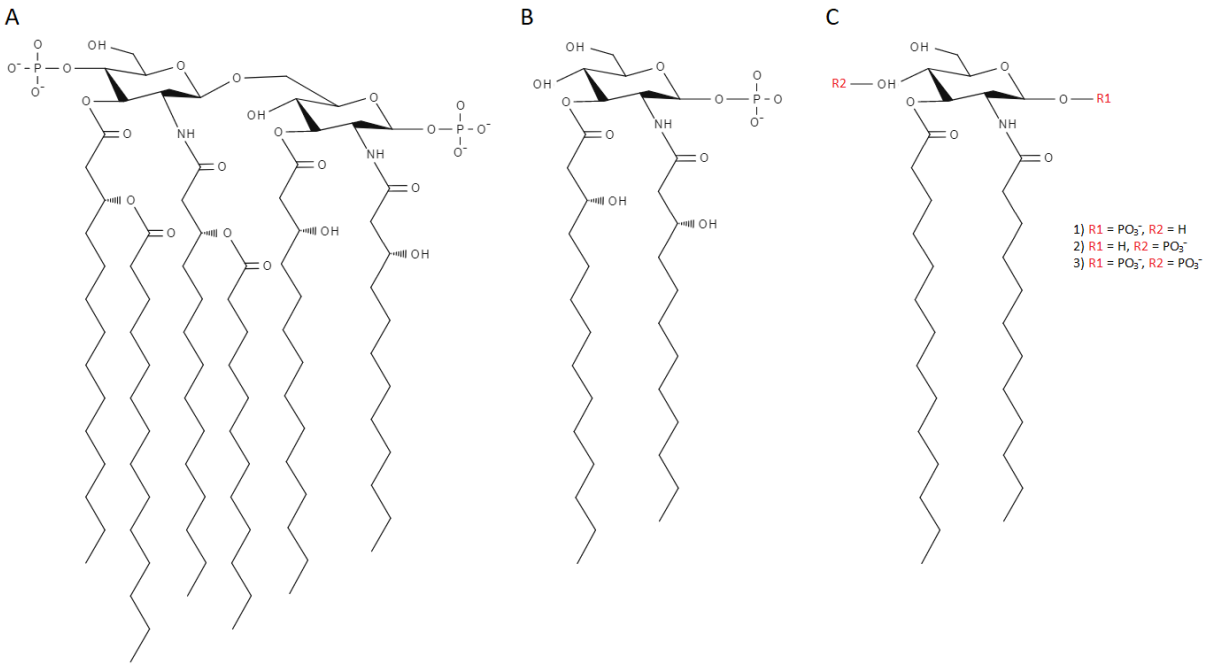

Figure 9. Lipid A mimetic molecules. Comparative structure of A) E. coli lipid A, B) lipid X and C) lipid A mimetic molecule structures. R1/R2 differ between molecule (1-3). C 3 = FP7 (Cighetti et al*., 2014).*

The importance of acyl chain interactions is a factor in antagonistic effects of LPS and lipid A mimetics. Eritoran is a modified structural variant of *Rhodobacter sphaeroides* Lipid A. While penta-acylated *E.coli* LPS fills the MD-2 pocket with five of its six acyl chains, the excess chain remains outside in an interaction with a hydrophobic patch on the opposing TLR4 receptor (Park *et al.*, 2009). In contrast, the four chains of eritoran are all located within the MD-2 pocket without external interactions (Kim *et al.*, 2007; Ohto *et al.*, 2012). This protrusion of *E.coli* lipid A might explain the increased antagonism of highly acylated LPS molecules compared to less acylated variants. Furthermore, higher acylation of Lipid A is associated with stronger response to the ligand (Stöver *et al.*, 2004). Thus, less acylated synthetic molecules are likely to show more antagonistic effects.

FP7 series molecules, including FP10, FP12, FP7 and FP116 are structural analogues of Lipid X. They are comprised of a monosaccharide backbone with two phosphate groups and two acyl chains which vary slightly in length between molecules (Facchini et al., 2018). FP7 has previously been shown to

inhibit TLR4-dependent NF-κB signalling in HEK-blue cells and TNFα in mouse macrophages (Cighetti *et al.*, 2014). In addition, FP7 has been shown to prevent viral activation of TLR4 in response in dendritic cells. IL-8, IL-6 and MIP-1β levels were reduced in FP7 treated samples, as compared to LPS alone and can inhibit LPS/TLR4 upregulation of glycolysis (Perrin-Cocon *et al.*, 2017). FP7 may remove CD14 from the cell surface by stimulating internalisation of CD14 but does not facilitate internalisation of MD-2/TLR4 (Cighetti *et al.*, 2014). The compound has been proposed to interact with CD14 and MD-2 in a similar way to Lipid A and Eritoran, whereby the acyl chains of FP7 occupy the hydrophobic pocket of the co-receptor (Cighetti *et al.*, 2014). FP7 functions as a strong antagonist of LPS induced TLR4 signalling, but is incapable of preventing activation of other TLRs, TLR1/TLR2, TLR2/TLR6 or TLR3 in response to PAM3CSK4, peptidoglycan or dsRNA respectively (Perrin-Cocon *et al.*, 2017). FP7 is therefore thought to compete with LPS for occupation of MD-2, an effect which may be compounded by removal of CD14 from the cell surface through endocytosis (Cighetti *et al.*, 2014). In addition, aggregative states of these molecules may also affect activity. LPS, Lipid A and FP7 form aggregates at a critical point. Antagonistic properties as associated with lamellar aggregates, while non-lamellar cubic symmetry is associated with agonistic properties (Mueller *et al.*, 2004; Gutsmann, Schromm and Brandenburg, 2007). However, while FP10 and FP12 present as lamellar structures, FP7 appears to form non-lamellar structures, which may affect antagonistic activity (Facchini *et al.*, 2018). Conversely, Structure-activity analysis of FP11 predicted positioning of one chain into the channel of MD-2, and FP11 and FP18 phosphate groups were predicted to interact with MD-2 residues linked to agonistic effects of *E.coli* LPS (Facchini *et al.*, 2021). FP7, FP10 and FP12 are predicted to have favourable binding positions for antagonism, while FP11 and FP18 interactions are predicted to confer agonistic behaviour (Facchini *et al.*, 2018, 2021).

FP7 has also been shown to have an effect on inflammation induced by sterile ligands. FP7 exhibits protective effects against influenza in mice, whereby lethality is thought to be the product of DAMPs such as oxPAPC and HMGB1 inducing subsequent cytokine storm. In this regard, FP7 was capable of reducing influenza-dependent expression of IL-1β, TNFα, IFNβ, RANTES, KC and IL-6 RNA (Perrin-

Cocon *et al.*, 2017). Further to this, FP7 was shown to protect against LPS induced ALS dependent motoneuronal death in mouse models (de Paola *et al.*, 2016). FP7 may then be capable of regulating TLR4 signalling resultant of other ligands of sterile inflammation. It is important to consider the translatability of findings in cell-based models into future animal and human studies. Differences in TLR4/MD-2 structures between species may affect binding and subsequent impact of molecules on TLR4 signalling in different species (Chebrolu *et al.*, 2015). Therefore, cross-species comparisons are required to determine the effects of lipid A mimetic molecules on human vs mouse TLR4 activity. Further to this, lipid A mimetic molecules as a result of poor solubility in organic solvents may require alternative methods of delivery. A lipid nanocarrier, Lipodiq, has previously been used and shown to be effective in facilitating FP7 delivery in mice via subcutaneous co-delivery (Charys Palmer *et al.*, 2018). There is a growing amount of evidence towards the potential of FP7 as a potent TLR4 antagonist. As such, FP7 represents a good candidate for further preclinical and clinical investigation.

2. Aims and objectives

The main aim of the study was to investigate the effects of novel TLR4 modulators (FP7, FP10, FP12, FP116, FP11, FP111, FP18) on TLR4 signalling pathways in human and mouse monocytes/macrophages cell models and to determine their potential to modulate the TLR4-mediated inflammatory responses.

The main objectives of this project were as follows:

- To investigate the effect of FP7, FP7 derivatives and ligands (LPS, oxLDL) on THP-1 monocyte/macrophages and RAW264 macrophage cell viability.
    - Ideally all small molecules should be well tolerated by monocytes/macrophages. Optimal concentrations for treatments were selected based on results.

- To carry out an initial screening in THP-1 monocytes to select potent TLR4 modulators from a series of FP7 and derivatives.
    - FP7, FP10, FP12 and FP116 have been designed to act as antagonists of TLR4. Previous data suggests that FP7 can downregulate TLR4-dependent signalling in reporter cells (Facchini *et al.*, 2018). If this is the case, a reduction will be seen in TLR4-dependent IL-8 production following LPS exposure as TLR4 signalling pathways are inhibited. The opposite effect will be seen for FP11 and FP111 which are designed to act as TLR4 agonists.

- To identify therapeutic targets for selected FP7 candidate(s) in TLR4 signalling in human THP-1 monocytes/macrophages. Two readouts (TLR4-dependent signalling mediator activation and release of proinflammatory proteins) to monitor the effect of FP compounds were used.
    - If TLR4-dependent IL-8 production is downregulated following treatment FP7 or other candidates, then downregulation of NF-κB or MAPK (TLR4 signalling mediators) signalling pathways will be observed following LPS stimulation. This has already been seen in reporter cell lines for NF-κB so it seems reasonable that the same effect would be seen in THP-1 macrophages.

- To determine cross-species translatability of findings between human and mouse cell models.
    - FP7 has been shown to protect against influenza lethality and motoneuron death in ALS models in mice (de Paola *et al.*, 2016; Perrin-Cocon *et al.*, 2017). It is reasonable to assume that antagonistic effects seen in human cells should translate into mouse models. Therefore, TLR4 induced cytokine production should be downregulated in FP7 and FP12 treated mouse macrophages.

- To investigate the potential of FP7 to modulate TLR4 proinflammatory signalling in response to ligands of sterile inflammation (oxLDL) which are associated with vascular diseases.
    - LPS is accepted as a specific ligand for TLR4. OxLDL have been shown to trigger sterile inflammation via different receptors including TLR4. As the ability of FP7 to inhibit LPS/TLR4 induced signalling, it is expected that this small molecule could modulate TLR4 induced signalling in response to oxLDL as well.

- To determine if FP7 and FP12 have the potential of affecting alternative pathway of TLR4 via TRIF signalling pathways in human THP-1 macrophages.
    - As FP7 and FP12 interact with MD-2 and cause internalisation of CD14 (Cighetti *et al.*, 2014)and TRIF-dependent signalling is more dependent on CD14 than MyD88-dependent signalling, FP7 and FP12 should be capable of preventing LPS induced TLR4 signalling via TRIF. FP7 and FP12 should therefore cause a reduction of activation of TRIF-dependent signalling molecules TBK1 and IRF-3 and downregulation of production of interferons.

- To determine the potential of FP11 and FP18 to activate TLR4-dependent MyD88 and TRIF-dependent signalling in human THP-1 macrophages.
    - TLR4 agonists FP11 and FP18 were expected increase phosphorylation of TLR4/MyD88-dependent NF-κB or MAPK, as well as the production of associated downstream proinflammatory proteins.

3. Materials and Methods

Human THP-1 monocytes and mouse RAW264 Macrophages were used as an *in vitro* model of vascular disease for investigation into the effects of FP7 and derivative molecules on LPS and oxLDL induced activation of TLR4 proinflammatory signalling pathways.

Functional activity of TLR4 was measured on two levels; activation of TLR4 induced signalling mediator recruitment and endpoint production of proinflammatory proteins. For this, Western blot, ELISA, and a proinflammatory protein antibody array were used to analyse samples obtained from the intracellular and extracellular environment.

3.1. FP compound preparation

Prof Francesco Peri (University of Milano-Bicocca, Milan, Italy) is an internationally recognised expert in medicinal chemistry and drug discovery and development. In last 10 years, his research has been focused on the development of novel molecules as lead compounds for pharmacological intervention on Toll-Like Receptors (TLRs). In particular, his group developed new synthetic glycolipids able to modulate the activity of TLR4 in agonistic (FP11. 18 etc) and antagonistic manner (FP7 etc) From 2015 to 2018, he coordinated the Horizon2020-funded MSCA-ETN European project TOLLerant (Toll-Like Receptor 4 activation and function in diseases: an integrated chemical-biology approach).

The FP series TLR4 antagonises share structural homology to Lipid X (Figure 9). They are synthetic glycolipids with a single sugar backbone with two attached phosphate groups and two acyl chains which vary in length between the different molecules. As the acyl chains of Lipid A and mimetic compounds are associated with binding to the hydrophobic pocket of MD-2 differences in acyl chain length may result in different strength of interaction and antagonism between these molecules.

Computational modelling was used to confirm docking of FP7, FP10, FP12 and FP116 in CD14 and MD-2 (Facchini *et al.*, 2018)

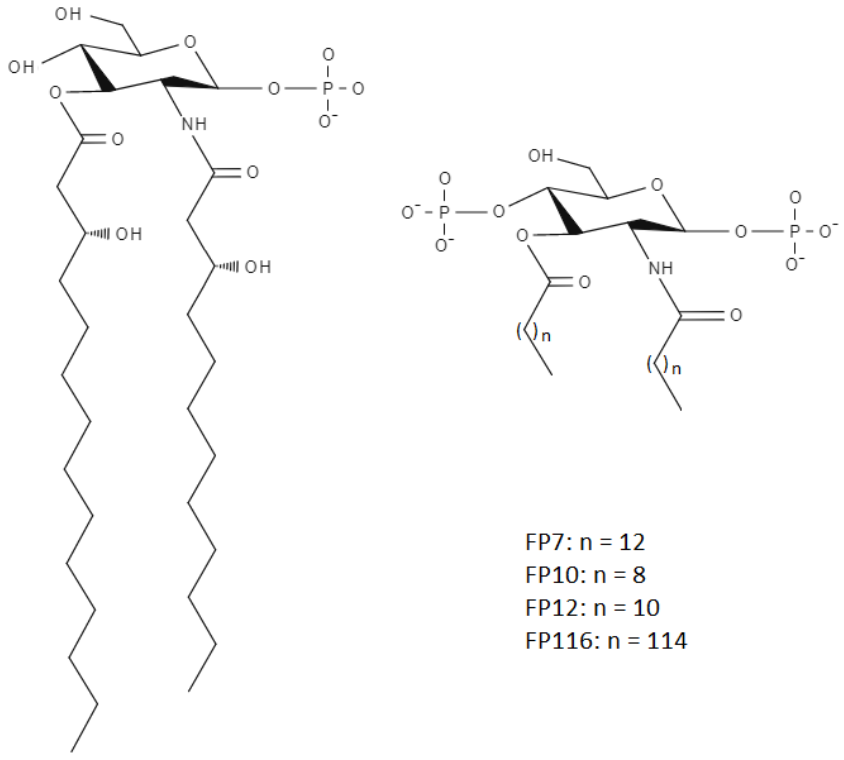

Figure 10. Structure of antagonists; FP7, FP10, FP12 and FP111 are monosaccharide synthetic glycolipids which share structural similarity to Lipid X (Left) They differ from one another by variation in the length of the two acyl chains associated with MD-2 pocket binding (Facchini et al.*, 2018)*

Agonist series FP compounds differ structurally to the antagonist series. Activity of these molecules was predicted to switch from antagonism to agonism based on the ratio of phosphate groups to acyl chains (Facchini *et al.,* 2021). FP11 and FP18 are also single sugar molecules but carry an additional carbon chain in place of the second phosphate group seen in the antagonist series, while FP111 has a second phosphate group in place of hydrogen (Figure 10). In much the same way as with the

antagonist series, FP11 and FP18 vary structurally in the length of acyl chains. MPLA was used as a second comparison along with LPS in experiments involving FP antagonists.

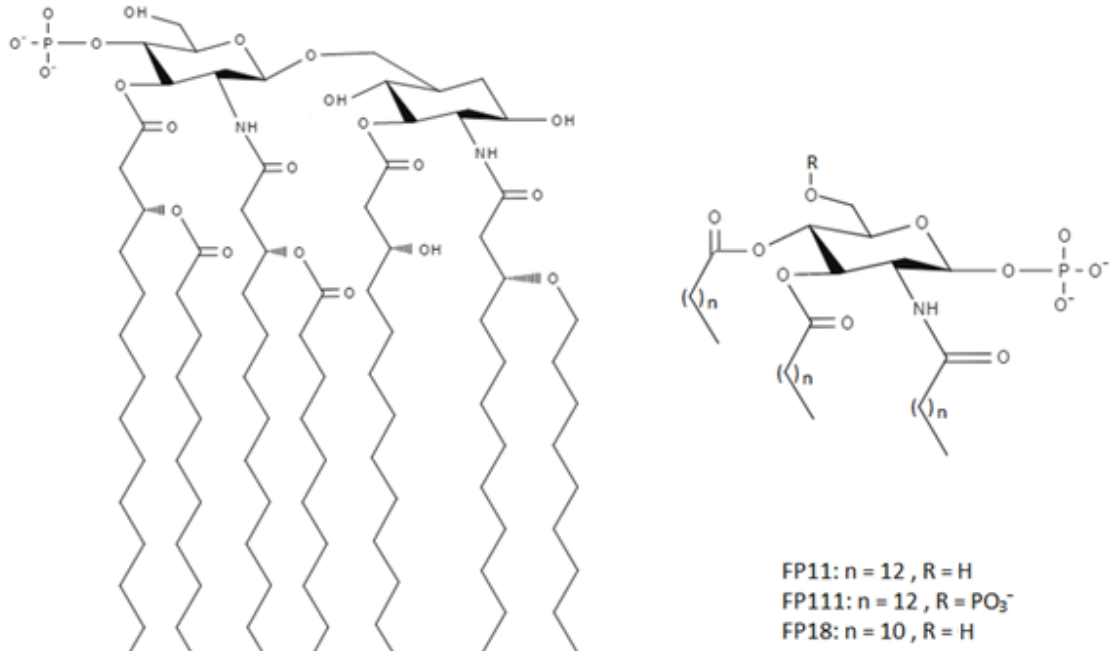

Figure 11. Structure of agonists; FP11, FP111, FP18 share some structural similarity with MPLA (left).

Lyophilised FP compounds were prepared at the University of Milano and delivered to Anglia Ruskin University. Compounds were reconstituted in either DMSO alone, DMSO/Ethanol at a 1:1 ratio (Appendix I), or water with heat and sonication used to aid in solubilisation of compounds where required.

3.2. Production of hydroperoxide and oxysterol-rich oxLDL forms

Oxidation of LDL was carried out following the protocol previously described for production of lipid hydroperoxide or oxysterol-rich oxLDL (Gerry, Satchell and Leake, 2008). In the presence of copper ions, hydroperoxide and oxysterol forms of LDL may be produced. At higher temperatures there is an initial increase in the levels of hydroperoxides created, which subsequently degrade leaving mainly oxysterol forms of oxLDL. At lower temperatures, the process is lengthened, allowing for the development of hydroperoxides and negligible levels of oxysterols. As such, temperature control in the oxidation process allows for the creation of two distinct forms of oxLDL: hydroperoxide-rich and oxysterol-rich oxLDL.

Native LDL (nLDL) was ethically obtained from healthy volunteer donors at Reading University. Oxidation was carried out through a series of dialysis steps including against copper sulphate. 2mg/mL nLDL (measured via Lowry assay) was used during the process. Overnight steps were carried out at 4^{o}C in IL buffer per 10mg LDL unless otherwise stated. EDTA present from the extraction process was removed, as this would chelate Cu2+, through overnight dialysis against Phosphate buffer (140mM NaCl, 8.1mM Na2HPO, 1.9mM NaH2PO4, pH7.4). This was followed by a further overnight dialysis against MOPS buffer (10mM MOPS, 150mM NaCl, pH7.4, treated with washed chelex-100). Another overnight dialysis was carried in MOPS buffer with $CuSO_4$ (10µM) added to the dialysis bag and in the surrounding buffer. This step was carried out at either 4^{o}C for production of hydroperoxide-rich oxLDL or 37^{o}C for oxysterol-rich oxLDL. Oxidation was stopped via addition of 1mM EDTA to the dialysis bag and dialysed overnight against phosphate buffer containing 100µM EDTA to chelate Cu^{2+}. The final products were filter sterilised (0.2µm). Oxidation was confirmed by HPLC analysis and concentration was determined via Lowry assay (Gerry, Satchell and Leake, 2008). Experiments were carried out within two weeks.

3.3. Tissue culture

Human THP-1 and mouse RAW264 macrophages were used as an *in vitro* model of vascular disease for investigation into the effects of FP7 and FP12 on LPS induced activation of TLR4 proinflammatory signalling pathways. All cells were recovered and maintained for at least one week in T-75 flasks from frozen storage prior to any experimental procedures.

3.3.1. **THP-1 monocytes and macrophage maintenance and differentiation**

THP-1 cells were cultured in Gibco™ Roswell Park Memorial Institute (RPMI) 1640 medium (+10% heat inactivated fetal bovine serum (HiFBS), +1% Glutamine, +1% Penicillin/Streptomycin). Cells were split 2-3 times weekly and maintained at a density of ~300x106 cells/ml. For differentiation of THP-1 cells 25nM of PMA (Phorbol 12-myristate 13-acetate) was added to plated cells for 3 days before washing 3x with fresh medium. Cells were then left to rest for overnight before treatment.

3.3.2. **PBMC isolation, maintenance, and differentiation**

Human monocytes were isolated from blood, ethically obtained from voluntary donors at Reading University. Monocytes were grown in RPMI (+10% human serum) for 10-14 days, changed every 3-4 days, prior to differentiation into macrophages via stimulation with GM-CSF (25ng/ml) for 3 days.

3.3.3. **RAW264 maintenance**

Raw 264 cells were cultured in DMEM (+10% HiFBS, +1% Penicillin/Streptomycin) in T-75 flasks. Medium was changed 3 times per week and cells split after reaching 60-70% confluence.

3.3.4. **Plating cells**

THP-1 cell suspensions were prepared for plating at a density of 5x105 cells/mL while RAW264 were prepared at 2x105 cells/ml. For each plating, the same volume of medium was used each time to give relative cell density. 96 well plates used 100µl of cell suspension, 24 wells used 500µl, 12 wells used 1.5mL and 6 wells used 3ml.

3.3.5. **MTT cell viability assays**

The assess the effect of oxysterol-rich oxLDL, hydroperoxide-rich oxLDL, FP7 and FP7 derivatives, on cell viability, MTT assay was used. THP-1 and Raw264 cells were seeded on 96 well plates. Cells were treated with each drug at concentrations in the range of 0-20µM for FP7 and 0-10µM for each derivative in three to six repeats. Plates were then placed in an incubator overnight (18 hours) prior to application of MTT reagent for 2 hours. 10% SDS was used to lyse cells and solubilise formazan crystals overnight. Plates were read at 620nm.

3.4. Sample treatment and collection

Cells were pre-treated with FP7 or FP12 using concentrations ranging from 0.1-10 µM in presence or absence of LPS (Provided by Innaxon – See Appendix II). 100 ng/mL LPS was used for activation of THP-1 or RAW264 cells. Samples absent compounds were treated with vehicle only (DMSO or 1:1 Ethanol/DMSO). Collection time points were ranged between 30 minutes and 6 hours for lysates and 2 hours to 24 hours (overnight) for culture supernatant after ligand exposure dependent on specific purpose.

3.4.1. **FP compound screening and preliminary investigation in monocytes and macrophages**

THP-1 monocytes were prepared on 6 well plates and left overnight to settle. Cells were treated with FP7, FP10, FP12, FP116, FP11 or FP111 at concentrations of 0.1, 1, 5 and 10µM for 1 hour, following which 100ng/mL LPS was added to medium to stimulate TLR4, or FP7 remained in absence of LPS. Medium was collected from wells after 24-hour exposure to LPS to measure cytokines present in the medium. Further investigations of FP7 and FP12 in Monocytes and macrophages follow a similar design. ELISA was used downstream to determine cytokine concentration in culture medium.

3.4.2. **Measurement of P65 NF-κB, P38 MAPK, JNK, ERK, TBK1 and STAT-1 phosphorylation**

Timing was determined for LPS induced P38 MAPK, p65/NF-κB, JNK, ERK, TBK1 and STAT-1 phosphorylation in THP-1 and RAW264 cells from timing experiments up to 6 hours. Selected collection times ranged from 15 minutes to 2.5 hours. Cells were treated with FP7 concentration ranging from 0.1-10µM, or 10µM if only one concentration was used, prior to 100ng/mL LPS and

collected at appropriate intervals. Cell lysates were processed and carried forward for Western Blot Analysis.

3.4.3. **Pre- post and simultaneous FP/LPS experimental design**

Treatment timing was investigated to determine if FP7 or FP12 could affect LPS induced TLR4 signalling at different time points before and after LPS exposure. Cells were either pre-treated with 10μM FP7/FP12 for 30 minutes prior to LPS exposure, at the same time as, or 30 minutes after LPS had been added to wells. Culture medium was collected from plates and used for analysis of cytokine levels after overnight exposure.

3.4.4. **OxLDL experiments**

THP-1 macrophages were exposed to oxLDL at concentrations of 10-100μg/ml. Following results obtained from MTT experiments for safe concentrations for use in THP-1 cells. THP-1 macrophages were exposed to either oxysterol-rich oxLDL at 10-50μg/ml, hydroperoxide-rich oxLDL at 10-100μg/mL or nLDL 50μg/mL with or without 1 hour pre-treatment of 10 μM FP7. Culture medium was collected after overnight exposure for cytokine analysis.

3.4.5. **LPS chemotype selection experiments**

Initial LPS selection experiments were carried out with *S.minnesota* S-form, Re-form, Ra-form, Lipid A and MPLA and *E.coli* Ra-Form and Lipid A (Appendix II), alongside IFNγ as positive STAT-1 activator. Later experiments used only *S.minnesota* variants. Lysates were collected from 0-6 hours for STAT-1 and TBK1 detection and at 2, 6 and 24 hours post LPS for IFNβ and IP10 analysis.

3.4.6. **Interferon alpha/beta receptor blocking experiments**

To prevent IFNβ signalling through IFNAR, a blocking antibody was used to prevent receptor activation. Initial experiments were carried out to confirm optimal concentration and collection time for IFNβ induced STAT-1 phosphorylation. A range of concentrations from 1-20ng IFNβ were used and timepoints of 5, 15, 30, 45 and 60 minutes to optimise experiments. An IFNAR blocking antibody was then tested at concentrations of 0.25, 1, 3 and 5 ng/mL to determine appropriate concentrations for IFNAR neutralisation. IFNAR blocking antibody exposure was carried out 30 minutes prior to IFNβ Further investigation was carried out into the effects of IFNAR blocking on LPS induced STAT-1 phosphorylation and IFNβ was used to confirm effectiveness of IFNAR blocking.

3.4.7. **Collection of medium**

Culture supernatant was collected before lysis of cells from 2 hours onwards and placed in a pre-labelled Eppendorf or falcon tubes. Monocytes were removed via centrifugation 5 minutes at 1000rpm and set aside for lysis. The remaining supernatant was further centrifuged at 3000 or 13000rpm for 5 minutes to remove any remaining debris and subsequent supernatant was retained for analysis.

3.4.8. **Protein extraction**

Cell lysates were collected 30 minutes to 6 hours after LPS exposure. THP-1 medium and cells were transferred from plates to pre-labelled falcon tubes and centrifuged at 1000rpm for 5 minutes. Culture medium was collected for further analysis, and pellet washed using 10mL cold PBS. Cells were then centrifuged again at 3000rpm for 3 minutes to remove PBS. For RAW264 cells, medium was removed from plates containing PBS added to each well to wash cells. Cells were scraped from the bottom of wells and transferred to a 15mL centrifuge tube. These were then centrifuged at

3000rpm for 3 minutes. PBS was then discarded, and tubes blotted to remove excess liquid. Lysis (RIPA) buffer was then added. All samples were sonicated in an ice cooled water bath for 10 minutes before being left to stand on ice for 20 minutes. Cells were centrifuged at 13,000rpm for 20 minutes and supernatant transferred into a new tube. A BCA assay was used to determine the concentration of protein in cell lysates.

3.4.9. **Measurement of protein concentration**

A bicinchoninic acid (BCA) assay (Pierce) was used to determine the protein concentration of each lysate sample for carrying forward to ELISA or Western Blot. A standard set of concentrations was prepared using BSA from 0-6mg/mL for comparison to unknown samples. 5µl of each sample and standard preparations were added to wells in duplicate. Plates were then left develop at 37^{o}C for 30 minutes or until sufficient colour development was observed. Absorbance was read at 620nm using TECAN microplate reader. Concentrations determined from the standard curve formula produced in Excel (Microsoft).

3.5. ELISA

Pro-inflammatory Protein production was measured using ELISA. For this, IL-8 was selected for initial screening of compounds in THP-1 cells. FP7 was further analysed for effects on IL-6, IL-8, MIP-1α and I-CAM-1 in THP-1 monocytes, IL-6, IL-8 and IL-1β and KC and IL-6 in RAW264 Macrophages.

Standards were prepared as per kit protocol (RayBiotech inc.) using provided reagents. 100µl of each standard was loaded onto the plate and an optimisation was carried out for each experiment to determine the appropriate volume of medium required for samples to fall within the standard range. Samples shaken gently for 2 hours at room temperature.

Following this, wells were washed 4 times before 1 hour application of 100µl biotinylated antibody (IL-6/IL-8/MIP-1α/I-CAM-1/IL-1β/KC), with gentle shaking. Plates were then washed again and 100µl Horseradish peroxidase (HRP)-Streptavidin conjugate secondary antibody applied for 45 minutes. A final wash was carried out before addition of 100µl TMB (tetramethylbenzidine) substrate solution to each well. TMB is oxidised in by HRP in the presence of hydrogen peroxide producing a blue colour. Plates were shaken gently in the dark while colour developed. 0.2M sulfuric acid was used to stop the reaction, changing this to a yellow colour and resulting in greater sensitivity. Plates were read at 450nm.

IFNβ was measured as a representative TRIF-dependent TLR4 activation. For this, an R&D systems pre-coated plate ELISA kit was used. The assay was carried out and all standards prepared as per Human IFNβ Quantikine ELISA kit protocol (R&D Systems, USA) using the reagents provided. An optimisation experiment was carried out to determine necessary dilution for medium before a full test.

3.6. SDS-Page and Western blot

20-50 µg protein was taken from cell lysates, calculated from each sample using BCA assay results, and used for Western blot analysis. Blue loading buffer (187.5mM Tris-HCL, 6% SDS, 30% Glycerol, 0.03% Bromophenol Blue) + 125mM DTT (Cell Signalling: 77225) was added 1:3 to each sample at and tubes were heated to 80^{o}C for 5 minutes. Prepared samples were then loaded onto a precast 10% polyacrylamide gel (Bio-Rad). Gels were run at 200v for 30 minutes.

Western blot analysis was used to ascertain the relative level of p65/NF-κB, P38 MAPK, ERK, JNK and STAT-1 phosphorylation following LPS stimulation and treatment. Cell lysates were processed as previously described and stored at on ice for immediate use, or at -20 for later analysis. BCA was used to measure protein in lysates before preparation of samples.

Trans-Blot Turbo transfer system (Bio-Rad) was used to transfer proteins onto PVDF membranes. 5% skimmed milk in tris-buffered-saline (TBS) was prepared and used as a blocking solution. Membranes were blocked with 5% milk for 1 hour before exposure to the primary antibodies from Cell Signalling: phospho-P38 (4511), phospho-p65 (3031), phospho-TBK1 (5483), phospho-IRF-3 (4947) or phospho-STAT-1 (9167). Phospho-antibodies were prepared in 2% milk + TBS and membranes were incubated at 4^{o}C for 28-48hrs. Membranes were then washed with TBS-T 3 times before application of the secondary antibody (anti-mouse or anti-rabbit HRP). Cell signalling β-actin (12262) was used as a loading control for each blot. Incubation was for 1 hour with β-Actin, then 1 hour with anti-mouse antibody.

Following antibody incubation, ECL substrate was used to visualise the blots and the signal was detected either by digital imaging through Genesys (Synaptics, UK) or using photographic film in the darkroom. Densitometry analysis was carried out via GeneTools (Syngene) to give quantifiable data, normalised to β-Actin loading controls.

3.7. Human inflammation antibody array

A proinflammatory protein antibody array was used to provide an overview of protein production following stimulation with LPS and FP antagonist series compounds, or subsequent downregulation of LPS stimulated proinflammatory protein production with FP antagonist series compounds. Human and mouse proinflammatory protein arrays were purchased from RayBiotech (RayBio, USA - C-Series Human Inflammation Antibody Array) and carried out as per protocol instructions. THP-1 cells were exposed to 100ng/mL LPS in presence or absence of 10 µM antagonist series compounds, or 10µM agonist series compounds alone. Medium was collected from plates and cells or cell debris removed through centrifugation at 13000rpm for 5 minutes. Samples were stored at 4^{o}C for immediate use or stored at -20^{o}C for later processing. Initial optimisation of sample dilution from a comparison between untreated and LPS treated samples suggested that no dilution of culture medium was required for assessment of most proinflammatory proteins produced by THP-1 macrophages. Array membranes were blocked using the kit provided blocking solution and 1mL of undiluted medium atop each membrane overnight at 4^{o}C to measure the concentration of proteins released by THP-1 macrophages in culture supernatants. Membranes were washed and exposed to a biotinylated antibody cocktail, followed by secondary HRP-conjugate before application of detection solution as per kit instructions. Membranes were then sandwiched between two thin plastic sheets for visualisation via Genesys (Synaptics, UK). Relative concentrations were quantified using GeneTools (Synaptics, UK) software from dot blots (Example in Appendix III – Figure S1) and are shown in tables as fold increase versus the levels obtained from control blots.

3.8. Quantification and statistical analysis

Statistical analysis and graph production were carried out in GraphPad Prism 6 software (GraphPad Software, Inc). Statistical analysis was carried out via analysis of variance (ANOVA) with a post hoc test (Tukey). Results are expressed as the mean +/- Standard Deviation. Quantification of all data from Western blot and antibody array were carried out via GeneTools (Syngene). Concentration was calculated from ELISA using Excel (Microsoft) or GraphPad Prism 6 (GraphPad Software, Inc).

4. Results

FP compounds are a series of Lipid A mimetic molecules, structurally similar to Lipid A, developed and produced by Francesco Peri (University of Milano). Preliminary success of FP7 in initial studies prompted the formulation of further variants, which have previously been assessed for docking potential *in situ* via AutoDock. All four antagonists showed potential to interact with TLR4/MD-2 in an antagonistic formation (Facchini et al., 2018). The antagonist series molecules are comprised of a single sugar backbone with two acyl chains and two phosphate groups attached. They are similar in structure to Lipid X and differ from each other only in the length of acyl chains. TLR4 agonist series molecules, FP11 and FP18 more closely resemble MPLA and may thereby exhibit comparable effects. However, further analysis was required in order to validate and understand the mechanisms behind the agonist and antagonistic properties of these novel series of mono-sugar synthetic glycolipids.

4.1. Investigating the effects of FP7 and FP7 derivative molecules on MyD88-dependent TLR4-driven signalling in THP-1 human monocytes and macrophages

The THP-1 cell line are an immortalised human monocytic cell line, regularly used as a representation of monocyte and macrophage behaviour. They provide a general representation of human TLR4 signalling in monocytes and macrophages, though THP-1 cells may produce a weaker response to endotoxic challenge than primary human equivalents (Bruckmeier *et al.*, 2012; Schildberger *et al.*, 2013). As such, THP-1 cells provide a good representative model for human TLR4 activity in monocytes and macrophages, but additional experiments to confirm results in primary and *in vitro* models may also be necessary.

THP-1 monocytes were treated with concentrations of each drug ranging from 1-10 μM for each experiment. In each case and MTT assay was carried out to determine the effects of each compound on THP-1 monocyte viability following treatment. ELISA was used to assess the potential of FP7 and derivatives to block activation of TLR4. IL-8 was selected as a TLR4 specific marker to assess the potential of each molecule to downregulate TLR4 signalling following LPS exposure. FP7 was assessed further in IL-6, MIP-1α, and iCAM-1 production following initial positive results seen in IL-8 screening.

4.1.1. **FP7 and FP7 derivatives do not negatively impact THP-1 human monocyte or macrophage cell viability**

THP-1 monocytes were selected for initial screening experiments involving FP compounds. An initial assessment was necessary to evaluate any possible toxicity of compounds within each system. To determine the effect of FP7 on viability of THP-1 monocytes and macrophages an MTT assay was carried out (Figure 12).

MTT Assay results suggested there was no significant reduction in cell viability after exposure to FP7 or derivatives up to 10 µM for each derivative in THP-1 monocytes. From these results, it was determined than that FP7 and derivatives were safe for use in THP-1 monocytes up to the maximum testes. A safe range of 0-10 µM was accepted as suitable for use in further experiments.

For further investigation into the effects of FP compounds on TLR4 signalling, THP-1 Macrophages were utilised as a model system. In THP-1 macrophages FP7, FP12, FP11 and FP18 were all well tolerated up to 10µM concentrations (Figure 13). 0-10µM was therefore also acceptable for future experiments involving THP-1 macrophages.

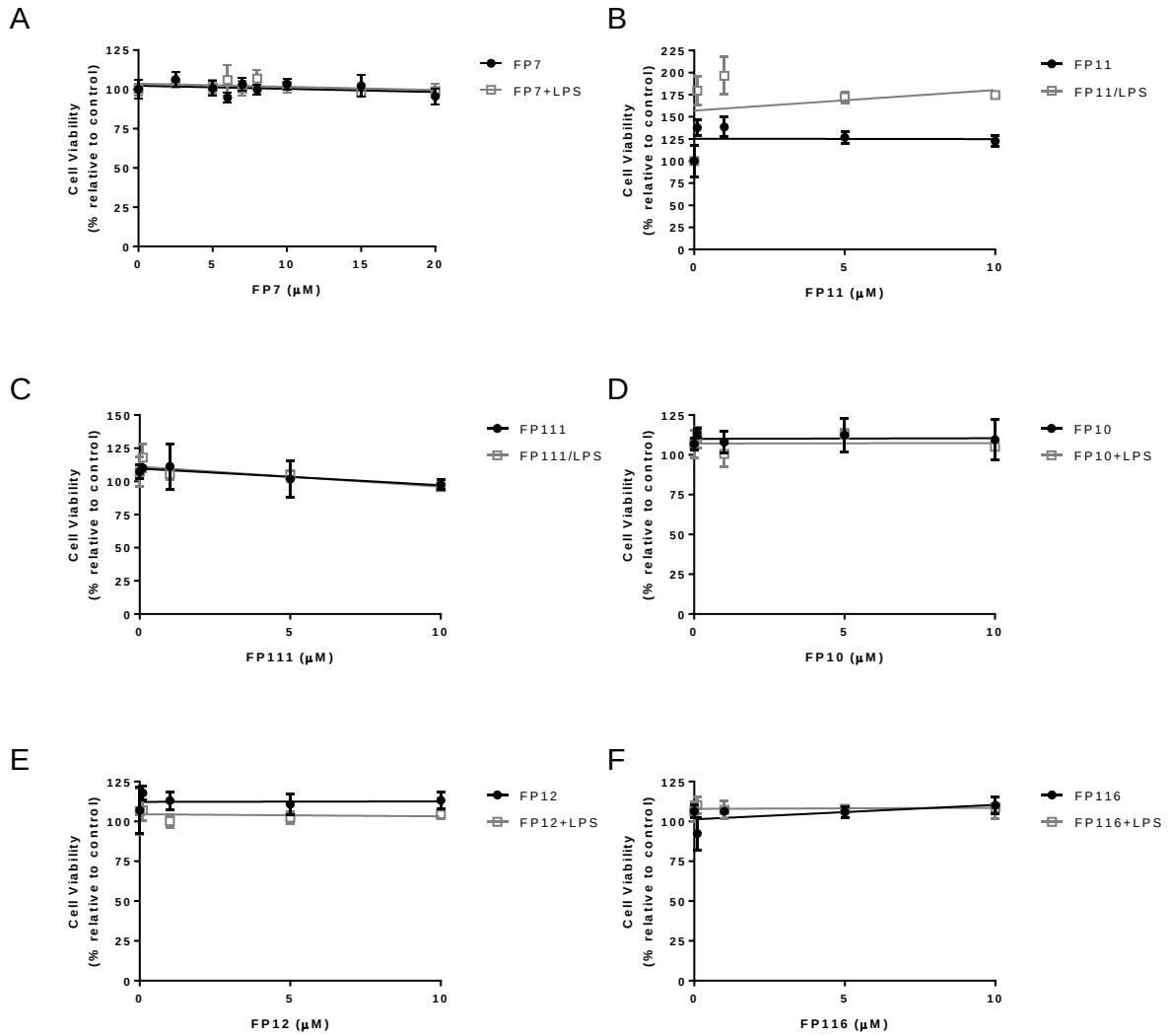

Figure 12. The effect of FP series Lipid A mimetics on THP-1 monocyte viability. Cells were treated with FP7 (0-20 μM) or 5 derived molecules (0-10 μM) in presence (grey) and absence (black) of LPS (100ng/ml). Controls (0) were treated with vehicle only. Plates were incubated overnight (18 hours) prior to addition of MTT reagent. Results are shown as mean +/- SD of 6 repeats expressed as a percentage relative to the control set. (0 μM/no drug) +/- SD of three separate treatments. Statistical comparison between different groups was found to be non- significant. (A) FP7 (B) FP11 (C) FP111 (D) FP10 (E) FP12 (F) FP116.

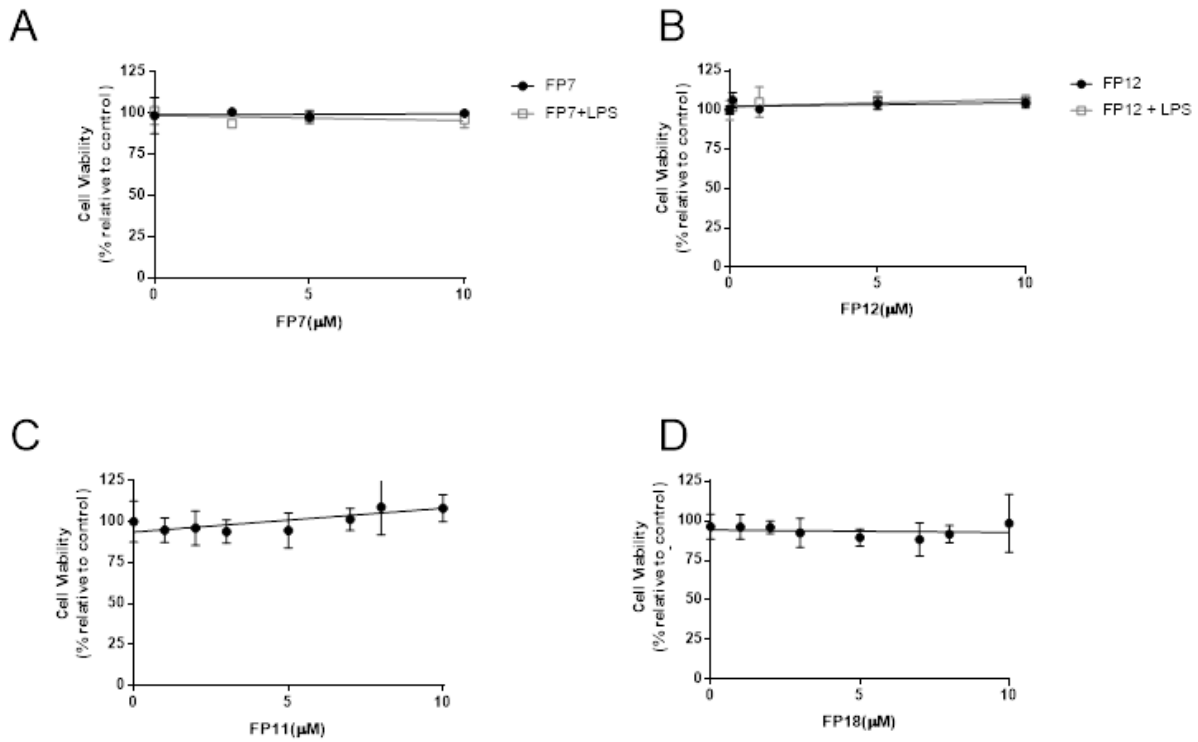

Figure 13. The effect of FP series Lipid A mimetics on THP-1 macrophage viability. Cells were treated with FPF, FP12, FP11 or FP18 (0-10 μM) in presence (grey) and absence (black) of LPS (100ng/ml). Controls (0) were treated with vehicle only. Plates were incubated overnight (18 hours) prior to addition of MTT reagent. Results are shown as mean +/- SD of 6 repeats expressed as a percentage relative to the control set. (0 μM/no drug) +/- SD of three separate treatments. Statistical comparison between different groups was found to be non- significant. (A) FP7 (B) FP12 (C) FP11 (D) FP18.

4.1.2. **Differential effects of FP7 derivatives on TLR4-dependent IL-8 production in THP-1 monocytes**

FP7 derivatives were screened using for effects on TLR4-dependent IL-8 production in THP-1 monocytes in order to select potential antagonists to use further in this project. Preliminary data based on *in silico* analysis obtained from collaborator Professor Peri suggested FP10, FP12 and FP116 may have antagonistic properties, while FP11 and FP111 were expected to act as agonists of the receptor.

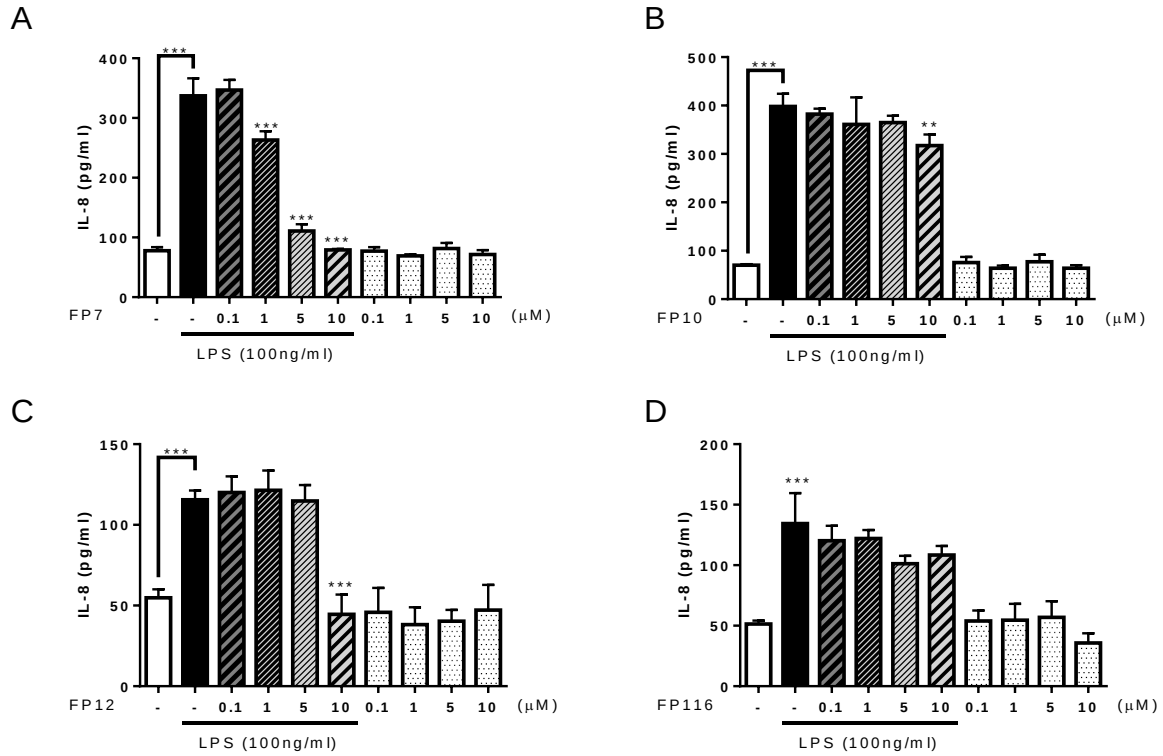

*Figure 14. The effect of FP7 (A), FP10 (B), FP12 (C) and FP116 (D) on IL-8 production in THP-1 monocytes. Cells were pre-treated with FP7, FP10, FP12 or FP116 (0-10μM) for 1 hour prior to exposure to LPS (100ng/ml). Untreated control (-) is with vehicle only. Culture medium was collected after 18 hours and IL-8 production was measured via ELISA. Results are shown as mean ±SD of three separate treatments. Statistically significant results indicated as *** P<0.001 ** P<0.01 *P<0.05 for control vs LPS and LPS vs LPS+FP treated samples.*

FP7 demonstrated a dose-dependent effect on IL-8 production (Figure 14a) with 10µM FP7 downregulating IL-8 production to levels comparable to the control and IC50 of approximately 3µM. FP10 and FP12 (Figure 14B and Figure 14C) both produced a similar, but less pronounced effect on IL-8 production. However, while FP12 has an IC50 around 8µM, FP10 is >10µM. FP116 had no significant impact on IL-8 production up to 10µM (Figure 14D). The effect was not as potent as for FP7 and was only significant at the highest concentration of 10µM. No significant effect was seen for FP116 in THP-1 monocytes. FP7 and FP12 were shown to be more effective in downregulating IL-8 production than FP10 or FP116, for which the IC50 would be >10µM. Thus, FP7 and FP12 were selected for further investigation, while FP10 and FP116 were disregarded for the remainder of the project.

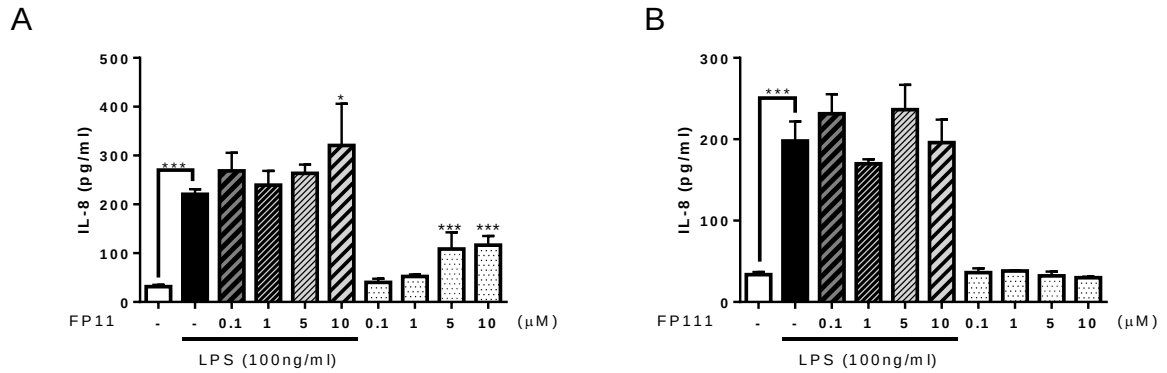

Figure 15. The effect of FP11 (A) and FP111 (B) on IL-8 production in THP-1 monocytes. Cells were pre-treated with FP11 or FP111 (0-10µM) for 1 hour prior to exposure to LPS (100ng/ml). *Untreated control (-) is with vehicle only.* Culture medium was collected after 18 hours and IL-8 production was measured via ELISA. Results are shown as mean ±SD of three separate treatments. Statistically significant results are indicated as *** P<0.001 ** P<0.01 *P<0.05 for control vs LPS and LPS vs LPS+FP treated samples.

From the agonist series, only FP11 appeared to have a significant effect in increasing IL-8 production in THP-1 monocytes, both in the presence or absence of LPS at 10 µM and 5-10µM respectively (Figure 15). Comparatively, FP111 does not appear to increase IL-8. FP11 can function as a mild agonist of TLR4. FP11 was carried forward for further investigation.

4.1.3. **FP7 negatively regulates the production of TLR4-dependent proinflammatory proteins (IL-1β, IL-8, IL-6, MIP-1α, ICAM-1) in THP-1 human monocytes**

Further evaluation of FP7 was required to understand the effect on TLR4 signalling. ELISA was used to investigate the effects of FP7 on TLR4 signalling. Measurements were made for the effect of FP7 on cytokine production and release taken from medium and cell lysate samples. Results indicated a dose-dependent downregulation of inflammatory proteins produced in response to LPS. FP7 significantly reduced levels of IL-8 in cell lysate at 1-5µM and culture medium at 5µM (Figure 16). However, these did not reach comparable levels to control and FP7 only samples. The difference between expression and secretion could be related to biphasic IL-8 release dependent on autocrine effects via TNFα and IL-1β (Cassatella *et al.*, 1993); for which downregulation by FP7 might also impact late phase IL-8 production. This suggests that FP7 can partially inhibit IL-8 production in THP-1 monocytes at this concentration.

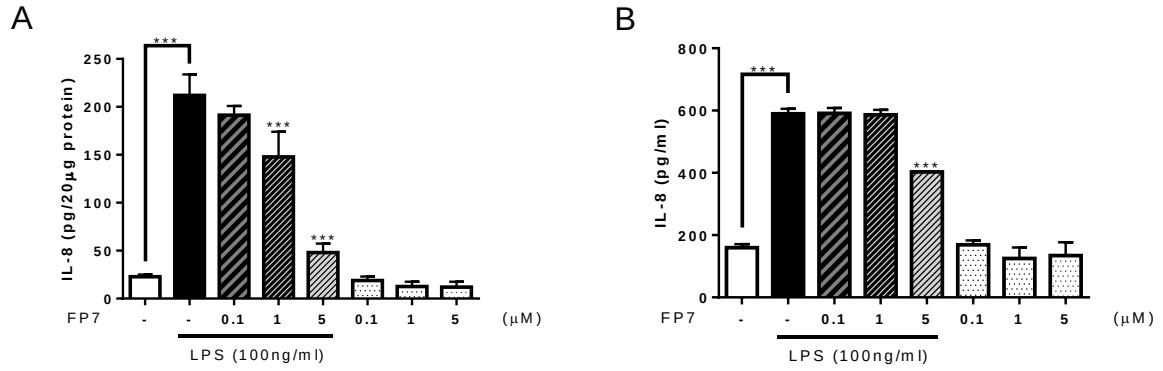

*Figure 16. The effect of FP7 on IL-8 production in THP-1 monocytes. Cells were pre-treated with FP7 (0-5µM) for 1 hour prior to exposure to LPS (100ng/ml). Untreated control (-) is with vehicle only. IL-8 production in culture cell lysates (A) and culture medium (B) were measured using ELISA after 18 hours (RayBiotech). Results are shown as mean ±SD of three separate treatments. Statistically significant results are indicated as *** P<0.001 ** P<0.01 *P<0.05 for control vs LPS and LPS vs LPS/FP7 treated samples.*

Treatment with 5µM FP7 also significantly reduced levels of IL-6 release in medium following LPS stimulation. This was to a level comparable to control samples (Figure 17). However, the level of IL-6 present within cells was unaffected by LPS after 18 hours, suggesting the effect of LPS on IL-6 production is no longer active at this point.

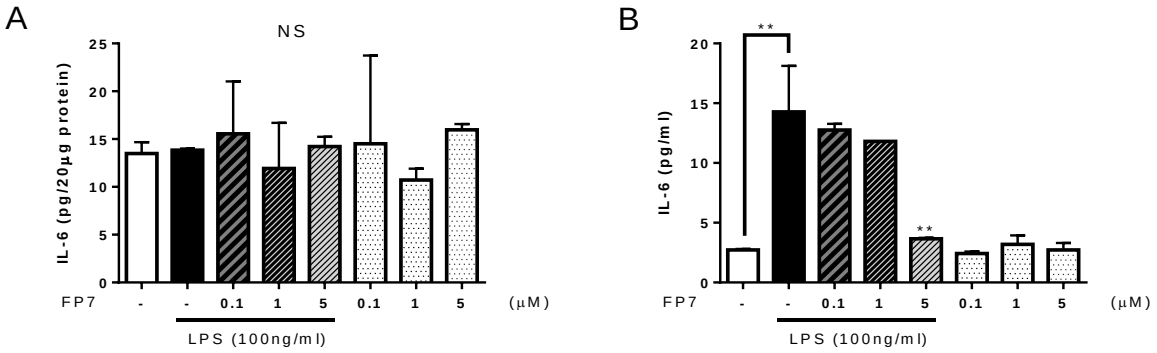

*Figure 17. The effect of FP7 on IL-6 production in THP-1 monocytes cells were pre-treated with FP7 (0-5µM) for 1 hour prior to exposure to LPS (100ng/ml). Untreated control (-) is with vehicle only. IL-6 production in culture cell lysates (A) and culture medium (B) were measured using ELISA after 18 hours (RayBiotech). Results are shown as mean ±SD of three separate treatments. Statistically significant results are indicated as *** P<0.001 ** P<0.01 *P<0.05 for control vs LPS and LPS vs LPS/FP7 treated samples.*

LPS showed no effect on IL-12 production following overnight treatment in either cell lysate or culture medium. FP7 had no significant effect in the presence of absence of LPS (Figure 18). IL-12 may therefore not be an appopriate target for evaluating the effect of TLR4 antagonists.

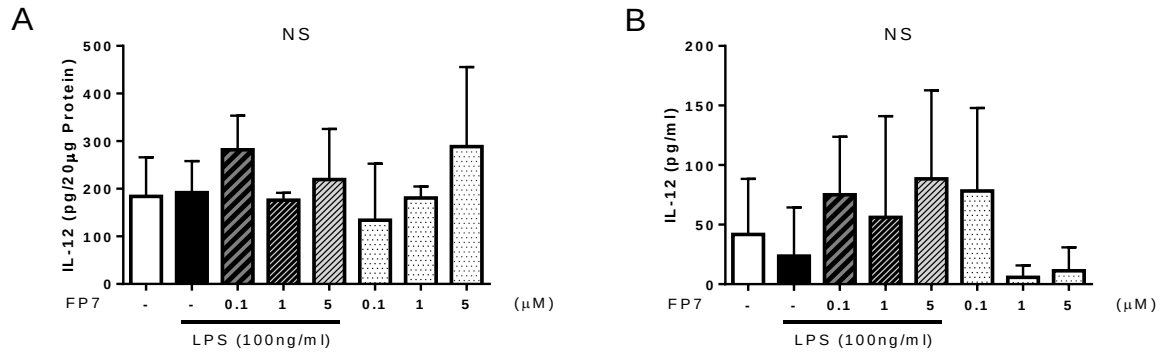

*Figure 18. The effect of FP7 on IL-12 production in THP-1 monocytes. Cells were pre-treated with FP7 (0-5µM) for 1 hour prior to exposure to LPS (100ng/ml). Untreated control (-) is with vehicle only. IL-12 production in culture cell lysates (A) and culture medium (B) were measured using ELISA after 18 hours (RayBiotech). Results are shown as mean ±SD of three separate treatments. Statistically significant results are indicated as *** P<0.001 ** P<0.01 *P<0.05 for control vs LPS and LPS vs LPS/FP7 treated samples.*

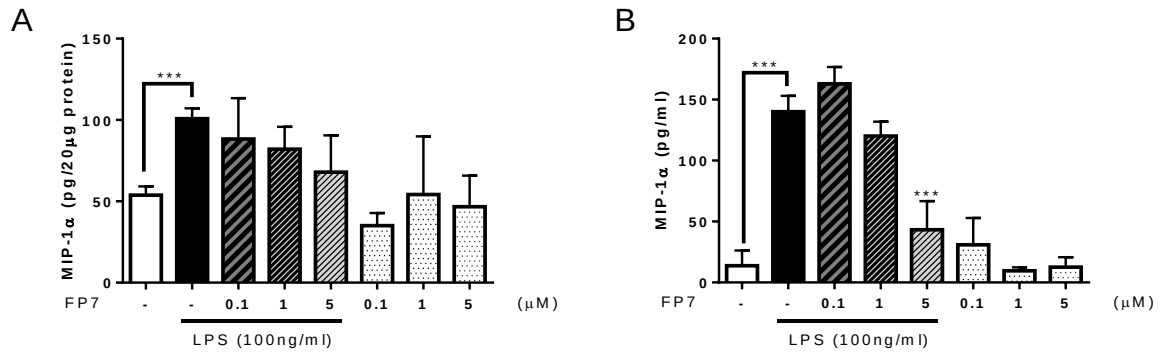

*Figure 19. The effect of FP7 on MIP-1α production in THP-1 monocytes. Cells were pre-treated with FP7 (0-5µM) for 1 hour prior to exposure to LPS (100ng/ml). Untreated control (-) is with vehicle only. MIP-1α production in culture cell lysates(A) and culture medium (B) were measured using ELISA after 18 hours (RayBiotech). Results are shown as mean ±SD of three separate treatments. Statistically significant results are indicated as *** P<0.001 ** P<0.01 *P<0.05 for control vs LPS and LPS vs LPS/FP7 treated samples.*

MIP-1α was downregulated by FP7 in medium MIP-1α (Figure 19B) while internal levels were unaffected (Figure 19A). FP7 significantly reduced MIP-1α at 10μM in medium, but cell lysates showed no significant reduction after overnight exposure. This suggests FP7 prevented release of MIP-1α.

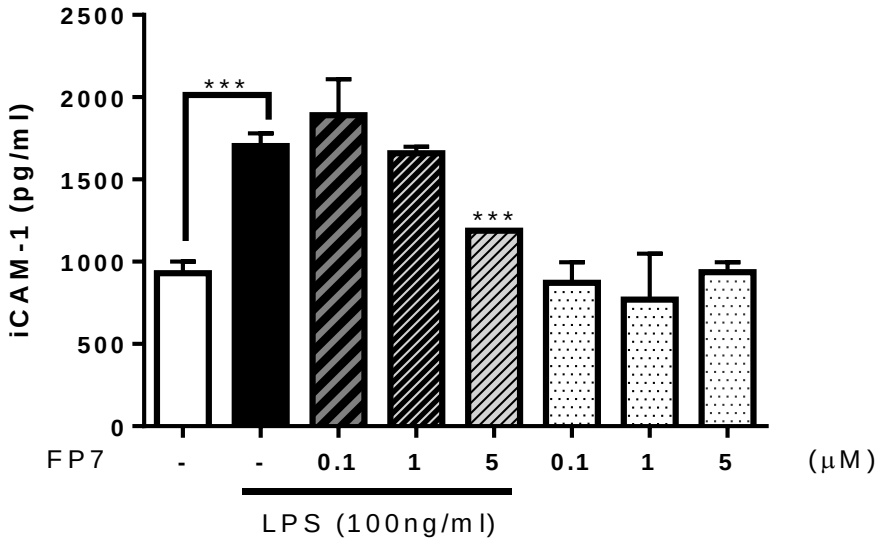

*Figure 20. The effect of FP7 on iCAM-1 production in THP-1 monocytes. Cells were pre-treated with FP7 (0-5μM) for 1 hour prior to exposure to LPS (100ng/ml). Untreated control (-) is with vehicle only. iCAM-1 production in culture medium was measured using ELISA after 18 hours (RayBiotech). Results are shown as mean ±SD of three separate treatments. Statistically significant results are indicated as *** P<0.001 ** P<0.01 *P<0.05 for control vs LPS and LPS vs LPS/FP7 treated samples.*

Further measurements of culture medium showed a similar effect in ICAM and IL-1β. FP7 was capable of negatively regulating LPS stimulated production of ICAM-1 (Figure 20) and IL-1β (Figure 21). In this regard, FP7 showed the most pronounced effect in IL-1β production as compared to any other.

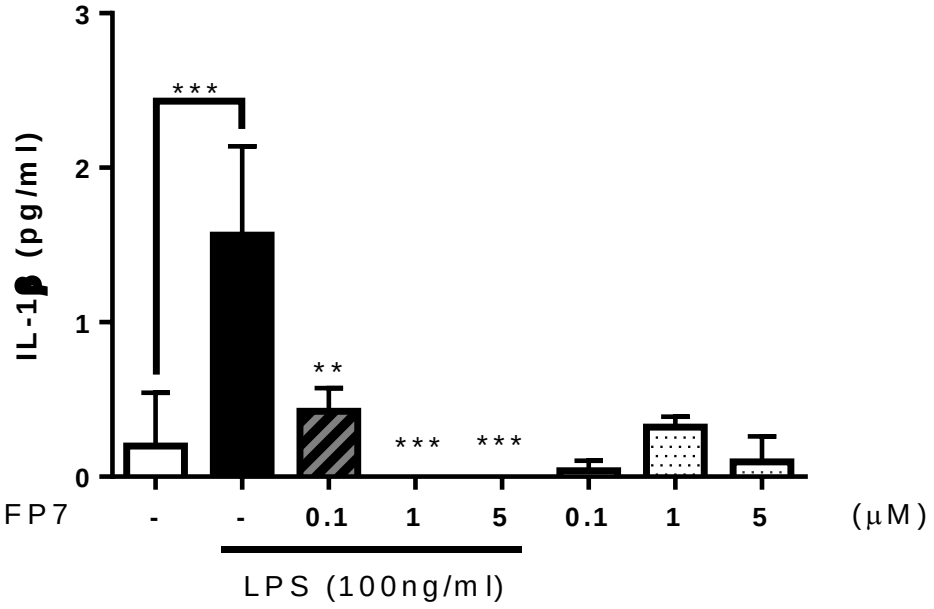

*Figure 21. The effect of FP7 on IL-1b production in THP-1 monocytes. Cells were pre-treated with FP7 (0-5μM) for 1 hour prior to exposure to LPS (100ng/ml). Untreated control (-) is with vehicle only. IL-1β production in culture medium was measured using ELISA after 18 hours (RayBiotech). Results are shown as mean ±SD of three separate treatments. Statistically significant results are indicated as *** P<0.001 ** P<0.01 *P<0.05 for control vs LPS and LPS vs LPS/FP7 treated samples.*

While these proinflammatory proteins are all dependent on LPS and TLR4 signalling, various secondary effects come into action following initial signalling by TLR4. For example, NLRP3 inflammasome activation is required for IL-1β activation , subsequent autocrine activation of cytokine receptor, for example by TNFα and IL-1β, which can affect levels of IL-8 expression (Cassatella *et al.*, 1993) and further synergistic effects may occur, as seen in VEGF production dependent IL-6 signalling which can be increased in conjunction with IL-1β or TNFα (Nakahara *et al.*, 2003). This effect effects may account for the different levels of upregulation of different cytokines following TLR4 signalling.

4.1.4. **FP7 negatively regulates the production of TLR4/MyD88-dependent proinflammatory signalling in THP-1 derived human macrophages**

Monocytes are mobile and circulate in the blood and are recruited to specific locations via chemoattractant molecules. In atherosclerosis, monocytes cross the endothelial barrier and differentiate into more static macrophages within plaques where they contribute to the sustained inflammatory response. The response of macrophages may differ from monocytes in the level of cytokine production. Both cell types, monocytes, and macrophages, express TLR4 on the cell surface. Following LPS exposure, macrophages were polarised as M1 type macrophages and produced release various cytokines and chemokines in the process of inflammatory response.

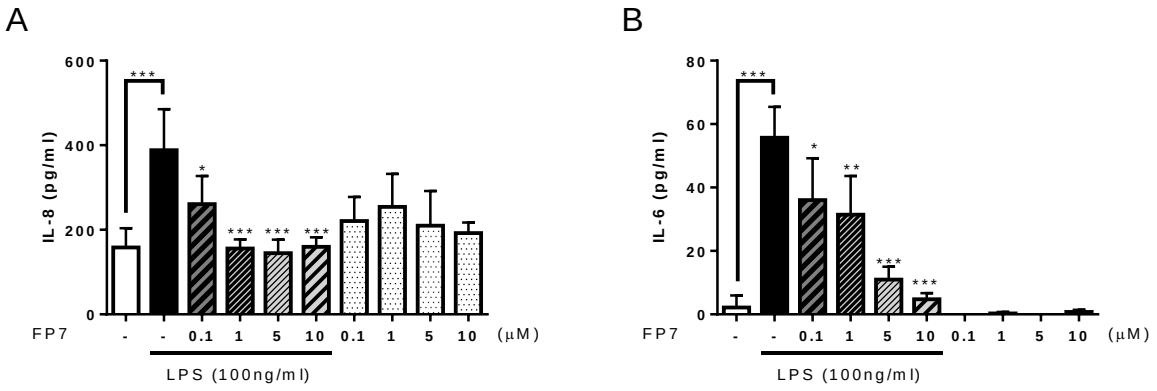

*Figure 22. The effect of FP7 on IL-8 and IL-6 production in THP-1 macrophages. Cells were pre-treated with FP7 (0-10μM) for 1 hour before being exposed to LPS (100ng/ml) and incubated overnight. Untreated control (-) is with vehicle only. Levels of (A) IL-8 and (B) IL-6 present in culture medium was measured using ELISA (Ray-Biotech, USA). Results are shown as mean ±SD of three separate treatments. Statistically significant results are indicated as *** P<0.001 ** P<0.01 *P<0.05 for control vs LPS and LPS vs LPS+FP7 treated samples.*

The effect of FP7 on LPS induced proinflammatory signalling was explored further in THP-1 macrophages. ELISA was used to determine levels of IL-6 and IL-8 release in the culture medium following LPS stimulation. A similar pattern was seen, as in THP-1 monocytes. LPS induced production of IL-6 and IL-8 was downregulated following LPS stimulation of cells (Figure 22). FP7 significantly reduced IL-6 production with as little as 0.1 µM compared to LPS activated samples and reduced this to a level almost comparable to the control at 10 µM treatment. IL-8 was downregulated to a level comparable to the control following treatment with only 1 µM FP7. This suggests that FP7 can reduce TLR4 induced cytokine production in THP-1 derived macrophages as well as monocytes.

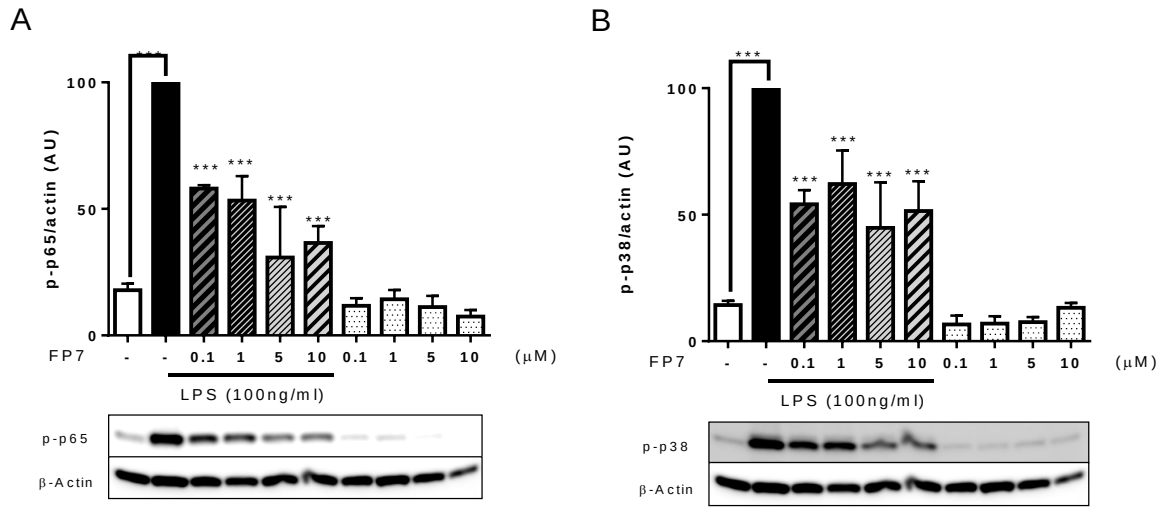

*Figure 23. The effect of FP7 on P65/NF-κB and P38 MAPK phosphorylation in THP-1 macrophages. Cells were pre-treated with FP7 (0-10µM) for 1 hour before being exposed to LPS (100ng/ml) for 1 hour. Untreated control (-) is with vehicle only. Cell lysate was analysed via Western blot to determine levels of P65/NF-κB and P38 MAPK phosphorylation. Results are shown as mean ±SD of three separate treatments. Statistically significant results are indicated as *** P<0.001 ** P<0.01 *P<0.05 for control vs LPS and LPS vs LPS+FP7 treated samples.*

TLR4 activations results in downstream phosphorylation of P65/NF-κB and P38 MAPK, which facilitate transcription and release of proinflammatory proteins. To determine the effect of FP7 on MyD88-dependent TLR4 signalling in THP-1 macrophages, the effect on P65/NF-κB (Figure 23A) and P38 MAPK (Figure 23B) phosphorylation were measured via Western Blot analysis. Both signalling mediators were significantly downregulated in the presence of FP7. Again, this effect was seen with as little as little as 0.1μM FP7. This suggests FP7 can affect TLR4 signalling and downstream proinflammatory protein release through a partial inhibition of NF-κB and P38 MAPK activation (C. Palmer et al., 2018)

4.1.5. **FP12 negatively regulates the production of TLR4-dependent proinflammatory proteins (IL-1 β, IL-6) and activation of signalling mediators in THP-1 human macrophages**

FP7 and FP12 are structurally similar, where FP12 only differs in a shorter acyl chain length. This may confer some differences in functional activity of the molecules, though FP12 is still designed and expected to function as an antagonist. Initial screening showed that FP12 could represent another good candidate from the antagonist series to investigate further. Preliminary toxicity and IL-8 screening results indicated that FP12 was safely tolerated in THP-1 monocytes and macrophages and downregulated TLR4 induced IL-8 production. To further assess the potential of FP12 as a novel TLR4 antagonist, ELISA and Western blot analysis were carried in both monocytes and macrophages.

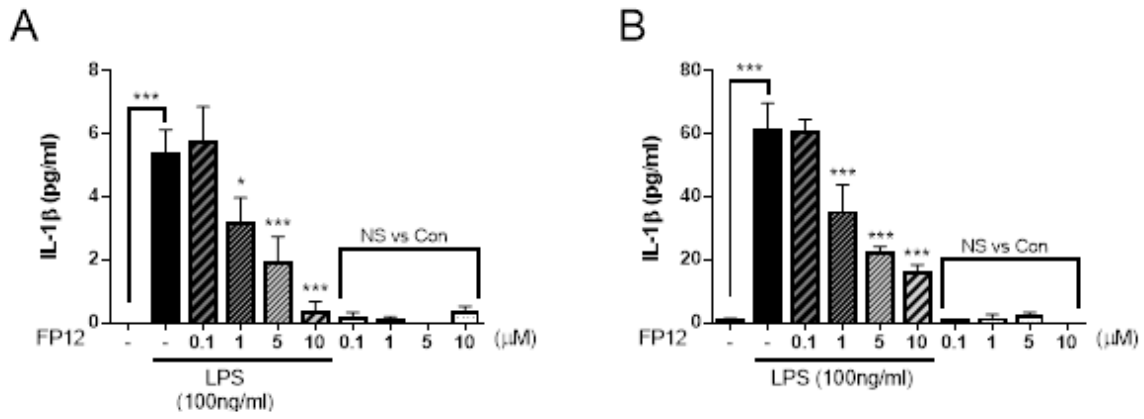

*Figure 24. The effect of FP12 on IL-1β production in THP-1 monocytes (A) and macrophages (B). Cells were pre-treated with FP12 (0-10μM) for 1 hour prior to exposure to LPS (100ng/ml). Untreated control (-) is with vehicle only. IL-1β production in culture medium was measured using ELISA after 18 hours (RayBiotech). Results are shown as mean ±SD of three separate treatments. Statistically significant results are indicated as *** P<0.001 ** P<0.01 *P<0.05 for control vs LPS and LPS vs LPS/FP12 treated samples.*

Following overnight activation of TLR4 signalling with LPS, IL-1β production was significantly increased as compared to the control. Monocytes (Figure 24A) exhibited a smaller increase in IL-1β production as compared to macrophages (Figure 24B), however 1μM FP12 was sufficient to produce a significant reduction in IL-1β production in both cases.

IL-6 production was significantly upregulated by LPS and negatively regulated by FP12 in both THP-1 monocytes and macrophages (Figure 25). As with IL-1β, macrophages (Figure 25B) were more responsive to LPS stimulation than monocytes (Figure 25A). FP12 was efficient in downregulating IL-6 production in macrophages at as little as 0.1μM in macrophages, though was only sufficient to produce a significant reduction in monocytes at 5μM.

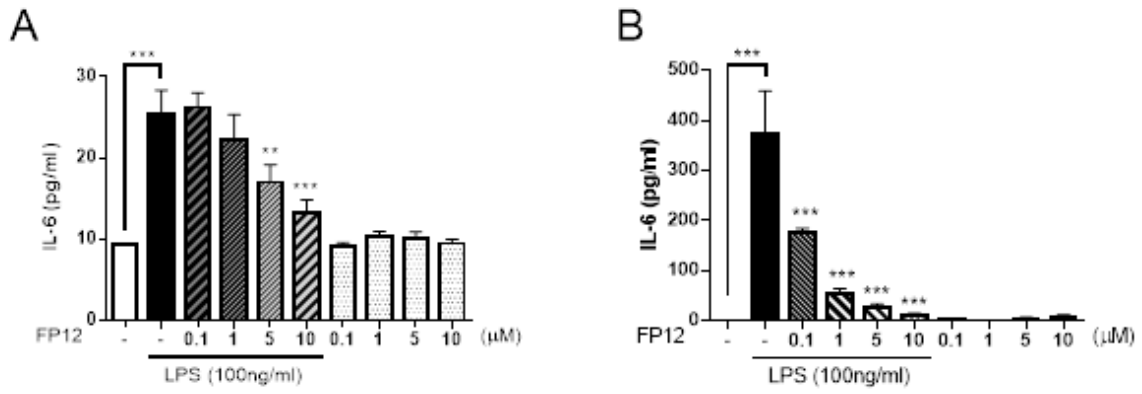

*Figure 25. The effect of FP12 on IL-6 production in THP-1 monocytes (A) and macrophages (B). Cells were pre-treated with FP12 (0-10μM) for 1 hour prior to exposure to LPS (100ng/ml). Untreated control (-) is with vehicle only. IL-6 production in culture medium was measured using ELISA after 18 hours (RayBiotech). Results are shown as mean ±SD of three separate treatments. Statistically significant results are indicated as *** P<0.001 ** P<0.01 *P<0.05 for control vs LPS and LPS vs LPS/FP12 treated samples.*

While initial experiments suggested FP7 may be more effective in downregulating IL-8 production as compared to FP12, the data for IL-6 suggested a more potent regulatory effect in response to FP12. Both FP12 and FP7 required at least 5µM to significantly reduced IL-6 production in macrophages and were more effective in reducing cytokine release in macrophages. However, FP12 produced a more significant reduction in IL-6 production at lower concentrations as compared to FP7 in this case.

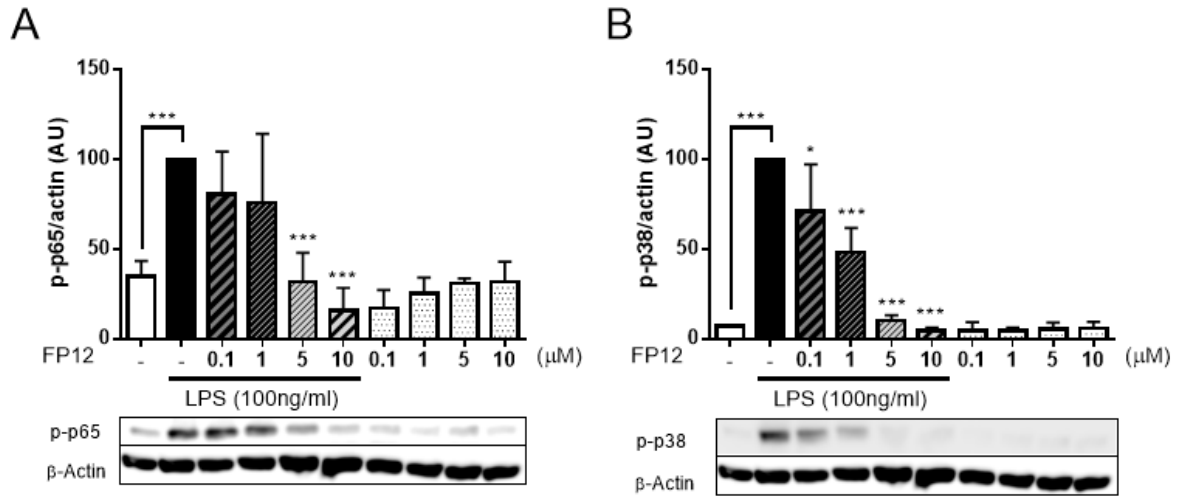

*Figure 26. The effect of FP12 on NF-κB and P38 MAPK phosphorylation in THP-1 macrophages. Cells were pre-treated with FP7 (0-10µM) for 1 hour before being exposed to LPS (100ng/ml) for 1 hour. Untreated control (-) is with vehicle only. Cell lysate was analysed via Western blot to determine levels of NF-κB and P38 MAPK phosphorylation using the same blot. Results are shown as mean ±SD of three separate treatments. Statistically significant results are indicated as *** P<0.001 ** P<0.01 *P<0.05 for control vs LPS and LPS vs LPS+FP12 treated samples.*

Further analysis of the signalling pathways involved in FP12 inhibition of LPS/TLR4 signalling was carried out via Western blot analysis. P65/NF-κB and P38 MAPK phosphorylation were significantly

reduced in pre-treated samples. While P38 MAPK phosphorylation was significantly reduced at 0.1µM, P65/NF-κB was only significantly downregulated at 5µM (Figure 26). As compared to FP7, FP12 appears to show a slightly less pronounced effect on P65/NF-KB and P38 MAPK at lower concentrations but can significantly downregulate both to a level comparable to the control at 10µM.

4.1.6. **FP7 and FP12 negatively regulate LPS-stimulated proinflammatory proteins production in THP-1 macrophages.**

A proinflammatory protein antibody array was used to give an overview of proinflammatory protein production in response to LPS and subsequent suppression when pre-treated with FP7 or FP12. This gave an overview to identify the relevant targets for further investigation (Table 2). Values are representative of fold difference versus untreated cells (vehicle only), the value of which was taken to be 1 for each protein listed. Therefore, values for LPS can be taken as a fold increase relative to the control. Values that exceeded a two-fold increase were considered potentially of interest and highlighted in green. Values for samples pre-treated with FP7 or FP12 were considered of interest if a 50% reduction was seen relative to LPS.

LPS stimulation of THP-1 macrophages produced greater than two-fold increase in an array of proinflammatory mediators including EOTAXIN--1, GCSF, GM-CSF, iCAM-1, I-309, IL-1α, IL-1β, IL-4, IL-6, IL-10, IL-11, IL-13, IP10, MCP-1, MCP-2, MIP-1α, MIP-1β and TNFα. In most cases, proteins upregulated by LPS are negatively regulated by FP7 and FP12. An exception to this, for both FP7 and FP12, Is MIP-1β which does not appear to show any reduction. In addition to this, FP12 does not appear to have as pronounced effect on GCSF, I-309 or MIP-1α. This gives the appearance that FP7 may be more effective overall at downregulating proinflammatory protein production than FP12. However, array data may not be the best direct representation of protein levels in the medium. While IL-8 was previously shown to be elevated significantly in response to LPS in previous experiments, it does not appear to increase when measured via array due to the levels being too high and subsequent signal being saturated, making any differences indistinguishable. Additionally, IL-6 appears to produce a 300-fold increase following LPS production, which was not as pronounced previously on ELISA. While the array can give a reasonable overview, any protein of interest must be verified by more quantitative means of analysis such as ELISA in order to draw conclusions from.

Table 2. FP7 and FP12 negatively regulate LPS induced proinflammatory protein production in THP-1 macrophages. THP-1 macrophages were treated with FP7 or FP12 (10μM) for 1 hour prior to LPS (100ng/ml) exposure. Culture medium collected after 18-hour incubation. A human inflammation array (RayBiotech). Green = 2-fold increase vs untreated control (vehicle only). Red = 50% decrease vs LPS.

Inflammatory Proteins	LPS	LPS/ FP7	LPS/ FP12	Inflammatory Proteins	LPS	LPS/ FP7	LPS/ FP12
1. EOTAXIN-1	5.95	0.97	1.10	**21. IL-13**	2.38	0.91	0.49
2. EOTAXIN-2	1.06	1.20	1.36	**22. IL-15**	0.71	0.58	0.83
3. GCSF	2.08	1.03	1.61	**23. IL-16**	1.53	1.09	1.34
4. GM-CSF	3.71	0.46	0.35	**24. IL-17A**	0.88	0.83	0.76
5. iCAM-1	3.21	1.59	2.09	**25. IP10**	8.09	1.49	1.57
6. IFNγ	0.19	0.19	0.32	**26. MCP-1**	19.82	3.33	9.01
7. I-309	5.63	1.96	4.12	**27. MCP-2**	146.71	1.66	4.43
8. IL-1α	3.71	0.19	1.82	**28. M-CSF**	0.59	0.98	1.00
9. IL-1β	7.86	1.72	2.60	**29. MIG**	0.23	0.36	1.53
10. IL-2	0.45	1.09	1.18	**30. MIP-1α**	15.53	5.37	13.07
11. IL-3	1.03	1.05	1.07	**31. MIP-1β**	2.54	2.21	2.73
12. IL-4	4.69	0.84	0.95	**32. MIP-1δ**	1.39	0.68	0.44
13. IL-6	299.34	0.65	3.86	**33. RANTES**	0.98	0.83	0.85
14. IL-6R	1.60	2.03	2.30	**34. TGFβ**	0.83	0.86	0.89
15. IL-7	1.00	1.00	2.84	**35. TNFα**	15.88	0.93	1.31
16. IL-8	1.36	1.07	1.26	**36. TNFβ**	0.75	1.42	1.80
17. IL-10	7.00	1.42	3.34	**37. TNF RI**	1.31	1.27	1.19
18. IL-11	2.35	0.48	0.43	**38. TNFII**	1.39	1.10	1.15
19. IL-12p40	1.73	1.26	1.15	**39. PGDFBB**	0.25	2.31	0.93
20. IL-12p70	1.43	0.13	1.01	**40. TIMP-2**	1.02	1.09	0.98

4.1.7. **FP7 and FP12 downregulate LPS/TLR4-induced proinflammatory cytokines irrespective of time of administration (in pre, simultaneous and post LPS treatment)**

LPS binds to MD-2/TLR4 causing receptor dimerisation and activation of MyD88-dependent signalling via adaptor protein Mal. This triggers the signalling cascade, resulting in release of downstream transcriptional molecules within 30-60 minutes of LPS exposure. FP7 and FP12 are thought to interact with MD-2 in a similar manner to LPS and may prevent LPS binding to the receptor through a mechanism of competitive exclusion (Cighetti *et al.*, 2014; Facchini *et al.*, 2018) and might then be expected to show reduced effects after LPS is already occupying the hydrophobic pocket.

Previous results were based on pre-treatment of cells with TLR4 antagonists before LPS exposure. To better understand interaction of FP7 and FP12 timing on signalling, THP-1 cells were treated at 30 minutes before, simultaneously and 30 minutes after LPS exposure. Following stimulation, levels of TNFα, IL-1β and IL-6 are significantly downregulated by FP7 and FP12 in all treatments (Figure 27). These data confirm previous results seen in array analysis (Table 4). This suggests FP7 and FP12 negatively affect TLR4-dependent proinflammatory protein production in THP-1 macrophages.

Unexpectedly, FP7 and FP12 downregulated cytokine production even after LPS exposure. This could suggest that these molecules have a higher binding affinity to the hydrophobic pocket of MD-2 than does LPS, resulting in displacement of LPS by FP7 or FP12. However, signal transduction would be expected to have occurred at this point and the exact mechanism of action is unclear. Further investigation is required to fully understand this interaction.

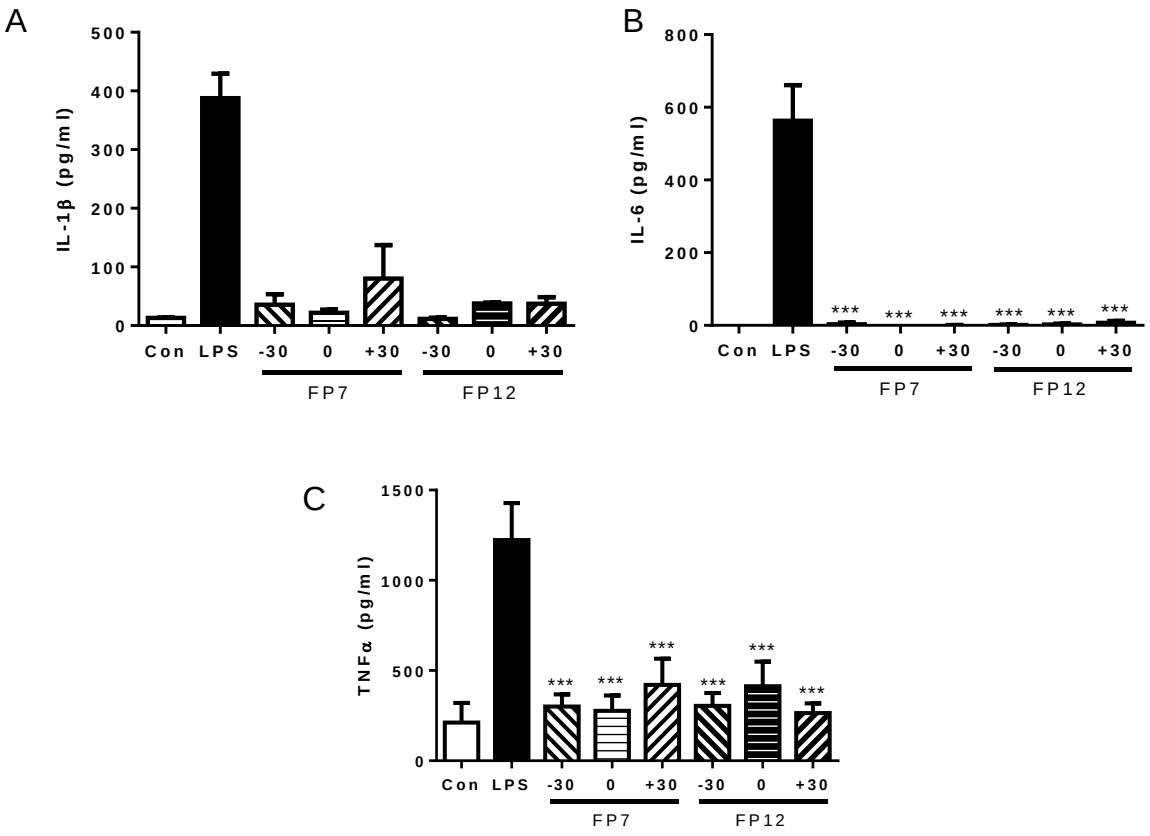

*Figure 27. FP7 and FP12 downregulate MyD88-dependent cytokines at different treatment timings. (A) IL-1β, (B) IL-6 and (C) TNFα. THP-1 macrophages were treated with FP7 or FP12 (10μM) 30 minutes prior (-30), simultaneously (0) or 30 minutes after (+30) LPS (100ng/ml) exposure. Untreated control is with vehicle only. Culture supernatants were then collected at 2 or 18 hours and IL-6, IL-1β, TNFα and IFNβ levels measured via ELISA. Results are shown as mean ±SD of three separate treatments. Statistically significant results are indicated as *** P<0.001 ** P<0.01 *P<0.05 for control vs LPS and LPS vs LPS+FP12 treated samples.*

4.1.8. **FP7 downregulates LPS/TLR4-induced proinflammatory cytokines in primary PBMC derived macrophages**

THP-1 derived macrophages and primary derived macrophages respond differently to stimulus. Although cytokine production is generally comparable between THP-1 macrophages and primary cells, PBMCs are known to have a higher response to LPS stimulation as compared to THP-1 cells which could impact antagonistic activity of FP7 or FP12. Additionally, the expression of M1 and M2 phenotype markers can differ between cell types. PBMC derived macrophages were prepared from isolated PBMCs in 10% FCS for 7 days at Reading University. Macrophages were treated with FP7 in presence or absence of LPS to determine the comparability of results obtained in THP-1 macrophages to primary cell response.

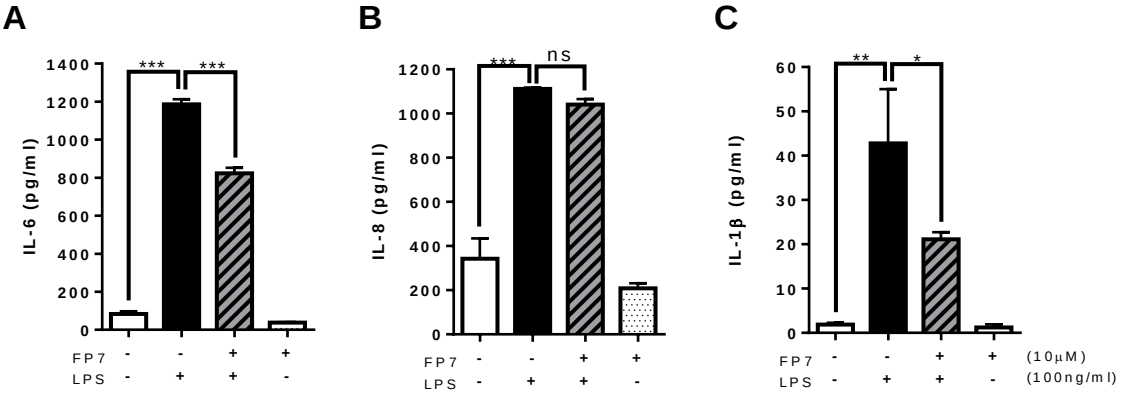

*Figure 28. The effect of FP7 on IL-6, IL-8 and IL-1β production in PMBC-derived macrophages. Cells were pre-treated with FP7 (10μM) for 1 hour prior to exposure to LPS (100ng/ml). Untreated control, FP7 (-). is with vehicle only . Culture medium was collected and expression of (A) IL-6, (B) IL-8 and (C) IL-1β were measured using ELISA after 18 hours (RayBiotech). Results are shown as mean ±SD of two separate treatments. Statistically significant results are indicated as *** P<0.001 ** P<0.01 *P<0.05 for control vs LPS and LPS vs LPS/FP7 treated samples.*

FP7 and FP12 negatively regulated LPS/TLR4 induced cytokine production in primary macrophages (Figure 28). Primary macrophages produced a significant increase in cytokine levels in response to LPS. IL-6, IL-8 and IL-1β levels were substantially elevated in LPS treated samples as compared to the controls. 1 hour pre-treatment with FP7 was sufficient to bring about a reduction in LPS/TLR4 induced IL-6 and IL-1β production, but not IL-8. However, FP7 did not bring down levels of any cytokine to that of the control samples. This preliminary data suggests that FP7 is capable of partial inhibition of TLR4-dependent cytokine production in PMBC-derived macrophages. in response to LPS as the same concentration as was used to treat THP-1 macrophages.

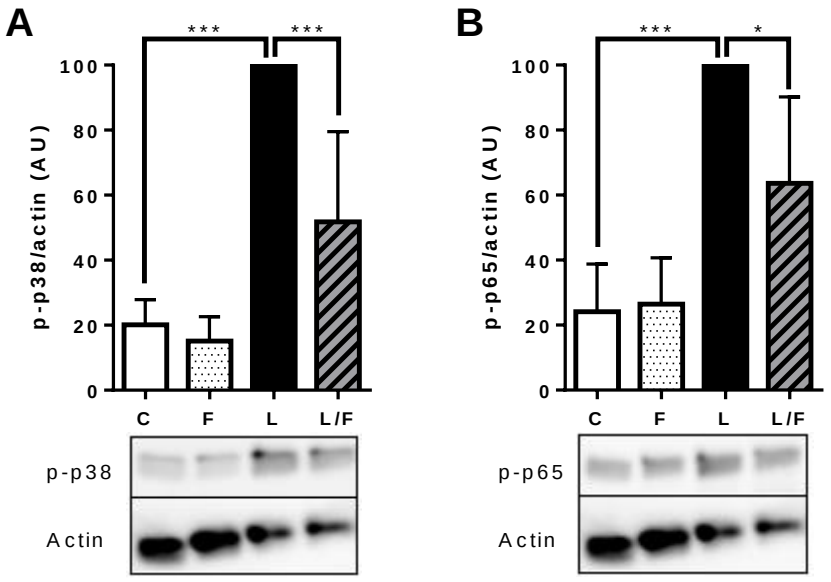

*Figure 29. The effect of FP7 on p-p65/NF-κB and p-P38 activation in PBMC derived macrophages. Cells were pre-treated with FP7 (10μM) for 1 hour prior to exposure to LPS (100ng/ml). Untreated control is with vehicle only. Cell lysates were collected after 1 hour and analysed via Western blot for relative levels of P65 and P38 phosphorylation using the same blot. Results are shown as mean ±SD of three separate treatments. Statistically significant results are indicated as *** P<0.001 ** P<0.01 *P<0.05 for control vs LPS and LPS vs LPS/FP7. C = control, L = LPS, F = FP7.*

Western blot was used to determine level of NF-κB/P65 and P38 phosphorylation in primary macrophages (Figure 29). LPS produced a similar increase in p-P38 and p-p65 as seen in THP-1 macrophages at 1 hour. FP7 was capable of significantly reducing phosphorylation of these signalling molecules in response to LPS. These results suggest that FP7 may have a comparable antagonistic effect on MyD88-dependent signalling and endpoint pro-inflammatory protein production in primary human macrophages to that seen previously in THP-1 derived macrophages.

Primary cells represent human physiology more effectively than immortalised cell lines and can help to provide greater validity to previous results. While THP-1 cells and PBMCs are somewhat comparable, LPS stimulated TLR4 activity in THP-1 macrophages may be lower than that observed in PBMCs. It is therefore important to see if results obtained with FP7 in THP-1 macrophages can translate into PBMCs.

While the effect of FP7 on PBMCs and THP-1 macrophages show similar antagonistic effects following LPS stimulation, FP7 appears to be less effective overall in PBMCs. While 10μM FP7 was high enough to produce a significant decrease in IL-8, this was not the case for PBMC where 10μM FP7 had no effect on IL-8 production. The effect of FP7 on IL-6 and IL-1β were also markedly lower, with limited data suggesting an IC50 of 10μM or higher. This reduced effect is also seen in signalling mediators NF-κB and P38. Higher concentrations or alternative delivery methods may be necessary to increase the effects of FP7 or FP12 in PBMCs.

4.1.9. **FP7 downregulates oxLDL-mediated TLR4 activation in THP-1 macrophages**

In many inflammatory based diseases, LPS is not thought the be the activator of TLR4 signalling and called sterile ligands of inflammation have been proposed in the absence of endotoxin. OxLDL is thought to play a part in cardiovascular pathology and has been suggested to affect TLR4 signalling. It is therefore necessary to understand the potential of FP7 and FP12 to negatively regulate TLR4 activation in response to sterile ligands, which may function through unconventional means.

OxLDL can be produced in the presence of transition ions. Hydroperoxide oxLDL is produced rapidly at room temperature, and subsequently degrades at higher temperatures. Oxysterol oxLDL is produced more slowly than hydroperoxide-rich oxLDL. Lower temperatures (4^{o}c) allow the formation of hydroperoxide-rich oxLDL by slowing production and degradation processes and stopping the reaction while hydroperoxides peak and oxysterol levels remain low. Conversely, higher temperature (37^{o}c) allows for rapid formation of oxysterols, while hydroperoxides degrade, to create oxysterol-rich oxLDL. These variant forms may have different biological effects and are of interest to explore further.

To investigate the effect of FP7 on oxLDL induced proinflammatory signalling, a series of experimental optimisation steps were required. Cell viability screening was carried out to determine safe concentration for use in future experiments. From this, 0-50 µg/mL oxysterol and 100 µg/mL hydroperoxide-rich oxLDL concentrations were selected for investigation. Cytokine production and signalling mediator activation was measured following exposure to 10, 50 and 100 µg/mL oxLDL, with and without pre-treatment with 10 µM of FP7.

First, to assess the effect of oxLDL onTHP-1macrophages cell viability, an MTT assay was carried out using oxLDL ranging from 0-100 µg/ml. Both forms of oxLDL were well tolerated in THP-1 macrophages. However, a significant reduction in cell viability was seen in cells exposed to oxysterol-rich oxLDL at concentrations greater than 50 µg/mL in THP-1 macrophages, but not hydroperoxide-

rich oxLDL up to 100 µg/ml.

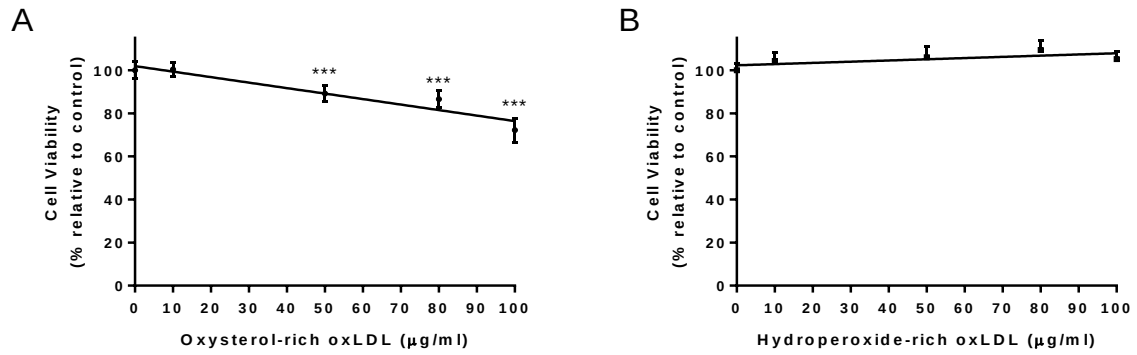

Figure 30. The effect of oxLDL on THP-1 macrophage cell viability. Cells were treated with 0-100 µg/mL oxysterol or hydroperoxide-rich oxLDL and incubated overnight (16h) prior to addition of MTT reagent. Results are shown as mean +/- SD of n=6 and expressed as a percentage relative to the control set.

Results obtained from MTT analysis of cell viability were used to inform concentrations used for future experiments (Figure 30). Hydroperoxide-rich oxLDL was well tolerated by THP-1 macrophages at all tested concentrations, whereas oxysterol-rich oxLDL significantly reduced viability following treatment >50 µg/ml. Concentrations which caused a reduction in viability of greater than 80% were considered unsuitable for further experiments. 0-50 µg/mL oxysterol and 0-100 µg/mL hydroperoxide-rich oxLDL were selected for activation of TLR4 induced signalling in THP-1 macrophages.

THP-1 macrophages were exposed to 10, 50 or 100 µg/mL hydroperoxide-rich oxLDL and 10 or 100 µg/mL oxysterol-rich oxLDL with or without pre-treatment with FP7 (10 µM). Statistical analysis was carried out on control vs oxLDL only groups, and oxLDL groups vs oxLDL+FP7 groups.

Both hydroperoxide and oxysterol-rich oxLDL types were capable of producing a significant increase in IL-1β (Figure 31), IL-6 (Figure 32) and IL-8 (Figure 33) after overnight exposure in a concentration-

dependent manner. However, nLDL did not produce a comparable response. Following oxLDL treatment, IL-1β levels were significantly increased in response to higher concentrations (>50µg/ml) of both hydroperoxide- and oxysterol- rich oxLDL (Figure 31). Pre-treatment of cells with FP7 (10µM) resulted in a significant reduction of IL-8 following stimulation in all treated groups.

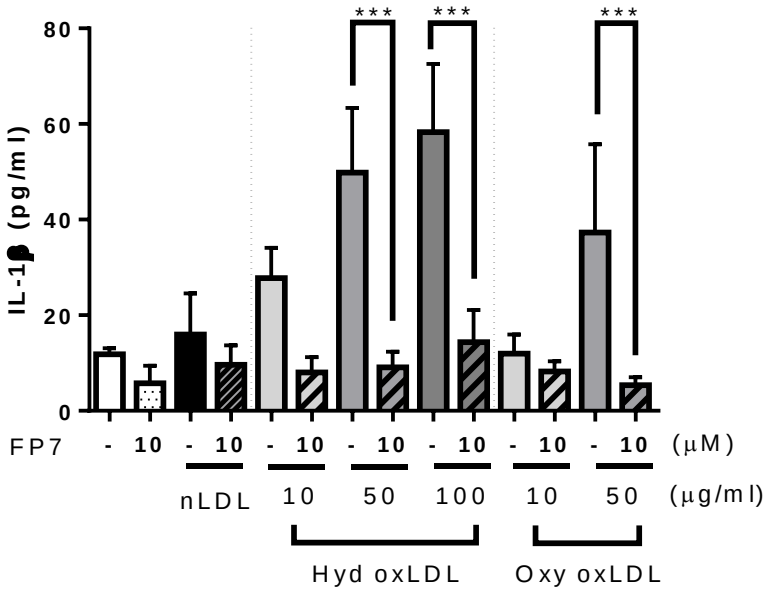

*Figure 31. FP7 downregulates IL-1β secretion in response to oxLDL in THP-1 derived macrophages. THP-1 derived macrophages were treated with a specific TLR4 antagonist FP7 (10 µM) or vehicle (Ethanol/DMSO) for 1 hour before overnight (16hr) incubation with hydroperoxide-rich oxLDL (0-100 µg/ml), oxysterol-rich oxLDL (0-50 /ml) or native LDL (50 µg/ml). (-) are with vehicle only. Culture medium was collected and retained for analysis. IL-1β secretion was measured via ELISA. Results are expressed as mean +/- standard deviation from three separate treatments. Significant results are shown as * = p<0.05, ** = p<0.01 *** = p<0.001.*

OxLDL produced a similar increase in IL-6 as seen with IL-1β whereby 50-100µg/mL produced the most effective response (Figure 32). However, 10µg/mL of hydroperoxide-rich oxLDL was also enough to induce a smaller, but significant, increase in IL-6 following stimulation. FP7 (10µM) was capable of counteracting IL-6 secretion in response to oxLDL.

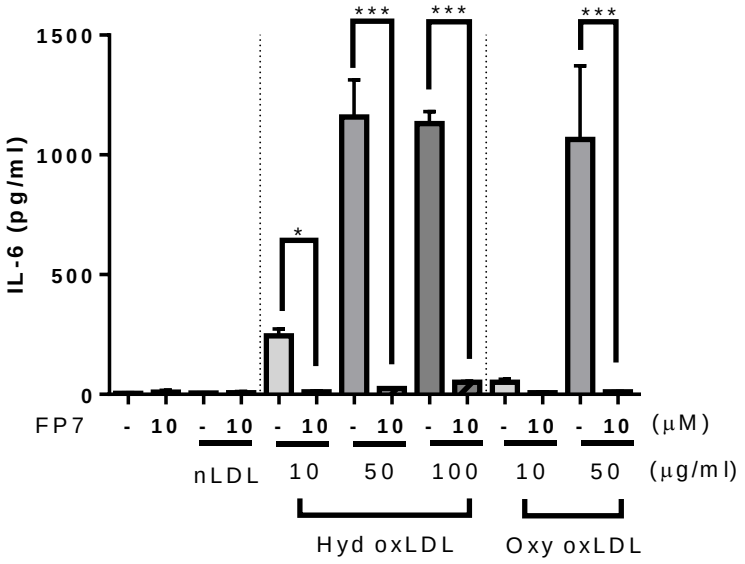

*Figure 32. FP7 downregulates IL-6 secretion in response to oxLDL in THP-1 derived macrophages. THP-1 derived macrophages were treated with a specific TLR4 antagonist FP7 (10 µM) or vehicle (Ethanol/DMSO) for 1 hour before overnight (16hr) incubation with hydroperoxide-rich oxLDL (0-100 µg/ml), oxysterol-rich oxLDL (0-50 µg/ml) or native LDL (50 µg/ml). (-) are with vehicle only. Culture medium was collected and retained for analysis. IL-6 secretion was measured via ELISA. Results are expressed as mean +/- standard deviation from three separate treatments. Significant results are shown as * = p<0.05, ** = p<0.01 *** = p<0.001.*

IL-8 was induced by oxLDL to a lesser extent an showed a dose-dependent response to hydroperoxide-rich, but not oxy-sterol rich oxLDL (Figure 33). Hydroperoxide-rich oxLDL caused a significant increase in IL-8 secretion at higher concentrations (>50µg/ml) which were substantially muted in presence of FP7 (10µM).

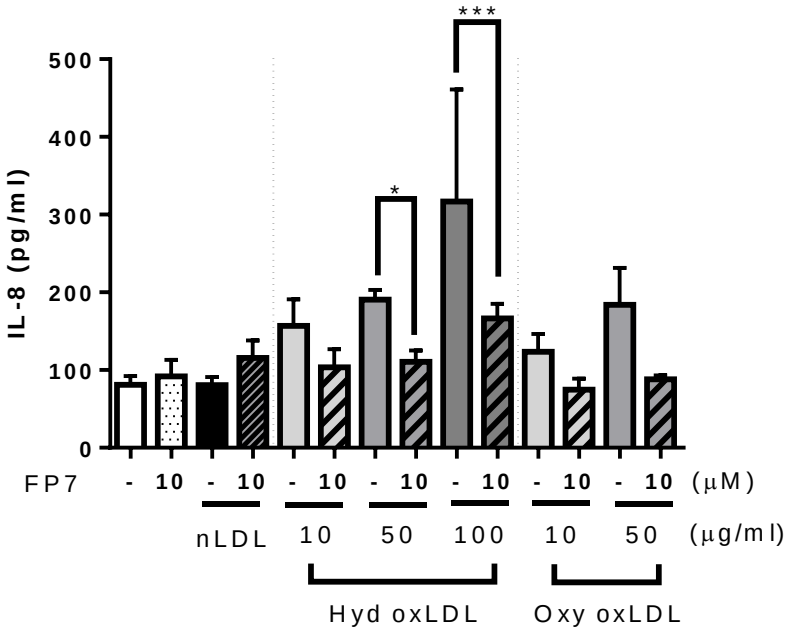

*Figure 33. FP7 downregulates IL-8 secretion in response to oxLDL in THP-1 derived macrophages. THP-1 derived macrophages were treated with a specific TLR4 antagonist FP7 (10 µM) or vehicle (Ethanol/DMSO) for 1 hour before overnight (16hr) incubation with hydroperoxide-rich oxLDL (0-100 µg/ml), oxysterol-rich oxLDL (0-50 µg/ml) or native LDL (50 µg/ml). (-) are with vehicle only. Culture medium was collected and retained for analysis. IL-8 secretion was measured via ELISA. Results are expressed as mean +/- standard deviation from three separate treatments. Significant results are shown as * = p<0.05, ** = p<0.01 *** = p<0.001.*

4.1.10. **FP11 and FP18 function as agonists, upregulating TLR4-dependent proinflammatory signalling in THP-1 macrophages**

FP11 was selected from initial candidates as an agonist of TLR4 in THP-1 monocytes. Initial screening of FP11 showed a tolerance in THP-1 monocytes and macrophages up to exposure of 10μM. IL-8 was significantly elevated at 5-10 μM concentrations of FP11. FP18 (FP11 derivative) was a later addition and thus not present at IL-8 screening stage.

FP11 is designed to function as an agonist of TLR4. Initial screening data here suggested FP11 was capable of inducing IL-8 production in THP-1 monocytes. To further investigate the effect of FP11 on TLR4 signalling, the capacity of FP11 to upregulate MyD88 and TRIF-dependent TLR4 signalling was explored in comparison to LPS and MPLA. Synthetic MPLA is a monophosphorylated lipid A with 6 acyl chains that is comparable, in part, to the two agonists FP11 and FP18 (Figure 10).

An initial a proinflammatory protein antibody array was used to give an overview of the inflammatory protein expression profile of FP11 and FP18 (Table 3). A two-fold increase in levels compared to the control was considered of interest for identification of suitable target. LPS produced an increase in EOTAXIN-1, GM-CSF, iCAM-1, IL-1β, IL-4, IL-6, IL-11, IL-13, IP10, MCP-1, and TNFα, while MPLA only appeared to upregulate IL-1α, IL-2, IL-8 and MIG to a level above the threshold, though GM-CSF iCAM-1 and I309 fall just short of this. Interestingly, the effects of FP11 and FP18 on proinflammatory protein expression appeared to be more comparable to that of LPS rather than MPLA. Of interest are ICAM-1, I309, IL-1β, IL-6, IP10 and MCP2 which are all around 2-4-fold increases, with exception of IL-6 for which FP18 produces a 10-fold increase compared to the control. These represent potential avenues of interest that require further investigation to confirm.

Table 3. FP11 and FP18 upregulate inflammatory protein expression in THP-1 Macrophages. THP-1 macrophages were treated with LPS (100ng/ml), MPLA (100ng/ml), FP11 or FP18 (10μM). Culture medium collected after 18-hour incubation. A human inflammation array (RayBiotech). Green = 2-fold increase vs control.

Inflammatory Proteins	LPS	MPLA	FP11	FP18	Inflammatory Proteins	LPS	MPLA	FP11	FP18
1. EOTAXIN-1	2.36	1.39	0.71	1.18	**21. IL-13**	1.26	3.40	1.04	0.78
2. EOTAXIN-2	1.44	1.25	1.17	1.19	**22. IL-15**	1.98	1.16	0.79	0.8
3. GCSF	1.69	1.27	0.54	0.73	**23. IL-16**	1.24	1.08	0.98	0.91
4. GM-CSF	4.21	1.96	1.56	1.83	**24. IL-17A**	2.79	1.14	0.65	1.03
5. iCAM-1	2.43	1.91	2.33	2.29	**25. IP10**	7.42	1.53	4.69	4.85
6. IFNγ	1.46	1.41	1.41	1.34	**26. MCP-1**	1.25	1.10	0.96	0.98
7. I-309	1.93	1.91	2.42	2.54	**27. MCP-2**	5.19	1.21	2.46	2.92
8. IL-1α	1.19	2.56	1.16	1.25	**28. M-CSF**	1.25	1.18	1.11	1.05
9. IL-1β	5.53	1.46	2.41	2.64	**29. MIG**	2.95	2.96	1.01	1.49
10. IL-2	1.06	2.05	1.96	0.72	**30. MIP-1α**	1.15	1.15	1.05	0.97
11. IL-3	1.26	1.18	1.05	1.02	**31. MIP-1β**	0.74	1.05	0.78	0.73
12. IL-4	2.36	0.76	0.60	0.90	**32. MIP-1δ**	1.58	0.92	1.06	0.92
13. IL-6	99.41	1.22	3.41	10.61	**33. RANTES**	1.16	1.24	0.99	0.98
14. IL-6R	1.39	1.21	1.12	1.14	**34. TGFβ**	0.23	0.35	0.70	0.97
15. IL-7	1.00	1.00	1.00	1.00	**35. TNFα**	2.32	1.39	1.19	1.02
16. IL-8	0.96	1.32	1.03	0.97	**36. TNFβ**	1.92	1.52	1.20	1.34
17. IL-10	1.57	1.37	1.45	1.25	**37. TNF RI**	1.23	1.04	1.21	1.00
18. IL-11	2.18	0.58	0.91	0.49	**38. TNFII**	1.5	1.12	1.06	1.01
19. IL-12p40	1.66	1	1.19	0.92	**39. PGDFBB**	1.38	1.55	0.93	1.37
20. IL-12p70	1.36	1.13	0.9	0.92	**40. TIMP-2**	1.15	1.03	0.95	0.84

The effect of FP11 and FP18 on TLR4 MyD88-dependent signalling was further investigated via measurement of NF-κB activation via Western blot and endpoint IL-1β production was verified via ELISA. THP-1 cells were treated with FP11 or FP18 and P65/NF-κB phosphorylation compared to levels induced by LPS or MPLA. Both LPS and MPLA produced a significant increase in P65/NF-κB phosphorylation, as in previous experiments. FP11 was also capable of activating NF-κB after 1 hour exposure (Figure 34). While FP18 appeared to cause a slight increase in activation of NF-κB, however this was not found to be significant.

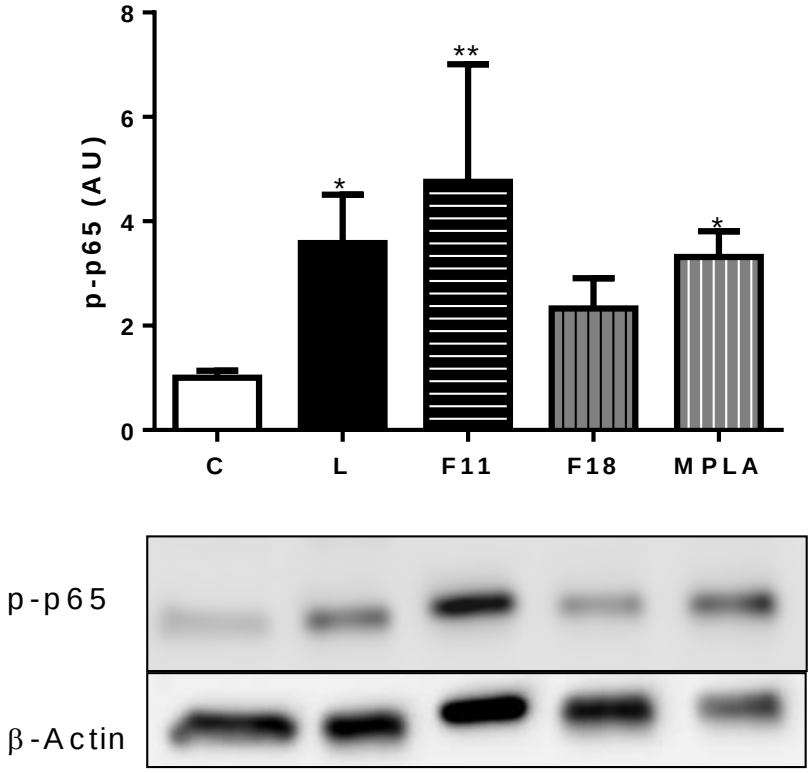

*Figure 34. FP11 upregulates NF-κB phosphorylation. THP-1 macrophages were treated with FP11 (10μM), FP18 (10μM) LPS or MPLA (100ng/ml). Untreated control is with vehicle only. Cell lysates were collected and analysed after 2.5 hours via Western blot. Results are expressed as mean +/- standard deviation from three separate treatments. Significant results are shown as * = p<0.05, ** = p<0.01 *** = p<0.001. F11 = FP11, F18 = FP18.*

However, both FP11 and FP18 had the potential to induce downstream production of IL-1β after overnight exposure. 10μM of either FP11 or FP18 caused a significant increase in IL-1β production of comparable levels to one another (Figure 35). This was comparable to levels produced by MPLA and double that produced in response to 100ng/mL LPS. These results provide further validation of previous data obtained via array which suggest that1 FP11 and FP18 can upregulate the production of MyD88-dependent cytokine production.

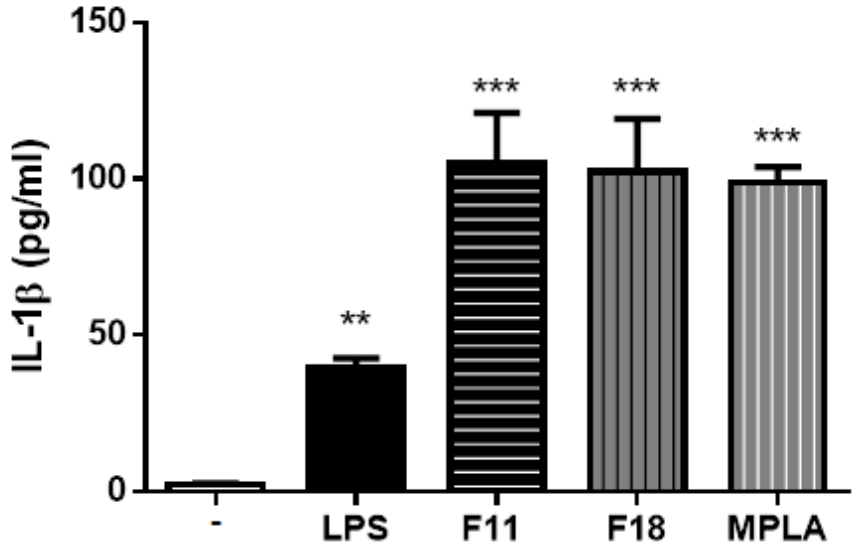

*Figure 35. FP11 and FP18 upregulate IL-1β production in THP-1 macrophages. THP-1 macrophages were treated with FP11 (10μM), FP18 (10μM) LPS or MPLA (100ng/ml). Untreated control (-) is with vehicle only . Culture medium was collected and analysed after 18 hours via ELISA. Results are expressed as mean +/- standard deviation from three separate treatments. Significant results are shown as * = p<0.05, ** = p<0.01 *** = p<0.001. F11 = FP11, F18 = FP18.*

4.1.11. **Summary**

Initial screening results highlighted FP7 and FP12 as potential TLR4 antagonists of interest. FP116 had no effect on LPS induced cytokine production. While FP10 produced some effect on IL-8, this was less pronounced than FP7 and FP12 and subsequently not followed up on. FP11 worked as expected, with a significant though slightly lower agonism than 100ng/mL LPS whereas FP111 had no effect.

FP7 experiments carried out in monocytes and macrophages further expand upon the initial screening and show that FP7 is capable downregulating a variety of proinflammatory proteins when produced in response to LPS stimulation. IL-6, IL-8, MIP-1α and iCAM-1 production were all significantly reduced following treatment with 5 μM of FP7 in THP-1 monocytes. In addition, IL-6 and IL-8 were significantly downregulated following treatment with as little as 0.1-1 μM of FP7 in macrophages. Further tot this, FP7 produced a reduction in LPS/TLR4 induced P65/NF-KB and P38 MAPK phosphorylation in THP-1 macrophages. These results provide evidence towards FP7 as an antagonist of TLR4 signalling. Further investigation into FP12 showed similar effects in monocytes and macrophages. Additionally, both FP7 and FP12 appear to be capable of downregulating LPS induced cytokine production when added to medium up to half an hour after LPS exposure. Data confirmed the antagonistic properties of FP12 in response to LPS induced IL-6 and IL-1β production as well as NF-κB. Both FP7 and FP12 can both negatively regulate LPS induced pro-inflammatory proteins. However, array results suggest FP7 may have a broader effectiveness than FP12. These therefore represent good candidates for further investigation.

FP7 demonstrated potential to reduce TLR4 activation induced by two forms of oxLDL. TLR4-dependent cytokine production following oxLDL exposure was significantly reduced by FP7. This may be through downregulation of NF-κB and P38 pathways. This suggests that FP7 may also function as an antagonist in alternative activation of TLR4 signalling induced by ligands of sterile inflammation.

FP11 and FP18 were shown to upregulate a range of proinflammatory proteins in THP-1 macrophages. This was further validated for MyD88-dependent TLR4 pathways via activation of NF-κB and subsequent production of IL-1β in response to FP11 and FP18. MPLA had only a small effect on proteins detected via array and FP compounds showed more similarity to the profile of LPS than to MPLA from the array data. However, ELISA showed a more comparable IL-1β response between MPLA and FP11 or FP18. These combined data show that FP11 and FP18 function as agonists of TLR4 MyD88-dependent signalling in THP-1 macrophages.

4.2. FP7 downregulates LPS/TLR4-induced proinflammatory proteins production in both human (THP-1) and murine (RAW264) macrophages

While the structure of TLR receptor complexes is relatively conserved between mammalian species, some small variations are present and can present differential activation of the receptor following exposure to TLR ligands. In this regard, some molecules which have been shown to act as antagonists of TLR4 signalling in humans may act as agonists in mice or vice versa. As mice are typically used as a representative model for human drug response, it is important to understand the comparability of this to real human responses.

A set of experiments were carried out to assess the consistency of response between human and mouse TLR4 for FP7, RAW264 cells were used to model the mouse response. Results suggest that FP7 still functioned antagonistically in mouse cells (C. Palmer et al., 2018). Following this initial investigation, a proinflammatory protein antibody array was used to give an overview of the comparability of human and murine response in FP12 (Table S2 – Appendix IV).

After overnight exposure to LPS, FP7 downregulated LPS induced IL-6 and IL-8 so the equivalent measurements were carried out via ELISA in murine macrophages. In this regard, IL-6 and KC were measured. KC is an analogue of IL-8 in mice. Further to this, activation of NF-κB and P38 were also carried out for comparison. These results implied some comparability in antagonistic effects between human and murine models, although some differences were observed suggesting that outcomes of studies in mice may directly represent FP7 efficacy in humans.

4.2.1. **FP7 has no effect on mouse RAW264 mouse macrophage cell viability**

MTT as used to assess potential toxic effects of FP7 on RAW264 cells in presence or absence of LPS. Assay results for RAW264 cells showed that FP7 did not affect significantly reduce cell viability at 0-10 µM concentrations (Figure 36). As such, FP7 was deemed to be suitable for use in the same range (0-10 µM) as used in THP-1 monocytes and macrophages.

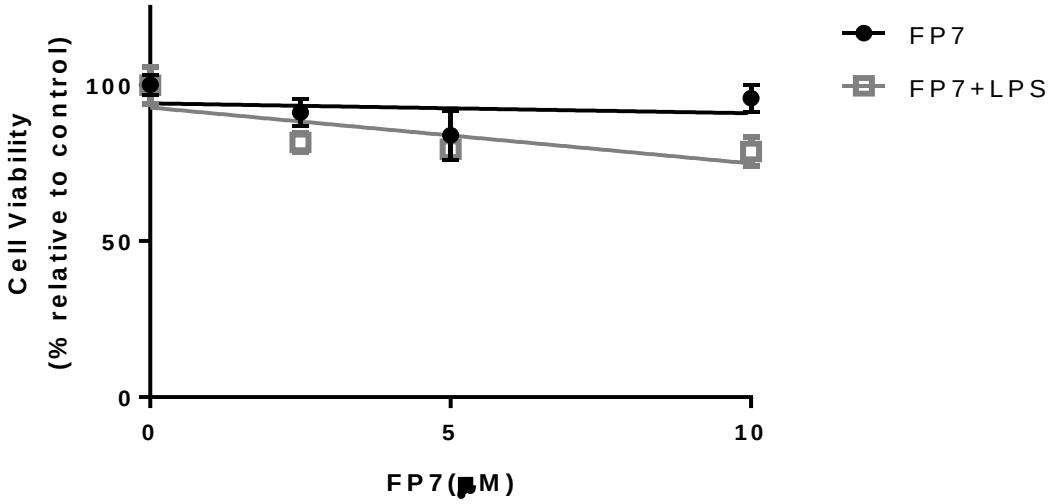

Figure 36.The effect of FP7 on RAW264 cell viability. Cells were treated with FP7 (0-20 µM) in presence (grey) and absence (black) of LPS (100ng/ml). Controls (0) were treated with vehicle only. Plates were incubated overnight (18 hours) prior to addition of MTT reagent. Results are shown as mean +/- SD of 6 repeats expressed as a percentage relative to the control set. (0 µM/no drug) +/- SD of three separate treatments. Statistical comparison between different groups was found to be non-statistically significant.

4.2.2. **FP7 negatively regulates the production of TLR4-dependent IL-6 but not KC in RAW264 mouse macrophages**

ELISA was used to investigate the effects of FP7 on TLR4 signalling in mouse macrophages. Measurements were made for the effect of FP7 on cytokine production in medium only for IL-6 and KC (Mouse Equivalent to IL-8) (Figure 37). FP7 was shown to significantly decrease levels of TLR4 induced IL-6 production, but not KC following stimulation upregulation by LPS (100ng/ml).

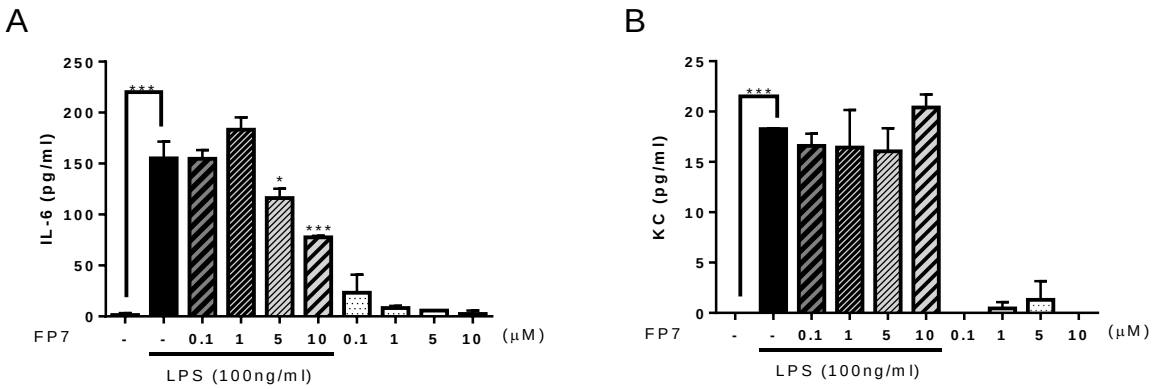

*Figure 37. The effect of FP7 on KC and IL-6 production in RAW264 macrophages. Cells were cultured in full-serum medium and treated with FP7(0.1, 1, 5 and 10 μM) for 1 hour prior to LPS exposure (100ng/ml). Untreated control (-) is with vehicle only . Cells were incubated overnight (18hours) before collection of culture medium. KC and IL-6 production in culture medium was measured using ELISA (Ray-Biotech, USA). Results show mean data ±SD of three independent experiments. Statistically significant results are indicated as *** P<0.001 ** P<0.01 *P<0.05 for control vs LPS and LPS vs LPS+FP7 treated samples.*

4.2.3. **FP7 differentially modulates activation of TLR4 signalling pathways in RAW264 mouse macrophages**

Intracellular signalling following TLR4 activation is facilitated by a series of protein-protein interactions which result in activation of signalling mediators. To determine the mechanism by which activation is inhibited following FP7 exposure Western blot analysis was used. Investigation was carried out on cell lysates collected after 15 mins – 1 hour of ligand exposure.

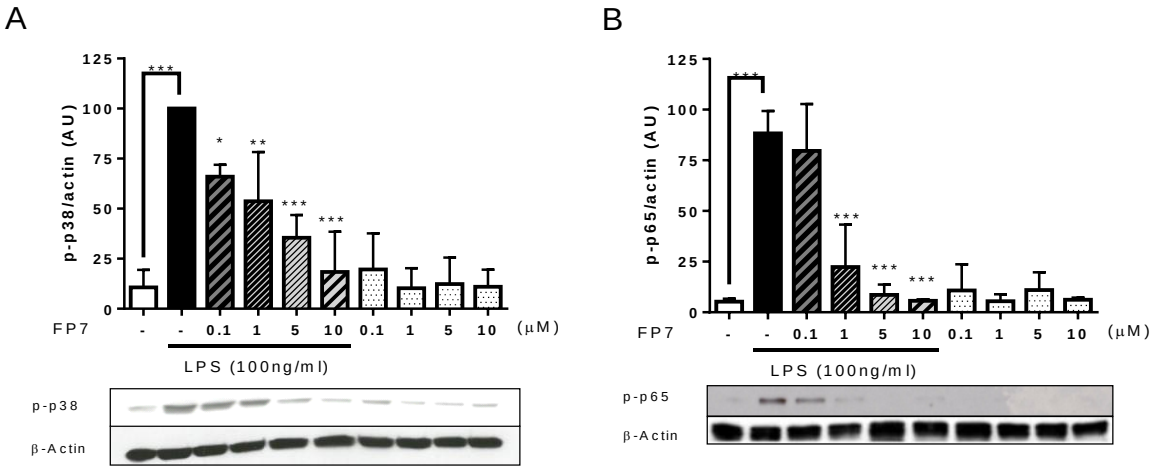

*Figure 38. The effect of FP7 on LPS induced P38 MAPK (A) and NF-κB (B) activation in RAW264 macrophages. Raw264 cells (mouse macrophages) were pre-treated with FP7 (0.1, 1, 5 and 10 μM) for 1 hour, prior to exposure to LPS (100 ng/ml). Untreated control (-) is with vehicle only . Cell lysates were collected after 15 minutes and processed. 20 μg protein was used for Western blot analysis of p-p65/NF- κB or P38 MAPK phosphorylation. Samples are normalised to β-Actin and displayed as mean +/- SD. Statistically significant results are indicated as *** P<0.001 ** P<0.01 *P<0.05 for control vs LPS and LPS vs LPS+FP7 treated samples.*

Analysis after 15 minutes of LPS treatment FP7 was shown to significantly reduce P38 MAPK phosphorylation in a dose-dependent manner (Figure 38A). A significant reduction was seen from 1 µM treatment of FP7 and 10 µM treatment resulted in a reduction to levels comparable to the control.

Further to this, p65/NF-κB phosphorylation was supressed in a similar manner to following treatment. A significant reduction was seen in LPS induced phosphorylation at 1 µM, reaching levels comparable to the control at 5 µM and 10 µM (Figure 38B).

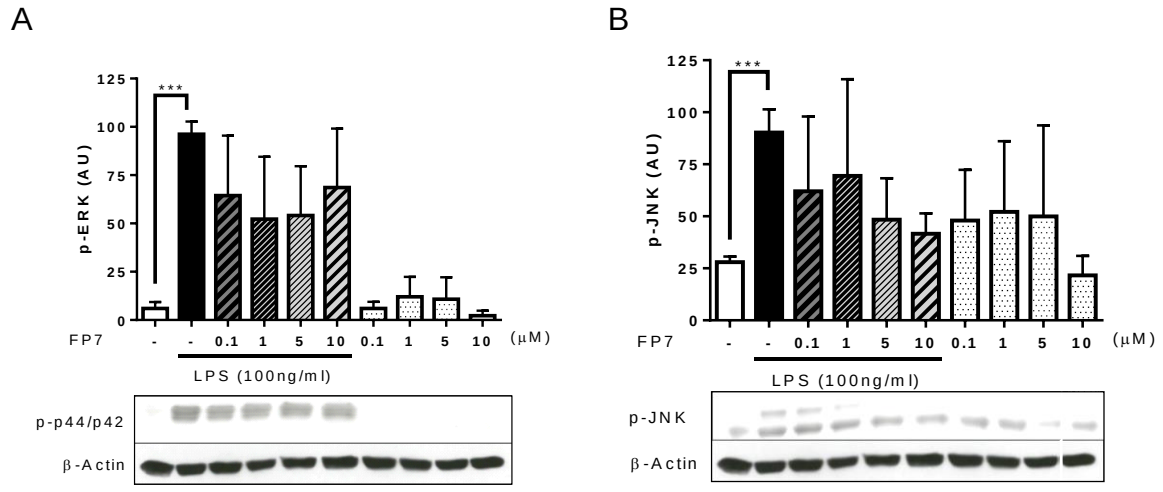

*Figure 39. The effect of FP7 on LPS induced ERK (A) and JNK (B) activation in RAW264 macrophages. Raw264 cells (mouse macrophages) were pre-treated with FP7 (0.1, 1, 5 and 10 µM) for 1 hour, prior to exposure to LPS (100ng/ml). Untreated control (-) is with vehicle only . Cell lysates were collected after 60 minutes and processed. 20 µg protein was used for Western blot analysis of JNK and ERK phosphorylation using the same membrane. Each treatment is displayed as mean +/- SD. Statistically significant results are indicated as *** P<0.001 ** P<0.01 *P<0.05 for control vs LPS and LPS vs LPS+FP7 treated samples.*

FP7 was shown to negatively regulate phosphorylation of NF-κB and P38 MAPK in RAW264 cells, but not ERK or JNK (Figure 39). LPS treatment resulted in a significant increase in ERK phosphorylation, but no significant decrease was seen in FP7 treated samples. No significant effect was seen in SAPK following LPS stimulation. These results demonstrated that FP7 can differentially affect the activation of signalling mediators in mouse LPS/TLR4 signalling.

4.2.4. **Summary**

Cross species comparison data suggests some antagonism across human and mouse TLR4. Results of RAW264 cell studies demonstrate that FP7 can reduce TLR4 induced proinflammatory signalling in a murine cell line. This was shown to be through inhibition of TLR4-dependent NF-κB and P38 MAPK pathways, as seen in THP-1 cells, but not ERK or JNK pathways. This suggests that FP7 can function as an antagonist to both human and mouse TLR4.

4.3. FP synthetic glycolipids can modulate TRIF-dependent TLR4 signalling in THP-1 macrophages

FP7 and FP12 have previously been shown to downregulate TLR4 associated signalling, here (4.1.1-3.1.9) and elsewhere (Cighetti et al., 2014; Facchini et al., 2018). However, previous data is specific to the MyD88-dependent pathways activated in response to LPS and less is known about the interactions of FP compounds on TRIF dependent signalling. It was therefore also necessary to further evaluate effects on MyD88-independent (TRIF-dependent) signalling.

An initial set of optimisation experiments were carried out to determine the correct method of activating TRIF dependent signalling for further experiments. The initial form of LPS used in previous MyD88 dependent signalling did not appear to activate TRIF-dependent signalling in THP-1 macrophages. It was therefore necessary to find an alternative method to activate TRIF-dependent signalling in order to test the ability of FP compounds to influence this pathway.

4.3.1. **LPS variants differentially activate TRIF-dependent signalling in THP-1 macrophages**

An issue was encountered initially with measurement of TRIF-dependent signalling as LPS from *S.minnesota* R595 did not appear to activate associated markers to relevant levels. In other studies, *S.minnesota* had been shown to be more appropriate for TRIF-dependent pathway activation. However, the *S.minnesota* (Re) LPS used in previous FP7 and FP12 MyD88-dependent experiments was not sufficient to produce consistent response for TRIF-dependent activation. Different forms of LPS were investigated to determine if these could activate TRIF-dependent signalling in THP-1 macrophages. A series of from *S.minnesota* and *E.coli* LPS variants were provided by Innaxon for testing on TRIF-dependent signalling pathways (See Appendix II).

LPS can exist in different forms, referred to as smooth (S) or rough (R) forms. S-form LPS, the wild-type form of LPS is made up of a full O-antigen and polysaccharide chain region, while R forms lack the O-antigen region and may have varying levels of reduction in number of sugar residues from the polysaccharide chain (Huber et al., 2006). These modifications may arise as a mechanism to circumvent immune detection and thus result in different degrees of recognition by TLR4.

To determine which, if any, of these ligands could elicit TRIF-dependent response in THP-1 macrophages, a Western blot analysis was carried out to investigate STAT-1 phosphorylation following ligand exposure. 3 variants from *S.minnesota*; one smooth and two rough (S, Re and Ra) were investigated alongside *E.coli* (Ra) and Lipid-A variants from both E-coli and *S.minnesota*.

STAT-1 phosphorylation was measured at 0-6 hours in three LPS variants. was carried out to determine the best point for collection. For this, three of the LPS variants were selected; *S.minnesota* (S), *E.coli* (Ra) and *S.minnesota* (Re). STAT-1 activation was shown to be elevated at 1.5-6 hours peaking at 2.5-3 hours for both *E.coli* (Ra) and *S.minnesota* (S) (Figure 40). While *S.minnesota* (Re) showed some increase in STAT-1 activity at 3 hours and was substantially lower compared to the other two LPS tested.

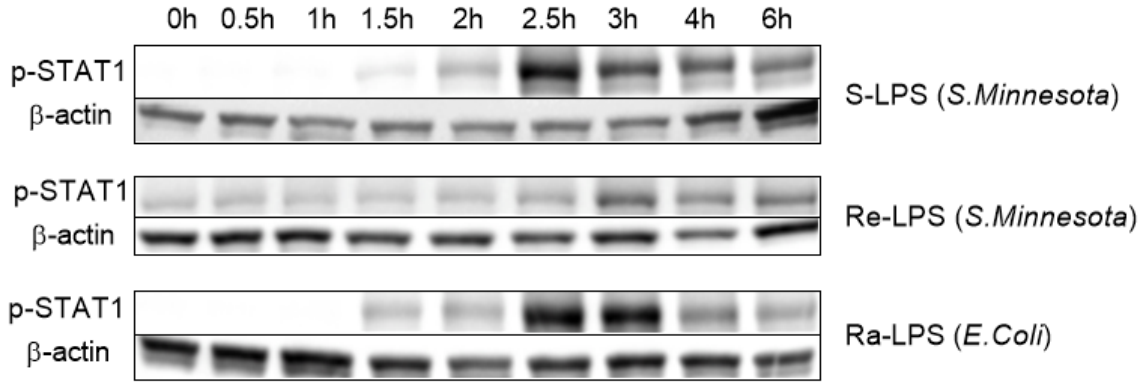

Figure 40. Timing of LPS induced TLR4 signalling by three different LPS. THP-1 macrophages were exposed to LPS (100ng/ml) or three different sources (S.minnesota S-form, S.minnesota R-form and E.coli R-form). cell lysates were then collected at 0–6-hour time points and levels of STAT-1 phosphorylation measured via Western blot. Results were from a single experiment.

LPS and Lipid A were used and compared to IFNγ which was known to activate STAT-1. In response to all three forms of *S.minnesota* LPS, as well as *E.coli* LPS, STAT-1 phosphorylation was elevated to a greater or lesser extent (Figure 41). Of these, the *S.minnesota* S-form LPS was found to be most effective at producing a response. In contrast, Lipid A alone does not appear to be enough to induce STAT-1 activation after 2.5 hours. This reiterates the assertion that *S.minnesota* (S) and *E.coli* (Re) are more appropriate for TRIF-dependent studies while suggesting both are also suitable for MyD88-dependent experiments.

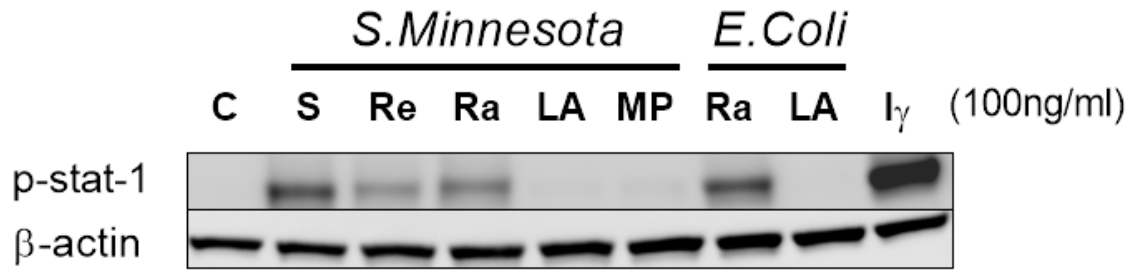

Figure 41. LPS variants differentially activate STAT-1. THP-1 macrophages were treated with LPS (100ng/ml) or Lipid A. Cell lysates were collected and analysed after 3 hours via Western blot. Data is from only 1 set. S = S.minnesota (S-form), Re = S.minnesota (Re-form), Ra = S.minnesota (Ra), LA = S.minnesota (Lipid A), MP = S.minnesota (MPLA), Ra = E.coli (Ra), LA = E.coli (Lipid A), Iγ = IFNγ. Results are from a single experiment

Preliminary data suggests LPS activation of STAT-1 peaks at 2.5-3 hours. To confirm this timing, a further set of timing experiments were carried out. *S.minnesota* S-form LPS was used to confirm provisional data for STAT-1 and additionally investigate TRIF-dependent TBK1 phosphorylation timing.

Following exposure to LPS, levels of both p-STAT-1 (Figure 42A) p-TBK1 (Figure 42B) are increased. p-TBK1 results suggest an early activation time at 30 minutes following LPS treatment, whereas p-STAT-1 remains below a significant threshold until a 2.5-3-hour peak. From this, 30 minutes was selected for further experiments involving TBK1 activity and 2.5-3 hours for STAT-1. The later activation of STAT-1 following early TBK-1 suggested a possible autocrine effect, following interferon release.

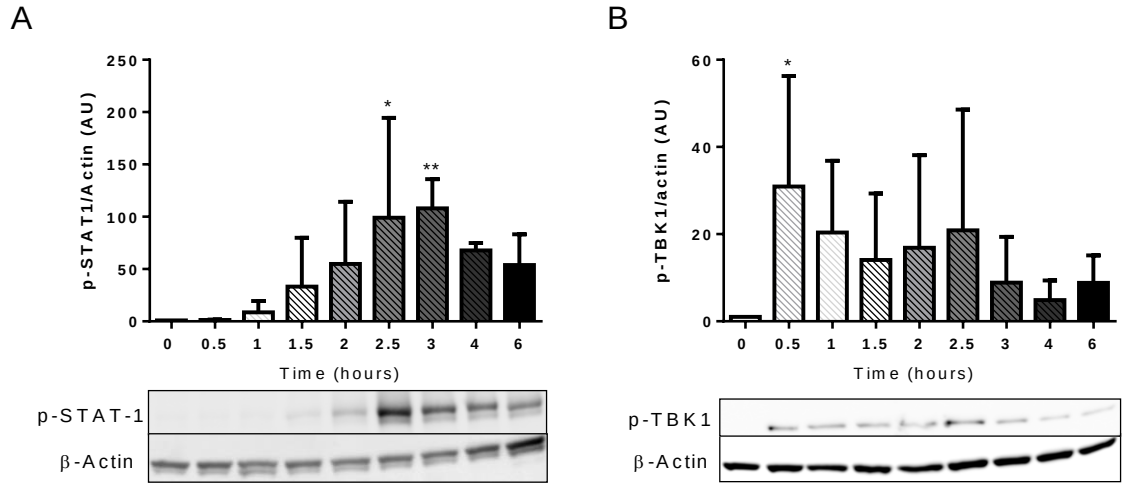

*Figure 42. Timing of LPS induced TLR4/TRIF-dependent signalling by S.minnesota (S-Form) LPS. THP-1 macrophages were exposed to LPS (100ng/ml) for 0-6 hours. Cell lysates were collected, and p-STAT or p-TBK1 was measured via Western blot. Results were expressed as mean +/- standard deviation from three separate treatments. Significant results are shown as * = p<0.05, ** = p<0.01 *** = p<0.001.*

To determine the best time for measurement of IP10 and IFNβ, three timepoints were selected for investigation; 2 hours, 6 hours, and 24 hours. Results from ELISA suggest, as with STAT-1 and TBK1, that S and Ra-Forms of LPS were more effective in stimulating production of these proteins. Again, Re, Lipid A and MPLA showed no significant effect. LPS (S) was most effective in producing a response.

Interestingly only LPS (S) produced a significant increase in IFNβ following exposure (Figure 43 A). This consisted of an initial increase in IFNβ production at two to six hours ($P<0.05$). However, levels of IFNβ fall later and are not significantly elevated after 24 hours. 2-hour collection time was selected for IFNβ measurement. This early release of IFNβ prior to the 3-hour peak in STAT-1 phosphorylation prompted investigation into the role of IFNAR signalling.

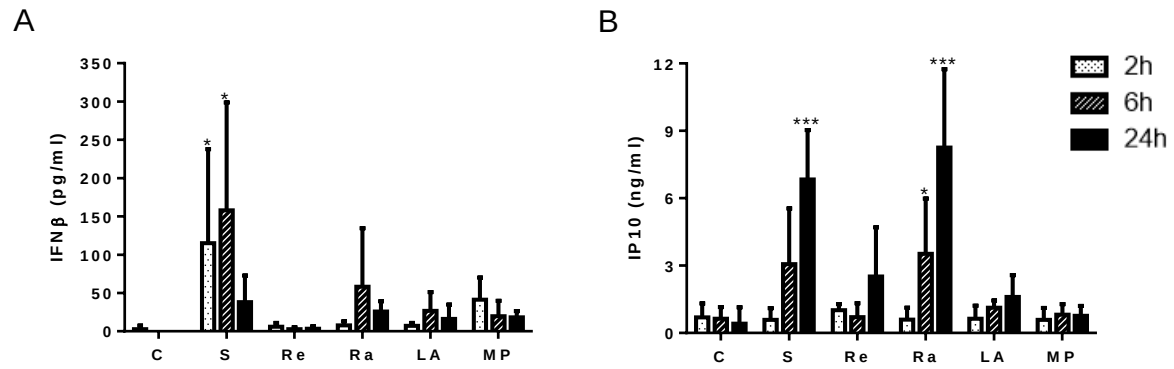

*Figure 43. Timing of IFNb and IP10 production following LPS stimulation in THP-1 macrophages. THP-1 macrophages were exposed to LPS (100ng/ml) of three different sources; S.minnesota (S), S.minnesota (Re), E.coli (Ra), S.Minnesota lipid A (LA) and MPLA (MP). C = Untreated cells. Culture medium supernatants were then collected at 2-, 6- and 24-hour time points after exposure and levels of (A) IFNβ or (B) IP10 measured via ELISA. Results were expressed as mean +/- standard deviation from three separate treatments. Significant results are shown as * = p<0.05, ** = p<0.01 *** = p<0.001.*

IP10 production was a considerably later event than IFNβ. Following LPS stimulation, IP10 was released within 6 hours of LPS exposure and increases up to 24 hours (Figure 43 B). While IFNβ production was more significantly affected by *S.Minnesota S*-form LPS, IP-10 was also significantly elevated following *E.Coli* Ra-form LPS. 24-hour collection was selected for measurement of IP10.

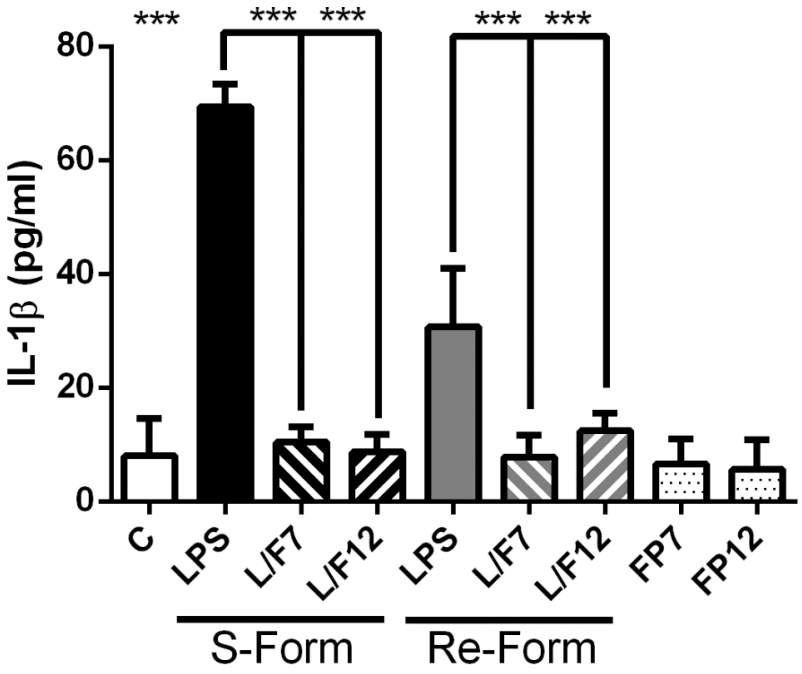

*Figure 44. FP7 and FP12 display similar antagonistic effects on THP-1 macrophages stimulated by S-form S. Minnesota LPS as previously used Re-form S.minnesota LPS. Thp-1 macrophages were treated with FP7 or FP12 (10µM) for 1 hour prior to LPS exposure. Control (C) is with vehicle only. Medium was collected after 18 hours and IL-1ɓ production was measured via ELISA. Results were expressed as mean +/- standard deviation from three separate treatments. Significant results are shown as * = p<0.05, ** = p<0.01 *** = p<0.001. C=Control L=LPS, F7=FP7, F12=FP12.*

Endpoint production of IFNs is associated with TLR4/TRIF signalling. An initial investigation of the effect of LPS chemotypes on IFNβ production following exposure was carried out in addition. IFNβ was seen to be present in culture medium at as soon as 2 hours following exposure to LPS. *S.minnesota* (S) and *E.coli* (Ra) are both capable of inducing IFNβ production, but not *S.minnesota* (Ra). This elevation in IFNβ following activation subsequently decreases over time and there are

substantially lower levels at 24 hours as compared to 2 hour and 6-hour time points. Thus, earlier collection of medium, between two and 6 hours, medium is required for measurement of IFNβ.

To verify that FP7 and FP12 function as antagonists in response to both S and previously used Re-Forms of LPS used in this study, the ability of FP7 and FP12 to downregulate LPS induced IL-1β production was measured via ELISA. Previously used Re-form *S.minnesota* LPS and S-form *S.minnesota* LPS were compared and both produced a significant increase in IL-1β after overnight exposures. The S-form LPS produced a greater increase in IL-1β than previously seen with Re-form LPS (Figure 44). FP7 and FP12 showed similar effects to that previously seen and were capable of downregulating IL-1β production by both forms of LPS to a comparable level to the control.

4.3.2. **FP7 and FP12 negatively regulate TRIF-dependent TBK1 phosphorylation in THP-1 macrophages**

TBK1 and STAT-1 are associated with TLR4/TRIF-dependent signalling. Following upregulation in response to LPS, both FP7 and FP12 significantly reduce p-TBK1 (Figure 45), to a level comparable to the controls. Results suggest FP7 and F12 can negatively regulate TRIF-dependent TBK1 activation and thereby TRIF-dependent signalling.

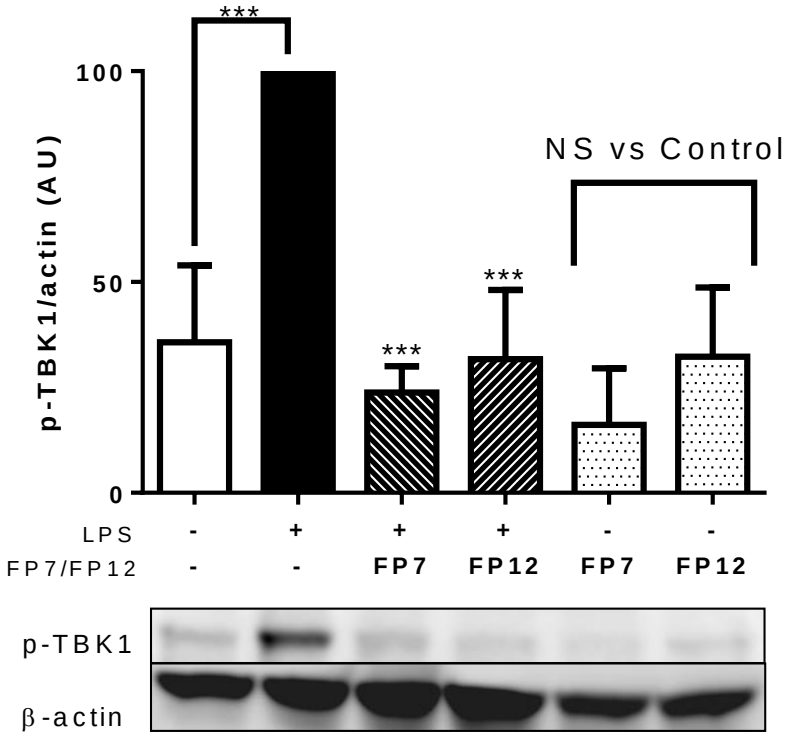

*Figure 45. FP7 and FP12 negatively regulate LPS induced TBK1 phosphorylation. THP-1 macrophages were treated with FP7 or FP12 (10μM) for 1 hour prior to LPS (100ng/ml) exposure. Untreated control (-) is with vehicle only . Cell lysates were collected and analysed after 30 minutes via Western blot. Results are expressed as mean +/- standard deviation from three separate treatments. Significant results are shown as * = p<0.05, ** = p<0.01 *** = p<0.001.*

4.3.3. **FP7 and FP12 negatively regulate LPS-induced IP10 and IFNβ production in THP-1 macrophages**

TRIF-dependent signalling is characterised by internalisation of the receptor complex and downstream production of IFNs. IFNβ and IP10 have previously been associated with TRIF-dependent signalling. As such, measurement of these were selected for measurement of endpoint TRIF-dependent activation. To determine the effect of FP7 and FP12 on IFNβ production, culture supernatants were analysed via ELISA.

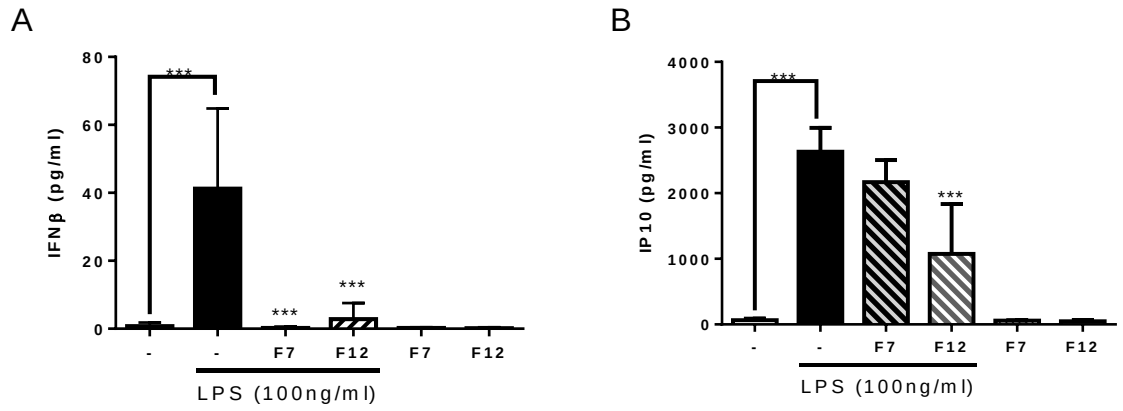

*Figure 46. FP7 and FP12 negatively regular LPS induced IFNβ and IP10. THP-1 macrophages were treated with FP7 or FP12 for 1 hour prior to LPS (100ng/ml) exposure. Untreated control (-) is with vehicle only . Culture supernatants were then collected at 2.5 hours or 24 hours and levels of IFNβ and IP10 measured via ELISA. Results are expressed as mean +/- standard deviation from three separate treatments. Significant results were shown as * = p<0.05, ** = p<0.01 *** = p<0.001.*

Following exposure to *S.minnesota* (S) LPS, IFNβ levels were shown to increase significantly. FP7 and FP12 were both capable of downregulating this LPS induced IFNβ production to a level comparable to the control (Figure 46A). Both FP7 and FP12 were less successful in affecting IP10 production in

response to LPS (Figure 46B). FP7 did not have a strong enough effect to cause a significant decrease in IP10, while FP12 caused a small reduction in production, but did not reduce to control levels.

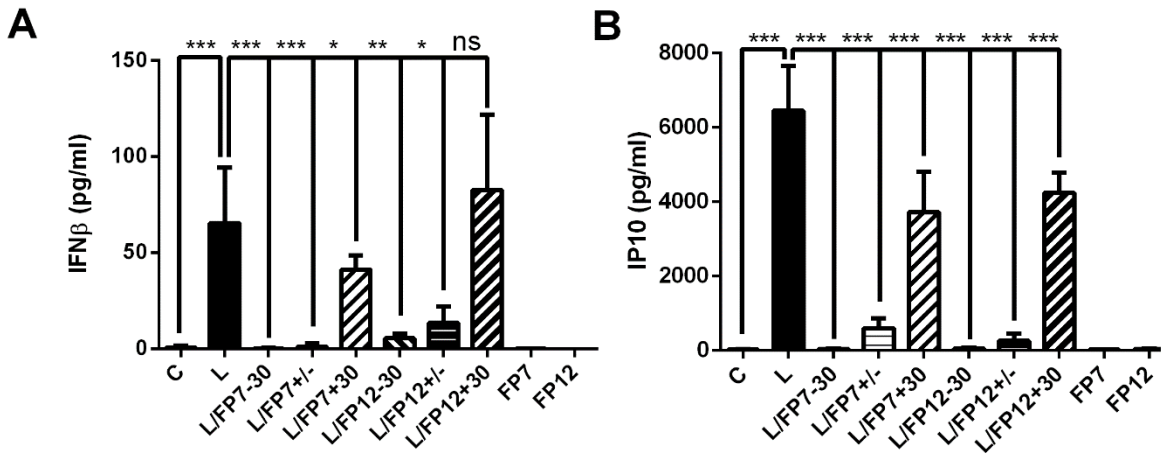

*Figure 47. FP7 (A) and FP12 (B) downregulate IFNß and IP10 production in prior and simultaneous treatments. THP-1 macrophages were pre-treated with FP7 or FP12 30 minutes before, at the same time as, and 30 minutes after LPS (100ng/ml) exposure. Control (C) is with vehicle only. Medium was collected at 2.5 hours and 18 hours for IFNß and IP10 respectively and measured via ELISA. Results were expressed as mean +/- standard deviation from three separate treatments. Significant results are shown as * = p<0.05, ** = p<0.01 *** = p<0.001. C=Control L=LPS, F7=FP7, F12=FP12.*

Previous evidence suggested FP7 and FP12 can down regulate MyD88-dependent cytokine production in THP-1 macrophages when applied before, simultaneously, and even post LPS exposure. Following the same experimental approach, the results showed the ability of FP7 to reduce TLR4-dependent IFNβ (Figure 47) or IP10 (Figure 47) irrespective of time of the administration of LPS. While FP12 significantly reduced IFNβ production in prior and simultaneous treatments, post LPS treatment with FP12 did not affect IFNβ production (Figure 47A). Similarly, to

FP7, FP12 negatively regulated IP10 production, though to a lesser extent in post-factum treatments as compared to prior and simultaneous treatment (Figure 47B). TBK-1 has been shown to phosphorylate within 30 minutes of LPS exposure (Figure 42), suggesting that TRIF-dependent signalling is already underway by this time. In addition, endocytosis of TLR4, which is important for TRIF-dependent signalling, occurs within 30 minutes of receptor activation (Kagan *et al.*, 2008), removing the receptor from the cell surface and potentially preventing FP7 or FP12 from interacting with the receptor after this time. This data suggests that FP7 and FP12 are negative modulators of type I interferons production, however FP12 was found less effective on TRIF-dependent TLR4 signalling post factum.

4.3.4. **FP7 and FP12 negatively regulate IFNAR signalling via TLR4**

A set of optimisation experiments were carried out to determine the most appropriate time and concentration for experiments involving IFNβ and an IFNAR blocking antibody. Results indicate that IFNβ is enough to induce STAT-1 phosphorylation alone, most likely via IFNAR (Figure 48A). 1ng/mL was sufficient to upregulate STAT-1 phosphorylation and was selected to activate IFNAR signalling for further experiments. This interaction with the receptor is particularly quick, with STAT-1 phosphorylation occurring as soon as 5 minutes after exposure and peaking around 30-45 minutes before tapering off (Figure 48B). 30 minutes was selected for future experiments. The IFNAR blocking antibody could prevent IFNβ/IFNAR induced STAT-1 phosphorylation with a minimum of 1µg/mL (Figure 48).

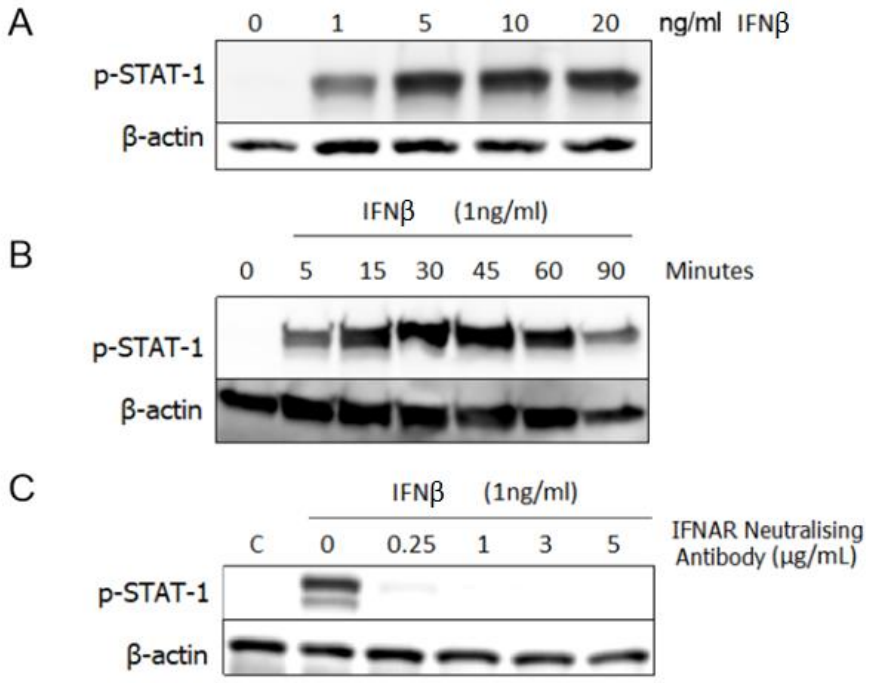

Figure 48. Optimisation of IFNβ and IFNAR blocking antibody concentration and timings. THP-1 macrophages were either (A) Exposed to 0-20 ng/mL IFNβ for 30 minutes. (B) Exposed to 1ng/mL IFNβ over a time course of 90 minutes. (C) Pre-treated with IFNAR blocking antibody for 1 hour prior to 1ng/mL IFNβ exposure. STAT-1 phosphorylation was measured via Western blot. Results are from a single experiment.

Optimisation data suggested that IFNβ is produced via TRIF-dependent signalling can activate IFNAR which in turn lead to the downstream STAT-1 phosphorylation. To confirm that the effect of FP7 and FP12 is TLR4-dependent and not associated with interaction with IFNAR, the effect of FP7 and FP12 in an IFNβ induced event was investigated in comparison to LPS.

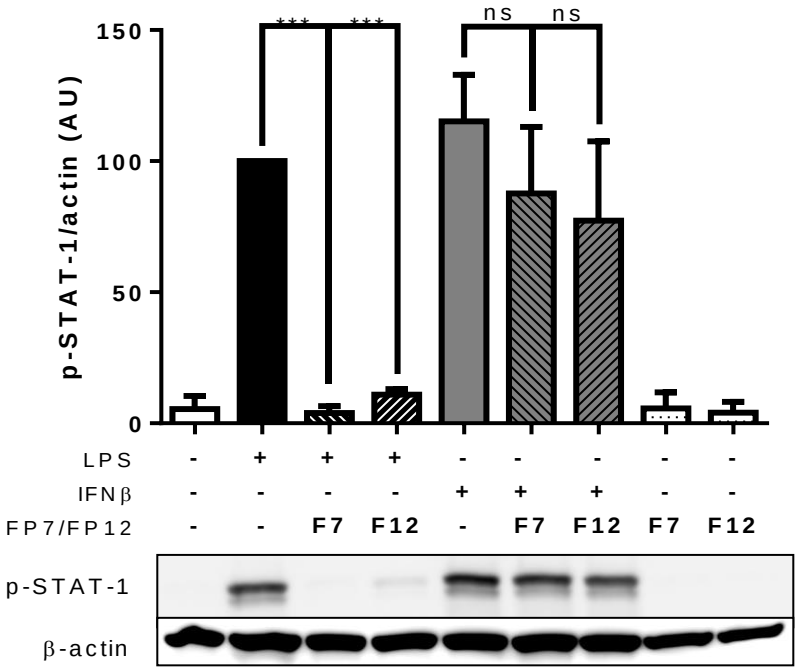

*Figure 49. FP7 and FP12 negatively regulated LPS induced STAT-1 phosphorylation. THP-1 macrophages were treated with FP7 or FP12 (10μM) for 1 hour prior to IFNβ (1ng/ml) or LPS (100ng/ml). Untreated control (-) is with vehicle only . Cell lysates were collected and analysed after 2.5 hours (LPS) and 30 min (IFNβ) via Western blot. Results are expressed as mean +/- standard deviation from three separate treatments. Significant results were shown as * = p<0.05, ** = p<0.01 *** = p<0.001.*

IFNβ can lead to activation of STAT-1 following via IFNAR signalling. To determine if STAT-1 activation following LPS exposure is the result of an autocrine effect following production IFNβ, a

blocking antibody was used to prevent IFNAR activation. STAT-1 is activated at 2.5 hours in response to LPS, but peaks at 30 minutes following IFNβ exposure via IFNAR. To compare these, exposure of IFNβ to THP-1 macrophages was initiated 2 hours after LPS and antagonists in comparison with LPS induced IFNβ. The data shows FP7 and FP12 produce a significant reduction in p-STAT-1 following LPS exposure, but not in response to IFNβ (Figure 50).

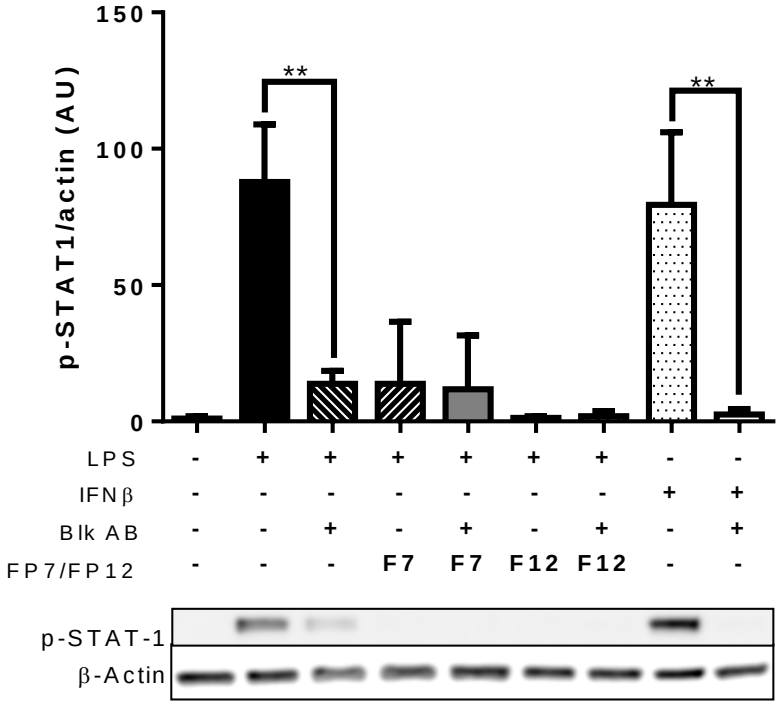

*Figure 50. FP7 and FP12 negatively regulated LPS induced STAT-1 phosphorylation. THP-1 macrophages were treated with FP7 or FP12 (10μM) for 1 hour prior to LPS (100ng/ml) alone in the presence or absence of IFNAR blocking antibody (Blk AB). IFNβ (1ng/ml) was used as a positive control for activation of IFNAR. Untreated control (-) is with vehicle only . Cell lysates were collected and analysed after 2.5 hours (LPS) and 30 min (IFNβ) via Western blot. Results are expressed as mean +/- standard deviation from three separate treatments. Significant results were shown as * = p<0.05, ** = p<0.01 *** = p<0.001.*

Following LPS or direct IFNβ stimulation, a significant increase is seen in p-STAT-1. This increase can be countered by blocking IFNAR in either case, suggesting TLR4 induced STAT-1 is dependent on IFNAR activation and as such is likely to be dependent on IFNβ (Figure 50). Both FP7 and FP12 can significantly reduce TLR4-dependent p-STAT-1 following LPS exposure. However, these did not affect IFNβ induced IFNAR signalling in THP-1 cells. STAT-1 phosphorylation following LPS exposure is the result of IFNAR activation, most likely by IFNβ. Subsequent downregulation by FP7 and FP12 is an indirect effect via inhibition of TLR4/TRIF-dependent signalling.

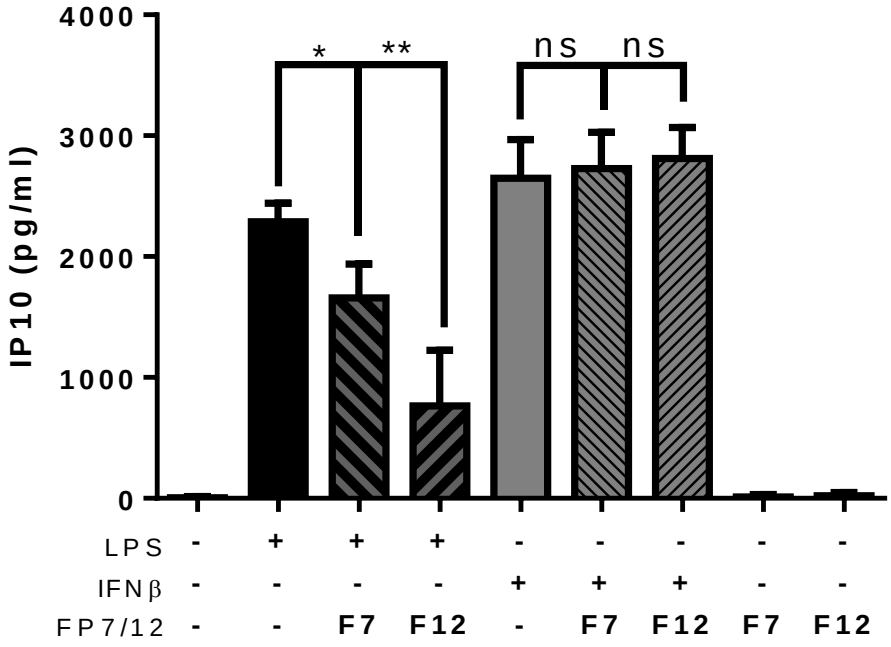

*Figure 51. FP7 and FP12 negatively regulate LPS induced IP10 production. THP-1 macrophages were treated with FP7 or FP12 (10μM) for 1 hour prior to IFNβ (1ng/ml) or LPS (100ng/ml). Untreated control (-) is with vehicle only . Culture medium was collected and analysed after Overnight incubation and analysed via ELISA. Results were expressed as mean +/- standard deviation from three separate treatments. Significant results are shown as * = p<0.05, ** = p<0.01 *** = p<0.001.*

Like STAT-1, IP10 was also shown to have an association with IFNAR signalling and can be induced by IFNβ (Figure 51) which FP7 and FP12 had no effect on. While blocking IFNAR appears to reduce levels of IP10, it is only a partial effect in response to LPS or IFNβ (Figure 52). FP7 and FP12 appear to be more effective when combined with the IFNAR blocking antibody. This suggests alternate pathways may be involved in IP10 regulation in response to LPS and IFNβ.TLR4 agonists FP11 and FP18 upregulate STAT-1 but not TBK1 phosphorylation.

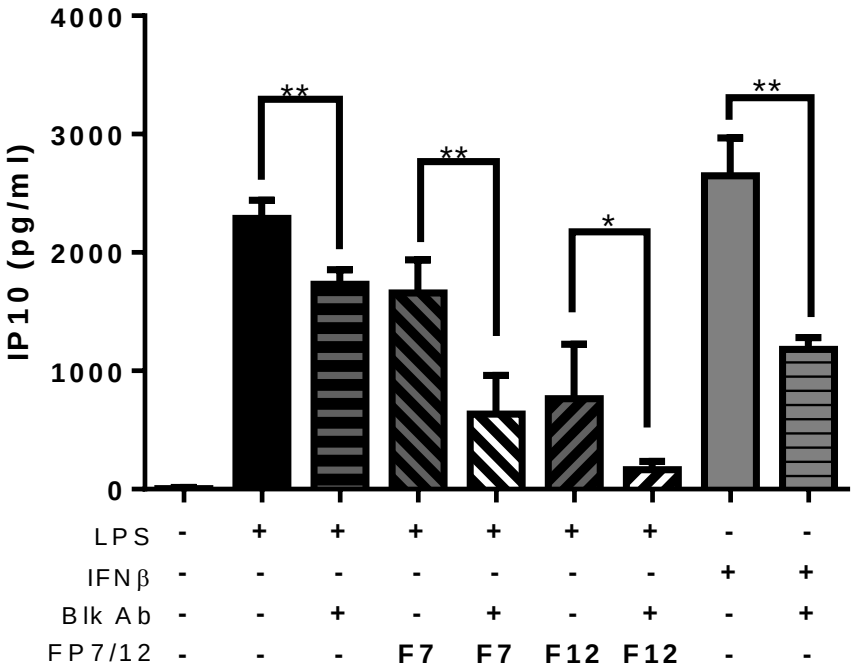

*Figure 52. FP7 and FP12 negatively regulated LPS induced STAT-1 phosphorylation. THP-1 macrophages were treated with FP7 or FP12 (10μM) for 1 hour prior to LPS (100ng/ml) in the presence or absence of IFNAR blocking antibody (Blk AB). IFNβ (1ng/ml) was used as a positive control for activation of IFNAR. Untreated control (-) is with vehicle only . Cell lysates were collected and analysed after overnight incubation via ELISA. Results are expressed as mean +/- standard deviation from three separate treatments. Significant results were shown as * = p<0.05, ** = p<0.01 *** = p<0.001.*

The effect of FP11 and FP18 on TRIF-dependent signalling is unknown. To understand the effect of FP11 and F18 on TRIF-dependent signalling, Western blot analysis was used to assess TBK1 and STAT-1 phosphorylation in relation to FP11, FP18, LPS and MPLA. Both FP11 and FP18 were capable of modulating activity of some, but not all, proteins involved in TRIF-dependent signalling in THP-1 macrophages.

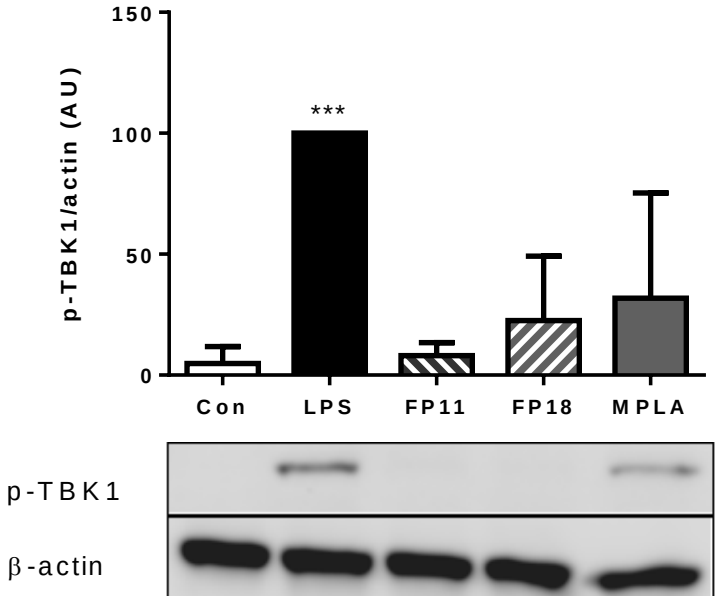

*Figure 53. FP11 and FP18 have no effect on TBK1 phosphorylation. THP-1 macrophages were treated with FP11 (10μM), FP18 (10μM) LPS or MPLA (100ng/ml). Untreated control (Con) is with vehicle only. Cell lysates were collected and analysed after 30 minutes via Western blot. Results are expressed as mean +/- standard deviation from three separate treatments. Significant results are shown as * = p<0.05, ** = p<0.01 *** = p<0.001.*

TRIF-dependent activation of TBK was measured at 30 minutes post stimulation. However, FP11 and FP18 had no effect of TBK1 phosphorylation after 30 minutes (Figure 53). While both FP18 and MPLA produced a small increase, this effect was inconsistent and did not produce a significant increase of TBK1 phosphorylation.

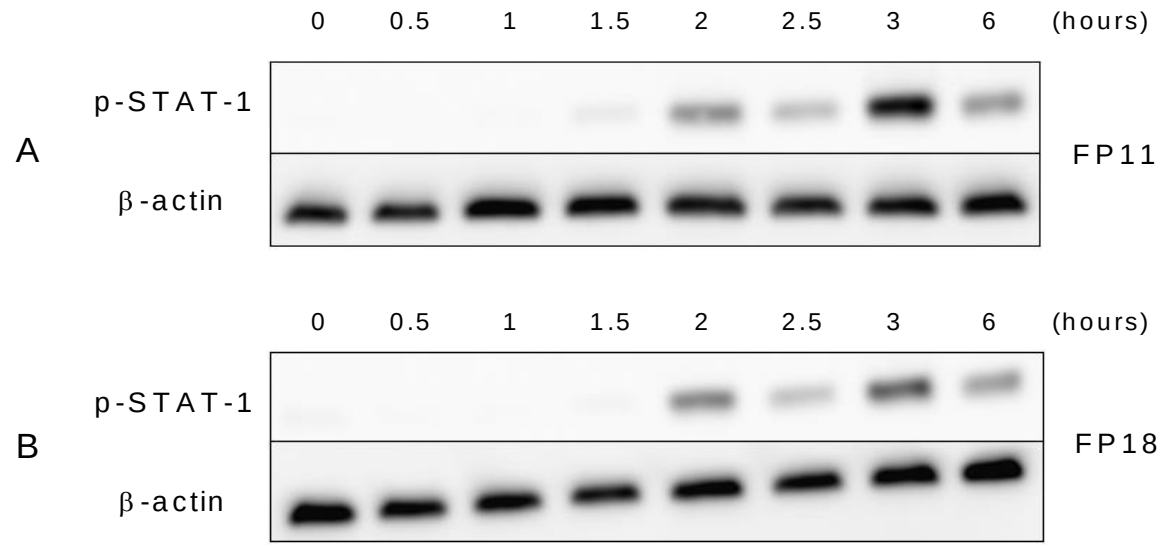

Figure 54. Timing of (A) FP11 and (B) FP18 induced STAT-1 phosphorylation. THP-1 macrophages were exposed to FP11 or FP18 (10μM). cell lysates were then collected at 0–6-hour time points and levels of STAT-1 phosphorylation measured via Western blot. Results are from a single experiment.

Despite negative results in relation to TBK1, both FP11 and FP18 were capable of inducing STAT-1 phosphorylation. A timing investigation was carried out to determine in FP11 and FP18 follow the same pattern of STAT-1 activation as LPS (Figure 54). This confirmed the correct collection time for measurement to be around 3 hours, though STAT-1 phosphorylation began as early as 2 hours after exposure to either FP11 or FP18. 2.5 hours is therefore suitable for comparison between LPS and FP11 or FP18.

Stimulation of THP-1 macrophages with LPS, FP11 or FP18 caused subsequent phosphorylation of STAT-1 (Figure 55). Similar to LPS, FP11 and FP18 positively regulated STAT-1 phosphorylation. THP-1 macrophages were treated with FP11 (10µM), FP18 (10µM) LPS or MPLA (100ng/ml). Cell lysates were collected and analysed after 2.5 hours via Western blot. However, MPLA had no significant effect on STAT-1 following stimulation. This corresponds to previous data from LPS variants where Lipid A and MPLA were found to be inadequate to activate TRIF-dependent signalling. This data confirms results from the initial timing experiments that FP11 and FP18 are consistently causing activation of STAT-1 despite not having greater impact than LPS on TRIF-dependent signalling.

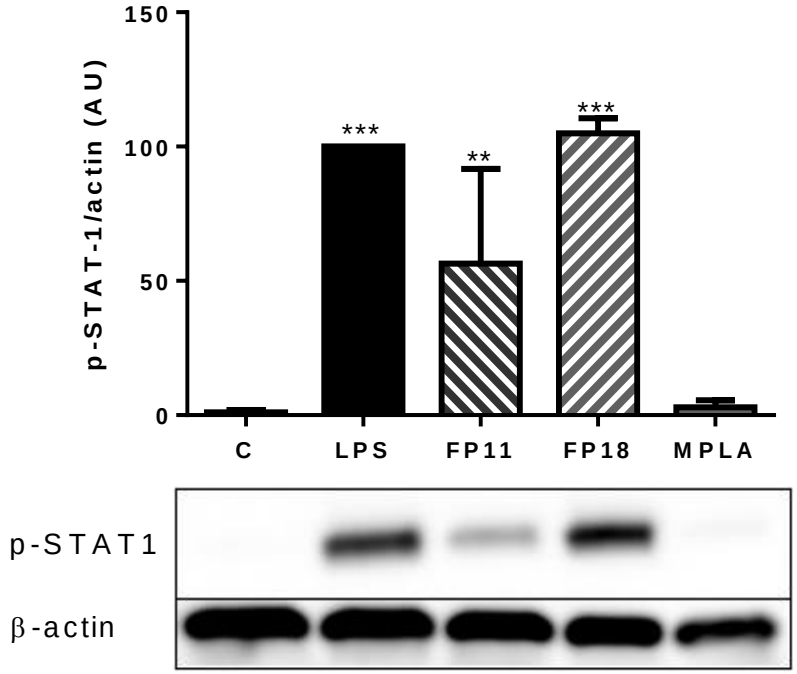

*Figure 55. FP11 and FP18 upregulate STAT-1 phosphorylation. THP-1 macrophages were treated with FP11 (10µM), FP18 (10µM) LPS or MPLA (100ng/ml). Untreated control is with vehicle only. Cell lysates were collected and analysed after 2.5 hours via Western blot. Results are expressed as mean +/- standard deviation from three separate treatments. Significant results are shown as * = p<0.05, ** = p<0.01 *** = p<0.001.*

TBK1 results were reflected again in IFNβ released by THP-1 macrophages. Both LPS and MPLA were shown to significantly elevate IFNβ production after 2.5 hours. However, there was no significant increase in production of IFNβ after 2.5 hour of treatment by either FP11 or FP18 (Figure 56). These results suggest that FP11 and FP18 do not affect TRIF-dependent signalling in similar manner as LPS or MPLA.

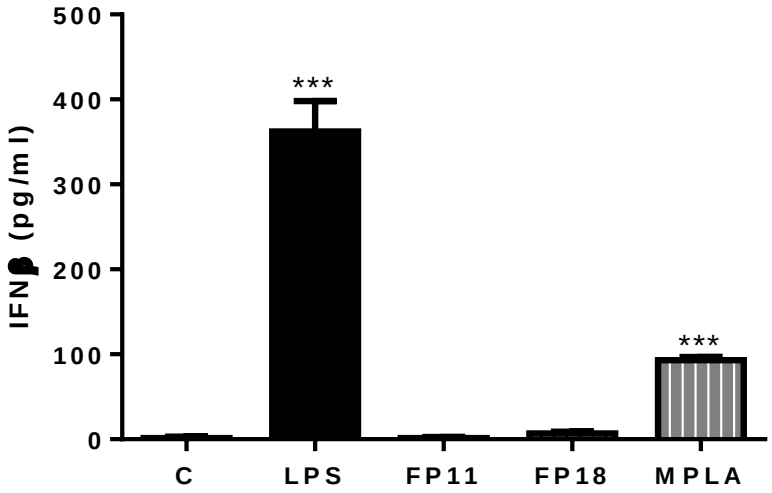

*Figure 56. FP11 and FP18 have no significant effect on IFNβ production. THP-1 macrophages were treated with FP11 (10μM), FP18 (10μM) LPS or MPLA (100ng/ml). Control (C) is with vehicle only. Culture medium was collected and analysed after 2.5 hours via ELISA. Results are expressed as mean +/- standard deviation from three separate treatments. Significant results are shown as * = p<0.05, ** = p<0.01 *** = p<0.001.*

Following array results, to validate the capture proteins of interest an ELISA was carried out for IP10 production. However, while IP10 appeared to be elevated in array data, this result was not reproducible via ELISA (Figure 57). This corresponds with IFNβ and TBK1 results, suggesting that FP11 and FP18 do not necessarily affect TRIF-dependent pathways in LPS-dependent manner.

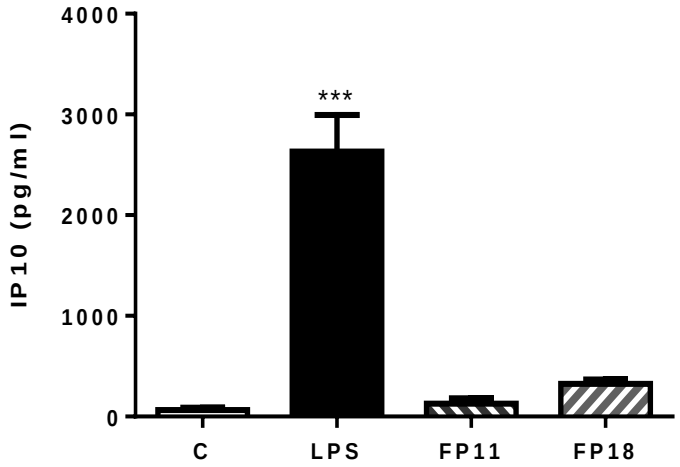

*Figure 57. FP11 and FP18 have no significant effect on IP10 production. THP-1 macrophages were treated with FP11 (10μM), FP18 (10μM) LPS or MPLA (100ng/ml). Control (C) is with vehicle only. Culture medium was collected and analysed after 18 hours via ELISA. Results are expressed as mean +/- standard deviation from three separate treatments. Significant results are shown as * = p<0.05, ** = p<0.01 *** = p<0.001.*

4.3.5. **IFNAR blocking antibody inhibits activation of FP11 and FP18 induced-STAT-1 phosphorylation**

Previous data suggested LPS caused STAT-1 activation via IFNAR (4.3.4). This effect was likely the result of IFNβ production through TRIF-dependent pathways which subsequently activates IFNAR. However, the mechanism by which FP11 and FP18 are capable of upregulating STAT-1 phosphorylation is unknown as TBK1 and IFNβ production were not significantly upregulated following stimulation with either agonist. To determine if IFNAR is involved in facilitating downstream phosphorylation of STAT-1, blocking experiments were carried out.

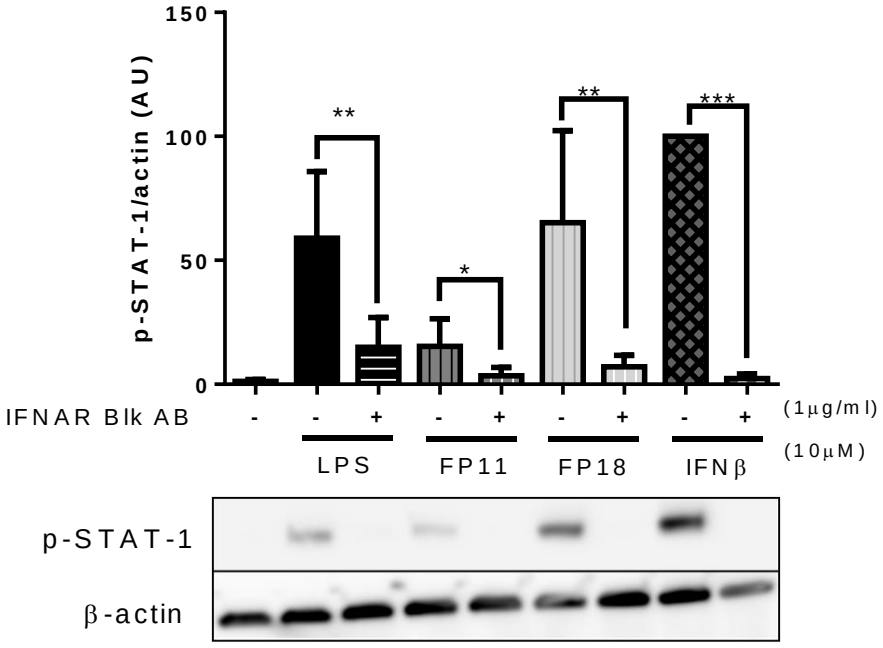

*Figure 58. FP11 and FP18 indirectly activate STAT-1 via IFNAR. THP-1 macrophages were treated with 1μg/mL IFNAR blocking antibody 1 hour prior to LPS (100ng/ml), IFNβ (1ng/ml), FP11 (10μM) or FP18 (10μM) treatment. Presence or absence of blocking antibody is indicated as + (present) or – (absent). Cell lysates were collected after 30 minutes for IFNβ, or 2.5 hours for all other treatments and analysed via Western blot. Results are expressed as mean +/- standard deviation from three separate treatments. Significant results are shown as * = p<0.05, ** = p<0.01 *** = p<0.001.*

The same experimental design was performed utilising IFNAR blocking antibody (as used for FP7 and FP12 experiments) was added to culture medium before FP11 and FP18 exposure. In response to IFNAR blocking, the increase of STAT-1 phosphorylation caused by FP11 and FP18 is reduced to a level comparable to the control (Figure 58). This appears to suggest that FP11 and FP18 cause STAT-1 activation via IFNAR without activating TRIF-dependent TBK1 signalling leading to production of IFNβ.

4.3.6. **Summary**

FP7 and FP12 negatively regulate TRIF-dependent signalling in response to LPS. FP7 and FP12 were shown to downregulate TBK1 and STAT-1 activation by LPS, as well as endpoint IFNβ and to a lesser extent IP10 production. Unlike MyD88-dependent signalling, post factum LPS treatment with FP7 or FP12 was enough to affect endpoint production of IFNβ. FP7 and FP12 have an indirect effect on IFNAR through downregulations of IFNβ which is produced through TLR4/TRIF-dependent signalling. These had no effect of IFNβ induced p-STAT-1 phosphorylation or IP10 production in absence of LPS.

FP11 and FP18 were not capable of producing a significant increase in TRIF-associated TBK1 activation and IP10 production. However, STAT-1 was activated in response to both molecules. STAT-1 was shown again to be activated via IFNAR in response to FP11 and FP18, despite absence of TBK1 activation beforehand. These results require further investigation to determine the specific interactions between FP11, FP18, receptors and signalling pathways.

5. Discussion

TLR an integral part of the innate immune response to exogenous threats such as bacteria and viruses. These recognise and respond to danger signals in the form of pathogen and damage associated molecular patterns. TLR mediated release of cytokines and chemokines in response to such threats provide a mechanism of protection against such invading pathogens. However, the protective effects conferred by release of such molecules can also be detrimental to host tissues and processes within the body. As such, many inflammatory based diseases have been associated with TLRs. The absence of TLR4 in mice has been shown to protect against a range of conditions and may protect against kidney disease (Souza *et al.*, 2015) and cardiovascular diseases (Michelsen *et al.*, 2004; Owens *et al.*, 2011). Alternatively, TLR based immunotherapeutic strategies have been postulated as novel means of treatment for various types of cancer (Huang, Xu and Peng, 2018). Thus, the potential to modulate TLR signalling pathways in either direction is currently of great interest for the development of novel therapeutics.

Manipulation of TLR4 mediated immune response has potential value for use in treatment of a range of conditions. Antagonism of TLR4 may be relevant to inflammatory based diseases. However, only two TLR4 antagonists, Eritoran and TAK242, have previously reached clinical trials for treatment of sepsis. Unfortunately, these were both discontinued in the late phase (Rice *et al.*, 2010; Salluh and Póvoa, 2010; Kalil *et al.*, 2011; Opal *et al.*, 2013). On the other hand, TLR4 agonists may be of value as adjuvants in cancer immunotherapies (Kuai *et al.*, 2018; Chou *et al.*, 2020). These may aide in activation antigen recognition response through T-cell proliferation and downregulation of immunosuppressive effects associated with cancers (Zhang *et al.*, 2011). MPLA has been shown to exhibit adjuvant activity which can be enhanced through synergistic effects in co-delivery with TLR9 stimulating CpG via lipid nanocarriers (Kuai *et al.*, 2018). As such the development of both novel agonists and antagonists is of clinical interest.

Recently a group headed by our collaborator professor Peri (University of Milano) have been developing a series of novel TLR4 antagonist, FP7 and IAXO compounds (Piazza *et al.*, 2009) These molecules are homologous to X and the Lipid A domain of LPS, known to be the active component recognised by TLR4. The formulation of these compounds allows for interaction with CD14 and the MD-2 co-receptor of TLR4 in a similar way to Lipid A. However, where most LPS Lipid A cause a conformational change in MD-2 which confers an antagonistic effect, mimetic molecules have potential to bind in an alternate agonistic confrontation with MD-2, as seen in complex with Lipid-IVA (Ohto *et al.*, 2012). FP7 and IAXO compounds have been shown to potential to bind to the CD14/MD-2/TLR4 complex but affect signalling in an antagonistic manner (Peri *et al.*, 2010; Cighetti *et al.*, 2014; Huggins *et al.*, 2015; Facchini *et al.*, 2018). Preliminary evidence suggests FP7 and derivative molecules may be suitable for further investigation with potential value for pharmacological intervention in inflammatory based diseases. However, in further development it is possible that higher concentrations, or alternative methods of delivery may be required to achieve the same effects.

5.1. Screening and identification of potential FP targets

Initial screening was carried out to determine which molecules wees carried out to identify potent antagonists of the TLR4. The effects of each molecule on cell viability and TLR4 induced IL-8 signalling was used to screen and select potent antagonists from the group. FP7 and derivative molecules were shown to have non-significant impact in safety screening assays at the concentrations used. Interestingly, FP11, particularly in conjunction with LPS treatment, appears to increase viability, perhaps through stimulating proliferation or metabolism of treated cells. Conversely FP111 produced a slight reduction in viability with increasing concentration, but this was not significant effect. However, all expected antagonists (FP7, FP10, FP12 and FP111) were well tolerated by THP-1 monocytes and macrophages and did not have any apparent negative impact on cell viability up to 10 or 20µM. FP7 and its derivative molecules were thus considered suitable for further use in THP-1 cells at concentrations between 0-10µM.

In initial screening, FP7 and two derivatives were shown to significantly downregulated TLR4-dependent cytokine production. FP7 was shown to reduce IL-8 production by LPS stimulation back down to a level comparable to the control. This corresponds with previous evidence that suggests that FP7 functions as an antagonist of TLR4-dependent P65/NF-κB signalling in HEK-blue cells and IL-8 production in murine models (Cighetti *et al.*, 2014; Perrin-Cocon *et al.*, 2017) Screening of FP7 derivatives yielded two further antagonists, FP10 and FP12. These were both shown to reduce IL-8 production by THP-1 cells in response to LPS at 10 µM. However, FP7 was a more potent inhibitor of IL-8 production than either FP10 or FP12. This is somewhat in contrast to other data, which suggests FP12 to be the more potent antagonist in HEK-Blue and RAW-Blue P65/NF-κB activation experiments (Cighetti *et al.*, 2014; Perrin-Cocon *et al.*, 2017; Facchini *et al.*, 2018). However, FP7 was also shown to have a slightly greater potential to displace LPS than FP12 than that of FP12. FP10 and FP116 were less effective in this regard, and likely accounts for the reduced activity of these two molecules as

compared to FP7 and FP12 (Facchini *et al.*, 2018). From the antagonist series, FP7 and FP12 were selected for further analysis while investigations of FP10 and FP116 were discontinued.

5.2. FP7 and FP12 function as antagonists in MyD88-dependent TLR4 signalling

The effect of selected FP compounds, FP7 and FP12, on TLR4 signalling was further expanded on and examined in both a human monocytes and macrophages. Both molecules performed well in initial investigations in THP-1 cell and showed positive ability to downregulate LPS induced cytokines in both monocytes and macrophages.

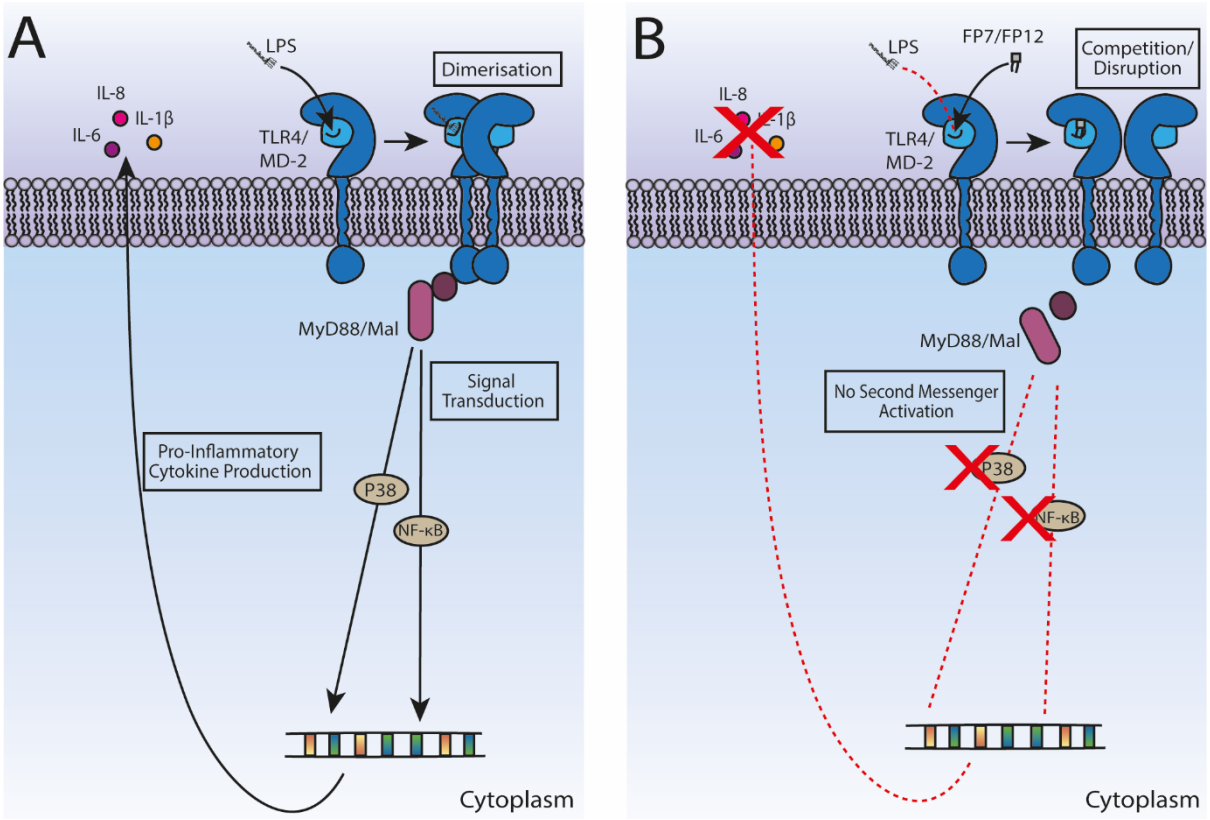

Figure 59. FP7 and FP12 negatively regulate LPS induced MyD88-dependent signalling via NF-κB and P38 phosphorylation, preventing downstream transcription and production of proinflammatory cytokines. (A) LPS activated MyD88-dependent signalling via TLR4. (B) FP7 and FP12 prevent LPS activation of TLR4/MyD88-dependent signalling pathways.

Two readouts were used to monitor the modulating effects of FP compounds; signalling mediator activation (NF-κB, P38 MAPK phosphorylation) was measured via Western blot, and endpoint cytokine production (IL-8, IL-6, IL-1β) production was measured via ELISA. In THP-1 monocytes FP7

was shown to negatively regulate LPS induced TLR4 signalling. Results suggested that higher concentrations (10 μM) of FP7 could negatively regulated cytokine production in THP-1 monocytes. FP7 could inhibit LPS induced production of IL-8, Il-6, IL-1β, MIP-1α, and iCAM-1. IL-12 was not produced in response to LPS at significantly detectable levels. Interestingly, IL-6 was present in the medium after 18 hours but was not significantly increased within cells by this time. This suggests an early production and release of IL-6 without further production within the cell.

FP7 also substantially decreased MIP-1α production. In this regard, FP7 appeared to be more effective in bringing about a reduction in of MIP-1α release into culture medium. The level of MIP-1α within the cell remains upregulated by LPS but is not significantly reduced in presence of FP7. This suggests that there is a difference between MIP-1α production and release from the cells. FP7 can affect MIP-1α release only, therefore this compound is capable of partial inhibition of MIP-1α release following LPS exposure.

Both FP7 and FP12 negatively regulated TLR4-dependent signalling in macrophages (Figure 59). FP7 and FP12 significantly reduced IL-8 production at as little as 0.1μM. IL-6 was downregulated at lower concentrations with FP7 than with FP12. Further to this, signalling mediators P65/NF-κB and P38 MAPK were downregulated following pre-treatment with both compounds but did not reach the same levels as controls in FP7. This may explain the apparent higher potency of FP12 in studies with reporter cell lines which focused on P65/NF-κB activation and inhibition and showed greater potency of FP12 (Facchini et al., 2018). This was not so apparent in screening, and further cytokine analysis seems to suggest FP7 is more effective in most cases. FP12 exhibits a more obvious dose dependent effect on signalling targets than does FP7, for which the dose dependent effect is less apparent. This could indicate a partial antagonism of FP7 as previously proposed (Cighetti et al., 2014). To further investigate and compare the effects of FP7 and FP12 in macrophages, a proinflammatory protein antibody array was used to give an overview of proinflammatory proteins production. The results of this suggested that FP7 was capable of downregulating most LPS-induced proinflammatory proteins

production, while FP12 appears to have a slightly lesser effect. In this regard, i309 and MIP-1α were downregulated by FP7, but not FP12. Also, levels of certain proteins such as MCP-1 in FP12 treated samples were higher than FP7. IL-8 production is not possible to measure via array due to high concentrations of IL-8 in medium saturating the signal and making it appear as though there is no difference between control, LPS and compound treated samples. These data together suggest that while FP12 may be a more potent antagonist of TLR4-dependent P65/NF-κB and P38 MAPK signalling, FP7 shows greater effects on downstream proinflammatory production. The reason for these differences between signalling and endpoint production is not entirely clear here. It is possible that post-transcriptional degradation of proteins could occur, but this would need to be further investigated through comparative mRNA analysis.

FP7 and FP12 are known to affect TLR4 through interactions with MD-2 and CD14. In this regard, FP7 is thought to competitively exclude LPS from binding to MD-2 and cause internalisation of CD14, further impairing the delivery mechanisms that aid delivery of LPS to the co-receptor (Cighetti *et al.*, 2014). However, results from this project suggest that FP7 and FP12 are capable of downregulating LPS induced signalling post-factum. This suggests that FP7 and FP12 can modulate MyD88-dependent signalling after LPS has bound to the pockets. Further modelling would be required to ascertain in FP7 and FP12 can bind to the pocket in conjunction with LPS. Alternatively, these molecules may interact with a different location on the receptor complex and perhaps destabilise the agonistic conformation of MD-2. It is unlikely that FP7 can affect MyD88 directly (Perrin-Cocon *et al.*, 2017). Previous studies have shown that FP7 does not affect TLR1/TLR2 or TLR2/TLR6 signalling. As these receptors utilise MyD88 in signalling, it is unlikely that FP7 is affecting this intracellular protein. However, this does not discount an alternative internal interaction with Mal or the TLR4 signalling domain, similar to how TAK242 affects TLR4 signalling (Takashima *et al.*, 2009). At present there is no evidence of FP7 or FP12 being associated with such an interaction.

FP7 and FP12 were shown to negatively regulate LPS induced TLR4 signalling. Results suggested that higher concentrations (10μM) of FP7 could negatively regulate cytokine production in THP-1 monocytes. The current work corresponds with other findings of dose-dependent antagonistic effects of FP7 (Cighetti *et al.*, 2014).

Preliminary data from primary PBMC derived macrophages suggested that FP7 can also negatively regulate TLR4 induced cytokines IL-6 and IL-1β and signalling via p-p65NFκB/p-P38. FP7 has also recently been shown to be effective in reducing IL-6 and IL-8 production primary dendritic cells in influenza induced cytokine expression (Perrin-Cocon *et al.*, 2017). Further to this, FP7 was shown to protect against LPS induced motoneuronal death in a mouse derived ALS model (de Paola *et al.*, 2016). FP12 represent an alternative mimetic molecule with similar structure and activity to FP7. Data obtained here suggests that FP12 may also be of value for investigation further in primary and *in vivo* models. This provides growing amount of evidence towards the potential of FP7 and FP12 as potent TLR4 antagonists. As such, both FP7 and FP12 represent good candidates for investigations into treatment of inflammatory based diseases.

5.3. FP7 negatively regulates TLR4 signalling in response to ligands of sterile Inflammation

Atherosclerosis is characterised by endothelial dysfunction and accumulation of lipids which lead to a sustained inflammatory response and recruitment of various immune cells into arterial walls (Shimada *et al.*, 2012). OxLDL in atherosclerotic plaques can be endocytosed by macrophages and leads to formation of foam cells. While TLR4 activation is known to play a part in progression of atherosclerosis, the means by which this is achieved is not clear. Sterile ligands have been proposed as a possible means by which TLR4 may be activated within atherosclerotic plaques. In this regard, oxLDL has been shown to activate TLR4 associated signalling mediator and downstream proinflammatory protein production (Chávez-Sánchez *et al.*, 2014; Kiyan *et al.*, 2014). As such, antagonistic effects on TLR4 response to oxLDL is of interest.

Previous data suggested that FP7 can downregulate LPS-induced proinflammatory signalling (Cighetti et al., 2014; Facchini et al., 2018). However, endogenous ligands such as oxLDL may activate the inflammatory response via different mechanisms to LPS, involving receptor clusters and activation of alternative pathways (Choi *et al.*, 2012; Chávez-Sánchez *et al.*, 2014). FP7 has been proposed to protect mice from influenza lethality through blocking TLR4-dependent sterile inflammation caused by DAMPs such as oxPAPC and HMGB1 (Perrin-Cocon *et al.*, 2017). Oxidised lipids may play an adverse role within the body and disease and can contribute to angiogenesis formation of foam cells which build up in atherosclerotic plaque (Miller and Shyy, 2017). OxLDL have been shown to upregulate TLR4-dependent signalling and may function via alternate activation mechanisms such as heterodimerisation of TLR4 and other TLRs and CD36 (Stewart *et al.*, 2010). As such, it was necessary to translate findings from LPS related studies into a model of sterile inflammation relevant for cardiovascular disease.

The method by which oxLDL is produced may result in different products and subsequently, a different effect of the product on TLR4 signalling (Choi *et al.*, 2012; Chávez-Sánchez *et al.*, 2014). Two different forms of oxLDL were produced using methods previously described involving dialysis

against copper sulphate at different temperatures (Gerry, Satchell and Leake, 2008). Native LDL was used as an additional control which should not activate TLR4. MTT assay was carried out to determine any cytotoxic effects of oxLDL and suggested both were well tolerated in monocytes. However, oxysterol-rich oxLDL appeared to reduce cell viability at higher concentrations in macrophages. This resulted in lower concentrations of oxysterol-rich LDL being used in later investigation than hydroperoxide-rich oxLDL.

For cytokine analysis in response to oxLDL, two different concentrations of oxysterol and three concentrations of hydroperoxide-rich oxLDL were used. Results obtained suggest that both oxysterol and hydroperoxide-rich oxLDL can initiate TLR4 induced cytokine production at 50μg/mL or higher, which is downregulated when cells are exposed to FP7. IL-6, IL-8 and IL-1β production was significantly increased following oxLDL exposure, as has been shown previously (Miller *et al.*, 2012; Chávez-Sánchez *et al.*, 2014). Previous evidence suggests that reduction in TLR4/NF-κB and TLR4/MAPK pathways can reduce oxLDL induced cytokine production in THP-1 macrophages (Zhang *et al.*, 2015). FP7 has been shown here to downregulate both P65/NF-κB and P38 MAPK in response to LPS and are likely to be suitable targets to measure by which FP7 can also prevent oxLDL-dependent cytokine production.

Interestingly, FP7 can bring cytokine production down to a level comparable to that seen in control samples in most cases despite potential involvement of alternate receptors besides TLR4. Previous experiments using TLR4 blocking antibodies did not produce as strong response in oxLDL stimulation (Chávez-Sánchez *et al.*, 2014). A 96.7-fold increase in IL-6 production was described following oxLDL stimulation with a 2-fold decrease when TLR4 was blocked, suggesting that oxLDL-dependent signalling does not rely entirely on TLR4 binding (Chávez-Sánchez *et al.*, 2014). As FP7 is thought to interact with the pocket of MD-2, it is possible that this is more important in facilitating other receptor interactions in oxLDL signalling than expected. MD-2 may therefore also be important in

heterodimer formation with TLR4 in response to oxLDL. This could also suggest an alternate effect of FP7 beyond competitive exclusion of ligands from the MD-2 pocket.

At present, there is no other data for the effect of FP7 in response to oxLDL activation of TLR4 signalling. Evidence collected here suggests that FP7 can negatively regulate TLR4 induced cytokine production following stimulation. Further investigation is required to understand the mechanisms by which TLR4 signalling is affected. Preliminary data suggests downregulation of P65/NF-κB and P38 MAPK activation may play a critical role in TLR4 signalling. However, other MAPKs, ERK and JNK pathways are also known to be activated in response to oxLDL (Choi *et al.*, 2012; Zhang *et al.*, 2015) Preliminary evidence obtained here suggests FP7 may function as an antagonist of TLR4 in response to sterile inflammation, though further mechanistic studies are required.

5.4. Comparability of FP7 effects between human and murine TLR4

While there is much conservation of TLR structure between species, slight variation in TLR4 may affect comparable functionality of the receptor. In some cases, modulators have been known to cause opposing effects in mouse compared to human TLR4. Transfection of human MD-2 into mouse cell lines is possible and has been shown to enhance responsiveness to human ligands (Akashi *et al.*, 2001). It is therefore important to consider the *in vivo* model in which FP7 and FP12 compounds may be tested in in future and consider if adaptation may be required. In such cases, consideration may be made to use transgenic animals for *in vivo* verification TLR4 modulators which produce species specific effects. As such, comparative studies of TLR4 modulators in both human and mouse cell lines could help inform design of future *in vivo* studies.

FP7 showed strong antagonism in human macrophages, but while FP7 could significantly downregulate LPS/TLR4 dependent cytokine production in mouse macrophages, the effect appeared to be slightly less. The greater antagonistic effect of FP7 in human macrophages may be due to acyl chain length of the molecule. Shorter chained molecules are known to confer greater antagonism in mouse TLR4 signalling and thus longer chained molecules may be less effective at blocking receptor activity due to the differences in the shape and space available in the pocket of MD-2 in mouse TLR4 (Chebrolu *et al.*, 2015). Previously there has been shown some comparability between the effects of FP7 on human and mouse TLR4 signalling. There is evidence to suggest that FP7 can supress IL-1β production in mouse embryo derived microglia cultures (de Paola *et al.*, 2016). Similarly, in the current work, FP7 demonstrated antagonistic properties in both human (THP-1) and mouse (RAW264) cell lines. IL-6 and IL-8 have previously been investigated in THP-1 cells and FP7 was shown to significantly downregulate both cytokines. As such, IL-6 and KC were further investigated in RAW264 macrophage models via ELISA. In line with previous results, FP7 showed some ability to downregulate IL-6 production from LPS exposure, showing that FP7 could significantly downregulate LPS-induced IL-6 production in both human THP-1 macrophages and in RAW264 macrophages.

Despite this, IL-6 levels in RAW264 macrophages did not decrease to the same extent in mice relative to LPS stimulated levels. In addition, while KC was significantly raised by LPS, but FP7 did not downregulate KC production at all. Further investigation into phosphorylation of signalling mediators revealed a differential effect between signalling molecules. P38 MAPK and P65/NF-KB were significantly downregulated following FP7 treatment, while ERK and JNK do not appear to be affected by FP7 treatment. This suggests that FP7 may only partially block TLR4-dependent signalling in mice.

It is possible that the differences seen between FP7 in human and FP7 in mouse models are related to the differences in interaction of acyl chains with mouse MD-2. Previously, there have shown to be differences in human and mouse TLR4 activation by ligands such as Lipid IVa, which exhibits an antagonistic effect in human TLR4, but a weak agonistic effect in mouse TLR4. This is thought to be associated with the way in which the acyl chains of Lipid A interact with the MD-2 pocket (Ohto *et al.*, 2012; Chebrolu *et al.*, 2015). The shorter length of acyl chains is associated with higher affinity of mouse MD-2 pocked and could offer explanation of the reduced antagonism of FP7 seen in RAW264 macrophages.

5.5. Differential effect of LPS from different bacterial strains on TLR4 signalling and link to antagonistic effects of FP compounds

LPS is characterised by three main regions: Lipid A, Oligosaccharide core and O-antigen regions. Previously, variations in ligand structure can affect the dynamic and potency of its effect TLR4 signalling and LPS from different sources have been shown differentially affect MyD88 and TRIF - dependent signalling. In this regard, structural differences in the Lipid A region of LPS were thought to be of importance in MyD88 or TRIF-dependent signalling (Zughaier *et al.*, 2005). Further to this, differences in the polysaccharide chain have been shown to be important in TLR4 induced responses (Huber *et al.*, 2006). As such, understanding how different LPS affect signalling can be vital to future experimental design.

Most wild-type LPS take the form of smooth LPS. This smooth LPS is considered the complete in that it has a full o-antigen or polysaccharide chain present. Conversely, rough LPS forms (Re, Rd1, Rd2, Rc, Rb, Ra) are typically the product of mutants which exhibit a reduction in sugar residues to varying extents (Figure 5). However, R-form constitute to a variable degree the S-form of LPS, depending upon for example on the bacterial growth conditions and selective pressure by immune surveillance. These structural alterations of S- and R-type LPS have been shown to elicit different responses in mast cells and macrophages cells depending on the specific form (Huber *et al.*, 2006).

While previous evidence suggests *S.minnesota* LPS was most effective at upregulating TLR4/TRIF-dependent signalling (Zughaier *et al.*, 2005), initial experiment involving *S.minnesota* (Re) did not consistently yield positive results for STAT-1 phosphorylation. Following further investigation into the capacity of LPS to upregulate p-STAT-1, stimulation with *S.minnesota* (Re) showed a much lower level of expression compared to *E.coli* (Ra) LPS. At the same time, the smooth variant of *S.minnesota* LPS was shown to upregulate p-STAT-1 more so than *E.coli*. Further to this, IFNβ levels were substantially higher in *S.minnesota* (S) than (Re) treated cells. This effect was also seen in MyD88-dependent IL-1β production in response to S and Re-form LPS. S-form LPS produced a greater

response in MyD88-dependent signalling. However, FP7 and FP12 were capable of causing comparable levels of downregulation of this response across both S and previously used Re LPS chemotypes. Lipid A and MPLA alone were ineffective at stimulating TRIF-dependent signalling. Evidence provided here suggests *S.minnesota* (S) is more effective at upregulating TRIF-dependent TLR4 signalling than *S.minnesota* (Re) and is thus more appropriate for investigation of this signalling pathway.

Timing experiments were carried out to optimise experimental design. To determine the correct time for activation signalling molecules, phosphorylation was measured by Western blot from 0-6 hours and IFNβ and IP10 production measured via ELISA at 2, 6 and 24h. From this, TBK1 was found to be upregulated at 30 minutes, while STAT-1 was substantially later at 2.5-3 hours. While IP10 production increased steadily over the course of the experiment, levels of IFNβ initially increased at 2 and 6 hours, before disappearing from medium. This may be due to degradation of the molecule, although interferons are typically quite stable. Alternatively, IFNβ may be removed from medium following receptor interactions. As such, an earlier collection time is required from measurements of IFNβ in the medium. These effects were consistent relatively consistent between different forms of LPS.

Lipid A is the active part of LPS which interacts with MD-2 and facilitates receptor dimerisation. These differential effects LPS chemotypes suggest that the polysaccharide chain plays a greater role in receptor activation, and specific response of these receptors to ligands. Evidence presented here suggested STAT-1 activation, IFNβ and IP-10 production were more greatly elevated in response to complete forms of LPS than to Lipid A alone, suggesting this could be important for TRIF-dependent signalling. However, further experiments would be necessary to confirm any relationship.

5.6. FP7 and FP12 negatively regulate TRIF-dependent TLR4 signalling

Further investigation into alternate TLR4 activation was also explored in TRIF-dependent signalling pathways. It seems logical to assume that FP7 and FP12 would affect both MyD88 and TRIF-dependent signalling as these molecules are affecting MD-2 which should be involved in both pathways. Additionally, FP7 can cause internalisation of CD14, removing this from the cell surface (Cighetti *et al.*, 2014). As TRIF-dependent signalling is more reliant on CD14 than is MyD88-dependent signalling (Tsukamoto *et al.*, 2018), FP7 should be capable of preventing TRIF-dependent signalling just as well, if not more effectively than MyD88.

Initial results confirmed the ability of both FP7 and FP12 molecules have to be capable of downregulating LPS mediated IL-8 production and LPS/TLR4 induced P38 MAPK and NF-κB activation in THP-1 cells. FP7 and FP12 have previously been shown both here and in alternative models by others to be effective in reducing TLR4-dependent signalling and cytokines (Facchini et al., 2018; C. Palmer et al., 2018). However, thus far there is not much information regarding the specific effects of these molecules in TLR4/TRIF-dependent signalling molecules and interferon release. Investigating pathway specific interactions of FP derivatives would provide further evidence as to the relevance of these molecules as TLR4 modulators of one or both TLR4-dependent signalling pathways.

Upon recognition of LPS, TLR4 initiates a response via MyD88 or TRIF-dependent signalling pathways. Investigation was carried out in relation to the TRIF-mediated response. The presence of proinflammatory M1 phenotype macrophages in atherosclerotic plaques has been associated with plaque instability (Cho *et al.*, 2013). THP-1 macrophages polarised to M1 phenotype via LPS and IFNγ exposure show elevated levels of TRIF and IRF-3 expression (Shi *et al.*, 2016). Further to this, IFNβ may be a contributing factor in cardiovascular disease (Schirmer, Bot and …, 2010). As TRIF-dependent signalling has been found to be upregulated following M1 polarisation, modulation of this pathway is of interest.

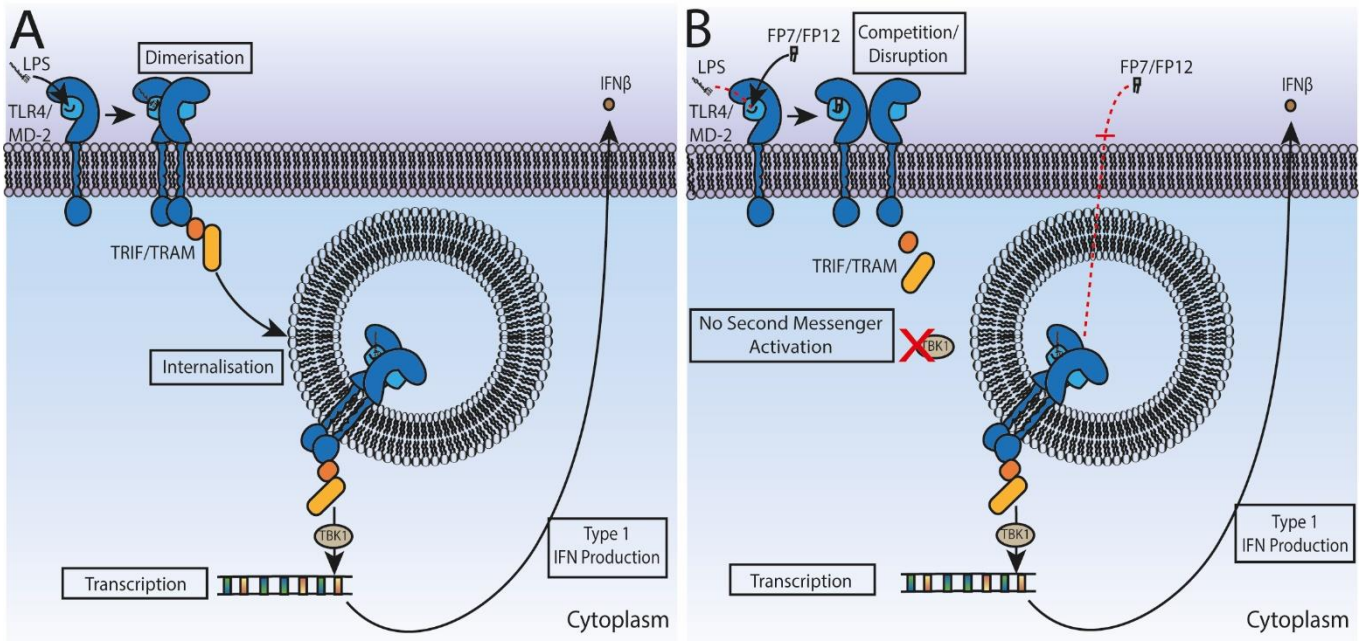

Figure 60. FP7 and FP12 can negatively regulate TRIF-dependent cytokine production in response to LPS. However, post factum treatment has no effect on TRIF dependent IFNβ production, perhaps as a result of receptor internalisation. (A) LPS activates TRIF-dependent signalling via TLR4, resulting in receptor internalisation and activation of TBK1 with downstream production of type 1 interferons. (B) FP7 and FP12 can block TRIF-dependent signalling prior to TBK-1 activation but may not have an effect on TRIF-dependent signalling after receptor internalisation.

Evidence obtained through the course of this study suggests FP7 and FP12 are also capable of downregulating TRIF-dependent signalling in THP-1 macrophages. Following LPS stimulation, both FP7 and FP12 have been shown to significantly reduce LPS mediated p-STAT-1, p-TBK and IFNβ production. Interestingly, while FP7 and FP12 appear capable of downregulating MyD88-dependent cytokines following post-LPS treatment, IFNβ is not affected in the same way. While prior and co-treatment with FP7 or FP12 can downregulate IFNβ production, post LPS does not produce any decrease in IFNβ for FP12 but not for FP7. This may be the result of receptor endocytosis, which occurs within 30 minutes of receptor activation (Kagan *et al.*, 2008). As shown here (Figure 42), TBK1

is already phosphorylated at 30 minutes, signal transduction is already well underway and thus assumedly TLR4 is no longer on the surface of cells (Figure 60). As FP7 interacts with MD-2 (Facchini *et al.*, 2018), this may suggest that interactions are extracellular and FP7 does affect TLR4 once internalised. However, further experiments would be required to determine localisation of FP12 following treatment timing.

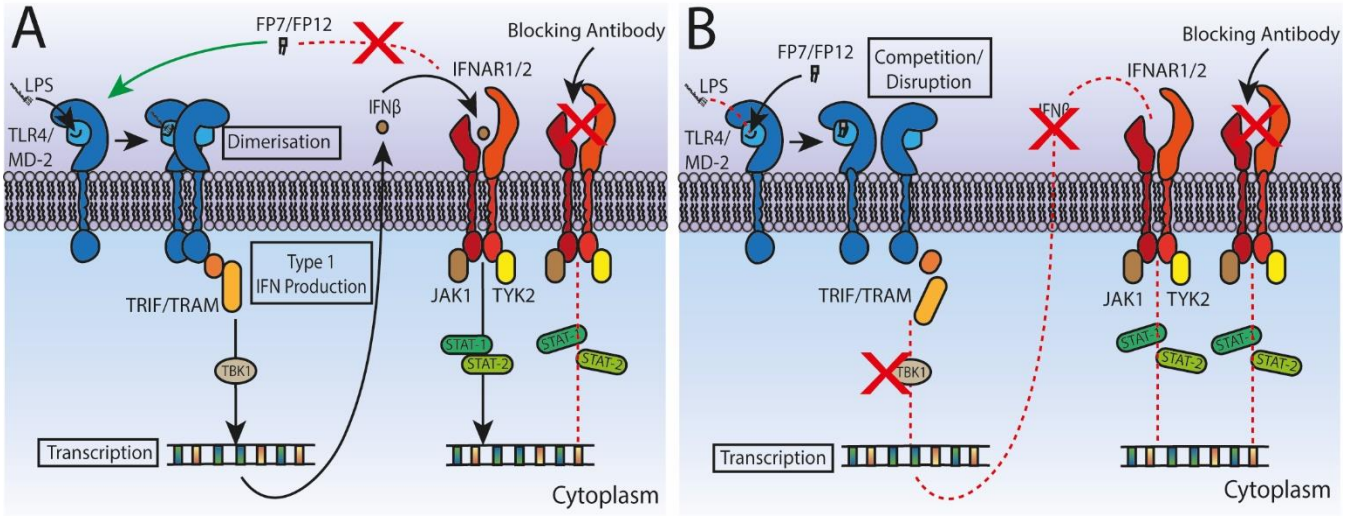

Figure 61. FP7 and FP12 indirectly downregulate STAT-1 phosphorylation via IFNAR. (A) TLR4 induced production of IFNβ results in subsequent activation of IFNAR and downstream phosphorylation of STAT-1. IFNAR blocking antibodies prevent TLR4 dependent activation of STAT-1 via IFNAR. FP7 and FP12 can affect IFNAR signalling by TLR4-dependent IFNβ, but not direct IFNAR activation by IFNβ. (B) FP7 and FP12 downregulate TRIF-dependent production if IFNβ, preventing IFNβ activation of IFNAR and thus downregulating IFNAR dependent STAT-1 phosphorylation.

Additional studies of the relationship between LPS, FP7, IFNβ and IFNAR were carried out. Following LPS stimulation of TLR4, STAT-1 can be activated via cytokine-dependent signalling pathways. IFNAR signalling is associated with JAK/STAT pathways. To determine how much STAT-1 activation is dependent on IFNβ and confirm specificity of FP7 and FP12, a series of experiments utilising an

IFNAR blocking antibody were carried out to assess these elements. LPS upregulated levels of IFNβ, which are suppressed when cells are pre-treated with FP7 or FP12. LPS caused an increase in STAT-1 phosphorylation, peaking around 3 hours after exposure which FP7 and FP12 can also downregulate . Exposure of cells to IFNβ produced a rapid increase in STAT-1 phosphorylation in THP-1 macrophages which was not significantly affected by FP7 or FP12. FP7 and FP12 were capable of downregulating LPS, but not IFNβ dependent STAT-1 phosphorylation. IFNAR blocking prevented STAT-1 phosphorylation in response to LPS or IFNβ. These results suggested that STAT-1 was entirely dependent on IFNAR signalling and FP7 and FP12 exert an indirect inhibition of IFNAR via downregulation of TLR4-dependent IFNβ production (Figure 61). This data in conjunction with previous evidence obtained through the project provides evidence towards FP7 and FP12 antagonism of both MyD88 and TRIF-dependent TLR4 signalling.

5.7. FP11 and FP18 upregulate MyD88 and TRIF-dependent TLR4 signalling

From the initial series of FP compounds, FP11 was the only antagonist to show positive initial effects on IL-8 production. While also a monosaccharide synthetic glycolipid, FP11 shares structural similarity to part of MPLA. As such, it may be expected to exhibit some similar effects to MPLA on proinflammatory protein production. A proinflammatory protein antibody array was used to compare LPS, MPLA, FP11 and an additional predicted agonist, FP18, which was structurally similar to FP11 but with shorter acyl chain length.

Results from array suggested that FP11 and FP18 were more comparable in effect to one another, and to LPS, than they were to MPLA. Both compounds produced a significant elevation in many proinflammatory cytokines, though they were less potent than LPS. Interestingly, MPLA appears to stimulate production of a different set of cytokines. It is worth noting that the LPS used for FP7/FP12 array was Re-form, while the FP11, FP18 array was S-form. This appears to give a different expression profile which may relate to MyD88/TRIF affinity of LPS chemotypes (Huber *et al.*, 2006)

FP11, FP18 and MPLA were compared for their potential to induce TLR4-dependent signalling in THP-1 cells. Previous data suggested FP11 exhibits some agonistic effect in THP-1 macrophages. Interestingly, while FP18, FP11 nor MPLA appeared capable of inducing TRIF-dependent TBK1 phosphorylation or IP10 production, these all still produced elevated levels of STAT-1 phosphorylation. For MPLA this effect is much later and to a lesser extent than with LPS, FP11 or FP18. Further blocking experiments show that FP11 and FP18-dependent p-STAT-1 is activated via IFNAR. Preliminary data suggested that these do not produce IFNβ. However, IFNAR can be activated other type-1 interferons. At this time, the exact mechanism or means by which IFNAR is activated by FP11 and FP18 is difficult to consider. To first prove that this is not a direct interaction, further TLR4 blocking experiments may be necessary for future consideration. To elucidate the exact role of IFNs and potential alternative mechanisms of IFNAR induced STAT-1 activation, further experiments are required.

5.8. Limitations and Future Work

The current work provides an overview of FP7 and FP12 activity in human and mouse TLR4 signalling. Both MyD88 and TRIF-dependent signalling were studied, and both compounds were found to be capable of affecting both pathways. However, some understanding of these interactions requires some further investigation understand exact mechanisms of FP7 and FP12 antagonism.

FP7 and FP12 are capable of downregulating TLR4-dependent MyD88, but not TRIF pathways in post factum treatment by FP12. The exact means by which this is possible is not currently understood. Speculations can be made, but further computational modelling of how FP7 or FP12 can interact with different signalling molecules after LPS binding is necessary to get a clearer understanding. Additionally, experiments to determine the distribution of FP7 and FP12 following treatment could help to understand if this is present and capable of interactions within the cell, or if effects are limited to external interactions.

In comparison studies between mouse and human macrophages, only one candidate was measured at this time. The effects of FP7 in IL-6 and KC provided some interesting evidence for cross-species translatability and potential differences between species, however further investigation into the effects of FP7 on other cytokines may provide a broader overview of the similarities and differences between species. Preliminary evidence for FP12 (Table S2 – Appendix IV) suggests that FP12 may show some interesting effects in mouse macrophages. In this regard, most cytokines are shown to be downregulated following FP12 treatment. However, it appears from array that FP12 may also upregulate IL-10 and IFNγ. These results need to be evaluated via ELISA in the future in order to confirm their validity in a quantifiable manner. Therefore, further investigation of FP12 is also required to determine cross-species similarities and differences. Further verification of targets via ELISA in human and mouse would help to bolster the validity to these initial findings and provide greater understanding of cross-species comparability.

The mechanism of STAT-1 phosphorylation in FP11, FP18 and MPLA receptor activation requires further investigation. These appear to signal through IFNAR, implying involvement of type 1 interferons. However, TRIF signalling is not activated by any of these molecules. As IFNAR is involved, it is unlikely that alternative cytokines are responsible for STAT-1 activation. Therefore, the means by which IFNAR is activated remains unclear and further investigation may be required to confirm this is not a direct effect. Blocking TLR4 may help to confirm specificity in this regard and additional TRIF receptor internalisation or blocking experiments may confirm whether TRIF signalling can be activated by FP11, FP18 and MPLA in absence of downstream TBK1 activation. Further experiments are required to determine the comparability of these effects on TRIF-dependent signalling between THP-1 macrophages and PBMC derived macrophages.

6. Conclusions

FP7 and FP12 were shown to function as antagonists in both human and mouse cell lines and downregulate both MyD88- and TRIF-dependent TLR4 signalling in THP-1 macrophages. These both brought about significant reductions in TLR4-indused proinflammatory proteins production in response to LPS and ligands of sterile inflammation such as oxLDL. Additional preliminary experiments in PBMC derived macrophages suggested that MyD88-dependent antagonistic effects of FP7 and FP12 observed in THP-1 macrophages are reproducible in primary cell models. Furthermore, P65/NF-κB and P38 MAPK activation was substantially downregulated by both compounds. Further to this, both FP7 and FP12 significantly reduce TLR4/TRIF-dependent TBK1 and STAT-1 phosphorylation and IP10 and IFNβ production following stimulation with LPS and affect subsequent IFNAR signalling through indirect downregulation of IFNβ. This proved that FP7 and FP12 can antagonistically affect TLR4-dependent signalling.

Additional cross species investigation showed some translatability of finding into murine TLR4. FP7 downregulated LPS-mediated proinflammatory protein production and signalling mediator activation in both human and mouse macrophages. However, this effect was less pronounced in mouse macrophages. This preliminary evidence suggests that FP7 can function antagonistically in both human and mouse macrophages.

FP11 and FP18 agonists were shown to upregulate TLR4-dependent signalling in THP-1 macrophages. While neither were capable of affecting TRIF-dependent signalling via TBK1, both were able to upregulate STAT-1 phosphorylation. Blocking experiments suggest that this is via IFNAR, although IFNβ and downstream IP10 levels were not significantly upregulated by either FP11 or FP18. These results suggest that FP11 is capable of functioning as an agonist of TLR4/MyD88 signalling, but the exact mechanism by which FP11 or FP18 might affect TRIF-dependent IFN signalling remains unclear and required further investigation. In addition, the effect of FP11 on TLR4 signalling was not

necessarily comparable to MPLA despite structural similarities. Ideally more specific experiments should be carried out to variety targets from array and further work is required to understand FP11 and FP18 activity on TRIF-dependent signalling.

Two antagonists and one agonist were selected from initial screening and tested in various scenarios. Data collected offered further evidence towards the preclinical validation of these small molecule antagonists for further preclinical/clinical investigations and provided evidence towards mechanism of TLR4 signalling inhibition. However, further experiments are necessary to better understand the interactive dynamics of these molecules with TLR4/MD-2 and LPS.

7. References

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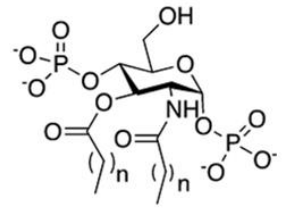
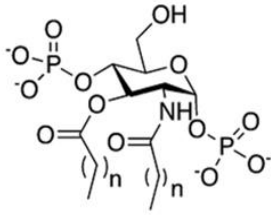
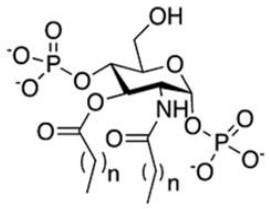
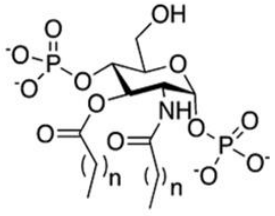
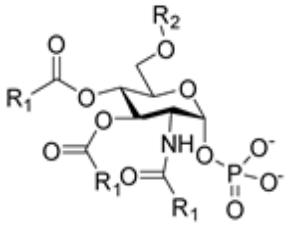
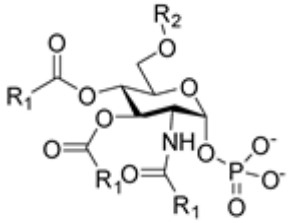
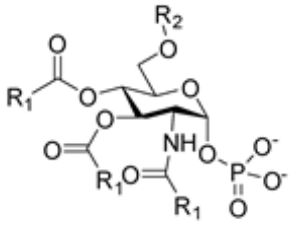
APPENDIX I – FP Compounds Preparations

FP Compounds were provided by Francesco Peri (University Milano). Compounds were delivered in lyophilised form and working stocks of each compound were prepared as follows, and used within one year of reconstitution – stored at 4◦c.

Compound/Structure	Preparation
 FP10: n = 8	Concentration: 5mM DMSO/Ethanol (1:1)
 FP12: n = 10	Concentration: 5mM DMSO/Ethanol (1:1)
 FP7: n = 12	Concentration: 2.5mM DMSO/Ethanol (1:1)
 FP116: n = 14	Concentration: 5mM DMSO/Ethanol (1:1)

Compound/Structure	Preparation
 **FP11**:$R_1 = C_{13}$, $R_2 = H$	Concentration: 5mM DMSO or Water
 **FP111**: $R_1 = C_{13}$, $R_2 = PO_3^{-}$	Concentration: 5mM DMSO/Ethanol (1:1)
 **FP18**: R1 = C_{11}, $R_2 = H$	Concentration: 5mM DMSO or Water

APPENDIX II – LPS Variants

Lipopolysaccharide and endotoxin free water were provided by INNAXON (Tewkesbury). LPS, Lipid A and MPLA were delivered pre-prepared in sterile endotoxin free water at stock concentrations of 0.5-1mg/mL and stored at 4^{o}C until use. The following reagents were used throughout the course of experiments.

- *S.minnesota (S-form)* #IAX-100-020
- *S.minnesota* R595 (Re) #IAX-100-008
- *S.minnesota* R60 (Ra) #IAX-100-016
- *S.minnesota* R595 (Re) #IAX-100-001
- *S.minnesota* R595 (Re) #IAX-100-002
- *E.coli* EH100 (Ra) #IAX-100-010
- *E.coli* R515 (Re) #IAX-100-004

APPENDIX III – Array Dot Blots

A proinflammatory protein antibody array was used to provide an overview of proinflammatory protein production in response to LPS and FP agonist series compounds, or subsequent downregulation by FP antagonist series compounds. Human inflammation array membranes are pre-prepared with 40 prebound spots of antibody in duplicate (see format from Table S1).

Table S1. Layout of antibody array membranes for RayBiotech human inflammation array C3

	A	B	C	D	E	F	G	H	I	J	K	L
1	Pos	Pos	Neg	Neg	Eotaxin 1	Eotaxin 2	GCSF	GM-CSF	iCAM-1	IFNγ	I-309	IL-1α
2	Pos	Pos	Neg	Neg	Eotaxin 1	Eotaxin 2	GCSF	GM-CSF	iCAM-2	IFNγ	I-310	IL-1α
3	IL-1β	IL-2	IL-3	IL-4	IL-6	IL-6R	IL-7	IL-8	IL-10	IL-11	IL-12 p40	IL-12 p70
4	IL-1β	IL-2	IL-3	IL-4	IL-6	IL-6R	IL-7	IL-8	IL-10	IL-11	IL-12 p40	IL-12 p70
5	IL-13	IL15	IL-16	IL-17A	IP-10	MCP-1	MCP-2	M-CSF	MIG	MIP-1α	MIP-1β	MIP-1δ
6	IL-13	IL15	IL-16	IL-17A	IP-11	MCP-1	MCP-2	M-CSF	MIG	MIP-1α	MIP-1β	MIP-1δ
7	RANTES	TGFβ1	TNFα	TNFβ	TNF RI	TNF RII	PDGF-BB	TIMP-2	Blank	Blank	Neg	Pos
8	RANTES	TGFβ2	TNFα	TNFβ	TNF RI	TNF RII	PDGF-BB	TIMP-3	Blank	Blank	Neg	Pos

THP-1 macrophages were activated with LPS and either pre-treated with FP7 or FP12 in the presence of LPS, or alone in the case of FP11 and FP18. A single membrane was produced for each treatment (See Figure S1). Each membrane was normalised to the average value of positive controls on the blot and blank values subtracted before calculation of relative fold-increase versus control samples. This gives a rough approximation of the level of each protein present and the potential difference between samples to help identify potential proteins of interest for further investigation.

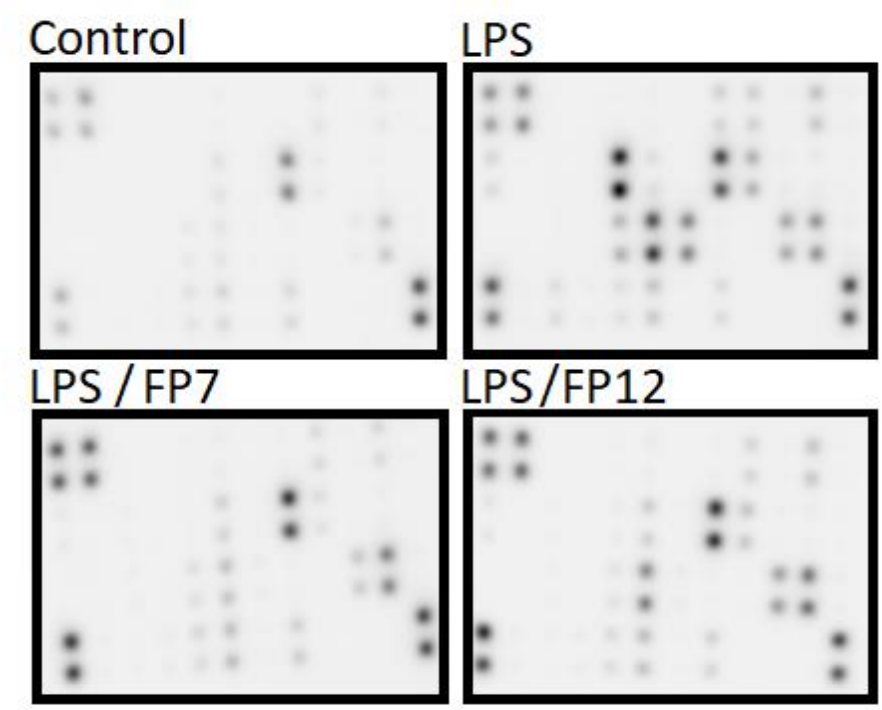


Figure S1. Dot blots produced for human inflammation Array: Control, LPS, FP7 and FP23. THP-1 macrophages were pre-treated with FP7 or FP12 for 1 hour prior to overnight LPS (100ng/ml) exposure. Culture medium was collected and centrifuged to remove cell debris before applying to array membranes overnight.

APPENDIX IV – Additional Data (Mouse)

An overview of the effects of FP12 on LPS induced mouse TLR4 signalling suggests that FP12 may downregulate some cytokines, while others appear to be upregulated. However, further verification of this data is required as array data do not provide quantitative data and alone do not provide an accurate enough representation of what may be happening with different cytokines.

Table S2. FP12 negatively regulate LPS induced proinflammatory protein production in RAW264 mouse macrophages. RAW264 macrophages were treated with FP12 (10μM) for 1 hour prior to S.minnesota LPS (Re) (100ng/ml) exposure. Culture medium collected after 18-hour incubation. A human inflammation array (RayBiotech) was used to assess relative levels of cytokine expression between samples. Results values are expressed as fold-increase relative to control samples.

Inflammatory Proteins	LPS	LPS/FP12	Inflammatory Proteins	LPS	LPS/FP12
1. CD30	2.39	0.62	**21. I-TAC**	1.54	1.02
2. EOTAXIN-1	2.60	1.80	**22. KC**	1.43	0.99
3. EOTAXIN-2	2.21	1.18	**23. Leptin**	1.00	1.49
4. Fas Ligand	0.96	1.75	**24. LIX**	0.74	1.21
5. Fractalkine	0.34	2.34	**25. XCL1**	1.44	0.59
6. GCSF	4.37	1.84	**26. MCP-1**	2.43	1.20
7. GM-CSF	4.36	1.21	**27. M-CSF**	1.06	1.03
8. IFNγ	0.96	2.33	**28. MIG**	0.90	0.32
9. IL-1α	1.99	0.71	**29. MIP-1α**	1.12	1.34
10. IL-1β	1.13	1.35	**30. MIP-1γ**	0.49	1.39
11. IL-2	1.73	0.86	**31. RANTES**	2.50	1.40
12. IL-3	2.43	0.57	**32. SDF-1α**	0.98	1.57
13. IL-4	0.74	1.32	**33. I-309**	1.09	0.87
14. IL-6	79.87	2.60	**34. TECK**	5.40	1.46
15. IL-9	0.94	1.21	**35. TIMP-1**	3.33	1.17
16. IL-10	1.02	3.12	**36. TIMP-2**	1.45	0.64
17. IL-12 p40/70	0.90	1.32	**37. TNFα**	2.55	9.27
18. IL-12 p70	1.45	0.39	**38. TNF RI**	1.01	1.23
19. IL-13	1.28	0.93	**39. TNF RII**	2.23	1.05
20. IL-17A	3.49	0.81			